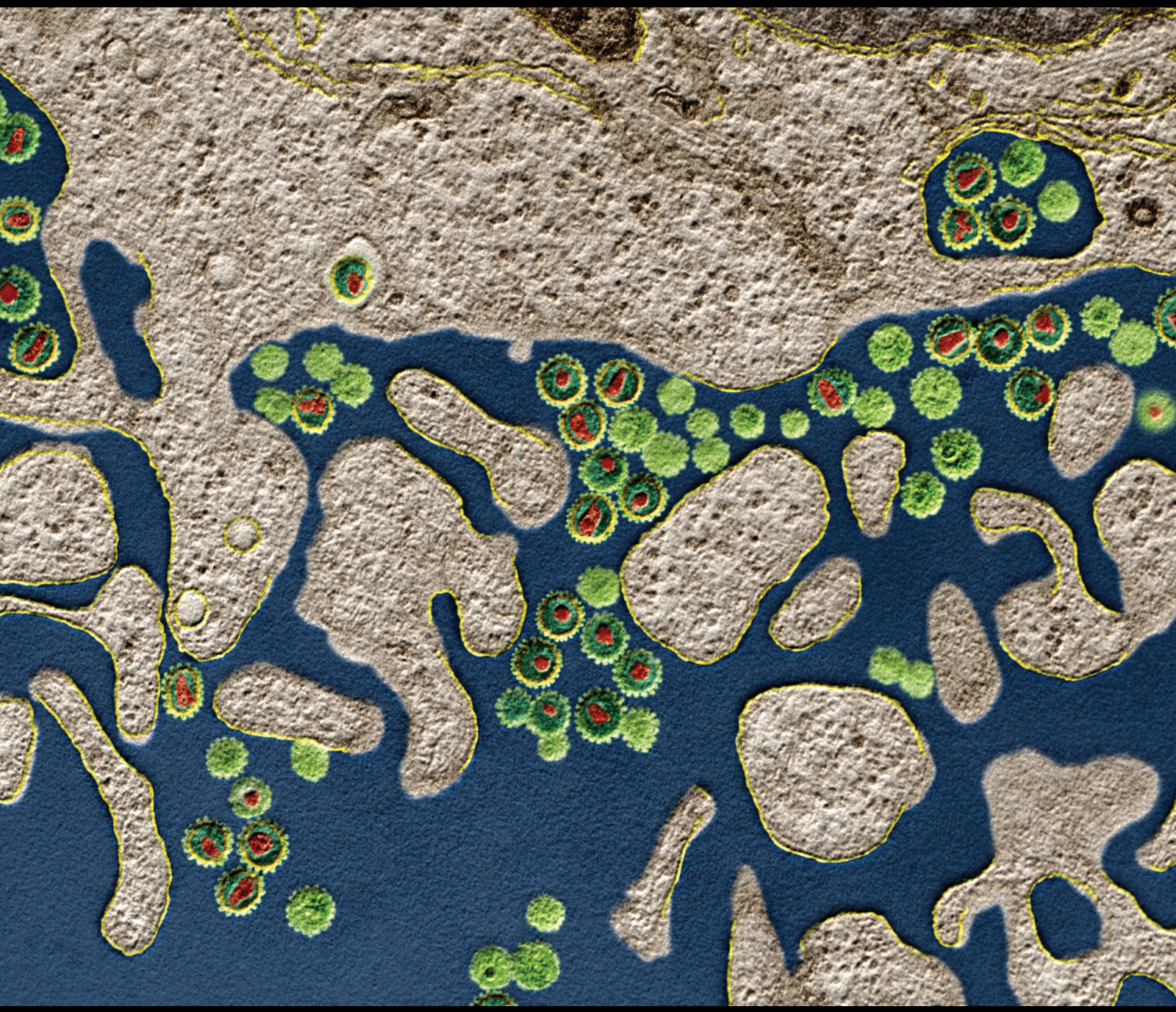
Journal of Immunology Research

Inflammation in the Tumor Microenvironment

Lead Guest Editor: Qing Lin
Guest Editors: Shi Jin, Mei Han, Wenxin Zheng, Jiaming Liu, and Xiaolong Wei



Inflammation in the Tumor Microenvironment

Journal of Immunology Research

Inflammation in the Tumor Microenvironment

Lead Guest Editor: Qing Lin
Guest Editors: Shi Jin, Mei Han, Wenxin Zheng, Jiaming Liu, and Xiaolong Wei

Editorial Board

Contents

Hindawi
Journal of Immunology Research
Volume 2018, Article ID 1965847, 2 pages
https://doi.org/10.1155/2018/1965847

Editorial

Inflammation in the Tumor Microenvironment

Qing Lin[1], **Shi Jin**[2], **Mei Han**[3], **Wenxin Zheng**[4], **Jiaming Liu**[5] and **Xiaolong Wei**[6]

[1]*Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*
[2]*Division of Gastroenterology, Department of Pediatrics, University of Florida, Gainesville, FL 32610, USA*
[3]*McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*
[4]*Ludwig Center for Metastasis Research, Department of Radiation and Cellular Oncology, The University of Chicago, Chicago, IL 60637, USA*
[5]*Department of Orthopedic Surgery, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, China*
[6]*Shantou University Medical College, Shantou, Guangdong 515041, China*

Correspondence should be addressed to Qing Lin; qlin2@jhmi.edu and Jiaming Liu; liujiamingdr@hotmail.com

Received 18 March 2018; Accepted 18 March 2018; Published 24 June 2018

The objective of this special issue is to provide a better understanding of the tumor-associated inflammatory signaling and their microenvironmental cross talk and network, which is crucial for elucidating tumorigenesis mechanisms and for cancer therapy, prevention, and risk assessment. A substantial number of manuscripts were submitted, and after a thorough peer review process, eleven papers were accepted for inclusion in this special issue. These papers investigate the involvements of the essential microenvironmental elements including inflammatory mediators and cellular effectors in the context of cancer-related inflammatory processes; they also offer new insights into tumor immunotherapy, which will help explore new targets or approaches for cancer treatment, by either boosting antitumor immunity or disrupting tumor-educated immunosuppression.

The paper by S. Ye et al. investigates the immunoregulatory properties of enavatuzumab, a humanized anti-TWEAK receptor monoclonal antibody. This preclinical study demonstrates that enavatuzumab exerted its potent antitumor activity by actively recruiting and activating myeloid effectors to kill tumor cells. The findings of enavatuzumab-induced chemokines warrant further evaluation in clinical studies as potential biomarkers for such activity.

The paper by M. Janiczek et al. presents a systematic review on immunotherapy as a promising treatment for prostate cancer. The authors summarize the recent advances and trends on three categories of immunotherapies: checkpoint inhibitors, cytokines, and therapeutic cancer vaccines, providing better understanding on the complexity of tumor cells and their interactions with the surrounding inflammatory microenvironment.

The paper by W. Zeng et al. tries to dissect the mechanism underlying the effects of methylation agent decitabine (DAC) treatment on myeloid myelodysplastic syndromes (MDS) by in vitro study with the MDS cell line SKM-1. Their results show that DAC could rescue FOXO3A, a potentially tumor-suppressive transcription factor. The reactivation of FOXO3 signaling is also critical for the anti-MDS therapeutic effects of DAC.

The paper by M. Liu et al. conducts bioinformatics analysis on differentially expressed genes (DEGs) between tumor samples and normal samples to identify key genes and pathways in glioma. Some identified hub genes may be important for regulating inflammatory responses, and CDK17, GNA13, PHF21A, and MTHFD2 might be important and potentially valuable in the prognosis and treatment of glioma. These identified genes and pathways would provide a more detailed molecular mechanism for underlying glioma initiation and development.

The paper by G.-Y. Liou focuses on cytokine signaling of tumor-facilitated immune cells and of cancer cells that lead to tumor initiation, progression, and metastasis of pancreatic cancer and prostate cancer. The review of the complex cytokine network among tumor cells, immune cells, and other

types of cells including stromal cells and endothelial cells would provide information on developing effective immunotherapies for treating related tumors.

The paper by G. D'Arena et al. summarizes published data on the prognostic significance of regulatory T cells in hematological malignancies. The authors attribute the variability reported by different groups to the heterogeneity of the experimental approaches, and emphasize the need to apply standardized approaches in the study of regulatory T cells in hematological malignancies and in cancer in general.

S. Chen et al. conducted a large cohort study to validate the involvement of four genes, STAT1, IGF1, RAC1, and MDM2, in the recurrence of a giant-cell tumor of the bone. Their findings suggest the potential of these genes to serve as biomarkers for the recurrence of the giant-cell tumor of the bone. Moreover, data presented also indicate that immune response mediated by these four genes through their interacting proteins might play important roles in the recurrence of the giant-cell tumor of the bone.

The paper by A. Aponte-López et al. discusses the evidence supporting protumoral and antitumoral roles of mast cells in breast cancer progression, highlighting recent findings placing mast cells as important drivers of tumor progression, as well as the potential use of these cells or their mediators as therapeutic targets. The paper calls for more work to clarify the role of mast cells in breast cancer and for a better understanding of mast cell communication with tumor cells and other immune cells within the tumor stroma.

The paper by G. Chen et al. compared the clinicopathologic features of gastric cardia cancer (GCC) and esophageal cancer (EC). Results showed that GCC in the Chaoshan high-risk area in China displays clinicopathologic characteristics different from those of EC, although they share genetic risk factors and similar geographic aggregation. Studies also detected toll-like receptor- (TLR-) 4 expression in gastric cardia epithelial cells and demonstrate upregulated TLR4 expression in gastric cardia inflammation and GCC, suggesting that TLR4 plays a role in GCC carcinogenesis.

The paper by Y. Xie et al. briefly reviews the mechanism underlying immunosuppression in hepatocellular carcinoma (HCC), followed by a summary of major immunotherapeutic approaches, including adoptive cell therapy, tumor vaccines, immune checkpoint inhibitors, and oncolytic virotherapy, for HCC with their current advances. The pros and cons of each option for HCC treatment are also discussed in this review article.

The paper by Q. Xu introduces a novel inflammation-based prognostic score, the fibrinogen/albumin ratio, which could predict prognoses of HCC patients undergoing curative resection. With a retrospective study, their data demonstrate that an elevated fibrinogen/albumin ratio significantly correlates with poorer survival and a higher risk of recurrence in HCC patients. The potential of the fibrinogen/albumin ratio to be a promising serum biomarker for predicting HCC prognoses is indicated in this study.

All the topics highlighted above, we believe, would be of particular interest to the readers, especially the basic and clinician scientists who specialize in immunology and immunooncology. Inflammation in the tumor microenvironment affects every aspect of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis, as well as response to therapy. In turn, to elucidate the signaling network of an inflammatory tumor microenvironment would be also crucial for developing a novel effective therapy for some complex inflammatory diseases, such as the pulmonary arterial hypertension (PAH) which is a fatal and multifactorial disease caused by vascular inflammation. Given the striking pathogenic analogies between cancer and PAH, as pulmonary vascular cells acquired cancer traits including apoptosis-resistant excessive proliferation during PAH development, the cancer theory of PAH is recently developed [1], and application of antineoplastic drugs thus may be a promising way to tackle established PAH [1]. Nevertheless, there is still a long way to go before a clear picture of the inflammation-tumor cross talk can be accomplished, and this special issue is adding a few new points in the picture being painted.

Acknowledgments

We would like to thank all the authors who contributed to this special issue and also thank all the expert reviewers who provided vital constructive feedback and criticism throughout the review process. The lead guest editor Dr. Qing Lin's research is supported by the "Stimulating and Advancing ACCM Research (StAAR)" grant from the Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University, during editing this special issue. Dr. Lin is also grateful to Dr. Roger Johns for his gracious support.

Qing Lin
Shi Jin
Mei Han
Wenxin Zheng
Jiaming Liu
Xiaolong Wei

References

[1] O. Boucherat, G. Vitry, I. Trinh, R. Paulin, S. Provencher, and S. Bonnet, "The cancer theory of pulmonary arterial hypertension," *Pulmonary Circulation*, vol. 7, no. 2, pp. 285–299, 2017.

Hindawi
Journal of Immunology Research
Volume 2018, Article ID 4925498, 11 pages
https://doi.org/10.1155/2018/4925498

Research Article

A Novel Inflammation-Based Prognostic Score: The Fibrinogen/Albumin Ratio Predicts Prognoses of Patients after Curative Resection for Hepatocellular Carcinoma

Qiaodong Xu,[1,2,3] **Yongcong Yan**,[1,2] **Songgang Gu**,[3] **Kai Mao**,[1,2] **Jianlong Zhang**,[1,2] **Pinbo Huang**,[1,2] **Zhenyu Zhou**,[1,2] **Zheng Chen**,[1,2] **Shaodong Zheng**,[3] **Jiahong Liang**,[3] **Zhihua Lin**,[3] **Jie Wang**,[1,2] **Jiang Yan**,[3] **and Zhiyu Xiao**[1,2]

[1]*Guangdong Province Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Research Center of Medicine, Sun Yat-sen Memorial Hospital, No. 107 Yanjiang Western Road, Guangzhou 510120, China*
[2]*Department of Hepatobiliary Surgery, Sun Yat-sen Memorial Hospital, No. 33 Yingfeng Road, Guangzhou 510289, China*
[3]*Department of Biliary-Pancreatic Minimally Invasive Surgery, The First Affiliated Hospital of Shantou University Medical College, No. 57 Changping Road, Shantou 515041, China*

Correspondence should be addressed to Jie Wang; jiewsysu@163.com, Jiang Yan; styanjiang@sina.com, and Zhiyu Xiao; xzysurgeon@163.com

Received 24 August 2017; Revised 18 January 2018; Accepted 4 February 2018; Published 22 May 2018

Academic Editor: Jiaming Liu

Background. Inflammation is an important hallmark of cancer. Fibrinogen and albumin are both vital factors in systemic inflammation. This study investigated the prognostic value of the fibrinogen/albumin ratio in HCC patients who underwent curative resection. *Methods*. HCC patients ($n = 151$) who underwent curative resection were evaluated retrospectively. The optimal cutoff value for the fibrinogen/albumin ratio was selected by receiver operating characteristic (ROC) curve analysis. Correlations between preoperative fibrinogen/albumin ratios and clinicopathologic characteristics were analyzed by χ^2 test. The area under the receiver operating characteristic curve (AUC) was calculated to compare the prognostic value of the fibrinogen/albumin ratio with other prognostic scores (neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ratio (PLR), and albumin-bilirubin (ALBI) score). The overall survival (OS) and time to recurrence (TTR) were assessed by the log-rank test and the Cox proportional hazard regression model. *Results*. An optimal cutoff value of the preoperative fibrinogen/albumin ratio (0.062) was determined for 151 patients who underwent curative resection for HCC via a ROC curve analysis. Fibrinogen/albumin ratio > 0.062 was significantly associated with microvascular invasion, an advanced BCLC stage, and ALBI grade. Multivariate analyses revealed that fibrinogen/albumin ratio was an independent predictor for OS ($P = 0.003$) and TTR ($P = 0.035$). The prognostic ability of fibrinogen/albumin ratio was comparable to other prognostic scores (NLR, PLR, and ALBI score) by AUC analysis. Patients with a fibrinogen/albumin ratio > 0.062 had lower 1-, 3-, and 5-year OS rates (66.0%, 41.8%, and 28.2% versus 81.9%, 69.3%, and 56.1%, resp., $P < 0.001$) and higher 1-, 3-, and 5-year recurrence rates (60.9%, 79.2%, and 90.5% versus 49.5%, 69.1%, and 77.1%, resp., $P = 0.008$) compared with patients with fibrinogen/albumin ratio ≤ 0.062. *Conclusion*. The preoperative fibrinogen/albumin ratio is an effective prognostic factor for HCC patients who underwent curative resection. An elevated fibrinogen/albumin ratio significantly correlates with poorer survival and a higher risk of recurrence in HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths and the sixth most prevalent cancer worldwide [1]. Currently, surgery remains the main treatment for patients with HCC. Although surgical treatments have markedly improved the overall survival of HCC patients, the long-term survival rate is still unsatisfactory. Approximately 60% of patients experience recurrence or distant metastasis within 5 years, even after curative resection [2]. Some prognostic factors, including microvascular invasion, tumor-related factors, the Child–Pugh classification, and albumin-bilirubin (ALBI) score, have been reported as prognostic indicators in HCC patients who underwent hepatectomy [3–5]. However, effective prognostic factors are still absent, especially serum biomarkers.

Inflammation is an important hallmark of cancer [6]. Numerous clinical and experimental studies have convincingly supported the concept that inflammation is an important component of tumor progression [7, 8]. Recently, inflammation-based index and scoring systems, such as the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), C-reactive protein (CRP)/albumin (Alb) ratio, Glasgow Prognostic Score (GPS), and modified GPS, have been reported as useful prognostic indicators in HCC [9–13].

Fibrinogen (Fib) is a glycoprotein synthesized by hepatocytes. It is produced in response to proinflammatory cytokines. Similar to CRP, fibrinogen belongs to the positive acute-phase-response group of proteins, which are characterized by elevated levels during systemic inflammation [14]. After being converted to fibrin, it plays a significant role in the progression of blood coagulation. It has been reported that plasma fibrinogen levels are predictive of a poor prognosis in various cancers, including HCC [15–19]. Albumin is produced by the liver and is considered a negative acute-phase protein. The reduction of albumin levels during inflammation is likely associated with the effect of cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Serum albumin has been shown to have protective properties, such as maintaining physiological homeostasis, antioxidant activity, anti-inflammatory effects, and the prevention of apoptosis [20]. Several inflammation-based prognostic indexes (e.g., CRP/Alb ratio, GPS, and modified GPS), which have been reported to have prognostic value for HCC, take preoperative serum albumin levels into consideration.

Recently, it was reported that the Fib/Alb ratio correlated with patient prognosis in esophageal squamous cell carcinoma [21]. However, whether the Fib/Alb ratio is associated with the prognosis of HCC patients after curative resection has not been elucidated.

Herein, we performed a retrospective study to investigate whether the Fib/Alb ratio has prognostic value in patients undergoing curative resection for HCC.

2. Materials and Methods

2.1. Patients and Specimens. A retrospective study was conducted in a primary cohort that included a total of 181 patients who underwent curative resection of HCC (defined as the complete removal of the tumor without residual cancer) in the department of Hepatobiliary Surgery of Sun Yat-Sen Memorial Hospital between 2006 and 2010. Of these, 13 patients who showed clinical evidence of infection or other inflammatory conditions and hematological diseases were excluded, and 17 patients were excluded due to a lack of clinical data. There were no relevant drugs and interventions used that may have directly influenced peripheral hematological components. In total, 151 patients were finally enrolled and evaluated.

The diagnosis of each patient was pathologically confirmed. Patients did not have signs of distant metastases nor had they received anticancer therapies before surgery. The patients' characteristics, clinicopathological factors, and postoperative survival and recurrence rates were recorded. Tumor stages were determined according to the Barcelona Clinic Liver Cancer (BCLC) staging classification [22]. Tumor differentiation was graded based on the Edmondson grading system [23]. The study was approved by the Sun Yat-Sen Memorial Hospital Research Ethics Committee, and informed consent was obtained from all participants.

2.2. Fibrinogen/Albumin Ratio, NLR, PLR, and ALBI Score. In all patients included in the study, blood samples were collected and routine laboratory analyses of plasma fibrinogen and albumin levels were performed during routine workup to exclude coagulation disorders or the presence of acute infections before surgery or diagnostic interventions. The total bilirubin, white blood cell count, neutrophil, lymphocyte, platelet count, and α-fetoprotein level (AFP) were measured as well. The Fib/Alb ratio was calculated by dividing the plasma fibrinogen level by the albumin level. The NLR was calculated by dividing the neutrophil count by the lymphocyte count [10]. The PLR was calculated by dividing the platelet count by the lymphocyte count [10]. The ALBI score was calculated from the formula, ALBI score = $(\log_{10}\text{bilirubin} \times 0.66) + (\text{albumin} \times -0.085)$, where bilirubin is in μmol/L and albumin in g/L. Specific cutoffs were then applied to generate three prognostic groups: ALBI score ≤ -2.60 (ALBI grade 1), >-2.60 to ≤ -1.39 (ALBI grade 2), and ALBI score > -1.39 (ALBI grade 3) [24].

2.3. Treatment and Follow-Up. All patients were observed over a median observation time of 33.8 months (range, 1 to 86 months). Patients were monitored by examining serum alpha-fetoprotein (AFP) levels and performing an abdominal ultrasound every 2 months during the first year after the operation and every 3 to 4 months thereafter. Magnetic resonance imaging or computed tomography, together with chest radiographic examination, was performed every three to six months in the first two years and every six to twelve months thereafter. Upon suspicion of recurrence or metastasis, chest computed tomography, magnetic resonance imaging, and bone scintigraphy were performed for confirmation. Overall survival (OS) and time to recurrence (TTR) were considered as the primary endpoints. OS was defined as the interval between surgery and death or between surgery and the last follow-up time for surviving patients.

Table 1: Relationship between preoperative Fib/Alb ratio and clinicopathological characteristics.

Clinical and pathologic indexes	Cases $n = 151$	Fib/Alb ratio >0.062 ($n = 101$)	Fib/Alb ratio ≤0.062 ($n = 50$)	P value
Gender				
Male	128	89	39	0.147
Female	23	12	11	
Age (years)				
>50	76	49	27	0.605
≤50	75	52	23	
HBs antigen				
(+)	132	88	44	1.00
(−)	19	13	6	
Cirrhosis				
Yes	121	82	39	0.668
No	30	19	11	
AFP (μg/L)				
>20	104	69	35	1.00
≤20	47	32	15	
Tumor size (cm)				
>5	89	65	24	0.078
≤5	62	36	26	
Tumor encapsulation				
Complete	69	46	23	1.00
None	82	55	27	
Microvascular invasion				
Yes	59	46	13	0.022*
No	92	55	37	
Tumor differentiation				
I-II	105	67	38	0.263
III-IV	46	34	12	
BCLC stage				
0 + A	32	16	16	0.033*
B + C	119	85	34	
ALBI grade				
1	89	51	38	0.003*
2 + 3	62	50	12	

*$P < 0.05$.

TTR was defined as the interval between surgery and recurrence or between surgery and the last follow-up time for patients without recurrence.

2.4. Statistical Analysis. Statistical analyses were performed using SPSS for Windows (version 19.0). A receiver operating characteristic (ROC) curve analysis was performed to select the optimal cutoff value for the Fib/Alb ratio. The χ^2 test was used to compare categorical variables. Cumulative survival and recurrence rates were calculated using a Kaplan–Meier analysis and log-rank test. A Cox proportional hazard regression model was used to assess prognostic factors. Factors with a $P < 0.05$ in the univariate analysis were included in the multivariate analysis. The final multivariate analysis was performed using a forward stepwise procedure for variable selection. $P < 0.05$ was considered statistically significant.

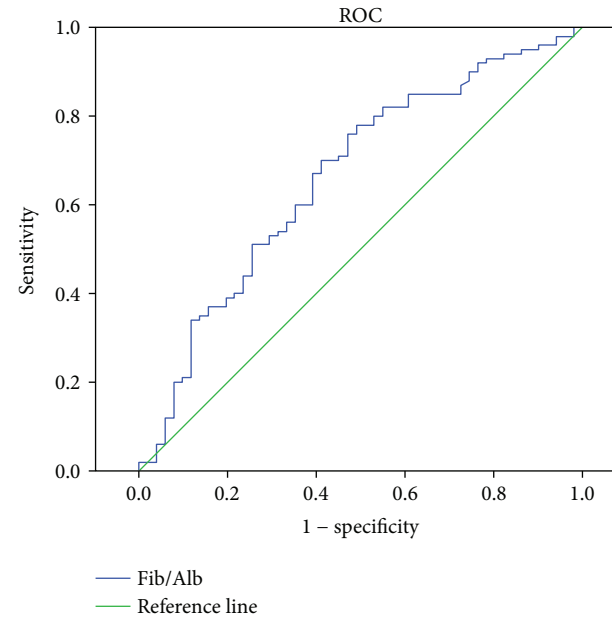


Figure 1: Determination of the cutoff value for the Fib/Alb ratio in patients undergoing curative resection for HCC by ROC analysis.

3. Results

3.1. Patient Characteristics. The median age of the patients was 51 (range 22–78) years, 128 (84.8%) were males and 23 (15.2%) were females, and 132 (87.4%) were positive for hepatitis B surface antigen (HBs-Ag) and 19 (12.6%) were negative for HBs-Ag. The detailed clinical characteristics of the 151 patients are summarized in Table 1.

3.2. Determination of Optimal Cutoff Value of Fib/Alb Ratio. The cutoff value of the preoperative Fib/Alb ratio was determined using a ROC curve (Figure 1). The Youden index was calculated as (sensitivity + specificity) − 1 for each cutoff point. The highest Youden index for Fib/Alb ratio was 0.279 at 0.062 (sensitivity = 75%, specificity = 52.9%). As a result, 0.062 was selected as the cutoff of fibrinogen/albumin ratio (the area under ROC curve: 0.663, 95% CI: 0.570–0.756, $P = 0.001$).

3.3. Relationship between Preoperative Fib/Alb Ratios and the Clinicopathological Characteristics of HCC. According to the Fib/Alb ratio cutoff value determined from the ROC curve, the 151 patients were divided into two groups (Fib/Alb ratio > 0.062, $n = 101$, and Fib/Alb ratio ≤ 0.062, $n = 50$). The relationship between preoperative Fib/Alb ratios and the clinicopathologic variables of patients with HCC was investigated, and the data showed that the preoperative Fib/Alb ratio was associated with microvascular

invasion ($P = 0.022$), BCLC stage ($P = 0.033$), and ALBI grade ($P = 0.003$). ALBI grade is a novel score with powerful prognostic value of HCC based on liver function [24]. For the reason that only two patients in our study fell into ALBI grade 3, patients that fell into ALBI grade 2 and ALBI grade 3 were grouped together. No significant relationship was found between the Fib/Alb ratio and other clinicopathologic features (Table 1).

3.4. Independent Prognostic Factors of OS and TTR for HCC Patients Receiving Curative Resection. To further identify predictors of postoperative OS and TTR, Fib/Alb ratios, NLR, PLR, ALBI grade, and other clinicopathologic parameters were evaluated via a Cox proportional hazard regression analysis. The selected cutoff value was 2.81 for NLR [9] and 115 for PLR [25]. In the univariate analysis, liver cirrhosis ($P = 0.025$), tumor encapsulation ($P = 0.041$), microvascular invasion ($P < 0.001$), tumor differentiation ($P < 0.001$), BCLC stage ($P = 0.006$), preoperative Fib/Alb ratio ($P = 0.001$), ALBI grade ($P = 0.006$), and NLR ($P = 0.037$) were responsible for the OS. A multivariate regression analysis was performed on all 8 factors that were shown to make a statistically significant difference in OS in the univariate analysis. The results show that liver cirrhosis, microvascular invasion, tumor differentiation, and the Fib/Alb ratio were the independent prognostic predictors of OS (Table 2).

Similarly, the univariate analysis showed that AFP ($P = 0.044$), tumor size ($P = 0.002$), tumor encapsulation ($P = 0.019$), microvascular invasion ($P < 0.001$), tumor differentiation ($P < 0.001$), BCLC stage ($P = 0.003$), preoperative Fib/Alb ratio ($P = 0.009$), NLR ($P = 0.005$), and PLR ($P = 0.043$) were associated with TTR. In a multivariate analysis, microvascular invasion, tumor differentiation, and the Fib/Alb ratio were independent risk factors for TTR (Table 3).

3.5. Comparison of the Areas under the Curves for Fib/Alb Ratio, NLR, PLR, ALBI Grade, and Other Clinical Indexes. The discrimination ability of the Fib/Alb ratio, two inflammation-based prognostic scores (NLR, PLR), a novel prognostic score of HCC based on liver function (ALBI grade), and other clinical indexes was compared by the AUC (Figure 2). The AUC for the Fib/Alb ratio (dichotomized) was 0.635 (95% CI, 0.539–0.731), which was higher than that for other indexes (cirrhosis, AFP, tumor size, tumor encapsulation, microvascular invasion, tumor differentiation, BCLC stage, NLR, PLR, ALBI grade, and tumor number) for predicting overall survival in HCC patients after curative resection (Table 4).

3.6. Association of Fib/Alb Ratios with OS and Recurrence Rates. To evaluate the prognostic ability of Fib/Alb ratios in predicting OS and recurrence, the 151 HCC patients were divided into two groups: Fib/Alb ratio > 0.062 ($n = 101$) and Fib/Alb ratio ≤ 0.062 ($n = 50$). Cumulative survival and recurrence rates were calculated using a Kaplan–Meier analysis. In terms of OS, patients with a Fib/Alb ratio > 0.062 had lower 1-, 3-, and 5-year OS rates (66.0%, 41.8%, and 28.2% versus 81.9%, 69.3%, and 56.1%, resp., $P < 0.001$) and a shorter OS (median, 26 months versus 69.9 months) compared with patients with a Fib/Alb ratio ≤ 0.062. (Figure 3(a)). In terms of recurrence, patients with a Fib/Alb ratio > 0.062 had higher 1-, 3-, and 5-year recurrence rates (60.9%, 79.2%, and 90.5% versus 49.5%, 69.1%, and 77.1%, resp., $P = 0.0081$) and a shorter TTR (median, 5.9 months versus 14.4 months) compared with patients with a Fib/Alb ratio ≤ 0.062 (Figure 3(b)). Therefore, the result revealed that elevated preoperative Fib/Alb ratios were associated with poor prognoses in HCC patients after curative resection.

3.7. Prognostic Significance of the Fib/Alb Ratio in HCC Patients without Microvascular Invasion. We further investigated the prognostic significance of the Fib/Alb ratio in the subgroup of HCC patients without microvascular invasion and found that it was significantly correlated with OS ($P = 0.0069$) and TTR ($P = 0.0359$) (Figures 4(a) and 4(b)). The results reveal that the Fib/Alb ratio was a prognostic factor for OS and TTR in patients without microvascular invasion.

4. Discussion

In the 19th century, the observation of leukocytes within tumors by Rudolf Virchow provided the first indication of a possible link between inflammation and cancer. Currently, it is widely accepted that cross talk exists between the inflammatory response and cancer development. Inflammation impacts every step of tumorigenesis, from initiation through tumor promotion and all the way to metastatic progression [7]. In addition, the secretion of growth factors and chemokines by tumors modulates the inflammatory environment and causes a systemic inflammatory response [6, 26]. Numerous data has revealed that the outcome of cancer is influenced not only by tumor-related factors but also by host-related factors, particularly the systemic inflammatory response, which is usually reflected by a variety of biochemical or hematological markers. As a result, several inflammation-based scores, such as NLR, PLR, GPS, and mGPS scoring systems, have been reported to have prognostic value in regard to HCC [9, 12, 13, 25].

Fibrinogen is not only an acute-phase-response protein that reflects systemic inflammatory response but also a vital factor that participates in the maintenance of hemostasis. Both systemic inflammatory response and hemostatic system closely connect with cancer development [7, 27]. Based on its ability to directly bind to members of the vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) families, fibrinogen has been reported to play a critical role in cell proliferation, the epithelial-to-mesenchymal transition (EMT), angiogenesis, and the hematogenous metastasis of tumor cells [28–30]. In addition, some clinical studies have shown that high pretreatment plasma fibrinogen levels are related to poorer prognoses in a variety of tumors [16, 31–33]. Perisanidis et al. proposed that adjunct treatments lowering plasma fibrinogen concentration may hold promise for prolonging survival in patients with solid tumors [34]. What is more, it is suggested that therapies targeting fibrinogen-dependent interactions may

Table 2: Univariate and multivariate Cox regression analyses of prognostic factors for overall survival (OS).

Variables	Univariate analyses		Multivariate analyses	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender				
Male	0.779 (0.442–1.374)	0.389		
Female				
Age (years)				
>50	0.864 (0.583–1.279)	0.465		
≤50				
HBs antigen				
(+)	0.913 (0.519–1.607)	0.753		
(−)				
Cirrhosis				
Yes	1.876 (1.082–3.250)	0.025*	1.994 (1.145–3.475)	0.015*
No				
AFP (μg/L)				
>20	1.458 (0.942–2.258)	0.091		
≤20				
Tumor size (cm)				
>5	1.444 (0.961–2.169)	0.077		
≤5				
Tumor encapsulation				
Complete	1.509 (1.018–2.237)	0.041*		
None				
Microvascular invasion				
Yes	2.236 (1.503–3.326)	<0.001*	1.552 (1.009–2.389)	0.046*
No				
Tumor differentiation				
I-II	2.539 (1.676–3.846)	<0.001*	2.189 (1.394–3.437)	0.001*
III-IV				
BCLC stage				
0 + A	2.181 (1.256–3.789)	0.006*		
B + C				
Fib/Alb ratio				
>0.062	2.146 (1.353–3.404)	0.001*	2.015 (1.266–3.207)	0.003*
≤0.062				
ALBI grade				
1	1.751 (1.178–2.604)	0.006*		
2 + 3				
NLR				
≥2.81	1.546 (1.027–2.327)	0.037*		
<2.81				
PLR				
≥115	1.221 (0.817–1.823)	0.330		
<115				

HR: hazard ratio; 95% CI: 95% confidence interval; BCLC: Barcelona Clinic Liver Cancer. Cox regression analysis, *$P < 0.05$.

make a positive contribution to the treatment of some kinds of malignancies [35, 36].

Albumin is the most abundant plasma protein accounting for about 50% of the total protein content [37]. The low concentration of albumin may reflect bad nutritional status and performance status. Malnutrition may weaken the immune system and negatively impact the prognosis of cancer patients [38]. Furthermore, albumin is also an important

Table 3: Univariate and multivariate Cox regression analyses of prognostic factors for time to recurrence (TTR).

Variables	Univariate analyses		Multivariate analyses	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender				
Male	1.319 (0.806–2.160)	0.271		
Female				
Age (years)				
>50	0.705 (0.49–1.014)	0.06		
≤50				
HBs antigen				
(+)	0.728 (0.440–1.203)	0.215		
(−)				
Cirrhosis				
Yes	1.160 (0.740–1.819)	0.517		
No				
AFP (μg/L)				
>20	1.510 (1.012–2.254)	0.044*		
≤20				
Tumor size (cm)				
>5	1.822 (1.245–2.666)	0.002*		
≤5				
Tumor encapsulation				
Complete	1.556 (1.076–2.248)	0.019*		
None				
Microvascular invasion				
Yes	2.104 (1.442–3.072)	<0.001*	1.584 (1.037–2.421)	0.033*
No				
Tumor differentiation				
I-II	2.326 (1.529–3.539)	<0.001*	1.718 (1.081–2.730)	0.022*
III-IV				
BCLC stage				
0 + A	2.034 (1.269–3.261)	0.003*		
B + C				
Fib/Alb ratio				
>0.062	1.704 (1.142–2.543)	0.009*	1.555 (1.031–2.346)	0.035*
≤0.062				
ALBI grade				
1	1.131 (0.783–1.632)	0.512		
2 + 3				
NLR				
≥2.81	1.723 (1.177–2.523)	0.005*		
<2.81				
PLR				
≥115	1.466 (1.013–2.121)	0.043*		
<115				

HR: hazard ratio; 95% CI: 95% confidence interval; BCLC: Barcelona Clinic Liver Cancer. Cox regression analysis, *$P < 0.05$.

factor that participates in the systemic inflammatory response. The prognostic value of pretreatment albumin has been reported in various human malignancies, including renal cell carcinoma [39], head and neck cancers [40], non-small cell lung cancer [41], ovarian cancer [42], and adenocarcinoma of the gastric cardia [43]. In addition, serum albumin is one of the components of the Child–Pugh classification system that reflects liver function. It has also been

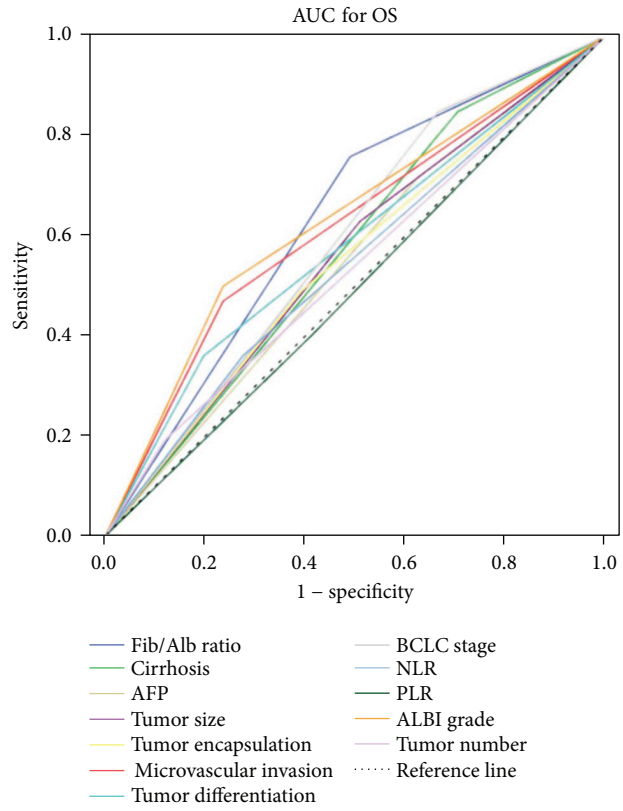


Figure 2: Comparison of the areas under the curves for the Fib/Alb ratio, NLR, PLR, ALBI grade, and other clinical indexes. The discrimination ability of the Fib/Alb ratio, NLR, PLR, ALBI grade, and other clinical indexes was compared by the AUC for OS.

Table 4: Comparison of the areas under the curves for Fib/Alb ratio, NLR, PLR, ALBI grade, and other clinical indexes.

Variables	AUC	95% CI	*P* value
Fib/Alb ratio (dichotomized)	0.635	0.539–0.731	0.007*
Cirrhosis (dichotomized)	0.572	0.473–0.671	0.148
AFP (dichotomized)	0.546	0.448–0.645	0.353
Tumor size (dichotomized)	0.56	0.463–0.658	0.228
Tumor encapsulation (dichotomized)	0.549	0.452–0.646	0.326
Microvascular invasion (dichotomized)	0.617	0.525–0.710	0.019*
Tumor differentiation (dichotomized)	0.582	0.488–0.676	0.1
BCLC stage (dichotomized)	0.592	0.493–0.691	0.066
NLR (dichotomized)	0.543	0.446–0.639	0.391
PLR (dichotomized)	0.494	0.396–0.592	0.906
ALBI grade (dichotomized)	0.632	0.540–0.724	0.008*
Tumor number (dichotomized)	0.536	0.440–0.632	0.468

*$P < 0.05$.

reported that hypoalbuminemia is an independent prognostic factor associated with poor outcomes in patients with HCC [44].

It was reported that the fibrinogen/albumin ratio was a novel blood tool of cancer prognosis in esophageal squamous cell carcinoma recently [21]. What is more, Kinoshita et al. found the prognostic value of C-reactive protein (CRP)/albumin ratio in patients with HCC [11]. Similar to CRP, fibrinogen belongs to the positive acute-phase-response proteins, characterized by its elevation during systemic inflammation [45]. It has been reported that there was a strong positive correlation between fibrinogen and CRP levels in thoracic malignancies [14, 46]. Inspired by these, we try to investigate whether fibrinogen/albumin ratio has a prognostic value in HCC patients after curative resection.

We report here for the first time, to the best of our knowledge, that the Fib/Alb ratio is an independent prognostic factor for HCC following curative hepatectomy. Hepatitis B or C infections and alcohol consumption are major risk factors of HCC. Patients with HCC are typically characterized by impaired liver function due to concomitant intrahepatic chronic inflammation and liver cirrhosis. Since fibrinogen, albumin, and CRP are synthesized by hepatocytes, impaired liver function will influence the accuracy of making assessments based on fibrinogen or albumin alone and of the GPS and mGPS scoring systems, which score serum CRP and albumin levels separately, in predicting a prognosis for patients with HCC. In contrast, the Fib/Alb ratio reflects the ratio of the fibrinogen and albumin levels, thus reducing the influence of poor liver function. It may reflect the association between cancer progression and the host's inflammatory environments more effectively in patients with HCC. We also compare the Fib/Alb ratio with a novel prognostic score of HCC based on liver function (ALBI grade). The Fib/Alb ratio was associated with the ALBI grade ($P = 0.003$, Table 1). The AUC for OS of the Fib/Alb ratio was 0.635, which was comparable to the ALBI grade (0.632) (Figure 2, Table 4). Meanwhile, Fib/Alb ratio measurements are based on standard laboratory measurements of fibrinogen and albumin, which are routinely performed in clinical practice. Hence, the Fib/Alb ratio is a promising and convenient biomarker for predicting HCC prognoses.

In our study, we first determined that the optimal cutoff value of the Fib/Alb ratio was 0.062 using a ROC curve analysis. Then, the relationship between preoperative Fib/Alb ratios and the clinicopathological variables of HCC patients was investigated, and the data showed that elevated Fib/Alb ratio was positively associated with microvascular invasion, BCLC stage, and ALBI grade. Furthermore, a multivariate Cox regression analysis revealed that microvascular invasion, tumor differentiation, and the Fib/Alb ratio were independent prognostic predictors of OS and TTR. Moreover, the ROC analysis demonstrated that the Fib/Alb ratio (dichotomized) had a higher AUC value than NLR, PLR, ALBI grade, and other clinical indexes for predicting OS. It suggested that the Fib/Alb ratio was comparable to that of other established prognostic scores (NLR, PLR, and ALBI grade) in terms of its prognostic ability. The prognostic value of the Fib/Alb ratio was further analyzed, and we found that patients with a Fib/Alb > 0.062 had lower 1-, 3-, and 5-year OS rates and shorter OS times, as well as higher 1-, 3-, and 5-year recurrence rates and shorter TTRs compared with patients with

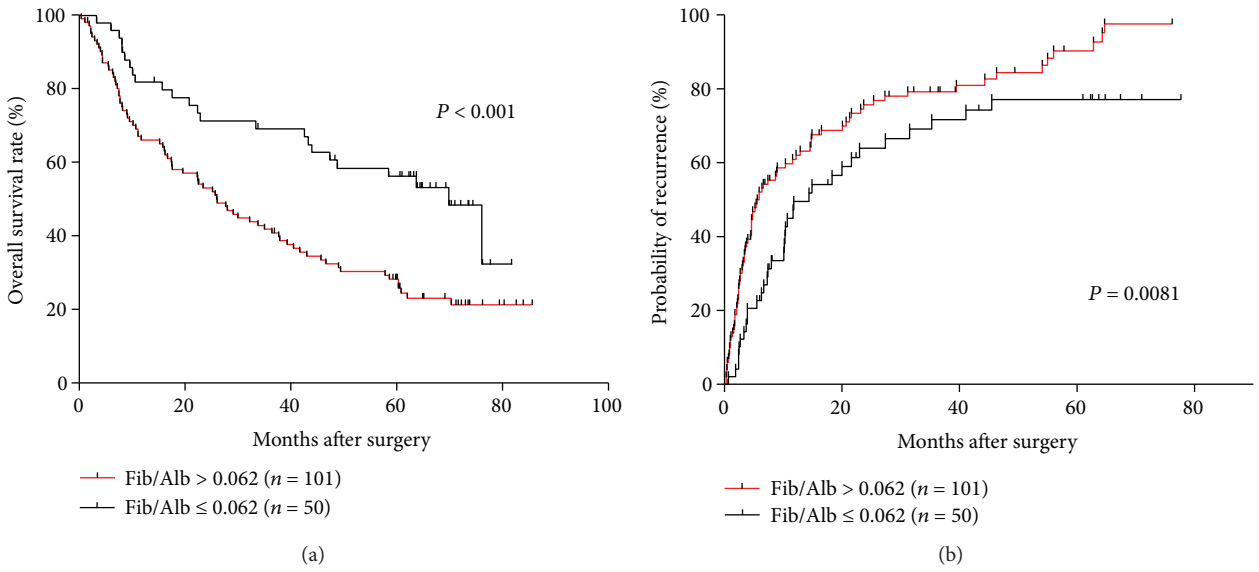


Figure 3: Kaplan–Meier curves of overall survival and recurrence are shown for HCC patients. (a) OS of patients with a Fib/Alb ratio > 0.062 was significantly shorter than that of those with a Fib/Alb ratio ≤ 0.062 ($P < 0.001$, log-rank test). (b) Recurrence rate of patients with a Fib/Alb ratio > 0.062 was significantly higher than that of those with a Fib/Alb ratio ≤ 0.062 ($P = 0.0081$, log-rank test).

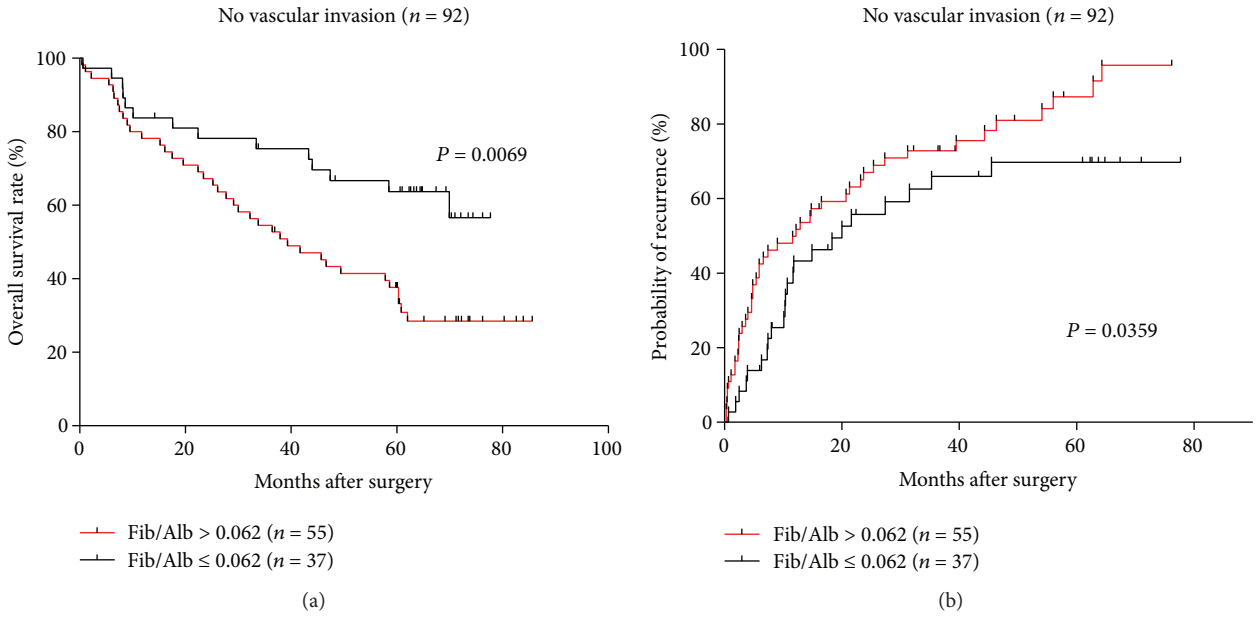


Figure 4: Kaplan–Meier survival curves of HCC patients without microvascular invasion. Fib/Alb ratio > 0.062 was significantly correlated with a shorter OS (a) and higher recurrence rate (b).

a Fib/Alb ≤ 0.062. Moreover, the Fib/Alb ratio had significant prognostic value for both OS and TTR in patients without microvascular invasion.

Predicting recurrence following curative resection is critical for the management of HCC. Up to now, there are still no highly reliable and convenient predictive biomarkers for the recurrence of HCC. Although AFP is widely used, its predictive effectiveness is poor as 30% to 40% of patients with HCC have normal serum AFP levels after surgery [47]. Encouragingly, our study provides the Fib/Alb ratio as an alternative option for predicting recurrence. Furthermore, microvascular invasion was recognized as an independent predictor of early recurrence in HCC patients who underwent curative surgery [48]. In clinical practice, it is difficult to predict which individuals will experience tumor relapse after surgical treatment in the absence of microvascular invasion. Finding a predictor to discriminate at-risk patients from this subpopulation is therefore important. In the current study, after stratifying the patient cohort according to the presence and absence of microvascular invasion, we found that the prognostic significance of the Fib/Alb ratio was still strong in HCC patients without microvascular invasion. Consequently, patients with higher Fib/Alb ratios in the subgroup of HCC patients without microvascular invasion

may require a closer follow-up since they are more likely to suffer from tumor recurrence. If recurrence is predicted early and prevented in a timely manner, these patients will achieve a favorable outcome.

There are several limitations to the current study. First, it was a retrospective, single-center study with a small sample size, and a well-designed, prospective study with a larger number of patients is needed. Second, owing to the relatively small number of patients, we did not divide the patients into a training cohort and a test cohort for statistical validation. Third, many other factors affect Fib/Alb ratios, such as acute undetected infections and hematological diseases, which affect the accuracy of prognostic predictions based on this ratio. Fourth, the different postoperative treatments that patients received were not included into the prognostic factor evaluation. Furthermore, many patients received multiple treatment measures due to tumor recurrence during their follow-up periods, which affects OS. Fifth, as 87.4% of the patients in our study were hepatitis B virus positive, the prognostic significance of the Fib/Alb ratio needs to be validated in HCC patients from the United States and Europe since hepatitis C is the most common risk factor for developing HCC in these geographic areas.

In conclusion, we demonstrated for the first time that the preoperative Fib/Alb ratio is a useful prognostic factor for assessing HCC patients following curative resection, particularly for patients without microvascular invasion. Its utility, convenience, and low cost make the Fib/Alb ratio a promising serum biomarker for predicting HCC prognoses and may support the therapeutic decisionmaking process for HCC patients in the future.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Qiaodong Xu, Yongcong Yan, Songgang Gu, and Kai Mao contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 81572407); the Natural Science Foundation of Guangdong Province, China (no. 2015A030313096); Grant [2013]163 from the Key Laboratory of Malignant Tumor Molecular Mechanism and Translational Medicine of Guangzhou Bureau of Science and Information Technology; and Grant KLB09001 from the Key Laboratory of Malignant Tumor Gene Regulation and Target Therapy of Guangdong Higher Education Institutes.

Supplementary Materials

Supplementary Figure 1: the ROC analysis of NLR (continuous), PLR (continuous), and ALBI grade (dichotomized) in 151 HCC patients who underwent curative resection. *(Supplementary Materials)*

References

[1] L. A. Torre, F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent, and A. Jemal, "Global cancer statistics, 2012," *CA: a Cancer Journal for Clinicians*, vol. 65, no. 2, pp. 87–108, 2015.

[2] J. Bruix, G. J. Gores, and V. Mazzaferro, "Hepatocellular carcinoma: clinical frontiers and perspectives," *Gut*, vol. 63, no. 5, pp. 844–855, 2014.

[3] S. Roayaie, I. N. Blume, S. N. Thung et al., "A system of classifying microvascular invasion to predict outcome after resection in patients with hepatocellular carcinoma," *Gastroenterology*, vol. 137, no. 3, pp. 850–855, 2009.

[4] A. Cucchetti, M. Vivarelli, F. Piscaglia et al., "Tumor doubling time predicts recurrence after surgery and describes the histological pattern of hepatocellular carcinoma on cirrhosis," *Journal of Hepatology*, vol. 43, no. 2, pp. 310–316, 2005.

[5] Y. Y. Wang, J. H. Zhong, Z. Y. Su et al., "Albumin–bilirubin versus Child–Pugh score as a predictor of outcome after liver resection for hepatocellular carcinoma," *British Journal of Surgery*, vol. 103, no. 6, pp. 725–734, 2016.

[6] A. Mantovani, "Cancer: inflaming metastasis," *Nature*, vol. 457, no. 7225, pp. 36-37, 2009.

[7] S. I. Grivennikov, F. R. Greten, and M. Karin, "Immunity, inflammation, and cancer," *Cell*, vol. 140, no. 6, pp. 883–899, 2010.

[8] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, no. 6917, pp. 860–867, 2002.

[9] Y. Mano, K. Shirabe, Y. Yamashita et al., "Preoperative neutrophil-to-lymphocyte ratio is a predictor of survival after hepatectomy for hepatocellular carcinoma: a retrospective analysis," *Annals of Surgery*, vol. 258, no. 2, pp. 301–305, 2013.

[10] A. Kinoshita, H. Onoda, N. Imai et al., "Comparison of the prognostic value of inflammation-based prognostic scores in patients with hepatocellular carcinoma," *British Journal of Cancer*, vol. 107, no. 6, pp. 988–993, 2012.

[11] A. Kinoshita, H. Onoda, N. Imai et al., "The C-reactive protein/albumin ratio, a novel inflammation-based prognostic score, predicts outcomes in patients with hepatocellular carcinoma," *Annals of Surgical Oncology*, vol. 22, no. 3, pp. 803–810, 2015.

[12] A. Kinoshita, H. Onoda, N. Imai et al., "The Glasgow Prognostic Score, an inflammation based prognostic score, predicts survival in patients with hepatocellular carcinoma," *BMC Cancer*, vol. 13, no. 1, p. 52, 2013.

[13] J. Huang, L. Xu, Y. Luo, F. He, Y. Zhang, and M. Chen, "The inflammation-based scores to predict prognosis of patients with hepatocellular carcinoma after hepatectomy," *Medical Oncology*, vol. 31, no. 4, p. 883, 2014.

[14] B. Ghanim, M. A. Hoda, T. Klikovits et al., "Circulating fibrinogen is a prognostic and predictive biomarker in malignant pleural mesothelioma," *British Journal of Cancer*, vol. 110, no. 4, pp. 984–990, 2014.

[15] L. Tang, K. Liu, J. Wang, C. Wang, P. Zhao, and J. Liu, "High preoperative plasma fibrinogen levels are associated with distant metastases and impaired prognosis after curative resection in patients with colorectal cancer," *Journal of Surgical Oncology*, vol. 102, no. 5, pp. 428–432, 2010.

[16] H. J. Son, J. W. Park, H. J. Chang et al., "Preoperative plasma hyperfibrinogenemia is predictive of poor prognosis in patients with nonmetastatic colon cancer," *Annals of Surgical Oncology*, vol. 20, no. 9, pp. 2908–2913, 2013.

[17] S. Polterauer, C. Grimm, V. Seebacher et al., "Plasma fibrinogen levels and prognosis in patients with ovarian cancer: a multicenter study," *The Oncologist*, vol. 14, no. 10, pp. 979–985, 2009.

[18] V. Seebacher, S. Polterauer, C. Grimm et al., "The prognostic value of plasma fibrinogen levels in patients with endometrial cancer: a multi-centre trial," *British Journal of Cancer*, vol. 102, no. 6, pp. 952–956, 2010.

[19] W. Gan, Y. Yi, Y. Fu et al., "Fibrinogen and C-reactive protein score is a prognostic index for patients with hepatocellular carcinoma undergoing curative resection: a prognostic nomogram study," *Journal of Cancer*, vol. 9, no. 1, pp. 148–156, 2018.

[20] M. H. Seo, M. Choa, J. S. You et al., "Hypoalbuminemia, low base excess values, and tachypnea predict 28-day mortality in severe sepsis and septic shock patients in the emergency department," *Yonsei Medical Journal*, vol. 57, no. 6, pp. 1361–1369, 2016.

[21] Z. Tan, M. Zhang, Q. Han et al., "A novel blood tool of cancer prognosis in esophageal squamous cell carcinoma: the fibrinogen/albumin ratio," *Journal of Cancer*, vol. 8, no. 6, pp. 1025–1029, 2017.

[22] J. Bruix and J. M. Llovet, "Prognostic prediction and treatment strategy in hepatocellular carcinoma," *Hepatology*, vol. 35, no. 3, pp. 519–524, 2002.

[23] H. A. Edmondson and P. E. Steiner, "Primary carcinoma of the liver. A study of 100 cases among 48,900 necropsies," *Cancer*, vol. 7, no. 3, pp. 462–503, 1954.

[24] P. J. Johnson, S. Berhane, C. Kagebayashi et al., "Assessment of liver function in patients with hepatocellular carcinoma: a new evidence-based approach—the ALBI grade," *Journal of Clinical Oncology*, vol. 33, no. 6, pp. 550–558, 2015.

[25] W. Song, K. Wang, F. P. Zhong, Y. W. Fan, L. Peng, and S. B. Zou, "Clinicopathological and prognostic significance of platelet-to-lymphocyte ratio in patients with hepatocellular carcinoma," *Oncotarget*, vol. 7, no. 49, pp. 81830–81838, 2016.

[26] E. Elinav, R. Nowarski, C. A. Thaiss, B. Hu, C. Jin, and R. A. Flavell, "Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms," *Nature Reviews Cancer*, vol. 13, no. 11, pp. 759–771, 2013.

[27] O. Konigsbrugge, I. Pabinger, and C. Ay, "Risk factors for venous thromboembolism in cancer: novel findings from the Vienna Cancer and Thrombosis Study (CATS)," *Thrombosis Research*, vol. 133, Supplement 2, pp. S39–S43, 2014.

[28] C. A. Staton, N. J. Brown, and C. E. Lewis, "The role of fibrinogen and related fragments in tumour angiogenesis and metastasis," *Expert Opinion on Biological Therapy*, vol. 3, no. 7, pp. 1105–1120, 2003.

[29] M. M. Martino, P. S. Briquez, A. Ranga, M. P. Lutolf, and J. A. Hubbell, "Heparin-binding domain of fibrin(ogen) binds growth factors and promotes tissue repair when incorporated within a synthetic matrix," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 12, pp. 4563–4568, 2013.

[30] E. Witsch, M. Sela, and Y. Yarden, "Roles for growth factors in cancer progression," *Physiology*, vol. 25, no. 2, pp. 85–101, 2010.

[31] Q. Qi, Y. Geng, M. Sun, H. Chen, P. Wang, and Z. Chen, "Hyperfibrinogen is associated with the systemic inflammatory response and predicts poor prognosis in advanced pancreatic cancer," *Pancreas*, vol. 44, no. 6, pp. 977–982, 2015.

[32] L. Q. Tang, Q. Y. Chen, S. S. Guo et al., "The impact of plasma Epstein–Barr virus DNA and fibrinogen on nasopharyngeal carcinoma prognosis: an observational study," *British Journal of Cancer*, vol. 111, no. 6, pp. 1102–1111, 2014.

[33] L. Sheng, M. Luo, X. Sun, N. Lin, W. Mao, and D. Su, "Serum fibrinogen is an independent prognostic factor in operable nonsmall cell lung cancer," *International Journal of Cancer*, vol. 133, pp. 2720–2725, 2013.

[34] C. Perisanidis, A. Psyrri, E. E. Cohen et al., "Prognostic role of pretreatment plasma fibrinogen in patients with solid tumors: a systematic review and meta-analysis," *Cancer Treatment Reviews*, vol. 41, no. 10, pp. 960–970, 2015.

[35] J. S. Desgrosellier and D. A. Cheresh, "Integrins in cancer: biological implications and therapeutic opportunities," *Nature Reviews Cancer*, vol. 10, no. 1, pp. 9–22, 2010.

[36] K. A. Steinbrecher, N. A. Horowitz, E. A. Blevins et al., "Colitis-associated cancer is dependent on the interplay between the hemostatic and inflammatory systems and supported by integrin $\alpha_M\beta_2$ engagement of fibrinogen," *Cancer Research*, vol. 70, no. 7, pp. 2634–2643, 2010.

[37] H. J. Xu, Y. Ma, F. Deng, W. B. Ju, X. Y. Sun, and H. Wang, "The prognostic value of C-reactive protein/albumin ratio in human malignancies: an updated meta-analysis," *OncoTargets and Therapy*, vol. 10, pp. 3059–3070, 2017.

[38] J. A. Virizuela, M. Camblor-Alvarez, L. M. Luengo-Perez et al., "Nutritional support and parenteral nutrition in cancer patients: an expert consensus report," *Clinical and Translational Oncology*, vol. 20, no. 5, pp. 619–629, 2017.

[39] Z. Chen, Y. Shao, K. Wang et al., "Prognostic role of pretreatment serum albumin in renal cell carcinoma: a systematic review and meta-analysis," *OncoTargets and Therapy*, vol. 9, pp. 6701–6710, 2016.

[40] D. Danan, D. C. Shonka Jr, Y. Selman, Z. Chow, M. E. Smolkin, and M. J. Jameson, "Prognostic value of albumin in patients with head and neck cancer," *The Laryngoscope*, vol. 126, no. 7, pp. 1567–1571, 2016.

[41] E. Espinosa, J. Feliu, P. Zamora et al., "Serum albumin and other prognostic factors related to response and survival in patients with advanced non-small cell lung cancer," *Lung Cancer*, vol. 12, no. 1-2, pp. 67–76, 1995.

[42] V. Asher, J. Lee, and A. Bali, "Preoperative serum albumin is an independent prognostic predictor of survival in ovarian cancer," *Medical Oncology*, vol. 29, no. 3, pp. 2005–2009, 2012.

[43] Y. C. Lien, C. C. Hsieh, Y. C. Wu et al., "Preoperative serum albumin level is a prognostic indicator for adenocarcinoma of the gastric cardia," *Journal of Gastrointestinal Surgery*, vol. 8, no. 8, pp. 1041–1048, 2004.

[44] Y. K. Cho, J. W. Chung, J. K. Kim et al., "Comparison of 7 staging systems for patients with hepatocellular carcinoma undergoing transarterial chemoembolization," *Cancer*, vol. 112, no. 2, pp. 352–361, 2008.

[45] A. Duvoix, J. Dickens, I. Haq et al., "Blood fibrinogen as a biomarker of chronic obstructive pulmonary disease," *Thorax*, vol. 68, no. 7, pp. 670–676, 2013.

[46] S. T. Gulen, F. Karadag, A. B. Karul et al., "Adipokines and systemic inflammation in weight-losing lung cancer patients," *Lung*, vol. 190, no. 3, pp. 327–332, 2012.

[47] B. Hu, X. R. Yang, Y. Xu et al., "Systemic immune-inflammation index predicts prognosis of patients after curative resection for hepatocellular carcinoma," *Clinical Cancer Research*, vol. 20, no. 23, pp. 6212–6222, 2014.

[48] J. Bruix, M. Reig, and M. Sherman, "Evidence-based diagnosis, staging, and treatment of patients with hepatocellular carcinoma," *Gastroenterology*, vol. 150, no. 4, pp. 835–853, 2016.

Hindawi
Journal of Immunology Research
Volume 2018, Article ID 8740976, 8 pages
https://doi.org/10.1155/2018/8740976

Review Article

Immunotherapy for Hepatocellular Carcinoma: Current Advances and Future Expectations

Yingjun Xie,[1,2] **Yien Xiang**,[1] **Jiyao Sheng**,[1] **Dan Zhang**,[1,2] **Xiaoxiao Yao**,[1] **Yongsheng Yang**,[1,2] **and Xuewen Zhang**[1,2]

[1]*Department of Hepatobiliary and Pancreatic Surgery, Second Hospital of Jilin University, Changchun 130041, China*
[2]*Jilin Engineering Laboratory for Translational Medicine of Hepatobiliary and Pancreatic Diseases, Changchun 130041, China*

Correspondence should be addressed to Yongsheng Yang; ysyang@jlu.edu.cn and Xuewen Zhang; zhangxw@jlu.edu.cn

Received 24 August 2017; Revised 2 January 2018; Accepted 21 January 2018; Published 26 March 2018

Academic Editor: Qing Lin

Primary liver cancer is a common kind of digestive cancers with high malignancy, causing 745,500 deaths each year. Hepatocellular carcinoma is the major pathological type of primary liver cancer. Traditional treatment methods for patients with hepatocellular carcinoma have shown poor efficacy in killing residual cancer cells for a long time. In recent years, tumor immunotherapy has emerged as a promising method owing to its safety and efficacy with respect to delaying the progression of advanced tumors and protecting postoperative patients against tumor relapse and metastasis. Immune tolerance and suppression in tumor microenvironments are the theoretical basis of immunotherapy. Adoptive cell therapy functions by stimulating and cultivating autologous lymphocytes ex vivo and then reinfusing them into the patient to kill cancer cells. Cancer vaccination is performed using antigenic substances to activate tumor-specific immune responses. Immune checkpoint inhibitors can reactivate tumor-specific T cells and develop an antitumor effect by suppressing checkpoint-mediated signaling. Oncolytic viruses may selectively replicate in tumor cells and cause lysis without harming normal tissues. Here, we briefly introduce the mechanism of immunosuppression in hepatocellular carcinoma and summarize the rationale of the four major immunotherapeutic approaches with their current advances.

1. Introduction

Primary liver cancer is the sixth most common type of cancer and the second most common cause of cancer-related deaths worldwide, with an extremely high malignancy such that the number of deaths (745,500) is similar to that of new cases (782,500) every year [1]. Hepatocellular carcinoma (HCC) is a predominant type of primary liver cancer. Traditional therapeutic approaches for HCC include radical or palliative liver resection, radioactive seed implantation, transarterial chemoembolization (TACE), radiofrequency ablation (RFA), and liver transplantation. Although these approaches effectively address local lesions, they fail to completely eliminate residual cancer cells, which lead to tumor recurrence and metastasis. In recent years, tumor immunotherapy has emerged as a promising method for inhibiting tumor progression, relapse, and metastasis [2]. The rationale of this method is to activate tumor-specific immune responses and disrupt immune tolerance by enhancing cellular or humoral immunity. To date, some immunotherapeutic drugs for treating hematological malignancies, melanomas, and lung cancers have been proven to be efficacious in phase III trials and have been approved by FDA. Furthermore, recently, studies on immunotherapeutic approaches for HCC are rapidly increasing. In this study, we briefly reviewed the mechanism underlying immunosuppression and summarized major immunotherapeutic approaches for HCC (Table 1).

2. Mechanism Underlying Immunosuppression in HCC

T cells are activated through a double signaling pathway that requires the interaction of T cell receptors (TCR) with major histocompatibility complex (MHC)/peptide complexes on antigen-presenting cells (APCs) and expression of costimulatory molecules (CMs) on T cells and APCs. Downregulation

Table 1: Major immunotherapeutic approaches for HCC.

Approaches	Subsets	Targets and applications
ACT	CIK cells	CIK with valproate, DC-CIK with TACE
	TILs	
	NK cells	NK with K562-mb15-41BBL, sorafenib, and NKG2D
	CAR T cells (generations 1–4)	Targeting GPC3, targeting GPC3 and ASGR1
HCC vaccines	Cell vaccines	HCC cells with GM-CSF
	Antigen peptide vaccines	AFP, GPC3, SSX-2, NY-ESO-1, hTERT, HCA587, and MAGE-A
	DC vaccines	TCL-loaded DCs with nifuroxazide
Immune checkpoint inhibitors	CTLA-4 inhibitors	Tremelimumab, Tremelimumab with RFA
	PD-1 inhibitors	Nivolumab, Pembrolizumab, and Pidilizumab
	PD-L1 inhibitors	PD-L1 inhibitor with DNMT1 inhibitor
Oncolytic viruses		CVV, JX-594, GLV-1h68, and G47delta

of MHC class I molecules on tumor cells induces impairment of tumor antigen processing and presentation [3, 4]. Furthermore, reduced expression of CMs, such as B7-1 and B7-2, in HCC [4] leads to T cell anergy.

Immune checkpoints normally protect humans from uncontrolled autologous immunity by preventing excessive activation of T cells. However, tumor cells can overexpress immune checkpoint molecules that bind their receptors on T cells and inhibit T cell activation. The upregulation of immune checkpoint pathways in most patients with HCC impairs the effector function of cellular immune responses [5–7].

Immunosuppression in HCC can also be achieved via impairment of $CD4^+$ T cells [8]. MHC class II genes are among the most frequently expressed genes in HCC tumors, and overexpression of MHC class II molecules leads to $CD4^+$ T cell anergy in the absence of suitable CMs [9]. Also, immunosuppressive cells, including T regulatory cells (Tregs) [10], myeloid-derived suppressor cells (MDSCs) [5], and regulatory dendritic cells (DCs) [11], are important immunosuppressive factors in cancer patients, and an increase in the number of immunosuppressive cells, such as Tregs, may contribute to disease progression and poor prognosis. A Th1/Th2-like cytokine shift in the liver microenvironment of HCC patients with venous metastases has been previously reported [12]. Moreover, the upregulation of anti-inflammatory/immunosuppressive Th2-like cytokines and downregulation of proinflammatory/immunogenic Th1-like cytokines in adjacent noncancerous hepatic tissues indicate that disordered immune responses in tumor microenvironments [13–15] are key predictors of HCC metastasis.

3. Adoptive Cell Therapy

Adoptive cell therapy (ACT) is an immunotherapeutic approach that kills cancer cells using patients' own lymphocytes. It functions by stimulating or loading autologous lymphocytes with cytokines or tumor antigens, cultivating them ex vivo and then reinfusing them into the patient [16–18]. Adoptive immunotherapy for HCC includes cytokine-induced killer (CIK) cells, tumor-infiltrating lymphocytes (TILs), natural killer (NK) cells, and chimeric antigen receptor (CAR) T cells. The feasibility and safety of ACT in patients with HCC have been evaluated in many experiments, thus laying a foundation for its clinical application.

3.1. CIK Cells. CIK cells are a heterogeneous MHC-independent cell population mainly comprising $CD3^{+-}$ $CD56^+$, $CD3^+CD56^-$, and $CD3^-CD56^+$cells [19–21]. CIK cells are derived from peripheral mononuclear cells and stimulated by IL-1, IL-12, interferon- (IFN-) γ, and anti-CD3 antibodies ex vivo [22]. In a phase III study of adjuvant CIK therapy after radical resection for HCC, patients were randomized to receive four cycles of CIK therapy or no treatment. The median time to recurrence (TTR) was 13.6 months in the CIK group and 7.8 months in the control group ($p = 0.01$), indicating the safety and efficacy with respect to prolonging TTR of CIK therapy in patients with HCC. However, there were no statistically significant differences between the groups in disease-free survival (DFS) and overall survival (OS) [23]. A combination therapy with CIK cells and valproate in mice demonstrated a synergistic effect in controlling tumor growth [24], warranting further assessment of this combination therapy through clinical trials. In addition, a meta-analysis of 693 patients with HCC demonstrated that a combination of dendritic cell- (DC-) CIK cells and TACE improves 1- and 2-year OS, overall response rate (ORR), disease control rate (DCR), and the quality of life [25].

3.2. TILs. TILs are derived from tumor tissues and are cultured and induced using IL-2 and anti-CD3 antibodies ex vivo [26–28]. Thus, reinfusion of autologous TILs, which possess tumor-specific immunity, may target multiple tumor antigens. Low toxicity of autologous TILs was verified in a phase I study involving patients with HCC, suggesting a novel treatment option [29]. However, this study included only 15 patients and lacked control groups, thus failing to prove the efficacy of TILs. To date, TILs have not been well characterized, mainly due to difficulties in purifying and expanding them.

3.3. NK Cells. NK cells belong to the innate immune system and can directly kill tumor cells and infected cells without preliminary sensitization or MHC restriction. However, they

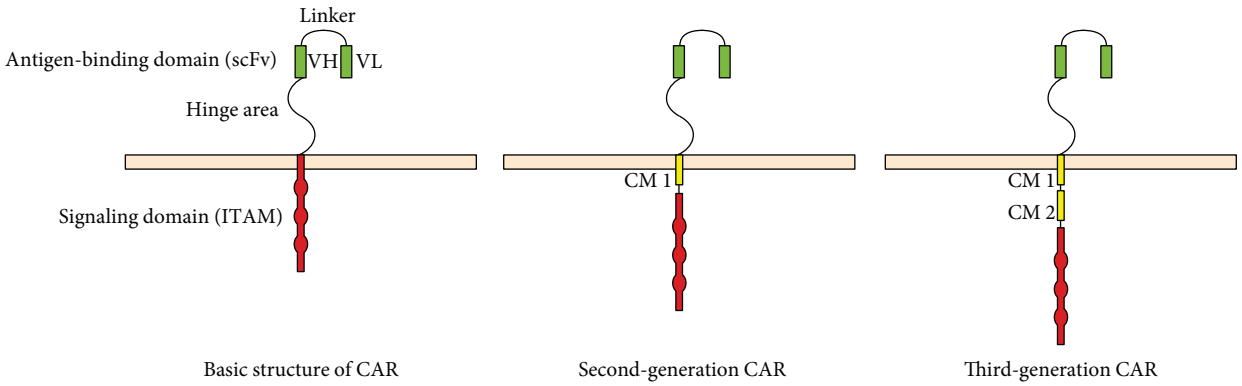


Figure 1: Structure of CAR.

lack the ability to target tumor cells and can injure normal liver tissues. In a previous series of experiments, the cytotoxicity of NK cells against HCC cells was enhanced [30] by first generating a new hepatoma cell line, K562-mb15-41BBL, which achieved a more efficient stimulation of NK cells in vitro. Second, HCC cells exposed to 5 μmol/L sorafenib for 48 h showed high sensitivity to NK cells. Finally, NKG2D, an engineered NK-cell-activating receptor, was tested in vitro and in mice. All of the outcomes were positive in increasing the cytotoxicity of NK cells, providing the possibility of further clinical trials for HCC.

3.4. CAR T Cells. CAR T cells are genetically modified T lymphocytes that specifically target tumor-associated antigens (TAAs) and kill cancer cells in a MHC-independent manner [31, 32]. CARs consist of three major components—the extracellular antigen-binding domain, the intracellular signaling domain, and the hinge area [33, 34] (Figure 1). The antigen-binding domain is a single chain fragment variable (scFv) region that comprises a heavy (VH) and a light (VL) chain derived from monoclonal antibodies (mAbs), which are connected by a linker fragment. The signaling domain involves immunoreceptor tyrosine-based activation motifs (ITAMs), such as CD3ζ and FcεRIγ. The above two domains are connected by the hinge area which imparts high flexibility for the movement of the antigen-binding domain. The first-generation CARs lacked the structure of CMs and led to poor replication, survival, and cytotoxicity of T cells. In contrast, the second- and third-generation CARs (Figure 1), with the addition of CMs, led to high proliferative capacity, long-term persistence, and potent cytotoxicity of T cells [35]. In the second-generation CARs, a CM (CM1), such as CD28, was engineered into the signaling region [36], whereas in the third generation, additional CMs (CM2), such as CD27, CD137/4-1BB, and CD134/OX40, were included [37]. In some tumors with a tremendous phenotypic heterogeneity, CAR T cells could target the tumor antigen and cause antigen-positive cell death, while antigen-negative cancer cells may induce tumor relapse. Recently, CAR T cells with a transgenic "payload," also called the "fourth-generation" CAR T cells, were designed [38]. The fourth-generation CAR T cells work by releasing inducible cytokines such as IL-12 which will augment T cell activation and further activate innate immune system to kill antigen-negative cancer cells. Recently, CAR T cell therapy has received much attention as an immunotherapy for tumors, and a good efficacy has been reported in some clinical trials of leukemia and lymphoma. CAR T cell therapy is also being investigated for solid tumors, such as HCC. Glypican3 (GPC3) is a TAA that is specifically overexpressed in 70%–81% of HCC tumors and has been correlated with poor prognosis [39]. Moreover, the ability of GPC3-targeted CAR T cells to eliminate GPC3-positive HCC cells was confirmed both in vivo and in vitro, and the survival of mice with HCC xenografts was evidently prolonged with CAR T cell therapy in vivo [40]. In another experiment, T cells with two complementary CARs against GPC3 and asialoglycoprotein receptor 1 (ASGR1) decreased the risk of on-target, off-tumor toxicities and demonstrated potent antitumor immune responses targeting GPC3+ ASGR1+ HCCs both in vivo and in vitro [41]. However, to date, the related studies conducted have been predominantly basic, and more clinical trials are required to prove the efficacy of CAR T cells against HCC. Complications of CAR T cell therapy include on-target, off-tumor toxicities [42], tumor lysis syndrome (TLS) [43], and cytokine release syndrome (CRS) [44]. Traditional solutions include nonspecific immunosuppression, complete elimination of T cells, and introduction of inducible suicide genes into CAR T cells. However, the best method to prevent these hazards could be the application of ideal tumor-specific antigens (TSAs) expressed only in tumor cells and not in normal cells. As for the difficulty of seeking for more appropriate TSAs, some techniques, such as the utilization of inhibitory CAR (iCAR) [45] and combinatorial antigen recognition by CAR and chimeric costimulatory receptor (CCR) [46], were employed to prevent on-target, off-tumor toxicities. In addition, CARs could be used to modify other lymphocytes, such as NK cells [47] and $\gamma\delta$T cells [48], which may highlight the use of HCC immunotherapy in the future.

4. HCC Vaccines

Cancer vaccination is performed using antigenic substances to activate tumor-specific immune responses that can reduce tumor load and prevent tumor relapse. HCC vaccines include cancer cells, antigen peptides, DCs, and DNA-based

vaccines, and some of these effectively inhibit tumor recurrence and metastasis.

4.1. HCC Cell Vaccines. Autologous or allogenic HCC cells or lysates that are physically or chemically disposed to eliminate pathogenicity could be used as immunogens for tumor-specific immune responses. In a phase I trial, bi-shRNA/granulocyte macrophage colony-stimulating factor-(GM-CSF-) augmented autologous tumor cells were tested in eight patients with advanced HCC. Three of these patients presented evident immune responses to the reinfused tumor cells, and long-term follow-up demonstrated a survival of 319, 729, 784, 931+, and 1043+ days after treatment [49]. However, the efficacy of HCC cell vaccines remains uncertain due to their weak immunogenicity.

4.2. Antigen Peptide Vaccines. Peptide-based TAAs, such as alpha-fetoprotein (AFP), GPC3, SSX-2, NY-ESO-1, human telomerase reverse transcriptase (hTERT), HCA587, and melanoma antigen gene-A (MAGE-A), are excellent vaccine targets for the treatment of HCC [50]. AFP, which normally originates from embryonic liver cells, can be overexpressed on HCC cell surfaces. However, immune responses to AFP are limited due to acquired immune tolerance during the development of the immune system. To break up this immune tolerance, a research group investigated the use of a recombinant rat AFP to induce cross-reactions between xenografts and endogenous molecules in animals and observed modest cellular and humoral immune responses [51]. In a phase II trial of GPC3-derived peptide vaccine for HCC, 25 patients received 10 vaccinations over one year after surgery. Recurrence in patients who underwent both surgery and vaccination was specifically lower than that in 21 patients who underwent surgery only (24% versus 48% and 52.4% versus 61.9% at 1 and 2 years, $p = 0.047$ and 0.387, resp.), indicating the efficacy of the GPC3-derived vaccine [52].

4.3. DC Vaccines. DCs, the most powerful APCs, are responsible for absorption, processing, and presentation of tumor antigens. They maintain high expression levels of MHCs and CMs, such as B7-1 and B7-2. They also elicit antitumor effects by the way of inducing primary T cells, releasing IFN-γ that suppresses tumoral angiogenesis and producing immune memory [53]. During vaccine preparation, DCs are initially activated by cytokines, such as rhGM-CSF and rhIL-4, then mature in the presence of tumor necrosis factor-(TNF-) α and are finally sensitized by autologous tumor cells or antigens [50]. Some gene-transfected DCs persistently express endogenous tumor antigens or cytokines that enhance their own functions. In a recent study, mice with HCC were treated with a combination of tumor cell lysate-(TCL-) loaded DCs and nifuroxazide, which is an inhibitor of signal transducer and activator of transcription 3 (STAT3). This combination increased the survival rate, limited tumor growth, and elevated antitumor immune response [54]. A phase I/IIa study using tumor antigen-pulsed DCs for HCC patients after primary treatment demonstrated that DC vaccination is an effective adjuvant treatment for such patients [55]. In addition, the safety and tolerance of DC vaccines have been confirmed in patients with HCC [56].

5. Immune Checkpoint Inhibitors

As mentioned above, the negative regulatory target-immune checkpoints are often overexpressed in tumors to escape the host immune surveillance. Immune checkpoint inhibitors can reactivate tumor-specific T cells and develop an antitumor effect by suppressing checkpoint-mediated signaling [57]. Common immune checkpoint proteins include cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed cell death protein-1 (PD-1), programmed cell death ligand 1 (PD-L1), VISTA, TIM-3, LAG-3, and OX40 [58, 59]. CTLA-4 and PD-1 inhibitors have been well characterized and have been approved by FDA for treating melanomas, with some progress in their application in treating HCCs.

5.1. CTLA-4 Inhibitors. CTLA-4 is predominantly expressed in activated T cells and NK cells [60]. It binds ligands B7-1 and B7-2 with much higher affinity than CD28 [61]. Moreover, CTLA-4 inhibitors prevent the binding of CTLA-4 to B7-1 and B7-2, thereby promoting the activation of T cells. In 2011, FDA approved a fully human anti-CTLA-4 mAb-Ipilimumab for the treatment of metastatic melanoma. In a phase II study of an anti-CTLA-4 mAb-Tremelimumab in patients with advanced HCC and hepatitis C, partial response rate (17.6%), disease control rate (76.4%), and time to progression (6.48 months) improved. Moreover, viral loads of HCC were significantly decreased, and no patients experienced immune-related adverse events (irAEs) or evident hepatotoxicity. These studies demonstrated that Tremelimumab treatment is a safe antitumor and antiviral method for hepatitis C-induced HCC [62]. In a noncomparative clinical trial involving patients with advanced HCC, a combination therapy with Tremelimumab and RFA increased the number of intratumoral $CD8^+$T cells and reduced HCV viral loads [63].

5.2. PD-1 Inhibitors. PD-1 is expressed in T cells, B cells, NK cells, mononuclear cells, and DCs [64]. PD-1 inhibitors block the receptor binding of PD-L1 and PD-L2, resulting in the activation of immune cells [65]. Some PD-1 inhibitors, such as Nivolumab, Pembrolizumab, and Pidilizumab, have been investigated for cancer treatment. A phase I/II study demonstrated the safety and antitumor effect of Nivolumab in patients with advanced HCC. In this study, of the 41 qualified patients who were intravenously administered 0.1–10 mg/kg Nivolumab, 29 (71%, 17% grade 3/4) endured drug-related AEs, two (5%) showed complete responses (CRs), and seven (18%) showed partial responses (PRs). Moreover, response durations for CR, PR, and stable disease (SD) were 14–17+ months, <1–8+ months, and 1.5–17+ months, respectively, and the OS rate at 6 months was 72%. These data indicated that Nivolumab activates sustained tumor-specific immune responses with manageable AEs [66]. A recent open-label, noncomparative, phase I/II dose escalation and expansion trial of Nivolumab involving 262 patients with advanced

HCC confirmed the safety and potential of this PD-1 inhibitor in treating HCCs [67].

5.3. PD-L1 Inhibitors. Cancer cells can evade immune surveillance by overexpressing PD-L1 and activating PD-L1/PD-1 signaling [68]. High PD-L1 expression has been observed in HCC tissues [69]. However, no clinical trials involving the use of PD-L1 inhibitors for treating HCC have been conducted. A recent experiment showed that contemporary inhibition of PD-L1 and DNA methyltransferase 1 (DNMT1) significantly suppressed the growth of sorafenib-resistant HCC cells in vitro, further suggesting a novel effective treatment option for sorafenib-resistant HCC [70].

6. Oncolytic Virotherapy

Oncolytic viruses are wild-type or engineered viruses that selectively replicate in tumor cells and cause lysis without harming normal tissues [71, 72]. The mechanism underlying the antitumor activity of oncolytic viruses involves direct killing of cancer cells by expanding in them and causing cell lysis. Most viruses can expand in cancer cells to a rather great extent due to the impairment of the tumor's defense mechanisms against viral infection [73]. In addition, tumor antigens and viruses in cell lysates activate immune responses against adjacent cancer cells [74–77]. The targeting mechanisms of oncolytic viruses are as follows. First, wild-type viruses that specifically infect tumors like reoviruses, varicella viruses, and Sindbis viruses [78] could be chosen. Second, viral genes that are crucial for replication in normal cells but have no functions in cancer cells are deleted by engineering [76]. Third, viral transcription is limited in cancer cells by applying tumor-specific promoters, such as the promoter of human telomerase reverse transcriptase, before crucial viral genes [79]. Finally, after modification by TAA-specific receptors, viruses effectively target tumor cells. For example, an oncolytic vaccinia virus engineered with antiangiogenic genes can specifically inhibit tumor angiogenesis [80]. The efficacy of an evolutionary cancer-favoring engineered vaccinia virus (CVV) was investigated in an animal model of metastatic HCC. In this study, animals were randomized into sorafenib, CVV, and sorafenib with CVV groups. Metastatic regions were fewer in the CVV-treated groups than in the sorafenib-treated group. The result suggested that CVV can be a promising virus targeting metastatic HCC [81]. JX-594, an engineered vaccinia virus with a mutation in the TK gene, which controls cancer cell-specific replication, and an insertion in the human GM-CSF gene, which increases antitumor immune responses [82], is stable and safe in humans and extremely toxic to cancer cells. A phase II randomized open-label study of JX-594 in patients with advanced HCC confirmed the safety and efficacy of the oncolytic virotherapy. This treatment was well tolerated at both high and low doses, with an intrahepatic response rate of 62% and one CR. In addition, the OS rate was higher in the high-dose group than in the low-dose group (median, 14.1 months versus 6.7 months; hazard ratio, 0.39; $p = 0.020$) [83]. To date, various oncolytic viruses, such as GLV-1h68 [84] and G47delta [85], have been studied for the treatment of HCC. Researchers should attach more importance to the dangers of viral infection and the insertional mutations that may activate oncogenes or damage tumor suppressor genes.

7. Brief Summary

The four major immunotherapeutic approaches for HCC have their own preponderances and defects.

CAR T cell therapy has been a star of immunotherapeutic researches in recent years. With its accurate targeting toward HCC and MHC independence, CAR T cells could directively kill HCC cells, like precision-guided missiles. The efficacy of CAR T cells has also been elevated after several generations. However, this favored method is not almighty. The lack of HCC-associated TSAs makes it difficult to construct more efficacious CARs. Meanwhile, more strategies should be designed to overcome the on-target, off-tumor effect. Other methods of adoptive cell therapy, like CIK cells, TILs, and NK cells, are being out of sight due to the nonspecificity and difficulty of extraction.

Immune checkpoint inhibitor is another hot topic. It breaks up tumor immune tolerance and causes reactivation of innate immune system, which may redirect and eliminate HCC cells as a result. It is a relatively simple process preparing for immune checkpoint inhibitors. Meanwhile, many clinical researches indicate the safety of this method. So, we may focus on how to improve its efficacy and test more practical combinatorial therapeutic methods in the future.

Tumor vaccines, because of tumor immune tolerance and lack of TSAs, did not show great value in HCC treatment, while DC vaccines may be a promising method in this realm, due to their potent capacity of antigen presenting. Researches of oncolytic viruses are quite few. Safety of viruses is the most important, while efficacy is the second. So the very much difficulty is to balance safety and toxicity of oncolytic viruses.

8. Future Expectations

As a new therapeutic approach for malignancies beyond traditional operations, chemotherapy and radiotherapy, immunotherapy has shown its efficacy in delaying the progression of advanced tumors and protecting postoperative patients against cancer relapse and metastasis. Although no drugs have been officially approved, numerous studies on immunotherapy for HCC are being conducted and some have already obtained important results. Future studies are required to identify more specific immune targets, such as TAAs/TSAs, novel immune checkpoints, and oncolytic viruses. These will enhance the intensity of tumor-specific immune responses and avoid unnecessary on-target, off-tumor toxicities. Meanwhile, the Aes should be valued, especially in clinical trials. The safety of a new treatment is as important as its efficacy. Furthermore, individualized treatment plans for patients with HCC will enhance the efficacy of immunotherapy and likely become a future trend. Taken together, the promising therapeutic approach certainly will bring the treatment for HCC to a brand new period.

Conflicts of Interest

The authors confirm no conflicts of interest regarding the publication of this article.

Acknowledgments

This work was supported by grants from the Finance Department of Jilin Province (SCZSYZ01502, SCZSYZ01521, and SCZSYZ01522).

References

[1] L. A. Torre, F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent, and A. Jemal, "Global cancer statistics, 2012," *CA: A Cancer Journal for Clinicians*, vol. 65, no. 2, pp. 87–108, 2015.

[2] L. X. Qin, "Inflammatory immune responses in tumor microenvironment and metastasis of hepatocellular carcinoma," *Cancer Microenvironment*, vol. 5, no. 3, pp. 203–209, 2012.

[3] N. Tsuchiya, Y. Sawada, I. Endo, Y. Uemura, and T. Nakatsura, "Potentiality of immunotherapy against hepatocellular carcinoma," *World Journal of Gastroenterology*, vol. 21, no. 36, pp. 10314–10326, 2015.

[4] K. Fujiwara, T. Higashi, K. Nouso et al., "Decreased expression of B7 costimulatory molecules and major histocompatibility complex class-I in human hepatocellular carcinoma," *Journal of Gastroenterology and Hepatology*, vol. 19, no. 10, pp. 1121–1127, 2004.

[5] F. Arihara, E. Mizukoshi, M. Kitahara et al., "Increase in $CD14^{+}HLA\text{-}DR^{-/low}$ myeloid-derived suppressor cells in hepatocellular carcinoma patients and its impact on prognosis," *Cancer Immunology, Immunotherapy*, vol. 62, no. 8, pp. 1421–1430, 2013.

[6] Z. Chen, S. Shen, B. Peng, and J. Tao, "Intratumoural GM-CSF microspheres and CTLA-4 blockade enhance the antitumour immunity induced by thermal ablation in a subcutaneous murine hepatoma model," *International Journal of Hyperthermia*, vol. 25, no. 5, pp. 374–382, 2009.

[7] B. Hoechst, T. Voigtlaender, L. Ormandy et al., "Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor," *Hepatology*, vol. 50, no. 3, pp. 799–807, 2009.

[8] A. Alisa, A. Ives, A. A. Pathan et al., "Analysis of $CD4^{+}$ T-cell responses to a novel α-fetoprotein-derived epitope in hepatocellular carcinoma patients," *Clinical Cancer Research*, vol. 11, no. 18, pp. 6686–6694, 2005.

[9] I. N. Crispe, M. Giannandrea, I. Klein, B. John, B. Sampson, and S. Wuensch, "Cellular and molecular mechanisms of liver tolerance," *Immunological Reviews*, vol. 213, no. 1, pp. 101–118, 2006.

[10] K. J. Chen, S. Z. Lin, L. Zhou et al., "Selective recruitment of regulatory T cell through CCR6-CCL20 in hepatocellular carcinoma fosters tumor progression and predicts poor prognosis," *PLoS One*, vol. 6, no. 9, article e24671, 2011.

[11] Y. Han, Z. Chen, Y. Yang et al., "Human $CD14^{+}CTLA\text{-}4^{+}$ regulatory dendritic cells suppress T-cell response by cytotoxic T-lymphocyte antigen-4-dependent IL-10 and indoleamine-2,3-dioxygenase production in hepatocellular carcinoma," *Hepatology*, vol. 59, no. 2, pp. 567–579, 2014.

[12] A. Budhu, M. Forgues, Q. H. Ye et al., "Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment," *Cancer Cell*, vol. 10, no. 2, pp. 99–111, 2006.

[13] J. Couzin-Frankel, "Breakthrough of the year 2013. Cancer immunotherapy," *Science*, vol. 342, no. 6165, pp. 1432-1433, 2013.

[14] F. M. Buonaguro and L. Buonaguro, "Cancer vaccines for hepatocellular carcinoma: future directions," *Immunotherapy*, vol. 8, no. 4, pp. 391–393, 2016.

[15] L. Buonaguro, A. Petrizzo, M. Tagliamonte, M. L. Tornesello, and F. M. Buonaguro, "Challenges in cancer vaccine development for hepatocellular carcinoma," *Journal of Hepatology*, vol. 59, no. 4, pp. 897–903, 2013.

[16] Z. Jixia, Z. Chengyan, and W. Pingli, "Advances in application of adoptive T-cell therapy for cancer patients," *Zhejiang Da Xue Xue Bao Yi Xue Ban*, vol. 46, no. 2, pp. 211–217, 2017.

[17] O. Yeku, X. Li, and R. J. Brentjens, "Adoptive T-cell therapy for solid tumors," *American Society of Clinical Oncology Educational Book*, vol. 37, pp. 193–204, 2017.

[18] E. N. Baruch, A. L. Berg, M. J. Besser, J. Schachter, and G. Markel, "Adoptive T cell therapy: an overview of obstacles and opportunities," *Cancer*, vol. 123, no. S11, pp. 2154–2162, 2017.

[19] V. Longo, A. Gnoni, A. Casadei Gardini et al., "Immunotherapeutic approaches for hepatocellular carcinoma," *Oncotarget*, vol. 8, no. 20, pp. 33897–33910, 2017.

[20] X. Gao, Y. Mi, N. Guo et al., "Cytokine-induced killer cells as pharmacological tools for cancer immunotherapy," *Frontiers in Immunology*, vol. 8, p. 774, 2017.

[21] J. J. Mata-Molanes, M. Sureda Gonzalez, B. Valenzuela Jimenez, E. M. Martinez Navarro, and A. Brugarolas Masllorens, "Cancer immunotherapy with cytokine-induced killer cells," *Targeted Oncology*, vol. 12, no. 3, pp. 289–299, 2017.

[22] F. S. Wang, M. X. Liu, B. Zhang et al., "Antitumor activities of human autologous cytokine-induced killer (CIK) cells against hepatocellular carcinoma cells in vitro and in vivo," *World Journal of Gastroenterology*, vol. 8, no. 3, pp. 464–468, 2002.

[23] L. Xu, J. Wang, Y. Kim et al., "A randomized controlled trial on patients with or without adjuvant autologous cytokine-induced killer cells after curative resection for hepatocellular carcinoma," *OncoImmunology*, vol. 5, no. 3, article e1083671, 2016.

[24] D. H. Lee, J. Y. Nam, Y. Chang et al., "Synergistic effect of cytokine-induced killer cell with valproate inhibits growth of hepatocellular carcinoma cell in a mouse model," *Cancer Biology & Therapy*, vol. 18, no. 1, pp. 67–75, 2017.

[25] Y. Su, Y. Yang, Y. Ma et al., "The efficacy and safety of dendritic cells co-cultured with cytokine-induced killer cell therapy in combination with TACE-predominant minimally-invasive treatment for hepatocellular carcinoma: a meta-analysis," *Clinical Laboratory*, vol. 62, no. 4, pp. 599–608, 2016.

[26] U. Toh, T. Fujii, N. Seki, F. Niiya, K. Shirouzu, and H. Yamana, "Characterization of IL-2-activated TILs and their use in intrapericardial immunotherapy in malignant pericardial effusion," *Cancer Immunology, Immunotherapy*, vol. 55, no. 10, pp. 1219–1227, 2006.

[27] L. Yuan, S. He, C. Guan, and Q. Pang, "The preparation and study on hepatic targeting tendency of galactosyl-anti-CD3-McAb in mice," *Hua Xi Yi Ke Da Xue Xue Bao*, vol. 32, no. 3, pp. 424–426, 2001.

[28] T. Kikuchi, M. Watanabe, and T. Ohno, "Cytological characteristics of human glioma-infiltrating lymphocytes stimulated

with recombinant interleukin 2 and an anti-CD3 antibody," *Japanese Journal of Cancer Research*, vol. 82, no. 3, pp. 339–345, 1991.

[29] S. S. Jiang, Y. Tang, Y. J. Zhang et al., "A phase I clinical trial utilizing autologous tumor-infiltrating lymphocytes in patients with primary hepatocellular carcinoma," *Oncotarget*, vol. 6, no. 38, pp. 41339–41349, 2015.

[30] T. Kamiya, Y. H. Chang, and D. Campana, "Expanded and activated natural killer cells for immunotherapy of hepatocellular carcinoma," *Cancer Immunology Research*, vol. 4, no. 7, pp. 574–581, 2016.

[31] M. Chmielewski, A. A. Hombach, and H. Abken, "Antigen-specific T-cell activation independently of the MHC: chimeric antigen receptor-redirected T cells," *Frontiers in Immunology*, vol. 4, p. 371, 2013.

[32] T. Li, H. T. Wang, and Z. G. Liu, "CAR technology and its application in treatment of multiple myeloma–review," *Zhongguo Shi Yan Xue Ye Xue Za Zhi*, vol. 24, no. 1, pp. 279–284, 2016.

[33] C. A. Ramos and G. Dotti, "Chimeric antigen receptor (CAR)-engineered lymphocytes for cancer therapy," *Expert Opinion on Biological Therapy*, vol. 11, no. 7, pp. 855–873, 2011.

[34] S. J. Priceman, S. J. Forman, and C. E. Brown, "Smart CARs engineered for cancer immunotherapy," *Current Opinion in Oncology*, vol. 27, no. 6, pp. 466–474, 2015.

[35] G. Dotti, B. Savoldo, and M. Brenner, "Fifteen years of gene therapy based on chimeric antigen receptors: "are we nearly there yet?"," *Human Gene Therapy*, vol. 20, no. 11, pp. 1229–1239, 2009.

[36] S. J. C. van der Stegen, M. Hamieh, and M. Sadelain, "The pharmacology of second-generation chimeric antigen receptors," *Nature Reviews Drug Discovery*, vol. 14, no. 7, pp. 499–509, 2015.

[37] X. Y. Tang, Y. Sun, A. Zhang et al., "Third-generation CD28/4-1BB chimeric antigen receptor T cells for chemotherapy relapsed or refractory acute lymphoblastic leukaemia: a non-randomised, open-label phase I trial protocol," *BMJ Open*, vol. 6, no. 12, article e013904, 2016.

[38] M. Chmielewski and H. Abken, "TRUCKs: the fourth generation of CARs," *Expert Opinion on Biological Therapy*, vol. 15, no. 8, pp. 1145–1154, 2015.

[39] H. Shirakawa, H. Suzuki, M. Shimomura et al., "Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma," *Cancer Science*, vol. 100, no. 8, pp. 1403–1407, 2009.

[40] H. Gao, K. Li, H. Tu et al., "Development of T cells redirected to glypican-3 for the treatment of hepatocellular carcinoma," *Clinical Cancer Research*, vol. 20, no. 24, pp. 6418–6428, 2014.

[41] C. Chen, K. Li, H. Jiang et al., "Development of T cells carrying two complementary chimeric antigen receptors against glypican-3 and asialoglycoprotein receptor 1 for the treatment of hepatocellular carcinoma," *Cancer Immunology, Immunotherapy*, vol. 66, no. 4, pp. 475–489, 2017.

[42] G. Gross and Z. Eshhar, "Therapeutic potential of T cell chimeric antigen receptors (CARs) in cancer treatment: counteracting off-tumor toxicities for safe CAR T cell therapy," *Annual Review of Pharmacology and Toxicology*, vol. 56, no. 1, pp. 59–83, 2016.

[43] D. T. Teachey, S. F. Lacey, P. A. Shaw et al., "Identification of predictive biomarkers for cytokine release syndrome after chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia," *Cancer Discovery*, vol. 6, no. 6, pp. 664–679, 2016.

[44] X. J. Xu and Y. M. Tang, "Cytokine release syndrome in cancer immunotherapy with chimeric antigen receptor engineered T cells," *Cancer Letters*, vol. 343, no. 2, pp. 172–178, 2014.

[45] V. D. Fedorov, M. Themeli, and M. Sadelain, "PD-1– and CTLA-4–based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses," *Science Translational Medicine*, vol. 5, no. 215, article 215ra172, 2013.

[46] C. C. Kloss, M. Condomines, M. Cartellieri, M. Bachmann, and M. Sadelain, "Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells," *Nature Biotechnology*, vol. 31, no. 1, pp. 71–75, 2013.

[47] H. J. Pegram, J. T. Jackson, M. J. Smyth, M. H. Kershaw, and P. K. Darcy, "Adoptive transfer of gene-modified primary NK cells can specifically inhibit tumor progression in vivo," *Journal of Immunology*, vol. 181, no. 5, pp. 3449–3455, 2008.

[48] M. Rischer, S. Pscherer, S. Duwe, J. Vormoor, H. Jurgens, and C. Rossig, "Human $\gamma\delta$ T cells as mediators of chimaeric-receptor redirected anti-tumour immunity," *British Journal of Haematology*, vol. 126, no. 4, pp. 583–592, 2004.

[49] J. Nemunaitis, M. Barve, D. Orr et al., "Summary of bi-shRNA^{furin}/GM-CSF augmented autologous tumor cell immunotherapy (FANG™) in advanced cancer of the liver," *Oncology*, vol. 87, no. 1, pp. 21–29, 2014.

[50] T. Y. Sun, W. Yan, C. M. Yang et al., "Clinical research on dendritic cell vaccines to prevent postoperative recurrence and metastasis of liver cancer," *Genetics and Molecular Research*, vol. 14, no. 4, pp. 16222–16232, 2015.

[51] W. Zhang, J. Liu, Y. Wu et al., "Immunotherapy of hepatocellular carcinoma with a vaccine based on xenogeneic homologous α fetoprotein in mice," *Biochemical and Biophysical Research Communications*, vol. 376, no. 1, pp. 10–14, 2008.

[52] Y. Sawada, T. Yoshikawa, K. Ofuji et al., "Phase II study of the GPC3-derived peptide vaccine as an adjuvant therapy for hepatocellular carcinoma patients," *OncoImmunology*, vol. 5, no. 5, article e1129483, 2016.

[53] K. Takakura, M. Kajihara, Z. Ito, T. Ohkusa, J. Gong, and S. Koido, "Dendritic-tumor fusion cells in cancer immunotherapy," *Discovery Medicine*, vol. 19, no. 104, pp. 169–174, 2015.

[54] T. Zhao, H. Jia, Q. Cheng et al., "Nifuroxazide prompts antitumor immune response of TCL-loaded DC in mice with orthotopically-implanted hepatocarcinoma," *Oncology Reports*, vol. 37, no. 6, pp. 3405–3414, 2017.

[55] J. H. Lee, Y. Lee, M. Lee et al., "A phase I/IIa study of adjuvant immunotherapy with tumour antigen-pulsed dendritic cells in patients with hepatocellular carcinoma," *British Journal of Cancer*, vol. 113, no. 12, pp. 1666–1676, 2015.

[56] M. El Ansary, S. Mogawer, S. A. Elhamid et al., "Immunotherapy by autologous dendritic cell vaccine in patients with advanced HCC," *Journal of Cancer Research and Clinical Oncology*, vol. 139, no. 1, pp. 39–48, 2013.

[57] J. B. Haanen and C. Robert, "Immune checkpoint inhibitors," *Progress in Tumor Research*, vol. 42, pp. 55–66, 2015.

[58] T. Hato, L. Goyal, T. F. Greten, D. G. Duda, and A. X. Zhu, "Immune checkpoint blockade in hepatocellular carcinoma: current progress and future directions," *Hepatology*, vol. 60, no. 5, pp. 1776–1782, 2014.

[59] X. Meng, Z. Huang, F. Teng, L. Xing, and J. Yu, "Predictive biomarkers in PD-1/PD-L1 checkpoint blockade immunotherapy," *Cancer Treatment Reviews*, vol. 41, no. 10, pp. 868–876, 2015.

[60] M. D. Vesely, M. H. Kershaw, R. D. Schreiber, and M. J. Smyth, "Natural innate and adaptive immunity to cancer," *Annual Review of Immunology*, vol. 29, no. 1, pp. 235–271, 2011.

[61] A. V. Collins, D. W. Brodie, R. J. C. Gilbert et al., "The interaction properties of costimulatory molecules revisited," *Immunity*, vol. 17, no. 2, pp. 201–210, 2002.

[62] B. Sangro, C. Gomez-Martin, M. de la Mata et al., "A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C," *Journal of Hepatology*, vol. 59, no. 1, pp. 81–88, 2013.

[63] A. G. Duffy, S. V. Ulahannan, O. Makorova-Rusher et al., "Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma," *Journal of Hepatology*, vol. 66, no. 3, pp. 545–551, 2017.

[64] L. Shi, S. Chen, L. Yang, and Y. Li, "The role of PD-1 and PD-L1 in T-cell immune suppression in patients with hematological malignancies," *Journal of Hematology & Oncology*, vol. 6, no. 1, p. 74, 2013.

[65] S. Dai, R. Jia, X. Zhang, Q. Fang, and L. Huang, "The PD-1/PD-Ls pathway and autoimmune diseases," *Cellular Immunology*, vol. 290, no. 1, pp. 72–79, 2014.

[66] A. B. El-Khoueiry, I. Melero, T. S. Crocenzi et al., "Phase I/II safety and antitumor activity of nivolumab in patients with advanced hepatocellular carcinoma (HCC): CA209-040," *Journal of Clinical Oncology*, vol. 33, no. 18, article LBA101, 2015Supplement, 2015.

[67] A. B. El-Khoueiry, B. Sangro, T. Yau et al., "Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial," *Lancet*, vol. 389, no. 10088, pp. 2492–2502, 2017.

[68] Y. Iwai, M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, and N. Minato, "Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 19, pp. 12293–12297, 2002.

[69] F. Zhong, X. Cheng, S. Sun, and J. Zhou, "Transcriptional activation of PD-L1 by Sox2 contributes to the proliferation of hepatocellular carcinoma cells," *Oncology Reports*, vol. 37, no. 5, pp. 3061–3067, 2017.

[70] J. Liu, Y. Liu, L. Meng, K. Liu, and B. Ji, "Targeting the PD-L1/DNMT1 axis in acquired resistance to sorafenib in human hepatocellular carcinoma," *Oncology Reports*, vol. 38, no. 2, pp. 899–907, 2017.

[71] A. M. Hammill, J. Conner, and T. P. Cripe, "Oncolytic virotherapy reaches adolescence," *Pediatric Blood & Cancer*, vol. 55, no. 7, pp. 1253–1263, 2010.

[72] M. G. Bourke, S. Salwa, K. J. Harrington et al., "The emerging role of viruses in the treatment of solid tumours," *Cancer Treatment Reviews*, vol. 37, no. 8, pp. 618–632, 2011.

[73] L. C. Platanias, "Mechanisms of type-I- and type-II-interferon-mediated signalling," *Nature Reviews Immunology*, vol. 5, no. 5, pp. 375–386, 2005.

[74] H. L. Kaufman, F. J. Kohlhapp, and A. Zloza, "Oncolytic viruses: a new class of immunotherapy drugs," *Nature Reviews Drug Discovery*, vol. 14, no. 9, pp. 642–662, 2015.

[75] V. Schirrmacher, "Oncolytic Newcastle disease virus as a prospective anti-cancer therapy. A biologic agent with potential to break therapy resistance," *Expert Opinion on Biological Therapy*, vol. 15, no. 12, pp. 1757–1771, 2015.

[76] E. A. Chiocca and S. D. Rabkin, "Oncolytic viruses and their application to cancer immunotherapy," *Cancer Immunology Research*, vol. 2, no. 4, pp. 295–300, 2014.

[77] S. T. Workenhe and K. L. Mossman, "Oncolytic virotherapy and immunogenic cancer cell death: sharpening the sword for improved cancer treatment strategies," *Molecular Therapy*, vol. 22, no. 2, pp. 251–256, 2014.

[78] M. M. Stanford, J. C. Bell, and M. J. V. Vähä-Koskela, "Novel oncolytic viruses: riding high on the next wave?," *Cytokine & Growth Factor Reviews*, vol. 21, no. 2-3, pp. 177–183, 2010.

[79] W. Zhang, K. Ge, Q. Zhao et al., "A novel oHSV-1 targeting telomerase reverse transcriptase-positive cancer cells via tumor-specific promoters regulating the expression of ICP4," *Oncotarget*, vol. 6, no. 24, pp. 20345–20355, 2015.

[80] C. J. Breitbach, R. Arulanandam, N. de Silva et al., "Oncolytic vaccinia virus disrupts tumor-associated vasculature in humans," *Cancer Research*, vol. 73, no. 4, pp. 1265–1275, 2013.

[81] S. Y. Yoo, S. N. Jeong, D. H. Kang, and J. Heo, "Evolutionary cancer-favoring engineered vaccinia virus for metastatic hepatocellular carcinoma," *Oncotarget*, vol. 8, no. 42, pp. 71489–71499, 2017.

[82] K. A. Parato, C. J. Breitbach, F. le Boeuf et al., "The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers," *Molecular Therapy*, vol. 20, no. 4, pp. 749–758, 2012.

[83] C. J. Breitbach, A. Moon, J. Burke, T. H. Hwang, and D. H. Kirn, "A phase 2, open-label, randomized study of Pexa-Vec (JX-594) administered by intratumoral injection in patients with unresectable primary hepatocellular carcinoma," *Methods in Molecular Biology*, vol. 1317, pp. 343–357, 2015.

[84] J. W. Ady, J. Heffner, K. Mojica et al., "Oncolytic immunotherapy using recombinant vaccinia virus GLV-1h68 kills sorafenib-resistant hepatocellular carcinoma efficiently," *Surgery*, vol. 156, no. 2, pp. 263–269, 2014.

[85] J. Wang, L. Xu, W. Zeng et al., "Treatment of human hepatocellular carcinoma by the oncolytic herpes simplex virus G47delta," *Cancer Cell International*, vol. 14, no. 1, p. 83, 2014.

Hindawi
Journal of Immunology Research
Volume 2018, Article ID 7132868, 10 pages
https://doi.org/10.1155/2018/7132868

Research Article

Clinicopathological Features and Increased Expression of Toll-Like Receptor 4 of Gastric Cardia Cancer in a High-Risk Chinese Population

Guangcan Chen,[1] Muming Xu,[2] Jingyao Chen,[3] Liangli Hong,[4] Wenting Lin,[3] Shukun Zhao,[3] Guohong Zhang,[3] Guo Dan,[3] and Shuhui Liu[3]

[1]*Department of Gastrointestinal Surgery, The First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province 515041, China*
[2]*Department of Abdominal Surgery, The Tumor Hospital of Shantou University Medical College, Shantou, Guangdong Province 515041, China*
[3]*Department of Pathology, Shantou University Medical College, Shantou, Guangdong Province 515031, China*
[4]*Department of Pathology, The First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province 515041, China*

Correspondence should be addressed to Shuhui Liu; liushuhuistu@126.com

Received 26 July 2017; Revised 14 November 2017; Accepted 28 December 2017; Published 18 February 2018

Academic Editor: Qing Lin

The incidence of gastric cardia cancer (GCC) is high in China. However, the clinicopathological characteristics and the carcinogenesis of GCC are unclear. Toll-like receptor 4 (TLR4) is an important innate immunity receptor and has a role in non-GCC (NGCC). We compared the clinicopathological characteristics of GCC patients from a high-risk area in China to esophageal cancer (EC) patients. Immunohistochemistry for TLR4 was performed in 201 histological samples of normal gastric cardia mucosa ($n = 11$), gastric cardia inflammation ($n = 87$), and GCC ($n = 103$). We included 84 patients with EC and 99 with GCC. GCC tissue was more poorly differentiated than EC tissue and more invasive, with more histomorphologic variation. Lymph node metastasis was more frequent in GCC than in EC. The *Helicobacter pylori* infection rate was higher but not significantly with GCC than EC. Survival was shorter with lymph node metastasis. We found a statistically significant trend for progressive increase of TLR4 expression from normal mucosa to inflammation in GCC. GCC in this high-risk area displays clinicopathologic characteristics different from those of EC and different from those of gastroesophageal junction carcinomas in other countries, although this was not analyzed statistically. Increased TLR4 expression in gastric cardia lesions may be associated with GCC tumorigenesis.

1. Introduction

Gastric cancer is the fifth most common malignancy, with an estimated 952,000 new cases in 2012 worldwide [1]. Although gastric cancer is still a major contributor to the global cancer burden, its incidence has decreased over the past decades [1].

Generally, gastric cancers can be classified into two categories: gastric cardia cancer (GCC) arising in the area of the stomach adjacent to the gastroesophageal junction (GEJ) and non-GCC (NGCC) arising from more distal regions of the stomach [2]. The incidence and risk factors for GCC and NGCC vary considerably. The decreasing incidence of gastric cancer is due mostly to the declining trend of NGCC; however, the incidence of GCC may be increasing [3–5]. In addition to different epidemiology, GCC and NGCC are thought to have different risk factors, clinicopathologic

features, and gene expression [6–8]. Thus, current research is addressing GCC and NGCC as separate diseases.

From the *Cancer Incidence in Five Continents, Vol. X* (CI5X) and "GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11" [9, 10], Colquhoun et al. estimated 260,000 cases of GCC worldwide in 2012, comprising 27% of the total gastric cancer cases, most of which occurred in Eastern/Southeastern Asia (59%), followed by Central Asia (15%). More than half of the total cases occurred in China (135,000; 52%) [2]. In high-risk areas in Asia [11–13], the incidence of GCC shows a characteristic geographic aggregation with esophageal cancer (EC). China has six high-risk EC regions, including the Taihang Mountain area, the Qingling Mountain area, North of Sichuan Province, the Dabie Mountain area, East of Guangdong Province, and the Subei area [5]. The Chaoshan high-risk area east of Guangdong is the only littoral high-risk area. Our previous epidemiological study showed an extremely high incidence of EC (74.47/100,000) and GCC (34.81/100,000) on Nan'ao Island in the Chaoshan area from 1995 to 2004 [11].

EC and GCC used to be treated as a single disease because of the coincidence of the two cancers, similar clinical symptoms, and limited techniques to distinguish them. Since the 1990s, with widespread use of endoscopy and a new classification by the World Health Organization (WHO), GCC is diagnosed as a disease different from EC in high-risk areas in China; however, the tumorigenesis, pathogenesis, development, and prognosis of GCC from high-risk areas are poorly understood. Although GCC has the same geographic distribution as EC in China [14], previous study showed that they differ in histopathology, although this was not analyzed statistically [15]. Recent epidemiological data have shown that the incidence of EC is declining as compared with an increasing trend of GCC in high-risk areas [16]. GCC in high-risk areas is also different from carcinomas of the GEJ studied primarily in Caucasian populations, which are believed to be related to gastroesophageal reflux disease (GERD) [3] and Barrett's esophagus (BE) [17, 18]. BE-related GCC are rare in China [19–21]. GERD prevalence in China was reported in some areas of China, and it is reported GERD rates in Hong Kong, China, have risen over the last decade. However the incidence rate of GERD in China is still much lower than that reported in Western countries [22–24].

Helicobacter pylori (H. pylori) infection has been confirmed as an important factor for distal gastric cancer [25]. Its relationship with GCC remains controversial [26–29]. Our previous study suggested that persistent *H. pylori* infection and the related chronic inflammation may contribute to the high incidence of GCC in the Chaoshan high-risk area [29]. Toll-like receptors are essential for *H. pylori* recognition and they initiate inflammatory pathways that may acquire oncogenic potential [30–32]. Toll-like receptor 4 (TLR4) can recognize lipopolysaccharide, a component of the bacterial cell wall [33]. The relationship between TLR4 and gastric cancer has been well studied [34–38]. However, most of these studies did not distinguish GCC from NGCC. No study has addressed TLR4 protein expression restricted to gastric cardia subtype.

In the present study, we compared the clinicopathologic features of GCC and EC in the Chaoshan high-risk area in China. TLR4 expression was evaluated by immunohistochemistry in normal gastric cardia mucosa, chronic gastric cardia inflammation, and GCC, to better understand the potential role of TLR4 in gastric cardia carcinogenesis. Despite different classifications and definitions of cancers originating around the GEJ [39, 40], we defined GCC as carcinoma in which the epicentre is ≤2 cm below the GEJ, the most accepted definition in China [16].

2. Results

2.1. Clinicopathological Results. We included 84 patients with EC (male : female ratio 3 : 1 and mean age 57.14 ± 10.28 years) and 99 with GCC (male : female ratio 5.6 : 1 and mean age 62.7 ± 7.79 years). Patients with EC were younger than those with GCC. Although more GCC than EC patients were male, the difference in the male : female ratio between the two groups was not considerable (Table 1).

The median tumor size was larger with GCC than that with EC (5.92 ± 2.22 versus 4.98 ± 1.51 cm; $P = 0.001$) (Table 1). For 66 EC tumors (78.57%), the epicentres were in the middle thoracic part of the esophagus (Table 1). Representative gross images of EC and GCC are in Figures 1 and 2. The microscopic features of the tumors are summarized in Table 2. Compared with EC tumors, GCC tumors were significantly more poorly differentiated ($P < 0.001$) and exhibited a wider histopathological spectrum. All 84 EC tumors (100%) were squamous cell carcinoma. In contrast, 75.8% of GCC tumors were tubular adenocarcinoma; the remainder were mucinous carcinoma (16.2%), adenosquamous carcinoma (4%), small-cell undifferentiated carcinoma ($n = 2$, 2%), squamous cell carcinoma ($n = 1$, 1%), or a tumor of neuroendocrine phenotype ($n = 1$, 1%). Representative histology images are in Figures 1 and 2.

A significantly higher proportion of patients with GCC than EC showed advanced-stage disease ($P < 0.001$). In addition, a higher proportion of patients with GCC showed lymph node metastasis and deep infiltration ($P = 0.002$ and $P = 0.009$, respectively, Table 2). The tumor tissues from 25/44 patients with EC (56.81%) and 49/83 with GCC (59.03%) were positive for *H. pylori* cytotoxin-associated gene A (CagA), a virulence factor that may damage the gastric mucosa and cause inflammation and cell death. Almost all of the East Asian *H. pylori* strains are CagA positive. Detecting CagA can be used to detect CagA-positive *H. pylori* infection [41]. We found no significant difference in the rate of *H. pylori* infection between EC and GCC groups ($P = 0.59$) (Table 3).

2.2. Patient Survival. Survival did not differ among patients who received surgery alone and both surgery and adjuvant therapy, so we chose all 84 EC patients and 99 GCC patients who underwent surgery for survival analysis. The mean survival was shorter with EC than that with GCC (32.14 months, 95% CI 26.796–37.477 versus 43.05 months, 95% CI 34.933–51.165) but not significantly ($P = 0.731$) (Figure 3(a)). The cumulative survival with EC was better without than with

Table 1: Clinical and gross features of tumor patients with esophageal cancer (EC) and gastric cardia cancer (GCC).

Features	EC $n = 84$	GCC $n = 99$	P
Male : female ratio	63 : 21	84 : 15	0.095
Age, years, mean ± SD	57.14 ± 10.28	62.7 ± 7.79	0.003
Size, cm, mean ± SD	4.98 ± 1.51	5.92 ± 2.22	0.001
Epicentre location, n (%)			
Upper thoracic part	9 (10.71)	—	
Middle thoracic part	66 (78.57)	—	
Lower thoracic part	9 (10.71)	—	
Gastric cardia	—	99	

lymph node metastasis (38.83 ± 4 versus 25.45 ± 3.4, $P = 0.029$) (Figure 3(b)). The cumulative survival of patients with GCC was better with higher than lower TNM stage (76.4 ± 20.27 and 67.7 ± 15.28 versus 37.86 ± 4.09, $P = 0.041$; Figure 3(c)) and without than with lymph node metastasis (65.67 ± 8.75 versus 34.19 ± 4.17, $P = 0.001$; Figure 3(d)). On multivariable analysis, lymph node metastasis was independently associated with survival with GCC (HR 2.02, 95% confidence interval [CI] 1.058 to 3.837, $P = 0.033$).

The 3-year survival rate was better for patients with EC than that with GCC (40.5% versus 34.3%), but the 5-year survival rate was poorer with EC than that with GCC (22.6% versus 25.3%). Neither of these differences was statistically significant ($P = 0.392$ and $P = 0.678$, resp.).

2.3. TLR4 Expression in Gastric Cardia Specimens. In Table 4 and Figures 4 and 5, TLR4 expression in gastric cardia tissue is shown. Among the 98 nonmalignant gastric cardia tissues examined, 11 normal epithelia without inflammation had the lowest score for TLR4 expression, and TLR4 was not detectable in 5 of them. On immunohistochemistry, TLR4 expression was higher in inflamed epithelia than that in gastric cardia mucosa (Figure 4) but did not differ between mild and severe inflammation. TLR4 expression was detectable in most of the 97/103 GCC cases (94.17%). Moreover, strong TLR4 staining was found in well- and moderately differentiated GCC cases with tubular structures but weak or negative TLR4 staining in poorly differentiated tumors (Figure 5). In mucosa with inflammation, TLR4 was expressed in a polarized manner, particularly at the basolateral membrane. In contrast, cancer cells expressed TLR4 diffusely throughout the cytoplasm even in the nucleus. We found a statistically significant trend for increasing TLR4 expression from normal mucosa to gastric cardia inflammation and carcinoma ($P < 0.05$) (Table 4).

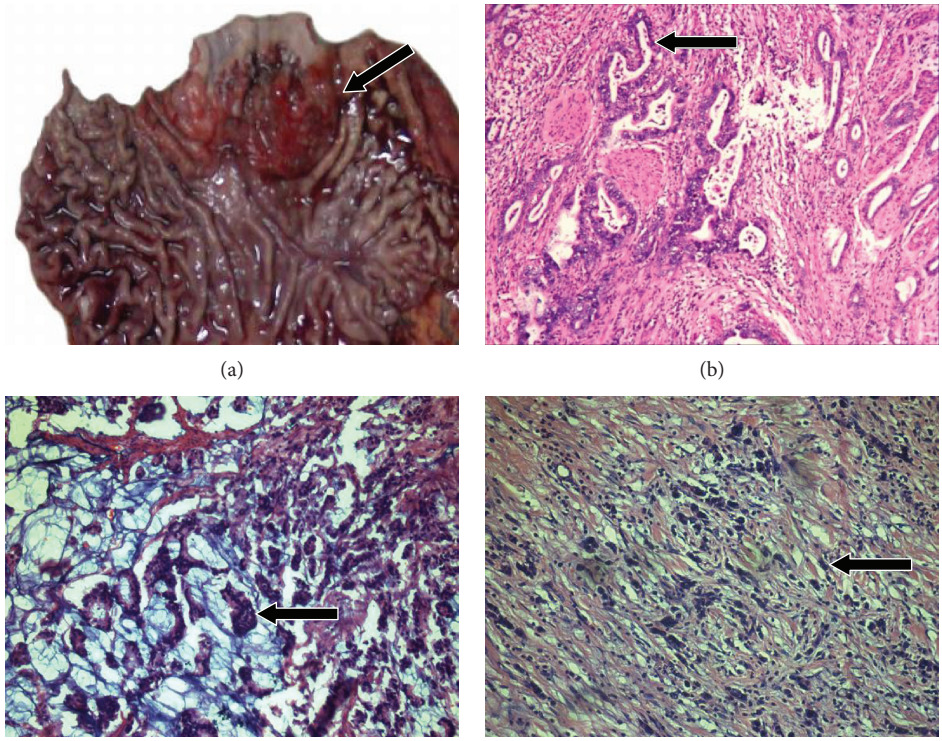
3. Discussion

In the present study, we compared clinical-pathological features between EC and GCC and described for the first time TLR4 expression restricted to the gastric cardia epithelium. Patients with GCC were significantly older and had a higher male : female ratio than patients with EC, although the difference in the latter variable was not considerable. 92.92% (92/99) of GCC patients were older than 50 years, and 72.62% (61/84) of EC patients were older than 50 years, indicating middle age and elderly people are high-risk group for both GCC and EC, especially for GCC. The mean age for GCC increased compared to the mean age of patients in the 1980s and 1990s [42]. It might be related to the Chinese social aging. A male predominance in the incidence of GCC and EC has been reported worldwide [11, 42, 43], and the male predominance is weaker in EC than that in GCC in this study. An assessment in China high-risk area for GCC indicated male : female ratio ranged from 1.68 to 5.6 [15, 16]. The reason for male predominance in GCC is still unclear. Although tobacco smoking is more prevalent in men than in women in China, the male predominance of GCC is unlikely to relate to this factor. A cohort study following 2 million person-years at risk indicated that the male predominance in GCC was similar among smokers and nonsmokers [44]. In China high-risk area, the male predominance in GCC may be caused by some sex-related genetic factors which need to be further studied.

GCC tumors were, on average, larger and more poorly differentiated, were of higher pathological stage, and were more likely to have lymph node metastasis and deeper invasion than EC tumors. Patients with GCC were more likely to have *H. pylori* infection, although the difference did not reach statistical significance. The GCC group had more histological variants than the EC group. GCC included adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, small-cell undifferentiated carcinoma, and neuroendocrine carcinoma. In contrast, in ECs, all tumors were squamous epithelial cell carcinoma. The most common location for EC in our sample was the middle thoracic part of the esophagus, whereas in the Western countries, EC originates mainly from the lower part of the esophagus, and the most common histological type is adenocarcinoma [17, 45]. Survival was poorer for both EC and GCC patients with than without lymph node metastasis. We found similar overall survival among our EC and GCC patients, even though the latter had a significantly higher proportion of lymph node metastasis and stage 3 and 4 tumors at the time of diagnosis. In light of these observations, GCC in high-risk areas in China has clinical and pathological features that differ from those of EC from the same area.

Although different studies have shown some shared genetic risk factors between EC and GCC from high-risk areas in China [14, 46–48], the different histology and surrounding anatomical structure of the esophagus and gastric cardia and respective risk factors indicate differences between EC and GCC [49].

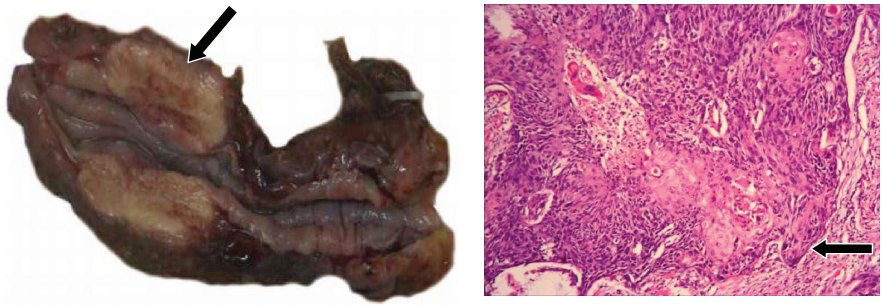
Huang et al. performed a study of the clinical and pathological features of GEJ carcinomas in Chinese and US patients. In terms of their data from the United States, patients with GCC in our group showed larger tumor size (5.92 ± 2.22 cm versus 3.5 ± 2.2 cm), lower 3-year (34.3% versus 43%) and 5-year (25.3% versus 28%) survival, and higher disease stage than the US patients [45]. Compared with adenocarcinoma of the GEJ in patients with BE mucosa from America, our patients with GCC showed deeper invasion,



(a) (b)

(c) (d)

Figure 1: Representative images of gastric cardia cancer (GCC). (a) Gross image of GCC below the gastroesophageal junction (arrow). (b) Histology of GCC with tubular formation (arrow). (c) Histology of mucinous GCC, arrow indicating tumor cells. (d) Histology of small-cell undifferentiated carcinoma, arrow indicating tumor cells.



(a) (b)

Figure 2: Representative images of esophageal cancer (EC). (a) Gross image of EC (arrow). (b) Histology of squamous cell EC, arrow indicating cancer nest.

higher disease stage, more lymph node metastases, and lower 5-year survival [50]. When comparing data on adenocarcinoma of the GEJ in patients from Japan, with the center located between 1 cm above and 2 cm below the GEJ [51], for our patients, the depth of tumor invasion was deeper, nodal metastases were more frequent, and the differentiation and 5-year survival were worse. In a meta-analysis, the 5-year survival for patients with cancer of the gastric cardia varied from 35% to 54.6% in China [52, 53]. All of these studies reported higher survival rates than we found in our study. Although these studies had discrepancies and overlapping descriptions of tumor location, careful consideration of these results suggests that GCC in the Chaoshan high-risk area in China is more aggressive with a worse prognosis.

Adenocarcinoma of the distal esophagus and Barrett's esophagus-related diseases remain uncommon in China. A large-scale longitudinal clinical and histological data was analyzed on 5401 esophageal cancer (EC) patients diagnosed during 10-year period (2002–2011) at Henan Taihang Mountain high risk area in China. All 217 esophageal adenocarcinoma (EAC) patients from these 5401 EC patients were examined, and EAC was relatively rare and accounted for approximately 5% of all esophageal cancers. Only 10 out of 217 (4.6%) EAC cases were detected to have any evidence

Table 2: Comparison of histopathology and pathological staging.

Microscopic features	EC $n = 84$	GCC $n = 99$	P
Tumor differentiation			<0.001
Well	29 (34.5)	5 (5.1)	
Moderate	50 (59.5)	49 (49.5)	
Poor	5 (6.0)	45 (45.5)	
Histology type			
Tubular adenocarcinoma	0 (0)	75 (75.8)	<0.001
Mucinous carcinoma	0 (0)	16 (16.2)	
Small-cell undifferentiated carcinoma	0 (0)	2 (2.0)	
Squamous cell carcinoma	84 (100)	1 (1.0)	
Adenosquamous	0 (0)	4 (4.0)	
Neuroendocrine carcinoma	0 (0)	1 (1.0)	
Lymph node metastasis	42 (50)	72 (72.7)	0.002
Serosal invasion	67 (70.8)	92 (92.9)	0.009
TNM stage			
0	0 (0)	0 (0)	<0.001
1	0 (0)	5 (5.1)	
2	40 (47.62)	10 (10.1)	
3	42 (50)	84 (84.8)	
4	2 (2.4)	0 (0)	

Data are n (%).

Table 3: *Helicobacter pylori* infection.

H. pylori infection	EC $n = 44$	GCC $n = 83$	P
Positive	19 (43.18)	40 (48.19)	0.59
Negative	25 (56.81)	43 (51.81)	

Data are n (%).

of Barrett's esophagus [20]. Though the GERD prevalence in GCC high-risk area in China is unclear, the prevalence of GERD symptoms in South China has varied from 2.3% to 3.8%, much lower than that in the Western countries [22, 23]. Patients with GCC in China and those in the Western countries might have different genetic polymorphisms, lifestyles, diet, and environmental influences [45]. *H. pylori* infection is the primary risk factor for distal gastric carcinomas in the Chinese population [54]. A case–cohort study with long-term follow-up in the Linzhou high-risk area found a strong association of GCC with *H. pylori* infection [55]. Our previous study found that *H. pylori* infection may contribute to the high incidence of GCC and esophageal squamous cell carcinoma in the Chaoshan region [29, 56]. Gastric carcinomas develop on the background of chronic active *H. pylori* gastritis via the epithelial precursor lesions. It is believed that a virulent bacterium in a genetically susceptible host is associated with more severe chronic inflammation, and this long-term inflammation may lead to cancer [31, 57]. Observing the adjacent tissue of GCC, we found that most cases showed chronic inflammatory cell infiltration. We speculated the carcinogenesis of GCC in high-risk area might be related to chronic inflammation similar to the NGCC.

Previous studies suggested that TLR4 expression might be the link between *H. pylori* infection and cancer [31, 37, 58], and this expression pattern is not significantly changed after the eradication of bacteria [59]. In the present study, TLR4 expression was evaluated in a cohort of gastric cardia tissues. To our knowledge, our study is the first to evaluate TLR4 protein expression from normal mucosa to different degree of inflammation and carcinoma restricted to gastric cardia tissue. TLR4 expression gradually increased from normal mucosa to gastric cardia inflammation and carcinoma, thereby providing pathological evidence that TLR4 expression is involved in GCC inflammation and carcinogenesis. Similar to other studies, our study showed that normal gastric cardia cancer has a very low expression of TLR4 [58, 60]. TLR4 expression was greatly increased during chronic inflammation, and there was no significant difference between mild inflammation and severe inflammation, suggesting changes in innate immune activation between normal and mild inflammation. As we have seen, GCC has the highest and diffuse TLR4 expression. TLR4 expression was not only in the cytoplasm but also in the nucleus. It was speculated that at this phase, the presence of infection was not absolutely necessary for epithelial stimulation [58]. Though we did not detect TLR4 expression in EC tissue, previous studies showed TLR4 appeared important to the pathogenesis of esophageal squamous cell carcinoma [61]. Confirming the potential role of TLR4 in the progression of gastric lesions, some studies associated TLR4 polymorphisms with the risk of gastric cancer [34, 62–64]. TLR4+896A>G polymorphism was reported as a risk factor for NGCC and its precursors. In contrast, prevalence of TLR4+896G was not significantly increased in GCC [34]. Considering that GCC has different characteristics from NGCC, more molecular and functional studies about TLR4 in GCC are necessary, and distinguishing GCC from NGCC is encouraged.

In summary, this study showed that GCC carcinomas are biologically different from EC carcinomas in the Chaoshan high-risk area in China, although they share genetic risk factors and similar geographic aggregation. GCC in this high-risk area displays different characteristics from those of GEJ carcinomas in developed countries as well. We detected TLR4 expression in gastric cardia epithelial cells and demonstrate a progressive increase in TLR4 expression from normal gastric cardia tissue, gastric cardia inflammation, and GCC, which suggests that TLR4 plays a role in GCC carcinogenesis.

4. Methods

4.1. Study Group. All surgical pathology reports with a final diagnosis of EC and GCC were collected from the Tumor Hospital and the First Affiliated Hospital of Shantou University Medical College in China. Not all patients underwent radiotherapy and chemotreatment postoperatively. All cases were divided into EC and GCC groups based on the location of the tumor epicentre. Inclusion criteria were (1) surgical

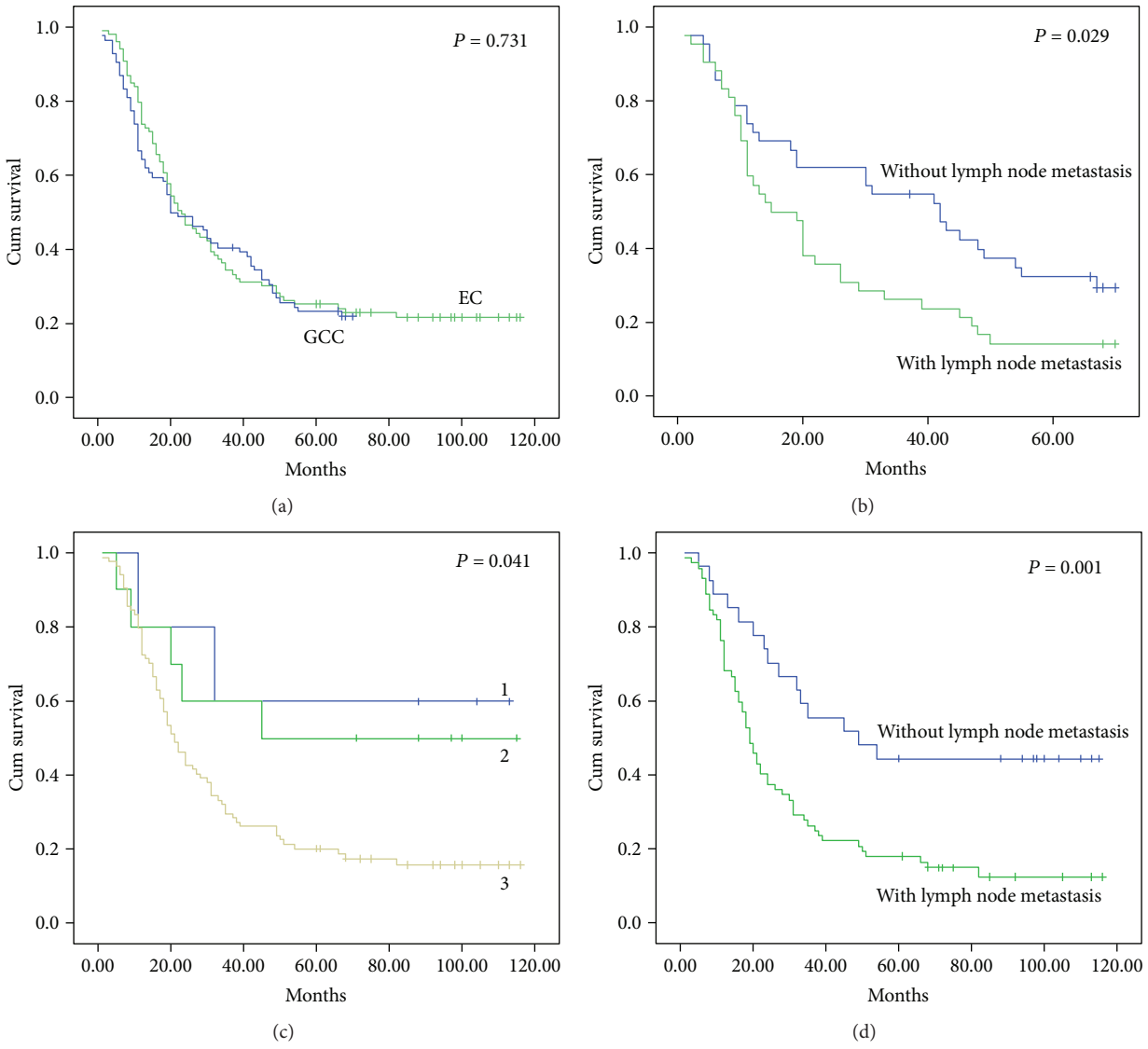


Figure 3: Kaplan-Meier survival curves for patients with GCC and EC. (a) Overall survival. (b) Cumulative survival with EC with and without lymph node metastasis. (c) Cumulative survival with GCC by TNM stage. 1: stage 1, 2: stage 2, 3: stage 3. (d) Cumulative survival with GCC with and without lymph node metastasis.

Table 4: Immunohistochemical evaluation of TLR4 expression.

	Normal $n = 11$	Mild carditis $n = 44$	Severe carditis $n = 43$	Carcinoma $n = 103$
TLR4	12.72 ± 15.71	$28.63 \pm 21.65^{*}$	$25.58 \pm 22.23^{*}$	$63.67 \pm 39.61^{*\#}$

TLR4: Toll-like receptor 4. Data are mean ± SD; $^{*}P < 0.05$ versus normal mucosa; $^{\#}P < 0.05$ versus mild or severe inflammation.

resection of tumors with lymph node dissection and (2) for GCC, the center of cancer within 2 cm of the GEJ on the gastric side. The GEJ was as defined by the WHO [46]. Most of the GCC tumors belonged to the AEG type II according to the Siewert classification [65]. Surgical details were collected principally from surgical notes and pathology findings of the resection specimen. GCCs were staged by the gastric TNM system, and ECs were staged by the esophageal TNM system, according to the American Joint Committee on Cancer Staging Manual [66]. Patients were followed for survival status by telephone or personal interview with the patient or family members. Patient consent for surgery and follow-up visit was obtained in all cases before surgical resection was performed. Informed consent was obtained from all patients. The methods were carried out in accordance with the approved guidelines. The Medical Ethics Committee of Shantou University Medical College approved the study protocol.

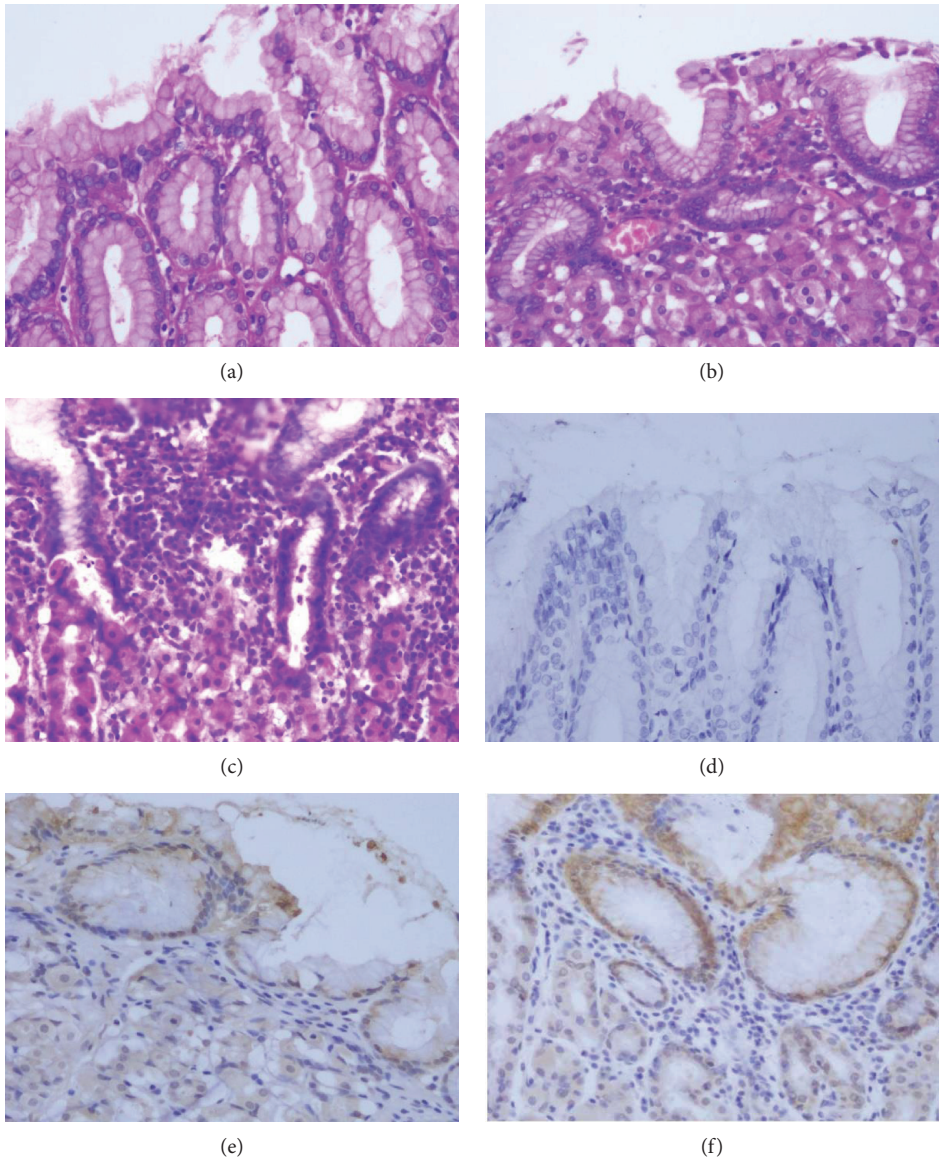


Figure 4: Representative IHC staining for TLR4 in gastric cardia mucosae with different degrees of chronic inflammation: (a) normal, (b) mild inflammation, (c) severe inflammation, (d) no immunostaining in normal mucosae, (e) weak positive staining in mucosae with mild inflammation, and (f) moderate positive staining in mucosae with severe inflammation.

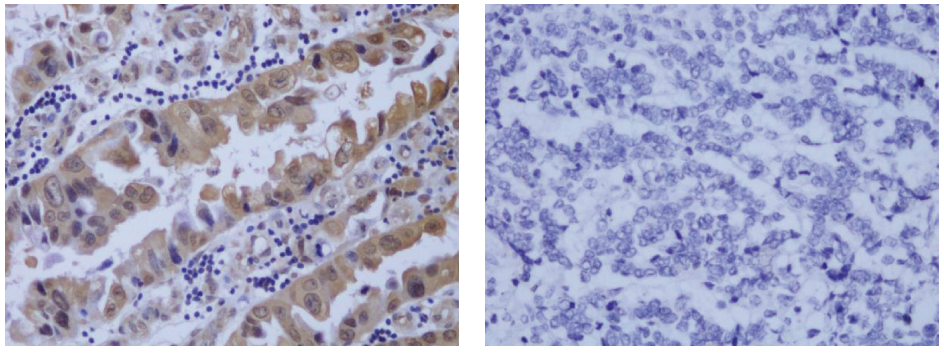


Figure 5: IHC staining for TLR4 in GCC tissue. (a) Strong positive staining in well-differentiated GCC cases with tubular structure. (b) Negative TLR4 staining in poorly differentiated tumor.

4.2. DNA Extraction and Amplification for Detecting H. pylori Infection. A total of 127 gastrointestinal mucosal tissue samples were collected from 83 patients with GCC and 44 with EC in the Tumor Hospital of Shantou University Medical College in China. Tissue DNA was extracted using a commercially available kit (Pure Link Genomic DNA Mini Kit, Invitrogen) according to the manufacturer's instructions [67]. The primers used were derived from the internal 300 bp fragment of the CagA as described [68]. The primer sequences used to detect CagA were 5′-ACCCTAGTCGG TAA TGGG-3′ and 5′-GCA AT TT TGT TAATCCGG TC-3′. These yielded a DNA fragment of 300 base pairs. A reaction mixture contained 3 μL extracted DNA, 4 μL primer, 3 μL of 10x PCR buffer, 0.3 μL AmpliTaq DNA polymerase, and 3 μL dNTP. The amplification cycle consisted of an initial denaturation at 94°C for 3 min, followed by 34 cycles at 94°C for 30 s, 50°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide.

4.3. Immunohistochemistry. Immunohistochemical (IHC) staining involved the Envision Labeled Peroxidase System (Dako, Carpinteria, CA). Paraffin-embedded samples were sectioned at 4 μm. Each sample was deparaffinized in xylene, rehydrated in a graded ethanol series, then preincubated with 3% hydrogen peroxide for 10 min. Antigen retrieval was performed by microwave heating. Following incubation in 10 mmol/L citrate buffer for 20 min, sections were incubated with primary antibody for TLR4 (rabbit, 1 : 100, Proteintech) at 4°C overnight, then horseradish peroxidase-conjugated goat antirabbit IgG antibody at 37°C for 30 min, and counterstained with haematoxylin. Images were captured under a Leica IM50 microscope (Imagic Bildverarbeitung AG, Wetzlar, Germany).

IHC slides were evaluated by two experienced pathologists in a blinded manner. Staining intensity score (0–3) was considered according to a subjective evaluation of the intensity of marked cells (0: no immunostaining; 1: weak positive staining; 2: moderate positive staining; 3: strong positive staining). The overall staining intensity (0–3) was multiplied by the proportion of positive cells (0–100%), and all values were added to generate a final score ranging from 0 to 300 [69].

4.4. Chronic Inflammation Grading. Inflammation in the gastric cardia tissue was graded as normal, mild, and severe according to the updated Sydney System [70]. The normal gastric mucosa contains only individual (0–5) scattered inflammatory cells in the lamina propria. Mild inflammation contains 5 to 30 inflammatory cells in the lamina propria per high-power (×40 objective) microscopic field or between the foveolae. More than 30 inflammatory cells per high-power field was considered severe inflammation.

4.5. Statistical Analysis. Differences in frequencies between the two groups were analyzed by using chi-square or Fisher's exact test, as appropriate. Survival was estimated by the Kaplan-Meier method with a log-rank test. Cox regression analysis was used to identify risk factors for overall survival. t-test for trend was used to evaluate the trend in increase or decrease in expression. All statistical analyses involved the use of SPSS v16 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by the Department of Education, Guangdong Government under the Top-tier University Development Scheme for Research and Control of Infectious Diseases, the Natural Science Foundation of China (Grant no. 81572684), the Natural Science Foundation of Guangdong Province (Grant no. 2014A030310139), and the National Natural Science Foundation of China (Grant no. 81702717).

References

[1] D. M. Parkin, S. L. Whelan, J. Ferlay, L. Teppo, and B. D. Thomas, *Cancer Incidence In Five Continents*, IARC Sci Publ No. 155. IARC, Lyon, 2002.

[2] A. Colquhoun, M. Arnold, J. Ferlay, K. J. Goodman, D. Forman, and I. Soerjomataram, "Global patterns of cardia and non-cardia gastric cancer incidence in 2012," *Gut*, vol. 64, no. 12, pp. 1881–1888, 2015.

[3] S. J. Crane, Richard Locke G3rd, W. S. Harmsen et al., "The changing incidence of oesophageal and gastric adenocarcinoma by anatomic sub-site," *Alimentary Pharmacology and Therapeutics*, vol. 25, no. 4, pp. 447–453, 2007.

[4] J. Shi, Q. Sun, B. Y. Xu et al., "Changing trends in the proportions of small (≤2 cm) proximal and non-proximal gastric carcinomas treated at a high-volume tertiary medical center in China," *Journal of Digestive Diseases*, vol. 15, no. 7, pp. 359–366, 2014.

[5] Z. C. Z. DONG, "Incidence and geographic characteristics of esophagus-gastric junction adenocarcinoma," *Chinese Journal of Clinical Oncology*, vol. 38, no. 1, pp. 57–60, 2011.

[6] F. Carneiro and P. Chaves, "Pathologic risk factors of adenocarcinoma of the gastric cardia and gastroesophageal junction," *Surgical Oncology Clinics of North America*, vol. 15, no. 4, pp. 697–714, 2006.

[7] R. D. Odze, "Pathology of the gastroesophageal junction," *Seminars in Diagnostic Pathology*, vol. 22, no. 4, pp. 256–265, 2005.

[8] J. Olsen, M. L. M. Espersen, P. Jess, L. T. Kirkeby, and J. T. Troelsen, "The clinical perspectives of CDX2 expression in colorectal cancer: a qualitative systematic review," *Surgical Oncology*, vol. 23, no. 3, pp. 167–176, 2014.

[9] D. Forman, F. Bray, D. H. Brewster et al., *Cancer Incidence in Five Continents, Vol. X (Electronic Version)*July 2014, http://ci5.iarc.fr.

[10] J. Ferlay, I. Soerjomataram, M. Ervik et al., "GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11," July 2014, http://globocan.iarc.fr.

[11] M. Su, M. Liu, D. P. Tian et al., "Temporal trends of esophageal cancer during 1995–2004 in Nanao Island, an extremely high-

risk area in China," *European Journal of Epidemiology*, vol. 22, no. 1, pp. 43–48, 2007.

[12] M. Babaei, F. Pourfarzi, A. Yazdanbod et al., "Gastric cancer in Ardabil, Iran – a review and update on cancer registry data," *Asian Pacific Journal of Cancer Prevention*, vol. 11, no. 3, pp. 595–599, 2010.

[13] N. Taghavi, D. Nasrollahzadeh, S. Merat et al., "Epidemiology of upper gastrointestinal cancers in Iran: a sub site analysis of 761 cases," *World Journal of Gastroenterology*, vol. 13, no. 40, pp. 5367–5370, 2007.

[14] L. D. Wang, F. Y. Zhou, X. M. Li et al., "Genome-wide association study of esophageal squamous cell carcinoma in Chinese subjects identifies a susceptibility locus at *PLCE1*," *Nature Genetics*, vol. 42, no. 9, pp. 759–763, 2010.

[15] C. L. Fu, M. Su, and D. P. Tian, "Clinipathologic analyses of 16954 cases with malignant tumor at esophageal and oesophagogastric junction in Chaoshan area, Guangdong Province," *China Cancer*, vol. 17, no. 5, pp. 421–425, 2008.

[16] C. W. Peng Xiabiao, C. Zhifeng, L. Zhiheng, O. Zhixiong, and W. Kuangrong, "Epidemiology of gastric cardia cancer in China," *Chinese Archives of General Surgery*, vol. 8, no. 2, pp. 156–159, 2014.

[17] P. Chandrasoma, K. Wickramasinghe, Y. Ma, and T. DeMeester, "Adenocarcinomas of the distal esophagus and "gastric cardia" are predominantly esophageal carcinomas," *The American Journal of Surgical Pathology*, vol. 31, no. 4, pp. 569–575, 2007.

[18] B. A. Whitson, S. S. Groth, Z. Li, R. A. Kratzke, and M. A. Maddaus, "Survival of patients with distal esophageal and gastric cardia tumors: a population-based analysis of gastroesophageal junction carcinomas," *The Journal of Thoracic and Cardiovascular Surgery*, vol. 139, no. 1, pp. 43–48, 2010.

[19] P. H. Tseng, Y. C. Lee, H. M. Chiu et al., "Prevalence and clinical characteristics of Barrett's esophagus in a Chinese general population," *Journal of Clinical Gastroenterology*, vol. 42, no. 10, pp. 1074–1079, 2008.

[20] S. Liu, J. Y. Dai, L. Yao et al., "Esophageal adenocarcinoma and its rare association with Barrett's esophagus in Henan, China," *PLoS One*, vol. 9, no. 10, article e110348, 2014.

[21] Q. Huang, D. C. Fang, C. G. Yu, J. Zhang, and M. H. Chen, "Barrett's esophagus-related diseases remain uncommon in China," *Journal of Digestive Diseases*, vol. 12, no. 6, pp. 420–427, 2011.

[22] M. Chen, L. Xiong, H. Chen, A. Xu, L. He, and P. Hu, "Prevalence, risk factors and impact of gastroesophageal reflux disease symptoms: a population-based study in South China," *Scandinavian Journal of Gastroenterology*, vol. 40, no. 7, pp. 759–767, 2005.

[23] V. P.-Y. Tan, B. C. Y. Wong, W. M. Wong et al., "Gastroesophageal reflux disease: cross-sectional study demonstrating rising prevalence in a Chinese population," *Journal of Clinical Gastroenterology*, vol. 50, no. 1, pp. e1–e7, 2016.

[24] H. B. El-Serag, "Time trends of gastroesophageal reflux disease: a systematic review," *Clinical Gastroenterology and Hepatology*, vol. 5, no. 1, pp. 17–26, 2007.

[25] C. de Martel, J. Ferlay, S. Franceschi et al., "Global burden of cancers attributable to infections in 2008: a review and synthetic analysis," *The Lancet Oncology*, vol. 13, no. 6, pp. 607–615, 2012.

[26] Helicobacter and Cancer Collaborative Group, "Gastric cancer and *Helicobacter pylori*: a combined analysis of 12 case control studies nested within prospective cohorts," *Gut*, vol. 49, no. 3, pp. 347–353, 2001.

[27] F. Kamangar, S. M. Dawsey, M. J. Blaser et al., "Opposing risks of gastric cardia and noncardia gastric adenocarcinomas associated with *Helicobacter pylori* seropositivity," *Journal of the National Cancer Institute*, vol. 98, no. 20, pp. 1445–1452, 2006.

[28] S. J. Cho, I. J. Choi, C. G. Kim et al., "Helicobacter pylori seropositivity is associated with gastric cancer regardless of tumor subtype in Korea," *Gut and Liver*, vol. 4, no. 4, pp. 466–474, 2010.

[29] Y. Wang, S. Liu, Y. Zhang et al., "*Helicobacter pylori* infection and gastric cardia cancer in Chaoshan region," *Microbes and Infection*, vol. 16, no. 10, pp. 840–844, 2014.

[30] S. Akira and K. Takeda, "Toll-like receptor signalling," *Nature Reviews Immunology*, vol. 4, no. 7, pp. 499–511, 2004.

[31] P. Pimentel-Nunes, N. Gonçalves, I. Boal-Carvalho et al., "*Helicobacter pylori* induces increased expression of Toll-like receptors and decreased Toll-interacting protein in gastric mucosa that persists throughout gastric carcinogenesis," *Helicobacter*, vol. 18, no. 1, pp. 22–32, 2013.

[32] N. Castaño-Rodríguez, N. O. Kaakoush, A. L. Pardo, K.-L. Goh, K. M. Fock, and H. M. Mitchell, "Genetic polymorphisms in the toll-like receptor signalling pathway in *Helicobacter pylori* infection and related gastric cancer," *Human Immunology*, vol. 75, no. 8, pp. 808–815, 2014.

[33] K. Takeda, T. Kaisho, and S. Akira, "Toll-like receptors," *Annual Review of Immunology*, vol. 21, no. 1, pp. 335–376, 2003.

[34] G. L. Hold, C. S. Rabkin, W. H. Chow et al., "A functional polymorphism of toll-like receptor 4 gene increases risk of gastric carcinoma and its precursors," *Gastroenterology*, vol. 132, no. 3, pp. 905–912, 2007.

[35] H. Huhta, O. Helminen, J. H. Kauppila et al., "The expression of Toll-like receptors in normal human and murine gastrointestinal organs and the effect of microbiome and cancer," *The Journal of Histochemistry & Cytochemistry*, vol. 64, no. 8, pp. 470–482, 2016.

[36] G. Li, Z. Wang, J. Ye et al., "Uncontrolled inflammation induced by AEG-1 promotes gastric cancer and poor prognosis," *Cancer Research*, vol. 74, no. 19, pp. 5541–5552, 2014.

[37] X. Yuan, Y. Zhou, W. Wang et al., "Activation of TLR4 signaling promotes gastric cancer progression by inducing mitochondrial ROS production," *Cell Death & Disease*, vol. 4, no. 9, article e794, 2013.

[38] B. Schmaußer, M. Andrulis, S. Endrich, H.-K. Müller-Hermelink, and M. Eck, "Toll-like receptors TLR4, TLR5 and TLR9 on gastric carcinoma cells: an implication for interaction with *Helicobacter pylori*," *International Journal of Medical Microbiology*, vol. 295, no. 3, pp. 179–185, 2005.

[39] L. Wang Lidong, Z. Yanxia, J. Xinying et al., "Analysis on the occurrence site of gastric cardia adenocarcinoma in high incidence area in Henan," *Journal of Zhengzhou University*, vol. 42, no. 3, pp. 389–393, 2007.

[40] M. J. Blaser and D. Saito, "Trends in reported adenocarcinomas of the oesophagus and gastric cardia in Japan," *European Journal of Gastroenterology & Hepatology*, vol. 14, no. 2, pp. 107–113, 2002.

[41] M. Hatakeyama and H. Higashi, "*Helicobacter pylori* CagA: a new paradigm for bacterial carcinogenesis," *Cancer Science*, vol. 96, no. 12, pp. 835–843, 2005.

[42] B. Zheng, Y.-B. Chen, Y. Hu, J.-Y. Wang, Z.-W. Zhou, and J.-H. Fu, "Trend analysis for clinical characteristics and prognosis of adenocarcinoma of cardia," *Chinese Journal of Cancer*, vol. 29, no. 1, pp. 94–97, 2010.

[43] J. Falk, H. Carstens, L. Lundell, and M. Albertsson, "Incidence of carcinoma of the oesophagus and gastric cardia. Changes over time and geographical differences," *Acta Oncologica*, vol. 46, no. 8, pp. 1070–1074, 2007.

[44] N. D. Freedman, M. H. Derakhshan, C. C. Abnet, A. Schatzkin, A. R. Hollenbeck, and K. E. L. McColl, "Male predominance of upper gastrointestinal adenocarcinoma cannot be explained by differences in tobacco smoking in men versus women," *European Journal of Cancer*, vol. 46, no. 13, pp. 2473–2478, 2010.

[45] Q. Huang, X. Fan, A. T. Agoston et al., "Comparison of gastro-oesophageal junction carcinomas in Chinese versus American patients," *Histopathology*, vol. 59, no. 2, pp. 188–197, 2011.

[46] D. Wen, B. Shan, S. Wang et al., "A positive family history of esophageal/gastric cardia cancer with gastric cardia adenocarcinoma is associated with a younger age at onset and more likely with another synchronous esophageal/gastric cardia cancer in a Chinese high-risk area," *European Journal of Medical Genetics*, vol. 53, no. 5, pp. 250–5, 2010.

[47] C. C. Abnet, N. D. Freedman, N. Hu et al., "A shared susceptibility locus in *PLCE1* at 10q23 for gastric adenocarcinoma and esophageal squamous cell carcinoma," *Nature Genetics*, vol. 42, no. 9, pp. 764–767, 2010.

[48] C. Wu, Z. Hu, Z. He et al., "Genome-wide association study identifies three new susceptibility loci for esophageal squamous-cell carcinoma in Chinese populations," *Nature Genetics*, vol. 43, no. 7, pp. 679–684, 2011.

[49] S. A. Savage et al., "Variants of the *IL8* and *IL8RB* genes and risk for gastric cardia adenocarcinoma and esophageal squamous cell carcinoma," *Cancer Epidemiology, Biomarkers & Prevention*, vol. 13, no. 12, pp. 2251–2257, 2004.

[50] G. Portale, J. H. Peters, J. A. Hagen et al., "Comparison of the clinical and histological characteristics and survival of distal esophageal–gastroesophageal junction adenocarcinoma in patients with and without Barrett mucosa," *Archives of Surgery*, vol. 140, no. 6, pp. 570–574, 2005.

[51] S. Hasegawa, T. Yoshikawa, H. Cho, A. Tsuburaya, and O. Kobayashi, "Is adenocarcinoma of the esophagogastric junction different between Japan and western countries? The incidence and clinicopathological features at a Japanese high-volume cancer center," *World Journal of Surgery*, vol. 33, no. 1, pp. 95–103, 2009.

[52] W. L. Fang, C. W. Wu, J. H. Chen et al., "Esophagogastric junction adenocarcinoma according to Siewert classification in Taiwan," *Annals of Surgical Oncology*, vol. 16, no. 12, pp. 3237–3244, 2009.

[53] S. Y. Law, M. Fok, S. W. Cheng, and J. Wong, "A comparison of outcome after resection for squamous cell carcinomas and adenocarcinomas of the esophagus and cardia," *Surgery, Gynecology & Obstetrics*, vol. 175, no. 2, pp. 107–112, 1992.

[54] Q. Huang, J. Shi, Q. Sun et al., "Clinicopathological characterisation of small (2cm or less) proximal and distal gastric carcinomas in a Chinese population," *Pathology*, vol. 47, no. 6, pp. 526–532, 2015.

[55] F. Kamangar, Y. L. Qiao, M. J. Blaser et al., "*Helicobacter pylori* and oesophageal and gastric cancers in a prospective study in China," *British Journal of Cancer*, vol. 96, no. 1, pp. 172–176, 2007.

[56] W.-s. Li, D.-p. Tian, X.-y. Guan et al., "Esophageal intraepithelial invasion of *Helicobacter pylori* correlates with atypical hyperplasia," *International Journal of Cancer*, vol. 134, no. 11, pp. 2626–2632, 2014.

[57] M. R. Amieva and E. M. El-Omar, "Host-bacterial interactions in *Helicobacter pylori* infection," *Gastroenterology*, vol. 134, no. 1, pp. 306–323, 2008.

[58] P. Pimentel-Nunes, L. Afonso, P. Lopes et al., "Increased expression of toll-like receptors (TLR) 2, 4 and 5 in gastric dysplasia," *Pathology & Oncology Research*, vol. 17, no. 3, pp. 677–683, 2011.

[59] A. C. T. Cadamuro, A. F. T. Rossi, J. M. Biselli-Périco et al., "Effect of Helicobacter pylori eradication on TLR2 and TLR4 expression in patients with gastric lesions," *Mediators of Inflammation*, vol. 2015, Article ID 481972, 9 pages, 2015.

[60] B. Schmausser, M. Andrulis, S. Endrich et al., "Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection," *Clinical and Experimental Immunology*, vol. 136, no. 3, pp. 521–526, 2004.

[61] I. Sheyhidin, G. Nabi, A. Hasim et al., "Overexpression of TLR3, TLR4, TLR7 and TLR9 in esophageal squamous cell carcinoma," *World Journal of Gastroenterology*, vol. 17, no. 32, pp. 3745–3751, 2011.

[62] B. R. Achyut, U. C. Ghoshal, N. Moorchung, and B. Mittal, "Association of Toll-like receptor–4 (Asp299Gly and Thr399Ileu) gene polymorphisms with gastritis and precancerous lesions," *Human Immunology*, vol. 68, no. 11, pp. 901–907, 2007.

[63] A. Hishida, K. Matsuo, Y. Goto et al., "*Toll-like* receptor *4* +3725 G/C polymorphism, *Helicobacter pylori* seropositivity, and the risk of gastric atrophy and gastric cancer in Japanese," *Helicobacter*, vol. 14, no. 1, pp. 47–53, 2009.

[64] T. H. Zou, Z. H. Wang, and J. Y. Fang, "Positive association between Toll-like receptor 4 gene +896A/G polymorphism and susceptibility to gastric carcinogenesis: a meta-analysis," *Tumour Biology*, vol. 34, no. 4, pp. 2441–2450, 2013.

[65] J. R. Siewert and H. J. Stein, "Classification of adenocarcinoma of the oesophagogastric junction," *British Journal of Surgery*, vol. 85, no. 11, pp. 1457–1459, 1998.

[66] American Joint Committee on Cancer, "Esophagus and esophagogastric junction," in *AJCC Cancer Staging Manual*, pp. 129–144, Springer, New York, NY, USA, 7th edition, 2009.

[67] P. I. Hsu, I.r. Hwang, D. Cittelly et al., "Clinical presentation in relation to diversity within the *Helicobacter pylori cag* pathogenicity island," *The American Journal of Gastroenterology*, vol. 97, no. 9, pp. 2231–2238, 2002.

[68] G. D. Liu, L. H. He, Y. X. Gu, N. M. Yang, and J. Z. Zhang, "Using PCR with different primers to detect Helicobacter pylori in gastric biopsy specimens," *Journal of Pathogen Biology*, vol. 6, no. 6, pp. 401–410, 2011.

[69] Q. Xu, Y. Xu, B. Pan et al., "TTK is a favorable prognostic biomarker for triple-negative breast cancer survival," *Oncotarget*, vol. 7, no. 49, pp. 81815–81829, 2016.

[70] J. Powell and C. C. McConkey, "Increasing incidence of adenocarcinoma of the gastric cardia and adjacent sites," *British Journal of Cancer*, vol. 62, no. 3, pp. 440–3, 1990.

Hindawi
Journal of Immunology Research
Volume 2018, Article ID 2584243, 11 pages
https://doi.org/10.1155/2018/2584243

Review Article

Mast Cell, the Neglected Member of the Tumor Microenvironment: Role in Breast Cancer

Angélica Aponte-López,[1,2] Ezequiel M. Fuentes-Pananá,[1] Daniel Cortes-Muñoz,[3] and Samira Muñoz-Cruz[4]

[1]*Unidad de Investigación en Virología y Cáncer, Hospital Infantil de México Federico Gómez, Dr. Márquez 162, Doctores, Cuauhtémoc, 06720 Ciudad de Mexico, Mexico*
[2]*Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM), Ciudad de México, Mexico*
[3]*Programa de Servicio Social en Investigación en Salud, Departamento de Inmunología y Reumatología, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga 15, Tlalpan, 14080 Ciudad de Mexico, Mexico*
[4]*Unidad de Investigación Médica en Enfermedades Infecciosas y Parasitarias, Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, Avenida Cuauhtémoc 330, Doctores, Cuauhtémoc, 06720 Ciudad de Mexico, Mexico*

Correspondence should be addressed to Samira Muñoz-Cruz; mcsamira@yahoo.com.mx

Received 25 August 2017; Revised 8 November 2017; Accepted 26 November 2017; Published 5 February 2018

Academic Editor: Shi Jin

Mast cells are unique tissue-resident immune cells that secrete a diverse array of biologically active compounds that can stimulate, modulate, or suppress the immune response. Although mounting evidence supports that mast cells are consistently infiltrating tumors, their role as either a driving or an opposite force for cancer progression is still controversial. Particularly, in breast cancer, their function is still under discussion. While some studies have shown a protective role, recent evidence indicates that mast cells enhance blood and lymphatic vessel formation. Interestingly, one of the most important components of the mast cell cargo, the serine protease tryptase, is a potent angiogenic factor, and elevated serum tryptase levels correlate with bad prognosis in breast cancer patients. Likewise, histamine is known to induce tumor cell proliferation and tumor growth. In agreement, mast cell depletion reduces the size of mammary tumors and metastasis in murine models that spontaneously develop breast cancer. In this review, we will discuss the evidence supporting protumoral and antitumoral roles of mast cells, emphasizing recent findings placing mast cells as important drivers of tumor progression, as well as the potential use of these cells or their mediators as therapeutic targets.

1. Introduction

The association between chronic inflammation and cancer has long been recognized. Inflammation evolved as part of the body's defense against internal and external stimuli that disrupt tissue homeostasis. It aims to eliminate the stimuli, repair the damaged tissue, and reestablish homeostasis. When inflammation is maintained for a short period of time, it usually comes with therapeutic consequences; however, when it is chronically sustained, it has the potential to enhance or promote the emergence of malignancies [1–3]. Virchow proposed a link between chronic inflammation and cancer as early as the 19th century, and he hypothesized that inflamed tissues were the primed sites in which cancer lesions were initiated [4]. Indeed, mounting evidence supports that chronic inflammation provides conditions that lead to malignant transformation. Immune cells persistently infiltrating tissues are actively inducing oxidative stress and releasing soluble mediators, such as cytokines, chemokines, and growth factors, which alter genes and proteins involved in cell cycle, DNA repair, and apoptosis [5, 6]. Besides initiation, chronic inflammation seems to be continually important during tumor progression, creating a favorable microenvironment that contributes to tumor

cell proliferation, survival, invasion, migration, tissue remodeling, and angiogenesis, ending in cancer metastasis [7].

Epidemiological data estimate that at least one-third of all cancers are associated with chronic infections or with evident long-lasting unresolved inflammation [8, 9]. Some of the well-described infection- and inflammation-associated cancers are gastric, colorectal, cervical, and hepatocellular carcinoma [3, 10]. Breast cancer has also been associated with chronic inflammation, although the inflammatory stimulus is less clear. The stroma of breast tumors is generally enriched with a great variety of inflammatory cells, which however do not seem to be protective. Moreover, several studies indicate that tumor cells can evade the immune responses and enhance inflammation favoring cancer evolution to aggressive stages [11, 12]. Among the best characterized immune cell populations present in the stroma of breast cancers are the tumor-associated macrophages, which have been linked to cancer aggressive features, such as angiogenesis, degradation of extracellular matrix (ECM) proteins, and invasion [13]. Likewise, it has become evident that other immune cells, such as neutrophils and mast cells, are consistently found in the breast cancer stroma, most likely contributing to the inflammatory microenvironment that shapes cancer behavior [13, 14]. In this review, we will discuss the evidence supporting protumoral and antitumoral roles of mast cells in breast cancer progression.

2. Mast Cell Biology

Mast cells are granulated innate immune cells characterized by their cargo of inflammatory mediators, comprised of a wide array of preformed bioactive molecules stored in cytoplasmic granules, which are released upon encountering the appropriate stimuli and have beneficial roles in immunological responses against pathogens, including intestinal helminths, bacteria, and viruses. Mast cell-derived mediators also participate in tissue physiological processes, such as wound healing and tissue repair, and in some pathological conditions [15]. For instance, IgE-induced mast cell degranulation triggers the immediate hypersensitivity reactions that play a central role in the pathogenesis of allergic diseases [16].

Mast cells are distributed in diverse tissues throughout the body, but a considerable number of them are located close to blood vessels, nerves, and mucosal surfaces. Some of the tissues in which they are most prominent are the dermis, hypodermis, and the respiratory and gastrointestinal tract [17, 18]. Like other immune cells, mast cells originate in the bone marrow from hematopoietic stem cells via a multipotent progenitor, which can become a committed mast cell progenitor (MCP) that exits the marrow and migrates to peripheral tissues to complete maturation. Early mast cell progenitors in bone marrow do not contain cytoplasmic granules and do not express FcεRI on their surface. A slightly more differentiated MCP, identified in tissues in mice and in bone marrow in rats, contains few small cytoplasmic granules, express high levels of integrin $\beta 7$, and can often also express the FcεRI. This seems to be the mast cell progenitor that leaves the bone marrow [19–22]. The MCPs arrive to diverse peripheral tissues by transendothelial migration in which they complete their differentiation under the control of microenvironmental cytokines and growth factors [23, 24]. Over the last years, several models for mast cell development have been proposed; however, the ontogenesis of mast cells in mice and humans is only beginning to be understood, and knowledge of the specific signals that modulate progenitor recruitment and differentiation is still limited. Mast cell development in mice and humans share some similarities but also exhibit major differences. In mice, different mast cell progenitor populations have been described depending on the particular strain [19–22]. So far, very few studies have attempted to characterize the mechanisms involved in human mast cell development [25–27]. Outstandingly, a recent study has identified a blood-derived human mast cell progenitor population that gives rise exclusively to mast cells. These cells express the FcεRI and integrin $\beta 7$ and display a mast cell-like phenotype, although with a limited cell division capacity *in vitro* [28]. In both humans and mice, a complex network of signaling molecules and transcription factors regulates formation of MCPs in bone marrow and their migration to tissues in which they develop into fully competent mature mast cells. Figure 1 illustrates a simplified overview of mast cell development and heterogeneity.

Mast cell differentiation, growth, and survival are strongly regulated by local tissue environmental factors. Stem cell factor (SCF), the ligand of the c-Kit receptor, and IL-3 are among the best-characterized factors. SCF is mainly secreted by fibroblasts and other mesenchymal cells and has an important role in survival, development, and expansion of mast cells [29, 30]. While, IL-3 is considered the main cytokine responsible for the T cell-induced proliferation and differentiation of mast cells, at least in rodents [31, 32]. Other endogenous factors contributing to mast cell maturation and function in rodents and humans are IL-4, IL-6, IL-9, IL-10, IL-33, nerve growth factor (NGF), and transforming growth factor-β (TGF-β) [31–34].

At least two major populations of mature mast cells have been described in humans based on their protease content. Mast cells containing only tryptase are termed MC_T, while those containing tryptase, chymase, carboxypeptidase A, and cathepsin G are named MC_{TC}. These mast cell subsets differ in their tissue localization; for instance, the MC_{TC} is the predominant type found in normal skin and small bowel submucosa, whereas the MC_T is almost the exclusive type found in small bowel mucosa and in bronchial/bronchiolar areas [35]. These mast cell subtypes also seem functionally different, since MC_{TC} responds to various nonimmunological stimuli such as compound 48/80 and substance P, while MC_T does not [36]. Similarly, two major populations of mature mast cells have been described in rodents, defined mainly according to the tissue in which they reside. Connective tissue mast cells (CTMCs) are preferentially located around venules and nerve endings of skin, peritoneal cavity, and the digestive tract muscularis propria, whereas mucosal mast cells (MMCs) are mainly found in the intestinal and respiratory mucosa [23, 37]. Some of the factors involved in the development and proliferation of the CTMC subtype

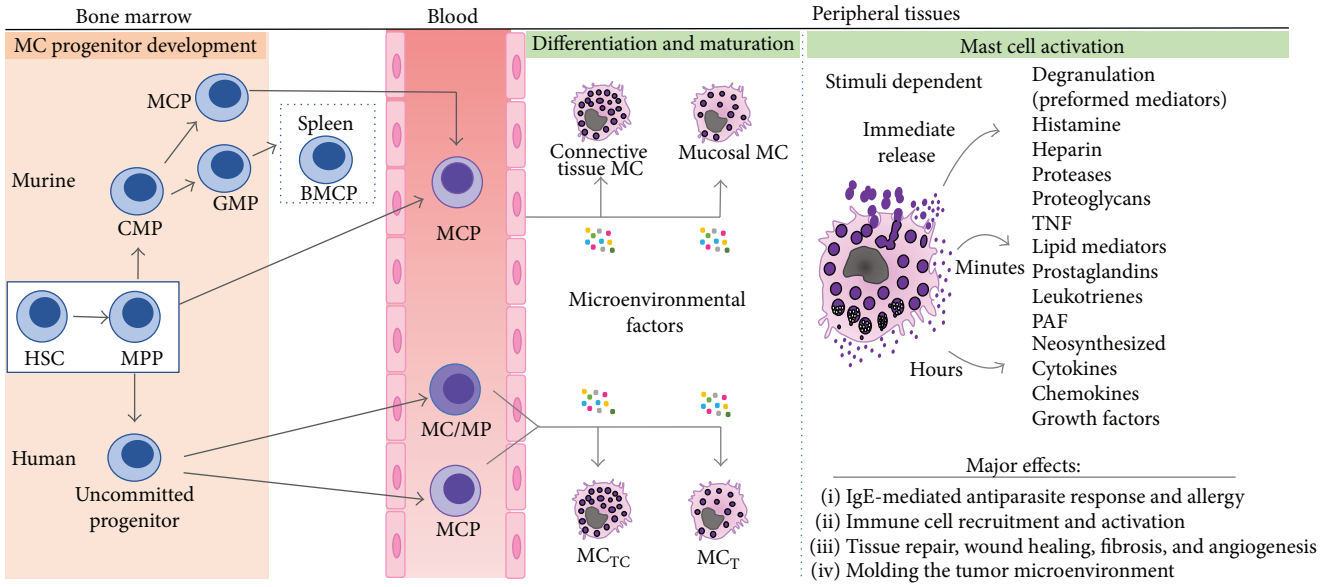


Figure 1: Overview of mast cell development, heterogeneity, and activation. Mast cells arise in the bone marrow from hematopoietic stem cells (HSC) via a multipotent progenitor (MPP), which can become a mast cell-committed progenitor (MCP) that exits the bone marrow and migrates to peripheral tissues to complete maturation. Several pathways have been described for murine and human mast cell origin. In mice, MCPs may be derived directly from MPPs or from common myeloid progenitors (CMP). Mast cells may also be derived from the granulocyte/monocyte progenitor (GMP) via an intermediate progenitor (BMCP), identified only in the spleen of C57BL6 mice, which gives rise to basophils and mast cells. In humans, it has been postulated that mast cells originate from a yet unidentified uncommitted progenitor that gives rise to a mast cell/monocyte-committed progenitor (MC/MP) in bone marrow. Alternatively, an MCP population that gives rise exclusively to mast cells has recently been identified in blood. Final differentiation occurs in peripheral tissues, where microenvironmental factors determine the phenotype of the mature mast cells. Mast cells exhibit marked phenotypic and functional heterogeneity. Two major subtypes have been described in both rodents and humans, in the former as mucosal and connective tissue mast cells and in the latter as tryptase- and chymase-rich mast cells (MC_{TC}) and those that mainly contain tryptase (MC_T). The right end diagram illustrates the distinct classes of bioactive molecules and their temporality of release upon mast cell activation in tissues. See text for a more detailed explanation.

are SCF, NGF, and IL-4, while MMCs require SCF, IL-3, IL-9, IL-10, and TGFβ. These latter factors are importantly secreted by T lymphocytes; hence, MMCs are usually considered T cell dependent [31, 32]. MMCs and CTMCs also differ in size and in their content of intragranular histamine, proteoglycans, and proteases. Specifically, MMCs are smaller than CTMCs, contain fewer granules with less histamine, and express mouse mast cell proteases-2 (mMCP-2), mMCP-4, mMCP-5, and mMCP-6, whereas CTMCs express primarily mMCP-1 and mMCP-2 [38, 39]. Additionally, MMCs contain proteoglycans with poorly sulfated glycosaminoglycans, such as chondroitin sulfate, while CTMCs contain highly sulfated glycosaminoglycans, such as heparin [40]. Taken together, these data support that there are different subtypes of mast cells, most likely fulfilling different functions and whose maturation is importantly shaped by their tissue location and the local stimuli provided by other resident immune cells [38, 41, 42].

Mast cell activation can lead to release of three distinct classes of bioactive molecules, depending on the type of stimuli and receptor involved: preformed mediators stored in their granules that are rapidly released (within seconds to minutes); de novo synthesized lipid mediators, prostaglandins, and leukotrienes (minutes); and a variety of cytokines and chemokines that are produced following their transcription and translation (hours). The most studied mechanism of mast cells activation is the response mediated through their high-affinity IgE receptor (FcεRI), which after their cross-linkage results in the rapid release of the granule content into the extracellular space, a process known as degranulation (Figure 1). This response also leads to the generation and release of the lipid inflammatory mediators derived from arachidonic acid, which are involved in leukocyte recruitment and activation, vasodilation, angiogenesis, and mitogenesis [33, 43].

Mast cell degranulation is also observed in many IgE-independent processes, such as degranulation induced by thrombin, IgG complexes, neuropeptides, and complement-derived anaphylatoxins [44–47]. Furthermore, mast cells have numerous other receptors on their plasma membrane, and the nature of the mast cell response is dependent on the stimulating ligand. For instance, mast cell activation by pathogen-associated molecular patterns (PAMPS) through Toll-like receptors (TLRs) triggers the differential and selective release of proinflammatory cytokines and chemokines with or without degranulation. For example, peptidoglycan (a TLR-2 ligand) can cause mast cell degranulation, while lipopolysaccharide (a TLR-4 ligand) does not. Moreover, a study showed that TLR-4 and TLR-6 elicit similar patterns of increased synthesis of GM-CSF, IL-8, and IL-10, whereas TLR-8 preferentially induces IL-8, MIP-1α, and TNF-α, and TLR-2 only IL-8 [47–50]. In addition to the rapid and

Table 1: Studies analyzing the participation of mast cells in breast cancer.

Study type	BC specimen	MC detection method	Prognosis in BC	Association	Ref.
D/C/E	Tumor tissue and blood *xenotransplanted mice	Tryptase	Positive	Decreased blood clotting and hypoxia	[94]
D/C	Tumor tissue	c-kit (CD117)	Positive	Greater survival	[95]
D/C	Tumor tissue from IDC	c-kit (CD117)	Positive	Greater survival	[87]
D/C	Tumor tissue	Giemsa and Alcian blue	Positive	BC subtype	[88]
D/C	Lymph nodes	Toluidine blue	Positive	Greater survival	[91]
D/C	Tumor tissue from IDC	Tryptase and chymase	Positive	BC subtypes	[89]
D/C	Sentinel lymph nodes	Tryptase	Negative	Angiogenesis and micrometastasis	[90]
D/C	Tumor tissue	Tryptase	Negative	Angiogenesis	[92]
D/C	Tumor tissue and sera to measure tryptase levels	Tryptase	Negative	Angiogenesis	[93]
D/C	Tumor tissue and lymph nodes from IDC patients	Toluidine blue	Negative	Angiogenesis	[98]
D/C	Tumor tissue from IDC	Toluidine blue	Negative	BC grade	[99]
D/C/E	Benign growths and tumor tissues Cell line treated with tryptase	Tryptase	Negative	BC grade and metastasis	[104]
E	*Mast cell-deficient BC-prone mice	Toluidine blue	Negative	Progression, metastasis, and angiogenesis	[105]
C/E	Tumor tissue from cimetidine treated patients	Toluidine blue	None	None	[111]

BC: breast cancer; IDC: invasive ductal carcinoma; D: descriptive study; C: correlative; E: experimentally tested; positive: antitumoral role; negative: protumoral role. *Studies also performed in mice.

massive release of granule content through exocytosis, there is significant evidence showing that mast cells can release granule compounds selectively by a process known as piecemeal degranulation, which involves vesicle transport from the granule to the plasma membrane, and it is the most prevalent form of mast cell mediator secretion identified in situ in several chronic diseases [51–56]. Taken together, these data highlight the phenotypic and functional plasticity of mast cells.

3. Overview of Mast Cells in Cancer

In 1878, Paul Ehrlich was the first to report the presence of mast cells in human tumors. Since then, there has been increasing evidence that mast cells, termed tumor-associated mast cells (TAMCs), infiltrate a variety of solid and hematological tumors. Examples of cancers with peritumoral or intratumoral high mast cells density are thyroid, stomach, pancreas, prostate, melanoma, and breast cancer [14, 57–60]. Puzzling, mast cells in these neoplasias have been reported as protumorigenic, antitumorigenic, or just as innocent bystanders [14]. Thus, increased accumulation of mast cells has been correlated with poor prognosis in gastric, pancreatic, and colorectal tumors [61–64]. Whilst in breast cancer, mast cell accumulation and function is still controversial (see Table 1).

The accumulation of TAMCs in different cancers may occur in response to various chemotactic factors secreted by tumor cells or immune cells in the tumor microenvironment. These can include SCF, monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF), angiopoietin 1 (Ang1), IL-8, CCL2, CXCL1, CXCL10, and osteopontin (OP), which in addition to recruit mast cell progenitors are also able to induce their maturation and activation [65–69]. Activated mast cells have been detected infiltrating angiosarcomas by electron microscopy; some of them exhibited anaphylactic degranulation while others exhibited piecemeal release [55]. However, tumor cells in close contact with activated mast cells did not show evidence of apoptotic or necrotic changes, thus concluding that it was unlikely that mast cells were battling cancer cells to contribute to the improvement of the clinical outcome [70]. This observation suggested that mast cell-mediator release by piecemeal could contribute to the selective release of protumorigenic mediators. Indeed, several protumorigenic functions for TAMCs have been reported, such as tumor cell proliferation, lymphatic and blood vessel formation, promotion of tumor cells invasion, and extravasation of diverse cytokine-producing cells [71–73]. TAMCs have also been shown to play a central role in angiogenesis of various types of tumors. In fact, mast cells can promote angiogenesis and lymphangiogenesis through the production not only of the classical proangiogenic mediators VEGF, fibroblast growth factor (FGF), IL-8, heparin, and metalloproteases but also of nonclassical factors, such as tryptase, chymase, and other serine proteases [65, 74, 75]. In melanoma, mast cell accumulation has been correlated with VEGF overexpression, increased neovascularization, enhanced tumor aggressiveness, and poor prognosis [76]. Moreover, mast cell production of tryptase correlates with local angiogenesis and tumor progression in skin tumors [71].

TAMCs can also promote tumor growth through the secretion of IL-8 and histamine, which function as chemotactic factors for immune cells and as tumor mitogens

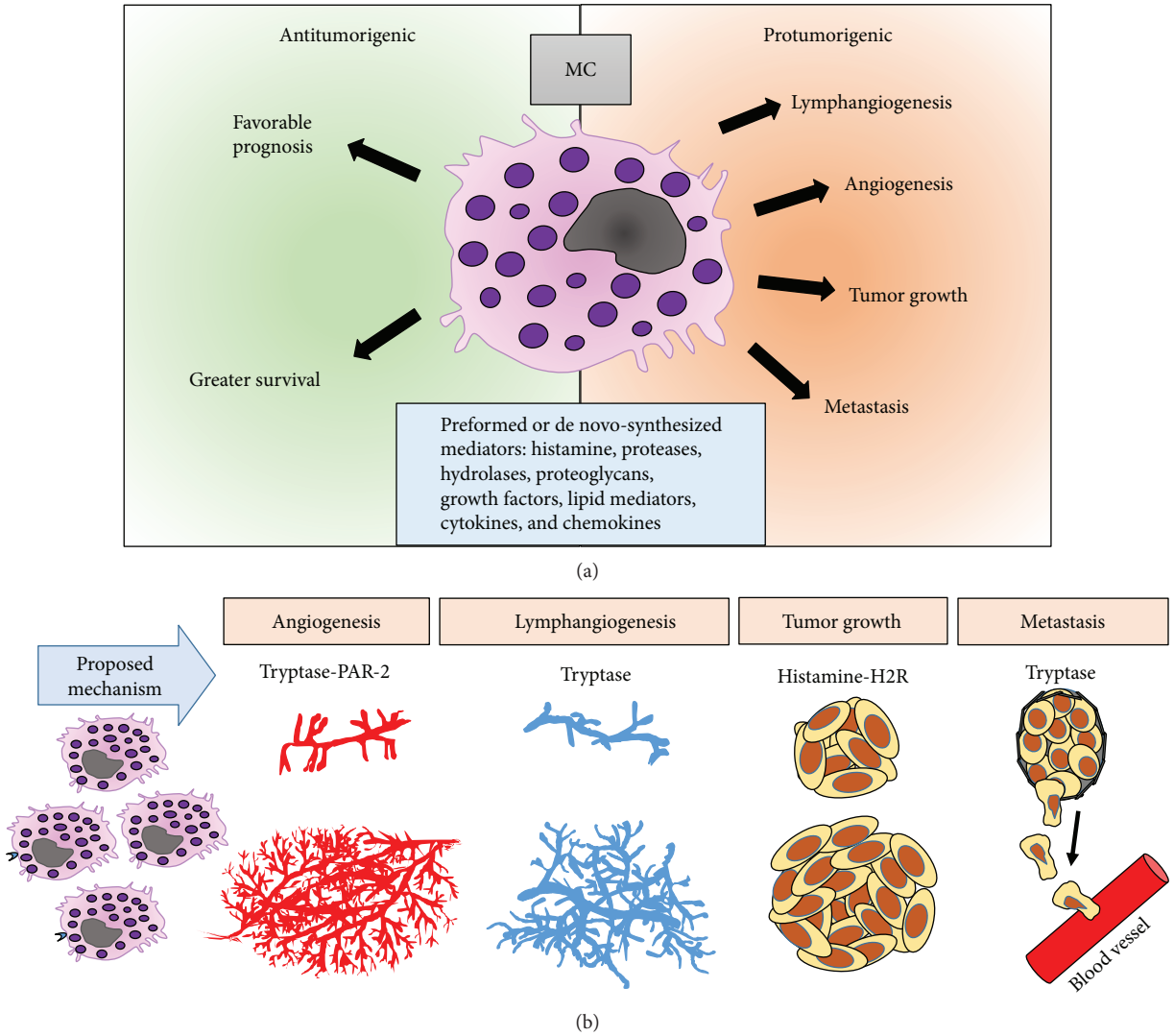


Figure 2: Role of mast cells in breast cancer. (a) The influence of mast cells in breast cancer prognosis is still a matter of discussion. Mast cells contain a great variety of bioactive components that may exert both pro- and antitumor effects. On the one hand, *in vitro* and *in vivo* studies support that mast cells exhibit protumor activity through promotion of lymphatic and blood vessel formation, tumor growth, and metastasis (orange right side). On the other hand, several population studies also associate mast cells with a greater survival and favorable prognosis (green left side). (b) Some bioactive molecules of mast cells documented to have protumorigenic effects are tryptase through its receptor PAR-2 and histamine through H2 receptor. The cancer processes in which these compounds have been associated are indicated.

[77]. Furthermore, the production of different matrix metalloproteinases (e.g., MMP-9) and proteases (tryptase and chymase) by TAMCs can regulate the proteolysis of ECM proteins and disturb the physiological communication between stroma and epithelium, favoring detachment of cancer cells, migration, and invasion [61, 73, 78]. All these TAMC protumoral activities are in line with mast cell homeostatic functions related to wound healing and tissue repair [79–81]. On the other hand, TAMCs antitumor activities have also been documented. In this regard, using a murine model of intestinal carcinogenesis, one study demonstrated that mast cell-deficient mice developed more abundant and larger tumors than mast cell competent littermates [82]. Similarly, it has been reported that TAMCs can mediate tumor cell apoptosis through the production of IL-4, TNF, and reactive peroxides [83–85].

4. Mast Cells in Breast Cancer

Breast cancer is one of the most common causes of mortality and morbidity among women worldwide [86]. As in other cancers, mast cells are frequently observed in the tumor stroma of breast cancers, and their accumulation and prognostic significance have been a source of heated discussion with evidence of both pro- and antitumoral roles (Figure 2 and Table 1). To date, there is not yet a clear verdict on this ongoing debate.

4.1. Evidence of Mast Cell Antitumoral Function. Different clinical studies support a protective role for mast cells infiltrating breast tumors. Using a multivariate analysis, one study found that the presence of stromal mast cells was a positive prognostic factor, showing a strong correlation with

survival curves, particularly for those cancers that still did not show evidence of lymph node invasion [87]. Compellingly, this study was further expanded to include a cohort of 4444 invasive breast cancer patients and a longer follow-up of up to 18.4 years. The conclusions reached were highly similar, with the authors proposing that mast cells could be used as a good prognostic marker, independent of age, tumor grade, tumor size, lymph node, and molecular subtype [88]. Naik et al. also found that higher numbers of mast cells in the axillary lymph nodes correlated with a better prognosis [89], although the pattern of distribution of mast cells in the different anatomic locations of the nodes was not different between the patients that survived and those that did not survive.

Perhaps, also very relevant is the specific participation of the MC_T and MC_{TC} subtypes for cancer progression. Unfortunately, this has only been addressed in one recent study in which no differences were found between both mast cell subtypes and prognosis; both MC_T and MC_{TC} cells were found infiltrating breast tumors, and both were associated with less aggressive cancer types (the luminal immunophenotype). Therefore, increased numbers of any of the mast cell subtypes correlated with a better disease prognosis [90]. Naik et al. also found a close association of mast cells with lymph node areas of high T cell density, similar to the location observed for T cell-dependent murine mucosal mast cells [89].

Another important factor that should be equated when studying the contribution of mast cells in breast cancer is their location within the tumor. Mast cells have been observed in either or both the intratumoral and peritumoral areas. More commonly, mast cells are almost exclusively found in the periphery of the tumor, frequently colonizing perivascular areas. Their potential role at these particular sites has been more difficult to elucidate, but the study of della Rovere et al. documented that peritumoral mast cells seemed to have a cytolytic activity against tumor cells [91].

4.2. Mast Cell Protumoral Function: Promotion of Angiogenesis and Metastasis. Similar to the evidence existing for other human cancers, several studies carried on with breast cancer patients have found a positive correlation between TAMCs and tumor angiogenesis. For example, one study found that high mast cell numbers correlated with increased microvascular density (MVD) in primary tumors [92]. However, the number of TAMCs did not correlate with other clinicopathological features of aggressive cancers, hampering the interpretation of their contribution to prognosis. Similarly, Samoszuk and Marech in 2003 and 2014, respectively, showed that TAMC numbers and tryptase levels in serum of breast cancer patients strongly correlated with MVD, supporting the involvement of mast cell-derived tryptase in tumor angiogenesis [93, 94]. Moreover, it has also been shown that microvessel counts increase in parallel to the number of tryptase-positive mast cells in lymph nodes from breast cancer patients and that their values are significantly higher in lymph nodes with micrometastasis compared with those without metastasis [95]. Therefore, it has been suggested that mast cells contribute, at least partially, to the micrometastasis that occur at early stages of tumor development and to the angiogenesis that supports it [92, 95].

Formation of lymphatic vessels or lymphangiogenesis is also a reliable predictor of lymph node metastasis [96, 97]. A recent study by Keser et al. found that mast cells were present in all invasive primary tumors and in the metastatic lymph nodes [98]. In previous studies, mast cells were generally observed in the stroma adjacent to the neoplastic cells and near vascular structures [87, 88, 99]. Confusingly, while mast cells were detected in all metastatic lymph nodes, not all enlarged lymph nodes with evidence of immune reactivity (reactive lymph nodes) showed evidence of their presence. Still, mast cell count was better correlated with metastatic lymph nodes than with reactive lymph nodes, which could indicate a specific mast cell role in metastasis of breast cancer cells. Although the study by Keser et al. did not find a correlation between mast cell density and disease-free/overall survival, the authors reported a significant link between lymphatic vessel density (LVD) and other poor prognostic parameters, such as tumor diameter, tumor volume, tumor nuclear grade, perineural invasion, metastatic lymph node count, and tumor stage [98]. Interestingly, LVD also correlated with a shorter period of disease-free survival. This apparently confusing data could be explained by a multifactorial influence on the clinical parameters measured in the study and/or by and indirect effect of mast cells in disease prognosis.

In support of the clinical correlation between cancer prognosis and tryptase serum levels, several *in vitro* studies have supported a direct effect of mast cell-derived tryptase on angiogenesis and lymphangiogenesis. Tryptase functions as an agonist of the proteinase-activated receptor-2 (PAR-2) in vascular endothelial cells stimulating their proliferation [100, 101]. Tryptase also induces angiogenesis by releasing stored angiogenic factors bound to the ECM, such as cytokines and metalloproteinases [102–104]. A study in 2009 showed that the peritumoral levels of tryptase augmented with the grade of the tumor, and this correlated positively with lymph node metastasis [104]. In agreement, MDA-MB-231 cells, a breast cancer cell line, increased migration and invasion in response to tryptase in transwell assays [104].

Concerning the tumor location of mast cells, one study found that intratumoral mast cells were better associated with lymphatic and perineural invasion, and this was an adverse prognostic parameter [98]. Contrary to della Rovere et al.'s study, indicating a potential positive role for peritumoral mast cells, other studies have found that these cells secrete proteases that facilitate vascular invasion and accelerate metastatic spread [92, 95, 98]. Thus, peritumoral mast cells seem to contribute to breast cancer progression. Fakhrjou et al. also found a positive association between the number of mast cells and the histopathological grade of the disease, particularly in invasive ductal carcinoma [99].

Experimental data using mast cell-deficient mice have also provided a strong support for a positive correlation between mast cells in mammary tumors and metastasis. In the study by He et al., mast cell-deficient mice ($Kit^{W\text{-}sh/W\text{-}sh}$), a c-Kit knockout strain, was crossed with mice that spontaneously develop breast cancer (PyMT

strain). Although the number and the onset of tumors was not affected in the offspring, the size of the tumor and their metastatic potential were significantly reduced in the c-Kit-deficient mice compared with their littermate controls [105]. Moreover, histological examination of tumors revealed a marked decreased in angiogenesis, thus supporting the fact that mast cells are not as important for tumor initiation as they are for tumor progression and that their contribution is strongly due to their ability to promote tumor vascularization.

5. Mast Cells Are Potential Targets for Anticancer Therapy

Experimental studies in mice have suggested that mast cell inhibitors could reduce the number and activity of the cells in certain types of cancers improving disease outcomes [106]. For instance, in murine models of prostate adenocarcinoma, treatment with cromolyn (sodium cromoglicate), a well-known mast cell degranulation inhibitor, blocked prostate tumor growth. Paradoxically, treated mice developed highly malignant neuroendocrine cancers, a fatal collateral event that should be more deeply studied before proposing the use of cromolyn or the targeting of mast cells as therapy [106]. On the other hand, in a preclinical study involving pancreatic cancer patients treated with the drug masitinib, a tyrosine-kinase inhibitor that has inhibitory activity against c-Kit compromising mast cell survival, it was shown that patients receiving a combination of masitinib plus standard chemotherapy had an increased survival compared with patients receiving chemotherapy alone [107]. However, it is important to note that the study did not distinguish whether the increased survival was directly related to mast cell activity.

Other *in vitro* and *in vivo* studies using mast cell stabilizers or mast cell-depleting agents have shown controversial results. For instance, depletion of mast cells with imatinib enhanced tumor growth in a murine model of breast carcinoma [108], supporting an antitumoral role for mast cells. In agreement, mice treated with cromolyn showed mammary tumors with extensive hypoxic hemorrhagic regions and clots, which were not observed in the control group, suggesting that mast cells play an important role in inhibiting blood clotting and maintaining blood perfusion in breast cancer, probably through secretion of heparin, plasminogen activator, chymase, and tryptase [94].

Histamine, one of the most important components of mast cell granules, has been shown to be critical for development of the normal rat mammary gland [110]. Likewise, histamine has been implicated in promoting tumor cell proliferation and enhancing growth of experimental mammary carcinomas, particularly acting through H2 receptors, and treatment with H2 receptor antagonists significantly inhibited tumor cell proliferation and tumor growth [109, 110]. However, a human clinical trial testing the H2 receptor antagonist cimetidine (Tagamet), found no relationship between the preoperative drug administration and breast cancer growth [111].

6. Conclusions

We have recently understood that tumor infiltration by immune cells is not necessarily a good sign for defense and protection. Tumor-associated macrophages have been the torch bearers to help us recognize how the immune system contributes to cancer progression often as early as precancerous stages. Although we have learned a good deal about the role of mast cells in cancer, we still lag behind and are far from understanding their potential protective or harmful influence. This, in spite of the wide range of bioactive molecules inherent to the activity of mast cells, potentially places them in a broad number of cancer-associated biological processes.

Most studies agree that breast tumors are infiltrated by mast cells. However, there is conflicting data about the meaning of that observation in terms of disease prognosis. The source of discrepancy may have different origins, from technical to biological, for instance, the markers and methods used to identify and count mast cells or the clinical parameters used to give correlative associations. Furthermore, cancer in general, and breast cancer in particular, is a highly heterogeneous disease with a great variety of genetic/histological/clinical subtypes, with each subtype also exhibiting a high heterogeneity within itself. It is possible that mast cell contribution, either positive or negative, is specific to certain breast cancer subtypes or that mast cells and the inflammatory microenvironment influence each other independently of other histological features. Indeed, mast cells are highly reactive cells that express a great variety of receptors and respond to a great variety of stimuli influencing their maturation, density, and activation, polarization into different subtypes, content of biomolecules, and immediate or persistent release mechanisms [112–114]. Although two mast cell-derived factors, tryptase and histamine, seem to perform a protumorigenic role in breast cancer, there are multiple other mast cell biomolecules for which we do not know much about their possible participation in cancer progression. For instance, arachidonic acid-derived lipid mediators, which are also involved in angiogenesis and mitogenesis. More work is needed to clarify the role of mast cells in breast cancer and for a better understanding of the mechanisms of mast cell communication with tumor cells and other immune cells within the tumor stroma.

Conflicts of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

Acknowledgments

This study was funded by Consejo Nacional de Ciencia y Tecnologia (CONACYT), México, Grant 223678. Angélica Aponte-López is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 482586/284102 from CONACYT. Samira Muñoz-Cruz is

truly thankful to be a Fundación IMSS A.C. research scholarship recipient.

References

[1] J. F. Bermejo-Martin, I. Martin-Loeches, and S. Bosinger, "Inflammation and infection in critical care medicine," *Mediators of Inflammation*, vol. 2014, Article ID 456256, 2 pages, 2014.

[2] R. Medzhitov, "Inflammation 2010: new adventures of an old flame," *Cell*, vol. 140, no. 6, pp. 771–776, 2010.

[3] D. B. Vendramini-Costa and J. E. Carvalho, "Molecular link mechanisms between inflammation and cancer," *Current Pharmaceutical Design*, vol. 18, no. 26, pp. 3831–3852, 2012.

[4] R. Virchow, *Cellular pathology as based upon physiological and pathological histology*, J. B. Lippincott and Co., Philadelphia, PA, USA, 1863.

[5] N. Eiro and F. J. Vizoso, "Inflammation and cancer," *World Journal of Gastrointestinal Surg*ery, vol. 4, no. 3, pp. 62–72, 2012.

[6] J. V. Fernandes, R. N. Cobucci, C. A. Jatoba, T. A. Fernandes, J. W. de Azevedo, and J. M. de Araujo, "The role of the mediators of inflammation in cancer development," *Pathology & Oncology Research*, vol. 21, no. 3, pp. 527–534, 2015.

[7] T. P. Raposo, B. C. Beirao, L. Y. Pang, F. L. Queiroga, and D. J. Argyle, "Inflammation and cancer: till death tears them apart," *The Veterinary Journal*, vol. 205, no. 2, pp. 161–174, 2015.

[8] S. P. Hussain and C. C. Harris, "Inflammation and cancer: an ancient link with novel potentials," *International Journal of Cancer*, vol. 121, no. 11, pp. 2373–2380, 2007.

[9] A. Mantovani, P. Allavena, A. Sica, and F. Balkwill, "Cancer-related inflammation," *Nature*, vol. 454, no. 7203, pp. 436–444, 2008.

[10] L. B. Meira, J. M. Bugni, S. L. Green et al., "DNA damage induced by chronic inflammation contributes to colon carcinogenesis in mice," *The Journal of Clinical Investigation*, vol. 118, no. 7, pp. 2516–2525, 2008.

[11] A. Hasan, H. Ghebeh, C. Lehe, R. Ahmad, and S. Dermime, "Therapeutic targeting of B7-H1 in breast cancer," *Expert Opininion on Therapeutic Targets*, vol. 15, no. 10, pp. 1211–1225, 2011.

[12] X. Jiang and D. J. Shapiro, "The immune system and inflammation in breast cancer," *Molecular and Cellular Endocrinology*, vol. 382, no. 1, pp. 673–682, 2014.

[13] M. R. Galdiero, C. Garlanda, S. Jaillon, G. Marone, and A. Mantovani, "Tumor associated macrophages and neutrophils in tumor progression," *Journal of Cellular Physiology*, vol. 228, no. 7, pp. 1404–1412, 2013.

[14] G. Varricchi, M. R. Galdiero, S. Loffredo et al., "Are mast cells MASTers in cancer?," *Frontiers in Immunology*, vol. 8, p. 424, 2017.

[15] S. Wernersson and G. Pejler, "Mast cell secretory granules: armed for battle," *Nature Reviews Immunology*, vol. 14, no. 7, pp. 478–494, 2014.

[16] M. Frieri, K. Kumar, and A. Boutin, "Role of mast cells in trauma and neuroinflammation in allergy immunology," *Annals of Allergy, Asthma & Immunology*, vol. 115, no. 3, pp. 172–177, 2015.

[17] E. Crivellato, C. A. Beltrami, F. Mallardi, and D. Ribatti, "The mast cell: an active participant or an innocent bystander?," *Histology and Histopathology*, vol. 19, no. 1, pp. 259–270, 2004.

[18] A. Weber, J. Knop, and M. Maurer, "Pattern analysis of human cutaneous mast cell populations by total body surface mapping," *British Journal of Dermatology*, vol. 148, no. 2, pp. 224–228, 2003.

[19] J. S. Dahlin, Z. Ding, and J. Hallgren, "Distinguishing mast cell progenitors from mature mast cells in mice," *Stem Cells and Development*, vol. 24, no. 14, pp. 1703–1711, 2015.

[20] M. C. Jamur, A. C. Grodzki, E. H. Berenstein, M. M. Hamawy, R. P. Siraganian, and C. Oliver, "Identification and characterization of undifferentiated mast cells in mouse bone marrow," *Blood*, vol. 105, no. 11, pp. 4282–4289, 2005.

[21] M. C. Jamur, A. N. Moreno, L. F. Mello et al., "Mast cell repopulation of the peritoneal cavity: contribution of mast cell progenitors versus bone marrow derived committed mast cell precursors," *BMC Immunology*, vol. 11, no. 1, p. 32, 2010.

[22] O. Schmetzer, P. Valentin, M. K. Church, M. Maurer, and F. Siebenhaar, "Murine and human mast cell progenitors," *European Journal of Pharmacology*, vol. 778, pp. 2–10, 2016.

[23] M. F. Gurish and K. F. Austen, "Developmental origin and functional specialization of mast cell subsets," *Immunity*, vol. 37, no. 1, pp. 25–33, 2012.

[24] Y. Kitamura, K. Oboki, and A. Ito, "Molecular mechanisms of mast cell development," *Immunology and Allergy Clinics of North America*, vol. 26, no. 3, pp. 387–405, 2006.

[25] A. S. Kirshenbaum, S. W. Kessler, J. P. Goff, and D. D. Metcalfe, "Demonstration of the origin of human mast cells from CD34+ bone marrow progenitor cells," *The Journal of Immunology*, vol. 146, no. 5, pp. 1410–1415, 1991.

[26] A. S. Kirshenbaum, J. P. Goff, T. Semere, B. Foster, L. M. Scott, and D. D. Metcalfe, "Demonstration that human mast cells arise from a progenitor cell population that is $CD34^+$, c-kit^+, and expresses aminopeptidase N (CD13)," *Blood*, vol. 94, no. 7, pp. 2333–2342, 1999.

[27] K. Maaninka, J. Lappalainen, and P. T. Kovanen, "Human mast cells arise from a common circulating progenitor," *The Journal of Allergy and Clinical Immunology*, vol. 132, no. 2, pp. 463–469.e3, 2013.

[28] J. S. Dahlin, A. Malinovschi, H. Ohrvik et al., "Lin- $CD34^{hi}$ $CD117^{int/hi}$ $FcepsilonRI^+$ cells in human blood constitute a rare population of mast cell progenitors," *Blood*, vol. 127, no. 4, pp. 383–391, 2016.

[29] T. Ito, D. Smrz, M. Y. Jung et al., "Stem cell factor programs the mast cell activation phenotype," *The Journal of Immunology*, vol. 188, no. 11, pp. 5428–5437, 2012.

[30] C. Moller, J. Alfredsson, M. Engstrom et al., "Stem cell factor promotes mast cell survival via inactivation of FOXO3a-mediated transcriptional induction and MEK-regulated phosphorylation of the proapoptotic protein Bim," *Blood*, vol. 106, no. 4, pp. 1330–1336, 2005.

[31] H. N. Lee, C. H. Kim, G. G. Song, and S. W. Cho, "Effects of IL-3 and SCF on histamine production kinetics and cell phenotype in rat bone marrow-derived mast cells," *Immune Network*, vol. 10, no. 1, pp. 15–25, 2010.

[32] C. M. Westerberg, E. Ulleras, and G. Nilsson, "Differentiation of mast cell subpopulations from mouse embryonic stem cells," *Journal of Immunological Methods*, vol. 382, no. 1-2, pp. 160–166, 2012.

[33] J. Douaiher, J. Succar, L. Lancerotto et al., "Development of mast cells and importance of their tryptase and chymase

serine proteases in inflammation and wound healing," *Advances in Immunology*, vol. 122, pp. 211–252, 2014.

[34] M. C. Jamur and C. Oliver, "Origin, maturation and recruitment of mast cell precursors," *Frontiers in Bioscience*, vol. S3, no. 4, pp. 1390–1406, 2011.

[35] A. A. Irani, N. M. Schechter, S. S. Craig, G. DeBlois, and L. B. Schwartz, "Two types of human mast cells that have distinct neutral protease compositions," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 12, pp. 4464–4468, 1986.

[36] A. D. Befus, N. Dyck, R. Goodacre, and J. Bienenstock, "Mast cells from the human intestinal lamina propria. Isolation, histochemical subtypes, and functional characterization," *The Journal of Immunology*, vol. 138, no. 8, pp. 2604–2610, 1987.

[37] T. Nakano, T. Sonoda, C. Hayashi et al., "Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell-deficient W/Wv mice. Evidence that cultured mast cells can give rise to both connective tissue type and mucosal mast cells," *Journal of Experimental Medicine*, vol. 162, no. 3, pp. 1025–1043, 1985.

[38] Y. Kitamura, "Heterogeneity of mast cells and phenotypic change between subpopulations," *Annual Review of Immunology*, vol. 7, no. 1, pp. 59–76, 1989.

[39] Y. Kitamura, K. Oboki, and A. Ito, "Development of mast cells," *Proceedings of the Japan Academy, Series B*, vol. 83, no. 6, pp. 164–174, 2007.

[40] H. R. Katz, R. L. Stevens, and K. F. Austen, "Heterogeneity of mammalian mast cells differentiated in vivo and in vitro," *Journal of Allergy and Clinical Immunology*, vol. 76, no. 2, pp. 250–259, 1985.

[41] R. H. Dougherty, S. S. Sidhu, K. Raman et al., "Accumulation of intraepithelial mast cells with a unique protease phenotype in T_H2-high asthma," *Journal of Allergy and Clinical Immunology*, vol. 125, no. 5, pp. 1046–1053.e8, 2010.

[42] W. Xing, K. F. Austen, M. F. Gurish, and T. G. Jones, "Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue," *Proceedings of the National Academy of Sciences of the Unites States of America*, vol. 108, no. 34, pp. 14210–14215, 2011.

[43] T. Ishizaka and K. Ishizaka, "Activation of mast cells for mediator release through IgE receptors," *Chemical Immunology and Allergy*, vol. 34, pp. 188–235, 1984.

[44] S. G. El-Lati, C. A. Dahinden, and M. K. Church, "Complement peptides C3a- and C5a-induced mediator release from dissociated human skin mast cells," *Journal of Investigative Dermatology*, vol. 102, no. 5, pp. 803–806, 1994.

[45] O. Malbec and M. Daeron, "The mast cell IgG receptors and their roles in tissue inflammation," *Immunological Reviews*, vol. 217, no. 1, pp. 206–221, 2007.

[46] H. Vliagoftis, "Thrombin induces mast cell adhesion to fibronectin: evidence for involvement of protease-activated receptor-1," *The Journal of Immunology*, vol. 169, no. 8, pp. 4551–8, 2002.

[47] Y. Yu, B. R. Blokhuis, J. Garssen, and F. A. Redegeld, "Non-IgE mediated mast cell activation," *European Journal of Pharmacology*, vol. 778, pp. 33–43, 2016.

[48] J. Suurmond, A. L. Dorjee, E. F. Knol, T. W. Huizinga, and R. E. Toes, "Differential TLR-induced cytokine production by human mast cells is amplified by FcvarepsilonRI triggering," *Clinical & Experimental Allergy*, vol. 45, no. 4, pp. 788–796, 2015.

[49] S. Varadaradjalou, F. Feger, N. Thieblemont et al., "Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells," *European Journal Immunology*, vol. 33, no. 4, pp. 899–906, 2003.

[50] H. Matsushima, N. Yamada, H. Matsue, and S. Shimada, "TLR3-, TLR7-, and TLR9-mediated production of proinflammatory cytokines and chemokines from murine connective tissue type skin-derived mast cells but not from bone marrow-derived mast cells," *The Journal of Immunology*, vol. 173, no. 1, pp. 531–541, 2004.

[51] A. M. Dvorak and S. Kissell, "Granule changes of human skin mast cells characteristic of piecemeal degranulation and associated with recovery during wound healing in situ," *Journal of Leukocyte Biology*, vol. 49, no. 2, pp. 197–210, 1991.

[52] J. A. Flores, S. Balseiro-Gomez, and E. Ales, "Emerging roles of granule recycling in mast cell plasticity and homeostasis," *Critical Reviews in Immunology*, vol. 36, no. 6, pp. 461–484, 2016.

[53] T. Hugle, "Beyond allergy: the role of mast cells in fibrosis," *Swiss Medical Weekly*, vol. 144, article w13999, 2014.

[54] T. C. Moon, A. D. Befus, and M. Kulka, "Mast cell mediators: their differential release and the secretory pathways involved," *Frontiers in Immunology*, vol. 119, no. 8, pp. 5555–5562, 2014.

[55] C. A. Oskeritzian, "Mast cell plasticity and sphingosine-1-phosphate in immunity, inflammation and cancer," *Molecular Immunology*, vol. 63, no. 1, pp. 104–112, 2015.

[56] K. V. Vukman, A. Forsonits, A. Oszvald, E. A. Toth, and E. I. Buzas, "Mast cell secretome: soluble and vesicular components," *Seminars in Cell & Developmental Biology*, vol. 67, pp. 65–73, 2017.

[57] S. Ch'ng, R. A. Wallis, L. Yuan, P. F. Davis, and S. T. Tan, "Mast cells and cutaneous malignancies," *Modern Pathology*, vol. 19, no. 1, pp. 149–159, 2005.

[58] E. Dundar, U. Oner, B. C. Peker, M. Metintas, S. Isiksoy, and G. Ak, "The significance and relationship between mast cells and tumour angiogenesis in non-small cell lung carcinoma," *Journal of International Medical Research*, vol. 36, no. 1, pp. 88–95, 2008.

[59] A. Johansson, S. Rudolfsson, P. Hammarsten et al., "Mast cells are novel independent prognostic markers in prostate cancer and represent a target for therapy," *The American Journal of Pathology*, vol. 177, no. 2, pp. 1031–1041, 2010.

[60] B. Tuna, K. Yorukoglu, M. Unlu, M. U. Mungan, and Z. Kirkali, "Association of mast cells with microvessel density in renal cell carcinomas," *European Urology*, vol. 50, no. 3, pp. 530–534, 2006.

[61] M. Ammendola, R. Sacco, G. Donato et al., "Mast cell positivity to tryptase correlates with metastatic lymph nodes in gastrointestinal cancer patients treated surgically," *Oncology*, vol. 85, no. 2, pp. 111–116, 2013.

[62] D. Z. Chang, Y. Ma, B. Ji et al., "Mast cells in tumor microenvironment promotes the *in vivo* growth of pancreatic ductal adenocarcinoma," *Clinical Cancer Research*, vol. 17, no. 22, pp. 7015–7023, 2011.

[63] K. Kondo, M. Muramatsu, Y. Okamoto et al., "Expression of chymase-positive cells in gastric cancer and its correlation

with the angiogenesis," *Journal of Surgical Oncology*, vol. 93, no. 1, pp. 36–42, 2006.

[64] Y. Ma, R. F. Hwang, C. D. Logsdon, and S. E. Ullrich, "Dynamic mast cell-stromal cell interactions promote growth of pancreatic cancer," *Cancer Research*, vol. 73, no. 13, pp. 3927–3937, 2013.

[65] A. Detoraki, R. I. Staiano, F. Granata et al., "Vascular endothelial growth factors synthesized by human lung mast cells exert angiogenic effects," *The Journal of Allergy and Clinical Immunology*, vol. 123, no. 5, pp. 1142–1149.e5, 2009.

[66] A. D. Giannou, A. Marazioti, M. Spella et al., "Mast cells mediate malignant pleural effusion formation," *Journal of Clinical Investigation*, vol. 125, no. 6, pp. 2317–2334, 2015.

[67] B. Huang, Z. Lei, G. M. Zhang et al., "SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment," *Blood*, vol. 112, no. 4, pp. 1269–1279, 2008.

[68] N. Prevete, R. I. Staiano, F. Granata et al., "Expression and function of Angiopoietins and their tie receptors in human basophils and mast cells," *Journal of Biological Regulators and Homeostatic Agents*, vol. 27, no. 3, pp. 827–839, 2013.

[69] C. Visciano, F. Liotti, N. Prevete et al., "Mast cells induce epithelial-to-mesenchymal transition and stem cell features in human thyroid cancer cells through an IL-8-Akt-Slug pathway," *Oncogene*, vol. 34, no. 40, pp. 5175–5186, 2015.

[70] T. Demitsu, T. Inoue, M. Kakurai, T. Kiyosawa, K. Yoneda, and M. Manabe, "Activation of mast cells within a tumor of angiosarcoma: ultrastructural study of five cases," *The Journal Dermatology*, vol. 29, no. 5, pp. 280–289, 2002.

[71] D. A. de Souza Jr., V. D. Toso, M. R. Campos, V. S. Lara, C. Oliver, and M. C. Jamur, "Expression of mast cell proteases correlates with mast cell maturation and angiogenesis during tumor progression," *PLoS One*, vol. 7, no. 7, article e40790, 2012.

[72] D. Ribatti and E. Crivellato, "Mast cells, angiogenesis, and tumour growth," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1822, no. 1, pp. 2–8, 2012.

[73] H. Yuan, Y. H. Hsiao, Y. Zhang et al., "Destructive impact of T-lymphocytes, NK and Mast cells on basal cell layers: implications for tumor invasion," *BMC Cancer*, vol. 13, no. 1, p. 258, 2013.

[74] R. J. Blair, H. Meng, M. J. Marchese et al., "Human mast cells stimulate vascular tube formation. Tryptase is a novel, potent angiogenic factor," *Journal of Clinical Investigation*, vol. 99, no. 11, pp. 2691–2700, 1997.

[75] D. Ribatti, E. Crivellato, A. M. Roccaro, R. Ria, and A. Vacca, "Mast cell contribution to angiogenesis related to tumour progression," *Clinical & Experimental Allergy*, vol. 34, no. 11, pp. 1660–1664, 2004.

[76] A. M. Dvorak, M. C. Mihm Jr, J. E. Osage, and H. F. Dvorak, "Melanoma. An ultrastructural study of the host inflammatory and vascular responses," *Journal of Investigative Dermatology*, vol. 75, no. 5, pp. 388–393, 1980.

[77] T. C. Theoharides and P. Conti, "Mast cells: the Jekyll and Hyde of tumor growth," *Trends in Immunology*, vol. 25, no. 5, pp. 235–241, 2004.

[78] D. Baram, G. G. Vaday, P. Salamon, I. Drucker, R. Hershkoviz, and Y. A. Mekori, "Human mast cells release metalloproteinase-9 on contact with activated T cells: juxtacrine regulation by TNF-α," *The Journal of Immunology*, vol. 167, no. 7, pp. 4008–4016, 2001.

[79] J. L. Pennock and R. K. Grencis, "The mast cell and gut nematodes: damage and defence," *Parasites and Allergy*, vol. 90, no. 4, pp. 128–140, 2006.

[80] S. M. Thakurdas, E. Melicoff, L. Sansores-Garcia et al., "The mast cell-restricted tryptase mMCP-6 has a critical immunoprotective role in bacterial infections," *The Journal of Biological Chemistry*, vol. 282, no. 29, pp. 20809–20815, 2007.

[81] Z. Wang, Y. Lai, J. J. Bernard et al., "Skin mast cells protect mice against vaccinia virus by triggering mast cell receptor S1PR2 and releasing antimicrobial peptides," *The Journal of Immunology*, vol. 188, no. 1, pp. 345–357, 2012.

[82] M. J. Sinnamon, K. J. Carter, L. P. Sims, B. Lafleur, B. Fingleton, and L. M. Matrisian, "A protective role of mast cells in intestinal tumorigenesis," *Carcinogenesis*, vol. 29, no. 4, pp. 880–886, 2008.

[83] J. L. Gooch, A. V. Lee, and D. Yee, "Interleukin 4 inhibits growth and induces apoptosis in human breast cancer cells," *Cancer Research*, vol. 58, no. 18, pp. 4199–4205, 1998.

[84] W. R. Henderson, E. Y. Chi, E. C. Jong, and S. J. Klebanoff, "Mast cell-mediated tumor-cell cytotoxicity. Role of the peroxidase system," *Journal of Experimental Medicine*, vol. 153, no. 3, pp. 520–533, 1981.

[85] S. Latti, M. Leskinen, N. Shiota, Y. Wang, P. T. Kovanen, and K. A. Lindstedt, "Mast cell-mediated apoptosis of endothelial cells in vitro: a paracrine mechanism involving TNF-α-mediated down-regulation of bcl-2 expression," *Journal of Cellular Physiology*, vol. 195, no. 1, pp. 130–138, 2003.

[86] WHO, *World Health Organization*, 2012, http://wwwwhoint/cancer/es/.

[87] S. Dabiri, D. Huntsman, N. Makretsov et al., "The presence of stromal mast cells identifies a subset of invasive breast cancers with a favorable prognosis," *Modern Pathology*, vol. 17, no. 6, pp. 690–695, 2004.

[88] A. B. Rajput, D. A. Turbin, M. C. Cheang et al., "Stromal mast cells in invasive breast cancer are a marker of favourable prognosis: a study of 4,444 cases," *Breast Cancer Research and Treatment*, vol. 107, no. 2, pp. 249–257, 2008.

[89] R. Naik, P. Baliga, R. Bansal, and M. Pai, "Distribution of mast cells in the axillary lymph nodes of breast cancer patients," *Journal of Indian Medical Association*, vol. 95, no. 12, pp. 606-607, 1997.

[90] A. Glajcar, J. Szpor, A. Pacek et al., "The relationship between breast cancer molecular subtypes and mast cell populations in tumor microenvironment," *Virchows Archiv*, vol. 470, no. 5, pp. 505–515, 2017.

[91] F. della Rovere, A. Granata, D. Familiari, G. D'Arrigo, B. Mondello, and G. Basile, "Mast cells in invasive ductal breast cancer: different behavior in high and minimum hormone-receptive cancers," *Anticancer Research*, vol. 27, no. 4B, pp. 2465–2471, 2007.

[92] G. Ranieri, M. Ammendola, R. Patruno et al., "Tryptase-positive mast cells correlate with angiogenesis in early breast cancer patients," *International Journal of Oncology*, vol. 35, no. 01, pp. 115–120, 2009.

[93] I. Marech, M. Ammendola, R. Sacco et al., "Serum tryptase, mast cells positive to tryptase and microvascular density evaluation in early breast cancer patients: possible translational significance," *BMC Cancer*, vol. 14, no. 1, p. 534, 2014.

[94] M. Samoszuk and M. A. Corwin, "Mast cell inhibitor cromolyn increases blood clotting and hypoxia in murine breast

cancer," *International Journal of Cancer*, vol. 107, no. 1, pp. 159–163, 2003.

[95] D. Ribatti, N. Finato, E. Crivellato et al., "Angiogenesis and mast cells in human breast cancer sentinel lymph nodes with and without micrometastases," *Histopathology*, vol. 51, no. 6, pp. 837–842, 2007.

[96] M. A. Al-Rawi, R. E. Mansel, and W. G. Jiang, "Lymphangiogenesis and its role in cancer," *Histology and Histopathology*, vol. 20, no. 1, pp. 283–298, 2005.

[97] D. Ribatti and G. Ranieri, "Tryptase, a novel angiogenic factor stored in mast cell granules," *Experminetal Cell Research*, vol. 332, no. 2, pp. 157–162, 2015.

[98] S. H. Keser, N. O. Kandemir, D. Ece et al., "Relationship of mast cell density with lymphangiogenesis and prognostic parameters in breast carcinoma," *The Kaohsiung Journal of Medical Sciences*, vol. 33, no. 4, pp. 171–180, 2017.

[99] A. Fakhrjou, M. Naghavi-Behzad, V. Montazeri, F. Karkon-Shayan, L. Norouzi-Panahi, and R. Piri, "The relationship between histologic grades of invasive carcinoma of breast ducts and mast cell infiltration," *South Asian Journal of Cancer*, vol. 5, no. 1, pp. 5–7, 2016.

[100] Y. Liu and B. M. Mueller, "Protease-activated receptor-2 regulates vascular endothelial growth factor expression in MDA-MB-231 cells via MAPK pathways," *Biochemical and Biophysical Research Communications*, vol. 344, no. 4, pp. 1263–1270, 2006.

[101] M. S. Stack and D. A. Johnson, "Human mast cell tryptase activates single-chain urinary-type plasminogen activator (pro-urokinase)," *Journal of Biological Chemistry*, vol. 269, no. 13, pp. 9416–9419, 1994.

[102] D. R. Morris, Y. Ding, T. K. Ricks, A. Gullapalli, B. L. Wolfe, and J. Trejo, "Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells," *Cancer Research*, vol. 66, no. 1, pp. 307–314, 2006.

[103] V. M. Shpacovitch, T. Brzoska, J. Buddenkotte et al., "Agonists of proteinase-activated receptor 2 induce cytokine release and activation of nuclear transcription factor kappaB in human dermal microvascular endothelial cells," *Journal of Investigative Dermatology*, vol. 118, no. 2, pp. 380–385, 2002.

[104] M. Xiang, Y. Gu, F. Zhao, H. Lu, S. Chen, and L. Yin, "Mast cell tryptase promotes breast cancer migration and invasion," *Oncology Reports*, vol. 23, no. 3, pp. 615–619, 2010.

[105] L. He, Z. Zhu, S. Chen, Y. Wang, and H. Gu, "Mammary tumor growth and metastasis are reduced in c-Kit mutant Sash mice," *Cancer Medicine*, vol. 5, no. 6, pp. 1292–1297, 2016.

[106] P. Pittoni, C. Tripodo, S. Piconese et al., "Mast cell targeting hampers prostate adenocarcinoma development but promotes the occurrence of highly malignant neuroendocrine cancers," *Cancer Research*, vol. 71, no. 18, pp. 5987–5997, 2011.

[107] E. Mitry, P. Hammel, G. Deplanque et al., "Safety and activity of masitinib in combination with gemcitabine in patients with advanced pancreatic cancer," *Cancer Chemotherapy Pharmacology*, vol. 66, no. 2, pp. 395–403, 2010.

[108] M. Samoszuk and M. A. Corwin, "Acceleration of tumor growth and peri-tumoral blood clotting by imatinib mesylate (Gleevec™)," *International Journal of Cancer*, vol. 106, no. 5, pp. 647–652, 2003.

[109] G. P. Cricco, C. A. Davio, G. Martin et al., "Histamine as an autocrine growth factor in experimental mammary carcinomas," *Agents and Actions*, vol. 43, no. 1-2, pp. 17–20, 1994.

[110] C. A. Davio, G. P. Cricco, G. Martin, C. P. Fitzsimons, R. M. Bergoc, and E. S. Rivera, "Effect of histamine on growth and differentiation of the rat mammary gland," *Agents and Actions*, vol. 41, Supplement 1, pp. C115–C117, 1994.

[111] P. F. Bowrey, J. King, C. Magarey et al., "Histamine, mast cells and tumour cell proliferation in breast cancer: does preoperative cimetidine administration have an effect?," *British Journal of Cancer*, vol. 82, no. 1, pp. 167–170, 2000.

[112] E. Z. da Silva, M. C. Jamur, and C. Oliver, "Mast cell function: a new vision of an old cell," *Journal of Histochemistry Cytochemistry*, vol. 62, no. 10, pp. 698–738, 2014.

[113] T. Gebhardt, A. Lorentz, F. Detmer et al., "Growth, phenotype, and function of human intestinal mast cells are tightly regulated by transforming growth factor β1," *Gut*, vol. 54, no. 7, pp. 928–934, 2005.

[114] T. C. Moon, C. D. St Laurent, K. E. Morris et al., "Advances in mast cell biology: new understanding of heterogeneity and function," *Mucosal Immunology*, vol. 3, no. 2, pp. 111–128, 2010.

Hindawi
Journal of Immunology Research
Volume 2018, Article ID 4564328, 10 pages
https://doi.org/10.1155/2018/4564328

Research Article

STAT1, IGF1, RAC1, and MDM2 Are Associated with Recurrence of Giant Cell Tumor of Bone

Shuxin Chen,[1] Zepeng Du,[2] Bingli Wu,[3] Huiyang Shen,[1] Chunpeng Liu,[1] Xueli Qiu,[1] Yufeng Zhang,[1] Liyan Xu,[4] Enmin Li,[3] and Zhigang Zhong[1]

[1]*Department of Orthopedic Surgery, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, China*
[2]*Department of Pathology, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, China*
[3]*Department of Biochemistry and Molecular Biology, Shantou University Medical College, Shantou 515041, China*
[4]*Institute of Oncologic Pathology, Shantou University Medical College, Shantou 515041, China*

Correspondence should be addressed to Zhigang Zhong; stzzg@163.com

Received 16 August 2017; Revised 8 November 2017; Accepted 28 November 2017; Published 31 January 2018

Academic Editor: Qing Lin

Background. In our previous study, mouse double minute 2 homolog (MDM2), insulin-like growth factor 1 (IGF1), signal transducer and activator of transcription 1 (STAT1), and Rac family small GTPase 1 (RAC1) were correlated with the recurrence of giant cell tumor of bone (GCT). The aim of this study is to use a large cohort study to confirm the involvement of these four genes in GCT recurrence. *Methods*. The expression of these four genes was detected and compared between GCT patients with or without recurrence. The correlation between the expression of these four genes and clinical characteristics was evaluated. Protein-protein interaction (PPI) network was constructed for functional enrichment analysis. *Results*. It showed that the expression levels of MDM2, IGF1, STAT1, and RAC1 in GCT patients with recurrence were significantly higher than those in GCT patients without recurrence ($P < 0.05$). Multivariate logistic regression analysis suggested that several clinical characteristics may influence prognosis. A PPI network was constructed using the four genes as hub genes. Functional enrichment analysis showed that this network involves many important biological progress mediated by these four genes, including immune response. *Conclusion.* MDM2, IGF1, STAT1, and RAC1 are associated with GCT recurrence, which might serve as biomarkers for GCT recurrence.

1. Introduction

Giant cell tumor of bone (GCT) is an aggressive osteolytic tumor with a high rate of recurrence. Recurrence after surgery treatment has been reported in about 50% of GCT patients. To date, surgery including wide resection and curettage is the major approach used for the treatment of GCT. While wide resection significantly reduces the recurrence of GCT [1], it destroys bone structure and limits joint function. In addition, the histological origin of GCT is not clear and the biological behavior of GCT is complex and varied, which causes difficulties for both surgical planning and the evaluation of the efficacy of surgery for GCT. Therefore, accurate evaluation of the biological behavior of GCT is important for the treatment of GCT. However, the traditional evaluation of GCT behavior, which is based on the histological classification proposed by Jaffe [2], is insufficient to predict tumor progression. The latter evaluation method, which is based on imaging examination results [3] and clinical stage [4], is still controversial. Currently, no efficient biomarkers are used to evaluate the biological behavior of GCT.

There is increasing evidence suggesting that mouse double minute 2 homolog (MDM2), insulin-like growth factor 1 (IGF1), signal transducer and activator of transcription 1 (STAT1), and Rac family small GTPase 1 (RAC1) are

involved in tumor progression. MDM2 is considered as a negative regulator of p53 [5]. Amplification of MDM2 genes and/or enhanced expression of MDM2 is observed in a variety of tumors including GCT [6]. IGF-1, which regulates cell proliferation, plays a key role in osteoblast proliferation and bone formation [7]. STAT1 is a STAT family member and is associated with human breast cancer, melanoma, leukemia, and lymphoma cancers [8]. RAC1 is also involved in tumor invasion, metastasis, and angiogenesis [9]. However, it is currently unclear whether these four genes are associated with GCT recurrence.

Previously, we evaluated differentially expressed genes and analyzed recurrence-related subpathways in the recurrent GCT patients. Based on microarray results and subpathway analyses, four genes (IGF1, MDM2, STAT1, and RAC1) were identified to play an important role in GCT of bone recurrence [10]. In this study, the aim is to verify, by immunohistochemistry, the expression of IGF1, MDM2, STAT1, and RAC1 and investigate their potential relationship with GCT recurrence, in a large cohort of patients with a giant cell tumor of the bone.

2. Materials and Methods

2.1. Specimens. A total of 75 formalin-fixed, paraffin-embedded GCT tissue blocks were included in the present study. These specimens were obtained from surgically dissected bone tissues from 75 GCT patients who were treated, between January 2000 and June 2012, in the Department of Orthopedics, Shantou Central Hospital affiliated with Zhongshan University. The diagnosis of GCT was confirmed based on histopathological examination by pathologists in the Department of Pathology, Shantou Central Hospital. The 75 primary GCT cases included both postoperative cases with recurrence ($n = 20$) and nonrecurrence ($n = 55$). The mean age of the 36 male and 39 female patients was 31 years (15–65 years). The clinical characteristics of these patients are shown in Table 1. All human studies have been approved by The Institutional Review Board of Shantou Hospital of Zhongshan University and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study.

2.2. Immunohistochemistry (IHC). Mouse anti-human monoclonal antibodies against MDM2, IGF1, STAT1, and RAC1 were purchased from DAKO. Immunohistochemistry SP kits were purchased from Fuzhou Maixin Company. IHC was conducted on paraffin sections using the SP kit according to the manufacturer's instructions. Slides were stained using DAB with hematoxylin contrast staining. The negative controls were incubated with PBS to substitute for the primary antibody.

IHC results were scored based on both staining intensity (no staining, score = 0; weak staining, score = 1; medium staining, score = 2; and strong staining, score = 3) and the percentage of positively stained cells (≤5%, score = 0; 6%~25%, score = 1; 26~50%, score = 2; 51 ~ 75%, score = 3; and >75%, score = 4). The final IHC score for individual tumors was determined by the product of the scores for intensity and percentage of positively stained cells. The final IHC score, for either patients with or patients without recurrence, was determined by the sum of the scores of all individual tumor staining within the group. For example, in three specimens where 25%, 25%, and 50% of tumor cells exhibited medium, weak, and no staining, respectively; the scores, for each individual tumor, were 2 (1×2), 1 (1×1), and 0 (2×0), respectively. The IHC score for the group was 3 ($2 + 1 + 0$).

Table 1: Clinical characteristics of the 75 GCT cases included in the present study.

Clinical characteristics	Postoperative recurrence	Nonrecurrence after surgery	Incidence of postoperative recurrence
75	20	55	26.6%
Gender			
Male	9	27	25%
Female	11	28	28.2%
Tumor location			
Distal femur	9	23	28.1%
Proximal tibia	2	6	25%
Distal radius	4	4	50%
Proximal humerus	0	6	0
Proximal femur	3	4	42.9%
Other sites	2	12	14.3%
Pathological fracture			
Yes	3	12	20%
No	17	43	28.3%
Campanacci grade			
I	3	12	20%
II	12	31	27.9%
III	5	12	29.4%
Surgery			
Wide resection	6	17	26.0%
Curettage	13	39	25%
Amputation	0	0	0

2.3. Protein-Protein Interaction Network Construction. To explore the correlation between four genes (MDM2, IGF1, STAT1, and RAC1), a protein-protein interaction (PPI) network was constructed by the method as we described before [11]. Briefly, the protein interaction data validated by experiment was obtained from BioGRID (https://thebiogrid.org/) and HPRD (http://www.hprd.org/) to form a parental PPI network. A child PPI was constructed by mapping the four genes as the seed proteins in the parental PPI network to extract their first class interacting proteins by Cytoscape [12].

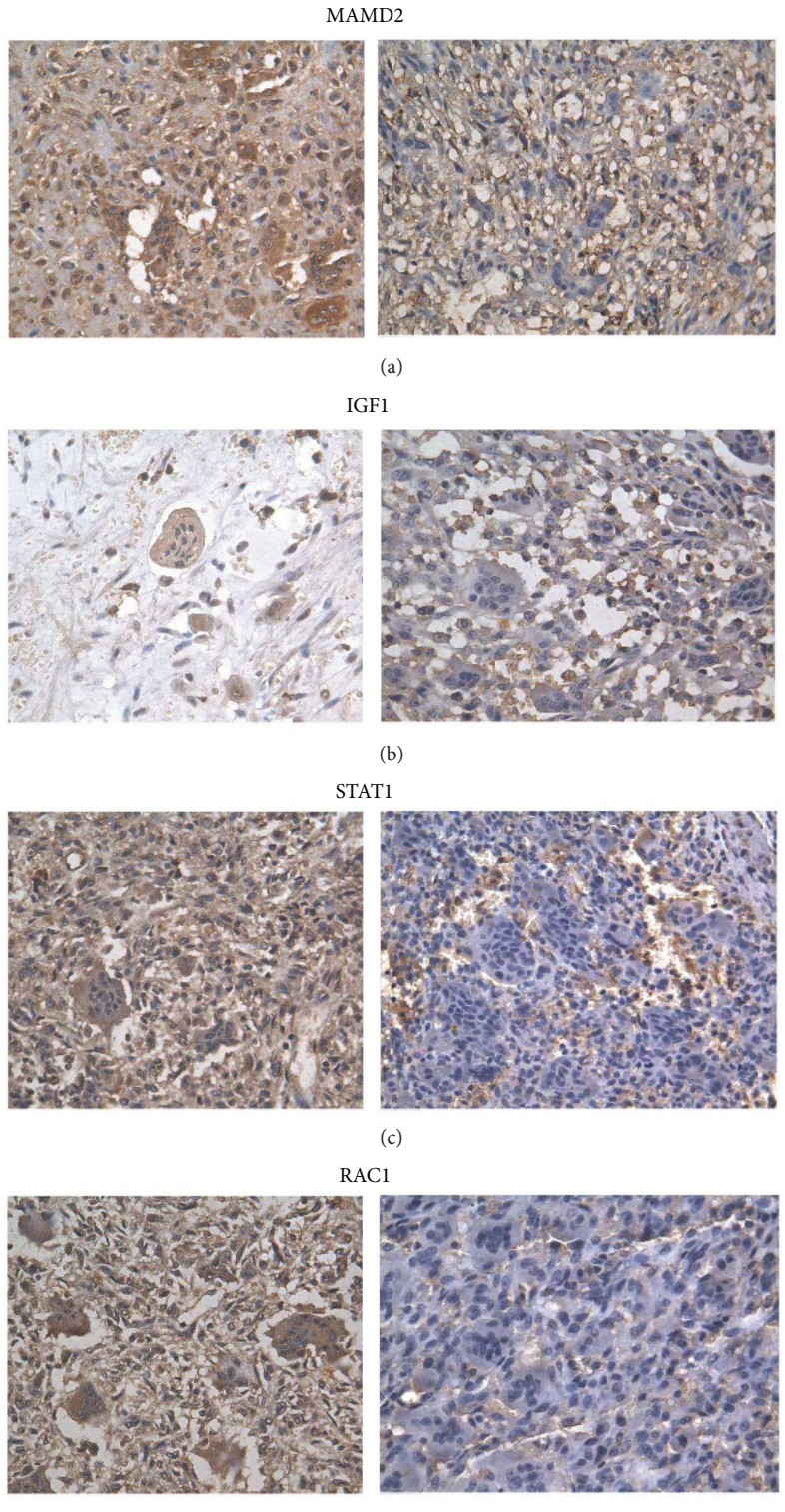


Figure 1: Expression of MDM2, IGF1, STAT1, and RAC1 in GCT tissues (400X, scale bar = 50 μm). (a) Expression of MDM2. Positive staining of MDM2 in the tumor tissue of a recurrent GCT (left). Negative staining of MDM2 in the tissue of a primary GCT from a patient without recurrence (right). (b) Positive staining of IGF1 in the tumor tissue of recurrent GCT (left). Negative staining of IGF1 in the primary tumor tissue of a GCT patient without recurrence (right). (c) Positive staining of STAT1 in the tumor tissue of recurrence GCT (left). Negative staining of STAT1 in the primary tumor tissue of a GCT patient without recurrence (right). (d) Positive staining of RAC1 in the tumor tissue of recurrence GCT (left). Negative staining of RAC1 in the tumor tissue of primary GCT without recurrence (right). Positive staining for all genes was located in the cytoplasm and nuclei of multinucleated giant cells and mononuclear stromal cells.

Table 2: Association between expression of MDM2, IGF1, STAT1, and RAC1 and the recurrence of GCT.

	MDM2		IGF1		STAT1		RAC1	
Staining intensity	—	+	—	+	—	+	—	+
Without recurrence	39	16	36	19	41	15	40	15
With recurrence	6	14	6	14	7	12	6	14
P value	0.003		0.009		0.006		0.001	

Table 3: Logistic regression analysis of the association between clinical characteristics and GCT recurrence.

		Score	Difference	Significance
Variables	Gender	0.783	1	0.376
	Age	0.511	1	0.475
	C grade	0.282	2	0.869
	C grade (1)	0.275	1	0.600
	C grade (2)	0.140	1	0.709
	Pathological fracture	0.078	1	0.781
	Treatment	0.739	1	0.390
	Adjuvant	0.168	1	0.682
	Burr	1.345	1	0.246
	Lung metastasis	2.641	1	0.104
Overall statistics		16.352	10	0.090

2.4. Functional Enrichment Analysis of PPI Network. Gene ontology (GO) annotation of the four genes, PPI networks, was performed using the ClueGO plugin, which could produce a functional enrichment group GO terms in the form of a network [13]. Only GO terms with a P value <0.001 were considered significant. A kappa score reflecting the connection between the GO terms was based on their overlapping genes, which was set to 0.5 as the threshold.

2.5. Statistical Analyses. Statistical analyses of the experimental results were conducted using SPSS software (Version 13.0). The correlation between gene expression (of MDM2, IGF1, STAT1, and RAC1) and clinical characteristics was conducted using the Kendall's tau-b test. The association of MDM2, IGF1, STAT1, and RAC1 and tumor recurrence was analyzed using chi-square analysis. Clinical characteristics that may be associated with GCT recurrence were analyzed using logistic regression, and an α value of 0.05 was used as a standard for regression analysis.

3. Results

3.1. MDM2, IGF1, STAT1, and RAC1 Expression in GCT Tissue. Immunohistochemistry was performed for MDM2, IGF1, STAT1, and RAC1 to determine their expression patterns in GCT patients with recurrence. As shown in Figure 1, the staining of MDM2, IGF1, STAT1, and RAC1 was mainly found in the cytoplasm of GCT tissues, but nuclear staining was also observed in some cells. Both multinucleated giant cells and mononuclear stromal cells were positively stained for these markers. Generally, the staining of MDM2 and RAC1 was stronger than IGF1 and STAT1 staining. The percentage of positively stained cells was similar for these four markers, which exhibited similar and consistent staining patterns.

3.2. Expression of MDM2, IGF1, STAT1, and RAC1 and GCT Patients Is Associated with Recurrence. Immunohistochemistry analyses combined with statistical analyses were conducted to confirm the association between the expression of the four genes and GCT recurrence. As shown in Table 2, the expression of MDM2, IGF1, STAT1, and RAC1 in GCT patients with recurrence was significantly higher than that in GCT patients without recurrence ($P < 0.05$). Positive staining of MDM2 was observed in 16 of 55 GCT tissues without recurrence and 14 of 20 GCT tissues with recurrence. A chi-square test demonstrated that the expression of MDM2 in GCT tissues with recurrence was significantly higher than that in GCT tissues without recurrence ($P = 0.012$) (Table 2). Positive staining for IGF1 was observed in 19 of 55 GCT tissues without recurrence and 14 of 20 GCT tissues with recurrence, with chi-square test suggesting that the expression of IGF1 in GCT tissues with recurrence was significantly higher than that in GCT tissues without recurrence ($P = 0.033$) (Table 2). Positive staining for STAT1 was observed in 15 of 55 GCT tissues without recurrence and 12 of 19 GCT tissues with recurrence. The chi-square test suggested that the expression of STAT1 in GCT tissues with recurrence was significantly higher than that in GCT tissues without recurrence ($P = 0.026$) (Table 2). Positive staining for RAC1 was observed in 15 of 55 GCT tissues without recurrence and 14 of 20 GCT tissues with recurrence, and chi-square test suggested that the expression of RAC1 in

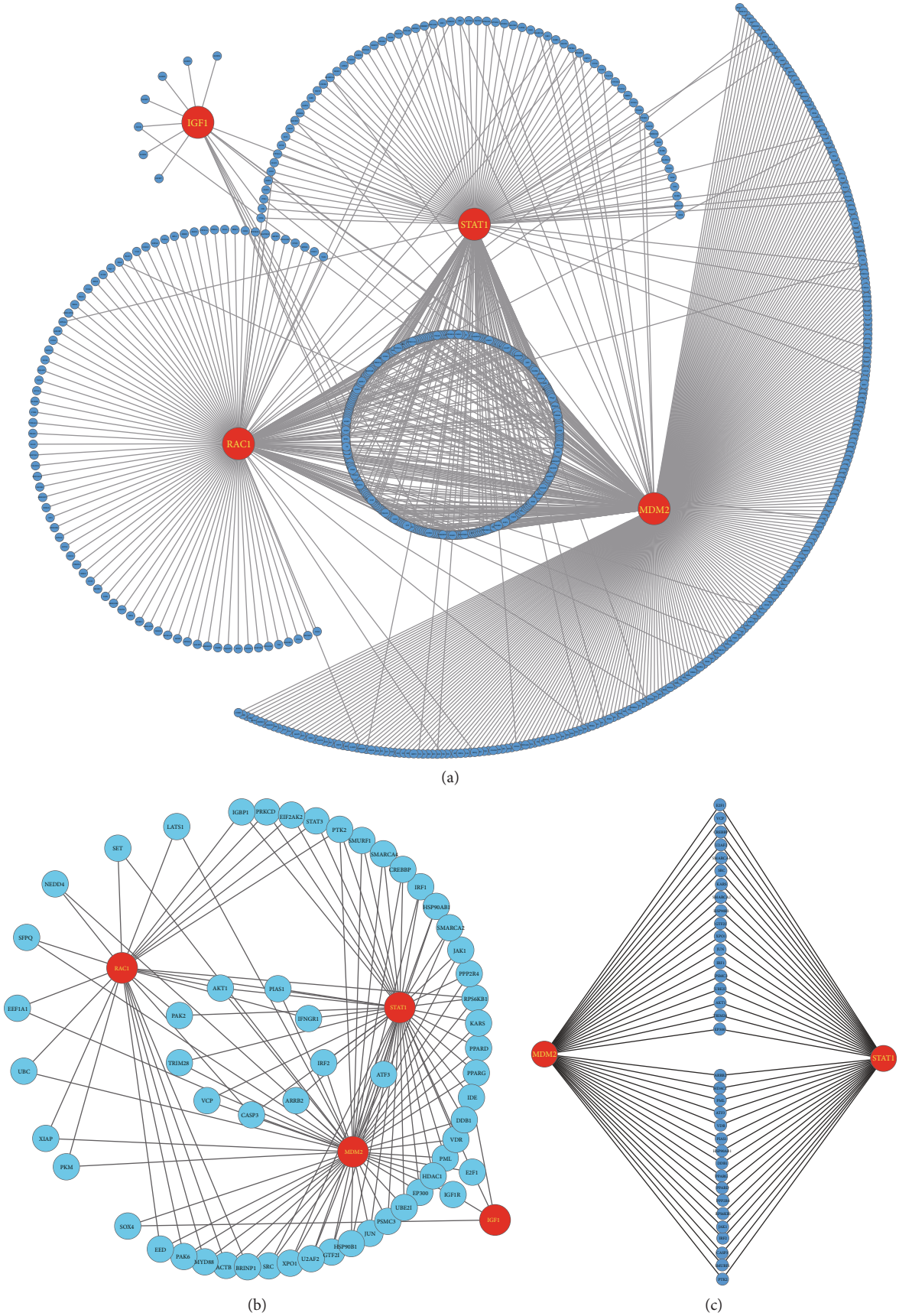


Figure 2: Continued.

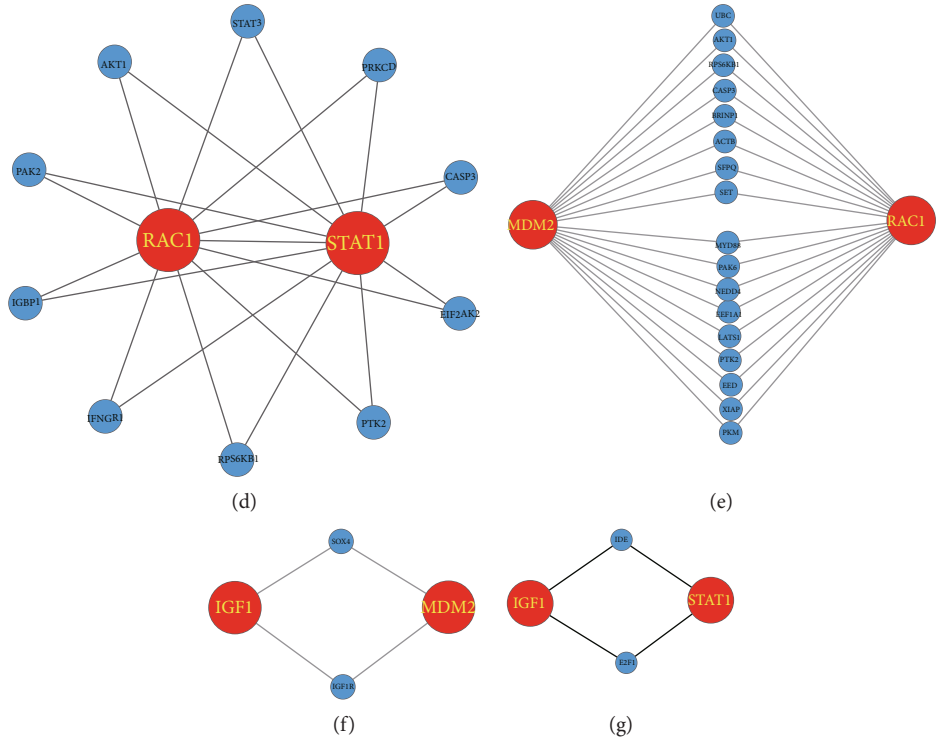


Figure 2: The protein-protein interaction network for MDM2, IGF1, STAT1, and RAC1. (a) MDM2, IGF1, STAT1, and RAC1 are hub proteins in the PPI network as they have hundreds of interacting proteins. (b) MDM2, IGF1, STAT1, and RAC1 are connected by at least two or more shared interacting proteins. (c–g) The interactions of every two genes between MDM2, IGF1, STAT1, and RAC1.

GCT tissues with recurrence was significantly higher than that in GCT tissues without recurrence ($P = 0.007$) (Table 2).

3.3. Expression of MDM2, IGF1, STAT1, and RAC1 Is Not Associated with Clinical Characteristics of GCT Patients with Recurrence. Association of the expression of MDM2, IGF1, STAT1, and RAC1 and clinical characteristics such as age, gender, Campanicci grade, pathological fracture, and lung metastasis was also analyzed using the Kendall's tau-b test. No significant association was observed between the expression of the four markers and any clinical characteristics, including age, gender, Campanicci grade, pathological fracture, and lung metastasis.

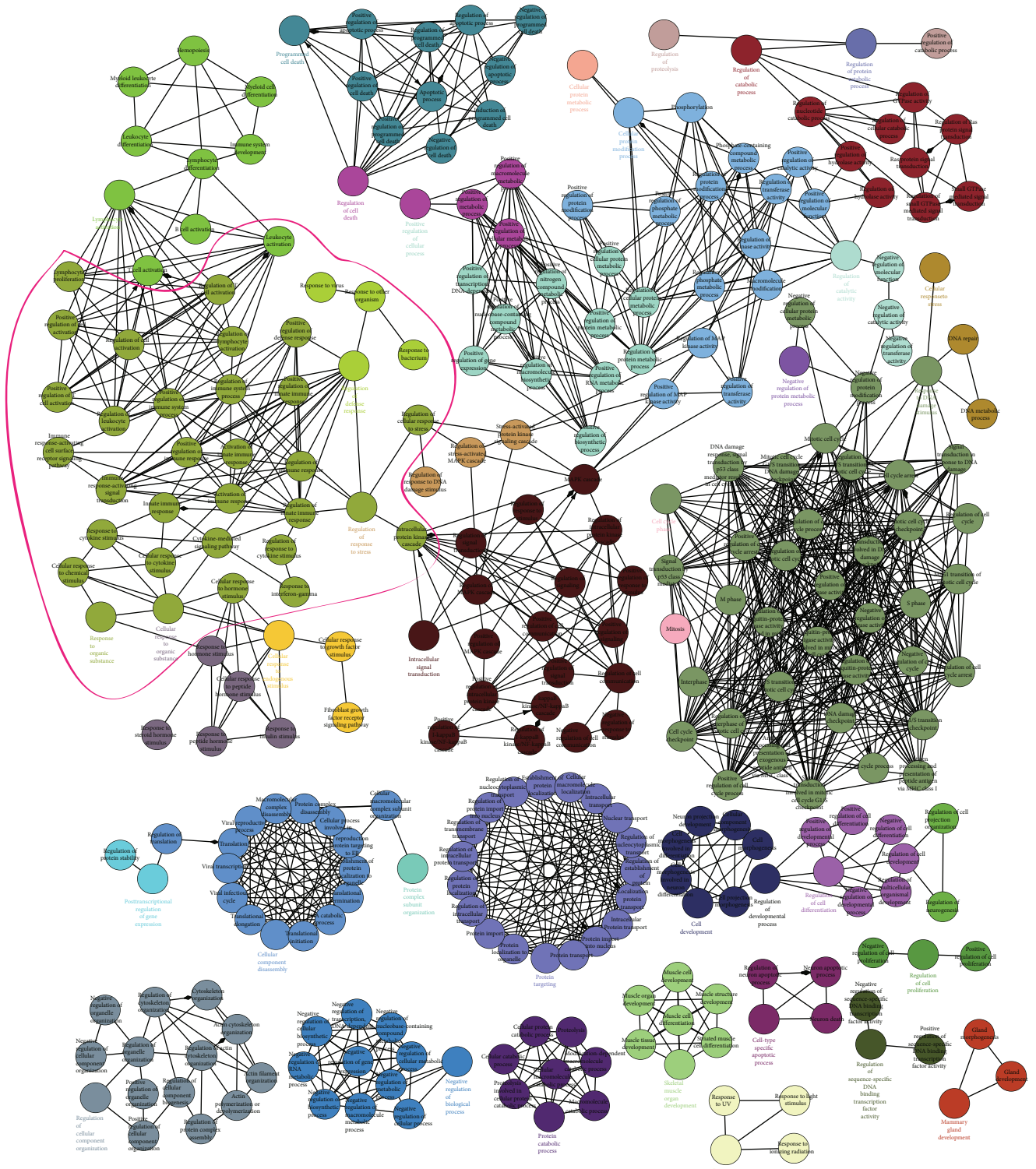
Furthermore, clinical characteristics including gender, age, tumor duration, tumor location, pathologic fracture, and surgical procedures, which may influence prognosis, were analyzed using multivariate logistic regression analysis. No clinical characteristics were identified to be significantly associated with GCT recurrence (Table 3).

3.4. PPI Network and Functional Enrichment. In order to gain insight into how these MDM2, IGF1, STAT1, and RAC1 are connected, a full screen of their interacting proteins would provide important clues of their links and functions. The PPI network in this study contains 768 nodes (proteins) and 830 edges (interactions) as shown in Figure 2(a), suggesting these four genes are hub proteins in the cellular network through the interactions with hundreds of their interacting proteins. To better illustration, only the direct interaction among the four genes was shown (Figures 2(b)–2(g)). We found that MDM2, IGF1, STAT1, and RAC1 are connected with at least two or more shared interacting proteins. RAC1 and STAT1 could interact directly.

A map containing 290 significant GO terms was generated by the functional enrichment analysis of the four genes, PPI network. In this map, the nodes represent as the protein-enriched GO terms, with the edges connecting the GO terms indicative of proteins shared between GO terms (Figure 3(a)). Several GO terms that were related to known functions were found, such as "intracellular signal transduction," "cell cycle phase," and "cell development." Moreover, we found a big group of immunity-related terms, including "regulation of immune system process," "activation of immune response," "immune response-activating signal transduction," "positive regulation of immune response," "immune response-activating cell surface receptor signaling pathway," and "regulation of innate immune response" (Figure 3(b)). These results suggested that the four genes involved in GCT recurrence might participate in immune response. The significant immune-related-enriched functional terms are listed in Table 4.

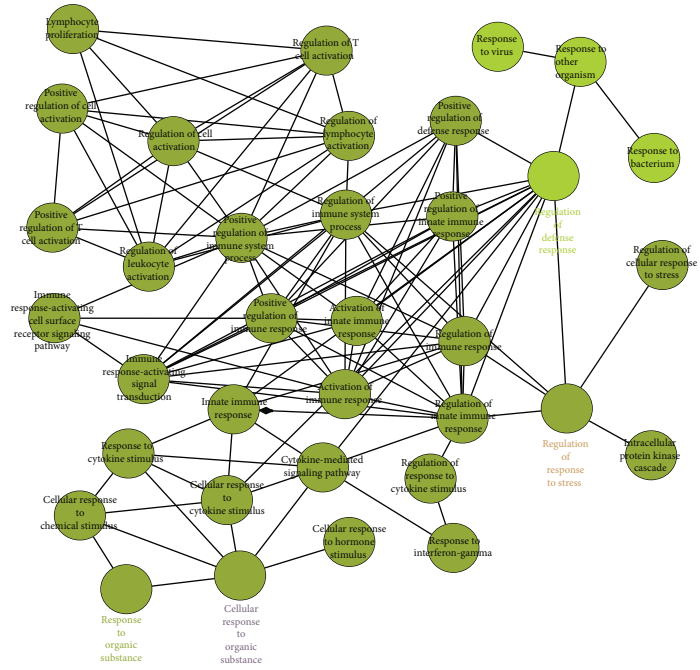
4. Discussion

In a previous study, we compared the expression profiles, based on microarray data, of a large number of genes between GCT with and GCT without recurrence [10] and identified six signaling pathways that may play important roles in the development of GCT. In the present study, we further analyze whether the differentially expressed genes



(a)

Figure 3: Continued.



(b)

Figure 3: Functional enrichment analysis of the PPI network of MDM2, IGF1, STAT1, and RAC1. (a) 290 significant GO terms were generated by the functional enrichment analysis of the four genes, PPI network. GO terms are connected when they shared enriched genes above the threshold of kappa score. (b) The group of immunity-related terms, as indicated in the pink circle in (a).

STAT1, IGF1, RAC1, and MDM2 are associated with GCT recurrence.

Based on the IHC results, significantly higher expression levels of MDM2, IGF1, STAT1, and RAC1 are found in the patients with recurrent GCT, compared to primary patients, which is consistent with our previous microarray study [10]. Subsequent statistical analyses suggested that these four genes are closely correlated with GCT recurrence, whereas no significant association with clinical characteristics, such as age, gender, Campanicci grade, pathological fracture, and lung metastasis. These findings indicate that MDM2, IGF1, STAT1, and RAC1 may serve as potential biomarkers for prediction of GCT patients at a high risk of recurrence. Nevertheless, researches based on the panel of these four genes in human disease have not been reported so far.

Many reports have confirmed that these four genes play important roles in tumors. Upregulation of MDM2 has also been observed in a variety of sarcoma [14]. Previous studies suggest that upregulation of MDM2 is associated with tumor initiation and metastasis of GCT [15]. MDM2 gene amplification in osteosarcoma is often associated with p53 inactivation [16] or osteosarcoma of metastasis and recurrence; we thus propose that MDM2 is closely related to GCT recurrence and may contribute to tumor cell proliferation. Evidence shows that IGF1 promotes tumor cell differentiation and growth through autocrine or paracrine means [17]. IGF1 is also closely associated with bone metabolism and plays a key role in growth factor promotion of osteoblast proliferation and bone formation [7]. In addition to these findings, IGFI is found to function in stimulating osteoblast proliferation and bone formation and inhibiting bone collagen degradation [18]. It has been recently reported that STAT1 expression is upregulated in osteoporotic bone tissue in mice [19]. Chen et al. reported that the expression of STAT1 in circulating monocytes is essential for bone metabolism, suggesting an important role for the STAT1-mediated IFN pathway in osteoporosis [20]. STAT1 plays an important role in bone growth and bone formation [21], which suggests that STAT1 might be involved in the development of GCT, especially tumor invasiveness. RAC1 expression is upregulated in the osteosarcoma cell lines and tissues, and its ectopic expression promotes the proliferation, migration, and invasion of MG-63 cells [22]. It has been reported that a novel ERBB4-PI3K-AKT-FAK-RAC1 pathway associated with an aggressive disease in Ewing sarcoma [23].

This study is the first time to confirm that MDM2, IGF1, STAT1, and RAC1 might link together involved in GCT recurrence. Proteins do not work alone, but interact with other proteins or molecules to perform their specific functions. MDM2, IGF1, STAT1, and RAC1 serve as hub genes in the PPI network. Functional enrichment result shows that this network involves many biological progress, including immune response. In recent years, the immune activity in bone giant cell has attracted more attention. Al-Sukaini et al. found that locally aggressive giant cell lesions are associated with low HLA class 1 antigen expression, low CD8 + T cell infiltration, and high expression of the immune modulator B7-H3 [24]. On the other hand, most of GCT of bone cases can show variable immunoreactivity for CD10 and CD138 [25]. These results suggest that immune response,

Table 4: The significant immune-related-enriched functional terms.

Significant GO list	Term name	*P* value corrected with Bonferroni
GO:00713100	Cellular response to organic substance	1.23*E*-28
GO:00072430	Intracellular protein kinase cascade	4.40*E*-27
GO:00100330	Response to organic substance	1.81*E*-26
GO:00192210	Cytokine-mediated signaling pathway	3.80*E*-19
GO:00713450	Cellular response to cytokine stimulus	1.23*E*-17
GO:00801350	Regulation of cellular response to stress	3.38*E*-14
GO:00026820	Regulation of immune system process	7.62*E*-14
GO:00450880	Regulation of innate immune response	8.01*E*-14
GO:00328700	Cellular response to hormone stimulus	9.06*E*-14
GO:00027570	Immune response-activating signal transduction	5.49*E*-10
GO:00026840	Positive regulation of immune system process	1.02*E*-09
GO:00022530	Activation of immune response	3.26*E*-09
GO:00607590	Regulation of response to cytokine stimulus	6.86*E*-09
GO:00508650	Regulation of cell activation	1.11*E*-08
GO:00517070	Response to other organism	2.13*E*-08
GO:00507780	Positive regulation of immune response	2.14*E*-08
GO:00512490	Regulation of lymphocyte activation	4.19*E*-08
GO:00450890	Positive regulation of innate immune response	1.86*E*-07
GO:00024290	Immune response-activating cell surface receptor signaling pathway	2.62*E*-07
GO:00508700	Positive regulation of T cell activation	2.07*E*-06
GO:00508670	Positive regulation of cell activation	3.30*E*-06
GO:00313490	Positive regulation of defense response	3.42*E*-06
GO:00096150	Response to virus	4.46*E*-06
GO:00466510	Lymphocyte proliferation	1.73*E*-05
GO:00096170	Response to bacterium	2.17*E*-05

as well as other biological functions, mediated by MDM2, IGF1, STAT1, and RAC1 through their interacting proteins, might play an important role in GCT recurrence.

In summary, we provide evidence that MDM2, IGF1, STAT1, and RAC1 are associated with GCT recurrence. MDM2, IGF1, STAT1, and RAC1 may serve as biomarkers to predict GCT recurrence. In addition, the molecular mechanism of these markers in the development of GCT in a panel model should also be explored for better use of these markers in the evaluation of the prognosis of GCT.

Conflicts of Interest

All authors declare that they have no conflict of interests to state.

Authors' Contributions

Shuxin Chen and Zepeng Du contributed equally to this work.

Acknowledgments

The authors thanked Dr. Stane Lin for proofreading the manuscript and constructive suggestions. This work was supported by grants from the National Natural Science Foundation of China (no. 81672473, no. 81502138), the Science and Technology Program of Guangdong (no. 2014A030310390, no. 2017A030313181), and the Department of Education, Guangdong Government under the Top-tier University Development Scheme for Research and Control of Infectious Diseases (2016034).

References

[1] K. A. Raskin, J. H. Schwab, H. J. Mankin, D. S. Springfield, and F. J. Hornicek, "Giant cell tumor of bone," *Journal of the American Academy of Orthopaedic Surgeons*, vol. 21, no. 2, pp. 118–126, 2013.

[2] M. Wülling, C. Engels, N. Jesse, M. Werner, G. Delling, and E. Kaiser, "The nature of giant cell tumor of bone," *Journal of Cancer Research and Clinical Oncology*, vol. 127, no. 8, pp. 467–474, 2001.

[3] C. J. Chakarun, D. M. Forrester, C. J. Gottsegen, D. B. Patel, E. A. White, and G. R. Matcuk Jr, "Giant cell tumor of bone: review, mimics, and new developments in treatment," *Radiographics*, vol. 33, no. 1, pp. 197–211, 2013.

[4] Y. Levav-Cohen, Z. Goldberg, K. H. Tan et al., "The p53-Mdm2 loop: a critical juncture of stress response," in *Subcellular Biochemistry*, vol. 85, pp. 161–186, Springer, Dordrecht, 2014.

[5] L. T. Chow, "Giant cell rich osteosarcoma revisited—diagnostic criteria and histopathologic patterns, Ki67, CDK4, and MDM2 expression, changes in response to bisphosphonate

and denosumab treatment," *Virchows Archiv*, vol. 468, no. 6, pp. 741–755, 2016.

[6] J. Reckenbeil, D. Kraus, H. Stark et al., "Insulin-like growth factor 1 (IGF1) affects proliferation and differentiation and wound healing processes in an inflammatory environment with p38 controlling early osteoblast differentiation in periodontal ligament cells," *Archives of Oral Biology*, vol. 73, pp. 142–150, 2017.

[7] G. Mazziotti, A. M. Formenti, R. A. Adler et al., "Glucocorticoid-induced osteoporosis: pathophysiological role of GH/IGF-I and PTH/VITAMIN D axes, treatment options and guidelines," *Endocrine*, vol. 54, no. 3, pp. 603–611, 2016.

[8] K. Meissl, S. Macho-Maschler, M. Müller, and B. Strobl, "The good and the bad faces of STAT1 in solid tumours," *Cytokine*, vol. 89, pp. 12–20, 2017.

[9] T. Zou, X. Mao, J. Yin et al., "Emerging roles of RAC1 in treating lung cancer patients," *Clinical Genetics*, vol. 91, no. 4, pp. 520–528, 2017.

[10] S. Chen, C. Li, B. Wu et al., "Identification of differentially expressed genes and their subpathways in recurrent versus primary bone giant cell tumors," *International Journal of Oncology*, vol. 45, no. 3, pp. 1133–1142, 2014.

[11] B. Wu, C. Li, Z. Du et al., "Network based analyses of gene expression profile of *LCN2* overexpression in esophageal squamous cell carcinoma," *Scientific Reports*, vol. 4, article 5403, 2014.

[12] G. Su, J. H. Morris, B. Demchak, and G. D. Bader, "Biological network exploration with Cytoscape 3," *Current Protocols in Bioinformatics*, vol. 47, pp. 8.13.1–8.13.24, 2014.

[13] G. Bindea, B. Mlecnik, H. Hackl et al., "ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks," *Bioinformatics*, vol. 25, no. 8, pp. 1091–1093, 2009.

[14] M. Guérin, J. Thariat, M. Ouali et al., "A new subtype of high-grade mandibular osteosarcoma with *RASAL1/MDM2* amplification," *Human Pathology*, vol. 50, pp. 70–78, 2016.

[15] M. F. Shaikh, W. F. Morano, J. Lee et al., "Emerging role of MDM2 as target for anti-cancer therapy: a review," *Annals of Clinical & Laboratory Science*, vol. 46, no. 6, pp. 627–634, 2016.

[16] F. Jin, Y. Wang, X. Wang et al., "Bre enhances osteoblastic differentiation by promoting the Mdm2-mediated degradation of p53," *Stem Cells*, vol. 35, no. 7, pp. 1760–1772, 2017.

[17] N. Rajbhandari, W. C. Lin, B. L. Wehde, A. A. Triplett, and K. U. Wagner, "Autocrine IGF1 signaling mediates pancreatic tumor cell dormancy in the absence of oncogenic drivers," *Cell Reports*, vol. 18, no. 9, pp. 2243–2255, 2017.

[18] Y. Wang, A. Menendez, C. Fong, H. Z. ElAlieh, W. Chang, and D. D. Bikle, "Ephrin B2/EphB4 mediates the actions of IGF-I signaling in regulating endochondral bone formation," *Journal of Bone and Mineral Research*, vol. 29, no. 8, pp. 1900–1913, 2014.

[19] Z. Feng, W. Zheng, Q. Tang et al., "Fludarabine inhibits STAT1-mediated up-regulation of caspase-3 expression in dexamethasone-induced osteoblasts apoptosis and slows the progression of steroid-induced avascular necrosis of the femoral head in rats," *Apoptosis*, vol. 22, no. 8, pp. 1001–1012, 2017.

[20] C. Seeliger, L. Schyschka, Z. Kronbach et al., "Signaling pathway STAT1 is strongly activated by IFN-β in the pathogenesis of osteoporosis," *European Journal of Medical Research*, vol. 20, no. 1, p. 1, 2015.

[21] L. Xiao, T. Naganawa, E. Obugunde et al., "Stat1 controls postnatal bone formation by regulating fibroblast growth factor signaling in osteoblasts," *Journal of Biological Chemistry*, vol. 279, no. 26, pp. 27743–27752, 2004.

[22] S. Geng, L. Gu, F. Ju et al., "MicroRNA-224 promotes the sensitivity of osteosarcoma cells to cisplatin by targeting Rac1," *Journal of Cellular and Molecular Medicine*, vol. 20, no. 9, pp. 1611–9, 2016.

[23] A. Mendoza-Naranjo, A. El-Naggar, D. H. Wai et al., "ERBB4 confers metastatic capacity in Ewing sarcoma," *EMBO Molecular Medicine*, vol. 5, no. 7, pp. 1087–1102, 2013.

[24] A. Al-Sukaini, F. J. Hornicek, Z. S. Peacock, L. B. Kaban, S. Ferrone, and J. H. Schwab, "Immune surveillance plays a role in locally aggressive giant cell lesions of bone," *Clinical Orthopaedics and Related Research*, vol. 475, no. 12, pp. 3071–3081, 2017.

[25] M. A. Al-Abbadi, M. J. Al-Yousef, M. M. Yousef, S. S. Sheikh, N. M. Almasri, and S. S. Amr, "CD10 and CD138 can be expressed in giant cell tumor of bone: an immunohistochemical study," *Avicenna Journal of Medicine*, vol. 6, no. 3, pp. 69–74, 2016.

Hindawi
Journal of Immunology Research
Volume 2017, Article ID 1832968, 13 pages
https://doi.org/10.1155/2017/1832968

Review Article

Regulatory T Cells and Their Prognostic Relevance in Hematologic Malignancies

Giovanni D'Arena,[1] Candida Vitale,[2,3] Marta Coscia,[2,3] Agostino Festa,[4] Nicola Matteo Dario Di Minno,[5] Vincenzo De Feo,[6] Michele Caraglia,[4] Gioacchino Calapai,[7] Luca Laurenti,[8] Pellegrino Musto,[9] Giovanni Di Minno,[5] and Daniela Fenoglio[10]

[1]*Hematology and Stem Cell Transplantation Unit, IRCCS Cancer Referral Center of Basilicata, Rionero in Vulture, Italy*
[2]*Division of Hematology, AOU Città della Salute e della Scienza di Torino, University of Torino, Torino, Italy*
[3]*Department of Molecular Biotechnology and Health Sciences, University of Torino, Torino, Italy*
[4]*Dipartimento di Biochimica, Biofisica e Patologia Generale, University of Campania "Luigi Vanvitelli", Napoli, Italy*
[5]*Department of Clinical Medicine and Surgery, Regional Reference Centre for Coagulation Disorders, "Federico II" University, Napoli, Italy*
[6]*Department of Pharmacology, University of Salerno, Salerno, Italy*
[7]*Department of Biomedical and Dental Sciences and Morphological and Functional Sciences, University of Messina, Messina, Italy*
[8]*Hematology Institute, Catholic University of Sacred Heart, Rome, Italy*
[9]*Scientific Direction, IRCCS Cancer Referral Center of Basilicata, Rionero in Vulture, Italy*
[10]*Centre of Excellence for Biomedical Research, Department of Internal Medicine, IRCCS Azienda Ospedaliero Universitaria San Martino-IST-Istituto Nazionale per la Ricerca sul Cancro, University of Genoa, Genoa, Italy*

Correspondence should be addressed to Giovanni D'Arena; giovannidarena@libero.it

Received 17 August 2017; Accepted 14 November 2017; Published 21 December 2017

Academic Editor: Wenxin Zheng

Regulatory T cells (Tregs) have a fundamental function in monitoring the immune homeostasis in healthy individuals. In cancer and, in particular, in hematological malignancies, Tregs exert a major immunosuppressive activity, thus playing a critical role in tumor cell growth, proliferation, and survival. Here, we summarize published data on the prognostic significance of Tregs in hematological malignancies and show that they are highly conflicting. The heterogeneity of the experimental approaches that were used explains—at least in part—the discordant results reported by different groups that have investigated the role of Tregs in cancer. In fact, different tissues have been studied (i.e., peripheral blood, bone marrow, and lymph node), applying different methods (i.e., flow cytometry versus immunohistochemistry, whole blood versus isolated peripheral blood mononuclear cells versus depletion of $CD25^+$ cells, various panels of monoclonal antibodies, techniques of fixation and permeabilization, and gating strategies). This is of relevance in order to stress the need to apply standardized approaches in the study of Tregs in hematological malignancies and in cancer in general.

1. Introduction

Regulatory T cells (Tregs) constitute a small-size subpopulation of $CD4^+$ T cells, accounting for 1–4% of circulating $CD4^+$ lymphocyte in humans, specialized in suppressive functions that control unwanted immune responses not only toward self-antigens but also toward foreign antigens in the context of the immune tolerance [1].

Gershon and Kondo from Yale University first proposed the existence of $CD8^+$ T cells with suppressive activity more than 40 years ago [2]. However, after the initial great interest following this first report, due to the fact that a precise

definition of Tregs lacked for several years, no further advances in the study of this cell population were made for decades. In 1995, Sakaguchi and coworkers identified Tregs in mouse as $CD4^+$ T cells expressing surface interleukin-2 (IL-2) receptor α-chain (CD25) [3]. Baecher-Allan and coworkers, using flow cytometry and analyzing sorted cells *in vitro*, identified a very small subset of T cells with high expression of CD25 and regulatory function in humans [4]. However, CD25 is not exclusively restricted to Tregs, and its surface expression is also seen on effector T lymphocytes after activation [5]. The intracytoplasmic Forkhead helix box P3 (FoxP3), a transcription factor required for the development, maintenance, and function of Tregs was subsequently identified [6, 7]. The central role of this transcription factor is confirmed by the fact that a FoxP3 single gene mutation on the X chromosome induces in Scurfy mice a severe autoimmune/inflammatory disease. In humans, the same mutation causes a disease called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), characterized by autoimmune manifestations in multiple endocrine organs, such as diabetes and thyroiditis, inflammatory bowel disease, and severe allergies [8]. Finally, the absence of the heterodimeric IL-7 receptor (CD127) combined with CD4, CD25, and FoxP3, has been shown to better identify Tregs, avoiding the contamination from other cell populations such as activated effector T cells [9, 10].

2. Regulatory T Cells and Prognostic Significance in Cancer

The role of Tregs in cancer appears to be relevant by promoting tumor progression and suppressing effective antitumor activity [11–13]. Overall, the large majority of studies report that the frequency and the suppressive function of Tregs are increased in cancer patients as compared to healthy subjects. However, some issues are still a matter of debate, in particular the prognostic significance of this cell subpopulation. In general, Tregs predict poor outcome in cancer patients [12], but some reports have shown that higher Treg numbers and preserved activity are associated with a better prognosis [14–16].

This review stems from the need to reassess the topic of prognostic relevance of Tregs in cancer, focusing on patients with hematologic malignancies. For this purpose, we reviewed a large body of published papers conducting a PubMed literature search (keywords: Regulatory T cells, Hodgkin lymphoma, non-Hodgkin lymphoma, chronic lymphocytic leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, acute myeloid leukemia, multiple myeloma, monoclonal gammopathies, myelofibrosis, essential thrombocythemia, polycythemia vera, and Ph1-negative chronic myeloproliferative neoplasms).

3. Regulatory T Cells in Chronic Lymphocytic Leukemia

The accumulation of monoclonal B lymphocytes in the bone marrow, lymphoid organs, and peripheral blood is the hallmark of chronic lymphocytic leukemia (CLL), the most common form of leukemia in Western countries [17]. The importance of T cell dysregulation in the pathogenesis and development of CLL is now well established [18, 19], and in this setting, the role of Tregs has also been investigated [20, 21]. As shown in Table 1, several authors reported data on Tregs in CLL showing in the majority of cases an expansion of this population [22–31]. In addition, a correlation between higher Treg numbers and more aggressive clinical-biological features and adverse prognosis of CLL has been described.

As previously discussed [20], the reported percentage of Tregs in CLL is highly variable. According to the majority of reports, the percentage of Tregs is higher in CLL patients than in normal controls, and when the absolute number is considered, Tregs are always found to be significantly greater in CLL as compared to healthy donors.

Interestingly, based on their experimental work, Jak et al. speculated that the accumulation of Tregs in CLL is due to an increased proliferation induced by CD27/CD70 interaction in the lymph node proliferation centers and to a decreased sensitivity to apoptosis [22].

Dasgupta et al. tried to establish an optimal threshold level for prognostic purpose [28]. The cut-off was assessed by receiver operating characteristic (ROC) analysis. A cut-off of 5.7% and 35 cells/μL for percentage and absolute Treg count, respectively, were determined as optimal in patients with CLL, along with a median Tregs percentage of 15.5% used to separate low- and high-risk patients. Using the same approach in the setting of Rai stage 0 CLL patients, our group found that the absolute number of Tregs was an independent predictor of time to the first treatment, with the best predictive cut-off being 41 cells/μL [24]. Overall, these data show that the absolute Treg number is able to identify Rai stage 0 CLL patients at higher risk of requiring therapy.

Rissiek et al., applying a multidimensional scaling analysis to assess the composition of the circulating T cell populations, generated T cell scores showing that suppressive T cell profiles emerge early during monoclonal B cell lymphocytosis (MBL), the well-recognized pre-CLL stage [31–33]. As the disease evolves from MBL to overt and advanced CLL, specific sequential changes in T cells appear, progressively compromising the effector T cells function and contributing to disease progression [30].

In our hands too, the absolute number of Tregs in MBL patients was lower compared to CLL patients, but slightly higher than healthy controls [30]. In addition, the absolute Treg cell number directly correlated with more advanced CLL clinical stages and higher circulating B cell numbers. Of note, the absolute number of Tregs was lower in MBL patients as compared to early-stage CLL patients (0/A according to Rai/Binet stage). In summary, Treg numbers increase gradually from normal subjects to "clinical" MBL patients and are significantly higher in CLL patients as compared to MBL patients.

Regarding the functional properties, some authors reported a reduced inhibitory function of Tregs in CLL [27, 34]. On the contrary, Piper et al. showed that in CLL patients Tregs retain their function and are not influenced by chemotherapy [35]. A correlation between a higher circulating Treg numbers and dysfunctional Vγ9Vδ2 T cells

Table 1: Most relevant published studies investigating frequency, function, and prognostic significance of Tregs in CLL.

Reference	Patients/controls evaluated	Samples tested	Marker panel used in Treg evaluation by flow cytometry	Treg frequency	Functional studies	Impact on prognosis
Beyer et al. [34]	CLL/controls	PB	CD4/CD25	Increased*	Reduced inhibitory function	Extended disease (Binet stage)
Giannopoulos et al. [73]	CLL/controls	PB	CD4/CD25/FoxP3	Increased	Not performed	Binet stage
Jak et al. [22]	CLL/controls	PB	CD4/CD25/CD127	Increased	More resistant to drug-induced apoptosis than controls	Not evaluated
D'Arena et al. [23, 24]	CLL/controls	PB	CD4/CD25/CD127	Increased with a gradual variation from normal subjects to clinical MBL to CLL	Not performed	Rai stage, lymphocytosis, LDH, first time to treatment
Weiss et al. [25]	CLL/controls	PB	CD4/CD25/FoxP3	Increased	Not performed	Unmutated IgVH, CD38, chromosomal aberrations
Lad et al. [26]	CLL/controls	PB and FNA	CD4/CD25/CD127/IL-10	Reduced both Treg and IL-10 expressing Treg; higher absolute number	Not performed	Correlation with LDT (Tregs but not $CD45RA^+$ Tregs and $CD8^+$ Tregs were lower in $CD38^+ZAP70^+$ CLL group (with respect to $CD38^-ZAP70^-$)
Biancotto et al. [27]	CLL/controls	PB	CD4/CD25/FoxP3	Increased	Slightly reduced suppressive activity	Correlation with ZAP-70 and CD38 expression
Dasgupta et al. [28]	CLL/controls	PB	CD4/CD25/CD127/FoxP3	Increased	Not performed	Correlated with ZAP70 and CD38 expression
Mpakou et al. [29]	CLL/controls	PB	CD4/CD25/CD127	Increased	Suppression of T effector cells	Advanced stage
D'Arena et al. [30]	Clinical MBL/ CLL/controls	PB	CD4/CD25/CD127	Reduced as % but increased as absolute number with a gradual variation from normal subjects to clinical MBL to CLL	Not performed	
Rissiek et al. [31]	MBL/CLL/controls	PB	CD4/CD25/CD127/CD39	Expansion. Highly suppressive $CD39^+$ Treg subset increased in all disease stages	Increasingly suppressive regulatory function initiating at MBL stage; effector function impairment after transition to CLL; partially recovered after chemo-immunotherapy	Shorter time to treatment

*Increased at diagnosis; significantly reduced after fludarabine therapy. CLL: chronic lymphocytic leukemia; MBL: monoclonal B cell lymphocytosis; PB: peripheral blood; FNA: fine needle aspiration; LDT: lymphocyte doubling time.

in untreated CLL patients was also shown, thus corroborating the hypothesis that Tregs may not be only bystanders but have a functional role in this setting [36].

A normalization in Treg number was observed after fludarabine therapy [34], and also in CLL patients treated with lenalidomide, suggesting that such drugs are able to modulate cell-mediated immunity in CLL [37].

Finally, we also tested the ability of green tea, a popular beverage in China, Japan, and increasingly used in Western countries, to modulate Treg number in peripheral blood of CLL patients in the early phases of the disease, for which at the present time there is no effective intervention and a "wait and see" policy is generally adopted [38, 39]. We showed that the B cell lymphocyte count and the absolute circulating Treg number were reduced after 6-month consumption of oral green tea extract, suggesting that this compound can modulate circulating Tregs in CLL patients with early stage of disease and delay disease progression.

4. Regulatory T Cells in Lymphomas and Monoclonal Gammopathies

The neoplastic lymph nodes in Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) contain not only neoplastic B cells but also nontumoral T cells, macrophages, and dendritic cells, constituting the so-called tumor microenvironment. The importance of the microenvironment in the pathogenesis and progression of lymphomas is still a matter of debate and many studies have focused on the role of its different components, including Tregs. Tregs are increased in lymphoma tissues and are able to inhibit cytotoxic $CD8^+$ T cells exposed to lymphoma B cells [40].

Marshall et al. showed that HL-infiltrating lymphocytes are highly enriched in Tregs, which induce a profoundly immunosuppressive environment [41]. This was confirmed by Schreck et al. who demonstrated that in classical HL the microenvironment is dominated by Th2 cells and Tregs [42]. Moreover, a high ratio of Tregs over Th2 cells resulted in a significantly shortened disease-free survival.

However, conflicting results have been reported regarding the prognostic significance of Tregs infiltration in both HL and NHL. In fact, whereas in follicular lymphoma (FL), the most common form of low-grade NHL, germinal center (GC) diffuse large B cell lymphomas (DLBCL), and HL, an intrafollicular infiltration of Tregs, has a positive prognostic significance; this is not true in the case of non-GC-type DLBCL [43]. Moreover, as shown in Table 2, in some reports, a higher number of Tregs correlates with a good prognosis, while in other, it does not [43–49]. Of interest, Kim et al. evaluating Tregs on node biopsy of extranodal natural killer/T cell lymphomas showed that patients with poor performance status and with non-upper aerodigestive tract had a decreased number of Tregs ($<50/0.40\,mm^2$), while an increased number ($>50/0.40\,mm^2$) was associated with prolonged overall survival and progression-free survival [48]. Finally, Carreras et al. reported that the median Treg number in patients with FL at diagnosis had a median cell percentage of 10.5% [49]. Furthermore, patients were classified as having Tregs > 10%, 5–10%, and <5% with a 5-year overall survival of 80%, 74%, and 50%, respectively. Patients with transformed DLBCL showed lower Treg number with respect to patients with grades 1–3 FL.

Regarding the frequency and prognostic significance of Tregs, conflicting results have also been obtained in the field of monoclonal gammopathies (Table 3). In some reports, Tregs were found to be increased in frequency, while in others they were reduced or comparable with respect to healthy subjects [50]. Again, some authors reported a correlation with tumour burden and with worse prognosis, but this was not consistent among different publications [50–57]. We recently published our data on the flow cytometric evaluation of Tregs in multiple myeloma (MM) and monoclonal gammopathies of undetermined significance (MGUS) [51]. We found no differences in Treg frequency in MM and MGUS with respect to normal controls, and no correlations with main clinical and laboratory features in this disease setting were observed.

5. Regulatory T Cells in Acute Leukemias, Chronic Myeloid Leukemia, and Ph1-Negative Chronic Myeloproliferative Neoplasms

Few studies have been published regarding the role of Tregs in acute myeloid and lymphoid leukemias (Table 4) [58–61]. In a study by Bhattacharya et al., an increased number of Tregs was found in patients with B cell acute lymphoblastic leukemia (B-ALL), and a correlation with disease progression was highlighted [58].

Regarding chronic myeloid leukemia (CML), an interesting paper has been published by Zahran and Badrawy, in which Tregs were found increased in the peripheral blood of affected individuals as compared to controls. Moreover, Tregs frequency correlated with the level of BCR/ABL, basophil number, blast cell count, and Sokal score, and Treg number was higher in accelerated and blastic phase with respect to chronic phase [62]. Of note, Treg frequency declined after therapy with imatinib. Rojas et al. found a lower Treg number in patients who achieved a complete cytogenetic response [63], while higher Treg frequencies were found after stem cell transplant compared to normal controls and newly diagnosed patients [64]. Finally, the correlations with Sokal score and basophil number were validated by other studies [65, 66], whereas the impact of treatment has not been confirmed, since no changes in Treg frequency was observed after 6 months of tyrosine kinase inhibitors therapy [65]. Table 5 summarizes the results of studies analyzing Tregs in CML.

Hasselbalch et al. studied patients with Ph1-negative chronic myeloproliferative neoplasms and found that circulating Tregs were significantly expanded in patients treated with IFN-α2 with respect to healthy donors and in patients treated with hydroxyurea [66]. Kovacsovics-Bankowski et al. analyzed patients with polycythemia vera (PV) and essential thrombocythemia (ET) and found increased numbers of circulating Tregs and an enrichment in highly suppressive subsets (defined as $CD39^+/HLA\text{-}DR^+$) in patients treated with PegIFN-α with respect to those treated with hydroxyurea [67].

Table 2: Most relevant published studies investigating the frequency and the prognostic significance of Tregs in lymphomas.

Reference	Patients/controls evaluated	Samples tested	Marker panel used in Treg evaluation by flow cytometry	Treg frequency	Functional studies	Impact on prognosis
Tzankov et al. [43]	Lymphomas	Node biopsy	FoxP3 (IHC)	Increased	Not performed	Correlation with disease-specific and failure-free survival in FL and disease-specific survival in germinal center-like DLBCL and OS and failure-free survival in classical HD, but negative prognostic effect in nongerminal center DLBCL. Independent prognostic significance for failure-free survival in classical HD and of borderline significance for OS in classical HD and disease-specific survival in germinal center-like and nongerminal center DLBCL
Alvaro et al. [44]	Classical HL	Node biopsy	FoxP3 (IHC)	Not reported	Not performed	Small number influenced negatively EFS and DFS
Schreck et al. [42]	Classical HL/hyperplastic tonsils	Node biopsy	FoxP3 (IHC)	Increased	Not performed	Increased DFS and EFS; high Tregs/Th2 ratio correlated with shortened DFS
Garcia et al. [45]	Gastric MALT lymphoma	Gastric biopsy	FoxP3 (IHC)	Increased with respect to DLBCL but similar to chronic gastritis	Not performed	Higher number correlated with response to antibacterial eradication therapy
Koreishi et al. [47]	Relapsed/refractory HL	Node biopsy	FoxP3 (tissue microarray)	Not reported	Not performed	Lower Tregs correlated with poor OS
Chang et al. [46]	DLBCL/normal	Node biopsy	$CD4^+$ $CD25^+$	Increased	Not performed	Higher with poor survival and IPI
Kim et al. [48]	Extranodal natural killer/T cell lymphoma	Node biopsy	FoxP3 (IHC)	Heterogenous expression	Not performed	The decreased number of Tregs was more common in patients with poor performance status or in those presented in nonupper aerodigestive tract. Patients with increased numbers of Tregs showed prolonged OS and PFS.
Carreras et al. [49]	FL at diagnosis and relapse	Node biopsy	FoxP3 (IHC)	Not reported	Not performed	Inversely correlated with OS. Patients with very low numbers of Tregs (<5%) presented more frequently with refractory disease. No correlation with FLIPI. Patients with transformed DLBCL had lower Treg percentages than FL grades 1, 2, or 3

HL: Hodgkin's lymphoma; FL: follicular lymphoma; DLBCL: diffuse large B cell lymphoma; MALT: mucosa-associated lymphoid tissue; IHC: immunohistochemistry; OS: overall survival; DFS: disease-free survival, EFS: event-free survival.

Table 3: Most relevant published studies investigating the frequency and the prognostic significance of Tregs in monoclonal gammopathies.

Reference	Patients/controls evaluated	Samples tested	Marker panel used in Treg evaluation by flow cytometry	Treg frequency	Functional studies	Impact on prognosis
Prabhala et al. [50]	MGUS/MM/controls	PBMC	CD4/FoxP3	Decreased	Unable to suppress anti-CD3-mediated T cell proliferation	Not evaluated
Beyer et al. [52]	MGUS/MM/controls	PBMC	CD4/CD25/FoxP3 (% of $CD4^+$ cells)	Increased in MM versus MGUS (trend without statistical significance)	Strong inhibitory function	Not evaluated
Feyler et al. [53]	MGUS/MM/controls	PBMC and BM	CD4/CD25/FoxP3	Increased in PBMC but not in BM	Not evaluated	Correlation with disease burden (paraprotein)
Gupta et al. [54]	MM	PBMC	CD4/CD25/CD127/ FoxP3 (% of $CD4^+$ cells)	Reduced in untreated which increased after treatment with lenalidomide	Able to inhibit proliferation of $CD4^+CD25^-$ T cells	Increase of Tregs in responding patients to therapy; decrease correlation with ISS I + II
Muthu Raja et al. [55]	MGUS/SMM/MM	PB/BM whole	CD4/CD25/CD127/ CD45RA (% of $CD4^+$ cells)	Increased in MM but not in SMM and MGUS	Able to inhibit proliferation of $CD4^+$ T cells and the secretion of IFN-γ	Correlation with adverse clinical features (hypercalcemia, lower normal PC, and IgA subtype); no correlation with ISS; predict time to progression; MM patients with ≥5% of Tregs had inferior time to progression
Giannopulos et al. [56]	MM/controls	PBMC	CD4/CD25/FoxP3	Increased	Not evaluated	Correlation with shorter overall survival
Foglietta et al. [57]	MM/MGUS/controls	Fresh PB and frozen BM	CD4/CD25/FoxP3	Similar	Effective suppressor function	No correlation with the pattern of BM infiltration
D'Arena et al. [51]	MM/MGUS/controls	PB whole	CD4/CD25/CD127 (% and absolute number)	Similar	Effective suppressor function	No correlation with laboratory and clinical variables; no correlation with outcome

MM: multiple myeloma; MGUS: monoclonal gammopathy of uncertain significance; SMM: smoldering multiple myeloma; ISS: international staging system; PB: peripheral blood; PBMC; peripheral blood mononuclear cells; BM: bone marrow.

Table 4: Most relevant published studies investigating the frequency and the prognostic significance of Tregs in acute leukemias.

Reference	Patients/controls evaluated	Samples tested	Marker panel used in Treg evaluation by flow cytometry	Treg frequency	Functional studies	Impact on prognosis
Bhattacharya et al. [58]	B-ALL	PBMC /BM	CD4/CD25/CD127/FoxP3	Decreased	Higher suppressive capability on $CD4^+CD25^-$ regulatory T cells than controls	Increased frequency with disease progression
Wu et al. [59]	B-ALL/T-ALL/ controls	PB	CD4/CD25	Higher	Not performed	Not evaluated
Wang et al. [60]	AML/controls	PBMC /BM	CD4/CD25	Higher	Inhibition of proliferation and cytokine production (IL2, IFN-γ) of $CD4^+CD25^-$ T cells; improved IL-10 production under coculture of both subsets with stimulation	Not evaluated
Idris et al. [61]	B-ALL/controls	PB and BM	CD4/CD25/CD127	Increased	Not performed	Correlation with age

ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; PB: peripheral blood; BM: bone marrow; PBMC: peripheral blood mononuclear cells; IL: interleukin; IFN: interferon.

Table 5: Most relevant published studies investigating the frequency and the prognostic significance of Tregs in chronic myeloid leukemia.

Reference	Patients/controls evaluated	Samples tested	Marker panel used in Treg evaluation by flow cytometry	Treg frequency	Functional studies	Impact on prognosis
Zahran and Badrawy [62]	CML/controls	PB	CD4/CD25/FoxP3	Increased	Not performed	Correlations with the level of BCR/ABL, basophils and blast cells. Significantly higher in accelerated phase and blastic phase than in chronic phase and with high Sokal score. Reduction of Tregs after therapy with IM
Hus et al. [65]	CP CML/controls	PB	CD4/CD25/FoxP3	Increased	Not performed	Correlation with higher basophiles. No change in frequency after 6 months of TKI inhibitors
Bachy et al. [74]	CP CML/controls	CD4+ enriched PBMC cells	CD4/CD25/ CD127/FoxP3	Increased in PB. Increased in BM of patients on IM compared to healthy volunteers.	No difference in inhibition	Correlation with Sokal risk score
Rojas et al. [63]	CP CML/controls	PBMC	CD4/CD25/ CD127/CD62L/ FoxP3	Lower in patients in complete cytogenetic response	Enhanced proliferative response to purified protein derivative	Not evaluated
Nadal et al. [64]	CP CML/controls	PBMC	CD4/CD25/ CD127/FoxP3/ CTLA-4	Higher frequencies after transplant than normal controls and newly diagnosed patients	Purified Tregs from SCT patients had a more potent suppressive activity than those from healthy volunteers	Not evaluated

CP: chronic phase; BM: bone marrow; IM: imatinib; PB: peripheral blood; PBMC; peripheral blood mononuclear cells; BM: bone marrow; SCT: stem cell transplant; IM: imatinib; TKI: tyrosine kinase inhibitor.

Table 6: Most relevant published studies investigating the frequency and the prognostic significance of Tregs in Ph1-negative chronic myeloproliferative neoplasms.

Reference	Patients/controls evaluated	Samples tested	Marker panel used in Treg evaluation by flow cytometry	Treg frequency	Functional studies	Impact on prognosis
Hasselbalch et al. [66]	PV ET PMF Controls	PBMC	CD4/CD25/CD127	Not increased	Inhibitory activity preserved	Marked expansion of Tregs in patients treated with IFN-α2 with than treated with hydroxyurea
Kovacsovics-Bankowski et al. [67]	PV ET	PB	CD4/CD25/FoxP3/Ki-67	Not reported	Not performed	Tregs (including highly suppressive $CD39^+$ $HLA\text{-}DR^+$) increase in patients treated with PegINFα
Massa et al. [68, 69]	PMF	PB	CD4/CD25/CD127/FoxP3	Reduced	Not performed	In patients with CALR mutation genotype association with longer disease duration and hemoglobin concentration

PV: polycythemia vera; ET: essential thrombocythemia; PMF: primary myelofibrosis; PB: peripheral blood; PBMC: peripheral blood mononuclear cells; CALR: calreticulin; IFN: interferon.

Moreover, molecular nonresponder patients showed a trend towards increased frequency of Tregs compared to responder patients, but no changes were observed in terms of absolute numbers of Tregs. Overall, a positive correlation between proliferating Tregs (Ki-67$^+$), highly suppressive Tregs (CD39$^+$/HLA-DR$^+$), and JAK2^{V617F} allelic burden was found, thus suggesting that the lack of ability of PegIFN-α treatment to decrease circulating Tregs predicts a poor molecular response.

Primary myelofibrosis (PMF) is a clonal disease of the hematopoietic stem cell characterized by a variable degree of bone marrow fibrosis, splenomegaly, and an increased risk of leukemic transformation. Contradictory data about Tregs in PMF have been published (Table 6). Massa et al. reported a reduced frequency and absolute number of Tregs in PMF than in normal controls [68]. No association with clinical-biological features of the disease was found, but a correlation between reduced Treg frequency and longer disease duration in patients with CALR mutation genotype was described. In these patients, a higher Treg frequency is significantly associated with advanced disease, higher IPSS/DIPSS score, and lower hemoglobin concentration. The same authors later documented the effect of ruxolitinib on Treg frequency, showing that the treatment with this small-molecule JAK1/2 inhibitor leads to a profound and long-lasting reduction in the frequency of circulating Tregs [69]. Wang et al. found no significant differences in the number of Tregs in patients with primary or post-ET myelofibrosis [70]. However, they reported that ruxolitinib significantly inhibits the release of sIL2-Rα, an inflammatory cytokine produced by Tregs, contributing to the clinical improvement of constitutional symptoms induced by the drug. These data have been further confirmed by an *in vitro* study in which the JAK1/2 inhibition by ruxolitinib was able to prevent Treg differentiation [71]. Table 6 summarizes the results of studies analyzing Tregs in Ph1-negative chronic myeloproliferative neoplasms.

6. Conclusions

Tregs have a fundamental function in maintaining the immune homeostasis in healthy individuals. In cancer and in particular in hematological malignancies, Tregs exert a major immunosuppressive activity, thus playing a critical role in tumor cell growth, proliferation, and survival. Published data on the prognostic significance of the Treg number in hematological malignancies show conflicting results. In our opinion, this variability reported by different groups is most likely explained by the heterogeneity of the experimental approaches that are used. In fact, different tissues have been studied (i.e., peripheral blood, bone marrow, and lymph node) and different analytic methodologies have been applied (i.e., flow cytometry versus immunohistochemistry). Moreover, while some authors studied the whole blood compartment, others evaluated the Treg population in isolated peripheral blood mononuclear cells or in a CD25-depleted subpopulation. Finally, various panels of markers, different techniques of fixation and permeabilization, and several gating strategies have been applied. This is of relevance to stress the need to apply standardized approaches in the study of Tregs in hematological malignancies and in cancer in general.

In perspective, in light of the increasing evidence of the important role of Tregs in immune evasion mechanism exerted by tumor cells, therapeutic interventions targeting intratumoral Treg infiltrates may be conceived in order to fight cancer. Treg inhibition or depletion, the latter uses monoclonal antibodies targeting surface antigens on Tregs such as CD25, is currently under investigation [72].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] S. Sakaguchi, K. Wing, and M. Miyara, "Regulatory T cells – a brief history and perspective," *European Journal of Immunology*, vol. 37, Supplement 1, pp. S116–S123, 2007.

[2] R. K. Gershon and K. Kondo, "Cell interactions in the induction of tolerance: the role of thymic lymphocytes," *Immunology*, vol. 18, no. 5, pp. 723–737, 1970.

[3] S. Sakaguchi, N. Sakaguchi, M. Asano, M. Itoh, and M. Toda, "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chain (CD25). breakdown of a single mechanism of self-tolerance causes various autoimmune disease," *Journal of Immunology*, vol. 155, pp. 1151–1164, 1995.

[4] C. Baecher-Allan, J. A. Brown, G. J. Freeman, and D. A. Hafler, "CD4$^+$CD25^{high} regulatory cells in human peripheral blood," *Journal of Immunology*, vol. 167, no. 3, pp. 1245–1253, 2001.

[5] R. J. Robb, A. Munck, and K. A. Smith, "T cell growth factor receptors. Quantitation, specificity, and biological relevance," *Journal of Experimental Medicine*, vol. 154, no. 5, pp. 1455–1474, 1981.

[6] S. Hori, T. Numura, and S. Sakaguchi, "Control of regulatory T cell development by the transcription factor *FoxP3*," *Science*, vol. 299, no. 5609, pp. 1057–1061, 2003.

[7] J. D. Fontenot, M. A. Gavin, and A. Y. Rudensky, "FoxP3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells," *Nature Immunology*, vol. 4, no. 4, pp. 330–336, 2003.

[8] C. L. Bennett, J. Christie, F. Ramsdell et al., "The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3," *Nature Genetics*, vol. 27, no. 1, pp. 20-21, 2001.

[9] N. Seddiki, B. Santner-Nanan, J. Martinson et al., "Expression of interleukin (IL)-2 and IL-7 receptors discriminate between human regulatory and activated T cells," *Journal of Experimental Medicine*, vol. 203, no. 7, pp. 1693–1700, 2006.

[10] W. Liu, A. L. Putnam, Z. Xu-Yu et al., "CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4$^+$ Treg cells," *Journal of Experimental Medicine*, vol. 203, pp. 1701–1711, 2006.

[11] M. Beyer and J. L. Schultze, "Regulatory T cells in cancer," *Blood*, vol. 108, no. 3, pp. 804–811, 2006.

[12] T. L. Whiteside, "The role of regulatory T cells in cancer immunology," *ImmunoTargets and Therapy*, vol. 4, pp. 159–171, 2015.

[13] Y. Takeuchi and H. Nishikawa, "Roles of regulatory T cells in cancer immunity," *International Immunology*, vol. 28, no. 8, pp. 401–409, 2016.

[14] J. Wang and X. Y. Ke, "The Four types of Tregs in malignant lymphomas," *Journal Hematological & Oncology*, vol. 4, p. 50, 2011.

[15] P. Farinha, A. Al-Tourah, K. Gill, R. Klasa, J. M. Connors, and R. D. Gascoyne, "The architectural pattern of FOXP3-positive T cells in follicular lymphoma is an independent predictor of survival and histologic transformation," *Blood*, vol. 115, no. 2, pp. 289–295, 2010.

[16] R. Droeser, I. Zlobec, E. Kilic et al., "Differential pattern and prognostic significance of $CD4^+$, $FOXP3^+$ and IL-7^+ tumor infiltrating lymphocytes in ductal and lobular breast cancers," *BMC Cancer*, vol. 12, no. 1, p. 134, 2012.

[17] N. Chiorazzi, K. R. Rai, and M. Ferrarini, "Chronic lymphocytic leukemia," *New England Journal of Medicine*, vol. 352, no. 8, pp. 804–815, 2005.

[18] F. Forconi and P. Moss, "Perturbation of the normal immune system in patients with CLL," *Blood*, vol. 126, no. 5, pp. 573–581, 2015.

[19] M. E. Wallace, M. B. Alcantara, M. Yosuke, G. Kannourakis, and S. P. Berzins, "An emerging role for immune regulatory subsets in chronic lymphocytic leukaemia," *International Immunopharmacology*, vol. 28, no. 2, pp. 897–900, 2015.

[20] G. D'Arena, V. Simeon, F. D'Auria et al., "Regulatory T-cells in chronic lymphocytic leukemia: actor or innocent bystander?," *American Journal of Blood Research*, vol. 3, no. 1, pp. 52–57, 2013.

[21] G. D'Arena, G. Rossi, B. Vannata et al., "Regulatory T-cells in chronic lymphocytic leukemia and autoimmune diseases," *Mediterranean Journal of Hematology and Infectious Diseases*, vol. 4, no. 1, article e2012053, 2012.

[22] M. Jak, R. Mous, E. B. Remmerswaal et al., "Enhanced formation and survival of $CD4^+$ $CD25^{hi}$ $FoxP3^+$ T-cells in chronic lymphocytic leukemia," *Leukemia & Lymphoma*, vol. 50, no. 5, pp. 788–801, 2009.

[23] G. D'Arena, L. Laurenti, M. M. Minervini et al., "Regulatory T-cell number is increased in chronic lymphocytic leukemia patients and correlates with progressive disease," *Leukemia Research*, vol. 35, no. 3, pp. 363–368, 2011.

[24] G. D'Arena, F. D'Auria, V. Simeon et al., "A shorter time to the first treatment may be predicted by the absolute number of regulatory T-cells in patients with Rai stage 0 chronic lymphocytic leukemia," *American Journal of Hematology*, vol. 87, no. 6, pp. 628–631, 2012.

[25] L. Weiss, T. Melchardt, A. Egle, C. Grabmer, R. Greil, and I. Tinhofer, "Regulatory T cells predict the time to initial treatment in early stage chronic lymphocytic leukemia," *Cancer*, vol. 117, no. 10, pp. 2163–2169, 2011.

[26] D. P. Lad, S. Varma, N. Varma, M. U. Sachdeva, P. Bose, and P. Malhotra, "Regulatory T-cells in B-cell chronic lymphocytic leukemia: their role in disease progression and autoimmune cytopenias," *Leukemia and Lymphoma*, vol. 54, pp. 1012–1019, 2013.

[27] A. Biancotto, P. K. Dagur, J. C. Fuchs, A. Wiestner, C. B. Bagwell, and J. P. McCoy Jr., "Phenotypic complexity of T regulatory subsets in patients with B-chronic lymphocytic leukemia," *Modern Pathology*, vol. 25, no. 2, pp. 246–259, 2012.

[28] A. Dasgupta, M. Mahapatra, and R. Saxena, "A study for proposal of use of regulatory T cells as a prognostic marker and establishing an optimal threshold level for their expression in chronic lymphocytic leukemia," *Leukemia & Lymphoma*, vol. 56, no. 6, pp. 1831–1838, 2015.

[29] V. E. Mpakou, H. D. Ioannidou, E. Konsta et al., "Quantitative and qualitative analysis of regulatory T cells in B cell chronic lymphocytic leukemia," *Leukemia Research*, vol. 60, pp. 74–81, 2017.

[30] G. D'Arena, G. Rossi, M. M. Minervini et al., "Circulating regulatory T cells in "clinical" monoclonal B-cell lymphocytosis," *International Journal of Immunopathology and Pharmacology*, vol. 24, no. 4, pp. 915–923, 2011.

[31] A. Rissiek, C. Schulze, U. Bacher et al., "Multidimensional scaling analysis identifies pathological and prognostically relevant profiles of circulating T-cells in chronic lymphocytic leukemia," *International Journal Cancer*, vol. 135, pp. 2370–2379, 2014.

[32] D. Rossi, E. C. Sozzi, A. Puma et al., "The prognosis of clinical monoclonal B cell lymphocytosis differs from prognosis of Rai 0 chronic lymphocytic leukemia and is recapitulated by biological risk factors," *British Journal of Haematology*, vol. 146, no. 1, pp. 64–75, 2009.

[33] G. D'Arena and P. Musto, "Monoclonal B-cell lymphocytosis," *Translational Medicine*, vol. 8, pp. 75–79, 2014.

[34] M. Beyer, M. Kochanek, K. Darabi et al., "Reduced frequencies and suppressive function of $CD4^+CD25^{hi}$ regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine," *Blood*, vol. 106, no. 6, pp. 2018–2025, 2005.

[35] K. P. Piper, M. Karanth, A. McLarnon et al., "Chronic lymphocytic leukaemia cells drive the global $CD4^+$ T cell repertoire towards a regulatory phenotype and leads to the accumulation of $CD4^+$ forkhead box $P3^+$ T cells," *Clinical & Experimental Immunology*, vol. 166, no. 2, pp. 154–163, 2011.

[36] M. Coscia, C. Vitale, S. Peola et al., "Dysfunctional $V\gamma9V\delta2$ T cells are negative prognosticators and markers of dysregulated mevalonate pathway activity in chronic lymphocytic leukemia cells," *Blood*, vol. 120, no. 16, pp. 3271–3279, 2012.

[37] B. N. Lee, H. Gao, E. N. Cohen et al., "Treatment with lenalidomide modulates T-cell immunophenotype and cytokine production in patients with chronic lymphocytic leukemia," *Cancer*, vol. 117, no. 17, pp. 3999–4008, 2011.

[38] G. D'Arena, V. Simeon, L. De Martino et al., "T-cell modulation by green tea in chronic lymphocytic leukemia," *International Journal Immunopathology Pharmacology*, vol. 26, pp. 117–125, 2013.

[39] G. D'Arena, L. Laurenti, M. Coscia et al., "Complementary and alternative medicine use in patients with chronic lymphocytic leukemia: an Italian multicentric survey," *Leukemia & Lymphoma*, vol. 55, no. 4, pp. 841–847, 2014.

[40] Z.-Z. Yang, A. J. Novak, S. C. Ziesmer, T. E. Witzig, and S. M. Ansell, "Attenuation of $CD8^+$ T-cell function by $CD4^+CD25^+$ regulatory T cells in B-cell non-Hodgkin's lymphoma," *Cancer Research*, vol. 66, no. 20, pp. 10145–10152, 2006.

[41] N. A. Marshall, L. E. Christei, L. R. Murro et al., "Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma," *Blood*, vol. 103, no. 5, pp. 1755–1762, 2004.

[42] S. Schreck, D. Friebel, M. Buettner et al., "Prognostic impact of tumour-infiltrating Th2 and regulatory T cells in classical Hodgkin lymphoma," *Hematological Oncology*, vol. 27, no. 1, pp. 31–39, 2009.

[43] A. Tzankov, C. Meier, P. Hirschmann, P. Went, S. A. Pileri, and S. Dirnhofer, "Correlation of high numbers of intratumoral

FOXP3$^+$ regulatory T cells with improved survival in germinal center-like diffuse large B-cell lymphoma, follicular lymphoma and classical Hodgkin's lymphoma," *Haematologica*, vol. 93, no. 2, pp. 193–200, 2008.

[44] T. Alvaro, M. Lejeune, M. T. Salvado et al., "Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells," *Clinical Cancer Research*, vol. 11, no. 4, pp. 1467–1473, 2005.

[45] M. Garcia, B. Bellosillo, B. Sánchez-González et al., "Study of regulatory T-cells in patients with gastric Malt lymphoma: influence on treatment response and outcome," *PLoS One*, vol. 7, no. 12, article e51681, 2012.

[46] C. Chang, S. Y. Wu, Y. W. Kang et al., "High levels of regulatory T cells in blood are a poor prognostic factor in patients with diffuse large B-cell lymphoma," *American Journal Clinical Pathology*, vol. 144, pp. 935–944, 2015.

[47] A. F. Koreishi, A. J. Saenz, D. O. Persky et al., "The role of cytotoxic and regulatory T-cells in relapsed/refractory Hodgkin lymphoma," *Apllied Immunohistochemistry & molecular Morphology*, vol. 18, pp. 206–211, 2010.

[48] W. Y. Kim, Y. K. Jeon, T. M. Kim et al., "Increased quantity of tumor-infiltrating FOXP3-positive regulatory T cells is an independent predictor for improved clinical outcome in extranodal NK/T-cell lymphoma," *Annals Oncology*, vol. 20, pp. 1688–1696, 2009.

[49] J. Carreras, A. Lopez-Guillermo, B. C. Fox et al., "High numbers of tumor-infiltrating FOXP3-positive regulatory T-cells are associated with improved overall survival in follicular lymphoma," *Blood*, vol. 108, no. 9, pp. 2957–2964, 2006.

[50] R. H. Prabhala, P. Neri, J. E. Bae et al., "Dysfunctional T regulatory cells in multiple myeloma," *Blood*, vol. 107, no. 1, pp. 301–304, 2006.

[51] G. D'Arena, G. Rossi, L. Laurenti et al., "Circulating regulatory T-cells in monoclonal gammopathies of uncertain significance and multiple myeloma: in search of a role," *Journal of Immunology Research*, vol. 2016, Article ID 9271469, 7 pages, 2016.

[52] M. Beyer, M. Kochanek, T. Giese et al., "In vivo peripheral expansion of naive CD4$^+$ CD25^{high} *FoxP3*$^+$ regulatory T cells in patients with multiple myeloma," *Blood*, vol. 107, no. 10, pp. 3940–3949, 2006.

[53] S. Feyler, M. von Lilenfeld-Toal, S. Jarmin et al., "CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cells are increased whilst CD3$^+$ CD4$^-$ CD8$^-$ $\alpha\beta$TCR$^+$ double negative T cells are decreased in the peripheral blood of patients with multiple myeloma which correlates with disease burden," *British Journal of Haematology*, vol. 144, no. 5, pp. 686–695, 2009.

[54] R. Gupta, P. Ganeshan, M. Hakim, R. Verma, A. Sharma, and L. Kumar, "Significantly reduced regulatory T cell population in patients with untreated multiple myeloma," *Leukemia Research*, vol. 35, no. 7, pp. 874–878, 2011.

[55] K. R. Muthu Raja, L. Rihova, L. Zahradova, M. Klincova, M. Penka, and R. Hajek, "Increased T regulatory cells are associated with adverse clinical features and predict progression in multiple myeloma," *PLoS One*, vol. 7, no. 10, article e47077, 2012.

[56] K. Giannopoulos, W. Kaminska, I. Hus, and A. Dmoszynska, "The frequency of T regulatory cells modulates the survival of multiple myeloma patients: detailed characterization of immune status in multiple myeloma," *British Journal of Cancer*, vol. 106, no. 3, pp. 546–552, 2012.

[57] M. Foglietta, B. Castella, S. Mariani et al., "The bone marrow of myeloma patients is steadily inhibited by a normal-sized pool of functional regulatory T cells irrespective of the disease status," *Haematologica*, vol. 99, no. 10, pp. 1605–1610, 2014.

[58] K. Bhattacharya, S. Chandra, and C. Mandal, "Critical stoichiometric ratio of CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cells and CD4$^+$ CD25$^-$ responder T cells influence immunosuppression in patients with B-cell acute lymphoblastic leukaemia," *Immunology*, vol. 142, pp. 124–139, 2013.

[59] C.-P. Wu, X. Quing, H. Zhu, and H.-Y. Zhou, "Immunophenotype and increased presence of CD4$^+$CD25$^+$ regulatory T cells in patients with acute lymphoblastic leukemia," *Oncology Letters*, vol. 3, no. 2, pp. 421–424, 2012.

[60] X. Wang, J. Zheng, J. Liu et al., "Increased population of CD4$^+$CD25^{high} regulatory T cells with their higher apoptotic and proliferating status in peripheral blood of acute myeloid leukemia patients," *European Journal of Haematology*, vol. 75, no. 6, pp. 468–476, 2005.

[61] S. Z. Idris, N. Hassan, L. J. Lee et al., "Increased regulatory T cells in acute lymphoblastic leukemia patients," *Hematology*, vol. 20, no. 9, pp. 523–529, 2015.

[62] A. M. Zahran, H. Badrawy, and A. Ibrahim, "Prognostic value of regulatory T cells in newly diagnosed chronic myeloid leukemia patients," *International Journal of Clinical Oncology*, vol. 19, no. 4, pp. 753–760, 2014.

[63] J. M. Rojas, L. Wang, S. Owen, K. Knight, S. J. Watmough, and R. E. Clark, "Naturally occurring CD4$^+$ CD25$^+$ FOXP3$^+$T-regulatory cells are increased in chronic myeloid leukemia patients not in complete cytogenetic remission and can be immunosuppressive," *Experimental Hematology*, vol. 38, no. 12, pp. 1209–1218, 2010.

[64] E. Nadal, M. Garin, J. Kaeda, J. Apperley, R. Lechlir, and F. Dazzi, "Increased frequencies of CD4$^+$CD25^{high} Tregs correlate with disease relapse after allogeneic stem cell transplantation for chronic myeloid leukemia," *Leukemia*, vol. 21, no. 3, pp. 472–479, 2007.

[65] I. Hus, J. Tabarkiewicz, M. Lewandowska et al., "Evaluation of monocyte-derived dendritic cells, T regulatory and Th17 cells in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors," *Folia Histochemistry Cytobiologica*, vol. 49, no. 1, pp. 153–160, 2011.

[66] C. R. Hasselbalch, M. K. Jensen, K. M. Brimnes et al., "Increase in circulating CD4$^+$CD25$^+$Foxp3$^+$ T cells in patients with Philadelphia-negative chronic myeloproliferative neoplasms during treatment with IFN-α," *Blood*, vol. 118, pp. 2170–2173, 2011.

[67] M. Kovacsovics-Bankowski, T. W. Kelley, O. Efimova et al., "Changes in peripheral blood lymphocytes in polycythemia vera and essential thrombocythemia patients treated with pegylated-interferon alpha and correlation with *JAK2*^{V617F} allelic burden," *Experimental Hematology & Oncology*, vol. 5, p. 28, 2016.

[68] M. Massa, R. Campanelli, G. Fois et al., "Reduced frequency of circulating CD4$^+$CD25^{bright}CD127^{low}FOXP3$^+$ regulatory T cells in primary myelofibrosis," *Blood*, vol. 128, no. 12, pp. 1660–1662, 2016.

[69] M. Massa, V. Rosti, R. Campanelli, G. Fois, and G. Barosi, "Rapid and long-lasting decrease of T-regulatory cells in patients with myelofibrosis treated with ruxolitinib," *Leukemia*, vol. 28, no. 2, pp. 449–451, 2014.

[70] J. C. Wang, H. Sindhu, C. Chen et al., "Immune derangements in patients with myelofibrosis: the role of Treg, Th17, and sIL2Rα," *PLoS One*, vol. 10, no. 3, article e0116723, 2015.

[71] S. P. Yajnanarayana, T. Stubig, I. Cornez et al., "JAK1/2 inhibition impairs T cell function *in vitro* and in patients with myeloproliferative neoplasms," *British Journal of Haematology*, vol. 169, no. 6, pp. 824–833, 2015.

[72] K. Wang and A. T. Vella, "Regulatory T cells and cancer: a two-sided story," *Immunology Investigation*, vol. 45, no. 8, pp. 797–812, 2016.

[73] K. Giannopoulos, M. Schmitt, M. Kowal et al., "Characterization of regulatory T cells in patients with B-cell chronic lymphocytic leukemia," *Oncology Report*, vol. 20, pp. 677–682, 2008.

[74] E. Bachy, J. Bernaud, P. Roy, D. Rigal, and F. E. Nicolini, "Quantitative and functional analyses of $CD4^+CD25^+FoxP3^+$ regulatory T cells in chronic phase chronic myeloid leukaemia patients at diagnosis and on imatinib mesylate," *British Journal of Haematology*, vol. 153, pp. 129–143, 2011.

Hindawi
Journal of Immunology Research
Volume 2017, Article ID 7979637, 10 pages
https://doi.org/10.1155/2017/7979637

Review Article

Inflammatory Cytokine Signaling during Development of Pancreatic and Prostate Cancers

Geou-Yarh Liou

Center for Cancer Research & Therapeutic Development and Department of Biological Sciences, Clark Atlanta University, 223 James P. Brawley Drive SW, Atlanta, GA 30314, USA

Correspondence should be addressed to Geou-Yarh Liou; gliou@cau.edu

Received 28 July 2017; Revised 31 October 2017; Accepted 8 November 2017; Published 12 December 2017

Academic Editor: Shi Jin

Inflammation is essential for many diseases including cancer. Activation and recruitment of immune cells during inflammation result in a cytokine- and chemokine-enriched cell environment, which affects cancer development. Since each type of cancer has its unique tumor environment, effects of cytokines from different sources such as tumor-infiltrating immune cells, stromal cells, endothelial cells, and cancer cells on cancer development can be quite complex. In this review, how immune cells contribute to tumorigenesis of pancreatic and prostate cancers through their secreted cytokines is discussed. In addition, the cytokine signaling that tumor cells of pancreatic and prostate cancers utilize to benefit their own survival is delineated.

1. Introduction

Cancers develop because of a dysfunctional immune system of the body, which is unable to detect or eliminate precancerous cells at an early stage. Hijacking the immune system, which eliminates pathogens and unwanted cells such as senescent, damaged, or immature cells under normal physiological conditions, is a very common strategy that cancers utilize for benefitting their long-term growth and survival against locally limited resources, for example, less oxygen in solid tumor tissues. To overcome this shortage of resource, cancer cells express several cytokines, growth factors, and receptors for cytokines and growth factors to become independent of the mitogens that are supplied other than themselves. Another mechanism, which cancer cells utilize, is to recruit immune cells such as macrophages, neutrophils, B cells, and so on, and these tumor-infiltrating immune cells interact with cancer cells, cancer-associated fibroblasts, and themselves. Meanwhile, tumor-associated immune cells secrete certain cytokines, chemokines, and proteases, for example, TGFβ to dampen T cells, natural killer (NK) cells, and dendritic cells; all of which are engaged in eradicating cancer cells. The indirect effects of immune cells among themselves also create a tumor-favorable outcome. For example, IL-10 secreted by $\gamma\delta$ Treg cells diminished the cytotoxic activity of $CD8^+$ T cells and NK cells, resulting in tumor growth [1]. It is certainly complex that how different types of tumor-infiltrating immune cells affect their biological functions among each other through self-secreted cytokines and chemokines, leading to specific and unique tumor environments in different types of cancer. Moreover, the neighboring cell types around cancer cells also contribute to distinct tumor environments in different types of cancer. For example, breast cancer is neighbored by adipose tissue; pancreatic cancer is accompanied by massive desmoplasia. Given that the same cytokine can result in opposite biological effects in different types of cancer, that is, tumor promoting versus tumor inhibiting due to their unique tumor environment, it is important to comprehensively review cytokine signaling and its functions during cancer development in each type of cancer for shedding light on cancer therapies by altering the tumor environment. In this review, I will mainly focus on cytokine signaling of tumor-facilitated immune cells and of cancer cells that lead to tumor initiation, progression, and metastasis of pancreatic cancer and prostate cancer.

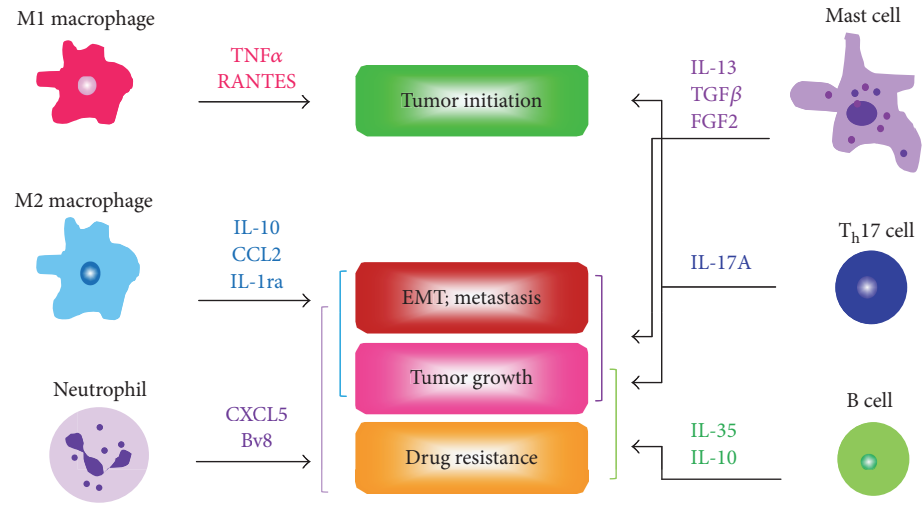


Figure 1: Effects of the cytokines secreted by different types of immune cells on tumor initiation, progression, and dissemination. Cytokines secreted by macrophages, neutrophils, mast cells, T_h17 cells, and B cells directly lead to initiation and development of pancreatic and prostate cancers. IL: interleukin; RANTES: regulated on activation, normal T cell expressed and secreted; TGFβ: transforming growth factor β; FGF2: fibroblast growth factor 2; EMT: epithelial mesenchymal transition.

2. Pancreatic and Prostate Cancers

Pancreatic cancer is the most lethal type of cancer with an approximately 7% 5-year survival rate and is projected to become the 2nd leading cause of all cancer-related death by 2030. Pancreatic ductal adenocarcinoma (PDAC) represents over 90% of pancreatic cancer cases. Oncogenic Kras mutations are present in almost all PDAC patients and are required to initiate and develop PDAC. Another unique feature of PDAC is severe desmoplasia/fibrosis, which occurs at the very early stage of the disease. Pancreatic stellate cells, fibroblasts, and enriched extracellular matrix (ECM) components from desmoplasia orchestrate with other types of cells including immune cells and endothelial cells to promote pancreatic cancer growth and survival.

Prostate cancer is the most common type of cancer in men. In addition, it is also the 2nd leading cause of cancer fatality in men. Unlike PDAC, which is a genetic disorder of oncogenic Kras, key genetic mutations lead to prostate cancer remain unclear. Although several genetically engineered mouse models are generated for studying prostate cancer initiation, development, and dissemination *in vivo*, none of them fully recapitulates this disease in humans. Most *in vivo* studies, especially for testing potential therapeutic drugs on prostate cancer, mainly rely on xenograft mouse models using human prostate cancer cell lines or patient tissue samples. It restrains the advancements of how these drugs also affect immune cells and stromal cells, which can contribute to drug efficacy during cancer therapy.

Although pancreatic and prostate cancers are quite different and face different challenges for cancer therapies, the patients of these two cancers share certain features among other cancer patients which include (1) late-onset diseases (in patients' late 60), (2) a gender preference which males rather than females are prone to the diseases, and (3) an ethnic preference which African Americans rather than other racial groups are more susceptible to these cancers. Of note, both cancer types are tightly associated with old age, implicating a pivotal role of aging-related inflammation in these two types of cancer. Herein, an overview of how the cytokine signaling from immune cells and cancer cells affects tumorigenesis of the pancreas and the prostate is provided below.

3. Cytokine Signaling from Immune Cells for Modulating Tumorigenesis

The immune system is consisted of many types of immune cells, organs, and antibodies to protect and defend our body. Immune cells can be majorly categorized to phagocytes and lymphocytes which secrete cytokines and chemokines to alter a local cell microenvironment. It has been demonstrated that several cytokines from both phagocytes and lymphocytes potentiate tumor initiation, proliferation, and metastasis of pancreatic and prostate cancers (Figure 1). How these cytokines secreted by different types of immune cells to directly influence cancer cells or indirectly affect nearby immune cells during cancer development are described below in details.

3.1. Cytokines Secreted by Phagocytes and Their Functions. Phagocytes include macrophages, neutrophils, mast cells, and dendritic cells and are capable of ingesting microorganisms, cellular debris, and foreign particles. A large and convincing body of evidence demonstrates the increased infiltrating macrophages and their role in cancer progression and dissemination of PDAC and prostate adenocarcinoma. IL-6 from infiltrating macrophages in the pancreata of PDAC transgenic mice activated transcription factor STAT3 in pancreatic tumor cells, promoting tumor development [2]. Macrophages are categorized to M1 and M2 subtypes, which are also known as classically activated (M1) and alternatively activated (M2) according to their activations (see details in review [3]). M1 macrophages are activated by lipopolysaccharide (LPS), interferon gamma (IFNγ), virus, and so on

and participate in killing pathogens and clearing up dying cells to protect the host, known as inflammation. During this process, M1 macrophages secrete inflammatory cytokines and generate nitric oxide (NO). However, M2 macrophages stimulated by interleukin-4 (IL-4), IL-13, IL-10, and so on produce extracellular matrix components, angiogenic and chemotactic factors, and IL-10. M2 macrophages are engaged in wound healing, tissue remodeling, allergy, and immunoregulation. Based on the characters of M1 and M2 macrophages described above, several markers such as iNOS, CD38 (for M1 macrophages), Egr2, and CD163 (for M2 macrophages) have been utilized for studying their functions on cancer initiation, development, and dissemination [4–6]. Increased numbers of M2-polarized tumor-associated macrophages (TAM) were reported to correlate with a poor prognosis of PDAC [7, 8]. Of note, M1 proinflammatory macrophages, which generally are thought to be antitumor, have been demonstrated to initiate PDAC development [9–11]. TNFα and RANTES secreted by M1 macrophages upregulate expressions and activities of matrix metalloproteinase 9 (MMP9) of pancreatic acini, resulting in transdifferentiating these cells to a progenitor duct-like phenotype, which later can further become PDAC cells. This piece of evidence also provides a plausible and common scenery of how inflammation-related conditions such as pancreatitis, obesity, diabetes, aging, and so on increase the risk of getting PDAC.

In a pdx1^{cre}:Kras^{G12D}:Trp53^{R172H} (KPC) transgenic mouse model that recapitulates human metastatic PDAC, depletion of macrophages by pharmacological compound clodronate liposomes at an early stage of PDAC reduced metastatic cancer cells in the liver and lungs [12]. M2-polarized macrophages secreted high levels of CCL2 and IL-1ra around preinvasive pancreatic intraepithelial neoplasia (PanIN) lesions *in vivo*, resulting in elevated proliferation of PanIN cells through enhanced ERK signaling [13]. In addition, prevention of macrophage polarization to a M2 subtype by an IL-13 neutralizing antibody diminished fibrosis, which is associated with pancreatic tumor proliferation and progression. In a coculture system, in addition to elevated cancer cell proliferation and migration, IL-4-polarized M2 macrophages also induced epithelial-mesenchymal transition (EMT) in human pancreatic cancer cell lines [14]. Furthermore, depletion of toll-like receptor 4 (TLR4) or neutralization of IL-10 in M2 macrophages blocked the EMT ability of cancer cells. Very recently, it has been demonstrated that inflammasome component protein NLRP3 expressed by M2 macrophages (CD206$^+$MHCII$^-$) is essential for their proliferation in PDAC [15]. In addition, these NLRP3$^+$ macrophages also resulted in increased populations of T_h17 and Treg cells and an inactivation of cytotoxic CD8$^+$ T cells. Interference with inflammasome by targeting its component proteins through pharmacological compounds or NLRP3 knockout reduced PDAC formation *in vivo*.

Accumulating evidence indicated an increased recruitment of M2 immunosuppressive macrophages in prostate cancer, associating with cancer growth, metastasis, and drug resistance, thus leading to worse outcomes for prostate cancer patients [16–20]. However, to date, research studies in prostate cancer field majorly are focused on how prostate cancer affects macrophage polarization and infiltration due to several discrepant clinical data of inflammation/macrophages in prostate cancer, which may be due to varied markers of different immune cell types and quality of specimens.

Several lines of evidence have demonstrated involvement of neutrophils in cancer initiation, progression, and dissemination. These processes are mediated by neutrophil-derived products including cytokine, chemokines, proteases, reactive oxygen species (ROS), and so on (see detailed reviews in [21, 22]). However, the detailed mechanisms of how these tumor-associated neutrophils (TAN) promote cancer development and metastasis, especially in varied tumor environments of different types of cancer, remain unclear. A neutrophil-expressed glycoprotein lipocalin 2, known for combatting bacterial infection, was present in preinvasive PanIN lesions and PDAC and was suggested as a marker for early diagnosis of PDAC [23]. In addition, the ratio of neutrophil to lymphocyte found in the peripheral blood was correlated to the overall survival of PDAC patients [24, 25]. TAN secretes several cytokines and chemokines such as CXCL5, TGFβ, and TNF during tumor development and metastasis. In transgenic KPC mice of PDAC, high levels of CXCL5 in the tumor stroma were detected [26]. Moreover, knockout of CXCL5 receptor CXCR2 in PDAC cells dramatically blocked liver metastases. In a xenograft mouse model of human pancreatic cancer AsPC-1 cells, administering an inhibitor of chemokine Bv8, which is secreted by TAN, reduced tumor size and enhanced gemcitabine-induced cytotoxicity of cancer cells [27].

Mast cells participate in many physiological processes such as immune tolerance, angiogenesis, wound healing, and defense against pathogens. Dysregulation of mast cell activation associates with many disorders including cancer. Increased infiltration of mast cells was reported in PDAC patient samples and correlates with a high grade of tumor as well as a poor prognosis [28, 29]. Implantation of murine cancer Pan02 cells into the pancreas tissues of mast cell-deficient mice failed to develop PADC *in vivo* [30]. It has been shown that neutralization of IL-13 secreted by mast cells hindered proliferation of human pancreatic stellate cells (PSC) [31]. Interestingly, blockade of IL-13 also decreased TGFβ expression and activation of Smad2 in PSC, suggesting that TGFβ signaling as downstream of IL-13 promotes PSC proliferation.

Similar to that in PDAC patients, more mast cells were present in prostate adenocarcinoma tissues and further increased their recruitment to castrate-resistant prostate tumors [32]. Less mast cells present within prostate tumors may indicate a high recurrence rate of the disease [33]. In an orthotopic rat model of prostate cancer, coinjection of mast cells with cancer AT-1 cells increased tumor growth [32]. Moreover, increased mRNA levels and protein expressions of fibroblast growth factor 2 (FGF2) were detected in mast cell-infiltrating prostate tumors. In a xenograft mouse model, coimplantation of human prostate cancer cells with mast cells enhanced tumor metastasis to the diaphragm

[34]. In addition, cultured mast cells downregulated mRNA levels of androgen receptor of human prostate cancer cell lines, leading to increased invasiveness and migration ability of cancer cells.

3.2. Cytokines Secreted by Lymphocytes and Their Functions. Lymphocytes include T cells, B cells, and natural killer (NK) cells. Dysfunctional T cells generated through different molecular and cellular mechanisms such as T cell anergy, exhaustion, and senescence allow tumor growth by escaping immune surveillance. In addition, presence of regulatory T cells (Treg), which suppress self-activation of T cells, also contributes to immunosuppressive tumor microenvironments, blocking antitumor immune responses, and subsequent cancer progression. In tumor tissue samples of PDAC patients, more $CD4^+CD25^+Foxp3^+$ Treg cells were detected and linked to a poor prognosis [35, 36]. This increase in Treg cells also correlates with less cytotoxic $CD8^+$ T cells and $CD4^+$ T helper cells in PDAC and precancerous PanIN lesions. Reprogramming of Treg cells by a CD25 neutralizing antibody daclizumab results in an increase in $CD56^{bright}$ natural killer cells and functional T cells such as cytotoxic $CD8^+$ T cells and $CD4^+$ T helper cells in a small clinical trial of metastatic breast cancer patients who receive a cancer vaccine [37]. Results from a heterotopic mouse model of PDAC using nonmetastatic murine Pan02 cancer cells and an orthotopic xenograft mouse model using metastatic human Panc-1 cancer cells revealed that pancreatic cancer expresses CCL5 to recruit $CD4^+Foxp3^+$ Treg cells, which have high levels of CCR5 [38, 39]. Interruption of this interaction between CCL5 and CCR5 by knocking down CCL5 in cancer cells that were used for cancer cell transplantation in mice impedes cancer growth. This suppression effect can also be achieved by administering a CCR5 inhibitor TAK-779- or a CCL5-neutralizing antibody in mice. IL-10 secreted by $CD4^+Foxp3^+$ Treg cells renders T cell anergy (unresponsive to IL-10 restimulations) (see review in [40, 41]). IL-10 from Treg can also prevent T cell expansion by directly suppressing IL-2 production in T cells. In addition to induction of IL-10 expression in $Foxp3^+$ Treg cells, TGFβ from $CD4^+Foxp3^+$ Treg cells and pancreatic cancer also stimulates transcription factor Foxp3 expression in $Foxp3^-$ naïve T cells, leading to differentiation of $Foxp3^+$ Treg cells [42, 43]. Whether targeting Treg cells, for example, through CD25 blockade or Treg-secreted cytokines as mentioned previously is sufficient to overcome immune resistance of pancreatic cancer *in vivo* remains to be evaluated. In prostate cancer, a high density of Treg cells was detected in cancer tissues and associated with an advanced tumor stage as well as a low survival rate [44, 45]. In prostate cancer with bone metastasis, Treg cells expressing high levels of CXCR4 were recruited to bone marrow through an interaction with CXCL12, which is enriched in the bone marrow [46]. Meanwhile, the Treg cells in the bone marrow upregulated RANKL expression to increase their proliferation, which is mediated by $RNAK^+$ dendritic cells.

T helper 17 (T_h17) cells are T helper cells producing IL-17 and involved in autoimmune and inflammatory disorders. In response to different stimuli including IL-6, TGFβ, CCL20, IL-23, IL-1β, and so on, especially under cancer microenvironments, Th17 cells can mediate tumor regression or tumor promotion [47–49]. In a PDAC heterograft mouse model, increased infiltration of Th17 cells was observed in IL-6-expressed tumors that cause high mortality in mice [50]. In a transgenic mouse model of PDAC, oncogenic $Kras^{G12D}$ induced IL17A-expressed T_h17 cells, leading to tumor initiation and progression of precancerous PanIN lesions [48]. This initiation and promotion effects by T_h17 cells are mediated through their interaction with tumor-initiating cells and PanIN cells, both expressing IL-17 receptor A (IL-17RA). In addition, manipulation of IL-17A by overexpression of IL-17A in the pancreas or knockout of IL-17A in hematopoietic cells interfered initiation and development of pancreatic tumor *in vivo*. In a xenograft mouse model of human pancreatic cancer CFPAC-1 cells, depletion of IL-17RB impedes cancer growth and metastasis to the liver and lungs [51]. The effect of IL-17RB on PDAC proliferation and dissemination was mediated by upregulation of CCL20, CXCL1, and IL-8 cytokines through ERK signaling in PDAC cancer cells. In prostate cancer, more T_h17 cells and higher levels of its secreted cytokine IL-17A are linked to a worse outcome of patients [52, 53]. Knockout of IL-17RC, a receptor for IL-17A, decreased invasive prostate tumor growth of $PTEN^{-/-}$ transgenic mice through downregulation of MMP7 and increased expressions of MMP inhibitors TIMP1, 2, and 4 [54]. Interference with T_h17 cells by administration of SR1001, a small molecule inhibitor targeting T_h17 transcription factors RORγt and RORα, or with T_h17 cell-secreted IL17 by an IL-17-neutralizing antibody, results in reduced cancer proliferation, angiogenesis, and inflammation of $PTEN^{-/-}$ mouse prostate tissues [55].

Microarray study using human samples of PDAC demonstrated increased mRNA expressions of CD20, a marker for B cells [56], suggesting an association of B cells with PDAC development. Infiltration of protumorigenic B cells is associated with hypoxia and fibroblast-enriched stroma [57, 58]. Depletion of B cells, such as use of a CD20 neutralizing antibody or B cell-deficient mice, significantly delays the growth of PDAC and its precursor pancreatic intraepithelial neoplasia (PanIN) in transgenic and orthotopic mouse models [56–58]. Furthermore, reconstitution of functional B cells obtained from wildtype mice through adoptive transfer method rescued pancreatic tumor growth. Among these protumorigenic B cells, which are $CD1d^{hi}CD5^+$, increased levels of IL-35 were observed and speculated to be responsible for pancreatic tumor growth [58].

Although an increased density of B cell within prostate tumors has been reported in prostate cancer patient samples [59, 60], so far, only a handful publications indicate the function of tumor-infiltrating B cells on tumorigenesis of prostate cancer. In castration-resistant prostate cancer from the xenograft mice, release of IL-1α from the necrotic primary cancer cells results in CXCL3 secretion, which, in turn, recruits B cells. These tumor-infiltrating B cells produce lymphotoxin to stimulate cancer growth under hormone-independent conditions [61]. In Oxaliplatin-resistant prostate cancers of

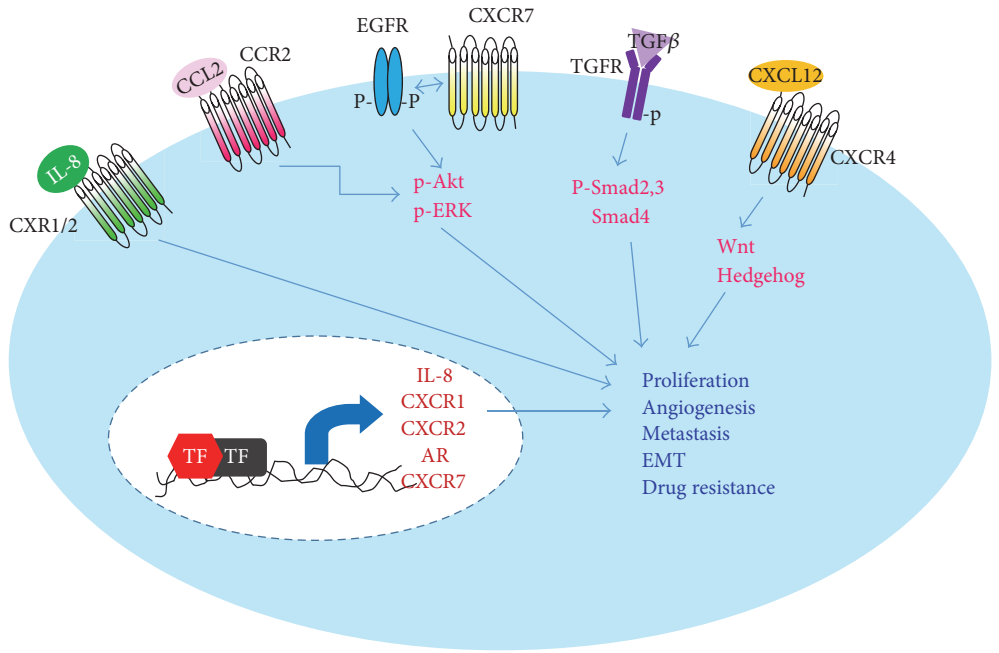


Figure 2: Cytokine signaling of cancer cells on modulation of cell proliferation, angiogenesis, metastasis, and drug resistance. Cytokine signaling pathways utilized by pancreatic and prostatic tumor cells through an autocrine-signaling mechanism to elevate their own growth, angiogenesis, and drug resistance. In addition, upregulation of these signaling molecules also renders tumor cells a more mesenchymal-like phenotype, which in turn, promotes metastasis. TF: transcription factor; IL-8: interleukin-8; AR: androgen receptor; EMT: epithelial mesenchymal transition; p: phosphorylated.

TRAMP transgenic mice, increased numbers of B lymphocytes were detected [59]. These infiltrating B cells expressed high levels of IL-10, IgA, and programmed death ligand 1 (PD-L1), leading to the development of oxaliplatin drug resistance over time. Moreover, ablation of IL-10, PD-L1, or B cells reinstated prostate cancer sensitivity to Oxaliplatin-induced cell death.

4. Cytokine Signaling from Tumor Cells for Modulating Tumor Growth and Proliferation

A large and still continuously increasing body of evidence demonstrated that cancer cells upregulate expressions of numerous cytokines to benefit their own survival. This is achieved by modulating surrounding cells to create a tumor-promoting environment. For example, pancreatic tumor cells expressed cytokine IL-13 to repolarize the nearby macrophages to a tumor-promoting/M2 subtype [13]. In addition, cancer cells also express receptors for the upregulated cytokines to persistently support their own growth (known as autocrine-signaling mechanism, see Figure 2) and become more independent on limited exogenous growth factors as well as mitogens. Insights into these cytokine-signaling pathways of cancer cells could shed light on developing a better cancer therapy for PDAC and castrate-resistant prostate adenocarcinoma.

Increased levels of mRNA and protein of IL-8 were detected in human pancreatic cancer cell lines and PDAC patient tissues ([62, 63]; see reviews in [64]). Elevated expression of IL-8 in PDAC tumor under hypoxic conditions associated with cancer metastatic ability in xenograft mice [65]. In addition, downregulation of IL-8 by expressing a specific antisense oligonucleotide against IL-8 in PDAC cells before implantating in mouse pancreas hindered tumor vascularization, leading to smaller tumors. Upregulation of IL-8 in PDAC is mediated by transcription factors AP-1 and NF-κB; both positively modulate IL-8 promoter upon oncogenic Kras mutation [66]. Besides, administration of an IL-8-neutralizing antibody, which blocks IL-8 signaling, in xenografted mice resulted in elevated tumor necrosis and decreased angiogenesis without affecting tumor proliferation. In addition to IL-8, its receptors CXCR1 and CXCR2 were also reported in human PDAC Capan-1 cells to regulate cancer cell growth, migration, invasion, and its stem cell-like features [62, 63]. In human androgen-independent prostate adenocarcinoma, stronger expressions of IL-8 were present and correlated with advanced stages of the disease [67, 68]. Depletion of IL-8 by shRNA technique in cultured prostate cancer cells, which are derived from human castrate-resistant prostate adenocarcinoma, led to reduced cell proliferation and migration and enhanced cytotoxicity in response to chemotherapeutic drugs such as docetaxel [69]. Several signaling pathways involved in potentiating cell proliferation of prostate cancer by IL-8 have been delineated. Increased IL-8 of prostate cancer cells signals through CXCR1 and CXCR2 to induce transcription of androgen receptor (AR) and to activate ERK and Akt, in turn, promoting cancer growth and survival [68, 70]. In addition, IL-8 of the prostate cancer cells also upregulated EGFR-ERK signaling through elevating CXCR7 transcripts and protein levels [71]. However, the mitogenic function of CXCR7 in prostate cancer is ligand independent.

Higher expressions of TGFβ have been detected in human PDAC tissue samples and associated with a better prognosis of PDAC patients [72–74]. In human pancreatic cancer Panc-1 cells, results from knockdown of TGFβ signaling mediators Smad2 and Smad3 demonstrated an opposite function of Smad2 versus Smad3 on TGFβ-induced cell migration [75]. Silencing Smad4 in Panc-1 cells impeded epithelial-to-mesenchymal transition ability of cells (increased E-cadherin; decreased N-cadherin and vimentin), and this alteration of EMT is mediated by loss of nestin, a protein expressed in stem cells during development [76]. In the KPC transgenic mouse model of PDAC, cancer precursors, which are acinar-to-ductal metaplasia (ADM) and PanIN, and PDAC expressed higher levels of activated Smad2 [77]. Moreover, suppression of TGFβ by a TGFβ-neutralizing antibody in these mice expedited PDAC proliferation and malignant progression and caused death, suggesting an inhibitory effect of TGFβ during PDAC growth and development. In addition to stromal cells as well as the cells of benign prostatic hyperplasia (BPH) and of prostatic intraepithelial neoplasia (PIN), increased mRNA and protein levels of both TGFβ and its receptors were detected in cancer cells of human and rat prostate adenocarcinoma tissues [78, 79]. In an allotransplantation model of rat prostate cancer cells that overexpressed TGFβ, these tumors grew faster and metastasized to the lungs and lymph nodes. Treating these animals with a TGFβ-neutralizing antibody rescued the phenotype [80]. Moreover, when the TGFβ-expressed cancer cells were cultured *in vitro* in 2D, their proliferation were inhibited as numerous cases reported in cultured human prostate cancer cell lines. This result suggested the importance of cell environment regarding the impact of TGFβ expressed by cancer cells on their own proliferation and malignancy.

Increased CCL2 expression was present in PDAC tissues and a portion of human PDAC cancer cell lines [13, 81]. Moreover, no CCR2, a receptor for CCL2, was detected in the tested human CCL2-expressed PDAC cell lines. This may be due to a low sensitivity of Northern blotting to detect CCR2 transcripts or cell culture conditions such as 2D culture system versus 3D culture system, hypoxic environment, and so on. Although it is unclear whether tumor cells of PDAC tissues have CCR2, presence of CCR2 was shown in preinvasive PanIN lesions of transgenic KC mice [13]. In addition, in a 3D culture system of murine duct-like cells derived from KC mouse pancreas, exogenous CCL2 treatment promoted cell proliferation through activation of ERK. These results suggested an autocrine mechanism of CCL2 used by pancreatic tumor cells to benefit their growth. Similar to pancreatic cancer, expressions of CCL2 and CCR2 were present in prostate cancer tissues and led to increased cancer proliferation and invasion through Akt signaling [82, 83]. Interference with this pathway by administration of CCR2 antagonist, CCL2 neutralizing antibody, or PI3 kinase inhibitor or by knockdown of CCL2 in prostate cancer PC3 cells all resulted in decreased tumor formation, smaller tumor size, and less metastases *in vivo* [82–84].

CXCR4 and its ligand CXCL12 were present in PanIN lesions and PDAC tissues and demonstrated to result in tumor proliferation, cancer progression, angiogenesis, and metastasis [85–87]. CXCR4-CXCL12-caused cell proliferation was mediated via EGFR-Src-PI3 kinase signaling pathway, which subsequently activates ERK [87–89]. For CXCR4-CXCL12-mediated invasion and metastasis of human pancreatic cancer cell lines, activation of Wnt, and Hedgehog signaling pathways were required and led to an EMT phenotype [90, 91]. Interestingly, inhibition of Wnt, Hedgehog, or CXCL12-CXCR4 signaling by pharmacological compounds resensitized cancer cells to gemcitabine-induced cytotoxicity, suggesting the importance of CXCL12-CXCR4 in PDAC drug resistance [92–94]. Similar to the functions of CXCR4-CXCL12 in PDAC, prostate cancer cells express high levels of CXCR4 and CXCL12 and utilize the same signaling pathways described above to modulate their proliferation, angiogenesis, drug resistance, and metastasis (see review in [95, 96]).

Cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) can affect tumor immune response positively or negatively depending on the local tumor microenvironment, which is distinct among cancer types [97]. Accumulating evidence supports a role of GM-CSF in promoting rather than inhibiting the development of pancreatic cancer. Higher expressions of GM-CSF were detected in human and mouse PDAC tissues and have been shown to modulate development of Gr-1$^+$CD11b$^+$ myeloid cells, which inactivate cytotoxic CD8$^+$ T cells [98, 99]. In addition, blockade of GM-CSF derived from PDAC by either a GM-CSF-neutralizing antibody or a specific knockdown of GM-CSF in pancreatic cancer cells suppressed tumor growth with less infiltrating Gr-1$^+$CD11b$^+$ cells *in vivo*. Interestingly, GM-CSF secreted by cancer-associated mesenchymal stem cells (CA-MSC) was required for cell invasion, metastasis, and transendothelial migration of human pancreatic cancer [100]. In addition, it also modulated EMT and stemness of PDAC. In contrast, despite of unknown mechanism of how GM-CSF inhibits metastatic prostate cancer, GM-CSF has been in the clinical trials for prostate cancer patients since 2001. It has been shown that systemic GM-CSF given to the patients in the clinical trials altered immune cell populations with an increase in CD4$^+$ T cells, CD8$^+$ T cells, and mature myeloid dendritic cell and a decrease in Treg cells [101]. Interestingly, expressions of receptors for GM-CSF are reported in cultured human prostate carcinoma LNCaP cells [102].

Activation of Fas ligand (FasL) and its receptor (Fas) leads to cell apoptosis. In pancreatic cancer, expressions of FasL and Fas were present in human PDAC tissues and cultured cancer cell lines [103–105]. It has been demonstrated that FasL-expressed PDAC induced apoptosis of the infiltrating lymphoid cells, thus eliminating tumor-killing immune cells [103, 105]. Meanwhile, pancreatic cancer cells were resistant to Fas-induced apoptosis by downregulation of Fas or upregulation of Fas-associated phosphatase 1 (FAP-1), which is mediated by JNK and p38 MAPK [104]. Similar to the functions and expressions of FasL in pancreatic cancer, human prostate cancer cells were also resistant to FasL-induced apoptosis. In addition, soluble FasL (sFasL) was consistently secreted through a cleavage on membrane-

bound FasL by MMPs in prostate cancer [106]. Tumor exosomes of human prostate carcinoma LNCaP cells expressed FasL and caused cytotoxic $CD8^+$ T cell apoptosis [107]. It has been shown that interaction of FasL with Fas in the intracellular compartment resulted in cancer cell apoptosis of human prostate carcinoma cells that are resistant to anti-Fas antibody CH-11 [108].

5. Conclusion

Increased cytokine signaling in both tumor-infiltrating immune cells and cancer cells potentiates tumor growth, metastasis, and drug resistance of pancreatic and prostate cancers. Many cytokines upregulated in cancer cells are mediated through autocrine signaling. Meanwhile, cancer cells also use paracrine-signaling mechanism to recruit immune cells to the tumor site. These infiltrating immune cells then produce more cytokines to directly or indirectly support tumor growth. Certain downstream effectors of cytokines, especially upregulated by cancer cells, modulate stem cell-like properties such as Notch, Wnt, and Hedgehog. Through controlling these pathways, cancer cells are capable of proliferating and surviving even when under harsh conditions, for example, cancer therapy. A comprehensive view of this complex cytokine network among tumor cells, immune cells, and other types of cells including stromal cells, endothelial cells, and so on will provide invaluable information on best strategies to defeat cancer-caused death, for example, use of cytokine inhibitor cocktails in the future.

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this article.

Acknowledgments

This work was supported by a grant from the Center for Cancer Research and Therapeutic Development (CCRTD) at Clark Atlanta University funded by NIH G12MD007590 to Geou-Yarh Liou.

References

[1] N. Seo, Y. Tokura, F. Furukawa, and M. Takigawa, "Down-regulation of tumoricidal NK and NK T cell activities by MHC K^b molecules expressed on Th2-type gammadelta T and alphabeta T cells coinfiltrating in early B16 melanoma lesions," *Journal of Immunology*, vol. 161, no. 8, pp. 4138–4145, 1998.

[2] M. Lesina, M. U. Kurkowski, K. Ludes et al., "Stat3/Socs3 activation by IL-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer," *Cancer Cell*, vol. 19, no. 4, pp. 456–469, 2011.

[3] F. O. Martinez and S. Gordon, "The M1 and M2 paradigm of macrophage activation: time for reassessment," *F1000Prime Reports*, vol. 6, p. 13, 2014.

[4] C. Caux, R. N. Ramos, G. C. Prendergast, N. Bendriss-Vermare, and C. Ménétrier-Caux, "A milestone review on how macrophages affect tumor growth," *Cancer Research*, vol. 76, no. 22, pp. 6439–6442, 2016.

[5] K. A. Jablonski, S. A. Amici, L. M. Webb et al., "Novel markers to delineate murine M1 and M2 macrophages," *PLoS One*, vol. 10, no. 12, article e0145342, 2015.

[6] S. R. Nielsen and M. C. Schmid, "Macrophages as key drivers of cancer progression and metastasis," *Mediators of Inflammation*, vol. 2017, Article ID 9624760, 11 pages, 2017.

[7] Y. C. Hou, Y. J. Chao, H. L. Tung, H. C. Wang, and Y. S. Shan, "Coexpression of CD44-positive/CD133-positive cancer stem cells and CD204-positive tumor-associated macrophages is a predictor of survival in pancreatic ductal adenocarcinoma," *Cancer*, vol. 120, no. 17, pp. 2766–2777, 2014.

[8] H. Kurahara, H. Shinchi, Y. Mataki et al., "Significance of M2-polarized tumor-associated macrophage in pancreatic cancer," *The Journal of Surgical Research*, vol. 167, no. 2, pp. e211–e219, 2011.

[9] G. Y. Liou, H. Döppler, B. Necela et al., "Mutant KRAS-induced expression of ICAM-1 in pancreatic acinar cells causes attraction of macrophages to expedite the formation of precancerous lesions," *Cancer Discovery*, vol. 5, no. 1, pp. 52–63, 2015.

[10] G. Y. Liou, H. Döppler, B. Necela et al., "Macrophage-secreted cytokines drive pancreatic acinar-to-ductal metaplasia through NF-kappaB and MMPs," *The Journal of Cell Biology*, vol. 202, no. 3, pp. 563–577, 2013.

[11] G. Y. Liou and P. Storz, "Inflammatory macrophages in pancreatic acinar cell metaplasia and initiation of pancreatic cancer," *Oncoscience*, vol. 2, no. 3, pp. 247–251, 2015.

[12] H. Griesmann, C. Drexel, N. Milosevic et al., "Pharmacological macrophage inhibition decreases metastasis formation in a genetic model of pancreatic cancer," *Gut*, vol. 66, no. 7, pp. 1278–1285, 2017.

[13] G. Y. Liou, L. Bastea, A. Fleming et al., "The presence of interleukin-13 at pancreatic ADM/PanIN lesions alters macrophage populations and mediates pancreatic tumorigenesis," *Cell Reports*, vol. 19, no. 7, pp. 1322–1333, 2017.

[14] C. Y. Liu, J. Y. Xu, X. Y. Shi et al., "M2-polarized tumor-associated macrophages promoted epithelial-mesenchymal transition in pancreatic cancer cells, partially through TLR4/IL-10 signaling pathway," *Laboratory Investigation*, vol. 93, no. 7, pp. 844–854, 2013.

[15] D. Daley, V. R. Mani, N. Mohan et al., "NLRP3 signaling drives macrophage-induced adaptive immune suppression in pancreatic carcinoma," *The Journal of Experimental Medicine*, vol. 214, no. 6, pp. 1711–1724, 2017.

[16] S. J. Ellem, H. Wang, M. Poutanen, and G. P. Risbridger, "Increased endogenous estrogen synthesis leads to the sequential induction of prostatic inflammation (prostatitis) and prostatic pre-malignancy," *The American Journal of Pathology*, vol. 175, no. 3, pp. 1187–1199, 2009.

[17] T. Fujii, K. Shimada, O. Asai et al., "Immunohistochemical analysis of inflammatory cells in benign and precancerous lesions and carcinoma of the prostate," *Pathobiology*, vol. 80, no. 3, pp. 119–126, 2013.

[18] K. Gollapudi, C. Galet, T. Grogan et al., "Association between tumor-associated macrophage infiltration, high grade prostate cancer, and biochemical recurrence after radical prostatectomy," *American Journal of Cancer Research*, vol. 3, no. 5, pp. 523–529, 2013.

[19] J. Escamilla, S. Schokrpur, C. Liu et al., "CSF1 receptor targeting in prostate cancer reverses macrophage-mediated resistance to androgen blockade therapy," *Cancer Research*, vol. 75, no. 6, pp. 950–962, 2015.

[20] M. Lanciotti, L. Masieri, M. R. Raspollini et al., "The role of M1 and M2 macrophages in prostate cancer in relation to extracapsular tumor extension and biochemical recurrence after radical prostatectomy," *BioMed Research International*, vol. 2014, Article ID 486798, 6 pages, 2014.

[21] S. B. Coffelt, M. D. Wellenstein, and K. E. de Visser, "Neutrophils in cancer: neutral no more," *Nature Reviews Cancer*, vol. 16, no. 7, pp. 431–446, 2016.

[22] A. D. Gregory and A. M. Houghton, "Tumor-associated neutrophils: new targets for cancer therapy," *Cancer Research*, vol. 71, no. 7, pp. 2411–2416, 2011.

[23] N. Moniaux, S. Chakraborty, M. Yalniz et al., "Early diagnosis of pancreatic cancer: neutrophil gelatinase-associated lipocalin as a marker of pancreatic intraepithelial neoplasia," *British Journal of Cancer*, vol. 98, no. 9, pp. 1540–1547, 2008.

[24] I. Bhatti, O. Peacock, G. Lloyd, M. Larvin, and R. I. Hall, "Preoperative hematologic markers as independent predictors of prognosis in resected pancreatic ductal adenocarcinoma: neutrophil-lymphocyte versus platelet-lymphocyte ratio," *American Journal of Surgery*, vol. 200, no. 2, pp. 197–203, 2010.

[25] X. An, P. R. Ding, Y. H. Li et al., "Elevated neutrophil to lymphocyte ratio predicts survival in advanced pancreatic cancer," *Biomarkers*, vol. 15, no. 6, pp. 516–522, 2010.

[26] C. W. Steele, S. A. Karim, J. D. G. Leach et al., "CXCR2 inhibition profoundly suppresses metastases and augments immunotherapy in pancreatic ductal adenocarcinoma," *Cancer Cell*, vol. 29, no. 6, pp. 832–845, 2016.

[27] V. F. Curtis, H. Wang, P. Yang et al., "A PK2/Bv8/PROK2 antagonist suppresses tumorigenic processes by inhibiting angiogenesis in glioma and blocking myeloid cell infiltration in pancreatic cancer," *PLoS One*, vol. 8, no. 1, article e54916, 2013.

[28] M. Ammendola, R. Sacco, G. Sammarco et al., "Mast cells density positive to tryptase correlates with angiogenesis in pancreatic ductal adenocarcinoma patients having undergone surgery," *Gastroenterology Research and Practice*, vol. 2014, Article ID 951957, 7 pages, 2014.

[29] M. J. Strouch, E. C. Cheon, M. R. Salabat et al., "Crosstalk between mast cells and pancreatic cancer cells contributes to pancreatic tumor progression," *Clinical Cancer Research*, vol. 16, no. 8, pp. 2257–2265, 2010.

[30] D. Z. Chang, Y. Ma, B. Ji et al., "Mast cells in tumor microenvironment promotes the in vivo growth of pancreatic ductal adenocarcinoma," *Clinical Cancer Research*, vol. 17, no. 22, pp. 7015–7023, 2011.

[31] Y. Ma, R. F. Hwang, C. D. Logsdon, and S. E. Ullrich, "Dynamic mast cell-stromal cell interactions promote growth of pancreatic cancer," *Cancer Research*, vol. 73, no. 13, pp. 3927–3937, 2013.

[32] A. Johansson, S. Rudolfsson, P. Hammarsten et al., "Mast cells are novel independent prognostic markers in prostate cancer and represent a target for therapy," *The American Journal of Pathology*, vol. 177, no. 2, pp. 1031–1041, 2010.

[33] H. A. Hempel, N. S. Cuka, I. Kulac et al., "Low intratumoral mast cells are associated with a higher risk of prostate cancer recurrence," *Prostate*, vol. 77, no. 4, pp. 412–424, 2017.

[34] L. Li, Q. Dang, H. Xie et al., "Infiltrating mast cells enhance prostate cancer invasion via altering LncRNA-HOTAIR/PRC2-androgen receptor (AR)-MMP9 signals and increased stem/progenitor cell population," *Oncotarget*, vol. 6, no. 16, pp. 14179–14190, 2015.

[35] N. Hiraoka, K. Onozato, T. Kosuge, and S. Hirohashi, "Prevalence of FOXP3$^+$ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions," *Clinical Cancer Research*, vol. 12, no. 18, pp. 5423–5434, 2006.

[36] Y. Tang, X. Xu, S. Guo et al., "An increased abundance of tumor-infiltrating regulatory T cells is correlated with the progression and prognosis of pancreatic ductal adenocarcinoma," *PLoS One*, vol. 9, no. 3, article e91551, 2014.

[37] A. J. Rech, R. Mick, S. Martin et al., "CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patients," *Science Translational Medicine*, vol. 4, no. 134, article 134ra62, 2012.

[38] M. C. Tan, P. S. Goedegebuure, B. A. Belt et al., "Disruption of CCR5-dependent homing of regulatory T cells inhibits tumor growth in a murine model of pancreatic cancer," *Journal of Immunology*, vol. 182, no. 3, pp. 1746–1755, 2009.

[39] X. Wang, M. Lang, T. Zhao et al., "Cancer-FOXP3 directly activated CCL5 to recruit FOXP3$^+$Treg cells in pancreatic ductal adenocarcinoma," *Oncogene*, vol. 36, no. 21, pp. 3048–3058, 2017.

[40] D. K. Sojka, Y. H. Huang, and D. J. Fowell, "Mechanisms of regulatory T-cell suppression - a diverse arsenal for a moving target," *Immunology*, vol. 124, no. 1, pp. 13–22, 2008.

[41] M. Saraiva and A. O'Garra, "The regulation of IL-10 production by immune cells," *Nature Reviews. Immunology*, vol. 10, no. 3, pp. 170–181, 2010.

[42] C. L. Maynard, L. E. Harrington, K. M. Janowski et al., "Regulatory T cells expressing interleukin 10 develop from Foxp3$^+$ and Foxp3$^-$ precursor cells in the absence of interleukin 10," *Nature Immunology*, vol. 8, no. 9, pp. 931–941, 2007.

[43] T. A. Moo-Young, J. W. Larson, B. A. Belt et al., "Tumor-derived TGF-beta mediates conversion of CD4$^+$Foxp3$^+$ regulatory T cells in a murine model of pancreas cancer," *Journal of Immunotherapy*, vol. 32, no. 1, pp. 12–21, 2009.

[44] A. Flammiger, L. Weisbach, H. Huland et al., "High tissue density of FOXP3+ T cells is associated with clinical outcome in prostate cancer," *European Journal of Cancer*, vol. 49, no. 6, pp. 1273–1279, 2013.

[45] A. M. Miller, K. Lundberg, V. Ozenci et al., "CD4$^+$CD25^{high} T cells are enriched in the tumor and peripheral blood of prostate cancer patients," *Journal of Immunology*, vol. 177, no. 10, pp. 7398–7405, 2006.

[46] E. Zhao, L. Wang, J. Dai et al., "Regulatory T cells in the bone marrow microenvironment in patients with prostate cancer," *Oncoimmunology*, vol. 1, no. 2, pp. 152–161, 2012.

[47] N. Martin-Orozco, Y. Li, Y. Wang et al., "Melanoma cells express ICOS ligand to promote the activation and expansion of T-regulatory cells," *Cancer Research*, vol. 70, no. 23, pp. 9581–9590, 2010.

[48] F. McAllister, J. M. Bailey, J. Alsina et al., "Oncogenic Kras activates a hematopoietic-to-epithelial IL-17 signaling axis in preinvasive pancreatic neoplasia," *Cancer Cell*, vol. 25, no. 5, pp. 621–637, 2014.

[49] S. Wu, K. J. Rhee, E. Albesiano et al., "A human colonic commensal promotes colon tumorigenesis via activation of

T helper type 17 T cell responses," *Nature Medicine*, vol. 15, no. 9, pp. 1016–1022, 2009.

[50] J. L. Gnerlich, J. B. Mitchem, J. S. Weir et al., "Induction of Th17 cells in the tumor microenvironment improves survival in a murine model of pancreatic cancer," *Journal of Immunology*, vol. 185, no. 7, pp. 4063–4071, 2010.

[51] H. H. Wu, W. W. Hwang-Verslues, W. H. Lee et al., "Targeting IL-17B–IL-17RB signaling with an anti–IL-17RB antibody blocks pancreatic cancer metastasis by silencing multiple chemokines," *The Journal of Experimental Medicine*, vol. 212, no. 3, pp. 333–349, 2015.

[52] K. S. Sfanos, T. C. Bruno, C. H. Maris et al., "Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing," *Clinical Cancer Research*, vol. 14, no. 11, pp. 3254–3261, 2008.

[53] G. E. Steiner, M. E. Newman, D. Paikl et al., "Expression and function of pro-inflammatory interleukin IL-17 and IL-17 receptor in normal, benign hyperplastic, and malignant prostate," *Prostate*, vol. 56, no. 3, pp. 171–182, 2003.

[54] Q. Zhang, S. Liu, D. Ge et al., "Interleukin-17 promotes formation and growth of prostate adenocarcinoma in mouse models," *Cancer Research*, vol. 72, no. 10, pp. 2589–2599, 2012.

[55] Q. Zhang, S. Liu, D. Ge et al., "Targeting Th17-IL-17 pathway in prevention of micro-invasive prostate cancer in a mouse model," *Prostate*, vol. 77, no. 8, pp. 888–899, 2017.

[56] A. J. Gunderson, M. M. Kaneda, T. Tsujikawa et al., "Bruton tyrosine kinase-dependent immune cell cross-talk drives pancreas cancer," *Cancer Discovery*, vol. 6, no. 3, pp. 270–285, 2016.

[57] K. E. Lee, M. Spata, L. J. Bayne et al., "*Hif1a* deletion reveals pro-neoplastic function of B cells in pancreatic neoplasia," *Cancer Discovery*, vol. 6, no. 3, pp. 256–269, 2016.

[58] Y. Pylayeva-Gupta, S. Das, J. S. Handler et al., "IL35-producing B cells promote the development of pancreatic neoplasia," *Cancer Discovery*, vol. 6, no. 3, pp. 247–255, 2016.

[59] S. Shalapour, J. Font-Burgada, G. di Caro et al., "Immunosuppressive plasma cells impede T-cell-dependent immunogenic chemotherapy," *Nature*, vol. 521, no. 7550, pp. 94–98, 2015.

[60] J. R. Woo, M. A. Liss, M. T. Muldong et al., "Tumor infiltrating B-cells are increased in prostate cancer tissue," *Journal of Translational Medicine*, vol. 12, no. 1, p. 30, 2014.

[61] M. Ammirante, J. L. Luo, S. Grivennikov, S. Nedospasov, and M. Karin, "B-cell-derived lymphotoxin promotes castration-resistant prostate cancer," *Nature*, vol. 464, no. 7286, pp. 302–305, 2010.

[62] L. Chen, J. Fan, H. Chen et al., "The IL-8/CXCR1 axis is associated with cancer stem cell-like properties and correlates with clinical prognosis in human pancreatic cancer cases," *Scientific Reports*, vol. 4, p. 5911, 2014.

[63] H. Takamori, Z. G. Oades, R. C. Hoch, M. Burger, and I. U. Schraufstatter, "Autocrine growth effect of IL-8 and GROalpha on a human pancreatic cancer cell line, Capan-1," *Pancreas*, vol. 21, no. 1, pp. 52–56, 2000.

[64] Y. Y. Yako, D. Kruger, M. Smith, and M. Brand, "Cytokines as biomarkers of pancreatic ductal adenocarcinoma: a systematic review," *PLoS One*, vol. 11, no. 5, article e0154016, 2016.

[65] Q. Shi, J. L. Abbruzzese, S. Huang, I. J. Fidler, Q. Xiong, and K. Xie, "Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic," *Clinical Cancer Research*, vol. 5, no. 11, pp. 3711–3721, 1999.

[66] A. Sparmann and D. Bar-Sagi, "Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis," *Cancer Cell*, vol. 6, no. 5, pp. 447–458, 2004.

[67] S. Araki, Y. Omori, D. Lyn et al., "Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer," *Cancer Research*, vol. 67, no. 14, pp. 6854–6862, 2007.

[68] C. Murphy, M. McGurk, J. Pettigrew et al., "Nonapical and cytoplasmic expression of interleukin-8, CXCR1, and CXCR2 correlates with cell proliferation and microvessel density in prostate cancer," *Clinical Cancer Research*, vol. 11, no. 11, pp. 4117–4127, 2005.

[69] R. K. Singh and B. L. Lokeshwar, "Depletion of intrinsic expression of Interleukin-8 in prostate cancer cells causes cell cycle arrest, spontaneous apoptosis and increases the efficacy of chemotherapeutic drugs," *Molecular Cancer*, vol. 8, no. 1, p. 57, 2009.

[70] A. Seaton, P. Scullin, P. J. Maxwell et al., "Interleukin-8 signaling promotes androgen-independent proliferation of prostate cancer cells via induction of androgen receptor expression and activation," *Carcinogenesis*, vol. 29, no. 6, pp. 1148–1156, 2008.

[71] R. K. Singh and B. L. Lokeshwar, "The IL-8-regulated chemokine receptor CXCR7 stimulates EGFR signaling to promote prostate cancer growth," *Cancer Research*, vol. 71, no. 9, pp. 3268–3277, 2011.

[72] G. Bellone, C. Smirne, F. A. Mauri et al., "Cytokine expression profile in human pancreatic carcinoma cells and in surgical specimens: implications for survival," *Cancer Immunology, Immunotherapy*, vol. 55, no. 6, pp. 684–698, 2006.

[73] K. Hashimoto, Y. Nio, S. Sumi et al., "Correlation between TGF-beta1 and p21 (WAF1/CIP1) expression and prognosis in resectable invasive ductal carcinoma of the pancreas," *Pancreas*, vol. 22, no. 4, pp. 341–347, 2001.

[74] H. Yue, B. Yang, H. Zhang et al., "Clinical significance of TGF-beta1 and beta-glucuronidase synchronous detection in human pancreatic cancer," *Hepatobiliary & Pancreatic Diseases International*, vol. 1, no. 2, pp. 309–311, 2002.

[75] H. Ungefroren, S. Groth, S. Sebens, H. Lehnert, F. Gieseler, and F. Fändrich, "Differential roles of Smad2 and Smad3 in the regulation of TGF-beta1-mediated growth inhibition and cell migration in pancreatic ductal adenocarcinoma cells: control by Rac1," *Molecular Cancer*, vol. 10, no. 1, p. 67, 2011.

[76] H. T. Su, C. C. Weng, P. J. Hsiao et al., "Stem cell marker nestin is critical for TGF-beta1-mediated tumor progression in pancreatic cancer," *Molecular Cancer Research*, vol. 11, no. 7, pp. 768–779, 2013.

[77] A. F. Hezel, V. Deshpande, S. M. Zimmerman et al., "TGF-beta and alphavbeta6 integrin act in a common pathway to suppress pancreatic cancer progression," *Cancer Research*, vol. 72, no. 18, pp. 4840–4845, 2012.

[78] M. R. Cardillo, E. Petrangeli, L. Perracchio, L. Salvatori, L. Ravenna, and F. di Silverio, "Transforming growth factor-beta expression in prostate neoplasia," *Analytical and Quantitative Cytology and Histology*, vol. 22, no. 1, pp. 1–10, 2000.

[79] M. S. Steiner, Z. Z. Zhou, D. C. Tonb, and E. R. Barrack, "Expression of transforming growth factor-beta 1 in prostate cancer," *Endocrinology*, vol. 135, no. 5, pp. 2240–2247, 1994.

[80] M. S. Steiner and E. R. Barrack, "Transforming growth factor-beta 1 overproduction in prostate cancer: effects on growth in vivo and in vitro," *Molecular Endocrinology*, vol. 6, no. 1, pp. 15–25, 1992.

[81] P. Monti, B. E. Leone, F. Marchesi et al., "The CC chemokine MCP-1/CCL2 in pancreatic cancer progression: regulation of expression and potential mechanisms of antimalignant activity," *Cancer Research*, vol. 63, no. 21, pp. 7451–7461, 2003.

[82] Y. Lu, Z. Cai, D. L. Galson et al., "Monocyte chemotactic protein-1 (MCP-1) acts as a paracrine and autocrine factor for prostate cancer growth and invasion," *Prostate*, vol. 66, no. 12, pp. 1311–1318, 2006.

[83] Y. Lu, Q. Chen, E. Corey et al., "Activation of MCP-1/CCR2 axis promotes prostate cancer growth in bone," *Clinical & Experimental Metastasis*, vol. 26, no. 2, pp. 161–169, 2009.

[84] R. D. Loberg, C. Ying, M. Craig et al., "Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo," *Cancer Research*, vol. 67, no. 19, pp. 9417–9424, 2007.

[85] T. Koshiba, R. Hosotani, Y. Miyamoto et al., "Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression," *Clinical Cancer Research*, vol. 6, no. 9, pp. 3530–3535, 2000.

[86] D. Saur, B. Seidler, G. Schneider et al., "CXCR4 expression increases liver and lung metastasis in a mouse model of pancreatic cancer," *Gastroenterology*, vol. 129, no. 4, pp. 1237–1250, 2005.

[87] R. M. Thomas, J. Kim, M. P. Revelo-Penafiel, R. Angel, D. W. Dawson, and A. M. Lowy, "The chemokine receptor CXCR4 is expressed in pancreatic intraepithelial neoplasia," *Gut*, vol. 57, no. 11, pp. 1555–1560, 2008.

[88] D. D. Billadeau, S. Chatterjee, P. Bramati et al., "Characterization of the CXCR4 signaling in pancreatic cancer cells," *International Journal of Gastrointestinal Cancer*, vol. 37, no. 4, pp. 110–119, 2006.

[89] X. Shen, A. Artinyan, D. Jackson, R. M. Thomas, A. M. Lowy, and J. Kim, "Chemokine receptor CXCR4 enhances proliferation in pancreatic cancer cells through AKT and ERK dependent pathways," *Pancreas*, vol. 39, no. 1, pp. 81–87, 2010.

[90] X. Li, Q. Ma, Q. Xu et al., "SDF-1/CXCR4 signaling induces pancreatic cancer cell invasion and epithelial-mesenchymal transition in vitro through non-canonical activation of hedgehog pathway," *Cancer Letters*, vol. 322, no. 2, pp. 169–176, 2012.

[91] Z. Wang, Q. Ma, Q. Liu et al., "Blockade of SDF-1/CXCR4 signalling inhibits pancreatic cancer progression *in vitro* via inactivation of canonical Wnt pathway," *British Journal of Cancer*, vol. 99, no. 10, pp. 1695–1703, 2008.

[92] J. Cui, W. Jiang, S. Wang, L. Wang, and K. Xie, "Role of Wnt/beta-catenin signaling in drug resistance of pancreatic cancer," *Current Pharmaceutical Design*, vol. 18, no. 17, pp. 2464–2471, 2012.

[93] K. P. Olive, M. A. Jacobetz, C. J. Davidson et al., "Inhibition of hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer," *Science*, vol. 324, no. 5933, pp. 1457–1461, 2009.

[94] S. Singh, S. K. Srivastava, A. Bhardwaj, L. B. Owen, and A. P. Singh, "CXCL12-CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells: a novel target for therapy," *British Journal of Cancer*, vol. 103, no. 11, pp. 1671–1679, 2010.

[95] U. M. Domanska, R. C. Kruizinga, W. B. Nagengast et al., "A review on CXCR4/CXCL12 axis in oncology: no place to hide," *European Journal of Cancer*, vol. 49, no. 1, pp. 219–230, 2013.

[96] M. Cojoc, C. Peitzsch, F. Trautmann, L. Polishchuk, G. D. Telegeev, and A. Dubrovska, "Emerging targets in cancer management: role of the CXCL12/CXCR4 axis," *OncoTargets and Therapy*, vol. 6, pp. 1347–1361, 2013.

[97] I. S. Hong, "Stimulatory versus suppressive effects of GM-CSF on tumor progression in multiple cancer types," *Experimental & Molecular Medicine*, vol. 48, no. 7, article e242, 2016.

[98] L. J. Bayne, G. L. Beatty, N. Jhala et al., "Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer," *Cancer Cell*, vol. 21, no. 6, pp. 822–835, 2012.

[99] Y. Pylayeva-Gupta, K. E. Lee, C. H. Hajdu, G. Miller, and D. Bar-Sagi, "Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia," *Cancer Cell*, vol. 21, no. 6, pp. 836–847, 2012.

[100] M. Waghray, M. Yalamanchili, M. Dziubinski et al., "GM-CSF mediates mesenchymal-epithelial cross-talk in pancreatic cancer," *Cancer Discovery*, vol. 6, no. 8, pp. 886–899, 2016.

[101] X. X. Wei, S. Chan, S. Kwek et al., "Systemic GM-CSF recruits effector T cells into the tumor microenvironment in localized prostate cancer," *Cancer Immunology Research*, vol. 4, no. 11, pp. 948–958, 2016.

[102] C. I. Rivas, J. C. Vera, F. Delgado-López et al., "Expression of granulocyte-macrophage colony-stimulating factor receptors in human prostate cancer," *Blood*, vol. 91, no. 3, pp. 1037–1043, 1998.

[103] A. Elnemr, T. Ohta, A. Yachie et al., "Human pancreatic cancer cells express non-functional Fas receptors and counterattack lymphocytes by expressing Fas ligand; a potential mechanism for immune escape," *International Journal of Oncology*, vol. 18, no. 1, pp. 33–39, 2001.

[104] M. Kornmann, T. Ishiwata, J. Kleeff, H. G. Beger, and M. Korc, "Fas and Fas-ligand expression in human pancreatic cancer," *Annals of Surgery*, vol. 231, no. 3, pp. 368–379, 2000.

[105] T. Ohta, A. Elnemr, H. Kitagawa et al., "Fas ligand expression in human pancreatic cancer," *Oncology Reports*, vol. 12, no. 4, pp. 749–754, 2004.

[106] Q. Y. Liu, M. A. Rubin, C. Omene, S. Lederman, and C. A. Stein, "Fas ligand is constitutively secreted by prostate cancer cells in vitro," *Clinical Cancer Research*, vol. 4, no. 7, pp. 1803–1811, 1998.

[107] A. J. Abusamra, Z. Zhong, X. Zheng et al., "Tumor exosomes expressing Fas ligand mediate $CD8^+$ T-cell apoptosis," *Blood Cells, Molecules & Diseases*, vol. 35, no. 2, pp. 169–173, 2005.

[108] M. L. Hyer, C. Voelkel-Johnson, S. Rubinchik, J. Y. Dong, and J. S. Norris, "Intracellular Fas ligand expression causes Fas-mediated apoptosis in human prostate cancer cells resistant to monoclonal antibody-induced apoptosis," *Molecular Therapy*, vol. 2, no. 4, pp. 348–358, 2000.

Hindawi
Journal of Immunology Research
Volume 2017, Article ID 1278081, 9 pages
https://doi.org/10.1155/2017/1278081

Research Article

The Identification of Key Genes and Pathways in Glioma by Bioinformatics Analysis

Mingfa Liu,[1] Zhennan Xu,[1] Zepeng Du,[2] Bingli Wu,[3] Tao Jin,[1] Ke Xu,[1] Liyan Xu,[4] Enmin Li,[3] and Haixiong Xu[1]

[1]*Department of Neurosurgery, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, China*
[2]*Department of Pathology, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-sen University, Shantou 515041, China*
[3]*Department of Biochemistry and Molecular Biology, Shantou University Medical College, Shantou 515041, China*
[4]*Institute of Oncologic Pathology, Shantou University Medical College, Shantou 515041, China*

Correspondence should be addressed to Haixiong Xu; xuhaix@126.com

Received 30 July 2017; Accepted 16 October 2017; Published 6 December 2017

Academic Editor: Jiaming Liu

Glioma is the most common malignant tumor in the central nervous system. This study aims to explore the potential mechanism and identify gene signatures of glioma. The glioma gene expression profile GSE4290 was analyzed for differentially expressed genes (DEGs). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied for the enriched pathways. A protein-protein interaction (PPI) network was constructed to find the hub genes. Survival analysis was conducted to screen and validate critical genes. In this study, 775 downregulated DEGs were identified. GO analysis demonstrated that the DEGs were enriched in cellular protein modification, regulation of cell communication, and regulation of signaling. KEGG analysis indicated that the DEGs were enriched in the MAPK signaling pathway, endocytosis, oxytocin signaling, and calcium signaling. PPI network and module analysis found 12 hub genes, which were enriched in synaptic vesicle cycling rheumatoid arthritis and collecting duct acid secretion. The four key genes CDK17, GNA13, PHF21A, and MTHFD2 were identified in both generation (GSE4412) and validation (GSE4271) dataset, respectively. Regression analysis showed that CDK13, PHF21A, and MTHFD2 were independent predictors. The results suggested that CDK17, GNA13, PHF21A, and MTHFD2 might play important roles and potentially be valuable in the prognosis and treatment of glioma.

1. Introduction

Among the various histological subtypes of brain tumor, glioma is the most common malignant tumor in the central nervous system [1]. Established by the World Health Organization (WHO), it can be classified from grade I to grade IV based on histopathological and clinical criteria [2]. During invasive growth, most gliomas extend processes, resulting in a lack of clear borders between tumor and normal brain tissue, making surgical resection of the entire carcinoma difficult. Currently, imageological examination is the most important diagnostic method, as well as the evaluation of the postoperative curative effect. However, imaging is influenced by many factors, such as radiation injury and surgery that result in poor specificity. It is difficult to achieve early diagnosis and treatment of glioma due to a lack of specificity of auxiliary examination indices, so that many patients can lose the chance for radical excision, thereby increasing the risk for poor prognosis. The 5-year overall survival (OS) of patients with glioblastoma is less than 10% [3]. Therefore, the identification of sensitive and specific biological markers that would help identify patients at a higher or lower risk of death from glioma is of vital importance, not only for a better understanding of the molecular and cellular processes involved in tumorigenesis but also for more effective diagnosis, suitable treatment, and improved prognosis.

Gene expression profiling analysis is a useful method with broad clinical application for identifying tumor-related

genes in various types of cancer, from molecular diagnosis to pathological classification, from therapeutic evaluation to prognosis prediction, and from drug sensitivity to neoplasm recurrence [4–6]. However, the use of microarrays in clinical practice is limited by the overwhelming number of genes identified by gene profiling, lack of both repeatability and independent validation, and need for complicated statistical analyses [7]. Therefore, in order to put these expression profiles in clinical practice, it is necessary to identify a suitable number of genes and develop a method that can be operated by routine assay. In this study, we downloaded original data from the glioma microarray in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), an online public collection database for registration, which is not only for saving microarray data but also for helping the user query and download. We compare gene expression profiles of tumor cells with normal brain cells in order to identify differentially expressed genes (DEGs). Subsequently, the identified DEGs were screened by using Morpheus online software, followed by gene ontology (GO) and pathway enrichment analysis. After analyzing their biological functions and pathways, we further explored the potential biomarkers for diagnosis and prognosis by survival analysis in two independent datasets in order to gain insight on glioma development and progression at the molecular level.

2. Materials and Methods

2.1. Microarray Data. We downloaded the gene expression profiles in GSE4290, GSE4412, and GSE4271 from the GEO database. GSE4290 has a total of 180 samples, including 157 cases of glioma (26 astrocytomas, 50 oligodendrogliomas, and 81 glioblastomas) and 23 cases of normal brain tissue, based on the GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array) by Fine HA et al. Using the GPL96 platform (Affymetrix Human Genome U133A Array), the GES4412 dataset containing 85 cases of glioma was submitted by Nelson SF; and the GES4271 dataset, containing 100 samples that included 77 cases of primary tumor samples and 23 cases of recurrence, was submitted by Phillips HS et al.

2.2. Screen Genes of Differential Expression. The analysis was carried out by using GEO2R, an online analysis tool, for the GEO database, based on R language. We applied analysis to classify the sample into two groups that had similar expression patterns in glioma and normal brain tissue. We defined DEGs as differentially expressed with logFC > 2 or logFC < −2, a criteria as described in the references [8, 9]. An adj. P value < 0.05 was considered statistically significant. In addition, we used visual hierarchical cluster analysis to show the two groups by Morpheus online analysis software (https://software.broadinstitute.org/morpheus/) after the relative raw data of TXT files was downloaded.

2.3. Gene Ontology and KEGG Pathway Analysis of DEGs. With functions including molecular function, biological pathways, and cellular component, gene ontology (GO) analysis annotates genes and gene products [10]. KEGG comprises a set of genome and enzymatic approaches and a biological chemical energy online database [11]. It is a resource for systematic analysis of gene function and related high-level genome functional information. DAVID (https://david.ncifcrf.gov/) can provide systematic and comprehensive biological function annotation information for high-throughput gene expression [12]. Therefore, we applied GO and KEGG pathway analyses to the DEGs by using DAVID online tools at the functional level. A $P < 0.05$ was considered to have significant differences.

2.4. Integration of Protein-Protein Interaction (PPI) Network and Module Analysis. The STRING database is an online tool for assessment and integration of protein-protein interactions, including direct (physical), as well as indirect (functional) associations. STRING version 10.0 covers 9,643,763 proteins from 2031 organisms [13]. We drew DEGs using STRING in order to assess the interactional relationships among the DEGs, then used the Cytoscape software to build a PPI network, employed the plug-in Molecular Complex Detection (MCODE) to screen PPI network modules, and established scores > 3 and nodes > 4 in the MCODE module, function, and pathway enrichment analysis. A $P < 0.05$ was considered statistically significant.

2.5. Identification of Biomarkers. Based on the information in the individual MCODE modules, the node with the highest score was selected as the hub gene in GSE4290. Every hub gene was also found in two independent datasets (generation dataset GSE4412, primary tumor samples $n = 85$, and validation dataset GSE4271, primary tumor samples $n = 77$) based on the downloaded raw data files, including the information of gene expression value, overall survival time (OS), and survival state. Statistical analyses were performed using SPSS version 20.0 for Windows (IBM, Chicago, IL). We divided expression values into two groups, high expression and low expression, according to X-tile [14]. The Kaplan-Meier method was used to determine the probability of survival and analyzed by the log-rank test. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of DEGs. A comparison of 157 cases of glioma samples with 23 cases of normal brain tissue in GSE4290 by using the GEO2R online analysis tool resulted in the identification of the DEGs listed in Figure 1. Based on GEO2R analysis, using an adjusted $P < 0.05$ and log (fold change) (logFC) ≥ 2.0 criteria, there were 775 downregulated genes identified. We further validated the results by using the Morpheus online tool, resulting in a DEG expression heat map, of the top 50 downregulated genes, shown in Figure 1.

3.2. Gene Ontology Enrichment Analysis. We used the DAVID online analysis tool to identify statistically significantly enriched GO terms and KEGG pathways after uploading all downregulated genes. GO analysis results showed that downregulated DEGs were significantly enriched in molecular function (MF), including small molecule binding, nucleoside phosphate binding, and carbohydrate derivative binding

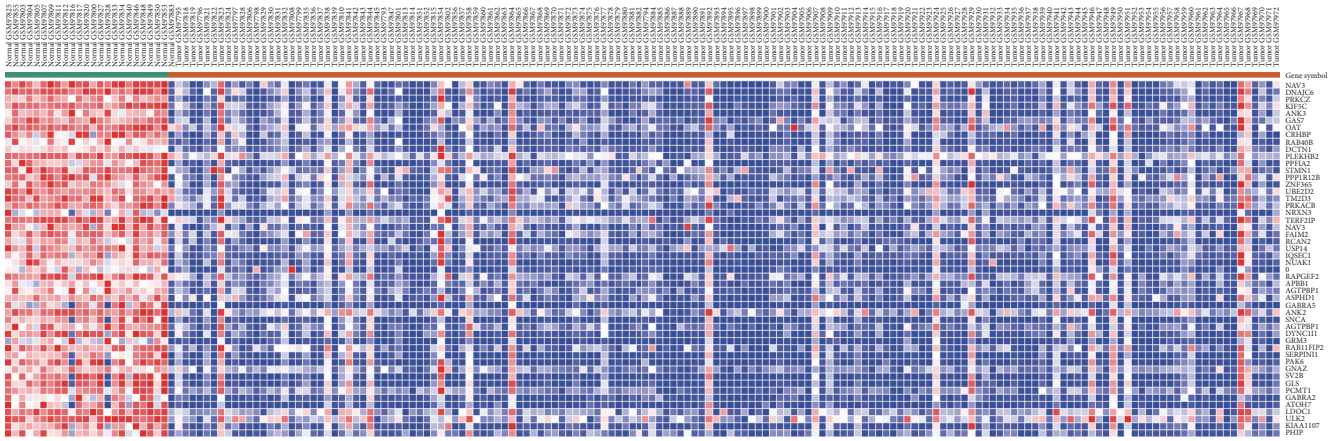


Figure 1: Heat map of the top 50 downregulated genes (red: upregulated; purple: downregulated).

Table 1: Gene ontology analysis of downregulated genes associated with glioma.

Category	Term/gene function	Count	%	P value
GOTERM_MF_FAT	GO:0036094~small molecule binding	119	0.125	$2.76E-06$
GOTERM_MF_FAT	GO:0000166~nucleotide binding	116	0.121	$3.45E-07$
GOTERM_MF_FAT	GO:1901265~nucleoside phosphate binding	116	0.121	$3.52E-07$
GOTERM_MF_FAT	GO:0097367~carbohydrate derivative binding	100	0.105	$1.00E-04$
GOTERM_MF_FAT	GO:0019899~enzyme binding	94	0.098	$2.24E-07$
GOTERM_MF_FAT	GO:0017076~purine nucleotide binding	92	0.096	$9.44E-06$
GOTERM_MF_FAT	GO:0032555~purine ribonucleotide binding	91	0.095	$1.26E-05$
GOTERM_MF_FAT	GO:0032553~ribonucleotide binding	91	0.095	$1.76E-05$
GOTERM_BP_FAT	GO:0036211~protein modification process	148	0.155	$8.31E-04$
GOTERM_BP_FAT	GO:0006464~cellular protein modification process	148	0.155	$8.31E-04$
GOTERM_BP_FAT	GO:0023051~regulation of signaling	140	0.147	$3.36E-07$
GOTERM_BP_FAT	GO:0010646~regulation of cell communication	139	0.146	$2.25E-07$
GOTERM_BP_FAT	GO:0033036~macromolecule localization	138	0.145	$5.25E-09$
GOTERM_BP_FAT	GO:0006793~phosphorus metabolic process	134	0.140	$1.05E-05$
GOTERM_BP_FAT	GO:0006796~phosphate-containing compound metabolic process	133	0.139	$1.53E-05$
GOTERM_BP_FAT	GO:0008104~protein localization	124	0.130	$6.40E-09$
GOTERM_CC_FAT	GO:0005829~cytosol	163	0.171	$4.20E-08$
GOTERM_CC_FAT	GO:0031988~membrane-bounded vesicle	152	0.159	$2.84E-04$
GOTERM_CC_FAT	GO:0005654~nucleoplasm	130	0.136	$2.88E-04$
GOTERM_CC_FAT	GO:0097458~neuron part	91	0.095	$1.38E-11$
GOTERM_CC_FAT	GO:0030054~cell junction	75	0.078	$1.15E-05$
GOTERM_CC_FAT	GO:0043005~neuron projection	66	0.069	$1.72E-08$
GOTERM_CC_FAT	GO:0005794~Golgi apparatus	66	0.069	0.008029
GOTERM_CC_FAT	GO:0016023~cytoplasmic, membrane-bounded vesicle	64	0.067	$7.15E-05$

(Table 1). For biological processes (BP), the downregulated genes were enriched in cellular protein modification, regulation of cell communication, and regulation of signaling (Table 1). In addition, GO cell component (CC) analysis also displayed that the downregulated DEGs were significantly enriched in the cytosol, membrane-bounded vesicles, and nucleoplasm (Table 1).

3.3. KEGG Pathway Analysis. The significant enriched pathways of the downregulated DEGs, analyzed by KEGG analysis, are shown in Table 2. The downregulated genes were enriched in the MAPK signaling pathway, endocytosis, oxytocin signaling pathway, calcium signaling pathway, proteoglycans in cancer, purine metabolism, cAMP signaling pathway, and regulation of the actin cytoskeleton.

Table 2: KEGG pathway analysis of downregulation genes associated with glioma.

Term	Pathway	Gene count	%	P value	Genes
hsa04010	MAPK signaling pathway	22	0.023	$6.35E-05$	MEF2C, BRAF, MAP2K1, NLK, MAP2K4, TP53, PPP3R1, PTPRR, CACNG3, PRKCG, PRKCB, CDC42, RASGRF2, MAPK9, MAPK8IP3, STMN1, PAK1, PRKACB, RAPGEF2, CACNA1C, DUSP7, CACNA1B
hsa04144	Endocytosis	20	0.021	$5.98E-04$	SH3GL3, PARD3, CLTB, PSD3, PIP5K1C, HLA-E, EPS15, RAB11FIP4, MVB12A, CDC42, AP2A2, RAB31, SH3GLB2, NEDD4, ARPC5L, ARF3, KIAA1033, NEDD4L, IQSEC1, F2R
hsa04921	Oxytocin signaling pathway	15	0.015	$5.25E-04$	MEF2C, ADCY2, CAMK1G, MAP2K1, PPP1R12B, PPP3R1, CACNG3, PRKCG, CAMKK1, PRKCB, CAMKK2, CAMK2B, GUCY1B3, PRKACB, CACNA1C
hsa04020	Calcium signaling pathway	14	0.014	0.0048	SLC8A1, ADCY2, PTGER3, CCKBR, PPP3R1, PRKCG, PRKCB, ATP2B1,PDE1A, CAMK2B, PRKACB, CACNA1C, CACNA1B, F2R
hsa05205	Proteoglycans in cancer	14	0.014	0.012	CDC42, WNT10B, HIF1A, MAP2K1, BRAF, ANK3, PPP1R12B, TP53, PRKCG, CAMK2B, PAK1, PRKACB, TIMP3, PRKCB
hsa00230	Purine metabolism	13	0.013	0.0109	NME4, ADSS, GDA, ADCY2, POLR1E, RRM2, PDE1A, PRIM2, AK5, GUCY1B3, ENTPD4, HPRT1, GART
hsa04024	cAMP signaling pathway	13	0.013	0.0253	PPARA, ADCY2, PTGER3, BRAF, MAP2K1, ATP2B1, NPY, MAPK9, CAMK2B, PRKACB, PAK1, CACNA1C, F2R
hsa04810	Regulation of actin cytoskeleton	13	0.013	0.0387	GNA13, PAK6, CDC42, ENAH,MAP2K1, BRAF, ARHGEF6, PPP1R12B, ARPC5L, WASF2, PIP5K1C, PAK1, F2R

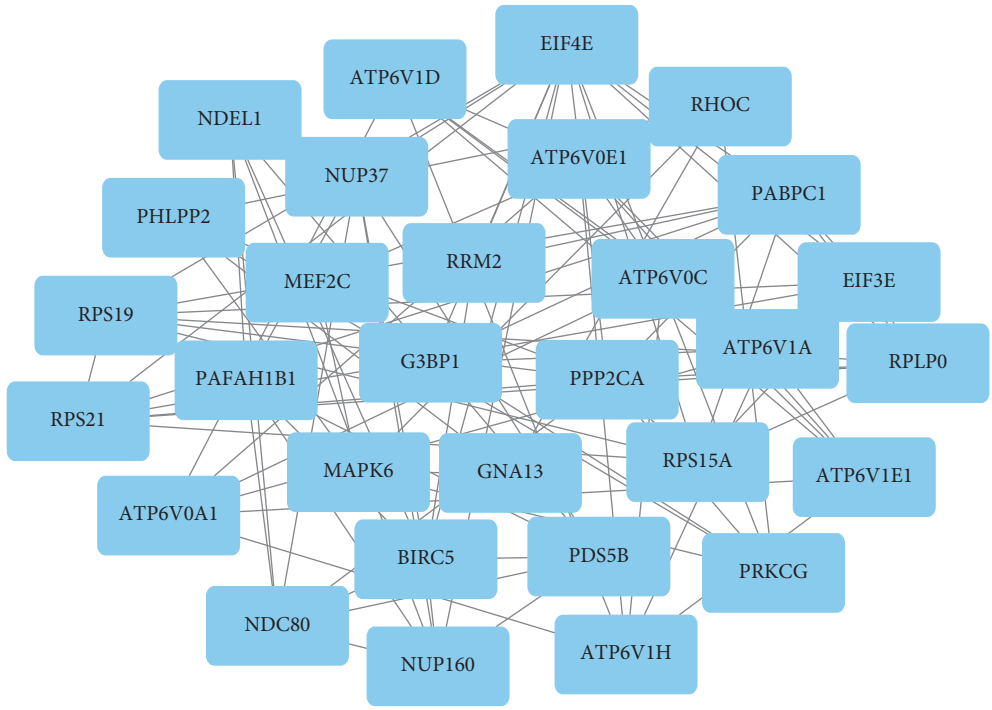


Figure 2: Highest module selected from the PPI network.

3.4. Module Analysis and Hub Gene Selection in the PPI Network. Based on the information in the STRING database, the highest module was shown by using the MCODE plug-in, and the functional annotation of the genes involved in the module was analyzed (Figure 2). Enrichment pathway analysis showed that the genes in the module were related to synaptic vesicle cycling, rheumatoid arthritis, and collecting duct acid secretion. Moreover, the 12 hub nodes of the highest score were screened in all modules. The hub genes included PDS5 cohesin associated factor B (PDS5B), chromodomain helicase DNA binding protein 5 (CHD5), cyclin-dependent kinase 17 (CDK17), eukaryotic translation initiation factor 3 subunit E (EIF3E), ATPase H+ transporting V1 subunit H (ATP6V1H), G protein subunit alpha 13 (GNA13), PHD

Table 3: The enriched pathways for genes in the highest module.

Pathway	P value	FDR	Nodes
Synaptic vesicle cycle	$3.36E-08$	$3.58E-05$	ATP6V0C, ATP6V1A, ATP6V0E1, ATP6V1E1, ATP6V1H, ATP6V0A1, ATP6V1D
Rheumatoid arthritis	$3.43E-07$	$3.66E-04$	ATP6V0C, ATP6V1A, ATP6V0E1, ATP6V1E1, ATP6V1H, ATP6V0A1, ATP6V1D
Collecting duct acid secretion	$2.29E-08$	$2.44E-05$	ATP6V0C, ATP6V1A, ATP6V0E1, ATP6V1E1, ATP6V0A1, ATP6V1D

finger protein 21A (PHF21A), methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2 (MTHFD2), lipoprotein lipase (LPL), adenylosuccinate synthase (ADSS), Wnt family member 10B (WNT10B), and serine and arginine-rich splicing factor 1 (SRSF1). The enriched pathways for genes in the highest module were shown in Table 3.

3.5. Identification of Biomarkers. In order to identify biomarkers, we calculated the survival rate for two groups of 12 hub genes in the generation dataset (GSE4412) and compared the result with the validation dataset (GSE4271) through Kaplan-Meier analysis and log-rank test. According to the analysis, we found that only the downregulation of CDK17, GNA13, PHF21A, and MTHFD2 was closely associated with a decreased overall survival among patients with glioma (Figures 3 and 4). The remaining 8 biomarkers had no statistical significance between gene expression and clinical outcome of glioma or no recoverability in the validation dataset. Furthermore, using the Cox proportional hazards model, a multivariate analysis was performed identifying that expression levels of CDK17, PHF21A, and MTHFD2 were independent prognostic factors (Table 4).

4. Discussion

In the present study, we identified DEGs between glioma and normal samples and used a series of bioinformatics analyses to screen key gene and pathways associated with cancer. However, GSE4290 dataset contains only limited number of control samples, 23 out of 180 samples. In order to improve the statistical power of DEG, we defined that the absolute value of the logarithm (base 2) fold change (logFC) greater than 2 and 775 DEGs was obtained. Bioinformatics analysis on DEGs, including GO enrichment, KEGG pathway, PPI network, and survival analyses, found glioma-related genes and pathways that play an important role in cancer initiation and progression.

GO term enrichment analysis demonstrated that 775 downregulated DEGs were significantly enriched in functions involving cellular protein modification, regulation of cell communication, and regulation of signaling. Many studies found that the cellular protein modification, including phosphorylation, ubiquitination, and acetylation, can change the cell biology function, influence disease phenotype, affect glioma cell proliferation, invasion, and apoptosis, and regulate the development of glioma [15–17]. And glioma cells can regulate cell communication, through the information passed to the target cells, interact with the receptor, resulting in specific biological effect such as cell proliferation and cytoskeleton changes, and promote glioma progression and angiogenesis [18]. KEGG pathway analysis indicated that the functions of the downregulated genes were enriched in MAPK signaling, endocytosis, oxytocin signaling, and calcium signaling. Zhang et al. [19] demonstrated that the MAPK signaling pathway induces cell apoptosis in glioma cells and the calcium signaling pathway is involved in quiescence, maintenance, proliferation, and migration in glioma cells [20, 21]. PPI network and module analysis found that the first gene module significantly was enriched in synaptic vesicle cycling. Some results indicate that interference synaptic vesicle cycling could disrupt synaptic function and homeostasis, which would lead to cognitive decline and neurodegeneration in Alzheimer's disease [22]. Therefore, monitoring these signaling pathways may help in the prediction of tumor occurrence and progression.

Since no survival data about GSE4290 could be available, two independent glioma datasets GSE4412 and GSE4271 were applied to detect whether the hub gene could affect the survival time of glioma patients. Survival analysis found that CDK17, GNA13, PHF21A, and MTHFD2 are closely associated with glioma. CDK17 is a member of the cyclin-dependent kinase family. Chaput et al. [23] found that the expression levels of CDK17 are significantly increased in Alzheimer's disease and are associated with the mechanism to promote AD neurodegeneration and may inhibit the pathology development in AD, and Demirkan et al. [24], through a genome-wide association study, found that the CDK17 can be mapped to the glycerophospholipid metabolism pathway. GNA13, one member of the G protein family, is involved in metastasis of tumor cells [25], angiogenesis, and cellular responses to chemokines [26]. In neuronal cells, GNA13 affects neurite outgrowth together with Rho, which is closely related with cell motility and differentiation [27]. Furthermore, GNA13 is coupled to brain-specific angiogenesis inhibitor-1 (BAI1), which is an adhesion-related GPCR, and regulates synaptic function via Rho signaling [28]. PHF21A (also known as BHC80), a plant homeodomain finger-containing protein, can affect the neurofacial and craniofacial development and suppression of the latter and lead to both craniofacial abnormalities and neuronal apoptosis [29]. Moreover, PHF21A specifically binds H3K4me, which is a transcribed genomic locus of regulated posttranslational modification, and implicated the development and maintenance of neural connections [30]. MTHFD2 (methylenetetrahydrofolate dehydrogenase 2) is a mitochondrial enzyme with methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activities and has an effect on cancer cell proliferation [31], migration, and invasion [32]. In the expression level of human tumors, MTHFD2 is overexpressed in most cancer types, but exceptions are found in glioma [33], similar to our results. Up to now, the biological functions of CDK17, GNA13, PHF21A, and MTHFD2 in

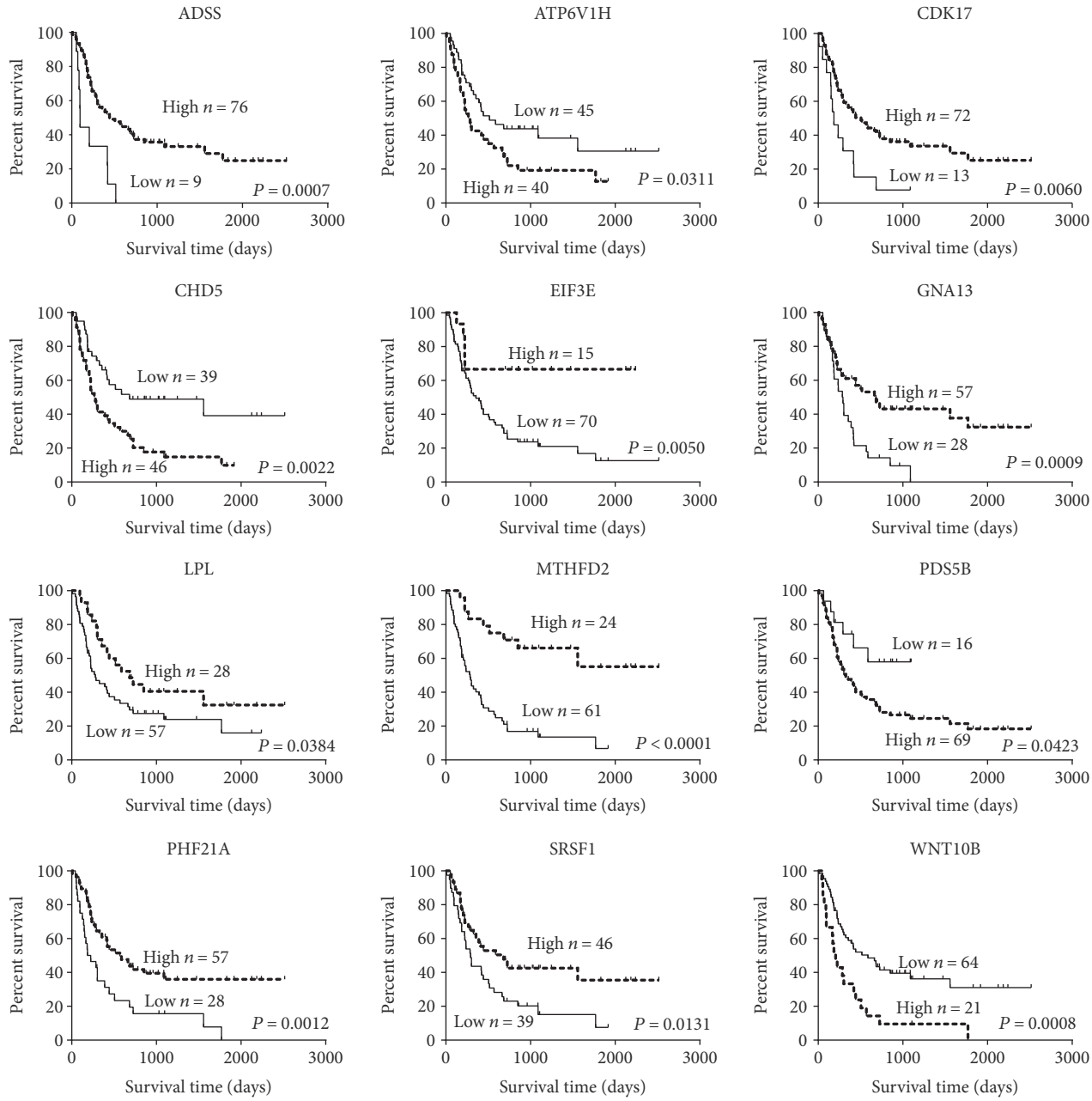


Figure 3: Kaplan-Meier analysis of overall survival for 12 hub genes in the generation dataset of 85 cases.

glioma have remained unclear. However, our study shows that the expression level of four key genes are all downregulated in glioma, after comparison with normal brain tissue, and the downregulation is associated with poorer prognosis, as the patients with extended survival time have increased expression of these genes.

At the moment, there are some related bioinformatic research reports of GSE4290 in glioma. Some studies have shown that different enrichment pathway analyses of DEGs can be classified according to their degrees of differential expression during tumor progression in order to explore the deterioration of low into high grade glioma [34]. Some research finds that the DEGs are regulated by transcription factors in glioblastoma [35] and microarray technology has been used to identify the DEGs and their functions in the development of three types of glioma (astrocytoma, glioblastoma, and oligodendroglioma) [36]. Different from these, our study selects the node of the highest score from each module as hub genes in MCODE after comparing nontumor samples with glioma samples. These hub nodes are the key genes of interaction, in the PPI network, that may play important roles in the occurrence and development of glioma. Moreover, hub gene identification is more persuasive, since we validate the association of hub genes and glioma by using survival analysis in two independent datasets to identify four genes that may be cancer biomarkers for glioma. Though not all hub genes associated with the survival of glioma patients, but some hub genes play important roles in immune or

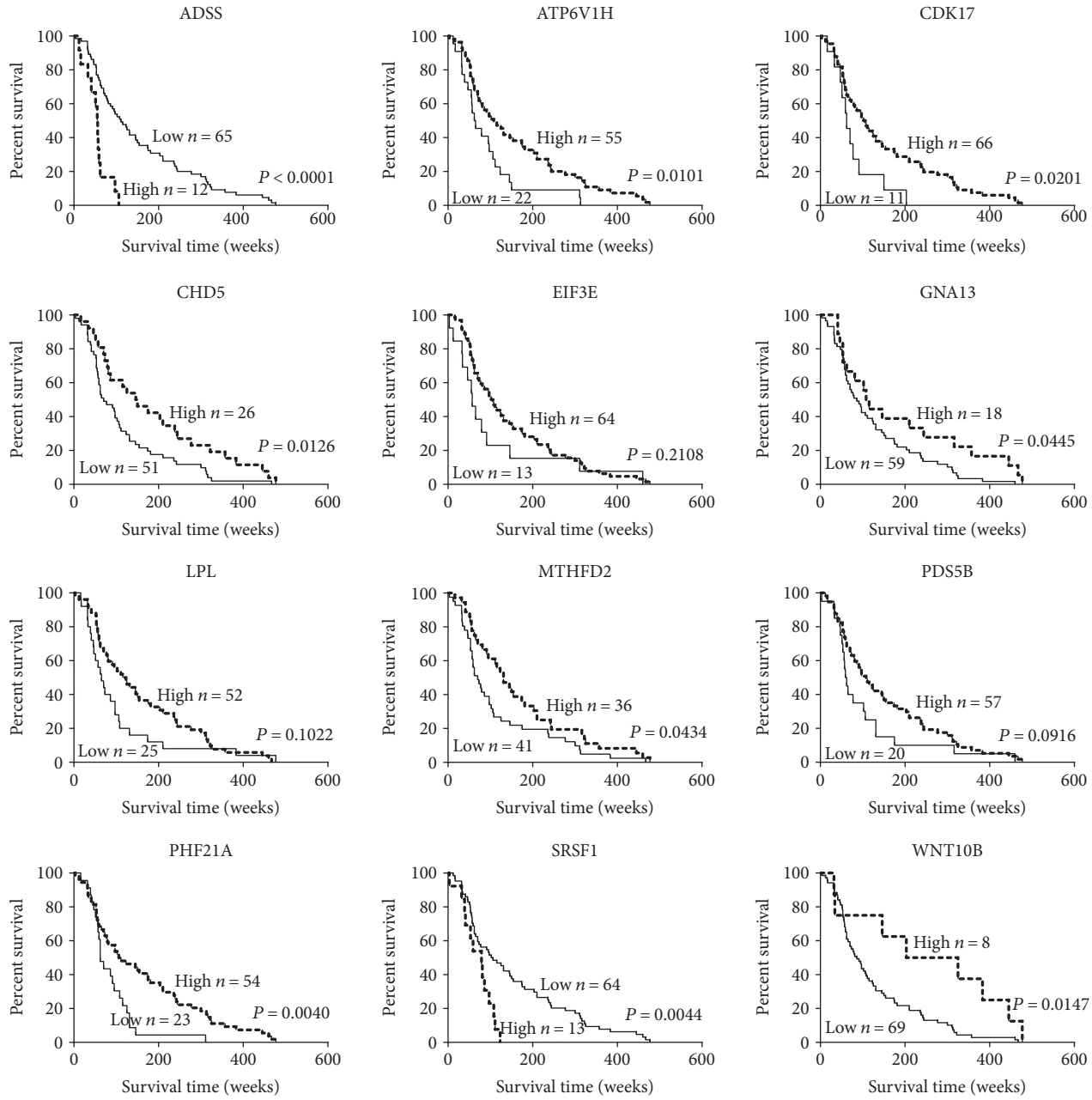


Figure 4: Kaplan-Meier analysis of overall survival for 12 hub genes in the validation dataset of 77 cases.

Table 4: Cox multivariate analyses of biomarkers associated with OS in the generation and validation datasets.

Dataset	Parameter	Regression coefficient	*P* value	Risk ratio	95% confidence interval
Generation	CDK17	−0.882	0.011	0.414	0.210 ~ 0.815
	MTHFD2	−1.264	0.001	0.283	0.133 ~ 0.598
	PHF21A	−0.671	0.018	0.511	0.293 ~ 0.891
Validation	CDK17	−0.847	0.016	0.429	0.215 ~ 0.856
	MTHFD2	−0.482	0.046	0.617	0.384 ~ 0.992
	PHF21A	−0.620	0.024	0.538	0.314 ~ 0.921

inflammation. For example, WNT10B plays an important role in regulating asthmatic airway inflammation through modification of the T cell response [37].

In conclusion, we presumed these key genes identified by a series of bioinformatics analyses on DEGs between tumor samples and normal samples, probably related to the development of glioma. These hub genes could also affect the survival time of glioma patients as validated from two independent datasets. These identified genes and pathways provide a more detailed molecular mechanism for underlying glioma initiation and development. According to the study, downregulation of CDK17, GNA13, PHF21A, and MTHFD2 can be considered as biomarkers or therapeutic targets for glioma. However, further molecular and biological

experiments are required to confirm the functions of the key genes in glioma.

Conflicts of Interest

All authors declare that they have no conflict of interests to state.

Authors' Contributions

Mingfa Liu and Zhennan Xu contributed equally to this work.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (nos. 81672473 and 81502138), the Science and Technology Program of Guangdong (nos. 2014A030310390 and 2017A030313181), the Department of Education of Guangdong Government under the Top-tier University Development Scheme for Research and Control of Infectious Diseases (2016034).

References

[1] Q. T. Ostrom, H. Gittleman, P. Liao et al., "CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2007–2011," *Neuro-Oncology*, vol. 16, pp. v1–v63, 2014.

[2] D. N. Louis, A. Perry, G. Reifenberger et al., "The 2016 World Health Organization classification of tumors of the central nervous system: a summary," *Acta Neuropathologica*, vol. 131, no. 6, pp. 803–820, 2016.

[3] R. Stupp, M. E. Hegi, W. P. Mason et al., "Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial," *The Lancet Oncology*, vol. 10, no. 5, pp. 459–466, 2009.

[4] W. A. Freije, F. E. Castro-Vargas, Z. Fang et al., "Gene expression profiling of gliomas strongly predicts survival," *Cancer Research*, vol. 64, no. 18, pp. 6503–6510, 2004.

[5] K. De Preter, J. Vermeulen, B. Brors et al., "Accurate outcome prediction in neuroblastoma across independent data sets using a multigene signature," *Clinical Cancer Research*, vol. 16, no. 5, pp. 1532–1541, 2010.

[6] Y. W. Kim, T. J. Liu, D. Koul et al., "Identification of novel synergistic targets for rational drug combinations with PI3 kinase inhibitors using siRNA synthetic lethality screening against GBM," *Neuro-Oncology*, vol. 13, no. 4, pp. 367–375, 2011.

[7] W. Cheng, X. Ren, C. Zhang et al., "Bioinformatic profiling identifies an immune-related risk signature for glioblastoma," *Neurology*, vol. 86, no. 24, pp. 2226–2234, 2016.

[8] A. Ward, A. Balwierz, J. D. Zhang et al., "Re-expression of microRNA-375 reverses both tamoxifen resistance and accompanying EMT-like properties in breast cancer," *Oncogene*, vol. 32, no. 9, pp. 1173–1182, 2013.

[9] M. Saura, S. Marquez, P. Reventun et al., "Oral administration of bisphenol A induces high blood pressure through angiotensin II/CaMKII-dependent uncoupling of eNOS," *The FASEB Journal*, vol. 28, no. 11, pp. 4719–4728, 2014.

[10] Gene Ontology Consortium, "The gene ontology (GO) project in 2006," *Nucleic Acids Research*, vol. 34, no. 90001, supplement 1, pp. D322–D326, 2006.

[11] M. Kanehisa and S. Goto, "KEGG: Kyoto Encyclopedia of Genes and Genomes," *Nucleic Acids Research*, vol. 28, no. 1, pp. 27–30, 2000.

[12] G. Dennis, B. T. Sherman, D. A. Hosack et al., "DAVID: database for annotation, visualization, and integrated discovery," *Genome Biology*, vol. 4, no. 5, article P3, 2003.

[13] D. Szklarczyk, A. Franceschini, S. Wyder et al., "STRING v10: protein-protein interaction networks, integrated over the tree of life," *Nucleic Acids Research*, vol. 43, no. D1, pp. D447–D452, 2015.

[14] R. L. Camp, M. Dolled-Filhart, and D. L. Rimm, "X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization," *Clinical Cancer Research*, vol. 10, no. 21, pp. 7252–7259, 2004.

[15] Y. D. Yoo, D. H. Lee, H. Cha-Molstad et al., "Glioma-derived cancer stem cells are hypersensitive to proteasomal inhibition," *EMBO Reports*, vol. 18, no. 9, p. 1671, 2017.

[16] X. Li, W. Yu, X. Qian et al., "Nucleus-translocated ACSS2 promotes gene transcription for lysosomal biogenesis and autophagy," *Molecular Cell*, vol. 66, no. 5, pp. 684–697.e9, 2017.

[17] X. Fang, W. Zhou, Q. Wu et al., "Deubiquitinase USP13 maintains glioblastoma stem cells by antagonizing FBXL14-mediated Myc ubiquitination," *The Journal of Experimental Medicine*, vol. 214, no. 1, pp. 245–267, 2017.

[18] B. Kigel, N. Rabinowicz, A. Varshavsky, O. Kessler, and G. Neufeld, "Plexin-A4 promotes tumor progression and tumor angiogenesis by enhancement of VEGF and bFGF signaling," *Blood*, vol. 118, no. 15, pp. 4285–4296, 2011.

[19] B. Zhang, T. Wu, Z. Wang et al., "p38MAPK activation mediates tumor necrosis factor-α-induced apoptosis in glioma cells," *Molecular Medicine Reports*, vol. 11, no. 4, pp. 3101–3107, 2015.

[20] C. Leclerc, J. Haeich, F. J. Aulestia et al., "Calcium signaling orchestrates glioblastoma development: facts and conjunctures," *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1863, no. 6, pp. 1447–1459, 2016.

[21] D. Wypych and P. Pomorski, "Calcium signaling in glioma cells – the role of nucleotide receptors," *Advances in Experimental Medicine and Biology*, vol. 986, pp. 61–79, 2013.

[22] V. Lazarevic, S. Fieńko, M. Andres-Alonso et al., "Physiological concentrations of amyloid beta regulate recycling of synaptic vesicles via alpha7 acetylcholine receptor and CDK5/calcineurin signaling," *Frontiers in Molecular Neuroscience*, vol. 10, p. 221, 2017.

[23] D. Chaput, L. Kirouac, S. M. Stevens Jr., and J. Padmanabhan, "Potential role of PCTAIRE-2, PCTAIRE-3 and P-histone H4 in amyloid precursor protein-dependent Alzheimer pathology," *Oncotarget*, vol. 7, no. 8, pp. 8481–8497, 2016.

[24] A. Demirkan, C. M. van Duijn, P. Ugocsai et al., "Genome-wide association study identifies novel loci associated with circulating phospho- and sphingolipid concentrations," *PLoS Genetics*, vol. 8, article e1002490, 2012.

[25] S. A. K. Rasheed, C. R. Teo, E. J. Beillard et al., "MicroRNA-31 controls G protein alpha-13 (GNA13) expression and cell invasion in breast cancer cells," *Molecular Cancer*, vol. 14, no. 1, p. 67, 2015.

[26] K. K. Sivaraj, M. Takefuji, I. Schmidt, R. H. Adams, S. Offermanns, and N. Wettschureck, "G_{13} controls

angiogenesis through regulation of VEGFR-2 expression," *Developmental Cell*, vol. 25, no. 4, pp. 427–434, 2013.

[27] A. Nurnberg, A. U. Brauer, N. Wettschureck, and S. Offermanns, "Antagonistic regulation of neurite morphology through G_q/G_{11} and G_{12}/G_{13}," *The Journal of Biological Chemistry*, vol. 283, no. 51, pp. 35526–35531, 2008.

[28] J. R. Stephenson, K. J. Paavola, S. A. Schaefer, B. Kaur, E. G. van Meir, and R. A. Hall, "Brain-specific angiogenesis inhibitor-1 signaling, regulation, and enrichment in the postsynaptic density," *The Journal of Biological Chemistry*, vol. 288, no. 31, pp. 22248–22256, 2013.

[29] H. G. Kim, H. T. Kim, N. T. Leach et al., "Translocations disrupting *PHF21A* in the Potocki-Shaffer-syndrome region are associated with intellectual disability and craniofacial anomalies," *American Journal of Human Genetics*, vol. 91, no. 1, pp. 56–72, 2012.

[30] C. N. Vallianatos and S. Iwase, "Disrupted intricacy of histone H3K4 methylation in neurodevelopmental disorders," *Epigenomics*, vol. 7, no. 3, pp. 503–519, 2015.

[31] P. M. Tedeschi, A. Vazquez, J. E. Kerrigan, and J. R. Bertino, "Mitochondrial methylenetetrahydrofolate dehydrogenase (MTHFD2) overexpression is associated with tumor cell proliferation and is a novel target for drug development," *Molecular Cancer Research*, vol. 13, no. 10, pp. 1361–1366, 2015.

[32] L. Lehtinen, K. Ketola, R. Mäkelä et al., "High-throughput RNAi screening for novel modulators of vimentin expression identifies MTHFD2 as a regulator of breast cancer cell migration and invasion," *Oncotarget*, vol. 4, no. 1, pp. 48–63, 2013.

[33] R. Nilsson, M. Jain, N. Madhusudhan et al., "Metabolic enzyme expression highlights a key role for MTHFD2 and the mitochondrial folate pathway in cancer," *Nature Communications*, vol. 5, p. 3128, 2014.

[34] G. Hu, B. Wei, L. Wang et al., "Analysis of gene expression profiles associated with glioma progression," *Molecular Medicine Reports*, vol. 12, no. 2, pp. 1884–1890, 2015.

[35] B. Wei, L. Wang, C. Du et al., "Identification of differentially expressed genes regulated by transcription factors in glioblastomas by bioinformatics analysis," *Molecular Medicine Reports*, vol. 11, no. 4, pp. 2548–2554, 2015.

[36] G. Li, W. Pan, X. Yang, and J. Miao, "Gene co-expression network and function modules in three types of glioma," *Molecular Medicine Reports*, vol. 11, no. 4, pp. 3055–3063, 2015.

[37] J. Trischler, T. Shiomi, D. L. Turner et al., "Immune modulation of the T cell response in asthma through Wnt10b," *American Journal of Respiratory Cell and Molecular Biology*, vol. 54, no. 4, pp. 584–593, 2016.

Hindawi
Journal of Immunology Research
Volume 2017, Article ID 4302320, 12 pages
https://doi.org/10.1155/2017/4302320

Research Article

Decitabine-Induced Changes in Human Myelodysplastic Syndrome Cell Line SKM-1 Are Mediated by FOXO3A Activation

Wen Zeng,[1] Hanjun Dai,[1] Ming Yan,[1] Xiaojun Cai,[1] Hong Luo,[1] Min Ke,[1] and Zeming Liu[2]

[1]*Department of Ophthalmology, Zhongnan Hospital of Wuhan University, Wuhan, China*
[2]*Department of Breast and Thyroid Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*

Correspondence should be addressed to Min Ke; keminyk@163.com and Zeming Liu; 6myt@163.com

Received 9 April 2017; Revised 12 July 2017; Accepted 20 July 2017; Published 16 October 2017

Academic Editor: Mei Han

The epigenetic silencing of tumor suppressor genes in myelodysplastic syndromes (MDS) can potentially confer a growth advantage to individual cellular clones. Currently, the recommended treatment for patients with high-risk MDS is the methylation agent decitabine (DAC), a drug that can induce the reexpression of silenced tumor suppressor genes. We investigated the effects of DAC treatment on the myeloid MDS cell line SKM-1 and investigated the role of FOXO3A, a potentially tumor-suppressive transcription factor, by silencing its expression prior to DAC treatment. We found that FOXO3A exists in an inactive, hyperphosphorylated form in SKM-1 cells, but that DAC both induces FOXO3A expression and reactivates the protein by reducing its phosphorylation level. Furthermore, we show that this FOXO3A activation is responsible for the DAC-induced differentiation of SKM-1 cells into monocytes, as well as for SKM-1 cell cycle arrest, apoptosis, and autophagy. Collectively, these results suggest that FOXO3A reactivation may contribute to the therapeutic effects of DAC in MDS.

1. Introduction

FOXO3A, also known as forkhead in rhabdomyosarcoma-like protein 1 (FKHRL1), is a transcription factor with important roles in embryonic development, differentiation, and tumorigenesis [1]. It is characterized by the presence of the distinctive forkhead DNA binding domain, a highly conserved winged helix motif, and regulates the transcription of genes involved in a variety of processes, including cell cycle regulation [2, 3], apoptosis [4, 5], DNA repair [6], and autophagy [7–9]. FOXO3A function is regulated by post-translational modifications such as phosphorylation, acetylation, and ubiquitination, which ultimately affect its nuclear/cytoplasmic transport and hence cellular location [10–12]. FOXO3A is considered to be a potential tumor suppressor gene and is involved in the regulation of differentiation in various cell types [13–16]. Furthermore, FOXO3A is inactivated, and its target genes are downregulated, following phosphorylation by oncogenic kinases such as AKT, MAPK1, and IKK, which are upregulated in many tumors [17–19]. Interestingly, the reexpression and activation of FOXO3A in tumor cells reportedly have potential in antitumor treatment [20].

A previous study showed that the epigenetic silencing of tumor suppressor genes could confer a growth advantage to a subgroup of myelodysplastic syndrome (MDS) cell clones. Such epigenetic modifications are reversible, and the silenced genes can be reactivated using methyltransferase inhibitors such as decitabine (DAC). Indeed, high doses of DAC are known to impair gene methylation, resulting in the activation of various cellular processes such as apoptosis [21]. On the other hand, at low doses, DAC is incorporated into newly synthesized double-stranded DNA during the S phase of the cell cycle without affecting elongation and induces cell cycle arrest and cellular differentiation [22, 23]. Such S phase-specific DAC incorporation may be responsible for a plateau in DAC activity that was observed in AML cell lines, wherein cellular activity could not be lowered beyond 40% even when the DAC concentration was increased to 50 μM [24]. However, it is possible to enhance the impact of DAC on cellular activity by extending the treatment duration; a reduction to just 15% of the original activity has been

observed after 6 days of DAC treatment [24]. Other reports have confirmed that DAC-induced inhibition of cellular proliferation depends mainly on treatment duration. Additionally, DAC-induced differentiation has been primarily observed at low doses, while high doses tend to be cytotoxic [25]. The optimal dosage of DAC has been explored; Cmax values of 0.3–1.6 μM have been reported in human plasma [26–28], while the reported EC_{50} for a variety of AML cell lines reportedly ranged from 0.4 to 0.8 μM [24, 29].

In this study, we investigated the effects of DAC on differentiation, apoptosis, cell cycle arrest, and autophagy using the myeloid MDS tumor cell line SKM-1. Our model used a low concentration of DAC (0.5 μM) and a duration of treatment of 6 days. Furthermore, we investigated the role of FOXO3A in DAC-dependent processes by measuring the expression levels and activity of this gene and its downstream targets following DAC treatment.

2. Materials and Methods

2.1. Cell Culture and DAC Treatment. Human myelodysplastic syndrome cell line SKM-1 was a gift from Professor Li Chunrui at the Department of Hematology, Tongji Medical College, Tongji Hospital, China. SKM-1 cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco Life Technologies, Grand Island, NY, USA) containing 10% FBS (Gibco Life Technologies) and 1% penicillin-streptomycin at 37°C in 5% CO_2. When cells reached the logarithmic growth phase, they were seeded at a density of 5×10^5 cells/well in 6-well plates and treated with 0.5 μM DAC (Xian-Janssen Pharmaceutical, Xian, China) for 6 days. Every 48 hours, the medium in the wells was replaced with fresh medium containing 0.5 μM DAC.

2.2. Cellular Transfection. Cells were transfected with either *Silencer*® Select FOXO3a (cat. number 4392420) or *Silencer* Select Negative Control (cat. number 4390843) siRNAs, both of which were purchased from Ambion (Thermo Fisher Scientific, Waltham, MA, USA), and Lipofectamine® 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, SKM-1 cells were washed twice in phosphate-buffered saline (PBS) before being resuspended in Opti-MEM medium (Gibco Life Technologies) at a density of 1×10^6 cells/ml. Five hundred microliters of this cell suspension was then diluted 2-fold in Opti-MEM medium and added to 6-well plates, giving 5×10^5 cells/well. To prepare the siRNA liposomes, 3.75 μl Lipofectamine 3000 reagent and 75 pg of the appropriate siRNA were each gently mixed with individual 125 μl aliquots of Opti-MEM, before being combined, and incubated at room temperature for 5 minutes. All 250 μl of the liposome mixture was then added to each well, and cells were incubated at 37°C in 5% CO_2. After 24 hours of transfection, the medium was replaced with fresh DMEM. The transfected cells were either cultured or treated with 0.5 μM DAC for 48 hours as required.

2.3. Assessment of Apoptosis Using Hoechst 33342 Staining. To examine the degree of apoptosis, DAC-treated cells were collected and washed once in PBS, before being counted and resuspended in PBS at a concentration of 1×10^7 cells/ml. Cells were then dried naturally onto antioff slides (ZSGB-BIO, Beijing, China) at room temperature, before being fixed in 4% paraformaldehyde at room temperature for 15 minutes and washed three times for 5 minutes in PBS. One hundred microliters of Hoechst 33342 stain (Beyotime Institute of Biotechnology, Nanjing, Jiangsu, China) was added to each slide, and the slides were incubated for 5 minutes at room temperature in darkness within a humidity chamber. Finally, slides were washed three times for 5 minutes in PBS; then, within 1 hour, they were visualized and photographed using a fluorescence microscope (Olympus TH4-200).

2.4. Assessment of Apoptosis Using Flow Cytometry. Apoptosis assays were performed using an Annexin V-FITC/PI apoptosis kit (Beyotime Biotech) according to the manufacturer's instructions. Briefly, the DAC-treated cells were collected and washed three times in PBS before being counted and resuspended 2×10^5 in 200 μl binding buffer. Ten microliters of Annexin V-FITC and 5 μl propidium iodide (PI; Beyotime Biotech) was then added to the cell suspensions, and samples were incubated in darkness for 15 minutes. Within 1 hour, apoptotic cells were detected by flow cytometry using a flow cytometer (FACSCanto II, BD Biosciences, Franklin Lakes, NJ, USA). Experiments were performed in triplicate.

2.5. Assessment of the Cell Cycle Using PI Staining. The cell cycle stages of the SKM-1 cells were assessed using a PI staining assay kit (Beyotime Biotech) according to the manufacturer's instructions. Briefly, collected cells were washed once in cold PBS and resuspended in 75% ethanol that had been precooled to −20°C. The cells were counted and their concentration was adjusted to 2×10^6 cell/tube using 75% ethanol, and they were fixed at −20°C between 1 and 24 hours. Cells were then pelleted by centrifugation at 2000g for 5 minutes, washed twice with cold PBS, and resuspended in 200 μl PBS. RNase was then added to each tube to a final concentration of 100 μg/ml, mixed, and incubated at 37°C for 30 mins. Next, 235 μl PBS and 60 μl PI (50 μg/ml final concentration) were added to each tube, mixed, and incubated in darkness at 37°C for 30 minutes. Cell cycle stages were examined by flow cytometry within an hour using a flow cytometer (FACSCanto II, BD Biosciences). Experiments were performed in triplicate.

2.6. Detection of Cell Surface Markers. To assess the expression of cell surface markers, DAC-treated cells were collected and washed three times in PBS before being counted and resuspended in 200 μl PBS at a concentration of 1×10^6 cells/tube. Next, either 10 μl FITC-conjugated anti-CD14 or APC-conjugated anti-CD11b antibodies or FITC- or APC-conjugated isotype controls (Becton Dickinson) were added to the cells as appropriate, mixed gently, and incubated in darkness at room temperature for 15 minutes. Cells were then washed once in PBS and resuspended in 200 μl PBS,

and within 1 hour, the surface markers were analyzed by flow cytometry using a flow cytometer (FACSCanto II, BD Biosciences). Experiments were performed in triplicate.

2.7. Western Blot Analysis. Proteins were extracted from cells using RIPA buffer with 1× protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions, and the concentration of total protein was determined using the BCA Protein Assay kit (Beyotime Biotech) according to the manufacturer's protocol. Thirty micrograms of total protein was separated using a sodium dodecyl sulfate polyacrylamide gel electrophoresis kit (Beyotime Biotech) according to the manufacturer's instructions, before being transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) in transfer buffer (200 mM glycine, 40 mM Tris, and 20% methanol) at 240 mA for between 30 and 90 minutes, depending on the molecular weight of the proteins. Membranes were then blocked with 5% BSA (CST) in TBS with 0.1% TBST (CST) for 1 hour at 37°C before incubating with the described primary antibodies (Supplementary Table 1 available online at https://doi.org/10.1155/2017/4302320; diluted according to instructions) overnight at 4°C. Membranes were then washed three times for 5 minutes in 0.1% TBST and then incubated for 1 hour at 37°C with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technology) at a dilution of 1 : 3000. Membranes were washed a further three times for 5 minutes in 0.1% TBST before ECL Chemiluminescent Substrate reagent (Cell Signaling Technology) was added and images were obtained using Bio-Rad ChemiDoc™ XRS+ Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of bands was quantified using Image Lab software version 2.0 (Bio-Rad Laboratories, Hercules, CA, USA), and GAPDH was used as an internal standard.

2.8. Statistical Analysis. Results were obtained from three independent replicate experiments and are expressed as mean ± standard deviation. Data were analyzed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). The significance of differences between groups was assessed using Student's *t*-test, and statistical significance was defined as $P < 0.05$.

3. Results

3.1. FOXO3A Contributes to DAC-Induced SKM-1 Cell Differentiation. The impact of DAC treatment on SKM-1 cell differentiation was examined by measuring the cell surface levels of both the monocyte differentiation marker CD14 and the myeloid cell differentiation marker CD11b before and after treatment. While we observed no CD14 expression on the surface of SKM-1 cells, more than half of the cells expressed CD11b on their surface (59.71% ± 3.80%). This CD11b expression remained constant throughout DAC treatment, whereas CD14 expression gradually increased on exposure to DAC, with the proportion of CD14-positive cells reaching a maximum of 37.19% ± 9.44% ($P < 0.05$) after 6 days of treatment (Figures 1(a) and 1(b)). FOXO3A expression in SKM-1 cells was very low in the absence of DAC, but increased statistical significance on days 3 and 6 following the initiation of treatment (Figure 1(c)). Expression of the inactive, phosphorylated form of FOXO3A (p-FOXO3A) was predominant in SKM-1 cells, indicating that FOXO3A exists primarily in the inactive form. Interestingly, DAC treatment significantly reduced the relative expression of the inactive p-FOXO3A form, resulting in a consequent increase in the FOXO3A/p-FOXO3A ratio (Figure 1(c)) and strongly indicating that DAC induces FOXO3A activation in SKM-1 cells.

Next, we investigated the role of FOXO3A in the observed DAC-induced differentiation of SKM-1 cells, by silencing FOXO3A expression with targeted siRNAs prior to DAC treatment. FOXO3A expression increased 1.77-fold in negative control siRNA-treated SKM-1 cells following treatment with DAC. Conversely, no significant difference in FOXO3A expression was seen following DAC treatment of cells treated with siRNAs targeting FOXO3A ($P = 0.729$); the expression of FOXO3A following DAC treatment was significantly lower in cells treated with the siRNA targeting FOXO3A than in cells that did not undergo NT-siRNA treatment (Figure 1(f)). These data confirm that FOXO3A expression was inhibited by the siRNA.

No significant differences in the surface expression of CD14 and CD11b were observed between SKM-1 cells where FOXO3A was silenced and nonsilenced controls (Figure 1(e)), suggesting that transient inhibition of FOXO3A expression does not affect the basal differentiation of SKM-1 cells. Interestingly, however, when FOXO3A siRNA-SKM-1 cells were treated with DAC, the observed increase in CD14-positive cells was approximately 50% lower than in cells carrying the negative control siRNA (Figure 1(g)), suggesting that silencing FOXO3A expression before DAC treatment impairs, but does not abolish, DAC-induced SKM-1 cell differentiation into monocytes. Thus, it appears that FOXO3A activation contributes to DAC-induced SKM-1 cell differentiation.

3.2. DAC Induces Cell Cycle Arrest in SKM-1 Cells. Having shown that DAC treatment induced differentiation in SKM-1 cells, we next investigated its impact on the cell cycle. Following DAC treatment, the proportion of cells in the S phase reduced while the proportions of cells in both G0/G1 and G2/M phases were increased, suggesting the induction of cell cycle arrest via blocks at G0/G1 and G2/M (Figures 2(a) and 2(b)). However, silencing FOXO3A before DAC treatment significantly attenuated the DAC-induced reduction of cells in the S phase (Figure 2(f)) and partly reversed the DAC-induced G0/G1 and G2/M blocks, indicating that FOXO3A is important for DAC-induced SKM-1 cell cycle arrest.

We next considered the impact of DAC treatment on gene expression. CDKN1A and CDKN1B are targeted by FOXO3A [30] and are downregulated in a variety of tumors. As shown in Figure 2(c), protein expression of both of these genes was rare in untreated SKM-1 cells, but increased after DAC treatment, especially CDKN1A ($P < 0.01$). While, compared with control siRNA, silencing FOXO3A had no

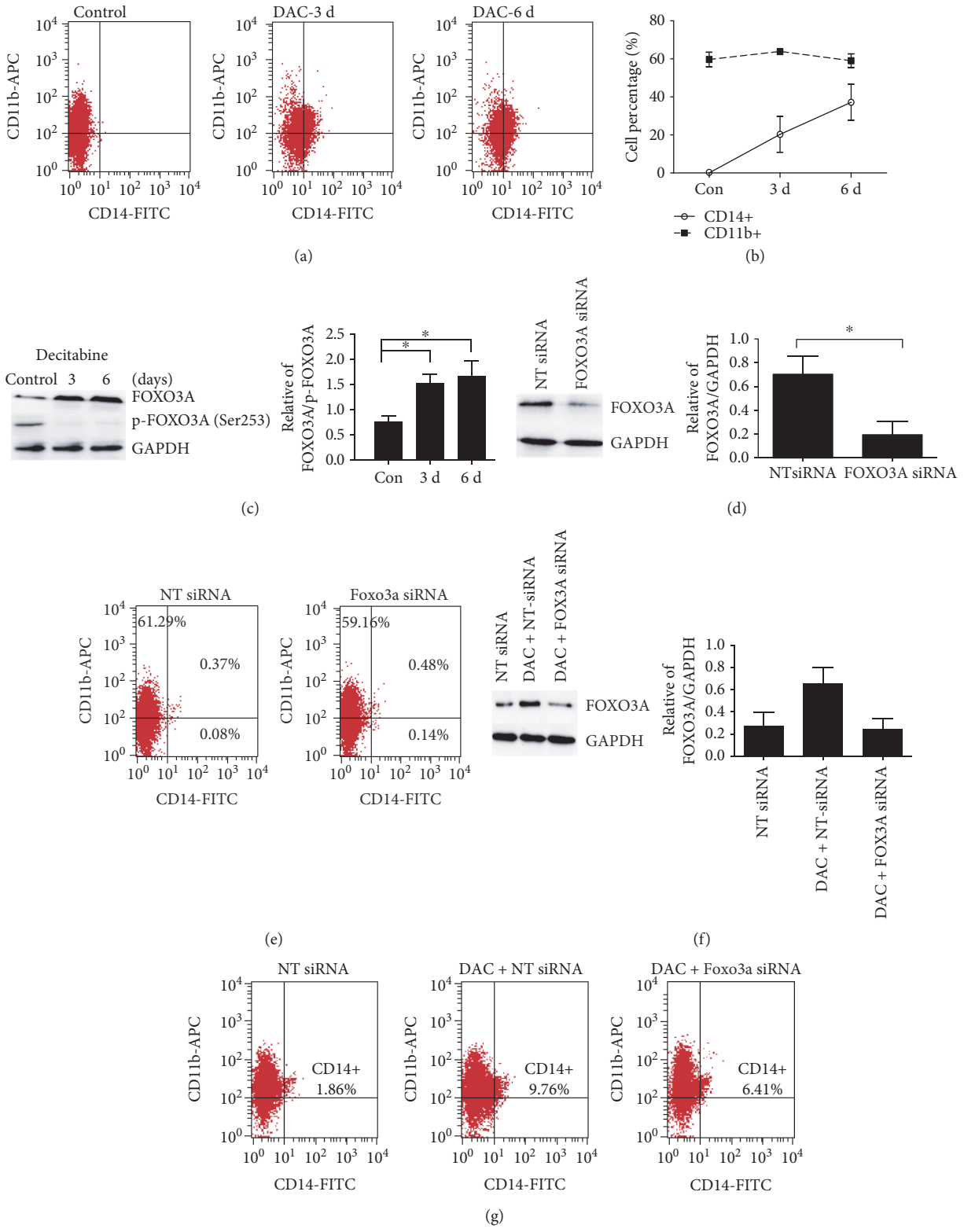


FIGURE 1: FOXO3A contributes to DAC-induced SKM-1 cell differentiation. (a, b) CD14 (marker for monocytes), but not CD11b (marker for myeloid cells), was significantly induced in SKM-1 cells treated with 0.5 μM DAC for 3 and 6 days. (c) Western blot showed that DAC treatment increases FOXO3A expression and FOXO3A/p-FOXO3A ratio. (d) Western blot showed that siRNA targeting FOXO3A decreased FOXO3A expression about 70% compared to negative control siRNA. (e) Surface CD14 and CD11b expression had clearly no change between FOXO3A siRNA and negative control siRNA in SKM-1 cells. (f) Western blot showed that FOXO3A siRNA could decrease FOXO3A expression in DAC-treated SKM-1 cells. (g) Surface CD14 expression was impaired by about 50% in FOXO3A siRNA compared with negative control siRNA in DAC-treated SKM-1 cells. *Two tailed $P < 0.05$.

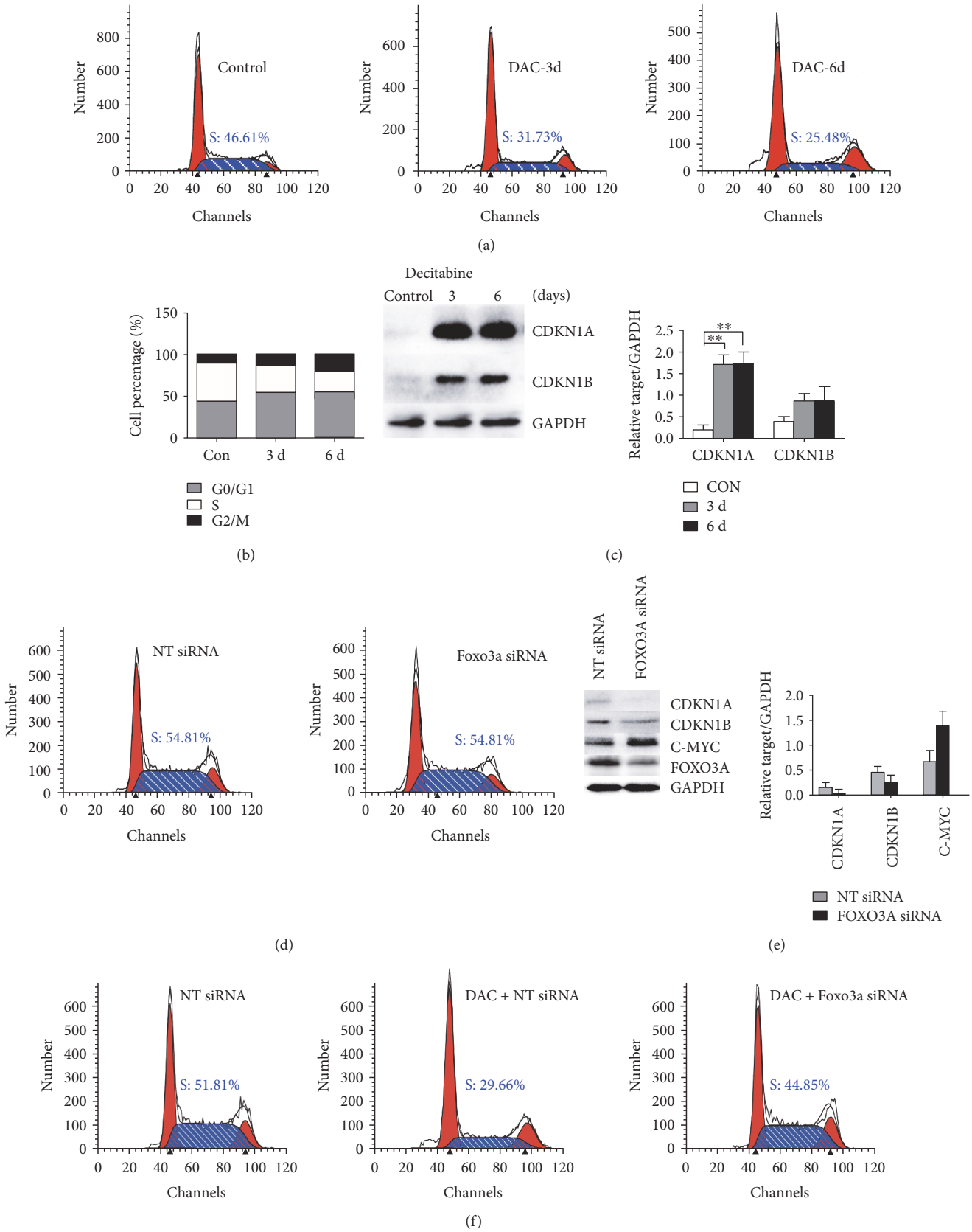


Figure 2: Continued.

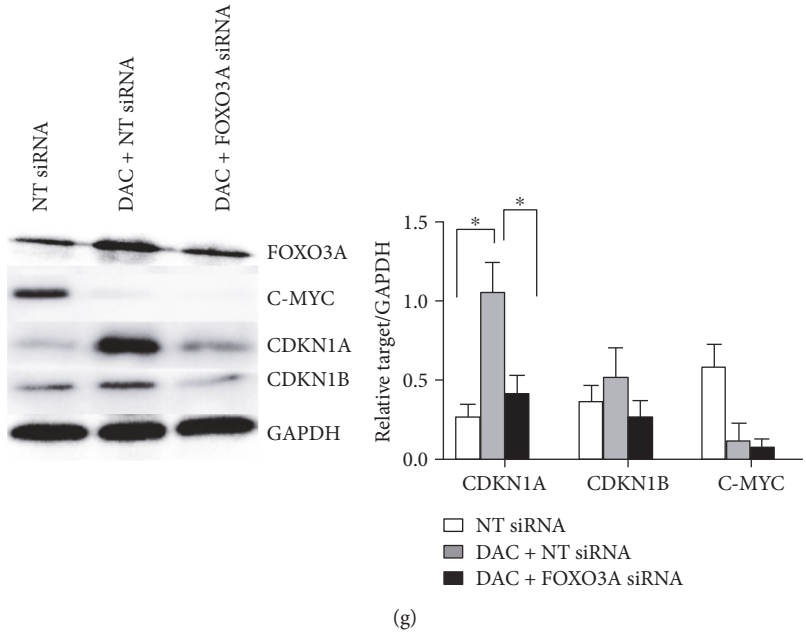


Figure 2: DAC induces cell cycle arrest in SKM-1 cells. (a, b) DAC treatment could decrease cells in S phase and arrest SKM-1 cells in G0/G1 and G2/M phases. (c) CDKN1A and CDKN1B levels examined in Western blot were both increased after DAC treatment in SKM-1 cells. The histogram summarizes the target/GAPDH ratio. (d) Cell cycle had clearly no change comparing FOXO3A siRNA with control siRNA. (e) Western blot showed that CDKN1A and CDKN1B levels in SKM-1 cells were decreased, but MYC was increased after FOXO3A silencing. (f) DAC-induced reduction of cells in the S phase was significantly attenuated after silencing FOXO3A. (g) DAC could not increase CDKN1A or CDKN1B protein expression in the presence of FOXO3A silencing. **Student's t-test $P < 0.01$ and $^{*}P < 0.05$.

significant effect on the cell cycle (Figure 2(d)), expression of the FOXO3A downstream targets CDKN1A and CDKN1B was decreased, with the effect on CDKN1A being particularly striking (Figure 2(e)). MYC is another key transcription factor that plays an important role during the G1/S phase of the cell cycle. Some studies have reported MYC to be a downstream target of FOXO3A, and, indeed, MYC activity can be inhibited by FOXO3A via the MXI1-SRα variant [31]. Consistent with this, MYC protein expression was promoted in cells where FOXO3A was silenced compared to in controls (Figure 2(e)). In the presence of FOXO3A silencing, DAC had no effect on either CDKN1A or CDKN1B protein expression, but MYC protein expression was downregulated (Figure 2(g)).

Overall, this suggests that DAC induces cell cycle arrest in SKM-1 cells and that the observed upregulation of CDKN1A and CDKN1B is dependent on FOXO3A, but that DAC-induced MYC downregulation is not dependent on active FOXO3A.

3.3. DAC Induces Apoptosis in SKM-1 Cells. Having shown that DAC could induce cell cycle arrest in SKM-1 cells, we investigated the effect of DAC treatment on SKM-1 apoptosis. First, DAC-treated cells were stained with Hoechst 33342 and examined using a fluorescence microscope. Apoptosis was visible from 3 days after treatment, with the apoptotic fraction reaching approximately two-thirds of the whole population by day 6 (Figure 3(a)). This trend was confirmed using Annexin-V-FITC/PI double labeling, which also showed a progressive increase in apoptosis on days 3 and 6, with the rate of increase in early apoptotic cells being most notable (Figures 3(b) and 3(c)).

Next, the expression of apoptosis-related FOXO3A downstream target proteins was examined. Detectable expression of the apoptosis-associated proteins BCL2L11 and FASLG was observed in untreated SKM-1 cells (Figure 3(d)). While DAC treatment had no significant effect on FASLG expression, BCL2L11 protein expression increased significantly after treatment, with the strongest expression seen on day 6. These results suggest that DAC induces apoptosis in SKM-1 cells primarily through the mitochondrial apoptosis pathway and that death receptor-mediated apoptosis, which features FASLG, is less important in these cells.

Annexin-V-FITC/PI double labeling indicated that silencing FOXO3A did not significantly affect apoptosis in SKM-1 cells (Figure 3(e)), although Western blot analysis did suggest that expression of the proapoptotic molecule BCL2L11 was significantly decreased after FOXO3A silencing (Figure 3(f)). Interestingly, FOXO3A silencing inhibited the accumulation of SKM-1 cells in the later stages of apoptosis that was observed following DAC treatment, but not the corresponding accumulation of cells in early apoptosis (Figures 3(d) and 3(e)), suggesting that FOXO3A might be required for the later stages, but not the early stages, of DAC-induced apoptosis. The reduction in BCL2L11 expression observed following FOXO3A silencing was not reversed by subsequent DAC treatment, suggesting a role in apoptosis that is downstream of FOXO3A activation (Figure 3(g)). As our data suggest that FOXO3A is involved only in the later

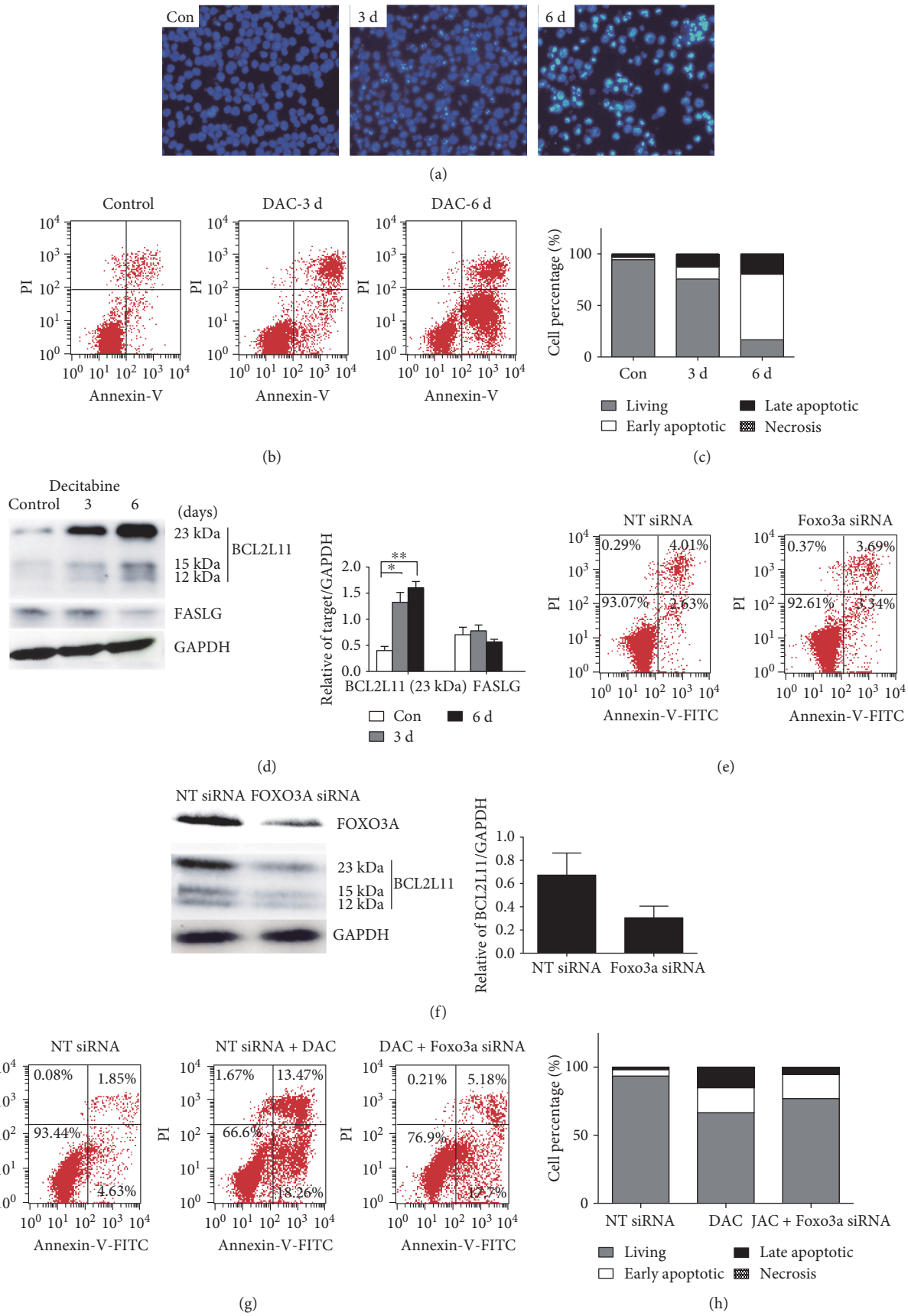


Figure 3: Continued.

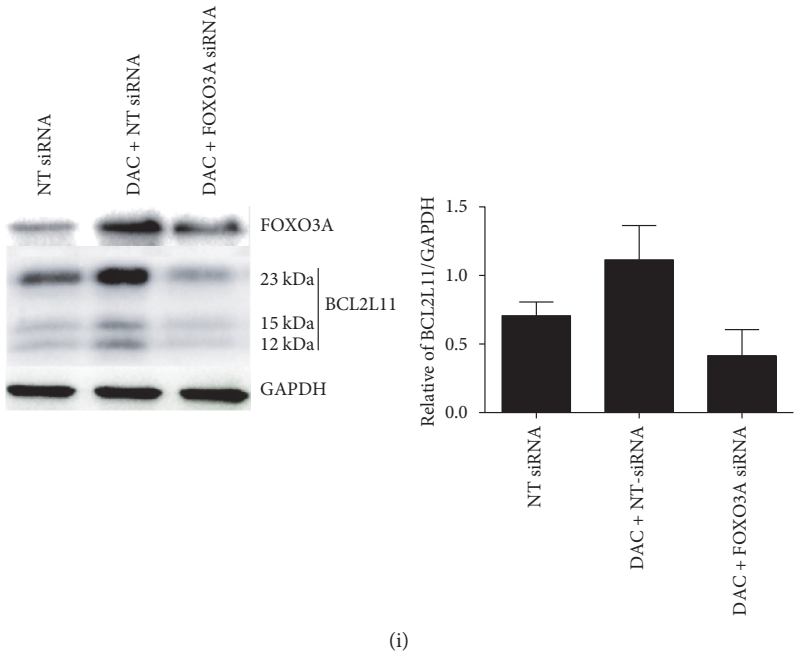


Figure 3: DAC induces apoptosis in SKM-1 cells. (a) Hoechst 33342 assay detected that DAC treatment could induce SKM-1 cell apoptosis. (b, c) Annexin-V-FITC/PI assay showed that apoptosis occurred in SKM-1 cells after DAC treatment. (d) BCL2L11 and FASLG proteins were examined in Western blot. BCL2L11, not FASLG, was increased after DAC treatment in SKM-1 cells. The histogram summarizes the target/GAPDH ratio. (e) Cell activity had clearly no change when compared FOXO3A siRNA with control siRNA. (f) Western blot found that BCL2L11 in SKM-1 cells were decreased after FOXO3A silencing. (g, h) FOXO3A silencing decreased the accumulation of late apoptosis of SKM-1 cells that was observed following DAC treatment. (i) BCL2L11 expression was inhibited after FOXO3A silencing and could not be induced by subsequent DAC treatment. **Student's t-test $P < 0.01$ and $^{*}P < 0.05$.

stages of DAC-induced apoptosis, it is likely that BCL2L11 also plays a role at this stage.

3.4. FOXO3A Contributes to DAC-Mediated Autophagy in SKM-1 Cells. The conversion of the nonlipidated LC3 form LC3-I to the lipidated LC3-II form is a common marker of autophagic activity [32]. Western blot analysis showed that LC3-I expression was slightly higher than LC3-II expression in SKM-1 cells and that while DAC treatment induced expression of both LC3-I and LC3-II, the induction of LC3-II was more prominent, giving the increased LC3-II/LC3-I ratio that is indicative of autophagy induction (Figure 4(a)). Furthermore, the autophagy initiation protein BECN1 and the ATG5-ATG12-ATG16 complex proteins, which are associated with nucleation and elongation of the autophagosome, were both upregulated following DAC treatment, confirming the DAC-mediated induction of autophagy in SKM-1 cells (Figure 4(b)).

Following FOXO3A silencing, the expression of both LC3-I and LC3-II was reduced, but the LC3-II/LC3-I ratio was preserved. Treating the knockdown cells with DAC could not rescue expression of LC3-I but could rescue LC3-II expression to an extent; expression was restored to baseline levels but was still significantly lower than was seen in nonsilenced cells treated with DAC (Figure 4(d)). While the LC3-II/LC3-I ratio in FOXO3A-silenced cells treated with DAC was higher than in untreated silenced cells, indicating the partial induction of autophagy, this ratio remained significantly lower than in DAC-treated cells.

Further to the LC3 results, FOXO3A silencing was associated with the downregulation of ATG12, ATG16, ATG5, and BECN1 protein expression (Figure 4(c)). In contrast, DAC treatment of nonsilenced SKM-1 cells upregulated expression of these autophagy-related proteins, but this upregulation was attenuated on FOXO3A silencing resulting in lower protein expression levels. Overall, these data indicate that FOXO3A contributes to DAC-induced autophagy in SKM-1 cells via the induction of the autophagy-related genes ATG12, ATG16, ATG5, and BECN1, as silencing FOXO3A leads to a reduction in downstream effector protein expression and a partial attenuation of autophagy.

4. Discussion

MDS is a highly heterogeneous myeloid malignant disease that is characterized by ineffective hematopoiesis and cytopenia and that can occasionally progress into acute myeloid leukemia. While the exact mechanisms underpinning this transformation are unknown, the accumulation of genetic or epigenetic abnormalities is likely to play a role. In particular, the epigenetic silencing of tumor suppressor genes could confer a growth advantage and accelerate clonal evolution in abnormal MDS cells. In this study, we have demonstrated that FOXO3A, a potential tumor suppressor gene, is hyperphosphorylated and thus inactivated in SKM-1 cells. However, DAC treatment activated FOXO3A by both increasing expression and reducing phosphorylation, leading to the upregulation of the downstream effectors CDKN1A,

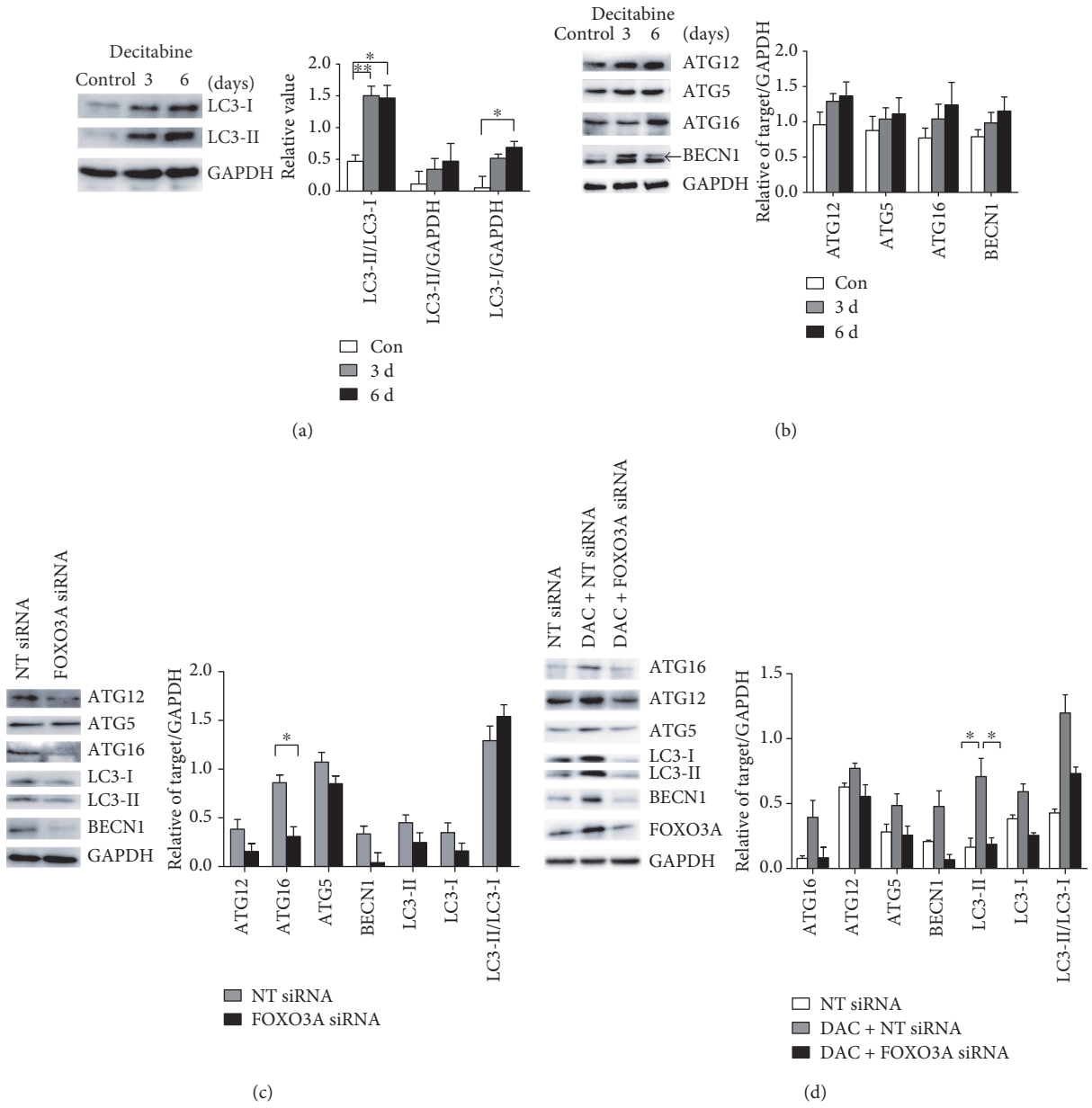


Figure 4: FOXO3A contributes to DAC-mediated autophagy in SKM-1 cells. (a) LC3-I and LC3-II were both increased after DAC treatment as well as the LC3-II/LC3-I ratio. (b) BECN1 and ATG5-ATG12-ATG16 complex proteins were both upregulated following DAC treatment. (c) LC3-I, LC3-II BECN1, and ATG5-ATG12-ATG16 complex proteins were decreased after FOXO3A silencing. (d) In DAC-treated SKM-1 cells, LC3-I, LC3-II BECN1, and ATG5-ATG12-ATG16 complex proteins were decreased after FOXO3A silencing. **Student's t-test $P < 0.01$ and $^{*}P < 0.05$.

CDKN1B, BCL2L11, BECN1, ATG5, ATG12, and ATG16. Consequently, DAC-induced differentiation of SKM-1 cells into monocytes, SKM-1 cell cycle arrest, apoptosis, and autophagy were observed.

We found that DAC greatly increased the expression of CDKN1A from a trace basal level in SKM-1 cells. While the CDKN1A promoter region is reportedly surrounded by CpG islands, a study of leukemic cells detected no methylation of these CpG motifs [4], suggesting that gene silencing caused by hypermethylation is not responsible for CDKN1A inactivation in leukemia cells and that other mechanisms may be involved in DAC-induced CDKN1A expression. In the ML-1 and BV-173 leukemia cell lines and the HCT116 colon cancer cell line, both of which contain wild-type TP53; DAC-induced apoptosis occurs in parallel with a TP53-dependent, but DNMT1-independent, upregulation of CDKN1A. This effect is not seen in the TP53-null HL-60 cell line, leading to the suggestion that DAC-induced CDKN1A upregulation is dependent on a DNA damage/ATM/TP53 axis [4]. As the SKM-1 cell line used in this study carries an inactive mutant form of TP53 [33], it is likely that DAC induces CDKN1A expression via other mechanisms. We observed that the expression and activity of FOXO3A, a gene that is involved in the regulation of

the G0/G1 phase of the cell cycle, increased significantly after DAC treatment. We therefore speculate that the upregulation of CDKN1A, a FOXO3A target gene, is associated with the DAC-mediated activation of FOXO3A in SKM-1 cells; this theory is supported by our FOXO3A silencing data.

The protooncogenes MYC and CDKN1B play a crucial role in the control of cell cycle progression, and the induction of CDKN1B transcription by FOXO3A leads to cell cycle arrest and apoptosis. Both FOXO3A and MYC interact functionally with the forkhead binding element in the CDKN1B proximal promoter [34], meaning that MYC may inhibit the activation of CDKN1B by FOXO3A in tumor cells, potentially leading to the uncontrolled proliferation and invasiveness of a variety of tumors. MYC expression increased markedly when FOXO3A was silenced in SKM-1 cells, which could indicate that MYC is a downstream target of FOXO3A and that the tumor-suppressive properties of FOXO3A may be related to MYC inhibition. Furthermore, there is considerable overlap in the genes regulated by both FOXO3A and MYC, both in those related to growth promotion such as *CCND2*, *CDK4*, and *CCNE2* and in those related to growth inhibition such as *BBC3*, *CDKN1B*, and *GADD45A* [35, 36], suggesting that the expression of such genes is regulated by both FOXO3A and MYC in an antagonistic manner. We found that FOXO3A silencing did not affect the MYC downregulation induced by DAC, which could explain why FOXO3A silencing Foxo3a could only partially reverse DAC-induced apoptosis and cell cycle arrest in SKM-1 cells.

BCL2L11 (also known as Bim) is a proapoptotic factor containing a BH3 domain that can bind to and neutralize the antiapoptotic protein BCL2. Activated FOXO3A regulates the transcription of the *BCL2L11* gene by translocating to the nucleus, binding to the *BCL2L11* promoter, and inducing expression [37, 38]. High doses of 5-azacitidine (5-AZA; 2 μM) can reportedly activate FOXO3A and thus upregulate *BCL2L11* expression and trigger apoptosis in AML cells [39]. We also observed the induction of BCL2L11 expression using low doses of DAC, although this treatment had no significant effect on FASLG expression. It is therefore likely that the apoptosis induced by low doses of DAC was mediated primarily through the mitochondrial pathway, with the death receptor pathway, which involves FASLG, playing no role in SKM-1 cells.

Autophagy, a vital mechanism for maintaining energy balance and metabolic homeostasis in cells, has a dual role in cellular biology that it can promote either cell survival or cell death. The effect of demethylation agents on tumor cell autophagy has been investigated previously, and azacitidine (AZA) treatment was found to induce apoptosis and autophagy in SKM-1 cells [40], with similar observations reported in CML cell lines [41]. In the latter study, autophagy occurred first and it was followed by apoptosis [41]. These studies, together with our results, show that demethylating agents activate cellular stress responses, eventually leading to autophagy and apoptosis. Previously, it was reported that cell death in BCR-ABL-positive CML cells was significantly increased following either treatment with an autophagy inhibitor or the silencing of the autophagy genes ATG5 and ATG7. Furthermore, the simultaneous inhibition of the Hedgehog signaling pathway and autophagy significantly reduced the activity of, and induced apoptosis in, BCR-ABL-positive CML cells, irrespective of whether they were sensitive or resistant to imatinib [42, 43]. Interestingly, silencing LC3 in MDS cell lines significantly increased AZA-induced cell death, indicating that AZA-induced autophagy may be a protective, rather than cytotoxic, mechanism in cells [44] and that the AZA-induced autophagy observed by Cluzeau et al. [40] may not be contributing to cell death. It is possible that the autophagy occurring with other traditional chemotherapy drugs, as with AZA, may constitute a compensatory mechanism to protect cells from drug-induced damage and apoptosis. It is therefore not possible to determine whether the autophagy that is observed in SKM-1 cells in response to DAC treatment is a drug-induced protective stress response or a cytotoxic response.

5. Conclusion

This study showed that silencing FOXO3A expression impaired DAC-induced cellular differentiation, cell cycle arrest, and apoptosis, potentially because of the observed downregulation of CDKN1B, CDKN1A, and BCL2L11. Interestingly, DAC-induced MYC downregulation was not reversed by FOXO3A silencing, which could explain the partial reversal of DAC-induced cell cycle arrest and apoptosis that is observed when FOXO3A expression is lost. The upregulation of the autophagy-related proteins BECN1, ATG5, ATG12, and ATG16 in SKM-1 cells following DAC treatment, as well as the consequent increase in autophagy, was found to be related to the DAC-induced upregulation and activation of FOXO3A. Collectively, these results suggest that DAC-reactivation of FOXO3A has potential in MDS therapeutics.

Conflicts of Interest

The authors declare that they have no competing interests.

References

[1] M. Wang, X. Zhang, H. Zhao, Q. Wang, and Y. Pan, "FoxO gene family evolution in vertebrates," *BMC Evolutionary Biology*, vol. 9, no. 1, pp. 1–15, 2009.

[2] H. M. Korashy, O. M. Belali, M. A. Ansar, and N. O. Alharbi, "FoxO3a is essential for the antiproliferative and apoptogenic effects of sunitinib in MDA-MB231 cell line," *Anticancer Research*, vol. 36, no. 11, pp. 6097–6108, 2016.

[3] K. Qian, G. Wang, R. Cao et al., "Capsaicin suppresses cell proliferation, induces cell cycle arrest and ROS production in bladder cancer cells through FOXO3a-mediated pathways," *Molecules*, vol. 21, no. 10, p. 1406, 2016.

[4] W. Qiang, F. Sui, J. Ma et al., "Proteasome inhibitor MG132 induces thyroid cancer cell apoptosis by modulating the activity of transcription factor FOXO3a," *Endocrine*, vol. 56, no. 1, pp. 98–108, 2017.

[5] L. T. Nguyen, Y. H. Lee, A. R. Sharma et al., "Quercetin induces apoptosis and cell cycle arrest in triple-negative breast cancer cells through modulation of Foxo3a activity," *The*

Korean Journal of Physiology & Pharmacology, vol. 21, no. 2, pp. 205–213, 2017.

[6] G. Nestal de Moraes, L. Bella, S. Zona, M. J. Burton, and E. W. Lam, "Insights into a critical role of the FOXO3a-FOXM1 axis in DNA damage response and genotoxic drug resistance," *Current Drug Targets*, vol. 17, no. 2, pp. 164–177, 2016.

[7] H. J. Kim, S. Y. Lee, C. Y. Kim, Y. H. Kim, W. Ju, and S. C. Kim, "Subcellular localization of FOXO3a as a potential biomarker of response to combined treatment with inhibitors of PI3K and autophagy in PIK3CA-mutant cancer cells," *Oncotarget*, vol. 8, no. 4, pp. 6608–6622, 2017.

[8] L. Sun, M. Zhao, Y. Wang et al., "Neuroprotective effects of miR-27a against traumatic brain injury via suppressing FoxO3a-mediated neuronal autophagy," *Biochemical and Biophysical Research Communications*, vol. 482, no. 4, pp. 1141–1147, 2017.

[9] Y. Chen, L. Lv, H. Pi et al., "Dihydromyricetin protects against liver ischemia/reperfusion induced apoptosis via activation of FOXO3a-mediated autophagy," *Oncotarget*, vol. 7, no. 47, pp. 76508–76522, 2016.

[10] L. P. Van Der Heide, M. F. Hoekman, and M. P. Smidt, "The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation," *Biochemical Journal*, vol. 380, Part 2, pp. 297–309, 2004.

[11] A. M. Brownawell, G. J. Kops, I. G. Macara, and B. M. Burgering, "Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX," *Molecular and Cellular Biology*, vol. 21, no. 10, pp. 3534–3546, 2001.

[12] W. H. Biggs 3rd, W. K. Cavenee, and K. C. Arden, "Identification and characterization of members of the FKHR (FOX O) subclass of winged-helix transcription factors in the mouse," *Mammalian Genome*, vol. 12, no. 6, pp. 416–425, 2001.

[13] G. Marfe, M. Tafani, F. Fiorito, U. Pagnini, G. Iovane, and L. De Martino, "Involvement of FOXO transcription factors, TRAIL-FasL/Fas, and sirtuin proteins family in canine coronavirus type II-induced apoptosis," *PLoS One*, vol. 6, no. 11, article e27313, 2011.

[14] E. Wattel, C. Preudhomme, B. Hecquet et al., "p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies," *Blood*, vol. 84, no. 9, pp. 3148–3157, 1994.

[15] Y. Fang, Y. Yang, C. Hua et al., "Rictor plays a pivotal role in maintaining quiescence as well as stemness of leukemia stem cells in MLL-driven leukemia," *Leukemia*, vol. 31, no. 2, pp. 414–422, 2017.

[16] C. Hu, Z. Ni, B. S. Li et al., "hTERT promotes the invasion of gastric cancer cells by enhancing FOXO3a ubiquitination and subsequent ITGB1 upregulation," *Gut*, vol. 66, no. 1, pp. 31–42, 2015.

[17] N. Chapuis, S. Park, L. Leotoing et al., "IκB kinase overcomes PI3K/Akt and ERK/MAPK to control FOXO3a activity in acute myeloid leukemia," *Blood*, vol. 116, no. 20, pp. 4250–4250, 2010.

[18] M. J. Stankiewicz and J. D. Crispino, "AKT collaborates with ERG and Gata1s to dysregulate megakaryopoiesis and promote AMKL," *Leukemia*, vol. 27, no. 6, pp. 1339–1347, 2013.

[19] L. Q. Tubi, S. C. Nunes, A. Brancalion et al., "Protein kinase CK2 regulates AKT, NF-κB and STAT3 activation, stem cell viability and proliferation in acute myeloid leukemia," *Leukemia*, vol. 31, no. 2, pp. 292–300, 2016.

[20] P. Ranganathan, X. Yu, R. Santhanam et al., "Decitabine priming enhances the antileukemic effects of exportin 1 (XPO1) selective inhibitor selinexor in acute myeloid leukemia," *Blood*, vol. 125, no. 17, pp. 2689–2692, 2015.

[21] K. Schmelz, M. Wagner, B. Dorken, and I. Tamm, "5-Aza-2′-deoxycytidine induces p21WAF expression by demethylation of p73 leading to p53-independent apoptosis in myeloid leukemia," *International Journal of Cancer*, vol. 114, no. 5, pp. 683–695, 2005.

[22] J. M. Covey, M. D'Incalci, E. J. Tilchen, D. S. Zaharko, and K. W. Kohn, "Differences in DNA damage produced by incorporation of 5-aza-2′-deoxycytidine or 5,6-dihydro-5-azacytidine into DNA of mammalian cells," *Cancer Research*, vol. 46, no. 11, pp. 5511–5517, 1986.

[23] L. Schermelleh, A. Haemmer, F. Spada et al., "Dynamics of Dnmt1 interaction with the replication machinery and its role in postreplicative maintenance of DNA methylation," *Nucleic Acids Research*, vol. 35, no. 13, pp. 4301–4312, 2007.

[24] P. W. Hollenbach, A. N. Nguyen, H. Brady et al., "A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines," *PLoS One*, vol. 5, no. 2, article e9001, 2010.

[25] O. Alcazar, S. Achberger, W. Aldrich et al., "Epigenetic regulation by decitabine of melanoma differentiation in vitro and in vivo," *International Journal of Cancer*, vol. 131, no. 1, pp. 18–29, 2012.

[26] G. Marcucci, L. Silverman, M. Eller, L. Lintz, and C. L. Beach, "Bioavailability of azacitidine subcutaneous versus intravenous in patients with the myelodysplastic syndromes," *Journal of Clinical Pharmacology*, vol. 45, no. 5, pp. 597–602, 2005.

[27] A. F. Cashen, A. K. Shah, L. Todt, N. Fisher, and J. DiPersio, "Pharmacokinetics of decitabine administered as a 3-h infusion to patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS)," *Cancer Chemotherapy and Pharmacology*, vol. 61, no. 5, pp. 759–766, 2008.

[28] W. Blum, R. B. Klisovic, B. Hackanson et al., "Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia," *Journal of Clinical Oncology*, vol. 25, no. 25, pp. 3884–3891, 2007.

[29] K. P. Ng, Q. Ebrahem, S. Negrotto et al., "p53 independent epigenetic-differentiation treatment in xenotransplant models of acute myeloid leukemia," *Leukemia*, vol. 25, no. 11, pp. 1739–1750, 2011.

[30] D. L. McClelland Descalzo, T. S. Satoorian, L. M. Walker, N. R. Sparks, P. Y. Pulyanina, and N. I. Zur Nieden, "Glucose-induced oxidative stress reduces proliferation in embryonic stem cells via FOXO3A/β-catenin-dependent transcription of $p21^{cip1}$," *Stem Cell Reports*, vol. 7, no. 1, pp. 55–68, 2016.

[31] B. Gan, C. Lim, G. Chu et al., "FoxOs enforce a progression checkpoint to constrain mTORC1-activated renal tumorigenesis," *Cancer Cell*, vol. 18, no. 5, pp. 472–484, 2010.

[32] H. J. Shin, H. Kim, S. Oh et al., "AMPK-SKP2-CARM1 signalling cascade in transcriptional regulation of autophagy," *Nature*, vol. 534, no. 7608, pp. 553–557, 2016.

[33] T. Cluzeau, A. Dubois, A. Jacquel et al., "Phenotypic and genotypic characterization of azacitidine-sensitive and resistant SKM1 myeloid cell lines," *Oncotarget*, vol. 5, no. 12, pp. 4384–4391, 2014.

[34] V. Chandramohan, N. D. Mineva, B. Burke et al., "c-Myc represses FOXO3a-mediated transcription of the gene encoding the p27Kip1 cyclin dependent kinase inhibitor,"

Journal of Cellular Biochemistry, vol. 104, no. 6, pp. 2091–2106, 2008.

[35] C. Bouchard, J. Marquardt, A. Brás, R. H. Medema, and M. Eilers, "Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins," *EMBO Journal*, vol. 23, no. 14, pp. 2830–2840, 2004.

[36] S. Amente, J. Zhang, M. L. Lavadera, L. Lania, E. V. Avvedimento, and B. Majello, "Myc and PI3K/AKT signaling cooperatively repress FOXO3a-dependent PUMA and GADD45a gene expression," *Nucleic Acids Research*, vol. 39, no. 22, pp. 9498–9507, 2011.

[37] H. You, M. Pellegrini, K. Tsuchihara et al., "FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal," *The Journal of Experimental Medicine*, vol. 203, no. 7, pp. 1657–1663, 2006.

[38] P. F. Dijkers, R. H. Medema, J. W. Lammers, L. Koenderman, and P. J. Coffer, "Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1," *Current Biology*, vol. 10, no. 19, pp. 1201–1204, 2000.

[39] S. Thepot, E. Lainey, T. Cluzeau et al., "Hypomethylating agents reactivate FOXO3A in acute myeloid leukemia," *Cell Cycle*, vol. 10, no. 14, pp. 2323–2330, 2011.

[40] T. Cluzeau, G. Robert, A. Puissant et al., "Azacitidine-resistant SKM1 myeloid cells are defective for AZA-induced mitochondrial apoptosis and autophagy," *Cell Cycle*, vol. 10, no. 14, pp. 2339–2343, 2011.

[41] M. Schnekenburger, C. Grandjenette, J. Ghelfi et al., "Sustained exposure to the DNA demethylating agent, 2′-deoxy-5-azacytidine, leads to apoptotic cell death in chronic myeloid leukemia by promoting differentiation, senescence, and autophagy," *Biochemical Pharmacology*, vol. 81, no. 3, pp. 364–378, 2011.

[42] X. Zeng, H. Zhao, Y. Li et al., "Targeting Hedgehog signaling pathway and autophagy overcomes drug resistance of BCR-ABL-positive chronic myeloid leukemia," *Autophagy*, vol. 11, no. 2, pp. 355–372, 2015.

[43] L. S. Hart, J. T. Cunningham, T. Datta et al., "ER stress–mediated autophagy promotes Myc-dependent transformation and tumor growth," *The Journal of Clinical Investigation*, vol. 122, no. 12, pp. 4621–4634, 2012.

[44] G. Kroemer, G. Marino, and B. Levine, "Autophagy and the integrated stress response," *Molecular Cell*, vol. 40, no. 2, pp. 280–293, 2010.

Hindawi
Journal of Immunology Research
Volume 2017, Article ID 4861570, 6 pages
https://doi.org/10.1155/2017/4861570

Review Article

Immunotherapy as a Promising Treatment for Prostate Cancer: A Systematic Review

Marlena Janiczek,[1,2] Łukasz Szylberg,[1] Anna Kasperska,[1] Adam Kowalewski,[1] Martyna Parol,[1] Paulina Antosik,[1] Barbara Radecka,[2] and Andrzej Marszałek[3]

[1]*Department of Clinical Pathomorphology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Torun, Poland*
[2]*Department of Clinical Oncology, Oncology Center in Opole, Opole, Poland*
[3]*Department of Oncologic Pathology and Prophylactics, Poznan University of Medical Sciences and Greater Poland Cancer Center, Poznan, Poland*

Correspondence should be addressed to Marlena Janiczek; janiczek.marlena@gmail.com

Received 7 June 2017; Revised 4 September 2017; Accepted 14 September 2017; Published 3 October 2017

Academic Editor: Jiaming Liu

Prostate cancer treatment is currently based on surgical removal, radiotherapy, and hormone therapy. In recent years, another therapeutic method has emerged—immunological treatment. Immunotherapy modulates and strengthens one's immune responses against cancer. Neoplastic cells naturally escape from the control of the immune system, and the main goal of immune therapy is to bring the control back. Satisfying outcomes after treatment of advanced melanoma and lung cancer suggest a great potential of immunotherapy as an approach for other tumors' treatment, especially in patients primarily introduced to palliative care. After initial clinical trials, immunotherapy seems to have different side effects than chemotherapy. Prostate cancer was the first neoplasm in which a specific vaccine significantly improved survival. There is a tremendous potential for synergistic combinations of immunotherapy with conventional cancer treatments. A combination of several drugs or methods can be a key in radical treatment of metastatic prostate cancer as demonstrated by preliminary studies.

1. Introduction

Prostate cancer (PC) is the second leading cause of cancer death in men, behind only lung cancer [1]. The American Cancer Society estimates that over 180,000 new cases of prostate cancer will be diagnosed in 2016 [2]. Additionally, a review of almost 800,000 cases revealed that the annual incidence of metastatic prostate cancer increased significantly in recent years [3]. Prostate cancer usually does not show any signs until it has progressed to an advanced stage. Prostate-specific antigen (PSA) has been used as a tumor marker for many years; however, the US Preventive Services Task Force (USPSTF) recommends against any routine PSA-based screening for prostate cancer [4]. Researchers pointed the possibility of overtreatment based on the aforementioned screening method with an increasing risk of side effects. Standard treatments of PC include surgical removal, radiation, and hormone therapy. In the last years, immunotherapy as an alternative method has gained increasing interest. Immunotherapy appears promising and probably will improve therapeutic strategy for patients with PC, resulting in increased quality and quantity of life. Immunotherapies fall into three categories such as checkpoint inhibitors, cytokines, and therapeutic cancer vaccines [5] (Table 1). There are numerous clinical trials on immune checkpoint therapy and therapeutic vaccines for PC. Moreover, immunotherapy has already been used in clinical trials for other malignant neoplasms, and positive clinical outcomes were observed in colon, renal, and lung cancer and in metastatic melanoma [6]. A key to the successful management of

Table 1: Types of cancer immunotherapy.

Blockade of immune checkpoint	Cytokines	Therapeutic vaccines
Disrupts signals that allow cancer immune evasion	Direct the immune system against cancer cells	Enhance host's natural immune response against cancer

metastatic castrate-resistant prostate cancer (CRPC) is to understand the complexity of tumor cells and their interactions with the surrounding microenvironment, in particular with infiltrating macrophages and lymphocytes [7]. During the course of the disease, neoplastic cells develop a mechanism of an immune escape and develop resistance to proapoptotic signals, for example, by blocking immune checkpoints in the PD-1, PD-L1, PD-L2, and CTLA-4 axes [8].

Table 2: Prostate cancer vaccines.

Drug	Agent description-based vaccine
Sipuleucel-T	Targets prostatic acid phosphatase (PAP)
Prostvac-VF	Fowlpox virus
GVAX	Expressing GM-CSF
DCVAC/PCa	Poly I:C

2. The Evolving Role of Immunotherapy in Prostate Cancer

2.1. Prostate Cancer Vaccines. In contrast to other solid tumors, cancer of the prostate was seen as an inflammatory disease for a long time. Recent studies on murine models revealed that chronic inflammation is preceded by endothelial changes that allow immune cell extravasation. Many studies have evaluated the relationship between specific immune cells and prostate cancer. In prostate cancer, we have learned to use significant amounts of vaccines, but they are still behind the results observed in other solid tumors such as melanoma, bladder and kidney cancer, and non-small-cell lung cancer. Currently, several vaccines for prostate cancer are available; however, most of them fail to meet expectations.

PC cells usually proliferate slowly, providing the time needed to elicit an immune response, even in patients with advanced disease. Hence, the PC represents an ideal target for cancer vaccines [9] (Table 2). Sipuleucel-T is an autologous vaccine in which the patient's peripheral blood mononuclear cells are retrieved via leukapheresis [3]. Vaccine's target is prostatic acid phosphatase (PAP), a glycoprotein enzyme synthesized in the prostate epithelium that significantly increases as cancer progresses. PAP is elevated in patients with bone metastasis and correlates with poor prognosis [10]. According to the phase III clinical trial known as Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT), treatment with sipuleucel-T resulted in a 4.1-month overall survival (OS) benefit and a 22% relative risk reduction of mortality in patients with metastatic CRPC [11]. Data from IMPACT also revealed that the greatest benefit occurs in patients with a lower disease burden [9, 12] indicating the importance of early screening and diagnosis of PCa. Sipuleucel-T is approved by the Food and Drug Administration (FDA); however, the treatment is currently cost-prohibitive [13]. Despite the survival benefits, only minimal antineoplastic responses were observed. It turned out that PC compared to melanoma responds to checkpoint inhibitors in a totally different manner. Vaccines seem to have a subtle impact on immunological microenvironment. There is evidence that antigen-specific B and antigen-specific T cell responses may be generated early, for example, after the first infusion, and can be restimulated in vitro. Many cytokines are involved in T cell activation including IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, interferon gamma (IFN-γ), and tumor necrosis factor-alpha. All of them can be detected in cell culture fluids after the second and third signal [14, 15].

Prostvac-VF is a viral-based vaccine based on a combination of two viral vectors, vaccinia that is a potent immunologic priming agent and fowlpox that is used as a boosting agent [9]. Each vector encodes for PSA and three immune costimulatory molecules including intracellular adhesion molecule 1, costimulatory molecule for T cells (B7–1), and lymphocyte function-associated antigen 3 [16]. The virus infects antigen-presenting cells (APCs), promoting cell surface protein expression and interaction with T cells, in consequence facilitating targeted immune response and cell-mediated tumor cell destruction [17]. In the phase II clinical trial, Prostvac-VF was well tolerated and improved OS compared with control vectors (25.1 months versus 16.6 months) in patients with minimally symptomatic CRPC [17]. Patients with aggressive or end-stage disease exhibited lower benefits [17, 18]. In contrast to preparation of sipuleucel-T, this construct is based on the inherent immunogenicity of the vaccinia virus. Induced T cell response directed against PSA can have other antigens that can activate other T cells. Results of phase I were encouraging for a phase II study, suggesting that the benefits of survival are comparable with the values of sipuleucel-T. However, the results of phase III trials are eagerly awaited [17]. GVAX is an allogeneic whole cell-based prostate cancer vaccine. In this approach, autologous or allogeneic tumor cells are genetically modified to bear GM-CSF [3]. GM-CSF induces the recruitment of APCs invoking a cascade of immune responses [9]. The whole tumor cell is used as an antigen that consequently facilitates both humoral and cellular immune responses. Although phase I and II studies confirmed clinical activity and safety, phase III was stopped due to increased mortality and futility analysis [3, 9].

DCVAC/PCa is an autologous dendritic cell-based vaccine composed of poly I:C-activated DCs pulsed with killed LNCaP prostate cancer cell line. Phase I and II trials showed that chemoimmunotherapy combined with DCVAC and docetaxel resulted in a 7.2-month OS benefit with no significant complications [19]. Currently, a phase III clinical trial

(NCT02111577) is being conducted to evaluate the efficacy and safety of DCVAC/PCa versus placebo in men with metastatic CRPC eligible for first-line chemotherapy.

DNA-based vaccines consist of genetically engineered DNA containing the coding sequence of a targeted antigen. This sequence can be taken up by cells which subsequently express the genes that induce an immune response [20]. Phase I trials have been done targeting various tumor-associated antigens, including PSA, PSMA, PAP, and the cancer-testis antigen NY-ESO-1. Little clinical efficacy has been demonstrated to date; however, most trials have demonstrated immunologic activity [21].

3. Novel Therapies in Castration-Resistant Prostate Cancer: The Blockade of Immune Checkpoints

3.1. CTLA-4-Based Immunotherapy. The first monoclonal antibody (ipilimumab, Yervoy™) directed against the control molecule, CTLA-4 [22], was approved for melanoma to improve survival and increase antitumor efficacy. CTLA-4 is a protein receptor that belongs to the immune checkpoints. It downregulates immune responses. CTLA-4 appears on the surface of T lymphocytes activated by contact with the antigen and acts to inhibit further lymphocyte response. T cells require two signals to become fully activated. CD28 and CTLA-4 are T cell receptors that play a decisive role in initial activation and subsequent control of cellular immunity. CD28 transmits a stimulatory signal to T cells. CTLA-4 is homologous to CD28. Both molecules bind to B7 on APCs. CTLA-4 binds to B7 with a greater affinity and avidity than CD28 thus enabling it to outcompete CD28 for its ligands. Ipilimumab is a fully human monoclonal antibody that decreases the binding of CTLA-4 to B7, which results in enhanced antitumor immunity [23]. A phase III trial in which men with CRPC that had progressed after docetaxel chemotherapy were treated with radiation therapy to a bone metastasis followed by either ipilimumab or placebo indicated that ipilimumab can prolong median OS in a select subset of patients lacking visceral disease and with favorable laboratory values [24]. However, patients who did not receive docetaxel did not achieve overall survival benefit, but it was suggested that patients with visceral metastases had poorer prognosis [22]. Combining ipilimumab with prostate cancer vaccines appears even more beneficial for the patients [24, 25].

3.2. Roles of the PD-1, PD-L1, and PD-L2 Pathway in Healthy Hosts. PD-1 (programmed cell death receptor-1) (also known as CD279) belongs to the CD28 (or B7) family and is coded by the PDCD-1 gene (programmed cell death genes) which is localized on chromosome 2 (2q37). PD-1 is a type I transmembrane glycoprotein composed of 288 amino acids [26]. PD-1 is expressed on the cellular surface of activated T cells (cytotoxic T lymphocytes (CTLs)) and B cells and on the activated monocytes, dendritic cells and natural killer (NK) cells, NKT cells, dendritic cells (DCs), and macrophages [27]. PD-1 is responsible for regulating immune responses and programmed cell death. PD-1 participates in induction and supports peripheral T cell immunity [28]. The crucial role of this immune checkpoint receptor in the inflammatory process is to reduce T cell activity in peripheral tissue, preventing autoimmunity [29].

Table 3: The blockade of immune checkpoints in prostate cancer.

Drug	Agent description
Ipilimumab	The inhibition of CTLA-4
Pembrolizumab (previously known as MK-3475 and lambrolizumab)	The inhibition of PD-1
Pidilizumab	The inhibition of PD-1

PD-1 can bind to one of its two ligands, programmed cell death protein ligand 1 (PD-L1) or programmed cell death protein ligand 2 (PD-L2). Both of them are expressed on the surface of tumor cells and correlate with patient prognosis [30]. PD-1 signaling inhibits allogeneic activation of T cells and may promote inducible regulation of T cell development. Furthermore, it influences several control points of the cell cycle [31–33]. Ligands for the PD-1 receptor are located on the surface of APCs and target cells (tumor cells). Interaction of PD-L1 or PD-L2 with the PD-1 antigen located on the surface of lymphocyte causes inhibition of their activity and leads to the blockade of immune response [34].

Recent studies reported successful use of an anti-PD-1 antibody in the treatment of advanced melanoma (FDA-approved pembrolizumab in September 2014) and metastatic melanoma (FDA-approved nivolumab in December 2014) [35, 36]. Moreover, nivolumab was approved for metastatic or advanced non-small-cell lung cancer in March 2015 [37]. There are only two clinical trials with anti-PD-L1 and anti-PD-L2 for prostate cancer. Pembrolizumab is being investigated in a phase II study in metastatic CRPC after androgen deprivation therapy (NCT02312557) [38]. Pidilizumab in combination with sipuleucel-T and cyclophosphamide is being studied in metastatic CRPC (NCT01420965) [39] (Table 3).

3.3. Cytokines in Prostate Cancer Immunotherapy. Stimulation of the immune system by affecting the cytokines may result in a strong antitumor immunity [40]. It is associated with the activity of innate and adaptive immune system [41]. Studies on PC demonstrated elevated levels of numerous interleukins such as IL-1α, IL-2, IL-4, IL-6, IL-7, IL-11, IL-12, IL-15, IL-17, IL-18, IL-27, and IL-35 [42–51]. The levels of interleukins usually correlate with the progression of PC including metastasis [42–51]. Recent studies focus on the possibility of blocking interleukins or their receptors. Cheng et al. showed that mesenchymal stem cells pretreated with IL-1α promoted the growth of prostate RM-1 mouse cancer cell line [42]. Dieli et al. in a phase I clinical trial investigated implications of the $\gamma\delta$ T cell agonist zoledronate with or without IL-2 for metastatic hormone-resistant prostate cancer. Most patients who received only zoledronate had progressive clinical deterioration, while a combination with IL-2 induced better clinical response [44]. Mackiewicz et al. demonstrated that vaccination with TRAMP-H6 (vaccines modified with hyper-IL-6) and TRAMP-H11 (vaccines

modified with hyper-IL-11) extends OS of mice with PC [45]. Recent studies indicate that the inflammatory process initiated by IL-17 may induce the progression of PC [52–54]. Yang et al. showed that expression of PD-1 and its ligands was higher in IL-17rc wild-type mouse PCs than IL-17rc-knockout mouse PCs. Furthermore, PD-1 expression was found primarily in the infiltrating inflammatory cells, while that of PD-L1 and PD-L2 was found in the neoplastic epithelial cells. It is suggested that elevated expression of PD-1 and its ligands promotes murine PC progression [7].

3.4. The Multitude of Different Potential Treatment Combinations for Prostate Cancer. The heterogeneity of prostate cancer, treatment resistance, and the growing need for individual therapy guide the latest research into combining different approaches. Although various drugs are available now in clinical practice, the potential toxicity resulting from their interactions has to be assessed [55, 56].

There are ongoing studies on combination vaccines with androgen deprivation therapy (ADT) and radiotherapy (RT). RT induces vaccination on T cells by releasing tumor antigens and soluble proinflammatory mediators. ADT, on the other hand, promotes lymphopoiesis, immune cell trafficking, and tumor penetration. Both strategies may be used in conjunction with immunotherapy. Clearly, maximum synergy can be achieved by thoroughly investigating each intervention at the exact phase of the immune response induced by therapy. The immune modulation is rather difficult and depends on many factors such as ADT type, RT strategy (type, dose, and duration), and administered immunotherapeutic agent. In a pilot study of intratumoral DC administration, patients remaining in the therapy of androgen suppression (GnRH agonist and bicalutamide) underwent external beam radiotherapy (EBRT) [55]. One patient had transient preexisting T cell responses to PSA, PSMA, and Her2/neu. Larger studies are needed to determine the optimal use of DC-based immunotherapy with RT-induced apoptosis and inflammatory responses [57].

A multicenter randomized phase II study tests active DCVAC/PCa cell immunotherapy in patients with localized high-risk prostate cancer after primary RT. The purpose of this study is to determine whether DCVAC/PCa can improve PSA progression times. This study is ongoing. The estimated completion date for the study is scheduled for September 2018 [58].

Another randomized phase II clinical trial investigates the role of ^{153}Sm-EDTMP (Quadramet) with or without a PSA/TRICOM vaccine in men with androgen-insensitive metastatic prostate cancer. Patients treated with both PSA-TRICOM and ^{153}Sm-EDTMP were found to have an increase in PSA-specific T cell lymphocytes and lower levels of circulating myeloid-derived suppressor cell (MDSC) subgroups compared to patients in the ^{153}Sm-EDTMP alone after 60 days of therapy. Although a statistically significant difference in overall survival was observed, patients receiving ^{153}Sm-EDTMP and PSA-TRICOM experienced more than twice the disease progression compared to those receiving ^{153}Sm-EDTMP alone (3.7 months versus 1.7 months, resp.). This vaccination strategy resulted in a strong immunological response in tumor biopsy, with a marked prolongation of PSA doubling time [59].

The alternative approach addresses CTLA-4, PD-1, PD-L1, and PD-L2. McNeel et al.6 tested tremelimumab in combination with bicalutamide in biochemically recurrent PC after local therapy. Eleven patients were enrolled and completed at least 1 year of follow-up. Monotherapy of ipilimumab resulted in decreased PSA level; however, the primary endpoint was not reached [60]. The early results of phase II studies on pembrolizumab in combination with enzalutamide showed a complete PSA response in 3/10 patients. Tumor regression indicates a great potential for a combination of immune checkpoint blockades for PC [61].

4. Conclusions

Science's editors have chosen cancer immunotherapy as the breakthrough of the year for 2013 [62]. Neoplastic cells naturally escape from the control of the immune system, and the main goal of immune therapy is to bring the control back.

Satisfying outcomes after treatment of advanced melanoma and lung cancer suggest a great potential of immunotherapy as an approach for other tumors' treatment, especially in patients primarily introduced to palliative care.

After initial clinical trials, immunotherapy seems to have different side effects than chemotherapy [63, 64]. Prostate cancer was the first neoplasm in which a specific vaccine significantly improved survival. Preliminary studies on new drugs blocking the immune checkpoints in CRPC indicate that it might be a solution for these patients. There is a tremendous potential for synergistic combinations of immunotherapy with conventional cancer treatments [65]. A combination of several drugs or methods can be a key in radical treatment of metastatic prostate cancer as demonstrated by preliminary studies. We definitely need more studies to clearly define the role of immunotherapy in the treatment of advanced prostate cancer.

Conflicts of Interest

The authors declare no conflict of interests for this article.

References

[1] http://www.cancer.org/cancer/prostatecancer/detailedguide/prostate-cancer-key-statistics.

[2] http://www.cancer.org/acs/groups/content/@research/documents/document/acspc-047079.pdf.

[3] A. B. Weiner, R. S. Matulewicz, S. E. Eggener, and E. M. Schaeffer, "Increasing incidence of metastatic prostate cancer in the United States (2004–2013)," *Prostate Cancer and Prostatic Disease*, vol. 19, pp. 395–397, 2016.

[4] R. Ren, M. Koti, T. Hamilton et al., "A primer on tumour immunology and prostate cancer immunotherapy," *Canadian Urological Association Journal*, vol. 10, no. 1-2, pp. 60–65, 2016.

[5] G. T. Wurz, C.-J. Kao, and M. W. DeGregorio, "Novel cancer antigens for personalized immunotherapies: latest evidence and clinical potential," *Therapeutic Advances Medical Oncology*, vol. 8, no. 1, pp. 4–31, 2016.

[6] J. R. Brahmer, C. G. Drake, I. Wollner et al., "Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates," *Journal of Clinical Oncology*, vol. 28, no. 19, pp. 3167–3175, 2010.

[7] S. Yang, Q. Zhang, S. Liu, A. R. Wang, and Z. You, "PD-1, PD-L1 and PD-L2 expression in mouse prostate cancer," *American Journal of Clinical and Experimental Urology*, vol. 4, no. 1, pp. 1–8, 2016.

[8] M. Black, I. B. Barsoum, P. Truesdell et al., "Activation of the PD-1/PD-L1 immune checkpoint confers tumor cell chemoresistance associated with increased metastasis," *Oncotarget*, vol. 7, no. 9, pp. 10557–10567, 2016.

[9] I. Silvestri, S. Cattarino, S. Giantulli, C. Nazzari, G. Collalti, and A. Sciarra, "A perspective of immunotherapy for prostate cancer," *Cancers*, vol. 8, no. 7, 2016.

[10] L. Matera, "The choice of the antigen in the dendritic cell-based vaccine therapy for prostate cancer," *Cancer Treatment Reviews*, vol. 36, no. 2, pp. 131–141, 2010.

[11] P. W. Kantoff, C. S. Higano, N. D. Shore et al., "Sipuleucel-T immunotherapy for castration-resistant prostate cancer," *The New England Journal of Medicine*, vol. 363, no. 5, pp. 411–422, 2010.

[12] P. F. Schellhammer, G. Chodak, J. B. Whitmore, R. Sims, M. W. Frohlich, and P. W. Kantoff, "Lower baseline prostate-specific antigen is associated with a greater overall survival benefit from sipuleucel-T in the Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) trial," *Urology*, vol. 81, no. 6, pp. 1297–1302, 2013.

[13] P. Holko and P. Kawalec, "Economic evaluation of sipuleucel-T immunotherapy in castration-resistant prostate cancer," *Expert Review of Anticancer Therapy*, vol. 14, no. 1, pp. 63–73, 2014.

[14] N. A. Sheikh, D. Petrylak, P. W. Kantoff et al., "Sipuleucel-T immune parameters correlate with survival: an analysis of the randomized phase 3 clinical trials in men with castration-resistant prostate cancer," *Cancer Immunology Immunotherapy*, vol. 62, no. 1, pp. 137–147, 2013.

[15] P. F. Mulders, M. De Santis, T. Powles, and K. Fizazi, "Targeted treatment of metastatic castration-resistant prostate cancer with sipuleucel-T immunotherapy," *Cancer Immunology Immunotherapy*, vol. 64, no. 6, pp. 655–663, 2015.

[16] P. M. Arlen, L. Skarupa, M. Pazdur et al., "Clinical safety of a viral vector based prostate cancer vaccine strategy," *The Journal of Urology*, vol. 178, pp. 1515–1520, 2007.

[17] P. W. Kantoff, T. J. Schuetz, B. A. Blumenstein et al., "Overall survival analysis of a phase II randomized controlled trial of a poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer," *Journal of Clinical Oncology*, vol. 28, no. 7, pp. 1099–1105, 2010.

[18] J. L. Gulley, P. M. Arlen, R. A. Madan et al., "Immunologic and prognostic factors associated with overall survival employing a poxviral-based PSA vaccine in metastatic castrate-resistant prostate cancer," *Cancer Immunology Immunotherapy*, vol. 59, no. 5, pp. 663–674, 2010.

[19] M. Podrazil, R. Horvath, E. Becht et al., "Phase I/II clinical trial of dendritic-cell based immunotherapy (DCVAC/PCa) combined with chemotherapy in patients with metastatic, castration-resistant prostate cancer," *Oncotarget*, vol. 6, no. 20, pp. 18192–18205, 2015.

[20] G. Cole, J. McCaffrey, A. A. Ali, and H. O. McCarthy, "DNA vaccination for prostate cancer: key concepts and considerations," *Cancer Nanotechnology*, vol. 6, no. 1, p. 2, 2015.

[21] V. T. Colluru, L. E. Johnson, B. M. Olson, and D. G. McNeel, "Preclinical and clinical development of DNA vaccines for prostate cancer," *Urologic Oncology*, vol. 34, no. 4, pp. 193–204, 2016.

[22] F. S. Hodi, S. J. O'Day, D. F. McDermott et al., "Improved survival with ipilimumab in patients with metastatic melanoma," *The New England Journal of Medicine*, vol. 363, pp. 711–723, 2010.

[23] D. Hannani, M. Vétizou, D. Enot et al., "Anticancer immunotherapy by CTLA-4 blockade: obligatory contribution of IL-2 receptors and negative prognostic impact of soluble CD25," *Cell Research*, vol. 25, no. 2, pp. 208–224, 2015.

[24] E. D. Kwon, C. G. Drake, H. I. Scher et al., "Ipilimumab versus placebo after radiotherapy in patients with metastatic castration-resistant prostate cancer that had progressed after docetaxel chemotherapy (CA184-043): a multicentre, randomised, double-blind, phase 3 trial," *The Lancet Oncology*, vol. 15, no. 7, pp. 700–712, 2014.

[25] R. A. Madan, C. R. Heery, and J. L. Gulley, "Combination of vaccine and immune checkpoint inhibitor is safe with encouraging clinical activity," *OncoImmunology*, vol. 1, no. 7, pp. 1167-1168, 2012.

[26] T. Okazaki, A. Maeda, H. Nishimura, T. Kurosaki, and T. Honjo, "PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 24, pp. 13866–13871, 2001.

[27] L. Chen, "Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity," *Nature Reviews Immunology*, vol. 4, pp. 336–347, 2004.

[28] G. J. Freeman, A. J. Long, Y. Iwai et al., "Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation," *The Journal of Experimental Medicine*, vol. 192, no. 7, pp. 1027–1034, 2000.

[29] D. M. Pardoll, "The blockade of immune checkpoints In cancer immunotherapy," *Nature Reviews Cancer*, vol. 12, no. 4, pp. 252–264, 2012.

[30] B. T. Fife, K. E. Pauken, T. N. Eagar et al., "Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal," *Nature Immunology*, vol. 10, no. 11, pp. 1185–1192, 2009.

[31] N. Patsoukis, J. Brown, V. Petkova, F. Liu, L. Li, and V. A. Boussiotis, "Selective effects of PD-1 on Akt and Ras pathways regulate molecular components of the cell cycle and inhibit T cell proliferation," *Science Signaling*, vol. 5, no. 230, p. 46, 2012.

[32] P. Chatterjee, N. Patsoukis, G. J. Freeman, and V. A. Boussiotis, "Distinct roles Of PD-1 Itsm and ITIM in regulating interactions with SHP-2, ZAP-70 and Lck, and PD-1-mediated inhibitory function," *Blood*, vol. 122, p. 191, 2013.

[33] M. A. Postow, M. K. Callahan, and J. D. Wolchok, "Immune checkpoint blockade in cancer therapy," *Journal of Clinical Oncology*, vol. 33, no. 17, pp. 1974–1982, 2015.

[34] X. Zhang, J. C. Schwartz, X. Guo et al., "Structural and functional analysis of the costimulatory receptor programmed death-1," *Immunity*, vol. 20, no. 3, pp. 337–347, 2004.

[35] P. Sharma and J. P. Allison, "Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential," *Cell*, vol. 161, no. 2, pp. 205–214, 2015.

[36] L. A. Raedler and M. Writer, "Opdivo (nivolumab): second PD-1 inhibitor receives FDA approval for unresectable or metastatic melanoma," *American Health & Drug Benefits*, vol. 8, pp. 180–183, 2015.

[37] D. Kazandjian, D. L. Suzman, G. Blumenthal et al., "FDA approval summary: nivolumab for the treatment of metastatic non-small cell lung cancer with progression on or after platinum-based chemotherapy," *The Oncologist*, vol. 21, no. 5, pp. 634–642, 2016.

[38] *ClinicalTrials.gov Identifier: NCT02312557, Pembrolizumab in Treating Patients With Metastatic Castration Resistant Prostate Cancer Previously Treated With Enzalutamide*.

[39] https://clinicaltrials.gov/ct2/show/NCT01420965.

[40] D. W. Kowalczyk, P. J. Wysocki, and A. Mackiewicz, "Cancer immunotherapy using cells modified with cytokine genes," *Acta Biochemica Polonica*, vol. 50, no. 3, pp. 613–624, 2003.

[41] G. P. Dunn, L. J. Old, and R. D. Schreiber, "The immunobiology of cancer immunosurveillance and immunoediting," *Immunity*, vol. 21, no. 2, pp. 137–148, 2004.

[42] J. Cheng, L. Li, Y. Liu, Z. Wang, X. Zhu, and X. Bai, "Interleukin-1α induces immunosuppression by mesenchymal stem cells promoting the growth of prostate cancer cells," *Molecular Medicine Reports*, vol. 6, no. 5, pp. 955–960, 2012.

[43] F. Dieli, D. Vermijlen, F. Fulfaro et al., "Targeting human $\gamma\delta$ T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer," *Cancer Research*, vol. 67, no. 15, pp. 7450–7457, 2007.

[44] K. Fujio, M. Watanabe, H. Ueki et al., "A vaccine strategy with multiple prostatic acid phosphatase-fused cytokines for prostate cancer treatment," *Oncology Reports*, vol. 33, no. 4, pp. 1585–1592, 2015.

[45] J. Mackiewicz, U. Kazimierczak, M. Kotlarski et al., "Cellular vaccines modified with hyper IL6 or hyper IL11 combined with docetaxel in an orthotopic prostate cancer model," *Anticancer Research*, vol. 35, no. 6, pp. 3275–3288, 2015.

[46] S. Zhang, G. Zeng, D. S. Wilkes et al., "Dendritic cells transfected with interleukin-12 and pulsed with tumor extract inhibit growth of murine prostatic carcinoma in vivo," *The Prostate*, vol. 55, no. 4, pp. 292–298, 2003.

[47] J. C. Morris, C. A. Ramlogan-Steel, P. Yu et al., "Vaccination with tumor cells expressing IL-15 and IL-15Rα inhibit murine breast and prostate cancer," *Gene Therapy*, vol. 21, no. 4, pp. 393–401, 2014.

[48] E. Derhovanessian, V. Adams, K. Hähnel et al., "Pretreatment frequency of circulating IL-17$^+$CD4$^+$ T-cells, but not Tregs, correlates with clinical response to whole-cell vaccination in prostate cancer patients," *International Journal of Cancer*, vol. 125, no. 6, pp. 1372–1379, 2009.

[49] B. W.-C. Tse, P. J. Russell, M. Lochner, I. Förster, and C. A. Power, "IL-18 inhibits growth of murine orthotopic prostate carcinomas via both adaptive and innate immune mechanisms," *PLoS One*, vol. 6, no. 9, article e24241, 2011.

[50] E. Di Carlo, C. Sorrentino, A. Zorzoli et al., "The antitumor potential of interleukin-27 in prostate cancer," *Oncotarget*, vol. 5, no. 21, pp. 10322–10341, 2013.

[51] B. M. Olson, E. Jankowska-Gan, J. T. Becker, D. A. A. Vignali, W. J. Burlingham, and D. G. McNeel, "Human prostate tumor antigen-specific CD8$^+$ regulatory T cells are inhibited by CTLA-4 or IL-35 blockade," *Journal of Immunology*, vol. 189, no. 12, pp. 5590–5601, 2012.

[52] X. Chen, M. J. Churchill, K. K. Nagar et al., "IL-17 producing mast cells promote the expansion of myeloid-derived suppressor cells in a mouse allergy model of colorectal cancer," *Oncotarget*, vol. 6, pp. 32966–32979, 2015.

[53] H. Lee, B. Kim, Y. H. Choi et al., "Inhibition of interleukin-1β-mediated interleukin-1 receptor-associated kinase 4 phosphorylation by zinc leads to repression of memory T helper type 17 response in humans," *Immunology*, vol. 146, no. 4, pp. 645–656, 2015.

[54] Y. Zeng, Q. Zhang, H. Wang et al., "Prognostic significance of interleukin-17 in solid tumors: a meta-analysis," *International Journal of Clinical and Experimental Medicine*, vol. 8, no. 7, pp. 10515–10536, 2015.

[55] F. Van Rhee, J. Szymonifka, E. Anaissie et al., "Total therapy 3 for multiple myeloma: prognostic implications of cumulative dosing and premature discontinuation of vtd maintenance components, bortezomib, thalidomide, and dexamethasone, relevant to all phases of therapy," *Blood*, vol. 116, pp. 1220–1227, 2010.

[56] J. G. Lohr, P. Stojanov, S. L. Carter et al., "Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy," *Cancer Cell*, vol. 25, pp. 91–101, 2014.

[57] J. W. Hodge, H. Sabzevari, A. G. Yafal, L. Gritz, M. G. Lorenz, and J. Schlom, "A triad of costimulatory molecules synergize to amplify T-cell activation," *Cancer Research*, vol. 59, pp. 5800–5807, 1999.

[58] "NCT02107430—phase II study of DCVAC/PCa after primary radiotherapy for patients with high risk localized prostate cancer," November 2016, https://clinicaltrials.gov/ct2/show/NCT02107430.

[59] "NCT00450619—153Sm-EDTMP with or without a PSA/TRICOM vaccine to treat men with androgen-insensitive prostate cancer," November 2016, https://clinicaltrials.gov/ct2/show/NCT00450619.

[60] J. N. Graff, J. J. Alumkal, C. G. Drake et al., "Early evidence of anti-PD-1 activity in enzalutamide-resistant prostate cancer," *Oncotarget*, vol. 25, pp. 4–13, 2016.

[61] "NCT00861614—study of immunotherapy to treat advanced prostate cancer," November 2016, https://clinicaltrials.gov/ct2/show/NCT00861614.

[62] S. L. Topalian, F. Stephen Hodi, J. R. Brahmer et al., "Safety, activity, and immune correlates of anti–PD-1 antibody in cancer," *The New England Journal of Medicine*, vol. 366, no. 26, pp. 2443–2454, 2012.

[63] B. S. CiRen, X. Wang, and Z. Long, "The evaluation of immunotherapy and chemotherapy treatment on melanoma: a network meta-analysis," *Oncotarget*, vol. 7, pp. 81493–81511, 2016.

[64] T. Zhang, J. Xie, S. Arai et al., "The efficacy and safety of anti-PD-1/PD-L1 antibodies for treatment of advanced or refractory cancers: a meta-analysis," *Oncotarget*, vol. 7, pp. 73068–73079, 2016.

[65] J. Couzin-Frankel, "Cancer immunotherapy," *Science*, vol. 342, no. 6165, pp. 1432-1433, 2013.

Hindawi
Journal of Immunology Research
Volume 2017, Article ID 5737159, 14 pages
https://doi.org/10.1155/2017/5737159

Research Article

Enavatuzumab, a Humanized Anti-TWEAK Receptor Monoclonal Antibody, Exerts Antitumor Activity through Attracting and Activating Innate Immune Effector Cells

Shiming Ye,[1] Melvin I. Fox,[1] Nicole A. Belmar,[1] Mien Sho,[1] Debra T. Chao,[1] Donghee Choi,[1] Yuni Fang,[1] Vivian Zhao,[1] Stephen F. Keller,[1] Gary C. Starling,[2] and Patricia A. Culp[3]

[1]*AbbVie Biotherapeutics Inc., Redwood City, CA 94063, USA*
[2]*Merck, Palo Alto, CA 94304, USA*
[3]*Alector LLC, South San Francisco, CA 94080, USA*

Correspondence should be addressed to Shiming Ye; shiming.ye@abbvie.com

Received 19 May 2017; Accepted 27 July 2017; Published 17 September 2017

Academic Editor: Qing Lin

Enavatuzumab is a humanized IgG1 anti-TWEAK receptor monoclonal antibody that was evaluated in a phase I clinical study for the treatment of solid malignancies. The current study was to determine whether and how myeloid effector cells were involved in postulated mechanisms for its potent antitumor activity in xenograft models. The initial evidence for a role of effector cells was obtained in a subset of tumor xenograft mouse models whose response to enavatuzumab relied on the binding of Fc of the antibody to Fcγ receptor. The involvement of effector cells was further confirmed by immunohistochemistry, which revealed strong infiltration of $CD45^+$ effector cells into tumor xenografts in responding models, but minimal infiltration in nonresponders. Consistent with the xenograft studies, human effector cells preferentially migrated toward *in vivo*-responsive tumor cells treated by enavatuzumab *in vitro*, with the majority of migratory cells being monocytes. Conditioned media from enavatuzumab-treated tumor cells contained elevated levels of chemokines, which might be responsible for enavatuzumab-triggered effector cell migration. These preclinical studies demonstrate that enavatuzumab can exert its potent antitumor activity by actively recruiting and activating myeloid effectors to kill tumor cells. Enavatuzumab-induced chemokines warrant further evaluation in clinical studies as potential biomarkers for such activity.

1. Introduction

Functional antibodies targeting cell surface receptors with the ability to induce signaling represent a relatively new class of therapeutic antibodies. Members of the TNF receptor super family (TNFRSF) are attractive targets for developing functional antibodies since stimulating this class of receptors potently regulates a wide variety of biological functions. Several antibodies targeting the members of TNFRSF, such as anti-TRAILR, anti-OX40, anti-CD40, and anti-4-1BB, have been developed and evaluated in preclinical studies or in clinical trials [1]. Enavatuzumab (also called PDL192) is a humanized IgG1 monoclonal antibody targeting the receptor of TNF-like weak inducer of apoptosis (TWEAK), one of the TNFRSF members, also known as Fn14 or TNFRSF12A [2, 3]. TWEAK is the natural ligand of the TWEAK receptor (TweakR), which stimulates multiple cellular responses, including proliferation, differentiation, apoptosis, and migration, as well as wound repair and inflammation [4, 5].

Although TWEAK has pleiotropic functions, it was initially identified as a weak inducer of apoptosis [6]. Additional studies further indicated that TWEAK can induce multiple cancer cell lines to undergo caspase-dependent apoptosis, and cell death can be further enhanced when combined with TNFα or IFNγ treatment [7–9]. Since TweakR is overexpressed in multiple tumors, such as breast cancer, lung cancer, ovarian cancer, glioma, and endometrial cancer [10–15], several functional anti-TweakR antibodies have been investigated for treating cancers [16]. Due to the relatively low expression of TweakR in normal tissues, an

immunotoxin-conjugated TweakR antibody has been tested in preclinical cancer models [17, 18]. We also reported previously that the antitumor activity of enavatuzumab has been attributed to three distinct mechanisms of action: (1) direct killing of tumor cells by inducing caspase-3/7 activation, (2) growth inhibition of tumor cell lines through p21-mediated cell cycle arrest, and (3) via antibody dependent cell-mediated cytotoxicity (ADCC) [2, 19].

Depletion of target cells through ADCC has been implicated as a major mechanism for therapeutic antibodies, including rituximab, alemtuzumab, and trastuzumab in treating both hematologic malignancies and solid tumors [20]. In addition to this conventional role in mediating ADCC, the interaction of Fc and the Fcγ receptor (FcγR) also provides a means to crosslink the antibodies, which for agonist antibodies enhances their signaling potential upon binding to the target antigen, as was recently confirmed for anti-DR5 antibodies [21]. The availability of Fc receptor-positive cells is therefore critical for antibodies to function through ADCC as well as through targets. Results from clinical studies in breast cancer patients indicate that increased numbers of lymphocytes within the tumor correlates with improved response to trastuzumab [22]. Thus, the presence of effector cells within the tumor microenvironment may be required for antibodies to best achieve their antitumor activities. Although ADCC function has been extensively studied preclinically for many therapeutic antibodies, very few studies have focused on whether therapeutic antibodies can actively recruit FcγR-positive cells into the tumor microenvironment, which may enhance ADCC or agonistic activity of therapeutic mAb *in vivo*.

As previously described, enavatuzumab induces tumor growth inhibition through direct TweakR signaling and ADCC. However, this may be an oversimplification of the mechanisms of this antibody given the pleiotropic nature of TweakR-mediated signaling. In the current study, we focused on the interaction of enavatuzumab with tumor cells and immune effector cells. We found that enavatuzumab treatment results in activation of immune effector cells and infiltration of immune cells into the tumors in mice bearing xenograft tumors sensitive to the antibody. We also showed that enavatuzumab stimulates migration of human immune cells *in vitro* toward tumor cells sensitive to enavatuzumab and that MCP-1 is a key driver of this migration. MCP-1 was also found to be increased in the serum of mice and in human patients after enavatuzumab treatment, suggesting that the preclinical findings may translate into the clinical setting.

2. Methods

2.1. Cell Lines and Therapeutic Antibodies. Tumor cell lines H520, A375, HCT116, and DLD-1 cells were obtained from ATCC, while SN12C was purchased from NCI. H520 lung cancer cells, SN12C renal cancer cells, and HCT116 and DLD-1 colorectal cancer cells were maintained in RPMI, and A375 melanoma cells were maintained in DMEM. H520 cells were transfected with a TweakR expression construct to generate H520-TweakR cell line. All cells were maintained and assays were done in the appropriate growth media containing fetal bovine serum (10%), unless otherwise indicated. All cell culture media and serum were purchased from Hyclone (Thermo Fisher Scientific).

Enavatuzumab and the human IgG1 isotype control (MSL109) have been described previously [2]. The enavatuzumab Fc mutant 1 is on a human IgG1 backbone that contains the L234A/L235A mutations in the Fc region (huIgG1-LALA), while the enavatuzumab Fc mutant 2 variant is a human IgG2 isotype containing the V234A/G236A mutations (hIgG2-VAGA).

2.2. Animal Models. Tumor cells were inoculated subcutaneously into the right flank of 6-week old severe combined immunodeficient (SCID) mice (IcrTac:ICR-Prkdc$^{<scid>}$, Taconic, Germantown, NY) at 1×10^7 cells per mouse. Animals were randomized into groups when the mean tumor volume reached 110–160 mm^3. Antibodies were administered intraperitoneally at 10 mg/kg, unless otherwise indicated.

For efficacy studies, tumor volumes ($L \times W \times H/2$) were generally measured on each dosing day; the group means $\pm$ SEM is displayed. Groups were removed from the study when at least one tumor in the group reached the allowable limit (1500 mm^3). The statistical significance of the differences between groups was determined by *t*-test using SAS statistical software (version 9). Mean tumor volumes between groups were considered significantly different if $p < 0.05$.

For tumor samples collected for immunohistochemistry, animals were administered antibody on days 0 and 2 or 3, and tumors were harvested on day 4.

For cytokine measurements, A375 tumor-bearing mice were given a single dose of antibody, and blood samples were taken up to 14 days after antibody dose. Cytokine levels were measured in serum by Luminex® (Millipore, Billerica, MA), according to the manufacturer's instructions.

All animal work was carried out under NIH guidelines "Guide for the Care and Use of Laboratory Animals" using AbbVie Biotherapeutics IACUC approved protocols.

2.3. Phenotyping of Mouse Splenocytes. SN12C or HCT116 tumor-bearing mice were given 7 or 9 doses, respectively, of enavatuzumab or a control antibody (10 mg/kg three times per week). Three days after the last antibody dose, spleens were harvested from 5–7 mice in each group, and isolated splenocytes were stained with conjugated staining antibodies from BD Bioscience (San Jose, CA): CD45-FITC, CD11b-APC-Cy7, DX5-PE, and biotinylated CD27. FACS data were collected by FACSCanto™ (BD Biosciences, San Jose, CA) and analyzed with Flowjo (Tree Star, Ashland, OR).

2.4. Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay. The ADCC activity of enavatuzumab wild-type or mutant antibodies was measured by Cr-51 release as described previously [2] using human peripheral blood mononuclear cells (PBMCs) as effectors and TweakR-positive tumor cells as targets. In brief, target cells were labeled with 50 μCi of Cr-51 (Perkin Elmer, Waltham, MA) per 1×10^6 cells for 1 hour (hr) at 37°C. Labeled target cells were mixed and incubated with serially diluted antibody for

30 min at 4°C. PBMCs were prepared from fresh whole blood using a Ficoll-Paque Plus gradient (GE Healthcare Biosciences, Pittsburgh, PA). PBMCs were then added to the opsonized cells at a E : T ratio at 40 : 1 and incubated for 4 hrs at 37°C in a CO_2 incubator. Antibody-independent cell-mediated cytotoxicity (AICC) was measured by incubating effector and target cells in the absence of antibody. Maximum release (MR) was measured by adding 2% Triton X-100 to target cells. Spontaneous release (SR) was measured by incubating target cells in the absence of antibody. After 4 hrs, the plates were gently centrifuged and Cr-51 release was measured by counting 100 μl of cell-free supernatant in a Wizard 1470 gamma counter (Perkin Elmer). The percent cytotoxicity was calculated as $[(\text{Sample} - \text{SR})/(\text{MR} - \text{SR})] \times 100$.

2.5. In Vitro Coculture Assay. PBMCs from healthy human donors were added to 24-well plates, either alone or into wells that contained SN12C cells that had been plated 24 hrs previously. The cultures were incubated with enavatuzumab or a control antibody (10 μg/mL) for 24 hrs, after which the immune cells were removed to measure activation markers by flow cytometry. Immune cells were stained for monocytes and nature killer (NK) cells with fluorochrome-conjugated antibodies purchased from BD Biosciences: CD3-FITC, CD54-PE, CD16-PerCP Cy5.5, CD14-PE Cy7, CD56-APC, and CD69-APC Cy7. In some experiments, tumor cell cytotoxicity was assessed after 24 hr culture by measuring the level of cytokeratin18 in the supernatant by M65® ELISA (Peviva, Bromma, Sweden), according to the manufacturer's instructions.

2.6. In Vitro Migration Assay. A total of 6×10^4 tumor cells were plated into the bottom well of 24-well Transwell® plates (Corning Inc., Corning, NY) and incubated with antibodies (10 μg/mL). Twenty-four hours later, 2×10^5 PBMCs from healthy donors were added to the top well (5 μm) of the Transwell plate and incubated for additional 4 hrs. Wells containing no tumor cells in the bottom chamber were used to quantify spontaneous migration. In some experiments, anti-human MCP-1 or anti-human IL-8 (R&D Systems, Minneapolis, MN) was added 30 min before the addition of PBMC. The total number of immune cells that had migrated into the bottom well was quantified by FACS using polystyrene beads (Polysciences, Warrington PA). Specific migration was calculated by $100 \times ((\text{number of cells that migrated toward tumor cells} - \text{number of cells that migrated spontaneously})/\text{number of PBMCs seeded})$. In some experiments, the supernatants from antibody-treated tumor cells were measured for cytokine production by Luminex, and immune cells that had migrated were phenotyped by collecting migrated cells and staining them with antibodies from BD Biosciences: CD3-FITC, CD56/CD16-PE, CD4-PerCP Cy5.5, CD14-PE Cy7, CD11c-APC, and CD20-APC Cy7. Analysis was performed on a FACSCanto flow cytometer.

2.7. Immunohistochemistry Staining. Tumor xenografts were harvested, fixed in 10% buffered formalin, and embedded in paraffin. Five-micrometer sections were deparaffinized, and antigen retrieval was performed in BORG solution (Biocare Medical, Concord, CA), followed by blocking with Background Sniper (Biocare Medical). Slides were incubated with anti-mouse CD45 antibody (rat IgG2b) or a control rat IgG2b antibody (BD Bioscience) and detected using the Rat on Mouse HRP detection system (Biocare Medical). Slides were then incubated with diaminobenzidine for 5 minutes and were counterstained with hematoxylin. Mouse effector cells were identified by CD45-positive staining.

2.8. Cytokine Measurements in Human Serum Samples. Serum samples were collected from patients in the enavatuzumab phase 1 study according to the study protocol. A 12-plex Luminex assay from Millipore was validated to measure cytokine/chemokine/growth factor levels in human serum. The assay kit contains capture antibodies for each analyte covalently bound to distinct color-coded microsphere subsets distinguished by differing dye ratios. Calibrators, controls, and study samples were incubated with microspheres in the wells of 96-well plates. The assay signal for each individual analyte was determined by measuring orange fluorescence produced by a complex of biotinylated analyte-specific antibodies and streptavidin-phycoerythrin as fluorescence intensity (FI) using a Luminex XMAP instrument. The concentration of each analyte was determined by using the FI value to extrapolate against the calibration curve generated from the regression of the FI values of the calibrators and their corresponding nominal concentrations.

2.9. Statistical Analysis. Data were analyzed using SAS statistical software (version 9) and GraphPad Prism software, version 4.03. They were subjected to one-way (treatment) ANOVA. When ANOVA revealed a significant effect of treatment, differences between treatments were tested using Duncan's multiple range tests.

3. Results

3.1. Enavatuzumab Exerted Potent ADCC on TweakR-Positive Tumor Cells through Activating Immune Effector Cells In Vitro. Enavatuzumab is a humanized IgG1 antibody, and Fc-mediated effector cell killing has been proposed as one of the mechanisms driving its antitumor activity. We previously reported that enavatuzumab ADCC on cells transfected with TweakR [2]. To further confirm the ability of enavatuzumab to induce ADCC *in vitro*, ADCC was evaluated on multiple endogenous TweakR-expressing tumor cell lines as targets [2]. Enavatuzumab showed potent tumor cell killing on all TweakR-positive tumor cells tested, including the renal carcinoma cell line SN12C, the melanoma cell line A375, and the colorectal cancer cell lines HCT116 and DLD-1 (Figure 1(a)).

ADCC is generally thought to be mediated by the activation of immune effector cells. To explore this further, immune cell activation by enavatuzumab was assessed in *in vitro* cocultures of human PBMCs and tumor cells. When PBMCs were cultured, enavatuzumab treatment did not alter the expression of CD54 and CD16 on monocytes or NK cells.

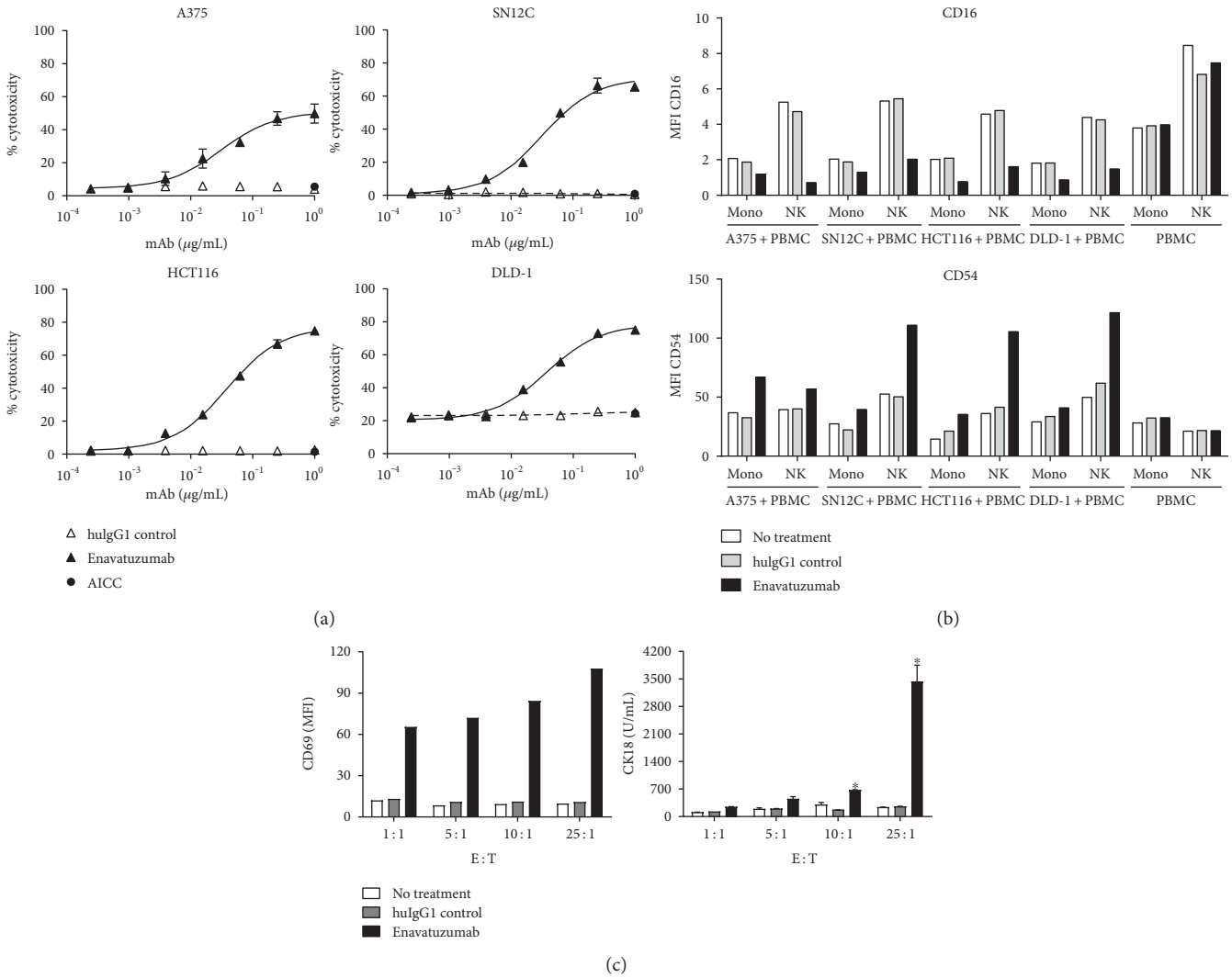


Figure 1: Enavatuzumab induces effector cell activation and tumor cell killing *in vitro*. (a) Human PBMCs were coincubated with Cr-51-labeled tumor target cells with E : T at 40 : 1 for 4 hr in the presence of enavatuzumab. Tumor cell cytotoxicity was calculated based on Cr-51 released into culture supernatant. Antibody-independent cell cytotoxicity (AICC) was also calculated. Experiments were performed with PBMCs from 4 donors. Representative data collected from one donor are shown. (b) Human PBMCs, cultured either alone or with indicated tumor cells at a 10 : 1 ratio, were treated with enavatuzumab or a control antibody. 24 hrs later, CD54 and CD16 levels were assessed on both monocytes (Mono) and NK cells by flow cytometry. Experiments were performed with PBMCs from 8 donors. Representative data collected from one donor were shown. (c) PBMCs were cultured with SN12C cells at the indicated ratios for 24 hr in the presence of enavatuzumab or a control antibody, after which CD69 was assessed on NK cells by flow cytometry (left) or cytokeratin18 levels were quantified in cell supernatants as a measure of tumor cell cytotoxicity (right). Enavatuzumab treatment increased CD69 levels at all E : T ratios tested (representative data from 4 donors) but significantly stimulated cytotoxicity only at 10 : 1 and 25 : 1 ratios ($n = 4$, $^*p < 0.05$).

In contrast, enavatuzumab treatment of cocultures of PBMCs with any of the four TweakR-expressing tumor cell lines resulted in activation of both monocytes and NK cells, as defined by upregulation of CD54 and downregulation of CD16 on both cell types (Figure 1(b)). To determine whether effector cell activation is sufficient to mediate tumor cell killing, we compared enavatuzumab-induced effector cell activation and tumor cell killing across a range of effector : target ratios (E : T). In cocultures of PBMCs and SN12C cells, NK activation, indicated by CD69 upregulation, was observed over a range of E : T, from 1 : 1 to 25 : 1 (Figure 1(c)). In contrast, enavatuzumab induced cytotoxicity of SN12C cells, indicated by CK18 production, started at E : T of 10 : 1, with marked cytotoxicity observed only at the highest E : T tested (25 : 1, Figure 1(c)). At high E : T, enavatuzumab stimulated cytotoxicity of all TweakR-expressing cells equivalently *in vitro* (Figure 1(a)). These data suggest that the quantity of effector cells is also essential for mediating tumor cell killing in addition to effector cell activation.

3.2. Enavatuzumab Induced Diverse Responses on TweakR-Positive Xenograft Tumors by Differentially Activating Immune Effector Cells In Vivo. We next attempted to translate the ability of enavatuzumab to kill a range of tumor cell

lines via ADCC *in vitro* into antitumor activity *in vivo*. Enavatuzumab has previously been shown to exhibit potent antitumor activity on human xenograft tumors implanted into ICR-SCID mice. ICR-SCID mice maintain a largely intact innate immune system and produce effector cells that express multiple Fcγ receptors. Enavatuzumab has been shown to induce ADCC *in vitro* using ICR-SCID mouse splenocytes as effector cells [2], suggesting that the human IgG1 Fc is able to bind effectively to mouse Fcγ receptors [23]. This binding was confirmed in flow cytometry assays measuring enavatuzumab binding on mouse CD11b^{high} splenocytes (Figure 2(a)). This interaction is likely through Fc-FcγR, as TweakR expression has not been detected on lymphoid cell types, regardless of their activation state [2, 24]. Moreover, enavatuzumab does not bind the mouse ortholog of human TweakR (data not shown). When tested on a range of xenograft tumor models, however, not all TweakR-positive tumors showed similar *in vivo* responses to enavatuzumab treatment. Some TweakR-expressing tumor cell lines, such as HCT116 and DLD-1, were not sensitive to enavatuzumab treatment *in vivo* (Figure 2(b)), though both cell lines were effectively killed via ADCC *in vitro* with enavatuzumab. Other TweakR-expressing cells, such as SN12C and A375, were sensitive to enavatuzumab treatment both *in vivo* and *in vitro*. However, the *in vivo* responses to enavatuzumab treatment in these cell lines appeared to rely on different mechanisms of action. In the SN12C model, an enavatuzumab Fc mutant variant, Fc mutant 2 (hIgG2-VAGA), with no binding to FcγR and no ADCC capability (Figure 2(a)), was unable to inhibit the growth of tumors, suggesting a critical role for Fc-FcγR interaction and/or ADCC in this model (Figure 2(b)). In contrast, a different Fc mutant variant, Fc mutant 1 (hIgG1-LALA), which was unable to induce ADCC, but retained some binding to mouse FcγR-expressing cells (Figure 2(a)), inhibited the growth of A375 tumors to a similar extent as wild-type enavatuzumab (Figure 2(b)), suggesting that cell death signaling through TweakR, and not ADCC, is critical for the antitumor activity of enavatuzumab in this model.

The finding that not all xenograft tumors tested were sensitive to enavatuzumab is not consistent with *in vitro* results showing that enavatuzumab was able to induce ADCC and immune cell activation efficiently on all TweakR-positive tumor cell lines tested. This raised the question of whether enavatuzumab might differentially activate effector cells *in vivo*. To address this, splenocytes isolated from tumor-bearing mice after enavatuzumab treatment were assessed for levels of activation markers. NK-like and monocyte-like cells were gated based on cell size and CD11b expression (Figure 3(a)). The activation markers DX5 and CD27 on monocyte-like cells (CD11b high) and DX5 on NK-like cells (CD11b low) were found to be up-regulated after enavatuzumab treatment in responder xenografts that rely on ADCC, such as SN12C tumor-bearing mice, but not in mice bearing HCT116 tumors which do not respond to treatment *in vivo* (Figures 3(b) and 3(c)).

In SN12C tumor-bearing mice, up-regulation of activation markers after enavatuzumab treatment was observed on splenocytes, indicating systemic activation of immune cells. As this activation is unlikely to be mediated by enavatuzumab through mouse TweakR on the immune cells, the function of enavatuzumab on mouse immune effector cells is likely through Fc-FcγR ligation; such "bridging" between the FcγR on immune cells and TweakR on the tumor target cells, as suggested in the PBMC-tumor cell co-culture studies, is probably required for triggering effector cell activation *in vivo*.

3.3. Enavatuzumab Promoted Infiltration and Migration of Effector Cells into Responder Tumor Xenografts as well as toward Tumor Cells Cultured In Vitro. To better understand the interaction between tumor cells and immune cells mediated by enavatuzumab *in vivo*, we assessed the xenograft tumors for the presence of immune cells by immunohistochemistry. While few immune cells were observed within the tumors after treatment with a control antibody, enavatuzumab treatment stimulated marked immune cell infiltration into SN12C and A375 tumors, both of which were sensitive to enavatuzumab (Figure 4(a)). The infiltration of immune cells into these tumors was not dependent on ADCC capability, as enavatuzumab and Fc mutant variants stimulated immune cell infiltration to similar extents (Figure 4(b)). In contrast, enavatuzumab did not stimulate infiltration of immune cells into HCT116 or DLD-1 tumors (Figure 4(a)), both of which were resistant to enavatuzumab *in vivo*. These results suggested that enavatuzumab treatment of sensitive tumors, but not resistant tumors, stimulated the migration of immune cells into the tumor. To simulate the effector cell infiltration observed in animal models, we used a Transwell assay to assess the ability of enavatuzumab to stimulate the migration of human immune cells toward tumor cells. Enavatuzumab treatment of both SN12C and A375 cells resulted in significantly increased migration of immune effector cells toward the tumor cells (Figure 5(a)). In contrast, treatment of HCT116 or DLD-1 cells with enavatuzumab did not stimulate immune cell migration. Phenotyping the immune cells that had migrated toward SN12C or A375 cells showed that monocytes were the predominant migrating immune cell type, as seen by the significant increase in this population relative to the starting PBMCs (Figure 5(b)). NK cells, dendritic cells (DC), and B cells were also enriched in the migrated population. However, only monocyte migration was significantly increased by enavatuzumab treatment of tumor cells (Figure 5(c)).

3.4. Chemokines Released from Enavatuzumab-Treated Tumor Cells Were Critical for Effector Cell Migration. The observed ability of enavatuzumab to stimulate migration of immune cells is likely mediated by cytokines released from the tumor cells. TWEAK has been shown to stimulate the release of cytokines and chemokines from a number of cell types [4, 5]; as a TweakR agonist, enavatuzumab would also be expected to have this function. Indeed, enavatuzumab stimulated release of multiple chemokines from A375 cells, including GM-CSF, IL-8, IL-6, and MCP-1 (Figure 6(a)). Treatment of SN12C with enavatuzumab had a more limited effect, resulting in increased GM-CSF and IL-8 levels,

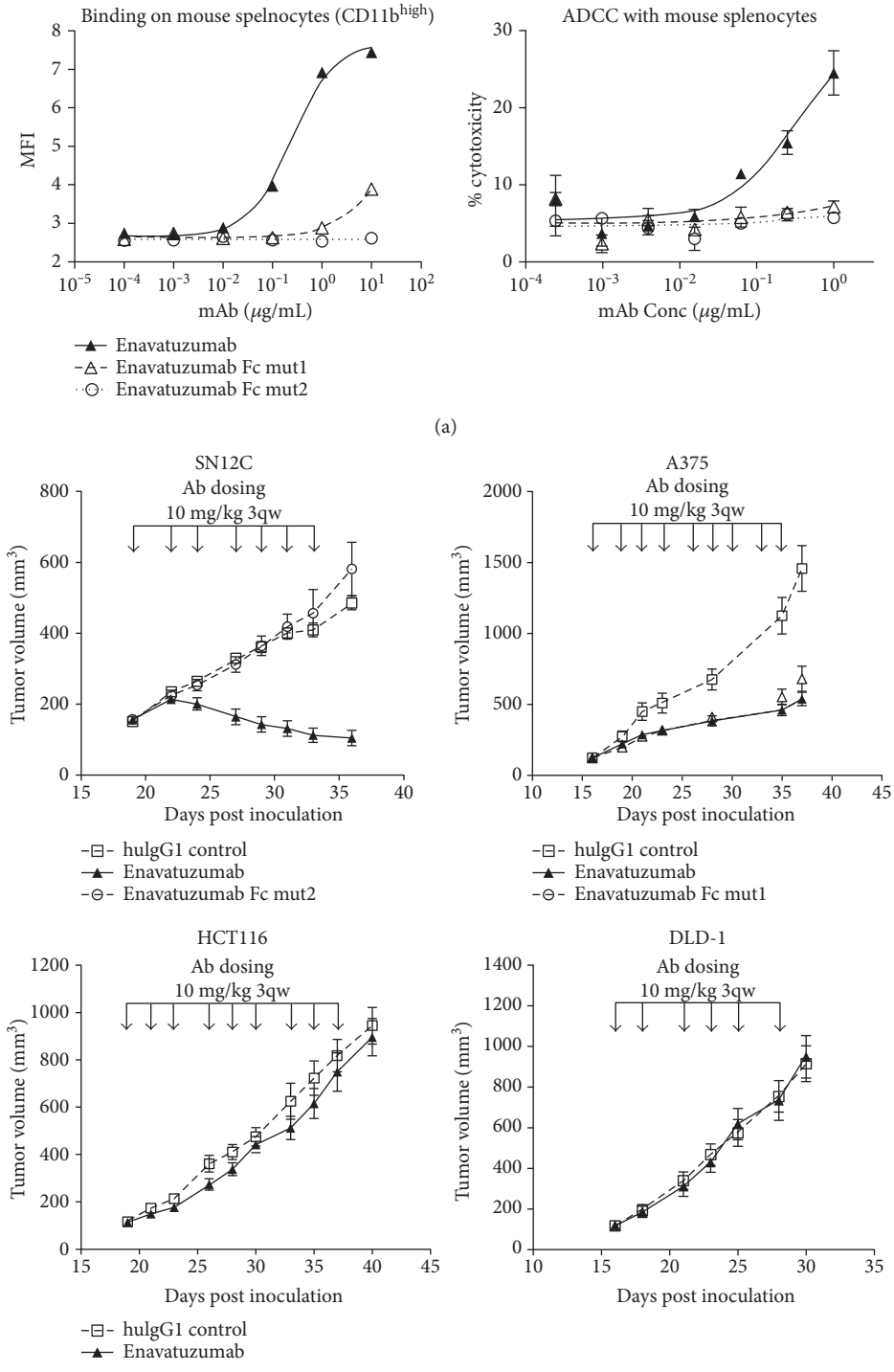


Figure 2: Enavatuzumab showed diverse antitumor activities on different xenograft tumors. (a) Enavatuzumab or two variants of enavatuzumab containing mutations in the Fc region were incubated with mouse splenocytes. Binding to CD11b^{high} cells was measured by FACS. ADCC activities of enavatuzumab and its Fc mutant variants were evaluated by Cr-51 release with H520-TweakR cells as targets and mouse splenocytes as effectors at a ratio of 1 : 40. (b) Established SN12C tumors were treated with enavatuzumab, a variant of enavatuzumab with no FcγR binding, or a control antibody (10 mg/kg) three times a week for a total of seven doses, with dosing days indicated by the arrows above the graph. Dosing groups contained 10 animals each. Treatment with enavatuzumab, but not the Fc mutant, resulted in significant tumor growth inhibition on days 24–36 ($p < 0.05$). A375 tumors were similarly administered with nine doses of enavatuzumab, an Fc mutant variant, or a control antibody. Dosing groups contained 8 animals each, and significant growth inhibition was observed with both enavatuzumab and the Fc mutant on days 21–37 ($p < 0.05$). HCT116 and DLD-1 xenograft tumors were treated with enavatuzumab or a control antibody for nine or six doses ($n = 8$ or 10). Enavatuzumab treatment resulted in no tumor growth inhibition in either models.

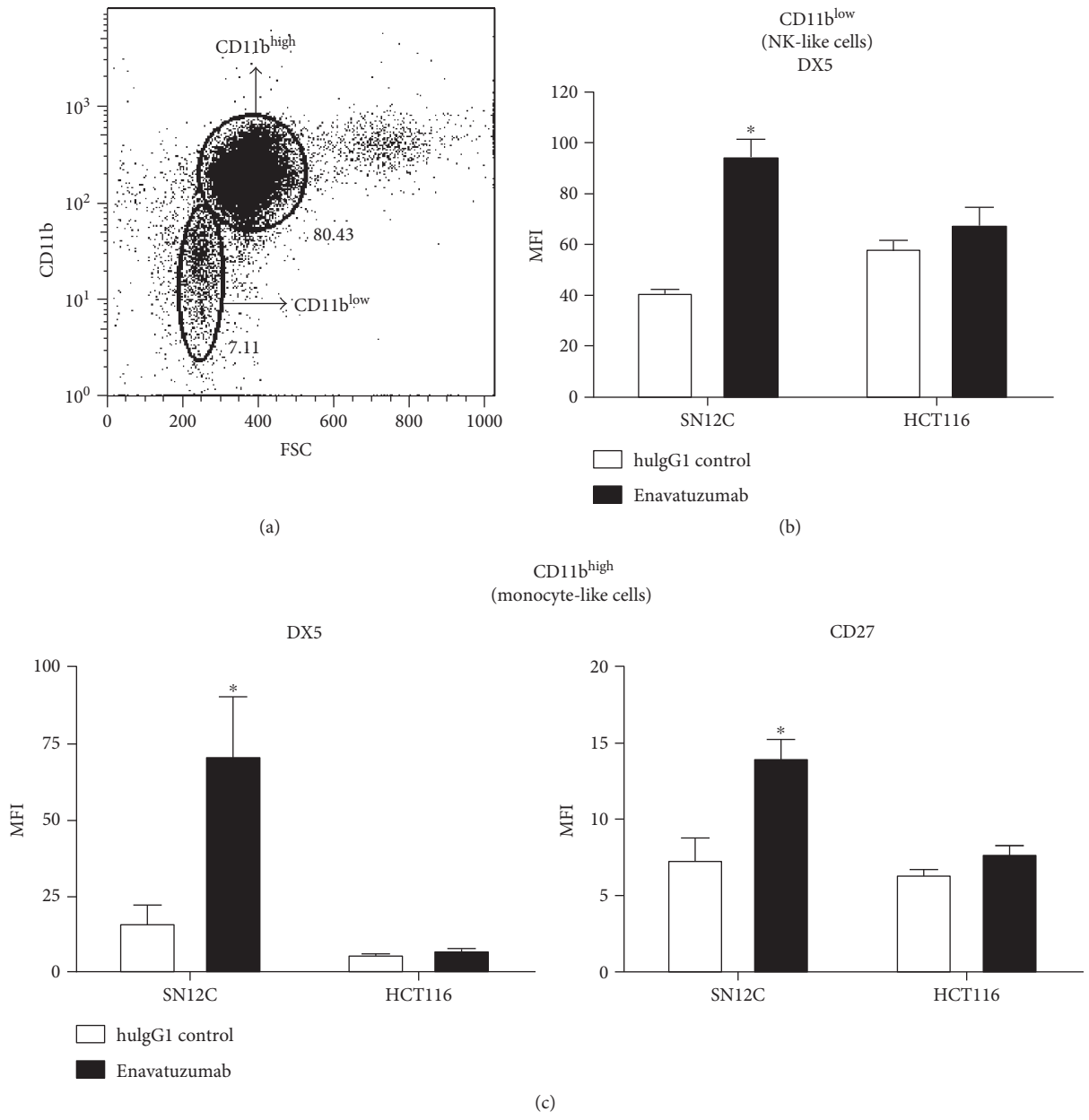


Figure 3: Enavatuzumab activated immune effector cells in responding tumor xenograft model. Splenocytes were isolated from tumor-bearing mice after enavatuzumab treatment and phenotyped by flow cytometry. (a) $CD45^+$ cells were gated out from dead cells and other cell types. Of the live $CD45^+$ cells, monocyte ($CD11b^{high}$) and NK-like ($CD11b^{low}$) cells were gated based on cell size and CD11b expression. (b, c) Splenocytes from SN12C or HCT116 tumor-bearing mice were assessed for DX5 and CD27 on monocyte-like cells ($CD45^+CD11b^{high}$) and for DX5 on NK-like cells ($CD45^+CD11b^{low}$) after enavatuzumab treatment. Enavatuzumab treatment significantly upregulated expression of both markers in SN12C tumor-bearing mice ($n = 6$, $^*p < 0.05$).

although untreated SN12C endogenously expressed high levels of MCP-1. Enavatuzumab had a much reduced effect on cytokine release by HCT116 or DLD-1, with only an increase in GM-CSF released by DLD-1 in response to enavatuzumab (Figure 6(a)).

To test the hypothesis that chemokines mediated enavatuzumab-induced migration of immune cells toward tumor cells, we tested the ability of enavatuzumab to stimulate immune cell migration in the presence of chemokine-blocking antibodies. An IL-8-blocking antibody had no effect on the migration of immune cells toward A375 cells; however, an anti-MCP-1 antibody prevented enavatuzumab-stimulated immune cell migration in a dose-dependent manner (Figure 6(b)), with a marked reduction in monocyte population (Supplemental Figure 1 available online at https://doi.org/10.1155/2017/5737159).

Having shown that enavatuzumab treatment of tumor cells stimulated release of multiple cytokines and chemokines *in vitro* and that MCP-1 appeared to be a key functional chemokine in immune cell migration, we next tested whether

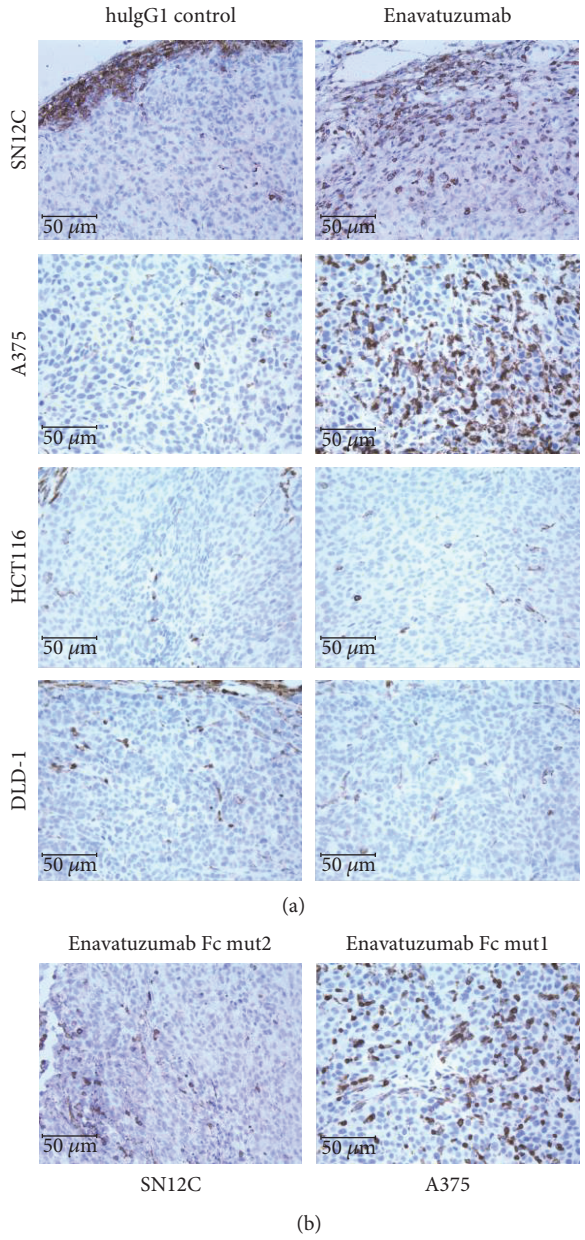


Figure 4: Enavatuzumab treatment results in increased infiltration of $CD45^+$ cells into tumors sensitive to antibody treatment. Established xenograft tumors were treated with enavatuzumab or a control antibody (a) or Fc mutants (b) on day 0 and day 2 or 3, and the tumors were harvested on day 4 for immunohistochemical staining for mouse CD45.

enavatuzumab stimulated release of MCP-1 *in vivo*. A375 tumor-bearing mice were administered a single dose of enavatuzumab, after which serum from mice was assessed for human or mouse MCP-1 levels. Enavatuzumab treatment resulted in a marked increase in circulating human MCP-1, which peaked 6 hrs after antibody injection (Figure 7(a)). Enavatuzumab stimulated a modest elevation in mouse MCP-1 at a single time point, 6 hrs after treatment. Treatment of mice bearing SN12C tumors with enavatuzumab also resulted in elevated circulating human and mouse MCP-1 levels (Supplemental Figure 2).

To translate the preclinical finding that enavatuzumab stimulated cytokine release from tumor cells *in vitro* and *in vivo* as a mechanism to mediate the migration of immune effector cells into tumors, we next assessed whether enavatuzumab might also have an effect on circulating chemokine levels in cancer patients. MCP-1 was measured in the serum from phase 1 subjects at various times after enavatuzumab treatment. At all dose levels tested (0.1–1.5 mg/kg), postdose elevations in MCP-1 were observed, with 13/30 patients exhibiting at least a 2-fold increase over baseline at 5 hr and/or 24 hr after the first infusion (Figure 7(b)). The increased level of MCP-1 could be of value as a potential biomarker for enavatuzumab biological activity in patients.

4. Discussion

Enavatuzumab is a functional anti-TweakR human IgG1 antibody that inhibits tumor cell growth through direct signaling and also kills tumor cells through ADCC. The current study confirmed that enavatuzumab can induce cytotoxicity of all TweakR-positive tumor cells tested by ADCC *in vitro*. However, not all TweakR-positive cells which can be lysed by ADCC *in vitro* were sensitive to enavatuzumab treatment in mouse xenograft tumor models. We hypothesize that tumor cells sensitive to enavatuzumab treatment *in vivo* actively recruit immune effector cells and the presence of immune effector cells within the tumor is critical for antibody-mediated tumor cell killing.

Immunohistochemistry (IHC) staining demonstrated strong presence of $CD45^+$ leukocytes within xenograft tumors sensitive to enavatuzumab treatment, and very few $CD45^+$ cells were found in nonresponding tumors. The infiltration of leukocytes into sensitive tumors was rapid and occurred within 3 days after the first dose; thus, it is unlikely that the leukocytes were recruited as a result of tumor necrosis at later time points (Supplemental Figure 3). In the *in vitro* Transwell assay, enavatuzumab stimulated the responder tumor cell lines to produce a number of cytokines and chemokines which attracted human leukocytes. In contrast, nonresponding lines produced few chemokines after exposure to enavatuzumab. In experiments using conditioned media, MCP-1 was found to be the major chemokine responsible for leukocyte infiltration into tumors. These data suggest that stimulation of TweakR signaling by enavatuzumab not only leads to tumor growth inhibition as previously reported [2] but can also result in chemokine release and leukocyte infiltration. The ability to stimulate leukocyte infiltration into tumors may explain the potent antitumor activity of enavatuzumab in responding tumor models. In xenograft models conducted in immune-deficient mice, the major leukocytes are neutrophils, monocytes, and NK-like cells, all of which express FcγR [25]. The presence of FcγR-positive leukocytes within the tumor would allow the antibody to bridge leukocytes and tumor targets through binding to FcγR and TweakR, respectively, thus

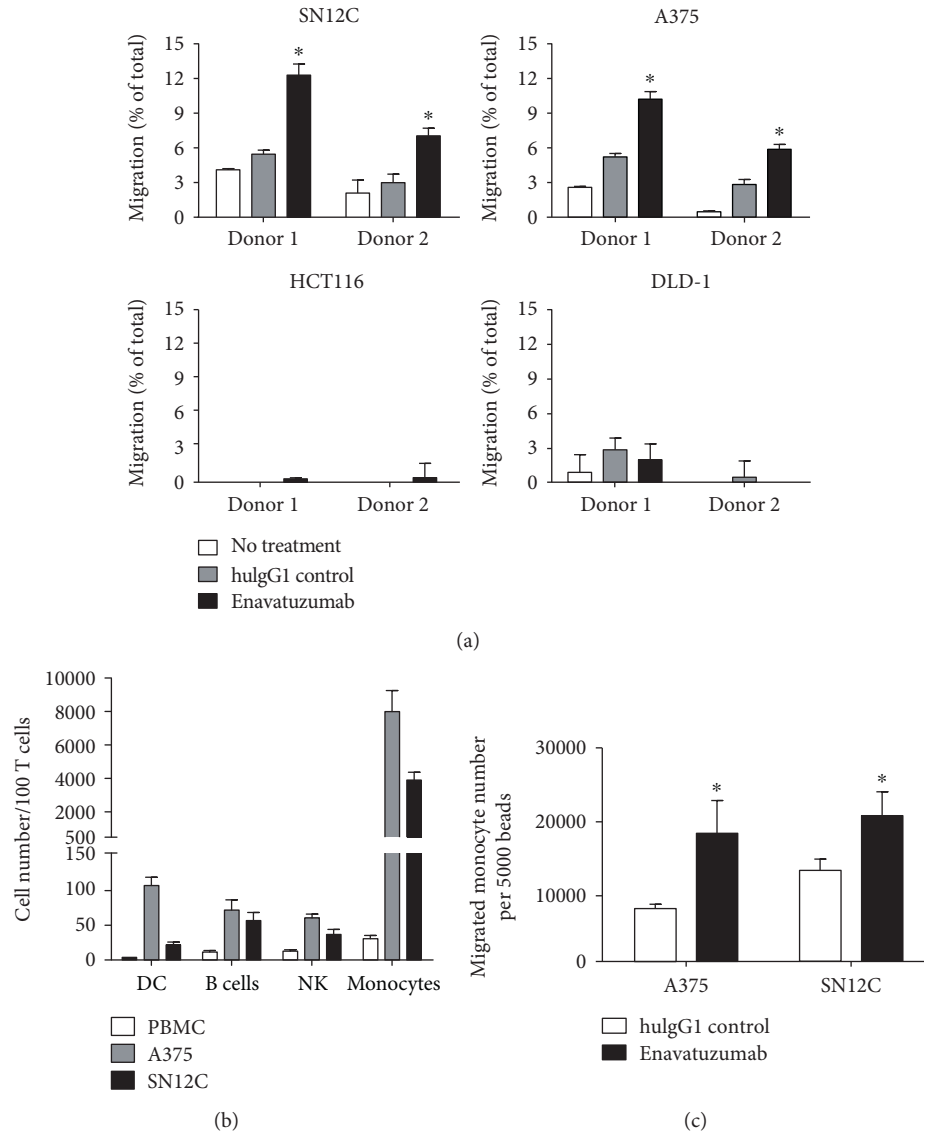


Figure 5: Human effector cells can migrate toward enavatuzumab-treated tumor cells in culture. (a) SN12C, A375, HCT116, and DLD-1 cells were plated into the bottom well of 24-well Transwell plates and treated with enavatuzumab or a control antibody. 24 hr later, PBMCs were added to the top of the Transwell and incubated for an additional 4 hr, after which the number of PBMCs that had migrated to the bottom chamber was quantified by flow cytometry and is represented as a percentage of the total number of PBMCs added. Enavatuzumab significantly increased PBMC migration toward SN12C and A375 cells ($*$, $n = 4$, $p < 0.05$) but had no effect on migration toward HCT116 or DLD-1 cells. (b) The phenotype of PBMCs prior to migration was compared to that of immune cells that had migrated toward A375 and SN12C cells after enavatuzumab treatment. The numbers of dendritic cells ($CD11c^+CD3^-CD20^-CD56^-CD16^-CD14^-$), B cells ($CD20^+$), NK cells ($CD3^-CD56/CD16^+$), and monocytes ($CD14^+$) were quantified and are expressed relative to the number of T cells in each population. (c) The absolute number of monocytes that migrated toward A375 and SN12C cells after antibody treatment was quantified and is expressed as the number per 5000 counting beads ($*$, $n = 4$, $p < 0.05$).

simultaneously modulating the functions of both tumor target cells and leukocytes.

FcγR binding has been demonstrated to drive direct signaling in target cells for antibody targeting members of TNFRSF, such as anti-DR5 [21]. It is well-known that members of the TNFRSF need to be oligomerized to initiate downstream signaling. Antibodies clustered by FcγR expressed on neighboring cells likely facilitate target receptor oligomerization and subsequent downstream signaling. A recent study on anti-TweakR antibodies confirmed this concept and provided evidence that antibodies can enhance TweakR-mediated signaling through FcγR binding [26]. One of the biological responses resulting from TweakR direct signaling is cytokine or chemokine production [4, 5]. Crosslinking provided by leukocytes can enhance TweakR signaling; thus, the subsequent cytokine or chemokine production from tumor cells could further increase leukocyte recruitment. This positive feedback loop created by

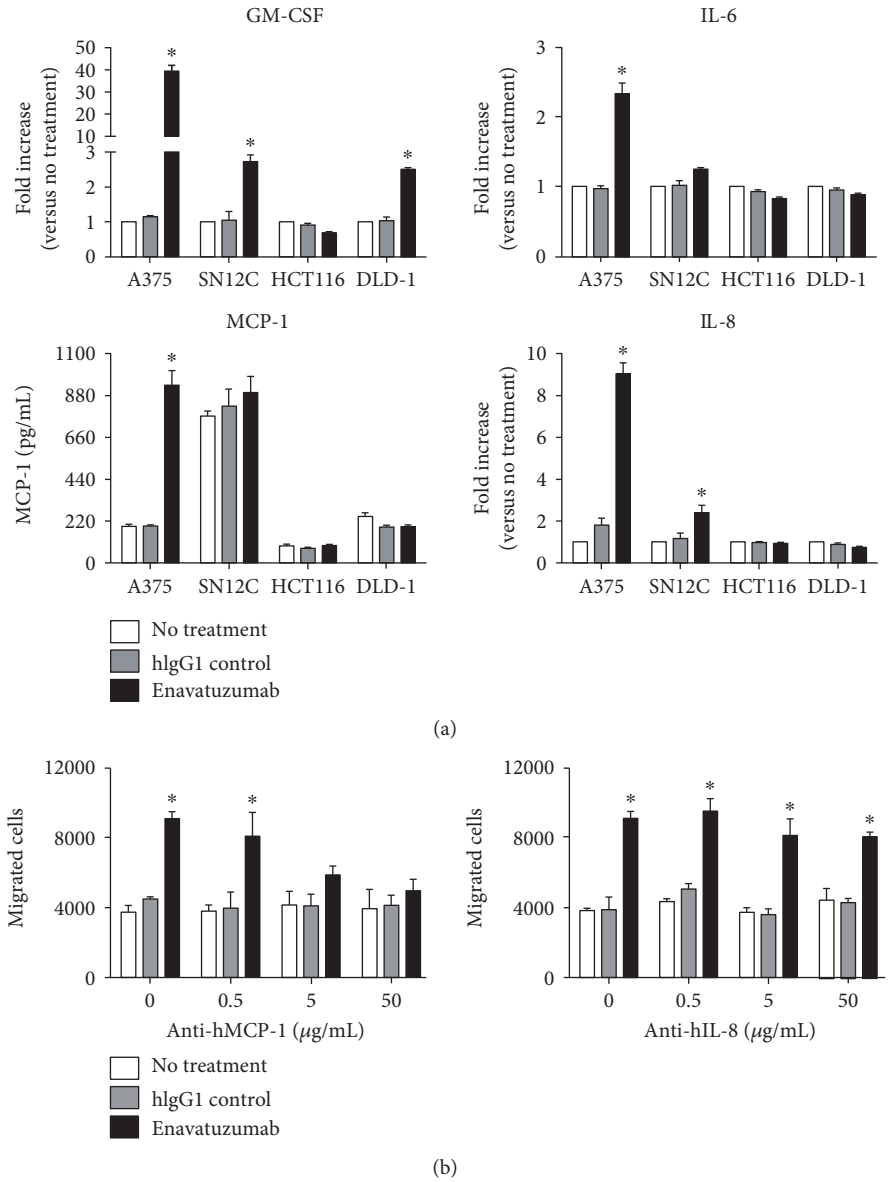


Figure 6: Enavatuzumab stimulates an increase in cytokine levels in some tumor cell cultures. (a) A375, SN12C, HCT116, and DLD-1 cells were treated with enavatuzumab or a control antibody for 24 hr, after which the levels GM-CSF, IL-6, IL-8, and MCP-1 were measured in the supernatants by Luminex multiplex assays ($n = 4$, $^*p < 0.05$). (b) A375 cells were plated into the bottom of Transwell plates and treated with enavatuzumab or a control antibody for 24 hr, after which antibodies blocking MCP-1 (left) or IL-8 (right) were added for 0.5 hr. PBMCs were then added to the top of the Transwell; 4 hr later, the number of immune cells that had migrated toward the tumor cells was quantified by flow cytometry ($n = 4$, $^*p < 0.05$).

leukocyte infiltration can therefore amplify the biological activity of enavatuzumab. The other biological response from TweakR direct signaling is tumor growth inhibition or cell death [7–9]. We have shown that crosslinking the Fc region of enavatuzumab using a soluble secondary antibody or by immobilizing enavatuzumab enhanced growth inhibition in responding tumor cell lines *in vitro* [10, 19]. It is possible that tumor-infiltrating leukocytes provide similar crosslinking through Fc-FcγR interactions and therefore enhance the antitumor activity of enavatuzumab *in vivo*. This antitumor activity of enavatuzumab is independent of its ADCC function. Such activity is likely a major mechanism driving the antitumor activity in the A375 model, where enavatuzumab and an ADCC-null Fc mutant (hIgG1-LALA) of enavatuzumab exhibited equivalent antitumor activity. This Fc mutant retained some mouse FcγR binding which might be sufficient to provide the crosslinking required to promote TweakR signaling, leading to tumor growth inhibition.

By bridging leukocytes and tumor cells, enavatuzumab can not only stimulate TweakR signaling on tumor cells but also enhance the activation of FcγR-bearing leukocytes.

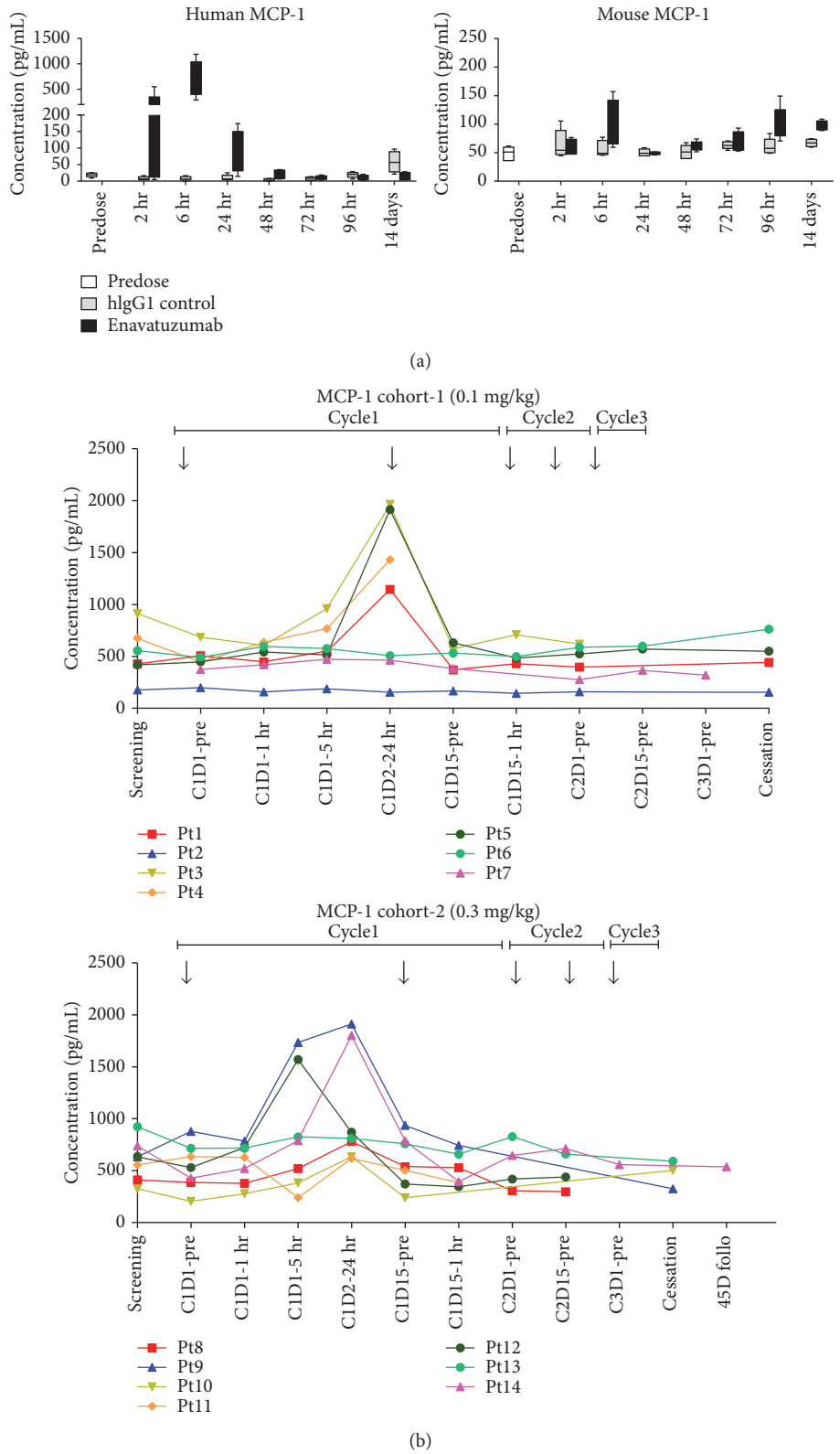


Figure 7: Enavatuzumab treatment stimulates MCP-1 secretion in mouse xenograft models and in human cancer patients. (a) Mice bearing established A375 tumors were given a single dose of enavatuzumab or a control antibody. Human MCP-1 (left) and mouse MCP-1 (right) were measured by Luminex in the serum of 5 mice in each dosing group at the indicated times after treatment. Data from 5 mice are shown as median and interquartile range at each time point in the graph. (b) Patients were treated with enavatuzumab at 0.1 mg/kg (top) or 0.3 mg/kg (bottom) every two weeks, with dosing days indicated by the arrows above each graph. MCP-1 was measured in serum samples by Luminex. Each colored line represents data from one patient and patient (Pt) numbers are also listed.

Human IgG1 antibodies' binding to activating FcγR and engagement of activating FcγR by antibody-antigen complexes may lead to leukocyte activation, cytokine release, and ADCC stimulation [20, 27]. Consistent with this notion, enavatuzumab activated NK cells and monocytes by modulating expression of cell surface markers when human PBMCs were cocultured with tumor cells. Since neither NK cells nor monocytes express TweakR [2, 24], activation of these cells is mediated by Fc-FcγR interactions and only when PBMCs were cocultured with TweakR-expressing tumor cells. In the absence of antigen engagement on the tumor cells, enavatuzumab treatment did not alter the expression of activation markers on PBMCs. These *in vitro* data suggested that crosslinking through tumor target binding was required for FcγR-mediated effector cell activation and is analogous to the ability of crosslinking through FcγR engagement to promote signaling through TweakR as discussed above. Enavatuzumab also activated leukocytes *in vivo*, as evidenced by the upregulation of activation markers expressed on the leukocytes from the spleens of mice bearing xenograft tumors sensitive to enavatuzumab. Given that the sensitive tumors were capable of attracting leukocytes after treatment, leukocytes might be activated while circulating through tumor xenografts in the presence of the antibody. Further evidence of *in vivo* effector cell activation by enavatuzumab comes from the observation that the production of mouse cytokines was enhanced by enavatuzumab in sensitive tumor models (Figure 7(a), Supplemental Figure 2). Since enavatuzumab does not bind to mouse TweakR, the mouse cytokines were likely secreted from host leukocytes activated by antibody FcγR engagement. Assessing cell surface marker expression and cytokine production associated with *in vivo* effector cell activation provided feasible ways to monitor biomarkers reflecting the biological activity of enavatuzumab.

Many studies have suggested that the activation of effector cells is sufficient to induce target cell killing via ADCC [28, 29]. In this study, however, we showed that tumor cell killing also required a sufficient quantity of activated effector cells, since optimal cell cytotoxicity mediated by enavatuzumab could only be achieved at higher E : T ratios than that was required for optimal activation of effector cells in culture. Consistent with this finding, a previous study assessing the ADCC capacity of effector cells derived from trastuzumab-treated patients showed that response to trastuzumab correlated with the numbers of $CD56^+$ or $CD16^+$ lymphocytes in PBMCs and with the ability of the PBMCs to lyse target cells [30]. Thus, the quantity of effector cells is as important as their functionality in mediating ADCC, suggesting that the active recruitment of leukocytes into tumors may further enhance the antitumor activity of enavatuzumab via ADCC.

Taken together, these data suggest a model to describe the various mechanisms by which enavatuzumab exerts its antitumor activity in xenograft models. Binding of enavatuzumab to its target on tumor cells such as SN12C or A375 initiates signaling through TweakR which leads to cytokine and chemokine production. The tumor-derived cytokines and chemokines then trigger a cascade of biological responses, including leukocyte infiltration, enhanced leukocyte activation by antibody-tumor target engagement, and enhanced tumor signaling through antibody crosslinking via effector cells. The increased number of activated effector cells within the tumor facilitates tumor cell killing through ADCC, while the enhanced tumor cell signaling through TweakR further promotes direct tumor growth inhibition.

MCP-1 was identified as a chemokine likely responsible for enavatuzumab-stimulated leukocyte infiltration in xenograft models. MCP-1 is known to attract monocytes and has previously been shown to be secreted by tumor cells in response to TweakR stimulation [11, 31]. It has also been reported that monocytes and macrophages at the tumor site may contribute to tumor growth inhibition through the release of soluble TWEAK [11, 32]. The results described in this manuscript demonstrate recruitment of monocytes and NK cells toward tumor cells upon MCP-1 release after enavatuzumab treatment, resulting in activation of these innate effector cells and subsequent ADCC or ADCP on tumor cells. Immune-deficient mice carrying xenograft tumors provide a relevant model to test enavatuzumab in contact with innate immune cells. In an immune-competent environment, there are studies showed that macrophage plays an important role in depleting target cells [33, 34] as well as in mediating antitumor immunity [35]. However, other studies showed that chronic activation of macrophage recruited into tumor may suppress adaptive antitumor immunity in syngeneic setting [36, 37]. The precise role of macrophages in the tumor microenvironment depends on multiple factors, including the phenotype of the cells at a given time, the timing of treatment, and the tumor model in which the studies are performed [38]. To study the effects of acute recruitment and activation of monocytes/macrophages by functional anti-TweakR mAb on tumor cells, surrogate murine antibody will be required for testing in mouse-syngeneic tumor models, which could provide a better translation to understand the role of MCP-1 induced by enavatuzumab in cancer patients. Nevertheless, the rapid induction of MCP-1 by enavatuzumab in clinical studies suggests the potential for this molecule to be a biomarker of biological activity for enavatuzumab. Monitoring this marker may provide a better understanding of the relationship of pharmacokinetics, pharmacodynamics and toxicity profile of enavatuzumab, which might facilitate the development of an appropriate dosing regimen in clinical studies.

Conflicts of Interest

Shiming Ye, Melvin I. Fox, Nicole A. Belmar, Mien Sho, Debra T. Chao, Donghee Choi, Yuni Fang, Vivian Zhao, and Stephen F. Keller are employees of AbbVie. Gary C. Starling and Patricia A. Culp were employees of AbbVie at the time of the study. The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

Authors' Contributions

Shiming Ye and Patricia A. Culp conceived and designed the study. Shiming Ye and Vivian Zhao developed the methodologies of the study. Shiming Ye, Melvin I. Fox, Nicole A. Belmar, Mien Sho, Donghee Choi, and Vivian Zhao acquired the data (provided animals, acquired and managed patients, provided facilities, etc.). Shiming Ye, Debra T. Chao, Yuni Fang, and Patricia A. Culp analyzed and interpreted the data (e.g., statistical analysis, biostatistics, and computational analysis). Shiming Ye, Gary C. Starling, and Patricia A. Culp wrote, reviewed, and/or revised the manuscript. Shiming Ye, Stephen F. Keller, Gary C. Starling, and Patricia A. Culp supervised the study.

Acknowledgments

This study was supported by AbbVie Biotherapeutics Inc. The authors thank AbbVie Biotherapeutics Inc. FACS facility core for cell sorting, Protein Science Group for antibody expression and purification, and Susan Rhodes from the Molecular Cloning group for generating the mutant enavatuzumab constructs.

References

[1] A. E. Moran, M. Kovacsovics-Bankowski, and A. D. Weinberg, "The TNFRs OX40, 4-1BB, and CD40 as targets for cancer immunotherapy," *Current Opinion in Immunology*, vol. 25, pp. 230–237, 2013.

[2] P. A. Culp, D. Choi, Y. Zhang et al., "Antibodies to TWEAK receptor inhibit human tumor growth through dual mechanisms," *Clinical Cancer Research*, vol. 16, pp. 497–508, 2010.

[3] S. R. Wiley, L. Cassiano, T. Lofton et al., "A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis," *Immunity*, vol. 15, pp. 837–846, 2001.

[4] L. C. Burkly, J. S. Michaelson, K. Hahm, A. Jakubowski, and T. S. Zheng, "TWEAKing tissue remodeling by a multifunctional cytokine: role of TWEAK/Fn14 pathway in health and disease," *Cytokine*, vol. 40, pp. 1–16, 2007.

[5] J. A. Winkles, "The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting," *Nature Reviews Drug Discovery*, vol. 7, no. 5, pp. 411–425, 2008.

[6] Y. Chicheportiche, P. R. Bourdon, H. Xu et al., "TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis," *The Journal of Biological Chemistry*, vol. 272, pp. 32401–32410, 1997.

[7] C. A. Wilson and J. L. Browning, "Death of HT 29 adenocarcinoma cells induced by TNF family receptor activation is caspase-independent and displays features of both apoptosis and necrosis," *Cell Death and Differentiation*, vol. 9, pp. 1321–1333, 2002.

[8] P. Schneider, R. Schwenzer, E. Haas et al., "TWEAK can induce cell death via endogenous TNF and TNF receptor 1," *European Journal of Immunology*, vol. 29, pp. 1785–1792, 1999.

[9] M. Nakayama, K. Ishidoh, N. Kayagaki et al., "Multiple pathways of TWEAK-induced cell death," *Journal of Immunology*, vol. 168, pp. 734–743, 2002.

[10] D. T. Chao, M. Su, S. Tanlimco et al., "Expression of TweakR in breast cancer and preclinical activity of enavatuzumab, a humanized anti-TweakR mAb," *Journal of Cancer Research and Clinical Oncology*, vol. 139, pp. 315–325, 2013.

[11] L. Gu, L. Dai, C. Cao et al., "Functional expression of TWEAK and the receptor Fn14 in human malignant ovarian tumors: possible implication for ovarian tumor intervention," *PLoS One*, vol. 8, no. 3, article e57436, 2013.

[12] N. L. Tran, W. S. McDonough, P. J. Donohue et al., "The human Fn14 receptor gene is up-regulated in migrating glioma cells in vitro and overexpressed in advanced glial tumors," *The American Journal of Pathology*, vol. 162, pp. 1313–1321, 2003.

[13] G. S. Watts, N. L. Tran, M. E. Berens et al., "Identification of Fn14/TWEAK receptor as a potential therapeutic target in esophageal adenocarcinoma," *International Journal of Cancer*, vol. 121, pp. 2132–2139, 2007.

[14] A. L. Willis, N. L. Tran, J. M. Chatigny et al., "The fibroblast growth factor-inducible 14 receptor is highly expressed in HER2-positive breast tumors and regulates breast cancer cell invasive capacity," *Molecular Cancer Research*, vol. 6, pp. 725–734, 2008.

[15] T. G. Whitsett, E. Cheng, L. Inge et al., "Elevated expression of Fn14 in non-small cell lung cancer correlates with activated EGFR and promotes tumor cell migration and invasion," *The American Journal of Pathology*, vol. 181, pp. 111–120, 2012.

[16] E. Cheng, C. L. Armstrong, R. Galisteo, and J. A. Winkles, "TWEAK/Fn14 axis-targeted therapeutics: moving basic science discoveries to the clinic," *Frontiers in Immunology*, vol. 4, p. 473, 2013.

[17] H. Zhou, S. Ekmekcioglu, J. W. Marks et al., "The TWEAK receptor Fn14 is a therapeutic target in melanoma: immunotoxins targeting Fn14 receptor for malignant melanoma treatment," *The Journal of Investigative Dermatology*, vol. 133, pp. 1052–1062, 2013.

[18] H. Zhou, W. N. Hittelman, H. Yagita et al., "Antitumor activity of a humanized, bivalent immunotoxin targeting fn14-positive solid tumors," *Cancer Research*, vol. 73, pp. 4439–4450, 2013.

[19] J. W. Purcell, H. K. Kim, S. G. Tanlimco et al., "Nuclear factor κB is required for tumor growth inhibition mediated by enavatuzumab (PDL192), a humanized monoclonal antibody to TweakR," *Frontiers in Immunology*, vol. 4, p. 505, 2014.

[20] L. M. Weiner, R. Surana, and S. Wang, "Monoclonal antibodies: versatile platforms for cancer immunotherapy," *Nature Reviews Immunology*, vol. 10, pp. 317–327, 2010.

[21] N. S. Wilson, B. Yang, A. Yang et al., "An Fcγ receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells," *Cancer Cell*, vol. 19, pp. 101–113, 2011.

[22] S. Loi, S. Michiels, R. Salgado et al., "Tumor infiltrating lymphocytes is prognostic and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial," *Annals of Oncology*, vol. 25, pp. 1544–1550, 2014.

[23] M. B. Overdijk, S. Verploegen, A. Ortiz Buijsse et al., "Crosstalk between human IgG isotypes and murine effector cells," *Journal of Immunology*, vol. 189, pp. 3430–3438, 2012.

[24] S. L. Feng, Y. Guo, V. M. Factor et al., "The Fn14 immediate-early response gene is induced during liver regeneration and highly expressed in both human and murine hepatocellular carcinomas," *The American Journal of Pathology*, vol. 156, pp. 1253–1261, 2000.

[25] F. Nimmerjahn and J. V. Ravetch, "Fcγ receptors as regulators of immune responses," *Nature Reviews Immunology*, vol. 8, pp. 34–47, 2008.

[26] S. Salzmann, A. Seher, J. Trebing et al., "Fibroblast growth factor inducible (Fn14)-specific antibodies concomitantly display signaling pathway-specific agonistic and antagonistic activity," *The Journal of Biological Chemistry*, vol. 288, pp. 13455–13466, 2013.

[27] J. A. Bowles and G. J. Weiner, "CD16 polymorphisms and NK activation induced by monoclonal antibody-coated target cells," *Journal of Immunological Methods*, vol. 304, pp. 88–99, 2005.

[28] I. Tassi, J. Klesney-Tait, and M. Colonna, "Dissecting natural killer cell activation pathways through analysis of genetic mutations in human and mouse," *Immunological Reviews*, vol. 214, pp. 92–105, 2006.

[29] L. Zamai, M. Ahmad, I. M. Bennett, L. Azzoni, E. S. Alnemri, and B. Perussia, "Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells," *The Journal of Experimental Medicine*, vol. 188, pp. 2375–2380, 1998.

[30] S. Varchetta, N. Gibelli, B. Oliviero et al., "Elements related to heterogeneity of antibody-dependent cell cytotoxicity in patients under trastuzumab therapy for primary operable breast cancer overexpressing Her2," *Cancer Research*, vol. 67, pp. 11991–11999, 2007.

[31] M. Bhattacharjee, R. Raju, A. Radhakrishnan et al., "A bioinformatics resource for TWEAK-Fn14 signaling pathway," *Journal of Signal Transduction*, vol. 2012, Article ID 376470, 10 pages, 2012.

[32] Y. Kaduka, K. Takeda, M. Nakayama, K. Kinoshita, H. Yagita, and K. Okumura, "TWEAK mediates anti-tumor effect of tumor-infiltrating macrophage," *Biochemical and Biophysical Research Communications*, vol. 331, pp. 384–390, 2005.

[33] Q. Gong, Q. Ou, S. Ye et al., "Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy," *Journal of Immunology*, vol. 174, pp. 817–826, 2005.

[34] T. R. Simpson, F. Li, W. Montalvo-Ortiz et al., "Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma," *The Journal of Experimental Medicine*, vol. 210, pp. 1695–1710, 2013.

[35] G. L. Beatty, E. G. Chiorean, M. P. Fishman et al., "CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans," *Science*, vol. 331, pp. 1612–1616, 2011.

[36] J. B. Mitchem, D. J. Brennan, B. L. Knolhoff et al., "Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses," *Cancer Research*, vol. 73, pp. 1128–1141, 2013.

[37] E. S. Nakasone, H. A. Askautrud, T. Kees et al., "Imaging tumor-stroma interactions during chemotherapy reveals contributions of the microenvironment to resistance," *Cancer Cell*, vol. 21, pp. 488–503, 2012.

[38] K. Franciszkiewicz, A. Boissonnas, M. Boutet, C. Combadière, and F. Mami-Chouaib, "Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response," *Cancer Research*, vol. 72, pp. 6325–6332, 2012.