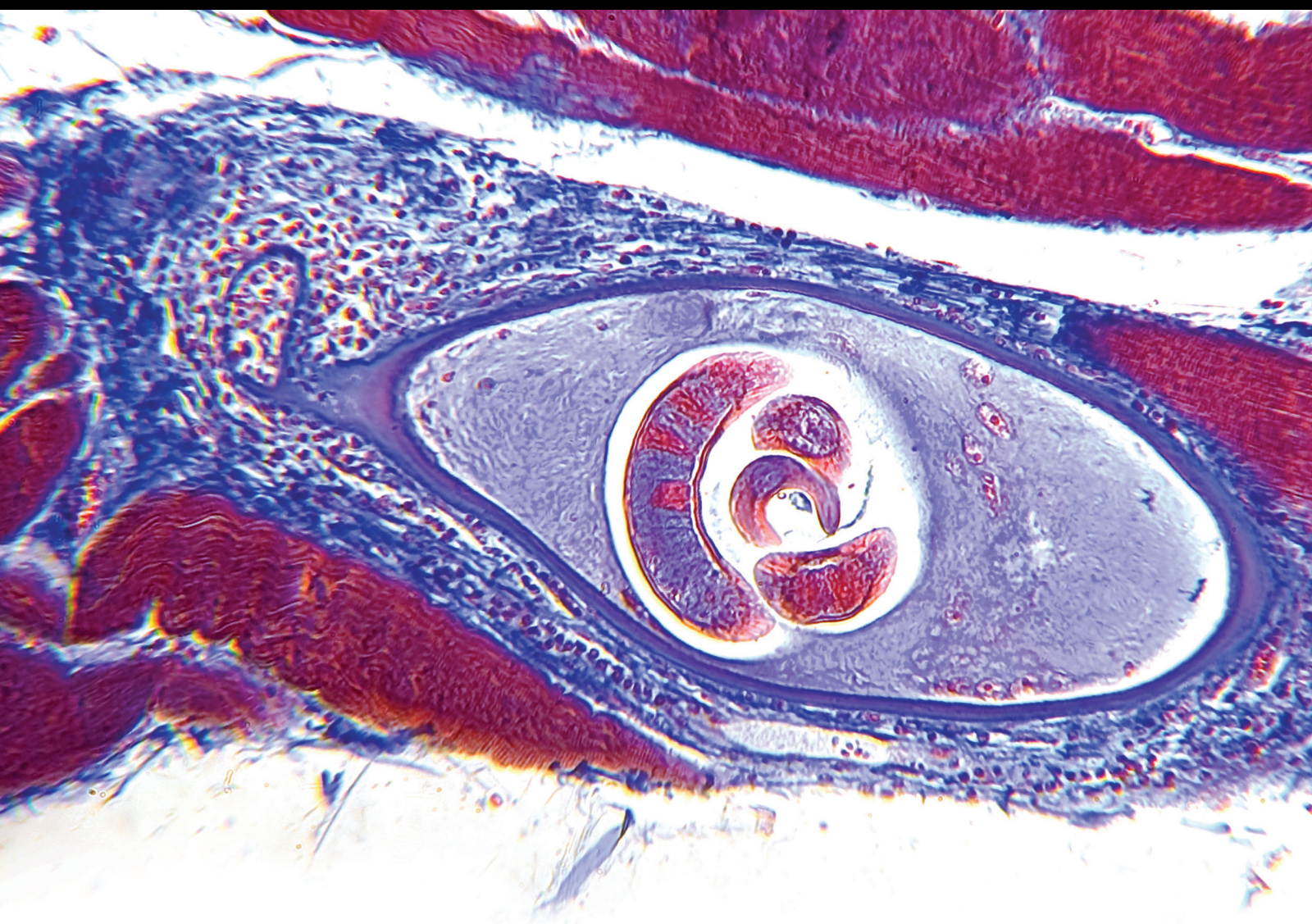
Gastroenterology Research and Practice

Translational Research in Colorectal Cancer: Current Status and Future Perspectives of Multimodal Treatment Approach

Special Issue Editor in Chief: Luigi Marano
Guest Editors: Gianluca Pellino, Christos Kontovounisios, Valerio Celentano, and Matteo Frasson



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Editorial

Translational Research in Colorectal Cancer: Current Status and Future Perspectives of Multimodal Treatment Approach

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Despite significant improvements in medical as well surgical technologies over the last two decades, colorectal malignancies remain a serious challenge to human health globally, representing one of the leading causes of cancer-related deaths worldwide.

High-quality research and advances in technology have contributed to the elucidation of molecular pathways underlying disease progression and have stimulated many clinical studies testing tailored managements. Similar to many other malignancies, colorectal cancer is a heterogeneous disease, making it a clinical challenge for optimization of treatment modalities in reducing the morbidity and mortality associated with this disease. Actually, it appears essential to classify tumors based on the underlying oncogenic pathways and to develop biological as well as genotype-based molecular therapies acting on individual tumors, redefining deeply the natural history of the colorectal cancer and establishing a new standard for diagnostic, stadiative, and therapeutic tools.

This special issue is devoted to current and emerging multimodal treatment approaches in colorectal cancer on the basis of bench to bedside philosophy, focusing on some selected topics that are both interesting and challenging: molecular biology, genetic and epigenetic factors, noninvasive molecular biomarkers, and metagenomic study on colorectal microbiota.

X. He and colleagues performed a retrospective analysis to investigate the impact of tumor location on survival outcomes in a total of 377,849 colon cancer patients. Furthermore, they also examined whether the prognostic role of cancer location was influenced by different groups of age, stage, year of diagnosis, and therapies. This clinical-based study demonstrated that left colon cancer was associated with better prognosis in terms of overall as well as cancer-specific survival. The data reported in this study provide us with new insights into the relationship between tumor location and survival outcomes, stimulating future studies to explore underlying mechanisms.

S. H. Kim et al. focused on the role of cyclooxygenase-2 (COX-2) expression on stage I to III primary colorectal cancer tissues and the impact on the biologic behavior of recurrent disease after curative resection. Interestingly, they

found that the mean time to recurrence was significantly longer in the elevated expression group, concluding that COX-2 expression was an independent factor associated with late recurrence (>3 years after surgery) during the follow-up period after surgery. Therefore, the positive COX-2 patients should be considered candidates for more frequent testing after 3 years of follow-up and extend follow-up period longer than 5 years after surgery. This study will help us to identify optimal surveillance methods and follow-up intervals.

The ideal colorectal cancer biomarker should be easily and quantitatively measured, highly specific, and sensitive, as well as reliable and reproducible. It should be able to stratify between different risk-based populations, selecting patients who really need a second-line test (endoscopic and radiologic investigations). Ideally, this aim can be achieved with a noninvasive and inexpensive method, using easily available biological samples such as urine, breath, serum, and feces. G. Pellino and colleagues reviewed the role of the newer noninvasive or minimally invasive biomarkers of colorectal cancer with evidence from currently available literature. They discussed imaging and biomolecular diagnostics ranging from their potential usefulness to obtain early and less-invasive diagnosis to their potential implementation in the development of a bespoke treatment of colorectal cancer. This paper will provide us with a comprehensive knowledge on noninvasive biomarkers and their roles in colorectal cancer management.

Another paper from A. O. Alomair et al. is aimed at investigating microbiota in colorectal cancer patients by means of metagenomic studies. Their results indicated that the colorectal cancer cases had significant enrichment of eleven genera compared to those in the control group. The metagenomic sequencing showed that specific species, such as Fusobacterium nucleatum, Peptostreptococcus stomatis, and Parvimonas micra, were present in significantly greater quantities in the CRC patients than those in the controls. The data reported in this study provide us with new insights into the relationship between microbiome alterations and susceptibility of colorectal cancer, suggesting strategies for early diagnosis, preventive measures, and curative therapies.

C. Bucci et al. provided a systematic review and a meta-analysis on current knowledge on the same-day bowel preparation prior colonoscopy. They proved that the same-day regimen is equivalent to the split preparation in terms of colon cleaning ability, on equal compliance and less sleep disturbance, giving to the clinicians the evidence to recommend this when the split preparation is unfeasible or does not fit the patients' needs.

In summary, the contributions of this special issue could stimulate the spread of novel molecular targets in colorectal malignancies and share some strategies to optimize diagnostic and curative approaches.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

Acknowledgments

Finally, we wish to express our appreciation to all the authors for their contribution and to the editors for their work on this issue.

Luigi Marano
Gianluca Pellino
Christos Kontovounisios
Valerio Celentano
Matteo Frasson

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Review Article

Same-Day Regimen as an Alternative to Split Preparation for Colonoscopy: A Systematic Review with Meta-Analysis

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Background. Split bowel preparation is the best regimen for colonoscopy. However, the same-day regimen can represent a valid alternative, but its use is limited by concerns about its cleansing ability, and to date, no convincing data support its use for routine colonoscopies. *Aim*. To evaluate the cleansing, compliance, and adverse event rates of the same-day compared to the split regimen. *Results*. A systematic literature search and meta-analysis was performed. Ten studies were included for a total of 1807 patients (880 in the same-day group and 927 in the split group). Overall, 85.3% patients in the same-day group vs. 86.3% in the split group had an adequate cleansing. Compliance was high for both, although patients were more compliant with the split than with the same-day prep (89.7% for same-day vs. 96.6% for split regimen). Sleep disturbance was more frequent in the split group, while nausea and vomit were more frequent in the same-day group. In the subgroup analysis, polyethylene glycol obtained a better cleansing rate when given as a split dose, with similar compliance and adverse events rates with both regimens. *Conclusion*. Split and same-day regimens are both useful in bowel cleaning before colonoscopy with a different pattern of adverse events and better compliance for split preparations. Endoscopists can consider the same-day preparation as a valid alternative, especially when the split preparation does not fit the patients' needs.

1. Introduction

Colonoscopy is considered the gold standard for the screening of colorectal cancer for its ability to identify and remove precancerous lesions [1]. Nonetheless, some adenomas or even cancers are missed during colonoscopy with a significant impact on the development of interval cancers [2, 3]. Reasons for this flaw detection rate may depend upon the operator's insufficient experience, inaccurate mucosal inspection, short withdrawal time, and substandard cleaning of the colon [4, 5]. Recent guidelines suggest using the split preparation for colonoscopy for its higher cleaning rate [6, 7] compared to the traditional previous-day regimen; however, concerns on the optimal bowel preparation still remain as some patients have an inadequate bowel cleansing also after this type of preparation.

Regardless of the regimen adopted, one of the critical factors for a clean colon is the time lapse between the end of the purge assumption and the beginning of colonoscopy. Ideally, this interval should not be longer than 5 hours to optimize the effects of the laxative [7, 8]. In this view, the same-day preparation has a shorter time lapse, providing the biological plausibility for an improved cleansing ability versus the split method, but it is recommended only for afternoon exams, as published studies were underpowered or provided controversial results.

Therefore, we aimed to evaluate the rate of adequate colon cleansing prior to colonoscopy by comparing the split regimen, the actual standard of care, to the same-day regimen. The secondary endpoints were to assess the rate of compliance and adverse events in the two groups.

2. Methods

2.1. Study Selection. We performed a systematic review of all the published articles (1960-2015) in which the same-day regimen was compared to the split one, following the methods as described in Bucci et al. [7]. Abstracts were included if the following inclusion criteria were fulfilled: (i) clinical trials, (ii) same-day vs. split regimens, or (iii) age > 18 years. Abstracts were excluded if not meeting the inclusion criteria or if the population was made up of a restricted group of patients, such as paediatrics or elderly patients. Of those selected, the full texts were obtained and the following data were extracted: study design, author, year of publication, patients' age, gender, diet prior to prep, time of colonoscopy, use of cathartics, compliance to the laxative (e.g., percentage of patients who took at least 75% of the prescribed dose), type, dose and regimen of prep, scale used to evaluate the colon cleaning, number of patients for each treatment arm with adequate/inadequate colon cleansing (grouping excellent-good vs. poor-fair), nausea, vomit, abdominal pain, bloating, and sleep disturbances. If one or more variables were not immediately inferable, principal investigators were contacted via e-mail. If the primary outcome was not available, the study was then excluded.

We assessed the studies quality using the Cochrane risk of bias assessment tool [9]. Studies were considered as having a low risk of bias if all risk domains were evaluated as low risk, a high risk of bias if at least one domain was assessed as high risk, or an unclear risk of bias if at least one domain was evaluated as vague without any high-risk domains.

2.2. Statistics. Percentage difference in the degree of colon cleaning between the same-day and the split preparation was the primary measure of the treatment effect. The meta-analysis was performed by computing the percentage difference using a random-effects model if heterogeneity was present. Quantitative analysis was performed on an intention-to-treat basis. Percentage difference and 95% confidence intervals (95% C.I.) for each treatment arm and pooled effect estimated were calculated. A forest graph was provided for the outcomes. Heterogeneity was calculated including the measures of consistency I^2 for each pooled datum; a p value < 0.05 was considered as significant. Egger regression asymmetry test for publication bias and Funnel plot asymmetry were used for assessing the risk of bias at outcome level [10]. All measures were performed using the STATA software 11.2 version (StataCorp 2009, Stata Statistical Software: Release 11. College Station, TX: StataCorp LP).

3. Results

Starting from 122 abstracts initially examined, ten full-text studies were retrieved, and 12 treatment arms were analysed for a total of 1807 patients [880 in the same-day regimen group (SDG) and 927 in the split regimen group (SG)]. The mean age of patients was 58.2 [±11.4 standard error (S.E.)] in the SDG and 57.8 (±11.9 S.E.) in the SG ($p = 0.89$), with a prevalence of 51.2% (95% C.I. 43.3-59.1) males in the SDG and 52.6% (95% C.I. 47.3-57.9) in the SG ($p = 0.78$). The studies' characteristics are summarized in Table 1. As for the bias, six trials were scored at low risk of bias [11–16], one trial was at unclear risk [17], and three trials were at high risk of bias [18–20] (Figure 1). For each study, bias was classified as represented in Figure 2.

A polyethylene glycol (PEG) laxative as same-day regimen was compared to split PEG into 8 studies [11–13, 15–17, 20], one study used same-day sodium picosulphate (PicoNa) versus split PEG [14], one compared PicoNa in both regimens [18], and one study tested same-day PEG vs. split 2 sachets of PicoNa or vs. split 3 sachets of PicoNa [19]. Six studies prescribed a 3-day low-residue diet before endoscopy [11, 14, 17–20] and in 2 studies a 1-day liquid diet was used [13, 15], while this information was unknown in the rest of the studies. Three studies used the Boston Bowel Preparation Scale to judge the degree of cleaning [11, 12, 20], 5 used the Ottawa [14–17, 19], and two used their own scale [13, 18]. Data on cleaning degrees were then categorized as adequate (good and excellent preparations) and inadequate (fair and poor preparations) cleaning.

Data on the number of patients with an adequate colon preparation and compliance were available in 9 out of 12 treatment arms. Overall, 569/667 patients in the same-day group vs. 619/717 in the split group had an adequate bowel cleansing with a pooled weighted rate difference (RD) of 2% [(95% C.I. -6% to 1%), $p = 0.55$; heterogeneity chi – squared = 13.83, $p = 0.061$; I – squared = 46.3%, Figure 3].

Data on compliance were available in 7 studies for a total of 586 patients in the same-day group and 674 in the split group. Although it was high for both regimens, patients were more compliant with the split preparation [RD 6% (95% C.I. 1-11%), $p = 0.030$; I – squared = 89.1%, $p \leq 0.001$, Figure 4]. The wide heterogeneity can be attributable to a different magnitude of the observed effect among the studies [19, 20] but also to the effect direction [13]. In the study by Matro et al., all patients in the same-day preparation group underwent colonoscopy in afternoon sessions, making the preparation easier to drink in a prolonged time and thus more patient-friendly [13].

All studies reported similar adverse events, which were extracted and compared (nausea, vomiting, bloating, abdominal pain/discomfort, and sleep disturbance). Nausea and vomit were more frequent in those who took the same-day prep [respectively, RD for nausea 10.5% (95% C.I. 2.4-18.6%), $p = 0.011$; RD for vomit 5% (95% C.I. 0.7-11%), $p = 0.087$]. In contrast, sleep disturbances were more frequent, even if not statistically significant, in the split regimen group [13.7% (95% C.I. -2.7 to 30%), $p = 0.10$]. No differences were noted for bloating and abdominal pain that were similar in the two groups [respectively, RD for bloating 2.3% (95% C.I. -5.9 to 10.6%), $p = 0.624$; RD for abdominal pain 1.7% (95% C.I. 4.6 to 8%), $p = 0.595$] (Figure 5).

Table 1: Characteristics of the studies included.

Author	Journal	Year	Laxative	Type of study	Scale	Same-day (total pts)	Split (total pts)	Compliance same-day	Compliance split	Same-day good prep	Same-day fair prep	Split good prep	Split fair prep
Matro et al.	AJG	2010	PEG vs. PEG	RCT	Own	61	54	52 (85.2%)	39 (72.2%)	56	5	51	3
Longcroft-Wheaton and Bhandari	J CLIN GASTR	2012	PicoNA vs. PicoNA	Prospective-NR	Own	132	95	UK	UK	130	2	85	10
de Leone et al.	WJGE	2013	PEG + bisacodyl vs. PEG	RCT	B	78	76	70 (89.7%)	71 (93.4%)	70	8	70	6
Seo et al.	Digestion	2013	PEG vs. PEG	RCT	O	97	100	97 (100%)	100 (100%)	72	25	75	25
Kim et al.	Scand J Gastr	2014	PEG vs. PEG	RCT	O	50	50	39 (78%)	47 (94%)	41	9	42	8
Kim et al.	Scand J Gastr	2014	PEG vs. PicoNA	RCT	O	50	50	39 (78%)	48 (96%)	41	9	46	4
Kim et al.	Scand J Gastr	2014	PEG vs. PicoNA	RCT	O	50	50	39 (78%)	50 (100%)	41	9	41	9
Kotwal et al.	J CLIN GASTR	2014	PEG vs. PEG	RCT	O	51	52	43 (84.3%)	48 (92.3%)				
Kang et al.	IR	2014	PicoNA vs. PEG	RCT	O	97	99	97 (100%)	99 (100%)	60	37	71	28
Chan et al.	WJG	2014	PEG vs. PEG	RCT	B	152	143	143 (94%)	142 (99.3%)	140	12	138	5
Tellez-Avila	DE	2014	PEG vs. PEG	RCT	B	59	61	UK	UK	UK	UK	UK	UK
Shah et al.	WJGE	2014	PEG vs. PEG	RCT	O	103	97	UK	UK	UK	UK	UK	UK

PicoNA = sodium picosulphate; PEG = polyethylene glycol; PTS = patients; RCT = randomized clinical trial; NR = nonrandomized; SCALE B = Boston Bowel Preparation Scale; O = Ottawa Bowel Preparation Scale; UK = unknown.

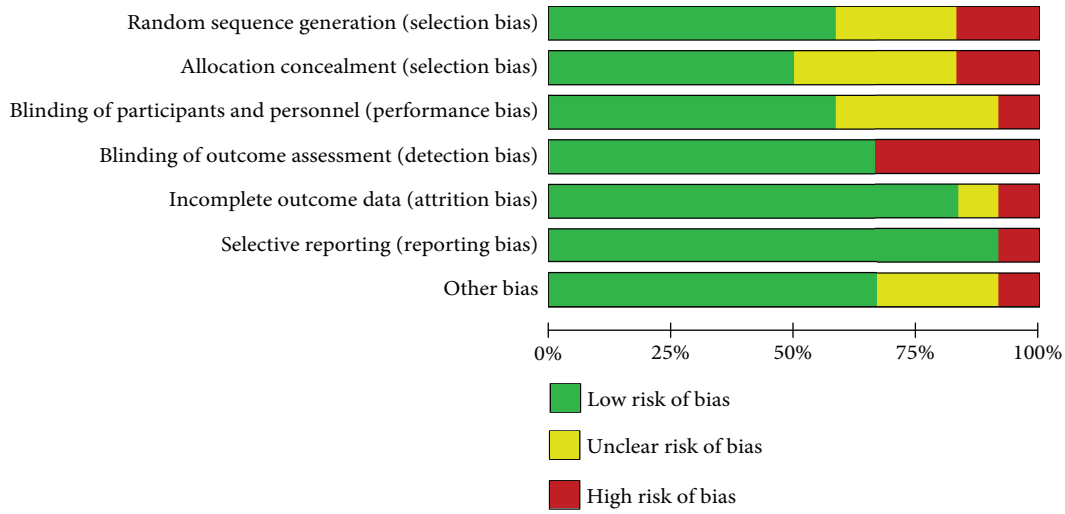

Figure 1: Risk of bias graph: a review of authors' judgments about each "risk of bias" item presented as percentages across all included studies.

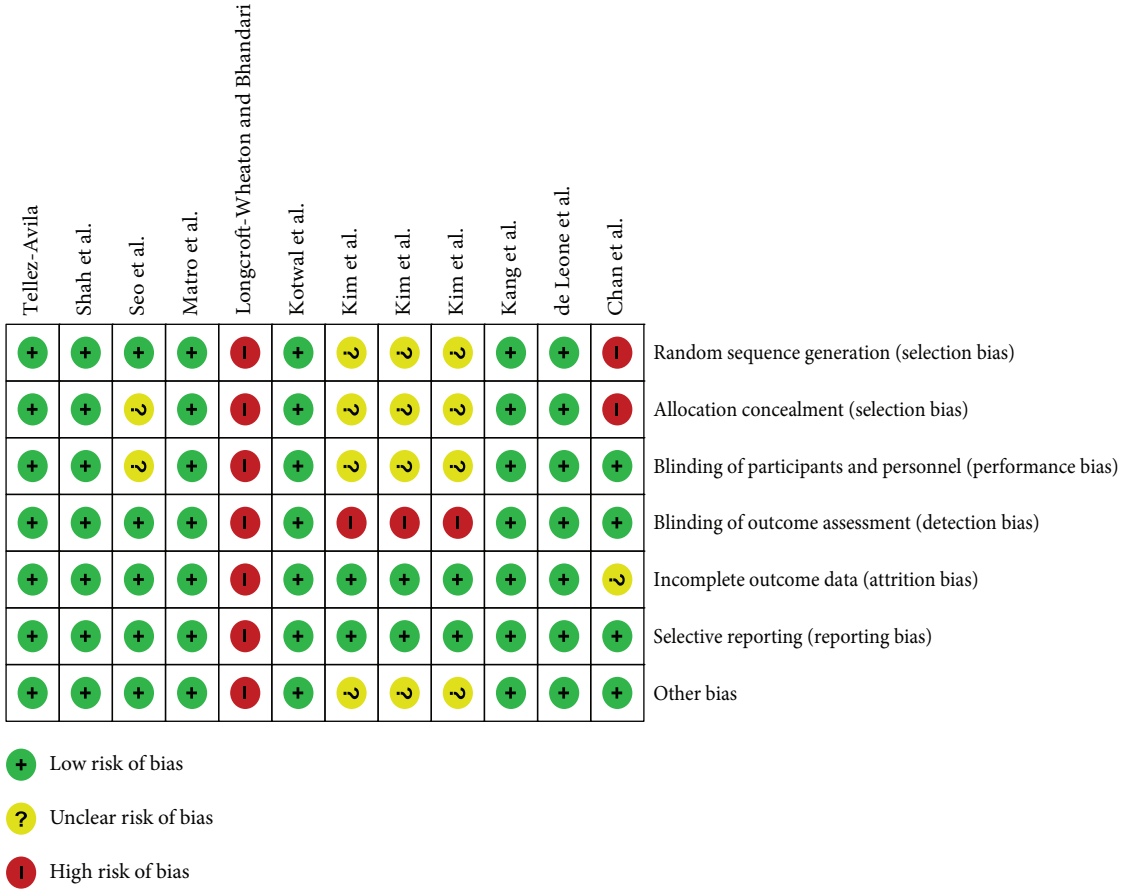

Figure 2: Risk of bias summary: a review of the authors' judgments about each risk of bias item for each included study.

3.1. Subgroup Analysis

3.1.1. Same-Day PEG vs. Split PEG. Eight studies compared the PEG laxatives within different regimens. Three of them compared two low-volume preparations [13, 15, 20], two compared two high-volume solutions [16, 19], and three compared a low-volume same-day vs. a high-volume split preparation [11, 12, 17]. 652 patients were prescribed a same-day preparation and 633 a split one. The cleaning rate within the SDG was 90% (95% C.I. 87-93%) and 93% within the SG (95% C.I. 91-96%). With this laxative, a better, thus not statistically significant, cleaning rate was obtained with the split regimen [RD 3.3% (95% C.I. -7 to 0.5%), $p = 0.086$]. Compliance was comparable between the two regimens

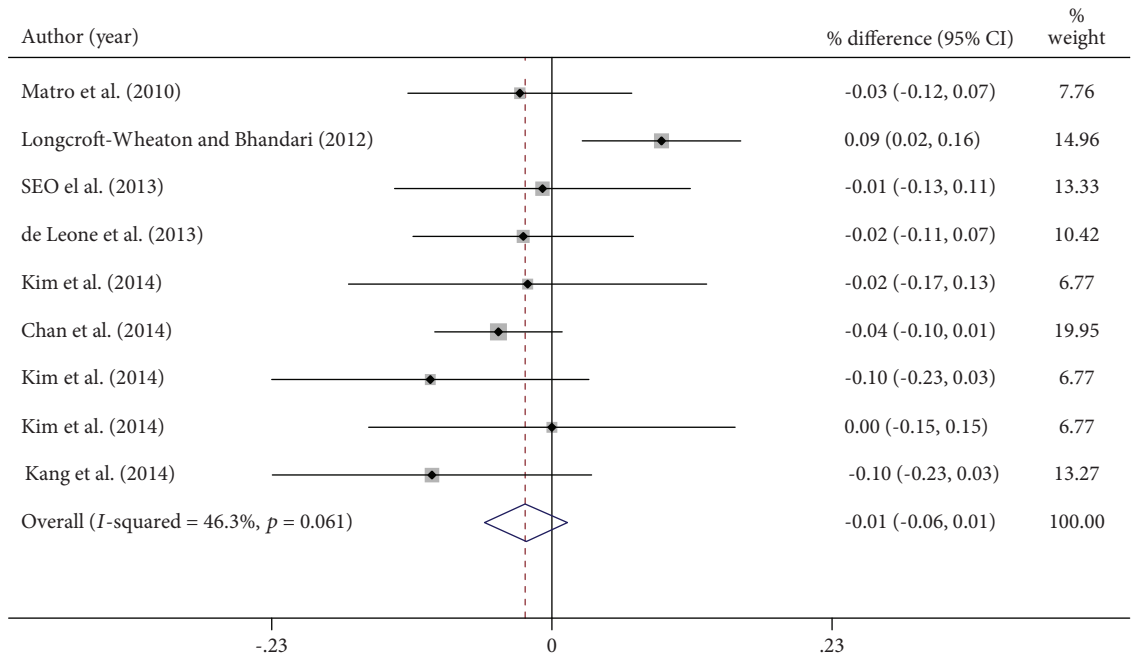

Figure 3: Percentage difference (expressed as rate difference, RD) of colon cleansing in patients assuming same-day or split bowel preparation. RD = rate difference.

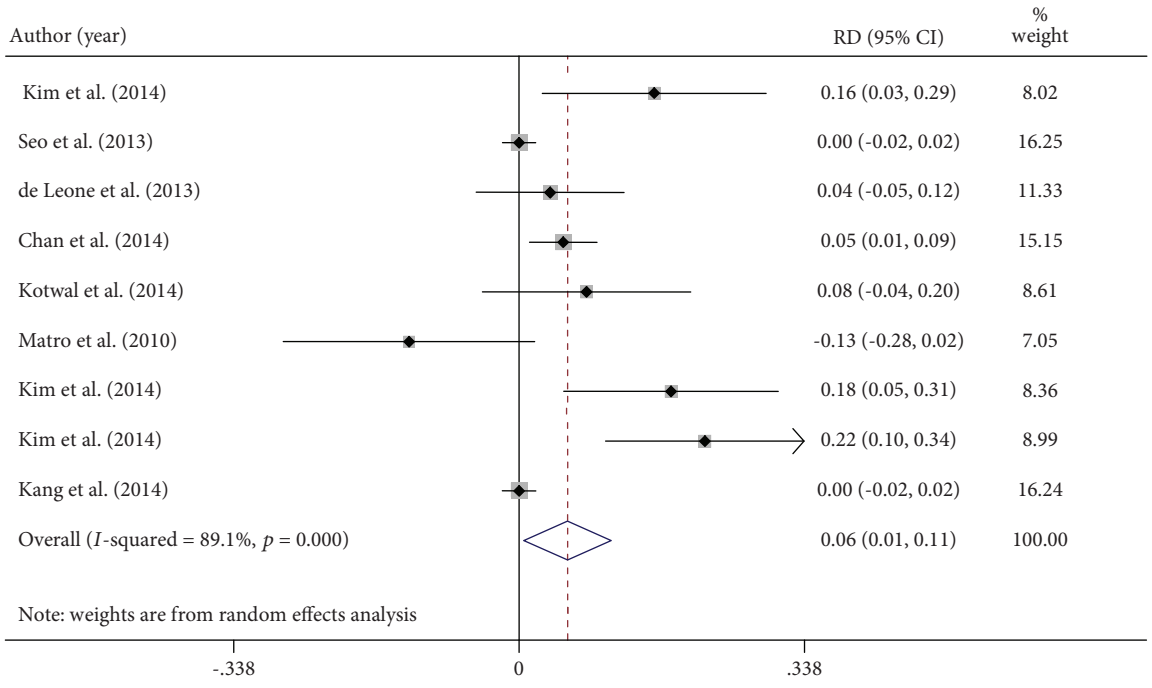

Figure 4: Percentage difference (expressed as rate difference, RD) of compliance of patients consuming same-day or split bowel preparation. RD = rate difference.

[RD -1.7% (95% C.I. -4.7 to 1.2%), $p = 0.24$]. Studies' heterogeneity was significant for compliance (24.42, $p \leq 0.001$, I – squared = 71.3%), but not for the cleaning rate (52%, $p = 0.972$, I – squared = 0.0%). Adverse events were similar with both preparations except for nausea that was more frequent in the SDG [RD for nausea 7.7%, $p = 0.020$ (95% C.I. 1.2 to 14.1%); chi – squared = 15.30, $p = 0.032$; I-squared 54.2%].

3.1.2. Same-Day Picosulphate vs. Split PEG. In the study by Kang et al., the authors tested a PicoNa morning only (97 patients) vs. 4 L PEG split preparation (99 patients) showing a slightly better, thus not statistically different, improvement in the cleaning rate with the PEG solution [respectively, 59/97 (61.5%) vs. 71/99 (71.3%), $p = 0.13$] [14]. Compliance was similar, but adverse events were overall lower with the same-day picosulphate ($p \leq 0.001$), with a

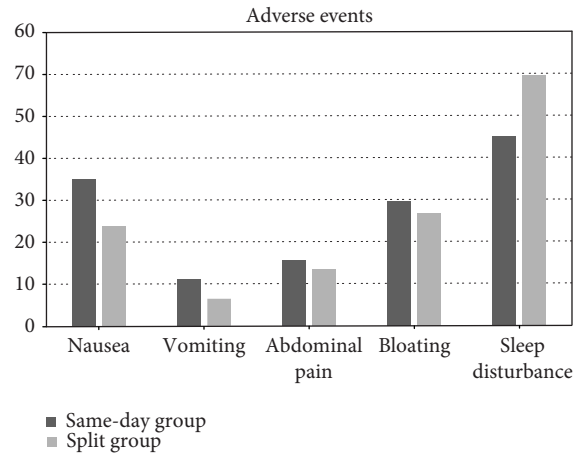

Figure 5: Distribution of adverse events in the same-day and split group, expressed as percentage.

particular emphasis on sleep disturbances that were significantly fewer in the same-day group ($p \leq 0.001$).

3.1.3. Same-Day Picosulphate vs. Split Picosulphate. In this prospective nonrandomized study, Longcroft-Wheaton and Bhandari compared picosulphate in two different regimens [18]. Results showed that the same-day preparation was more effective than the split dose [130/132 (98%) same-day vs. 85/95 (89%) split, $p = 0.007$] with fewer adverse events ($p \leq 0.001$), on equal compliance.

3.1.4. Same-Day PEG vs. Split Picosulphate. In this three-arm randomized clinical trial, the cleaning efficacy of the same-day 4 L PEG (50 patients, group A) was compared to split doses of 2 picosulphate sachets (50 patients, group B) or to split doses of 3 picosulphate sachets (50 patients, group C) [19]. Patients with an excellent/good preparation were 82% (41/50) in the PEG group, 80% (40/50) in the two-sachet group, and 92% (46/50) in the three-sachet group ($p = 0.325$). Compliance was higher for picosulphate [RD -20.1% (95% C.I. -28.8 to -11.5%), $p \leq 0.001$; heterogeneity chi − squared = 10.83, $p = 0.650$; I − squared = 0.0%]. In general, adverse events occurred less frequently with picosulphate preparations with the exception of sleep interruption that was uniformly distributed among groups [nausea RD = 37.1%, (95% C.I. 24.3 to 49.8%), $p \leq 0.001$; vomit RD = 25%, (95% C.I. 16.1 to 33.9%), $p \leq 0.001$; sleep disturbances RD = 16%, (95% C.I. 4.2 to -7.5%), $p = 0.480$; bloating RD = 46.5%, (95% C.I. 26.8 to 66.3%), $p \leq 0.001$; and abdominal pain RD = 24.7%, (95% C.I. 13 to 36.5%), $p \leq 0.001$].

4. Discussion

According to current guidelines, endoscopists should report the quality of the bowel preparation for screening colonoscopy and repeat within a year or less all those procedures classified as inadequate to visualize polyps < 5 mm in size [21, 22]. As a result, international societies published recommendations on bowel preparation for screening colonoscopies, agreeing on the superiority of the split preparation over other cleaning regimens [7, 23] and suggesting tailoring the type of purge to the patients' characteristics according to specific clinical scenarios (in patients, elderly, nephropathic, etc.). The split preparation is associated with higher compliance compared to the traditional previous-day regimen and a better quality of bowel preparation allowing for a substantial increase in the detection of advanced and serrated lesions [23, 24]. One of the main advantages of split preparation over the previous-day regimen is a shorter time lapse between the end of the preparation ingestion and the start of the procedure. It has been shown that the shorter the time lapse, the better the cleaning of the colon and hence the better mucosal visualization. On the other hand, the same-day preparation, which has by definition the shortest interval until the start of the procedure, has been relegated only for afternoon sessions of colonoscopy because the studies published so far are underpowered and provided conflicting results both for cleaning rate and compliance.

In our systematic review, the same-day preparation was compared to the split regimen with a total of 1807 patients included. Results showed that same-day regimens are not inferior to the split, as the overall cleaning efficacy was equivalent between the two and therefore should be taken into account as a valuable alternative regimen. Also, the subgroup analysis (i.e., per type of purge) confirmed the result of the primary analysis and suggested that PEG-based laxatives have their best use within the split regimen rather than the same-day. Conversely, from the patients' prospective, compliance was higher for split prep that was better accepted than the same-day. One could speculate that the reason for this different compliance could be sought in the type of laxative prescribed, as 8 out of 10 studies used a high- or low-volume PEG solution that is hard to drink in a few hours if compared with the same volume to be ingested in two separate doses, but definite conclusions cannot be drawn. Also, the compliance rate was heterogeneous among different studies as a result of a different magnitude and direction of the observed effect (while the cleaning rate was uniform in all but one study) [18]. Such heterogeneity may be due to the lack of use of clinical standards to describe the patients' experience. It would be advisable for future research to use dichotomous variables to describe the patients' experience as well as standardized scales for describing colon cleansing (such as Boston Bowel or Ottawa preparation scales instead of personal scales) to make results easily comparable.

When we compared the adverse events which occurred, sleep disturbance showed a nonsignificant trend toward significance in the split regimen group, while more cases of nausea and vomit were reported for the patients who took the same-day preparation. In our opinion, these differences could be attributable to the specific time-based characteristics of the regimen adopted rather than to the type of purge. Thus, they should be taken into account to optimize the patients' compliance when endoscopists prescribe the bowel preparation.

As for the publication bias, no significant asymmetry was observed in the meta-analysis, and neither significant was the Egger-Harbord regression (Figure 6).

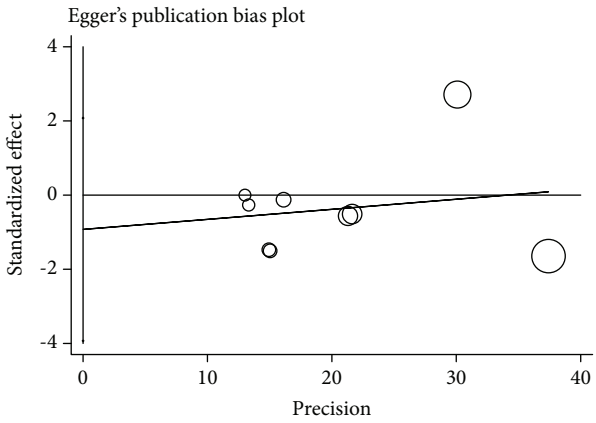

Figure 6: Egger's publication bias plot.

The results of the meta-analysis show that the overall cleaning efficacy of the two regimens was comparable, but slightly better compliance favors the split prep.

As previously demonstrated, this datum confirms two critical elements: firstly, the cleaning efficacy is driven by the time (and thus a short time interval until the beginning of colonoscopy), more than by the type of laxative used. In other words, the second dose of laxative should be considered as the critical factor for an optimal bowel preparation, irrespective of the timing of the first dose, which consequently can be taken the evening before the exam or in the same morning, depending on the subject's need and endoscopy service organization; secondly, more efforts should be made to optimize the patients' compliance that should be regarded as a surrogate marker of quality in endoscopy. Patients' willingness is essential to get a good compliance and to increase participation in bowel screening programs as confirmed by the fact that two of the top ten patients' complaints for CRC screening are the bowel prep and the time spent for the test (e.g., missed work), more than the procedure itself [25, 26]. Also, in a recent publication by Radaelli et al. [27] who assessed patients' attitude toward two cleaning regimens (split vs. previous-day), the authors demonstrated that although split regimen was an independent predictor of adequate colon cleansing (OR 3.34) and polyp detection (OR 1.46) with no statistically different risk of travel interruption and fecal incontinence, appointment before 10:00 a.m. and travel time to endoscopy service > 1 h shift patients' preferences toward the previous-day regimen. Moreover, one should consider that patients might sometimes have personal or professional problems that make split preparation unsuitable in specific circumstances (e.g., evening dose unfeasible for work/personal situations). In our opinion, instead of using the same regimen for all patients, it could be advisable for endoscopy services to tailor the appropriate bowel preparation on the patients' needs and on the time of the scheduled colonoscopy, giving that overall the split and the same-day are comparable for the bowel cleaning. Still, patients should be clearly informed about pros (e.g., palatability and cleaning efficacy) and cons (adverse events, time needed, etc.) of the different regimens to improve their acceptance and reduce social barriers toward this screening tool.

In conclusion, although split preparation should be regarded as the best cleaning regimen for its high rate of efficacy and tolerability, the same-day preparation can be a valid alternative when the split preparation does not fit the patients' needs or when an afternoon colonoscopy is scheduled, provided that patients are informed on the different prevalence of side effects.

Conflicts of Interest

Authors have no conflicts of interest to declare.

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Research Article

Different Anatomical Subsites of Colon Cancer and Mortality: A Population-Based Study

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Background. In terms of incidence and pathogenesis, right-sided colon cancer (RCC) and left-sided colon cancer (LCC) exhibit several differences. However, whether existing differences could reflect the different survival outcomes remains unclear. Therefore, we aimed to ascertain the role of location in the prognosis. *Methods*. We identified colon cancer cases from the Surveillance, Epidemiology, and End Results database between 1973 and 2012. Differences among subsites of colon cancer regarding clinical features and metastatic patterns were compared. The Kaplan-Meier curves were conducted to compare overall and disease-specific survival in relation to cancer location. The effect of tumour location on overall and cancer-specific survival was analysed by Cox proportional hazards model. *Results*. A total of 377,849 patients from SEER database were included in the current study, with 180,889 (47.9%) RCC and 196,960 (52.1%) LCC. LCC was more likely to metastasize to the liver and lung. Kaplan-Meier curves demonstrated that LCC patients had better overall and cancer-specific survival outcomes. Among Cox multivariate analyses, LCC was associated with a slightly reduced risk of overall survival (HR, 0.92; 95% CI, 0.92-0.93) and cancer-specific survival (HR, 0.92; 95% CI, 0.91-0.93), even after adjusted for other variables. However, the relationship between location and prognosis was varied by subgroups defined by age, year at diagnosis, stage, and therapies. *Conclusions*. We demonstrated that LCC was associated with better prognosis, especially for patients with distant metastasis. Future trails should seek to identify the underlying mechanism.

1. Introduction

Colorectal cancer remains the third common malignancy in males and the second in females worldwide, respectively [1]. In 2012, it was estimated that approximately 1,400,000 individuals were diagnosed with colorectal cancer, with accounting for 694,000 deaths globally [1]. Notably, screening tests and comprehensive treatments of colorectal cancer had contributed to the better prognosis in the past decades [2].

It is well acknowledged that colon and rectal cancer share several similarities; however, some important differences exist as well [3, 4]. Recently, colon cancer subsites, in terms of right- or left-sided origins, had aroused great public interests [5–11]. Several studies had investigated the influence of different anatomic sites in clinical features and survival outcomes of colon cancer [6, 8, 9, 12, 13]. Back in 1990, Bufill.et al. [14] firstly proposed that tumour located in the distal and proximal colon location might possess different biologic and genetic properties. Subsequently, accumulating evidence had indicated that right- and left-sided colon cancer (RCC and LCC) not only located on different sites simply but also presented distinct clinical symptoms and molecular profiles [10, 15–19]. According to previous studies, patients with RCC always presented with subtle or occult symptoms,

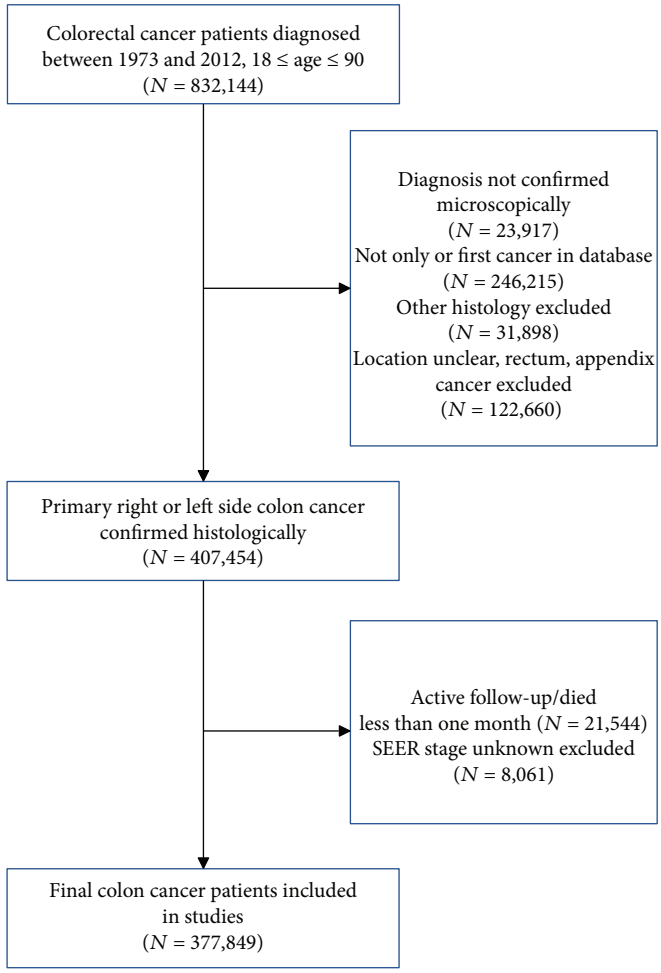

Figure 1: Flowchart for include patients from the Surveillance, Epidemiology, and End Results database.

higher tumour stage, poor differentiated, higher percentage of CIMP, MSI, and BRAF mutation positive [18, 20–23]. As opposed to RCC, patients with LCC frequently exhibited evident symptoms, lower tumour stages, and chromosomal instability [23–26]. The underlying mechanisms for these discrepancies were still uncertain, yet, different embryologic origins might partly account for those [12]. However, whether those differences could translate into different survival outcomes needs further exploration. Several studies had attempted to assess the prognostic role of cancer location in patients with colon cancer [5, 7, 11–13, 23]. However, the current results remained conflicting and might need to be further addressed. [13, 17, 27]. For example, in 2011, Weiss and colleagues [17] observed no overall survival difference between RCC and LCC among patients with combined I–III stages. In 2014, another study also reported that no survival advantage was observed among patients with either right- or left-sided stage II cancer [27]. The inconsistent association might reflect the complexity of this issue, limited sample size, and high degree of heterogeneity across studies.

In light of those conflicting results, we, therefore, performed a retrospective analysis to investigate the impact of tumour location on survival outcomes in a population-based study. Furthermore, we also examined whether the prognostic role of cancer location was influenced by different groups of age, stage, year of diagnosis, and therapies.

2. Results

2.1. Patient Characteristics. A total of 377,849 cases with colon cancer during 1973 to 2012 were included in our studies. The detailed selection diagram of the population was presented in Figure 1. Among populations, there were 180,889 (47.9%) patients with RCC and 196,960 (52.1%) patients with LCC. The proportion of patients with RCC increased faster from 1973–1982 to 2003–2012 compared with LCC. Patients with RCC were older, female, and poorly differentiated. Table 1 summarized the baseline characteristics with respect to primary cancer location.

2.2. Metastasis Pattern of Colorectal Cancer by Subsites. The SEER database only included detailed main information of four metastatic sites about the bone, brain, liver, and lung since 2010^{+}. Therefore, we included patients with clear metastatic information from 2010 to 2012 and made use of those population to analyse the synchronous metastasis distribution according to primary cancer location. The most frequent distant metastasis site of colon cancer was the liver, followed

Table 1: Baseline patient and disease characteristics of patients by primary tumour location.

Characteristic	Right-sided tumours	Left-sided tumours	*P*
Year at diagnosis			*<0.001*
1973–1982	18,670 (10.3%)	26,094 (13.2%)	
1983–1992	25,442 (14.1%)	33,380 (16.9%)	
1993–2002	51,278 (28.3%)	54,272 (27.6%)	
2003–2012	85,499 (47.3%)	83,214 (42.2%)	
Age at diagnosis[†]			*<0.001*
<50 y	13,333 (7.4%)	22,511 (11.4%)	
50–69 y	67,385 (37.3%)	93,511 (47.5%)	
≥70 y	100,171 (55.4%)	80,938 (41.1%)	
Gender			*<0.001*
Male	80,436 (44.5%)	103,612 (52.6%)	
Female	100,453 (55.5%)	93,348 (47.4%)	
Race			*<0.001*
White	149,172 (82.5%)	159,284 (80.9%)	
Black	20,626 (11.4%)	18,880 (9.6%)	
Other (AP, AI/AN)	10,545 (5.8%)	17,772 (9.0%)	
Unknown	546 (0.3%)	1024 (0.5%)	
Insurance status			*<0.001*
Insured	49,043 (27.1%)	45,911 (23.3%)	
Uninsured	1520 (0.8%)	1991 (1.0%)	
Unknown	130,326 (72.0%)	149,058 (75.7%)	
Marital status			*<0.001*
Married	97,678 (54.0%)	115,912 (58.9%)	
Unmarried	76,752 (42.4%)	73,159 (37.1%)	
Unknown	6459 (3.6%)	7889 (4.0%)	
Tumour stage			*<0.001*
Localized	65,978 (36.5%)	78,611 (39.9%)	
Regional	76,694 (42.4%)	76,269 (38.7%)	
Distant	38,217 (21.1%)	42,080 (21.4%)	
Tumour grade[‡]			*<0.001*
I	16,562 (9.2%)	22,181 (11.3%)	
II	104,036 (57.5%)	120,896 (61.4%)	
III	38,575 (21.3%)	24,897 (12.6%)	
IV	2790 (1.5%)	1546 (0.8%)	
Unknown	18,926 (10.5%)	27,440 (13.9%)	
Surgery			*<0.001*
Surgery	173,582 (96.0%)	187,853 (95.4%)	
No surgery	7202 (4.0%)	8993 (4.6%)	
Unknown	105 (0.1%)	114 (0.1%)	
Radiation			*<0.001*
Radiation	3453 (1.9%)	15,658 (7.9%)	
No radiation	175,810 (97.2%)	179,168 (91.0%)	
Unknown	1626 (0.9%)	2134 (1.1%)	
Chemotherapy			*<0.001*
Yes	50,568 (28.0%)	59,160 (30.0)	
No	130,321 (72.0%)	137,800 (70.0%)	

[†]Year; [‡]I means well differentiated; II means moderately differentiated; III means poorly differentiated; IV means undifferentiated. AP: Asian or Pacific Islander; AI/AN: American Indian/Alaska Native.

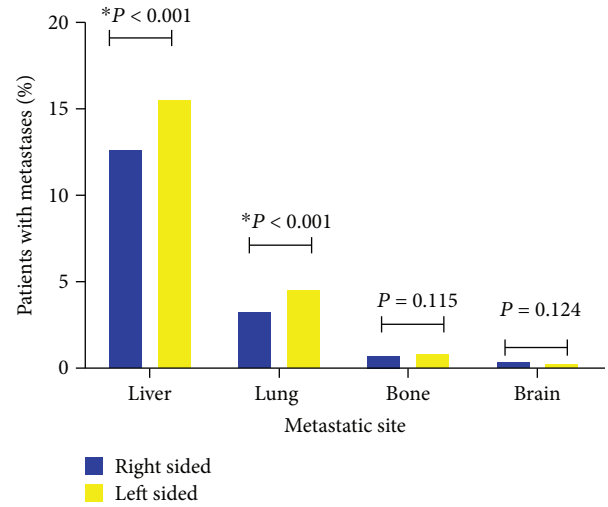

Figure 2: Metastatic distribution patterns between right- and left-sided colon cancers. *$P < 0.01$.

by the lung, bone, and brain, in respective of subsites. As shown in Figure 2, patients with LCC were more likely to have liver and lung metastasis at the time of diagnosis (both $P < 0.01$), which were main distant metastases for colon cancer. However, there were no significant difference between RCC and LCC for bone and brain metastases (both $P > 0.05$). Furthermore, we analysed the OS and CSS between RCC and LCC stratified by different metastases. As shown in Figure 3, there was no OS and CSS difference among RCC and LCC for bone and brain metastases. For liver and lung metastasis, patients with LCC enjoyed a better OS and CSS in relation to those with RCC.

2.3. Survival Analysis of Cancer Subsites by Stage, Age, Year of Diagnosis, and Therapies. Kaplan-Meier curves firstly demonstrated a significant difference of OS and CSS between RCC and LCC patients (both $P < 0.001$, Figure 4). The overall five-year survival rates for RCC and LCC patients were 50.6% and 54.4%, respectively. Multivariable Cox analyses demonstrated that cancer location was an independent prognostic factor for OS and CSS, even after adjusting for other variables, such as age, year, gender, race, insurance, marital status, tumour stage, grade, surgery, radiation, and chemotherapy (RCC as reference, OS, LCC HR = 0.92, 95% CI, 0.92-0.93; CSS, adjusted HR = 0.92, 95% CI, 0.91-0.93) (Table 2). Furthermore, we analyses overall survival differences in subgroups defined by the age (i.e., <50 y, 50–69 y, ≥70 y), year at diagnosis (i.e., 1973–1982, 1983–1992, 1993–2002, and 2003–2012), SEER tumour stage (i.e., regional, localized, and distant), and therapies (surgery, radiation, and chemotherapy). Interestingly, we found that the prognostic effect of subsites was inconsistent across subgroups (Table 3). The benefits associated with cancer location were more pronounced in colon cancer diagnosed at 2003–2010. There were no significant survival advantages among localized and regional stages, while survival benefits associated with left-sided cancer were obvious among distant stage. By contrast, in older age group (≥70 y), patients with RCC even

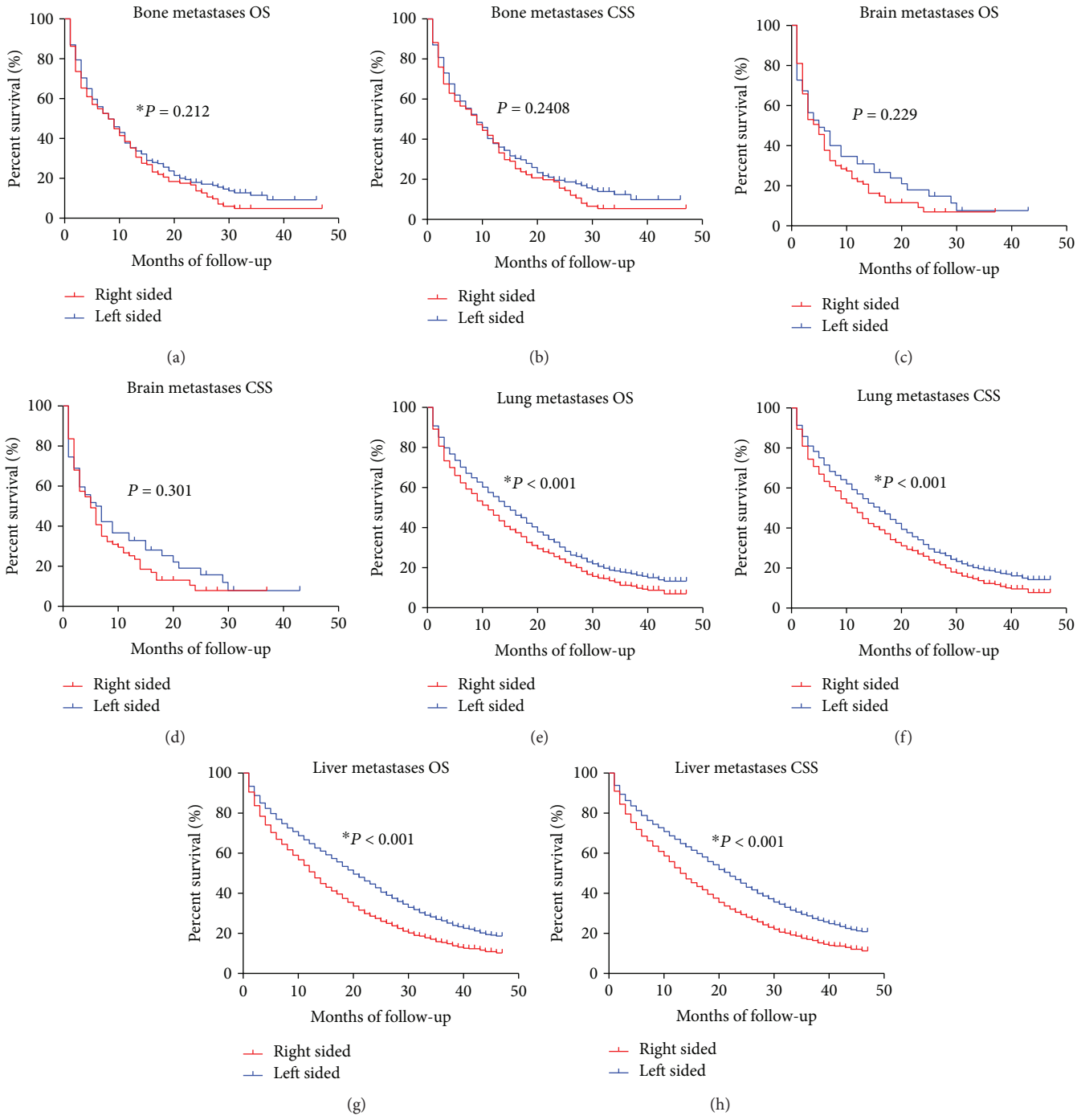

Figure 3: Kaplan-Meier survival analysis of patients with different metastases. (a) Overall survival for bone metastases. (b) Cancer-specific survival for bone metastases. (c) Overall survival for brain metastases. (d) Cancer-specific survival for brain metastases. (e) Overall survival for lung metastases. (f) Cancer-specific survival for lung metastases. (g) Overall survival for liver metastases. (h) Cancer-specific survival for liver metastases.

had a decreased risk of mortality in relation to LCC. For different therapies, the prognostic role of tumour location was consistent.

3. Discussion

Utilizing population-based database from SEER, we observed that different metastatic distribution and prognosis among right- and left-sided cancers. In accordance with previous studies [12], liver and lung metastases were more likely to present in left-sided carcinomas. It was reported that peritoneal metastases were in right-sided carcinomas predominately [12]. Since lacking other distant metastatic information, we were unable to examine this pattern. Subsequently, we demonstrated that LCC was significantly associated with better OS and CSS, even after adjusted for multiple variables. Our

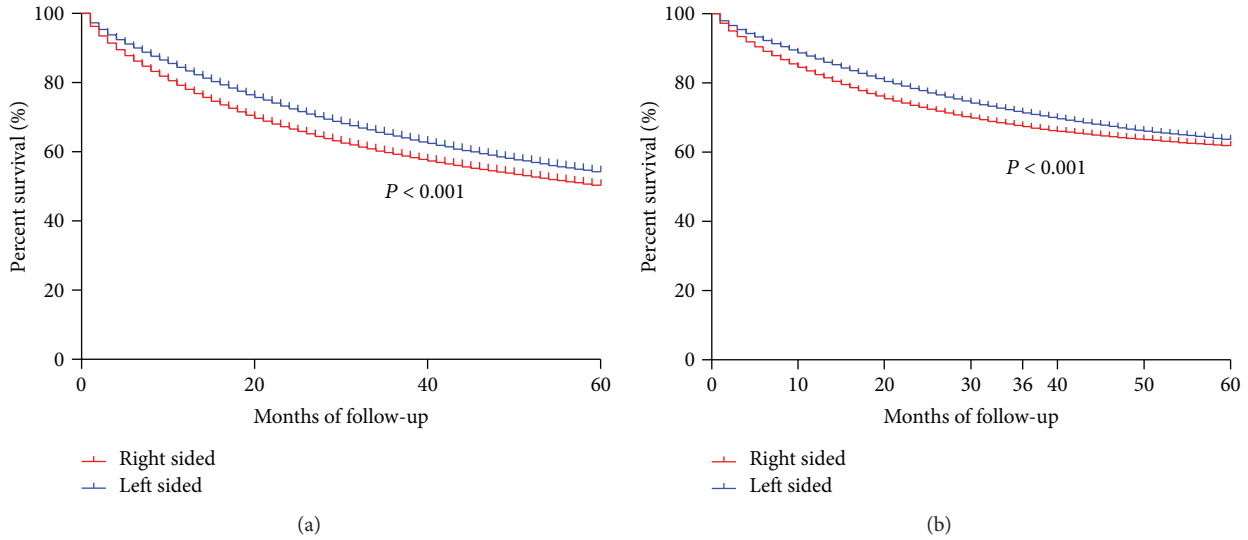

Figure 4: Overall and cancer-specific analysis between right- and left-sided colon cancers. (a) Overall survival. (b) Cancer-specific survival.

result was consistent with a large body of research, which indicated potential survival benefits existed among patients with left-sided cancer, although several studies could not confirm this result [17, 27]. Whether other factors influenced the prognostic effect of primary cancer location was less well studied. Therefore, we performed subgroup analysis to explore other factors. Notably, we observed the association was varied across different age, stage, year at diagnosis, and therapies. Patients with left-sided cancer were significantly associated with favourable overall survival, especially for patients with distant metastases. The clear underlying causes remained unknown. Different tumour biology and therapies might be partly accounted for those.

Recently, differences between RCC and LCC has aroused considerable attentions [3, 4, 11, 14, 23]. An increasing amount of evidence showed that RCC and LCC had differences in clinical presentation, pathology, and molecular signatures. According to Missiaglia and colleagues [28], microsatellite instable-high (MSI) and BRAF mutation were predominate among proximal (right-sided) tumours, while distal cancers (left-sided) were characterised by chromosome instable, high expression of epiregulin, and human epidermal growth factor receptor 2 (HER2) amplification [24, 29, 30]. Another large genome-scale analysis of colorectal cancer tissues conducted by the Cancer Genome Atlas Network also revealed some differences between cancers originating from the right colon and all other sites [31]. It is conceivable that underlying molecular base might drive the observed survival difference between RCC and LCC.

Taking colon cancer as a heterogeneous group with different genetic and epigenetic changes into account, appropriate classification of colorectal cancer is increasingly important for clinical practice, especially for therapies chosen in the exciting age of precision medicine [25]. Although molecular classification is promising, huge cost impedes its wide application. Whether primary cancer location could be considered as a surrogate marker for prognosis attracts huge interests, although the significance of cancer location on prognosis was still a dispute. Chemotherapy is an important part of advanced cancer treatments. It was reported that RCC and LCC exhibited different response to chemotherapy and targeted treatments, which might influence therapy selection. In 2013, a retrospective analysis of two independent cohorts indicated that only metastatic CRC patients with left-side cancer might benefit from bevacizumab in combination with capecitabine and oxaliplatin. [32] Further study by Loupakis et al. [6] validated this finding in three independent cohorts (PROVETTA, AVF2107g, and NO16966). However, in 2015, Brulé et al. [13] reanalysed the results of NCIC CO.17 trial and the results showed that tumour location was only predictive of progression-free survival benefit from cetuximab in refractory metastatic colon cancer, although location alone was absent of prognostic effect on survival in best supportive care group. In ASCO 2016, Venook and colleagues reanalysed CALGB/SWOG 80405 (Alliance) study and unexpectedly found that survival benefits associated with the side of colon appeared to be far greater in metastatic stage than previously considered [33]. A striking survival difference was observed in subgroups among RCC and LCC. Cetuximab was superior to bevacizumab for overall survival when the primary tumour was on the left side [33]. It indicated that the response of cetuximab and bevacizumab were dependent on the location of the primary tumours, which reminds us that anatomical location may promisingly indicate optimal therapy regimen selection. A recent meta-analysis by Petrelli et al. [34] including 66 studies demonstrated that left-sided colon cancer was associated with better prognosis (HR, 0.82; 95% CI, 0.79-0.84; $P < 0.001$) and colon cancer location should be considered as a prognostic criterion when making treatment decisions.

As a retrospective study, several intrinsic limitations of this study should be considered. Firstly, SEER dataset lacks of detailed information on specific chemotherapy regimens, especially for biotarget therapies. Therefore, we were unable to adjust this important effect on survival. Secondly, genetic or molecular marker statuses were not available in this

Table 2: Univariate and multivariate analysis of overall and cancer-specific survival in patients with colon cancer.

Variable	Overall survival				Cancer-specific survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Year of diagnosis								
1973–1982	Reference		Reference		Reference		Reference	
1983–1992	0.88 (0.87, 0.89)	<0.001	0.87 (0.86, 0.89)	<0.001	0.82 (0.80, 0.83)	<0.001	0.82 (0.81, 0.84)	<0.001
1993–2002	0.74 (0.73, 0.75)	<0.001	0.73 (0.72, 0.74)	<0.001	0.67 (0.66, 0.68)	<0.001	0.64 (0.63, 0.66)	<0.001
2003–2012	0.59 (0.58, 0.59)	<0.001	0.62 (0.61, 0.63)	<0.001	0.54 (0.53, 0.55)	<0.001	0.52 (0.51, 0.53)	<0.001
Age at diagnosis								
<50 y	Reference		Reference		Reference		Reference	
50–69 y	1.34 (1.32, 1.37)	<0.001	1.48 (1.46, 1.51)	<0.001	1.02 (1.00, 1.04)	0.091	1.19 (1.17, 1.21)	<0.001
≥70 y	2.45 (2.41, 2.49)	<0.001	2.87 (2.82, 2.92)	<0.001	1.17 (1.15, 1.19)	<0.001	1.58 (1.55, 1.61)	<0.001
Gender								
Male	Reference		Reference		Reference		Reference	
Female	0.93 (0.93, 0.94)	<0.001	0.81 (0.80, 0.81)	<0.001	0.93 (0.92, 0.94)	<0.001	0.89 (0.88, 0.90)	<0.001
Race								
White	Reference		Reference		Reference		Reference	
Black	1.08 (1.07, 1.10)	<0.001	1.15 (1.13, 1.16)	<0.001	1.20 (1.18, 1.22)	<0.001	1.18 (1.17, 1.20)	<0.001
Other (AP, AI/AN)	0.77 (0.76, 0.78)	<0.001	0.86 (0.85, 0.88)	<0.001	0.84 (0.82, 0.85)	<0.001	0.91 (0.89, 0.93)	<0.001
Primary site								
Right	Reference		Reference		Reference		Reference	
Left	0.86 (0.85, 0.87)	<0.001	0.92 (0.92, 0.93)	<0.001	0.93 (0.92, 0.94)	<0.001	0.92 (0.91, 0.93)	<0.001
Insurance status								
Insured	Reference		Reference		Reference		Reference	
Uninsured	1.21 (1.14,1.28)	<0.001	1.21 (1.15,1.28)	<0.001	1.43 (1.35, 1.52)	<0.001	1.11 (1.04, 1.18)	<0.001
Marital status								
Unmarried	Reference		Reference		Reference		Reference	
Married	1.37 (1.36, 1.39)	<0.001	1.26 (1.25, 1.27)	<0.001	1.18 (1.17, 1.19)	<0.001	1.14 (1.12, 1.15)	<0.001
Tumour stage								
Localized	Reference		Reference		Reference		Reference	
Regional	1.59 (1.57, 1.60)	<0.001	1.62 (1.60, 1.63)	<0.001	3.34 (3.29, 3.40)	<0.001	3.22 (3.16, 3.27)	<0.001
Distant	6.60 (6.53, 6.67)	<0.001	6.99 (6.91, 7.08)	<0.001	17.98 (17.68, 18.28)	<0.001	16.98 (16.68, 17.29)	<0.001
Tumour grade[‡]								
I	Reference		Reference		Reference		Reference	
II	1.15 (1.14, 1.17)	<0.001	1.07 (1.06, 1.09)	<0.001	1.40 (1.37,1.43)	<0.001	1.16 (1.14, 1.18)	<0.001
III	1.75 (1.72, 1.78)	<0.001	1.38 (1.35, 1.40)	<0.001	2.54 (2.49, 2.60)	<0.001	1.64 (1.60, 1.67)	<0.001
IV	1.86 (1.78, 1.93)	<0.001	1.54 (1.48, 1.60)	<0.001	2.65 (2.53, 2.78)	<0.001	1.82 (1.73, 1.91)	<0.001
Chemotherapy								
Yes	Reference		Reference		Reference		Reference	
No	0.83 (0.83, 0.84)	<0.001	1.13 (1.12, 1.15)	<0.001	1.85 (1.83, 1.87)	<0.001	1.06 (1.05, 1.07)	<0.001
Surgery								
Yes	Reference		Reference		Reference		Reference	
No	4.41 (4.43, 4.49)	<0.001	2.59 (2.54, 2.63)	<0.001	5.42 (5.32, 5.52)	<0.001	2.62 (2.56, 2.67)	<0.001
Radiation therapy								
Yes	Reference		Reference		Reference		Reference	
No	0.82 (0.80, 0.83)	<0.001	0.91 (0.90, 0.93)	<0.001	0.61 (0.59, 0.62)	<0.001	0.86 (0.84, 0.88)	<0.001

[‡]I means well differentiated; II means moderately differentiated; III means poorly differentiated; IV means undifferentiated. AP: Asian or Pacific Islander; AI/AN: American Indian/Alaska Native.

Table 3: Crude and adjusted hazard ratios for overall survival between right- and left-sided cancers by year, age, stage, and therapy.

Cohort	Crude HR (95% CI)	*P*	Adjusted HR* (95% CI)	*P*
Year at diagnosis				
All	0.86 (0.85, 0.87)	*<0.001*	0.92 (0.92, 0.93)	*<0.001*
1973–1982	0.88 (0.86, 0.90)	*<0.001*	0.96 (0.94, 0.98)	*<0.001*
1983–1992	0.91 (0.89,0.94)	*<0.001*	0.98 (0.97,1.00)	*0.059*
1993–2002	0.89 (0.87,0.91)	*<0.001*	0.95 (0.94,0.97)	*<0.001*
2003–2012	0.83 (0.81, 0.84)	*<0.001*	0.91 (0.89,0.92)	*<0.001*
Age at diagnosis				
All	0.86 (0.85, 0.87)	*<0.001*	0.92 (0.92, 0.93)	*<0.001*
<50 y	0.89 (0.86, 0.92)	*<0.001*	0.88 (0.85, 0.91)	*<0.001*
50–69 y	0.87 (0.86, 0.88)	*<0.001*	0.89 (0.88, 0.91)	*<0.001*
70 y	1.00 (0.99, 1.01)	*0.423*	1.02 (1.00, 1.03)	*0.006*
SEER stage				
All	0.86 (0.85, 0.87)	*<0.001*	0.92 (0.92, 0.93)	*<0.001*
Localized	0.81 (0.79, 0.82)	*<0.001*	0.99 (0.98,1.01)	*0.323*
Regional	0.90 (0.89,0.91)	*<0.001*	0.99 (0.98,1.00)	*0.156*
Distant	0.80 (0.79,0.82)	*<0.001*	0.81 (0.79,0.82)	*<0.001*
Surgery				
Yes	0.85 (0.85–0.86)	*<0.001*	0.92 (0.92, 0.93)	*<0.001*
None	0.81 (0.75–0.88)	*<0.001*	0.82 (0.79–0.85)	*<0.001*
Radiation				
Yes	0.54 (0.52–0.56)	*<0.001*	0.69 (0.66–0.72)	*<0.001*
None	0.86 (0.85–0.86)	*<0.001*	0.94 (0.93–0.94)	*<0.001*
Chemotherapy				
Yes	0.87 (0.86–0.88)	*<0.001*	0.83 (0.82–0.84)	*<0.001*
None/unknown	0.85 (0.84–0.85)	*<0.001*	0.96 (0.95–0.97)	*<0.001*

HR: hazard ratio; CI: confidence interval. *Adjusted for age, gender, race, year, insurance status, marital status, grade, surgery, radiation, and SEER stage. Right-sided cancer as conference.

dataset. We failed to examine the effect of molecular difference on survival of right- and left-sided cancers.

In summary, subsites of colon cancer could be potentially considered as an independent prognostic factor for OS and CSS. Additional further prospective research should verify this association and seek to elucidate the underlying biological mechanisms. We hope our finding could provide some evidence for further studies.

4. Methods

4.1. Data Sources and Cohort Definition. We identified patients with primary colon cancer from the Surveillance, Epidemiology, and End Results (SEER) between 1973 and 2012. Primary cancer site was identified by the International Classification of Diseases for Oncology (ICD-O-3) site codes (C18.0, C18.2 to C18.7, and C19.9), and adenocarcinoma type was identified by the ICD-O-3 histology codes (8140 to 8147, 8210 to 8211, 8220 to 8221, 8260 to 8263, 8480 to 8481, and 8490). In this analysis, we adopted the SEER historic staging system instead of the American Joint Committee on Cancer (AJCC) system because of its advantage that recorded consistently from 1973 to 2012. According to previous studies [6], we discriminated right and left-sided cancers by splenic flexure as the cut-off. Therefore, C18.0 (cecum), C18.2 (ascending colon), C18.3 (hepatic flexure of colon), and C18.4 (transverse colon) were considered as right-sided colon cancers, and C18.5 (splenic flexure of colon), C18.6 (descending colon), C18.7 (sigmoid colon), and 19.9 (recto-sigmoid) were defined as left-sided colon cancers. We defined any cause of deaths as events and alive as censored events in overall survival analysis. In cause-specific survival analysis, we defined deaths due to colon cancer as events and deaths from any other causes as censored events. In addition, we included patients who were diagnosed with colon cancer during 2010–2012 to analyse metastatic pattern. The following cases were excluded in our study: colon cancer was not the primary cancer; cases diagnosed at autopsy or by death certificate only and without histological confirmation; patients who died less than one month. This study was approved by the review board of the Sir Run Run Shaw Hospital, Zhejiang University School Medicine, Zhejiang, China.

4.2. Statistical Analysis. We conducted chi-square tests to compare the clinical characteristics and metastatic pattern between RCC and LCC. Kaplan-Meier curves were conducted to compare overall and cancer-specific survival

between RCC and LCC within different metastasis sites. The multivariable Cox analyses were adopted to calculate corresponding hazard ratios (HRs) and 95% confidence intervals (CIs). According to previous studies, we selected several prognostic variables and confounders into Cox proportional hazards, such as age, year, gender, race, insurance, marital status, tumour stage, grade, surgery, radiation, and chemotherapy. Two-sided P values at the $P < 0.05$ level was considered to be statistically significant. All analyses were performed with SPSS version 20.0 (SPSS, Chicago, Illinois, USA).

Abbreviations

SEER: Surveillance, Epidemiology, and End Results
HR: Hazard ratio
CI: Confidence interval
RCC: Right-sided colon cancer
LCC: Left-sided colon cancer
OS: Overall survival
CSS: Cancer-specific survival.

Data Availability

All data used to support the findings of this study are public.

Conflicts of Interest

No potential conflicts of interest to declare.

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Research Article

Cyclooxygenase-2 Expression Is a Predictive Marker for Late Recurrence in Colorectal Cancer

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Introduction. Cyclooxygenase-2 (COX-2) expression is elevated in colorectal cancer (CRC). However, data about the relation between COX-2 expression and the impact on the biologic behavior of recurrent disease are inconclusive as yet. The aim of this study is to investigate the relationship between the status of COX-2 expression in the primary CRC and the characteristics of recurrence after curative resection of stage I to III CRC. *Materials and Methods*. Ninety-eight patients with recurrence in 376 CRC patients, who underwent curative surgery between January 1991 and August 2001, were retrospectively assessed. Immunohistochemical staining, performed for the presence of COX-2 on tissue microarrays, was analyzed. *Results*. Forty-six patients showed elevated COX-2 expression, and 52 patients did not. The mean time to recurrence was significantly longer in the positive group than in the negative group (34.1 months ± 30.0 versus 21.9 months ± 17.4; $P = 0.019$). Positive COX-2 expression was correlated with late recurrence (>3 years after surgery) [43.5% versus 13.5%; $P = 0.001$]. In multivariate analysis, COX-2 expression was an independent factor associated with late recurrence (OR 4.656; 95% CI, 1.696 to 12.779; $P = 0.003$). Recurrence pattern and postrecurrence survival were not different between the two groups. *Conclusions*. Elevated COX-2 expression in itself is not a prognostic factor, but COX-2 expression in tumor tissue may be an independent predictive marker of late recurrence for patients with stage I to III CRC.

1. Introduction

The mainstay of colorectal cancer (CRC) treatment is curative resection, and tumor recurrence is a major concern after surgery. There have been several attempts to identify molecular markers that can predict recurrence and survival rates, but still, none is approved for clinical application.

Cyclooxygenase-2 (COX-2) is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandins and thromboxanes. These products play crucial roles in cell proliferation, immune response, angiogenesis, and inflammatory reaction, which may involve tumor development and progression [1, 2]. Previous studies have reported that COX-2 overexpression is detected in colorectal, gastric, breast, pulmonary, esophageal, and pancreatic cancer [3–7]. Increased COX-2 gene expression has been reported in human colorectal adenocarcinoma and in carcinogen-induced rat colonic tumors [8–12]. However, the molecular mechanisms by which COX-2 contributes to CRC progression and metastasis remain unclear. In addition, it remains controversial whether COX-2 expression is a prognostic factor for the survival of CRC patients or not [13–15].

The aim of this study is to investigate the relationship between the status of COX-2 expression and the characteristics of recurrent disease in stage I–III CRC patients after curative resection.

2. Methods

2.1. Selection of Patients. We retrospectively reviewed, between January 1991 and August 2001, 492 patients with the diagnosis of CRC and treated in one tertiary care center. 44 patients were excluded by the inclusion criteria. The inclusion criteria were stage I–III patients with curative resection

(R_0). Patients with (a) distant metastasis ($n = 29$), (b) incomplete resection ($n = 2$), and (c) metachronous cancers ($n = 3$) were excluded. The patients who expired due to other causes ($n = 10$) were also excluded. As a result, formalin-fixed paraffin-embedded samples from 376 patients were available. Patients with lymph node-positive disease received 5-FU-based adjuvant chemotherapy, and none of the patients received preoperative chemotherapy or radiotherapy. The median follow-up period was 56 months (range, 3 to 192 months). Of the 376 patients, COX-2 expression was elevated in 211 patients (56.0%, 211 patients), and the overall recurrence rate was 26.0% (98/376 patients).

2.2. Tissue Microarrays (TMAs). After the histological examination of H&E-stained samples by an experienced pathologist, parts containing a high proportion of tumor cells were assembled. TMAs were constructed with a tissue arrayer (AccuMac Arrayer, ISU ABXIS Co. Ltd., Seoul, Korea). The assembled TMAs were held in an X-Y position guide with 1 mm increments between individual samples and a 3 mm punch-depth stop device. Briefly, this instrument was utilized to make holes in a recipient block with defined array cores, and a solid stylet, which fitted the needle closely, was used to transfer the tissue cores into the recipient block. Due to the limited size of representative areas of the tumors, triplicate 1 mm diameter tissue cores were made from each donor block.

2.3. Immunohistochemical Staining. We obtained multiple 4 μm cut sections using a Leica microtome in immunohistochemical staining. The obtained sections were shifted to adhesive-coated slides. Dewaxing was performed with the TMA slides by heating at 55°C for 30 min and by three washes, of 5 min each, with xylene. Rehydration was done with the tissues by 5 min washes in 100%, 90%, and 70% ethanol and phosphate-buffered saline (PBS). Antigen was retrieved by heating the samples for 4 min 20 s in a microwave at full power in 250 ml 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase for 20 min. The sections were incubated with primary goat polyclonal anti-COX-2 antibody (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 100 in goat serum at room temperature for 1 h. After three washes of 2 min each with PBS, the sections were incubated with biotinylated anti-goat secondary antibody for 30 min (DAKO, Carpinteria, CA, USA). After three further washes with PBS, horseradish peroxidase streptavidin (DAKO) was added to the section for 30 min, followed by another three washes. The samples were developed for 1 min with 3,3′-diaminobenzidine substrate (Vector Laboratories, Burlington, Ontario, Canada) and counterstained with Mayer's hematoxylin. They were dehydrated according to standard procedures and closed with coverslips.

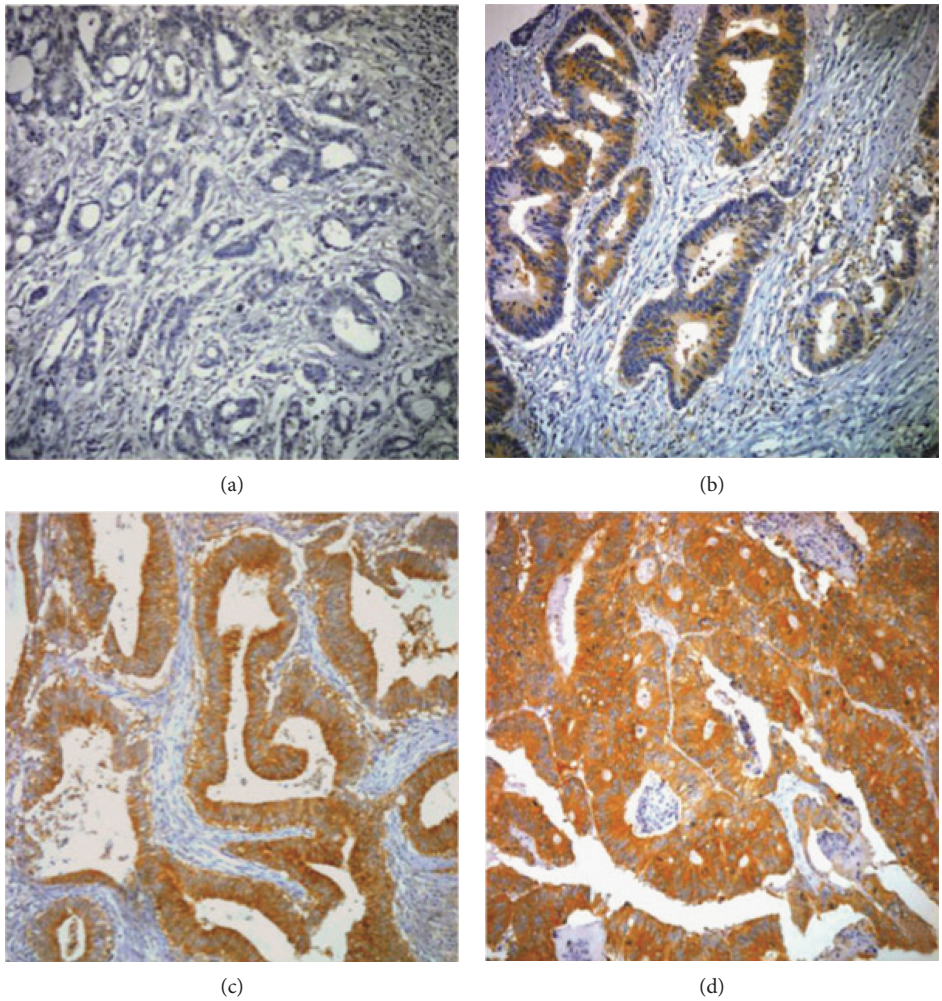
2.4. Interpretation of COX-2 Expression. COX-2 expression was interpreted independently by two experienced pathologists (SS Paik and SH Jang) on the basis of staining intensity and extent. Three punches per case were evaluated and considered a whole. Staining intensity was scored as 0 to 3 (0 = negative; 1 = weak; 2 = moderate; and 3 = strong) (Figure 1). Staining extent was scored as 0 to 4 based on the percentage of positive-stained cells (0 = 0%; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100%). The final staining score was determined with a sum of the intensity and extent score. We divided all cases into four expression groups based on their sum of scores (0 = negative; 1–3 = low; 4-5 = moderate; and 6-7 = high). If the sum of scores was ≥4, we classified the cases as elevated COX-2 expression (positive). If the sum of scores was ≤3, we classified the cases as COX-2 negative. When there was a disagreement between the two pathologists, reinvestigation of the slide was performed with a multiheaded microscope and the final agreement was achieved.

2.5. Statistical Analysis. Statistical analysis was conducted with SPSS ver. 19.0 (SPSS Inc., Chicago, IL, USA). The chi-square test and Student's t-test were used to examine the association between COX-2 expression and clinical and pathological features including age, gender, tumor location, tumor size, gross type, cell type, differentiation, lymphatic invasion, vascular invasion, T category, N category, AJCC stage, and time to recurrence. Survival curves were constructed using the Kaplan-Meier method, and survival differences were analyzed by the log-rank test. P values < 0.05 were considered statistically significant.

3. Results

3.1. COX-2 Expression Is Related with Time to Recurrence. The clinical and pathological characteristics of the 376 patients are described in Table 1 according to the COX-2 expression status. In univariate analysis, the COX-2-positive group was different from the COX-2-negative group in terms of differentiation, lymphatic invasion, N category, AJCC stage, and recurrence rate. The clinical and pathologic findings of the 98 patients with tumor recurrence are included in Table 2. There were no statistically significant differences in terms of age, gender, tumor location, tumor size, gross type, cell type, differentiation, lymphatic invasion, vascular invasion, T category, N category, or AJCC stage between the two groups except for the time to recurrence. This was significantly longer in the COX-2-positive group than in the COX-2-negative group (34.1 months ± 30.0 versus 21.9 months ± 17.4, $P = 0.019$).

71 out of 98 patients with recurrence experienced early recurrence (72.4%), and 27 patients had late recurrence (27.6%). In univariate analysis, lymphatic invasion and positive COX-2 expression were significantly different between the two groups ($P = 0.012$ and $P = 0.001$, resp.) (Table 3). Positive lymphatic invasion was significantly correlated with early recurrence ($P = 0.012$), while positive COX-2 expression was significantly related with late recurrence ($P = 0.001$). Multivariate analysis revealed that lymphatic invasion was an independent factor for the early recurrence (OR 0.309; 95% CI, 0.103 to 0.924; $P = 0.036$), and positive COX-2 expression was an independent factor for the late recurrence (OR 4.656; 95% CI, 1.696 to 12.779; $P = 0.003$) (Table 3).



(a) (b) (c) (d)

Figure 1: The microphotographs of COX-2 immunostaining by intensity in colorectal cancer: (a) negative, (b) weak, (c) moderate, and (d) strong.

3.2. Recurrence Patterns and Postrecurrence Survival according to the COX-2 Expression Status. Thirty-two (8.5%) of the 376 patients experienced local recurrence, and 66 (17.5%) had distant metastasis. The most common site of distant metastasis was the liver ($n = 25$, 6.6%) followed by the lung ($n = 19$, 5.1%). The patterns of recurrence in the positive and the negative COX-2 expression groups were not different (Table 4). In the 98 patients with recurrence, there was no relation on postrecurrence survival according to the COX-2 expression status (Figure 2).

4. Discussion

Our results suggest that COX-2 expression in CRC is associated with late recurrence (>3 years after surgery) during the postsurgery follow-up period, which may not mean that COX-2 expression prevents early recurrence. In this study, lymphatic invasion was a significant factor for the early recurrence but COX-2 expression was a significant factor for the late recurrence. We assumed that positive COX-2 expression do not prevent early recurrence but induce late recurrence. Maybe there are other mechanisms which contribute to recurrence in the positive COX-2 expression group different from the lymphatic invasion group. A number of mechanisms may be involved in the process of late recurrence. COX-2 overexpression increases the migration and proliferation of intestinal epithelial cell and inhibits programmed cell death, so prolonging the survival of abnormal cells [16]. Interestingly, another study found that COX-2 overexpression was correlated with elevated intracellular telomerase and reduced apoptosis [17]. In nonsmall cell lung cancer, COX-2 overexpression has been shown to stabilize survivin, an inhibitor of apoptosis [18]. In breast cancer, the presence of cytoplasmic survivin positively correlates with COX-2 expression [19]. COX-2 and survivin are overexpressed and positively correlated in endometrial adenocarcinoma [20]. Although our data do not have a bearing on molecular mechanisms, we suspect that the relation between COX-2 overexpression and late recurrence may be due to a decreased rate of apoptosis of surviving tumor cells. We suspect that surviving tumor cells in the stromal compartment may grow and migrate to other organs, and time may be required for surviving tumor cells to acquire resistance to adjuvant treatment.

Table 1: Clinical and pathological characteristics according to COX-2 expression in 376 patients with R0 resection.

Variable	Total patient ($n = 376$)	COX-2 negative ($n = 165$)	COX-2 positive ($n = 211$)	P value
Age (yr)				0.496
<70	311 (82.7%)	134 (81.2%)	177 (83.9%)	
≥70	65 (17.3%)	31 (18.8%)	34 (16.1%)	
Gender				0.436
Male	208 (55.3%)	95 (57.6%)	113 (53.6%)	
Female	168 (44.7%)	70 (42.4%)	98 (46.4%)	
Tumor location				0.209
Right colon	82 (21.8%)	34 (20.6%)	48 (22.7%)	
Left colon	113 (30.1%)	43 (26.1%)	70 (33.2%)	
Rectum	181 (48.1%)	88 (53.3%)	93 (44.1%)	
Tumor size				0.961
<5 cm	118 (31.4%)	52 (31.5%)	66 (31.3%)	
≥5 cm	258 (68.6%)	113 (68.5%)	145 (68.7%)	
Gross type				0.297
Fungating	180 (44.9%)	83 (50.3%)	97 (46.0%)	
Infiltrative	170 (46.9%)	69 (41.8%)	101 (47.8%)	
Unknown	26 (8.2%)	13 (7.9%)	13 (6.2%)	
Cell type				0.303
Nonmucinous	356 (94.7%)	154 (93.3%)	202 (95.7%)	
Mucinous	20 (5.3%)	11 (6.7%)	9 (4.3%)	
Differentiation				0.032
WD	10 (2.7%)	5 (3.0%)	5 (2.4%)	
MD	301 (80.1%)	141 (85.5%)	160 (75.8%)	
PD	65 (17.3%)	19 (11.5%)	46 (21.8%)	
Lymphatic invasion				0.004
Absent	180 (47.9%)	65 (39.4%)	115 (54.5%)	
Present	196 (52.1%)	100 (60.6%)	96 (45.5%)	
Vascular invasion				0.634
Absent	372 (98.9%)	164 (99.4%)	208 (98.6%)	
Present	4 (1.1%)	1 (0.6%)	3 (1.4%)	
T category				0.089
T1	7 (1.9%)	1 (0.6%)	6 (2.8%)	
T2	32 (8.5%)	9 (5.5%)	23 (10.9%)	
T3	330 (87.8%)	152 (92.1%)	178 (84.4%)	
T4	7 (1.9%)	3 (1.8%)	4 (1.9%)	
N category				0.019
N0	185 (49.2%)	69 (41.8%)	116 (55.0%)	
N1	90 (23.9%)	41 (24.8%)	49 (23.2%)	
N2	101 (26.9%)	55 (33.3%)	46 (21.8%)	
Stage				0.003
I	31 (8.2%)	7 (4.2%)	24 (11.4%)	
II	158 (42.0%)	62 (37.6%)	96 (45.5%)	
III	187 (49.7%)	96 (58.2%)	91 (43.1%)	
Recurrence				0.001
Early (≤3 yrs)	71 (18.9%)	45 (27.4%)	26 (12.3%)	
Late (>3 yrs)	27 (7.2%)	7 (4.3%)	20 (9.4%)	
No	278 (73.9%)	112 (67.9%)	166 (78.7%)	

Table 2: Clinical and pathological characteristics according to COX-2 expression in patients with recurrence.

Variable	Total patient ($n = 98$)	COX-2 negative ($n = 52$)	COX-2 positive ($n = 46$)	P value
Age (yr)				0.200
<70	80 (81.6%)	40 (76.9%)	40 (87.0%)	
≥70	18 (18.4%)	12 (23.1%)	6 (13.0%)	
Gender				0.267
Male	56 (57.1%)	27 (51.9%)	29 (63.0%)	
Female	42 (42.9%)	25 (48.1%)	17 (37.0%)	
Tumor location				0.725
Right colon	25 (25.5%)	13 (25.0%)	12 (26.1%)	
Left colon	16 (16.3%)	9 (17.3%)	7 (15.2%)	
Rectum	57 (58.2%)	30 (57.7%)	26 (58.7%)	
Tumor size				0.286
<5 cm	33 (33.7%)	20 (38.5%)	13 (28.3%)	
≥5 cm	65 (66.3%)	32 (61.5%)	33 (71.7%)	
Gross type				0.680
Fungating	44 (44.9%)	22 (42.3%)	22 (47.8%)	
Infiltrative	46 (46.9%)	25 (48.0%)	21 (45.6%)	
Unknown	8 (8.2%)	5 (9.6%)	3 (6.5%)	
Cell type				1.000
Nonmucinous	93 (94.9%)	49 (94.2%)	44 (95.7%)	
Mucinous	5 (5.1%)	3 (5.8%)	2 (4.3%)	
Differentiation				0.291
WD	4 (4.1%)	2 (3.8%)	2 (4.3%)	
MD	75 (76.5%)	43 (82.7%)	32 (69.6%)	
PD	19 (19.4%)	7 (13.5%)	12 (26.1%)	
Lymphatic invasion				0.190
Absent	20 (20.4%)	8 (15.4%)	12 (26.1%)	
Present	78 (79.6%)	44 (84.6%)	34 (73.9%)	
Vascular invasion				1.000
Absent	98 (100%)	52 (100%)	46 (100%)	
Present	0 (0%)	0 (0%)	0 (0%)	
T category				0.840
T1	0 (0%)	0 (0%)	0 (0%)	
T2	3 (3.1%)	1 (1.9%)	2 (4.3%)	
T3	92 (93.9%)	49 (94.2%)	43 (93.5%)	
T4	3 (3.1%)	2 (3.8%)	1 (2.2%)	
N category				0.479
N0	22 (22.4%)	11 (21.2%)	11 (23.9%)	
N1	27 (27.6%)	17 (32.7%)	10 (21.7%)	
N2	49 (50.0%)	24 (46.2%)	25 (54.3%)	
Stage				0.713
I	1 (1%)	0 (0%)	1 (2.2%)	
II	22 (22.4%)	11 (21.2%)	11 (23.9%)	
III	75 (76.6%)	41 (78.8%)	34 (73.9%)	
Time to recurrence (month)	27.6 ± 24.8	21.9 ± 17.4	34.1 ± 30.0	0.019
Recurrence type				0.001
Early (≤3 yrs)	71 (72.4%)	45 (86.5%)	26 (56.5%)	
Late (>3 yrs)	27 (27.6%)	7 (13.5%)	20 (43.5%)	

Table 3: Univariate and multivariate analysis of independent risk factors associated with late recurrence.

Variable	Early recurrence (<3 yr) ($n = 71$)	Late recurrence (≥3 yr) ($n = 27$)	Univariate analysis (P value)	Multivariate analysis (OR, 95% CI, P value)
Age (yr)			0.382	
<70	56 (78.9%)	24 (88.9%)		
≥70	15 (21.1%)	3 (11.1%)		0.726 (0.174–3.024) 0.660
Gender			0.473	
Male	39 (54.9%)	17 (63.0%)		0.996 (0.361–2.747) 0.993
Female	32 (45.1%)	10 (37.0%)		
Tumor location			1.000	
Right colon	18 (25.3%)	7 (25.9%)		
Left colon	12 (16.9%)	4 (14.8%)		
Rectum	41 (57.7%)	16 (59.3%)		
Tumor size			0.965	
<5 cm	24 (33.8%)	9 (33.3%)		
≥5 cm	47 (66.2%)	18 (66.7%)		
Gross type			0.280	
Fungating	30 (42.3%)	14 (51.9%)		
Infiltrative	36 (50.7%)	10 (37.0%)		
Unknown	5 (7.0%)	3 (11.1%)		
Cell type			0.614	
Nonmucinous	68 (95.8%)	25 (92.6%)		
Mucinous	3 (4.2%)	2 (7.4%)		
Differentiation			0.818	
WD	3 (4.2%)	1 (3.7%)		
MD	53 (74.6%)	22 (81.5%)		
PD	15 (21.1%)	4 (14.8%)		
Lymphatic invasion			0.012	
Absent	10 (14.1%)	10 (37.0%)		
Present	61 (85.9%)	17 (63.0%)		0.309 (0.103–0.924) 0.036
Vascular invasion			1.000	
Absent	71 (100%)	27 (100%)		
Present	0 (0%)	0 (0%)		
T category			0.193	
T1	0 (0%)	0 (0%)		
T2	1 (1.4%)	2 (7.4%)		
T3	67 (94.4%)	25 (92.6%)		
T4	3 (4.2%)	0 (0%)		
N category			0.102	
N0	12 (16.9%)	10 (37.0%)		
N1	21 (29.6%)	6 (22.3%)		
N2	38 (53.5%)	11 (40.7%)		
Stage			0.051	
I	0 (0%)	1 (3.7%)		
II	13 (18.3%)	9 (33.3%)		
III	58 (81.7%)	17 (63.0%)		1.315 (0.256–6.753) 0.743
COX-2 expression			0.001	
Negative	45 (63.4%)	7 (25.9%)		
Positive	26 (36.6%)	20 (74.1%)		4.656 (1.696–12.779) 0.003

Table 4: Recurrence pattern according to the status of COX-2 expression in the 98 patients with recurrence.

	COX-2 negative ($n = 52$)	COX-2 positive ($n = 46$)	P value
			0.256
Local recurrence	14 (26.9%)	18 (39.1%)	
Liver	13 (25.0%)	12 (26.1%)	
Lung	9 (17.3%)	10 (21.7%)	
Peritoneal seeding	5 (9.6%)	3 (6.5%)	
Others (brain, bone, skin)	11 (21.2%)	3 (6.5%)	

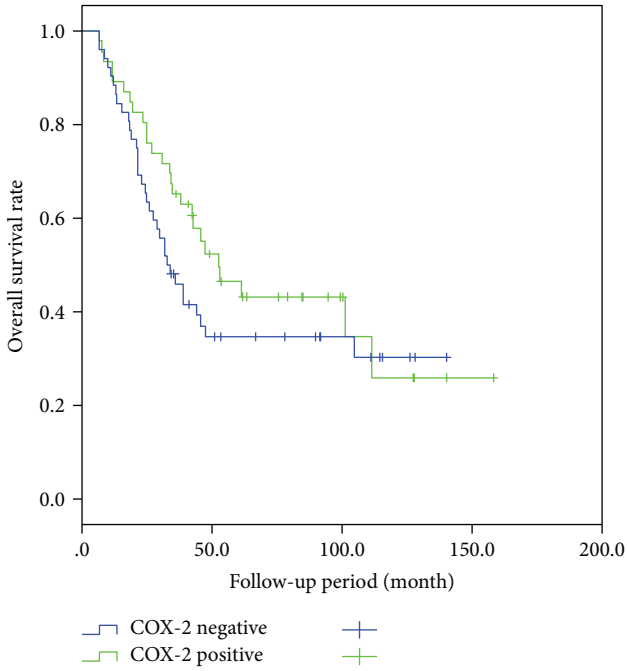


Figure 2: Postrecurrence survival was not significantly different according to the COX-2 status of the 98 patients with recurrence ($P = 0.230$).

Some studies have suggested that elevated COX-2 expression of CRC patients is related with reduced survival [15, 21]. However, others found that the elevated expression of COX-2 protein had no significant impact on disease-specific survival and overall survival in CRC patients [22, 23]. We observed no significant postrecurrence survival difference according to COX-2 expression status. Thus, COX-2 expression is not likely a prognostic factor for postrecurrence in CRC.

After curative surgery, CRC patients with positive COX-2 expression have an increased probability of late tumor recurrence based on the result of this study. Therefore, the positive COX-2 patients should be considered candidates for more frequent testing after 3 years of follow-up and extend follow-up period longer than 5 years after surgery. In protocols for postsurgery surveillance, there is a tendency for the frequency of follow-up and testing to be reduced after 3 years. We suggest that since COX-2 expression may be a marker for late recurrence, the frequency of follow-up and testing should not be reduced after 3 years. Furthermore, suspending follow-up after 5 years from the initial operation may be inappropriate especially in COX-2-positive patients. A further prospective randomized study is required to identify optimal surveillance methods and follow-up intervals.

As smoking habit and body mass index may modify the risk of CRC in COX-2 genotype, this bias could affect our conclusions regarding the predictive marker [24]. A further well-designed, large, sample-sized study is mandatory.

A limitation of this study was its retrospective design, which is subject to selection bias. Also, the cases were all from a single institution. As no molecular biological study was performed, it was not clear how COX-2 expression contributed to late recurrence. But it is meaningful that the study revealed a novel finding about the relationship between elevated COX-2 expression and late recurrence of CRC: we were able to demonstrate the possibility of COX-2 expression as a biologic marker predicting late recurrence in CRC patients.

5. Conclusions

Elevated COX-2 expression in itself is not a prognostic factor, but COX-2 expression in tumor tissue may be an independent predictive marker of late recurrence for patients with stage I to III CRC. A further well-designed study is required to demonstrate the regulatory mechanism of COX-2 expression on CRC recurrence.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Sung Hoo Kim contributed to the collection of data, analysis, interpretation, and writing the article. Byung Kyu Ahn contributed to the critical revision of the article. Seung Sam Paik contributed to the critical revision of the article. Kang Hong Lee contributed to the conceiving of the design and approved the final version of the manuscript.

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Review Article

Noninvasive Biomarkers of Colorectal Cancer: Role in Diagnosis and Personalised Treatment Perspectives

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Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide. It has been estimated that more than one-third of patients are diagnosed when CRC has already spread to the lymph nodes. One out of five patients is diagnosed with metastatic CRC. The stage of diagnosis influences treatment outcome and survival. Notwithstanding the recent advances in multidisciplinary management and treatment of CRC, patients are still reluctant to undergo screening tests because of the associated invasiveness and discomfort (e.g., colonoscopy with biopsies). Moreover, the serological markers currently used for diagnosis are not reliable and, even if they were useful to detect disease recurrence after treatment, they are not always detected in patients with CRC (e.g., CEA). Recently, translational research in CRC has produced a wide spectrum of potential biomarkers that could be useful for diagnosis, treatment, and follow-up of these patients. The aim of this review is to provide an overview of the newer noninvasive or minimally invasive biomarkers of CRC. Here, we discuss imaging and biomolecular diagnostics ranging from their potential usefulness to obtain early and less-invasive diagnosis to their potential implementation in the development of a bespoke treatment of CRC.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer among men and women and the third leading cause of cancer-related deaths in the world, with an incidence of 1.2 million new cases and 608,700 deaths annually [1].

Metastasis accounts for approximately 90% of CRC-related deaths; this is mainly due to the absence of an ideal method of screening [2]. Detection of CRC at an early stage may confer a 90% 5-year survival rate, compared to 12% if distant metastasis occurs [3, 4].

One of the primary targets of screening is the identification of advanced colorectal adenomas.

The currently available screening modalities, such as the guaiac-based faecal occult blood test (gFOBT) and carcinoembryonic antigen (CEA) test, are effective but limited by low specificity and sensitivity. Sigmoidoscopy and colonoscopy are invasive, have certain morbidity risks, and require cumbersome preparatory procedures that lead to a low participation rate.

The gFOBT has been associated with a reduction of 15–33% in CRC-related mortality, particularly if the test is performed every 1 or 2 years [5, 6]. Despite being noninvasive, inexpensive, and easily applicable, it has low accuracy, particularly regarding the detection of preneoplastic lesions; it also has a low specificity rate leading to a high number of unnecessary colonoscopies [7, 8]. The new, more sensitive version of an antibody-based globin test, known as immunochemical FOBT or faecal immunochemical test (FIT), is inconvenient because the specimen needs to be sent to a laboratory for testing [9]. Nowadays, colonoscopy is the gold standard for the early diagnosis of CRC [10], but it has several risks such as bleeding, perforation, missed adenoma/cancer, and related death.

The ideal CRC biomarker should be easily and quantitatively measured, highly specific, and sensitive, as well as reliable and reproducible [11]. It should be able to stratify between different risk-based populations, selecting patients who really need a second-line test (endoscopic and radiologic investigations). Ideally, this aim can be achieved with a noninvasive and inexpensive method, using easily available biological samples such as urine, breath, serum, and faeces.

Despite the advances made over the last years, no single test is currently able to diagnose and monitor the posttreatment course of CRC patients. Herein, we review the current status of noninvasive biomarkers in CRC and provide insights for their implementation in the clinical management of patients.

2. Circulating Biomarkers and Eliminated Metabolites

2.1. Genetic and Epigenetic Alterations and CRC. Genetic and epigenetic changes characterizing the carcinogenesis of CRC are essential for the identification and development of an ideal biomarker [12]. Genetic markers are based on the identification of mutations in a subset of genes, including p53, APC, KRAS, NRAS, and DNA repair genes such as hMSH1 (human mutS homolog 1) or hMLSH2 [13, 14]. Unfortunately, this approach has a modest diagnostic sensitivity for invasive cancers and advanced benign tumors [15]. Epigenetic alterations include DNA methylation, microRNA (miRNA) expression, histone modification, and chromatin remodelling. They represent inheritable changes in gene expression without modifications to the DNA sequence.

DNA methylation consists in the enzymatic addition of a methyl group to cytosine in 5-position. The process is catalyzed by DNA methyltransferases and usually entails a covalent linkage within a CG dinucleotide sequence, termed CpG transcription [16].

Owing to their high tissue specificity and critical role in oncogenesis, miRNAs have the potential to be reliable biomarkers for the diagnosis and classification of CRC as well as for predicting treatment outcomes in the near future [17–19]. Several studies have recently demonstrated the role of miRNAs obtained from different body fluids (such as plasma, serum, urine, saliva, and tissues) in the pathogenesis of CRC (including metastasis spread [20]) with subsequent implications on treatment and prognosis [21].

The improvement of validated protocols and the discovery of new technologies such as next-generation sequencing (NGS) [22] allow a very careful evaluation of the whole miRNAome in different samples.

miRNAs are small single-stranded, noncoding RNAs, discovered in 1993 as developmental regulators in *Caenorhabditis elegans* [23, 24], with a length of 18–25 nucleotides [25]. Their aberrant expression patterns have been detected in various types of malignancies including breast cancer, lung cancer, pancreatic cancer, ovarian cancer, and CRC [26–28], playing an essential role as posttranscriptional regulators of carcinogenesis, progression, invasion, angiogenesis, and metastasis [25, 29–34].

They suppress translation or induce mRNA degradation by binding to the $3'$ untranslated region (UTR) of their target genes [35]. More than 50% of the discovered human miRNA genes are localized in fragile chromosomal regions that are susceptible to amplification, deletion, or translocation during the natural history of CRC [36, 37]. This makes them the most promising future predictive markers for the diagnosis and prognosis of CRC; additionally, they could aid to determine the therapeutic response to chemotherapeutic drugs.

After several enzymatic reactions, the mature miRNA is integrated into the RNA-induced silencing complex (RISC) to then negatively regulate the expression of hundreds of target mRNAs by translation inhibition or mRNA degradation. This is achieved by the recognition of complementary sites on the target mRNA [38]. Consequently, miRNAs are able to give us more prognostic and diagnostic information than mRNAs. miRNAs also modulate T and B lymphocyte activation (both the innate and adaptive immune responses [39]), thus helping cancer cells avoid recognition by the immune system in the blood/lymph vessels.

Lastly, they target inflammatory signalling molecules, thereby inducing or inhibiting chronic inflammation and inflammation-related cancers. This is confirmed by studies on the expression of KRAS, which is inversely correlated with miR 143 [40] and c-Myc, which can promote tumoral growth via miR 17-92 [41]. Both miR 324-5p and miR 122 are

involved in the regulation of TNF-alfa [42], CUEDC2 [43], and NOD2 [44].

2.1.1. Plasmatic miRNAs. Several miRNAs are dysregulated in the plasma of patients with CRC [45, 46]. They can either circulate freely or be in exosomes. Thanks to their small size, miRNAs are well protected from endogenous degradation [47–49] and can remain stable for a long period of time, in contrast to the fast degradation of mRNAs and proteins. Furthermore, cancer cells secrete some miRNAs into systemic circulation [48], confirming their central role in CRC screening.

The number of aberrantly expressed miRNAs in CRC tissues has rapidly grown due to the increasing number of studies on the topic [28, 50]. As an example, miR 17-92a has an oncogenic function because it is upregulated during the well-known adenoma-to-carcinoma sequence [51, 52].

miR 21, one of the most extensively investigated oncogenic miRNAs, is highly correlated with CRC cell proliferation, invasion, lymph node metastasis, and advanced clinical stage [53–55]. It is overexpressed in colorectal adenomas when compared with normal colonic mucosa [54]. It participates in the multistep process of CRC carcinogenesis, regulates several pathways such as MAPK and WNT/Beta-catenin [56–58], and its level decreases after surgical removal of CRC [59].

Other evaluated miRNAs are linked with hepatic metastasis. These can also be useful for early detection of CRC, as predictors of recurrence of CRC (stages II and III), and to determine the probability of resistance to preoperative chemoradiotherapy (CRT) in a CRC cell line [46, 60–67]. miR 92a is overexpressed in serum, plasma, and stool of patients with advanced adenoma, when compared to controls [66–68].

2.1.2. Faecal miRNAs. Exfoliated faecal colonocytes or tumor-secreted miRNAs are directly and continuously released from tumors into the intestinal lumen, providing a rationale for a stool-based miRNA test for the diagnosis of CRC [11, 69]. Furthermore, miRNAs in faeces correlate with the grading of the tumor [70]. Faecal miR 135b is elevated in CRC and adenomatous tissue samples in contrast to adjacent healthy tissue [71], whereas miR 106a can decrease the number of false negatives when using a gFOBT [72]. Unfortunately, the stool environment is much more complex (compared to plasma) and its testing requires a certain volume and density of the sample for each assay.

2.1.3. miRNAs, Diet, and Lifestyle. In CRC, there is a clear link between lifestyle, diet, and epigenetic factors expressed in an aberrant way. However, it is still debated whether changes in lifestyle can modify epigenetic mechanisms and reduce the risk of CRC progression.

Tarallo et al. [73] demonstrated the modulatory effect of different dietary habits on a panel of miRNAs; the highest differences in the expression (in stool and plasma samples) of miR 17-92 cluster among people with vegan, vegetarian, or omnivorous diet habits.

Several recent studies show that fish; oil-fed animals; vitamins A, D, and E; and minerals such as selenium and resveratrol (trans-3,4′,5′-trihydroxystilbene) can modify the levels of expressed miRNAs [73–79].

Various recent studies demonstrate a clear relationship between miRNA expression and CRC [80]. These new diagnostic possibilities are highly influencing the current research in the CRC field.

Endogenous miRNAs, packed and protected from the action of RNase (in contrast with rapid degradation of mRNA and proteins), allow us to discriminate normal colonic mucosa, colon adenomas, and carcinomas. The possibility of miRNA-based therapies, inhibiting oncogenic miRNAs or restoring tumor suppressor miRNAs, could open a new scenario in the treatment of CRC, despite the bias on the different methods for evaluating the population, methodology of collection of the used samples, and quantification methods.

2.1.4. Methylated DNA. Increased concentrations of circulating methylated DNA have been reported in the blood of cancer patients [81–83]. *SEPT9* is one of the most widely studied genes with an important role in the early diagnosis of CRC as well as in metastatic CRC.

In the PRESEPT study, Church and coworkers [84] found 666 (9.7%) advanced and 2359 (34.3%) nonadvanced adenomas in the 6874 patients who underwent colonoscopy. Among them, circulating methylated *SEPT9* has been identified in 9.6% of the advanced adenomas and 7.7% of the nonadvanced adenomas.

Barault et al. [85] identified candidate biomarkers of CRC analysing the methylation profile of CRC cell lines. Methylated ctDNA enables, in association with CT scan, the tracking of tumor response in metastatic CRC patients treated with chemotherapy (FOLFOX, FOLFIRI, ± bevacizumab) and targeted agents (panitumumab). They validated its use in monitoring metastatic CRC response to therapy, including chemotherapy, targeted therapy, and temozolomide in a longitudinal study. Furthermore, several authors evaluated the diagnostic performance of SEPT9 assay along with other blood-based methylated genes. The association of SEPT9 with TAC1 methylation assay yielded a sensitivity of 73.1% and a specificity of 92.3% [86] while its association with *TMEFF2* and *ALX4* further increased both sensitivity (80.7%) and specificity (90.0%) [87].

2.2. Neurotensins and CRC. There is increasing recognition that cancers of the gastrointestinal tract (pancreas as well as other organs) express receptors for various endogenous host hormones. This raises the possibility that hormones can play a role in the proliferation of these cancers and therefore highlights the potential of these hormonal signalling pathways as targets for novel cancer diagnostic and therapeutic strategies. One of these promising candidates in CRC is the tridecapeptide neurotensin (NT) [88, 89]. NT was first isolated in 1973 from the bovine hypothalamus and digestive tract [90]. Its physiological functions are those of a neurotransmitter in the central nervous system and of a hormone in the periphery. There is increasing evidence of the role it plays in CRC.

Some colonic tumors synthesise and release NT, resulting in autocrine control and cellular proliferation [91]. Physiological levels of NT appear to stimulate the growth of many human CRC cell lines (SW480, SW620, HT29, HCT116, and Cl.19A) expressing the NT receptor 1 (NTR1) [92]. NT accelerates colonic cancer carcinogenesis in animal models. For example, rats injected with both a CRC carcinogen and NT demonstrate a significant increase in the number, size, and invasiveness of colon tumors [93]. Administration of NT by itself significantly stimulated growth in murine colon tumors as well as human colon cancers xenografted into mice; it also resulted in a significant decrease in survival [94].

In humans, NT mRNA, peptide, and receptor were found in resected CRC specimens as well as four well-known human cancer cell lines *in vitro*. In surgical specimens where NT was identified in cancer cells, it was absent in adjacent normal bowel mucosa [91].

Gui et al. examined NTR1 expression in human CRC by measuring NTR1 mRNA in normal colonic mucosa, adenomas, and colonic adenocarcinomas. NTR1 mRNA expression was undetectable in epithelial cells of normal colonic epithelium, but it was expressed in adenomas and adenocarcinomas. Higher expression levels were seen in adenocarcinomas when compared to adenomas. Tissue from lymphovascular invasion showed even higher expression levels of NTR1 than that from the rest of the tumor. These results suggest that increased NTR1 expression may be an early event during colonic tumorigenesis that can also contribute to tumor progression and aggressive behaviour in colonic adenocarcinomas [95].

Evaluation of blood NT levels in colorectal cancer was recently conducted using 56 colorectal cancer patients and 15 controls; early evidence suggests that NT levels could differentiate between cancer and noncancer patient groups.

2.3. Liquid Biopsy. Biopsies have a central role in disease management, particularly in cancer patients. They allow clinicians to diagnose, determine a treatment course, and evaluate prognosis. In addition to specifying the histological nature of the disease, tissue biopsies are used to determine the genetic features of the tumor. This information can be used to treat patients with drugs tailored to the genetic makeup of their tumor and to give predictive and prognostic information.

However, although tissue biopsies are critical in the decision-making process, they have limitations. A single biopsy represents a snapshot of the complexity of molecular tumor alterations and tends to underestimate the real intratumoral heterogeneity [96]. Moreover, molecular targeted therapies may require multiple biopsies to accurately evaluate both the intratumoral heterogeneity and the genome modifications occurring during treatment because cancer genomes are also unstable and tend to change over time. Obtaining multiple biopsies at baseline and during the treatments is challenging owing to patient discomfort, procedural complications, costs, tumor accessibility, and the potential risk of tumor seeding.

In the field of precision medicine, the term liquid biopsy (LB) refers to those genetic tests performed on a biological component extracted from body fluids, in particular, from whole blood. This sample can be used to obtain circulating tumor cells (CTC), circulating tumor DNA (ctDNA), and exosomes [97, 98]; these components represent a small fraction of the total biological elements actively or passively released into the blood through metastisation processes, necrosis, or apoptosis. Today, many clinical trials have aimed to investigate the role of LBs in the management of metastatic CRC (mCRC) patients, specifically by analysing the role of CTC, ctDNA, and exosomes as alternative biological sources to monitor tumor evolution and response in a dynamic manner, considering that cancer is not a "molecularly stable" disease [99–101]. In addition, an LB represents a key approach to analyse, through a noninvasive and simple blood test, the molecular heterogeneity among different tumor sites in the same patient (primary tumor versus distant metastasis) to define the best target for a therapeutic approach or to monitor patients with no clinically detectable disease after surgery and standard therapy [99–106].

Plasma DNA that is analysed on CTC or on ctDNA has been suggested as an alternative way to evaluate tumor genomes [106–112]. CTCs are cancer cells derived from tumors that are released into the bloodstream through a process known as the epithelial-mesenchymal transition (EMT), from either a primary or metastatic site. In the past years, the simple presence of CTCs was an indicator of a poorer prognosis in CRC and other cancer types, such as breast, prostate, and lung cancer. CTC molecular characterization represents an attractive real-time option to monitor metastatic diffusion before instrumental detection [98–100].

Plasma cell-free DNA (cfDNA) is the result of DNA fragments that are released into circulation from both normal and tumor cells. Since CTC and ctDNA are valid sources to evaluate tumor genomes and can be considered as "surrogates" of tissue biopsies, they can be defined as LB. Initially, both CTC and ctDNA were used as simple quantitative markers. The analysis of clinical relevant mutations can also be feasible in a simple way by using ctDNA, even if it represents a small fraction of the total cfDNA released into the blood by healthy cells or primary cancer cells or directly by CTCs. Additionally, the quantity of cfDNA has a prognostic role in mCRC patients, and, overall, cfDNA levels have been demonstrated to be higher in cancer patients, when compared to healthy controls [101, 103, 105, 106].

In another recent study, Strickler et al. [113] reported results from clinical cfDNA testing of 1397 patients with advanced CRC. They compared these results with three large CRC tumor tissue sequencing cohorts. Evidence of ctDNA in the blood was similar to recent studies in colon cancer. Furthermore, authors identified a previously unreported cluster of EGFR extracellular domain (ECD) mutations involving V441 and S442 that accounted for 25% of all ECD mutations, representing an important and novel mechanism of resistance to EGFR blockade.

Analysis of plasma samples may offer several advantages in determining KRAS mutation status in patients who have progressed on EFGR target therapy. Siena et al. [114] studied the mechanism of secondary resistance in EGFR inhibitor-treated patients finding new *KRAS* mutations in 7.1% and 57.1% of patients whose tumor genotype was determined

Table 1

Potential applications of LB in CRC
Early diagnosis
Assessment of molecular heterogeneity of overall disease
Identification of genetic alterations for targeted therapy
Evaluation of tumor response after preoperative treatments
Monitoring of minimal residual disease
Assessment of evolution of resistance in real time

using tissuebased and plasmabased analyses, respectively, during treatment with the combination of irinotecan and panitumumab.

Exosomes are very interesting, small endocytic membrane vesicles that are initially isolated from the peripheral circulation of cancer patients and play a central role in the communication processes among cells by activation of surface ligands or by transferring of molecules among the cells. Exosomes can either manipulate the local and systemic environment, allowing cancer growth and dissemination, or modulate the immune system to elicit or suppress an antitumor response. In addition, exosomes represent a good source of DNA fragments, proteins, mRNA, miRNA, and other biological molecules and are protected by a lipid bilayer membrane, which confers a high degree of stability. Recently, many studies on exosomes demonstrate either a prognostic or predictive value, emphasizing their potentiality in clinical practice. A genome-wide expression profile of miRNAs has been shown to be significantly different among primary lung cancers and corresponding noncancerous lung tissues and thus has shown to have a potential role as a diagnostic marker [98, 105].

Therefore, in the near future, an integrated analysis of ctDNA, CTC, and exosomes could be used in a clinical setting for mCRC to refine patient therapy selection and management.

Currently, there is an increasing interest in the evaluation of genomic features available from LB.

The concept behind LB and its possible clinical applications in CRC is summarized in Table 1.

2.3.1. LB as a Diagnostic Biomarker. The first effort towards the clinical use of cfDNA from LB was the simple quantitative evaluation of plasma DNA. A significant difference in cfDNA levels was found in healthy subjects compared to cancer patients. Heitzer et al. [115] found that, compared to healthy subjects, stage IV CRC patients ($n = 32$) showed higher cfDNA levels with substantial variability. Moreover, a third of patients had a biphasic size distribution of plasma DNA fragments, and this finding was associated with increased CTC numbers and an elevated concentration of KRAS-mutated plasma DNA fragments. Therefore, CRC patients show not only higher levels of cfDNA but also a specific pattern of tumor DNA fragmentation.

Frattini et al. [116] performed a quantitative analysis of plasma DNA in 70 CRC patients and 20 healthy subjects at baseline and during follow-up. In a subset of CRC patients, they also compared the KRAS mutation and the p16INK4a promoter hypermethylation in tissue samples and in cfDNA. They found that plasma DNA levels are useful for diagnosing cancer as well as determining disease-free status and the presence of recurrence.

Unfortunately, the fraction of ctDNA originating from tumor cells is between 0.01% and more than 90%, and it can vary greatly [117].

2.3.2. LB as a Prognostic Biomarker. Clinical, radiological, histopathological, and molecular factors are widely used as prognostic factors of rectal cancer. Tumor alterations in LB could have the potential to be associated with prognosis. Lecomte et al. [118] evaluated KRAS mutations and epigenetic alterations such as hypermethylation of a cyclin-dependent kinase inhibitor in cfDNA of 8 stage I, 21 stage II, 16 stage III, and 13 stage IV CRC patients. KRAS mutations and epigenetic alterations were found in 20 to 50% of these patients, and all of the patients without evidence of KRAS mutations or epigenetic alterations showed a 2-year survival rate. They also found an association between plasma ctDNA levels and the prognosis of CRC patients. These findings were confirmed by Diehl et al. [117], who demonstrated that CRC patients who relapsed within 1 year after surgery had higher ctDNA levels at the time of recurrence.

Bertorelle et al. [119] evaluated the association between RNA-hTERT (telomere-specific reverse transcriptase) plasma levels and the overall survival of stage II CRC patients, for whom the value of adjuvant chemotherapy is still debated. Compared to patients with low hTERT levels, those with high hTERT levels showed a significantly poorer survival rate (hazard ratio = 3.30, 95% CI 1.98–5.52), suggesting that hTERT levels could support the decision of performing adjuvant chemotherapy in stage II CRC patients.

In the CAPRI-GOIM trial, conducted by Normanno et al. [120], 340 KRAS exon-2 wild-type metastatic CRC patients received first-line cetuximab plus FOLFIRI. Tumor samples were analysed using NGS while BEAMing (digital PCR technology combines emulsion PCR with magnetic beads and flow cytometry for the highly sensitive detection and quantification of mutant tumor DNA molecules) has been used to search for KRAS and NRAS mutations in plasma samples. They concluded that ctDNA may replace tumor tissue analysis.

2.3.3. LB as a Predictive Biomarker. The prediction of tumor responses to a neoadjuvant therapy is clinically relevant because it can allow for treatment modifications before or during the treatment and, ultimately, to a tailored therapy that avoids inefficient, toxic, and costly approaches. Moreover, a treatment fails when resistance develops against chemotherapeutic agents, as observed for KRAS in CRC patients [121]. In this setting, LB could be preferable to tissue biopsies to monitor molecular changes throughout therapy (e.g., biological drugs), thus avoiding repeated tumor tissue sampling; it could also be useful to detect drug resistance before it becomes clinically evident. Kuo et al. [122] compared KRAS mutations in cfDNA and primary tumor tissues and demonstrated that the detection rate of KRAS mutations

Table 2: Imaging Biomarkers.

Modality	Parameters	Application
CT	Anatomical and functional imaging (DCET-CT)	Staging and treatment response
MRI	Anatomical and functional imaging (DWI, DCE-MRI, TA)	Diagnosis, local staging, prognostic evaluation, treatment response
PET-CI	Metabolic and anatomical imaging	Diagnosis, staging and treatment response
PET-MRI	Metabolic and anatomical imaging	Diagnosis, staging, prognostic evaluation, treatment response

was 50% in plasma and 28.8% in resected primary tumor tissue with an agreement of 78.8%. Diaz et al. [123] showed that, in CRC patients without KRAS mutations, treatment with panitumumab induced mutations in 38% of cases within 5 and 6 months following treatment. In a blind prospective study, Thierry et al. [109] compared KRAS and BRAF mutation statuses in tumor tissue and cfDNA of mCRC patients. They showed a 100% diagnostic specificity and sensitivity for the BRAF V600E mutation and a 98% specificity and 92% sensitivity for the KRAS mutation by cfDNA analysis. In 98 clinically stage II-III rectal patients who underwent neoadjuvant CRT, RNA-hTERT plasma levels were found to be a promising biomarker of tumor response [124]. The posttherapy levels of hTERT statistically decreased, and the difference of cfRNA levels between post- and preneoadjuvant therapy independently predicted tumor response. Agostini el al. [125] evaluated the role of cfDNA as a predictor of tumor response in rectal cancer patients who underwent neoadjuvant CRT. Based on the findings that cfDNA arising from tumor cells can be recognized on the basis of fragment lengths (compared to physiological cfDNA), they found that the longer fragments of cfDNA (derived from tumor cells) and, in particular, the ratio between long and short fragments (derived from apoptosis), were associated with tumor response to neoadjuvant therapy.

2.3.4. LB as a Biomarker of Tumor Relapse. Another promising clinical application of an LB is the detection of tumor relapse after a curative treatment. Currently, local or distant recurrence is detected by clinical data and radiological imaging. These methods are costly with a questionable cost-effective value. An LB has the potential to overcome this limitation. Diehl et al. [117] demonstrated that it was possible to detect disease recurrence by monitoring tumor-specific alterations in the plasma of CRC patients after surgery with almost 100% sensitivity and specificity. The persistence of tumor alterations in cfDNA after a radical surgery was associated with an incomplete resection, thus allowing clinicians a very early identification of residual disease in patients. Frattini et al. [116] reported the role of cfDNA as a promising biomarker of recurrence; however, CEA determination is currently, even with its limitations, the only widely accepted biomarker used in clinical practice.

Resection radicality is one of the most important predictors for local recurrence and overall survival. In the largest prospective trial of minimal residual disease (MRD) to date, Tie et al. [126] performed next-generation-sequencing of 1046 plasma samples from 230 patients with resected stage II colorectal cancer. Thanks to the early decreases in ctDNA amounts in patients with metastatic disease, they demonstrated that plasma tumor DNA is a better marker for recurrence than carcinoembryonic antigen (CEA), which is currently used in the clinical setting.

The biological principles behind an LB are widely accepted, and the future applications are appealing. Although many studies support the role of LB as a new noninvasive tool in cancer detection and cancer-treatment settings, few studies have focused on the impact of using LB in the diagnosis and treatment of rectal cancer. Moreover, because this research topic is still relatively new, it is quite difficult to translate early findings into clinical applications. In addition, there are many technical aspects that differ between studies with a lack of standardization, which makes clinical application even more difficult. These considerations led to the conclusion that, although there is a solid theoretical basis and increasing evidence for its potential clinical use, the inclusion of LB into the clinical decision-making process for CRC diagnosis and treatment will require more time.

3. Imaging

The use of imaging in CRC has significantly evolved over the last decade, playing a key role in providing answers concerning diagnosis, staging, treatment optimization, and follow-up [126–131]. The imaging modalities currently available for CRC assessment (Table 2) can be divided into two main types: anatomical and functional. Anatomical imaging modalities still remain the mainstay, with computed tomography (CT) imaging suited for colon tumor evaluation and magnetic resonance imaging (MRI) optimal for rectal tumor assessment. However, with the development of new tracer and contrast agents, the evolution of fusion technologies between fludeoxyglucose positron emission tomography (FDG-PET) and MRI and the development of functional MRI techniques may offer new perspectives into cancer perfusion, metabolic, and molecular phenotypes [132]. During recent years, MRI has gained wide acceptance in the assessment of CRC and is considered the first-choice imaging modality for the primary staging and restaging after CRT [133–135]. In particular, despite CT, MRI is an imaging technique that provides functional data in addition to structural and anatomic details. Diffusion-weighted MRI (DW-MRI) and dynamic contrast-enhanced MRI (DCE-MRI) tools can allow to evaluate biological and functional modifications induced by treatment, also aiming to predict clinical outcomes in the setting of adjuvant therapies [136].

DW-MRI investigates and highlights the random movement ("diffusion") of water protons in the extracellular space of biological tissues and derives its imaging contrast from these differences. The diffusion of water molecules in biological tissues depends on many factors and is mainly influenced by cellular density [134, 137]. In tissues with low cellularity, water molecules can freely diffuse resulting in a low DW-MRI signal. On the contrary, this mobility is impeded or "restricted" in tissues with high cellularity (e.g., a tumor) due to reduced extracellular space, resulting in a high DWI-MRI signal. Water proton diffusion can be quantified by the means of the apparent diffusion coefficient (ADC), which reflects the degree of restriction of water molecules (diffusion), indirectly reflecting tissue cellularity [134, 136].

Recognizing these properties, DW-MRI can be a useful tool to detect CRC in cases where the identification of cancer with conventional MRI sequences and CT may be difficult. This technique is useful in cases including malignant transformation within nonspecific mural thickening, desmoplastic reaction, fibrotic or inflammatory changes due to inflammatory bowel disease (IBD), pelvic extraintestinal malignancy, or radiotherapy [138–141]. The addition of DWI-MRI to the conventional T2-weighted sequences improves lesion conspicuity of rectal cancer with 96% sensitivity and a positive predictive value of up to 100% [142, 143]. In this regard, Barral et al. reported that DW-MRI is able to reveal malignant foci in rectal involvement by IBD [140]. Similarly, several studies suggested that DWI-MRI also increases the sensitivity for the diagnosis of colon cancer, with the ability to discriminate between colon cancer and acute diverticulitis in patients with uncertain CT findings (due to a pseudotumoral diverticulitis pattern [142–145]). The use of DWI-MRI could also aid to exceed the limitation of conventional MRI sequences in discriminating between T2 and T3 cancer because the former may present with a desmoplastic reaction resembling cancer invasion.

By quantitative analysis, the ADC value of rectal cancer is reported to be significantly lower than that of a normal rectal wall, with a threshold ADC value of $1.240 \times 10{-}3\,\mathrm{mm}^2/\mathrm{s}$ having a sensitivity of 94% and a specificity of 100% for the diagnosis of rectal cancer [146]. The ADC value has been proposed as a potential biomarker for rectal cancer because it seems to correlate with tumor aggressiveness [147, 148]. ADC values correlate with mesorectal fascia invasion, lymph node involvement, histological differentiation, CA 19-9 and Ki-67 levels, and AgNOR counts [148, 149]. It also helps to differentiate between mucinous carcinoma and tubular adenocarcinoma [150].

DW-MRI is now more commonly used to assess early tumor changes and response after treatment [151, 152]. Treatment-induced cellular death and vascular changes can precede tumor size variation; thus, ADC variations may be a useful biomarker of treatment outcome for drugs that induce apoptosis and neoadjuvant CRT in locally advanced cancer [151]. In the literature, although controversial, it has been found that rectal cancer with low ADC values ($<1.0 \times 10{-}3$ mm2/s) has a better response to CRT [153, 154]. Similarly, it has been demonstrated that liver metastasis with a high ADC baseline value shows a poor response to chemotherapy because it is commonly characterized by necrosis and cellular membrane disruption, suggesting an aggressive phenotype [155]. Another approach by Cai et al. showed that the signal intensity and signal intensity ratio of the tumor on DWI-MRI was more accurate than ADC measurements to assess complete tumor response [156].

Treatment-induced change is often preceded by perfusion alterations as changes of permeability, blood volume, and blood flow [157]. Because capillary perfusion influences the delivery of drugs to cancer cells, measurement of capillary perfusion by DCE-MRI is described as a surrogate marker for evaluating the efficacy of chemotherapy with bevacizumab [158]. Thanks to the fast imaging acquisition after intravenous contrast medium administration, DCE-MRI is an attractive modality for assessing antiangiogenic cancer treatments because it reveals changes in cancer vascularization and even predicts cancer shrinkage, otherwise reflecting a prognostic tumor phenotype [133, 158]. The improvement of postprocessing and the implementation of more complex algorithms of extraction of the signal decrease in DWI-MRI allow to separate tissue diffusivity and microcapillary perfusion. In detail, the biexponential model is based on the intravoxel incoherent motion (IVIM) theory introduced by Le Bihan et al., as a method useful to assess both perfusion and diffusion [159, 160]. This method could allow an early diagnosis of tumor response to CRT or new therapeutic agents like antiangiogenics [161, 162].

Heterogeneity is a well-recognized feature of malignancy associated with increased tumor aggression and treatment resistance. Texture analysis (TA) is an emerging image processing algorithm that can quantify heterogeneity of cancers. Recently, it has been reported that textural features of rectal cancer, assessed by textural analysis (TexRAD) using a filtration-histogram technique of T2-weighted pre- and post-CRT, can predict the outcome before undergoing surgery and could potentially select patients for individual therapy [163].

Malignant cells have high glucose metabolism, and the differential uptake of 18FDG by cancer cells can be used to detect both the short- and the long-term tumor responses, which either are not evident on CT or foresee a decrease in tumor size [164]. Integrated FDG-PET-CT provides complementary metabolic information that allows the detection of malignant disease in morphologically normal organs or at unexpected sites that can be easily overlooked on cross-sectional imaging [165]. The combination of metabolic and anatomical imaging increases sensitivity and specificity of cancer detection and is useful to evaluate treatment response [136, 166]. When assessed by FDG-PET-CT, metabolic response to therapy correlates with clinical response, tumor biology, and disease-free survival in CRC patients [166]. However, unlike CT scans, a validated scheme for assessing cancer response to therapy with FDG-PET-CT is not available [128]. In addition to FDG, other PET radiotracers can be employed to image intracellular processes targeted for therapy. Indicators of cellular proliferation include 18F-FLT, 11C-choline, and 18F-choline, whereas 15O-water and 18-F-FMISO indicate perfusion and hypoxia, respectively [136]. Hypoxia is known to contribute to CRT

resistance, leading to angiogenesis and potential development of metastasis [165]. An imaging biomarker for radioresistance, such as 18-F-FMISO, could be employed to determine any differentials within the cancer and used to modulate radiotherapy in order to appropriately vary the radiation field and also to identify resistant areas that can be selectively dose escalated [137].

A novel approach of molecular imaging using PET-CT is the employment of radiolabelled antibodies or antibody fragments, such as 89Zr-rituximab, which allows to assess the distribution and availability among cancer cells of the epidermal growth factor receptor [167, 168]. This may support decision making for the selection of patients likely to benefit from therapy, identification of dose-limiting tissue, and optimization therapeutic planning [168].

Recently, thanks to new fusion technologies, a hybrid PET-MRI machine became available allowing functional imaging with simultaneously acquired PET and MRI data, changing the management of cancer patients [169–172]. This hybrid tool exceeds some limitations of FDG-PET-CT by allowing better soft tissue evaluation, more accurate T-staging, and improved characterization of small liver lesions and by providing better anatomical details for surgical planning while minimizing radiation exposure [169]. By adding functional MRI to PET, PET/MRI may further improve diagnostic accuracy in the differentiation of scar tissue for recurrence of CRC [172].

4. Nanotechnology and CRC Diagnosis

In recent years, nanotechnologies have made striking improvements in the diagnosis and treatment of human cancers. Specifically, they have enabled the development of nanomedicine, a new branch of medicine based on the use of nanomaterials in different activities, research, and clinical settings to improve the diagnosis and treatment of diseases [173]. Their applications in medicine are possible because nanoparticles (NPs) are resistant to oxidation, are easy to generate, and are full of interesting optical properties [174]. Additionally, other important characteristics such as biocompatibility, adaptable toxicity, dimension and surface chemical features, and a good stability in biological fluids and tissues [169] permit us to use them as active nanosystems in biomedicine [175–178].

In addition, because they are very small (nanometric scale), they are able to directly interact with cell and subcellular structures, although in a nonselective manner [175–177]. In principle, this could limit the utilization of NPs for specific applications; however, the organic groups and molecules linked to the NP surface (functionalization) allow to overcome this problem [174], thus improving the quality of the NP (based on the chemical groups linked on its surface, it can be used in different applications [174]). Further improvements to NPs include linking polyethylene glycol (PEG) molecules to its surface (to avoid passive extravasation and to increase their half-life in the circulation [168–180]) and the optimization of functionalization protocols that otherwise tend to generate agglomeration and agglutination of NPs [181, 182]. Finally, the addition of specific fragments capable of recognizing particular cell surfaces allows the NPs to transfer, accumulate, and promote the internalization of NP in a specific manner by tumor cells [183, 184].

4.1. Nanotechnologies in Noninvasive Diagnosis. Nanobioconjugates produced as previously described have been successfully used in the diagnosis of colorectal cancer [185]; in particular, various types of applications lead to an effective improvement of diagnostic techniques.

First of all, we know that, although MRI represents an indispensable noninvasive tool for diagnosis (it does not use ionizing radiation like computer tomography), it is still not sufficiently adequate to achieve 3D resolutions in real time [174]. However, this method is undergoing several improvements thanks to the use of magnetic nanobioconjugates as a contrast medium (magnetic particle imaging (MPI)), which help to increase the specificity and sensitivity of detection [174]. Among the NPs used effectively in these imaging techniques, we include iron oxide and nanobioconjugates constituted by liposomes, micelles, and dendrimers carrying paramagnetic ions [183, 186]. In addition, NPs have been used in a new noninvasive imaging technique (not employing ionizing radiation) defined as photoacoustic tomography, which combines ultrasound with the optical contrast provided by nanocages, carbon nanotubes, and gold speckled silica particles [187, 188]. Magnetic nanocrystals have also been used for multimodal diagnosis employing MPI and fluorescence [175]. As far as CRC is concerned, successful applications have been made with quantum dots (QD) [185]. These NPs are constituted by nanocrystals of a semiconductor and are capable of emitting fluorescence following excitation. Their particular optical characteristics provide them a series of advantages in several applications for cancer, including CRC [189, 190]. In particular, one study [191] reported an improvement of the immunohistochemical evaluation using QD in the procedure (QD-IHC). This methodology could be also applied to the immunocytochemical evaluation of antigens on the surface of living cells in a noninvasive manner, opening new perspectives in the evaluation of CTC in clinical practice [191]. Other studies demonstrated their eminent role in *in vivo* MRI diagnosis [192] and in CRC targeting of QD linked to bevacizumab [193]. In the latter case, noninvasive nanoprobes were able to detect CRC expressing high levels of VEGF. Additional methodologies, which take advantage of the useful characteristics of QD, are currently being evaluated and applied in the field of CRC diagnosis.

Another class of NPs employed in the analysis of CRC is constituted by dendrimers [185]. These are macromolecular structures that, starting from a centre, are formed by the addition of several repeated and branching elements. Similar to other NPs, these features conferred them the opportunity to be used in several applications of cancer nanomedicine [194, 195].

5. Bespoke Treatment: Role of Biomarkers in Clinical Setting

For many years, chemotherapy for mCRC was based simply on the combination of 5-fluorouracil (5-FU) plus levamisole

(LV), a treatment that could improve median survival up to 11 months [196]. During the last 20 years, the association of oxaliplatin or irinotecan with 5-FU/LV led to an improvement in the outcome of patients affected by mCRC [197, 198]. Independently from the first-line therapy choice, patients receiving all available anti-CRC drugs may report a prolonged overall survival (OS) exceeding even 2 years [199]. The introduction of target therapies (bevacizumab, cetuximab, panitumumab, aflibercept, and regorafenib) has further ameliorated overall survival in the metastatic setting. As these agents are active on processes controlling cell growth, survival, angiogenesis, and spread following selective pathways, the efficacy of these drugs depends on a strict selection linked to particular molecular profiles.

The combination of mAbs binding to the vascular endothelial growth factor and EGFR with chemotherapy in mCRC has been shown to improve the efficacy, thus increasing treatment options [200, 201].

With the aim to optimize treatments, it is now well recognized that the variable responses among mCRC patients are influenced by the molecular profile of the tumor, which is specific and different among all individuals. Therefore, it is essential to individualize these different molecular aspects.

In the management of mCRC, several prognostic and predictive biomarkers have been identified over the past years and they can be used to define a personalised treatment for patients. Prognostic biomarkers identify patients regardless of treatment and may provide details about the disease prognosis. Predictive biomarkers help categorize patients potentially benefiting from a specific treatment or that show resistance [202] towards it. Thus, many analyses were conducted to identify tumor-related predictive factors aimed to suggest treatment responses [203].

The Erb family of cell membrane receptors includes HER1/erbB1 (EGFR), HER2/c-neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4) [204]. As the EGFR gene was initially identified as an oncogene, it has become progressively the main target of biologic agents, prompting the development of anti-EGFR mAbs and tyrosine kinase inhibitors (TKIs). mAbs cetuximab (an anti-IgG1) and panitumumab (an anti-IgG2) act by binding to the extracellular domain site of the receptor, whereas erlotinib and gefitinib, two EGFR TKIs, compete with the binding site of ATP to the TK portion of the receptor, resulting in the inhibition of EGFR autophosphorylation. Both strategies (mAb and TKI) suspend the intracellular downstream signalling transmission.

The first clinical trials exploring the efficacy of anti-EGFR mAbs enrolled patients whose tumors expressed high levels of EGFR; however, overall response rates (ORRs) were low [205], which suggested the need of identifying additional factors potentially affecting the response to these agents [206]. Lièvre et al. were the first to identify a relation between mutant KRAS and poor responsiveness to EGFR-targeted treatments [207]. Thirty patients treated with cetuximab combined with irinotecan as second/third-line treatment were considered. KRAS mutations were detected in 13 of the 30 (43%) patients. None of the responders (0/11) had KRAS mutations, whereas 68.4% (13/19) of nonresponders presented them ($p = 0.0003$). The OS was significantly higher in wild-type KRAS (KRAS-WT) patients than in patients carrying a KRAS mutation (median OS: 16.3 versus 6.9 months, respectively, $p = 0.016$).

The next challenge was to understand why KRAS-mutated tumors did not respond to anti-EGFR mAbs. In this context, studies focused on key signalling molecule downstream of EGFR, including mutations in the KRAS, NRAS, BRAF, and PIK3CA genes and PTEN protein expression.

In the EGFR/RAS/RAF/MEK/ERK kinase downstream path, the KRAS protein is a GTPase that normally binds to the interior fragment of the cell wall. It conveys external signals from the receptor to the nucleus, regulating cell cycle (growth, proliferation, and apoptosis). The KRAS gene is located on the short arm of chromosome 12. Patients harboring point mutations in the KRAS gene generally have mutations within codon 12 at exon 2 (82%–87%), codon 13 (13%–18%), codon 61 (exon 3), and codon 146 (exon 4) [208]. In wild-type subjects, the activity of anti-EGFR mAbs on the external part of the receptor causes conformational changes blocking the RAS/RAF/MEK/ERK transmission. KRAS mutations impede EGFR activity for the constitutive activation of the intracellular fragment of the KRAS protein. In mCRC patients, the incidence of KRAS mutations is about 30%–45% [209].

Two randomized clinical trials comparing panitumumab or cetuximab with no active care in pretreated and chemorefractory mCRC patients [121, 208] demonstrated that KRAS mutant patients do not benefit from anti-EGFR mAbs. In the CO.17 [121] and AMGEN [208] studies, only KRAS-WT patients treated with cetuximab (median OS: 9.5 months versus 4.8 months) or panitumumab (median PFS: 12.3 weeks versus 7.3 weeks) had a survival benefit over best supportive care. In the cohort of patients with KRAS mutations, mAbs did not prolong PFS or OS.

The Cetuximab Combined With Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer (CRYSTAL) phase III trial enrolled 599 patients to receive FOLFIRI plus cetuximab and 599 patients in the arm with FOLFIRI alone [210]. Sixty-four percent of the cases were exon 2-KRAS-WT; in these patients, both the risk of disease progression (HR of PFS: 0.68 [95% CI 0.50–0.94]) and that of death (HR of OS: 0.84 [95% CI 0.64 to 1.11] were lower in cetuximab-treated patients. No difference in PFS or OS was reported in the experimental arm in mutated patients.

The role of KRAS as a prognostic biomarker in CRC is quite controversial. The CO.17 study [121] analysed the prognostic involvement of KRAS status by assessing the interaction between KRAS status and survival in patients receiving best supportive care alone. There were no significant differences in median OS in either KRAS-WT or KRAS-MUT patients (4.8 months versus 4.6 months, resp.).

Similarly, Kim et al. [211] found that clinical outcomes did not differ between KRAS-WT and KRAS-MUT mCRC patients treated with chemotherapy alone. The RASCAL Collaborative Group evaluated the prognostic role of KRAS among thousands of patients with any-stage CRC [212, 213]. They found that KRAS-MUT patients presented shorter PFS and OS compared to wild-type patients. The RASCAL-2 study concluded that the G12 V mutation in

the KRAS gene at codon 12 increases the risk of relapse or death only in Dukes' C CRC [213].

The retrospective analysis performed on mCRC patients in the MRC FOCUS trial [214] showed that KRAS mutations have a modest negative prognostic impact on OS (HR = 1.24; 95% CI 1.06–1.46; $p = 0.008$), but not on PFS (HR = 1.14; 95% CI 0.98–1.36; $p = 0.09$).

Neuroblastoma-ras (NRAS) is a member of the RAS oncogene family and is located on chromosome 1. The product of this gene is a GTPase enzyme membrane protein that shuttles between the Golgi apparatus and the cellular membrane. KRAS, BRAF, and NRAS mutations are mutually exclusive [215]. In CRC, the NRAS mutation rate is 3%–5% [216]. NRAS mutations are associated with the lack of response to cetuximab treatment. In the study by De Roock et al., NRAS-MUT patients treated with either cetuximab or panitumumab (2.6% of 644 KRAS-WT subjects) had a significantly lower ORR than NRAS-WT patients (7.7% versus 38.1%). PFS and OS did not differ statistically between mutated and wild-type patients.

A retrospective evaluation of biomarkers from patients enrolled in the PRIME trial indicated that NRAS plays an important role in predicting the efficacy of panitumumab treatment. Among the 656 patients with KRAS-WT exon 2, 108 (17%) had other mutations in KRAS exon 3 or 4, in NRAS exons 2, 3, or 4, or in BRAF exon 15 [217]. Patients with KRAS-WT exon 2 tumors bearing any RAS mutation did not achieve any benefit from panitumumab (median OS 17.1 months versus 17.1 months; $p = 0.12$). "All RAS" wild-type tumors (namely, wild type for KRAS exons 2/3/4 and for NRAS exons 2/3/4) significantly benefited from the combination treatment (median OS 25.8 months versus 20.2 months HR = 0.77; 95% CI 0.64–0.94; $p = 0.009$).

To elucidate the mechanisms of resistance to anti-EGFR antibodies, Bertotti et al. [218] performed a whole-exome analysis of 129 tumors in patient-derived xenografts detecting mutations in ERBB2, EGFR, FGFR1, PDGFRA, and MAP2K1 that could be potential mechanisms of primary resistance to anti-EGFR antibody therapy in CRC. Furthermore, investigation showed that amplifications and mutations in the tyrosine kinase receptor adaptor gene (IRS2) may contribute to the increased sensitivity to anti-EGFR therapy, representing a potential biomarker to predict full response to anti-EGFR-related CRC therapy.

Following these results, retrospective subanalyses were done on all the surviving wild-type patients enrolled in the CRYSTAL and OPUS trials. The results confirmed the important role of RAS mutational status in the optimal management of mCRC. Mutated patients do not benefit from anti-EGFR treatment, to the extent that this treatment could even be detrimental compared with chemotherapy alone. Therefore, in a real-life setting, the RAS mutational analysis has become essential and mandatory before beginning an anti-EGFR-based treatment.

5.1. BRAF. The BRAF protein is a cytoplasmic serine-threonine kinase bearing mutations in approximately 8%–10% of sporadic CRC [219]. The BRAF protein is one of the main effectors of KRAS; it is located immediately after KRAS effectors and it must be phosphorylated by KRAS to be activated. The point mutation V600E causes a CTG to CAG substitution at codon 600, which leads to a constitutive activation of the RAS/RAF/MEK/ERK cascade, similar to KRAS mutations.

In the retrospective analysis performed by Di Nicolantonio and colleagues, 113 tumor samples treated with cetuximab or panitumumab (with or without chemotherapy) were analysed and 79 KRAS-WT patients were identified. In this cohort, 11 (13.9%) patients were BRAF mutants and none of them responded to treatment.

In the CRYSTAL study [210], 9% (59 of 625) of patients carried BRAF mutations and they reported limited benefits from treatment with a shorter median OS in both arms, compared with the KRAS-WT and BRAF-WT population whose survival was 21.6 and 25.1 months, respectively. BRAF-MUT status was unrelated to cetuximab efficacy; thus, the authors concluded that BRAF mutation is a negative prognostic biomarker and not a predictive factor. Moreover, in the combined analysis of the CRYSTAL and OPUS results [220], a BRAF mutation was considered a negative prognostic marker in conclusion. In fact, survival times were lower in the BRAF-MUT population irrespective of therapy administered.

A BRAF mutation was also considered a negative prognostic biomarker in the PRIME trial [217]. In fact, patients with RAS-WT but BRAF-MUT tumors had a worse PFS and OS compared to subjects with wild-type RAS and BRAF tumors. In the RAS-WT/BRAF-MUT subgroup, the addition of panitumumab to chemotherapy produced a small benefit (difference was not statistically significant) in term of DFS and OS ($p = 0.12$ and 0.76, resp.).

The negative prognostic role of BRAF was also explored in clinical trials that enrolled patients to receive an intensive chemotherapy regimen plus an anti-VEGF treatment. The TRIBE trial compared the effect of bevacizumab plus the standard chemotherapy FOLFIRI with the association of 5-fluorouracil, irinotecan, levamisole, and oxaliplatin (FOLFOXIRI schedule). Final results of this trial showed a better outcome with the experimental arm; results showed an improvement of median PFS, OS, and ORR. A subset analysis was also performed in BRAF-MUT patients. Among the assessed patients, 28 (7%) BRAF-MUT patients reported a median OS of 13.7 months, significantly short if compared with the 37.1 months calculated for all wild-type patients [221].

It has been recently debated if a BRAF mutation could be considered a negative predictive factor too. Two meta-analyses were published in 2015 highlighting BRAF function. Pietrantonio et al. [222] assessed the negative predictive role covered by this mutation, which was mainly exerted towards anti-EGFR treatment. Rowland et al. [223] concluded that a BRAF mutation could only be considered as a negative prognostic biomarker (based on a not-significant interaction test and on the absence of a sufficient amount of data).

In conclusion, a BRAF mutation should be considered a negative prognostic biomarker rather than a negative predictive factor influencing anti-EGFR mAbs. BRAF-MUT patients have a poorer prognosis than BRAF-WT patients, irrespective of schedule of chemotherapy. These patients

may benefit from anti-EGFR mAb treatment, but to a significantly lesser extent than BRAF-WT patients.

5.2. HER-2. The HER family of tyrosine kinase receptors consists of EGFR, HER2 (ErbB2), HER3, and HER4. They are responsible for cell survival and proliferation via signalling through the RAS-RAF-ERK and PI3K-PTEN-AKT pathways [224].

HER2 is a potential therapeutic target in patients with CRCs, and it is overexpressed in 25–35% of human breast cancers [225]. The level and incidence of HER2 overexpression in primary CRCs appear to be different.

In 2012, The Cancer Genome Atlas (TCGA) Network published the most comprehensive systematic molecular characterization of CRC to date, revealing genomic amplifications or mutations of the tyrosine kinase-encoding gene *ERBB2* in 7% of colorectal tumors, suggesting a novel potential therapeutic target for this cancer [226].

Several studies have assessed HER2 overexpression in CRC, with some reporting membranous expression, varying in the range 2.1–11% in [227–233], and others reporting cytoplasmic overexpression in the range 47.4–68.5% [230, 234, 235].

Kavuri and colleagues [236] studied the effect of ERBB2-targeted therapy in *ERBB2*-mutated CRC demonstrating that engineered intestinal cell lines that host *ERBB2* mutations are highly sensitive to irreversible EGFR/ERBB2 tyrosine kinase inhibitors, neratinib and afatinib, with these inhibitors inducing effective inhibition of ERBB2 and its downstream pathways.

Furthermore, xenografts from these cells lines were also sensitive to both neratinib and the combination of neratinib and trastuzumab. Interestingly, single-agent neratinib in a patient-derived xenograft (PDX) harboring *ERBB2* L866M mutation and amplification resulted in tumor stabilization and not in tumor regression as in the case of the combination of trastuzumab and neratinib. This result has been confirmed in another PDX harboring *ERBB2* S310Y mutation. Both PDX models were resistant to trastuzumab alone.

Anti-EGFR therapies, including cetuximab and panitumumab, have improved the prognosis of patients with CRC, particularly in the case of wild-type *KRAS* genes, in which these agents exhibit greater effectiveness [210, 237–239]. KRAS activities downstream the EGFR pathway and its spontaneous activation because mutation promotes cell proliferation despite the presence of anti-EGFR antibody [240].

In the previously described trial, sequencing CRC tumors with *ERBB2* mutation, Kavuri et al. [236] found that 50% (6/12) had a coccurring *KRAS* mutation.

Similarly, Kloth et al. [241] reported that three of 14 of *ERBB2*-mutated MSI CRC harbored *KRAS* mutation. Even though this cooccurrence could be justified in hypermutated MSI tumors, it is a surprising finding in the nonhypermutated tumors. Above all, several studies have exclusively evaluated mutant or amplified *ERBB2* as a target in tumors or models lacking such *KRAS* alterations. Further studies will be needed to better define both the etiology of this cooccurrence as well as the therapeutic consequences.

Based on promising preclinical studies in *ERBB2*-amplified CRC [242, 243], Siena et al. [244] conducted a phase II clinical trial of dual ERBB2 blockade. Patients with *ERBB2*-amplified, *KRAS* exon 2 wild-type, metastatic CRC who progressed after multiple lines of therapy, were treated with trastuzumab and lapatinib. Of 913 patients screened, 44 (4.8%) were found to be *ERBB2* amplified. Among 23 patients treated with dual anti-ERBB2 therapy, 8 (35%) patients had an objective response.

Similarly, trastuzumab and pertuzumab demonstrated response rates of 23% and disease control rates of 69% in the colorectal arm of a basket study [245]. These results were consistent with those of Valtorta et al [246].

Recent studies do suggest that HER2 overexpression by gene amplification may indeed be related to poor outcome in *KRAS* wt metastatic CRC patients treated with cetuximab or panitumumab [247].

In a study of 137 patient-derived xenograft (PDX) tumors, conducted by Hurwitz and colleagues, HER2 amplification was found in 13.6% of cases in patients with cetuximab-resistant, *KRAS* wild-type tumors [243].

Although patients with *HER2* amplification were resistant to anti-EGFR antibody therapy, other treatment strategies, including lapatinib or trastuzumab, can overcome cetuximab resistance in CRCs [242]. Finally, in addition to the previously reported trial conducted by Siena et al. [244], Deeken et al. [248] concluded that the combination of cetuximab and lapatinib provided a partial response in some patients with CRC who were resistant to anti-EGFR antibody therapy.

5.3. MSI-H. There are two molecular pathways in colorectal carcinogenesis. One is chromosomal instability (CIN) and the other is microsatellite instability (MSI) [249, 250].

High-level microsatellite instability (MSI-H) CRCs constitute approximately 15% of all CRCs in Western countries [251–253], more frequent in the early than the late stage of disease. The cause of MSI-H colorectal cancers is a deficiency of the DNA mismatch repair (MMR) system characterized by unstable microsatellites, a type of simple DNA sequence repeat. Its role consists in the postreplicative control of newly synthesised DNA strands and the correction of polymerase misincorporation events [254, 255].

MSI-H colorectal cancers can occur as sporadic tumors, because of methylation of promoter regions of the hMLH1 during tumorigenesis [256], or in the context of hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome (LS) [257] with mutations of DNA MMR genes, primarily hMLH1, hMSH2, hMSH6, and hPMS2.

A defect in MMR is not manifested until both alleles of an MMR gene are inactivated (even if LS is dominantly inherited, a second hit on the other allele is required). MSI status can be determined by DNA testing. In particular, five microsatellite markers recommended by the National Cancer Institute (NCI) workshop have been used for MSI analysis: BAT25, BAT26, D2S123, D5S346, and D17S250 [256].

Two or more of the five markers are required to confirm the presence of MSI-H. Conversely, a low level of MSI (MSI-L) is assigned when only one unstable markers is

detected. MSI-H CRC is known to have well-defined clinicopathological and molecular features. In fact, MSI-H CRC are preferentially located in the proximal colon and frequently associated with a less advanced cancer stage, extracellular mucin production, medullary carcinoma and poorly differentiated carcinoma, tumor-infiltrating lymphocytes, a Crohn's-like lymphoid reaction, and a *BRAF* V600E mutation [258–260]. Furthermore, it is associated with favorable survival in comparison with MSS/MSI-L. Interestingly, it is associated with chemotherapy resistance (i.e., adjuvant 5-FU-based chemotherapy) [261–265] but patients with metastatic disease are good candidates for immune-targeted therapy such nivolumab or pembrolizumab [266–268]. Conversely, several studies supporting MSI-H as a predictive factor for improved response to irinotecan- or irinotecan-based chemotherapy in CRC patients have been reported [269, 270].

6. Conclusive Remarks

CRC is a complex biological process involving multiple steps and genes, including genetic and epigenetic [271] factors, germline and somatic mutations, and chromosomal aberrations [272].

The three most important pathways of CRC carcinogenesis are the EGFR signalling pathway, with the involvement of KRAS and BRAF, the DNA mismatch repair (MMR), and the fields of epigenetics such aberrant hypermethylation and microRNAs (miRNAs) expression.

Over the recent years, several biomarkers of CRC have been proposed and encouraging progress has been made in our understanding the behaviour of CRC at a molecular level. Even if further validation studies are needed, assessing the role of biomarkers in experimental models and in patients could open new perspectives concerning a patient-tailored approach. Moreover, they could increase CRC screening uptake, given their limited invasiveness.

Conflicts of Interest

The authors have no conflict of interest to disclose for this manuscript.

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Research Article

Colonic Mucosal Microbiota in Colorectal Cancer: A Single-Center Metagenomic Study in Saudi Arabia

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Background and Aim. Because genetic and geographic variations in intestinal microbiota are known to exist, the focus of this study was to establish an estimation of microbiota in colorectal cancer (CRC) patients in Saudi Arabia by means of metagenomic studies. *Methods*. From July 2010 to November 2012, colorectal cancer patients attending our hospital were enrolled for the metagenomic studies. All underwent clinical, endoscopic, and histological assessment. Mucosal microbiota samples were collected from each patient by jet-flushing colonic mucosa with distilled water at unified segments of the colon, followed by aspiration, during colonoscopy. Total purified dsDNA was extracted and quantified prior to metagenomic sequencing using an Illumina platform. Satisfactory DNA samples ($n = 29$) were subjected to metagenomics studies, followed by comprehensive comparative phylogenetic analysis. An equal number of healthy age-matched controls were also examined for colonic mucosal microbiota. *Results*. Metagenomics data on 29 patients (14 females) in the age range 38–77 years were analyzed. The majority 11 (37%) of our patients were overweight (BMI = 25–30). Rectal bleeding was the presenting symptom in 18/29 (62%), while symptomatic anemia was the presenting symptom in 11/29 (37%). The location of colon cancer was rectal in 14 (48%), while cecal growth was observed in 8 (27%). Hepatic flexure growth was found in 1 (3%), descending colonic growth was found in 2 (6%), and 4 (13%) patients had transverse colon growth. The metagenomics analysis was carried out, and a total of 3.58G reads were sequenced, and about 321.91G data were used in the analysis. This study identified 11 genera specific to colorectal cancer patients when compared to genera in the control group. *Bacteroides fragilis* and *Fusobacterium* were found to be significantly prevalent in the carcinoma group when compared to the control group. *Conclusion*. The current study has given an insight into the microbiota of colorectal cancer patients in Saudi Arabia and has identified various genera significantly present in these patients when compared to those of the control group.

1. Introduction

Metagenomics is a molecular method of culture-independent microbiology, in which genetic material recovered directly from environmental samples is studied. It has emerged as one of the most robust sequence-driven approaches for studying the composition and the genetic potential of the mucosal gut microbiota. Metagenomics analysis has begun to demonstrate the breadth of the functional and metabolic potential of microbes. It has been used to demonstrate significant metabolic discrepancies between diseased and healthy individuals. Although the intestinal microbiota in individuals reflects great variations among people according to their age, geographic origin, state of health, and variations in diet, it tends to remain stable over long periods [1].

An alteration in gut microbiota can cause the development of inflammation within the colon, and such inflammation is implicated in colonic neoplastic development. Although the precise mechanisms through which the microbiota is involved in cancer development remain elusive,

the message is, however, clear: the microbiota contributes to cancer risk by influencing some fundamental host processes [2]. This implies that modifiable risk factor interventions to modulate gut microbiota can contribute to decreasing the morbidity and mortality rates in colon cancer.

The risk of developing colorectal cancer (CRC) varies markedly between and within populations and geographical regions [3]. Accordingly, various aspects of carcinogenesis in colon cancer have been addressed in Saudi Arabia by different investigators from time to time [4–8], but to the best of our knowledge and after a literature search, there appears to be no existing data regarding the role of microbiota in CRC in this part of the globe. Hence, we were prompted to undertake this study, first of its kind in Saudi Arabia, in which we have tried to introspect the role of mucosal intestinal microbiota in CRC patients.

It will be prudent to mention that the mucosal microbiota lives closer to the intestinal epithelium when compared to the luminal microbiota, and conceivably, it would be interacting more directly with the host immune system than would the luminal/fecal bacteria. It is quite possible that mucosal microbiota might be more directly involved in inducing colon carcinogenesis. In addition, the availability of nutrients in the mucus layer of the epithelium is also entirely different from that in the gut lumen environment. Substantial differences in mucosal and fecal microbial composition have been shown to exist [9, 10]. Hence, we choose mucosal samples and not fecal samples for this metagenomic study in CRC patients. The microbiota was compared with that in age- and gender-matched controls.

2. Material and Methods

This study was conducted in full compliance with the guidelines for good clinical practice of the World Medical Assembly Declaration of Helsinki and the research guidelines of the King Fahad Medical City (KFMC), Riyadh. The study was approved both by the Ethics Committee of King Abdulaziz City for Science and Technology and KFMC, Riyadh.

2.1. Inclusion Criteria for Study Participants. During the study period (July 2010 and November 2012), all colonoscopy patients at King Fahad Medical City (KFMC), Riyadh, were asked to provide specific written consent for possible inclusion in this study. Samples collected during the colonoscopy procedures in the following types of patient were then processed.

(1) Cases

   (a) Adult patients with diagnosed colorectal cancer whose diagnoses were based on endoscopic and histological criteria.

(2) Controls

   (a) Patients undergoing screening colonoscopic examination with normal colonoscopic procedures.

   (b) Patients undergoing colonoscopic procedures for lower GI bleed who had been proved to have hemorroids and anal fissures.

   (c) Patients with abdominal pain undergoing the colonoscopic procedure and who proved to have a normal colonoscopic examination

2.2. Exclusion Criteria. Patients with obstruction at presentation due to colon cancer were excluded. Patients who had used antibiotics two weeks prior to colonoscopy were excluded.

2.3. Data Collection. Demographic and clinical data were collected from all participants, including age, sex, and body mass index (BMI). Detailed medical histories, including diet, comorbidities, and the chronological order of any medications taken or procedures performed, were also obtained from each participant, and the data was entered in a Microsoft Excel file.

2.4. Bowel Preparation and Sample Collection. After a standard bowel preparation that included polyethylene glycol colonic preparation, a full-length colonoscopy was carried out. During the procedure, distilled water was pushed through the biopsy channel of the scope which was collected back by aspiration. The mucosal jet wash from unified segments of the colon (cecum, transverse, left, and rectal colon segments) was collected from all study participants. The healthy mucosa of each study participant was also sampled. Sites of macroscopic mucosal abnormality in any of the four selected segments were also included. Finally, 50 mL of the washes, along with the remaining colonic fluids, was aspirated through the working suction and biopsy channel. All segment samples obtained from each colonoscopy were collected in four 15 mL test tubes and immediately stored at −80°C for further processing.

2.5. DNA Extraction for Total Metagenomics Sequencing. All segment samples from each participant were pooled and considered representative of the whole colon to minimize technical errors and variations. DNA samples were centrifuged at 5000 ×g for 15 minutes, and the supernatants were discarded. The pellet was resuspended in 10 mL lysis buffer (0.5 M Tris-HCl; 20 mM EDTA; 10 mM NaCl; 0.1% SDS; pH 9.0), and the mixtures were homogenized by centrifuging and shaking for 5–10 minutes.

Samples were then diluted (1 : 2) with a 10 mL lysis buffer and homogenized for another 5 minutes. Genomic DNA (gDNA) was precipitated by adding 5 mL 7.5 M ammonium acetate and 25 mL ice-cold ethanol (95–100%). The samples were subsequently incubated at −20°C for 20–30 minutes, and gDNA was collected following centrifugation at 4500 ×g for 15 minutes at room temperature. DNA pellets were resuspended in 600 μL TE buffer (Tris-EDTA, pH 8.0) and incubated at 65°C for 15 minutes. An equal volume of phenol-chloroform : isoamyl alcohol solution was briefly mixed with the DNA, and the mixtures were centrifuged at 12,000 ×g for 5 minutes at room temperature. The supernatant aqueous phase was then transferred to a new tube, while

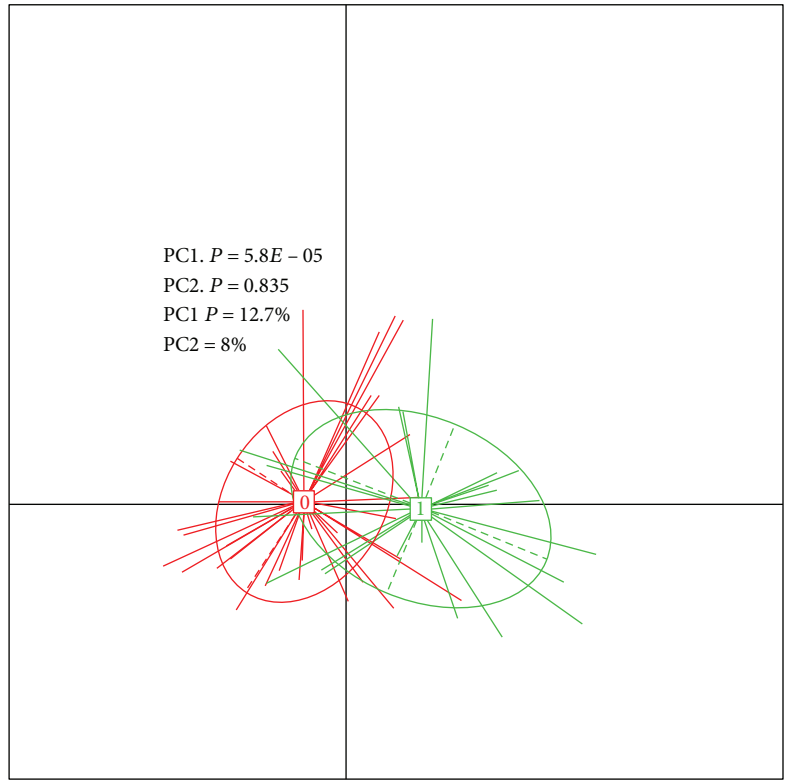

Figure 1: Principal coordinate analysis (PCoA) between cases and controls.

the interface and the organic phase were discarded. This step was repeated until no protein was visible at the interface. The final supernatant aqueous phase was then transferred to a new tube; twice the amount of ethanol was added to the aqueous phase, and the solution was stored overnight. The following day, the DNA was ethanol precipitated as described above. The resulting DNA pellet was resuspended in 50 μL TE buffer. The quality and concentration of the extracted gDNA were verified using 1% agarose gel electrophoresis and a Qubit fluorometer 3.0 (Life Technologies, Carlsbad, CA).

2.6. Metagenomics DNA Library Construction and Sequencing. Paired-end (PE) metagenomics DNA library construction was performed, based on the manufacturer's instructions (Sequencing Kits and Reagents, Illumina, San Diego, CA). High-quality reads were separated from low-quality reads with "N" bases, adapter contamination, or human DNA contamination from the Illumina raw data using the BWA-SW algorithm (Li H, Durbin R. 2010, Bioinformatics). On average, the proportion of high-quality reads in all samples was approximately 98.1%, and the insert sizes of our PE clones ranged from 313 bp to 381 bp. Sequencing and data processing were performed at the Beijing Genomics Institute, where Illumina GAIIx and HiSeq 2000 platforms were utilized to sequence the samples.

2.7. Gene Catalog Construction. The first 72 randomly chosen samples were combined to establish the nonredundant gene set. Predicted open reading frames (ORFs) of the 72 samples were aligned to each other, and gene pairs with higher than 95% identity were grouped. Groups with similar genes were merged, and the longest ORF in each group was used to represent that group. We therefore organized the nonredundant gene set from all predicted genes by excluding redundant ORFs. For 159 high-quality reads in stages I, II, and III, we performed de novo assembly and gene predictions using SOAPdenovo v1.06 (Luo et al.: "SOAPdenovo2: An Empirically Improved Memory-Efficient Short-Read De Novo Assembler". GigaScience 2012 1 : 18) and GeneMark v2.7 (Ter-Hovhannisyan, Vardges, et al. "Gene Prediction in Novel Fungal Genomes Using an Ab Initio Algorithm with Unsupervised Training"; Genome Research 18.12 (2008): 1979–1990.), respectively. All predicted genes were aligned pair-wise using BLAT, and genes that could be aligned (>90% of gene length) to another gene with more than 95% identity (no gaps allowed) were removed as redundancies, resulting in a nonredundant gene catalog. This catalog of colonic samples was further combined with the previously constructed Meta HIT gene catalog by eliminating redundancies in the same manner.

2.8. Bioinformatics Analysis Pipeline. We subjected multiple samples from the mucosal microbiota metagenome to comparative phylogenetic analyses to understand the ecology of cultivation-independent gut microbiota and the phylogenetic differences between samples (Figure 1). We aligned all high-quality reads to known bacterial, fungal, protozoal, or human gut gene databases from NCBI, RDP, or MetaHIT. For each

Table 1: Demographic data of study participants.

Demographic data	Age range	38–77 years
	Gender ratio M : F	15 : 14
	BMI > 30	11 (37%)
Symptoms at presentation	Rectal bleeding	18/29 (62%)
	Anemia	11/29 (37%)
Location of tumor	Rectal growth	14 (48%)
	Descending colon growth	2 (6%)
	Transverse colon growth	4 (13%)
	Hepatic flexure growth	1(3%)
	Cecal growth	8 (27%)

sample, we compared paired alignment and single alignments to the databases.

2.9. Phylogenetic Classification of ORFs. Taxonomic assignments were performed with the BLASTp alignment tool against the NR90 database. Alignment hits with E values greater than 1E-5 were removed, and significant matches with E values in the same order of the top hit were used for determining taxonomic groups. We assessed the taxonomic association of each gene by a lowest common ancestor-(LCA-) based algorithm implemented in MEGAN43.

2.10. Statistical Analysis. The module extracted from the ipath (reference) test was assessed by means of the Wilcoxon test between the control and the cancer groups. The Chao1 Richness Index, Shannon Index, and Simpson Diversity Index were used to describe the α diversity features of our bacterial community. A P value of <0.05 was considered significant.

Beta diversity was used to assess diversity between cases and controls. Principal coordinate analysis (PCoA) based on the unweighted UniFrac distance metrics was used to demonstrate that there was a difference in the mucosal bacterial communities between the cases and controls, which was confirmed by permutational multivariate analysis of variance (PERMANOVA) PCoA in the CRC patients.

3. Results

Thirty-two patients were enrolled for the CRC metagenomics study to begin with, but of these, three were excluded because of low DNA quantity in their samples. Finally, the data on 29 confirmed patients with CRC were analyzed along with a group of age- and gender-matched controls. In this study, there were 14 females and 15 males aged from 38 to 77 years.

The majority 11 (37%) of our patients were overweight (BMI = 25–30). Five (24%) patients were assigned to obesity class I (BMI = 35–40). Obesity class III BMI > 40 was observed in a 54-year-old male subject. Only 4 (13%) patients had normal BMI (BMI = 18–25). One patient in our study was observed to be mildly thin (BMI = 17-18.5), a 66-year-old female who had a BMI of just 15.66 as shown in Table 1.

Rectal bleeding was the commonest (18/29, 62%) presentation characteristic in the CRC group, and 11/29 or 37% of patients presented with symptomatic anemia. On colonoscopic examination, the CRC location was rectal in 14 (48%), and cecal growth was observed in 8 (27%). There was also hepatic flexure growth in 1 (3%) and descending colonic growth in 2 (6%), and 4 (13%) patients had transverse colon growth (Table 1). None of our patients had a familial colon cancer syndrome or any family history of colon cancer.

The histology of CRC was that of adenocarcinoma which was confirmed by two histopathologists experienced in GI histology in all patients.

The metagenomics analysis was carried out in cases and control as shown in Table 2, and in total, 3.58 G reads were sequenced, with about 321.91 G data being used in the analysis. The useful reads in each sample were aligned to 4.3 M gene set 1 by soap 2. On average, 70.33% reads can be mapped to the gene set; the max ration can reach 81.36%.

The Chao1 Richness Index, Shannon Index, and Simpson Diversity Index used to describe the α diversity features of our bacterial community are shown in Figures 2 and 3. The module extracted from the ipath (reference) test by the Wilcoxon test between the CRC and the control groups was 0.23.

All the results were mapped to 9.9 M gene sets 9 and 10. The difference between the groups in rarefaction was quite significant, as shown in Figure 1. However, the Shannon alpha diversity showed no significant difference between the diseased group and the control group, as shown in Figure 3.

Beta diversity showed significant diversity between cases and controls. Principal coordinate analysis (PCoA) based on the unweighted UniFrac distance metrics demonstrated that there was a separation in the mucosal bacterial communities between the cases and controls, which was confirmed by permutational multivariate analysis of variance (PERMANOVA) PCoA in colorectal cancer, as shown in Figure 1.

It was further observed that the CRC group had statistically significantly higher 11 genera compared to those in the controls, as shown in Table 3. These genera were *Atopobium*, *Beggiatoa*, *Burkholderia*, *Collinsella*, *Comamonas*, *Finegoldia*, *Fusobacterium*, *Gemella*, *Listeria*, *Methanobrevibacter*, *Parvimonas*, *Peptoniphilus*, *Peptostreptococcus*, *Porphyromonas*, *Selenomonas*, *Shuttleworthia*, *Solobacterium*, *Thermoanaerobacter*, *Verrucomicrobiales*, and *Yersinia.* The enrichment of bacteria in colorectal cases is shown in Figure 4.

Table 2: Metagenomic analysis in cases and controls.

Genus, phylum, class, order, family	*P* value	Mean rank sum		E
		Controls	Cases	
Abiotrophia, Firmicutes, Bacilli, Lactobacillales, Aerococcaceae	0.0066	24	37	0
Acidaminococcus, Firmicutes, Negativicutes, Selenomonadales, Acidaminococcaceae	0.0082	24	37	0
Akkermansia, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae	0.0005	22	38	0
Alcaligenes, Proteobacteria, Betaproteobacteria, Burkholderiales, Alcaligenaceae	0.0066	36	31	1
Anaerostipes, "Firmicutes," Clostridia, Clostridiales, Lachnospiraceae	0.0005	22	38	0
Azoarcus, Proteobacteria, Betaproteobacteria, Rhodocyclales, Rhodocyclaceae	0.0086	39	29	1
Bacteroides, Bacteroidetes, Bacteroidia, Bacteroidales, Bacteroidaceae	0.0072	24	37	0
Beggiatoa, Proteobacteria, Gammaproteobacteria, Thiotrichales, Thiotrichaceae	0.0003	44	26	1
Burkholderia, Proteobacteria, Betaproteobacteria, Burkholderiales, Burkholderiaceae	0.0028	42	27	1
Butyrivibrio, Firmicutes, Clostridia, Clostridiales, Lachnospiraceae	0.0005	22	38	0
Chlorobium, Chlorobi, Chlorobia, Chlorobiales, Chlorobiaceae	0.0027	39	29	1
Clostridium, Firmicutes, Clostridia, Clostridiales, Clostridiaceae	0.0002	21	39	0
Coprococcus, Firmicutes, Clostridia, Clostridiales, Lachnospiraceae	0.0052	24	37	0
Coriobacterium, Actinobacteria, Actinobacteria, Coriobacteriales, Coriobacteriaceae	0.0083	38	29	1
Coxiella, Proteobacteria, Gammaproteobacteria, Legionellales, Coxiellaceae	0.0066	36	31	1
Crenothrix, Bacteroidetes, Sphingobacteria, Sphingobacteriales, Crenotrichaceae	0.0013	42	27	1
Cryptobacterium, Actinobacteria, Actinobacteria, Coriobacteriales, Coriobacteriaceae	0.0027	39	29	1
Dethiobacter, Firmicutes, Clostridia, Clostridiales, Syntrophomonadaceae	0.0084	40	28	1
Enterobacter, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae	0.0004	22	39	0
Eubacterium, Firmicutes, Clostridia, Clostridiale, Eubacteriaceae	0.0026	23	38	0
Haemophilus, Proteobacteria, Gammaproteobacteria, Pasteurellales, Pasteurellaceae	0.0060	24	37	0
Haladaptatus, Archaea, Euryarchaeota, Halobacteria, Halobacteriales, Haladaptatus	0.0007	38	30	1
Holdemania, Firmicutes, Erysipelotrichi, Erysipelotrichales, Erysipelotrichidae	0.0004	22	39	0
Klebsiella, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae	0.0066	24	37	0
Listeria, Firmicutes, Bacilli, Bacillales, Listeriaceae	0.0008	41	28	1
Megasphaera, Firmicutes, Negativicutes, Selenomonadales, Veillonellaceae	0.0021	23	38	0
Mycoplasma, Tenericutes, Mollicutes, Mycoplasmatales, Mycoplasmataceae	0.0025	40	28	1
Paracoccus, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae	0.0075	41	28	1
Polaribacter, Bacteroidetes, Flavobacteria, Flavobacteriales, Flavobacteriaceae	0.0002	43	27	1
Roseburia, Firmicutes, Clostridia, Clostridiales, Lachnospiraceae	0.0057	24	37	0
Serratia, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae	0.0002	41	28	1
Sphaerochaeta, Spirochaetes, Spirochaetia, Brachyspirales, Sarpulinacea	0.0004	43	26	1
Sulfurovum, Proteobacteria, Epsilonproteobacteria	0.0027	42	27	1
Ureaplasma, Tenericutes, Mollicutes, Mycoplasmatales, Mycoplasmataceae	0.0034	39	29	1
Unclassified	0.0031	23	38	0

Further, the subanalysis of Enterobacteriaceae revealed that *enterotype* 1 was observed in 15 patients with CRC while *enterotype* 2 was found to be present in 2 cases. There were six patients with *enterotype* 3 positive among the CRC group. *Enterotype* 1 was observed to be more frequently present in the control subjects than in the CRC group (Figure 5).

4. Discussion

Carcinogenesis in colorectal cancer (CRC) represents a heterogeneous process with a differing set of somatic molecular alterations and can be influenced by a diet and environmental and microbial exposures. Recent evidence has shown a significant link between CRC and microbiota thus affirming the old ties of bacteria and colorectal carcinogenesis observed in the past. Various studies have indicated that the presence in the gut of *Bacteroides vulgatus*, *Bacteroides stercoris*, and Clostridia species have all been directly linked to a high risk of CRC [3]. It is hypothesized that some intestinal bacteria potentiate intestinal carcinogenesis by producing genotoxins, altering the immune response and intestinal microenvironment, and activating oncogenic signaling pathways [11].

In this study, we intentionally choose metagenomic studies on colonic washes rather than on fecal samples as the evidence in support of mucosal microbiota mapping is profound. The mucosal microbiota maintains a closer interaction with the intestinal epithelium than that of the

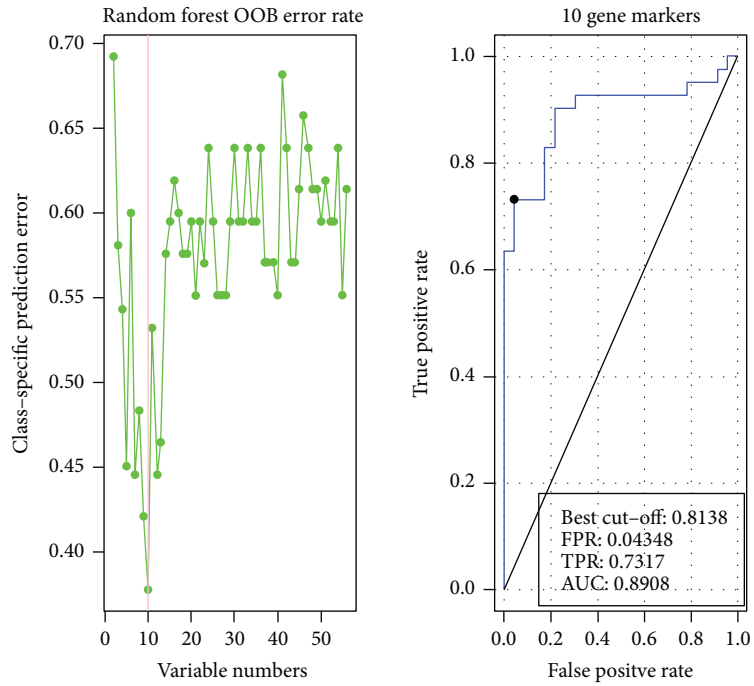

Figure 2: Classification based on mOTU marker in CRC cases and controls.

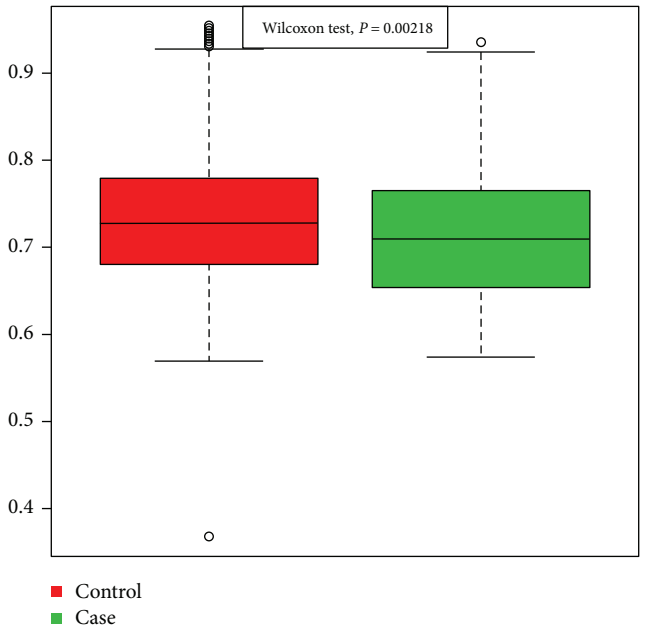

Figure 3: Alpha-Shannon Index in CRC cases and controls.

microbiota found in feces, and there is significant intersubject variability as well as differences between stool and mucosa community composition as has been demonstrated by Eckburg et al. [12].

The standard bowel prepration used in subjects in this study may be presumed to have altered the diversity of mucosa associated microbiota. Nevertheless, Harrell et al. observed that the taxonomic classification did not reveal significant changes at the phylum level, but only at the genus level. The authors of this study concluded that the degree of change underscores the importance of the need to consider the potentially influential effects of bowel preparation in experimental studies [13].

Our study showed that the CRC cases had significant enrichment of eleven genera compared to those in the control group, as shown in Table 3. The metagenomic sequencing showed that specific species, such as *Fusobacterium nucleatum*, *Peptostreptococcus stomatis*, and *Parvimonas micra*, were present in significantly greater quantities in the CRC patients than in the controls. *Fusobacterium nucleatum* has been identified to have a tumor-based immune evasion mechanism that is bacteria-dependent in the pathogenesis of CRC. Gur et al. have demonstrated that *Fusobacterium nucleatum*-bound tumors are protected from NK-mediated killing and immune cell attack due to an interaction between the fusobacterial protein Fap2 and the immune cell inhibitory receptor TIGIT on tumor-infiltrating lymphocytes including natural killer cells [14]. The multiplication of this genus in our population may possibly be a pointer to one of the target microbes for study in the future.

Another organism found to be quite significantly present in our study was *P. anaerobius*. It interacts with a toll-like receptor 2 (TLR2) and TLR4 on colon cells to increase the levels of reactive oxidative species, which promotes cholesterol synthesis and cell proliferation. Ni et al. have shown that the levels of *P. anaerobius* were found to be higher in human colon tumor tissues and adenomas when compared with nontumorous tissues. The authors of this study postulated that this bacterium increases colon dysplasia in a mouse model of CRC [15].

In animal studies, germ-free mice fed with stool from individuals with CRC developed significantly higher

Table 3: Significant enrichment in colorectal cases.

Genus, phylum, class, order, family	*P* value	Enrichment
Atopobium, Actinobacteria, Coriobacteriia, Coriobacteriales, Coriobacteriaceae	0.00047	CRC
Beggiatoa, Proteobacteria, Gammaproteobacteria, Thiotrichales, Thiotrichaceae	0.0002	CRC
Burkholderia, Proteobacteria, Betaproteobacteria, Burkholderiales, Burkholderiaceae	0.00011	CRC
Collinsella, Actinobacteria, Actinobacteria, Coriobacteriales, Coriobacteriaceae	0.00269	CRC
Comamonas, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae	0.00665	CRC
Finegoldia, Firmicutes, Clostridia, Clostridiales, Peptoniphilaceae	0.00726	CRC
Fusobacterium, Firmicutes, Clostridia, Clostridiales, Peptoniphilaceae	0.00751	CRC
Gemella, Firmicutes, Bacilli, Bacillales	0.00743	CRC
Listeria, Firmicutes, Bacilli, Bacillales, Listeriaceae	0.00657	CRC
Methanobrevibacter, Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae	$7.63E-05$	CRC
Peptostreptococcus, Firmicutes, Clostridia, Clostridiales, Peptostreptococcaceae	0.00014	CRC
Peptoniphilus, Firmicutes, Clostridia, Clostridiales	0.00023	CRC
Peptostreptococcus, Firmicutes, Clostridia, Clostridiales, Peptostreptococcaceae	0.00142	CRC
Porphyromonas, Bacteroidetes, Bacteroidetes, Bacteroidales, Porphyromonadaceae	0.0066	CRC
Selenomonas, Firmicutes, Negativicutes, Selenomonadales, Veillonellaceae	0.00343	CRC
Solobacterium, Firmicutes, Erysipelotrichi, Erysipelotrichales, Erysipelotrichidae	0.00904	CRC
Thermoanaerobacter, Firmicutes, Clostridia, Thermoanaerobacterales, Thermoanaerobacteraceae	0.00043	CRC
Verrucomicrobiales, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiacea	$1.68E-06$	CRC
Yersinia, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Yersiniacea	0.0095	CRC

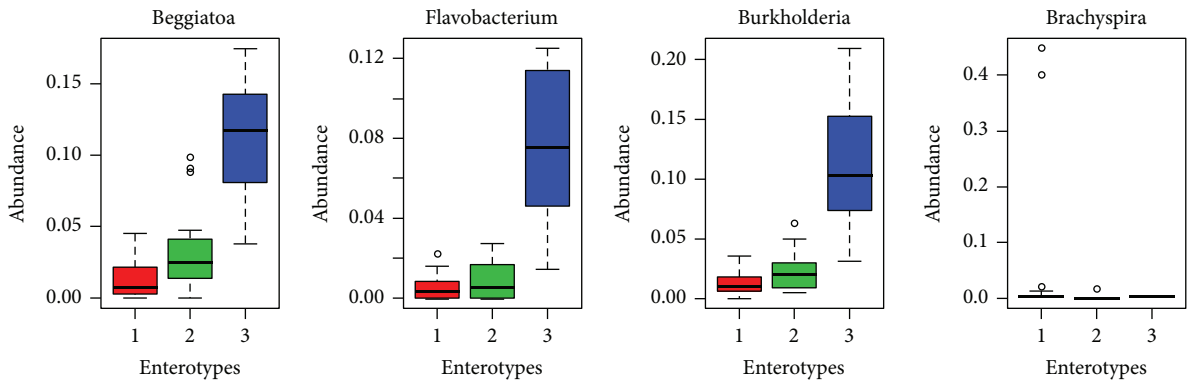

Figure 4: Principal coordinate analysis (PCA) in colorectal cancer patients.

proportions of high-grade dysplasia ($P < 0.05$) and macroscopic polyps ($P < 0.01$) than mice fed stools from controls [16]. This suggests that the fecal microbiota from patients with CRC can promote tumorigenesis in germ-free mice, connoting the CRC and microbiota relationship.

Fusobacterium, *Selenomonas*, and *Peptostreptococcus* were other genera which were present in significant quantities in our CRC patients. These butyrate-producing bacteria have been identified in colorectal cases by Hibberd et al. as well [17]. The presence of Firmicutes, Proteobacteria, and Bacteroidetes was again significantly higher in CRC patients as compared to controls, as shown in Table 3. In a study by Xu and Jiang [18], microbiota in the normal, cancer, and adenoma groups were observed. The authors found that bacteria with potential tumorigenesis, like *Bacteroides fragilis* and *Fusobacterium*, were more common in the carcinoma group, while some short-chain fatty acids (SCFA) producing microbes were more numerous in the healthy group. The commensal *Escherichia* were more abundant in the adenoma patients in their study. Authors describing the same research proposed that some bacteria, such as *Butyricicoccus*, *E. coli*, and *Fusobacterium* could possibly be used as potential biomarkers for the normal, adenoma, and cancer groups, respectively.

The majority of our study participants were obese. Obesity has been linked to colon cancer and also to diabetes. The role of microbiota in both of these conditions has been described [19]. It is possible that a sinister relationship between the two exists, and bearing in mind the global epidemics of obesity and diabetes, it may be prudent to mention that to mitigate the consequences of

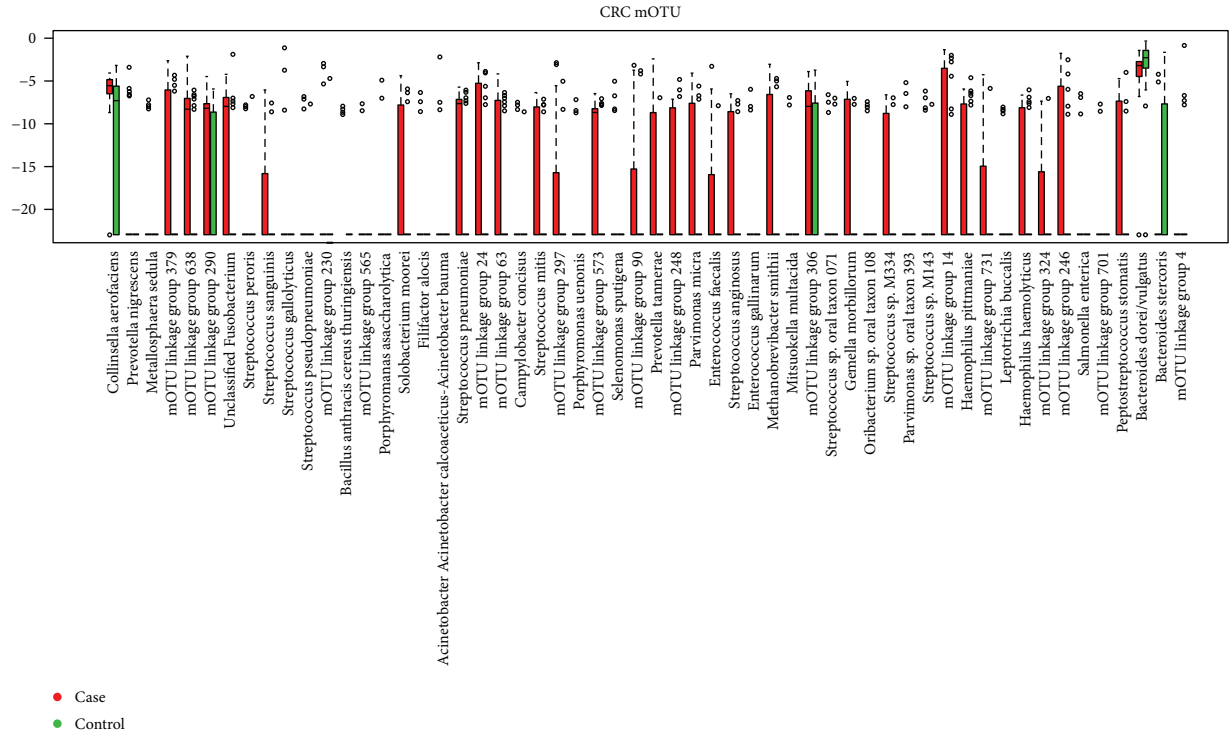

Figure 5: Control versus colorectal cancer case marker in mOTU.

colon cancer, both these modifiable factors should be addressed quite aggressively.

Genetic differences in intestinal microbiota in CRC patients were demonstrated by Goyal et al. [20], and the results of the current study identified changes in 11 genera in this sample of Saudi Arabian CRC patients. The limitation of our study is that the sample size was small but it nevertheless provides insights into the possible involvement of these gut microbes in CRC patients in this part of the globe.

The other side of the coin is whether knowledge of the potential role of microbiota in triggering CRC could suggest some protective interventions in colorectal carcinogenesis in the future. To address this issue, Hibberd et al. [17] observed how probiotic LGG exerted its beneficial effects and decreased the rate of CRC development. This probiotic intervention targeting microbiota could be used in humans in conjugation with other dietary supplements or drugs as part of prevention strategies for early-stage colon cancer, after further clinical validations.

To conclude, it is well known that CRC is one of the most treatable cancers, with a 5-year survival rate of approximately 64% [21]. These insights into the relationship between the microbiome, host genotype, and inflammation could suggest strategies for early diagnosis, preventive measures, and curative therapies for CRC. Further, it is anticipated that the study of microbiome dysbiosis may facilitate clinical application in CRC patient care. Hence, this study may be seen as a potential reference in this field when diagnostic tests for the early diagnosis of CRC, based on the analysis of gut microbiota, are finally discovered.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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