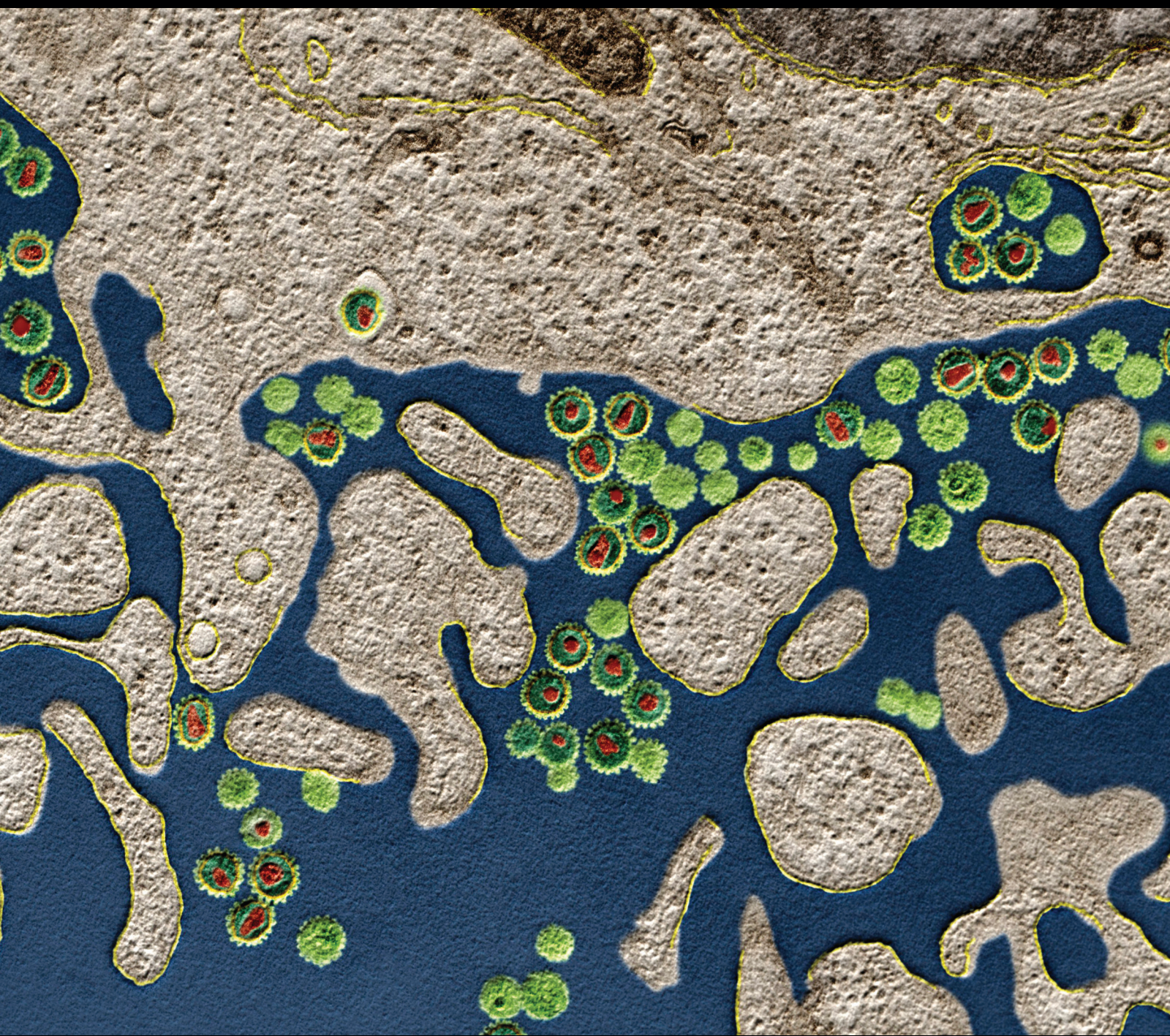
Journal of Immunology Research

Genetic Factors of Autoimmune Diseases

Guest Editors: Fulvia Ceccarelli, Carlo Perricone, and Nancy Agmon-Levin



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Editorial Board

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 3476023, 2 pages
http://dx.doi.org/10.1155/2016/3476023

Editorial

Genetic Factors of Autoimmune Diseases

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Received 21 December 2015; Accepted 24 December 2015

The multifactorial pathogenesis of autoimmune disease has been widely confirmed; indeed, several evidences underline the interaction between genetic and environmental factors in determining the development of autoimmunity [1]. The higher concordance ratio between monozygotic twins compared to dizygotic twins or other siblings confirmed the role of genetic factors in the pathogenesis of many autoimmune diseases. More recently, genome wide association studies have allowed the identification of several genetic loci associated not only with disease susceptibility, but also with specific clinical manifestations or outcomes [1]. The proteins encoded by genes associated with autoimmune diseases are involved in several inflammatory mechanisms, such as antigen presentation, type I interferon, Toll-like receptor and NF-κB signaling, B-cell and T-cell function, apoptosis, and clearance of cellular debris and immune complexes [2]. Genetic variants could induce proteins modifications, in terms of production rate and function, with possible changes in the related processes. Moreover, different autoimmune diseases are linked with the same genetic modifications, suggesting a shared genetic pathway to loss of tolerance and induction of autoimmunity [1, 2].

The present special issue includes 2 reviews and 12 research articles, focusing on aspects related to genetic factors in determining autoimmune diseases susceptibility and phenotype. Interestingly, in addition to classical autoimmune diseases, such as Systemic Lupus Erythematosus (SLE) and Sjögren's Syndrome (SjS), others have been investigated, making this special issue even more intriguing. Looking at the broad spectrum of genetics and autoimmunity, the role of HLA-DRB1 alleles has been evaluated in a large cohort of patients affected by different autoimmune diseases, identifying associations between specific alleles and different diseases and the HLA-DRB13 underrepresentation in all diseases evaluated [e.g., SLE, Psoriasis (PS), Psoriatic Arthritis (PsA), Rheumatoid Arthritis (RA), Systemic Sclerosis (SSc), Multiple Sclerosis (MS), and Myasthenia Gravis (MG)]. A very recent paper has confirmed such role of the HLA-DRB1*13 showing that some alleles are associated with protection from ACPA-positive RA, but not with significant protection from ACPA in individuals without RA. These data indicate that HLA-DRB1*13 mainly affects the onset of ACPA-positive RA in ACPA positive non-RA individuals [3].

Moreover, the special issue includes an interesting evaluation of familial aggregation of first-degree relatives as well as segregation analysis in families presenting with autoimmune diseases. Polyautoimmunity and multiple autoimmune syndrome seem to be dependent traits, while gender, age, and age of onset are interrelated factors that also influence autoimmunity.

Five studies addressed the role of genetic factors in SLE susceptibility and phenotypes. In particular, the association between the polymorphisms of the gamma-aminobutyric acid receptor subunit pi (GABRP) gene, neurological diseases, and SLE susceptibility has been investigated, revealing significant differences in terms of genotype frequencies (rs929763, rs732157, and rs3805455) among SLE patients compared with the control group. The first evidence for the role of cyclic AMP-responsive element modulator α (CREMα) polymorphisms in SLE susceptibility has been suggested in this issue. CREM proteins are members of the leucine zipper

protein superfamily of nuclear transcription factors and act as regulators of cAMP-mediated signal transduction. Moving from the evidence demonstrating CREMα overexpression in T cells from SLE patients, specific CREMα SNPs (rs2295415, rs1057108) seem to be associated with SLE susceptibility.

Genetic factors are associated not only with SLE susceptibility, but also with specific disease phenotypes: data published so far deriving from the analysis of small cohorts, which did not enable conclusive results. Remarkably, the majority of studies published so far on this linkage focused on the influence of genetic factors in determining renal manifestations. In this special issue, a relationship between ATG5 SNPs and lupus nephritis has been suggested, by using new systemic genetics approach.

Moreover, the expansion of $CD25^{high}$ $FoxP3^{high}$ B regulatory cells in SLE patients is an intriguing topic. This cell subset, characterized by high expression of IL-10, was found to be increased in SLE patients and in correlation with disease activity. This result suggests that these cells expansion could represent the attempt of the regulatory immune responses to maintain self-tolerance and to suppress SLE disease activity. Other systemic and organ specific autoimmune diseases have also been linked to genetic factors. In SjS and SjS-related lymphomagenesis, Sjögren's disease was linked with genetic variants in the major histocompatibility complex (MHC) locus. Moreover, genetic variants outside the MHC locus, such as those involving genes of the type I interferon pathway, NF-κB signaling, B-cell and T-cell function, and methylation processes, have also been described.

Two further studies evaluated pediatric autoimmune disease, such as juvenile idiopathic arthritis (JIA) and autoimmune hepatitis (AIH). An association between poor prognosis in JIA patients and the TRAF1/C5 gene locus was suggested, although larger studies are required to confirm this result. Furthermore, the role of IL-13, IL-4RA, and HLA-DRB1 polymorphisms in AIH type I has been evaluated, identifying an association with specific genetic variants.

In another liver disease, Primary Sclerosing Cholangitis (PSC), the expression of sulfotransferase 2A1 (SULT2A1) enzyme has been estimated, founding a modification of SULT2A1 expression, probably related to the impaired hepatoprotection. Moreover, miRNA analysis suggested a role for miR-378a-5p in the SULT2A1 expression.

A large number of SNPs in genes previously associated with skin autoimmune disease plaque psoriasis (PS) have been investigated in a large population. The distinction between type I (early-onset, <40 years) and type II (late-onset, ≥40 years) PS reveals an association between early-onset and polymorphisms of the CLMN, FBXL19, CCL4L, C17orf51, TYK2, IL-13, SLC22A4, CDKAL1, and HLA-B/MICA genes. Moreover, a significant association between age at onset and gene variants of PSORS6, TNF-α, FCGR2A, TNFR1, CD226, HLA-C, TNFAIP3, and CCHCR1 was also identified. These data suggested the role of genetic factors in determining the age of onset in patients with plaque psoriasis.

Another organ specific autoimmune disease, uveitis, has been investigated for the interaction between disease and IL-6 gene polymorphism and HLA-B27. A significantly higher frequency of the minor allele for rs1800795 in patients with intermediate uveitis compared to controls has been identified, suggesting a role of IL-6 as therapeutic target in patients with HLA-B27 associated uveitis. Last but not least, a study on 235 hemochromatosis probands was performed, demonstrating the presence of autoimmune conditions in high percentage of subjects, with Hashimoto's thyroiditis being most prevalent. Notably, the risk of autoimmunity in this scenario was not associated with any HLA haplotype.

In conclusion, the present special issue adds interesting data concerning genetic factors associated with different systemic and organ specific autoimmune diseases, evaluating the genetic impact both on disease susceptibility and on disease phenotypes. Conspicuously, there is an urgent need for more studies utilizing large cohorts that will broaden our knowledge as currently it is estimated that only about 15% of the genetic factors contributing to autoimmune disease susceptibility have been identified [4]. Moreover, besides genetic association studies, functional analyses aiming at unveiling the mechanistic role of each factor should be performed. Novel techniques, including next-generation sequencing studies, will further contribute and expand our understanding of the genetic basis of autoimmunity.

Fulvia Ceccarelli
Nancy Agmon-Levin
Carlo Perricone

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 745647, 11 pages
http://dx.doi.org/10.1155/2015/745647

Review Article

Genetic Factors in Systemic Lupus Erythematosus: Contribution to Disease Phenotype

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Received 30 July 2015; Accepted 29 November 2015

Academic Editor: Nejat K. Egilmez

Genetic factors exert an important role in determining Systemic Lupus Erythematosus (SLE) susceptibility, interplaying with environmental factors. Several genetic studies in various SLE populations have identified numerous susceptibility loci. From a clinical point of view, SLE is characterized by a great heterogeneity in terms of clinical and laboratory manifestations. As widely demonstrated, specific laboratory features are associated with clinical disease subset, with different severity degree. Similarly, in the last years, an association between specific phenotypes and genetic variants has been identified, allowing the possibility to elucidate different mechanisms and pathways accountable for disease manifestations. However, except for Lupus Nephritis (LN), no studies have been designed to identify the genetic variants associated with the development of different phenotypes. In this review, we will report data currently known about this specific association.

1. Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune disease with multifactorial etiology, in which genetic and environmental factors interplay determining disease susceptibility [1].

Starting from 1970, several genetic studies in various SLE populations have identified numerous susceptibility loci. However, the genetic variability so far identified accounts for less than half of the SLE heritability, with modest overall effect sizes (OR ~ 1.5 to 1.2) (Figure 1) [2–7]. It is well established that some specific genetic factors are not shared between all SLE patients, excluding a role in the disease susceptibility and suggesting an association with specific phenotypes (Table 1) [6, 8]. However, this discrepancy could be related to multiple mechanisms that can lead to SLE development.

As widely demonstrated, specific autoantibodies resulted in being associated with different disease-related manifestations, identifying distinctive subset in terms of morbidity and mortality and suggesting different underlying etiologies [9]. Similarly, in the last years, some studies have evaluated the relationships between SLE risk genes and disease phenotypes, in order to elucidate different mechanisms and pathways accountable for disease manifestations. However, except for Lupus Nephritis (LN), no studies have been specifically designed to evaluate the genetic risk factors associated with different manifestations. Therefore, these data could be extrapolated from studies evaluating disease susceptibility, which include a genotype-phenotype analysis.

2. Renal Involvement

Renal involvement could affect up to 60% of SLE patients, as initial manifestation or during disease course. Despite the improvement in terms of diagnostic accuracy and management, LN patients showed higher morbidity and mortality compared with those without this manifestation [10].

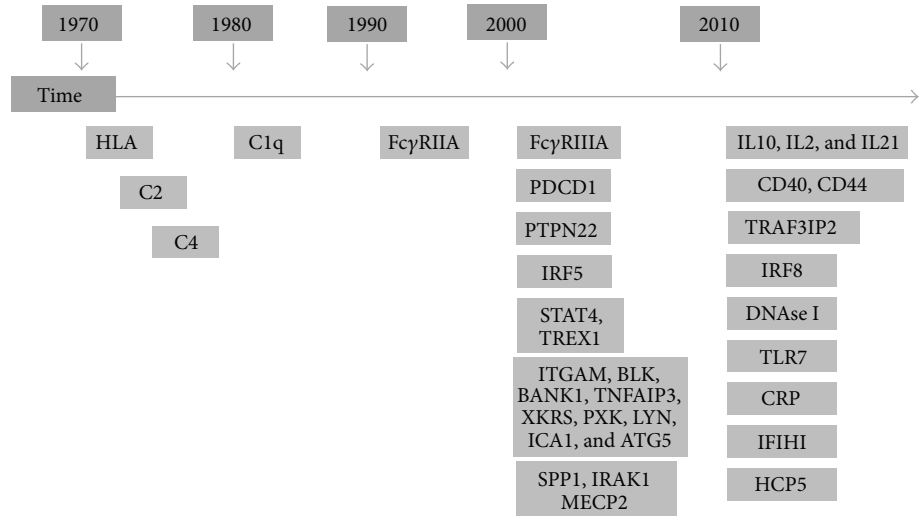

Figure 1: Schematic representation of genetic variants associated with SLE susceptibility identified from 1970.

Accordingly, the identification of markers able to identify most severe disease and to predict the end-stage renal disease (ESRD) development is a crucial topic. In particular, during the last years, numerous attempts have been made in order to identify serological and urinary biomarkers able to discriminate the different severity degree and to monitor response to treatment in LN patients, obtaining contrasting results [11]. Moreover, the use of resistive index (RI) as a severity marker in LN patients has been suggested in a recent study published by Conti et al. The authors identified a significant association between a pathologic RI (>0.7) and class IV glomerulonephritis, widely identified as the most severe [12].

In the context of biomarkers, genetic factors could have an important role in SLE patients with renal involvement in order to identify subject at risk to develop most severe and rapidly progressive forms. Moving from the genetic variants previously associated with disease susceptibility, several studies have verified the association of the same alleles with the presence of renal involvement.

The first genetic association described for SLE based on the case-control methodology was with the human leucocyte antigen (HLA) region at chromosome 6p21.3, encoding more than 200 genes, many of them with a specific immunological role. Seven HLA Class II alleles were demonstrated to be significantly associated with SLE and LN [13]. The HLA-DR2 and DR3 alleles resulted in being the most strongly associated with SLE susceptibility in African, Asian, European, and North, Central, and South American populations, even though HLA-DR3 tends to be more associated in European-derived populations [8]. In particular, the association between the disease susceptibility and highly conserved HLA-DRB1*03:01 and HLA-DRB1*15:01 haplotypes has been well established in European populations [8]. The punctual mechanism by which HLA-DR alleles determine an increased risk to develop SLE is not completely defined. The most reliable hypothesis suggests the influence of HLA-DR on the selection and enrichment of autoreactive T cells through the presentation of molecular mimics [14].

Moving from these premises, the association with renal involvement has been investigated, showing the primary role exerted by HLA-DR3 and DR-2 [15]. In particular, the results obtained from the study conducted by Taylor and colleagues in 2011 and by Bolin in 2013 showed the association between HLA-DR3 and LN (OR = 1.37 and $P < 1 \times 10^{-4}$, resp.) [16, 17]. Particularly, Bolin et al. found an association between HLA-DR3 allele and proliferative nephritis ($P < 0.001$) [17].

Numerous evidences identified an association between *signal transducer and activator of transcription 4* (STAT4) genetic variants and increased risk to develop SLE, suggesting a role of these genetic variants in influencing disease phenotype.

In 2008, Taylor and colleagues analyzed a large SLE population, obtained from four sources (UCSF Lupus Genetics Project; Autoimmune Biomarkers Collaborative Network; Multiple Autoimmune Disease Genetics Consortium; Pittsburgh Lupus Registry) in order to evaluate the association between SNP rs7574865 of STAT4 and the different SLE-related manifestations. The phenotype case-only analysis identified a significant association with the presence of severe nephritis, defined as ESRD, or histopathologic evidence of severe, progressive renal disease (OR = 1.50) [18]. Simultaneously, the study conducted by Kawasaki et al. in a Japanese population confirmed the association between this STAT4 risk allele and renal involvement. In particular, the authors identified the association with rs7574865 both in SLE patients with nephritis (OR = 1.85) and in those without (OR = 1.55), which was stronger in nephritis cases [19].

More recently, Bolin and colleagues evaluated a cohort constituted by 567 Swedish Caucasian SLE patients and 512 healthy controls to elucidate the genetic components of LN [17]. By performing a LN case/controls analysis, a significant association between the SNPs rs11889341, rs7574865,

Table 1: Genetic variants associated with disease manifestations.

Disease phenotypes	Genetic variants associated with related SNPs
Skin involvement	ITGAM rs1143679 FCGR2A rs1801274 IL-6 174G/C VDR rs1168268
Serositis	TRAF3IP2 rs33980500, rs13190932, and rs13196377 PTPN2 rs2542151
Kidney involvement	HLADR2, HLADR3 rs2187668 STAT4 rs7574865, rs11889341, rs7568275, and rs7582694 ITGAM rs1143683, rs1143679 IRF5 rs2004640, rs2079197, and rs10488631 IRF7 rs4963128 TNFS4 rs2205960 DNAse I Q222R
Neurologic disorder	TREX1 rs922075, rs6776700, rs6442123, rs2242150, and rs11797
Joint involvement	ITGAM rs1143679 FGCR2A, FCGR3A VDR rs3890733 Mir146a rs2910164
Hematological features	IL-21 rs907715 STK17A haplotype TAGTC
Immunologic disorders	Anti-dsDNA HLADR2, HLADR3 rs2187668 STAT4 rs7582694, rs7574865 ITGAM rs1143679, rs9888739 IRF5 rsrs10488631 SSA/SSB ITGAM rs1143679 IRF7 rs4963128 HCP5 rs3099855 HLADR3 rs2187668 RNP ITGAM rs1143679 Sm ITGAM rs7574865 aCL HCP5 rs3099844 C3 reduction Mir146a rs2910164 C4 reduction TRAF3IP2 rs33980500

rs7568275, and rs7582694 of STAT4 gene and the development of LN ($r^2 = 0.98$) was identified. In particular, the rs7582694 allele resulted in being associated with the presence of a proliferative nephritis (OR = 2.27) and with the development of a severe renal insufficiency (defined as a GRF < 30 mL/min/1.73 m^2 at follow-up) (OR = 3.61) [17]. This association was not always confirmed. In the study conducted by Li and colleagues in 2011 on a Northern Han Chinese SLE population, the SNP rs7574865 of STAT4 did not show any correlation with clinical manifestations [20].

Several hypothesis have been suggested to understand the mechanisms by which STAT4 could contribute to LN development. Interleukin-12 (IL-12), the main STAT4 activating cytokine, is able to induce the Th1 and Th17 differentiation with consequent production of IFN-γ and IL-17. These specific pathways seem to be crucial for the LN pathogenic mechanism: in particular, IL-17 could exert a direct role, as demonstrated by the identification of Th17 cells in kidney tissue and by the association between high IL-17 levels and less favorable outcome [21]. Moreover, SLE patients carrying the STAT4 risk allele rs7574865 showed an increased sensitivity to IFN-α signaling, as demonstrated by the overexpression of IFN-α regulated gene. Among these, TNFSF13B codifies the B lymphocyte stimulator (BLyS), which is able to promote B cell differentiation and autoantibody production [22].

The influence of ITGAM genetic variants on SLE susceptibility has been demonstrated in populations with different ethnicity: in particular, convincing data derived from European ancestry, but also from Hispanic, African-Americans, Mexicans, and Colombians cohorts [23]. Moving from these results, the association with specific clinical manifestations has been investigated. In 2009, Yang and colleagues identified a significant association between renal involvement and the ITGAM risk alleles rs1143683 and rs1143679 (OR = 3.35, OR = 2.05, resp.) in a Hong Kong SLE cohort [24]. Moreover, this association was confirmed in an analysis conducted on Finnish and Swedish population of patients affected by SLE. The authors observed increased risk in SLE patients with renal involvement, with an OR = 2.49 for the rs1143679 SNP [25].

The study conducted by Kim-Howard and colleagues in 2010 on a very large population, constituted by 2366 SLE patients and 2931 unaffected controls with European ancestry, confirmed the link between the genetic variant rs1143679 of ITGAM and renal disorders as defined by the American College of Rheumatology (ACR) criteria (OR = 1.39) [26]. To better assess the magnitude of this association, a comparison between patients with the specific ACR-criteria renal manifestations and healthy controls was performed. This statistical approach allowed the identification of a strong effect concerning the association with renal criteria (OR = 2.15) [26].

More recently, in 2011 Sanchez et al. confirmed this association (OR = 1.25) by evaluating a population constituted by 4001 European-derived, 1547 Hispanic, 1590 African-American, and 1191 Asian patients. This association seems to be driven prevalently by the European-derived cohort, as demonstrated by a higher OR in this specific subset (OR = 1.39) and by the lack of a significant association with African-American or Asian individuals [27].

ITGAM encodes the CD11b chain of the Mac-1 (alphaM-beta2; CD11b/CD18; complement receptor-3) integrin, a surface receptor protein implicated in the interaction of monocytes, macrophages, and granulocytes. The genetic variants of this molecule, resulting in amino acid substitution in the extracellular portion, could determine a dysfunctional integrin, not able to mediate cell adhesion to integrin ligands and phagocytosis. Moreover, this defective integrin does not

seem to be able to restrict the production of inflammatory cytokines in macrophages [28].

Under physiological conditions, ITGAM is expressed by endothelial cells of glomerular and peritubular capillaries of Bowman's capsule. It has been suggested that an increased expression of a defective molecule, due to the presence of a genetic variant, could be associated with a loss of clearance of glomerular deposits, with inflammatory process development [26]. Similarly, defective handling of immune complexes could be the mechanism explaining the association between genetic variants of FCGR3A and kidney involvement in SLE patients. A meta-analysis conducted by Karassa and colleagues in 2003 evaluated the study examining the association of the FCGR3A V/F158 polymorphism and LN, published until August 2002 [29]. Data deriving from the analysis on 16 studies demonstrated a significant overrepresentation of the low-binding F158 allele in patients with renal disease compared with those without ($P = 0.003$). Moreover, the presence of this allele seems to confer a 1.2-fold greater risk for renal disease development, irrespective of the ethnicity [29]. More recently, the PROFILE cohort, constituted by 1008 SLE patients, with renal involvement in 43.4% of the cases (438 patients), was evaluated in order to identify the association with FCGR3A polymorphism. The authors identified an overrepresentation of FCGR3A*GG in SLE patient developing ESRD (21.9%) compared with those who did not develop it (7.5%) ($P = 0.0175$) [30]. Interestingly, the evaluation of FCGR3A variants demonstrated different genetic association for the global lupus phenotype and for the renal involvement (FCGR3A*T and FCGR3A*GG, resp.). This observation confirms the hypothesis of different genetic background for susceptibility and disease phenotype, leading to different pathogenic mechanisms associated with the corresponding molecule [30].

FCGR plays a pivotal role in removing antigen-antibody complexes at the tissue and organ level. Allelic variants could alter this function, causing an inflammatory response with damage development. In particular, as widely demonstrated, a homozygosity condition for this FCGR3A SNP could lead to impaired handling of immune complexes, causing a proinflammatory status [29, 30]. This could justify the impact of genetic variants of FCGR3A in the determination of a specific phenotype, such as renal involvement.

In the last 20 years, the role of Interferon (IFN) signature in the SLE pathogenesis has been recognized, as demonstrated by the dysregulation in the expression of genes in the IFN pathway in more than half of SLE patients [31]. IFN pathway is involved in several pathologic mechanisms, involving Th1 and B cells activation and survival. Moreover, IFN acts as a bridge between innate and adaptive immune systems. Interferon Regulatory Factors (IRF) ensure the regulation of this complex pathway, by acting on signaling and immune cell development [31]. Genetic variants of IRF5, IRF7, and IRF8 genes have been associated with SLE susceptibility (OR = 1.88, OR = 0.78, and OR = 1.17, resp.) since they associated with increased levels of protein expression [32]. Starting from these evidences, the association with renal involvement in SLE patients has been investigated. The study conducted on 190 LN patients and 182 healthy Chinese blood donors demonstrated a significantly higher frequency of the T allele of IRF5 rs2004640 SNP in LN patients (OR = 1.60) [33].

The abovementioned study conducted by Bolin and colleagues in 2013 identified a strong association between LN and two nearly perfectly linked SNPs in IRF5 (rs2070197 and rs10488631, r^2 1.0). In particular, the risk allele C of rs10488631 was associated with proliferative nephritis (OR = 2.61) and severe renal insufficiency (OR = 3.03) [17]. More recently, an association between IRF7 rs4963128 and LN (OR = 2.69) has been identified in the study conducted by Li and colleagues in 2011 in a Northern Han Chinese population [34].

In 2011, for the first time, Sanchez and colleagues suggested a new interesting genetic factor related to renal disorders in SLE patients by identifying a significant association with rs2205960 TNFSF4 risk allele (OR = 1.14) [27]. TNFSF4, also called OX40L, is a member of the TNF superfamily, expressed prevalently on antigen-presenting cells; activated T cells express the receptor of this molecule (TNFSFR4 or OX40) [35]. The expression of TNFSF4 at the epithelial level of the glomerular capillary has been demonstrated in LN patients [36]. More recently, Zhou and colleagues demonstrated a modification of cytokine production in PBMC in LN patients after treatment with anti-CD134 monoclonal antibody [37]. Finally, significantly higher TNFSF4 serum levels have been demonstrated in SLE patients with renal involvement, compared with patients without nephritis, suggesting the role of this molecule as a marker. Moreover, the increased expression on CD4 positive T cells seems to be associated with LN and disease activity [38]. Taken together, these evidences could justify the link between a genetic variant in the TNFSF4 gene and renal involvement.

Several other genetic variants have been associated with kidney manifestations in SLE patients. Panneer and colleagues suggested the role of polymorphism in the gene codifying the DNAse I, an endonuclease involved in the cleavage and clearance of chromatin during apoptotic processes [39]. The reduction of the DNAse I function, related to the genetic modification, could alter this cleavage and the clearance of immune-complexes and NETs, resulting in the persistence of apoptotic debris [40]. By evaluating 300 South Indian Tamil SLE patients, the authors identified a significantly higher frequency of heterozygous genotype of Q222R polymorphism in patients with nephritis than in those without (67% versus 53%, OR = 1.93) [39]. Some interesting data concerning the association between LN development and polymorphism on the gene codifying C1q have been recently published [41, 42]. However, due to the small cohorts evaluated in these studies, their results should be confirmed in large populations.

3. Neuropsychiatric Manifestations

Neuropsychiatric SLE is a major disease manifestation, characterized by a wide heterogeneity in terms of clinical features, degrees of morbidity, and severity between patients [43]. A percentage of SLE patients ranging from 14 to 75% may refer to neurological symptoms: this wide variability is probably related to the great heterogeneity of this disease manifestation. Despite the relevance of this involvement,

studies focusing on genetic variants specifically associated with NPSLE have been rarely conducted. Nonetheless, Koga and colleagues in 2011 evaluated 282 Japanese SLE patients and 222 healthy controls in order to assess the cumulative number of risk alleles associated with SNPs of HLA-DRB1, IRF5, STAT4, BLK, TNFAIP3, TNIP1, FCGR2B, and TNFSF13 genes. SLE patients registered a significantly higher number of risk alleles compared with controls (8.07 ± 1.60 *versus* 7.02 ± 1.64, $P = 1.63 \times 10^{-12}$). Interestingly, when considering SLE patients carrying more than 10 risk alleles, the proportion of patients with neurological involvement was significantly higher compared with subjects with a number of risk alleles lower than 10 (OR = 2.30). This result could suggest that a higher number of risk alleles could determine most severe disease manifestations [44].

Genetic variants in TREX1 gene, codifying a threeprime repair exonuclease 1 (also known as DNAse III), have been considered a good candidate for NPSLE. de Vries and colleagues scanned genomic DNA of 60 NPSLE patients for exonic TREX1 mutations using direct sequencing. This study identified a novel heterozygous p.Arg128His mutation in one NPSLE patient, admitted to the hospital because of lethargy and progressive migraine-like headache [45]. The authors suggested that the p.Arg128His mutation is responsible for neurological manifestations at the light of the absence of this mutation in 400 control chromosomes and in 1712 healthy individuals, previously screened by Lee-Kirsch et al. [46]. More recently, this association has been confirmed in the study conducted by Namjou and colleagues in 2011. By evaluating the European population enrolled in the analysis, the authors identified a significant association between the presence of neurological manifestations (as defined by ACR criteria), especially seizure, and specific variants in TREX1 gene. In particular, the rs922075 (OR = 1.644); rs6776700 (OR = 1.689); rs6442123 (OR = 1.747); rs2242150 (OR = 1.638); rs11797 (OR = 1.714) SNPs resulted in being significantly associated [47].

4. Joint Involvement

Joint involvement is a frequent manifestation in patients with SLE and could affect up to 90% of patients. A wide heterogeneity, varying from arthralgia to erosive arthritis similar to rheumatoid arthritis, characterizes this manifestation [48]. Nevertheless, the number of reports is relatively scarce. Concerning the identification of specific genetic variants, few studies have evaluated the association with joint involvement.

ITGAM gene risk variants have been associated with arthritis in SLE patients. The study conducted by Warchoł et al. in 2011 in a Polish SLE population demonstrated an association between the rs1143679 genetic variant and occurrence of arthritis (OR = 3.486) [49].

A strong association with arthritis and Vitamin D Receptor (VDR) polymorphism was identified in the study conducted by de Azevêdo Silva and colleagues in 2013 [50]. Through the Vitamin D Receptor (VDR), Vitamin D exerts an immune-modulatory effect. In particular, it intervenes in downregulation of Th1 immune response, modulation of dendritic cells differentiation, depressing activated B cell proliferation, upregulation of regulatory T cells, and preserving immune response [50]. A number of evidences showed that patients with SLE often present reduced levels of Vitamin D suggesting an involvement of this molecule in disease pathogenesis [51]. There is still a debate concerning the precise role of VDR in SLE [52]. The study conducted by de Azevêdo Silva in 2013 did not identify any association between VDR polymorphism and SLE susceptibility. Conversely, the T/T genotype (rs3890733) resulted in being significantly associated with the presence of joint involvement (OR = 17.05). The authors underlined that this association should be interpreted with caution because the frequencies observed for this VDR polymorphism were not in Hardy-Weinberg equilibrium [50].

Other associations between genetic variants and joint involvement have been suggested: some data identified an association with C4 and ACP5 genetic variants, but no replication studies are available [53, 54]. Moreover, Ciccacci and colleagues identified an association between joint involvement and rs2910164 of mir146a gene (OR = 1.93) [55].

The association between arthritis and the FCGR2A and FCGR3A low copy number genotype has been identified in a cohort of Taiwan SLE patients [56, 57]. In particular, in the most recent study, the FCGR3A low copy number genotype was significantly enriched in SLE patients with arthritis ($P = 0.001$; OR = 1.56) [57].

Finally, the study conducted by Fonseca et al. in 2013 identified an association between arthritis and the SNP rs15866 of STK17A gene (OR = 2.92), encoding serine/threonine-protein kinase 17A [58]. The mechanism explaining this association is not clarified and replication studies are needed to confirm these results.

5. Skin Manifestations

Skin involvement represents a frequent manifestation in SLE patients (up to 75%), characterized by a great heterogeneity, including acute and chronic phenotypes. Some genetic variants have been associated with different skin manifestations in SLE cohorts.

ITGAM genetic polymorphisms are to date the most frequently associated variants with skin involvement. In 2010, Kim-Howard et al. have identified an association between malar rash and the polymorphism rs1143679 of ITGAM (OR = 1.27) [26]. Moreover, the presence of discoid rash resulted in being associated with ITGAM rs1143679 (OR = 1.20) in the study conducted by Sanchez et al. in 2011 [27].

Järvinen and colleagues conducted an analysis specifically designed to address the role of ITGAM genetic variants in a cohort of Finnish and Swedish patients with discoid LE, without signs of systemic disease. The analysis demonstrated a strong association between the allele rs1143679 and DLE (OR = 3.2). The authors identified a significant association also in SLE patients with discoid rash (OR = 3.76). Moreover, other variants in ITGAM resulted in being associated with these manifestations, but the authors hypothesized that the strong linkage disequilibrium with rs1143679 could explain this result [25].

The link between ITGAM and photosensitivity, frequently identified in patients with discoid LE, could explain this association. Ultraviolet- (UV-) B irradiation determines the activation of several proinflammatory events at the skin level, involving prevalently macrophages ITGAM-expressing and dendritic cells. Genetic-determined modification in the function of ITGAM could modify the processes regulating the dendritic cell differentiation, inducing inflammatory reactions in discoid LE patients [25]. On the other hand, the absence of CD11b seems to enhance the differentiation of naive T cells to IL-17 producing Th17 cells, determining the increase of IL-17 serum levels, identified in discoid LE and SLE patients with skin involvement [59].

Moreover, genetic variants of FCGR2A seem to be associated with skin manifestations. In particular, Sanchez and colleagues identified an association between malar rash and FCGR2A rs1801274 (OR = 1.11) [27].

The abovementioned study conducted by de Azevêdo Silva et al. in 2013 identified an association between the SNP rs11168268 of VDR and cutaneous alterations in a cohort of Brazilian SLE patients [50]. Photosensitivity, one of the most common cutaneous alterations described in SLE patients derives from the exposure to UV light, causing a macular or erythematous rash. After UV exposure, keratinocytes begin apoptotic process due to DNA damage with release of nuclear material. A defective clearance of apoptotic body could trigger an immune response. Vitamin D has proved to be able to reduce the UV-induced DNA damage and suppress cutaneous immunity, playing an important role in the maintenance of cell integrity after UV light exposure [60]. The presence of genetic variants in VDR, expressed in the skin epithelial cells, could modify this Vitamin D ability, promoting cutaneous alterations in SLE patients [61].

A recent meta-analysis identified a significant association between the IL-6-174 G/C polymorphism and discoid skin lesions by the evaluation of 15 studies (OR = 2.271). These results support the role of IL-6 in the pathogenesis discoid skin lesions [62].

6. Serositis

Few data are available in the literature about the genetic risk for the serositis development. The study published by Perricone et al. in 2013 identified an interesting correlation between the TRAF3IP2 SNPs and the development of pericarditis. The authors identified a significant association with the three TRAF3IP2 SNPs evaluated (rs33980500: OR = 2.59; rs13190932: OR = 2.38; rs13196377: OR = 2.44). Moreover, the authors analyzed the contribution of SLE antibody to the development of this specific manifestation, showing a significant association between the risk to develop pericarditis and anti-La/SSB positivity (OR = 2.65). A binary logistic regression analysis demonstrated that both TRAF3IP2 rs33980500 and anti-La/SSB could be independently associated with the development of pericarditis ($P = 0.006$ and $P = 0.032$, resp.) [63]. In this study, for the first time, the role of TRAF3IP2 genetic variants on SLE susceptibility has been ascertained. Interestingly, TRAF3IP2 polymorphism resulted also in being associated with a specific disease manifestation. TRAF3IP2 codifies the molecule Act1, which from one side is involved in the IL-17 pathways, but it is also a negative regulator of the CD40-mediated signaling pathway [64, 65].

The study conducted by Ciccacci and colleagues in 2014 identified a new association between the occurrence of pericarditis and the genetic variant rs2542151 of PTPN2 gene (OR = 2.49) [55]. PTPN2 codifies the enzyme tyrosine-protein phosphatase nonreceptor type 2, a member of the protein tyrosine kinases (PTP) superfamily. PTPN2 genetic variants have been previously associated with susceptibility to both Crohn's disease and ulcerative colitis and with an earlier onset of type 1 diabetes [66, 67]. The abovementioned study by Ciccacci and colleagues analyzed for the first time the role of PTPN2 genetic variants in the SLE susceptibility, without identifying significant differences between patients and healthy controls. Conversely, the SNP rs2542151 of PTPN2 resulted in being associated with serositis, and specifically with pericarditis [55]. The relevance of this association should be clarified by larger studies.

7. Hematological Manifestations

Similarly to the other SLE-related manifestations, few studies focusing on the association between genetic variants and hematological features have been conducted to date. The extrapolation from genotype-phenotype studies identified some associations. Among these, Sanchez and colleagues in 2011 identified an association between hematological features and IL-21 rs907715 (OR = 1.13). When the different ACR hematological criteria were analyzed, an association with leucopenia was confirmed (OR = 1.14). IL-21, primarily produced by activated CD4+ T cells, is involved in differentiation and functional activity of T and B cells [68–70]. This evidence could justify this association, by hypothesizing that a genetic variant of IL-21 could be related to a modification of this activity on B and T cells, influencing disease phenotype.

More recently, Fonseca et al. in 2013 described a significant association between hematological features and haplotype TAGTC of STK17A gene (OR = 0.03). The patients stratification according to ethnicity and gender suggested a protective role of this haplotype on hematological manifestations development (OR 0.37) [58]. Similarly to the association with arthritis, the mechanism explaining this association is not identified and other replication studies are needed to confirm these results.

8. Immunological Abnormalities

The production of a wide range of autoantibodies, resulting from polyclonal B cells activation, impaired apoptotic pathways, or idiotypic network dysregulation, characterizes SLE [1, 71]. Among these, the anti-double-stranded DNA antibodies (anti-dsDNA) are considered the most specific marker for SLE, due to their high frequency (ranging from 70% to 98%) and sensitivity and specificity (57.3% and 97.4%, resp.) [72, 73].

Several evidences suggested a role of genetic factors in autoantibodies determination [74]. The same genetic variants, previously described as associated with renal involvement, have been investigated in order to identify a link with anti-dsDNA production. Since 1998, Podrebarac and colleagues described the association between anti-dsDNA production and the presence of HLA-DRB1*1501 (DR2) allele [75]. More recently, the association between HLA-DR2 and DR3 with the presence of anti-dsDNA has been confirmed by several analysis [16, 76].

Starting from 2008, the association between the STAT4-risk allele of the SNP rs7582694 and positivity for anti-dsDNA has been identified by different studies [16, 18, 77]. Finally, ITGAM polymorphism has been also associated with the presence of anti-dsDNA. In particular, the study conducted by Kim-Howard and colleagues in 2010 identified an association with rs1143679 (OR 1.65) in a case-only analysis performed by comparing SLE patients positive and negative for anti-dsDNA [26].

Four years ago, Chung and colleagues conducted the first genome wide study focused to identify genetic factors associated with anti-dsDNA autoantibody production, by analyzing 1278 SLE cases and 3334 healthy controls of European descent [78]. Genetic variants STAT4 (rs7574865), IRF5 (rs10488631), ITGAM (rs9888739), and MHC (HLA-DR3, rs2187668) resulted in being strongly associated with anti-dsDNA positivity (OR = 1.77, OR = 1.92, OR = 1.80, and OR = 2.23, resp.). Moreover, the authors assessed the relationship between the anti-dsDNA autoantibody production and the cumulative genetic risk, calculated by counting the total number of risk alleles identified in a single subject. The mean SLE genetic risk was higher in SLE patients positive for anti-dsDNA (15.5 ± 3.1) compared with anti-dsDNA negative patients (14.5 ± 3.0) and healthy controls (13.1 ± 2.8), even though this difference was not significant [78]. The results of this study suggest that some genetic variants are more strongly associated with anti-dsDNA autoantibody production than with SLE susceptibility, and they could be described as "autoantibody propensity genes" [78].

Even though the majority of the studies have focused on the anti-dsDNA antibodies, some evidences demonstrated an association between genetic variants and production of other autoantibodies in patients affected by SLE.

Järvinen and colleagues in 2010 identified an association between the polymorphism rs1143679 in ITGAM gene and the presence of Ro/SSA autoantibodies in the Finnish (OR = 2.65) and Swedish (OR 1.62) populations [25]. The involvement of both Ro-autoantibodies and the ITGAM protein product in the same biological pathways of apoptosis and phagocytosis could explain this association, which remains mostly unknown [25].

The study conducted by Li and colleagues in 2011 suggested a new association between IRF7 rs4963128 polymorphism and anti-SSA/SSB (OR = 0.61) [20]. Moreover, the study conducted by Ciccacci and colleagues in 2014 identified for the first time an association between anti-Ro/SSA and HCP5 rs3099855 polymorphism (OR = 2.28) [55]. This SNP has been previously associated not only with Steven Johnson syndrome and toxic epidermal necrolysis susceptibility, but also with primary sclerosing cholangitis, another autoimmune condition [79, 80]. More interestingly, as demonstrated by a genome-wide association study, the same polymorphism resulted in being associated with cardiac manifestations of SLE, a clinical condition frequently associated with the presence of anti-Ro/SSA antibodies [81]. These data suggest a pathological link between anti-Ro/SSA antibodies and this HCP5 polymorphism, requiring further studies to clarify the specific underlying mechanisms. Moreover, both anti-Ro/SSA and anti-La/SSB autoantibodies resulted in being significantly associated with HLA-DRB1*03:01 (OR = 1.60, OR = 2.57, resp.), as demonstrated by the largest SLE subphenotype genetic association study conducted so far [82].

A recent study published by Niewold et al. in 2012 evaluated the association between IFR5 haplotype and different SLE-related manifestations. Interestingly, the authors identified a strong and strikingly distinct association between different autoantibodies and different IRF5 haplotypes. In particular, TACA haplotype was associated with anti-dsDNA and anti-Ro/SSA (OR = 1.5, OR = 1.51, resp.), TATA haplotype with anti-dsDNA (OR = 1.68), and TCTA haplotype with anti-La/SSB (OR = 3.51) [83]. These results suggest the possible role of IRF5 genotype to predispose the antibodies formation: IRF5 haplotypes could influence susceptibility to form particular antibodies. Immune complexes containing these antibodies are internalized into cells, and the nucleic acid component could trigger endosomal TLR7 and TLR9. The presence of IRF5 SLE-risk variants could increase IFN-α production in the setting of different antibodies, resulting in high serum IFN-α and subsequent SLE risk [83].

The studies published until now have suggested other associations between specific autoantibodies and genetic variants, among which are the associations between anti-RNP and rs1143679 of ITGAM (OR = 1.89), anti-RNP and rs56203834 of TREX1 in European populations (OR = 5.2), anti-Sm and rs7574865 of ITGAM (OR = 0.65), and anti-cardiolipin and rs3099844 of HCP5 (OR = 0.34) [20, 26, 47, 55]. However, all these associations should be confirmed in larger populations and the mechanisms explaining must be identified.

The reduction of C3 and/or C4 serum levels represents a frequent manifestation in patients affected by SLE and could correlate with disease activity [84]. A strong association was well established between homozygous hereditary deficiency of each of the early proteins of the classical pathway of complement activation and SLE development. The deficiency of the C1 complex proteins and of total C4 is recognized as the most prevalent and most severe disease. Indeed, more than 75% of all individuals with deficiency of one of these proteins develop SLE [84]. Conversely, the deficiency of C2 protein seems to be associated with lower prevalence of disease (10%), while C3 deficiency is rarely associated with SLE development, probably due to the rarity of homozygous C3 deficiency [84]. Even though the association between complement proteins deficiency and SLE development has been largely clarified, very few data are available concerning genetic variants associated with C3 and C4 levels reduction, extrapolated by studies not focusing on this specific aspect.

A correlation between C4 reduction and the SNP rs33980500 of TRAF3IP2 has been identified in the study conducted by Perricone et al. (OR = 1.96) [63]. Conversely, the reduction of C3 serum level was associated with the genetic variant mir146a rs2910164 (OR = 1.91) [55].

9. Age at Disease Diagnosis

The evaluation of the studies published so far identified interesting data concerning the influence of STAT4 genetic variants on age at diagnosis. The SNP rs7574865 of STAT4 resulted in being associated with age at diagnosis lower than 30 years (OR = 1.22) [16, 18]. The frequency of the same genetic variant resulted in being slightly higher in SLE Japanese patients with an age of onset lower than 20 years as compared with patients with age ≥ 20 years, although this difference was not statistically significant [19].

Moreover, rs2233945 of PSORS1C1 resulted in being associated with age at diagnosis. Ciccacci and colleagues observed that patients carrying the variant allele present a lower mean age at disease onset compared with those not carrying the variant (28.6 ± 11.57 years *versus* 32.2 ± 11.46 years, $P = 0.042$) [55].

10. Chronic Damage

The increase of survival of SLE patients determined the accrual of cumulative organ damage: adverse events of treatment, disease activity, and comorbidities seem to be the major risk factors. The prevention of damage development is a critical issue in the management of SLE patients, as underlined by the recent treat-to-target recommendations [85]. Consequently, the identification of specific biomarkers, able to identify SLE patients with a major risk to develop chronic damage, is an attractive topic. Among the different biomarkers, genetic variants could play a role. The study conducted by Carvalho and colleagues in 2015 suggested a role of VDR polymorphism [86]. The evaluation of 170 Portuguese SLE patients and 192 healthy controls demonstrated an association between different genetic variants and accrual damage. In particular, the frequency of VDR genotypes TaqI TT (rs731236) and Fok I CT (rs2228570) was higher in SLE patients with damage, evaluated by using the SLICC Damage Index (SDI) [86, 87].

The development of osteoporosis with fractures is considered chronic damage in SLE patients and is inserted in the SDI [87]. Bonfá and colleagues performed a case-control study by evaluating 211 premenopausal SLE patients and 154 healthy women, in order to evaluate the association between the RANKL, OPG, and RANK gene polymorphisms and bone parameters. A significantly lower frequency of the RANKL 290 G allele (AG/GG) was identified in the patients with vertebral fractures compared with those without ($P = 0.011$). In the logistic regression analysis, in addition to the age, only RANKL 290A>G remained as an independent risk factor for vertebral fractures in SLE patients [88]. The authors hypothesized that the protection against vertebral fractures that is associated with the AG/GG genotype could be a consequence of decreased osteoclast activation due to RANKL dysfunction or to a local reduction of this molecule in the bone [88].

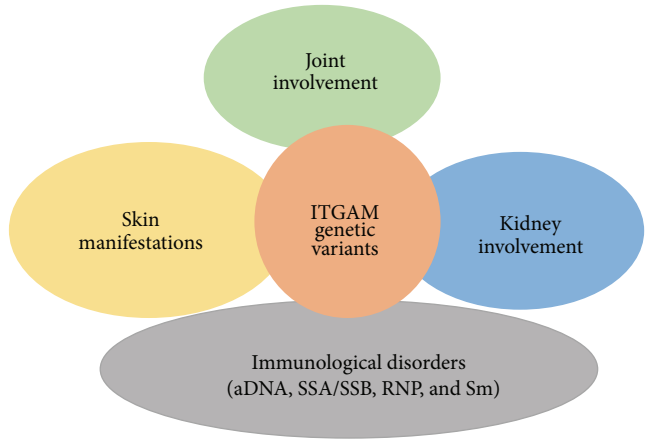

Figure 2: Disease manifestations associated with ITGAM genetic variants.

11. Conclusion

As widely demonstrated, genetic factors play a pivotal role in SLE pathogenesis. Moreover, in the last years several evidences suggested the role of genetic factors not only in disease susceptibility, but also in the development of specific disease phenotype. Several data are available to date concerning genetic variants involved in renal involvement, while fewer studies have been focused on SLE clinical and immunological manifestations. Interestingly, some genetic variants seem to be involved in the determination of different disease-related manifestations, suggesting a common pathogenetic mechanism, able to identify specific subset of patients. An example of this concept is represented by ITGAM genetic variants that resulted in being simultaneously associated with different disease manifestations (Figure 2). The recent progress leading to the discovery of novel methods to perform genetic studies will definitely allow clearly defining the associations between genes variability and SLE susceptibility and phenotype. Possibly, risk algorithms will be developed permitting a more personalized management of the disease.

However, small populations and lack of all clinical data characterize the studies evaluating the association between genetic and different disease phenotypes, not allowing a sufficient statistical power. Further studies specifically designed to evaluate this issue are needed to clarify the strongest associations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Fulvia Ceccarelli and Carlo Perricone equally contributed.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 572353, 10 pages
http://dx.doi.org/10.1155/2015/572353

Research Article

Familial Aggregation and Segregation Analysis in Families Presenting Autoimmunity, Polyautoimmunity, and Multiple Autoimmune Syndrome

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Received 17 August 2015; Accepted 29 October 2015

Academic Editor: Fulvia Ceccarelli

Studies documenting increased risk of developing autoimmune diseases (ADs) have shown that these conditions share several immunogenetic mechanisms (i.e., the autoimmune tautology). This report explored familial aggregation and segregation of AD, polyautoimmunity, and multiple autoimmune syndrome (MAS) in 210 families. Familial aggregation was examined for first-degree relatives. Segregation analysis was implemented as in S.A.G.E. release 6.3. Data showed differences between late- and early-onset families regarding their age, age of onset, and sex. Familial aggregation of AD in late- and early-onset families was observed. For polyautoimmunity as a trait, only aggregation was observed between sibling pairs in late-onset families. No aggregation was observed for MAS. Segregation analyses for AD suggested major gene(s) with no clear discernible classical known Mendelian transmission in late-onset families, while for polyautoimmunity and MAS no model was implied. Data suggest that polyautoimmunity and MAS are not independent traits and that gender, age, and age of onset are interrelated factors influencing autoimmunity.

1. Introduction

Autoimmune diseases (ADs) are responsible for a substantial amount of disability and morbidity worldwide. Although their epidemiology varies according to individual conditions, collectively, autoimmune prevalence is at least 5% in the general population and is one of the major causes of premature mortality in young and middle aged women [1].

As heterogeneous diseases, ADs develop from the cumulative effect of diverse events on the immune system [2]. It is clear that ADs do not begin at the time of clinical appearance but rather many years before. A common origin for diverse ADs is sustained by three levels of evidence [3]: the first comes from clinical observations indicating the possible shift from one disease to another or to the fact that more than one AD may coexist in a single patient (i.e., polyautoimmunity) [4–8] or in the same family (i.e., familial autoimmunity) [9]; a second level of evidence refers to known shared pathophysiological mechanisms between ADs [10, 11]. Epidemiological studies show correlations among certain ADs, linking epidemiological observations to physiopathological evidence for AD might contribute to our knowledge for the shared etiological and immunogenetic mechanisms [2]; and a third level of evidence corresponds to the evidence implying common genetic factors [7]. The importance of this concept focuses on the probability of having multiple ADs simultaneously in one patient, which goes beyond epidemiologic inferences.

Numerous genetic factors are established to be important contributors to susceptibility in developing ADs based on several findings including the examination of the concordance rates between relatives for many autoimmune diseases (ADs) [12]. However, due to their multifactorial and polygenic nature, accompanied by differential penetrance

influenced by environmental factors and genetic heterogeneity among populations [13, 14], untangling of the genetic determinants defining their outcome and onset has proven to be extremely challenging. Likewise, data showing the existence of different ADs within a single family or within the same individual suggest a combination of genetic defects that may predispose individuals to different ADs sharing common pathogenic pathways [15].

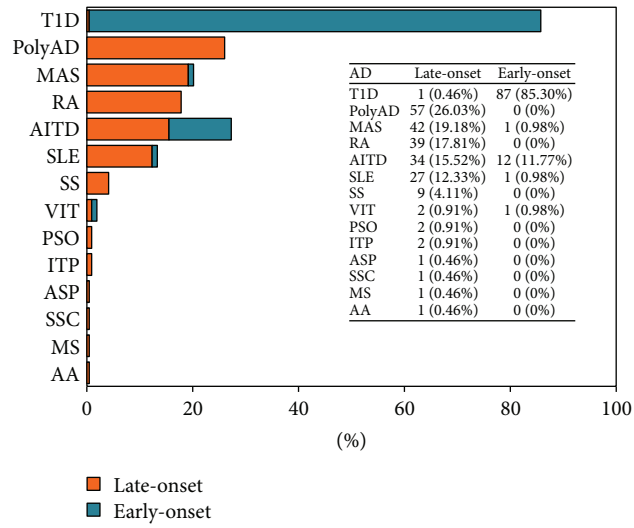
Therefore, family history of ADs should be considered when performing genetic analysis as this new approach incorporates all accepted pathologies for which evidence suggests an autoimmune origin. Families with multiple affected relatives appear to share common risk alleles with sporadic patients but may have a higher genetic load. A consequence of the polygenic model for complex diseases is that patients are inevitably highly heterogeneous in terms of the particular set of risk alleles they carry. It has been suggested that this may translate in different genetically determined disease mechanisms in subgroups of patients or a common disease mechanism that is complemented by additional pathways that are more or less predominant in different subgroups [16]. Familial approaches have documented the clustering of certain ADs among the relatives of individuals who have rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE), and type 1 diabetes mellitus (T1D) among other diseases [17–23].

ADs are not inherited in a simple, classical Mendelian way but have instead a complex or a yet uncharacterized mode of inheritance [13, 24]. Bias et al. were the first to consider a single major gene conferring susceptibility for autoimmunity and suggested an autosomal dominant inheritance pattern with penetrance of approximately 92% in females and 9% in males [13]. In addition, Arcos-Burgos et al. showed the presence of a dominant major gene and strong environmental effects as the most parsimonious model of segregation for VIT [25]. On the other hand, when analyzing RA together with other ADs, a mixed model fitted the data significantly better than the major gene or polygenic models [26].

The clinical evidence of the autoimmune tautology highlights the cooccurrence of distinct ADs within an individual [27]. ADs coexistence in a single individual has led researchers to consider different terms like autoimmune diathesis [28] or kaleidoscope of autoimmunity [29] both of which point to a common genetic background of ADs [6]. In an effort to understand and further support the commonality of autoimmunity as a trait among ADs, the present study examined the dynamics of familial aggregation and segregation in AD, polyautoimmunity, and multiple autoimmune syndrome (MAS) in well-defined and characterized patients and their relatives from Colombia, South America.

2. Materials and Methods

2.1. Study Population and Family Collection. This study sample consisted of multiplex families of varying size ascertained through patients treated at the Center for Autoimmune Diseases Research (CREA) in Medellin and Bogotá at the University of Rosario, Colombia (Table 1). (i) Each recruited family presented a proband with at least one AD according to validated international classification criteria; (ii) each recruited family presented at least one family member with polyautoimmunity (i.e., cooccurrence of distinct ADs within an individual); (iii) each recruited family presented evidence of familial autoimmunity (i.e., different ADs within members of a nuclear family); and (iv) each other affected individual presented a well-defined autoimmune phenotype (i.e., fulfillment of international classification criteria in first-degree relatives (FDRs)). Moreover, families in which the proband presented with T1D were included and used as early-onset AD families (Figure 1). FDRs were defined as parents and siblings.



AD	Late-onset	Early-onset
T1D	1 (0.46%)	87 (85.30%)
PolyAD	57 (26.03%)	0 (0%)
MAS	42 (19.18%)	1 (0.98%)
RA	39 (17.81%)	0 (0%)
AITD	34 (15.52%)	12 (11.77%)
SLE	27 (12.33%)	1 (0.98%)
SS	9 (4.11%)	0 (0%)
VIT	2 (0.91%)	1 (0.98%)
PSO	2 (0.91%)	0 (0%)
ITP	2 (0.91%)	0 (0%)
ASP	1 (0.46%)	0 (0%)
SSC	1 (0.46%)	0 (0%)
MS	1 (0.46%)	0 (0%)
AA	1 (0.46%)	0 (0%)

Figure 1: Frequency and distribution of autoimmune disease (AD) in late-onset and early-onset families included in this report. For analytical purposes, families were divided into two types: late-onset (i.e., families where a proband presents a late-onset AD) and early-onset (i.e., T1D families) (Figure 1).

Patients with AD, polyautoimmunity, and MAS fulfilled validated classification criteria and were part of a multicenter cohort followed at the CREA. Their information on demographics and cumulative clinical manifestations over the course of disease were obtained by both chart review and discussion with the patient and were collected in a standard data collection form. Only relatives of Colombian patients were included and interviewed, following the methodology described by Priori et al. [30], using a standardized questionnaire that incorporates demographics and medical information including a check-point list of 18 ADs [21]. In order to avoid ascertainment bias, the diagnosis of any AD was only considered reliable and consequently registered if made by a certified physician (i.e., internist, endocrinologist, or rheumatologist) and confirmed by chart review or verification during discussion with the relative. All patients fulfilled the diagnostic classification criteria proposed per disease as previously applied [6, 21].

In T1D families, recruited cases were children all of whom fulfilled the diagnostic classification criteria proposed by the American Diabetes Association (ADA) [31] and had been previously described [32] (Table 1). Their information on demographics and cumulative clinical manifestations over

Table 1: Characteristics of probands and families classified as late-onset and early-onset.

Characteristic	Late-onset				Early-onset		
	All	AD	PolyAD	MAS	All	AD	PolyAD/MAS
Age (yrs)	49.43	45.99	45.49	44.81	32.32**	19.54**	16
[Min, Max]	[11, 91]	[13, 83]	[16, 78]	[20, 64]	[3, 94]	[4, 70]	
Age at onset (yrs)	—	32.80	33.42	33.97	—	7.77**	11
[Min, Max]		[5, 62]	[5, 62]	[5, 62]		[1, 24]	
Male							
Aff (Unaff)	265	24 (156)	8 (172)	2 (178)	227	50 (141)	0 (191)
Female							
Aff (Unaff)	451	195 (216)	91 (320)	41 (370)	216	52 (152)	1 (203)
Number of Peds	127				83		
Mean size ± SD	5.64 ± 2.76				5.34 ± 2.94		
[Min, Max]	[3, 16]				[3, 20]		

AD: autoimmune disease; PolyAD: polyautoimmunity; MAS: multiple autoimmune syndrome. Data correspond to FDRs affected or unaffected and taking into account the analysis. Aff: affected; Unaff: unaffected.
** p value < 0.001 t-test when comparing late-onset versus early-onset variables.

the course of disease were obtained by both chart review and discussion with the patient and were collected in a standard data collection form. A total of 87 patients with T1D were analyzed and their relatives were included (Table 1).

For individuals (i.e., probands and FDR) with thyroid disorders, anti-thyroglobulin and anti-thyroperoxidase antibodies were measured by enzyme-linked immunosorbent assay (QUANTA Lite, INOVA Diagnostics, San Diego, CA, USA). Only patients with positive antibody profile for autoimmune thyroid disease (AITD) were included for analysis. Exclusion criteria were preexisting hematological diseases and hepatitis B virus, hepatitis C virus, or human immunodeficiency virus infections. As for the family characteristics in our population, most of them are nuclear and at least 30% are multigenerational [33, 34]. The great majority of our country households still contain related persons. In addition, all family members participating in this study were living in the same city and approved informed consent in order to participate in the present study. This research is being carried out in accordance with Resolution number 008430 of 1993 issued by the Ministry of Health of the Republic of Colombia and was classified as a minimal risk research. The Ethics Committee of Universidad del Rosario approved the present project.

2.2. Statistical and Genetic Data Analysis. Data was managed and stored using the R software version 3.1.1 [35] and Excel spreadsheets. Results are presented as means ± standard deviation (SD) and minimum/maximum and/or in percentages. Comparison between means was performed by Student's t-test and those between percentages by the χ^2 test and two-sided Fisher's exact test, where appropriate. A p value of less than 0.05 was considered as statistically significant.

The present study included information on (i) sex, (ii) autoimmunity affection status defined as affected, unaffected, or unknown for AD (i.e., having at least one AD), polyautoimmunity (i.e., having at least two ADs), and MAS (i.e., having three or more ADs), and (iii) family/pedigree relationships. Estimation of the distributions of relationship types and affection status among relatives pairs were performed using the Statistical Analysis for Genetic Epidemiology (S.A.G.E.) program PEDINFO, release 6.3 [36]. Where necessary, dummy individuals were added to families for the purpose of connecting relatives within pedigrees, and the affection status for such dummy individuals was set to missing and thus they were not used in the analyses.

Familial Aggregation Analysis. Recurrent risk ratios (λ_R) were calculated for first-degree relatedness (parent/offspring and sibling/sibling pairs) using the formula $\lambda_R = K_{\text{Relative}}/K$, where K_{Relative} (K_R) is the prevalence for a specific degree of relatedness in the sample and K is the mean prevalence in the population [37] and/or the previously reported K in specific pairs of relatives in the same population [21]. Information about the prevalence of ADs in our population is not clear and available; for this matter prevalence values in the range of 0.1%–0.5% were chosen as reported in the literature [1, 38–45]. Therefore, 0.5% (5/1000 individuals) for AD and 2.5% (25/1000 individuals) for all ADs taken together were selected as putative population prevalence as previously reported [1, 21, 38–45]. These methods were extended to ascertain whether or not clustering of two or more autoimmune disorders in relatives increased the probability or the risk for the presence of the disorder in the affected proband.

Familial Segregation Analysis. Analyses on 210 single ascertained pedigrees (Table 1) to identify the most plausible model explaining the segregation of AD, polyautoimmunity, and MAS in late-onset (non-T1D families) and early-onset families (T1D families) were performed for a binary trait as implemented in SEGREG S.A.G.E. release 6.3 (Table 2). SEGREG uses maximum-likelihood methods to estimate the parameters of mathematical models of disease occurrence in families. Each model assumes that the presence (or absence) of a putative disease allele influences susceptibility to the trait and applies the regressive multivariate logistic model allowing us to include available covariates into the fitted models.

Table 2: Parameter estimates from segregation analysis of autoimmune disease proband-ascertained pedigrees.

Model/parameter	Type susceptibilities			Transmission probabilities			Freq	Multifactorial/polygenic effect[b]
	β_{AA}	β_{AB}	β_{BB}	τ_{AA}	τ_{AB}	τ_{BB}	q_A	$\rho_{FM} = 0$[a]; $\rho_{F0} = \rho_{M0}$
(1) Random environmental	—	—	—	q_A	q_A	q_A	*	0
(2) Dominant	*	β_{AA}	*	1	0.5	0	*	0
(3) Dominant multifactorial	*	β_{AA}	*	1	0.5	0	*	*
(4) Recessive	*	β_{BB}	*	1	0.5	0	*	0
(5) Recessive multifactorial	*	β_{BB}	*	1	0.5	0	*	*
(6) Codominant	*	*	*	1	0.5	0	*	0
(7) Additive	*	$(1/2)(\beta_{AA} + \beta_{BB})$	*	1	0.5	0	*	0
(8) Mayor gene	*	*	*	*	*	*	*	0
(9) General transmission[c]	*	*	*	*	*	*	*	*

[*] Parameters freely estimated within an appropriate range; q_A: allele frequency; when $\tau_{AA} = 1.0$, $\tau_{AB} = 0.5$, and $\tau_{BB} = 0.0$, Mendelian transmission is assumed; when q_A is estimated under Mendelian transmission, Hardy-Weinberg proportions ($\psi_{AA} = q_A^2$; $\psi_{AB} = 2q_A^2(1 - 2q_A^2)$; $\psi_{BB} = q_B^2$) are assumed.

[a] Father-mother correlations, set to 0 assuming absence of assortative mating or consanguineous mating.

[b] Polygenic transmission effect inclusion assumes that the phenotype is determined by polygenic inheritance, so the phenotype has one distribution, and familial correlations can explain the familial aggregation of the trait.

[c] All parameters are estimated in Model 9. As a result, all other models are nested, and thus the general model is used as the baseline to compare all other models in this study.

Models Description. Random environmental model (Model 1) assumes that the trait segregation is caused purely by a random environmental factor and there is no transmission from generation to generation ($\tau_{AA} = \tau_{AB} = \tau_{BB} = q_A$). Pure major locus transmission models (Models 2, 4, 6, and 8) assume major locus transmission in a Mendelian mode, without multifactorial/polygenic inheritance. Major gene plus multifactorial/polygenic models (Models 3 and 5) assumes that both a major locus (transmitted in a Mendelian mode) and a multifactorial/polygenic effect influence the trait. The general model (Model 9) is the unrestricted full model, which subsumes all of the other models.

The fitted models assumed that the likelihood for any two individuals presenting with the phenotype and having the major type over nuclear families is independent. Consequently, the susceptibility (marginal probability) that any pedigree member has a particular phenotype is the same for all members who have the same values of any covariates in the model. This susceptibility is given the cumulative logistic function $\lambda = e^{\theta y}/(1 + e^{\theta y})$, where y is the affection status phenotype of ith individual and θ is the logit of the susceptibility for ith individual defined as $\theta(i) = \log[p(Y = 1)/1-p(Y = 1)] = \beta g+\varphi X$, where β is the baseline parameter, g is the susceptibility type and X is the covariate vector.

Analyses were performed by estimating the following parameters: type frequencies Ψ_u (u = AA, AB, BB): if the type frequencies were in Hardy-Weinberg equilibrium proportions, they were defined in terms of q_A (frequency of allele A); transmission probabilities τ_u (the probability that a parent of type u transmits allele A to an offspring: under Mendelian transmission, $\tau_{AA} = 1$, $\tau_{AB} = 0.5$, and $\tau_{BB} = 0$); and baseline parameter β, which can be sex dependent and/or type dependent. Sporadic/environmental and genetic models that were considered in assessing type of familial association and possible evidence of transmission of major effect are shown in Table 2.

Every model was tested against the likelihood of the general (unrestricted) model, in which all parameters were unrestricted and allowed to fit the empirical data. The estimated model hypotheses of transmission were as follows: major gene type, Mendelian dominant, Mendelian recessive, Mendelian additive, random environmental effect, codominant, and no transmission (Table 2). A likelihood ratio test (LRT) was used to test the significance of the departure from a specified null hypothesis model using the asymptotic properties of the LRT distributed as chi-square distribution with degrees of freedom equal to the difference in the number of parameters estimated in both models. Using this test, a significant chi-square test indicates that the submodel tested can be rejected at the given alpha level, which means the hypothesized model does not fit the data. Models were also compared using Akaike's information criterion (AIC), which is defined as AIC = $-2 \ln L + 2x$ (number of parameters estimated). A lower value of AIC represents a better fitting model.

3. Results

In this study, 127 late-onset diseases and 83 early-onset families were examined. The general statistics of the pedigrees are disclosed in Table 1. The mean pedigree size and standard deviation as well as the total number of relative pairs were obtained in order to calculate the prevalence for AD, polyautoimmunity, and MAS as main traits. Analyses were restricted to FDR. When early-onset and late-onset families age and age of onset were compared, the difference was statistically significant (p value < 0.001) as expected given their autoimmune disorder characteristics.

In total 716 and 443 individuals were included for the analyses, for late-onset and early-onset families, respectively (Table 1). Late-onset families included 37% males and 63% females while early-onset presented 51% males and 49% females. Moreover, females represented the most affected ones in late-onset families while in early-onset the ratio of the affected was close to 1 : 1 (male : female). In early-onset

Table 3: Familial aggregation (λ_R) of autoimmune disease (AD), polyautoimmunity, and multiple autoimmune syndrome (MAS) in late-onset and early-onset families.

Type of family	Pairs of relatives	Total pairs	Pairs	K (%)	$\lambda_R = K_R/K_{HI}$	$\lambda_R = K_R/K_{pop}$
Late-onset	AD			K_{AD}	λ_{HI}	λ_{pop}
	Parent/offspring	876	55/190/208	6.28	4.76	2.51
	Sibling/sibling	706	86/267/353	12.1	13.39	4.87
	Sister/sister	336	67/92/177	19.9	21.91	7.98
	Brother/brother	64	0/44/20	0.00	0.00	0.00
	Brother/sister	306	19/131/156	6.21	6.82	2.48
Late-onset	Polyautoimmunity			K_{PolyAD}	λ_{HI}	λ_{pop}
	Parent/offspring	876	8/333/112	0.91	0.69	0.37
	Sibling/sibling	706	23/450/233	3.26	3.58	1.30
	Sister/sister	336	20/181/135	5.95	6.54	2.38
	Brother/brother	64	0/59/5	0.00	0.00	0.00
	Brother/sister	306	3/210/93	0.98	1.08	0.39
Late-onset	MAS			K_{MAS}	λ_{HI}	λ_{pop}
	Parent/offspring	876	1/403/49	0.11	0.09	0.05
	Sibling/sibling	706	4/581/121	0.57	0.62	0.23
	Sister/sister	336	3/260/73	0.89	0.98	0.36
	Brother/brother	64	0/60/4	0.00	0.00	0.00
	Brother/sister	306	1/261/44	0.33	0.36	0.13
Early-onset	AD			K_{AD}	λ_{HI}	λ_{pop}
	Parent/offspring	498	9/199/155	1.81	1.37	0.72
	Sibling/sibling	245	9/130/106	3.67	4.04	1.47
	Sister/sister	61	3/30/28	4.92	5.40	1.97
	Brother/brother	60	2/33/25	3.33	3.66	1.33
	Brother/sister	120	4/67/53	3.33	3.66	1.33
Early-onset	Polyautoimmunity/MAS			K_{MAS}	λ_{HI}	λ_{pop}
	Parent/offspring	498	0/361/2	0.00	0.00	0.00
	Sibling/sibling	245	0/244/1	0.00	0.00	0.00
	Sister/sister	61	0/61/0	0.00	0.00	0.00
	Brother/brother	60	0/60/0	0.00	0.00	0.00
	Brother/sister	120	0/123/1	0.00	0.00	0.00

[a] Affected/unaffected/discordant pairs.

[*] K_{AD}, K_{PolyAD}, and K_{MAS} = prevalence for AD, polyautoimmunity, and MAS, respectively. K_{HI} = prevalence for AD in healthy individual's pedigrees as previously reported (K_{PO} = 1.32%; $K_{S/S}$ = 0.91%) [21]. K_{pop} = chosen prevalence for the general population. Recurrent risk ratio ($\lambda_R = K_R/(K_{HI}$ or $K_{pop})$), where R is the specific relative pair used (P/O = parent/offspring; SIB = sibling/sibling). The chosen population prevalence (K) for AD was considered as 25/1000 individuals [21]. Prevalence is given in percentages.

families, there was only one individual presenting with MAS among the 102 affected individuals.

3.1. Familial Aggregation (λ_R). The distribution of relationship types and total number of study subjects included in this study is presented in Table 3. No two probands belonged to the same family. Pairs of relatives discordant or concordant for AD, polyautoimmunity, and MAS were calculated in order to examine the family aggregation. Overall, the data is composed of 876 parent-offspring pairs and 706 different sib-pairs broken down to sister-sister (n = 336), sister-brother (n = 64), and brother-brother (n = 306) pairs (Table 3).

The prevalence of AD, polyautoimmunity, and MAS for each pair of relatives (parent/offspring [P/O], sibling/sibling [S/S]) is disclosed in Table 3. Previously reported prevalence values for familial pairs for AD in healthy individuals were taken into account for the examination of aggregation (K_{PO} = 1.32%; $K_{S/S}$ = 0.91%) [21]. Also, using a putative chosen prevalence for all AD taken together as trait (K_{pop} = 2.5%), λ_R were calculated (Table 3). Values supporting familial aggregation ($\lambda_R > 1.0$) were observed for AD in late-onset families in P/O (λ_{HI} = 4.76, λ_{pop} = 2.51) and S/S (λ_{HI} = 13.39, λ_{pop} = 4.87) pairs, with the highest familial aggregation within sister-pairs (λ_{HI} = 21.91, λ_{pop} = 7.98). For

Table 4: Parameter estimates from segregation analyses of early-onset families. For details in each model check Table 2. AD: autoimmune disease; PolyAD: polyautoimmunity; MAS: multiple autoimmune syndrome; ND: model not able to maximize.

Model/parameter	β_{AA}	β_{AB}	β_{BB}	q_A	ρ_{SS}	τ_{AA}	τ_{AA}	τ_{AA}	Sex	$-2\ln(L)$	d.f.	p value	AIC
AD													
Random environmental	—	—	—	0.19	1.43	q_A	q_A	q_A	2.36	698.079	3	<0.05	708.079
Dominant	1.21	β_{AA}	−109	0.05	0.00	1.00	0.50	0.00	2.16	707.994	5	<0.05	**715.994**
Dominant multifactorial	1.00	β_{AA}	−1.16	0.07	−0.06	1.00	0.50	0.00	2.19	706.492	4	<0.05	716.492
Recessive	−1.09	β_{BB}	1.21	0.95	0.00	1.00	0.50	0.00	2.16	707.994	5	<0.05	**715.994**
Recessive multifactorial	−1.15	β_{BB}	1.33	0.94	−0.05	1.00	0.50	0.00	2.24	706.653	4	<0.05	716.653
Codominant	−33.00	1.41	−1.21	0.06	0.00	1.00	0.50	0.00	2.35	707.529	5	<0.05	717.529
Additive	1.94	0.38	−1.18	0.10	−0.09	1.00	0.50	0.00	2.08	706.956	5	<0.05	716.956
Mayor locus	−0.73	1.54	−2.07	0.01	0.00	0.56	0.00	1.00	2.14	667.079	1	0.52	**679.079**
General transmission	−0.75	1.49	−2.07	0.02	−0.05	0.56	0.00	1.00	2.13	666.871		Ref.	680.871
PolyAD													
Random environmental	—	—	—	0.35	2.27	q_A	q_A	q_A	2.30	491.607	3	<0.05	501.607
Dominant	1.50	β_{AA}	−2.09	0.01	0	1	0.5	0	2.10	499.629	5	<0.05	**507.629**
Dominant multifactorial	−0.89	β_{AA}	−2.21	0.08	−0.22	1	0.5	0	1.89	499.127	4	<0.05	509.127
Recessive	−2.09	β_{BB}	1.51	0.99	0	1	0.5	0	2.10	499.629	5	<0.05	**507.629**
Recessive multifactorial	−2.29	β_{BB}	−0.98	0.90	−0.24	1	0.5	0	1.90	499.135	4	<0.05	509.135
Codominant	−48.15	1.44	−2.09	0.01	0	1	0.5	0	2.11	499.614	5	<0.05	509.614
Additive	−0.65	−1.56	−2.47	0.24	−0.27	1	0.5	0	1.85	499.233	5	<0.05	509.233
Mayor gene	−2.02	−0.44	−17.60	0.04	0	0.86	0.00	1.00	2.09	472.191	1	<0.05	**484.191**
General transmission	−66.10	−1.05	−3.47	0.00	1.86	1.00	0.00	0.39	1.86	459.356		Ref.	471.356
MAS													
Random environmental	—	—	—	0.51	2.65	q_A	q_A	q_A	2.73	286.846	3	<0.05	**296.846**
Dominant	ND	β_{AA}	ND	ND	0	1.00	0.50	0.00	ND	ND	5		
Dominant multifactorial	−2.27	β_{AA}	−4.32	0.25	−0.06	1.00	0.50	0.00	2.25	286.875	4	<0.05	296.875
Recessive	ND	β_{BB}	ND	ND	0	1.00	0.50	0.00	ND	ND	5		
Recessive multifactorial	−4.84	β_{BB}	−2.28	0.72	−0.05	1.00	0.50	0.00	0.72	286.856	4	<0.05	296.856
Codominant	−2.27	−2.27	−4.66	0.27	0	1.00	0.50	0.00	−0.98	286.838	5	<0.05	298.838
Additive	−2.27	−4.14	−6.07	0.66	−0.97	1.00	0.50	0.00	2.25	287.122	5	<0.05	297.122
Mayor gene	24.70	−14.29	−18.36	0.00	0	0.00	0.00	0.15	37.20	271.525	1	<0.05	**281.525**
General transmission	−152.53	−3.36	−1.91	0.96	4.42	0.42	0.12	0.99	1.95	260.304		Ref.	276.304

polyautoimmunity, familial aggregation was not observed for P/O pairs but for S/S pairs ($\lambda_{HI} = 3.58$, $\lambda_{pop} = 1.30$). In early-onset families, familial aggregation was observed for AD in P/O ($\lambda_{HI} = 1.37$) and in S/S ($\lambda_{HI} = 4.04$, $\lambda_{pop} = 1.47$). No aggregation for MAS was observed in any pair of relatives.

3.2. Segregation Analysis. The parameter estimates and test statistics from the segregation analyses for late- and early-onset families for AD, polyautoimmunity, and MAS are presented in Tables 4 and 5, respectively.

To determine support for familial or residual association in the data, initially we compared four no-transmission models, each having different type of familial association, to inspect whether the sibling (S) correlation equals the parent-offspring correlation (FO and/or MO, F: father, M: mother, and O: offspring). Four no major models were fitted and compared; each, respectively, assumed (1) ρFO; ρMO; ρSS-free; (2) ρFO = ρMO, ρSS-free; (3) ρFO = ρMO = ρSS; and (4) ρFO = ρMO = ρSS = 0 (the no multifactorial component model). ρFM was assumed to be 0 for all models. The model where both parent-offspring and sibling residual associations are equal (i.e., ρFO = ρMO = ρSS) fitted the data better than any of the other three models for AD, polyautoimmunity, and MAS for both late- and early-onset families (results not shown), thereby providing support for the existence of familial association in the data and inclusion and estimation of familial association parameters in the subsequent models. To determine whether sex should be included in the segregation models, two nontransmission models were initially fitted, one including the covariate and the other not, and then compared by AIC. Results showed that including sex as a covariate in the models allowed better model fitting (data not shown).

The hypothesis of no major gene was tested by comparing the random environmental (Model 1) and general transmission model (Model 9) (Table 2). The random transmission model was rejected in late-onset disease families, supporting the existence of a major gene in AD ($p < 0.05$, AIC = 708.08), polyautoimmunity ($p < 0.05$, AIC = 501.61), and MAS ($p < 0.05$, AIC = 296.46) (Table 4), while in early-onset

Table 5: Parameter estimates from segregation analyses of early-onset families. AD: autoimmune disease. For details in each model check Table 2.

Model/parameter	β_{AA}	β_{AB}	β_{BB}	q_A	ρ_{SS}	τ_{AA}	τ_{AA}	τ_{AA}	Sex	$-2\ln(L)$	d.f.	p value	AIC
AD													
Random environmental	—	—	—	0.01	−0.83	q_A	q_A	q_A	−0.02	426.292	3	0.55	438.292
Dominant	−1.05	β_{AA}	−1.05	0.02	0	1	0.5	0	−0.03	451.220	5	<0.05	459.22
Dominant multifactorial	−1.99	β_{AA}	−1.05	0.08	0.01	1	0.5	0	0.01	441.228	4	<0.05	451.228
Recessive	−1.07	β_{BB}	−1.05	0.00	0	1	0.5	0	−0.03	451.220	5	<0.05	459.22
Recessive multifactorial	−2.80	β_{BB}	−1.04	0.32	−0.53	1	0.5	0	0.01	440.46	4	<0.05	450.46
Codominant	−2.78	−1.05	−1.08	0.29	0	1	0.5	0	0.01	440.408	5	<0.05	452.408
Additive	−1.17	−1.17	−1.17	0.10	−0.48	1	0.5	0	0.01	441.265	5	<0.05	451.265
Mayor gene	115.3	21.2	−2.68	0.00	0	0.3	0.0	0.1	0.54	400.587	1	<0.05	412.587
General transmission	−9.57	−0.71	−0.91	0.32	−0.84	0.20	0.33	0.34	−0.005	427.342	0		443.342

families the model could not be rejected ($p = 0.55$, AIC = 438.29) (Table 5). Subsequently, the major gene hypothesis was further tested by comparing the major gene only model (Model 8) and the general transmission model (Model 9) (Table 2). For this comparison, the hypothesis for the major gene was rejected only for AD in late-onset families ($p < 0.05$, AIC = 679.08) (Table 4), while it was not rejected for late-onset families when taking polyautoimmunity and MAS as main traits, as well as in early-onset families for AD (Table 5). Of note, for early-onset families due to low frequency of polyautoimmunity and MAS, only models for AD as a main trait were estimated.

After having procured evidence for the segregation of major gene(s) in late-onset families with AD as the main trait and not for polyautoimmunity and MAS for late-onset and for AD in early-onset families, the hypothesis of Mendelian transmission was tested by comparing the Mendelian proposed models (Models 2, 4, 6, and 8) with the general transmission model (Model 9) (Table 2). Dominant, recessive, codominant, and additive Mendelian transmission models were rejected for late-onset families when taking AD as a trait. All the same, when a multifactorial/polygenic parameter was added to the dominant and recessive Mendelian models (Models 3 and 5, resp.) and compared with the Mendelian counterpart without the multifactorial component, no change in the rejection of the models was observed (Table 4).

4. Discussion

The commonality between ADs is the damage to tissues and organs arising from the loss of tolerance and in most cases a gender imbalance [46]. Research generally focuses on a single disease, although autoimmune phenotypes could represent pleiotropic outcomes of nonspecific disease genes underlying similar immunogenetic mechanisms [47]. While it is apparent that multiple cases of a single disease cluster within families [4], more striking are the individuals in those families afflicted with multiple ADs [3].

This report presents the familial aggregation and segregation analyses of AD, polyautoimmunity, and MAS in Colombian families. We have analyzed 210 families (i.e., 127 late-onset diseases and 83 early-onset ones) in Table 1, for which a total of 716 and 443 individuals were analyzed (Table 1). Each pedigree was ascertained through an affected proband fulfilling the inclusion criteria presented in Section 2. This study is restricted and takes into account AD, polyautoimmunity, and MAS as main traits presented in the recruited families (Figure 1). The recruited families were divided into two types of family given by the pathology presented in the proband (i.e., early-onset families are constituted mainly by T1D probands and late-onset families by AD known to develop later in life). Results show differences between late- and early-onset families regarding their age, age of onset, and sex distribution, which is expected given the particular and specific autoimmune disorder prevalence (Table 1, Figure 1).

Analyses of familial aggregation treat the family like any other unit of clustering. In addressing whether there is phenotypic aggregation within families, no attempt is made to determine the cause of any aggregation [48]. The observation and portrayal of familial autoimmunity and the outline of MAS have put aside the environmental aggregation and given a greater value towards the common/rare genetic component for diverse autoimmune phenotypes with a generally common background [4]. When considering the familial aggregation of AD, polyautoimmunity, and MAS for both types of families, values supporting the aggregation of AD in late- and early-onset families for P/O and S/S pairs, with the highest aggregation observed between sister-pairs of late-onset families, were observed (Table 3). For polyautoimmunity as a trait only aggregation was observed between S/S pairs in late-onset families. No familial aggregation for MAS was observed for any type of family. This suggests and confirms that polyautoimmunity and MAS are not AD independent traits and that gender, age, and age of onset represent factors that define and allow the study of the dynamics of the traits within the familial group.

Segregation analyses help to assess the possible genetic mode of segregation of a trait by consideration of relevant hypothesis-based mathematical models. Findings from segregation analyses are often used to formulate tailored research hypotheses for the trait under investigation and/or to decide the type of investigative effort to be put forward. This study was carried out to assess types of familial dependence in AD, polyautoimmunity, and MAS to investigate possible evidence

of transmission of major gene(s) and to determine the best mode of transmission for such major gene(s). The presented analyses indicate evidence for the familial transmission of major gene(s) with no clear discernible classical known Mendelian transmission in late-onset families when AD is taken as the main trait, while for polyautoimmunity and MAS familial transmission fails to be demonstrated. In early-onset families analyses did not demonstrate a major gene effect but a random environmental model explaining the presence of the phenotypes in the families. These results thus provide evidence for the genetic role in the etiology of AD in late-onset families by showing support for major gene(s) mode of segregation of susceptibility to AD, while for the early-onset families and perhaps by their relatively young status eludes a clear picture of autoimmunity segregation and aggregation in these families.

Previous segregation analyses have proposed models in families with more than one member affected by autoimmune hemolytic anemia and chronic thrombocytopenic purpura compatible with a Mendelian dominant trait [49]. In African Americans [50, 51] and EA [52] SLE families, presenting FAD, a dominant inheritance is reported, while in Chinese families segregation analyses describe a polygenetic model and major gene model, suggesting a polygenetic multifactorial disease [53]. Other analyses in VIT for Chinese families suggest a dominant inheritance model [54], while other reports suggest a non-Mendelian pattern supporting a multifactorial, polygenic inheritance [38]; even so other models describe a major dominant gene and the existence of strong environmental effects acting on a recessive genotype [25]. More generally, a Mendelian dominant genetic inheritance is proposed in many ADs, like SS [55] and T1D [56], while segregation is better explained by either dominant or codominant or polygenic models in APS [57], RA [26], and idiopathic inflammatory myopathies [58]. Others suggest that several major ADs result from pleiotropic effects of a single major gene on a polygenic background [26]. Finally, in traits such as MS segregation results are indeterminate and cannot be explained by a genetic model [59].

5. Conclusions

Overall, aggregation and segregation analyses in Colombian families enriched by autoimmunity as a trait show how ADs, polyautoimmunity, and MAS are not independent entities. Familial aggregation for ADs was observed between parents and offspring as well as in sibling pairs in late-onset families, while aggregation for polyautoimmunity and MAS was lesser given by the fact that both traits represent a more complex etiology with lower prevalence but still a common autoimmunity background. Segregation analyses were not able to discern a Mendelian transmission model but still suggested major gene(s) transmission for AD in late-onset families, while for early-onset families a stochastic model was suggested. Thus, a clinical defined individual AD, defined by symptoms and signs, might not be completely juxtaposed to the AD trait defined by environment and genetics, which makes the task to define and untangle disease mechanisms even more difficult. Last but not least, to further study and describe the familial dynamics of two or more cluster ADs, approaches such as familial coaggregation might find their place towards the exploration of common familial factors on top of studies taking into account AD, polyautoimmunity, and MAS as a trait in order to disentangle the common/rare genetic landscape of autoimmunity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by Universidad del Rosario and Colciencias (122254531722), Bogotá, Colombia. Some of the results of this paper were obtained by using the software package S.A.G.E., which was supported by a US Public Health Service Resource Grant (RR03655) from the National Center for Research Resources.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 679813, 9 pages
http://dx.doi.org/10.1155/2015/679813

Research Article

Autoimmune Hepatitis in Brazilian Children: IgE and Genetic Polymorphisms in Associated Genes

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Received 18 June 2015; Accepted 12 October 2015

Academic Editor: Fulvia Ceccarelli

Pediatric autoimmune hepatitis (AIH) patients present hypergammaglobulinemia, periportal $CD8^+$ cytotoxic T cell infiltration, and cirrhosis. Autoantibody profile defines AIH types 1 and 2 in addition to strong association with HLA-DRB1. We previously detected increased IgE serum levels and sought to compare clinical and histological features according to IgE levels in AIH ($n = 74$, ages 1–14 years) patients. Additionally, we typed 117 patients and 227 controls for functional polymorphisms of IL4, IL13, IL5, and IL4RA genes involved in IgE switching and eosinophil maturation that might contribute to overall genetic susceptibility to AIH. Serum IgE levels were high in 55% of AIH-1, but only in 12% of AIH-2 ($P = 0.003$) patients. Liver IgE was present in 91.3% of AIH-1 patients. The A alleles at both IL13 rs20541 and IL4RA rs1805011 were associated with AIH-1 ($P = 0.024$, OR = 1.55 and $P < 0.0001$, OR = 2.15, resp.). Furthermore, individuals presenting homozygosis for the A allele at IL4RA rs1805011 and HLA-DRB1*03 and/or *13 allele had sixfold greater risk to develop the disease (OR = 14.00, $P < 0.001$). The novel association suggests an additional role for IgE-linked immune response genes in the pathogenesis of AIH.

1. Introduction

Autoimmune hepatitis (AIH) is a chronic inflammatory disease characterized by progressive destruction of the hepatic parenchyma [1]. The disease displays female predominance and is considered rare in childhood, although it may occur in very young children [2]. The hallmark of the disease is the presence of circulating autoantibodies, defining two major subtypes: type 1 (AIH-1) [3, 4] and type 2 (AIH-2) [5]. Equally striking is the strong genetic susceptibility identified by specific MHC class II molecules, especially HLA-DRB1, which discriminates between the two types of AIH. Brazilian AIH-1 patients carry HLA-DRB1*13 and/or HLA-DRB1*03 whereas AIH-2 patients present mainly carry HLA-DRB1*07 [6].

Hypergammaglobulinemia is a diagnostic feature of AIH but other immunoglobulins may be altered as well. Low IgA levels are particularly common in AIH-2 [7] and we

have observed high IgE levels in children with AIH-1 [8]. Elevated serum IgE levels have been previously described in acute and chronic liver diseases usually linked to alcohol abuse or viral infection [9]. This phenomenon is traditionally linked to allergy, asthma, and atopy, but elevated IgE serum levels in specific autoimmune diseases have been increasingly acknowledged. To date, elevated IgE serum levels have been identified in Churg–Strauss vasculitis [10], sclerosing cholangitis [11], bullous pemphigus [12], autoimmune pancreatitis [13], and Grave's disease [14]. IgE seems also to play a role in the pathogenesis of rheumatoid arthritis contributing to the immune response against citrullinated proteins [15]. Atta et al. [16] also observed specific IgE antinuclear antibodies in systemic lupus erythematosus suggesting there is an important contribution to the pathogenesis of the disease. B lymphocyte switching to IgE is induced by IL4 and its neighbor gene IL13 [17], which form, together with IL5, a well-studied cytokine gene cluster (5q31.1) controlling TH2 type immune responses. IL4 is a pleiotropic cytokine essential for IgE synthesis by B cells and for T cell differentiation into a TH2 phenotype and upregulation of MHC class II expression. The functions of IL13 in immune surveillance and in TH2 type immune responses partially overlap with those of IL4. In addition to the classic TH2 pathway shared with IL4, IL13 has other important functions. IL13, together with IL5 [18], is a potent mediator of tissue fibrosis and tissue remodeling, as shown in experimental models of schistosomiasis [19]. A steadily increasing literature indicates that there is an important role for IL13 in the development of hepatic fibrosis, signaling through the IL13 receptor to induce collagen production by local fibroblasts [20] AIH-1 pediatric patients typically exhibit liver fibrosis, including most patients in our study. About 25% of AIH patients, despite treatment with corticosteroids, present progressive fibrosis, highlighting the importance of any gene which might be involved in this process [21]. In addition, both IL4 and IL13 genes harbor functionally relevant polymorphisms [22, 23].

Histological findings in AIH include typical piecemeal necrosis with infiltrating T lymphocytes. T cell-mediated cytotoxicity is believed to be the central mechanism responsible for hepatic damage, but other cells are involved. Typically, $CD4^+$ helper T and B cells gather around portal tracts, whereas $CD8^+$ cytotoxic T cells have a periportal distribution [24]. In addition to the abundant infiltrating mononuclear cells, plasma cells and eosinophils may also be present [1]. Interestingly, a previous study has highlighted the increased production of IL4 messenger RNA in AIH-1 liver biopsies in parallel with the expected increase in inflammatory interferon gamma and other proinflammatory cytokines [25]. These findings led us to try to identify additional factors involved in the autoimmune processes present in this liver disease, which might act either as prognostic disease markers or as novel targets for a therapeutic approach. To this end, we analyzed the major clinical manifestations and biopsies from Brazilian children grouped according to the AIH type and serum IgE levels. We also investigated, in the predominant AIH-1 group of patients, functional polymorphisms of the IL4, IL13, IL5, and IL4RA (IL4 receptor alpha chain) genes involved in IgE switching and eosinophil differentiation and maturation that we believe might contribute to overall genetic susceptibility to AIH.

2. Patients and Methods

A total of 141 patients diagnosed as AIH, according to the International Autoimmune Hepatitis Group Report [26], were studied. Patients were followed at the Pediatric Hepatology Unit of the Children's Institute, General Hospital, Faculty of Medicine, University of São Paulo in São Paulo, Brazil. Clinical, biochemical, and histological features of 74 AIH patients (61 with AIH-1 and 24 with AIH-2) aged 1 to 14 years were evaluated.

To increase statistical power for analysis of gene polymorphisms, we included a further 43 children with AIH-1 (a total of 117). Non-HLA matched siblings of bone marrow recipients from the same hospital and with similar social and ethnic background, without any autoimmune and/or other severe disease, were enrolled as healthy controls (HC, $n = 227$). Written informed consents were obtained from all participants and/or legal guardians, and the Internal Review Board of the University of São Paulo approved the study.

Laboratory liver tests, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, gamma glutamyl transpeptidase (γGT), albumin, γ-globulins, prothrombin, and total bilirubin, and autoantibody profiles were performed in all patients. Fecal samples collected in all patients were negative for parasitic infection. Radioallergosorbent test (RAST) for specific allergen against house dust, animal fur, food, and fungi was assayed by radioimmunoassay using Unicap100E (Pharmacia & Upjohn Company LLC, MI, USA) system. I. Immunoglobulins M, G, A, and E were assayed by nephelometry using a DADE Behring System Nephelometer BN 100 (Dade Behring Diagnostics Inc., Somerville, NJ). Serological tests for hepatitis A, B, and C were negative in all patients. Clinicians involved in this study ruled out other hepatic diseases such as alpha-1 antitrypsin deficiency and Wilson's disease.

Histological features of liver biopsies were graded semiquantitatively using the Brazilian Consensus for Histopathology of Chronic Hepatitis [27]. Specific monoclonal antibodies for IgE, CD3, CD4, CD8, CD20, and CD16 (BD Biosciences, San Jose, CA, USA) were used for immunohistochemistry [28].

Genomic DNA was extracted using a dodecyl/hexadecyltrimethylammonium bromide (DTAB/CTAB) method [29]. IL4 rs2243250, rs2070874, IL5 rs2069812, and IL13 rs20541 polymorphisms were typed by restriction fragment length polymorphism (RFLP). IL4 rs2070874 and IL13 rs20541 typing by RFLP is described elsewhere [30, 31]. The primers and restriction enzymes for IL4 rs2243250 and IL5 rs2069812 were 5′CCTAAACTTGGGAGAACATGGT, 3′TCCTCCTGGGGAAAGATAGA (AvaII) and 5′TTCCTGCTGCTCATGAACAGAATACGT, 3′CATTTTGATGGCTTCAGTGACTCTTCC (RsaI), respectively. IL4 rs2227284 and IL4RA rs1805011 polymorphisms were typed by ASPCR (allele-specific polymerase chain reaction). Primers for IL4RA rs1805011 have been described [32] and primers used for IL4 rs2227284 were 5′TTGGGTGGACAAGTAGTTGGAGCG,

Table 1: Clinical and laboratory findings of children with type 1 and type 2 autoimmune hepatitis.

	AIH-1 $n = 117$	AIH-2 $n = 24$
Clinical features		
Age onset; median (min–max)	8.2 (1.6–15.2)	4.8 (11.1–9.0)
Sex; *n* (F/M)	78/39	21/3
Onset; *n* (acute/insidious)	98/19	20/4
Concurrent autoimmune disease[1]; *n* (%)	15 (12.8)	3 (12.5)
Autoimmune diseases in relatives[2]; *n* (%)	23 (19.6)	11 (45.8)
Laboratory findings		
AA: type 1, SMA/ANA/SMA + ANA; type 2, LKM (*n*)	64/7/46	24
Alanine aminotransferase IU/L (× upper normal limit); median (min-max)	18 (2–128)	28 (4–85)
Albumin g/dL; median (min-max)	3.3 (2.2–5.1)	3.5 (2.6–4.7)
Bilirubin mg/dL; median (min-max)	3.3 (0.3–27.2)	5.8 (0.6–35)
γ-globulin g/dL; median (min-max)	3.4 (0.9–6.3)	3.3 (0.9–4.8)
IgE IU/mL; median (min-max)	96 (11–2245)	65 (6–560)
Histological features		
Cirrhosis; *n* (yes/no)	64/41	10/10
Not done	12	4

F = female; M = male; AA = autoantibody; SMA = smooth muscle antibody; ANA = antinuclear antibody; LKM = Liver Kidney Microsomal; *n* = number of individuals.
Normal albumin = 3.5–5.0 g/dL; normal bilirubin ≤ 1.1 mg/dL; normal γ-globulin = 0.7–1.6 g/dL; normal IgE = 20–100 IU/mL.
[1]Vitiligo, thyroiditis, diabetes mellitus, psoriasis, or Behçet's disease.
[2]First degree relatives.

5′TTGGGTGGACAAGTAGTTGGAGCT and 3′ATGTCCCATCCTGCCCAGGATAG.

2.1. Statistical Analysis. All statistical analyses were carried out using GraphPad Prism 5 or SPSS, v.13 Software. The clinical and laboratory parameters were analyzed using Student's *t*-test or Fisher's exact test, as well as the Mann-Whitney test where necessary. *P* values under 0.05 were considered as significant. The power was estimated for all studied SNPs and values ranged from 76 to 82%, indicating adequate sample size. In addition, all SNPs were in HWE and, as expected, Haploview analysis confirmed that the three studied IL4 SNPs were in linkage disequilibrium.

For the possible genetic associations, χ^2 or exact Fisher's test were applied. Unpaired *t*-test was used to evaluate associations between IgE and the genotypes of all studied SNPs. For regression analysis, variables presenting *P* value <0.100 in the univariate analysis were included. To identify possible gene-gene interactions, a binary logistic regression was performed considering changes in the OR.

3. Results

The majority of the AIH patients were classified as type 1 (85% versus 15% type 2). The median age of diagnosis was 8.2 and 4.8 years, respectively, for AIH-1 and AIH-2. In addition, 54% (13/24) of AIH-2 patients developed the disease before the age of 5 years, whereas this occurred only in 8/117 (7%) of AIH-1 patients ($P < 0.001$). Twenty-three (20%) AIH-1 and 11 (46%) AIH-2 patients ($P = 0.006$) had relatives presenting autoimmune diseases. In addition, median serum alanine aminotransferase values were higher in the AIH-2 group (28 versus 18 × upper normal limit; see Table 1).

Serum IgG, IgA, and IgE levels were significantly higher in AIH-1 in comparison to the AIH-2 group of patients (Figure 1). High IgE levels were observed in 50/91 (55%) of patients with AIH-1, but only in 2/17 (12%) of those with AIH-2 ($P = 0.003$) (Table 1).

Histopathology showed presence of cirrhosis in the majority of AIH-1 patients (57 out of 60) analyzed, usually accompanied by necroinflammatory activity corresponding to a score 3 and a score 4 panacinar necrosis. Liver cell rosettes were also present in almost 90% of livers, accompanied by infiltrating eosinophils and/or plasma cells, independently of patients IgE serum levels (Table 2). Importantly, in contrast to increased IgE serum levels present in about half of the patients, liver IgE was absent in only 4 of the 46 AIH-1 patients. Finally, most patients exhibited $CD8^+$ cytotoxic T cell and NK infiltrating cells, in some cases without detectable $CD4^+$ helper T cells (Table 3). However, irrespective of serum IgE levels, in most patients, moderate to high infiltration levels of $CD4^+$ helper T cells usually accompanied by moderately elevated liver NK cells were in fact present. In conclusion and in spite of having analyzed only a subgroup (46/60) of patients, our results clearly show that the well-known infiltrating proinflammatory cell profile coexists side by side with IgE, eosinophils, and the plasma cells possibly involved in IgE production. The reason for this mixed cell profile is currently unknown.

Among the studied SNPs in AIH-1, two functionally relevant SNPs present, respectively, in the IL13 gene and in its receptor IL4RA disclosed statistically significant increases.

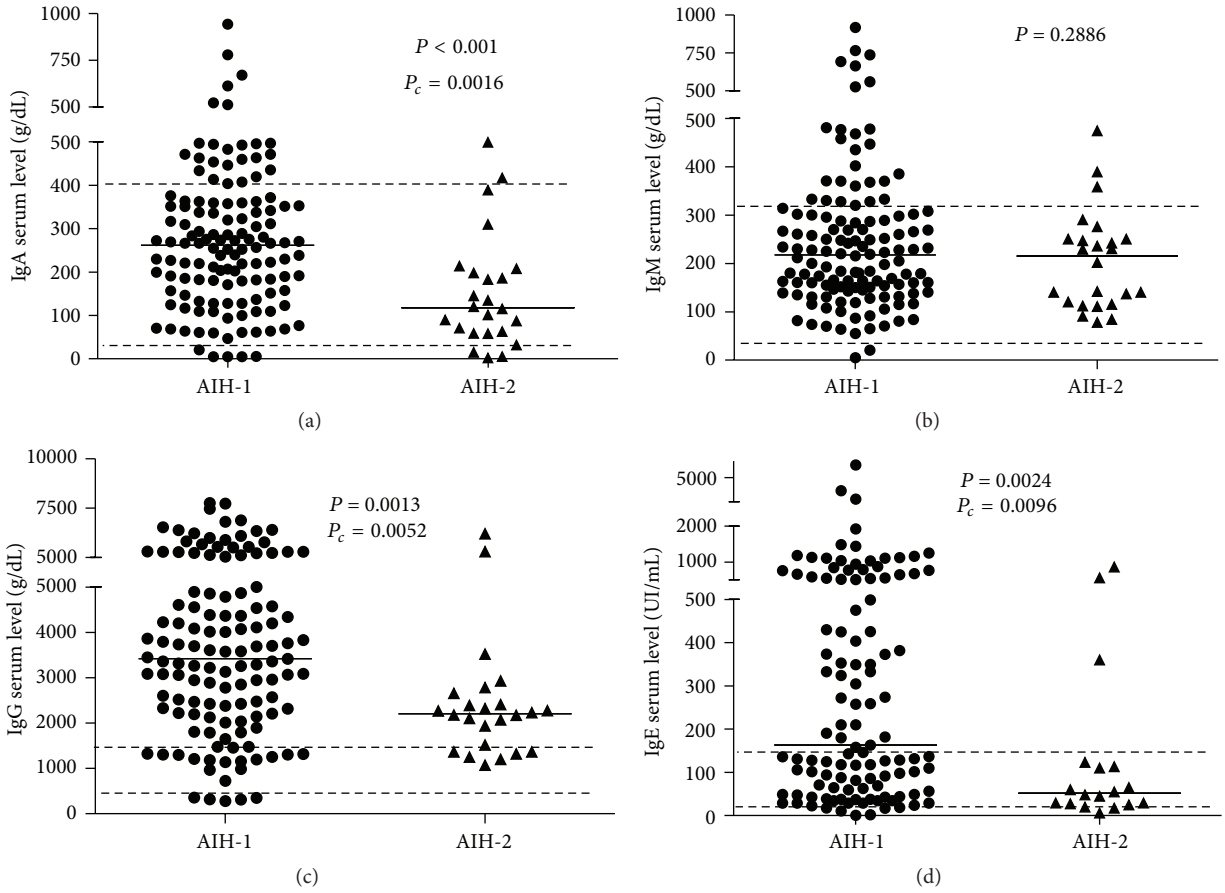

Figure 1: Immunoglobulins concentrations according to autoimmune hepatitis type. (a) IgA (g/dL); (b) IgM (g/dL); (c) IgG (g/dL), and (d) IgE (UI/mL). The immunoglobulins concentrations were assessed by nephelometry. Statistical analysis by Mann-Whitney nonparametric test (for medians).

The first SNP is IL13 rs20541 (31 versus 23% of HC; $P = 0.024$, OR = 1.55) and, moreover, homozygosis for the A allele at IL13 rs20541, known to impact upon receptor ligand affinity, was also significantly increased compared to healthy controls ($P < 0.001$, OR = 4.62). Increased frequencies were also found for A allele at IL4RA rs1805011 (68% versus 49%; $P < 0.0001$, OR = 2.15) and homozygosis for A (47% versus 19%; $P < 0.001$, OR = 3.75) (Table 4). The remaining polymorphisms did not show any relevant difference when AIH-1 and HC groups were compared (Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/679813).

Finally, we carried out analysis using a logistic regression model, which included allele carriage of the different SNPs as well as clinical and laboratory parameters. Three modes of analysis were tested. In the first mode (mode 1), presence of disease was considered as the dependent variable. The results confirmed the findings for both IL13 rs20541 (OR = 9.45 (95% 2.28–39.18) $P = 0.002$) and IL4RA rs1805011 (OR = 3.72 (95% 1.78–7.77) $P < 0.001$). To investigate a possible association of SNPs with pathogenesis of the disease, a second mode (mode 2) of analysis considered each SNP as the dependent variable. The T allele at IL5 rs2069812 showed association with treatment suspension (remission by clinical and laboratory standards) ($P = 0.004$) but was a very rare outcome, present only in 7 patients (7/117, 6%) where 5 achieved regression of fibrosis after treatment (grades IV to II). IL5 is directly involved in eosinophil activation and is a key molecule in allergy and eosinophilic inflammation [33], expressed by $CD4^+$ helper T and B cells, mast cells, and eosinophils. It remains to be seen if an extended analysis confirms this indication. Finally, IgE was considered as the dependent variable in another analysis (mode 3). The presence of the T allele at IL4 rs2227284 showed association with high IgE levels (OR = 7.42 (95% CI 1.33 to 41.34), $P = 0.02$) (Table 5), an expected result.

The genes individually associated with susceptibility to the disease were examined for potential gene-gene interactions. Gene-gene interactions considered grouped genotypes for IL4RA rs1805011, IL4 rs2243250, IL4 rs2070874, rs2227284, IL13 rs20541, and IL5 rs2069812 and the presence of *03 and/or *13 alleles at the HLA-DRB1 locus. Individuals presenting homozygosis for the A allele at IL4RA rs1805011 and HLA-DRB1*03 and/or *13 allele were at six times greater risk to develop the disease (OR = 14.00, $P < 0.001$) compared to the risks conferred by the same alleles individually (HLA-DRB1*03 and/or *13, OR = 8.28; IL4RA rs1805011, OR = 3.72). Individuals homozygous for the A allele at IL13 rs20541,

Table 2: Semiquantitative assessment of the histopathological variables by serum IgE levels in AIH-1 and AIH-2 patients.

Histopathological variables	Score	AIH-1 IgE		AIH-2 IgE	
		Normal $n = 27$ (%)	Increased $n = 33$ (%)	Normal $n = 11$ (%)	Increased $n = 2$ (%)
Structural changes	1-2	2 (7)	1 (3)	1 (9)	0 (0)
	3	2 (7)	2 (6)	1 (9)	1 (50)
	4	23 (86)	30 (91)	9 (82)	1 (50)
Portal inflammation	1-2	8 (30)	11 (33)	5 (45)	0 (0)
	3	15 (55)	11 (33)	4 (36)	1 (50)
	4	4 (15)	11 (33)	2 (18)	1 (50)
Periportal inflammation	1-2	6 (22)	7 (21)	3 (27)	0 (0)
	3	11 (41)	10 (30)	3 (27)	1 (50)
	4	10 (37)	19 (55)	5 (46)	1 (50)
Panacinar necrosis	Present	12 (44)	21 (64)	3 (27)	2 (100)
Plasmocytes	Present	20 (74)	27 (82)	8 (73)	1 (50)
Eosinophils	Present	16 (59)	19 (58)	7 (64)	1 (50)
Rosettes	Present	24 (89)	29 (88)	10 (91)	2 (100)

1 = minimal portal fibrosis; 2 = moderate portal fibrosis; 3 = bridging fibrosis; 4 = cirrhosis.

combined with HLA-DRB1*13 and/or *03 allele also showed a slightly greater risk to develop the disease (OR = 8.88, $P = 0.04$).

4. Discussion

The recurrent presence of plasmocytes and eosinophils in liver biopsies along with the unusual finding of increased circulating IgE antibodies in Brazilian pediatric patients with AIH was the basis for this retrospective study. To further understand if those cells might be disease markers for AIH-1, we investigated gene polymorphisms of cytokines involved in plasmocyte and eosinophil maturation and IgE production. Our hypothesis was that these SNPs might play an additional role in the development of AIH, a disease primarily caused by autoreactive T cells, acting as disease modifiers in synergy with the strongly associated MHC class II HLA-DRB1*13 and *03 alleles in the Brazilian admixed population [34]. Our cross-sectional analysis of laboratory and clinical parameters aimed to distinguish if the increased levels of circulating IgE are markers for the presence of an autoimmune process and therefore present in all patients irrespective of other markers or an indicator of a pathogenic role varying according to disease severity. It is also possible that IgE levels are simply an epiphenomenon caused by widespread inflammatory and immune activity.

The degree of portal inflammation and, especially, parenchymal lesions and interface necroinflammatory activity were remarkable in AIH-1 patients and occurred irrespective of IgE serum levels. A major feature in the present series of analysis was the finding of panacinar necrosis in about half of all patients, again regardless of IgE serum levels, eosinophil count, or other histology characteristics. In spite of liver-infiltrating eosinophils in about 60% of these patients, eosinophil count in peripheral blood of all patients was in the normal range (data not shown). This observation is in accordance with the observed lack of RAST reactivity in the patients. We concluded that despite the high IgE serum levels, the laboratory and clinical findings are not indicative of a concomitant allergy or atopy occurring in these children. In addition, eosinophils have a circulating half-life of only a few hours, with rapid removal of tissues by leukocyte extravasation [35]. In tissues, eosinophils live from 2 to 14 days, especially in liver and spleen. Eosinophils are not usually present in livers from healthy or CMV-infected patients, in contrast to liver transplanted patients, where eosinophil count correlates with degree of rejection [36]. In our patients, and arguably due to the widespread inflammation, not only were eosinophils present but also IgE was identified in most biopsies analyzed. In addition, plasma cells, T and B lymphocytes, and NK cells were also found in the liver of most patients, confirming the generalized inflammatory process. In AIH, T cell-mediated cytotoxicity is believed to be the central mechanism responsible for hepatic damage. In fact, the intriguingly mixed immune profile included also the clearly defined $CD8^+$ cytotoxic T cell periportal infiltration responsible for the piecemeal necrosis that is a hallmark of the disease whereas $CD4^+$ helper T cell and B cells gathered around portal tracts. Of note, in our group of patients, we observed a more modest score in the case of $CD4^+$ helper T cell infiltrating cells than described elsewhere [24].

Our data are similar to a recent study in adult AIH and drug-induced liver injury patients. Infiltrating liver cells were profiled and the presence of eosinophils was detected after standard staining in varying percentages in both groups of patients [37], but tissue IgE was not measured. Added to the unambiguous detection of eosinophils in the biopsies of our group of pediatric patients, we show that liver IgE is present in the vast majority of patients.

Table 3: Immunohistochemical analysis for tissue IgE, liver-infiltrating T and B lymphocytes, and NK cells in the liver of AIH-1 patients, grouped according to serum IgE levels.

Infiltrate	AIH-1 IgE serum levels	
	Normal	Increased
	n = 26 (%)	n = 20 (%)
	IgE	
Negative	3 (12)	1 (5)
Low	11 (42)	11 (55)
Moderate/elevated	8 (31)	8 (40)
Not done	4 (15)	0
	CD3	
Negative	0	0
Low	6 (23)	6 (30)
Moderate/elevated	20 (77)	14 (70)
Not done	0	0
	CD8	
Negative	0	1 (5)
Low	14 (54)	9 (45)
Moderate/elevated	10 (38)	9 (45)
Not done	2 (8)	1 (5)
	CD4	
Negative	6 (23)	6 (30)
Low	9 (35)	7 (35)
Moderate/elevated	11 (42)	6 (30)
Not done	0	1 (5)
	CD20	
Negative	1 (4)	1 (5)
Low	11 (42)	10 (50)
Moderate/elevated	14 (54)	8 (40)
Not done	0	1 (5)
	CD16	
Negative	0	0
Low	13 (50)	11 (55)
Moderate/elevated	13 (50)	5 (25)
Not done	0	4 (20)

Table 4: Genotype and allele frequencies of *IL13* rs20541 and *IL4RA* rs1805011 in children with type 1 autoimmune hepatitis (AIH-1) and in healthy controls (HC).

	AIH-1	HC	P	OR	95% CI
	n = 117	n = 160			
IL13 rs20541	n (%)	n (%)			
Genotype					
AA	18 (15)	6 (4)			
AG	37 (32)	60 (38)	0.003		
GG	62 (53)	94 (58)			
AA versus AG+GG			<0.001	4.62	1.77–12.04
Allele					
A	73 (31)	72 (23)	0.024	1.55	1.06–2.27
G	161 (69)	248 (77)			
IL4RA rs1805011	n = 88	n = 212			
Genotype					
AA	41 (47)	40 (19)			
AG	37 (42)	129 (61)	<0.001		
GG	10 (11)	43 (20)			
AA versus AG+GG			<0.001	3.75	2.18–6.45
Allele					
A	119 (68)	209 (49)	<0.001	2.15	1.49–3.11
G	57 (32)	215 (51)			

IL13 codon 110 (rs20541): A allele = Q (glutamic acid) and G allele = R (arginine); *IL4RA* codon 50 (rs1805011): A allele = I (isoleucine) and G allele = V (valine); n = number of individuals; OR = odds ratio; CI = confidence interval.

Taken together, beyond the characteristic portal and periportal inflammatory cell profile, the ubiquitous presence of IgE deposits, plasma cells, and eosinophils suggests a yet unidentified additional role in the pathogenesis of AIH. In rheumatoid arthritis, the involvement of eosinophils [38] has been linked to IL-5 and TGF-β1, profibrogenic cytokines that contribute to collagen accumulation in tissues [39]. It is possible that, likewise, the excess liver-infiltrating eosinophils take part in the development of the severe fibrosis typical of the disease in young children.

On the other hand, IL4 and IL13 are major cytokines involved in IgE synthesis by B cells [17] and exhibit overlapping functions due to the interaction with the type II receptor composed of the IL4Rα and IL13Rα1 expressed in nonhematopoietic cells and shared by both cytokines [40]. IL13 additionally impacts upon tissue eosinophilia, tissue remodeling, and fibrosis, especially in the liver [17]. We observed an association between presence of the IL13 codon 110 A allele (coding for glutamine) and susceptibility to AIH-1. This variant has been associated with increased IgE levels in both atopic and healthy children [41]. Association with the functional polymorphism coding for valine in the alpha chain of the IL4 receptor was also identified (see multivariate analysis, model 1). Chen et al. (2004) [42] have previously shown that the IL13 glutamine carrying variant displays increased activity compared to the wild type arginine variant. Furthermore, they showed that signal transduction by the variant was further enhanced when the IL4 receptor alpha chain carried valine in position 50. The results suggest that the joint presence of these two polymorphisms in AIH pediatric patients may indeed impact AIH pathology and contribute to disease severity. It is possible that the presence of higher circulating and liver IgE reflects an overall stimulus of the immune system that results in enhanced immunoglobulin levels, which could include target-driven autoantibodies. It remains to be seen if any specific autoantigen is recognized by these IgE antibodies, but without a defined target this analysis remains difficult to be achieved.

The IL4 rs2243250, rs2070874, and rs2227284 SNPs included in this study have been shown to impact IL4 transcriptional activity [43] and IL4 rs2227284 (G>T), which resides in a putative transcription factor binding site, may act independently to regulate IL4 transcription and IgE production. Furthermore, presence of the T allele at IL4

Table 5: Multivariate analysis of factors associated with AIH-1, using three different models.

	Dependent variable	*P*	OR	95% CI
	AIH-1			
Model 1				
*HLA-DRB1**	Different from 03 and/or 13 03 and/or 13	<0.001	8.28	3.46–19.82
IL13 rs20541	AG plus GG AA	0.002	9.45	2.28–39.18
IL4RA rs1805011	AG plus GG AA	0.001	3.72	1.78–7.77
	IL5 rs2069812			
Model 2				
Treatment suspension	Yes[a] No	0.004	6.41	1.83–22.44
	IgE levels			
Model 3				
IL4 rs2227284	TT and GT GG	0.022	7.42	1.33–41.34

Dependent variable in model 1: AIH-1 susceptibility.
Dependent variable in model 2: *IL5* rs2069812.
Dependent variable in model 3: IgE levels.
IL13 codon 110 (rs20541): A allele = Q (glutamic acid) and G allele = R (arginine); *IL4RA* codon 50 (rs1805011): A allele = I (isoleucine) and G allele = V (valine).
[a]Homozigosis for T allele.

rs2227284 has been associated with higher IgE levels in White, African-American, and Hispanic asthma patients [40]. In the multivariate analysis (see model 3), the same T allele was significantly associated with serum IgE levels strengthening our hypothesis of an additional role for the IL4, IL13 cytokine pathway in the pathogenesis of AIH.

5. Conclusion

In conclusion, in agreement with the recurrent observation of high serum IgE levels and presence of eosinophils, plasmocytes, and IgE in the liver of AIH-1 pediatric patients, we have identified novel associations with polymorphic variants of the IL13 gene and the functionally related IL4 receptor alpha chain which suggest IgE-linked immune responses may be involved in the overall susceptibility to AIH-1.

Abbreviations

AIH: Autoimmune hepatitis
MHC: Major Histocompatibility Complex
SNP: Single nucleotide polymorphism
TGF: Transforming growth factor
OR: Odds ratio
CI: Confidence interval
LD: Linkage disequilibrium
HWE: Hardy-Weinberg equilibrium.

Conflict of Interests

All authors declare no conflict of interests regarding the publication of this paper.

Authors' Contribution

Léa Campos de Oliveira designed the study, performed the analyses and interpreted the data, drafted the initial paper, and wrote the report. Anna Carla Goldberg and Gilda Porta designed and supervised the study and wrote the report. Maria Lucia Carnevale Marin performed the analyses and interpreted the data, drafted the initial paper, and wrote the report. Karina Rosa Schneidwind designed the study, performed the analyses, and drafted the initial paper. Amanda Farage Frade performed the analyses. Jorge Kalil supervised the study. Irene Kasue Miura, Renata Pereira Sustovich Pugliese, and Vera Lucia Baggio Danesi were in charge of patient follow-up and clinical data collection. All authors approved the decision to submit the final paper. Anna Carla Goldberg and Gilda Porta contributed equally to the study.

Acknowledgments

The authors thank Venâncio Avancini Ferreira Alves, M.D., Ph.D., and Evandro Sobroza de Mello, M.D., Ph.D., for histological and immunohistochemical analysis and thank Paulo Lisboa Bittencourt, M.D., Ph.D., for his review of the paper. Anna Carla Goldberg and Jorge Kalil are recipients of personal grants for scientific achievement from CNPq. All phases of this study were supported by the Brazilian National Council for Scientific and Technological Development (CNPq) and São Paulo State Research Foundation (FAPESP).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 174062, 5 pages
http://dx.doi.org/10.1155/2015/174062

Research Article

Analysis of a Functional IL-6 Gene Polymorphism in HLAB27 Associated and Intermediate Uveitis Gives New Insight in Disease Pathogenesis and Commonality with Other Autoimmune Diseases

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Received 17 June 2015; Accepted 22 October 2015

Academic Editor: Fulvia Ceccarelli

Purpose. Interleukin 6 (IL-6) plays a crucial role in both adaptive and innate immunity. The rs1800795 gene polymorphism of IL-6 is associated with various autoimmune diseases, like multiple sclerosis. *Methods*. 134 patients with HLAB27 positive iridocyclitis, 84 patients with intermediate uveitis, 132 controls, and 65 HLAB27 positive controls were recruited for the present case-control study. Main outcome measures were genotype distribution and allelic frequencies determined by polymerase chain reaction. *Results*. The frequency of carriers of the minor allele for rs1800795 was significantly higher in patients with intermediate uveitis compared to controls ($p = 0.04$; OR: 1.46; CI: 1.02–2.11). Frequencies of the minor allele for rs1800795 did not differ significantly in patients with HLAB27 associated uveitis when compared to controls ($p > 0.05$). *Conclusion*. These findings further deepen our understanding of the commonality between multiple sclerosis and intermediate uveitis. Given the functionality of the investigated polymorphism, new pathophysiological insights are gained that help to evaluate possible therapeutic targets.

1. Introduction

The treatment of endogenous uveitis can be very challenging because of side effects or lack of efficacy of standard immunomodulatory treatment. Biologicals, such as selective cytokine inhibitors, may offer a more tailored therapeutic venue [1]. However the lack of knowledge on the pathogenesis of uveitis and the differences in between the various uveitis entities, for instance, regarding the specific cytokine patterns, has so far made that approach difficult. Candidate gene analyses give new insights in the pathogenesis of uveitis and may help to find possible therapeutic targets. We for instance have recently found a genetic variant of interleukin 2 receptor alpha (IL2RA) associated with intermediate uveitis (IU), but not with HLAB27 acute anterior uveitis (HLAB27 AAU) [2], suggesting IL2RA as a possible therapeutic target in IU. Treatment with biologicals has to be evaluated with care and separately in each and every disease. TNF alpha-blockers used for the therapy of rheumatoid arthritis have been shown to worsen [3] and to initiate multiple sclerosis (MS) [4, 5]. Thus, the application of biologicals should be based on a thorough knowledge of the pathogenesis which is supported by gene analyses.

Interleukin 6 (IL-6) plays a pivotal role in the immune processes of many diseases. IL-6, together with transforming growth factor- (TGF-) β, induces differentiation of IL-17-producing T helper cells (Th17) [6] that play a crucial role in the development of various autoimmune diseases including systemic lupus erythematosus [7], Behçet's disease [8], or rheumatoid arthritis [9]. On the other hand IL-6 exerts also anti-inflammatory effects via the induction of interleukin-1 and tumor necrosis factor alpha antagonists [10] and it has

been shown that IL-6 deficiency leads to increased expression of other cytokines [11]. Tocilizumab blocks IL-6 signaling [12] and has been approved by the Food and Drug Administration (FDA) for use in selected patients with rheumatoid arthritis [13]. Results in spondyloarthritis were disappointing [14], which highlights the role to assess targets in each disease separately.

The IL-6 gene polymorphism (-174 G/C, rs1800795), which is functional [15, 16] with the G allele increasing IL-6 production, has been found to be associated amongst others with toxoplasmic retinochoroiditis [17] and multiple sclerosis [18]. Interestingly, this polymorphism is not associated with ankylosing spondylitis [19]. Given the fact that IU is the most common form of uveitis in multiple sclerosis patients [20] and since HLAB27 AAU is the most common extra-articular manifestation in ankylosing spondylitis [21], we wanted to evaluate the role of rs1800795 in IU and HLAB27 AAU.

2. Materials and Methods

Study participants were recruited at the Department of Ophthalmology, Medical University Graz, Austria. All participants were Caucasian and were living in the same geographic region in the south of Austria. After detailed explanation of the nature and possible consequences of the study, the patients signed a written informed consent. The study was conducted according to the tenets of the Declaration of Helsinki (2013) and was approved by the ethics committee of the Medical University of Graz.

Gender, age at presentation, age at onset of uveitis, systemic disease association, number of flares, duration of flares, duration between flares, and prevalence of severe ocular complications were recorded in order to characterize the study population. Significant cataract (greater than or equal to 2+ opacity) or secondary glaucoma was documented as complications. SUN criteria [22] were used to define HLAB27 AAU and IU. HLAB27 AAU patients were examined by a rheumatologist for clinical and radiographic signs and symptoms of spondyloarthropathy. In case of symptoms of inflammatory back pain or other symptoms compatible with spondyloarthropathy radiographs of the sacroiliac joints and the spine were made. In order to rule out radiologic signs in accordance with a possible diagnosis of MS such as presence and distribution of white matter lesions, MRI of the brain was obtained in all patients suffering from IU. In case of neurological symptoms patients were examined by a neurologist and a lumbar puncture with testing for oligoclonal bands was performed.

As controls 143 random, unrelated, healthy individuals attending our department for reasons other than ocular inflammation were included. Exclusion criteria were any history of intraocular inflammation, arthritis, lower back pain, autoimmune diseases, or malignancy. None of the controls showed any signs of past uveitis episodes (e.g., residual pigment on lens) in slit-lamp examination. The past medical history was collected following a routine questionnaire. Of course it cannot be ruled out that the controls will eventually develop autoimmune diseases or malignancies in the future. All control subjects were genotyped for HLAB27. Eleven HLAB27 positive controls, together with 54 HLAB27 positive healthy unrelated blood donors, whose DNA was provided by the Department of Blood Serology and Transfusion Medicine, served as the HLAB27 positive control group.

3. Genetics

DNA was extracted from peripheral lymphocytes using the nucleic isolation kit: QIAamp DNA Mini and Blood Kit (QIAGEN; Netherlands) following the manufacturers protocol and stored at −20°C.

Genotype determination was performed using high-resolution melting curve analysis on the LightCycler 480 PCR system. The samples were amplified in duplicate 20 μL reactions using the Light Cycler 480 High Resolution Melting Master kit (Roche Diagnostics, Vienna, Austria) and analyzed on a LC480 instrument I (Roche Diagnostics GmbH, Mannheim, Germany). The final reaction mixture contained 1x Master Mix, 3 mM $MgCl_2$, 4 μM forward and reverse primer, and 50 ng of genomic DNA. For PCR the following cycling conditions were chosen: one cycle of 95°C for 10 minutes followed by 45 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 20 seconds. The amplicons were then denaturated at 95°C for 1 minute, cooled down to 40°C for 1 minute, and then melted from 65°C to 95°C with 25 signal acquisitions per degree. To detect sequence variations the Gene Scanning Software version 1.5 (Roche Diagnostics GmbH, Mannheim, Germany) was used. Samples were automatically grouped because of their melting curves using the Auto Group mode.

4. Statistics

Statistical analysis was performed using PASW 22.0 (SPSS Inc., Chicago, IL). Means were compared using Mann-Whitney U test. Proportions of groups were compared by the χ^2 test. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated by logistic regression. The criterion for statistical significance was $p \leq 0.05$. Hardy-Weinberg equilibrium has been calculated using HW Diagnostics-Version 1 beta (Fox Chase Cancer Center, Philadelphia, PA).

5. Results

For this study 134 patients with HLAB27 AAU (59 female (44.03%)), 84 patients with IU (49 female (58.34%)), 132 HLAB27 negative controls (38 female (28.79%)), and 65 HLAB27 positive controls (31 female (47.70%)) were enrolled. The mean age was 44.81 ± 14.70 for patients with HLAB27 AAU, 30.82 ± 16.66 for patients with IU, 35.56 ± 12.10 for HLAB27 negative controls, and 37.75 ± 4.02 for HLAB27 positive controls. Differences in age between the groups were tolerated since polymorphisms do not change with age.

For rs1800795 an association has been found with the C allele significantly more prevalent in IU patients ($p = 0.04$, allelic OR: 1.46 (1.02–2.11)). No significant differences were found in genotype or allele distribution between patients

Table 1: Distribution of the investigated gene polymorphisms in patients and controls.

	HLAB27 AAU ($n = 134$)	IU ($n = 84$)	HLAB27 negative controls ($n = 132$)	HLAB27 positive controls ($n = 65$)	Controls combined ($n = 197$)
rs1800795					
G/G	40 (29.9%)	19 (22.6%)	51 (38.6%)	24 (36.9%)	75 (38.1%)
G/C	76 (56.7%)	50 (59.5%)	62 (47.0%)	31 (47.7%)	93 (47.2%)
C/C	18 (13.4%)	15 (17.9%)	19 (14.4%)	10 (15.4%)	29 (14.7%)

AAU: acute anterior uveitis.
IU: intermediate uveitis.

Table 2: Baseline ocular and systemic parameters.

Patient characteristics	HLAB27 AAU ($n = 134$)	IU ($n = 84$)
Mean age of onset ± SD (years)	35.64 ± 14.10	25.91 ± 14.59
Mean number of flares ± SD	6.93 ± 7.80	4.24 ± 7.17
Mean duration of flares ± SD (weeks)	4.00 ± 2.74	6.02 ± 6.39
Mean duration between flares ± SD (months)	21.19 ± 18.38	11.29 ± 12.10
Secondary cataract	21 (15.67)	11 (13.10)
Secondary glaucoma	5 (3.73)	2 (2.40)
Ankylosing spondylitis	57 (42.54)	0 (0.00)
Juvenile idiopathic arthritis	1 (0.75)	0 (0.00)
Undifferentiated spondyloarthritis	18 (13.43)	0 (0.00)
Reactive arthritis	5 (3.73)	0 (0.00)
Crohn's disease	1 (0.75)	1 (1.20)
Psoriatic arthritis	12 (9.00)	0 (0.00)
Multiple sclerosis	0 (0.00)	2 (2.40)

HLAB27 AAU: HLAB27 associated acute anterior uveitis.
IU: intermediate uveitis.
SD: standard deviation.
Values are *n* (%) unless otherwise indicated.

with HLAB27 AAU and HLAB27 positive or negative control subjects ($p \geq 0.05$; Table 1). Controls were divided into HLAB27 positive and negatives in order to rule out any confounding effect of HLAB27.

Observed genotype frequencies of both polymorphic markers were in accordance with the Hardy-Weinberg equilibrium (data not shown). No association was found between the investigated polymorphism and the investigated ocular parameters depicted in Table 2. Two patients with IU had MS and they were homozygous for the C allele.

6. Discussion

This is the first study to demonstrate an association of the rs1800795 gene variant and IU. Given the known effect of this polymorphism on IL-6 levels and its association with related diseases our findings give new insights in the pathophysiology of IU and are especially interesting concerning a potential use of IL-6 as therapeutic target.

The C allele at rs1800795 was associated with lower IL-6 levels. Lower plasma levels of IL-6 in healthy individuals [16] and lower LPS-stimulated IL-6 production *ex vivo* were found. Furthermore the C allele was found to bind nuclear protein less avidly than the G allele, which demonstrates possible molecular mechanisms of the investigated alterations [15].

Effects of rs1800795 have been found in a wide variety of conditions including cancer [23], psychiatric diseases [24], arthrosclerosis [25], and even sport performance [26]. In several autoimmune diseases an association with this polymorphism has been reported. Interestingly some diseases are associated with the G allele and some with the C allele. Besides its proinflammatory properties IL-6 has been shown to act anti-inflammatory as well by the induction of interleukin-1 and tumor necrosis factor alpha antagonists [10]. In our study we found the C allele to be the risk allele for IU which is in line with findings in type-1-diabetes [27], Hashimoto's thyroiditis [28], and most importantly MS [18]. Autoimmune conditions are apparently very distinct in their development which leads to two considerations. First, a specific therapy which addresses this heterogeneity might be very competitive compared with the standard regimen in terms of efficacy and safety. Secondly, a targeted treatment which is beneficial for one condition might be harmful in another as this was seen with TNF alpha-blockers [3–5]. Regarding anti-IL-6 treatment the FDA lists nervous system problems including multiple sclerosis as possible serious

side effects of tocilizumab [29]. Given the commonality between MS and IU and taking into account the findings presented here and in previous studies, which suggest parallel pathways of MS and IU, we suggest that evaluation of anti-IL6 in IU should be carried out with caution. A phase 2 clinical trial with Sarilumab, a high affinity IL-6 receptor antibody, in noninfectious uveitis will be completed this year (NCT01900431) and its results are awaited with high interest.

The following potential limitations should be kept in mind, when interpreting our results. Only one gene polymorphism in the IL-6 gene was analyzed, so we cannot rule out that the true causative variant lies somewhere else within the LD block; therefore further sequencing of the IL-6 gene may reveal further associations of other variants. Secondly, as our study population was of European descent our findings might not apply to populations other than Caucasian. Thirdly, we did not measure IL-6 levels in our samples. Furthermore, only two patients had intermediate uveitis and multiple sclerosis. A larger number of patients suffering from both diseases could help to discover further genetic commonalities.

7. Conclusion

In conclusion we found that the functional IL-6 polymorphism rs1800795 is associated with IU but not with HLAB27 AAU. Our findings further highlight the commonality between IU and MS and give new insights in the pathogenesis of IU. Since rs1800795 is known to be functional our results may help to evaluate a possible therapy targeting IL-6.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The authors thank Ms. Elschatti for her skillful technical assistance.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 602154, 6 pages
http://dx.doi.org/10.1155/2015/602154

Research Article

The Association of the *GABRP* Polymorphisms with Systemic Lupus Erythematosus

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Received 19 June 2015; Revised 21 August 2015; Accepted 25 August 2015

Academic Editor: Carlo Perricone

Gamma-aminobutyric acid receptor subunit pi (GABRP) is involved in inhibitory synaptic transmission in the central nervous system. This gene encodes multisubunit chloride channels and is also expressed in numerous nonneuronal tissues such as the uterus and the ovaries. This study was aimed to validate whether the polymorphisms in the GABRP gene are associated with the susceptibility to systematic lupus erythematosus (SLE). The genotype frequencies of the rs929763, rs732157, and rs3805455 of the *GABRP* gene in SLE patients were significantly different from those of the control group ($P < 0.0001$, $P = 0.05$ and 0.002, resp.). Additional analysis showed that the genotype of the rs929763 and rs3805455 of the *GABRP* gene were also significantly associated with female SLE patients ($P < 0.0001$, $P = 0.005$, resp.). Two haplotype frequencies including a major haplotype of *GABRP* SNPs were more significantly different between the SLE patients and the healthy controls ($P = 0.038$ and $4.2E - 24$, resp.). These results suggest that the polymorphisms in the *GABRP* gene might be associated with the susceptibility to SLE and the haplotype of *GABRP* SNPs is useful genetic marker for SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that can affect almost any organ system and has erratic manifestations and follows a relapsing and remitting course. More than 90% of cases of SLE occur in women, frequently starting at childbearing age. The exact cause of SLE is not known, but several factors have been associated with the disease including genetic, environmental, and hormonal factors [1, 2]. Although SLE is not linked to a certain gene, the people with SLE often have members of their family with other autoimmune conditions. The treatment for SLE is not curative but mainly focused on lessening the symptoms and reducing its long term complications. Treatment options can vary depending on the severity and the location of the body or organ that are affected. These include corticosteroids, immunosuppressants, or antimalarial drugs.

We previously made a customized 3K SNPs chip containing the presumable SNPs associated with various immune disorder, such as rheumatic arthritis (RA) and SLE, and carried out a pilot study using the genomic DNA samples of RA and SLE patients. We identified 16 candidate genes, including the gamma-aminobutyric acid receptor pi (*GABRP*), epithelial stromal interaction 1 (*EPSTI1*), bone morphogenetic protein 6 (*BMP6*), integrin beta 5 (*ITGB5*), spectrin repeat containing nuclear envelope 1 (*SYNE1*), and TIMP metallopeptidase inhibitor 3 (*TIMP3*), from SLE (Our not published data). These results led us to determine whether the SNPs of the *GABRP* gene are associated with the susceptibility of SLE.

The gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain, mediates neuronal inhibition by binding GABRP, a multisubunit chloride channel, and opening an integral chloride channel. Transcripts

of this subunit could be detected in several human tissues, especially in the uterus, in which the function of the receptor appears to be related to tissue contractility [3, 4]. Variations at the *GABA* receptor gene family are associated with susceptibility to neuropsychiatric bipolar schizoaffective disorder [5, 6]. The expression levels of *GABRP* genes are relatively high in normal and benign human epithelial cells of the breast compared to other normal tissues and even compared with neuronal tissues of the CNS. There is progressive downregulation in concordance with tumor progression in sporadic breast cancer tissues [7]. Expression of GABRP is also associated with the basal-like/triple negative subtype, brain metastases and poorer prognosis [8].

In this study, we analyzed the allelic and genotypic frequencies of *GABRP* SNPs between the SLE patients and the healthy controls. Furthermore, we investigated haplotype frequencies constructed by these SNPs in both groups.

2. Materials and Methods

2.1. Patients and DNA Samples. The DNA samples used in this study were provided by the Biobank of Wonkwang University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health and Welfare Affairs. On the basis of approval and informed consent from the institutional review board, we obtained the genomic DNAs from 164 SLE patients (12 males and 152 females) and 528 healthy controls (329 males and 199 females). Mean ages of SLE patients and healthy controls were 40.6 years and 34.4 years, respectively. Genomic DNA was extracted from peripheral blood leukocytes by using a standard phenol-chloroform method or by using a Genomic DNA Extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's directions. SLE patients were recruited from the outpatient clinic at Chonnam National University Hospital. SLE was diagnosed according to criteria of the American College of Rheumatology (ACR) [9]. Antinuclear antibody (ANA) levels in SLE patients were determined in a routine laboratory at Chonnam National University Hospital. The controls were recruited from the general population and had received comprehensive medical testing at the Wonkwang University Hospital.

2.2. SNP Selection and Genotype Analysis. We selected six SNPs (rs929763 [T/A, intron 1], rs732157 [C/T, intron 1], rs2303134 [Asn55Ser, A/G, exon 3], rs1063310 [Phe391Len, C/A, exon 10], rs3805455 [C/T, 3′-UTR], and rs3828619 [G/A, 3′-UTR]) of the *GABRP* based on their location, minor allele frequencies (MAF < 0.05), and linkage disequilibrium (LD) analysis from NCBI SNP database. Genotyping was performed by high resolution melting (HRM) analysis. The 10 μL reaction mixture was made up using 1x QuantiTect Probe PCR Kit (Qiagen, USA) and consisted of 50 ng of genomic DNA, 100 nM of each primer (Table 1), and 1x Evagreen solution (Biotium, USA). PCR cycling and HRM analysis were carried out using the Rotor-Gene thermal cycler RG6000 (Corbett Research, Australia). The PCR was performed in the following conditions: one cycle at 95°C for 15 min and 45 cycles of 95°C for 15 sec, 68°C for 10 sec, and 72°C for 30 sec. Optical measurements in the green channel (excitation at 470 nm and detection at 519 nm) were recorded during the extension step. After completion of 45 cycles, melting-curve data were generated by increasing the temperature from 77 to 95°C at 0.1°C per second and recording fluorescence. HRM curve analysis was performed using the software Rotor-Gene 1.7.40 and the HRM algorithm provided.

Table 1: Primer sequences used for genotyping of *GABRP* gene.

Primer	Sequence (5′ → 3′)	SNP
GABRP-F1	TCCAGGTGTCTGGAGAGAGG	rs929763
GABRP-R1	CAGCAGGGGCTCTAATCTTG	
GABRP-F2	TGGCAATAATGCCTTTCCTC	rs732157
GABRP-R2	TTGTCACCTCCAGCTTCCTT	
GABRP-F3	CCTGGCTTTGAGAACCTCAC	rs2303134
GABRP-R3	TAAAACCTCCCGAAACCCTC	
GABRP-F4	AGATCAGCTTTGCCAGCATT	rs1063310
GABRP-R4	CAACAATCCTGCCCATCTTT	
GABRP-F5	GCTGGCCCTGAGTACTGAAC	rs3805455
GABRP-R5	CTCTGGCTGGATTTGGAGAG	
GABRP-F6	GAGCCACAGGTTCTCATTCC	rs3828619
GABRP-R6	CCTCTTTTCACCAGCACTCC	

2.3. Statistical Analysis. SLE patients and healthy control groups were compared using case-control association analysis. The χ^2 test was used to estimate Hardy-Weinberg equilibrium (HWE). Allele frequency was defined as the percentage of individuals carrying the allele among the total number of individuals. Logistic regression analyses (SPSS 11.5) were used to calculate odds ratios (95% confidence interval) for SNP sites. Linkage disequilibrium (LD) analyses by pair-wise comparison of biallelic loci and haplotype frequencies of the *GABRP* gene for multiple loci were estimated using the expectation maximization (EM) algorithm with SNPAlyze software (DYNACOM, Yokohama, Japan). The ANOVA was applied to analyze differences between the genotype and ANA levels in SLE patients. A P value of less than 0.05 was considered an indication of statistical significance.

3. Results

The human *GABRP* gene is located on chromosome 5q35.1 and consists of 10 (NM_014211.2, isoform 1) or 9 exons (NM_001291985.1, isoform 2). The *GABRP* gene was identified as a candidate gene associated to SLE by our previous pilot study using customized 3K SNPs chip (our data not published).

To determine whether the six *GABRP* SNPs are associated with SLE susceptibility, the genotypes of the *GABRP* polymorphisms were analyzed by HRM method, and the genotype and allelic frequencies between the both groups were compared. All the genotype frequencies in both the healthy controls and the SLE patients were consistent with HWE, except for rs3828619 in SLE (data not shown). The genotype frequencies in four of the six *GABRP* SNPs, rs929763, rs732157, rs3805455, and rs3828619, in the SLE

Table 2: Genotype and allele analyses of the *GABRP* gene SNPs in SLE patients and healthy controls.

Position[a]	Genotype/allele	Control *n* (%)	SLE *n* (%)	Odds ratio[b] (95% CI)	P^c
rs929763	TT	260 (50.4)	68 (42.5)	1.00	**<0.0001**
	TA	222 (43.0)	61 (38.1)	1.05 (0.71–1.55)	
	AA	34 (6.6)	31 (19.4)	3.49 (2.00–6.07)	
	T	742 (71.9)	197 (61.6)	1.00	**0.0005**
	A	290 (28.1)	123 (38.4)	1.60 (1.23–2.08)	
rs732157	CC	261 (50.5)	96 (59.3)	1.00	**0.05**
	CT	218 (42.2)	57 (35.2)	0.71 (0.49–1.03)	
	TT	38 (7.4)	9 (5.5)	0.64 (0.30–1.38)	
	C	740 (71.6)	249 (76.9)	1.00	0.06
	T	294 (28.4)	75 (23.1)	0.76 (0.57–1.02)	
rs2303134	AA	518 (98.1)	162 (98.8)	1.00	0.57
	AG	10 (1.9)	2 (1.2)	0.64 (0.14–2.95)	
	GG	0 (0.0)	0 (0)	—	
	A	1046 (99.1)	326 (99.4)	1.00	0.57
	G	10 (0.9)	2 (0.6)	0.64 (0.14–2.94)	
rs1063310	CC	233 (45.0)	82 (50.9)	1.00	0.18
	CA	237 (45.8)	66 (41.0)	0.79 (0.55–1.15)	
	AA	48 (9.3)	13 (8.1)	0.77 (0.40–1.49)	
	C	703 (67.9)	230 (71.4)	1.00	0.23
	A	333 (32.1)	92 (28.6)	0.84 (0.64–1.11)	
rs3805455	CC	226 (43.9)	63 (40.4)	1.00	**0.002**
	CT	243 (47.2)	65 (41.7)	0.96 (0.65–1.42)	
	TT	46 (8.9)	28 (17.9)	2.18 (1.26–3.77)	
	C	695 (67.5)	191 (61.2)	1.00	**0.04**
	T	335 (32.5)	121 (38.8)	1.31 (1.01–1.71)	
rs3828619	GG	299 (57.2)	73 (48.7)	1.00	**0.003**
	GA	181 (34.6)	53 (35.3)	1.20 (0.80–1.79)	
	AA	43 (8.2)	24 (16.0)	2.29 (1.30–4.01)	
	G	779 (74.5)	199 (66.3)	1.00	**0.005**
	A	267 (25.5)	101 (33.7)	1.48 (1.12–1.95)	

[a]Calculated from the translation start site.
[b]Logistic regression analyses were used for calculating OR (95% CI; confidence interval).
[c]Value was determined by Fisher's exact test or χ^2 test from a 2 × 2 contingency table.

group were significantly different from the healthy control group ($P < 0.0001$, $P = 0.05$, 0.002, and 0.003, resp.; Table 2). The allele frequencies of *GABRP* SNPs, rs929763, rs3805455, and rs3828619, in the SLE group also were significantly different from those in the healthy control group ($P = 0.0005$, 0.04, and 0.005, resp.; Table 2). These results strongly suggest that the SNPs of *GABRP* appear to be associated with SLE susceptibility. Since SLE, like many other autoimmune diseases, affects females more frequently than males, at a rate of almost 9 to 1 [10], we independently analyzed the 152 female patients (92.7%) out of the total of 164 SLE patients recruited in the study, in order to find out if gender has an important role in the high susceptibility of the *GABRP* SNPs and SLE. The genotype frequencies of the rs929763, rs3805455, and rs3828619 in female SLE patients were statistically different from those of female healthy controls ($P < 0.0001$, $P = 0.005$ and 0.008, resp.; Table 3).

We further estimated the haplotype frequencies of SNPs (rs929763, rs732157, rs2303134, rs1063310, and rs3805455) of the *GABRP* gene between healthy controls and SLE patients (Table 4). Out of 32 possible haplotypes, three haplotypes (TCACC, ACACC, and ATAAT) were identified as the main haplotypes (>5%) in both groups (Table 4). The distribution of the major haplotype (TCACC) was significantly different in the SLE patients compared to that of the healthy controls ($P = 0.038$). Interestingly, the distribution frequency of the ACACT haplotype in SLE patients group was hugely different than that in healthy controls group ($P = 4.2E - 24$; Table 4). This ACACT haplotype is distributed very infrequently in healthy control groups (0.064%), compared to it being one

Table 3: Genotype and allele analyses of the *GABRP* gene polymorphisms in the female SLE patients and female healthy controls.

Position[a]	Genotype/allele	Control *n* (%)	SLE *n* (%)	Odds ratio[b] (95% CI)	P^c
rs929763	TT	109 (56.8)	63 (42.6)	1.00	**<0.0001**
	TA	73 (38.0)	56 (37.8)	1.33 (0.83–2.12)	
	AA	10 (5.2)	29 (19.6)	5.02 (2.29–10.98)	
	T	291 (75.8)	182 (61.5)	1.00	**<0.0001**
	A	93 (24.2)	114 (38.5)	1.96 (1.410–2.73)	
rs732157	CC	108 (54.8)	89 (59.3)	1.00	0.40
	CT	76 (38.6)	52 (34.7)	0.83 (0.53–1.30)	
	TT	13 (6.6)	9 (6.0)	0.84 (0.34–2.06)	
	C	292 (74.1)	230 (76.7)	1.00	0.44
	T	102 (25.9)	70 (23.3)	0.87 (0.61–1.24)	
rs2303134	AA	193 (97.0)	150 (98.7)	1.00	0.29
	AG	6 (3.0)	2 (1.3)	0.43 (0.09–2.16)	
	GG	0 (0)	0 (0)	—	
	A	392 (98.5)	302 (99.3)	1.00	0.29
	G	6 (1.5)	2 (0.7)	0.43 (0.09–2.16)	
rs1063310	CC	86 (43.9)	77 (51.7)	1.00	0.1
	CA	95 (48.5)	59 (39.6)	0.69 (0.44–1.09)	
	AA	15 (7.6)	13 (8.7)	0.97 (0.43–2.16)	
	C	267 (68.1)	213 (71.5)	1.00	0.34
	A	125 (31.9)	85 (28.5)	0.85 (0.61–1.19)	
rs3805455	CC	89 (44.9)	60 (41.7)	1.00	**0.005**
	CT	93 (47.0)	58 (40.3)	0.93 (0.58–1.47)	
	TT	16 (8.1)	26 (18.1)	2.41 (1.19–4.87)	
	C	271 (68.4)	178 (61.8)	1.00	**0.07**
	T	125 (31.6)	110 (38.2)	1.34 (0.97–1.84)	
rs3828619	GG	113 (57.4)	68 (48.9)	1.00	**0.008**
	GA	71 (36.0)	50 (36.0)	1.17 (0.73–1.87)	
	AA	13 (6.6)	21 (15.1)	2.68 (1.26–5.71)	
	G	297 (75.4)	186 (66.9)	1.00	**0.02**
	A	97 (24.6)	92 (33.1)	1.51 (1.08–2.13)	

[a]Calculated from the translation start site.
[b]Logistic regression analyses were used for calculating OR (95% CI; confidence interval).
[c]Value was determined by Fisher's exact test or χ^2 test from a 2 × 2 contingency table.

Table 4: Haplotype frequencies of *GABRP* SNPs in SLE patients and healthy controls.

Haplotype					Frequency[a]		Chi-square	P^b
rs929763	rs732157	rs2303134	rs1063310	rs3805455	Control	SLE		
T	C	A	C	C	0.406	0.345	3.580	**0.03**
A	C	A	C	C	0.228	0.220	0.091	0.803
A	T	A	A	T	0.223	0.185	1.942	0.166
A	C	A	A	T	0.037	0.057	2.516	0.334
T	C	A	A	T	0.044	0.026	1.893	0.431
A	C	A	C	T	6.4*E* − 4	0.105	102.6	**4.2E − 24**
Other					0.062	0.062	—	—

[a]Values were constructed by EM algorithm with genotyped SNPs.
[b]Values were analyzed by permutation test or Chi-square.

Table 5: Haplotype frequencies of *GABRP* SNPs in female SLE patients and female healthy controls.

Haplotype					Frequency[a]		Chi-square	P^b
rs929763	rs732157	rs2303134	rs1063310	rs3805455	Control	SLE		
T	C	A	C	C	0.645	0.514	11.31	**7.7E − 4**
A	T	A	A	T	0.195	0.186	0.099	0.753
T	C	A	A	T	0.097	0.070	0.585	0.444
A	C	A	C	T	3.2*E* − 30	0.113	44.36	**2.7E − 11**
A	C	A	C	C	0.013	0.043	5.917	**0.015**
Other					0.050	0.074	—	—

[a]Values were constructed by EM algorithm with genotyped SNPs.
[b]Values were analyzed by permutation test or Chi-square.

of the main haplotype in the SLE patient group (10.5%). This haplotype frequency difference of TCACC and ACACT in both groups is especially true when only the female subjects were analyzed ($P = 7.7E - 4$ and $2.7E - 11$, resp.; Table 5). These results suggest that *GABRP* polymorphisms might be an important genetic factor associated with SLE susceptibility.

Finally, to define a possible correlation between *GABRP* polymorphisms and the clinical features of SLE, we further analyzed the difference of the antinuclear antibody (ANA) levels according to each genotype of the SLE patients. We found that these SNPs in the SLE patients have no significant association with the levels of ANA (Table 6).

Table 6: The levels of ANA among the genotypes of polymorphisms of *GABRP* gene in SLE patients.

SNP	Genotype	ANA			P^a
		N	Mean	SD	
rs929763	TT	41	577.1	1.7*E* + 3	0.720
	TA	44	874.6	3.1*E* + 3	
	AA	27	471.1	6.3*E* + 2	
rs732157	CC	67	510.7	1.3*E* + 3	0.478
	CT	40	986.0	3.3*E* + 3	
	TT	7	205.7	2.0*E* + 2	
rs2303134	AA	115	652.5	2.2*E* + 3	0.880
	AG	1	320.0	—	
	GG	0	—	—	
rs1063310	CC	56	559.6	1.4*E* + 3	0.731
	CA	49	834.3	3.0*E* + 3	
	AA	8	300.0	2.3*E* + 2	
rs3805455	CC	39	621.0	1.7*E* + 3	0.684
	CT	47	863.0	3.1*E* + 3	
	TT	23	372.2	5.0*E* + 2	
rs3828619	GG	48	536.3	1.5*E* + 3	0.521
	GA	38	1031.6	3.4*E* + 3	
	AA	19	425.3	5.4*E* + 2	

[a]Values were analyzed by ANOVA.

4. Discussion

SLE is an autoimmune disorder with autoantibody-mediated tissue damage. SLE is clinically characterized by heterogeneous symptoms which involves almost all organs in the body. Management of this disease is complex and usually involves many different specialties for optimal patient management [11]. We previously reported that SNPs in the forkhead-box J1 (*FOXJ1*), interleukin coactosin-like 1 (*COTL1*), and thymic stromal lymphopoietin receptor (*TSLPR*) genes are associated with susceptibility to SLE in a Korean population [12–14]. In this study, we evaluated the association between *GABRP* polymorphisms and susceptibility to SLE.

We previously identified the several candidate genes including *GABRP* gene associated with SLE by our pilot study using customized 3K SNPs chip (our data not published). The mRNA expression levels of GABRP in 23 normal human tissues were diagrammatically presented in a study done by Zafrakas et al. [7]. Five out of the 23 normal tissues were directly or indirectly related to human lymphoreticular and immune system (lymph node, thymus, bone marrow, spleen, and liver). Among them, none of them showed any significant increase in *GABRP* mRNA expression. The main physiologic function of GABRP in nonneuronal tissue and its role in disease is not well known. Only a few manuscripts have been acknowledged for having association, and these are mostly limited to breast cancer and neuropsychiatric disorders. Our putative data has suggested that the polymorphism of the human *GABRP* gene is strongly associated with the susceptibility to SLE (Tables 2 and 3). It could be promptly argued that the expression levels of GABRP and its involvement in the immune system could be difficult to connect. Only a single study was published evaluating the relationship between acute and chronic rejections of renal allografts to 345 genes that provided potential relevance to renal allograft rejection. This study demonstrated that there was increased expression of GABRP along with 8 other genes in the grafts that failed [15].

We demonstrated that the genotype frequencies of *GABRP* polymorphisms (rs929763, rs732157, and rs3805455) in SLE patients were significantly different from that of the healthy control group to such a degree that defies any possibility of random chance (Table 2). Specifically the genotype and allele frequencies of rs929763 had very high associations ($P < 0.0001$ and 0.0005, resp.). This strong association is also true when the subjects are confined to the female population (Table 3). These results confidently led us to think that *GABRP* gene polymorphism have a strong influence on the susceptibility to SLE.

The distributions of the major haplotypes (TCACC) of the *GABRP* SNPs, rs929763, rs732157, rs2303134, rs1063310, and rs3805455, in the SLE patients were significantly different from that of the healthy controls ($P = 0.038$; Tables 4 and 5). The frequency of other haplotype (ACACT) of the *GABRP* SNPs was more profoundly different between both groups ($P = 4.2E - 24$). These results suggest that *GABRP* polymorphisms might be an important genetic factor associated with SLE susceptibility.

We also compared ANA levels among the genotypes of polymorphisms of *GABRP* gene in SLE (Table 6). The ANA test detects the autoantibodies present in an individual's blood serum. ANA titers are useful in diagnosis of various autoimmune disorders including SLE and monitoring levels help to predict the progression of disease [16, 17]. We could not find any correlation with ANA level between the genotypes of *GABRP* polymorphism, suggesting that the *GABRP* polymorphism is only linked to SLE susceptibility and is not associated with disease progression.

In conclusion, the results of this study strongly suggest that the *GABRP* gene might be a candidate gene associated with the susceptibility of SLE, and our result also indicates that the haplotypes of the *GABRP* polymorphisms might be one of the influential genetic markers for SLE susceptibility. Although it is not clear how the *GABRP* polymorphisms are related to the pathogenesis of SLE, our results could provide valuable resource for further functional studies of the *GABRP* gene and its relationship with other various autoimmune or inflammatory disorders. Actually *GABRP* gene is located in 5q3 locus.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Hun-Soo Kim and Eun-Heui Jin contributed equally to this work.

Acknowledgments

The DNA samples used in this study were provided by the Biobank of Wonkwang University Hospital, a member of the National Biobank of Korea; this Biobank is supported by the Ministry of Health and Welfare. This research was supported by Wonkwang University in 2013.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 101879, 8 pages
http://dx.doi.org/10.1155/2015/101879

Research Article

Polymorphisms Associated with Age at Onset in Patients with Moderate-to-Severe Plaque Psoriasis

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Received 18 June 2015; Accepted 15 September 2015

Academic Editor: Fulvia Ceccarelli

Psoriasis is a chronic skin disease in which genetics play a major role. Although many genome-wide association studies have been performed in psoriasis, knowledge of the age at onset remains limited. Therefore, we analyzed 173 single-nucleotide polymorphisms in genes associated with psoriasis and other autoimmune diseases in patients with moderate-to-severe plaque psoriasis type I (early-onset, <40 years) or type II (late-onset, ≥40 years) and healthy controls. Moreover, we performed a comparison between patients with type I psoriasis and patients with type II psoriasis. Our comparison of a stratified population with type I psoriasis ($n = 155$) and healthy controls ($N = 197$) is the first to reveal a relationship between the *CLMN*, *FBXL19*, *CCL4L*, *C17orf51*, *TYK2*, *IL13*, *SLC22A4*, *CDKAL1*, and *HLA-B/MICA* genes. When we compared type I psoriasis with type II psoriasis ($N = 36$), we found a significant association between age at onset and the genes *PSORS6*, *TNF-α*, *FCGR2A*, *TNFR1*, *CD226*, *HLA-C*, *TNFAIP3*, and *CCHCR1*. Moreover, we replicated the association between rs12191877 (*HLA-C*) and type I psoriasis and between type I and type II psoriasis. Our findings highlight the role of genetics in age of onset of psoriasis.

1. Introduction

Psoriasis is a chronic inflammatory skin disorder with a major genetic component. The prevalence of chronic plaque psoriasis is around 2% in the general population [1]. The many genetic studies performed in recent years showed that genes such as interleukin 23 receptor (*IL23R*) and *IL12B* and tumor necrosis factor alpha (*TNFα*) are closely associated with psoriasis and related diseases such as rheumatoid arthritis, psoriatic arthritis, and Crohn's disease [2]. Human leukocyte antigen C (HLA-C)*0602 is the allele most closely associated with this disease [3].

The age at onset of psoriasis follows a bimodal distribution [4]: type I psoriasis appears before the age of 40 years (early-onset), with a peak at 16–22 years; type II psoriasis appears after the age of 40 years (late-onset), with a peak at 57–60 years [5]. Type I psoriasis has been associated with several single-nucleotide polymorphisms (SNPs) in genes associated with the immune response (Table 1). For example, HLA-C*0602 is more strongly associated with type I psoriasis

Table 1: SNPs associated with type I (early-onset) and type II (late-onset) psoriasis: an update.

SNP	Gene	Function[†]	Association with			References
			Ps type I	Ps type II	Ps type I versus type II	
—	HLA-C*0602	Encodes a class I molecule which plays a central role in the immune system by presenting peptides derived from endoplasmic reticulum lumen	X	X		[6–10]
—	HLA-C*12:02			X		[8]
rs1265181	*HLA-C*		X		X	[11]
rs12191877			X*		X*	[7, 11]
rs4406273			X		X	[11]
rs2395029			X			[11]
rs10484554			X	X	X	[7, 10, 12]
rs13191099	*HLA-A*			X		[4]
rs10876882				X		[4]
rs33980500	*TRAF3IP2*	Encodes a protein involved in regulating responses to cytokines by members of the Rel/NF-kappa-B transcription factor family	X			[11]
rs71562288				X		[4]
rs2233278	*TNIP1*	Encodes A20-binding protein which plays a role in autoimmunity and tissue homeostasis through the regulation of nuclear factor kappa-B activation			X	[11]
rs17728338	*TNIP1*				X	[11]
rs1295685	*IL13*	Encodes a cytokine involved in several stages of B cell maturation and differentiation	X			[11]
rs17716942	*IFIH1*	Encodes an Asp-Glu-Ala-Asp box protein (putative RNA helicases)	X			[7]
rs1990760				X		[4]
rs27524	*ERAP1*	Encodes an aminopeptidase involved in trimming HLA class I-binding precursors	X			[6][#]
rs11209026	*IL23R*	Encodes a subunit of the receptor for IL23A/IL23	X			[6][#]
rs72676067				X		[4]
rs10876882	*IL23A*	Encodes a subunit of IL23 involved in immune responses		X		[4]
—	*LCE3B/LCE3C-del*	Encodes precursors of the cornified envelope of the stratum corneum	X			[6, 13][#]
rs2546890	*IL12B*	Encodes a subunit of IL12 that acts on T and natural killer cells	X	X		[4, 14]
rs60813083	*RNF114*	Encodes a protein that may play a role in spermatogenesis		X		[4]
rs887998	*IL1R1*	Encodes a receptor for IL1 involved in inflammatory responses		X		[4]
rs16944	*IL1B*	Encodes a cytokine produced by activated macrophages and is involved in immune responses, cell proliferation, differentiation, and apoptosis		X		[4, 15]
rs2853550				X		[4]
rs26653	*ERAP1*	Encodes an aminopeptidase involved in trimming HLA class I-binding precursors so that they can be presented on the MHC class I molecule	X			[10]
rs30187			X			[10]
rs2227473	*IL22*	Encodes an interleukin 22 that contributes to the inflammatory response	X			[16]
rs2227483			X			[16]
INDEL rs35774195/rs10784699			X			[16]
rs6822844	*IL2/IL21*	Encode cytokines that are important in the innate and adaptive immune responses by inducing differentiation, proliferation, and activity of multiple target cells including macrophages, natural killer cells, B cells, and cytotoxic T cells	X			[17]
rs2069778			X			[17]

Table 1: Continued.

SNP	Gene	Function[†]	Association with			References
			Ps type I	Ps type II	Ps type I versus type II	
rs6311	*HTR2A*	Encodes a receptor for neurotransmitter serotonin		X		[18]
rs12459358	*PSORS6*	Encodes genetic locus associated with susceptibility to psoriasis	X		*	[19]
rs1800629	*TNF-α*	Encodes a cytokine secreted by macrophages and involved in the regulation of cell proliferation, differentiation, and apoptosis, as well as in lipid metabolism and coagulation	X			[20, 21]
rs361525			X		*	[15, 20–24]
rs3733197	*BANK1*	Encodes a protein involved in B cell receptor-induced calcium mobilization from intracellular stores	X			[25]
rs755622	*MIF*	Encodes a lymphokine involved in cell-mediated immunity, immunoregulation, and inflammation		X	X	[26]
rs6693899	*IL10*	Encodes a cytokine produced by monocytes and lymphocytes and involved in immunoregulation and inflammation		X	X	[27]
rs1800896				X		[28]
rs4341	*ACE*	Encodes an enzyme involved in catalyzing the conversion of angiotensin I into a physiologically active peptide angiotensin II	X			[29]
SNPs at positions -1540, -1512, -1451, -460, and -152	*VEGFA*	Encodes a protein involved in angiogenesis, vasculogenesis, endothelial cell growth, promotion of cell migration, and inhibition of apoptosis		X		[30]
SNPs at positions -386 and -404	*CCHCR1*	Encodes a protein that may be a regulator of keratinocyte proliferation or differentiation	X			[31]
—	CCHCR1* WW allele		X			[9]

SNP: single-nucleotide polymorphism; Ps: psoriasis; [†]Information available at NCBI (http://www.ncbi.nlm.nih.gov/gene) or GeneCards (http://www.genecards.org/); [#]Study performed in pediatric-onset psoriasis (patients <18 years); *Association found in our study.

than with type II psoriasis [5]. Although several association studies have already been performed in psoriasis in both populations (type I or type II psoriasis patients), knowledge of age at onset remains limited and controversial (Table 1) [6]. Therefore, we performed a candidate gene study, where we evaluated genetic susceptibility to type I or type II psoriasis in patients with moderate-to-severe chronic plaque psoriasis. This approach may help us to identify SNPs previously associated with psoriasis or other autoimmune diseases [2] that are specific to type I or type II psoriasis. Furthermore, our genetic study could improve our understanding of psoriasis and of its etiology and pathogenesis.

2. Material and Methods

2.1. Experimental Design. We recruited 198 Caucasian patients with moderate-to-severe plaque type psoriasis (psoriasis area and severity index > 10) who attended the department of dermatology in four university hospitals in Madrid between 16/10/2007 and 17/12/2012. Five samples did not fulfill the quality criteria of the Human Genotyping Unit-CeGen (CEGEN, Spanish National Cancer Research Centre, Madrid, Spain), and 2 samples had insufficient volume. We also included 197 healthy volunteers (controls) recruited between 10/01/2011 and 14/12/2012 from the Clinical Pharmacology Service (Hospital Universitario de la Princesa, Madrid, Spain). All the volunteers were Caucasian and had no personal or family history of psoriasis (at least 2 generations).

The protocol fulfilled Spanish law on biomedical research and was approved by the Ethics Committee for Clinical Investigation of Hospital Universitario de la Princesa. All controls and patients gave their written informed consent to donate a sample for investigation. The samples are kept in the Clinical Pharmacology Service.

2.2. Selection of the Polymorphisms. We preselected 320 SNPs based on an extensive review of 449 articles describing the association between polymorphisms and psoriasis and response to biological drugs and psoriasis and related inflammatory diseases (rheumatoid arthritis, psoriatic arthritis, and Crohn's disease) [2]. We finally selected 192 SNPs based on minor allele frequency (≥0.05) and on the results of studies performed in Caucasians and psoriatic patients. Information on the 173 SNPs analyzed can be found in supplementary Table S1, which is published in [3].

2.3. Sample Processing. A 3-mL peripheral blood sample was extracted from each subject in EDTA tubes. DNA was obtained from samples using an automatic DNA extractor (MagNa Pure System, Roche Applied Science, USA) and its concentration was quantified in Nanodrop ND-1000 Spectrophotometer (Wilmington, USA). The extracted DNA was stored at −80°C in the Clinical Pharmacology Service until use.

2.4. Genotyping. A total of 196 samples from patients (2 samples of 198 cases had insufficient volume) and 197 samples from controls were sent to the Human Genotyping Unit-CeGen to genotype 192 SNPs. The analysis was performed using the Illumina Veracode genotyping platform. If fluorescence was low or the genotype clusters were undifferentiated, the SNPs were removed. In addition, if the call rate was less than 95% of the average of the 192 SNPs analyzed, the samples were removed. Since CEGEN quality criteria were not met in 19 SNPs and 5 patients, we finally analyzed 173 SNPs in 191 patients and 197 controls.

2.5. Statistical Analysis. The statistical analysis was performed to compare the following stratified populations: patients with type I psoriasis ($N = 155$) or type II psoriasis ($N = 36$) versus controls ($N = 197$) and patients with type I psoriasis versus cases with type II psoriasis. Hardy-Weinberg equilibrium was tested for all the SNPs analyzed using the SNPStats program [32]. Allele and genotype frequencies were also calculated using the SNPStats program. SNPs that were not in Hardy-Weinberg equilibrium in controls were removed from the subsequent analysis [33].

The univariate analysis was performed using R 3.0.2. (SNPassoc) [34]. We constructed various logistic regression models depending on the main types of inheritance (codominant, dominant, recessive, and additive). In the additive model, the presence of 2 mutant alleles confers double the risk of 1 mutant allele [33]. The results were adjusted for rs12191877 (SNP that is strongly associated with the HLA-C*0602 allele and is highly prevalent in our population) [3, 35]. The optimal model was selected using the lower Akaike Information Criterion (AIC). Subsequently, SNPs with $p < 0.1$ in the univariate analysis (adjusted for rs12191877) were included in a multivariate logistic regression model to adjust for relevant confounding factors (SPSS 15.0). The results of the univariate analysis were adjusted for rs12191877, except when we compared patients with type I psoriasis and patients with type II psoriasis (the influence of rs12191877 was not very relevant). We expressed the results as the odds ratio (OR), 95% confidence interval, and p value.

3. Results

3.1. Study Population. The study population included 155 patients with moderate-to-severe chronic plaque type I psoriasis (92 men and 63 women), 36 patients with type II psoriasis (19 men and 17 women), and 197 controls (98 men and 99 women). The mean age was 46.01 ± 13.11 years in patients with type I psoriasis (45.72 ± 11.69 in men and 46.43 ± 15.04 in women), 67.72 ± 11.85 years in patients with type II psoriasis (65.95 ± 11.18 in men and 69.71 ± 12.59 in women), and 24.51 ± 4.29 years in the controls (25.07 ± 4.94 in men and 23.95 ± 3.46 in women). The mean age at onset of psoriasis was 23.31 ± 8.52 in patients with type I psoriasis and 52.58 ± 10.45 in patients with type II psoriasis. Analysis of the effect of sex on our results revealed no significant association.

3.2. Genotyping Results. A total of 192 SNPs were analyzed (see supplementary Table S1 published in [3]). However, only 173 SNPs fulfilled the quality criteria. One SNP was monomorphic (rs165161 in the *JUNB* gene) and was excluded from the statistical analysis. The genotyping success rate was 89.82%, and the reproducibility rate was 100%.

Table 2: Results of univariate linear regression analysis (unadjusted and adjusted for rs12191877 in *HLA-C*) and multivariate linear regression analysis (155 patients with type I psoriasis versus 197 controls). In the multivariate analysis, we included the SNPs with $p < 0.1$ in the univariate analysis adjusted for *HLA-C*. Only polymorphisms that were significant in the multivariate analysis are shown.

SNP	Gene	Model	Risk genotype	Univariate unadjusted. Type I patients versus controls		Univariate adjusted for HLA-C		Multivariate	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
rs2282276	*CLMN*	A	CC/CT	1.74 (0.96–3.15)	0.066	1.95 (1.04–3.65)	0.037	3.90 (1.13–13.38)	0.031
rs10782001	*FBXL19*	A	GG/AG	1.58 (1.13–2.21)	0.007	1.59 (1.09–2.32)	0.016	2.10 (1.05–4.17)	0.035
rs1634517	*CCL4L*	D	AA/AC	0.89 (0.58–1.36)	0.590	0.64 (0.39–1.05)	0.073	0.34 (0.14–0.84)	0.019
rs1975974	*C17orf51*	A	GG/AG	0.80 (0.57–1.14)	0.220	0.66 (0.44–0.99)	0.040	0.48 (0.23–0.99)	0.048
rs12720356	*TYK2*	A	GG/GT	0.42 (0.21–0.81)	0.019	0.27 (0.13–0.58)	0.0003	0.10 (0.03–0.39)	0.001
rs1800925	*IL13*	R	TT	0.18 (0.02–1.45)	0.051	0.17 (0.02–1.49)	0.061	0.01 (0.00–0.73)	0.034
rs3792876	*SLC22A4*	A	TT/CT	1.57 (0.89–2.76)	0.110	1.87 (0.98–3.55)	0.057	3.75 (1.19–11.83)	0.024
rs6908425	*CDKAL1*	A	TT/CT	0.67 (0.47–0.97)	0.029	0.58 (0.39–0.89)	0.01	0.41 (0.20–0.85)	0.017
rs12191877	*HLA-C*	A	TT/CT	5.92 (3.83–9.15)	$2.50E-19$	—	—	30.54 (10.62–87.85)	0.000
rs13437088	*HLA-B/MICA*	D	TT/CT	2.17 (1.42–3.34)	$3.00E-04$	1.93 (1.19–3.13)	0.007	2.52 (1.01–6.31)	0.048

CLMN: calponin-like transmembrane gene; FBXL19: F-box and leucine-rich repeat protein 19; CCL4L: chemokine (C-C motif) ligand 4-like; C17orf51: chromosome 17 open reading frame 51; TYK2: nonreceptor tyrosine-protein kinase; IL13: interleukin 13; SLC22A4: solute carrier family 22 member 4; CDKAL1: cyclin-dependent kinase 5 regulatory subunit associated protein 1-like 1; HLA: major histocompatibility complex; MICA: major histocompatibility complex class I polypeptide-related sequence A; SNPs: single-nucleotide polymorphisms; OR: odds ratio of presenting type 1 psoriasis; CI: confidence interval; A: additive; R: recessive; D: dominant; —: no data.

All the minor allele frequencies were in Hardy-Weinberg equilibrium except 9 SNPs in the controls and 12 SNPs in the patients (see supplementary Table S1 published in [3]). The 9 SNPs which were not in Hardy-Weinberg equilibrium in controls were removed from the statistical analysis [33].

3.3. Association with Type I or Type II Psoriasis. Our findings showed an association between type I psoriasis and 10 SNPs ($N = 155$ versus $N = 197$ controls): rs1634517 (*CCL4L*), rs1975974 (*C17orf51*), rs12720356 (*TYK2*), rs1800925 (*IL13*), and rs6908425 (*CDKAL1*) decreased the risk of psoriasis 2.94-fold, 2.08-fold, 10-fold, 100-fold, and 2.44-fold, respectively; and rs2282276 (*CLMN*), rs10782001 (*FBXL19*), rs3792876 (*SLC22A4*), rs12191877 (*HLA-C*), and rs13437088 (*HLA-B/MICA*) increased the risk of psoriasis 3.90-fold, 2.10-fold, 3.75-fold, 30.54-fold, and 2.52-fold, respectively (Table 2). However, comparison of 36 patients with type II psoriasis and 197 controls revealed no significant association (results not shown).

Four SNPs were associated with significant decreases in the risk of type I psoriasis ($N = 155$) compared with type II psoriasis ($N = 36$), namely, rs191190 (*TNFR1*; 126.08-fold), rs361525 (*TNF-α*; 190.76-fold), and rs10499194 and rs6920220 (*TNFAIP3*; 155.02-fold and 19.14-fold, resp.). We also found 5 SNPs that were associated with a significant increase in the risk of type I psoriasis, namely, rs1801274 (*FCGR2A*; 5.26-fold), rs763361 (*CD226*; 33.3-fold), rs12459358 (*PSORS6*; 11.11-fold), rs12191877 (*HLA*-C; 12.5-fold), and rs1576 (*CCHCR1*; 166.66-fold) (Table 3).

4. Discussion

About 75% of patients with chronic plaque psoriasis have type I psoriasis before age 40 [4], whereas a lower number of patients develop psoriasis at around 50–60 years [11]. Our results are consistent with these findings, since 79.06% of our patients developed psoriasis before the age of 40.

When we compared patients with type I psoriasis and controls, we found 10 significant SNPs in *CLMN*, *FBXL19*, *CCL4L*, *C17orf51*, *TYK2*, *IL13*, *SLC22A4*, *CDKAL1*, *HLA-C*, and *HLA-B/MICA*.

The HLA-C*0602 allele is a risk factor for psoriasis [35] and has been associated with both type I [6–9] and type II psoriasis [10]. In one study, 85.3% of patients with type I psoriasis had this allele [5], whereas only 14.7% of patients with type II psoriasis were carriers [5]. Other authors found an association between rs10484554 (*HLA-C*) and type I psoriasis compared with type II psoriasis (OR = 3.24 in type I) [12]. rs10484554 has also been associated with type II psoriasis [10]. In a recent GWAS, the *HLA-C* gene was associated with type I psoriasis ($p = 2.97E-18$ for rs1265181, $p = 2.58E-15$ for rs12191877, $p = 1.84E-15$ for rs4406273, and $p = 1.10E-07$ for rs2395029), but not with type II psoriasis after application of the Bonferroni correction [11]. In addition, our results showed significant differences in rs12191877 (*HLA-C*) in patients with type I psoriasis ($p = 2.50E-19$). However, we did not find this association in patients with type II psoriasis, probably owing to the small sample size in this group ($N = 36$).

Munir et al. found an association between rs1295685 in the *IL13* gene and type I psoriasis ($p = 2.47E-03$) [11]. Our results showed an association between another SNP in *IL13* (rs1800925) and type I psoriasis ($p = 0.034$). In addition, Munir et al. did not obtain significant results when they compared controls with type II psoriasis or type I psoriasis with type II psoriasis [11]. Both SNPs in *IL13* have been associated with predisposition to psoriasis [36, 37].

Our comparison of patients with type I psoriasis and controls is the first to obtain significant results for a series

Table 3: Results of univariate and multivariate linear regression analyses (155 patients with psoriasis type I versus 36 cases with psoriasis type II). SNPs with $p < 0.1$ in the univariate analysis were included in the multivariate analysis. Only polymorphisms that were significant in the multivariate analysis are shown.

SNP	Gene	Model	Risk genotype	Univariate Ps patients type I versus type II		Multivariate	
				OR (95% CI)	p value	OR (95% CI)	p value
rs1801274	*FCGR2A*	A	CC/CT	1.96 (1.12–3.45)	0.016	5.26 (1.11–25)	0.037
rs191190	*TNFR1*	D	CC/CT	0.43 (0.17–1.11)	0.065	0.01 (1.44E − 04–0.44)	0.018
rs763361	*CD226*	D	TT/CT	2.08 (0.99–4.35)	0.056	33.33 (1.11–1000)	0.043
rs12459358	*PSORS6*	A	TT/CT	2.44 (1.32–4.55)	0.002	11.11 (1.32–100)	0.026
rs10499194	*TNFAIP3*	D	TT/CT	0.38 (0.17–0.90)	0.02	0.01 (6.77E − 05–0.61)	0.030
rs12191877	*HLA-C*	A	TT/CT	2.33 (1.23–4.35)	0.006	12.50 (1.06–100)	0.045
rs6920220	*TNFAIP3*	A	AA/AG	0.55 (0.30–1.03)	0.068	0.05 (0.003–0.90)	0.042
rs361525	*TNF-α*	C	AG	2.17 (0.62–7.69)	0.087	0.01 (5.48E − 05–0.50)	0.024
rs1576	*CCHCR1*	D	GG/GC	2.56 (1.22–5.26)	0.012	166.67 (2.32–1000)	0.019

FCGR2A: Fc fragment of IgG low affinity IIa receptor; TNFR1: tumor necrosis factor receptor 1; CD226: CD226 antigen; PSORS6: psoriasis susceptibility 6; TNFAIP3: tumor necrosis factor alpha-induced protein 3; HLA-C: major histocompatibility complex; TNFAIP3: tumor necrosis factor alpha-induced protein 3; TNF-α: tumor necrosis factor alpha; CCHCR1: coiled-coil alpha-helical rod protein 1; SNPs: single-nucleotide polymorphisms; OR: odds ratio of presenting type I psoriasis; CI: confidence interval; A: additive; D: dominant; C: codominant.

of SNPs in type I psoriasis, although the SNPs have already been associated with the risk of psoriasis. rs10782001 in *FBXL19* [38], rs1975974 in *C17orf51* [38], rs12720356 in *TYK2* [3, 39], rs3792876 in *SLC22A4* [3], rs6908425 in *CDKAL1* [40], and rs13437088 in *HLA-B/MICA* [35] have previously been associated with psoriasis, but not with type I psoriasis.

Furthermore, SNPs in *CLMN* (rs2282276) and *CCL4L* (rs1634517) have not been associated with psoriasis or age at onset.

We found no significant differences between patients with type II psoriasis and controls owing to the small sample size ($N = 36$).

Comparison between patients with type I psoriasis and patients with type II psoriasis revealed significant associations for the following genes: *FCGR2A*, *TNFR1*, *CD226*, *PSORS6*, *TNFAIP3*, *HLA-C*, *TNF-α*, and *CCHCR1*.

Polymorphisms in *CCHCR1* (−386 and −404, CCHCR1*WW allele) have been associated with type I psoriasis [9, 31]. We found significant differences between rs1576 in *CCHCR1* and age at onset. In a study comparing controls (54.8%) and patients with psoriasis type II (66.0%), Allen et al. showed a significant increase in the number of patients carrying rs1576 [41]. This SNP has been associated with psoriasis elsewhere [42].

Douroudis et al. analyzed rs763361 in *CD226* in patients with early-onset psoriasis and patients with late-onset psoriasis, although they found no associations [43]. We performed the same analyses and found significant differences between the groups. In addition, rs763361 in *CD226* has been associated with severity of psoriasis [43].

rs12459358 in *PSORS6* has been associated with type I psoriasis (G risk allele, OR = 1.47 and $p = 0.005$) [19]. In contrast, our data showed an association between the T allele and type I psoriasis (OR = 11.11; $p = 0.026$).

rs361525 (−238) in the *TNFα* gene has been associated with susceptibility to psoriasis [44], and the A allele was more frequent in male patients with type I psoriasis ($p = 2E - 07$) [15, 22]. We found significant results in rs361525 (*TNF-α*) when we compared patients with type I psoriasis and patients with type II psoriasis, although we found no gender differences. Other authors confirmed our association with type I psoriasis in Caucasian [20, 23] and Mongolian patients [24]. A meta-analysis showed an association between rs361525 and type I psoriasis [21]. Baran et al. found no significant differences between rs1800629 in the −308 promoter (*TNFα*) and type I or type II psoriasis [45].

Likewise, rs12191877 in *HLA-C* has been associated with increased risk of psoriasis [35]. Munir et al. [11] compared patients with type I psoriasis and patients with type II psoriasis and obtained significant results for rs1265181, rs4406273, and rs12191877 in *HLA-C*. We replicated these results in rs12191877 (T allele risk; $p = 0.045$).

rs191190 in *TNFR1* [46] and rs10499194 in *TNFAIP3* [3] have been associated with psoriasis, but not with age of onset. Moreover, rs1801274 in *FCGR2A* and rs6920220 in *TNFAIP3* have not been studied in patients with psoriasis according to age of onset. Given the small sample size in the group with type II psoriasis in our study, our results should be interpreted with caution.

Our results highlight the role of the immune system in psoriasis and enhance our understanding of pathogenic mechanisms. Such knowledge can help to optimize treatment.

Our study is subject to a series of limitations. First, mean age varied between the cases and the controls. Second, the sample size was limited by the number of study patients treated in the dermatology department, thus making it difficult to detect SNPs with a low probability of causing

psoriasis. Third, since the SNPs were selected based on a literature review, several major SNPs may not yet have been investigated.

In conclusion, our study confirmed an association between rs12191877 (*HLA-C*) and type I psoriasis and between type I and type II psoriasis patients. Ours is the first study to show an association between *CLMN*, *FBXL19*, *CCL4L*, *C17orf51*, *TYK2*, *IL13*, *SLC22A4*, *CDKAL1*, and *HLA-B/MICA* and type I psoriasis. Moreover, *CLMN* and *CCL4L* have not been previously described in psoriasis. In addition, *PSORS6* and *TNFα* have been described as more prevalent genes in type I psoriasis and we showed a significant association when we compared type I psoriasis and type II psoriasis. Ours is the first study to identify an association between *FCGR2A*, *TNFR1*, *CD226*, *TNFAIP3*, and *CCHCR1* and age at onset of psoriasis. Our results suggest that genetics could play a role in age at onset. However, further studies are needed to confirm our findings.

Conflict of Interests

E. Daudén has potential conflict of interests (advisory board member, consultant, grants, research support, participation in clinical trials, honoraria for speaking, and research support) with the following pharmaceutical companies: AbbVie (Abbott), Amgen, Janssen-Cilag, Leo Pharma, Novartis, Pfizer, MSD, and Celgene. F. Abad-Santos has been a consultant or investigator in clinical trials sponsored by the following pharmaceutical companies: Abbott, Alter, Chemo, Faes, Farmalíder, Ferrer, GlaxoSmithKline, Janssen-Cilag, Kern, Normon, Servier, Teva, and Zambon. P. de la Cueva has conflict of interests (advisory board member, consultant, grants, research support, participation in clinical trials, honoraria for speaking, and/or research support) with the following pharmaceutical companies: AbbVie (Abbott), Astellas, Janssen-Cilag, Leo Pharma, Novartis, Pfizer, MSD, Gebro, Isdin, and Lilly. J. L. López Estebaranz has conflict of interests (advisory board member, speaker, or participation in clinical trials) with AbbVie, Amgen, Pfizer, MSD, Janssen-Cilag, Lilly, Celgene. Ofelia Baniandrés has conflict of interests (participation in clinical trials and honoraria for speaking) with the following pharmaceutical companies: AbbVie (Abbott), Janssen-Cilag, Leo Pharma, Pfizer, and MSD.

Authors' Contribution

Rocío Prieto-Pérez and Guillermo Solano-López contributed equally to the paper.

Acknowledgments

This work was supported by Instituto de Salud Carlos III (FIS PI10/01740), Fundación Teófilo Hernando, and AbbVie. Rocío Prieto-Pérez has a grant from University Autónoma de Madrid (FPI Program 2013). The authors are grateful to Thomas O'Boyle for editorial assistance and to Cristina Carazo for helping to set up the patient databases.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 948723, 6 pages
http://dx.doi.org/10.1155/2015/948723

Research Article

The Protective Role of HLA-DRB1*13 in Autoimmune Diseases

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Received 10 August 2015; Accepted 20 October 2015

Academic Editor: Carlo Perricone

Autoimmune diseases (AIDs) are characterized by a multifactorial aetiology and a complex genetic background, with the MHC region playing a major role. We genotyped for HLA-DRB1 locus 1228 patients with AIDs-213 with Systemic Lupus Erythematosus (SLE), 166 with Psoriasis or Psoriatic Arthritis (Ps + PsA), 153 with Rheumatoid Arthritis (RA), 67 with Systemic Sclerosis (SSc), 536 with Multiple Sclerosis (MS), and 93 with Myasthenia Gravis (MG) and 282 unrelated controls. We confirmed previously established associations of HLA-DRB1*15 (OR = 2.17) and HLA-DRB1*03 (OR = 1.81) alleles with MS, HLA-DRB1*03 with SLE (OR = 2.49), HLA-DRB1*01 (OR = 1.79) and HLA-DRB1*04 (OR = 2.81) with RA, HLA-DRB1*07 with Ps + PsA (OR = 1.79), HLA-DRB1*01 (OR = 2.28) and HLA-DRB1*08 (OR = 3.01) with SSc, and HLA-DRB1*03 with MG (OR = 2.98). We further observed a consistent negative association of HLA-DRB1*13 allele with SLE, Ps + PsA, RA, and SSc (18.3%, 19.3%, 16.3%, and 11.9%, resp., versus 29.8% in controls). HLA-DRB1*13 frequency in the AIDs group was 20.0% (OR = 0.58). Although different alleles were associated with particular AIDs, the same allele, HLA-DRB1*13, was underrepresented in all of the six diseases analysed. This observation suggests that this allele may confer protection for AIDs, particularly for systemic and rheumatic disease. The protective effect of HLA-DRB1*13 could be explained by a more proficient antigen presentation by these molecules, favouring efficient clonal deletion during thymic selection.

1. Introduction

Autoimmune diseases (AIDs) are chronic disorders originated by the loss of immunological tolerance to self-antigens. This heterogeneous group of conditions present common genetic risk factors and share several pathophysiological mechanisms leading to overlapping clinical manifestations targeting specific organs or multiple organ systems [1]. There is evidence that they share similar immunogenetic mechanisms, even though they exhibit varying epidemiological features and clinical manifestations [2, 3]. Underlying these diverse clinical phenotypes is a deregulated immune system

with an enriched ability to respond against self-tissues. The fact that AIDs share several clinical signs and symptoms (i.e., subphenotypes) and also share physiopathological mechanisms and genetic factors has been called autoimmune tautology and indicates that they may have a common origin [4].

The immune system is in charge of the defence against external pathogens. For this purpose, T and B lymphocytes are responsible for the immune response through regulated cell-cell interactions and secretion of cytokines, chemokines, and other inflammatory mediators. This defence against external pathogens must occur without causing unnecessary harm to self. To achieve this delicate balance, the majority of self-reactive T and B lymphocytes are destroyed in the thymus and bone marrow through negative selection [5]. Nevertheless, this process is far from perfect, and self-reactive lymphocytes escape into the periphery. Consequently, peripheral tolerance mechanisms are necessary to keep these self-reactive cells in check [6]. Activated self-reactive T and B cells promote autoimmunity when the effector and regulatory balance of the immune response is disturbed [7].

Major histocompatibility complex (MHC) molecules are widely distributed surface membrane glycoproteins that present antigenic peptides to T cell receptors (TCRs). Developing thymocytes encounter a highly heterogeneous repertoire of self (endogenous) peptide-MHC (pMHC) complexes on thymic epithelial cells, the main thymus antigen presenting cells. The affinity/avidity with which these thymocyte TCRs bind self pMHC determines if it is destined to perish or if it will survive [8]. In this way, a repertoire of peripheral T cells that is essentially self-tolerant is generated [6, 9, 10].

Several hypotheses have been put forward to explain how MHC polymorphisms influence autoimmunity risk or protection. They must do so, somehow, by shaping the central or peripheral T cell repertoires toward autoimmune resistance or proclivity [8]. A protective MHC profile could achieve this by the selection of a T cell repertoire with diminished pathogenicity [11]. On the other hand, protective MHC molecules may keep autoimmunity in check by favouring the negative selection of particular self-reactive T cells [12–14].

The functional basis of the association between specific HLA alleles and development of AIDs can be classically explained by two possible etiopathogenic models [15].

The molecular mimicry hypothesis proposes that certain HLA alleles are more efficient in presenting pathogen epitopes that share structural features with self-peptides to mature T cells. Once the response to the pathogen is initiated the self-antigen is also recognized and disease ensues.

Central selection failure proposes that certain HLA alleles are less efficient at presenting self-peptides to developing T cells in the thymus, so negative selection fails.

A different hypothesis proposes that different alleles can modulate the immunologic profile of an individual, through antigen-independent mechanisms, resulting in either promoting a higher autoimmune predisposition or, in opposition, a more efficient immune regulation. Given the consistent association of HLA-DRB1 alleles with different autoimmune diseases (Table 1), we explored the idea that the same HLA-DRB1 alleles could be influencing several different autoimmune diseases. To this end we compared the immunogenetic profile in different AIDs. This study includes four autoimmune systemic diseases, namely, Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), Psoriasis or Psoriatic Arthritis (Ps + PsA), and Systemic Sclerosis (SSc). Patients with Multiple Sclerosis (MS) and Myasthenia Gravis (MG) were also included.

2. Patients and Methods

2.1. Patients and Controls. A total of 1228 patients with AIDs, 213 patients with SLE and 153 patients with RA diagnosed according to the American College of Rheumatology (ACR) criteria, 166 patients with Ps + PsA, 67 with SSc, 536 with definitive diagnosis of MS according to the revised McDonald criteria, and 93 with MG, were recruited from the Neurology and Medicine Outpatient Clinic of Centro Hospitalar do Porto-Hospital de Santo António (CHP-HSA). The HLA-DRB1 frequencies of patients were compared with the ones of a control group consisting of 282 unrelated individuals without disease and from the same geographic origin (north of Portugal).

2.2. HLA-DRB1 Genotyping. Peripheral blood samples (10 mL) were collected in EDTA. Genomic DNA was obtained from proteinase-K–treated peripheral blood leukocytes by using a Salting-Out procedure [27]. Low-resolution genotyping for HLA–DRB1 locus (i.e., 2-digit HLA nomenclature) was performed using polymerase chain reaction and sequence-specific primers (PCR-SSP), based on methods previously described [28]. In order to produce PCR-SSP reactions able to detect and discriminate each of the known HLA-DRB1 genes, primers were designed using sequence alignments comprising all HLA-DRB1 variants and were validated by the Twelfth International Histocompatibility Workshop. PCR products were visualized under ultraviolet light after running in a 1.5% agarose gel containing ethidium bromide.

2.3. Statistical Analysis. To identify the HLA-DRB1 genes contributing to the six different AIDs, we applied stepwise logistic regression on an allelic level, using forward selection which involves starting with no variables in the model, testing the addition of each variable using a chosen model comparison criterion, adding the variable (if any) that improves the model the most, and repeating this process until none improves the model. It should be noted that odds ratios (ORs) obtained in a multivariable logistic regression analysis are adjusted for all the other genes included in the model and therefore differ from those obtained when a given gene is compared with all other genes. The data were analysed using IBM SPSS 20 statistical software.

3. Results

A total of 1228 cases and 282 controls were analysed and different types of association between alleles and AIDs were found (Table 2). These included three risk alleles for two or more AIDs, two protective alleles for two or more AIDs, and three risk alleles for a particular AID.

Table 1: HLA-DRB1 alleles associated with SLE, Ps + PsA, RA, SSc, MS, and MG.

Autoimmune disease	HLA-DRB1 associated allele		References
	Susceptibility	Protection	
Systemic Lupus Erythematosus (SLE)	HLA-DRB1*03 HLA-DRB1*08 HLA-DRB1*15	HLA-DRB1*09 HLA-DRB1*13	[16–18]
Psoriasis or Psoriatic Arthritis (Ps + PsA)	HLA-DRB1*07	—	[19, 20]
Rheumatoid Arthritis (RA)	HLA-DRB1*01 HLA-DRB1*04 HLA-DRB1*10	HLA-DRB1*13	[21]
Systemic Sclerosis (SSc)	HLA-DRB1*01 HLA-DRB1*08 HLA-DRB1*11	HLA-DRB1*07 HLA-DRB1*15	[22, 23]
Multiple Sclerosis (MS)	HLA-DRB1*03 HLA-DRB1*08 HLA-DRB1*15	HLA-DRB1*10 HLA-DRB1*14	[24, 25]
Myasthenia Gravis (MG)	HLA-DRB1*03	—	[26]

Table 2: Associations between HLA class II and six AIDs: SLE, Ps + PsA, RA, SSc, MS, and MG.

	Controls ($n = 282$)	SLE ($n = 213$)	Ps + PsA ($n = 166$)	RA ($n = 153$)	SSc ($n = 67$)	MS ($n = 536$)	MG ($n = 93$)	Total ($n = 1228$)
HLA-DRB1*01	**66 (23.4%)**	40 (18.8%)	39 (23.5%)	**50 (32.7%) OR = 1.79 p = 0.017**	**28 (41.8%) OR = 2.28 p = 0.006**	100 (18.7%)	23 (24.7%)	280 (22.8%)
HLA-DRB1*03	**44 (15.6%)**	**73 (34.3%) OR = 2.49 $p = 4.2 \times 10^{-5}$**	25 (15.1%)	28 (18.3%)	11 (16.4%)	**123 (22.9%) OR = 1.81 p = 0.003**	**33 (35.5%) OR = 2.98 $p = 6.1 \times 10^{-5}$**	**293 (23.9%) OR = 1.51 p = 0.022**
HLA-DRB1*04	**69 (24.5%)**	42 (19.7%)	46 (27.7%)	**73 (47.7%) OR = 2.81 $p = 6 \times 10^{-6}$**	13 (19.4%)	128 (23.9%)	23 (24.7%)	325 (26.5%)
HLA-DRB1*07	**72 (25.5%)**	55 (25.8%)	**66 (39.8%) OR = 1.79 p = 0.006**	38 (24.8%)	14 (20.9%)	147 (27.4%)	23 (24.7%)	343 (27.9%)
HLA-DRB1*08	**24 (8.5%)**	21 (10.0%)	10 (6.0%)	**3 (2.0%) OR = 0.24 p = 0.026**	**15 (22.4%) OR = 3.01 p = 0.004**	**65 (12.1%) OR = 1.73 p = 0.033**	7 (7.5%)	121 (9.9%)
HLA-DRB1*09	**14 (5.0%)**	**2 (1.0%) OR = 0.18 p = 0.027**	5 (3.0%)	**0 (0.0%)* OR = 0.95 p = 0.003**	3 (4.5%)	**5 (1.0%) OR = 0.22 p = 0.004**	2 (2.2%)	**17 (1.4%) OR = 0.23 $p = 1 \times 10^{-4}$**
HLA-DRB1*13	**84 (29.8%)**	**39 (18.3%) OR = 0.58 p = 0.016**	**32 (19.3%) OR = 0.62 p = 0.050**	**25 (16.3%) OR = 0.58 p = 0.044**	**8 (11.9%) OR = 0.42 p = 0.035**	124 (23.1%)	17 (18.3%)	**245 (20.0%) OR = 0.58 p = 0.004**
HLA-DRB1*15	**56 (19.9%)**	55 (25.8%)	22 (13.3%)	17 (11.1%)	12 (17.9%)	**175 (32.7%) OR = 2.17 $p = 2 \times 10^{-5}$**	15 (16.1%)	296 (24.1%)

AIDs: autoimmune diseases; SLE: Systemic Lupus Erythematosus; Ps + PsA: Psoriasis or Psoriatic Arthritis; RA: Rheumatoid Arthritis; SSc: Systemic Sclerosis; MS: Multiple Sclerosis; MG: Myasthenia Gravis. *Fisher's exact test was used to calculate this value.

HLA-DRB1*13 was a protective allele for four AIDs: SLE (18.3% versus 29.8%, $p = 0.016$, OR = 0.58, and 95% CI = 0.37–0.90), Ps + PsA (19.3% versus 29.8%, $p = 0.050$, OR = 0.621, and 95% CI = 0.39–1.00), RA (16.3% versus 29.8%, $p = 0.044$, OR = 0.58, and 95% CI = 0.34–0.98), and SSc (11.9% versus 29.8%, $p = 0.035$, OR = 0.42, and 95% CI = 0.19–0.94).

There was a specific risk allele associated with three AIDs. HLA-DRB1*03 was found to be a risk factor for SLE (34.3% versus 15.6%, $p = 4.2 \times 10^{-5}$, OR = 2.49, and 95% CI = 1.61–3.86), MS (22.9% versus 15.6%, $p = 0.003$, OR = 1.81, and 95% CI = 1.23–2.67), and MG (35.5% versus 15.6%, $p = 6.1 \times 10^{-5}$, OR = 2.98, and 95% CI = 1.75–5.07). There were

two risk alleles associated with two AIDs: HLA-DRB1*08 was positively associated with MS (12.1% versus 8.5%, $p = 0.033$, OR = 1.73, and 95% CI = 1.05–2.87) and SSc (22.4% versus 8.5%, $p = 0.004$, OR = 3.01, and 95% CI = 1.43–6.31) and HLA-DRB1*01 was found to be a risk factor for RA (32.7% versus 23.4%, $p = 0.017$, OR = 1.79, and 95% CI = 1.11–2.88) and SSc (41.8% versus 23.4%, $p = 0.006$, OR = 2.28, and 95% CI = 1.27–4.09).

HLA-DRB1*09 was negatively associated with SLE (1.0% versus 5.0%, $p = 0.027$, OR = 0.18, and 95% CI = 0.04–0.83), MS (1.0% versus 5.0%, $p = 0.004$, OR = 0.22, and 95% CI = 0.08–0.63), and RA (0.0% versus 1.0%, $p = 0.003$, OR = 0.95, and 95% CI = 0.93–0.98).

Three risk disease-specific alleles were found: HLA-DRB1*04 for RA (47.7% versus 24.5%, $p = 6\times10^{-6}$, OR = 2.81, and 95% CI = 1.79–4.39), HLA-DRB1*07 for Ps + PsA (39.8% versus 25.5%, $p = 0.006$, OR = 1.79, and 95% CI = 1.18–2.72), and HLA-DRB1*15 for MS (32.7% versus 19.9%, $p = 2\times10^{-5}$, OR = 2.17, and 95% CI = 1.53–3.10).

Considering AIDs as a group, HLA-DRB1*03 frequency was significantly higher (23.9% versus 15.6%, $p = 0.022$, OR = 1.51, and 95% CI = 1.0–2.15) compared with controls; conversely HLA-DRB1*13 (20.0% versus 29.8%, $p = 0.004$, OR = 0.58, and 95% CI = 0.43–0.79) and HLA-DRB1*09 (1.4% versus 5.0%, $p = 1\times10^{-4}$, OR = 0.23, and 95% CI = 0.11–0.49) frequencies were significantly lower.

4. Discussion

Through a systematic review of published works, Cruz-Tapias and collaborators, in 2012, identified some common HLA class II alleles that contribute to susceptibility to AIDs in Latin Americans [3]. The present study is, to date and to the best of our knowledge, the only one that addresses the hypothesis that a HLA-DRB1 allele could influence different autoimmune diseases, using a new cohort, encompassing six different autoimmune diseases.

In this study we observed associations of different HLA-DRB1 alleles with several AIDs. We confirmed positive and negative associations in MS [24, 25], SLE [16–18], Ps + PsA [19, 20], RA [21], SSc [22, 23], and MG [26], previously reported in our or other populations.

When AIDs studied were considered as a group, HLA-DRB1*03 allele was significantly overrepresented, as already described [29]. It has been shown that this allele has low affinity for CLIP (class II-associated invariant chain peptide) and may not require HLA-DM to ensure peptide presentation, preventing efficient peptide selection and allowing the binding of low stability peptides [30]. Concerning the observed negative association with HLA-DRB1*09, we think that this is likely a spurious association, as this is a rare allele and the frequency found in controls is, for some reason, double the one reported for the Portuguese population [31].

Our observations suggest that the presence of HLA-DRB1*13 allele may confer protection for AIDs. HLA-DRB1*13 is a high frequency allele in the general population both in Portugal [31] and worldwide. Our results confirm that the lower frequency of HLA-DRB1*13 in every individual AIDs group is not secondary to the deviations granted by the concurrent positive associations. When the data obtained from previous studies are taken into consideration, the HLA-DRB1*13 allele seems to be a universal protective allele for RA. It was reported as protective against RA in Asian [32, 33], Turkish [34], and several European populations [35–37]. Recently this allele was also described to be protective in SLE in the Japanese population [18] and for ANCA-associated vasculitis in the Dutch population [38].

Subtle structural differences in the HLA molecule have functional implications at the protein level. Specific amino acid patterns at the peptide binding cleft are involved in disease susceptibility, such as the well-known shared epitope first described in the RA susceptibility alleles HLA-DRB1*01 and HLA-DRB1*04 [37, 39]. Similar to the shared epitope classification of susceptibility alleles, protective HLA-DRB1 alleles have been categorized according to several models. One of the most accepted classifications proposes that protection against RA is conferred by the DERAA sequence at positions 70–74 of the HLA-DRB1 allele [40]. Other models suggest that protection is conferred by an aspartic acid at position 70 (D70 allele) [41] or an isoleucine at position 67 (I67 allele) of the HLA-DRB1 molecule. Because it was unclear which HLA-DRB1 alleles were protective a meta-analysis was performed involving four European populations with >2,700 patients and >3,000 control subjects. The objective was to investigate exhaustively which HLA-DRB1 alleles were associated with protection against RA [36]. Interestingly, this study showed that the protective effect attributed to DERAA and D70 was no longer present after the exclusion of HLA-DRB1*13. The authors concluded that this evidence indicates that HLA-DRB1*13 rather than DERAA, D70, or I67 is associated with protection [36]. In a recent study van Heemst and collaborators identify citrullinated vinculin, present in the joints of $ACPA^+$ RA patients, as an autoantigen targeted by ACPA and $CD4^+$ T cells. These T cells recognize an epitope with the core sequence DERAA, which is also found in many microbes and in protective HLA-DRB1*13 molecules, presented by predisposing HLA-DQ molecules. Intriguingly, DERAA-directed T cells were not detected in HLA-DRB1*13^+ donors, indicating that the DERAA epitope from HLA-DRB1*13 could mediate thymic tolerance in these donors and explain the protective effects associated with HLA-DRB1*13. They suggest that subjects born with HLA-DRB1*13 will present the HLA-DRB1*13-derived DERAA-peptide in the thymus, leading to tolerization of the DERAA-reactive T cell response [42]. The negative association we describe here supports the idea that the HLA-DRB1*13 allele, possibly by its specific structural features, may as well confer resistance to several other AIDs. The protective effect of HLA-DRB1*13 could be explained by a more proficient antigen presentation by these molecules [43, 44], favouring an efficient thymic selection. As a result, negative selection and development of DR-driven autoreactive regulatory T cells are promoted [8].

A different model would relate HLA molecules with the presence of specific endophenotypes indirectly associated with autoimmunity. Other studies of our group suggest that the HLA genotype may primarily influence the general activation state of CD4 T cells [45]. The protective effect

of HLA-DRB1*13 could also be explained by this effect. Curiously, several reports have suggested an association of HLA-DRB1*13 and/or HLA-DQB1*06 with slow disease progression in human immunodeficiency virus (HIV) infected individuals, meaning that among HIV controllers there is an association between the presence of certain class II HLA alleles and strong CD4 T cell responses [46, 47].

Although different alleles are associated with particular AIDs, the same allele, HLA-DRB1*13, was underrepresented in all six diseases. This difference is statistically significant for the four rheumatic diseases studied. This observation suggests that this allele confers protection to AIDs in general and particularly to rheumatic diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 906086, 6 pages
http://dx.doi.org/10.1155/2015/906086

Research Article

Cyclic AMP-Responsive Element Modulator α Polymorphisms Are Potential Genetic Risks for Systemic Lupus Erythematosus

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Received 17 June 2015; Revised 23 September 2015; Accepted 5 October 2015

Academic Editor: Fulvia Ceccarelli

To investigate whether the cyclic AMP-responsive element modulator α (*CREM*α) polymorphisms are novel susceptibility factors for systemic lupus erythematosus (SLE), four tag SNPs, rs1057108, rs2295415, rs11592925, and rs1148247, were genotyped in 889 SLE cases and 825 healthy controls. Association analyses were performed on whole dataset or clinical/serologic subsets. Association statistics were calculated by age and sex adjusted logistic regression. The G allele frequencies of rs2295415 and rs1057108 were increased in SLE patients, compared with healthy controls (rs2295415: 21.2% versus 17.8%, OR 1.244, $P = 0.019$; rs1057108: 30.8% versus 27.7%, OR 1.165, $P = 0.049$). The haplotype constituted by the two risk alleles "G-G" from rs1057108 and rs2295415 displayed strong association with SLE susceptibility (OR 1.454, $P = 0.00056$). Following stratification by clinical/serologic features, a suggestive association was observed between rs2295415 and anti-Sm antibodies-positive SLE (OR 1.382, $P = 0.044$). Interestingly, a potential protective effect of rs2295415 was observed for SLE patients with renal disorder (OR 0.745, $P = 0.032$). Our data provide first evidence that *CREM*α SNPs rs2295415 and rs1057108 maybe novel genetic susceptibility factors for SLE. SNP rs2295415 appears to confer higher risk to develop anti-Sm antibodies-positive SLE and may play a protective role against lupus nephritis.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease which is characterized by complex immunological abnormalities and multiple tissue and organ damage [1]. The etiology of SLE has not been fully understood, but it is widely accepted that the interaction between genetic and environmental factors contributes to SLE pathogenesis [2, 3]. Previous studies have shown that genetic factors are the major determinants leading to the susceptibility of SLE, and to date more than 40 genetic loci have been proven to be associated with SLE [4, 5].

Cyclic AMP-responsive element modulator (CREM) proteins are members of the leucine zipper protein superfamily of nuclear transcription factors. They are key regulators of cAMP-mediated signal transduction. Human CREM proteins are encoded by the CREM gene, which comprised over 20 alternatively spliced isoforms, including CREMα. CREMα is a widely expressed transcriptional repressor that is important in regulation of T cell immune response [6, 7]. Evidence has shown that CREMα was overexpressed in T cells from patients with SLE [8, 9]. Previous study reported that CREMα mRNA expression was increased in T cells from SLE patients, though it did not correlate with clinical features, disease activity, or therapeutic effects; patients with high doses of corticosteroids had a trend to possess low CREMα mRNA levels [10]. Despite a number of immunological studies of CREMα in SLE, however, to date, there is no any report on genetic susceptibility of *CREM*α in SLE. This study therefore aimed to investigate whether the genetic variant(s) in human *CREM*α is associated with SLE susceptibility and to evaluate whether *CREM*α polymorphism(s) is associated with any clinical/serologic features in SLE. To the best of our knowledge, this is the first time we report that two tag single-nucleotide polymorphisms (SNPs) rs2295415 and rs1057108 from *CREM*α are novel susceptibility factors for SLE. SNP rs2295415 may confer increased risk to developing of anti-Smith (Sm) antibodies-positive SLE and may have a protective role in patients with renal disorder.

Table 1: Demographic and clinical characteristics of subjects.

Characteristics	SLE cases (n = 889)	Controls (n = 825)
Female (%)	90.0	88.5
Age (mean ± SD years)	36 ± 13	43 ± 9
Age of onset (mean ± SD years)	29.0 ± 0.6	—
Disease duration (mean ± SD years)	5.4 ± 0.3	—
Clinical manifestations (%)		
Rash (n = 392)	44.1	—
Arthritis (n = 386)	43.4	—
Renal disorder (n = 315)	35.4	—
Autoantibody positivity (%)		
Anti-dsDNA positivity (n = 364)	41.0	—
Anti-Sm positivity (n = 151)	17.0	—

SLE: systemic lupus erythematosus; anti-dsDNA: anti-double-stranded DNA antibody; anti-Sm: anti-Smith antibody; SD: standard deviation.

2. Materials and Methods

2.1. Study Subjects. A total of 889 patients with SLE and 825 nonrelated healthy controls were enrolled in the study. The patients with SLE were recruited from the Department of Rheumatology and Immunology from Peking University People's Hospital and People's Hospital of Xinjiang Province. The healthy controls were recruited from Health Care Centers of Peking University People's Hospital. In the Health Care Centers, thousands of residents come for annual regular physical examination from the local geographical areas. The healthy controls were selected from these residents without any disease records. All patients and healthy controls are Han Chinese.

The patients with SLE fulfilled 1997 revised American College of Rheumatology (ACR) classification criteria for SLE [11] and were selected without developing other rheumatic diseases. The patients with renal disorder were defined by the following criteria: (a) persistent proteinuria greater than 0.5 g per day or greater than +++ if quantization is not performed or (b) cellular casts which may be red cell, hemoglobin, granular, tubular, or mixed. Anti-double-stranded DNA (dsDNA) antibodies were measured with enzyme-linked immunosorbent assay (ELISA, Kexin Biotechnology Ltd., Shanghai, China). Values >100 IU/mL were assessed as positive. Anti-Sm antibodies were determined by an immunoblot method from Euroimmun (Lübeck, Germany), and results were reported as positive or negative in relation to reference sera. The data for the two autoantibodies were available for all the in-patients and part of out-patients due to the incomplete records in electronic system in out-patient department.

The characteristics of patients and healthy controls were detailed in Table 1. The study was approved by Medical Ethics Committee in Peking University People's Hospital and the informed consents were obtained from all participants.

2.2. TagSNP Selection and Genotyping. The tagSNPs were selected from the CHB panel (Han Chinese in Beijing) of HapMap project (the Phase II database, http://hapmap.ncbi.nlm.nih.gov/). The criterion for tagging was set at $D' > 0.8$ and minor allele frequency (MAF) > 0.05. By using Haploview 4.2 software, a total of 4 tagSNPs were identified: rs1057108, rs11592925, rs114824, and rs2295415 (Figures 1(a) and 1(b)).

Genotyping of SNPs rs1148247 and rs2295415 was performed using predesigned TaqMan SNP Genotyping Assays (C_8876200_10 and C_16189959_10, resp., Applied Biosystems, Foster City, California). Allelic discrimination was performed in ABI 7300 Real-Time PCR system (Applied Biosystems). The genotyping successful rate was 99.4%.

SNPs rs1057108 and rs11592925 were genotyped using Sequenom MassArray platform with primers and probes (Supplementary Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/906086). Briefly, DNA from case and control subjects was randomly assigned to the 96-well plates, and genotyping was performed, blind to the status of the samples. Genotyping was repeated in 5% of samples for validation and quality control. The genotype data had an error rate less than 0.1%.

2.3. Power Analysis. The power analyses were performed retrospectively for the available samples (cases and controls), according to MAF for each SNP, type I error P of 0.05, and an odds ratio (OR) of 1.40. The Power and Sample-size (PS) software (version 3.0.14) was used for the power calculation (available at http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize).

2.4. Statistical Analysis. Hardy-Weinberg equilibrium was assessed for each polymorphism, using Pearson's goodness-of-fit chi-square test. The chi-square tests with continuity correction were performed for the comparisons of allelic frequency differences and haplotypes between patients and controls. The linkage disequilibrium (LD) and haplotype were calculated using online software SHEsis (http://analysis.bio-x.cn/myAnalysis.php). ORs and 95% confidence intervals (CI) for alternative genetic model analysis were calculated using logistic regression, adjusting for age and sex. All statistical analyses were conducted using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). P values less than 0.05 were considered statistically significant.

3. Results

SNPs rs2295415, rs1057108, rs11592925, and rs1148247 were in Hardy-Weinberg equilibrium in both patients and controls ($P > 0.05$, Supplementary Table S2), illustrating that the subjects were collected from a random mating population. In control group, the allele frequencies of the four SNPs were similar to the data from HapMap CHB. The study has a statistical power of greater than 0.807 to detect the significant effect between the tagSNPs and SLE, except for rs11592925 (study power = 0.609).

3.1. Association of CREMα Polymorphisms with Susceptibility to SLE at Allele and Genotype Level. We first sought to determine whether there was an association between *CREMα*

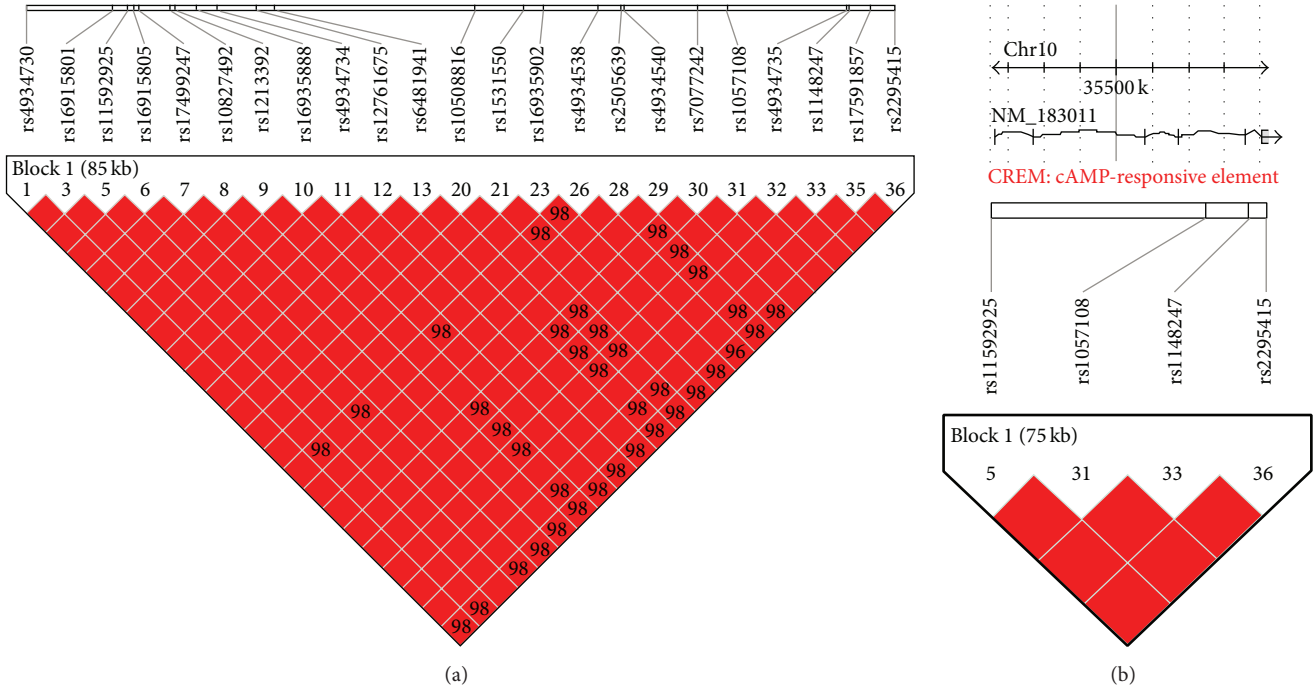

Figure 1: (a) Thirty-six *CREMα* SNPs were shown with linkage disequilibrium (LD); the intensity of LD is reflected in the color and digital value of each box. (b) The LD structure and location of four tagSNPs. Red represents strong linkage; white represents no linkage. Digital value in each box represents the D' values ×100 for linkage disequilibrium between the two corresponding SNPs; the maximum D' value is 1, which indicates complete linkage.

Table 2: Allele analysis of *CREMα* tagSNPs in SLE association.

SNPs	Allele	Allelic frequencies (cases versus controls)	MAF (cases versus controls)	OR (95% CI)	*P* values
rs1057108	G/T	542/1216, 444/1160	0.308, 0.277	1.165 (1.003–1.152)	**0.049**
rs2295415	G/A	343/1275, 265/1225	0.212, 0.178	1.244 (1.040–1.478)	**0.019**
rs11592925	T/C	169/1591, 159/1447	0.096, 0.099	0.967 (0.770–1.214)	0.772
rs1148247	A/G	574/1054, 536/948	0.353, 0.361	0.963 (0.832-1.116)	0.626

CREMα: cyclic AMP-responsive element modulator α; SNPs: single-nucleotide polymorphisms; MAF: minor allele frequency; OR: odds ratio; CI: confidence interval.

polymorphisms and SLE susceptibility. As shown in Table 2, at allele level, we observed significant higher minor allele frequencies of rs2295415 and rs1057108 in patients with SLE, compared with healthy controls (rs2295415: 21.2% versus 17.8%, OR 1.244, 95% CI 1.040–1.487, $P = 0.019$; rs1057108: 30.8% versus 27.7%, OR 1.165, 95% CI 1.003–1.152, $P = 0.049$). At genotype level, SNP rs2295415 displayed a significant association with SLE susceptibility (codominant model: OR = 1.241, 95% CI 1.009–1.527, $P = 0.041$, Table 3). A suggestive association was also found between rs1057108 and SLE (codominant model: OR = 1.185, 95% CI 0.999–1.405, $P = 0.052$). No association was observed for rs11592925 and rs1148247 in SLE susceptibility.

3.2. Association of CREMα Polymorphisms with Susceptibility to SLE at Haplotype Level. Haplotypes were constructed using the two susceptible SNPs rs1057108 and rs2295415 ($D' = 0.82$). As shown in Table 4, a total of 4 haplotypes were identified. Haplotype T-A was the major composition (63.8% versus 65.3%). The haplotype constituted by the two risk alleles "G-G" displayed strong risk effects contributed to SLE susceptibility (OR 1.454, 95%CI 1.175–1.799, and $P = 0.00056$). In contrast, other haplotypes showed no association.

3.3. Association of CREMα Polymorphisms with SLE Subphenotypes. Next, we sought to determine whether there was any association between the risk SNP rs2295415 and any specific clinical/serologic manifestations in SLE. Following stratification by clinical/serologic features, we found higher frequencies of rs2295415 G allele in anti-Sm antibodies-positive patients, compared with healthy controls (22.8% versus 17.8%, OR 1.382, 95% CI 1.015–1.883, and $P = 0.044$, Table 5). A similar result was also observed for anti-dsDNA

Table 3: Genotype analysis of *CREMα* tagSNPs in SLE association, adjusting for sex and age.

SNPs	Genotype	Cases (%)	Controls (%)	Codominant		Dominant		Recessive	
				OR (95% CI)	*P* values	OR (95% CI)	*P* values	OR (95% CI)	*P* values
rs1057108	TT	422 (48)	417 (52)	1.185 (0.999–1.405)	0.052	1.200 (0.966–1.491)	0.100	1.363 (0.916–2.028)	0.127
	TG	372 (42)	326 (41)						
	GG	85 (10)	59 (7)						
rs2295415	AA	498 (62)	497 (67)	**1.241** (1.009–1.527)	**0.041**	1.222 (0.964–1.549)	0.098	1.873 (0.967–3.625)	0.063
	AG	279 (34)	231 (31)						
	GG	32 (5)	17 (2)						
rs11592925	CC	719 (82)	654 (82)	1.049 (0.813–1.354)	0.712	1.055 (0.799–1.394)	0.704	1.046 (0.369–2.964)	0.933
	CT	153 (17)	139 (17)						
	TT	8 (1)	10 (1)						
rs1148247	GG	349 (43)	307 (41)	0.934 (0.794–1.10)	0.416	0.916 (0.729–1.150)	0.447	0.913 (0.657–1.629)	0.588
	GA	356 (44)	334 (45)						
	AA	109 (13)	101 (14)						

CREMα: cyclic AMP-responsive element modulator α; SNPs: single-nucleotide polymorphisms; SLE: systemic lupus erythematosus; OR: odds ratio; CI: confidence interval.

Table 4: Haplotype analysis between rs1057108 and rs2295415 in SLE association.

Haplotype	Cases (%)	Controls (%)	χ^2	*P* values	OR (95% CI)
G-G	244 (15.3)	160 (11.0)	11.927	**0.00056**	**1.454 (1.175~1.799)**
G-A	242 (15.1)	245 (16.8)	1.637	0.207	0.881 (0.726~1.070)
T-A	1021 (63.8)	949 (65.3)	0.770	0.380	0.936 (0.807~1.085)
T-G	93 (5.8)	100 (6.9)	1.351	0.245	0.841 (0.628~1.126)

SLE: systemic lupus erythematosus; OR: odds ratio; CI: confidence interval.

Table 5: Association analyses between rs2295415 and subphenotypes in SLE.

Subjects	MAF (%)	OR (95% CI)	*P* values
Controls ($n = 745$)	17.8		
Subphenotypes (positive)			
Rash ($n = 366$)	19.7	1.132 (0.904–1.418)	0.295
Arthritis ($n = 357$)	18.2	1.029 (0.816–1.298)	0.813
Renal disorder ($n = 299$)	13.9	**0.745** (0.570–0.973)	**0.032**
Anti-dsDNA ($n = 345$)	20.3	1.177 (0.937–1.478)	0.173
Anti-Sm ($n = 139$)	22.8	**1.382** (1.015–1.883)	**0.044**

SLE: systemic lupus erythematosus; anti-dsDNA: anti-double-stranded DNA antibody; anti-Sm: anti-Smith antibody; OR: odds ratio; CI: confidence interval.

antibodies-positive patients, though they did not reach the statistical significance (20.3% versus 17.8%, OR 1.177, 95% CI 0.937–1.478, and $P = 0.173$). Interestingly, a potential protective effect of rs2295415 was observed for SLE patients with renal disorder (OR 0.745, 95% CI 0.570–0.973, and $P = 0.032$).

4. Discussion

Although several genetic studies have showed that the SNPs rs2295415 and rs1057108 were significantly associated with ulcerative colitis and Crohn's disease [12–14] and the immunological studies have indicated that CREMα is implicated in the pathogenesis of SLE, to data, there are no genetic studies of *CREMα* in SLE susceptibility. Therefore, we undertook the current study to investigate whether human *CREMα* polymorphisms play a role in SLE susceptibility. Our results indicated that *CREMα* SNPs rs2295415 and rs1057108 may be novel genetic risk factors contributing to SLE susceptibility in Han population. SNP rs2295415 G allele conferred a potential risk to develop anti-Sm antibodies-positive SLE and appeared to have a protective role in patients with renal disorder.

The precise function of CREMα is unknown. However, the existing data suggest that CREMα can specifically bind to the IL-2 promoter, leading to a repression of IL-2 transcription [15, 16]. CREMα overexpression in SLE T cells resulted in enhanced binding of CREMα to IL-2 promoter and reduced IL-2 expression [17]. It is clear that IL-2 is a critical cytokine produced by T cells upon activation and is important for the generation of T regulatory cells and activation-induced cell death [18]. In SLE patients, T cells display decreased capacity to produce IL-2 [19]. Impaired IL-2 expression resulted in decreased generation of regulatory T lymphocytes [20] and

defect of activation-induced cell death [21, 22]. These findings suggest an important role of CREMα in regulation of IL-2 and in the pathogenesis of SLE.

The mechanism(s) underlying this genetic association remains elusive. It may be explained by the fact that rs2295415 is located in 3′ UTR of *CREMα*, the region implicated in the regulation of gene expression. A number of disease-associated polymorphisms have been mapped in the 3′ UTR of protein-coding genes [23, 24]. The significant association of SNP rs2295415 with SLE susceptibility and subphenotypes may be due to its effect on *CREMα* gene expression and/or its LD with a functional variant residing in a neighboring gene(s). However, in present study, we also showed that another tagSNP rs1057108 was also significantly associated with SLE. Thus, we currently cannot exclude that rs1057108 might be independent genetic risk contributing to SLE susceptibility, though the SNP may be a nonfunctional variant since it is resided in the intronic region and has not been annotated as any promoter, enhancer, repressor, or distant regulatory element (NCBI http://www.ncbi.nlm.nih.gov/ MAF Source: 1000 Genomes). The functional consequences of the SLE-risk SNPs need to be elucidated in the future studies.

Identification of genetic risk factors responsible for disease subsets/subphenotypes is important for the understanding of disease pathogenesis. It has been shown that certain genetic association was restricted to clinical and autoantibody subsets in SLE [25]. In present study, we found a suggestive association between rs2295415 G allele and anti-Sm antibodies-positive SLE and a trend association between rs2295415 G allele and anti-dsDNA antibodies-positive SLE. Another interesting finding of our work is the potential protective effect of rs2295415 G allele in patients with renal disorder. However, in present work, the case numbers were relatively small after stratification for clinical/serologic subsets. As a result of the modest sample size, there may be a risk that observed findings are due to chance. Additional studies with larger sample sizes are desired to confirm our findings.

In conclusion, our study provides the first evidence that *CREMα* SNPs rs2295415 and rs1057108 may be novel genetic susceptibility factors for SLE, especially at haplotype level. SNP rs2295415 appears to confer higher risk to develop autoantibody-positive diseases, such as anti-Sm antibodies-positive and anti-dsDNA antibodies-positive SLE, and may play a protective role against lupus nephritis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Qian Guo and Xuyong Chen have equally contributed to this work.

Acknowledgments

This study is supported in part by National Key Basic Research Program of China (973 Program) (no. 2014CB541901) and National Natural Science Foundation of China (no. 81172814, no. 81471600, and no. 31470875). The authors thank all the members and staff for recruiting patients and healthy controls and the technical assistance from Department of Rheumatology and Immunology, Peking University People's Hospital. The authors wish to thank all the patients and healthy volunteers for their cooperation and for giving consent to participate in this study.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 754825, 12 pages
http://dx.doi.org/10.1155/2015/754825

Review Article

Contribution of Genetic Factors to Sjögren's Syndrome and Sjögren's Syndrome Related Lymphomagenesis

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Received 21 July 2015; Accepted 17 September 2015

Academic Editor: Fulvia Ceccarelli

We aimed to summarize the current evidence related to the contributory role of genetic factors in the pathogenesis of Sjögren's syndrome (SS) and SS-related lymphoma. Genes within the major histocompatibility complex (MHC) locus previously considered conferring increased susceptibility to SS development have been also revealed as important contributors in recent genome wide association studies. Moreover, genetic variations outside the MHC locus involving genes in type I interferon pathway, NF-κB signaling, B- and T-cell function and methylation processes have been shown to be associated with both SS and SS-related lymphoma development. Appreciating the functional implications of SS-related genetic variants could provide further insights into our understanding of SS heterogeneity, allowing the design of tailored therapeutic interventions.

1. Introduction

Sjögren's syndrome (SS) is the second most common systemic autoimmune disease (after rheumatoid arthritis, RA), with a prevalence of about 0.5% in the general population. It occurs primarily in perimenopausal women (at a ratio of women to men of 9 : 1) [1, 2]. Key features of the disease include infiltration of exocrine glands (predominantly salivary and lacrimal) by lymphocytes, production of inflammatory cytokines, and the activation of B lymphocytes and the production of autoantibodies. At the level of exocrine glands, loss of secretory activity has been observed leading to the characteristic symptoms of dry mouth and dry eyes. Other systems or organs such as musculoskeletal system (arthralgias, myalgias, and nonerosive arthritis of small joints), lungs, liver, skin, and kidneys can also be involved [3]. Compared to the general population, patients with SS exhibit significantly increased risk of developing B-cell lymphoproliferative disorders (usually B-cell lymphoma involving the mucosa-associated lymphoid tissue (MALT)), which affects about 5–10% of patients leading to significant increased morbidity and mortality rates of these patients [4]. Several clinical and laboratory manifestations have so far been proposed as adverse predictors for malignant disease and include features related to deposition of immune complexes (palpable purpura, peripheral neuropathy, low levels of complement C4, and cryoglobulinemia), swelling of the parotid glands, as well as high lymphocytic scores, and the presence of ectopic germinal centers in minor salivary gland biopsies [5]. The causative mechanisms of SS have not been fully elucidated. However, based on the current pathogenetic model, the interaction of both genetic, epigenetic [6, 7] and environmental factors seems to contribute to disease development [8]. Viruses, especially the Epstein-Barr virus (EBV) [9] and Coxsackievirus [10], hormones (low estrogen levels seen in perimenopausal women), stress [11], and occupational exposures [12] have been all considered as the main environmental triggers for disease onset. In the current review article, we will mainly focus on the contributory role of genetic influences in the development of the SS as well as in SS-related lymphoproliferation, a major disease complication.

2. Genetic Factors Associated with Sjögren's Syndrome

Familial clustering and coaggregation with other autoimmune disorders in SS has been long considered [13–16]. In an Italian multicenter case control study [17], the risk of SS

development among first-degree relatives with autoimmune disease was sevenfold higher compared to controls. Of note, these first-degree relatives of SS patients had a higher risk of autoimmune disease compared to subjects without first-degree relative affected by SS. A large recent study in Taiwanese population confirmed previous observations showing that first-degree relatives of SS patients had an increased risk of SS development as well as of other autoimmune disorders, mainly systemic lupus erythematosus (SLE), RA, systemic sclerosis, and type 1 diabetes (T1D), compared to the general population [14]. Of interest, siblings of affected individuals demonstrated the highest relative risk for SS development compared to other first-degree relatives (parents and offsprings), implying both genetic influences and shared environmental exposures as contributory factors to disease development [14].

Since the 1970s, strong associations between specific alleles of the major histocompatibility complex (MHC) and SS development have been suggested [18, 19]. Over the last decade, high throughput technologies allowed the confirmation of the dominant role of MHC alleles in the pathogenesis of the disease with novel genetic variants outside the MHC locus emerging as susceptibility factors [20]. The latter seem to be involved in signaling pathways of natural and acquired immunity, inflammatory responses and cellular apoptosis.

2.1. The Role of MHC Complex. Though initially recognized as major determinants of tissue rejection, MHC genes have been soon after appreciated as critical contributors to the pathogenesis of autoimmune disorders as well. They encode components of the human leukocyte antigen (HLA) system [21] including HLA class I (A, B, and C) and class II (DR, DQ, and DP), which present endogenous and exogenous antigens to T lymphocytes, respectively. MHC class II molecules, especially those which encode HLA-DR and HLA-DQ antigens, have been proposed as the most important genes associated with SS susceptibility. The first genetic study on SS revealed an association between SS and HLA-DR3 (which was in linkage disequilibrium (LD) with the class I allele HLA-B8) in Caucasian SS patients [22]. Subsequent reports highlighted the association between SS and the HLA-D locus [18, 23], with a diverse distribution between primary SS (high frequency of the alleles HLA-DRw3 and HLA-B8) and secondary SS (increased frequency of allele HLA-DRw4) [24], as well as in patients with SS characterized by the presence of Raynaud's phenomenon (increased frequency of alleles HLA-DRw3 and HLA-DRw4) [25]. Subsequent studies in other ethnic populations confirmed HLA associations with SS susceptibility [26–41], with DRB1*04:05-DQB1*04:01 being a risk allele in Japanese populations [36], DRB1*08:03-DQB1*06:01 in Chinese populations [36], DR3 and DR11 in Spanish populations [42], and DRB1*11:01, DRB1*11:04, DQB1*03:01, and DQA1*05:01 in Israeli Jews and Greeks [43, 44] (Summarized in Table 1). In a subsequent meta-analysis [21], HLA class II alleles DRB1*03:01, DQA1*05:01, and QB1*02:01 were shown to predispose to disease development, while DQB1*05:01 exhibited a protective role [21]. Two recent large genome wide association studies (GWAS), in Caucasian [45] and Han-Chinese population [46], confirmed the strong influence of HLA locus on SS. Further studies are required on the functional role of the HLA polymorphic regions in SS pathogenesis as well as their possible associations with disease diagnosis and/or prognosis.

A functional deletion of 6.7 Kb in the gene leukocyte immunoglobulin-like receptor subfamily A member 3 (LILRA3) has been also associated with autoimmune diseases. LILRA3 is a soluble receptor of class I MHC antigens involved in the regulation of immune function. It has been found in SS patients of both Caucasian [47] and Chinese [48] origin, as well as in other autoimmune diseases including multiple sclerosis [49], RA [50], and SLE [48].

2.2. Genetic Factors Associated with SS outside the MHC Locus. In the following years, research has been directed to the investigation of single nucleotide polymorphisms (SNPs) in genes outside the MHC locus (summarized in Table 2 [51–68]), already found to be associated with other autoimmune diseases such as SLE [69]. These novel SS-associated genetic variants (outside the MHC locus) can be roughly classified into three main groups depending on the implicated signaling pathway [20]. The first group consists of variants in genes involved in the activation of the interferon (IFN) signaling pathway. The second group includes important genes affecting B-cell function and autoantibody production. Specific autoantibodies have been found in approximately two-thirds of SS patients and genetic contribution has been proposed. HLA class II SS-related phenotype has been associated with the presence of autoantibodies in various studies [33, 35, 40]. SNPs in the 5′ untranslated region of the BAFF [61] gene as well as in GTF2I [70] and genes implicated in the NF-κB pathway [45] have been also associated with the presence of autoantibodies. Finally, the third one contains apoptotic and inflammatory genes, which participate in the NF-κB signaling pathway.

2.2.1. Genes Associated with Interferon Pathways. Gene expression studies in SS patients over the last decade revealed upregulation of IFN-inducible genes (the so-called IFN signature) at the level of peripheral blood and affected salivary gland tissues in a substantial proportion of these individuals [71]. Moreover, recent data revealed that both type I (IFNα/β) and type II (IFNγ) IFN signatures are upregulated in both peripheral blood and minor salivary gland tissues derived from SS patients while the IFNγ/IFNα mRNA ratio in diagnostic salivary gland biopsies could predict the in situ lymphoma development in the setting of SS [72, 73]. While the mechanisms leading to this activation remain under investigation, several genetic variants in genes implicated in the IFN pathway have been designated as potential contributors [74].

Interferon Regulatory Factor 5 (IRF5). IRF5 is a transcription factor involved in type I IFN induction following TLR ligation [75]. Several polymorphisms of the IRF5 gene have been previously shown to either increase or decrease SLE susceptibility [74, 76]. Initial studies on SS revealed the IRF5 polymorphism rs2004640 (creates an alternate splice site (exon 1B) in the first exon) as predisposing factor

Table 1: Genetic associations of the HLA alleles with Sjögren's syndrome susceptibility.

Study	Year	Population	Sample size (patients/controls)	Associated HLA alleles
Chused et al. [18]	1977	American Caucasian	110 (19/91)	HLA-Dw3
Fye et al. [22]	1978	American Caucasian	115 (19/96)	HLA-Dw3-HLA-B8
Moutsopoulos et al. [23]	1978	American Caucasian	208 (24/184)	B lymphocytes immune response associated (Ia) antigens
Moutsopoulos et al. [24]	1979	American Caucasian	206 (22/184)	HLA-DRw3-HLA-B8
Manthorpe et al. [27]	1981	Danish	32 (32/—)	HLA-Dw2
Mann and Moutsopoulos [25]	1983	American Caucasian	52 (25/27)	HLA-DRw3-HLA-B8
Molina et al. [28]	1986	American Caucasian	694 (68/626)	HLA-B8 HLA-DR3 DRw52
Moriuchi et al. [29]	1986	Japanese	135 (21/114)	DRw53
Vitali et al. [30]	1986	Italian	90 (28/62)	DR3
Papasteriades et al. [26]	1988	Greek	218 (46/172)	DR-5
Pease et al. [31]	1989	British Caucasian	141 (41/100)	DR-3 DRw52
Morling et al. [41]	1991	Danish	19 (19/—)	DQA1*0501-DQB1*0201-DQA1*0301
Kang et al. [36]	1993	American Caucasian	210 (75/135)	DRB1*03-DRB3*0101-DQB1*0201-DQA1*0501
Kang et al. [36]	1993	Chinese	87 (45/42)	DRB1*0803-DQA1*0103-DQB1*0601
Kang et al. [36]	1993	Japanese	82 (33/49)	DRB1*0405-DRB4*0101-DQA1*0301-DQB1*0401
Roitberg-Tambur et al. [43]	1993	Jews (Israel)	275 (17/258)	DQA1*001-DQA1*0201-DQB1*0501
Roitberg-Tambur et al. [43]	1993	Greek	76 (22/54)	DQA1*0501
Portales et al. [42]	1994	Spanish	286 (30/256)	HLA-Cw7 DR3 DR11
Wang et al. [32]	1997	Chinese	206 (70/136)	DR3, DR52, DR2, DR5, and DR9
Jean et al. [38]	1998	French	242 (42/200)	DRBI*1501*-0301-DQB1*0201*-0602
Rishmueller et al. [33]	1998	Australian	244 (80/164)	DR3-DQA1*0501-DQB1*02
Bolstad et al. [34]	2001	Norwegian Caucasian	95 (31/64)	DRB1*03-DQB1*02-DQA1*0501
Nakken et al. [40]	2001	Norwegian Caucasian	210 (29/181)	DRB1*0301
Anaya et al. [39]	2002	Colombian	149 (73/76)	DRB1*0301
Gottenberg et al. [35]	2003	French	371 (149/222)	DRB1*03
Manoussakis et al. [44]	2004	Greek	301 (55/246)	DRB1*0301
Kovács et al. [37]	2006	Hungarian	98 (48/50)	DQB1*0201-DRB1*03-DQB1*0501
Cruz-Tapias et al. [21]	2012	Meta-analysis	7636 (1166/6470)	DQA1*0501-DQB1*0201-DRB1*0301-DQA1*0201-DQA1*0301-DQB1*0501
Li et al. [46]	2013	Chinese	5622 (1845/3777)	HLA class II locus
Lessard et al. [45]	2013	Caucasian	10916 (4712/6204)	HLA class II locus

to disease development in both Scandinavian and French cohorts [51, 52]. Another strong signal of association was observed between the insertion/deletion (in/del) of the CGGGG sequence in the IRF5 gene promoter in both SS and other autoimmune diseases [52, 53, 77]. This CGGGG in/del is part of a polymorphic repetitive DNA region, which includes either 3 or 4 CGGGG repeats; the insertion of an additional CGGGG unit (the 4 × CGGGG allele) is the risk allele associated with increased IRF5 transcription in peripheral blood mononuclear cells (PBMCs) and cultured epithelial cells derived from the salivary glands of SS patients, possibly through the addition of Sp1 binding site in the

Table 2: Associations of non-HLA genetic locus with Sjögren's syndrome.

Gene/chromosome	Polymorphism	Population	Sample size (patients/controls)	p value	Relative risk	Study/year
		Interferon pathways				
IRF5/Chr7	rs2004640	Caucasians	364 (210/154)	0.01	1.93	Miceli-Richard et al. 2007 [51]
	rs10488631	Norwegian/Swedish	1079 (368/711)	$2.4 * 10^{-5}$	1.49	Nordmark et al. 2009 [52]
	CGGGG promoter insertion/deletion	Caucasians	824 (385/439)	$6 * 10^{-6}$	2.00	Miceli-Richard et al. 2009 [53]
IRF5/TNPO3/Chr7	CGGGG promoter insertion/deletion	Norwegian/Swedish	1072 (540/532)	$5.5 * 10^{-6}$	1.70	Nordmark et al. 2011 [54]
	rs13246321	Caucasians	10916 (4712/6204)	$2.73 * 10^{-19}$	1.44	Lessard et al. 2013 [45]
	rs3757387	Caucasians	1232 (120/1112)	0.01	1.47	Korman et al. 2008 [55]
STAT4/Chr2	rs7574865	Norwegian/Swedish	1079 (368/711)	0.0014	1.41	Nordmark et al. 2009 [52]
	rs7582694	Colombian/German	800 (277/523)	$7.7 * 10^{-6}$	1.40	Palomino-Morales et al. 2010 [56]
	rs7574865	Norwegian/Swedish	1072 (540/532)	$7 * 10^{-4}$	1.40	Nordmark et al. 2011 [54]
	rs7582694	Chinese	5622 (1845/3777)	$1.77 * 10^{-17}$	1.44	Li et al. 2013 [46]
	rs10168266	Caucasians	10916 (4712/6204)	$6.8 * 10^{-15}$	1.43	Lessard et al. 2013 [45]
	rs10553577			$9.45 * 10^{-9}$	1.32	
	rs13426947	Caucasians	10916 (4712/6204)	$1.17 * 10^{-10}$	1.30	Lessard et al. 2013 [45]
IL12A/Chr3	rs485497			$9.88 * 10^{-9}$	1.27	
	rs583911	French/Scandinavian	1902 (1010/892)	0.0039	0.48	Rusakiewicz et al. 2013 [57]
NCR3/NKp30/Chr6	rs11575837			0.0019	0.56	
	rs2736191	French	3.55 (183/172)	ns	ns	Ittah et al. 2005 [58]
PTPN22/Chr1	rs2476601	Colombian	378 (70/308)	0.01	2.42	Gomez et al. 2005 [59]
		B-cell function				
BLK-FAM167A/Chr8	rs12549796	Norwegian/Swedish	1072 (540/532)	$4.7 * 10^{-4}$	1.37	Nordmark et al. 2011 [54]
	rs7812879	Chinese	1152 (555/597)	0.045	—	Sun et al. 2013 [60]
	rs2736345	Caucasians	10916 (4712/6204)	$4.97 * 10^{-10}$	1.30	Lessard et al. 2013 [45]
	rs2729935			$6.85 * 10^{-10}$	1.30	
	rs6998387			$7.96 * 10^{-8}$	1.26	
CXCR5/Chr11	rs7119038	Caucasians	10916 (4712/6204)	$1.0 * 10^{-8}$	0.74	Lessard et al. 2013 [45]
	4936443			$6.82 * 10^{-8}$	0.75	
BAFF/Chr13	−2841 T → C. −2704 T → C. −2701 T → A. −871 C → T	Caucasians	259 (123/136)	<0.001	—	Nossent et al. 2008 [61]
	rs1224141	Greek	330 (193/137)	<0.05	—	Nezos et al 2014 [62]
	rs12583006					
	rs9514828					
	rs1041569					
	rs9514827					
GTF2I/chr7	rs117026326	Chinese	5622 (1845/3777)	$1.31 * 10^{-53}$	2.20	Li et al. 2013 [46]
EBF1/Chr5	rs3843489	Norwegian/Swedish	1072 (540/532)	$9.9 * 10^{-5}$	1.68	Nordmark et al. 2011 [54]
	rs1234315	Norwegian/Swedish		$7.4 * 10^{-4}$	1.34	Nordmark et al. 2011 [54]
Ox40L-TNFSF4/Chr1	rs2205960	Chinese	643 (250/393)	<0.05	—	Kong et al. 2013 [63]
	rs1234313					

Table 2: Continued.

Gene/chromosome	Polymorphism	Population	Sample size (patients/controls)	p value	Relative risk	Study/year
		NF-κB pathway				
TNFAIP3/chr6	rs2230926	Caucasians	(18/397)	0.038	3.38	Musone et al. 2011 [64]
	rs5029939	Chinese	5622 (1845/3777)	$7.75 * 10^{-5}$	1.67	Li et al. 2013 [46]
	rs6933404	Caucasians	10916 (4712/6204)	$6.53 * 10^{-8}$	1.26	Lessard et al. 2013 [45]
	rs35926684			$7.21 * 10^{-8}$	1.26	
	rs2230926	Caucasians	1025 (574/451)	0.01	3.36	Nocturne et al. 2013 [65]
TNIP1/Chr5	rs6579837	Caucasians	10916 (4712/6204)	$3.30 * 10^{-8}$	1.43	Lessard et al. 2013 [45]
	rs7732451			$5.32 * 10^{-7}$	1.34	
	rs3792783	Scandinavian/British	5565 (1105/4460)	$3.4 * 10^{-5}$	1.33	Nordmark et al. 2013 [66]
	rs7708392			$1.3 * 10^{-3}$	1.21	
LTA/LTB/TNF gene clusters	rs1800629	Norwegian/Swedish	1060 (527/532)	$1.6 * 10^{-11}$	—	Bolstand et al. 2012 [67]
	rs909253			$4.42 * 10^{-8}$	—	
BAFF-R/Chr22	His159Tyr	Greek	427 (247/180)	0.01	2.75	Papageorgiou et al. 2015 [68]

gene promoter of the 4R allele [53, 77]. Moreover, reovirus infection of salivary gland epithelial cells from SS patients carrying the 4R allele further increased IRF5 expression at mRNA level [53]. Taken together, these findings suggest a possible association between the IRF5 gene variants and the induction of type I IFNs (through the induction of the IRF5 gene expression after a viral infection) that could lead to the robust activation of the immune system in salivary glands (target organ) as an early event in SS pathogenesis.

Recent studies revealed genetic association of Transportin-3 (TNPO3), an IRF5 neighboring gene that encodes a nuclear receptor involved in the import of splicing factors in the nucleus, with both SLE and SS susceptibility with specific variants spanning the IRF5-TNPO3 locus being identified [45, 76].

Signal Transducer and Activator of Transcription 4 (STAT4). The transcription factor STAT4 is primarily involved in the signal transduction induced by the cytokines interleukin- (IL-) 12 and IL-23 leading to differentiation of T helper (Th) naïve cells towards a Th1 phenotype and subsequent production of IFNγ [78]. STAT4 intronic variants, namely, rs7582694 and rs7574865, have been associated with SS development in four candidate gene association studies [52, 55, 56, 79]. Subsequent GWAS studies in SS patients with both European and Chinese descent confirmed STAT4 locus as an important determinant of SS susceptibility [45, 46]. While rs7582694 risk variant has been associated with increased expression of several IFN-inducible genes in SS patients [79], PBMC derived from lupus patients harboring the risk variant of rs7574865 demonstrated increased sensitivity to IFNα effects [78].

Interleukin 12A (IL12A). The recent GWAS in the Caucasian [45] but not Chinese population revealed an important association of IL12A gene polymorphisms with SS. The IL12A is a cytokine that forms a heterodimer with the IL12B subunit inducing through STAT4 the differentiation of naïve T-cells in T helper 1 cells which promotes immune response through IFNγ production by T helper 1 cells [80].

Natural Cytotoxicity Triggering Receptor 3 (NCR3). NCR3/NKp30 is a natural killer (NK) specific receptor regulating the cross talk between NK and dendritic cells as well as type II IFN secretion [81]. The minor allele of the rs11575837 polymorphism within the promoter of NCR3 gene has been found as protective allele for SS development that is associated with reduced NCR3 gene transcription. Compared to controls, SS patients who lacked this polymorphism demonstrated higher circulating levels of the NCR3 ligand and demonstrated higher focus scores in salivary gland biopsy [57].

Protein Tyrosine Phosphatase Nonreceptor 22 (PTPN22). PTPN22 gene encodes the protein lymphocyte tyrosine phosphatase (Lyp) previously shown to be implicated in both adaptive (inhibition of T-cell receptor (TCR) and B-cell receptor (BCR) signaling) and innate immune responses (type I interferon (IFN) production by myeloid cells through TLR ligation) [82]. A single nucleotide polymorphism (SNP) of the PTPN22 gene 1858C>T (rs2476601) leading to substitution of arginine (R) by tryptophan (W) at position 620 has been previously shown to increase susceptibility to several autoimmune diseases including T1D, SLE, and RA [83–85]. Although the underlying mechanisms leading to autoimmunity are not clearly delineated, a break in B- and T-cell tolerance through altered BCR and TCR signaling, enhancement of T helper follicular cells, and dampened type I IFN responses leading to a proinflammatory microenvironment have been all shown to contribute to autoimmune pathogenesis reviewed in [82].

In regard to SS, available data so far is rather conflicting. In contrast to a French report [58] in which no association with SS development was detected, studies in both Colombian [59] and Greek populations identified a strong association with SS susceptibility, particularly in patients characterized by low IFN signatures (manuscript in preparation). On this basis, we postulate that the apparent discrepancies between different studies are related to IFN status of SS patients included. This finding implies an additional shared etiological origin in autoimmune disorders, with a putative role of genetic contributors as determinants of distinct IFN patterns in patients with autoimmune diseases.

2.2.2. Genes Involved in B-Cell Function

B-Lymphocyte Kinase (Blk). The kinase Blk is a member of the family of the src tyrosine kinase, which seems to be involved in signaling and differentiation of B lymphocytes [86]. Common polymorphisms in the Blk and in the neighboring family with sequence similarity 167, member A (FAM167A) genes have been found to predispose to SLE [87], systemic sclerosis [88], RA [89], and recently SS [54, 60, 90]. Two main SNPs associated with SS development include rs12677843 (located in intron 1 of the Blk gene) and rs12549796 (second intron of FAM167A). These SNPs have been found in partial LD ($r^2 = 0.29$). The functional implication of these SNPs in SS remains unknown, although previous studies on SLE showed an association between the presence of risk alleles with decreased Blk mRNA levels and increased FAM167A mRNA levels in transformed B lymphocytes [87]. The association of Blk/FAM167A polymorphisms with SS was also suggested in a large GWAS study in Caucasian populations [45].

B-Cell Activating Factor (BAFF). BAFF is an important cytokine that promotes survival and proliferation of B-cells. Previously published data support a role for several haplotypes in the 5′ regulatory region of BAFF gene in autoantibody positive SS and increased serum BAFF levels [61] as well as in distinct (both low and high risk for lymphoma development) SS phenotypes [62].

Chemokine (C-X-C Motif) Receptor 5 (CXCR5). In a GWAS Caucasian study, chemokine receptor CXCR5 gene variants were found to confer protection against SS development [45]. The chemokine receptor CXCR5 detected in both circulating B-cells and activated $CD4^+$ cells contributes to B- and T-cell migration in peripheral lymphoid as well as in inflamed peripheral organs, upon ligation with the CXCL13

chemokine [91, 92]. The latter has been previously found to be upregulated in salivary gland tissues derived from SS patients leading eventually to preferential retention of memory $CXCR4^+CXCR5^+$ B-cells in the SS derived salivary gland infiltrates [93].

Early B-Cell Factor 1 (EBF1). In a large candidate gene association study in SS patients of Scandinavian origin [54], genetic variants of the *EBF1* gene (previously shown to be involved in antigen independent changes of B-cell differentiation [94]) have been found to confer increased risk for SS.

Ox40 Ligand/Tumor Necrosis Factor Superfamily 4 (Ox40L/TNFSF4). Ox40L (or TNFSF4), a TNF family ligand member, expressed on activated dendritic cells, endothelial cells, and the B-cell surface, has been previously shown to get involved in B-cell activation through interaction with Ox40-positive T-cells [95, 96]. Genetic variants of Ox40L have been previously associated with susceptibility to SLE (in association with increased transcript and protein levels) and scleroderma, but not with primary biliary cirrhosis or SS after Bonferroni corrections in a Han-Chinese population [63, 97, 98]. However, in a Scandinavian study, two SNPs (namely, rs1234315 and rs1234314) located in the 5′-untranslated region of the gene Ox40L have been found to be significantly associated with SS [54].

General Transcription Factor 2I (GTF2I). An interesting finding from a large study (GWAS) in Han-Chinese [46] but not in European population revealed that a polymorphism in the GTF2I gene (namely, rs117026326) (encodes a transcription factor involved in both T-cell signaling [99] and activation of immunoglobulin heavy-chain transcription upon B-lymphocyte activation [100]) is strongly associated with SS development with overall risk (OR) scores higher than other SS-associated identified genes including MHC-II genes, STAT4, and TNFAIP3 [46]. This finding was also confirmed in another study in Chinese population and was linked to the presence of anti-Ro/SSA autoantibodies [70].

2.2.3. Genes Involved in the NF-κB Pathway

Tumor Necrosis Factor-Alpha Induced Protein 3 (TNFAIP3). TNFAIP3 gene encodes the A20 protein, an enzyme with ubiquitination activity that appears to play an important role in the regulation of inflammation through the NF-κB pathway. A20 protein is expressed at low levels on most of the cells but is rapidly induced after activation of NF-κB, acting as a negative feedback regulating both inflammation and apoptosis [101]. Experiments on mice revealed that A20 is important for survival and normal development since A20-deficient mice fail to regulate TNF induced NF-κB activation and die early due to multiorgan inflammation and cachexia [102]. Several genetic variants of the TNFAIP3 gene have been associated with autoimmune diseases including SS [64]. The coding TNFAIP3 polymorphism, namely, rs2230926, which changes the amino acid sequence from phenylalanine (Phe) to cysteine (Cys) at position 127 has been previously found to confer increased risk for SLE [103]. Functional analysis showed that the rs2230926 minor allele, which predisposed to disease, is less effective in inhibiting the activity of NF-κB after induction by TNF [103]. The association of TNFAIP3 rs2230926 polymorphism with SS has been recently confirmed by two large studies (GWAS) in both Caucasian [45] and Chinese population [46].

TNFAIP3-Interacting Protein 1 (TNIP1). Of note, polymorphisms of the *TNIP1* gene, a molecule which interacts with the TNFAIP3 gene regulating the NF-κB activation, have been recently found to confer increased risk to SS [54, 66] and other autoimmune diseases [104, 105]. The role of TNIP1 polymorphisms in SS development was also confirmed in a large GWAS study in Caucasian population [45].

Lymphotoxin Gene A (LTA). Polymorphisms of the lymphotoxin gene A (LTA), located on locus LTA/LTB/TNF and related to the activation of the NF-κB pathway as well as inflammation, have been found to increase the risk of SS [67].

Chemokine (C-C Motif) Ligand 11 (CCL11). Finally, the CCL11 (eotaxin) is a chemokine with important role in SS. The expression of CCL11 has been found to be regulated by the NF-κB pathway and specific polymorphisms in the CCL11 gene have been associated with ectopic germinal center-like structures present in salivary gland tissues of a proportion of SS patients who are found to be at risk of lymphoma development [106].

2.3. Animal Models for the Study of Genetic Predisposition to SS.

Animal models are useful tools for elucidating the etiopathogenetic mechanisms of various autoimmune diseases including SS. Over the last decade, various murine models have been proposed in an attempt to explore the early initiating and subsequent events leading to disease development. Spontaneous or transgenic murine models which are prone to develop Sjögren's syndrome-like symptoms during lifetime include, among others (as reviewed recently in [107]), (NZB/NZW)F1, MRL, NOD, NOD-Aec1Aec2, Baff Tg, Opn Tg, and $Act1^{-/-}$. The latter has been recently found to develop a disorder which closely resembles Sjögren's syndrome in association with lupus. The Act1-deficient mice are characterized by marginal zone-like B-lymphocyte accumulations, salivary and lachrymal gland inflammation, and production of anti-Ro/SSA and anti-La/SSB autoantibodies [108]. Act1 is a negative regulator of BAFF and CD40 molecules (both implicated in B-cell survival and activation) while recent findings proposed it to be a critical component of the IL-17 signalling pathway [109]. Of interest, several SNPs around the TRAF3IP2 gene (which encodes the Act1 protein) have been recently found to confer increased risk to lupus and may play an important role in the induction of the interferon pathway (interferon-β, interferon inducible genes), which is relevant in the context of autoimmune diseases, like lupus and SS [110]. Taken together, these findings indicate the putative role of the SNPs in the TRAF3IP2 gene in the development of histological and serological features of SS.

2.4. Genetic Factors Associated with Sjögren's Syndrome Related Lymphomagenesis. Lymphocytic infiltration of the exocrine glands and ectopic formation of germinal centers have been considered as the sine qua non of lymphoma development. B-cell hyperactivity, the hallmark of SS, molecular events affecting B-cell function and survival, and the deregulation of the NF-κB pathway have been recently proposed as potential factors leading to lymphoma development [111]. Chronic antigenic stimulation of autoreactive B-cells and tumorigenic events such as chromosomal translocation and gene mutations/polymorphisms have been suggested as possible mechanisms underlying neoplastic diversion in the setting of SS. Regarding oncogenic mechanisms, the presence of the translocation $t(14; 18)$ (leading to overexpression of Bcl-2, an antiapoptotic gene promoting B-cell survival) has been detected in 5 of 7 salivary gland biopsies of patients with Sjögren's syndrome who developed lymphoma and in none of the 50 corresponding biopsies of patients with the syndrome not associated with lymphoma [112]. Furthermore, mutations of tumor suppressor gene p53 are possibly associated with the occurrence of lymphoma in patients with SS [113].

Additionally, somatic mutations and polymorphisms in the TNFAIP3 gene have been also reported in several types of lymphomas [114] including lymphomas of mucosal marginal zone (MALT), which is the major type of SS-related lymphoproliferative disease. In a recent study, the rs2230926 TNFAIP3 polymorphism along with other genetic alterations has been found to be associated with SS-related lymphoproliferation, especially of MALT type, while functional assays found that this polymorphism is associated with increased activation of the NF-κB pathway [65].

Another study failed to provide evidence for the presence of MyD88 L265P gene mutation (a nonsynonymous change at amino acid position 265 from leucine to proline (L265P)) in patients with SS with and without lymphoma [115]. MyD88 is an adaptor protein leading to NF-κB activation through TLR, IL-1R, and IL-18 signaling, which has been previously shown to be implicated in patients with Waldenström's macroglobulinemia (WM) and other haematological malignancies [116, 117]. The absence of mutation in SS patients with or without lymphoma suggests that probably there are different pathogenetic mechanisms in lymphoproliferation in the setting of SS [115].

Given that deregulation of B-cell activation has been postulated as fundamental event in both autoimmunity and B-cell lymphomagenesis, the BAFF/BAFF-R axis attracted our research interest. Specific haplotypes of the BAFF gene could discriminate SS patients with lymphoma from SS patients without lymphoma and healthy controls [62] and a functional mutation His159Tyr of the BAFF receptor (BAFF-R), previously found to confer an increased risk in patients with NHL through activation of the alternative NF-κB pathway [118], has been found to be more prevalent in SS population compared to healthy controls. Of interest, more than two-thirds of SS patients complicated by MALT type NHL with an age at SS diagnosis between 3rd and 4th decade carried this mutation [68].

The role of known polymorphisms of the methylenetetrahydrofolate reductase (MTHFR), gene, an enzyme necessary for the DNA synthesis and methylation, which have been previously associated with NHL development [119, 120] and autoimmune diseases [121], has been also investigated. MTHFR polymorphisms have been found to be associated with both SS and SS non-MALT NHL development in association with methylation alterations, implying genetic and epigenetic abnormalities as common pathogenetic pathways in both benign autoimmunity and malignant transformation (Fragioudaki et al., in preparation).

3. Discussion/Conclusions

While growing evidence over the last years supports a genetic contribution to SS susceptibility, the majority of genetic variants seem to have weak or moderate effect (except, perhaps, for HLA locus), implying an additional role for the environmental insults such as viruses, hormones, and stress in disease pathogenesis. Given that the vast majority of these genetic loci have been also detected as susceptibility factors in other autoimmune disorders, shared mechanisms leading to deregulation of the immune system imply a central role in autoimmune pathogenesis. Heterogeneity of SS clinical expression from local disease confined to exocrine glands to lymphoma development should be always taken into account when genetic studies are designed, since distinct operating immune pathways underlie distinct clinical phenotypes. Further multicenter efforts exploring genetic, epigenetic, and environmental interactions are warranted to further clarify the pathogenesis of the syndrome.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 153132, 7 pages
http://dx.doi.org/10.1155/2015/153132

Research Article

Detecting Genetic Associations between *ATG5* and Lupus Nephritis by *trans*-eQTL

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Received 21 April 2015; Revised 26 May 2015; Accepted 28 May 2015

Academic Editor: Fulvia Ceccarelli

Objectives. Numerous loci were identified to perturb gene expression in *trans*. As elevated *ATG5* expression was observed in systemic lupus erythematosus (SLE), the study was conducted to analyze the genome-wide genetic regulatory mechanisms associated with *ATG5* expression in a Chinese population with lupus nephritis (LN). *Methods*. The online expression quantitative trait loci database was searched for *trans*-expression single nucleotide polymorphisms (*trans*-eSNPs) of *ATG5*. Tagging *trans*-eSNPs were genotyped by a custom-made genotyping chip in 280 patients and 199 controls. For positive findings, clinical information and bioinformation analyses were performed. *Results*. Four *trans*-eSNPs were observed to be associated with susceptibility to LN ($P < 0.05$), including ANKRD50 rs17008504, AGA rs2271100, PAK7 rs6056923, and TET2 rs1391441, while seven other *trans*-eSNPs showed marginal significant associations ($0.05 < P < 0.1$). Correlations between the *trans*-eSNPs and *ATG5* expression and different expression levels of *ATG5* in SLE patients and controls were validated, and their regulatory effects were annotated. However, no significant associations were observed between different genotypes of *trans*-eSNPs and severity or outcome of the patients. *Conclusion*. Using the new systemic genetics approach, we identified 10 loci associated with susceptibility to LN potentially, which may be complementary to future pathway based genetic studies.

1. Introduction

A strong body of evidence has suggested the complex genetic basis of systemic lupus erythematosus (SLE), and to date more than 50 loci have been identified, largely improving our insights into the pathogenesis of SLE [1, 2]. However, approximately 80%–90% of the associated variants were observed to be located in noncoding regions, which may have effects on gene expressions [3, 4]. As the gene expression level has been suggested to be heritable, the expression quantitative trait loci (eQTL) have been widely studied. Up to date, a number of eQTL were observed to influence gene expression through *cis*-acting regulatory effects (with the variants located within or near the target gene) [5], significantly broadening our understanding of genetic pathogenesis of diseases [6, 7]. However, *trans*-acting regulatory effects (with the variants distal to the target gene or on different chromosomes) have been seldom addressed.

Autophagy is a phylogenetically ancient mechanism by which the cell can degrade and dispose of intracellular constituents or intracellular infectious agents in a regulated manner. Recently, genetic variants within or near *ATG5*—a gene product required for the formation of autophagosomes—have been identified to be associated with SLE by several genome-wide association studies (GWASs) [8, 9]. And elevated *ATG5* expression level was observed in the splenic and renal macrophages of lupus mice and in peripheral blood mononuclear cells (PBMC) of SLE patients [10]. However, no significant associations between variants within *ATG5* and SLE were observed in a Chinese population [9]. Also, in our

previous study [11], only variants in the *PRDM1-ATG5* intergenic region not within *ATG5* were detected to be associated with susceptibility to SLE, and they were detected to affect *ATG5* expression level through a *cis*-eQTL effect. Animal studies suggested that *ATG5*-null mice were lethal within 24 hours of birth, indicating the important role of *ATG5* for life. Thus, *ATG5* was likely to be a strong susceptibility gene to SLE and its abnormal expression may be a key determinant in susceptibility. As a strong body of evidence supported that numerous loci perturb gene expression in *trans* [12–14], we thus hypothesized that genetic polymorphisms of autophagy genes may also function at the upstream of *ATG5* by *trans*-eQTL effects to further modulate SLE susceptibility.

Lupus nephritis (LN), a major phenotype with poor prognosis of SLE, is possibly a kind of extreme phenotype. It was reported that autophagy inhibitions could decrease proteinuria levels, robustly reduced renal immune complex deposition, and remitted glomerulonephritis. Thus, by choosing *trans*-expression single nucleotide polymorphisms (*trans*-eSNPs) of *ATG5* at the genome-wide level, the present genetic association study was conducted to search for the regulatory mechanisms associated with the gene expression of *ATG5* in Chinese patients with LN.

2. Subjects and Methods

2.1. Subjects. A total of 280 LN patients from the Peking University First Hospital, who were of Han ethnicity living in north of China, were enrolled in the study. Their mean age was 33.2 ± 9.8 years and 247 of them were female. The controls were 198 geographically and ethnically matched healthy blood donors. Their mean age was 34.5 ± 10.3 years and 97 of them were female. One HapMap Han Chinese in Beijing (CHB) sample (NA18524) was included as a positive control for checking genotyping and no template control was taken as a negative control. All the patients met the revised SLE criteria of the American College of Rheumatology (ACR) [15] and were confirmed by renal biopsy using light microscopy, immunofluorescence, and electron microscopy.

The study was approved by the medical ethics committee of Peking University First Hospital and all the subjects gave written informed consents.

2.2. SNP Selection and Genotyping. The online eQTL database (mRNA by SNP Browser v. 1.0.1, http://www.sph.umich.edu/csg/liang/imputation/byGene.html) based on Epstein-Barr virus-transformed lymphoblastoid cell lines from 400 children was used to search for *trans*-eSNPs of *ATG5*. The inclusion criteria of the *trans*-eSNPs included (1) locating within the respective genes, (2) minor allele frequency (MAF) more than 5%, and (3) call rate more than 95%. In total, 1097 *trans*-eSNPs of *ATG5* were derived from the database and with the inclusion criteria 78 tagging *trans*-eSNPs (correlation coefficients between genotypes and *ATG5* expression ranged from 0.302 to 0.999,and P values ranged from 1.00×10^{-6} to 2.00×10^{-4}) were selected for genotyping.

The tagging *trans*-eSNPs were customized into a genotyping chip by an Illumina Solexa HiSeq 2000 platform (VC-201-0144). Beadpress Scanner and illumina Genomstudio were used for the analysis of chip data.

2.3. Statistical Analyses. For quality control analyses, *trans*-eSNPs were excluded if they had a call rate lower than 95% or a significant deviation from Hardy-Weinberg equilibrium in controls ($P < 0.01$). Similarly, we removed all the samples with a genotyping rate lower than 95% from further analysis. Principle component analysis (PCA) was used to detect population outliers in both cases and controls as previously described [16]. After quality control analyses for *trans*-eSNPs and samples, 68 *trans*-eSNPs in 279 LN patients and 199 controls were left for further genetic association analyses (Figure 1).

Allele frequencies were compared between cases and controls using chi-square tests and Fisher's exact test was used when necessary. For the positive *trans*-eSNPs, we evaluated the associations between their genotypes and disease severity and outcome. Results of the measurement data were expressed as mean ± SD, and t-tests or one-way analysis of variance were used to analyze the difference. Statistical analyses were performed with SPSS16.0 software (SPSS Inc., Chicago, IL). A two-tailed P value of less than 0.05 was considered statistically significant.

2.4. Bioinformation Analyses. For noncoding variants, RegulomeDB and HaploRegv3 databases were searched for their regulatory effects. For exon variants, PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) was used to predict possible impact of an amino acid substitution on the structure and function of the protein.

To validate the *trans*-eQTL effects of the positive *trans*-eSNPs, the gene expression profiling of EBV-transformed lymphoblastoid cell lines of 270 unrelated HapMap individuals from the Gene Expression Variation project (GENEVAR project, http://www.sanger.ac.uk/humgen/genevar/) was used. Besides, the different expression levels of *ATG5* were checked between SLE (E-GEOD-50772) and LN (E-GEOD-32592 and E-GEOD-32591) and healthy controls by using the ArrayExpress Archive database (http://www.ebi.ac.uk/arrayexpress/).

3. Results

3.1. Association Analyses of trans-eSNPs with LN. After quality control analyses, a total of 68 tagging *trans*-eSNPs were analyzed in 279 LN patients and 199 healthy controls. Four of them, including rs17008504 on *ANKRD50* (OR = 0.645, 95% CI = 0.476 to 0.875, $P = 0.005$), rs2271100 on *AGA* (OR = 1.630, 95% CI = 1.105 to 2.405, $P = 0.014$), rs6056923 on *PAK7* (OR = 0.546, 95% CI = 0.326 to 0.916, $P = 0.022$), and rs1391441 on *TET2* (OR = 1.345, 95% CI = 1.039 to 1.743, $P = 0.025$), were observed to be associated with LN in the current study (Table 1). And, marginal significance was observed between 7/68 *trans*-eSNPs and susceptibility to LN ($0.05 < P < 0.1$), including rs712377 on *SLC25A21* (OR = 1.283, 95% CI = 0.984 to 1.671, $P = 0.065$), rs1391438 on *TET2* (OR = 1.266, 95% CI = 0.978 to 1.638, $P = 0.073$), rs10878953 on *CPSF6* (OR = 1.268, 95% CI = 0.977 to 1.647, $P = 0.075$), rs7529592 on *AKNAD1*

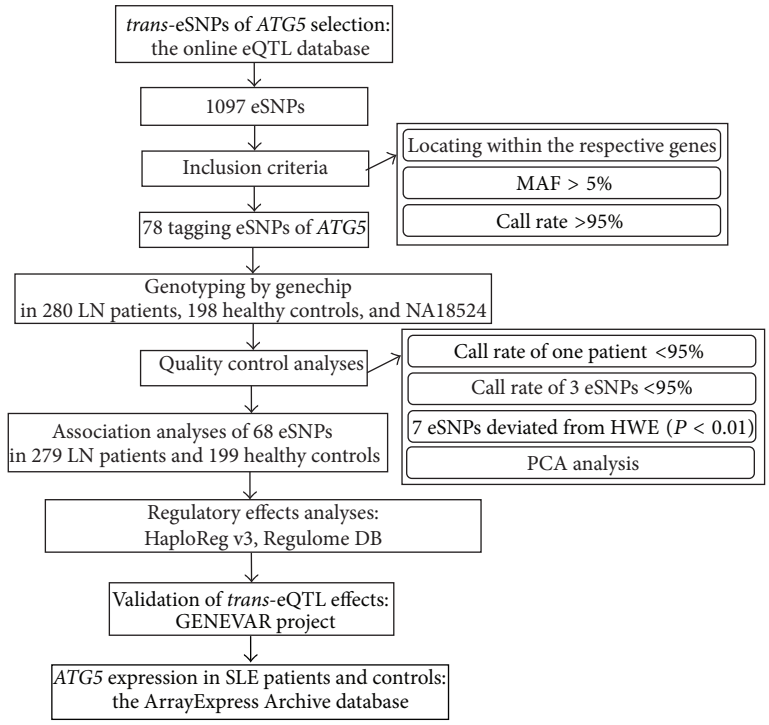

Figure 1: The workflow of genetic association study of *ATG5* by *trans*-eQTL. (a) eQTL: expression quantitative trait loci, eSNP: expression single nucleotide polymorphism, HWE: Hardy-Weinberg equilibrium, LN: lupus nephritis, MAF: minor allele frequency, PCA: principle component analysis, and SLE: systemic lupus erythematosus. (b) The online eQTL database: mRNA by SNP Browser v 1.0.1, http://www.sph.umich.edu/csg/liang/imputation/byGene.html. (c) HaploReg v3 database: http://www.broadinstitute.org/mammals/haploreg/haploreg_v3.php. RegulomeDB database: http://regulome.stanford.edu/. (d) GENEVAR project: the Gene Expression Variation project, http://www.sanger.ac.uk/humgen/genevar/. (e) The ArrayExpress Archive database: http://www.ebi.ac.uk/arrayexpress/.

(OR = 0.701, 95% CI = 0.463 to 1.062, $P = 0.094$), rs155098 on *ITGA4* (OR = 1.264, 95% CI = 0.960 to 1.665, $P = 0.095$), rs7751485 on *CDKAL1* (OR = 1.286, 95% CI = 0.975 to 1.698, $P = 0.096$), and rs7081173 on *LINP* (OR = 1.245, 95% CI = 0.962 to 1.611, $P = 0.096$). Thus, a total of 10 loci were suggested to play a potential role in the pathogenesis of LN through *trans*-eQTL effects.

To increase the detecting power, the genotype data of 136 CHB controls (except for the positive control NA18524) from the HapMap3 project, which were available for rs2271100, rs7529592, rs7081173, rs712377, rs1391438, and rs1391441, were downloaded. Using these data and our genotyping data, association analyses were performed. As can be seen in Table 1, almost all the loci showed more significant associations with combined data.

Besides, except for analyzing the association of the SNPs with LN susceptibility, we detected the association between *trans*-eSNPs genotypes and severity and outcome of LN patients in further, including their onset age, proteinuria, estimated glomerular filtration rate (eGFR), serum creatinine level, C3 level, systemic lupus nephritis disease activity index (SLEDAI) scores, percentage of crescent, different histological classes, response to treatment, and development of end stage renal disease (ESRD). Among them, response to treatment was measured by changes in proteinuria, and complete remission was defined as proteinuria <0.3 g per 24 hours while partial remission was defined as a decrease in proteinuria by at least 50% from the initial value and <3.5 g per 24 hours. Development of ESRD was defined as dialysis or death. However, no significant differences were observed between these clinical features and the different genotypes of the positive *trans*-eSNPs of *ATG5* (see supplementary Table 1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/153132), which may be due to the lower detecting power for subset analysis.

3.2. Functional Annotations by ENCODE Databases. RegulomeDB and HaploReg databases were used to annotate the regulatory effects of the positive *trans*-eSNPs. In RegulomeDB database, the *trans*-eSNPs with scores between 1 and 4 were listed (Supplementary Table 2); among them rs2271100, rs7081173, rs10788613, and rs11177577 showed the highest score (1f, eQTL + TF binding/DNase peak) (Supplementary Table 3). And in HaploReg v3 database, the positive *trans*-eSNPs were annotated to locate in the regions of promoter histone marks, enhancer histone marks, DNase-I hypersensitivity, protein binding, eQTL tissues, and regulatory motif, suggesting their potential roles for gene expression regulation (Supplementary Table 4). Besides, 1 missense variant rs10788611 (in strong linkage disequilibrium (LD) with rs7081173, $r^2 = 0.98$) on *LIPN* and 1 synonymous variant rs2305641 (in strong LD with rs10878953, $r^2 = 0.98$) on *CPSF6* were searched. However, their effects on protein function still need to be studied further.

Table 1: Allelic association analyses of the positive *trans*-eSNPs of *ATG5* in LN.

Chr	Position	Locus	SNP (minor allele)	MAF Case/control (%)	Allele OR by minor allele (95% CI)	Allele *P* values	
						Current population	Combined with HapMap CHB population
4	124718662	5′ of ANKRD50	rs17008504(A)	19.2/26.9	0.645 (0.476, 0.875)	0.005	—
4	177438525	AGA	rs2271100(G)	16.1/10.6	1.630 (1.105, 2.405)	0.014	**0.012**
20	9840271	5′ of PAK7	rs6056923(G)	4.9/8.5	0.546 (0.326, 0.916)	0.022	—
4	105207603	TET2	rs1391441(G)	49.8/42.3	1.345 (1.039, 1.743)	0.025	**0.002**
14	36867767	SLC25A21	rs712377(C)	42.3/36.4	1.283 (0.984, 1.671)	0.065	0.146
4	105230686	TET2	rs1391438(G)	51.6/45.7	1.266 (0.978, 1.638)	0.073	**0.009**
8	69280407	3′ of CPSF6	rs10878953(G)	45.0/39.2	1.268 (0.977, 1.647)	0.075	—
1	108823932	AKNAD1	rs7529592(G)	9.0/12.3	0.701 (0.463, 1.062)	0.094	**0.080**
2	181482227	ITGA4	rs155098(G)	35.3/30.2	1.264 (0.960, 1.665)	0.095	—
6	21127081	CDKAL1	rs7751485(G)	34.8/29.3	1.286 (0.975, 1.698)	0.096	—
10	88787819	3′ of LIPN	rs7081173(A)	50.2/44.7	1.245 (0.962, 1.611)	0.096	0.116

(a) CHB: Han Chinese in Beijing, 95% CI: 95% confidence interval, chr: chromosome, LN: lupus nephritis, OR: odds ratio, SNP: single nucleotide polymorphism.
(b) *P* values were calculated by chi-square test using 2 × 2 contingency tables based on allele frequencies.
(c) None of the genotypes in the controls or patients showed significant deviation from Hardy-Weinberg equilibrium.
(d) Chromosome positions were referred to GRCh38.

3.3. Validation of trans-eQTL Effects of the Positive trans-eSNPs and the Differential Expression Level of ATG5 in SLE. The 11 positive *trans*-eSNPs were significantly associated with the expression level of *ATG5* in our selective database of lymphoblastoid cell lines from 400 children (correlation coefficient ranged from 0.666 to 0.999, and *P* ranged from 0.00015 to 1.00×10^{-6}). Their *trans*-eQTL effects were validated in 270 unrelated HapMap individuals. As shown in Table 2, except for rs155098 and rs7751485, the significant associations between the positive *trans*-eSNPs and *ATG5* expression level were consistently verified.

Furthermore, we ascertained whether *ATG5* was expressed differently in SLE patients and healthy controls. The expression level of *ATG5* was significantly higher in SLE PBMC and LN tubulointerstitial samples than those of controls (1794.00 ± 240.22 versus 1541.73 ± 201.64, $P = 6.27 \times 10^{-5}$; 6.75 ± 0.19 versus 6.46 ± 0.20, $P = 1.82 \times 10^{-5}$) while in glomeruli samples of LN only marginal significant association was observed (7.86 ± 0.22 versus 7.73 ± 0.15, $P = 0.053$).

4. Discussion

Hypothesis-free GWASs have significantly broadened our views about genetic pathogenesis of SLE [1]. However, the majority of the associated variants were noncoding variants and with modest effects (OR = 1.1–1.5), which can only account for a small proportion of heritability of SLE. Gene expression level was suggested to be heritable, and the detection of the related eSNPs was considered to be an efficient way to reconstruct gene networks [17]. As a disease-predisposing gene, higher *ATG5* expression level [10, 11] and variants with *cis*-eQTL effects within or near *ATG5* [11] were observed in SLE. Thus, we suspect that any variants that perturb the expression of *ATG5* in *trans* would also be related to the susceptibility to SLE. Thus, the present study was conducted to analyze the genome-wide genetic regulatory mechanisms associated with the gene expression of *ATG5* in Chinese LN patients. By searching the online eQTL database, 78 tagging *trans*-eSNPs of *ATG5* were genotyped for the association study. The results showed that 4 *trans*-eSNPs, including rs17008504, rs2271100, rs6056923, and rs1391441, showed significant associations with susceptibility to LN ($P < 0.05$), while 7 *trans*-eSNPs, including rs712377, rs1391438, rs10878953, rs7529592, rs155098, rs7751485, and rs7081173, showed marginal significant associations ($0.05 < P < 0.1$). However, no significant associations between the SNPs and severity or outcome of patients with LN were observed in the current study. *In silicon* analysis suggested their regulatory effects. Besides, compared with healthy controls, higher expression level of *ATG5* was observed in PBMC, tubulointerstitial, and glomeruli samples of SLE patients, and

Table 2: Correlation between genotypes of the positive *trans*-eSNP with *ATG5* expression in public databases.

Gene	Children LCL (n = 400)		HapMap LCL (n = 270)		
	Positive *trans*-eSNPs	P	SNP[d]	r^2	P
ANKRD50	rs17008504	9.10×10^{-5}	rs1027497	0.92	0.001
AGA	rs2271100	1.20×10^{-4}	rs2271100	1	0.002
PAK7	rs6056923	1.10×10^{-4}	rs6056922	1	0.001
TET2	rs1391441	5.90×10^{-6}	rs1391441	1	0.005
TET2	rs1391438	7.10×10^{-6}	rs7655890	0.99	0.003
SLC25A21	rs712377	6.70×10^{-5}	rs712377	1	0.037
CPSF6	rs10878953	6.40×10^{-5}	rs7308481	0.99	0.030
AKNAD1	rs7529592	6.00×10^{-5}	rs7529592	1	0.001
ITGA4	rs155098	1.20×10^{-4}	rs155099	1	0.136
CDKAL1	rs7751485	1.00×10^{-6}	rs10946430	0.97	0.215
LIPN	rs7081173	1.50×10^{-4}	rs7081173	1	0.054

(a) LCL: lymphoblastoid cell lines, *trans*-eSNP: *trans*-expression single nucleotide polymorphism.
(b) Children LCL refers to lymphoblastoid cell lines from 400 children from families recruited through a proband with asthma.
(c) HapMap LCL refers to Epstein-Barr virus-transformed lymphoblastoid cell lines from 270 HapMap CEU, CHB, JPT, and YRI individuals (http://www.sanger.ac.uk/humgen/genevar/).
(d) Only the SNP on each locus with strongest associated significance with *ATG5* expression level was shown.

the correlation between the positive *trans*-eSNPs and *ATG5* expression level was validated in 270 unrelated HapMap individuals, suggesting the potential role of these loci in the pathogenesis of LN through perturbing the expression of *ATG5*.

Since *ATG5* was reported to be expressed in B cells, the eQTL database of lymphoblastoid cell lines from 400 children was chosen for searching *trans*-eSNPs of *ATG5*, for its relatively larger sample size and that environmental factors tend to have less effects on children. However, though the correlation between the *trans*-eSNPs and *ATG5* expression was validated in EBV-transformed lymphoblastoid B cell lines from the 270 unrelated HapMap individuals (JPT, CHB, CEU, and YRI), the association significance seemed to be weaker. This may be due to the smaller sample size, and also it was presumed that *trans*-eQTL effects are often more indirect and therefore weaker [14], indicating the necessity to replicate the associations in a larger cohort in the future.

In the present study, 4 loci, including *ANKRD50*, *AGA*, *PAK7*, and *TET2*, were identified to be associated with susceptibility to LN significantly. ANKRD50 (ankyrin repeat domain 50) was reported to have an essential role in the SNX27-retromer-mediated endosome-to-plasma-membrane recycling [18], but its exact mechanism was still needed to be studied further. AGA (aspartylglucosaminidase) is a lysosomal hydrolase that participates in the degradation of glycoproteins. Like other lysosomal enzymes, the deficiency of AGA leads to lysosomal storage disorder and *AGA* mutation was suggested to be weakly associated with chronic arthritis [19]. Thus, whether the dysfunction of AGA is associated with susceptibility to SLE by affecting autophagy—a degradation process conducted by lysosome—should be studied. PAK7 (p21 protein-activated kinase 7) is a member of Ser/Thr protein kinases, which has effects on cytoskeletal dynamics, cell proliferation, and survival [20]. For both ATG5 and microtubule associated light chain 3 (LC3) plays important roles in the extension of autophagosome membrane, PAK7 may regulate the expression of *ATG5* through microtubule system. TET2 (tet methylcytosine dioxygenase) is a methylcytosine dioxygenase that catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine. It was involved in chromatin modifications and other cellular processes through the interaction with O-linked β-N-acetylglucosamine transferase and it was also reported to be associated with several myeloproliferative disorders [21, 22]. Future studies are needed to uncover the mechanism of epigenetic and signaling networks wired with TET2 in SLE. Besides, another 6 loci with marginal associations, including *SLC25A21*, *CPSF6*, *AKNAD1*, *ITGA4*, *CDKAL1*, and *LINP*, were identified. SLC25A21 (solute carrier family 25 member 21) is a homolog of the *S. cerevisiae* ODC proteins. It is reported that HIV-1 Vif downregulates the expression of *SLC25A21* in Vif-expression T cells [23]. And, virus infection was closely associated with SLE, indicating the potential role of *SLC25A21* in SLE. CPSF6 (cleavage and polyadenylation specific factor 6) is one subunit of a cleavage factor required for 3′ RNA cleavage and polyadenylation processing. The interaction between CPSF6 and RNA is involved in the assembly of the 3′ end processing complex and facilitates the recruitment of other processing factors. AKNAD1 (AKNA domain containing 1) contains a domain found in an AT-hook-containing transcription factor and its alternative splicing can result in multiple transcript variants. ITGA4 (alpha 4 subunit of VLA-4 receptor) belongs to the integrin alpha chain family proteins, which can mediate adhesion of cells [24] and participate in B cell apoptosis. Besides, gene expression of integrins and their ligands was found to be upregulated in rheumatology arthritis [25]. *CDKAL1* (CDK5 regulatory subunit associated protein 1-like 1) is a member of the methylthiotransferase family and GWASs have linked intronic SNPs of *CDKAL1* with susceptibility to type 2 diabetes [26]. As autophagy is also strongly suggested to be associated with the pathogenesis of metabolic diseases including diabetes, the interaction between *CDKAL1* and *ATG5* could exist. LIPN (lipase, family member N) is

a lipase that is highly expressed in granular keratinocytes in the epidermis and plays a role in the differentiation of keratinocytes. Mutations in this gene were reported to be associated with lamellar ichthyosis type 4 [27]. In the present study, a missense variant rs10788611 within *LIPN* showed potential association with LN, which has caused the amino acid change from Threonine (T) to Asparagine (N) at position 244. However, its effects on protein function were seldom studied. Overall, the data above provided some clues for understanding the potential role of *ATG5* in the genetic pathogenesis of SLE.

However, there were still some limits in the present study. Due to the low allelic frequency of some *tran*-eSNPs and the relatively small sample size, the associations between *tran*-eSNPs and susceptibility to LN seemed to be weak, which could be enhanced by adding the referred CHB controls in HapMap. Besides, the smaller sample size of genotype subsets could be difficult to provide enough power for analyzing the association between *tran*-eSNPs genotypes and clinical features. Thus, to replicate the associations in a larger population is still needed in the future.

In summary, the present study established a relationship between *trans*-eSNPs of *ATG5* and LN in a northern Han population from China. By this new systemic genetics approach, 10 loci have been identified to be associated with LN potentially, widely broadening our understanding of the genetics role of *ATG5* in LN. Although these variants showed moderate associations, our approach allows for analysis of association data from a new perspective, and the results may be complementary to future pathway based genetic studies in SLE.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Yue-miao Zhang and Fa-juan Cheng contributed equally to this work.

Acknowledgments

The authors appreciate the work of all colleagues and they are grateful for the participation of all of the patients and healthy controls. This work was supported by grants from the National Natural Science Foundation of China (81200524), the Foundation of Ministry of Education of China (20120001120008), the Beijing Natural Science Foundation (7152148), the Major State Basic Research Development Program of China (973 program, 2012CB517700), the Research Fund of Beijing Municipal Science and Technology for the Outstanding Ph.D. Program (20121000110), and the Natural Science Fund of China to the Innovation Research Group (81021004). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 706515, 6 pages
http://dx.doi.org/10.1155/2015/706515

Research Article

Genetic Predictors of Poor Prognosis in Portuguese Patients with Juvenile Idiopathic Arthritis: Data from Reuma.pt

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Received 5 May 2015; Accepted 14 May 2015

Academic Editor: Carlo Perricone

Introduction. This study aimed to assess the genetic determinants of poor outcome in Portuguese patients with juvenile idiopathic arthritis (JIA). *Methods.* Our study was conducted in Reuma.pt, the Rheumatic Diseases Portuguese Register, which includes patients with JIA. We collected prospectively patient and disease characteristics and a blood sample for DNA analysis. Poor prognosis was defined as CHAQ/HAQ >0.75 at the last visit and/or the treatment with biological therapy. A selected panel of single nucleotide polymorphisms (SNPs) associated with susceptibility was studied to verify if there was association with poor prognosis. *Results.* Of the 812 patients with JIA registered in Reuma.pt, 267 had a blood sample and registered information used to define "poor prognosis." In univariate analysis, we found significant associations with poor prognosis for allele A of *TNFA1P3/20* rs6920220, allele G of *TRAF1/C5* rs3761847, and allele G of *PTPN2* rs7234029. In multivariate models, the associations with *TRAF1/C5* (1.96 [1.17–3.3]) remained significant at the 5% level, while *TNFA1P3/20* and *PTPN2* were no longer significant. Nevertheless, none of associations found was significant after the Bonferroni correction was applied. *Conclusion.* Our study does not confirm the association between a panel of selected SNP and poor prognosis in Portuguese patients with JIA.

1. Introduction

Juvenile idiopathic arthritis (JIA) is the most common childhood rheumatic disease [1]. Despite significant improvements in the management of children with JIA, the likelihood of long-term persistent disease activity remains high [2]. Published evidence demonstrates that clinical subtype, disease activity and duration, and response to treatment all influence the prognosis [3, 4]. In addition, diagnostic delay, severity and extension of arthritis at onset, symmetric disease, early hip or wrist involvement, involvement of cervical spine, the presence of rheumatoid factor (RF) or anticyclic citrullinated peptide, early age at onset, female gender, and family history of rheumatic disease were the best predictors of a poor outcome [3, 5–11]. However, in most studies of prognostic predictors in JIA, the authors are unanimous in concluding that there is considerable variability in results, making it harder to draw consistent conclusions [8].

Identifying earlier JIA worse prognosis cases is crucial to start appropriate treatment and to correctly inform patients and their parents. Much effort has already been done to elucidate prognosis predictors. Besides clinical factors, identification of genetic predictors of poor prognosis would be a significant contribution to the development of optimal treatment strategies for JIA.

Studies that evaluate nonclinical predictors, such as genetic or immunological parameters, hardly exist. Most of the genetic research aimed to identify variants that affect the risk of developing JIA or pathways modulating drug response in this disease. On the contrary, the goal of this study was to assess the genetic determinants of poor outcome in Portuguese patients with JIA. Our secondary objective was to find potential clinical predictors of poor prognosis.

2. Methods

2.1. Patient Population. Our study was conducted based on Reuma.pt, the Rheumatic Diseases Portuguese Register, which includes JIA patients treated with synthetic and biological Disease Modifying Antirheumatic Drugs (DMARDs) since June 2001. Patients registered up to December 2013 were included. The parent's consent and patient's assent (as appropriate) were obtained according to the declaration of Helsinki. The study was approved by local Ethics Committee. All patients fulfilled the ILAR criteria for the classification of JIA [12]. This study did not have any interference with patients' standard of care.

We analyzed the patients registered in Reuma.pt with the diagnosis of JIA, who had collected a blood sample for DNA analysis. The following data were collected at the time of the last visit to rheumatology clinics: gender, age, JIA subtype, disease duration, time until diagnosis (time since the beginning of the symptoms until the diagnosis of JIA), extra-articular manifestations of the disease, duration of therapy with DMARDs, corticosteroids and biological therapies, Childhood Health Assessment Questionnaire (CHAQ)/Health Assessment Questionnaire (HAQ) [13], patient's/parent's pain visual analogue scale (VAS), patient's/parent's disease global activity VAS, and physician's global disease activity VAS.

One of the barriers found in prognostic studies of JIA is that there is no universal definition of "poor prognosis." We have chosen to integrate in our definition of "poor prognosis" two variables: one instrument that combines disease activity and damage (CHAQ), dichotomized in accordance with other studies [14–19], using 0.75 as the cut-off point, combined with "the need for biological therapy," as a surrogate marker of disease severity and higher likelihood of a worse outcome. We have classified as patients in "need for biological therapy" all patients that were ever treated with biological agents for more than 3 months, due to articular or extra-articular manifestations of the disease. Thus, for the purpose of this study a patient was classified as having poor prognosis if CHAQ >0.75 and/or if the patient was ever treated with biological therapy for more than 3 months.

Genetic single nucleotide polymorphisms (SNPs) were studied to verify if there was any association with poor prognosis.

2.2. Genetic Analysis. The choice of SNP variants was based on information from previous studies of susceptibility and prognosis factors in JIA and included the following 32 SNPs of genes with a known function in the immune system: *PTPN22* rs2476601, *PTPRC* rs10919563, *TNFAIP3/A20* rs10499194, *TNFAIP3/A20* rs6920220, *TRAF1/C5* rs3761847, *ANGPT1* rs1010824, *ANGPT1* rs7151781, *AFF3* rs1160542, *AFF3* rs10865035, *CTLA4* rs3087243, *ERAP1/ARTS1* rs30187, *IL1* rs6712572, *IL1* rs2071374, *IL1* rs1688075, *IL10-1080GA* rs1800896, *IL10-819CT* rs1800871, *IL1R* rs12712122, *IL23R* rs11209026, *IL2-IL21* rs6822844, *IL2RA/CD25* rs2104286, *MIF-173CG* rs755622, *PTPN2* rs1893217, *PTPN2* rs7234029, *SLC26A2* rs1541915, *STAT4* rs3821236, *STAT4* rs7574865, *TNF-238* rs361525, *TNF-308* rs1800629, *VTCN1* rs10923223, *VTCN1* rs12046117, *WISP3* rs2280153, and *EYA4* rs17301249.

All samples were genotyped using Taqman SNP genotyping assays (Applied Biosystems, Foster City, USA) performed as described in the manufacturers' protocol. Genotyping reactions were carried out with an ABI 7500-fast thermocycler. The allele call was obtained by the AB software v2.0.5, by the analysis of allelic discrimination plots. SNPs with deviation from Hardy-Weinberg equilibrium ($P < 0.05$) or minor allele frequency (MAF) <1% were excluded from further analysis.

2.3. Statistical Analysis. We used the additive model to study the association between SNPs and poor prognosis, where homozygotes for the major allele were classified as zero, heterozygotes as 1, and homozygotes for the minor allele as 2. We report crude odds ratio (OR) based on a univariate logistic regression and adjusted OR from a multivariate model including significant clinical predictors. The following clinical variables were characterized: gender, disease category (classified into five groups including polyarticular JIA (RF negative, RF positive, and extended oligoarticular), persistent oligoarticular JIA, systemic arthritis, enthesitis-related arthritis (ERA), and psoriatic arthritis), time until diagnosis

Table 1: Distribution of the clinical characteristics of patients with and without poor prognosis.

Variable	Total	Patients with poor prognosis	Patients without poor prognosis	*P* value
Number	267	85	182	
Female gender *n* (%)	171 (64)	60 (22.59)	111 (41.6)	0.166
JIA categories (%):				
Polyarticular RF negative	48 (18)	19 (39.6)	29 (60.4)	
Polyarticular RF positive	25 (9.4)	19 (76)	6 (24)	
Extended oligoarticular	43 (16.1)	17 (39.5)	26 (60.5)	
Persistent oligoarticular	89 (33.3)	7 (7.9)	82 (92.1)	<0.001
Systemic	22 (8.2)	11 (50)	11 (50)	
Enthesitis-related arthritis	28 (10.5)	8 (28.6)	20 (71.4)	
Psoriatic arthritis	12 (4.5)	4 (33.3)	8 (66.7)	
Age at disease onset (median (IQR))	5.3 (2.2–9.7)	6.6 (3.1–11.6)	4.8 (2.1–8.8)	0.056
Age at diagnosis (median (IQR))	6.6 (2.8–11.6)	8.7 (3.4–13.7)	5.7 (2.5–10.6)	0.013
Time until diagnosis (median (IQR))	0.33 (1.14–1.00)	0.50 (0.17–1.0)	0.26 (0.14–0.88)	0.130
Age at last visit (median (IQR))	14.3 (8.9–18.3)	16.9 (13.1–24.1)	12.7 (6.9–13.3)	<0.001
Disease duration (median (IQR))	6.4 (3.1–12.0)	10.4 (5.2–16.0)	4.9 (2.3–10.4)	<0.001
CHAQ/HAQ (median (IQR))	0 (0–0.25)	0.25 (0-1)	0 (0–0.13)	<0.001
Patient's/parent's VAS (median (IQR))	5 (0–30)	10 (0–50)	0 (0–30)	0.017
Physician VAS (median (IQR))	0 (0–20)	10 (0–35)	0 (0–11.3)	<0.001
Extra-articular manifestations	98	41	57	0.011
Duration of DMARD use (median (IQR))	2.37 (0–5.8)	5.46 (3.02–9.54)	1.43 (0–4.02)	<0.001
Corticosteroid use (Y/N)	124	52	68	<0.001

F: female; M: male; JIA: juvenile idiopathic arthritis; RF: rheumatoid factor; IQR: interquartile range; CHAQ: Childhood Health Assessment Questionnaire; HAQ: Health Assessment Questionnaire; SD: standard deviation; VAS: visual analogue scale; DMARD: Disease Modifying Antirheumatic Drug; Y: yes; N: no. *P* values < 0.05 were considered statistically significant. Note: *P* values are from Pearson's chi-squared or Mann-Whitney tests, as appropriate.

(years), age at disease onset, disease duration (years), duration of DMARD treatment (years), corticosteroid treatment (ever or never), patient's/parent's disease global activity VAS, physician's global disease activity VAS, and extra-articular manifestations (yes or no). Continuous variables were modelled as linear. All clinical variables crudely associated with poor prognosis ($P < 0.20$) were included in a multivariate model. Then backward selection was applied to retain the clinical variables most associated with the outcome, using a significance level of 5%. Due to small sample size for most of the disease categories we carried out the analysis using all JIA categories combined. The stratified analysis was only possible for the polyarticular categories (polyarticular RF positive, polyarticular RF negative, and extended oligoarticular JIA) with 116 patients.

There was missing data for some of the variables, as follows: age at disease onset (1.5%), age at diagnosis (1.9%), patient's/parent's VAS (4.9%), and physician VAS (9.7%).

Statistical significance was considered at the 5% level. After Bonferroni correction for the 32 SNPs analyzed, results were considered significant for $P < 0.0016$.

Statistical analysis was made in R version 2.15.3 [20].

3. Results

Twenty-one centers and 77 rheumatologists and pediatricians contributed with data to Reuma.pt. Of the 812 patients with JIA registered in Reuma.pt (mean age 19.9 ± 11.3 years old, 65% females, and mean age at JIA onset 6.9 ± 4.7 years old), 291 had a blood sample to perform the genetic analysis and, from those, 267 had registered information about CHAQ/HAQ and/or the need for biological therapy used to define "poor prognosis." Of the 267 patients included, 85 had a poor prognosis, according to the definition: CHAQ/HAQ >0.75 and/or the treatment with biological therapy for more than 3 months. Nineteen patients had a CHAQ/HAQ >0.75 at the last appointment, 58 were treated with biological therapy, and 8 fulfilled both criteria.

Table 1 shows the distribution of the clinical characteristics of patients with and without poor prognosis.

3.1. Clinical Predictors of Poor Prognosis. Almost all the clinical variables, except gender, age at disease onset, and delay in diagnosis, were significantly different between the group of JIA patients with poor prognosis and the group who did not had poor prognosis (Table 1).

Clinical variables significantly associated with poor prognosis and included in the multivariate models were DMARD treatment (OR 1.17 [95% confidence interval 1.07–1.27]), higher physician VAS (1.03 [1.01–1.04]), and disease category. In particular, the persistent oligoarticular category had a much lower chance of worse prognosis (0.09 [0.04–0.22]) compared to the polyarticular category; ERA (0.44 [0.18–1.09]), systemic arthritis (1.11 [0.45–2.76]), and psoriatic arthritis (0.55 [0.16–1.94]) categories were not significantly different to the polyarticular group of JIA.

Table 2: Crude and adjusted *odds ratio* for the association between single nucleotide polymorphisms and poor prognosis.

	Minor allele	Crude		Adjusted†	
		OR (95% CI)	*P* value	OR (95% CI)	*P* value
All categories					
TNFA1P3/20 rs6920220 A/G	A	1.53 (1.01–2.33)	0.0436	1.67 (0.98–2.83)	0.0579
TRAF1/C5 rs3761847 A/G	G	1.49 (1.00–2.21)	0.0491	1.96 (1.17–3.3)	0.0110
PTPN2 rs7234029 A/G	G	1.86 (1.17–2.95)	0.0085	1.75 (0.99–3.1)	0.0540
Polyarticular categories					
CTLA4 rs3087243 A/G	A	1.98 (1.14–3.45)	0.0153	2.9 (1.39–6.08)	0.0047
PTPN2 rs7234029 A/G	G	3.08 (1.53–6.19)	0.0016	3.3 (1.48–7.37)	0.0035

†Clinical covariates included disease category, DMARD treatment, and physician VAS. Disease category was omitted from the model for the polyarticular categories of JIA.
OR: odds ratio; SNPs: single nucleotide polymorphisms. *P* values < 0.05 were considered statistically significant.

3.2. Genetic Predictors of Poor Prognosis. Crude and adjusted odds ratios for the association between studied SNPs and poor prognosis are shown in Table 2. In univariate analysis including all disease categories we found significant associations with poor prognosis for allele A of *TNFA1P3/20* rs6920220, allele G of *TRAF1/C5* rs3761847, and allele G of *PTPN2* rs7234029. In multivariate models adjusted for relevant clinical predictors (disease category, DMARD treatment, and physician VAS) the association for *TRAF1/C5* rs3761847 (1.96 [1.17–3.30]) remained significant at the 5% level while *TNFA1P3/20* rs6920220 (1.67 [0.98–2.83]) and *PTPN2* rs7234029 (1.75 [0.99–3.10]) were no longer significant.

In the univariate analysis for the polyarticular categories we found associations for allele A of *CTLA4* rs3087243 and allele G of *PTPN2* rs7234029. After adjusting for clinical factors the associations for *CTLA4* rs3087243 (2.90 [1.39–6.08]) and *PTPN2* rs7234029 (3.30 [1.48–7.37]) were still significant at the 5% level.

Nevertheless, none of associations found was significant after the Bonferroni correction ($P < 0.0016$).

4. Discussion

The aim of our study was to identify genetic and clinical predictors of poor outcome in JIA. In a Portuguese sample of patients with JIA, we have not found genetic associations with a poor outcome. Longer duration of DMARD treatment, higher physician VAS, and polyarticular categories of JIA had a significant association with poor prognosis.

A growing number of studies have been focused on susceptibility to JIA, including genome wide association studies [21]. However, studies on genetics of JIA outcomes are still scarce. In a recent systematic literature review of early predictors of prognosis in JIA [8], the authors concluded that demographic, clinical, and laboratory values were insufficient to predict the individual prognosis. The authors also pointed out that hardly any other potential predictors were evaluated, such as cytokine levels, cell characteristics, results of imaging obtained early in the disease course, or genetic evaluations, such as HLA and SNPs in genes with a known function in the immune system.

There are some examples of genetic research on JIA outcomes, including a study that suggests that the *MIF-173* polymorphism (*MIF-173*∗C allele) is a predictor of poor outcome in systemic-onset JIA [22], another study that found SNPs in the *IL6* gene associated with pain [14] and a correlation between *TGF-b1* gene codon 25 genotypes and early radiological damage [14], and, in the ERA subtype, a publication suggesting that the presence of *HLA-DRB1∗08* predicts failure to attain disease remission [23].

RA shares several clinical and pathological features with JIA and previous studies reported considerable overlap in genetic susceptibility loci for the two diseases [24–26]. JIA is a heterogeneous disease and genetic differences across the JIA categories and some category-specific effects have been identified [27, 28]. However, stratified analysis leads to small sample sizes for many of the categories. Larger cohorts of the ILAR categories are required to improve the power to detect any category-specific effects. We have stratified our analysis to investigate the polyarticular categories (polyarticular RF positive, polyarticular RF negative, and extended oligoarticular) which are the largest category in our sample.

We have found an association between a variant in the *TRAF1/C5* locus and poor prognosis in Portuguese with JIA regardless of the disease category. Only in the polyarticular category of JIA did we find an association between 2 variants in the *CTLA4* and *PTPN2* loci and a poor outcome. Nevertheless, none of the associations found was significant after the Bonferroni correction was applied ($P < 0.0016$).

The analysis of the clinical variables identified a number of parameters associated with poor outcome. Patients with a poor prognosis were more likely to have polyarticular categories of JIA (polyarticular RF negative, polyarticular RF positive, and extended oligoarticular), to be on treatment with DMARDs for a longer period, and to have higher values of physician VAS at the last visit. Additionally, patients with a poor prognosis were less likely to have persistent oligoarticular JIA. Our results are in accordance with other studies that revealed that children with persistent oligoarticular JIA have a substantially better outcome than those with either systemic or polyarticular JIA, as measured by attaining remission, degree of disability, and structural damage [8, 10, 11].

There are some limitations in our study, namely, the definition used to determine poor prognosis. There is no universal definition of "poor prognosis" in patients with JIA. We have chosen to integrate in our definition of "poor

prognosis" two variables: (1) an instrument that combines disease activity and damage (CHAQ/HAQ); (2) the need for biological treatment, because patients that do not respond to conventional DMARDs, namely, methotrexate, have a higher chance of a poor outcome. Regarding this last point, our study included patients at different phases of their disease and we are aware that the access to biological therapy could not have been the same for all patients, leading to a selection bias. In addition, some patients could have started biological therapy mainly for extra-articular manifestations of the disease (e.g., uveitis) and not due to joint disease. This could also have potentially confounded our results.

Another limitation of our study is the problem of multiple comparisons: our results may simply be attributable to chance. The sample size in our cohort was too small to adequately test replication and a further study in a larger cohort is still required in order to confirm or refute our findings.

5. Conclusions

In summary, our study does not confirm the association between a panel of selected SNPs and poor prognosis in Portuguese patients with JIA. A search for additional genetic variants is required. Moreover, combination of genetic factors together with environmental exposures should also be considered. Further studies, in different populations of JIA patients, should be performed to replicate these findings.

Abbreviations

JIA:	Juvenile idiopathic arthritis
DMARD:	Disease Modifying Antirheumatic Drug
CHAQ:	Childhood Health Assessment Questionnaire
SNP:	Single nucleotide polymorphism
VAS:	Visual analogue scale
MAF:	Minor allele frequency
OR:	Odds ratio
RF:	Rheumatoid factor
ERA:	Enthesitis-related arthritis.

Disclosure

There are no financial relationships relevant to the work.

Conflict of Interests

The authors have declared no conflict of interests.

Acknowledgments

This study would not have been possible without the collaboration of numerous clinicians, patients, and their parents. Funding for the genetic analysis was provided by an unrestricted grant from Abbvie, USA.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 571353, 8 pages
http://dx.doi.org/10.1155/2015/571353

Research Article

Liver Expression of Sulphotransferase 2A1 Enzyme Is Impaired in Patients with Primary Sclerosing Cholangitis: Lack of the Response to Enhanced Expression of PXR

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Received 19 May 2015; Accepted 11 June 2015

Academic Editor: Fulvia Ceccarelli

Background/Aim. Sulphotransferase 2A1 (SULT2A1) exerts hepatoprotective effects. Transcription of *SULT2A1* gene is induced by pregnane-X-receptor (PXR) and can be repressed by miR-378a-5p. We studied the PXR/SULT2A1 axis in chronic cholestatic conditions: primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC). *Materials/Methods*. Western-blot/PCRs for SULT2A1/PXR were performed in PSC ($n = 11$), PBC ($n = 19$), and control liver tissues ($n = 19$). *PXR* and *SULT2A1* mRNA was analyzed in intestinal tissues from 22 PSC patients. Genomic DNA was isolated from blood of PSC patients ($n = 120$) and an equal number of healthy volunteers. Liver miRNA expression was evaluated using Affymetrix-Gene-Chip miRNA4.0. *Results*. Increased PXR protein was observed in both PSC and PBC compared to controls and was accompanied by a significant increase of SULT2A1 in PBC but not in PSC. Decreased expression of *SULT2A1* mRNA was also seen in ileum of patients with PSC. Unlike PBC, miRNA analysis in PSC has shown a substantial increase in liver miR-378a-5p. *Conclusions*. PSC is characterized by disease-specific impairment of SULT2A1 expression following PXR activation, a phenomenon which is not noted in PBC, and may account for the impaired hepatoprotection in PSC. miRNA analysis suggests that SULT2A1 expression in PSC may be regulated by miR-378a-5p, connoting its pathogenic role.

1. Introduction

Primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) are both immune-mediated chronic cholestatic liver conditions [1]. PSC, which is frequently seen in association with inflammatory bowel disease [2], is a chronic biliary disease which may affect both the intra- and extrahepatic biliary tree while in PBC the main damage is noted in the small and medium size intrahepatic bile ducts. Many patients affected with PSC develop progressive biliary strictures, leading to recurrent cholangitis. Both PSC and PBC lead to hepatic and systemic accumulation of toxic biliary compounds,

resulting in progressive liver damage [3]. In response, several defense mechanisms are induced to prevent from liver injury. These comprise equilibrium changes in hepatobiliary transporters, downregulation of uptake systems, and induction of enzymes catalyzing detoxification processes [4–8]. The end readout of the complex interplay of these systems is prevention of and compensation for the deleterious accumulation of toxic bile acids. At the transcriptional level, the pivotal "fine-tuning" role for the homeostasis of these adaptive mechanisms is mainly played by members of the nuclear receptor (NR) family.

The pregnane X receptor (PXR; NR1I2) is a ligand-activated member of the nuclear receptor superfamily of transcription factors, which is highly expressed in human liver and gastrointestinal tract. It serves as a xenobiotic sensor which induces phase I (catalyzing hydroxylation) and phase II (catalyzing glucuronidation and sulfation) metabolism of many endogenous and exogenous compounds including bile acids (BA).

The primary bile acids in man are cholic and chenodeoxycholic acid. They are metabolized by enteric bacteria to produce the secondary bile acids deoxycholic and lithocholic, respectively. Lithocholic acid (LCA) is extremely lipophilic, rapidly partitions into membranes, and has a high potential for toxicity. Hydroxylation and sulphation of LCA greatly reduce its intestinal reabsorption, thus minimising its enterohepatic circulation and promoting its excretion in faeces. In contrast to the other bile acids which act as FXR ligands, LCA is a preferred ligand of PXR. Sonoda et al. have shown that LCA in micromolar amounts is a powerful inducer of the sulphotransferase (SULT2A1) responsible for its sulphation only in the presence of the RXR:PXR heterodimer [9]. Sulphation, a phase 2 activity, converts LCA to a less toxic and more water-soluble form which is readily excreted in faeces.

We had previously reported low levels of lithocholic acid sulphation in chronic cholestatic liver disease but could not distinguish between a causal and consequential linkage [10]. We postulated that failure of the liver's coordinated defense against lithocholic acid toxicity could be critically involved in the pathogenesis of PSC [11]. Recently, we reported that concentrations of plasma lithocholic acid sulphate were significantly reduced in PSC in comparison to patients with PBC and normal controls [12]. We postulated that an increased expression of SULT 2A1, in response to PXR activation, would be anticipated to occur as a hepatoprotective response to injurious cholestasis in conditions such as PSC and possibly PBC. To test our hypothesis, we analyzed the levels of PXR and its target gene SULT2A1 in PSC and PBC patients with comparable degrees of clinical cholestasis and sought out for potentially significant differences amongst PSC and PBC in this regard.

2. Materials and Methods

2.1. Patients Characteristic and Tissue Specimens. Liver tissue specimens were collected from explanted livers of patients with PSC ($n = 11$) and PBC ($n = 19$) who underwent liver transplantation. Control liver tissues ($n = 19$) were obtained from large margin liver resections of colorectal metastases with no microscopic changes of liver disease identified by a pathologist. Table 1 summarizes clinical and laboratory features of patients included in the analysis of liver expression of PXR and SULT2A1.

Table 1: Demographic and laboratory features of patients with primary PBC and PSC included in the part of the study analyzing expression of PXR and SULT2A1 in explanted livers.

	Liver	
	PBC ($n = 21$)	PSC ($n = 11$)
Gender (M/F)	1/20	7/4
Age (mean ± SD. range)	56 ± 9 (36–69)	48 ± 14 (17–62)
AST (U/L)	148 ± 128	204 ± 127
ALP (U/L)	447 ± 296	541 ± 265
Bilirubin (μmol/L)	114 ± 112	133 ± 102

Intestinal tissues were obtained from a group of 22 patients with PSC who underwent their routine colonoscopies. Eleven patients (8 males, 3 females; mean age 35 ± 17) had macroscopic features of ulcerative colitis (UC), called PSC + UC group, and 11 (9 males, 2 females; mean age 30 ± 9) had never been diagnosed with inflammatory bowel disease (called PSC group). All patients with PSC were treated with ursodeoxycholic acid with an average dose of 15 mg/kg. b.w. and patients with UC additionally received 5ASA (2-3 g/daily). Specimens were collected from ileum and ascending and sigmoid colon. For this part of the study, the control group comprised 14 (8 males, 6 females; mean age 50 ± 16) subjects who underwent their colonoscopies for various indications and who were found to have no macroscopic changes in their colons. Three tissue samples were obtained from each examined part of intestine. Then, biopsies were processed for future analyses, that is, either (i) stored in RNAlater for analysis of mRNA expression (AM7021; Applied Biosystems, Carlsbad, CA, USA), (ii) fixed in neutral-buffered formalin for histological assessment, or (iii) immediately frozen in liquid nitrogen for proteomic analyses. Histology was assessed by a pathologist (EU) who was blinded to clinical diagnoses of analyzed patients, according to histological grading scale introduced by Geboes et al. [13]. Briefly, according to this score, 6 histological features are assessed; these include (i) architectural changes; (ii) chronic inflammatory infiltrate; (iii) lamina propria neutrophils and eosinophils; (iv) neutrophils in epithelium; (v) crypt destruction; (vi) erosion or ulceration.

An informed consent was obtained from each patient participating in this study. The research protocol was approved by the Ethics Committee of Pomeranian Medical University and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

2.2. RNA Extraction and Quantification of Gene Expression. Total RNA were isolated using the RNeasy Mini kit (Qiagen, Valencia, USA), according to the manufacturer's protocol. cDNA synthesis was carried out using Superscript II RT kit

(Invitrogen, Carlsbad, CA, USA) according to the protocol previously described [14] and stored at −20°C. The expression of specific target genes was measured by quantitative real-time PCR using commercially available Gene Expression Assays and 7500 Fast Real-Time PCR System (Applied Biosystems). The following assays were used in the study: PXR (Hs01114267_m1); SULT2A1 (Hs00234219_m1); and control human GAPDH (Hs99999905_m1). A 20 μL reaction mixture contained 10 μL of TaqMan Gene Expression PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μL diluted cDNA template, and 1 μL of the probe/primer assay mix. The fluorescence data were analyzed with 7500 Software v2.0.2. (Applied Biosystems, Carlsbad, CA, USA). The expression of target genes was calculated using the ΔΔCt method of relative quantification.

2.3. Protein Expression Analysis. Proteins from frozen liver and intestine tissue were extracted through homogenization in an ice-cold RIPA buffer (50 mM Tris-HCl pH = 8, 150 mM NaCl, 1% NP-40, 0,5% NaDOC, 0,1% SDS, 1 mM EDTA, 100 mM PMSF, 100 mM NaF) containing protease inhibitor cocktail and PhosSTOP (Roche Diagnostics GmbH, Mannheim, Germany). Protein quantification was made using the bicinchoninic acid assay (Micro BCA Protein Assay Kit; Thermo Scientific, Waltham, MA, USA). Forty μg of protein extracts from each liver sample was electrophoresed in SDS polyacrylamide gels and subsequently blotted into PVDF membranes (Thermo Scientific, Rockford, IL, USA) under semidry transfer conditions. Membranes were blocked overnight at 4°C with TBST containing 5% (w/v) milk (Merck) and then probed using the following primary antibodies: PXR (sc-48403; Santa Cruz, 1 : 500), SULT2A1 (sc-8002 Santa Cruz, 1 : 200), and anti-α/β-tubulin (2148, Cell Signaling, 1 : 1000). For the detection of antigen-antibody complexes, peroxidase conjugated anti-rabbit secondary antibody (NA9340V, Amersham, GE Healthcare, UK; 1 : 5000 dilution) or anti-mouse secondary antibody (NA9310V, Amersham; 1 : 5000 dilution) was used. Protein expression was detected using an enhanced chemiluminescence detection system (Chemiluminescent HRP Substrate, Millipore, Billerica, MA, USA). Bands were visualized and quantified using MicroChemi 2.0 System and GelQuant software (Israel).

2.4. SULT2A1 Genotyping and Promoter Sequencing. Two SNPs (rs11569683 [A/G] and rs112433193 [C/G]), located near/within the PXR binding site within promoter region of *SULT2A1* gene, were analyzed in genomic DNA isolated from peripheral blood mononuclear cells of 151 PSC patients (109 males, 42 females; mean age 32 ± 13), (DNeasy Blood & Tissue Kit, Qiagen). PCR reactions contained 20 ng DNA, 900 nM of each primer, 12.5 μL of *TaqMan* Universal Master Mix, and 200 nM of VIC-labelled and FAM-labelled probes in 25 μL-reactions. Amplification conditions were as follows: 95°C for 10 min, 40 cycles of 92°C for 15 s, and 60°C for 1 min. Oligonucleotide primers and TaqMan probes for the *SULT2A1* polymorphisms were designed and synthesized by Applied Biosystems. The fluorescence data were analyzed with allelic discrimination 7500 Software v.2.0.2. Additionally, for the purpose of detecting a DNA sequence of *SULT2A1* promoter region within the PXR binding sites represented by IR2 and DR4 motifs, the PCR reaction was performed in samples of genomic DNA of 151 PSCs patients with the use of the following *primers: Fw* 5-GCACGATTGCAGGATTATTTAG-3′; Rv 5′AGAAATCGTCCGACATGATGAT-3′. The amplified DNA (436 pb) was purified with EXTRACTME *DNA GEL–OUT Kit (DNA, Gdansk, Poland)* followed by sequencing in the Laboratory of *DNA* Sequencing and Oligonucleotide Synthesis (Oligo.pl; Institute of Biochemistry and Biophysics, Polish Academy of Sciences). The location of primers used in the sequencing and PXR binding site within the promoter region of *SULT2A1* gene is showed in Figure 1.

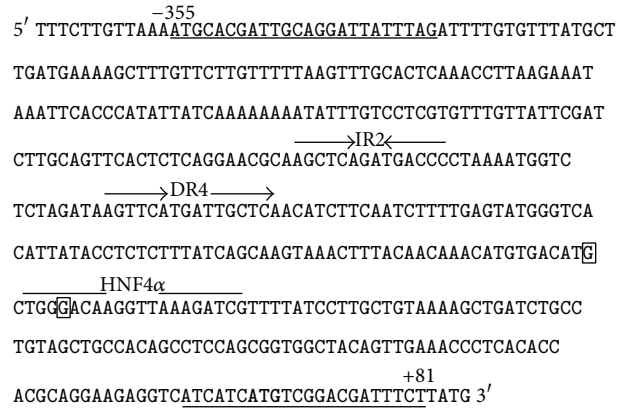

Figure 1: The DNA sequence within *SULT2A1* promoter region (−367 to +85). The positions of the studied SNPs, that is, rs11569683, rs112433193, are marked in squares. The PXR binding sites are represented by IR2 and DR4 motif. The HNF4 alpha binding site is marked. The location of primers used in the sequencing of HNF4alpha and PXR binding sites is underlined.

2.5. MicroRNA Assay. Total RNA from liver samples explanted from patients diagnosed with PSC (n = 4), PBC (n = 4) and aged and gender-matched control donors for each disease (n = 4 per experimental group) was isolated with the use of miRNeasy Mini Kit (Qiagen). Microarray analysis comprised Affymetrix GeneChip miRNA 4.0 arrays and was performed by Microarray Core Boston University (http://www.bumc.bu.edu/microarray/).

2.6. Statistics. Data were evaluated as mean ± standard error (SE) for continuous variables. Data were analyzed using Stat-View-5 Software (SAS Institute, Cary, NC, US) and included Fisher's exact and ANOVA analysis. Correlations were assessed by parametric tests (Pearson Correlation test). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Different Expression Patterns of PXR and SULT2A1 in Patients with PSC and PBC. In cirrhotic liver tissues expression of *PXR* mRNA was considerably enhanced in both PSC

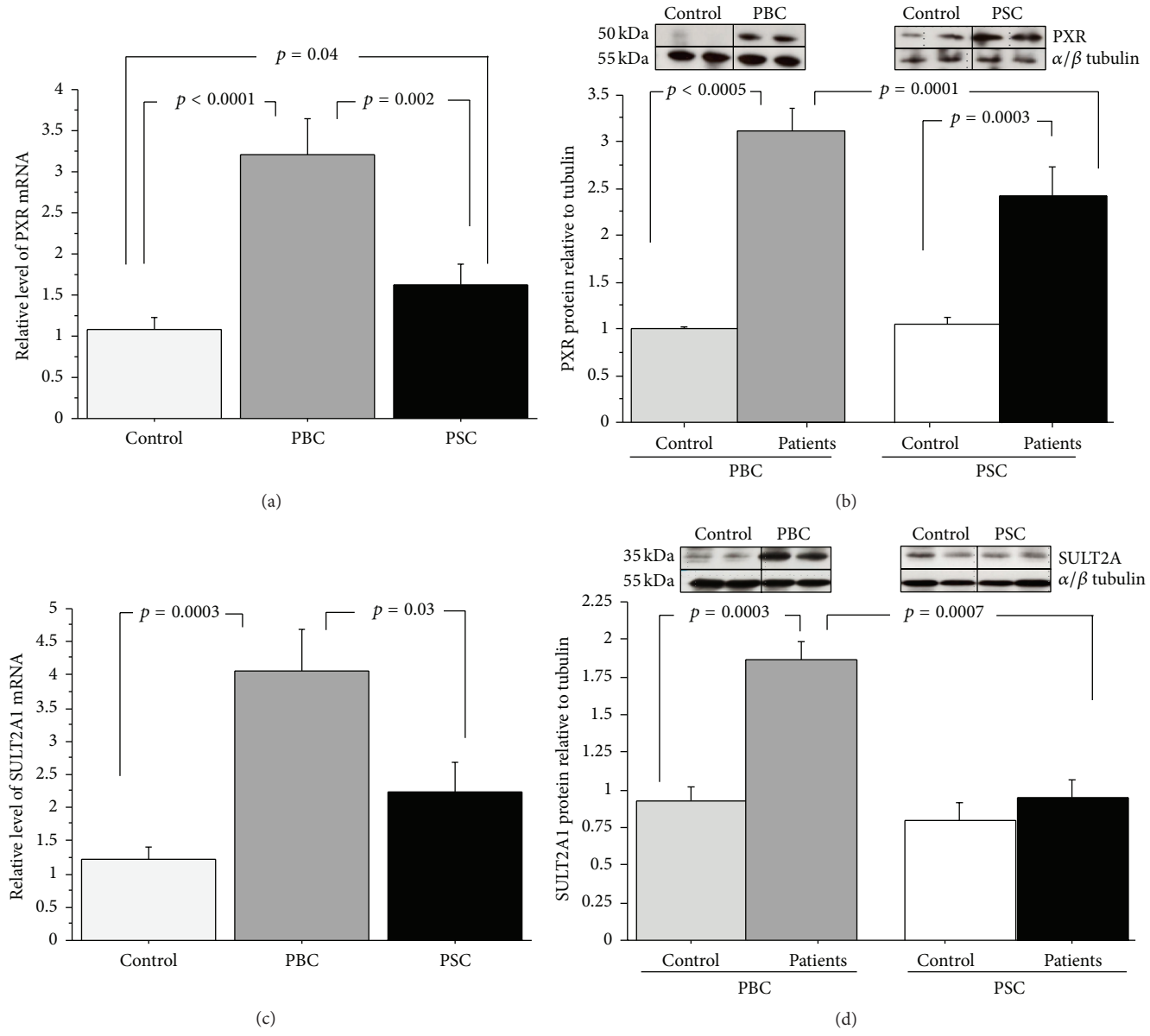

Figure 2: Liver expression of PXR and SULT2A1 in liver tissue from patients with cirrhotic PSC and PBC. (a) PXR mRNA, (b) PXR protein, (c) SULT2A1 mRNA, and (d) SULT2A1 protein. Levels of gene expression presented as fold-change relative to control were normalized with glyceraldehydes 3-phosphate dehydrogenase (GAPDH). Bars indicate the mean ± SEM. Changes in protein levels were determined by densitometry analyses after normalization to α/β tubulin as a control for loading. Bars indicate the mean ± SEM.

(1.6-fold, p = 0,04) and PBC (3-fold increase, $p < 0.0001$ versus controls; Figure 2(a)). Expression of *PXR* mRNA did not correlate with biochemical features of cholestasis (data not shown). Similarly, protein level of PXR was significantly augmented in PSC and PBC compared to control tissues (2.7 ± 0.3 versus 1.2 ± 0.2, $p = 0.0003$ and 3.1 ± 0.5 versus 1.1 ± 0.1, $p = 0.0005$ resp.; Figure 2(b)). The increase in PXR mRNA and protein levels was significantly less pronounced in PSC than PBC patients ($p = 0.002$ and $p = 0.0001$ versus PBC, for PXR mRNA and protein levels, resp.; Figures 2(a) and 2(b)). In PBC, the enhanced PXR expression was accompanied by the increased expression of *SULT2A1* mRNA (4-fold, p = 0.0003 versus controls; Figure 2(c)) and SULT2A1 protein level (1.9-fold, $p = 0.0003$ versus controls; Figure 2(d)). Such changes were not observed in livers from patients with PSC (Figures 2(c) and 2(d)).

3.2. Expression of SULT2A1 mRNA Is Suppressed in Small Intestine of Patients with PSC. Data on histological findings in colons in patients with PSC and controls are summarized in Table 2. Ileal tissues were not assessed by the pathologist as they are not included in the scoring system applied in this study. Expression of *PXR* mRNA was similar in patients with PSC and controls regardless of the examined part of colon. The level of *SULT2A1* mRNA was significantly lower in the ileum of patients: 0.37 ± 0.1 in PSC without UC and 0.41 ± 0.1

Table 2: Histological assessment of epithelial and inflammatory features in ascending colon (a) and sigmoid colon (b) of PSC patients without or with ulcerative colitis (PSC + UC). Values indicate the mean of scoring system ± SEM.

(a)

Features	Ascending Colon					
	Control	PSC	PSC + UC	PSC versus control	PSC + UC versus control	PSC versus PSC + UC
Architectural change	0.1 ± 0.1	0.4 ± 0.2	0.7 ± 0.2	NS	$p = 0.015$	NS
Chronic inflammatory infiltrate	0.4 ± 0.1	1.1 ± 0.2	1.5 ± 0.3	$p = 0.01$	$p = 0.0003$	NS
Lamina propria neutrophils and eosinophils	0.1 ± 0.1	0.5 ± 0.2	1.3 ± 0.3	NS	$p = 0.001$	$p = 0.038$
Neutrophils in epithelium	0.0	0.1 ± 0.1	0.3 ± 0.2	NS	NS	NS
Crypt destruction	0.0	0.0	0.5 ± 0.4	NS	NS	NS
Erosion or ulceration	0.0	0.4 ± 0.4	0.3 ± 0.3	NS	NS	NS
Total	**0.5 ± 0.2**	**2.1 ± 0.4**	**3.6 ± 0.8**	**$p = 0.025$**	**$p < 0.0001$**	**$p = 0.038$**

(b)

Feature	Sigmoid colon					
	Control	PSC	PSC + UC	PSC versus control	PSC + UC versus control	PSC versus PSC + UC
Architectural change	0.1 ± 0.1	0.2 ± 0.2	0.9 ± 0.3	NS	$p = 0.0015$	$p = 0.01$
Chronic inflammatory infiltrate	0.4 ± 0.1	0.4 ± 0.2	1.3 ± 0.1	NS	$p < 0.0001$	$p = 0.0003$
Lamina propria neutrophils and eosinophils	0.0	0.1 ± 0.1	0.9 ± 0.2	NS	$p < 0.0001$	$p = 0.0001$
Neutrophils in epithelium	0.0	0.0	0.1 ± 0.1	NS	NS	NS
Crypt destruction	0.0	0.0	0.5 ± 0.4	NS	NS ($p = 0.054$)	NS
Erosion or ulceration	0.0	0.0	0.4 ± 0.3	NS	NS	NS
Total	**0.5 ± 0.2**	**0.6 ± 0.3**	**2.9 ± 0.4**	**NS**	**$p < 0.0001$**	**$p < 0.0001$**

in PSC with UC versus 1.01 ± 0.2 in controls, $p = 0.02$ and $p = 0.03$, respectively. This decrease was not seen in either ascending or sigmoid colon. These data are summarized in Figure 3.

3.3. Lack of the Alterations within Promoter Region of SULT2A1 in PSC Patients. The genotyping analysis has shown that the examined SNPs, that is, rs11569683 and rs112433193, are not present among PSC patients. Furthermore, since earlier studies established that IR2 and DR4 motifs within promoter region of human *SULT2A1* are involved in the PXR-induced activity of the this gene [15], the detailed analysis of the nucleotide sequence of the promoter region of *SULT2A1* gene containing the PXR binding sites was carried out (Figure 1). The genomic analysis did not identify any changes within the examined region (data not shown).

3.4. Expression of miR-378a Is Considerably Enhanced in Livers of PSC Patients. A substantial increase in the level of microRNA miR-378a-5p in liver tissue of PSC patients (3,6-fold change; $p = 0.0047$ versus PBC) was seen. The observed changes were specific for PSC but not for PBC. The identified microRNA was predicted to target *SULT2A1* mRNA (http://mirdb.org/miRDB/).

4. Discussion

In the present study, we looked at the expression of pregnane-X-receptor (PXR) and sulphotransferase 2A1 (SULT2A1) in the livers of patients with PSC and PBC. We documented, for the first time, *in vivo* evidence of increased PXR expression in these conditions. We also found that, contrary to PBC, PXR activation is not accompanied by an enhanced expression of SULT2A1, suggesting a disease-specific impairment of SULT2A1 expression in PSC. Also, miRNA analysis suggested that SULT2A1 expression in PSC is likely regulated by miR-378a-5p, further indicating a pathogenic role for this miR in PSC.

Why PXR activation fails to boost SULT2A1 expression in PSC remains puzzling. PXR is a key member of the NRs family of ligand-modulated transcription factors. It binds (as a heterodimer with RXR) to response elements in the promoter region of target genes involved in stimulation of the bile acid detoxification machinery. Human PXR agonists include LCA, rifampicin, statins, and corticosteroids [16]. Although there is a wide evidence for NR interaction pathways in cholestatic conditions, most data derive from *in vitro* and animal studies in experimentally induced cholestasis. Data analyzing expression of NRs in patients with chronic cholestatic disorders are scarce.

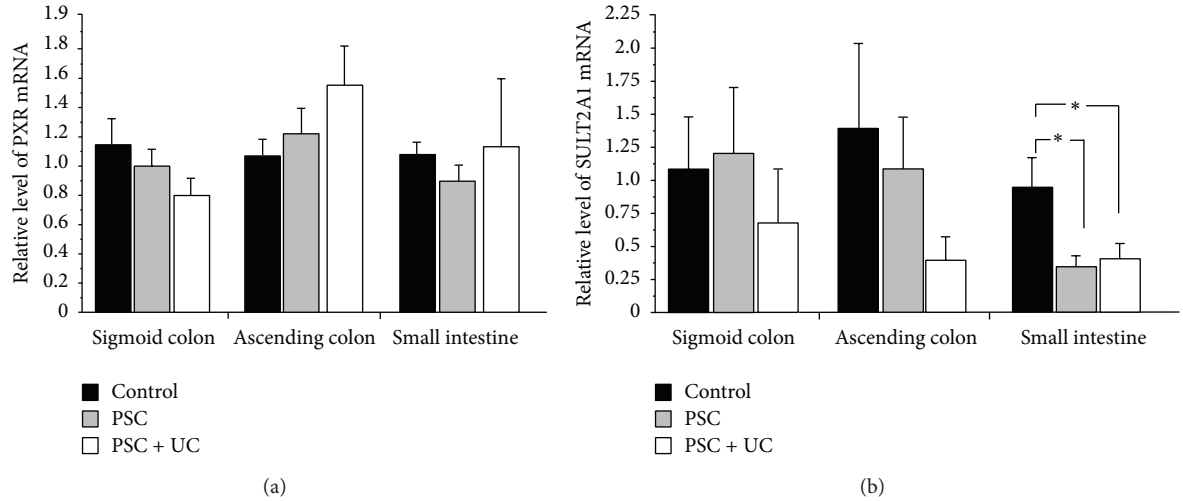

Figure 3: Gene expression of (a) PXR and (b) SULT2A1 mRNA in colon and small intestinal tissues of PSC patients without (PSC) or with ulcerative colitis (PSC + UC). Levels of gene expression presented as fold-change relative to control were normalized with glyceraldehydes 3-phosphate dehydrogenase (GAPDH). Bars indicate the mean ± SEM and $^{*}P < 0.05$.

SULT2A1 is a target gene for PXR and plays an important role in PXR-mediated detoxification. Little is known about SULT expression in cholestatic liver diseases. A comprehensive analyses of the BA profile in serum from cholestatic patients has shown significant reduction of the amount of sulfated LCA present in patients with PSC [12] and reduced LCA sulfotransferase activity was detected in human livers with PSC [10, 17]. Such data are in line with the diminished potential of SULT2A1 expression following PXR activation noted in PSC. Our study showed that *PXR* mRNA and protein expression were significantly enhanced in PSC and PBC livers. To our knowledge, *in vivo* evidence of increased expression of *PXR* has been obtained for the first time. This finding is in agreement with data obtained in experimental cholestatic injury induced in animals, as well as *in vitro* observations. These cumulative data confirm that in the course of cholestatic processes the expression of BA-activated PXR protein increases in order to protect against BA toxicity. PXR was reported to be involved in pathogenesis of ICP [18, 19] and PSC [19] as well as in adaptation to cholestatic liver diseases, for example, in obstructive cholestasis [20]. With regard to PBC, limited data were obtained by Zollner et al., who have shown a repression of *CYP7A1* mRNA and elevation of MRP4 protein level in patients with PBC compared to controls. However, that study has found that expressions of NRs including PXR were not significantly changed [6].

Although, in response to cholestatic insult, PXR expression was significantly increased in both conditions, this increase was markedly less effective in our patients with PSC. The pathophysiological significance of this finding requires further investigation. PXR participates in diverse ways in transcriptional regulation of cytosolic SULTs. Treatment of human intestinal Caco-2 cells with activators of PXR leads to the induction of SULT2A1 in a hepatocyte nuclear factor 4- (HNF4-) dependent manner [15]. In a rodent model, stimulation of PXR expression reduced liver injury triggered by LCA administration in a SULT2A1-dependent manner [21]. Radominska et al. demonstrated that SULT2A1 is the only enzyme responsible for bile-acid sulphation in the human liver [21, 22]. In human hepatocytes the basal levels of SULT2A1 expression are relatively high [23, 24]. Thus, in normal conditions, bile acid sulphation is a very efficient elimination process in humans, and LCA, the most hydrophobic and toxic bile acid, is rapidly sulphated already on the first pass through the liver [25, 26]. Taking into account the biological importance of SULT2A1 in protecting against the dysregulation of homeostasis caused by cholestasis, we considered that it is worthy to study its expression. In our study, the overexpression of PXR was accompanied by elevation of SULT2A1 mRNA and protein levels in patients with PBC, but not in PSC. In the latter group of patients, mRNA and protein levels were unchanged in comparison to controls samples, though an induction of PXR proteins was observed in this group. This observation may suggest that, in PSC, the positive regulation pathway between PXR and SULT2A1 is disturbed, resulting in an impairment of sulphation capacity. This finding is in line with our previous observations demonstrating a reduction of sulphation capacity in PSC but not in PBC and controls [12]. LCMS/MS analysis of the concentrations of LCA and its SULT2A1 metabolite-LCA-S in patients with PSC, PBC and controls clearly demonstrated a decrease of the sulphation potential of LCA in PSC with a metabolic LCA-S/LCA ratio reduction noted in PSC when compared to both PBC patients and controls. LCA-S/LCA ratio was 5.01 in PSC as compared to 1.94 in PBC and 1.75 in controls [12].

The data on SULT2A1 expression in human cholestatic tissues are very scanty. Analysis of human livers from various

chronic liver diseases such as PBC, PSC, autoimmune hepatitis, and alcoholic cirrhosis (but not in cryptogenic cirrhosis) has shown that SULT2A1 activity and concentration are significantly reduced when compared to normal livers. However, a detailed comparison among disease groups did not produce statistically significant results [10].

As already mentioned, the interplay between PXR and HNF4alpha has been shown to be of importance in regulation of *SULT2A1* gene expression [27]. Regardless of the enhanced expression of PXR, the transcript level of its target gene, that is, *SULT2A1*, was not changed in the livers of PSC patients. Therefore, we decided to perform the genomic analysis of *SULT2A1* promoter in order to find out whether the alteration in nucleotide sequence may be accountable for the lack of adequate level of this detoxification enzyme. We hypothesized that a possible change in DNA sequence within *SULT2A1* promoter region, more precisely in the proximity of HNF4alpha and PXR binding sites, could explain the lack of the increased expression of *SULT2A1* gene in PSC disease. However, our hypothesis proved to be wrong, as neither SNP genotyping assessments nor a detailed sequencing of *SULT2A1* promoter have provided evidence in support of our assumption.

Within the last decade, microRNA (miRNA) have emerged as a new class of small molecules that control intracellular gene expression at a posttranscriptional level. Increasing body of evidences confirms the fundamental role of miRNA in the physiological and pathological processes in the liver. Our study provide a new insight into SULT2A1-specific expression patterns that can be modulated by miR-378a-5p in PSC patients. The substantially increased expression of miR-378a-5p in PSC liver may be responsible for the observed lower level of SULT2A1 protein, as the identified microRNA was predicted to be involved in the regulation of *SULT2A1* gene expression. However further analysis is needed to understand the role of miR-378a-5p in cholestatic liver diseases like PSC.

In this study we have also analyzed expression of both *PXR* and *SULT2A1* mRNA in the intestine of patients with PSC. To our knowledge, expression of *PXR* in the intestinal tissue has not been analyzed so far in PSC. The tendency of colitis to primarily involve the caecum and right hemicolon in PSC patients is in contradistinction to non-PSC related ulcerative colitis in which the disease is always distal and supports the hypothesis that elements of the enterohepatic circulation are implicated in the pathogenesis of PSC-associated colitis [28]. We found a significantly decreased expression of *SULT2A1* mRNA but not *PXR* in the ileum of patients with PSC. This finding further supports the notion of impaired SULT2A1 function in PSC. On the other hand, we did not see any difference between analyzed groups in terms of expression of *PXR* and *SULT2A1* mRNA in the colon. As expected, patients with PSC with concomitant UC had significantly more pronounced inflammatory features on their histology; however, this did not appear to affect either *PXR* or *SULT2A1* mRNA. As already mentioned, there is no study in the literature dealing specifically with this issue. Thus, this finding requires further investigation and its interpretation is difficult at this point.

5. Conclusions

In conclusion, our results indicate disease-specificity of intrinsic PXR-coordinated hepatoprotective mechanism against BA toxicity. In contrast to PBC, PSC patients show an impaired signaling between PXR and SULT2A1. The observed increase in liver miR-378a-5p level, a negative post-transcriptional modulator of *SULT2A1* gene, could contribute to the pathogenic processes seen in this condition. Since most of the accessible research in this area was undertaken in a rodent model, which does not translate directly to humans, our data on PXR and SULT2A1 expressions in humans are novel and may have a future translational clinical repercussion. More research is needed to understand the enigmatic role of SULT2A1 in the development of liver disease [29, 30].

List of Abbreviations

HNF4: Hepatocyte nuclear factor 4
LCA: Lithocholic acid
NR: Nuclear receptor
PBC: Primary biliary cirrhosis
PSC: Primary sclerosing cholangitis
PXR: Pregnane-X-receptor
SULT2A1: Sulphotransferase 2A1.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

Ewa Wunsch was supported by the Foundation for Polish Science. Piotr Milkiewicz was supported by Grant nos. 2011/01/B/NZ5/04216 and 2011/02/A/NZ5/00321 from National Science Centre in Poland. Olivier Barbier was supported by grant from the Canadian Liver Foundation and CIHR salary award (new investigator award no. MSH95330). Dimitrios P. Bogdanos was supported by a CSL Award from the Higher Education Funding Council for England (HEFCE).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 453046, 11 pages
http://dx.doi.org/10.1155/2015/453046

Research Article

Autoimmune Conditions in 235 Hemochromatosis Probands with *HFE* C282Y Homozygosity and Their First-Degree Relatives

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Received 18 June 2015; Accepted 2 August 2015

Academic Editor: Fulvia Ceccarelli

We performed a retrospective study of autoimmune conditions (ACs) in 235 hemochromatosis probands at diagnosis by analyzing age, sex, ACs, history of first-degree family members with ACs (FH), diabetes, heavy ethanol consumption, elevated serum ALT/AST, nonalcoholic fatty liver disease, viral hepatitis, cirrhosis, iron removed to achieve iron depletion (QFe), and positivity for human leukocyte antigen (HLA) haplotypes A*01, B*08; A*02, B*44; A*03, B*07; A*03, B*14; and A*29, B*44. There were 138 men (58.7%). Median followup was 19.6 y. One or more of 19 ACs were diagnosed in each of 35 probands (14.9%). Prevalences of Hashimoto's thyroiditis, rheumatoid arthritis, and ankylosing spondylitis were 8.1% (95% CI: [5.1, 12.5]), 1.7% [0.6, 4.6], and 0.0085 [0.0015, 0.0337], respectively. Eighteen probands (7.7%) had a FH. Eight probands with ACs had 9 family members with ACs. In a logistic regression, ACs were less likely in men (odds ratio (OR) 0.3 [0.1, 0.6]) and more likely in probands with a FH (OR 4.1 [1.4, 11.8]). Overall ACs risk was not significantly associated with QFe or HLA haplotypes. Estimated survival of probands with and without ACs did not differ significantly. We conclude that ACs are common in hemochromatosis probands, especially women and probands with a FH.

1. Introduction

Hemochromatosis in whites of western European descent is an autosomal recessive disorder that increases the risk to develop iron overload. Severe iron overload can cause cirrhosis, primary liver cancer, diabetes mellitus, other endocrinopathies, and cardiomyopathy [1, 2]. By 1976, it was discovered that hemochromatosis in most whites is linked to human leukocyte antigen (HLA) A*03 or HLA haplotypes A*03, B*07 or A*03, B*14 [3, 4]. For two decades thereafter, the combination of iron phenotyping and HLA-A and HLA-B typing was used to diagnose hemochromatosis in probands and first-degree family members [5].

In 1996, it was discovered that ~90% of whites with hemochromatosis were homozygous for a common mutation (exon 4, c.845G>A, p.C282Y; rs1800562) in a previously undescribed nonclassical class I major histocompatibility complex (MHC) gene on chromosome 6p [6]. The gene, now known as *HFE*, occurs in linkage disequilibrium with the HLA-A locus [3, 7, 8]. The most common hemochromatosis ancestral haplotypes in many northwestern European and derivative populations include *HFE* C282Y linked to HLA-A*03 [7–10], HLA-A*03, B*07 [7–9], or HLA-A*03, B*14 [7–9, 11]. C282Y also occurs on other HLA haplotypes, including A*01, B*08 and A*29, B*44 [7, 8, 12, 13].

Autoimmune conditions (ACs) in persons with hemochromatosis have been documented predominantly in case reports, small case series, or studies of single ACs [14–19]. Our informal experience suggested that ACs are relatively common in adults with hemochromatosis. To study this further, we performed a retrospective analysis of ACs at diagnosis of hemochromatosis in 235 nonscreening probands with *HFE* C282Y homozygosity who had also undergone HLA-A and -B typing. Median followup was 19.6 y. We compared characteristics of probands with and without ACs, used univariable and multivariable methods to identify attributes significantly associated with ACs, and estimated survival of probands with and without ACs. We compiled a list of ACs previously

reported in adults with hemochromatosis. We discuss our observations in the context of prevalence estimates of ACs in general populations and the association of ACs with serum ferritin (SF) and iron removed by phlebotomy to achieve iron depletion (QFe).

2. Methods

2.1. Selection of Hemochromatosis Probands. The performance of this work was approved by the Institutional Review Board of Brookwood Medical Center. Obtaining informed consent was not required because information reported herein was documented as part of routine medical care. We performed computerized and manual searches of medical records to identify patients who were evaluated for hemochromatosis in the interval 1976–2014 by the first author because they had elevated values of transferrin saturation or SF detected in medical care (not as part of family or population screening). Each patient selected for this study (a) was a white adult (>18 years of age) and the first in his/her respective family to be diagnosed to have hemochromatosis (proband); (b) had *HFE* C282Y/C282Y; (c) had undergone HLA-A and -B haplotyping; and (d) resided in central Alabama. Each proband was evaluated for iron overload and associated complications, as appropriate [20].

2.2. Autoimmune Conditions in Probands. We studied ACs diagnosed before hemochromatosis was diagnosed. Probands with ACs were characterized by referring physicians, our queries regarding ACs, medication reviews, interpretation of tissue specimens, and immunologic characteristics. In probands diagnosed to have Hashimoto's thyroiditis (HT), we concurred with diagnoses of HT after reviewing evidence of goiter or hypothyroidism, autoantibodies for thyroid peroxidase, thyroglobulin or thyroid-stimulating hormone receptors, features of thyroid glands typical of HT on pathology reports, use of thyroid hormone supplements, and exclusion or absence of other causes of hypothyroidism. Probands with rheumatoid arthritis had typical clinical manifestations, positive RA latex turbidity tests, and elevated levels of cyclic citrullinated peptide antibodies. We performed confirmatory tests for ACs in some probands.

2.3. Family History of Autoimmune Conditions. Reports of first-degree relatives (parents, full siblings, and children) with ACs (family history, FH) were elicited from each proband and documented at the time of initial evaluation for hemochromatosis. We interviewed first-degree relatives as part of family evaluations and reviewed their medical records or both.

2.4. Diabetes Mellitus in Probands. Probands with diabetes were diagnosed and characterized by referring physicians, our queries regarding diabetes, and medication reviews. Diabetes was defined and subclassified according to the criteria of the American Diabetes Association [21]. We excluded a woman with diabetes because she had undergone pancreatectomy for management of adenocarcinoma of the pancreas.

2.5. Definition of Heavy Ethanol Consumption. Heavy ethanol consumption was defined as the self-reported consumption of ≥60 g ethanol/d for ≥5 y [22].

2.6. Definitions of Liver Conditions. Elevated serum alanine aminotransferase (ALT) or aspartate aminotransferase (AST) was defined as a level >2 SD above mean (>40 IU/L). Nonalcoholic fatty liver disease (NAFLD) was defined as steatosis or steatohepatitis detected on liver biopsy specimens or by typical increase of hepatic echogenicity detected by ultrasonography, in the absence of self-reports of heavy ethanol consumption [22]. Chronic hepatitis B or C was defined as positivity for HB_sAg or hepatitis C antibody, respectively, in association with other clinical or liver biopsy abnormalities consistent with chronic viral hepatitis [22]. Liver biopsy was performed in probands with SF > 1000 μg/L and in those suspected to have an undiagnosed non-iron liver disorder. Liver histology and intrahepatocytic iron were evaluated as previously described [22, 23]. Cirrhosis was defined by pathologists' interpretations of liver biopsy specimens [22].

2.7. Serum Ferritin and Iron Removed by Phlebotomy. SF values >300 μg/L in men and >200 μg/L in women were defined as elevated [20]. Iron depletion therapy, defined as the periodic removal of blood to eliminate storage iron, was complete when SF was ≤20 μg/L [20]. QFe was estimated as 200 mg Fe per unit of blood (450–500 mL) [20].

2.8. Survival. All probands designated as alive were so confirmed on May 1, 2015. Dates of death were determined by review of medical records, by computerized searches for obituary notices, and by the Social Security Death Index (http://ssdi.rootsweb.ancestry.com/ and https://www.dobsearch.com/death-records/search-ssn.php). Survival after diagnosis was computed using date of diagnosis of hemochromatosis and either May 1, 2015, or date of death, as appropriate. Overall survival was computed using date of birth and either May 1, 2015, or date of death, as appropriate.

2.9. Laboratory Methods. Levels of SF, ALT, AST, HB_sAg, HB_sAb, HB_cAb, and hepatitis C antibody were measured using automated clinical methods. *HFE* mutation analysis was performed as previously described [24]. HLA-A and -B alleles were detected using low-resolution DNA-based typing in probands and family members; haplotypes were ascertained using HLA analyses of appropriate first-degree family members as previously described [7].

2.10. Statistics. SF and QFe values were converted to natural logarithms (ln) to normalize them for analyses; antilns were computed to display mean values (95% confidence intervals (CI)). The Pearson correlation coefficient of lnSF with lnQFe was 0.4509 (two-tailed test; $p < 0.0001$). We used QFe as the independent variable representing iron overload severity. Phlebotomy data were incomplete in 20 probands (14 men

Table 1: Comparisons of hemochromatosis probands with and without autoimmune conditions[1].

Characteristic	Autoimmune condition (n = 35)	No autoimmune condition (n = 200)	Value of p[2]
Mean age, y	53 ± 13	48 ± 13	0.0747
Men, %	28.6 (10)	64.0 (128)	<0.0001
Family history, % (n)[3]	22.9 (8)	5.0 (10)	0.0002
Diabetes mellitus, % (n)	14.3 (5)	14.0 (28)	0.9642
Heavy ethanol, % (n)	5.7 (2)	14.0 (28)	0.1754
Elevated ALT, % (n)[4]	20.0 (7)	25.0 (50)	0.5243
Elevated AST, % (n)[4]	25.7 (9)	28.0 (56)	0.7803
NAFLD, % (n)	17.1 (6)	19.0 (38)	0.7950
Viral hepatitis, % (n)	2.9 (1)	5.0 (10)	0.4922
Cirrhosis, % (n)	14.3 (5)	10.0 (20)	0.4481
Mean SF, μg/L	551 [72, 4202]	807 [133, 4879]	0.0441
Mean FeQ, g[5]	1.7 [1.1, 2.5]	2.0 [1.7, 2.4]	0.3962
HLA-A*03 positivity, % (n)	77.1 (27)	71.5 (143)	0.4911
HLA-A*01, B*08 positivity, % (n)	14.3 (5)	11.0 (22)	0.5739
HLA-A*02, B*44 positivity, % (n)	11.4 (4)	9.0 (18)	0.4911
HLA-A*03, B*07 positivity, % (n)	48.6 (17)	41.0 (82)	0.4026
HLA-A*03, B*14 positivity, % (n)	22.9 (8)	15.5 (31)	0.2805
HLA-A*29, B*44 positivity, % (n)	0	5.5 (11)	0.1626

[1]ALT, alanine aminotransferase; AST, aspartate aminotransferase; NAFLD, nonalcoholic fatty liver disease; SF, serum ferritin; FeQ, iron removed by phlebotomy to achieve iron depletion; HLA, human leukocyte antigen. Mean results are displayed as mean ± SD or mean [95% CI].
[2]These are nominal values of p. Bonferroni correction for 18 comparisons yielded a revised p for significance of <0.0028.
[3]History of autoimmune condition(s) in one or more first-degree family members.
[4]Elevated values were defined as >40 IU/L.
[5]These observations represent 215 probands because phlebotomy data were incomplete in 20 probands.

and 6 women; 8.5%) and thus their QFe data were not used for analyses.

The final analytic data set consisted of observations on 235 probands. Logistic regressions were performed to identify independent variables significantly associated with ACs and HT. We did not use logistic technique to assess predictors of rheumatoid arthritis, ankylosing spondylitis, or other ACs because logistic models suffer from small sample bias [25]. Independent variables appropriate for most regression analyses were age, sex, FH, diabetes mellitus, heavy ethanol consumption, elevated ALT/AST, NAFLD, viral hepatitis, cirrhosis, QFe, and positivity for HLA haplotypes A*01, B*08; A*02, B*44; A*03, B*07; A*03, B*14; and A*29, B*44. We used backward stepwise multiple regression to identify predictors of SF. We used Kaplan-Meier technique (log-rank test) to estimate survival and Cox proportional hazards models to identify significant contributors to death.

General descriptive data are presented as enumerations, percentages, mean ±1 standard deviation (SD), or mean [95% CI]. Univariable comparisons between groups were evaluated using Student's t-test, Pearson's X^2 test, and Fisher's exact test, as appropriate. We expressed some results as odds ratios (OR [95% CI]). We computed the 95% CI of proportions with continuity corrections. Values of $p < 0.05$ were defined as significant. Bonferroni corrections were applied to control type I error rate at 0.05 for multiple comparisons of continuous and dichotomous data, as appropriate. Analyses were performed with a computer spreadsheet (Excel 2000, Microsoft Corp., Redmond, WA) and a statistical program (GB-Stat, v. 10.0, 2000, Dynamic Microsystems, Inc., Silver Spring, MD).

2.11. Literature Search. We performed a computerized search of the National Library of Medicine database to identify previous reports of ACs in persons with hemochromatosis. We used two terms per search: the first was the name of an AC and the second was hemochromatosis. Reports were reviewed to verify the diagnosis of hemochromatosis defined as an adult-onset condition typical of *HFE* hemochromatosis in whites of western European descent characterized by iron phenotyping, HLA typing or haplotyping, or *HFE* C282Y homozygosity.

3. Results

3.1. General Characteristics of 235 Probands. The mean age at diagnosis of hemochromatosis was 49 ± 13 y. There were 138 men (58.7%). One or more ACs were diagnosed in each of 35 probands (14.9%; [10.7, 20.2]). The proportion of men was lower among 35 probands with ACs than among 200 probands without ACs (Table 1). Eighteen probands (7.7%; [4.7, 12.0]) had a FH of ACs. The proportion of probands with a FH was greater among those with ACs (Table 1). Prevalences of other characteristics did not differ significantly between probands with and without ACs (Table 1). The previous diagnosis of primary biliary cirrhosis was confirmed in one of 102 probands who underwent liver biopsy at the time of

hemochromatosis diagnosis. No proband was diagnosed to have anterior pituitary or myocardial siderosis.

Mean SF at diagnosis of hemochromatosis was 761 μg/L [677, 858]. SF > 300 μg/L was observed in 131 men (94.9%). Of these, 124 (89.9%) achieved iron depletion. SF > 200 μg/L was observed in 86 women (88.7%). Of these, 91 (93.8%) achieved iron depletion. Phlebotomy data were incomplete in 20 probands (Table 1). Mean QFe in 215 probands was 2.0 g [1.7, 2.3]. Mean QFe in probands with and without ACs did not differ significantly (Table 1).

3.2. Autoimmune Conditions in 35 Probands. Nineteen different ACs were diagnosed in 35 probands. HT was diagnosed in 19 of 35 probands with ACs (54.3%) (Table 2). The female: male ratio among probands with HT was 2.8. The prevalence of HT was greater in women than men (14.4% versus 3.6%, resp.; $p = 0.0031$; OR 4.6 [1.6, 13.1]). Positivity for HLA-A*03 or HLA haplotypes did not differ significantly between 19 probands with HT and 216 probands without HT (data not shown). Among 216 probands without HT, 16 (7.4%; [4.4, 12.0]) had one or more ACs. No proband was diagnosed to have an autoimmune polyendocrine syndrome or diabetes mellitus type 1.

*3.3. Ankylosing Spondylitis and HLA-B*27 Positivity.* Heterozygosity for B*27 was detected in 16 probands (6.8%). Homozygosity for B*27 was not observed. Neither of two probands with ankylosing spondylitis was positive for B*27. Only one proband with an AC, a man with HT, was positive for B*27. Positivity for B*27 in the present 235 probands and in 1318 central Alabama white control subjects [7] did not differ significantly (0.0681 versus 0.0895, resp.; $p = 0.2808$). Eight probands had either two B*07 alleles, two B*14 alleles, or one of each, as part of ancestral hemochromatosis haplotypes. Accordingly, only 227 probands would have been "at risk" for inheritance of B*27. Even with this correction, the difference in positivity for B*27 in 227 probands and in 1318 control subjects was not significant (0.0705 versus 0.0895, resp.; $p = 0.3464$).

3.4. Autoimmune Conditions in First-Degree Family Members. Eighteen probands had 19 family members with ACs, among which HT was the most prevalent (Table 3). The proportion of female relatives with ACs (16/19; 84.2%) was greater than the proportion of male relatives with ACs (3/19; 15.8%) ($p < 0.0001$).

Eight probands with ACs had 9 family members who also had ACs. There was concordance for specific ACs in at least two generations in 7 of the 8 kinships: HT (four kinships) and Crohn's disease, Graves' disease, and sarcoidosis (one kinship each). In one kinship, both a female proband and her mother, also a C282Y homozygote, had HT.

3.5. Predictors of Autoimmune Conditions. We performed an initial regression on ACs using 16 appropriate independent variables. In a refined model that included only two variables, there was a negative association of male sex with ACs ($p = 0.0010$; OR 0.3 [0.1, 0.6]). There was a positive association of FH with ACs ($p = 0.0093$; OR 4.1 [1.4, 11.8]). This 2-factor model explained 11.0% of total deviance contributing to ACs ($X^2 = 21.69$; $p < 0.0001$).

Table 2: Autoimmune conditions in 35 hemochromatosis probands[1].

Condition	Percentage (n)
Hashimoto's thyroiditis[2]	54.3 (19)
Rheumatoid arthritis[3]	11.4 (4)
Ankylosing spondylitis[4]	5.7 (2)
Mixed connective tissue disorder	5.7 (2)
Myasthenia gravis	5.7 (2)
Pernicious anemia	5.7 (2)
Sarcoidosis	5.7 (2)
Ulcerative colitis	5.7 (2)
Autoimmune hemolytic anemia[5]	2.9 (1)
Crohn's disease	2.9 (1)
Elevated antinuclear antibody[6]	2.9 (1)
Graves' disease	2.9 (1)
Polymyalgia rheumatica	2.9 (1)
Primary biliary cirrhosis	2.9 (1)
Psoriasis	2.9 (1)
Raynaud's phenomenon	2.9 (1)
Scleroderma	2.9 (1)
Sjögren's syndrome	2.9 (1)
Systemic lupus erythematosus	2.9 (1)

[1]Six probands had two or more autoimmune conditions. Two men had both Hashimoto's thyroiditis and pernicious anemia. One woman had both Hashimoto's thyroiditis and Crohn's disease. One woman had Hashimoto's thyroiditis, polymyalgia rheumatica, and systemic lupus erythematosus. One woman had Hashimoto's thyroiditis, Raynaud's phenomenon, sarcoidosis, scleroderma, and Sjögren's syndrome. One woman had both ulcerative colitis and biliary cirrhosis.
[2]Of 235 probands, 14 women and 5 men had Hashimoto's thyroiditis (8.1% [5.1, 12.5]).
[3]Of 235 probands, 2 men and 2 women had rheumatoid arthritis (1.7% [0.6, 4.6]).
[4]Of 235 probands, 2 men had ankylosing spondylitis (0.0085 [0.0015, 0.0337]).
[5]Mediated by IgG and complement.
[6]This proband had anti-nuclear antibody >1 : 320 without other manifestation of autoimmunity. Elevated anti-nuclear antibodies (>1 : 80) in other probands were interpreted as part of broader autoimmune condition diagnoses named separately.

3.6. Predictors of Hashimoto's Thyroiditis. We performed an initial regression on HT using 16 appropriate independent variables. The variables age, male sex, FH, and QFe were used in a final model. There was a negative association with male sex ($p = 0.0216$; OR 0.3 [0.1, 0.8]). There was a positive association of HT with FH ($p = 0.0323$; OR 3.8 [1.1, 13.0]). This 4-factor model explained 13.0% of total deviance contributing to HT ($X^2 = 17.16$; $p = 0.0018$).

3.7. Predictors of Autoimmune Conditions other than Hashimoto's Thyroiditis. We performed a logistic regression on ACs after removing observations on 19 probands with HT from the analytic dataset. An initial regression included 16

Table 3: Autoimmune conditions in 18 first-degree relatives of 235 hemochromatosis probands[1].

Conditions in relatives	35 probands with autoimmune conditions	200 probands without autoimmune conditions	Value of p[2]
Hashimoto's thyroiditis	11.4 (4)	0.5 (1)	0.0019
Crohn's disease	5.7 (2)	0	0.0216
Graves' disease	2.9 (1)	0.5 (1)	0.2762
Rheumatoid arthritis	2.9 (1)	2.0 (4)	0.5569
Sarcoidosis	2.9 (1)	0	0.1489
Vitiligo	2.9 (1)	0	0.1489
Addison's disease	0	0.5 (1)	0.8511
Multiple sclerosis	0	0.5 (1)	0.8511
Pernicious anemia	0	0.5 (1)	0.8511
Scleroderma	0	0.5 (1)	0.8511

[1]Observations are displayed as % (n) of the proband subgroups who had first-degree relatives with the indicated conditions. Each of two relatives had two autoimmune conditions.
[2]These are nominal values of p. Bonferroni correction for 10 comparisons yielded a revised p for significance of <0.0050.

appropriate independent variables. Age, male sex, FH, and QFe were appropriate for a final model. The only significant (negative) predictor was male sex ($p = 0.0261$; OR 0.3 [0.1, 0.9]). This 4-factor model explained 9.7% of total deviance contributing to ACs other than HT ($X^2 = 11.12$; $p = 0.0252$).

3.8. Predictors of Serum Ferritin. We performed a backwards stepwise regression on SF using 16 independent variables. There were significant positive associations with male sex ($p < 0.0001$); diabetes ($p = 0.0403$); elevated AST ($p = 0.0375$); and cirrhosis ($p < 0.0001$). There was a significant negative association with HLA-A*01, B*08 ($p = 0.0047$). This 5-factor model explained 23.6% of total deviance contributing to SF (ANOVA $p = 0.0252$).

3.9. Survival. Median survival after diagnosis of hemochromatosis was 19.6 y (mean 18.5 ± 7.7 y). Median overall survival was 68.0 y (mean 66.8 ± 14.8 y). There were 40 deaths (27 men, 13 women) (17.0%). No death was attributed to direct or indirect consequences of ACs.

Kaplan-Meier estimates of survival after hemochromatosis diagnosis of probands with and without ACs were similar ($p = 0.6380$) (Figure 1). Cox proportional hazards analysis revealed two positive associations with death: age ($p = 0.0107$) and QFe ($p = 0.0032$). Overall survival of probands with and without ACs was also similar ($p = 0.1127$) (Figure 1). Cox proportional hazards analysis revealed two positive associations with death: heavy ethanol consumption ($p = 0.0228$) and cirrhosis ($p = 0.0096$). Comparison of survival after hemochromatosis diagnosis or overall survival of men with and without ACs and of women with and without ACs revealed no significant differences (data not shown).

3.10. Autoimmune Conditions Previously Reported in Persons with Hemochromatosis. These ACs are displayed in Table 4.

Table 4: Autoimmune conditions previously reported in persons with hemochromatosis[1].

Condition	Reference
Autoimmune hemolytic anemia	[14]
Celiac disease	[17, 19, 26–29]
Diabetes mellitus type 1[2]	[30]
Hashimoto's thyroiditis	[16, 31, 32]
Hyperthyroidism or Graves' disease	[16, 33–35]
Immune thrombocytopenia	[34]
Myasthenia gravis[3]	[36]
Pernicious anemia	[37, 38]
Psoriasis	[39]
Rheumatoid arthritis	[40, 41]
Sarcoidosis[4]	[18, 42]
Schnitzler's syndrome	[43]
Sclerosing cholangitis[5]	[44]
Ulcerative colitis[5]	[44]
Vitiligo	[15]

[1]Hemochromatosis was defined as an adult-onset condition typical of *HFE* hemochromatosis in European white adults characterized by iron phenotyping, HLA typing or haplotyping, or *HFE* C282Y homozygosity. One *HFE* C282Y homozygote had collagenous sprue, a rare condition of uncertain etiology [45].
[2]Autoimmunity was not mentioned in descriptions of study subjects.
[3]This man had pure red cell aplasia of undefined cause.
[4]The case of a woman with sarcoidosis included herein was reported previously [18].
[5]Ulcerative colitis and sclerosing cholangitis occurred in the same man.

4. Discussion

The present results demonstrate that 14.9% of 235 hemochromatosis probands with *HFE* C282Y homozygosity had one or more of 19 different ACs at diagnosis of hemochromatosis. ACs were more likely to occur in women and in probands with first-degree relatives who had ACs. HT was diagnosed in 54% of probands with ACs. Among 216 probands without HT, 7.4% had ACs that were positively associated with women. Overall risk of ACs was not significantly associated with QFe. ACs were not significantly associated with positivity for HLA-A*03 or HLA-A and -B haplotypes, including the A*01, B*08 haplotype associated elsewhere with ACs [46].

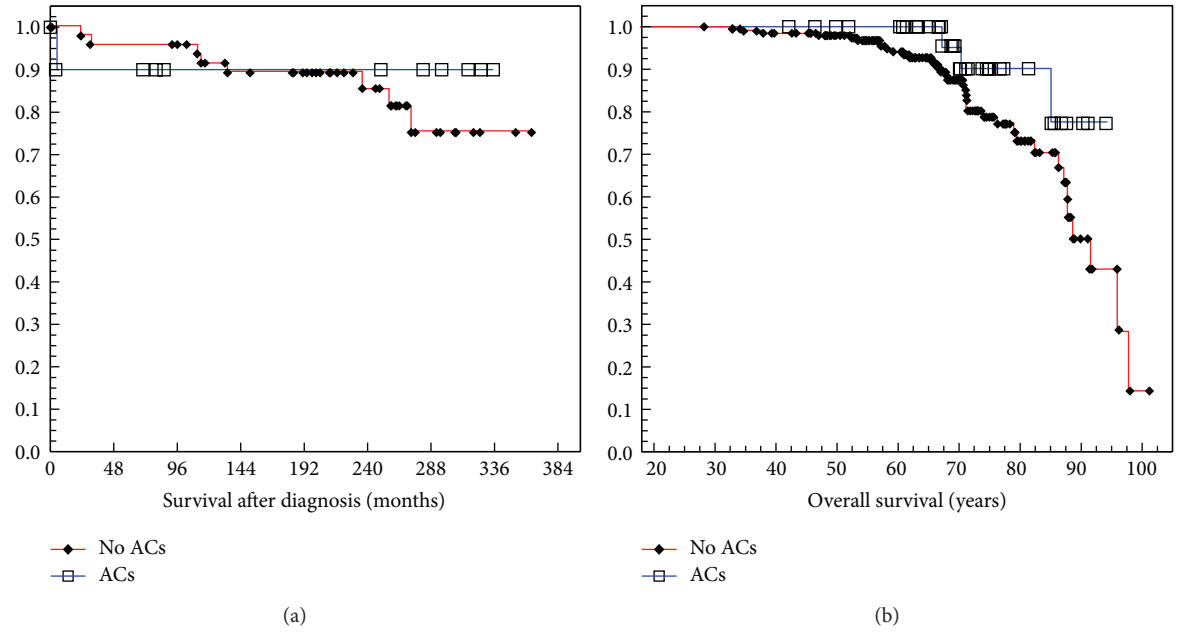

Figure 1: (a) Kaplan-Meier estimates of survival after hemochromatosis diagnosis in probands with and without autoimmune conditions (ACs) ($p = 0.6380$; log-rank test). (b) Kaplan-Meier estimates of overall survival of hemochromatosis probands with and without ACs ($p = 0.1127$; log-rank test).

Estimated survival of probands with and without ACs did not differ significantly.

Population prevalence rates for ACs vary for many reasons [47], including underrepresentation of certain ACs in study cohorts [47, 48], high prevalence of undiagnosed ACs [49], lack of prevalence data for some ACs [47], and differences across race/ethnicity groups or geographic regions [48]. The combined prevalence of 24 ACs in persons unselected for hemochromatosis diagnoses was 3.2% [50]. The combined prevalence of 31 ACs in hospitalized Danes was more than 5% [48]. In 2009, Cooper and colleagues, using "adjustments" to these prevalence assessments [48, 49], estimated that the overall population prevalence of ACs is 9.4% [47]. These observations suggest that the combined prevalence of ACs in the present probands of 14.9% (10.7, 20.2) is similar to that in populations not selected for hemochromatosis diagnoses.

The prevalence of HT in the present probands was 8.1% (5.1, 12.5). In women aged 20–49 y in a large population hemochromatosis screening program in Norway, 12.5% of *HFE* C282Y homozygotes and 3.0% of control participants reported having hypothyroidism [51]. In the Hemochromatosis and Iron Overload Screening Study of adult primary care participants in North America, 8.5% of 176 *HFE* C282Y homozygotes and 10.9% of 312 *HFE* wt/wt controls reported taking thyroid supplements ($p = 0.4019$) [52]. Prevalence estimates for untreated hypothyroidism did not differ significantly between C282Y homozygotes (1.7%) and controls (1.3%) or between male and female C282Y homozygotes and corresponding controls [52]. The prevalence of HT confirmed by cytology in 811 consecutive patients unselected for hemochromatosis diagnoses who underwent fine-needle aspiration of thyroid nodules was 13.4%, of whom 5.7% (4.1, 7.4) were clinically hypothyroid [53]. Although some differences in prevalence estimates of HT or presumed HT are due to dissimilarities in study design and case ascertainment, we infer that prevalences of HT in hemochromatosis probands diagnosed in medical care and in *HFE* C282Y homozygotes identified in screening are similar to those in other populations [53].

HT was significantly associated with the present female probands in univariable and multivariable analyses. The female: male ratio of HT was 2.8. In other studies, female: male ratios of autoimmune thyroiditis were 5.1–9.1 [54]. In a meta-analysis, skewed X-inactivation was significantly greater in women with HT than in control subjects. This could explain in part the preponderance of women in HT cohorts [55]. On the other hand, English gerontologist Joseph Sheldon did not describe thyromegaly, lymphocytic infiltrates, or other attributes of autoimmune thyroiditis in his 1935 monograph of 311 hemochromatosis cases [56]. It is plausible that gross and microscopic manifestations typical of autoimmune thyroiditis were obscured by thyroid fibrosis attributed to iron deposits or were not detected because women represented only 5% of the cases [56].

The prevalence of rheumatoid arthritis in the present probands (1.7% [0.6, 4.6]) is similar to that in the U.S. general adult population (0.5–1.0%) [57, 58]. Some manifestations of hand arthropathy due to hemochromatosis and rheumatoid arthritis are similar [59]. To distinguish these conditions, it is prudent to perform an immunologic evaluation for rheumatoid arthritis in patients with hemochromatosis and hand arthropathy [40, 41, 59].

The prevalence of ankylosing spondylitis in the present probands (15–337/10,000) is similar to that of 32/10,000 in

North American subjects unselected for hemochromatosis or *HFE* C282Y homozygosity [60]. HLA-B*27 positivity rates in the present probands, in white control subjects from central Alabama [7], and in Caucasian control subjects in the US [61] are similar (7–9%). Neither of the two present probands diagnosed to have ankylosing spondylitis was positive for B*27, whereas 80–95% of persons with ankylosing spondylitis worldwide are B*27-positive [62]. We attribute this difference to the small sample size of probands with ankylosing spondylitis in the present study. There are radiographic similarities and dissimilarities in spine manifestations of ankylosing spondylitis and hemochromatosis [63].

QFe was not significantly associated with ACs in the present study. In contrast, the prevalence of autoimmune thyroid disorders in 34 men homozygous for hemochromatosis alleles (8.8%) was higher than in the general male population [16]. Although it has been postulated that iron deposits in the thyroid glands of persons with hemochromatosis cause HT or Graves' disease [16, 31, 64], this postulate remains unproven. Iron deposits were detected in the thyroid glands of a majority of persons with hemochromatosis studied at autopsy [56, 64, 65]. Among 391 persons with hemochromatosis diagnosed in medical care, only 4.1% had primary hypothyroidism and only 0.3% had hyperthyroidism [16, 66–69]. Thus, it is unlikely that iron deposits in the thyroid gland contribute to the pathogenesis of hypothyroidism or hyperthyroidism in most persons with hemochromatosis. There are few well-documented hemochromatosis patients who had hypothyroidism due to impaired thyrotroph function. Most of them also had hypogonadotropic hypogonadism [70–74]. Iron deposits were visualized in a minority of thyrotrophs in some persons with severe iron overload due to hemochromatosis. The deposits were much less prominent than those in gonadotrophs [64, 75]. In rare cases, secondary hypothyroidism resolved after iron depletion [72, 74].

There was intraperson co-occurrence of two or more ACs in six probands, five of whom had HT. Persons with HT unselected for hemochromatosis diagnoses also have increased risk for co-occurrence of other ACs, including Addison's disease [76, 77]; autoimmune hepatitis [78]; celiac disease [76, 79]; diabetes mellitus type 1 [80–82]; mixed connective tissue disorder [83]; multiple sclerosis [33]; pernicious anemia [76, 77]; polymyositis/dermatomyositis [83]; rheumatoid arthritis [33, 83]; Sjögren's syndrome [83]; systemic lupus erythematosus [76, 83]; systemic sclerosis [83]; and vitiligo [76]. One of the present probands, a woman, had both ulcerative colitis and biliary cirrhosis. Co-occurrence of these conditions in the same individual is uncommon but well documented [84–86].

There was co-occurrence of specific ACs in two (or three) generations in seven of the present kinships, including four kinships with HT. Familial clusters of specific ACs occur, depending upon the proband's specific AC [47, 87]. There is strong familial clustering of autoimmune thyroid disease in kinships unselected for hemochromatosis diagnoses [88, 89]. It is also more feasible to detect statistically significant associations with autoimmune thyroid conditions than associations with ACs that are less common [47].

SF levels were elevated in 92% of the present probands and in some patients with ACs who were not selected for hemochromatosis diagnoses [90]. ACs were not significant predictors of SF in the present study. SF is a mixture of iron-rich ferritin and apoferritin [91, 92]. The iron composition of SF is increased in hemochromatosis and other iron overload disorders, consistent with the function of ferritin as an iron storage protein [91, 93]. It cannot be determined whether increased amounts of apoferritin were released into the blood of some of the present probands as acute phase reactants due to inflammation or interleukin-1 [92, 94].

Survival estimates in probands with and without ACs did not differ significantly. Although ACs are among the leading causes of death in young and middle-age women in the US [95], death of none of the 40 present probands was attributed to consequences of ACs. Decreased survival after diagnosis of hemochromatosis was significantly associated with age at diagnosis and QFe. Decreased overall survival was significantly associated with heavy ethanol consumption and cirrhosis. These outcomes are consistent with previous reports of survival of patients with hemochromatosis unselected for diagnoses of ACs [11, 22, 96].

ACs in two or more previous reports (HT, Graves' disease, rheumatoid arthritis, pernicious anemia, and sarcoidosis) mirror the relative prevalences of the corresponding ACs in the present probands. We may have missed other reports of ACs in persons with hemochromatosis because the search terms we used did not correspond to the key words for indexing the reports. There are multiple reports of the concurrence of hemochromatosis and celiac disease [17, 19, 26–29]. Although hemochromatosis was associated with an increased risk of celiac disease in one study [19], none of the present probands was diagnosed to have celiac disease.

There are uncertainties in the present results. Our enumerations and prevalence estimates of ACs are conservative because some probands may have failed to report or their medical records did not document that they had ACs or FH of ACs. Some persons with HT are euthyroid or have subclinical hypothyroidism [54] and thus their thyroiditis may have been unrecognized at the time of hemochromatosis diagnosis. Followup of each proband for the possible development of ACs after hemochromatosis diagnosis was beyond the scope of the present study, although we observed informally that two probands developed HT several years after they achieved iron depletion. Many ACs have been described in insufficient numbers of persons with hemochromatosis to permit meaningful prevalence estimates. The reliability of FH reports in previous studies of ACs [88, 89], especially when medical records were available [97], was excellent. Complete QFe data were not available in 20 of the present probands. Based on the results of univariable comparisons and regression analyses of QFe observations in the other 215 probands, it is unlikely that the present results would have differed significantly had QFe data been available for all probands. The present observations cannot exclude the possibility that MHC-linked genes, including some loci linked to HLA-A and -B [46, 98], contributed to the pathogenesis of ACs in the present probands. HLA characteristics of *HFE* C282Y homozygotes that reside in other geographic

areas may differ from those of the present probands. It is plausible but unproven that diagnosis and management of ACs increase the likelihood that a *HFE* C282Y homozygote would be diagnosed subsequently to have hemochromatosis.

5. Conclusions

We conclude that ACs are common in hemochromatosis probands, especially women and probands with a FH. Overall risk of ACs is not significantly associated with QFe or HLA haplotypes.

Abbreviations

ACs: Autoimmune conditions
ALT: Serum alanine aminotransferase
AST: Serum aspartate aminotransferase
CI: Confidence interval
FH: History of autoimmune conditions in first-degree family members
HLA: Human leukocyte antigen
HT: Hashimoto's thyroiditis
MHC: Major histocompatibility complex
OR: Odds ratio
QFe: Quantity of iron removed by phlebotomy to achieve iron depletion
SD: Standard deviation
SF: Serum ferritin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was supported in part by Southern Iron Disorders Center.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 254245, 6 pages
http://dx.doi.org/10.1155/2015/254245

Research Article

The Expansion of $CD25^{high}IL\text{-}10^{high}FoxP3^{high}$ B Regulatory Cells Is in Association with SLE Disease Activity

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Received 28 July 2015; Accepted 8 September 2015

Academic Editor: Carlo Perricone

B regulatory cells (Bregs) belong to a subgroup of activated B cells tasked with maintaining self-tolerance and preventing autoimmunity. While sharing similar regulatory mechanisms such as IL-10 dependency, they also defer in exhibiting their suppressive effects by expressing Fas-Ligand, TGF-beta, and PDL-1. In this study we show, for the first time, the expansion of $CD25^{high}FoxP3^{high}$ Bregs in systemic lupus erythematosus (SLE) patients compared to healthy individuals ($18.5 \pm 3.052\%$ versus $11.0 \pm 1.654\%$, $p < 0.001$, resp.). This expansion was also shown to correlate with SLE disease activity ($r = 0.75$). In addition, $CD25^{high}FoxP3^{high}$ Bregs were also $IL\text{-}10^{high}$ expressing and further expanded when stimulated with semaphorin 3A. In sum we show that $CD25^{high}FoxP3^{high}$ are an additional subtype of Bregs, involved in regulating SLE disease activity. Being IL-10 expressing, we may assume that they are one of the sources of increased serum IL-10 in SLE patients. Further studies are required in order to assess the relation between high serum IL-10 and $CD25^{high}FoxP3^{high}$ Breg cells.

1. Introduction

Among the many immune mediated responses involved in systemic lupus erythematosus (SLE) is the imbalance between T-helper cells (Th) subsets, namely, Th1/Th2/Th17, and both T and B regulatory (reg) cells [1]. Th1 proinflammatory cytokine levels such as IL-12, IL-6, and IFNs are usually increased in association with SLE disease activity index (SLEDAI). Th17 related cytokines such as IL-17 and IL-21 are also reported to be enhanced and contribute to inflammatory processes in SLE and other rheumatic diseases such as rheumatoid arthritis (RA) and psoriasis. Th2 related cytokines, that is, IL-4 and IL-10, are known for their ability in driving humoral immune responses, B cell overactivation, and the production of many specific autoantibodies [2–5]. Many studies during the last decade have reported on the failure of Treg cells to maintain self-tolerance, allowing the development of many autoimmune diseases. The failure in suppressing effector Th cell proliferation is mainly considered to be IL-10 dependent (lower expression and/or production of IL-10) due to the altered expression of FoxP3 and/or inhibitory molecules such as CTLA-4 in Treg cells [6]. Breg cells are involved in regulating/suppressing immune mediated inflammation but act earlier than Treg cells. They use similar suppressive modalities, that is, IL-10, TGF-beta, and the expression of proapoptotic membrane molecules which vary across different Breg subtypes [7]. Among these different subtypes, $CD19^{+}CD24^{high}CD38^{high}$ and $CD19^{+}CD25^{high}CD86^{high}CD1d^{high}$ were both described as being involved in suppressing autoimmune processes, both in an IL-10 dependent way and with an altered function in SLE [8, 9]. Breg cells have also been characterized as $CD5^{high}$, $FoxP3^{high}$, and Fas-Ligand expressing cells. $CD19^{+}CD5^{high}FoxP3^{high}$ Breg cells were reported to be involved in non-IgE-mediated food allergies, namely, in maintaining tolerance to milk allergies [10]. In addition to this subtype, Breg cells were defined as being

$CD19^+CD5^{high}$Fas-L^{high}, also called "killer B cells." Numerous researchers have reported that these cells participate in the escape of viral infections from the efficient cytotoxic T cell response [11]. The similarities and differences between all the above-mentioned Breg cells are not sufficiently understood. Are they similar in their regulatory effects? Do they express/produce similar amounts of IL-10 and TGF-beta? How do they react to various stimuli? (see [12]). In previous studies, we and others showed that Breg cell function was enhanced when stimulated by CpG and CD40L, increasing by this autologous Treg cell properties following their coculture [9, 13]. When cocultured with semaphorin 3A (sema3A), IL-10 and TGF-beta expression was enhanced in $CD19^+CD25^{high}$ Breg cells, suggesting that sema3A is a frontier factor in improving Breg cell function (unpublished data). Later, we reported on the ability of sema3A in enhancing Breg cell properties by increasing CD72 (a regulatory molecule) expression on B cells [14]. Expecting to find lower serum levels of IL-10 in some autoimmune diseases, namely, in SLE, the opposite was found. Paradoxically, serum IL-10 is reported to be increased in association with increased SLEDAI and with high titers of anti-dsDNA antibodies. The source of increased serum IL-10 in SLE is yet undefined, suggested to be overproduced by Th2 and/or by one of the Breg subtypes. In addition, the association of Atg5 rs573775 single nucleotide polymorphism (SNP) with SLE susceptibility and IL-10 serum levels was analyzed. Here, carriage of the rs573775 T allele was associated with IL-10 upregulation and clinical features of SLE, concluding that such mutated allele influenced both SLE susceptibility and IL-10 production [15]. In this study, we aim to evaluate the status of $CD19^+CD25^{high}FoxP3^{high}$ Breg cells, namely, whether they are IL-10 expressing. We will also assess the status of these cells in SLE patients when compared to healthy individuals. We speculate on their possible contribution to increased serum IL-10 in SLE patients. Finally, we will evaluate the response of this subtype of Breg cells to sema3A, to see if this coculture increases IL-10 expression as it does in other Breg cells.

2. Patients and Methods

2.1. Patients Population. This study examined 21 SLE patients (20 females and 1 male; age range 16–59 years; mean 30.5 ± 9.2). All patients are routinely followed up by well-trained rheumatologists and all fulfill the ACR criteria for the classification of SLE [16]. Clinical and serological data (skin involvement; arthritis; renal involvement; full cell blood count; serum complement levels; anti-dsDNA and other extractable nuclear autoantibodies) were all available, enabling the determination of SLEDAI. The serological work-up was performed at the Bnai Zion Medical Center by a single experienced technician to insure uniformity of all analyses, utilizing identical kits. Patients in whom SLEDAI was between 4 and 6 points were treated with hydroxychloroquine and in some patients prednisolone (2.5 mg/daily) was added. When SLEDAI was above 7 points, azathioprine was added, but only after analyzing specific serology and purifying B cells. When SLEDAI was above 12 points the addition of cyclophosphamide or MMF was considered again, only after performing SLE serology and purifying B cells. Twenty healthy controls, sex and age matched, were assessed and analyzed for all above-mentioned parameters. This study was approved by both the local Helsinki Committee of the Bnai Zion Medical Center and the Rambam Health Care Campus, Haifa, Israel.

2.2. B Cell Purification. B cells were purified from peripheral blood of healthy controls and SLE patients. To do so, peripheral blood mononuclear cells (PBMCs) were isolated on Lymphoprep (Axis-Shield, Oslo, Norway), and B lymphocytes were then twice purified by positive selection using CD22 microbeads ($20\,\mu L/10^7$ cells; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions, achieving by this >99% purity.

2.3. FoxP3 and IL-10 Expression in $CD19^+CD25^{high}$ B Cells. The expression of FoxP3 and IL-10 in $CD19^+CD25^{high}$ cells (considered as Breg cells) from healthy controls and SLE patients was initially assessed by staining purified B cells after 48 hours of activation with ODN-CpG and CD40L. The staining was performed by using monoclonal antibodies, human anti-CD19-BUV737 (BD Horizon, Becton Dickinson, NJ, USA) and human anti-CD25 BUV395 (BD Horizon, Becton Dickinson, NJ, USA) as outer membrane antibodies, and FoxP3 PE\CF594 and IL-10 APC (BD Horizon, Becton Dickinson, NJ, USA) as intracellular staining, using a "Fix and Perm" kit (Invitrogen, NY, USA) according to the manufacturer's instructions. The staining was evaluated using flow cytometry software (FC500 and CXP software, Beckman Coulter, Brea, CA, USA, and Becton Dickinson, NJ, USA). CD3 positive cells in the purified cell culture were determined by using monoclonal CD3 PerCP-Cy5.5 antibody (BD Pharmingen, Becton Dickinson, NJ, USA) and analyzed by Becton Dickinson FACS-Fortessa. The results are shown as % of $CD19^+CD25^{high}$ Breg cells expressing FoxP3 or IL-10, taking into consideration that the absolute number of Breg cells in all groups was found to be comparable. Standard deviation (STDEV) was used to quantify the amount of variation of a set of data values (e.g., percentage of Breg cells expressing FoxP3 among the patients in each indicated group of disease or normal control).

2.4. Semaphorin 3A Enhances FoxP3 Expression. Aiming to evaluate the effect of sema3A on FoxP3 expression, condition-media from HEK293− cells, which were infected by NSPI-CMV-FLAG lentivirus with or without human sema3A cDNA, a kind gift from Professor Gera Neufeld and Dr. Ofra Kessler, Ruth and Bruce Rappaport Faculty of Medicine, Technion, Israel, as previously described [17], were added to the above-mentioned purified B cells activated by ODN-CpG and CD40L and incubated for 48 hours. After incubation, $CD19^+CD25^{high}$ cells were analyzed for the possible change in FoxP3 expression using the above-mentioned specific monoclonal antibodies and evaluated using an FC500 flow

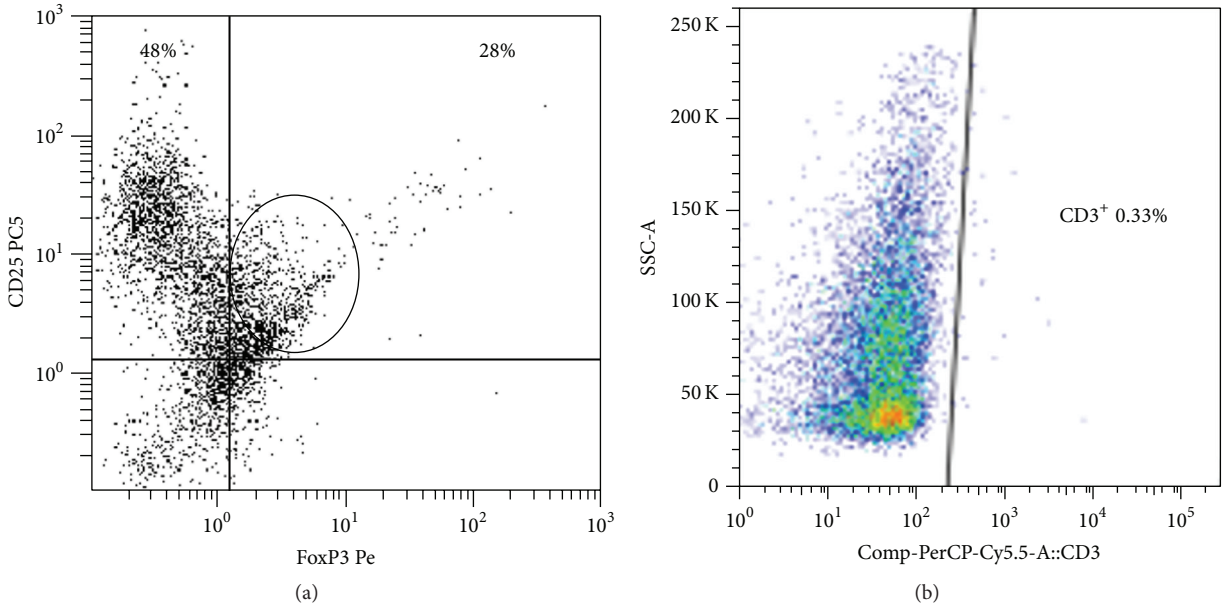

Figure 1: (a) A representative FACS analysis of purified B cells (expressing CD25^{high} FoxP3) following CpG-ODN and CD40L activation. Of note is that CD25$^{dim\text{-}low}$ B cells (upper left quadrant) do not express FoxP3. However, CD25^{high} B cells coexpress significant amount of FoxP3 (upper right quadrant). (b) A representative FACS analysis of purified activated B cells, showing that CD3$^+$ T cell contamination (gated CD3$^+$ T cells) is less than 0.5%.

cytometer and Becton Dickinson FACS-Fortessa. The results are shown as % of Breg cells expressing FoxP3, taking into consideration that the absolute number of Breg cells in all groups was found to be comparable.

2.5. Clinical Correlation and Statistical Analysis. Comparison of FoxP3 expression in B cells from SLE patients and healthy controls was done using the unpaired Student t-test. The correlation coefficient (r) of clinical correlation between SLEDAI score and % of Breg cells expressing FoxP3 was determined using the Pearson correlation test. A two-tailed p value of 0.05 or less was considered to be statistically significant.

3. Results

3.1. CD19$^+$CD25^{high} Activated B Cells Are FoxP3^{high}. First, we examined whether CD19$^+$CD25^{high} B regulatory cells are also FoxP3 expressing cells. Purified resting B cells (immediately following purification) were FoxP3^{dim} (weakly detectable) (data not shown). However, following their stimulation with CpG-ODN and CD40L for 48 h, CD19$^+$CD25^{high} B cells turned to become FoxP3^{high} (Figure 1(a)). As also seen, there are less than 0.5% gated CD3 T cells and therefore B cell contamination with CD3 is unlikely and FoxP3 expression in CD25^{high} B cells is very prominent (Figure 1(b)).

3.2. Activated CD19$^+$CD25^{high} FoxP3^{high} Are Also IL-10^{high}. Gating on activated CD25^{high}FoxP3^{high} one can see that most

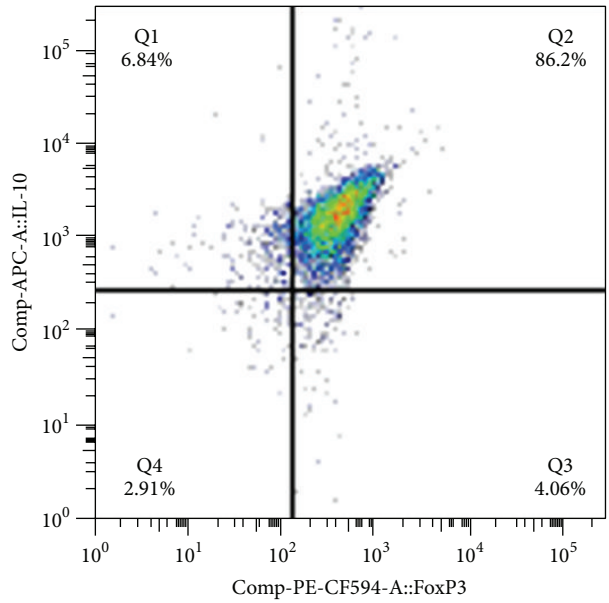

Figure 2: A demonstrative FACS analysis of purified B cells, showing that activated CD19$^+$CD25^{high}FoxP3^{high} are also IL-10^{high}. Of note is that CD25$^{dim\text{-}low}$\FoxP3$^{dim\text{-}low}$ B cells express very little IL-10.

of these cells (>85% of these cells) are IL-10^{high} (Figure 2) in contrast to B cells that are FoxP3^{dim} being also IL-10^{dim}.

3.3. CD19$^+$CD25^{high}FoxP3^{high} in SLE. The percentage of Breg cells (CD19$^+$CD25^{high} cells) in peripheral blood (highly

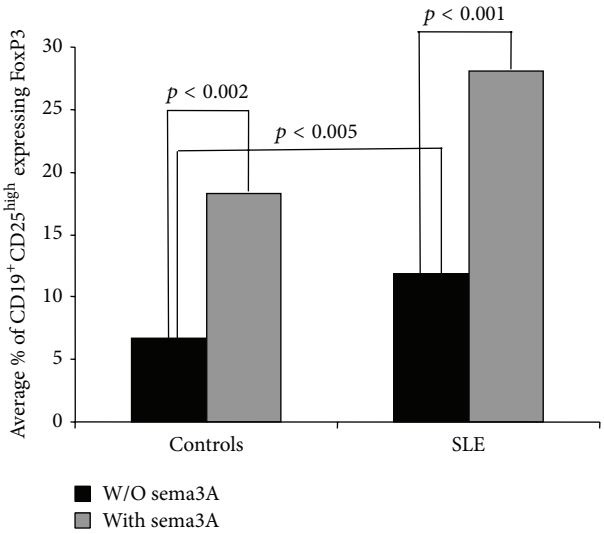

Figure 3: The percentage of $CD19^+CD25^{high}FoxP3^{high}$ Breg cells in normal controls ($n = 20$) and in patients suffering from SLE ($n = 21$). One can see that this subtype of B cells is significantly increased in SLE patients. In addition, the addition of sema3A to these cells increased significantly the percentage of these cells.

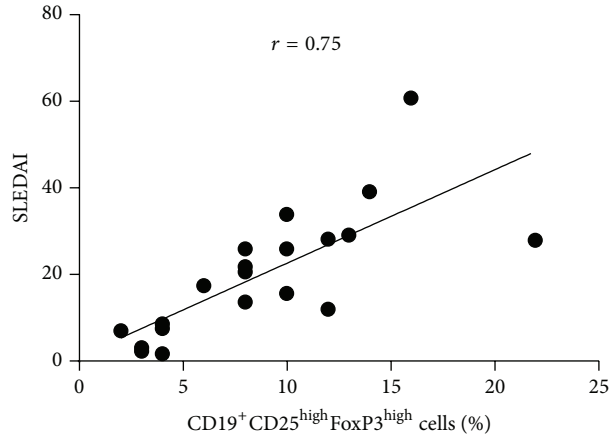

Figure 4: Clinical correlation between the percentage of $CD19^+CD25^{high}FoxP3^{high}$ cells of SLE patients and the SLEDAI score of these patients. The correlation was done using the Pearson correlation test.

expressing FoxP3) was significantly higher in SLE patients when compared to that of healthy individuals (18.5% ± 3.052 versus 11.0 ± 1.654%, resp., $p < 0.005$) (Figure 3).

3.4. Semaphorin 3A Increases FoxP3 Expression in Breg Cells. We then sought to determine if sema3A increases the expression of FoxP3 in these Breg cells. As is demonstrated in Figure 3, sema3A increases the percentage of Breg cells ($CD19^+CD25^{high}$ cells) in peripheral blood expressing FoxP3, in normal controls and to a higher extent in SLE patients (in normal controls up to 13.6 ± 1.806% from baseline, $p < 0.002$, and in SLE patients up to 28.5 ± 3.506%, $p < 0.0001$) (Figure 3).

3.5. FoxP3 Expression in B Cells Is Correlated with SLEDAI. Figure 4 demonstrates the correlation between the percentage of $CD19^+CD25^{high}FoxP3^{high}$ cells of SLE patients and the SLEDAI score of these patients. As can be seen, there is a positive correlation with an "r" Pearson coefficient of 0.75. This result is in line with the known correlation between IL-10 level in SLE patients and their SLEDAI.

4. Discussion

In most autoimmune diseases, immune mediated inflammatory damage is always the result of a net balance between the overactivity of self-reactive cells (T and B effector cells) and immune regulatory mechanisms (T and B regulatory cells). Most B regulatory cells are defined as being IL-10 expressing/producing cells; however, they have different subtypes, are heterogeneous, and have different mechanisms in diseases in which they are involved. Their homology to Treg subtypes, namely, Br1 cells (expressing IL-10), Br3 cells (mainly expressing TGF-beta), and B-FoxP3 positive cells, was recently mentioned. In this case, Breg cells were shown to initiate immune regulatory responses by facilitating the recruitment of Tregs and then disappearing once Tregs become dominant in the immune response [18]. As mentioned above, when $CD19^+CD24^{high}CD38^{high}$ B cells were evaluated in SLE, they had both a reduced ability to produce IL-10 and a reduced ability to suppress T cell cytokine production, although it is unclear if this latter defect is a cause or a consequence of SLE. In contrast to this finding, human IL-10 producing $CD24^{high}CD27^{high}$ Breg cells (found to suppress monocytes in an IL-10 dependent manner) were increased in patients with rheumatoid arthritis, SLE, and multiple sclerosis when compared to healthy individuals, suggesting this increase to be compensatory, aiming (with little success) to maintain self-tolerance [19]. The role of $CD5^{high}FasL^{high}$ "killer B cells" was assessed in lupus susceptible MRL/lpr mice. Being cytotoxic to T cells they were found to be increased, probably in attempt to suppress autoreactive T cells in these mice [20]. Focusing on $CD19^+CD25^{high}FoxP3^{high}$ Breg cells we first assessed their status in healthy individuals. Here, we show for the first time that both IL-10 and FoxP3 expressions were noticed mainly in activated $CD25^{high}$ B cells (activated with CpG and ODN) and that this expression was enhanced when these B cells were stimulated with add-on sema3A. In this case $CD25^{high}FoxP3^{high}$ Breg cells were characterized by being IL-10^{high} whereas $FoxP3^{dim}$ B cells were IL-10^{dim} as well. When analyzed in SLE patients, we found $CD19^+CD25^{high}FoxP3^{high}$ cells to be significantly increased as compared to healthy individuals. This was found to be in positive correlation with increased SLEDAI and in association with lupus nephritis. In a recent study and in line with our finding, $CD19^+CD25^{high}FoxP3^{high}$ B regulatory cells were found to be increased in the cerebrospinal fluid of active patients suffering from relapsing-remitting multiple sclerosis (MS) when compared to that of nonclinically active MS. This

expansion of B regulatory cells was attributed to the compensatory attempt of these cells to maintain immune regulatory processes [21]. In contrast to this study, rheumatoid arthritis patients had significantly lower proportions of peripheral blood $CD19^{+}FoxP3^{+}$ B cells as compared to healthy controls, particularly in patients with interstitial lung disease. This finding suggests that Breg phenotypes may have different functions in the pathogenesis of different rheumatic diseases [22]. The fact that serum IL-10 is increased in SLE and in association with SLE disease activity has been established in many previous studies. In one, increased IL-10 was shown to exhibit a modulatory effect by suppressing the differentiation and function of monocyte-derived dendritic cells [23]. In a recent study, increased IL-10 in the sera of SLE patients was capable of inducing Fas and FasL expression on $CD4^{+}$ T cell surfaces, promoting apoptosis of this cell subset, thus contributing to many other mechanisms of self-tolerance [24]. However, we still need to explain the mechanisms by which serum IL-10 is increased in SLE. In this regard, the expansion of IL-10 producing B cells was shown to be in part the result of increased B cell activating factor (BAFF). Enhanced serum BAFF in SLE was described in many studies as being associated with increased expression of TLR-9 and other markers of B cell activation [25, 26]. This may explain our finding of increased $IL\text{-}10^{high}FoxP3^{high}$ Breg cells as well as increased serum IL-10 in SLE. Another significance of $FoxP3^{high}$ B cells being increased in SLE is the possibility that by multiplying they also increase their IL-10 production improving by this their regulatory function. When B cells were cocultured with sema3A they responded by increasing their FoxP3 expression. This raises the possibility that if provided with the proper stimulation Bregs may develop higher regulatory properties and that by increasing their IL-10 production they may induce a better regulatory mechanism in SLE.

5. Conclusion

$CD25^{high}FoxP3^{high}$ Bregs (highly expressing IL-10) are significantly increased in SLE, in correlation with SLEDAI. Semaphorin 3A increases FoxP3 expression in Breg cells improving by this their regulatory properties. We assume that the expansion of these cells is the attempt of our regulatory immune responses to maintain self-tolerance and to suppress as much as possible SLE disease activity. Further studies are required in order to better understand the role of this subset of B cells in autoimmunity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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