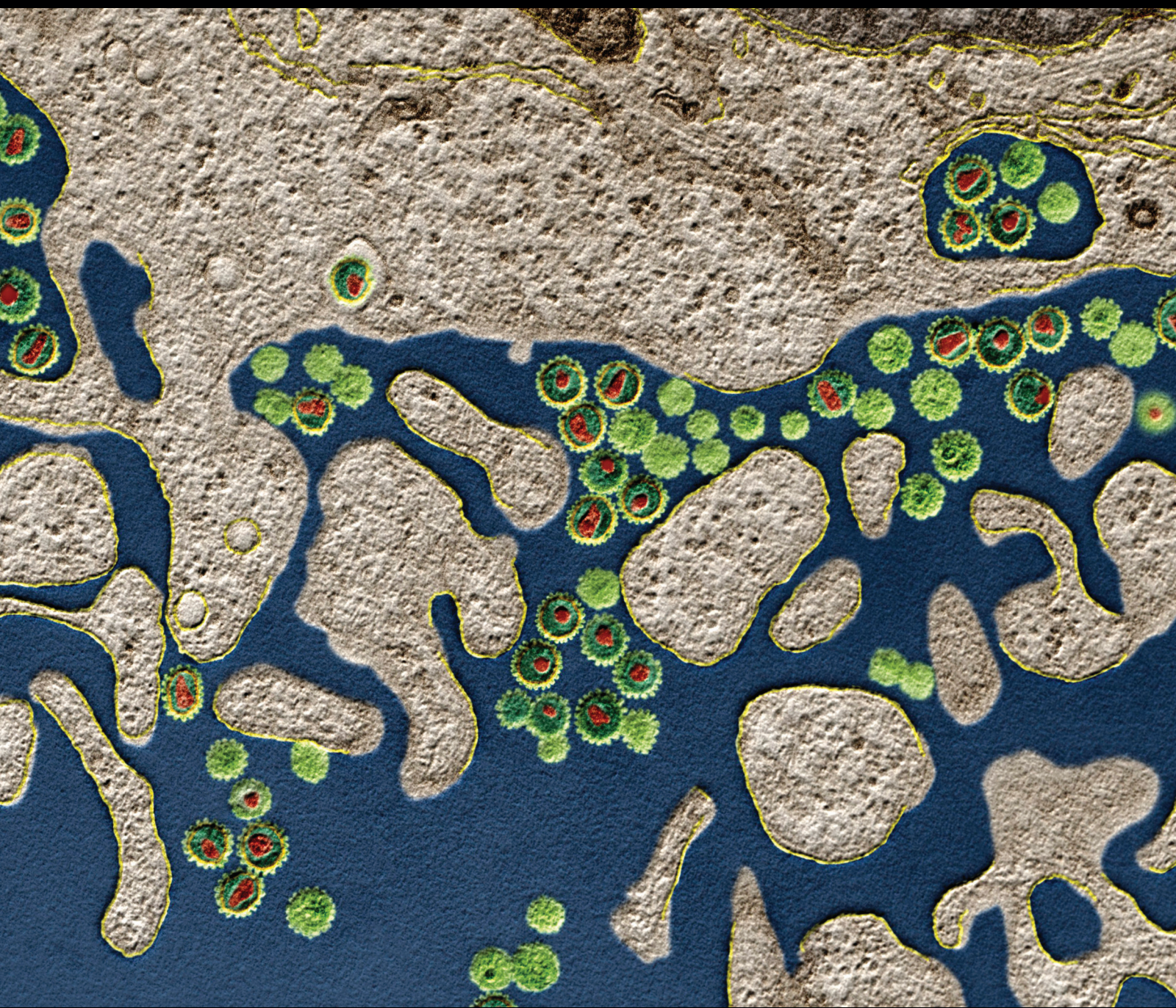
Journal of Immunology Research

Genetic Factors of Autoimmune Diseases 2017

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



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Editorial Board

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Editorial

Genetic Factors of Autoimmune Diseases 2017

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Autoimmune diseases are characterized by a multifactorial aetiology, in which genetic factors interplay with environmental factors. The different genetic factors are associated not only with disease susceptibility but also with specific autoantibodies and disease phenotypes.

Several studies have been conducted on this issue, identifying different genetic loci suspected to be involved in systemic autoimmune disease pathogenesis. Interestingly, autoimmune diseases share several risk loci, suggesting the involvement of common pathways to loss of tolerance.

Genetic factors associated with rheumatoid arthritis (RA) development have been widely investigated. Of these, the human leukocyte antigen (HLA) region contributes to approximately half of its genetic susceptibility, particularly in disease characterized by the presence of anti-citrullinated antibodies. Notably, a number of alleles in the epitope recognition part of the HLA molecule which is strongly associated with RA share a common string of amino acid residues, the so-called shared epitope. Next to HLA genes, other variants seem to be implicated in RA susceptibility such as the PTPN22, TRAF1-C5, PADI4, and STAT4 genes. Moreover, genetic factors seem to contribute to a disease phenotype, especially in terms of erosive damage. Recent data suggest an association between radiographic damage and polymorphisms of genes encoding TNF, IL-1, IL-6, IL-4, IL-5, OPN, and PRF1.

Considering systemic lupus erythematosus (SLE) genetic variabilities which were identified so far, it has been demonstrated that the latter accounts for less than half of this disease heritability, by the modest overall effect sizes. In this context, HLA loci as well as non-HLA risk loci (i.e., STAT4, PTPN22, IFIH1, and TRAF3IP2) have been associated with SLE susceptibility. Moreover, the disease heterogeneity in terms of clinical manifestations and outcomes has been demonstrated to be associated with specific genetic factors leading to the protean clinical picture. These evidences allow the possibility of elucidating different mechanisms and pathways accountable for disease manifestations. However, except for lupus nephritis associations with ITGAM and IRF gene polymorphisms, no studies have been designed to identify the genetic variants associated with the development of different SLE phenotypes.

In the last years, some studies investigated the genetic links to other autoimmune diseases, especially organ-specific ones. Among these, some data are available herein regarding the genetic background of primary biliary cholangitis (PBC), an autoimmune cholestatic liver disease, characterized by the antimitochondrial autoantibody positivity and by the accumulation of antigen-specific autoreactive B and T cells targeting biliary epithelial cells. The aetiology of PBC has yet to be fully defined; however, similar to other autoimmune diseases, the human leukocyte antigen (HLA) class II alleles have been significantly associated with disease susceptibility. More recently, genome-wide association studies have identified also non-HLA genes implicated in PBC development. Of these, the strongest associations were identified for genetic variants of IL12A and IL12RB2 genes, but other polymorphisms, such as STAT4 and CTLA4, have been also recognized. The recognition of specific genetic variants may be helpful in the elucidation of pathogenic mechanisms

underlying PBC development as well as in the identification of patients with a more aggressive disease course.

Another angle to the *scenario* of genetics and autoimmunity is related to the role of virus-derived microRNA in the genome stability. As widely demonstrated, infections play a crucial role as environmental factors affecting the development of autoimmune diseases. Viral infections, through noncoding RNAs termed microRNA, could interplay with the host genome, allowing modifications that could be responsible for a dysregulation of an immune response, as was reported for the Epstein-Barr virus.

In conclusion, in the last years, the focus of genetic studies shifted towards other autoimmune diseases and environmental factors that may modify the host genome. These new aspects of the mosaic between the gene environment and diseases may enable us to better understand the pathogenic mechanisms responsible for the loss of tolerance and the development of immune-related diseases.

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Research Article

Polymorphism rs2073618 of the *TNFRSF11B* (*OPG*) Gene and Bone Mineral Density in Mexican Women with Rheumatoid Arthritis

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Osteoporosis (OP) is highly prevalent in rheumatoid arthritis (RA) and is influenced by genetic factors. Single-nucleotide polymorphism (SNP) rs2073618 in the *TNFRSF11B* osteoprotegerin (*OPG*) gene has been related to postmenopausal OP although, to date, no information has been described concerning whether this polymorphism is implied in abnormalities of bone mineral density (BMD) in RA. We evaluated, in a case-control study performed in Mexican-Mestizo women with RA, whether SNP rs2073618 in the *TNFRSF11B* gene is associated with a decrease in BMD. RA patients were classified as follows: (1) low BMD and (2) normal BMD. All patients were genotyped for the *rs2073618* polymorphism by PCR-RFLP. The frequency of low BMD was 74.4%. Higher age was observed in RA with low BMD versus normal BMD (62 and 54 years, resp.; $p < 0.001$). Worse functioning and lower BMI were observed in RA with low BMD ($p = 0.003$ and $p = 0.002$, resp.). We found similar genotype frequencies in RA with low BMD versus RA with normal BMD (GG genotype 71% versus 64.4%, GC 26% versus 33%, and CC 3% versus 2.2%, resp.; $p = 0.6$). We concluded that in Mexican-Mestizo female patients with RA, the rs2073618 polymorphism of the *TNRFS11B* gene is not associated with low BMD.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by a chronic inflammation of the synovial joints, resulting in bone erosions, joint deformities, subluxations, and sequelae that might lead to functional disability [1]. Osteoporosis (OP) is observed in 31% in Mexican women with RA [2]. OP constitutes the main risk factor for the development of osteoporotic fractures observed in around 4–23% of patients with RA [3–5]. Additionally, the risk of OP in RA is 2-fold higher compared with that in the general population [6]. Because OP comprises a multifactorial complication in RA, the genetic characteristics comprise one of the main factors implicated in the development of OP.

Osteoprotegerin (OPG) is a tumor necrosis factor receptor superfamily member (TNFRSF) that exerts protective properties against OP. Under normal conditions, OPG inhibits the maturation and activation of osteoclasts, acting as a protector for OP [7, 8]. Additionally, OPG shares a similar molecular structure with receptor nuclear factor kappa B (RANK), which is a molecule required for the linking of receptor nuclear factor kappa B ligand (RANKL); the latter molecule is implicated in the development of osteoclastogenesis with subsequent increased bone resorption, leading to OP [9, 10]. OPG acts on linking with RANKL, avoiding the formation of the RANK-RANKL complex, leading to osteoclast apoptosis [8].

OPG is encoded in humans by the *TNFRSF11B* gene that is located at 8q24.12 and that contains several polymorphisms, including the rs2073618 G>C transversion in exon 1, resulting in changes from Lys3Asn, the third amino acid of the signal peptide from lysine (AAG), into asparagine (AAC) [11, 12].

Several studies disagree concerning the role of the polymorphism in rs2073618 in postmenopausal OP, whereas some of these have suggested that it might constitute a genetic factor for OP [13]; others have found no relationship with postmenopausal OP [12, 14, 15]. However, the role of this polymorphism in the *TNFRSF11B* gene has not, to our knowledge, been evaluated in RA-associated OP.

Therefore, the aim of the present study was to assess whether there is an association among single-nucleotide polymorphisms (SNP), the rs2073618 *TNFRSF11B* (*OPG*) gene, and OP in Mexican-Mestizo women with RA.

2. Materials and Methods

2.1. Study Design. This study is a case-control study.

2.2. Clinical Setting. This study included patients with RA referred from two secondary-care hospitals (Hospital General Regional 110 [HGR110] and Hospital General de Zona 45 [HGZ45] of the Mexican Institute for Social Security (IMSS)) for performing a bone mineral density (BMD) scan in the bone mineral densitometry area at a tertiary-care center (UMAE, Hospital de Especialidades, Centro Médico Nacional de Occidente [CMNO]) in Guadalajara, Mexico.

2.3. Subjects. One hundred seventy-six patients were included in the study complying with the following inclusion criteria: (a) female, (b) >40 years of age, (c) Mexican Mestizos defined according to the Mexican National Institute of Anthropology and History (INAH) as "individuals who were born in Mexico, of the 3rd generation including their own and who were descendants of the original autochthonous inhabitants of the region and individuals who were mainly Spaniards" [16], and (d) those who met the American College of Rheumatology (ACR) 1987 criteria for RA. Patients were excluded if they had overlapping syndrome or if they were receiving antiresorptive treatment, such as prednisone ≥ 15 mg/day, or other therapies with biologic disease-modifying antirheumatic drugs (bDMARDs). We also excluded patients with chronic infectious diseases including hepatitis B and C or HIV infections and patients with chronic renal failure, transaminasemia (>2-fold normal values), or cancer.

2.4. Clinical Assessments. All patients were assessed for clinical and sociodemographic characteristics. Disease activity was assessed using the 28-joint Disease Activity Score (DAS28), and functioning was evaluated with the Health Assessment Questionnaire-disability index (HAQ-Di). Rheumatoid factor (RF) and C-reactive protein (CRP) were quantified in serum using nephelometry, and erythrocyte sedimentation rate (ESR) was determined utilizing the Wintrobe method.

2.5. Bone Mineral Density (BMD) Measurements. BMD (g/cm^2) of the lumbar spine (L1–L4) and total hip was determined using dual-energy X-ray absorptiometry (DXA) with a Lunar Prodigy Advance densitometer (GE

Medical Systems Lunar ver. 8.8 software; GE Medical Systems, Madison, WI, USA). According to the *T*-score of the BMD results, we classified the latter into two groups employing the 1994 World Health Organization (WHO) criteria as follows: group 1—low bone density if BMD in the lumbar spine or total hip had a *T*-score of ≤1.0 standard deviation (SD) (cases), and group 2—normal central BMD if the *T*-score of these two regions was >1.0 SD (controls).

For obtaining Hardy-Weinberg equilibrium, we used a group of 80 healthy blood donors obtained from the previously mentioned hospitals.

2.6. Genotyping. Genomic DNA from 256 subjects was extracted from peripheral blood leukocyte samples using the modified Miller technique [17]. The genotype was screened by an approach based on polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs). The 147 bp PCR product was incubated at 55°C with 5 U of *SmlI* restriction endonuclease as described by Langdahl et al. [15]. The resulting fragments were analyzed by electrophoresis in 6% followed by silver staining. The resulting genotypes for polymorphism were classified into one of the following three categories: nonexcisable homozygote (GG), excisable homozygote (CC), and heterozygote (CG).

2.7. Quantification of Serum OPG. Serum OPG levels were quantified by the enzyme-linked immunosorbent assay (ELISA) using a commercial human monoclonal OPG kit (RayBioTech Inc., Norcross, GA, USA). Characteristics of the OPG kit include a 1.0–900 pg/mL detection range and minimal detectable doses of 1.0 pg/mL.

2.8. Other Laboratory Tests. We quantified the levels of rheumatoid factor (RF) (IU/mL) and C-reactive protein (CRP) (mg/mL) by nephelometry. We also quantified erythrocyte sedimentation rate (ESR) (mm/h). We also included the quantification titers of antibodies directed against citrullinated proteins (ACPAs): second-generation anticyclic citrullinated peptide/protein antibodies of the second generation (anti-CCP2) and antimutated citrullinated vimentin antibodies (anti-MCV). We were able to determine one of these ACPAs in 115 patients. We determined anti-CCP2 in 112 patients and anti-MCV in 109 patients. But, both ACPAs (anti-CCP2 and anti-MCV) were determined only in 106 patients.

2.9. Statistical Analysis. Qualitative variables were expressed as frequencies (%), while quantitative variables were indicated as means ± standard deviation (SD). We identified genotype frequencies by direct counting. Allele frequencies were determined by counting from the observed genotype frequencies. Comparisons in means between the two groups were computed using the independent sample Student *t*-test, and comparisons among three subgroups, including (a) RA + OP, (b) RA + osteopenia, and (c) RA with normal BMD, were performed with one-way ANOVA. Dunnett correction was used as the post hoc test in case of statistical significance in the ANOVA. Comparisons between proportions were carried out using the chi-square test (or the Fisher exact test if required). Odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated.

Hardy-Weinberg equilibrium in healthy subjects was determined by comparing the observed and expected data employing the chi-square test. Comparisons in OPG serum levels between each genotype of the rs2073618 polymorphism genotypes were performed by the Kruskal-Wallis test. In the present study, we employed, for comparison, the genotype frequencies of the recessive model GC + CC versus the frequencies of the GG genotype in each group.

A p value was considered significant at the $p \leq 0.05$ level. To identify the variables associated with low BMD, we performed a multivariate logistic regression analysis, where low BMD was the dependent variable. We included, as covariates, variables such as age, BMI, positive ACPAs, glucocorticoid dose, and genotypes. The covariates included in the final model had $p < 0.20$ on univariate comparison or variables with biological plausibility using the stepwise method to identify associated variables, excluded potential confounders. Data were analyzed with the SPSS ver. 23.0 statistical software program (SPSS Inc., Chicago, IL, USA), and OR and their 95% CI were obtained utilizing EPI-INFO ver. 7.2 software (Epi Info™; Atlanta, GA, USA).

2.10. Ethics. The study protocol was approved by the Research and Ethics Board of the Hospital (R2007-1301-1). All study participants voluntarily provided written informed consent. All procedures in the protocol were performed according to the Declaration of Helsinki guidelines.

3. Results

We assessed 176 women with RA. Low BMD was observed in 74.4% of these patients, whereas OP was detected in 46.6%. Genotype distributions of this SNP in the group of 80 healthy subjects were consistent with the Hardy-Weinberg equilibrium ($p > 0.64$).

Table 1 presents two different comparisons. The first comprises the comparison between RA with low BMD (group 1; $n = 131$) and RA with normal bone density (group 2; $n = 45$). In the first comparison, a higher age was observed in patients with low BMD compared with patients with normal BMD ($p < 0.001$). The majority of epidemiological and clinical characteristics related to RA were similar in both groups, except for more deteriorated functioning in the group with low BMD compared with that with normal BMD ($p = 0.003$) and lower BMI ($p = 0.002$). In relation to ACPAs, anti-CCP2 antibodies were determined only in 112 and anti-MCV only in 109 patients. Higher titers of anti-MCV were observed in patients with low BMD in comparison with patients with normal BMD ($p < 0.0001$). Instead, there were no differences observed in anti-CCP2 titers ($p = 0.37$). No differences were observed in currently administered doses of corticosteroids between these two groups ($p = 0.23$). The type of conventional synthetic DMARDs (cs-DMARDs), as well as the frequency of using the combination of cs-DMARDs (>1 cs-DMARD prescribed at the same time) had no statistical differences. The second comparison depicted in this table was among the following three subgroups of patients: (a) RA + OP ($n = 82$), (b) RA + osteopenia ($n = 49$), and

Table 1: Comparison of selected patient characteristics between rheumatoid arthritis (RA) with low bone mineral density (BMD) versus RA with normal BMD and among subgroups of patients with RA with osteoporosis (OP), osteopenia, and normal BMD.

	Low BMD ($n = 131$)	Normal BMD ($n = 45$)	p	Osteoporosis ($n = 82$)	Osteopenia ($n = 49$)	Normal BMD ($n = 45$)	p
Sociodemographic characteristics							
Age (yr), mean ± SD	62 ± 9	54 ± 7	**<0.0001**	62 ± 8	61 ± 10	54 ± 7** ***	**<0.0001**
BMI (kg/m^2), mean ± SD	27.5 ± 4.1	29.8 ± 4.3	**0.002**	26.9 ± 3.9	28.5 ± 4.1	29.8 ± 4.3**	**0.001**
Disease characteristics							
Disease duration (yr), mean ± SD	14 ± 10	13 ± 11	0.58	13 ± 10	14 ± 9	13 ± 10	0.75
DAS28, mean ± SD	3.5 ± 1.5	3.4 ± 1.4	0.58	3.7 ± 1.5	3.8 ± 1.5	3.4 ± 1.4	0.37
HAQ-Di score, mean ± SD	0.62 ± 0.60	0.38 ± 0.36	**0.003**	0.49 ± 0.52	0.60 ± 0.71	0.38 ± 0.36	0.11
ESR (mm/hr), mean ± SD	25 ± 10	23 ± 13	0.39	28 ± 12	24 ± 10	24 ± 13	0.18
CRP (mg/mL), mean ± SD	18.6 ± 32.6	12.6 ± 11.8	0.29	21.9 ± 39.6	13.0 ± 14.0	12.6 ± 11.8	0.22
RF (UI/mL), mean ± SD	192 ± 533	74 ± 118	0.24	177 ± 620	213 ± 378	74 ± 118	0.48
ACPAS (+), $n = 115$ (%)	74/90 (82.2)	15/25 (60.0)	0.03	47/56 (83.9)	27/34 (79.4)	15/25 (60.0)	0.06
Anti-CCP2 (RU/mL), mean ± SD	107 ± 126	82 ± 106	0.37	106 ± 120	108 ± 136	82 ± 106	0.67
Anti-CCP2 (+), n (%)	58/87 (66.7)	13/25 (52)	0.24	39/55 (70.9)	19/32 (59.4)	13/25 (52.0)	0.23
Anti-MCV (U/mL), mean ± SD	280 ± 363	82 ± 164	**<0.0001**	271 ± 343	295 ± 399	82 ± 164	0.06
Anti-MCV (+), n (%)	59/88 (67.0)	7/21 (33.3)	**0.005**	37/55 (67.3)	22/33 (66.7)	7/21 (33.3)	**0.02**
OPG serum levels (pg/mL), mean ± SD	104.0 ± 73.9	118.4 ± 61.1	0.24	115.0 ± 84.5*	85.6 ± 46.9	118.4 ± 61.1	**0.03**
Treatment characteristics							
cs-DMARD use, n (%)	106 (80.9)	36 (80.0)	1.00	66 (80.5)	40 (81.6)	36 (80.0)	0.66
Monotherapy, n (%)	35 (26.7)	16 (35.6)	0.47	24 (29.3)	11 (22.4)	16 (35.6)	0.66
Combinated therapy, n (%)	71 (54.2)	20 (44.4)		42 (51.2)	29 (59.2)	20 (44.4)	
Methotrexate, n (%)	72 (55.0)	23 (51.1)	0.73	44 (53.7)	30 (61.2)	23 (51.1)	0.57
Leflunomide, n (%)	43 (32.8)	13 (28.9)	0.71	24 (29.3)	19 (38.8)	13 (28.9)	0.51
Sulfasalazine, n (%)	38 (29.0)	9 (20.0)	0.25	26 (31.7)	12 (24.5)	9 (20.0)	0.35
Azathioprine, n (%)	20 (15.3)	7 (15.6)	1.00	13 (15.9)	7 (14.3)	7 (15.6)	1.00
Chloroquine, n (%)	17 (13.0)	8 (17.8)	0.46	12 (14.6)	5 (10.2)	8 (17.8)	0.57
Glucocorticoid use, n (%)	109 (83.2)	35 (77.8)	0.50	57 (69.5)	35 (71.4)	33 (73.3)	0.91
Glucocorticoid dose (mg), mean ± SD	6.6 ± 8.7	4.8 ± 3.3	0.23	4.7 ± 6.1	6.4 ± 10.2	4.8 ± 3.3	0.37

BMD: bone mineral density; anti-CCP2: 2nd-generation antibodies against citrullinated proteins; anti-MCV: antimutated citrullinated vimentin antibodies; ACPAs: antibodies against cyclic citrullinated peptides/proteins including anti-CCP2 (+) or anti-MCV (+); cs-DMARD: conventional synthetic disease-modifying antirheumatic drugs; DAS28: Disease Activity Score for 28 joints; HAQ-DI: Health Assessment Questionnaire-disability index; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RF: rheumatoid factor. Anti-CCP2 (+) was defined as >5 relative units (RU/mL); anti-MCV (+) was defined as >20 RU/mL; combined therapy was defined as the use of two or more cs-DMARDs. Qualitative variables were expressed in frequencies (%) and quantitative variables in means ± standard deviations (SD). Statistical tests: the chi-square test (or the Fisher exact test if applicable) was conducted for comparisons between proportions and independent sample Student t-tests were conducted for comparisons between means, and p values were obtained comparing low BMD versus normal BMD. Low BMD (including osteopenia or osteoporosis) (T-score ≤ −1 SD). Comparisons between differences in means were performed using one-way ANOVA. Dunnett correction was used as the post hoc test in case of statistical significance in the ANOVA. p values were obtained comparing OP (T-score ≤ −2.5 SD), osteopenia (T-score between −1.0 and −2.5 SD), and normal BMD (T-score ≥ −1 SD). *Statistical significance between OP and osteopenia groups ($p < 0.05$); **statistical significance between normal BMD and OP groups ($p < 0.05$); ***statistical significance between normal BMD versus osteopenia groups ($p < 0.05$).

(c) RA with normal BMD ($n = 45$). Utilizing ANOVA analysis, we observed that the group with normal BMD had lower age ($p < 0.0001$) and higher BMI ($p = 0.0001$). A trend toward lower anti-MCV titers was observed in patients with normal BMD, although this trend did not achieve statistical significance ($p = 0.06$). Moreover, higher OPG titers were observed in OP compared with osteopenia ($p = 0.03$), but not compared with normal BMD. Table 2 presents a comparison of clinical characteristics between RA patients with the GG genotype versus patients with the GC or CC genotype. No statistical differences were observed between GG genotype carriers versus GC or CC carriers in the majority of the clinical variables. GC or CC carriers had higher anti-MCV titers ($p = 0.04$) and a trend toward higher corticosteroid doses ($p = 0.09$). GG genotype carriers had similar OPG levels compared with GC or CC genotype carriers (111.3 versus 99.7 pg/mL; $p = 0.32$).

Table 3 compares the genotype frequencies between both RA groups that were similar in terms of low BMD and normal BMD ($p = 0.62$). Polymorphic genotype CC was observed in a low frequency in both groups (group 1, low BMD: 3%; group 2, normal BMD: 2.2%), whereas, as expected, GG was the most frequent genotype in both groups (71 and 64%, resp.). Also, Table 3 shows the OR and their

Table 2: Comparison of sociodemographic, clinical, and laboratory characteristics between GG genotype carriers and GC or CC genotype carriers in patients with rheumatoid arthritis (RA).

	GG ($n = 122$)	GC or CC ($n = 54$)	p
Sociodemographic characteristics			
Age (yr), mean ± SD	60 ± 9	58 ± 9	0.16
Body mass index (kg/m^2), mean ± SD	28.1 ± 4.4	28.1 ± 4.0	0.95
Disease characteristics			
Disease duration (yr), mean ± SD	14 ± 11	13 ± 8	0.70
DAS28 score, mean ± SD	3.5 ± 1.4	3.6 ± 1.6	0.85
HAQ-Di score, mean ± SD	0.49 ± 0.57	0.47 ± 0.52	0.81
Lumbar spine L1–L4 BMD (g/cm^2), mean ± SD	0.97 ± 0.17	1.00 ± 0.19	0.37
Femoral neck BMD (g/cm^2), mean ± SD	0.85 ± 0.17	0.86 ± 0.15	0.55
ESR (mm/hr), mean ± SD	25 ± 11	25 ± 11	0.86
CRP (mg/mL), mean ± SD	15.7 ± 25.9	19.1 ± 32.5	0.56
RF (UI/mL), mean ± SD	170.6 ± 524.8	129.7 ± 229.4	0.68
ACPAs (+), $n = 115$ (%)	63/82 (76.8)	26/33 (78.8)	1.00
Anti-CCP2 (RU/mL), mean ± SD	105 ± 131	92 ± 97	0.56
Anti-CCP2 (+), n (%)	48/79 (60.8)	23/33 (69.7)	0.40
Anti-MCV (U/mL), mean ± SD	201 ± 309	345 ± 403	**0.04**
Anti-MCV (+), n (%)	46/78 (59.0)	20/31 (64.5)	0.67
OPG serum levels (pg/mL), mean ± SD	111.3 ± 71.9	99.7 ± 68.9	0.32
Treatment characteristics			
cs-DMARD use, n (%)	98 (80.3)	44 (81.5)	1.00
Monotherapy, n (%)	36 (29.5)	15 (27.8)	0.93
Combinated therapy, n (%)	62 (50.8)	29 (53.7)	0.93
Methotrexate	66 (54.1)	31 (57.4)	0.74
Leflunomide	37 (30.3)	19 (35.2)	0.60
Sulfasalazine	33 (27.0)	14 (25.9)	1.00
Azathioprine	16 (13.1)	11 (20.4)	0.26
Chloroquine	16 (13.1)	9 (16.7)	0.64
Glucocorticoid use, n (%)	85 (69.7)	40 (74.1)	0.59
Glucocorticoid dose (mg), mean ± SD	4.54 ± 5.98	6.54 ± 9.83	0.09

RA: rheumatoid arthritis; BMD: bone mineral density; GG: homozygote genotype; GC: heterozygote genotype; CC: polymorphic homozygote genotype; anti-CCP2: 2nd-generation antibodies against citrullinated proteins; anti-MCV: antimutated citrullinated vimentin antibodies; ACPA: antibodies against cyclic citrullinated peptides/protein including anti-CCP2 (+) or anti-MCV (+); cs-DMARD: conventional synthetic disease-modifying antirheumatic drugs; DAS28: Disease Activity Score for 28 joints; HAQ-DI: Health Assessment Questionnaire-disability index; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RF: rheumatoid factor. Anti-CCP2 (+) was defined as >5 RU/mL; anti-MCV (+) was defined as >20 RU/mL; combined therapy was defined as the use of two or more cs-DMARDs. Qualitative variables were expressed in frequencies (%) and quantitative variables in means ± standard deviations (SD). Statistical tests: the chi-square test (or the Fisher exact test if applicable) was conducted for comparisons between proportions and independent sample Student t-tests were performed for comparisons between differences in means. p values were obtained comparing the GG genotype versus GC or CC genotype.

95% CI of the comparison between GG genotype carriers and those of other genotypes and dominant and recessive models, as well as the comparison of allele frequencies between patients with RA with low BMD and patients with RA with normal BMD. No differences in the risk of low BMD were observed in any of these comparisons.

Table 4 shows the risk factors associated with low BMD, in which it is noted that age is a risk (OR = 1.105; 95% CI = 1.041–1.173; $p = 0.001$). Also, positive ACPAS (anti-CCP-positive or anti-MCV-positive) are a risk for low BMD (OR = 3.755; 95% CI = 1.299–10.852; $p = 0.015$). We do not observe an association for risk of low BMD with the presence of any genotype of the *TNFRSF11B* gene rs2073618 polymorphism.

4. Discussion

In this study, we observed that the rs2073618 polymorphism of the *TNFRSF11B* gene does not confer a higher risk of low BMD. GG was the genotype most frequently observed in RA independent of the group. Previous studies have been evaluated in postmenopausal women without rheumatic diseases, observing a probable association between polymorphisms in the *TNFRSF11B* gene and

Table 3: Evaluation of the *rs2073618* polymorphism as a predictor of low bone mineral density (BMD) in patients with rheumatoid arthritis (RA).

Rheumatoid arthritis ($n = 176$)	Low BMD ($n = 131$)	Normal BMD ($n = 45$)	OR	95% CI	p
Genotype					
GG, $n = 122$ (%)	93 (71.0)	29 (64.4)	—	—	
GC, $n = 49$ (%)	34 (26.0)	15 (33.3)	—	—	0.62
CC, $n = 5$ (%)	4 (3.0)	1 (2.2)	—	—	
GG versus GC (as a referent)	—	—	1.41	0.68 to 2.95	0.18
GG versus CC (as a referent)	—	—	0.80	0.09 to 7.46	0.46
GC versus CC (as a referent)	—	—	0.57	0.02 to 5.00	0.35
GC versus GG (as a referent)	—	—	0.71	0.34 to 1.48	0.18
CC versus GG (as a referent)	—	—	1.25	0.03 to 11.6	0.46
CC versus GC (as a referent)	—	—	1.76	0.18 to 17.1	0.34
Genetic models					
Dominant model (GG versus CC + GC)	—	—	1.35	0.66 to 2.77	0.21
Recessive model (GG + GC versus CC)	—	—	0.72	0.07 to 6.63	0.42
Alleles, $2n = 352$	$2n = 262$	$2n = 90$			
G allele, $2n = 293$ (%)	220 (84.0)	73 (81.1)	1.22	0.65 to 2.27	0.26
C allele, $2n = 59$ (%)	42 (16.0)	17 (18.9)	0.82	0.44 to 1.53	0.26

RA: rheumatoid arthritis; BMD: bone mineral density; GG: homozygote genotype; GC: heterozygote genotype; CC: polymorphic homozygote genotype; OR: odds ratio risk; 95% CI: 95% confidence interval. p values were obtained comparing low BMD versus normal BMD. Low BMD was defined as osteopenia or osteoporosis (OP) (T-score ≤ 1 SD).

Table 4: Factors associated with low bone mineral density in the logistic regression.

Variables	OR	95% CI	p
Age, years	1.105	1.041–1.173	0.001
ACPAS, positives	3.755	1.299–10.852	0.015
BMI, kg/m^2	—	Not in the model	—
HAQ-DI, score	—	Not in the model	—
Glucocorticoid dose, mg/day	—	Not in the model	—
Genotype (GG + GC + CC)	—	Not in the model	—

ACPAS: antibodies against cyclic citrullinated peptides/proteins, including anti-CCP2 (+) or anti-MCV (+); HAQ-DI: Health Assessment Questionnaire-disability index; multivariate analysis: logistic regression analysis; dependent variable: low bone mineral density (low BMD). Using a stepwise method.

OP in different populations, with nonconclusive results. Some of these studies found an association of polymorphisms in the *TNFRSF11B* gene with osteoporotic changes [14, 15, 18–23], whereas other studies did not observe any association of this polymorphism with OP in postmenopausal women [24–26].

To date, to the best of our knowledge, this is the first study to observe the lack of association between this polymorphism and abnormalities in BMD in RA.

Xu et al., on investigating the association between rs2073618 and the presence of RA, found no significant association with an increase in the risk of RA in Chinese Han population [27]. Assmann et al. and Ye et al., in two separate studies, similarly reported no association between rs2073618 and the risk for RA in Caucasian and Chinese population, respectively [28, 29].

Our hypothesis regarding a possible role of *TNFRSF11B* in an increase in the frequency of OP in RA was based on evidence that OPG levels are related to a decrease in OP and that genetic factors producing conformational changes in serum levels might lead to an increase in the loss of BMD. Xu et al., for example, observed that patients with RA have lower serum OPG levels compared with controls [27]. Zhao et al. described that genetic differences in OPG expression can be important for the regulation of bone remodeling in postmenopausal women [12]. In this latter study, Zhao et al. observed a trend toward lower serum concentrations of OPG in GG genotype carriers compared with CC genotype carriers, although this trend failed to achieve statistical significance.

Although the present study is not related to genotypes for risk for low BMD, including OP, we obtained information about other factors related to low BMD. The presence of positive ACPAs in patients with RA could be an important factor. Guler et al. observed that the presence of high anti-CCP titers had an association with low BMD [30]. However, data have not, to our knowledge, been reported on the role of anti-MCV in BMD in patients with RA.

Our exploratory study had several limitations that must be taken into account. This study might reflect only the genetic characteristics of patients with RA from Western population of Mexico. Therefore, a multicenter study including patients from other regions of Mexico should be considered. On the other hand, we consider that although these multicenter studies are required, they probably would not modify our main conclusion that this polymorphism does not have a significant influence on BMD in RA.

In conclusion, the rs2073618 polymorphism of the *TNFRSF11B* gene does not confer a risk for lower BMD in RA. Instead, factors associated with low BMD, including

older age, BMI, and deteriorated functioning constituted the main factors for low BMD in patients with RA. However, given the relevance of genetic factors for the development of OP and low BMD in these patients, the search for other polymorphisms explaining the higher prevalence of low BMD in RA compared with other diseases continues to be ongoing.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Genetic Polymorphisms of Cytotoxic T-Lymphocyte Antigen 4 in Primary Biliary Cholangitis: A Meta-Analysis

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Background and Aim. The connection between gene polymorphisms of cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) and primary biliary cholangitis (PBC) is still vague and blurred. The purpose of this study is to precisely estimate the association of the polymorphisms of *CTLA4* with the risk of PBC by using a meta-analysis. *Methods*. PubMed and the Chinese National Knowledge Infrastructure (CNKI) database were used to search correlative literatures, and the documents which were about the relationships between the polymorphisms of *CTLA4* (rs231775, rs231725, rs3087243, and rs5742909) and PBC were collected as of June 2016. The strength of correlation based on odds ratios (ORs) and its 95% confidence intervals (95%CIs) was computed by STATA. *Results*. Generally, in rs231775, a significant risk was found in G allele, the value of OR was 1.32, and its 95%CI was 1.19 to 1.47. The same situation was found in A allele of rs231725, the value of OR was 1.33, and its 95%CI was 1.22 to 1.45. As genotypic level, different genotypic models were also found to have obvious relevance with PBC in rs231775 and rs231725. No obvious connections were found in other SNPs. *Conclusion*. This study indicated that the polymorphisms of rs231775 and rs231725 would be the risk factors of PBC.

1. Introduction

Autoimmune diseases are resulted from the dysfunction of the immune system, which generate immune response to autoantigens. Primary biliary cholangitis (PBC) is one kind of specific autoimmune diseases, which can cause progression of fibrosis and cirrhosis in the liver and lead to liver failure finally [1–4]. There are still no special treatments for primary biliary cholangitis in the world. At present, the most efficient therapy is to use ursodeoxycholic acid for those who are in early period of primary biliary cholangitis. However, ursodeoxycholic acid could not still stop the progression of the disease. When the final stage of PBC occurred, the only therapy is liver transplantation. Up to now, the definite etiology of PBC is still not clear.

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is expressed on the surface of activated T and transmits inhibitory signal, and it is also found on the surface of regulatory T cells. The functions of CTLA4 are to lower responses of T cell and maintain peripheral tolerance of T cell [5]. The abnormal costimulation between specific autoreactive T lymphocytes and CTLA4 in PBC patients causes the reaction of peripheral T lymphocyte not to be terminated, which might be one of the pathogens of PBC.

As to PBC, $CD8^+$ T cells are important factors in the pathogenesis [6]. $CD8^+$ T cells are sensitive to E2 components of pyruvate dehydrogenase complexes (PDC-E2) which are abnormally expressed on the surface of biliary epithelial cells (BECs) and would result in apoptosis for these epithelial cells and destruction of the small bile duct [7, 8]. As a coinhibitor signal, CTLA4 binds to CD80/CD86 on antigen-presenting cells (APCs) with higher affinity comparing with CD28 [9]. Binding with CD80/CD86 to deliver negative signal into T cells, CTLA4 can result T cell responses

in inhibition or termination [10]. Then, CTLA4 can regulate immune suppression and peripheral tolerance in $CD8^+$ T cells. Thus, CTLA4 could be involved in the regulation of pathological processes of PBC, which might be a therapeutic for PBC. Two studies demonstrated that treatment with CTLA4-Ig, which can reduce self-reactive T cell activation and liver inflammation significantly, could obviously reduce the level of portal inflammation and biliary cell damage in the mouse model [11, 12].

So, CTLA4 regulation plays an important role in the pathological process of PBC. Meanwhile, the results, which were shown in some researches about the treatment of *CTLA4*, have shown that *CTLA4* plays a unique role in the pathogenesis and treatment of PBC.

Recently, genetic factors are deemed to be an important role in PBC, which is mainly in favor of familial clustering of PBC [13]. Recent study showed that PBC was significantly associated with some concrete gene polymorphisms [14]. Since PBC displays characteristics of autoimmunity, more and more studies concentrated in associations between genetic polymorphism and variations of autoimmunity.

There are several evidences to prove the connections between polymorphisms of *CTLA4* and other autoimmune diseases in recent literatures [15–17]. In recent years, there are extensive researches about the links between *CTLA4* and PBC. rs231775, rs231775, rs3087243, and rs5742909 are the most common four single-nucleotide polymorphisms (SNPs) to be widely studied [18–22]. Thus, with changes of the function in these SNPs, the possibility of PBC might be increased. Because of inconclusive connections between the polymorphisms of *CTLA4* and the risks of PBC, those relative researches are necessary to be combined to conduct a meta-analysis. Several early systematic reviews which had been published mainly regarded the relationships between the polymorphisms in several SNPs and primary biliary cholangitis [23–25]. However, these studies either did not draw the clear conclusion or did not include some latest literatures. So, in this research, 16 studies are combined to analyze the correlation between the polymorphisms of *CTLA4* and risks of PBC [1, 18–22, 26–35].

2. Materials and Methods

2.1. Study Selection and Data Extraction. We used PubMed and the China Knowledge Resource Integrated database up to June 2016, and related literatures about the relationships between the polymorphisms of *CTLA4* and risks of PBC were researched on computer with retrieval words ("primary biliary cholangitis, PBC, cytotoxic T-lymphocyte antigen 4, Polymorphism, SNP, genetic variants"). At last, we found 26 studies contained relative contents about *CTLA4* and PBC.

The following conditions should be met in studies: firstly, literature should be a case-control study; secondly, outcome was about primary biliary cholangitis; and thirdly, odds ratio and its 95% confidence interval should be estimated with adequate data in literatures. Exclusive criteria: insufficient information for data extraction. At last, this meta-analysis included 16 literatures after 10 literatures were excluded.

According to the inclusion conditions mentioned above, two researchers extracted data independently. The researchers gathered these data from each study: SNPs, name of the first author, date of publication, ethnicity, number of allele, and genotype. Diversities among researchers were solved with discussion.

3. Statistical Analysis

The intensity of associations between PBC risk and *CTLA4* polymorphisms was assessed in a random-effect model or fixed-effect model by the estimated OR and its 95%CI. Assessing the difference between the *CTLA4* polymorphisms and the PBC risk in Caucasian and Asian was also conducted by using subgroup analyzing. *Z*-test was used to compute the significant difference of pooled OR. The p value of *Z*-test was calculated to access significance. Because multiple comparisons were conducted in this study, the threshold of p values was corrected with formula $1-(1-p)^{1/n}$ for the Bonferroni correction [36]. *Q*-test was used to assess heterogeneity and calculate I^2 statistic. When p was less than 0.05 or I^2 was more than 50%, the results among the studies indicated significant heterogeneity. In addition, possible publication biases were estimated by Begg's funnel plot and Egger's regression; p value was calculated to access bias. When p was less than 0.05, a publication bias was considered to be existence. The validity and reliability of a meta-analysis were evaluated by conducting sensitivity analysis [37]. All the statistics were performed by STATA 14 software.

In this study, linkage disequilibrium (LD) was used to measure the association among these four *CTLA4* SNPs after multiple comparison. The values of D' and r^2 were calculated by SHEsis software (http://analysis.bio-x.cn/myAnalysis.php) [38]. When $D' > 0.8$ or $r^2 > 0.4$, linkage disequilibrium could be considered.

4. Results

4.1. Literature Search. In total, the number of cases and controls were 4422 and 5210 in 16 studies, respectively (Table 1). As to *CTLA4*, the SNPs which were mostly consulted were rs231775, rs231725, rs3087243, and rs5742909. These SNPs were reported in 14, 6, 9, and 5 studies, respectively. The genotypes of controls were in line with Hardy-Weinberg equilibrium in most articles.

4.2. Meta-Analysis. Obvious heterogeneity was identified in *CTLA4* rs231775 polymorphism (G versus A: P(het) = 0.008, $I^2 = 54.1\%$; GG versus AA: P(het) = 0.032, $I^2 = 45.6\%$), rs3087243 polymorphism (GA versus GG: P(het) = 0.028, $I^2 = 53.5\%$; AA + GA versus GG: P(het) = 0.037, $I^2 = 51.2\%$), and rs5742909 polymorphism (T versus C: P(het) = 0.001, $I^2 = 69.4\%$; TC versus CC: P(het) = 0.01, $I^2 = 66.7\%$; and (TT + TC) versus CC: P(het) = 0.007, $I^2 = 69\%$). Therefore, we chose the random-effect model to generate extensive CIs in these genetic models, the rest of genetic models were used the fixed-effects model (Table 2).

Table 1: Basic characteristics of involved studies.

SNP	First author	Year	Race	Case					Control					P(HWE)
				A	B	AA	AB	BB	A	B	AA	AB	BB	
rs231775	Kosh	2000	C	131	115	35	61	27	278	122	99	80	21	0.613
	Paulo	2003	C	69	31	23	23	4	88	46	29	30	8	0.955
	Fan	2004	A	46	108	6	37	34	139	181	23	93	44	0.021
	Yukiko	2006	A	30	60	5	20	20	61	85	14	33	26	0.545
	Eckart	2007	C	206	154	58	90	32	243	161	78	87	37	0.149
	Peter	2007	C	389	243	40	29	4	497	285	54	35	10	0.716
	Brian-3	2008	C	414	288	122	170	59	352	206	111	130	38	0.995
	Raoul	2008	C	313	203	95	123	40	407	165	145	118	23	0.883
	Brian-2	2008	C	421	281	131	161	59	258	152	79	99	27	0.644
	Erin	2009	C	545	417	162	223	96	1562	934	493	577	178	0.661
	Satoru	2010	A	226	390	42	143	123	224	312	47	131	90	0.955
	Yoshihiro	2011	A	314	586	55	204	191	313	429	66	181	124	0.997
	Mantaka	2012	C	144	56	52	40	8	226	90	81	64	13	<0.001
	Li	2013	A	180	444	20	140	152	312	438	49	214	112	0.001
rs231725	Brian-3	2008	C	442	260	139	164	48	391	167	137	117	25	0.998
	Satoru	2010	A	250	368	51	149	108	250	286	58	133	77	0.968
	Brian-1	2010	C	1091	641	368	357	141	1032	490	350	332	79	0.984
	Yoshihiro	2011	A	351	549	68	214	168	347	395	81	185	105	0.977
	Li	2013	A	204	420	29	146	137	316	434	59	198	118	0.109
rs3087243	Sabine	2005	C	167	141	40	87	27	170	162	49	72	45	0.089
	Peter	2007	C	222	168	59	104	32	301	251	82	137	57	0.987
	Brian-3	2008	C	407	295	118	171	62	318	240	91	136	52	0.925
	Brian-2	2008	C	400	302	117	168	66	234	176	70	94	41	0.358
	Erin	2009	C	602	360	198	205	78	1335	1161	362	613	273	0.656
	Satoru	2010	A	454	162	167	120	21	372	164	129	114	25	0.979
	Yoshihiro	2011	A	689	211	264	161	25	515	227	179	157	35	0.946
	Mantaka	2012	C	107	93	32	43	25	158	158	37	84	37	0.426
	Li	2013	A	430	194	159	112	41	492	258	170	152	53	0.048
rs5742909	Fan	2004	A	138	16	63	12	2	274	46	122	30	8	0.003
	Brian-3	2008	C	646	56	297	52	2	502	56	226	50	3	0.9
	Raoul	2008	C	477	39	220	36	2	509	63	226	56	4	0.803
	Erin	2009	C	852	110	377	99	5	2291	205	1055	183	10	0.509
	Satoru	2010	A	550	66	245	59	4	470	66	206	58	4	0.971
	Li	2013	A	541	83	246	49	17	644	106	288	68	19	<0.001

C: Caucasian; A: Asian; P(HWE): p value of Hardy-Weinberg equilibrium for control.

The results of this analysis in the association of *CTLA4* polymorphisms (rs231775, rs231725, rs3087243, and rs5742909) with susceptibility to PBC are presented (Table 3).

The study identified that rs231775 polymorphism of *CTLA4* was significantly associated with PBC susceptibility. The ORs (95%CIs) of G versus A, GG versus AA, GA versus AA, (GG + GA) versus AA, and GG versus (AA + GA) were 1.32 (1.19–1.47), 1.72 (1.37–2.16), 1.27 (1.13–1.43), 1.38 (1.23–1.54), and 1.52 (1.35–1.71), respectively. As to each model, the p value was below 0.0001 (Figure 1). The rs231725 polymorphism also showed significant association with PBC susceptibility. The ORs with 95%CIs of A versus G, AA versus GG, GA versus GG, (AA + GA) versus GG, and AA versus (GG + GA) were 1.33 (1.22–1.45), 1.83 (1.52–2.21), 1.20 (1.04–1.38), 1.34 (1.17–1.53), and 1.57 (1.35–1.82), respectively. As to each genetic model, the p value was below 0.05 (Figure 2). Nevertheless, no association was identified between rs3087243 (Figure 3) and rs5742909 (Figure 4) polymorphisms and PBC susceptibility. Subgroup analysis showed that both rs231775 and rs231725 showed significant association with PBC susceptibility for Asians and for Caucasians.

4.3. Calculation of Linkage Disequilibrium. Based on the values of r^2, there was no obviously linkage disequilibrium among four SNPs (Table 4).

Table 2: The results of heterogeneity.

SNP	Genetic model	I^2 (%)	P(het)	Effect model
rs231775	G : A	54.1	0.008	Random
	GG : AA	45.6	0.032	Random
	GA : AA	0.0	0.616	Fixed
	(GG + GA) : AA	31.9	0.121	Fixed
	GG : (AA + GA)	37.3	0.079	Fixed
rs231725	A : G	0.0	0.674	Fixed
	AA : GG	0.0	0.815	Fixed
	GA : GG	6.9	0.368	Fixed
	(AA + GA) : GG	20.2	0.286	Fixed
	AA : (GG + GA)	0.0	0.858	Fixed
rs3087243	A : G	37.9	0.116	Fixed
	AA : GG	11.8	0.337	Fixed
	GA : GG	53.5	0.028	Random
	(AA + GA) : GG	51.2	0.037	Random
	AA : (GG + GA)	0.0	0.569	Fixed
rs5742909	T : C	69.4	0.001	Random
	TT : CC	0.0	0.814	Fixed
	TC : CC	66.7	0.01	Random
	(TT + TC) : CC	69.0	0.007	Random
	TT : (CC + TC)	0.0	0.864	Fixed

P(het): p value of Q-test for heterogeneity test; I^2: the proportion of total variation contributed among study variants; P(het) < 0.05 or $I^2 > 50\%$ indicated significant heterogeneity, using a random model; otherwise, using a fixed model.

Table 3: Results of *CTLA4* polymorphisms and PBC.

SNP	Genetic model	Asian			Caucasian			Overall		
		OR	95%CI	p	OR	95%CI	p	OR	95%CI	p
rs231775	G : A	1.47	1.27, 1.72	<0.0001	1.24	1.09, 1.42	0.001	1.32	1.19, 1.47	<0.0001
	GG : AA	2.10	1.55, 2.84	<0.0001	1.51	1.12, 2.04	0.006	1.72	1.37, 2.16	<0.0001
	GA : AA	1.39	1.08, 1.79	0.011	1.24	1.09, 1.42	0.001	1.27	1.13, 1.43	<0.0001
	(GG + GA) : AA	1.65	1.30, 2.10	<0.0001	1.31	1.16, 1.49	<0.0001	1.38	1.23, 1.54	<0.0001
	GG : (AA + GA)	1.66	1.40, 1.96	<0.0001	1.39	1.18, 1.65	<0.0001	1.52	1.35, 1.71	<0.0001
rs231725	A : G	1.39	1.22, 1.57	<0.0001	1.27	1.12, 1.44	<0.0001	1.33	1.22, 1.45	<0.0001
	AA : GG	1.92	1.47, 2.49	<0.0001	1.75	1.33, 2.29	<0.0001	1.83	1.52, 2.21	<0.0001
	GA : GG	1.38	1.07, 1.76	0.012	1.11	0.93, 1.33	0.236	1.20	1.04, 1.38	0.015
	(AA + GA) : GG	1.57	1.24, 1.99	<0.0001	1.23	1.04, 1.46	0.014	1.34	1.17, 1.53	<0.0001
	AA: (GG + GA)	1.52	1.27, 1.83	<0.0001	1.66	1.29, 2.14	<0.0001	1.57	1.35, 1.82	<0.0001
rs3087243	A : G	0.78	0.68, 0.89	<0.0001	0.83	0.76, 0.91	<0.0001	0.81	0.75, 0.88	<0.0001
	AA : GG	0.66	0.49, 0.90	0.008	0.70	0.58, 0.85	<0.0001	0.69	0.59, 0.81	<0.0001
	GA : GG	0.76	0.63, 0.91	0.003	0.90	0.68, 1.21	0.491	0.84	0.70, 1.00	0.051
	(AA + GA) : GG	0.74	0.62, 0.88	0.001	0.86	0.66, 1.11	0.250	0.80	0.68, 0.94	0.008
	AA : (GG + GA)	0.74	0.55, 1.00	0.050	0.78	0.66, 0.92	0.004	0.77	0.67, 0.89	0.001
rs5742909	T : C	0.87	0.70, 1.08	0.206	0.92	0.55, 1.55	0.766	0.89	0.68, 1.17	0.417
	TT : CC	0.90	0.51, 1.58	0.715	0.88	0.39, 1.99	0.762	0.89	0.56, 1.42	0.636
	TC : CC	0.84	0.64, 1.10	0.199	0.95	0.55, 1.64	0.846	0.90	0.67, 1.22	0.506
	(TT + TC) : CC	0.85	0.66, 1.09	0.200	0.93	0.53, 1.63	0.809	0.90	0.67, 1.20	0.463
	TT : (CC + TC)	0.93	0.53, 1.63	0.804	0.87	0.39, 1.97	0.743	0.91	0.58, 1.45	0.695

OR: odd ratio; 95%CI: 95% confidence interval; p: p value of Z-test for significance test of OR; using the Bonferroni correction, $p < 0.0127$ means statistically significant.

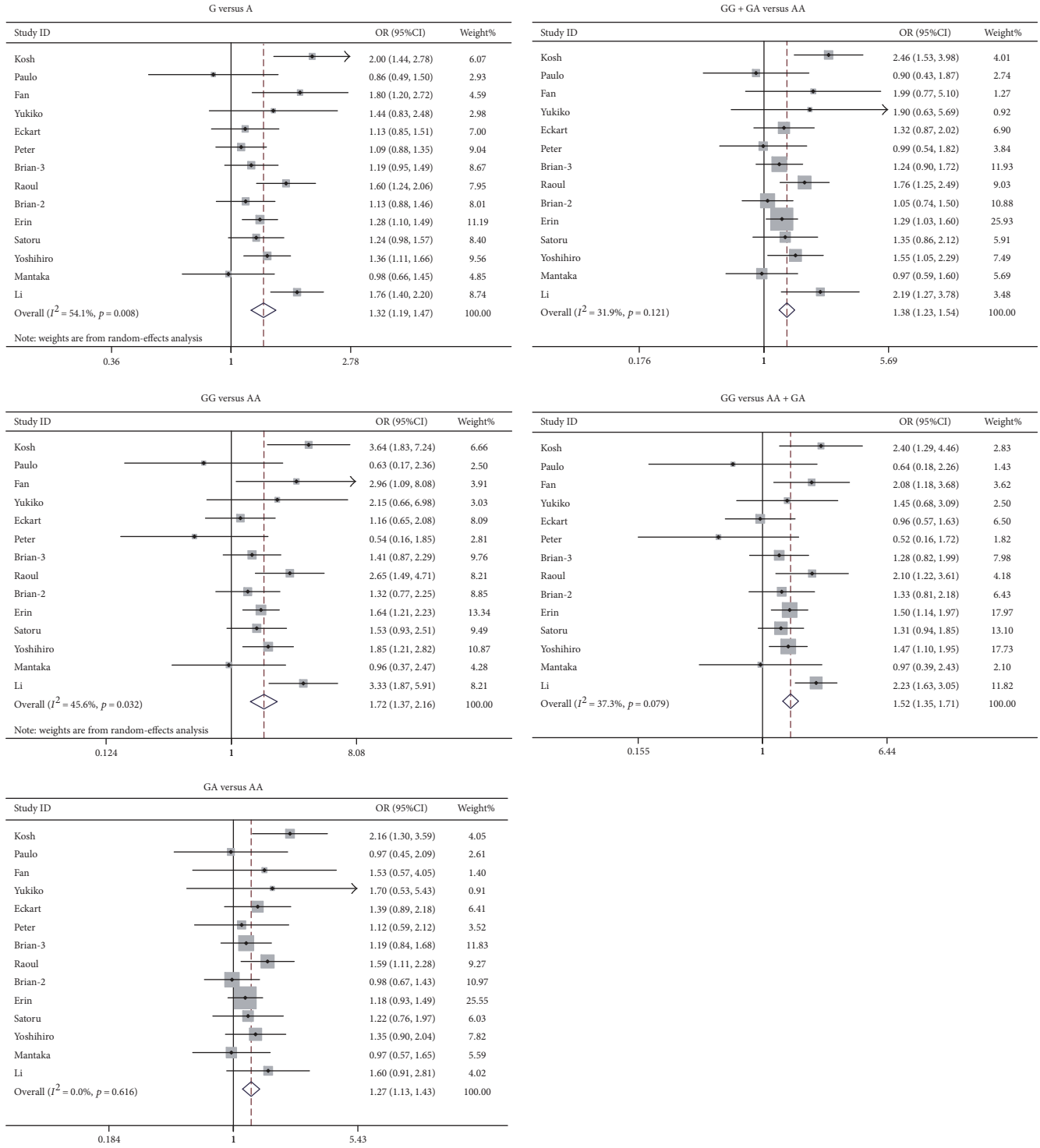

Figure 1: Odds ratio with its 95% confidence interval of PBC linked with *CTLA4* rs231775. The rhombus represented the pooled OR with 95%CI.

4.4. Sensitivity Analyses and Publication Bias. Sensitivity analysis was conducted by omitting each studies sequentially, suggesting that the results for the overall population were statistically stable and reliable (Figure 5). Publication bias was examined by using Egger's regression and Begg's funnel plot in our research. No obvious publication bias was identified. For Begg's test, p values of rs231775 (G versus A), rs231725 (A versus G), rs3087243 (A versus G), and rs5742909 (T versus C) were 0.661, 0.806, 0.466, and 0.060, respectively. For Egger's test, p values of genetic models mentioned above were 0.952, 0.186, 0.061, and 0.029, respectively (Figure 6).

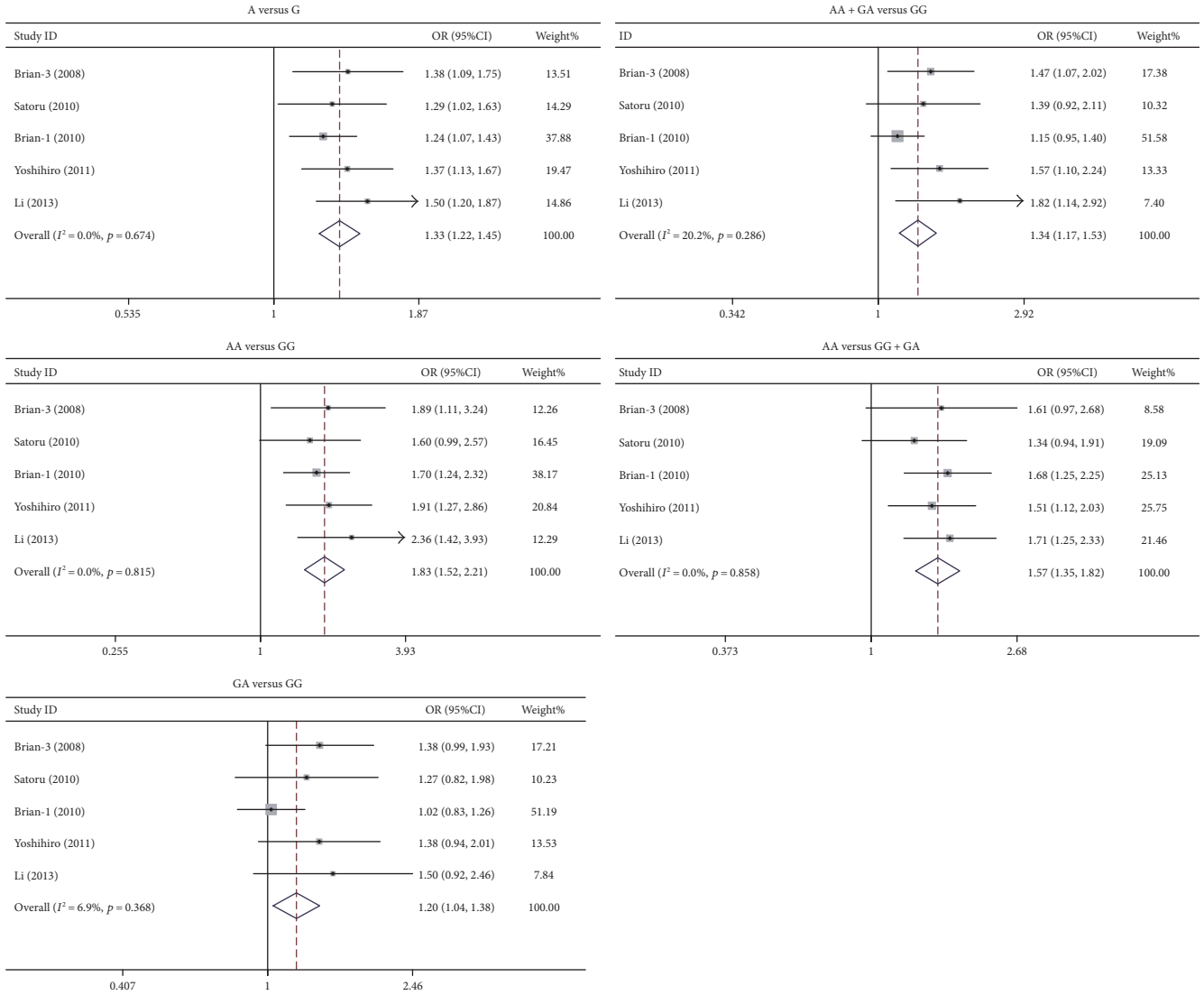

Figure 2: Odds ratio with its 95% confidence interval of PBC linked with *CTLA4* rs231725. The rhombus represented the pooled OR with 95%CI.

5. Discussion

Multiple comparisons were conducted in this study. To minimize the type I error, the threshold of p values was corrected by the Bonferroni correction. The Bonferroni correction compensates for that increase by testing each individual hypothesis at a significance level [36]. Then, the threshold of p value for the Bonferroni correction can be calculated with the corresponding critical values $1-(1-p)^{1/n}$. There are four SNPs in this study for multiple comparisons, and the original threshold of p value was 0.05. So, after calculating with above formula ($p = 0.05$, $n = 4$), $p < 0.0127$ was considered statistically significant.

As to the polymorphisms of rs231775 and rs231725, significant connections were found to be associated with PBC in all 5 genetic models. For patients in cases, the frequencies of allele and genotype in rs231775 and rs231725 were increased more significantly than those in controls. As to allele, the results of rs231775 were similar to the results of five published meta-analyses by Eskandari-Nasab et al. [39], Huang et al. [23], Miyake et al. [24], Li et al. [25], and Chen et al. [14]. Li and Miyake indicated that the G allele might be connected with PBC as a risk factor. On the contrary, meta-analyses from Chen and Huang proposed that the relationship between G allele and susceptibility of PBC was observed only in Asian.

As to rs3087243, our analysis showed that both allele and genotype were negative associations with PBC in overall populations. For rs5742909, in codominant, dominant, and recessive models, there were no connections with susceptibility of PBC in Caucasian and Asian. These results were consistent with those in one meta-analysis, which was conducted by Li et al. [25], including 12 studies.

Through subgroup analysis, GG homozygosity of rs231775 and AA homozygosity of rs231725 were associated with the susceptibility to PBC both in Asians and in Caucasians. AA homozygosity of rs3087243 was protective against PBC in Asians and Caucasians. On the other hand,

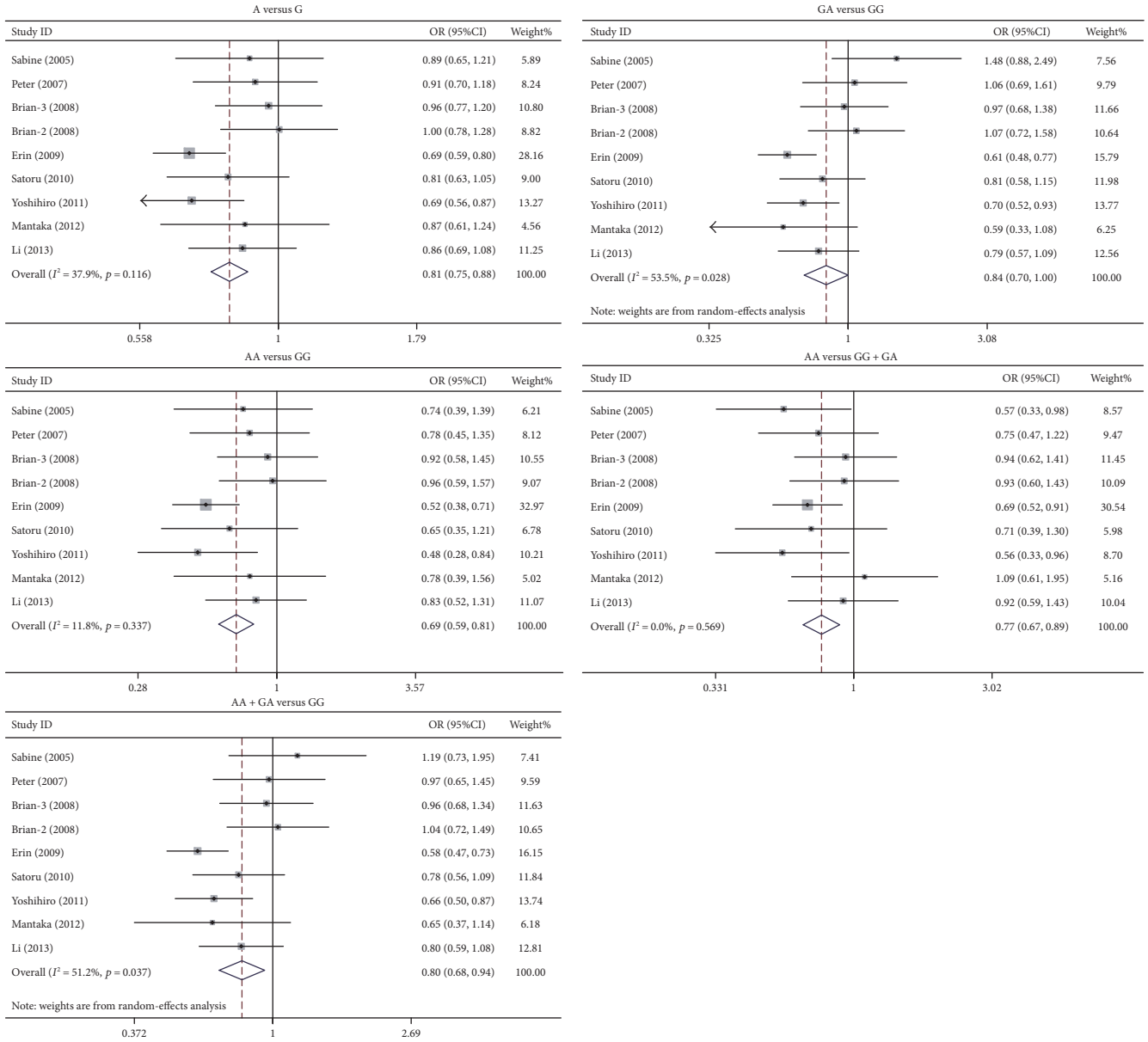

Figure 3: Odds ratio with its 95% confidence interval of PBC linked with *CTLA4* rs3087243. The rhombus represented the pooled OR with 95%CI.

GA heterozygosity of rs231725 was associated with the susceptibility to PBC in Asians although it was not in Caucasians. GA heterozygosity of rs3087243 was protective against PBC in Asians although it was not in Caucasians. Thus, there may be a little different between Asians and Caucasians in the relationship between SNP polymorphism of *CTLA4* and the susceptibility to PBC. In order to solve these problems, further studies in various ethnicities are required.

In linkage disequilibrium, coefficients D' and r^2 were frequently used. They have quite different characteristics and could be applied for different purposes. Typically, r^2 is useful in the context of association studies, D' is the measure of choice to assess recombination patterns in a given population [40]. It is indicated that the two loci were not recombined and were in a complete linkage disequilibrium when the value of D' is 1. But the significance of values would be difficult to interpret when $D' < 1$. Meanwhile, when the sample size is small and the frequency of SNPs is low, the estimate of D' would be too large. In this case, even the sites of linkage equilibrium can get larger D' value, the actual meaning of the D' could easily be exaggerated. Then, the value of r^2 could be more reliable under this condition. In this study, the frequency of genotypes in rs5742909 was much lower than the others (Table 1). Thus, the values of D' were much larger than the value of r^2. So, r^2 was chosen to assess the linkage disequilibrium. Based on the results, LD was not observed among four SNPs.

Heterogeneity may affect pooled results as one of possible factors. It can be categorized into heterogeneity of the genetic model and effect. In this study, a relatively moderate heterogeneity was heeded. Among 16 studies, HWE values of five studies were out. As to rs231775 and rs5742909, there were

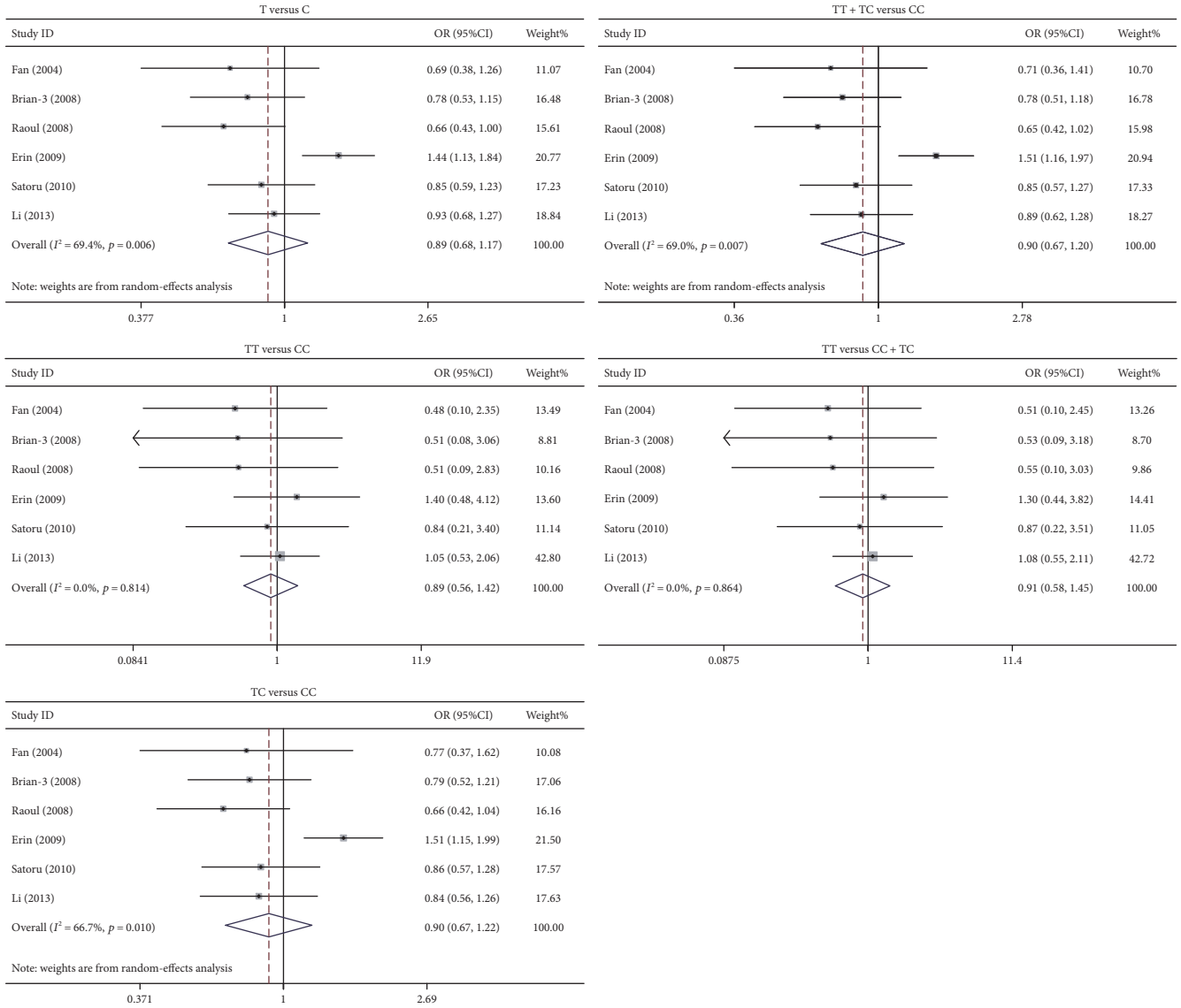

Figure 4: Odds ratio with its 95% confidence interval of PBC linked with *CTLA4* rs5742909. The rhombus represented the pooled OR with 95%CI.

Table 4: Results of linkage disequilibrium.

	rs231775	rs231725	rs3087243	rs5742909
rs231775	—	0.908	0.964	0.942
rs231725	0.030	—	0.924	0.921
rs3087243	0.017	0.031	—	0.914
rs5742909	0.020	0.074	0.030	—

Lower left areas are values of r^2. Upper right areas are values of D'.

three and two studies to be out of HWE, respectively. Thus, we conducted the sensitivity analysis in all studies. In the analysis of rs231775, the results of I^2values reduced when we removed the article by Li et al. [22]. The heterogeneity in Caucasians was larger than in Asians. This study showed that diversity of genetic ethnicities or methodological differences might be the sources of heterogeneity.

There are some characteristics in this meta-analysis. Comparing with other similar articles, we conducted four SNPs in one study, and each SNP included five different genetic models. We assessed subgroup by analyzing ethnicities and obtained more precise estimation of the relationships. We also performed sensitivity analysis to test the validity of the results.

To date, several genome-wide association studies (GWAS) and genome-wide meta-analysis on PBC have been performed. From these literatures [41–47], there were several genes to be identified as significant susceptibility loci for PBC. There were ethnic differences in genetic susceptibility loci such as *TNFSF15*, *POU2AF1*, *IL12A*, and *IL12RB2* and common pathogenic pathways such as B cell differentiation, IL-12 signaling, and T cell activation.

As to GWAS, the relationship between mutations of SNPs and occurrence of disease might not be a necessity

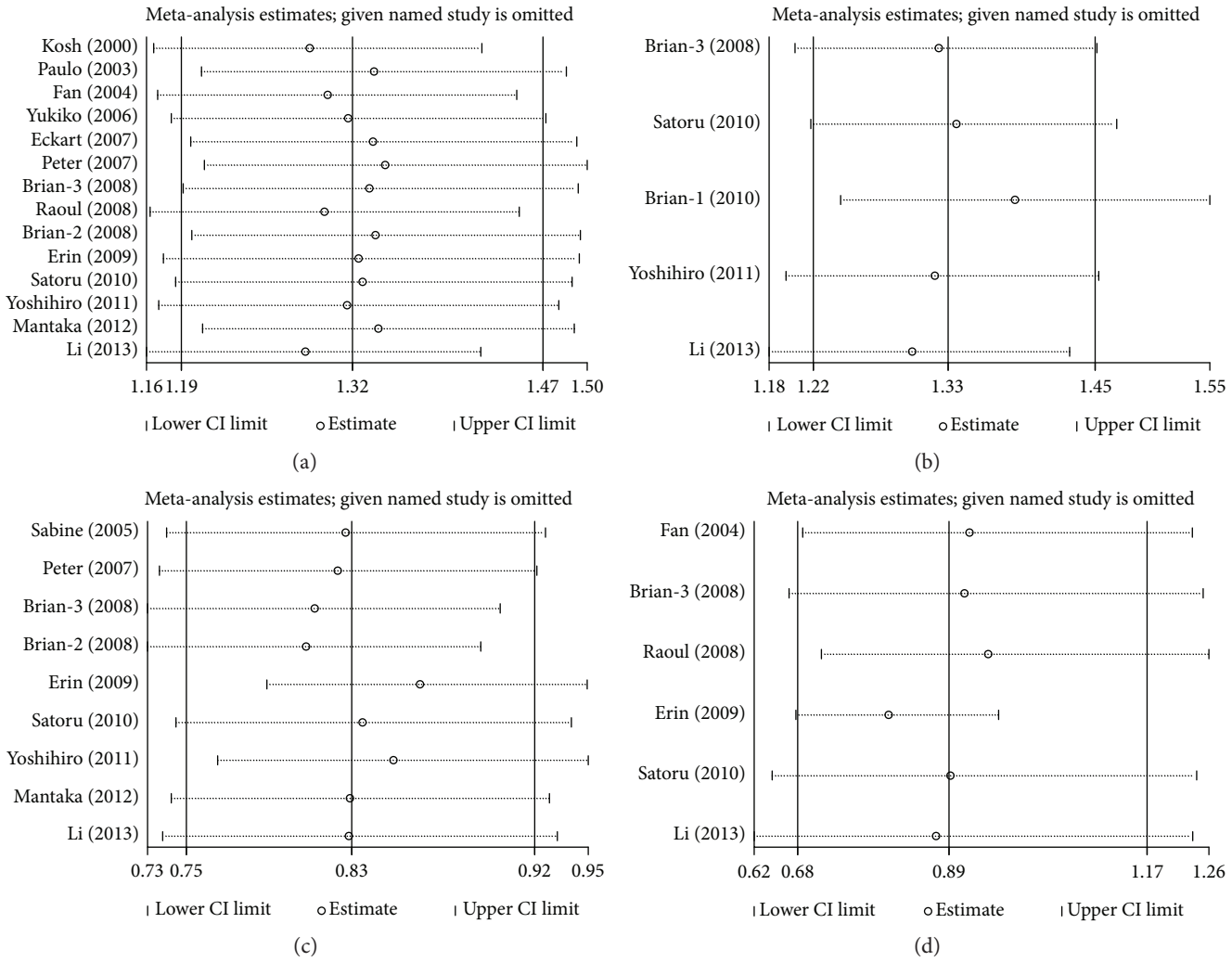

Figure 5: Sensitivity analysis for the effect of *CTLA4* and PBC: (a) rs231755 (G versus A), (b) rs231725 (A versus G), (c) rs3087243 (A versus G), and (d) rs5742909 (T versus C).

but a probability. So, a large number of samples should be analyzed for the association study between gene and disease. The number of cases which were enrolled in each publication of genome-wide association study of PBC was not enough comparing with the probability of gene mutation. Thus, some higher risk loci with lower mutation frequency could be concealed by lower risk loci with higher mutation frequency. Meanwhile, it was different from the GWAS that focused on the onset of disease, and the data of our meta-analysis might provide a point in the search for novel therapies that are urgently needed to improve outcomes for PBC patients.

On the one hand, GWAS efforts have focused on the identification of association of genetic variants with PBC, but not with specific properties of disease such as response of treatment [48]. As mentioned, the IL-12 pathway has been strongly implicated in the pathogenesis of PBC. The monoclonal antibody took the IL-12p40 subunit as the target and exerted its effect on both the IL-12/TH1 and IL-23/TH17 axes. While the monoclonal antibody has demonstrated therapeutic benefit in patients with Crohn's disease and psoriasis, none of the patients achieved the predefined primary endpoint of alkaline phosphatase reduction from baseline [10, 49]. Although, the data of *CTLA4* polymorphism and the association between *CTLA4* and PBC were not reported in the GWAS of PBC, *CTLA4* was the main focus of PBC in many candidate gene studies, and certain benefit results were obtained as a therapeutic target from *CTLA4*. CTLA4-Ig has been developed as an exciting outcome in mouse model of PBC [11, 12]. Based on these studies, a new clinical study has been set to determine the effect of abatacept in PBC patients who have no response to UDCA (NCT02078882).

On the other hand, IL-12 signal pathway may be an important role for PBC through Th1/2 differentiation among these loci from GWAS, but *CTLA4* could also impact Th1/2. Indeed, *CTLA4*-deficient mice and T cells were shown to be strongly trend a Th2 phenotype [50]. This is the control of Th1/Th2 differentiation, which was shown to depend both on the cytokine microenvironment and costimulatory

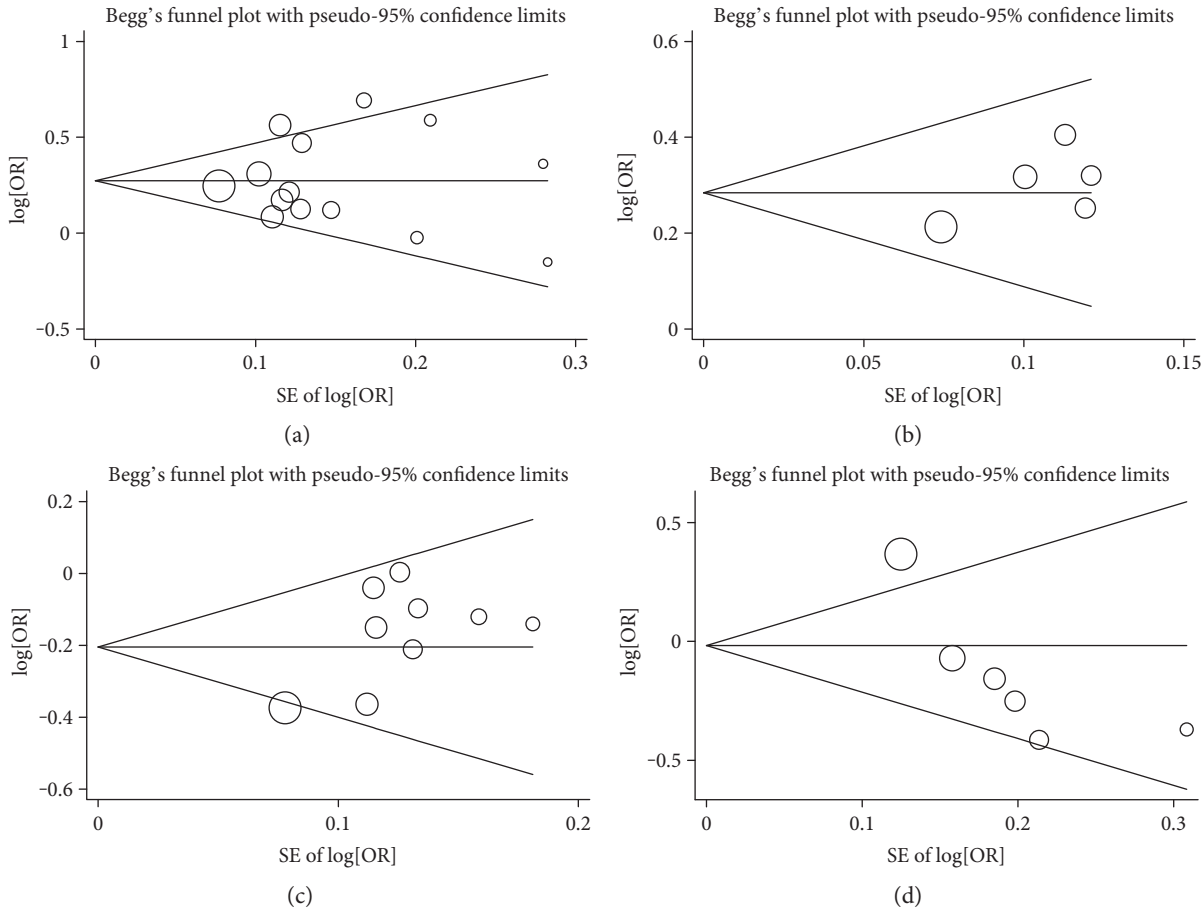

Figure 6: Publication bias for the influence of *CTLA4* and PBC: (a) rs231755 (G versus A), (b) rs231725 (A versus G), (c) rs3087243 (A versus G), and (d) rs5742909 (T versus C).

signals [51]. It is evidenced that some gene loci could be potential risk for PBC in GWAS, but these findings still have not translated into clinic. Although, the polymorphism of *CTLA4* could not be improved in GWAS, as to biliary cell damages in PBC, *CTLA4* could influence the effect of these inflammatory cytokines, such as IL-12, and IL-23 [52].

In view of the publication of the GWAS of PBC, our meta-analysis might be quite basic. However, we have collected sufficient documentations that have ever been published and analyzed four SNPs of *CTLA4* that have ever been reported in the publication of candidate gene studies. It should be said that this study was a more comprehensive meta-analysis of association between *CTLA4* and PBC. Our findings might illustrate that relevant research could still be gained from the candidate gene investigation.

In this meta-analysis, there were still some limitations to be existed. First, except race, there were other factors to be concerned, which included age, gender, and alcohol habit. It would be useful to understand that different risk factors might interact with the development of PBC as genetic variations. Secondly, some studies suggested that patients in PBC would also suffer other autoimmune diseases. Since, these literatures which we included did not mention whether other diseases were excluded in those patients, these situations may introduce errors during analyzing.

To sum up, this meta-analysis showed that the GG, GA genotype, and G allele of rs231775 and AA, GA genotype, and A allele of rs231725 in *CTLA4* may be risk factors for PBC in Asians and Caucasians. AA, GA genotype, and A allele of rs3087243 may be negatively associated with PBC in overall populations, especially in Asian. There was no significant connection with PBC in rs5742909 of *CTLA4*. Not only the impact on cytokine regulation but also the benefit results as therapeutic target, to some extent, *CTLA4* still plays a role which could not be completely ignored in PBC.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Genetics and Molecular Biology of Epstein-Barr Virus-Encoded BART MicroRNA: A Paradigm for Viral Modulation of Host Immune Response Genes and Genome Stability

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Epstein-Barr virus, a ubiquitous human herpesvirus, is associated through epidemiologic evidence with common autoimmune syndromes and cancers. However, specific genetic mechanisms of pathogenesis have been difficult to identify. In this review, the author summarizes evidence that recently discovered noncoding RNAs termed microRNA encoded by Epstein-Barr virus BARF (BamHI A right frame) termed BART (BamHI A right transcripts) are modulators of human immune response genes and genome stability in infected and bystander cells. BART expression is apparently regulated by complex feedback loops with the host immune response regulatory NF-κB transcription factors. EBV-encoded BZLF-1 (ZEBRA) protein could also regulate BART since ZEBRA contains a terminal region similar to ankyrin proteins such as IκBα that regulate host NF-κB. BALF-2 (BamHI A left frame transcript), a viral homologue of the immunoglobulin and T cell receptor gene recombinase RAG-1 (recombination-activating gene-1), may also be coregulated with BART since BALF-2 regulatory sequences are located near the BART locus. Viral-encoded microRNA and viral mRNA transferred to bystander cells through vesicles, defective viral particles, or other mechanisms suggest a new paradigm in which bystander or hit-and-run mechanisms enable the virus to transiently or chronically alter human immune response genes as well as the stability of the human genome.

1. Introduction: Overview of EBV Pathogenesis in Autoimmune Diseases

EBV (Epstein-Barr virus or human herpes virus 4), a ubiquitous human herpesvirus, is associated through epidemiologic evidence with common autoimmune syndromes and cancers suggesting that antiviral therapies or vaccines may be a therapy option [1]. Although specific molecular genetic mechanisms of pathogenesis have been difficult to identify, EBV and other common human viral pathogens such as herpes viruses and retroviruses are never cleared from the host and instead enter a latent state which may contribute to viral immunopathology. EBV exhibits complex regulatory interaction with the host genome during latency in part because the virus contains a phenocopy of the host immunoglobulin gene locus and immune response regulatory transcription factors [2–4]. Because of these shared immune response genes, changes in the microbiome, and other environmental factors, the host immune response to EBV and other herpes viruses may be evolving unpredictably with the host immune response (Figure 1).

Although most research on EBV has focused on EBV-infected B lymphocytes which harbor the latent viral genome, in fact, much of the immunopathology related to viral infection may occur due to effects on bystander cells through a variety of mechanisms including viral-encoded microRNA [5, 6]. The author previously suggested a paradigm of "gene sharing" between viral- and host-encoded immune-regulatory genes including viral-encoded cytokines or "virokines," viral mRNA and microRNA, and human endogenous retroviruses (HERV) which may play a role in immune dysregulation in autoimmune diseases [2]. The author has also previously suggested that EBV may also potentially affect the biology bystander cells such as T lymphocytes through a variety of mechanisms in autoimmune syndromes such as systemic lupus erythematosus (SLE) which are related to EBV

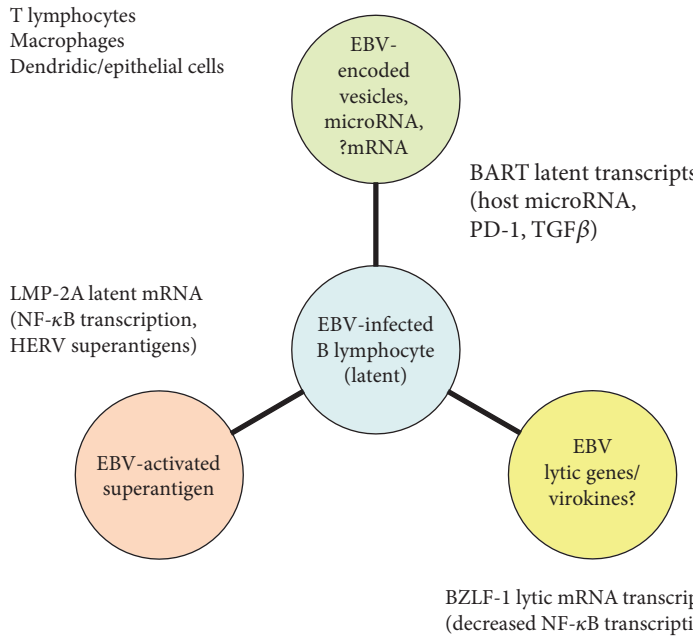

Figure 1: EBV viral genome expression (center) potential for effects on bystander cells of the human immune response are summarized. EBV-encoded BART microRNA may suppress the host immune response and destabilize host genomes in bystander cells through hit-and-run mechanisms during viral latency (top). EBV may also alter the immune response in bystander cells through induced expression of a HERV (human endogenous retrovirus) superantigen by the viral LMP2A protein also expressed during viral latency (left). Induction of viral lytic gene expression by the viral ZEBRA protein may also affect bystander cells through transient expression of viral mRNA as well as viral-encoded microRNA and viral recombinases and other factors altering host genome stability.

infection based on epidemiologic evidence [7]. Since numerous recent publications confirm that viral-encoded microRNA have a complex relationship with host-encoded immunoregulatory microRNA [8–16], and viral-encoded microRNA are themselves apparently coregulated by host and viral immune response transcription factors [17–26], it is reasonable to include viral and host microRNA in the "shared gene paradigm."

This review is focused upon current peer-reviewed literature regarding the genetics and molecular biology of recently discovered noncoding RNAs encoded by Epstein-Barr virus BARF (BamHI A right frame) termed BART (BamHI A right transcripts) which are suggested as a paradigm for further studies of virus-host interaction. The author will not attempt to review all of the known effects of EBV noncoding RNA, reviewed elsewhere, but instead focus on a specific well-characterized region of the EBV genome, the BamHI A fragment, as a potent source of dysregulation both of host and of bystander cells through as yet poorly understood mechanisms. A review of literature regarding expression of the EBV BamHI A and studies of lytic gene expression of the fragment performed by the author are also included.

A few points regarding EBV genome and nomenclature of the BamHI A fragment may be useful [27]. The virus has a large double-stranded DNA genome of approximately 180 kb with significant differences in substrains due to recombination of internally repeated regions, deletions, and recombinations relative to the canonical strain denoted "B95-8." Most EBV genome fragments are transcribed bidirectionally arbitrarily denoted "left" and "right." The large DNA EBV genome was originally digested with BamHI restriction endonuclease to generate a ladder of DNA fragments visible on electrophoresis for mapping studies, with the largest fragment denoted "A" and the smallest "Z," and this convention has been retained despite more advanced sequencing studies of the viral genome. The author has attempted to include most relevant peer-reviewed published studies available through online search of "PubMed" over the past 2 decades and regrets if inadvertently some references may have been omitted.

2. Effects of EBV-Encoded BART MicroRNA on EBV-Infected Cells

The author has chosen to focus upon a particular class of viral-encoded microRNA termed BART because these transcripts are not only expressed in latently infected cells but also found in noninfected cells as will be discussed later in more detail [5, 6]. EBV RNA transcripts from the EBV BamHI fragment termed BART (BamHI right transcripts) and other EBV noncoding RNAs were identified several decades ago and suggested to have regulatory effects on cells prior to the discovery of the host RISC (RNA interference-specific complex). RISC, a member of the DDE magnesium binding nuclease family, was identified as a site-specific nuclease that uses short "hairpin loop" structures in guide sequences termed microRNA to regulate multiple host cell mRNA transcripts. More recent studies confirm that BART form RISC-associated "hairpin loops" that play complex roles in cellular growth and development through inactivation and

Table 1: Summary of EBV gene products related to microRNA gene regulation. EBV gene products modulate levels of host-encoded factors shared with immune response regulatory factors such as NF-κB. Most EBV-encoded BART microRNAs are upregulated by NF-κB during viral latency and in turn increase stability of a large number of cellular transcripts that play a role in both resistance of cells to apoptosis and also evasion of the host immune response. EBV-encoded BZLF-1 protein (ZEBRA) downregulates NF-κB and promotes viral lytic growth and host cell apoptosis. Some BART which are homologues of host miR155 may counterregulate other BART and decrease NF-κB. ZEBRA-regulated factors may also upregulate other viral host gene products associated with genome instability (see text).

Transcript	+/−	Effects
BART (1, 2, 3...) microRNA (viral)	NF-κB	Resistance to apoptosis, immune surveillance, host genome instability
LMP2A protein	NF-κB	Resistance to apoptosis, immune surveillance, increased HERV expression
BZLF1 protein (ZEBRA)	NF-κB AP-1 Sp1 CREB	Increased apoptosis, viral replication proteins, viral replication Host genome instability?
BART5-5p miR155 (viral and host)	NF-κB?	Feedback regulation of BART, NF-κB?

cleavage of target mRNA by RISC-associated host microRNA homologous sequences in 3′ regulatory regions of the target mRNA [15].

Extensive studies of EBV BART have utilized immunoprecipitation of the RISC and microsequencing of RISC-associated transcripts including BART and host microRNA [15]. These studies have the advantage of providing a complete picture of the viral BART microRNA transcriptome and related host microRNA that may compete for binding sites both at the level of RISC binding and also at the level of binding to host mRNA 3′ regulatory regions. Specific host cell survival factors targeted by BART include the wnt transcription factors, cell cycle growth regulators, and host microRNAs such as miR155. miR155 and related BART have been suggested to be a part of a feedback loop in which most BART are associated with resistance of host cells to apoptosis, but some are opposing and promote apoptosis [28]. Effects of the host transcription factor denoted "NF-κB" which is modulated both by ZEBRA and by other viral latency-associated proteins and may function as a switch coordinated between host and viral genome and between viral lytic and latent growth are summarized (Table 1). NF-κB is also related to expression of human endogenous retroviruses (HERV) and HERV-associated superantigens that in turn may affect cell growth and effects on bystander cells.

As shown in Table 1, significant feedback loops exist in the regulation of BART microRNA since some BART may have effects that oppose cell growth and apoptosis.

Perhaps not surprisingly, the consensus of these studies is that the host BART in general upregulate host pathways providing resistance to apoptosis of the B lymphocyte cell in which the latent viral genome resides [28]. These studies are consistent with the overall effects of EBV latency-associated transcripts and gene products in prolonging the life of the host cell, in contrast to the effects of viral lytic growth factors such as the viral-encoded transcription factor ZEBRA (also termed BZLF-1-encoded protein or ZTA) that promote apoptosis of the host cell to permit viral particle release from apoptotic cells [29–31]. Since the lytic switch protein ZEBRA is coupled to both repression of NF-κB and expression of other transcription sites such as fos/jun, SP1, and CREB, all of these transcription factors in the host response may in turn influence expression of BART.

3. EBV-Encoded MicroRNA: A New Paradigm for Viral Immunopathology?

As noted above, significant progress has occurred in the past decade in characterizing effects of EBV on host cell-encoded immunoregulatory microRNA [8–16] and also expression of viral-encoded noncoding and microRNA [17–26]. However, the author and others have previously suggested that much of the immunopathology associated with EBV infection may involve "hit-and-run" effects of EBV gene products transiently expressed in noninfected bystander cells [32]. For example, EBV latent genome products activate expression of endogenous retroviruses or HERV which encode a "superantigen" affecting the host immune response in noninfected lymphocytes and the EBV-encoded ZEBRA protein promotes apoptosis of T lymphocytes required for control of viral replication [1].

Similarly, EBV-encoded BART microRNA as well as other viral-encoded mRNAs could potentially affect bystander cells such as T lymphocytes, macrophages, and epithelial cells through a "hit-and-run" mechanism. BART may be transferred to bystander cells through defective viral particles or vesicles. Bystander cells may have receptors for EBV although these cells are not capable of supporting viral latency. Remarkably, in support of this hypothesis, recent studies of noncoding RNA including microRNA in common human ovarian cancer have recently identified EBV BART in ovarian cancer whole transcriptome sequencing studies [5, 6].

In ovarian cancer, as in other common human cancers, EBV genomes infected cells that are present in malignant tissues but not in all malignant cells. Thus, BART should be included in the effects of EBV on bystander cells affecting the immune response. If in fact EBV-encoded BART can affect expression of other mRNAs in both EBV-infected cells and bystander cells contributing to pathology in common human autoimmune syndromes and cancers, a better understanding of host factors that regulate viral BART may be important, and transcriptional regulation of the EBV BamHI fragment will be the subject of the remainder of this review.

In addition to regulation by NF-κB during viral latency outlined above, the BamHI A fragment encoding BART

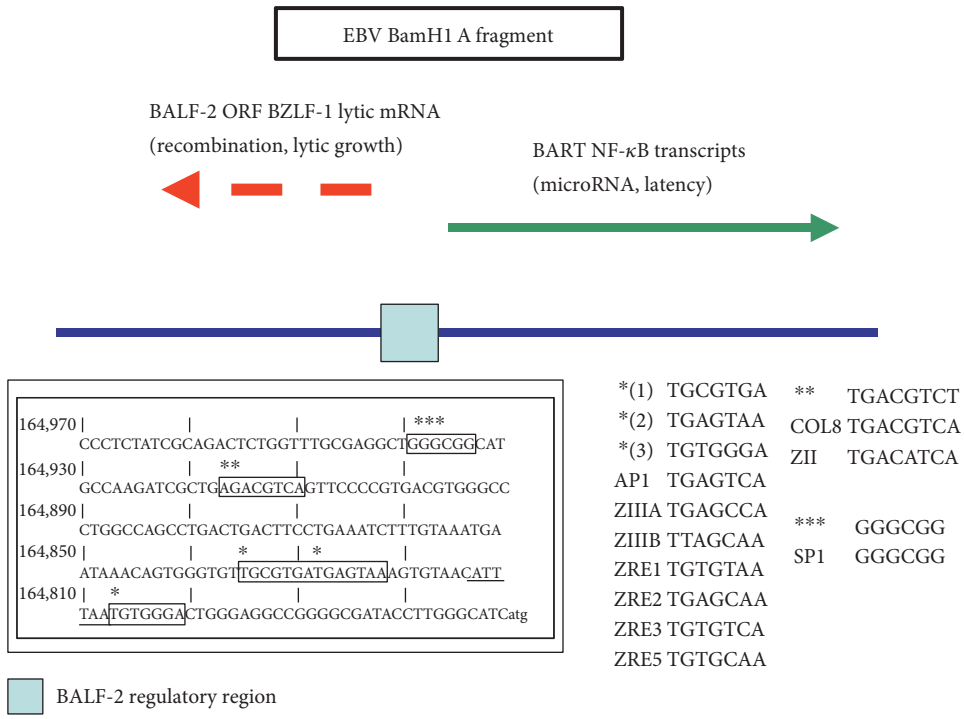

Figure 2: A region within the EBV BART microRNA encoding BamHI A fragment contains regulatory sequences for multiple EBV- and host-encoded immune response transcription factors. EBV genome sequences 164,810 to 164,970 are shown in inverted orientation, with sites for BART also indicated. Sites resembling consensus response elements for CREB, AP-1, and SP1 and a putative TATA box have been identified in the EBV BamHI fragment also encoding BART. AP-1 sites resemble binding sites for host fos/jun factors are similar to sites recognized by the EBV-encoded BZLF-1 (ZEBRA) transcription factor (see text).

may also be regulated by viral and host immune response transcription factors during EBV lytic growth (29–31). In contrast to BART which are transcribed in the direction arbitrarily termed "right" during lytic growth expression of the BamHI A fragment is also transcribed to the "left." Whether left and right transcription of the BamHI A fragment can occur in both directions simultaneously has not been determined since most studies of transcription have focused on latently infected lymphoblastoid cell lines that are stable in in vitro culture rather than in vivo or in primary infection of B lymphocytes in vitro [33, 34]. However, some detailed information is available regarding the transcription of the BALF-2; a left-transcribed open-reading frame adjacent to the BARF is available in peer-reviewed studies [35].

4. Regulation of EBV-Encoded MicroRNA BamHI A Fragment by Host and Viral Immune Response Transcription Factors

Studies of the BALF-2 promoter are important both because of the importance of the BamHI A fragment in regulation of BART and also because the BALF-2 promoter may be coregulated with the human RAG-1 (recombination-activating gene-1) required for generation of the immune repertoire [36, 37]. As shown in Figure 2, a region of the EBV BART microRNA-encoding BamHI A fragment contains regulatory sequences for multiple EBV- and host-encoded immune response transcription factors.

In Figure 2, EBV genome sequences 164,810 to 164,970 are shown in inverted orientation, with sites for BART also indicated. Regions of the EBV BamHI A fragment are located within approximately 10 kb of the BARF genome region of EBV-encoding BART which are transcribed in the opposite direction relative to the BALF-2 mRNA; although because B95-8 has a deletion near the BART locus, exact coordinates between the two strains are not shown. Sites resembling consensus response elements for CREB, AP-1, and SP1 and a putative TATA box have been identified in the EBV BamHI fragment also encoding BART. AP-1 sites resemble binding sites for host fos/jun factors are similar to sites recognized by the EBV-encoded BZLF-1 (ZEBRA) transcription factor.

In previously unpublished studies by the author, the promoter region of BALF-2, a 5′ region of the BamHI A fragment adjacent to the BALF-2 mRNA start site, has been cloned from B95-8 viral DNA into a firefly luciferase expression vector (PGL-3 (Promega), reporter vector denoted BAP52/32 BLUC), and sequences have been shown to be identical to those published for B95-8. Results of transient transfection of the putative BALF-2 minimal promoter into 3 EBV positive B lymphoblastoid cell lines (Akata, Namalwa, and Raji) are shown in Figure 3. Results shown are a representative of multiple unpublished preliminary results obtained by the author and are similar to published results using a similar region of the BALF-2 promoter in vivo. Results are expressed as the ratio of luciferase activity of BAP52/32 BLUC to control plasmid PGL3-B (denoted stimulation index SI).

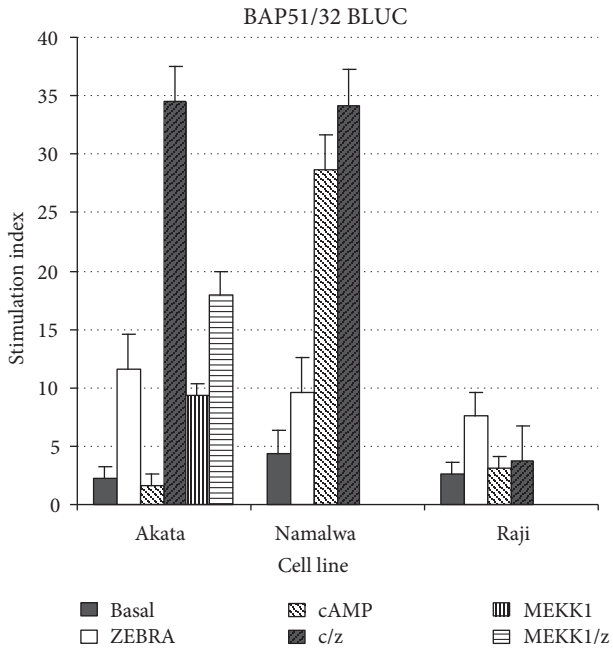

Figure 3: Detection of host immune response transcription factors including AP-1 binding fos/jun induced by cotransfection of cell cycle inducer MEKK1 and viral ZEBRA switch protein are shown in three EBV positive lymphoblastoid cell lines. The promoter region of BALF-2, a 5′ region of the BamHI A fragment adjacent to the BALF-2 mRNA start site, has been cloned from B95-8 viral DNA into a firefly luciferase expression vector (PGL-3 (Promega), reporter vector denoted BAP52/32 BLUC), and sequences have been shown to be identical to those published for B95-8. Results of transient transfection of the putative BALF-2 minimal promoter into 3 EBV positive B lymphoblastoid cell lines (Akata, Namalwa, and Raji) are shown (see text).

As shown, the BALF-2 promoter region of the BamHI A fragment cloned in BAP51/32 BLUC is a functional lytic promoter with SI of 3–5 in unstimulated B lymphoblastoid. BAP51/32 BLUC responds to transient transfection of BZLF-1 with SI of approximately 10 in B lymphoblastoid cells. BAP51/32 BLUC responds variably to activation of the endogenous CREB system (stimulation with dibutyrl cAMP 1 mM). Variable response to CREB activation contrasts with similar responses to BZLF-1 cotransfection in EBV positive B lymphoblastoid cells for unknown reasons. BAP51/32 BLUC also responds to activation of the endogenous AP-1 system (3–5-fold increase in transcription) in B lymphoblastoid cells.

These results demonstrate that the BamHI fragment not only encodes BART microRNA primarily associated with viral latency in one direction (denoted right) but also that transcription of the same region in the opposite direction (denoted left) encodes mRNA for a viral recombinase required for viral lytic replication. An important as yet unanswered question is whether lytic transcripts of the viral BamHI fragment such as BALF-2 and coregulated host RAG-1 transcripts induced by EBV lytic replication are inversely regulated with BART or whether both may be upregulated and expressed in a coordinate fashion by transcription factors such as NF-κB and other host DNA-binding immune response regulatory factors. Another related question is whether BALF-2 mRNA could be coexpressed and packaged into defective viral particles or vesicles and exported to bystander cells contributing to both altered cell growth and genome instability.

5. Viral Modulation of Host and Bystander Cell Genome Stability by BART: Implications for Genome Stability

EBV infection is associated with genomic instability of latently infected cells through a variety of previously reported mechanisms [38–41]. Lymphocyte malignancy associated with EBV may also be related in part to expression of EBV noncoding RNA and also an unusual feature of the vertebrate immune system; generation of a large repertoire of T and B lymphocyte receptors through breakage and rejoining of DNA in lymphocytes is termed V(D)J recombination [42–45]. While a full discussion of the role of V(D)J recombination and associated RAG (recombination-activating genes) is beyond the scope of this review, EBV infection induces expression of RAG in lymphocytes through unknown pathways [46].

ZEBRA mRNA is detected in cell-free EBV viral particles (unpublished observations by the author) and mRNA for ZEBRA or other viral gene products could be transiently expressed in immune response cells through defective viral particles or other mechanisms. Detection of BART in cells that are not EBV infected also supports the possibility that mechanisms such as defective viral particles and vesicles may actively facilitate transfer of both noncoding and coding RNA to bystander cells.

Remarkably, all herpes viruses share a recombinase termed the herpes DBP (major DNA-binding protein) in the DDE recombinase superfamily with the human RAG-1 recombinase, represented in EBV by the BALF-2 gene product [36]. DBP such as the BALF-2 protein are structurally conserved between widely divergent herpes virus strains and are also absolutely required for lytic replication of known herpes viruses including EBV. These observations support the hypothesis that the DBP shares common regulatory elements (CREB, AP-1, and possibly SP1) with the RAG proteins, providing a plausible mechanism for the activation of RAG-1 transcription associated with EBV infection [46].

The complex relationship between herpes recombinases and their relationship to the RAG-1 gene required for V(D)J recombination and gene sharing between herpes microRNA, recombinases, herpes recombination signals, and V(D)J recombination could provide a link between autoimmune syndromes and malignancy. As discussed in this review, the BALF-2 locus located on the BamHI fragment may also share regulatory signals with the BART locus encoding microRNA that are transported to bystander cells through unknown mechanisms. Thus, it is possible that cotranscription of BALF-2 mRNA with BART microRNA could simultaneously alter the growth and genomic stability of both the host and bystander cells. Since EBV infection of lymphocytes could both alter the immune repertoire of host

Table 2: Central role of BART and host transcription factor NF-κB in regulation of host and EBV shared immune response genes (summary).

(i) EBV BARF (BamHI A right frame) encodes multiple viral microRNAs (BamHI A fragment right transcripts) that are important for viral latency and immune evasion.
(ii) Viral LMP-2A, host NF-κB(+) positive regulator of BART, and resistance to apoptosis reversed by viral ZEBRA (BZLF-1) lytic protein.
(iii) BART are transferred to bystander cells by unknown mechanisms, possibly vesicles or defective viral particles.
(iv) Other EBV mRNAs may be transferred with BART microRNA to bystander cells by unknown mechanisms.
(v) Potential for multiple viral regulatory interactions with host and bystanders cell microRNA and gene transcription networks is important in immune surveillance, cell growth, and genome stability.

and bystander cells and also contribute to genome stability of host and bystander cells through expression of both viral induced RAG and viral expressed BART, the author suggests that additional studies of EBV as "hit-and-run" carcinogens are indicated. These studies could also provide new insights into mechanisms of EBV infection as a cofactor in common autoimmune syndromes and suggest new antiviral therapies and vaccine strategies [1].

6. Conclusions and Future Research Directions

Gene sharing between human chronic viral pathogens such as EBV microRNA and host cell microRNA may play a prominent role in autoimmune disease and malignancy in the human host (gene sharing between EBV and host proteins; microRNA regulation summarized in Table 2). Simultaneous molecular mimicry response to "altered self" and viral alteration of the host IR (immune response) by EBV could explain why the IR to EBV cross-reactive antigens are not eventually suppressed by the host suppressor cells and cytokines and instead propagate an apparently endless series of autoimmune antibodies and immune dysfunction in various autoimmune syndromes. For example, the author has previously reviewed evidence that common autoimmune syndromes such as SLE (systemic lupus erythematosus), RA (rheumatoid arthritis), and MS (multiple sclerosis) may result from complex interactions between infectious viruses and genes shared with endogenous retroviruses and host genes leading to autoreactive IgG and more recently suggested that chronic idiopathic urticaria, a common cutaneous syndrome with autoimmune features, may also result from similar mechanisms resulting in both autoreactive IgG and possibly other classes of immunoglobulin such as autoreactive IgE [47, 48].

As noted in this review, EBV-encoded BART provide another example of viral genes that are functional homologues of host genes. Unfortunately, gene sharing as a paradigm cannot be studied completely in animal models since EBV and other human herpes viruses do not infect other species, although humanized murine models and other animal models such as rabbits that can become infected with latent EBV may be useful in confirming basic observations such as mechanisms of transfer of BART viral microRNA to host bystander immune cells. Instead, the author suggests that gene sharing between unique human pathogens such as EBV and the human host must be considered as complementary to animal models that consider only host genomic factors rather than viral pathogens and the metagenome. The author suggests that gene sharing between the host IR and viral proteins expressed by latent herpes viruses such as EBV may trigger chaotic behavior in human autoimmune disease through unstable feedback loops and perturbations of immune tolerance associated with human herpes virus reactivation and thus in part explain the failure of animal models to adequately predict response of common autoimmune disease to therapy.

The combined and interactive system of the host genome, the virome, and the microbiome has been collectively termed the "metagenome." As in astronomy, a system such as the human metagenome with more than two independent variables can become inherently unstable or chaotic. Thus, in actual human autoimmune disease, unpredictable and chaotic interactions between the host genome, the viral genome, or metagenome, and other factors such as the microbiome are evident and must be considered in disease pathology as well as response to disease-modifying agents.

Disclosure

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Conflicts of Interest

The author declares that he has no conflicts of interest.

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Research Article

Polymorphisms of IL12RB2 May Affect the Natural History of Primary Biliary Cholangitis: A Single Centre Study

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Background. Recent GWAS in primary biliary cholangitis (PBC) showed strong associations with SNPs located within interleukin-12 receptor (IL12R) beta-2 *(IL12RB2)* gene. *Aims*. We assessed whether genetic variation of *IL12RB2* is associated with laboratory and clinical features of PBC. *Methods*. Genomic DNA was isolated from 306 patients with PBC and 258 age/gender-matched controls. PBC-specific anti-mitochondrial antibodies (AMA) were tested in all subjects by ELISA. Two SNPs, rs3790567 and rs6679356, of *IL12RB2* were genotyped using the MGB-TaqMan SNP assay. *Results*. Despite comparable age at diagnosis of cirrhotic and noncirrhotic PBC patients, allele A of rs3790567 and allele C of rs6679356 were overrepresented in the former rather than the latter group ($p = 0.0009$ and $p = 0.002$, resp.). The risk of cirrhosis at presentation increased when allele A and allele C coexisted. AMA-M2 titres were significantly higher in AA homozygotes of rs3790567 compared to GG homozygotes (132 ± 54 versus 103 ± 62, $p = 0.02$) and in rs6679356 when C allele was present ($p = 0.038$). There were no other significant associations between *IL12RB2* polymorphisms and laboratory or clinical features. *Conclusion*. In this first study analyzing phenotypic features of PBC carriers of the *IL12RB2* polymorphisms, we found that carriers are more frequently cirrhotic at diagnosis and have significantly higher titres of AMA.

1. Introduction

Interleukin-12 (IL-12) belongs to the IL-12 family cytokines along with IL-23, IL-27, and IL-35. IL-12 is a heterodimer comprised of two subunits: a 35 kDa (p35) light chain encoded by *IL12A* gene and a 40 kDa heavy chain (p40), encoded by *IL12B* gene. The synthesis and secretion of IL-12 occur *via* stimulation of Toll-like receptors expressed by antigen presenting cells (APCs) in response to microbial constituents [1, 2]. IL-12 exerts its immunobiological action through its binding to specific IL-12 receptors (IL-12Rs), termed IL-12Rβ1 and IL-12Rβ2, which are mapped on chromosomes 19p13.1 and 1p31.2, respectively [1–3]. Secreted IL-12 targets naive T cells, NK cells, and NKT cells and promotes their proliferation and differentiation. The most significant function of IL-12 is its involvement in the differentiation of T-helper 1 from naive CD4+ T cells resulting in the secretion of the proinflammatory interferon-γ (IFN-γ) cytokine [1–4].

Primary biliary cholangitis (PBC), formerly known as primary biliary cirrhosis [5, 6], is an autoimmune cholestatic liver disease, affecting up to 1 in 1000 women over 40 years of age with complaints such as fatigue and/or pruritus [7–9].

The hallmark of PBC is the presence of high-titre antimitochondrial autoantibodies (AMA) and the accumulation of antigen-specific autoreactive B and (CD4+ and CD8+) T cells targeting biliary epithelial cells [10]. Studies on patients with PBC, experimental work on animal models, and in vitro studies indicate a potential role of the IL-12 pathway in the development of autoimmune cholangitis [11, 12]. There is evidence for a pathogenic role for IL-12B in experimental murine model of PBC. It has been demonstrated that deletion of the IL-12B on the transforming growth factor β receptor II dominant negative (dnTGFβRII) murine model of PBC results in the amelioration of cholangitis [13].

Genetic studies provided additional support in favour of the instrumental role of IL-12 in the immunopathogenesis of PBC. Genome wide association studies (GWAS) provided evidence that PBC displays a strong association with SNPs located within the *IL12A* as well as within *IL12RB2* gene [14, 15]. The involvement of the IL-12 pathway in PBC could be indirectly supported by associations found between PBC and several SNPs across the *STAT4* gene, which encodes an effector that is essential for interleukin-12 signaling [16]. To better understand the extent of involvement of IL-12 in PBC, we analyzed the potential association between two SNPs located in the IL-12Rβ2 gene and phenotypic features of PBC in a well-defined cohort of patients with PBC.

2. Methods

2.1. Patients and Controls. All patients with PBC fulfilled the diagnostic criteria established by the European Association for the Study of Liver (EASL) [17]. The study was performed in a homogenous group of Caucasian patients with PBC who were treated in two Polish liver transplant centres, in Szczecin and in Warsaw. Patients recruited at both sites did not differ in terms of demographic, clinical, and biochemical characteristics (data not shown). One hundred and seven (36.5%) subjects were diagnosed with liver cirrhosis, by either liver biopsy or imaging screening. Data on cirrhosis status were not available in 28 patients. Clinical and laboratory features of analyzed subjects are summarized in Table 1. Health-related quality of life (HRQoL) was assessed with disease-specific questionnaires (PBC-40 and PBC-27) [18]. PBC-specific AMA were tested by ELISA (Inova Diagnostics, San Diego, CA, USA).

Control group was comprised of 258 age- and gender-matched subjects without liver diseases. These controls were also tested for AMA using the same ELISA.

2.2. Genotyping. Genomic DNA from peripheral blood mononuclear cells was isolated using the DNeasy Blood & Tissue Kit (Qiagen). Oligonucleotide primers and TaqMan probes for IL-12Rβ2 polymorphisms (rs3790567, rs6679356) were designed and synthesized by Applied Biosystems. The fluorescence data were analyzed with allelic discrimination 7500 Software v.2.0.2.

2.3. Ethics. Appropriate informed consent was obtained from each patient included in the study. The study protocol was approved by the Ethics Committee of Pomeranian Medical University and Medical School of Warsaw. They conformed to the Ethical Guidelines of the 1975 Declaration of Helsinki (6th revision, 2008).

2.4. Statistical Analysis. Data are shown as means and standard deviations. All statistical analyses (chi-square, odds ratios, and confidence intervals) were carried out using StatView software (Cary, NC, USA). The genotype and allelic frequencies were compared between patients and controls using Fisher's PLSD test. The analysis of genotype frequency within PBC patients in regard to the clinical characteristics was performed using Fisher's PLSD test. The general genotype-dependent survivals were estimated using the Kaplan-Meier survival curves.

3. Results

3.1. Distribution of IL12RB2 Polymorphisms in Patients with PBC and Controls. The allele A of rs3790567 and allele C of rs6679356 were overrepresented in PBC patients compared to controls, 30.4% versus 20.7% ($p = 0.0009$) and 25% versus 17.2% ($p = 0.0022$), respectively, confirming a significant impact of *IL12RB2* polymorphisms on PBC susceptibility (Table 2).

3.2. Association of rs3790567 and rs6679356 with Liver Cirrhosis. The frequency distributions of the rs3790567 and rs6679356 genotype in cirrhotic and noncirrhotic patients are shown in Table 3. Allelic analysis demonstrated that allele A of rs3790567 and allele C of rs6679356 were overrepresented in patients with histological and/or clinical features of liver cirrhosis (38% versus 25%, $p = 0.001$ and 31% versus 20.8%, $p = 0.013$, resp.). Moreover, subjects who were GG homozygote of the rs3790567 were overrepresented in noncirrhotic group ($p = 0.003$), similarly to subjects who were TT homozygote of rs6679356 ($p = 0.009$). The risk of cirrhosis increased when A (rs3790567) allele and C (rs6679356) allele coexisted ($p = 0.007$).

3.3. The Association of rs3790567 and rs6679356 with Features of PBC. A positive association between *IL12RB2* polymorphisms and the PBC-specific AMA-M2 concentration was found in patients with PBC. The AA-carriers of rs3790567 were characterized by an increased concentration of AMA-M2 in comparison to GG-carriers ($p = 0.0231$) (Table 4). The association of rs6679356 genotypes with higher AMA-M2 concentrations has not been observed. However, higher serum AMA-M2 was associated with the presence of allele C ($p = 0.0384$). No other associations between polymorphism distribution and clinical or laboratory features of PBC were found.

4. Discussion

IL-12 activates through its receptor's subunits a cascade of signaling factors such as NFKβ and STAT4 to promote the production of proinflammatory Th1-type cytokines including

Table 1: Clinical and laboratory data in patients with primary biliary cholangitis (PBC).

	PBC	PBC		p value (cirrhotic versus noncirrhotic)
		Noncirrhotic	Cirrhotic	
Age (years)	53 ± 11	51 ± 9	53 ± 10	NS
Gender (female/male)	277/29	152/6	89/18	$p = 0.004$
Liver cirrhosis (yes/no/unknown)	107/158/41	158	107	—
Death or OLTx (yes/no/unknown)	66/214/26	17/133	42/61	$p < 0.001$
UDCA response (yes/no/unknown)	81/120/105	46/47	24/56	$p = 0.01$
ALP (IU/l; mean ± SD; normal: 30–120)	363 ± 313	149 ± 306	102 ± 339	NS
ALT (IU/l; mean ± SD; normal: 3–30)	104 ± 122	110 ± 126	97 ± 123	NS
AST (IU/l; mean ± SD; normal: 3–30)	95 ± 112	85 ± 104	105 ± 123	NS
γ-GT (IU/l; mean ± SD; normal: 3–30)	365 ± 417	360 ± 437	342 ± 380	NS
Bilirubin (mg/dl; mean ± SD; normal: 0.2–1.0)	3.1 ± 5.6	1.6 ± 2.9	5.2 ± 7.9	NS

Table 2: Distribution of *IL12RB2* polymorphisms (rs3790567, rs6679356) in PBC patients and control subjects.

SNP	Genotype	Count of alleles/genotypes		Fisher exact p value	χ^2	OR
		Control ($n = 258$) (%)	PBC ($n = 306$) (%)			
rs3790567	A/G	107/409 (20.7/79.3)	186/426 (30.4/69.6)	0.0009	11.5	1.7 [1.3–2.2]
	AA	11 (4.2)	29 (9.5)	0.02	5.8	2.3 [1.1–4.8]
	AG	85 (33)	128 (42)	0.02	4.7	1,5 [1.0–2.1]
	GG	162 (62.8)	149 (48.5)	0.0009	11.3	0.6 [0.4–0,8]
rs6679356	C/T	89/427 (17.2/82.8)	153/459 (25/75)	0.002	9.5	1.6 [1.2–2.1]
	CC	11 (4.2)	22 (7.2)	NS	2.2	1,7 [0.8–3.6]
	CT	67 (26)	109 (35.6)	0.01	6.1	1,6 [1.1–2.3]
	TT	180 (69.8)	175 (57.2)	0.03	5.1	0,6 [0.4–0.8]

TNFα and IFNγ, as well as to enhance the cytotoxic response of NK, NKT, and CD8+ T cells [19]. Genetic association data that have emerged from GWAS highlighted a relationship between the IL-12/STAT4/Th1 pathway and PBC [14, 15, 20, 21]. Two SNPs, namely, rs3790567 and rs6679356, in the intronic regions downstream of *IL12RB2* showed strong associations with PBC in a Canadian PBC cohort when compared to controls [20]. Another GWAS on Italian PBCs confirmed disease's association with rs3790567 [14].

Such GWAS are by nature unable to assess whether SNPs are associated with features of the disease, namely, presence of cirrhosis at diagnosis, fibrosis stage, liver biochemical indexes, and response to treatment or magnitude of autoantibody response. In the present study, we evaluated the association between *IL12RB2* polymorphisms and PBC in a cohort of Caucasian individuals with PBC and unaffected controls. As clinicians are more interested to know whether specific genetic markers are associated with features of the disease, we analyzed the potential association between rs3790567 and rs6679356 and clinical or laboratory features of the disease in our PBC cohort.

Our study, including a homogenous cohort of more than 300 Caucasian patients with PBC, confirmed the GWAS findings; the allele A of rs3790567 and allele C of rs6679356 are indeed overrepresented in PBC compared to disease-free controls. In view of the aforementioned associations, we considered our cohort an adequate population to further investigate the potential relationship between such polymorphisms and laboratory as well as clinical features of the disease.

In PBC, small and medium-size intrahepatic bile ducts are destroyed by an autoimmune attack which if left untreated could lead to liver cirrhosis in a proportion of patients with PBC. At times, patients present with cirrhosis and a diagnosis of PBC is made at presentation, based on laboratory, immunological, and clinical features compatible with the autoimmune cholestatic nature of the disease. Our allelic analysis demonstrated that the allele A of rs3790567 and the allele C of rs6679356 significantly increased risk of cirrhosis

Table 3: The *IL12RB2* polymorphisms (rs3790567, rs6679356) association with cirrhosis in PBC.

SNP	Genotype	Cirrhosis (n = 276)		Fisher exact p value	χ^2	OR
		No (%)	Yes (%)			
rs3790567	A/G	83 (25%)/247 (75%)	84 (38%)/138 (62%)	0.001	10.12	1.8 [1.3–2.6]
	AA	12 (7.3%)	16 (14.4%)	NS	3.71	2.1 [0.9–4.7]
	AG	59 (35.7%)	52 (46.9%)	NS	3.39	1.6 [0.9–2.6]
	GG	94 (57%)	43 (38.7%)	0.003	8.82	0.5 [0.3–0.8]
rs6679356	C/T	69 (20.8%)/263 (79.2%)	68 (31%)/152 (69%)	0.013	7.27	1.7 [1.1–2.5]
	CC	10 (6%)	12 (10.9%)	NS	2.15	1.9 [0.8–4.6]
	CT	49 (29.5%)	44 (40%)	NS	3.25	1.6 [0.9–2.6]
	TT	107 (64.5%)	54 (49.1%)	0.009	6.43	0.5 [0.3–0.9]

Table 4: The *IL12RB2* polymorphisms (rs3790567, rs6679356) association with M2-AMA concentration in patients with PBC.

SNP	Genotype	Count of genotypes (n = 295)	AMA level-mean [IU/l]	p
rs3790567	A/G	177/413		NS
	AA	27	132 ± 54	
	AG	123	107 ± 62	AA versus GG p = 0.023
	GG	145	103 ± 62	
rs6679356	C/T	147/443		0.0384
	CC	21	128 ± 60	CC versus TT NS
	CT	105	113 ± 60	
	TT	169	101 ± 62	

AMA: anti-mitochondrial antibody.

at the diagnosis. The IL-12 signaling pathway is a key player in the effector mechanisms that lead to biliary destruction and subsequent liver cirrhosis. IL-12Rβ2 is upregulated by IFNγ triggered by IL-12; thus in this case cytokine-receptor interaction provides a positive feedback loop further promoting biliary epithelial cell damage. Since IL-12-dependent, self-perpetuating autoimmune mechanism appears to be responsible for cholangiocyte destruction, it is reasonable to expect a more progressive pace of the disease in patients who carry specific *IL12RB2* polymorphisms.

The characteristic immunological hallmark of PBC is the presence of disease-specific M2-AMA in serum of affected patients [22]. More than 90% of PBC patients have detectable AMA, but their titres vary amongst affected individuals. Our data show that analyzed *IL12RB2* polymorphisms were associated with significantly higher AMA-M2 titres. IL-12 is secreted by activated APCs. It is likely that their activation occurs *via* release of E2 subunit mitochondrial pyruvate dehydrogenase autoantigen (PDC-E2) during apoptosis of biliary epithelial cells (BECs) [23, 24]. PDC-E2 is endocytosed by APC and this endocytosis leads to APC maturation and IL-12 secretion which provides the cytokine milieu which is essential for the maintenance of the continuum of proinflammatory T-helper type 1 (Th1) induction arising from naive CD4+ T through antigen presentation; such antigen-specific CD4 T cells help plasmocytes to produce high-titre AMA targeting PDC-E2. IL-12Rβ2 appears to be pivotal for the continuation of this deleterious positive feedback. Though our study is the first to identify *IL12RB2* polymorphisms positively associated with the magnitude of AMA response, this is not the first time that an association between *IL12RB2* and high-titre autoantibodies has been found in autoimmune diseases, providing a link between the IL-12/IL-12R axis and the scale of the autoimmune attack at the humoral level. This is not the first time that an association between autoantibody status and IL12-IL12R/STAT4 polymorphisms has been reported. In fact, Joshita et al. [16] have found an association between three STAT4 PBC risk alleles (rs7574865, rs8179673, and rs10181656) with ANA status (but not with AMA positivity) in Japanese patients with PBC; IL12R polymorphisms do not appear to be risk alleles in non-Caucasian patients, and in particular those originated from Japan, and this may explain why associations were not reported by the former study between AMA or ANA with IL12R alleles. In systemic lupus erythematosus, the presence of anti-dsDNA antibodies is also associated with rs7574865 within *STAT4*, further highlighting the close relationship between the immunogenetic make-up of the autoimmune disease and pathognomonic immunological features, such as disease-related autoantibodies [25].

We found no association between analyzed polymorphisms and liver biochemistry indexes; this is not unexpected as liver cirrhosis is a "burnt out" condition characterized by low aminotransferase levels. We have recently published that patients with PBC have impaired HRQoL, irrespectively of whether they are cirrhotic or not [18]; thus an association

between the analyzed polymorphisms and HRQoL measures depicted by the PBC-40 or PBC-27 questionnaires would be rather unlikely; this appears to be the case, as our analysis failed to identify significant associations.

In conclusion, we have confirmed the relationship of *IL12RB2* polymorphisms with PBC susceptibility. Moreover, we have provided evidence that *IL12RB2* polymorphisms are associated with liver cirrhosis and an increased concentration of disease-specific AMA in sera of PBC patients. Larger, multicentre studies are warranted to assess the diagnostic and prognostic significance of our findings in a more comprehensive manner.

Competing Interests

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

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Review Article

Genetic Contribution to the Pathogenesis of Primary Biliary Cholangitis

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Formerly termed primary biliary cirrhosis, primary biliary cholangitis (PBC) is a chronic and progressive cholestatic liver disease characterized by the presence of antimitochondrial antibodies. Ursodeoxycholic acid (UDCA) therapy is the most effective and approved treatment for PBC and leads to a favorable outcome in the vast majority of cases. Although the etiology of PBC has not yet been elucidated, human leukocyte antigen (HLA) class II alleles have been consistently associated with disease onset for decades. Individuals in different geographic regions of the world may have varying susceptibility alleles that reflect indigenous triggering antigens. In this review, we describe the influence of HLA alleles and other gene polymorphisms on PBC along with the results of genome-wide association studies (GWAS) on this disease.

1. Introduction

Primary biliary cholangitis (PBC), formerly known as primary biliary cirrhosis [1, 2], is a liver-specific autoimmune disease characterized by female preponderance and destruction of intrahepatic bile ducts that often results in cirrhosis and hepatic failure [3–5]. The prevalence of PBC ranges from 20 to 40 cases per 100,000 persons [4–6], although the number of patients with PBC, specifically asymptomatic PBC, is on the rise due mainly to increased awareness and earlier detection by disease-specific antimitochondrial antibodies (AMAs). Ursodeoxycholic acid (UDCA) therapy is the most effective treatment for PBC and is recommended by most guidelines [7, 8]. The vast majority of patients with PBC show a favorable response to UDCA treatment despite some cases of disease progression via unknown mechanisms [9, 10]. Genetic factors are considered to play a prominent role in disease onset as higher concordance rates in monozygotic twins than in dizygotic twins and familial clustering of patients with PBC has been demonstrated in family and population studies [11–16]. However, the etiology of this disease has yet to be conclusively clarified; PBC is presumed to be a multifactorial polygenic condition caused by allelic triggers and environmental factors in genetically susceptible individuals, although epigenetic mechanisms, such as instability of X chromosome gene expression, may also participate in the disease's female predominance [17–19].

In the present article, we summarize the literature on human leukocyte antigen (HLA) involvement in PBC onset and GWAS findings from North American, European, and Japanese populations to explore the disease pathways of PBC pathogenesis.

2. Associations between HLA and PBC Susceptibility

Many significant susceptibility single nucleotide polymorphisms (SNPs), such as *CTLA4*, *TNF-a*, *STAT4*, *PTPN22*, and *VDR,* have been identified using candidate gene methods [20–24]. Among them, however, only *HLA* has consistently been associated with PBC in distinct patient cohorts across ethnicities.

Located on the most gene-dense genomic region on chromosomal position 6p21 [25], HLA genes are extremely polymorphic and play an essential role in numerous biologically and medically relevant processes. The products of the

Table 1: HLA haplotype associations with PBC.

Study	Population	HLA allele	p value	OR (95% CI)
Susceptibility				
Umemura et al. [26]	Japanese	DRB1*08:03-DQB1*06:01	0.000025	2.22 (1.53–3.20)
		DRB1*04:05-DQB1*04:01	0.044	1.38 (10.2–1.87)
Zhao et al. [27]	Chinese	DRB1*08:03-DQB1*06:01	<0.0001	3.17 (1.91–5.23)
		DRB1*07:01-DQB1*02:02	0.005	1.85 (1.20–2.83)
Donaldson et al. [28]	UK	DRB1*08:01-DQA1*04*01-DQB1*04:02	0.0027	2.9
	Italian	DRB1*08:01-DQA1*04*01-DQB1*04:02	0.0086	3.41
Protective				
Umemura et al. [26]	Japanese	DRB1*13:02-DQB1*06:04	0.00093	0.27 (0.12–0.60)
		DRB1*11:01-DQB1*03:01	0.03	0.37 (0.15–0.88)
Zhao et al. [27]	Chinese	DRB1*12:02-DQB1*03:01	0.015	0.43 (0.22–0.86)
Donaldson et al. [28]	UK	DRB1*11:01-DQA1*05:01-DQB1*03:01	0.086	0.47
	Italian	DRB1*13:01-DQA1*01:03-DQB1*06:03	0.0041	0.28

classical HLA class I (*A, B,* and *C*) and class II (*DR, DQ,* and *DP*) genes include cell-surface glycoproteins involved in the binding and presentation of self- or non-self-peptides to T-cell receptors (TCRs). Class I molecules present endogenous peptides derived from viruses to $CD8^+$ cytotoxic T cells, while class II molecules present processed peptides from exogenous pathogens to $CD4^+$ helper T cells. The extent of endogenous and exogenous peptide binding to HLA molecules depends on allelic polymorphisms. Additionally, both HLA class I and II molecules have functional roles in protein interactions, transcription regulation involved in the inflammatory response, and natural killer cell-cytokine interactions as part of innate immunity.

HLA polymorphisms have been extensively studied in immune-mediated diseases, revealing associations of particular alleles with ankylosing spondylitis (AS), Behçet's disease (BD), psoriasis, multiple sclerosis (MS), insulin-dependent diabetes mellitus (IDDM), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), rheumatoid arthritis (RA), narcolepsy, autoimmune hepatitis (AIH), and autoimmune pancreatitis (AIP) among others. Early investigations on associations between HLA polymorphisms and PBC were carried out more than a quarter-century ago [37]. Based on these findings, subsequent cumulative studies have provided evidence that PBC is associated with *DRB1*08* as predisposing and *DRB1*11* and *DRB1*13* as protective alleles [28, 38]. Li et al. conducted a meta-analysis to assess for relationships between HLA class II and disease susceptibility to PBC and demonstrated that *HLA DR*07* and **08* alleles were risk factors for PBC in certain populations, whereas *DR*11*, **12*, **13,* and **15* alleles were protective factors [39].

Several key reports [26–28] on the association between HLA haplotype and PBC susceptibility or resistance are summarized in Table 1. *HLA DR*08* alleles caused disease susceptibility, while *HLA DRB1*13* and **11* alleles conferred disease protection in haplotype analyses across ethnicities. Both protective *DRB1*11* and *DRB1*13* alleles have also been implicated *DRB1*11* against hepatitis C [40], human papilloma [41], and human immunodeficiency [42] and *DRB1*13* against hepatitis C [43], human papilloma [44], and human immunodeficiency [45] viruses along with malaria [44]. Thus, one of the pathogenic mechanisms in PBC may be bacterial infection as these protective HLA class II alleles play a functional role in blocking the invasion of infectious agents.

However, individuals harboring the above haplotypes constitute only a minority of patients with PBC, suggesting that other candidate genes and environmental cues evoke PBC pathogenesis. Umemura et al. [26] reported the possibility that the distribution of DRB1 amino acid residues encoded by different HLA *DRB1* alleles influenced the binding affinity to antigens, which might also be a predominant factor in PBC susceptibility.

3. GWAS on PBC

There have been extensive GWAS in patients with PBC, a number of which documenting significant associations with disease risk. To date, five GWAS [29–33], two Illumina immunochip studies [34, 35], and one genome-wide meta-analysis (GWMA) [36] on PBC have been performed on well-characterized cohorts in North American, European, and Japanese populations (Table 2). These investigations clarified that the HLA class II domain possessed the strongest association with disease susceptibility, particularly at the *HLA-DRB1*, *HLA-DQA1*, and *HLA-DQB1* loci. However, HLA alone does not explain the entire genetic predisposition to PBC, mainly since 80–90% of patients with the disease do not carry the most common HLA susceptibility alleles. In this regard, other genes apart from HLA loci are suggested to contribute to disease development. At present, GWAS have identified 39 non-HLA loci predisposing to PBC at a genome-wide level of significance (Table 3).

The first GWAS [29] in a North American cohort identified a significant association of PBC with genetic variants at *IL12A*, encoding IL-12 p35, and *IL12RB2,* encoding IL-12 receptor $\beta 2$. Modest ($p < 5.0 \times 10^{-5}$) genome-wide associations with disease risk for SNPs at the *signal transducer*

Table 2: GWAS on PBC.

Study	Year	Platform	Patients	Controls
Hirschfield et al. [29]	2009	Illumina HumanHap 370	1,031	2,713
Hirschfield et al. [30]	2010	Illumina HumanHap 370	1,351	4,700
Liu et al. [31]	2010	Illumina 610K	945	4,651
Mells et al. [32]	2011	Illumina 660W-Quad	1,840	5,163
Nakamura et al. [33]	2012	Affymetrix Axiom	1,274	1,091
Juran et al. [34]	2012	Immunochip	2,426	5,731
Liu et al. [35]	2012	Immunochip	2,861	8,514
Cordell et al. [36]	2015	GWMA	2,764	10,475

Table 3: Non-HLA risk loci identified through GWAS as associated with PBC at the genome-wide level of significance.

Chromosome	Locus	Study [reference #]	SNP	OR	p value	Candidate gene(s)	Disease(s) with shared risk loci
1	1p31	[35]	rs72678531	1.61	$2.47E-38$	*IL12RB2*	BD
	1p36	[30]	rs3748816	1.33	$3.15E-08$	*MMEL1*	MS
	1q31	[32]	rs12134279	1.34	$2.06E-14$	*DENND1B*	CD
2	2q12	[36]	rs12712133	1.14	$5.19E-09$	*IL1RL1, IL1RL2,*	
	2q12	[34]	rs10186746	1.21	$2.40E-05$	*IL1RL1, IL1RL2,*	
	2q32	[32]	rs10931468	1.50	$2.35E-19$	*STAT4, STAT1*	RA, SLE, Sjögren's, IBD, SSc, BD
	2q36	[36]	rs4973341	1.22	$2.34E-10$	*CCL20*	
3	3p24	[32]	rs1372072	1.20	$2.28E-08$	*PLCL2*	RA
	3q13	[35]	rs2293370	1.39	$6.84E-16$	*CD80*	MS, SLE, Celiac
	3q25	[35]	rs2366643	1.35	$3.92E-22$	*IL12A*	Celiac
4	4p16	[36]	rs11724804	1.22	$9.01E-12$	*DGKQ*	
	4q24	[32]	rs7665090	1.26	$8.48E-14$	*NFKB1*	UC
5	5p13	[35]	rs6871748	1.30	$2.26E-13$	*IL7R*	MS, UC
	5q21	[36]	rs526231	1.15	$1.14E-08$	*C5orf30*	
	5q33	[36]	rs2546890	1.15	$1.06E-10$	*IL12B, LOC285626*	
6	6q23	[36]	rs6933404	1.18	$1.27E-10$	*OLIG3, TNFAIP3*	
	6q23	[34]	rs6920220	1.29	$1.17E-06$	*OLIG3, TNFAIP3*	
7	7p14	[32]	rs6974491	1.25	$4.44E-08$	*ELMO1*	RA, Celiac
	7q32	[35]	rs35188261	1.52	$6.52E-22$	*IRF5*	RA, SLE, SSc, UC
8	8q24	[34]	rs2608029	1.23	$3.14E-06$	*PVT1, GSDMC*	
9	9p32	[33]	rs4979462	1.57	$1.85E-14$	*TNFSF15*	UC, CD
11	11q13	[32]	rs538147	1.23	$2.06E-10$	*RPS6KA4*	IBD
	11q13	[34]	rs10898201	1.31	$4.91E-06$	*NADSYN1*	
	11q23	[33]	rs4938534	1.38	$3.27E-08$	*POU2AF1*	
	11q23	[35]	rs80065107	1.39	$7.20E-16$	*CXCR5, DDX6*	RA, IBD, Celiac
12	12p13	[35]	rs1800693	1.27	$1.18E-14$	*TNFRSF1A, LTBR*	MS
	12q24	[35]	rs11065979	1.20	$2.87E-09$	*SH2B3*	RA, T1DM, Hyperthyroidism, Celiac
	12q24	[34]	rs7309325	1.26	$2.54E-05$	*SH2B3*	RA, T1DM, Hyperthyroidism, Celiac
13	13q14	[34, 35]	rs3862738	1.33	$2.18E-08$	*TNFSF11*	CD
14	14q24	[35]	rs911263	1.26	$9.95E-11$	*RAD51B*	RA
	14q32	[32]	rs8017161	1.22	$2.61E-13$	*TNFAIP2*	
16	16p13	[35]	rs12708715	1.29	$2.19E-13$	*CLEC16A, SOCS1*	MS, UC, T1DM
	16q24	[32]	rs11117432	1.31	$4.66E-11$	*IRF8*	MS, IBD, RA, SSc
17	17q12	[35]	rs17564829	1.26	$6.05E-14$	*IKZF3*	UC, CD, RA, T1DM
	17q21	[35]	rs17564829	1.25	$2.15E-09$	*MAPT*	
19	19p12	[35]	rs34536443	1.91	$1.23E-12$	*TYK2*	IBD, RA, SLE, psoriasis, T1DM
	19p13	[34]	rs73003205	1.35	$1.43E-05$	*KIAA1683*	
	19q13	[31]	rs3745516	1.46	$7.97E-11$	*SPIB*	
22	22q13	[35]	rs2267407	1.29	$1.29E-13$	*SYNGR1*	

CD, Crohn's disease; UC, ulcerative colitis; T1DM, type 1 diabetes mellitus.

and activator of transcription 4 (STAT4) and *cytotoxic T-lymphocyte-associated protein 4 (CTLA4)* loci were found as well. The second GWAS [31] confirmed the existence of additional risk loci, including *interferon regulatory factor 5 (IRF5)*, *transportin 3 (TNPO3)*, and *SPIB* encoding a transcription factor involved in B-cell receptor signaling and T-cell lineage decisions. A subsequent noteworthy GWAS from Japan showed that the *IL12A* and *IL12RB2* loci were not significantly associated with PBC, but rather that the *TNFSF15* and *POU2AF1* genes constituted novel risk loci in Japanese patients with PBC along with other non-HLA loci, including *IL7R*, *IKZF3*, *CD80*, *STAT4*, and *NFKB1*. This discrepancy among ethnicities indicated important differences in the pathogenesis of PBC despite several common key molecules and pathways, such as the IL-12 pathway to induce Th1 polarization of $CD4^+$ T cells. Our body of evidence suggests that there may be an inherited abnormality in immune regulation during PBC onset and perhaps an inability to suppress inflammatory attacks on small bile ducts once initiated.

It should be noted that Juran et al. identified risk-conferring epistatic interactions between *IL12RB2* and *IRF5* loci [34] as well as between *CTLA4* and *TNFα* loci in the pre-GWAS era [46]. Epistatic interactions between genes revealed by GWAS in the pathogenesis of PBC should be explored in future studies.

While gene associations are of considerable interest in the pathogenesis of PBC, virtually none have been translated into useful clinical testing. For instance, the importance of the IL-12 pathway in PBC onset has been highlighted in animal models and in the case of a child with a congenital IL-12 deficiency who developed PBC [47]. Although antibodies or drugs targeting the IL-12 pathway would seem to be effective, clinical trials using ustekinumab, a human monoclonal antibody directed against IL-12 and IL-23, have failed to produce effects in phase II trials [48]. One reason explaining the discrepancy between GWAS results and clinical testing may be that clinicians typically encounter patients who have already become complicated with cholestasis; in fact, the immunological destruction of cholangiocytes occurs in the very early stages of PBC. Thus, the mechanisms of disease progression should also be addressed to halt the deterioration of disease status and afford PBC patients an improved prognosis.

Lastly, it is particularly interesting that many genes implicated in PBC pathogenesis by GWAS have also been reported in other autoimmune diseases, such as SLE, systemic sclerosis (SSc), and Sjögren's syndrome (Table 3), suggesting a genetic overlap. Understanding the mechanisms involved in the onset and progression of certain autoimmune diseases may accordingly shed light on those in PBC.

4. Conclusions and Future Directions

The pathogenesis of PBC is incompletely understood but appears to involve genetic susceptibility and resistance alleles in HLA and other gene loci, with a possible overlap with several autoimmune diseases. It is also probable that genetically susceptible individuals develop PBC following environmental cues, leading to both adaptive and innate immune responses that result in portal inflammation and bile duct epithelial damage. In addition to susceptibility, the precise mechanisms of PBC progression should be addressed to improve patient prognosis and quality of life.

Competing Interests

The authors declare that they have nothing to disclose regarding funding from industries or conflict of interests with respect to this manuscript.

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