Clinical Investigations

Correlation Between HLA-DRB1, HLA-DQB1 Polymorphism and Autoantibodies Against Angiotensin AT_1 Receptors in Chinese Patients With Essential Hypertension

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ABSTRACT

Background: The autoantibodies (AAs) against angiotensin AT_1 receptors (AT_1-AAs) have been discovered in patients with preeclampsia, malignant hypertension, and essential hypertension (EH); however, the mechanism of AA production remains to be investigated.

Hypothesis: Polymorphisms of HLA-DRB1 or HLA-DQB1 are related to production of AAs in autoimmune diseases. We hypothesis that the polymorphisms of the HLA molecules are also associated with production of AT_1-AAs in patients with EH.

Methods: We enrolled 394 patients with EH and 224 normotensive subjects in this study. Autoantibodies in sera of donors were detected by enzyme-linked immunosorbent assay. The subjects' clinical data were collected, including gender, age, body mass index, blood pressure, smoking status, and diabetes. The patients and the normotensive subjects were classified respectively into AA-positive and AA-negative groups. Typing of DNA for HLA-DRB1 and HLA-DQB1 alleles was done by polymerase chain reaction amplification with sequence-specific primers.

Results: Thirteen HLA-DRB1 and 7 HLA-DQB1 alleles were found in this population. The frequencies of AT_1-AAs were related to blood pressure level. The frequency of AT_1-AAs in the EH group was higher than that in the normotensive group ($P = 0.0001$). The levels of AAs in different groups of EH show a significant difference ($P = 0.027$). In addition, HLA-DRB1*04 and HLA-DRB1*14 (odds ratio [OR]: 3.06, 95% confidence interval [CI]: 1.56–5.97, $P = 0.001$; and OR: 2.53, 95% CI: 1.080–5.91, $P = 0.033$, respectively) were related to AT_1-AA production in normotensive subjects after adjusting for covariants. The HLA-DRB1*04 allele might be related to AT_1-AA production in hypertensive subjects, and the P value was of baseline statistical significance after adjusting for blood pressure and other covariants (OR: 1.63, 95% CI: 0.95–2.78, $P = 0.070$).

Conclusions: These results suggest a difference in the immunogenetic background between the positive and negative AAs with hypertension or normotension. The HLA-DRB1*04 allele increases the risk for AT_1-AA production.

Introduction

Autoantibodies (AAs) against the AT_1 receptor (AT_1-AAs) have been described in patients with malignant, preeclamptic, and essential hypertension (EH).[1–4] The AT_1-AAs exhibit an agonistic action like angiotensin II when they bind to the AT_1 receptor. For example, they increase intracellular free calcium (Ca^{2+}) concentrations and the spontaneous heart rate in rats.[5,6] They increase tissue factor expression, induce intracellular AP-1 and nuclear factor κB activation, induce nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase–derived reactive oxygen species in vascular smooth muscle cells,[6] and even mediate the refractory vascular rejection by the AT_1 receptor.[7] In addition, AT_1-AAs are involved in myocardial tissue remodeling.[8] Therefore, autoimmune mechanisms have been proposed to play a role in the pathogenesis of hypertension.[9]

The first two authors contributed equally to this work.
This study was supported by a grant from the National Natural Science Foundation of China (No. 03600235). The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.20852
Received: March 12, 2010
Accepted with revision: September 28, 2010

The major histocompatibility complex locus, mainly class II polymorphism,[10,11] has been associated with more diseases than any other region of the human genome,[12] especially those diseases with AAs, such as systemic lupus erythematosus, rheumatoid arthritis, Graves disease, pernicious anemia, juvenile diabetes mellitus, myasthenia gravis, and others.[13] The production of antibodies requires the help of T cells, a process restricted by the human leukocyte antigen (HLA) class II presentation of antigen peptide.[14] HLA-DRB1 and HLA-DQB1 are functional genes of HLA class II[15] that are related to the prevalence of AAs in autoimmune diseases mentioned above.[16–18] In addition, the HLA system has been considered to have a possible relationship with EH in linkage studies.[19–21]

Based on these observations, and given the paramount importance of the HLA system in the immune response, we sought to clarify the possible relationship among HLA-DRB1 and HLA-DQB1 alleles and AAs, thus elucidating the mechanism of AA production and the possible interactions between HLA alleles and AAs with respect to risk of developing EH. We carried out a case-control study in which we typed HLA-DRB1 and DQB1 alleles and determined the serum AA levels of patients with EH and controls.

Methods

Subjects and Groups

In our study, all subjects were members of the Chinese Han ethnic group and came from the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology in Wuhan, China. The enrolled normotensive subjects came from the medical examination center in the hospital. The protocol was approved by the local bioethics committee, and informed written consent was obtained from each participant. All participants were subjected to complete blood counts, urinalysis, serum creatinine measurement, blood electrolytes, electrocardiography, and an echocardiogram. Candidates were considered ineligible for any of the following reasons: a known secondary cause of hypertension, autoimmune diseases (based on characteristic parameters including erythrocyte sedimentation rate, antinuclear antibody test, double-strand antinuclear antibody test, extractable nuclear antibody test, and anticentromere antibody test), liver disease, renal insufficiency (defined as a serum creatinine $\geq$1.5 mg/dL in men and 1.2 mg/dL in women, respectively), and concurrent life-threatening illness or severe illness requiring extensive systemic treatment. At enrollment, measurements were taken of gender, age, body mass index (BMI), blood pressure (BP), prevalence of smoking, and diabetes. The subjects underwent a 2-week washout phase if they had used an AT_1 blocker before AA detection. Three BP measurements were obtained from each participant by trained and certified observers according to a standard protocol recommended by the American Heart Association. Hypertension was defined as an average systolic blood pressure (SBP) of $\geq$140 mm Hg, and/or an average diastolic blood pressure (DBP) of $\geq$90 mm Hg. Grade 1 corresponds to SBP of 140–159 mm Hg and/or DBP of 90–99 mm Hg. Grade 2 corresponds to SBP 160–179 mm Hg and/or DBP 100–109 mm Hg. Grade 3 corresponds to SBP $\geq$180 mmHg and/or DBP $\geq$110 mm Hg. When a patient's SBP and DBP fell into different categories, the higher category applied.

Antigen Peptide Synthesis and Identification of Autoantibodies

The peptide corresponding to the sequence of the second extracellular loop of the human AT_1 receptor positions 165aa-191aa (I-H-R-N-V-F-F-I-E-N-T-N-I-T-V-C-A-F-H-Y-E-S-Q-N-S-T-L)[1] was synthesized by an automated, multiple solid-phase peptide synthesizer (PSSM-8 type; Shimadzu, Tokyo, Japan). The peptide was evaluated by high-performance liquid chromatography analysis on a Vydac C-18 column and <95% purity was achieved. The level of AT_1-AA was measured by enzyme-linked immunosorbent assay as we used previously, and the results were expressed as optical density (OD) values.[1,22,23] Briefly, AT_1 receptor peptide (10 μg/mL) in a 100-mM sodium carbonate solution (pH 9.6) was coated on microtiter well plates and left overnight. The wells were then saturated with phosphate-buffered saline (PBS; pH 7.4, 0.01 mol/L, PBS-Tween 20). After washing 3 times with PBS Tween 20, the sera 1:40 dilutions were added to the coated microtiter plates for 1 hour at 37°C. After 3 additional washings, horseradish peroxidase conjugated antihuman immunoglobulin G antibodies 1:2,000 (Huarui Biotech Co. Ltd., Zhuhai, China) were added for 1 hour at 37°C. The plates were then washed 3 more times, the substrate (0.01% hydrogen peroxide and 0.1% 3′-3′-5′-5′-tetramethyl benzidine) was added for 5 minutes, and the reaction was ended with 2 M sulfuric acid. The ODs were measured at 450 nm using an enzyme-linked immunosorbent assay reader (Spectra Max Plus; Molecular Devices, Sunnyvale, CA). We also calculated positive/negative ratio (the OD of sample−the OD of empty control)/(the OD of negative control−the OD of empty control) of each sample, and those samples with a positive/negative ratio of at least 2.1 were considered as AT_1-AA positive.

HLA-DRB1 and HLA-DQB1 Genotyping

Blood samples were collected in 5-mL vacuum tubes containing ethylenediamine tetra-acetic acid (Greiner, Linz, Austria). Genomic DNA was extracted using a TIANgen Blood DNA extraction kit (Tiangen Biotech, Beijing, China). The DNA was stored at −80°C until required for batch genotyping. The HLA genotyping was determined using polymerase chain reaction with sequence-specific primers (PCR-SSP), an HLA phototype method.[24] The specific primer sets that were used to subtype alleles of DRB1 and DQB1 are described elsewhere.[25,26] Briefly, 20 SSP reactions were used to amplify selectively alleles or groups of alleles from 1 mg of DNA. Each 13µl amplification mixture contained 67 mM Tris base (pH 8.8), 16 mM ammonium sulfate, 2 mM magnesium chloride, 0.01% (vol/vol) Tween 20, 200 mM of deoxyribonucleotide triphosphate, 1–3 mM of SSPs, 0.1 mM of DRB1 or DQB1 control primers, and 0.25 units Taq polymerase (Advanced Biotechnologies, London, UK). The PCR amplifications were carried out

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DOI:10.1002/clc.20852

in MJ Research 96V machines under the following cycling conditions: 1 minute at 96°C; 5 cycles of 25 seconds at 96°C, 45 seconds at 70°C, 45 seconds at 72°C; 21 cycles of 25 seconds at 96°C, 50 seconds at 65°C, 45 seconds at 72°C; 4 cycles of 25 seconds at 96°C, 60 seconds at 55°C, 120 seconds at 72°C. The PCR products were electrophoresed on 1.0% agarose gels and then visualized under ultraviolet illumination. Specificities in an allele-specific or group-specific manner were examined by using the same method, reagents, PCR parameters, and protocols for all loci as mentioned above. All samples were replicated at least 3 times.

Statistical Analysis

Analyses were performed using SPSS 10.0 software (SPSS Inc., Chicago, IL). Comparison of quantitative variables was carried out by unpaired Student t test and categorical ones using χ^2 statistics. Allele families, alleles of the HLA system, and the AAs, as dichotomous and the categorical variables, were tested for association with EH by univariate and multivariate logistic regression analyses. Alleles were analyzed by multiple comparisons, and covariates with significance $P < 0.1$ in stepwise regression were retained in the final model. Crude and adjusted odds ratio (OR) and 95% confidence interval (CI) were calculated from the univariate and multivariate logistic regression parameter estimates, respectively. Alleles were tested for deviation from Hardy-Weinberg equilibrium using the SHEsis software platform.[27] A P value <0.05 was considered statistically significant.

Results

Overview of the Study Population

A total of 667 subjects initially enrolled in the study (403 EH patients and 264 normotensive subjects). The blood samples of 643 subjects were collected and stored for DNA extraction. Of these, 608 subjects had sufficient DNA samples for genotyping (394 EH and 224 normotensive). The participants' ages ranged from 30 to 75 years. Hypertensive patients and normotensive subjects did not differ regarding sex distribution and smoking status. The EH patients had significantly higher BMIs. Diabetes was also more frequent in patients with EH than in the normotensive group, and the difference reached statistical significance ($P = 0.016$). The mean age of the EH subjects was 57 ± 13.55 years, and the mean age of the normotensive subjects was 49.14 ± 12.88 years. The demographic and clinical data of the study subjects are shown in Table 1. There were no significant differences between AA-positive groups and AA-negative groups, either in the EH group or the normotensive group, regarding DBP, BMI, smoking status, and the prevalence of diabetes. The sex distribution and age were different between the AT_1-AA–positive and AT_1-AA–negative groups among patients with EH patients, but not in normotensive subjects ($P < 0.1$ were retained in the final regression model, Table 2). Gender, age, and SBP, which were retained in the logistic model as covariates, were adjusted in EH in statistical analysis.

Table 1. Demographic and Clinical Data in EH Patients and Normotensive Subjects

Parameter	EH Patients (N = 394)	Normotensive Subjects (N = 224)
SBP (mm Hg)	155.23 ± 21.80	113.54 ± 13.03[a]
DBP (mm Hg)	93.28 ± 13.95	75.22 ± 9.38[a]
Age (y)	57 ± 13.55	49.14 ± 12.88[a]
Sex (male, %)	58.18	65.45
BMI (mean ± SD)	24.64 ± 3.38	22.74 ± 3.05[a]
Diabetes (%)	15.55	9.34[b]
Smoking(%)	49.44	42.78[b]

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; EH, essential hypertension; SBP, systolic blood pressure; SD, standard deviation. [a]$P < 0.001$ vs EH patients. [b]$P = 0.016$ vs EH patients.

Association With Frequency of AT_1-AAs and Blood Pressure

Frequency of AT_1-AAs was different according to BP, and the data are shown in Table 3. We took the titer serum dilution of 1:40 as a positive serum threshold value. The data indicated that the prevalence of AT_1-AAs was significantly higher in EH subjects than the proportion of AAs in normotensive subjects ($P = 0.0001$). Frequencies of AT_1-AAs were obvious differences between the groups ($P = 0.027$) according to BP from grade 1 to grade 3. The results indicated that the tendency toward higher BP was coincident with a higher level of AT_1-AAs. These results demonstrated that BP was a risk factor for expression of AT_1-AAs.

HLA-DQB1 and HLA-DRB1 Gene Associations With AT_1-AAs

The further analysis focused on AT_1-AA production and HLA gene polymorphism. The number of all alleles was 13 for DRB1 and 7 for DQB1 analyses, and only results with statistical significance were listed. The HLA alleles were found to be possible risk or protective factors ($P < 0.1$) or definite risk or protective factors (adjusted $P < 0.05$, after correction for confounding factors) for different groups. Allele families or alleles were evaluated for their statistical association with AT_1-AAs (Table 4). In the univariate analysis of HLA allele families, DQB1*06, DRB1*04, and DRB1*14 were significantly associated with AT_1-AAs in normotensive subjects. After adjusting for confounding variables, only DRB1*04 and DRB1*14 were still significantly related to the prevalence of AT_1-AAs (adjusted OR: 3.06, 95% CI: 1.56–5.97, $P = 0.001$; and OR: 2.53, 95% CI: 1.08–5.91, $P = 0.033$, respectively). This result demonstrates that the alleles DRB1*04 and DRB1*14 are independent risk factors for AT_1-AA production in normotensive subjects. In addition, we found that the HLA-DRB1*04 allele was also possibly related to AT_1-AA production in hypertensive subjects. The P value was 0.082 ($P < 0.1$) in the univariate analysis and entered the logistic regression model, and the adjusted P value was 0.07 (OR:

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DOI:10.1002/clc.20852

Table 2. Demographic and Clinical Data in EH Patients and Normotensive Subjects According to AT_1-AAs

	EH Patients (N = 394)			Normotensive Subjects (N = 224)		
Parameter	AT_1-AA(−) N = 162	AT_1-AA(+) N = 232	*P* Value	AT_1-AA(−) N = 173	AT_1-AA(+) N = 51	*P* Value
SBP (mm Hg)	152.72 ± 20.69	156.98 ± 22.53	0.053	112.74 ± 12.35	116.25 ± 12.90	0.079
DBP (mm Hg)	94.36 ± 13.21	92.37 ± 14.42	0.135	75.50 ± 9.66	74.89 ± 6.81	0.752
Age (y)	58.77 ± 13.16	56.91 ± 13.32	0.100	49.07 ± 12.57	48.53 ± 12.66	0.785
Sex (male, %)	65.43	56.89	0.088	67.05	60.78	0.407
BMI (mean ± SD)	24.30 ± 1.97	24.71 ± 2.14	0.264	22.46 ± 1.66	23.39 ± 1.49	0.801
Diabetes (%)	13.58	16.81	0.471	9.8	7.8	0.877
Smoking (%)	47.0	53.1	0.233	37.25	43.95	0.427

Abbreviations: AT_1-AA (+), autoantibody against AT_1-receptor positive; AT_1-AA(−), autoantibody against AT_1-receptor negative; BMI, body mass index; DBP, diastolic blood pressure; EH, essential hypertension; SBP, systolic blood pressure; SD, standard deviation. *P* value by the unpaired Student *t* test or κ^2 statistics: AT1-AA(+) vs AT1-AA(−) in EH patients or normotensive subjects.

Table 3. Association With Level of AT_1-AAs and Blood Pressure

	N	AT_1-AA(+), N (%)	*P* Value
Normotensive subjects	224	51 (22.76)	0.0001[a]
Patients with EH			
Grade 1	145	75 (51.72)	0.027[b]
Grade 2	152	90 (59.21)	
Grade 3	97	67 (69.07)	
Total	394	232 (58.88)	

Abbreviations: AT_1-AA, autoantibody against AT1-receptor; EH, essential hypertension. [a]Comparisons between EH patients and normotensive subjects. [b]Comparisons between groups of different blood pressure grades in EH.

1.63, 95% CI: 0.95–2.78). Allele HLA-DRB1*04 was positive for correlation with AT_1-AA production, although it was not an independent risk factor after adjusting for BP, age, and gender. The reason might be attributed to other genetic and environmental elements that may very well be influencing the production of AT_1-AAs in EH. The allele DRB1*14 had no significant correlation with AT_1-AAs in EH (data not shown). These results suggest that HLA-DRB1*04 is a possible risk factor for the prevalence of AT_1-AAs, both in normotensive and hypertensive subjects.

HLA-DRB1*04 and Blood Pressure

We further observed the relationship between HLA-DRB1*04 and BP. Table 5 shows the data; the allele HLA-DRB1*04 did not correlate with SBP and DBP in patients with EH or in normotensive subjects (all $P < 0.05$).

Discussion

Our data confirm that the prevalence of AT_1-AA was higher in EH patients than in normotensive subjects, as previously demonstrated.[1] This study showed that the prevalence of AAs was higher than that in our previous investigation, because we took the titer serum dilution of 1:40 as positive serum threshold value this time instead of 1:80 in our previous study.[1] The production mechanisms of AAs were not known. Recently, studies have implied that immune mechanisms are involved in EH, either secondary to the molecular mimicry and vascular damage, or as a primary abnormality.[28–30] In other words, the production of AAs in hypertension is associated with virus infection of homologous structure, autoantigen exposure, or is mediated by genetic background, which may harbor specific polymorphisms or predispose a certain subgroup of patients to autoimmune manifestations.

The hypertension could result in vascular damage and endogenous collagen exposure, and then induce activation of an autoimmune reaction. This study revealed that frequency of AT_1-AA was significantly higher in patients with EH than in normotensive subjects, and the level of AT_1-AA gradually increased with elevations in BP. Therefore, we took BP as one of the covariates in the logistic regression model about AT_1-AA production and adjusted it. The further study is to elucidate mechanisms regarding the individual difference of AT_1-AA production. The main finding of this study is that the risk for production of AAs is associated with allele HLA.

The previous reports had described that the allele HLA-DRB1*04 was significantly associated with the prevalence of type II collagen AA in arthritis.[31] Recently, the presence of HLA-DRB1*04 was found to be significantly higher in rheumatoid arthritis patients of Asian populations.[32] Similar associations between HLA-DRB1*04 and different AAs had also been reported in presumed autoimmune diseases in the cardiovascular system.[33,34] The HLA-DRB1*04 phenotype was associated with the susceptibility to preeclampsia[35] and anticardiolipin antibodies in preeclampsia.[36] The presence of AAs against the β_1-adrenergic receptor in patients with idiopathic dilated cardiomyopathy was also linked

DOI:10.1002/clc.20852

Table 4. Association of HLA Gene and Frequency of AT_1-AAs

	Univariate Model		Multivariate Model[a]	
Allele	Crude OR (95% CI)	*P* Value	Adjusted OR (95% CI)	*P* Value
Normotensive subjects				
HLA-DQB1*06	0.49 (0.23–0.86)	0.017	0.39 (0.07–2.33)	0.242[b]
HLA-DRB1*04	2.78 (1.44–5.34)	0.002	3.06 (1.56–5.97)	0.001[c]
HLA-DRB1*14	2.09 (0.93–4.75)	0.076	2.53 (1.08–5.91)	0.03[d]
Patients with EH				
HLA-DRB1*04	1.55 (0.93–2.59)	0.082	1.63 (0.95–2.78)	0.070[e]

Abbreviations: AT_1-AA, autoantibody against AT_1-receptor; CI, confidence interval; OR, odds ratio; SBP, systolic blood pressure. [a]Multiple logistic regression analysis. Covariates with significance $P < 0.1$ in stepwise regression were retained in the final model. [b]Adjusted for SBP, HLA-DQB1*04, and HLA-DRB1*14. [c]Adjusted for SBP, HLA-DQB1*06, and HLA-DRB1*14. [d]Adjusted for SBP, HLA-DQB1*06, and HLA-DRB1*04. [e]Adjusted for SBP, age, and gender.

Table 5. HLA-DRB1*04 Allele and Blood Pressure

	HLA-DRB1*04		
	Carries	Does Not Carry	*P* Value
Patients with EH			
SBP (mm Hg)	154.96 ± 21.01	155.88 ± 21.47	0.632
DBP (mm Hg)	92.39 ± 14.14	93.60 ± 14.03	0.441
Normotensive subjects			
SBP (mm Hg)	114.50 ± 12.68	113.88 ± 11.75	0.722
DBP (mm Hg)	75.63 ± 8.27	74.58 ± 9.71	0.424

Abbreviations: DBP, diastolic blood pressure; EH, essential hypertension; SBP, systolic blood pressure.

to HLA-DRB1*04.[36] These studies indicated that HLA-DRB1*04 was an important risk factor for autoimmune and AA production. The present results also showed that the DRB1*04 allele increased the production of the AT_1-AAs, especially in normotensive people.

The association between HLA-DRB1*04 molecules and AA production in normotensive and hypertensive subjects has implied the pathogenesis of this autoimmune phenomenon. Because the antigens are presented as a major histocompatibility complex–peptide complex by antigen-presenting cells, a crucial role of HLA molecules is indicated in distinguishing self and nonself cells. Extensive documentation exists associating HLA-DR molecules that share the HV3 region with the DRB1*0401 shared epitope (SE) to susceptibility to autoimmune diseases, especially rheumatoid arthritis.[37,38] It has been proposed that SE might function by determining the charge in peptide binding pockets.[37] Alternatively, this epitope might be responsible for shaping the T-cell repertoire in the thymus, thereby determining the outcome of disease when the relevant antigen is presented in the periphery.[37] Further study is needed about whether the HLA-DRB1*04 molecules have the positive relationship with these AAs according to the SE theory. Moreover, why are there different relationships between HLA alleles in groups with different AAs? We postulated that HLA partly determines the individual response to antigens because a set of HLA molecules expressed by an individual influences the functional T-cell repertoire due to the function of HLA molecules as antigen-presenting structures.

Why did DRB1 or DQB1 alleles not equally predispose or suppress the production of the AT_1-AAs in the EH and normotensive subjects? In this study, it was likely that they were in contact with significantly different environmental factors, such as BP, age, BMI, and diabetes complications. The immunologic derangement in EH could weaken predisposition of the HLA-DRB1*04 allele to AT_1-AAs, and it was only of baseline statistical significance in the logistic regression model in our study.

However, although the allele HLA-DRB1*04 could induce the higher prevalence of AT_1-AAs, it was not the risk factor for changes in BP. We could not conclude that HLA-DRB1*04 induced higher prevalence of AAs and consequently increased or decreased BP. The pathogenesis mechanism of EH was complicated, and the AAs were not unique stimulative factors for EH. In addition, the biological effect of AT_1-AAs could not directly induce increased BP, but stimulate the proliferation of vascular smooth muscle cells and increase intracellular Ca^{2+}, resulting in vascular remodeling.[2,3] The prevalence of AAs was higher in patients with hypertension than in normotensive subjects, and the level of AT_1-AA was relative to BP, from which we deduced that hypertension could be the important causative environmental agent on AA production and susceptive immunological genetic patterns would play a key role to elucidate AT_1-AA expression with individual differences. The present result indicated that HLA-DRB1*04 increased the prevalence of AAs, but there was not necessarily a correlation with change in BP.

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DOI:10.1002/clc.20852

In conclusion, EH is a complex genetic trait, as BP in each individual was due to the combined effects of variation at an indeterminate number of BP-determining genetic loci and environmental factors.[39] Furthermore, one of the genetic systems for which a possible relationship with EH has been considered is the HLA system, and this has been documented in linkage studies.[21,24] All individuals are tolerant of their own potentially antigenic substances, and failure of self-tolerance is the fundamental cause of AAs. Although the mechanisms by which this occurs are not entirely known, AA production is thought to result from a combination of genetic variants, acquired environmental triggers such as infections, and stochastic events.[40] We described for the first time a relationship between risk of AA production among EH patients and HLA gene polymorphism. These associations show AA expressions linked to allele HLA-DRB1*04, which will provide us with novel avenues to understand the epidemiological mechanisms of AA production, and further establish the role of immunology and genetics in the pathogenesis of EH.

Study Limitations

The major limitation of this study was the small number of study samples. Further studies in larger populations and including samples of other ethnic origins are necessary to confirm this finding. Large samples will increase power to detect a genetic effect attributable to reduced noise in the dataset, and maybe the new genetic effect will be found. But there was no effect in the analysis presented here.

Acknowledgments

The first 2 authors contributed equally to this work.

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