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Genetic polymorphisms of G protein-coupled receptor 65 gene are associated with ankylosing spondylitis in a Chinese Han population: A case-control study

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ABSTRACT

Objective: This study aimed to assess the association between two tag single nucleotide polymorphisms (SNPs) (rs68177277 and rs11624293) of G protein-coupled receptor 65 (GPR65) gene and ankylosing spondylitis (AS) susceptibility in a Chinese Han population.

Methods: 673 patients with AS diagnosed according to the modified New York criteria and 679 age- and gender-matched healthy controls were recruited. SNP genotyping for rs68177277 and rs11624293 polymorphisms were performed using the SNPscan technique. Disease activity was assessed by the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI).

Results: Genotype and allele distribution of rs11624293 but not rs68177277 were significantly different between AS and controls ($p = 0.004$ and $p = 0.002$). Compared to the wild-type T/T genotype and T allele at rs11624293, the frequencies of C/T genotype and C allele were significantly higher in AS than controls after adjusting for age and gender (OR = 1.527, 95%CIs: 1.190–1.958; OR = 1.515, 95%CIs: 1.183–1.942, respectively). Dominant and co-dominant model of rs11624293 were predictive of AS susceptibility. In AS patients, the genotype of rs11624293 was significantly associated with BASFI scores in those with low disease activity (BASDAI < 4, $p = 0.007$).

Conclusions: The results of our study suggest that rs11624293 polymorphism of GPR65 gene is associated with the susceptibility and severity of AS in Chinese Han population.

1. Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease and the most common form of spondyloarthropathies, which include a series of arthritis conditions including AS, reactive arthritis, enteropathic arthritis (in association with inflammatory bowel disease (IBD)) and psoriatic arthritis [1]. These diseases share several common aetiological features, especially genetic factors [2,3]. For example, it has been demonstrated that IBD shares some disease-associated risk loci with AS [4,5]. Although a strong association between AS and HLA (human leukocyte antigen)-B27 has been well documented, the pathogenesis of AS remains uncertain. Specifically, numerous evidence has demonstrated that a substantial number of non-major histocompatibility complex (MHC) genes may contribute to the onset of AS

Abbreviations: AS, ankylosing spondylitis; SNPs, single nucleotide polymorphisms; GPR65, G-protein coupled receptor 65; IBD, inflammatory bowel disease; TDAG-8, T cell death-associated gene 8; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; PsA, psoriatic arthritis; BASDAI, the Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; ACOS, asthma-chronic obstructive pulmonary disease; MS, multiple sclerosis; HIT, heparin-induced thrombocytopenia

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Table 1
SNP information and PCR primers for GPR65 gene polymorphisms.

SNP	Position	Allele	PCR primers	HWE
rs68177277	88008109	C/A	F: TGTGGGTAAAACAGATGTTTGTTTGACM R: AGATGCTCTACATGCCATCTCACA	$P = 0.1822$
rs11624293	88022477	C/T	F: GGTGGACAGCAGTGCTGCTGY R: GAACGTGCTAGGAACAGAGAATGAGC	$P = 0.9434$

F: forward.
HWE: Hardy-Weinberg equilibrium.
PCR: polymerase chain reaction.
SNP: single nucleotide polymorphism.
M and Y represents merged base (M, A/C; Y, C/T).

[6,7].

G protein-coupled receptor 65 (GPR65) gene, also known as T cell death-associated gene 8 (TDAG-8), maps to chromosome 14q31.3 and encodes a proton-sensing G protein-coupled receptor. Northern blot analysis indicated that GPR65 gene was primarily expressed in immune cells and leukocytes-rich tissues [8]. In addition, Onozawa et al. found that GPR65 gene regulated the function of immune cells in GPR65 knockout mice [9]. Therefore, GPR65 gene polymorphisms may play a role in autoimmune system, and this have been demonstrated in IBD [10,11], asthma-chronic obstructive pulmonary disease (ACOS) [12,13], multiple sclerosis (MS) [14], and heparin-induced thrombocytopenia (HIT) [15], but not in AS. A recent genome-wide association study (GWAS) conducted in Europe reported that variants of GPR65 gene achieved genome-wide significance in AS [16]. Thus, we hypothesized that GPR65 gene polymorphisms may be related to the susceptibility of AS in Chinese population.

Haplotype blocks (or tag single nucleotide polymorphisms (SNPs)) have been widely applied in genetic association studies [17], and they can represent the genetic information of closely linked SNPs and markedly decrease the cost of genotyping SNPs [18]. In this study, we selected two tag SNPs (rs68177277 and rs11624293) of GPR65 gene to explore the association between GPR65 gene variants and AS susceptibility in a Chinese population.

2. Materials and methods

2.1. Subjects

Six hundred seventy-three unrelated Chinese Han participants diagnosed with AS (548 males and 125 females) were consecutively recruited from outpatient clinics at the First Affiliated Hospital of Anhui Medical University, Hefei, China. All patients were diagnosed by skilled rheumatologists according to the modified 1984 New York Criteria [19]. Participants were excluded from this study if they were complicated by pulmonary tuberculosis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), psoriatic arthritis (PsA), psoriasis, IBD or other chronic inflammatory or immune diseases. Six hundred eighty-seven age- and gender-matched unrelated healthy blood donors (560 males and 127 females) were selected in this study as controls. We obtained informed consent from all patients and healthy controls, and the study was approved by the Ethics Committee of Anhui Medical University (Hefei, China).

Age, gender, and body mass index (BMI) of the patients were recorded. We also collected the following data from all patients to assess AS disease activity: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [20] and Bath Ankylosing Spondylitis Functional Index (BASFI) [21]. By definition, AS patients were considered high disease activity, if the BASDAI score was equal or greater than 4. In addition, finger to floor distance and occiput-wall distance were measured in the present study. Blood samples were obtained from all enrolled subjects using EDTA and stored at −20 °C until genomic DNA extraction.

2.2. SNPs selection

Two tag SNPs (rs68177277 and rs11624293) were selected using Haploview 4.2 software (Broad Institute, Cambridge, MA, USA) based on the HapMap databases for Chinese Han population in Beijing (CHB) (HapMap Data Rel 28 PhaseII + III, on NCBI B36 assembly, dpSNP b126). Candidate SNPs were identified as tag SNPs if the minimum minor allele frequency was larger than 5% and the r^2 value was equal or greater than 0.8.

2.3. DNA isolation and genotyping

Genomic DNA was extracted from peripheral blood lymphocytes from AS patients and healthy controls using a standard method (Qiagen, Hilden, Germany) based on the manufacturer's instructions. SNP genotyping for rs68177277 and rs11624293 of GPR65 gene were performed using SNPscan Kit (Cat#: G0104; Genesky Biotechnologies Inc., Shanghai, China). This kit was developed according to a patented SNP genotyping technology by Genesky Biotechnologies Inc., which was based on double ligation and multiplex fluorescence PCR. PCR products were separated and detected by capillary electrophoresis in an ABI3730XL sequencer. Raw data were analyzed according to the information obtained from the labeling dye color and the fragment size of the allele-specific ligation-PCR product. Genotyping was conducted by researchers who did not know the subjects' disease status (i.e., AS cases or healthy controls). For quality control, repeated analyses were accomplished by randomly choosing 4% of samples with high DNA quality. The success rates of genotyping were higher than 99% in Stage II samples, and the concordance rates were higher than 99% in the 4% duplicate samples. The details of genotyping are shown in Table 1.

2.4. Statistical analysis

Variables were expressed as mean ± SD (standard deviation) or median and inter-quartile range (IQR) as appropriate. Continuous data were compared using Student's *t*-test or Kruskal-Wallis *H* test. The distributions of allele and genotype of GPR65 gene between AS patients and controls were assessed by Chi-square tests. Odds ratio (OR) with 95% confidence intervals (95%CIs) was calculated using binary logistic regression model to analyze the association between genotype of GPR65 and AS susceptibility, with adjustment of age and gender. A two-sided *p-value* less than 0.05 was considered statistically significant. We also corrected for multiple comparisons using Bonferroni correction [22]. *P*-value for a truly significant result was set at 0.05/n, where n indicates the number of comparisons. In addition, Hardy-Weinberg equilibrium (HWE) tests were performed in controls using Haploview version 4.2 [23]. All analyses were performed in SPSS 23.0 (SPSS, Int., Chicago, IL, USA) and Haploview version 4.2 (Broad Institute, Cambridge, MA, USA). Besides, we used PASS 11 software (NCSS, LLC.

Table 2
Demographic and clinical indicators in AS patients and controls.

Variables	Patients	Healthy controls	t / χ^2	P
Age (years)	28.62 ± 7.76	28.58 ± 9.31	0.087	0.931
Sex ratio (M/F)	548/125	560/127	0.002	0.967
BMI (Kg/m^2)	22.27 ± 4.06	NA		
Disease duration (years)	3.3 (0.8, 8.0)	NA		
HLA-B27$^+$, n (%)	413 (61.4)	NA		
Finger to floor distance (cm)	5.00 (0.00, 17.00)	NA		
Occiput–wall distance (cm)	0.00 (0.00, 3.00)	NA		
ESR (mm/l)	15.00 (6.00, 33.00)	NA		
CRP (mg/l)	9.89 (2.81, 29.82)	NA		
BASDAI (cm)	2.00 (0.60, 3.80)	NA		
BASFI (cm)	0.90 (0.00, 2.60)	NA		

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BMI: body mass index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; HLA: human leukocyte antigen; M/F: male/female; NA: not available; Variables are expressed as mean ± standard deviation or median (inter-quartile range).

Kaysville, Utah, USA) [24] to test the power of our study. And the result revealed the power of our study was 0.86.

3. Results

3.1. Characteristics of subjects

The detailed demographic and clinical characteristics of all subjects are shown in Table 2. In the present study, 673 AS patients and 687 healthy controls were recruited. The mean ages of AS patients and controls were 28.62 ± 7.76 years and 28.58 ± 9.31 years, respectively. There was no significant difference between AS patients and healthy controls in age and gender ($p = 0.931$ and $p = 0.967$, respectively). In AS patients, 152 individuals with high disease activity were noted.

3.2. Genotype and allele analysis

The results indicated that rs68177277 and rs11624293 of GPR65 gene in controls were in agreement with Hardy-Weinberg equilibrium (HWE) ($p = 0.1822$ and $p = 0.9434$, respectively) (Table 1).

Genotype distribution and allele frequencies of GPR65 gene polymorphisms are shown in Table 3. The distribution of genotype and allele in rs68177277 polymorphism was not statistically different between AS patients and controls ($p = 0.290$ and $p = 0.836$). Besides, the dominant, recessive and co-dominant models also supported that the rs68177277 polymorphism was not significantly related with AS susceptibility. For rs11624293 polymorphism, the genotype and allele frequencies were significantly different between AS patients and controls ($p = 0.004$ and $p = 0.002$). Using binary logistic regression analysis adjusting for age and gender, we found that AS patients were more likely to have genotype C/T and C allele rather than wild-type (WT) T/T and T allele in GPR65 gene rs11624293 polymorphism (adjusted OR = 1.527, 95%CIs: 1.190–1.958; adjusted OR = 1.515, 95%CIs: 1.183–1.942; respectively). Moreover, rs11624293 polymorphism was significantly related to AS in the dominant model and co-dominant model (Both $p = 0.001$), and the detailed results are present in Supplementary material Table S1. In addition, these associations were still statistically significant after Bonferroni correction ($p < 0.05/2 = 0.025$).

Table 3
Distributions of genotype and allele of GPR65 gene in AS patients and controls.

		Cases	Controls	χ^2	P	Adjusted OR[a] (95% CIs)
rs68177277	CC	36	28	2.476	0.290	1.274 (0.746, 2.125)
	CA	198	224			0.876 (0.694, 1.105)
	AA	439	435			Reference
	C	270	280	0.043	0.836	0.862 (0.684, 1.85)
	A	1076	1094			Reference
rs11624293	CC	15	13	11.304	**0.004**	1.314 (0.619, 2.792)
	CT	195	146			1.527 (1.190, 1.958)
	TT	463	528			Reference
	C	225	172	9.613	**0.002**	1.515 (1.183, 1.942)
	T	1121	1202			Reference

a: Adjusted for age and gender, b: Only adjusted for age; OR: Odds ratio; CIs: Confidence intervals; $P < 0.025$ was considered statistically significant (based on the Bonferroni correction).

3.3. Genotype and clinical phenotypes

The associations between rs68177277 and rs11624293 genotypes and clinical phenotypes (included ESR, CRP, BASDAI, BASFI, finger to floor distance, and occiput-wall distance) of AS were also assessed in this study, but no significant association was observed (see in Table S2). In addition, we further investigated whether the BASDAI and BASFI scores were significantly different among rs11624293 genotypes after stratification of AS disease activity (i.e., BASDAI < 4 or ≥ 4). A significant association was observed between rs11624293 genotypes and BASFI scores in patients with low disease activity ($p = 0.007$), see in Table 4.

3.4. Linkage disequilibrium (LD) and haplotype analysis

We observed a D' = 1 and $r^2 = 0.043$ for the linkage between rs68177277 and rs11624293 polymorphisms in LD analysis (Fig. 1). Therefore, we did not perform haplotype analysis due to the small r^2 (i.e., < 0.8).

4. Discussion

The etiology of AS is still uncertain, but gene plays an important role in AS susceptibility. Although HLA-B27 is strongly related to AS heritability, only 1%-5% of HLA-B27 positive individuals develop AS, implying that non-MHC genes contribute to AS heritability [25]. G protein-coupled receptors are constituted by a large superfamily of receptors characterized by a seven transmembrane domain structure, and these receptors can activate intracellular transducer G proteins [26]. Among those receptors, GPR65 is predominantly expressed in lymphoid organs and is activated by extracellular protons. GWASs have demonstrated that the GPR65 gene loci were related to MS [27,28], type 1 diabetes [29], IBD [10,30], and rheumatoid arthritis (RA) [31]. Besides, an European GWAS found an association between GPR65 gene and AS [16], but the association has not been confirmed in Chinese population.

In the present study, the polymorphism of rs11624293 was significantly associated with AS susceptibility. After adjusting for age and gender, GPR65 gene C/T genotype and C allele contributed 1.527-fold and 1.515-fold of odds for developing AS. Interestingly, rs11624293 genotype was associated with BASFI scores in patients with low disease activity stratified by BASDAI (< 4 or ≥ 4). A plausible explanation for we did not observe the association in AS patients with higher BASDAI might be that only a small number of them had a high disease activity (22.58%).

Ahn et al. recently reported that the psychosine could inhibit osteoclastogenesis by increasing intracellular cAMP levels through GPR65 [29]. In addition, Appel et al. found that inflammation of AS simultaneously increased bone resorption and osteoproliferation leading to ossifying enthesitis and ankylosis of the sacroiliac joints and intervertebral discs [30]. Therefore, GPR65 may play a role in enthesitis and ankylosis in AS. Moreover, previous study has demonstrated that an

Table 4
BASDAI and BASFI in AS patients with genotype of rs11624293 under low or high disease activity.

rs11624293		Low disease activity[a]		High disease activity[b]	
		BASDAI (cm)	BASFI (cm)	BASDAI (cm)	BASFI (cm)
	CC	0.70 (0.00, 1.45)	0.20 (0.00, 1.30)	5.50 (5.40,5.60)	4.10 (3.60, 4.60)
	CT	1.50 (0.20, 2.80)	0.80 (0.00, 2.20)	5.00 (4.60, 5.60)	3.20 (1.30, 6.20)
	TT	1.50 (0.00, 2.60)	0.20 (0.00, 1.40)	5.10 (4.50, 6.00)	3.20 (1.80, 5.40)
	p	0.192	**0.007**	0.653	0.892

a: BASDAI < 4; b: BASDAI ≥ 4; All variables were expressed as median with IQR.

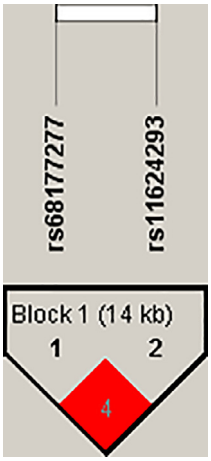

Fig 1. Pair-wise linkage equilibrium plot of GPR65 gene (rs68177277 and rs11624293) SNPs.

acidic microenvironment can regulate pro-inflammatory and anti-inflammatory responses in many cells, and GPR65 is activated by protons in extracellular acidic conditions [31,32]. Consistent with above findings, Onozawa et al. found that GPR65 inhibited inflammation by reducing the production of pro-inflammatory cytokines [33]. Taken together, it is possible that point mutation of rs11624293 may affect the regulation of inflammation and bone metabolism in patients with AS. In this study, our results indicated that BASFI was significantly associated with rs11624293 polymorphism at patients with low disease activity, suggesting the role of rs11624293 polymorphism in the regulation of inflammation.

Some limitations existed in our case-control study. First, we only selected two tag SNPs of GPR65 gene with the r^2 value less than 0.8; therefore, we cannot evaluate the association between the haplotype of SNPs and the susceptibility and severity of AS. It is necessary to identify the role of GPR65 gene in AS by studying more SNPs. Second, we did not collect specific information such as smoking status and alcohol drinking, which may be confounding factors for the association between GPR65 gene variants and AS severity. We acknowledge that this issue should be addressed in future studies. Finally, the male/female ratio in our study was about 4:1, and the number of females is too small to perform a subgroup analysis. Therefore, larger and multi-center studies are required.

In conclusion, the present study suggests an association of rs11624293 polymorphism of GPR65 gene with the susceptibility and severity of AS. These findings need be verified in other ethnicities and larger case-control studies.

We sincerely thank all people who helped us in this study.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2018.12.001.

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