No association of CTLA-4 polymorphisms with susceptibility to Behçet disease

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Accepted 29 April 2009
Published Online First 30 June 2009

ABSTRACT

Background: Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a key negative regulator of T lymphocytes and has been shown to be associated with a number of autoimmune diseases. The present study was performed to assess the association between CTLA-4 polymorphisms and Behçet disease (BD) in Chinese patients.

Methods: Two hundred and twenty-eight BD patients and 207 controls were analysed for four single nucleotide polymorphisms (SNPs) (−1661A/G, −318C/T, +49G/A and CT60G/A) in the CTLA-4 gene by PCR-restriction fragment length polymorphism (RFLP) analysis. The association between SNP +49A/G and BD in Chinese population as well as other ethnic groups was analysed by meta-analysis.

Results: No association could be detected between CTLA-4 SNPs or haplotypes and BD. Also, no association was observed between CTLA-4 polymorphisms and BD subgroups, stratified by clinical features. A meta-analysis showed that there was no heterogeneity between studies ($p = 0.60$, $I^2 = 0\%$) and that CTLA-4 SNP +49 was not associated with BD (overall effect: $Z = 0.26$, $p = 0.79$).

Conclusion: This study and a meta-analysis failed to demonstrate any association between the tested CTLA-4 polymorphisms and BD.

Behçet disease (BD) is a well-known refractory autoimmune disease, characterised by its classical triad (recurrent oral aphthous ulceration, genital ulceration and recurrent uveitis) and skin lesions. Although the precise aetiology and pathogenesis of BD are still unknown, a widely accepted hypothesis is that an infectious agent and immunological abnormalities in genetically susceptible individuals may be responsible for the initiation and maintenance of BD. Genetic susceptibility to BD has been evidenced by familial cases and a strong association with HLA-B51.[1–3] However, the contribution of HLA-B51 to the overall genetic susceptibility to BD is estimated to be less than 20%.[4] As a complex trait disease, BD may result from a combination of non-genetic factors and genetic risk factors with a contribution from a variety of different genes. Each individual gene may have a relatively modest effect on the risk of BD.

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), located on human chromosome 2q33, is a member of the CD28 gene family. It is a key negative regulator of T lymphocyte and is expressed predominantly in activated and regulatory T lymphocytes.[5 6] Numerous studies have revealed the association of CTLA-4 with autoimmune diseases mediated by T lymphocytes, such as type 1 diabetes mellitus, Hashimoto thyroiditis and Graves disease,[7 8] though there are also some discrepancies between studies.[9 10] Some studies have revealed an association of one particular single nucleotide polymorphism (SNP) (rs231775) in CTLA-4 with BD.[11 12] However, this association could not be confirmed by other studies.[13] In order to avoid the one-sidedness of association identified by one SNP, the present study investigated the association of four CTLA-4 SNPs (−1661A/G, rs4553808; −318C/T, rs5742909; +49G/A, rs231775; and CT60G/A, rs3087243) with BD in a cohort of Chinese patients. Additionally, a meta-analysis of the available data from the literature was also performed. The combined results failed to show any association between the currently known CTLA-4 polymorphisms with BD.

PATIENTS AND HEALTHY CONTROLS

Two hundred and twenty-eight unrelated patients were recruited from the Uveitis Study Center of Sun Yat-sen University, and the First Affiliated Hospital of Chongqing Medical University, China. All the patients fulfilled the International Study Group criteria for diagnosis of BD or the revised criteria from Behçet disease Research Committee of Japan if oral ulceration was not present.[14] Controls ($n = 207$) included age- and ethnic-matched healthy individuals. All patients and controls were Han Chinese. The clinical characteristics of the BD patients included in our study are presented in table 1. Informed consent was obtained from patients and controls. This study was approved by the Ethics Committee of Zhongshan Ophthalmic Center and the First Affiliated Hospital of Chongqing Medical University, and complied with the tenets of the Declaration of Helsinki.

DNA EXTRACTION

Peripheral blood samples were collected in EDTA tubes from all subjects and kept at −70°C until use. DNA was extracted from whole blood using standard proteinase K digestion and phenol–chloroform extraction.

CTLA-4 GENOTYPING

The CTLA-4 SNPs were examined by PCR-restriction fragment length polymorphism (RFLP) analysis with restriction endonucleases according to the method described previously.[15] Briefly, PCR products of −1661, −318 +49 and CT60 polymorphisms were respectively digested with 2 U of MseI, MseI, BstEII and NcoI restriction enzyme (MBI Fermentas, Vilnius, Lithuania) in a 10 µl reaction volume overnight. Digestion products were visualised on agarose gels of appropriate concentration

Table 1 Clinical findings of patients with Behçet disease

	Total no with Behçet disease (228)	Percentage (100%)
Male	189	82.9
Female	39	17.1
Age at onset (years (SD))	30.04 (8.95)	–
Uveitis	227	99.6
Oral ulcer	218	95.6
Genital ulcer	96	42.1
Hypopyon	52	22.8
Skin lesions	111	48.7
Positive pathergy test	80	35.1
Arthritis	60	26.3

and stained with GoldView (SBS Genetech, Beijing) (fig 1). Appropriate controls (no template and known genotype) were included in each typing run.

STATISTICAL ANALYSIS

SPSS software (version 10.0; SPSS, Chicago) was used to analyse the data. The software Haploview 3.32 was used for testing pairwise linkage disequilibrium and to estimate the haplotype frequency.[16] The default setting, confidence interval algorithm, was applied to our analysis. The allele and genotype distributions of SNPs in CTLA-4 were compared between patients and controls by χ^2 test and Fisher exact correction. Allele and genotype distributions were also analysed between patients with or without certain clinical features by χ^2 test. A p value of <0.05 was considered significant. A meta-analysis was performed using the Review Manager software package (version 4.2) (http://www.cc-ims.net/RevMan).

RESULTS

Allele and genotype frequencies of CTLA-4

All samples from 228 BD patients and 207 controls were genotyped for four SNPs in the CTLA-4 gene. The distribution of genotype frequencies of each SNP in our cohort was in Hardy–Weinberg equilibrium. Table 2 summarises the results from our study. There were no differences in the allele or genotype frequencies of the four SNPs between BD patients and controls. Furthermore, no association was observed when BD patients were subdivided according to sex, extraocular clinical features and HLA-B51 (data not shown).

Haplotype frequencies of CTLA-4

Haplotypes were calculated with the program Haploview 3.32. Four SNPs were in tight linkage disequilibrium with each other. Three haplotypes were obtained which have a frequency of more than 3% in the patient or control group (table 3). As reported previously,[15] the haplotype −1661A:−318C:+49G:CT60G was the most prevalent haplotype. There were no differences in haplotype frequencies between the patients and the controls (table 3). Stratification of patients by sex, extraocular manifestations and HLA-B51 did not reveal any correlation between these three haplotypes and subgroups of patients (data not shown).

Meta-analysis of CTLA-4 polymorphism

For a meta-analysis, we searched the Medline database and checked the reference lists of the retrieved articles for all studies which tested the association between CTLA-4 polymorphism and BD patients. Three studies were found and included in this meta-analysis. Two studies were from Turkey, and one study included Caucasian patients (table 4). The association between SNP +49 and BD patients was assessed using the data from these three studies as well as the present study. Genotype frequencies of SNP +49 in these four cohorts were in Hardy–Weinberg Equilibrium, and there was no heterogeneity among these studies ($p = 0.60$, $I^2 = 0\%$). The pooled OR was 0.98 (0.83 to 1.15) for fixed effects. The results showed no association between CTLA-4 SNP +49 and BD (overall effect: $Z = 0.26$, $p = 0.79$).

DISCUSSION

In this study, we examined the association between CTLA-4 polymorphisms and BD in a Chinese Han population. Our results showed no association between CTLA-4 SNPs and BD or between haplotypes and BD. There was also no association between CTLA-4 polymorphisms and BD after stratification by clinical features. Meta-analysis using data from four studies also showed no association between CTLA-4 SNP +49 and BD.

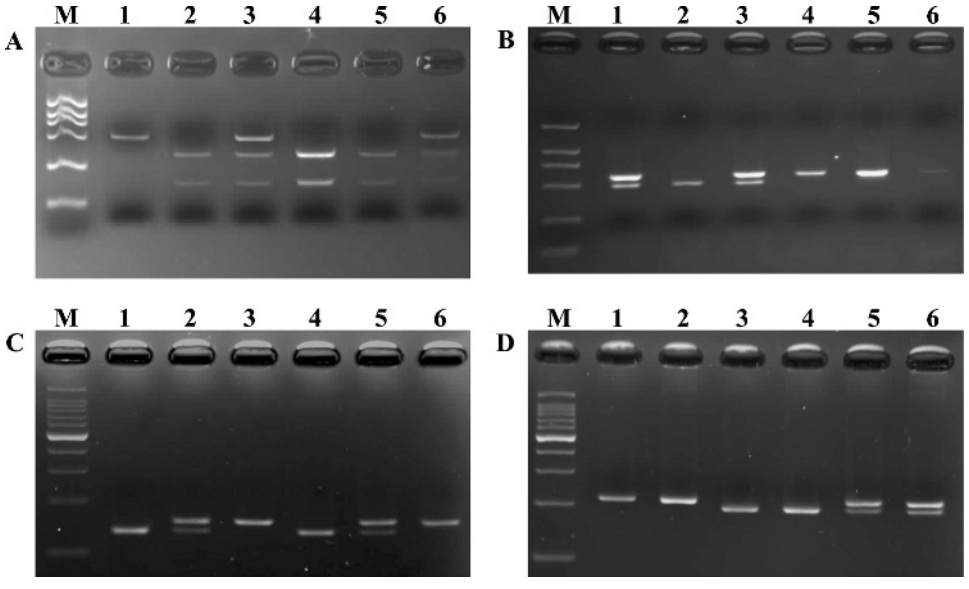

Figure 1 Example of the different genotype of four SNPs (A) −1661A/G: lane M, molecular size standard; lane 1, genotype GG (485 bp); lanes 2, 4 and 5, genotype AA (322 bp and 163 bp); and lanes 3 and 6, genotype AG (485 bp, 322 bp and 163 bp). (B) −318C/T: lanes 1 and 3, genotype CT (577 bp and 483 bp); lane 2, genotype TT (483 bp); and lanes 4, 5 and 6, genotype CC (577 bp). (C) CT60G/A: lanes 1 and 4, genotype AA (196 bp); lanes 2 and 5, genotype AG (216 bp and 196 bp); and lanes 3 and 6, genotype GG (216 bp). (D) +49G/A: lanes 3 and 4, genotype AA (130 bp); lanes 5 and 6, genotype AG (152 bp and 130 bp); and lanes 1 and 2, genotype GG (152 bp).

Table 2 Allele and genotype frequencies of cytotoxic T lymphocyte-associated antigen 4 polymorphisms in Han Chinese Behçet disease patients and controls

Single nucleotide polymorphism	Alleles and genotypes	Behçet disease no (%)	Controls no (%)	p Value	Odds ratio (95% CI)
−1661A/G	A	388 (85.8)	352 (85.9)	0.996	0.999 (0.681 to 1.466)
	G	64 (14.2)	58 (14.1)		1
	AA	165 (73.0)	149 (72.7)	0.935	1.031 (0.669 to 1.588)
	AG	58 (25.7)	54 (26.3)		1
	GG	3 (1.3)	2 (1.0)		1.397 (0.225 to 8.681)
−318C/T	C	390 (86.3)	352 (85.4)	0.721	1.072 (0.731 to 1.573)
	T	62 (13.7)	60 (14.6)		1
	CC	166 (73.4)	149 (72.3)	0.846	1.071 (0.731 to 1.573)
	CT	58 (25.7)	54 (26.2)		1
	TT	2 (0.9)	3 (1.5)		0.645 (0.104 to 3.998)
+49G/A	A	152 (33.3)	149 (36.2)	0.381	0.883 (0.667 to 1.168)
	G	304 (66.7)	263 (63.8)		1
	AA	20 (8.8)	26 (12.6)	0.429	0.666 (0.350 to 1.268)
	GA	112 (49.1)	97 (47.1)		1
	GG	96 (42.1)	83 (40.3)		1.002 (0.671 to 1.495)
CT60G/A	A	99 (21.7)	92 (22.2)	0.856	0.971 (0.704 to 1.338)
	G	357 (78.3)	322 (77.8)		1
	AA	9 (3.9)	11 (5.3)	0.766	0.707 (0.277 to 1.805)
	GA	81 (35.5)	70 (33.8)		1
	GG	138 (60.6)	126 (60.9)		0.947 (0.634 to 1.413)

Allele and genotypes frequencies were compared between Behçet disease and control by χ^2 tests. Single nucleotide polymorphism (SNP) −1661A/G was not identified successfully in four samples; SNP −318C/T was not identified successfully in three samples; and SNP +49G/A was not identified successfully in one sample.

All four SNPs assessed in our study have been previously shown to be associated with a variety of autoimmune diseases.[8 17 18] Sallakci *et al*[12] showed that the +49A allele and the AA genotype were significantly higher in BD patients with ocular or skin involvement. They did not find any association when comparing all BD patients with controls and concluded that CTLA-4 is rather a disease modifying than a susceptibility gene. Studies by Gunesacar *et al*[11] and Bye *et al*[13] however failed to demonstrate any correlation of CTLA-4 polymorphisms with BD subgroups that were classified according to clinical features. Our study also did not support the association between CTLA-4 polymorphisms and BD subgroup stratification by clinical features. The observed discrepancy between these studies may result from different genetic backgrounds in the populations analysed or from sampling error. Meta-analysis has been used to assess the association between gene polymorphisms and diseases in different races. Certain studies showed an association between CTLA-4 gene polymorphisms and systemic lupus erythematosus (SLE),[19] while other studies did not support these results.[20] Meta-analysis using the data from a variety of studies showed that there was an association between CTLA-4 gene polymorphism and SLE.[21] We therefore performed a meta-analysis using the data available in the literature[11–13] and those presented in our study. The results showed that there was no heterogeneity among the four studies and that CTLA-4 SNP +49 was not associated with BD. This difference in the results concerning association between CTLA-4 SNP +49 and certain autoimmune diseases may result from the different pathogenesis of these diseases. The Th1/Th2 paradigm varies significantly in different diseases. CTLA-4 has been regarded as a genetic master switch for autoimmunity and plays a critical role in the Th1/Th2 balance. Therefore, the different associations between CTLA-4 SNP +49 and autoimmune diseases may lead to the varied Th1/Th2 paradigms in different diseases.

In a recent study, we showed a significant association of the CTLA-4 haplotype −1661A:−318C:+49G:CT60G and the G allele at SNP +49 with the susceptibility to VKH syndrome, another common uveitis entity in China which is considered an autoimmune disease mediated by T cells.[15] Unexpectedly, however, no association was found between CTLA-4 polymorphisms and BD, another frequent uveitis in China. The conflicting results may be explained by the different nature of these two common autoimmune uveitis entities. VKH syndrome is a granulomatous inflammation, while BD is in fact a non-granulomatous inflammation. In addition, although Behçet disease is considered as an autoimmune disease mediated by T

Table 3 Frequency of the cytotoxic T lymphocyte-associated antigen 4 haplotypes (−1661, −318, +49 and CT60) constructed using the program Haploview 3.32

Haplotypes*	Behçet disease (%)†	Controls (%)†	χ^2‡	p Value‡	OR (95% CI)‡
ACGG	288.7 (63.3)	252.1 (60.9)	0.580	0.446	1.11 (0.846 to 1.464)
ACAA	85.3 (18.6)	80.3 (19.4)	0.066	0.797	0.96 (0.681 to 1.343)
GTAG	55.3 (12.1)	53.9 (13.0)	0.191	0.662	0.91 (0.612 to 1.366)

Haplotypes with a frequency less than 3% are not listed.
*Haplotypes were constructed from single nucleotide polymorphisms in the order of −1661A/G, −318C/T, +49G/A and CT60G/A using the Haploview 3.32 software based on an accelerated EM algorithm.
†Haplotype numbers and frequencies were estimated using the Haploview 3.32 software.
‡The percentages of Behçet disease and controls with a certain haplotype were compared with the percentages of Behçet disease and controls without this kind haplotype using the χ^2 test (2×2 table).

Table 4 Frequency of cytotoxic T lymphocyte-associated antigen 4 +49 alleles for Behçet disease patients and controls

	Behçet disease patients		Controls		
Study	**G**	**A**	**G**	**A**	**OR (95% CI)**
Sallakci *et al*[12]	33	85	63	135	0.832 (0.504 to 1.373)
Gunesacar *et al*[11]	69	177	109	249	0.891 (0.623 to 1.274)
Bye *et al*[13]	169	307	122	238	1.074 (0.805 to 1.432)
Present study	304	152	263	149	1.133 (0.857 to 1.499)

OR (95% CI), odds ratio (G allele vs A allele) 95% confidence interval.

cells, it is still proposed that it may represent an autoinflammatory condition developing in the background of enhanced innate immune reactivity.[22 23]

In conclusion, all results in our study failed to demonstrate any association of tested CTLA-4 gene polymorphisms with BD in Chinese patients.

Funding: This study is supported by the Fund for Key Project of Natural Science Foundation (30630064), National supporting project of PR China (2007BAI18B10), Clinical Key Project of Ministry of Health of PR China, Chongqing Key Laboratory of Ophthalmology (CSTC, 2008CA5003), Key project of Chongqing National Science Foundation (CSTC, 2009BA5037); Key project of Health Bureau (2008-1-15).

Competing interests: None.

Ethics approval: Ethics approval was provided by the Ethics Committee of Zhongshan Ophthalmic Center.

Patient consent: Obtained.

Provenance and peer review: Not commissioned; not externally peer reviewed.

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Br J Ophthalmol 2009 93: 1378-1381 originally published online June 30, 2009
doi: 10.1136/bjo.2008.156919