ORIGINAL ARTICLE

Cytotoxic T Lymphocyte-associated Antigen 4 Polymorphisms Correlated with Graves' Disease in Patients of Han Ethnicity in Yunnan, China

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ABSTRACT

Objective: *To investigate the correlations between polymorphisms at position 49 in exon 1 and position 318 in the promoter of the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) gene and autoimmune thyroid diseases in a Han Chinese population.*

Methods: *Polymerase chain reaction-restriction fragment length polymorphism was utilized. The MseI and BbvI restriction endonucleases were used to detect and analyse position 49 in exon 1 and position 318 in the promoter as well as the T/C alleles of the CTLA-4 gene in peripheral blood samples from 112 patients with Graves' disease (GD), 101 with Hashimoto's thyroiditis (HT) and 100 healthy individuals.*

Results: *At position 49 of exon 1, the frequencies of the GG genotype and the G allele in the GD group ($\chi^2 = 12.147$; p = 0.002) were statistically significantly higher than those in the control group ($\chi^2 = 9.925$; p = 0.002), while no statistically significant differences were found between the frequencies of the GG genotype and the G allele in the HT group ($\chi^2 = 1.195$; p = 0.550) and those in the control group ($\chi^2 = 0.984$; p = 0.321). No statistically significant differences in the promoter (-318) or the T/C alleles were observed among the three groups. Position 49 in the 17th codon of exon 1 of the CTLA-4 gene may be a candidate susceptibility marker in patients of Han ethnicity with GD.*

Conclusion: *This finding helps us to better understand the genetic risks for GD and provides a direction for targeted gene therapy.*

Keywords: Autoimmune thyroid disease, cytotoxic T lymphocyte-associated antigen 4, gene polymorphism

Polimorfismos del antígeno 4 del linfocito T citotóxico correlacionados con la enfermedad de Graves en pacientes de la etnia Han en Yunnan, China

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RESUMEN

Objetivo: *Investigar las correlaciones entre los polimorfismos en la posición 49 en el exón 1 y la posición 318 en el promotor del gen del antígeno 4 asociado al linfocito T citotóxico (CTLA-4), con las enfermedades autoinmunes de la tiroides en una población China de Han.*

Métodos: *Se utilizó la reacción en cadena de la polimerasa-polimorfismo de la longitud de los fragmentos de restricción. Las endonucleasas de restricción de MseI y BbvI se utilizaron para detectar y analizar la posición 49 en el exón 1 y la posición 318 en el promotor, así como los alelos T/C del gen CTLA-4 en muestras de sangre periférica de 112 pacientes con enfermedad de Graves (EG), 101 con tiroiditis de Hashimoto (TH) y 100 individuos sanos.*

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Resultados: *En la posición 49 de exón 1, las frecuencias del genotipo GG y el alelo G en el grupo de EG ($\chi^2 = 12.147$;* p *= 0.002) fueron estadísticamente significativamente más altas que las del grupo de control ($\chi^2 = 9.925$;* p *= 0.002), pero no se encontraron diferencias estadísticamente significativas entre las frecuencias del genotipo GG y el alelo G en el grupo de TH ($\chi^2 = 1.195$;* p *= 0.550) y las del grupo de control ($\chi^2 = 0.984$;* p *= 0.321). No se observaron diferencias estadísticamente significativas en el promotor (-318) ni en los alelos T/C entre los tres grupos. La posición 49 en el codón17.º del exón 1 del gen CTLA-4 puede ser un marcador de susceptibilidad candidato en pacientes de la etnia Han con EG.*

Conclusión: *Este hallazgo nos ayuda a comprender mejor los riesgos genéticos de la EG y ofrece una dirección para la terapia génica dirigida.*

Palabras clave: Enfermedad autoinmune de la tiroides, antígeno 4 del linfocito T citotóxico, polimorfismo genético

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INTRODUCTION

Autoimmune thyroid disease (AITD), including diffuse toxic goiter (Graves' disease, GD) and Hashimoto's thyroiditis (HT), is an organ-specific autoimmune disease. Autoimmune thyroid disease develops as a result of numerous complex genetic and environmental factors (1). In recent years, studies have shown that cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) plays an important role in the genetic susceptibility to GD (2–5).

Cytotoxic T lymphocyte-associated antigen 4, also known as CD152, is an important co-stimulatory transmembrane surface receptor on T cells. In immune responses, two types of signals are required for the activation of T cells. One signal involves the presentation of antigenic peptide-human leukocyte antigen complexes by antigen-presenting cells and recognition by specific T cell receptors; subsequently, the signal is passed to the T cells. The other signal is the co-stimulatory signal, which is generated by stimulatory molecules on antigen-presenting cells that bind to the corresponding specific receptors on the surface of T cells. If such a signal is insufficient, T cell activation will be impaired, leading to a non-reactive state or even apoptosis; conversely, excessive stimulation can activate auto-reactive T cells, leading to autoimmune disease. B7:CD28/CTLA-4 is the most important co-stimulatory pathway in this process (6–8). Cytotoxic T lymphocyte-associated antigen 4 shares the B7 ligand with CD28; CD28 recognizes B7-1 and B7-2 to provide positive co-stimulatory signals for T cells, but CTLA-4 binding to B7 negatively regulates T cell activation (7–9).

Various groups have studied the correlation between CTLA-4 gene polymorphisms and AITD. Kucharska *et al* found that the GG genotype at position 49 of CTLA-4 was statistically significantly more prevalent among Caucasian children from Poland with HT than among those in the control group, and that anti-Tg Ab titers were higher in patients who were homozygous for the G allele than in those with the AA genotype (3). Jin *et al* reported that in Chinese patients with Type 1 diabetes mellitus (T1DM) complicated with thyroid autoimmunity, the frequencies of both GG genotype and the G allele at position 49 of CTLA-4 were statistically significantly higher than in healthy controls (2). Zaletel *et al* found that genotypes containing the G allele were associated with a statistically significantly higher frequency of TPOAb and TgAb positivity compared with the AA genotype in Caucasian patients with AITD (10). In contrast, Pastuszak-Lewandoska *et al* reported no statistically significant difference in the frequency of the GG genotype at position 49 of CTLA-4 between patients with HT and GD and the control group in Poland; however, the CC genotype was statistically significantly less frequent in patients with HT and GD compared with the control group, suggesting that the AA genotype may be protective against HT and GD (11).

Therefore, we investigated the A/G polymorphism at position 49 in the 17th codon of exon 1 and the T/C polymorphism at position -318 of the promoter of the CTLA-4 gene in patients with GD and HT to explore further the correlation between CTLA-4 gene polymorphisms and AITD.

SUBJECTS AND METHODS

Patients with GD and HT seen at the Department of Endocrinology at Yan'an Affiliated Hospital of Kunming Medical University, China, from September 2012 to September 2014 were randomly selected for inclusion in

this study. After excluding patients with T1DM, systemic lupus erythematosus or other autoimmune diseases, 112 patients with GD (37 males and 75 females) and 101 patients with HT (40 males and 61 females) were enrolled. The diagnosis was made according to clinical manifestations, physical examination and laboratory tests (thyroid function test and thyroid ultrasound). The healthy control group included 100 patients (35 males and 65 females) from the medical centre of the Yan'an Affiliated Hospital of Kunming Medical University; these patients had no family history of thyroid disease or other autoimmune disease. All subjects were of Han ethnicity from the Yunnan region, China. This study was approved by the Ethics Committee of the Yan'an Affiliated Hospital of Kunming Medical University. All participants signed an informed consent form.

Detection of CTLA-4 gene polymorphisms

Sample collection and DNA extraction

A total of 2 ml of fasting blood (fasting for 12 hours) was collected from each subject into ethylenediaminetetraacetic acid anticoagulant tubes and stored at -80°C. Genomic DNA was extracted from blood samples using an AxyPrep blood genomic DNA extraction kit and stored at -80°C.

Target fragment processing via *polymerase chain reaction*

The primers were synthesized by Shanghai Biological Engineering Co Ltd, and the primer sequences were as follows: exon 1 (upstream primer: 5'-GCTCTACTTCCTGAAGACCT-3'; downstream primer: 5'-AGTCTCACTCACCTTTGCAG-3'; amplified fragment length: 162 bp) and promoter (upstream primer: 5'-AAATGAATTGGACTGGATGGT-3'; downstreamprimer: 5'-TTACGAGAAAGGAAGCCGTG-3'). The genomic DNA stock solution was diluted to 5–10 ng/L with the DNA solution for polymerase chain reaction (PCR). The total PCR volume was 15 µl, including 1.5 µl of 10× Taq amplification buffer, 0.3 µl of a mixture of the 4 dNTPs (2.5 mM each), 0.2 µl of the upstream primer, 0.2 µl of the downstream primer, 0.5 µl of template DNA, 0.1 µl of Taq DNA polymerase (5 U/µl), 1.2 µl of $MgCl_2$, and 11 µl of double-distilled water. The reactions were performed as follows: pre-denaturation at 95°C for five minutes; 20 cycles of denaturation at 95°C for 30 seconds, annealing at 67.5°C for 45 seconds, and extension at 72°C for 60 seconds; 20 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 40 seconds; and extension at 72°C for six minutes.

Detection of CTLA-4 gene polymorphisms using restriction endonucleases

After the PCR amplification products had been incubated with the BbvI restriction endonuclease at 37°C for 24 hours, the restriction fragments were analysed *via* 3% agarose gel electrophoresis. A band at 162 bp indicated the homozygous AA genotype; three bands at 162, 88 and 74 bp indicated the heterozygous AG genotype; and two bands at 88 and 74 bp indicated the homozygous GG genotype (12).

The PCR amplification products were incubated with the MseI restriction endonuclease at 37°C for 24 hours and then separated *via* 3% agarose gel electrophoresis. Cytotoxic T lymphocyte-associated antigen 4 (318) C/T has three genotypes: CC, CT and TT. The homozygous CC genotype produces two bands, at 226 and 21 bp. The heterozygous CT genotype produces four bands, at 226, 130, 96 and 21 bp. The homozygous TT genotype produces three bands, at 130, 96 and 21 bp; however, the small 21 bp fragment is difficult to recognize (13).

Statistical analysis

The frequency of genotypes and the distribution of alleles in each group were evaluated *via* the Hardy-Weinberg genetic equilibrium test. Measurement data with normal distributions were expressed as the mean ± standard deviation (X ± S); comparisons between two groups of measurement data were performed using the t test; and categorical data were compared using the Chi-square test. Statistical data analysis was performed with SPSS 21.0 statistical software, and $p < 0.05$ indicated a statistically significant difference.

RESULTS

Comparison of general information

No statistically significant differences in age or gender were found among the GD, HT and control groups ($p > 0.05$) (Tables 1 and 2).

Table 1: General information of the Graves' disease and control groups

	Graves' disease group	Control group	*p* value
Age	28.2 ± 6.3	28.9 ± 6.4	0.428
Male/Female	37/75	35/65	0.763

Table 2: General information of the Hashimoto's thyroiditis and control groups

	Hashimoto's thyroiditis group	Control group	*p* value
Age	27.9 ± 4.2	28.9 ± 6.4	0.207
Male/Female	40/61	35/65	0.500

Comparison of the genotype and allele distribution frequencies at position 49 of exon 1 of the CTLA-4 gene

The frequencies of each genotype at position 49 of exon 1 of CTLA-4 in the GD, HT and control groups were evaluated using the Hardy-Weinberg equilibrium test. There were no statistically significant differences between the actual values and the theoretical values calculated based on the Hardy-Weinberg law ($\chi^2 = 0.013$, $p = 0.908$; $\chi^2 = 2.659$, $p = 0.103$; $\chi^2 = 3.819$, $p = 0.051$). The frequencies of the three genotypes were in accordance with Hardy-Weinberg equilibrium, and the distribution of alleles exhibited specific group representation.

The frequencies of the GG genotype and the G allele at position 49 of exon 1 of the CTLA-4 gene were statistically significantly higher in the GD group ($\chi^2 = 12.147$, $p = 0.002$) than in the control group ($\chi^2 = 9.925$, $p = 0.002$), whereas the frequencies of the homozygous AA genotype and the A allele were statistically significantly lower in the GD group than in the control group (Table 3).

There were no statistically significant differences in the frequencies of the GG genotype and the G allele between the HT and control groups ($\chi^2 = 1.195$, $p = 0.550$; $\chi^2 = 0.984$, $p = 0.321$) (Table 4).

Comparison of the genotype and allele distribution frequencies at position 318 of the promoter of the CTLA-4 gene

The genotype frequencies at position 318 of the CTLA-4 promoter in the GD, HT and control groups were evaluated using the Hardy-Weinberg equilibrium test, which indicated no statistically significant differences between the actual values and the theoretical values calculated based on the Hardy-Weinberg law ($\chi^2 = 1.466$, $p = 0.226$; $\chi^2 = 2.204$, $p = 0.138$; $\chi^2 = 0.978$, $p = 0.323$). The frequencies of the three genotypes were all in accordance with Hardy-Weinberg equilibrium, and the distribution of alleles exhibited specific group representation. There were no statistically significant differences in genotype frequencies among the GD, HT and control groups (Tables 5 and 6).

Table 3: Genotype and allele distribution frequency analysis of position 49 of exon 1 of the CTLA-4 gene in the Graves' disease and control groups

Genotype	Graves' disease group	Control group	χ^2	*p* value	Odds ratio	95% confidence interval
Genotype			12.147	0.002		
AA	2 (1.79%)	3 (3.00%)				
GG	83 (74.11%)	51 (51.00%)				
AG	27 (24.10%)	46 (46.00%)				
Allele frequency			9.925	0.002	0.457	0.279, 0.749
A	31 (13.84%)	52 (26.00%)				
G	193 (86.16%)	148 (74.00%)				

Table 4: Genotype and allele distribution frequency analysis of position 49 of exon 1 of the CTLA-4 gene in the Hashimoto's thyroiditis and control groups

Genotype	Hashimoto's thyroiditis group	Control group	χ^2	*p* value	Odds ratio	95% confidence interval
Genotype			1.195	0.550		
AA	2 (1.98%)	3 (3.00%)				
GG	59 (58.42%)	51 (51.00%)				
AG	40 (39.60%)	46 (46.00%)				
Allele frequency			0.984	0.321	0.793	0.500, 1.255
A	44 (21.78%)	52 (26.00%)				
G	158 (78.22%)	148 (74.00%)				

Table 5: Genotype and allele distribution frequency analysis of position 318 of the CTLA-4 promoter in the Graves' disease and control groups

Genotype	Graves' disease group	Control group	χ^2	*p* value	Odds ratio	95% confidence interval
Genotype			0.086	0.770		
TT	0 (0%)	0 (3.00%)				
CC	89 (79.46%)	82 (82%)				
CT	23 (20.54%)	18 (18%)				
Allele frequency			0.194	0.659	1.157	0.605, 2.213
T	23 (10.27%)	18 (9.00%)				
C	201 (89.73%)	182 (91.00%)				

Table 6: Genotype and allele distribution frequency analysis of position 318 of the CTLA-4 promoter in the Hashimoto's thyroiditis and control groups

Genotype	Hashimoto's thyroiditis group	Control group	χ^2	*p* value	Odds ratio	95% confidence interval
Genotype			1.338	0.247		
TT	0 (0%)	0 (3.00%)				
CC	75 (74.26%)	82 (82%)				
CT	26 (25.74%)	18 (18%)				
Allele frequency			1.545	0.214	1.494	0.791, 2.821
T	26 (12.87%)	18 (9.00%)				
C	176 (87.13%)	182 (91.00%)				

DISCUSSION

The CTLA-4 gene is located at 2q31-33 and includes four exons and three introns. Position 49 within the 17th codon of exon 1 represents a site of CTLA-4 gene polymorphism; the other sites are located at position +1822 in intron 1 (C/T allele) (14, 15), position -318 in the promoter (T/C allele) (16), and position +642 in the 3' untranslated region ($(AT)_n$ variable repeats; exon 4) (17, 18).

Domestic and foreign findings vary regarding the correlation between the polymorphism at position 49 of the 17th codon of exon 1 of the CTLA-4 gene and AITD. In this study, we detected and analysed the genotype at position 49 of the 17th codon of exon 1 of the CTLA-4 gene in peripheral blood samples from 112 patients with GD and 101 with HT as well as 100 healthy individuals, and the results showed that the frequencies of the GG genotype and the G allele were statistically significantly higher in the GD group ($\chi^2 = 12.147$, $p = 0.002$) than in the control group ($\chi^2 = 9.925$, $p = 0.002$), whereas there were no statistically significant differences in the frequencies of the GG genotype and the G allele between the HT group ($\chi^2 = 1.195$, $p = 0.550$) and the control group ($\chi^2 = 0.984$, $p = 0.321$). This finding indicates that the risk of developing GD was higher for individuals carrying the GG genotype at position 49 of the 17th codon of exon 1 of the CTLA-4 gene, which is consistent with the findings of most scholars (2, 19), but not with those of Pastuszak-Lewandoska *et al* (11). The discrepant results from different regions and ethnic groups may be due to sampling error or racial differences.

The correlation between CTLA-4 gene polymorphisms and an increased risk for GD may exist because the different genotypes at position 49 of the 17th codon of exon 1 of CTLA-4 influence the expression and function of the CTLA-4 protein, leading to differences in individual susceptibility to autoimmune diseases. Further analysis of the CTLA-4 sequence revealed that a mononucleotide polymorphism at position 49 of the 17th codon of exon 1 may lead to threonine or alanine substitution at this position during protein translation, and such a substitution may cause hypo-expression and reduced glycosylation of the CTLA-4 protein (12). Moreover, some studies have found that the G allele polymorphism at this position could cause increased titers of thyroid antibodies, thyroglobulin, and thyroid peroxidase antibodies (10, 20). Linkage disequilibrium with other single nucleotide polymorphisms can also affect CTLA-4 mRNA stability. Furthermore, some studies have indicated that the G allele could reduce CTLA-4 expression and increase self-reactive T cell proliferation; after stimulation, higher CTLA-4 mRNA

and protein expression was observed in association with the AA genotype compared with the GG genotype (2, 3).

This study has some limitations. Firstly, this is a novel observation that requires confirmation in additional studies with more patients and control subjects. Secondly, functional studies are needed to elucidate the involvement of CTLA-4 in GD complications.

This study shows that CTLA-4 played an important role in GD. However, to elucidate the effect of CTLA-4 on the pathogenesis of GD, more in-depth studies are needed to investigate its genetic linkage and protein expression as well as its role in immunity.

AUTHORS' NOTE

YP Wang devised the study plan and led the writing of the article. BK Peng and LQ Zhu conducted the experiment and collected the data. XC Weng and Y Wang conducted the analysis. Z Tang supervised the whole process and gave constructive advice. This article was approved by all authors. The authors declare no conflicts of interest.

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