Association of cytotoxic T-lymphocyte antigen 4 gene with immune thrombocytopenia in Chinese Han children

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ABSTRACT

Objectives: To investigate the association of cytotoxic T lymphocyte-associated antigen 4 (CTLA4) with immune thrombocytopenia (ITP).

Methods: A case–control association analysis of 277 Chinese Han children was performed. The tagging variants rs11571315 and rs3087243 in the CTLA4 gene were detected using polymerase chain reaction-restriction fragment length polymorphism method. The expression quantitative trait loci (eQTL) analysis and quantitative real-time polymerase chain reaction were performed to determine the relationship of CTLA4 with ITP.

Results: Neither SNP was significantly different between case and control groups in either the genotypic or allelic distribution. The eQTL analysis results indicated that in the spleen, the rs3087243 was significantly different with the expression of CTLA4. The rs11571315 has similar results. Interestingly, the transcript level of CTLA4 was found to significantly decrease in patients with ITP.

Discussion: The autoimmune gene etiology is implicated in the pathogen of ITP. The CTLA4 is important for negative regulation of T-cell activation, and CTLA-4 gene has been identified as a risk factor for some autoimmune diseases. However, association studies of ITP and CTLA4 gene have obtained conflicting results. This is the first study to systematically investigate the association of CTLA4 with ITP in Chinese Han children.

Conclusions: The CTLA4 gene is suggested to correlate with ITP through its abnormal expression level instead of gene site mutation.

Introduction

Immune thrombocytopenia (ITP), one of the most common hematologic disorders in children, is an acquired organ-specific autoimmune hemorrhagic disease resulting in bleeding and a mortality rate of about 4% per year [1]. The clinical features of ITP are decreased platelet counts in the absence of any obvious cause and emerging of anti-platelet autoantibodies which mediate platelet destruction [2]. ITP is usually chronic in adults, while in children, most cases are acute with short duration and spontaneous remission occurring in six months. About 20% of children acute ITP cases become chronic (>six months) [3]. To date, the etiology of ITP is not clear yet. Although autoreactive B lymphocytes are viewed as the primary immunologic defect, dysfunction of T cells plays important roles in the pathophysiology of ITP based on the following evidences: 1. Autoantibody production in ITP is associated with both T-B cognate interaction and T-cell activation; 2. Abnormal cytotoxic T cells and regulatory T cells (Tregs) have been characterized in ITP; 3. Patients with ITP have enhanced T-cell reactivity against platelets [4–7]. Thus, antibody-mediated as well as T-cell mediated platelet destruction may occur in ITP [8,9].

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is a co-inhibitory molecule expressed by activated cytotoxic T cells, and also a specific surface marker of Treg. The CTLA4 gene, located on chromosome 2q33.2 between CD28 and ICOS genes, contains four exons encoding the cytoplasmic domain, transmembrane domain, ligand-binding domain, and leading peptide. CTLA-4 can compete with CD28 to bind to common ligands B7 with higher affinities than CD28 on antigen-presenting cells [10]. CTLA-4 has important independent inhibiting properties in maintaining homeostasis of inflammatory reactions, and the absence of CTLA4 precipitates lethal T-cell hyperactivity. Thus, it is considered as a potential candidate gene in the pathogenesis of autoimmune diseases. Abnormal expression of CTLA-4 has been found in
many autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [11,12]. In ITP patients, the CTLA4-immunoglobulin (CTLA4-Ig) can induce platelet-specific T-cell anergy [13]. Recently, several studies have studied the association of different CTLA4 variants with ITP, however, conflicting results are obtained [2,14,15].

In order to investigate the associations of the CTLA-4 gene polymorphisms with ITP, we explored the CTLA4 variants using 1000 genome data and performed a case–control association analysis with selected tagging SNPs in Chinese children. Furthermore, the quantitative real-time polymerase chain reaction (qRT-PCR)-based transcription level and eQTL of CTLA4 gene was analyzed between ITP cases and healthy controls.

Materials and methods

Study subjects

A total of 102 pediatric patients who were newly diagnosed treatment-naïve patients with ITP and 175 healthy controls were included in this study. The diagnosis of ITP was based on classic clinical and laboratory features defined in the third version of Diagnostic and therapeutic criteria for hematopathy. Briefly, the criteria for ITP have purpura, platelet count <100 × 10^9/l and no evidence of underlying disorder except the only hematological abnormality. Thrombocytopenia that resolves within 6 months was considered as acute ITP, persistence of thrombocytopenia for longer than 6 months was considered as chronic ITP. The characteristics of the subjects are listed in Table 1. The ethics committee of the first clinical medical college of Lanzhou university approved the study and informed consent was obtained from the patients’ legal guardians.

Genomic DNA Extraction

Five milliliter peripheral blood samples were collected from participants in vacuum tubes containing 5% EDTA. Genomic DNA was isolated and stored using the genomic DNA purification kit (Tiangen Biotech, Beijing, China) according to the instructions [16,17].

SNP genotyping

The tagging SNPs were selected based on the (LD) structure of the CTLA4 gene. The SNPs located within 2 kb upstream of the 5’ untranslated region and 2 kb downstream of the 3’ untranslated region of CTLA4 gene from 1000 genomes SNP databases (http://www.1000genomes.org) were used to construct the CTLA4 LD structure. Furthermore, these SNPs have a minor allele frequency (MAF) ≥ 0.05 and r^2 ≥ 0.80 in the Chinese Han population. The CTLA4 LD structure is shown in Figure 1. The SNPs rs11571315 and rs3087243 were sorted out for the genotyping.

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) detection method was used to genotype rs11571315 and rs3087243. The primers for PCR and restriction enzymes are listed in Table 2. The 80 µl PCR mixture consisting of 50–150 ng of genomic DNA, 10 pM of each primer and Premix Taq (Takara, Dalian, China) was processed under 94°C for 5 min, 35 cycles including 94°C for 30 s, annealing temperature for each SNP for 30 s and 72°C for 1 min; and then 72°C for 10 min. Cleavage was performed with restriction enzymes (New England BioLabs, Ipswich, MA, U.S.A.) according to the corresponding protocols. Digested fragments were separated on 2% agarose gels. At the end of electrophoresis, the bands were visualized by ethidium bromide staining and photographed under an ultraviolet transilluminator. To confirm the genotyping results, PCR products with special band patterns were purified for the Sanger sequencing.

Clinical tests

Platelet count was performed by manual optical microscopy on the whole blood.

Platelets (10^9) was attached to the bottom of each well of 96-well plate in a fixed dose. Enzyme-linked immunosorbent assay method was used to measure PAIgG by the corresponding kit according to the protocol (Sun Bio-tech, Shanghai, China). The content of PAIgG is calculated according to the following formula:

\[
\text{PAIgG} = \frac{\text{lg} G \mu g / ml}{\text{Platelets} \times 10^9 / \mu l} \times 1000. 
\]

QTL analysis and qRT-PCR of CTLA4

The genotype-tissue expression Portal (GTEx) (https://gtexportal.org/home/) was used to analyze the eQTL in CTLA4 gene. Now, the GTEx contains the gene expression and genotype data of 53 normal human tissues from 544 donors. Furthermore, the peripheral blood mononuclear cells (PBMCs) from each subject were isolated using Lympholyte H (Cedarlane Laboratories, Burlington, Canada). RNA was extracted and reverse transcribed into complementary DNA (cDNA). Then, the 384-well plate TaqMan real-time PCR (Thermo Fisher Scientific, Boston, U.S.A.) was used to determine the transcription level of full length CTLA4.
with an expression of β-actin as an internal standard. 
The primers for qRT-PCR were following: 5′-CATGGA-
CACGGGACTCTACAT-3′, 5′-GCACGGTTCTGGATCAT-
TACATA-3′. Cycling reaction was following: 95°C for
15 s, 60°C for 30 s and 72°C for 30 s. There are triplicate
for each reaction. The threshold cycle (CT) is de
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xed threshold. The expression of target
genes was normalized to that of the internal standard
gene (β-actin), and mRNA expression level was calcu-
lated with the 2^−ΔΔCt method.

Statistical analysis

The difference in the characteristics or transcription
level of CTLA4 between ITP cases and healthy controls
was detected using a student T-test. Hardy-Weinberg
equilibrium (HWE) for each of tagging SNPs was
con
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rmed using Fisher’s exact test. Chi-square test
was used to detect the difference of the genotypic or
allelic distribution between ITP cases and healthy con-
trols. Odds ratios (OR) and 95% confidence interval (CI)
were calculated. p < 0.05 was considered as statistical
significance. All statistical analyses were completed
using the R software version 3.1.3.

Results

The 16 SNPs in CTLA4 from 103 Chinese Han subjects of
1000 genomes project database had a MAF ≧0.05 and
r^2≧0.80. The clinical characteristics of all 277 subjects are pre-
sented in Table 2. Among them, 102 ITP patients and
175 healthy controls had no signi
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age and gender. However, the mean levels of platelet
were signi
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cantly increased in ITP patients than healthy controls
(p<0.0001 and p<0.0001, respectively).

Table 2. Clinical characteristic of ITP patients and healthy
controls.

| Items                        | Cases (n = 102) | Controls (n = 175) | p value |
|------------------------------|---------------|-------------------|---------|
| Age (year)                   | 5.43 ± 3.21   | 6.06 ± 3.77       | 0.16    |
| Males (%)                    | 30 (29.41)    | 52 (29.7)         | 0.95    |
| Platelet (×10^9/l)           | 11.74 ± 3.96  | 194.52 ± 27.59    | <0.0001 |
| The number of Acute ITP      | 78            | 0                 |         |
| The number of Chronic ITP    | 24            | 0                 |         |
| PAIgG, ng/10^7 platelet      | 94.15 ± 8.2   | 37.36 ± 3.7       | <0.0001 |

Figure 1. CTLA4 linkage blocks constructed by 16 SNPs from 1 the 1000 genomes project database. These SNPs have a MAF ≧0.05 and r^2≧0.80.
0.40 for rs3087243 and 0.32 for rs11571315, respectively).

The eQTL analysis results indicated that both rs3087243 and rs11571315 affect the expression of CTLA4 (Figure 2). In spleen, the allele A of rs3087243 can significantly increase the expression of CTLA4 ($p = 2.6E^{-3}$). Thus, we compared the expression level of CTLA4 gene between the ITP patients and healthy controls and found that CTLA4 gene has a lower transcription level in cases than in healthy controls ($p = 0.0019$, Figure 3).

**Discussion**

ITP is an acquired autoimmune disorder resulting from the interaction of genes and environmental factors. Identification of risk genes for ITP is important for understanding the etiology of this disease. Although over-production of anti-platelet autoantibodies is one of the characters of ITP, accumulating evidences support that T cells mediate the pathophysiology of this disease. It is estimated that one-third of patients with no detectable anti-platelet antibodies can harbor platelet and megakaryocyte-specific T cells involved in the development of ITP while two-thirds of patients with ITP have detectable antiplatelet antibodies produced by B cells [18]. However, CD20+ B-cell depletion therapy was found to suppress murine CD8+ T-cell-mediated ITP, which suggests that the direct interaction between B cells and T cells is essential for ITP induction [18]. The CTLA-4 has two main isoforms, a full-length and a soluble CTLA-4 without a transmembrane domain [19]. CTLA-4 is an important costimulatory molecule, which has suppressive function of activated T cells by transmitting the inhibitory signal. CTLA4−/− mice have a severe lymphoproliferative

| SNPs       | Genotypes | Cases (%) | Con (%) | X2   | p     | Alleles | Cases (%) | Con (%) | X2   | p     | OR (95%CI) |
|------------|-----------|-----------|---------|------|-------|---------|-----------|---------|------|-------|------------|
| rs11571315 | GG        | 36(35.29) | 68(38.86) | 1.03 | 0.31  | G       | 122(39.80) | 224(44.00) | 0.97 | 0.33  | ref.       |
|            | GA        | 50(49.02) | 88(50.29) |      |       | A       | 82(40.20)  | 126(36.00) |      |       | 1.20(0.84-1.7) |
|            | AA        | 16(15.69) | 19(10.86) |      |       |         |           |         |      |       |            |
| rs3087243  | GG        | 51(50.00) | 95(54.29) | 0.78 | 0.37  | G       | 146(71.57) | 262(74.86) | 0.72 | 0.4   | ref.       |
|            | GA        | 44(43.14) | 72(41.14) |      |       | A       | 58(28.43)  | 88(25.14)  |      |       | 1.18(0.57-1.24) |
|            | AA        | 7(6.86)   | 8(4.57)   |      |       |         |           |         |      |       |            |

OR, Odds ratios; CI, Confidence interval.

**Figure 2.** eQTL analysis of CTLA4 gene from the GTEx database. (A) rs11571315; (B) rs3087243.
disorder with massive autoimmune tissue destruction [20]. The CTLA-4 also has decreased expression in an animal model of autoimmune diabetes, i.e. nonobese diabetic mice. Therefore, CTLA4 is a candidate gene for the risk of ITP [21]. However, the genetic association studies of CTLA4 and ITP have obtained the conflicting results.

To our knowledge, the current study is the first one to explore CTLA4 gene structure and systematically and efficiently study tagging SNPs with ITP in the Chinese Han children. Two tagging SNPs of rs3087243 and rs11571315 were selected based on the LD structure of CTLA4 gene. Then, we used PCR-RFLP to genotype these two tag SNPs in 102 cases and 175 healthy controls. Furthermore, through a case–control association analysis of the two SNPs with ITP, we found that both variants were not significantly associated with ITP within current samples. These results are consistent with the Li’s study, which indicated that the rs3087243 is not associated with susceptibility to ITP in a Chinese population [14]. However, in a Japanese population study, the rs3087243 was found to be significantly associated with susceptibility to chronic ITP [22]. The controversial results obtained may be due to the different ethnic population studied.

Both tagging SNPs of CTLA4 can modulate CTLA4 gene expression in multiple tissues based on the GTEx database. Interestingly, in spleen, rs3087243 can significantly affect the expression of CTLA4. Spleen is a source of antiplatelet antibody synthesis and the primary site of platelet destruction. Both immune white pulp and phagocytic red pulp in the spleen contribute to the pathogenesis of ITP [23]. Thus, surgical splenectomy in patients with ITP was performed about a century ago. And it is necessary to measure the expression level of CTLA4 in patients with ITP.

We found that patients with ITP have a significant lower transcription level than healthy controls. This result agrees with a previous study which reports decreased level of CTLA4 in patients with acute ITP and suggests that CTLA4 could be an evaluation indicator for the treatment efficiency of acute ITP patients [2]. It is widely considered that immune dysregulation is involved in the pathogenesis of ITP. CTLA4 is a specific regulatory T cells (Tregs), whose low expression means decreased Tregs. Similar result has been found in a recent study that pre-treated ITP patients present low levels of Tregs and NK cells [24]. Since the tagging SNPs in CTLA4 are not associated with ITP, the dysregulation of CTLA4 gene expression in ITP may not be driven by CTLA4 gene site mutation. The dysregulation of CTLA4 gene expression in ITP may be driven by some environmental factors, such as viral infection. Thus, CTLA4 gene expression instead of gene mutation is one of the factors for ITP.

In conclusion, the CTLA4 gene is not found involved in the genetic predisposition to the ITP in Chinese Han children, while it is found that decreased expression of CTLA4 may be involved in the pathogenesis of ITP. These results support that ITP may not mainly be driven by genetic factors but by environmental factors. Further model studies on the CTLA4 are required to gain more insights on the effects of CTLA4 on ITP.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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