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*Original Article*

# Cytotoxic T lymphocyte-associated antigen-4 gene polymorphisms and biliary atresia susceptibility in Chinese children

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**Abstract:** Biliary atresia (BA) is a devastating liver disease of complex pathogenesis in neonates, characterized by an inflammatory and fibrosing obstruction of extrahepatic bile ducts. Cytotoxic T lymphocyte-associated antigen-4 (CTLA4) is expressed on the surface of a subset of regulatory T cells (Treg) and down regulates the human immune response. To investigate the possible association between CTLA4 gene polymorphisms and BA susceptibility, we conducted a case-control study in the Chinese children. Three single nucleotide polymorphisms (SNPs) in the CLTA4 gene (rs231725, rs231775 and rs3087243) were genotyped in 113 BA patients and 133 healthy controls. The statistical analysis revealed no significant difference between BA patients and healthy controls in allele or genotype frequencies (rs231725, P = 0.2718, OR = 0.814, 95% CI = 0.564-1.175; rs231775, P = 0.1599, OR = 1.316, 95% CI = 0.897-1.931; rs3087243, P = 0.0572, OR = 1.582, 95% CI = 0.984-2.543), neither in the distribution of haplotypes of these CTLA4 gene SNPs. The result of our study is the first one to provide the evidence that there is no significant association between CLTA4 gene polymorphisms and BA susceptibility in Chinese children.

**Keywords:** Biliary atresia, cytotoxic T lymphocyte-associated antigen-4, single nucleotide polymorphism

## Introduction

Biliary atresia (BA) is a major cause of liver transplantation in children. Characterized by progressive obliteration of the extrahepatic biliary system and fibrosis in liver, it will lead to fatal consequences if there is no prompt and proper treatment [1, 2]. The incidence of BA varies geographically and has a slight female predominance, while in Asia it is estimated to be 1 in 5000, higher than in western countries [2, 3]. Although surgical treatment such as Kasai hepatoportoenterostomy can remove the bile duct remnants and improve short-term outcome, many children suffered liver disease progression to end-stage cirrhosis, which indicates a multifaceted etiology of this disease [4].

In the past decades, many relative hypotheses of BA etiology have been established, which can be summarized to genetic causes, environmental factors and immune dysregulation [1, 3, 5]. Recently, studies investigating single nucleotide polymorphisms (SNPs) with BA susceptibility are gaining much more focus. Several genes have been discovered a significant association with BA, including adiponectin, adducin 3 (ADD3), X-prolyl aminopeptidase P1 (XPNPEP1) and intercellular adhesion molecule-1 (ICAM-1), showing that genetic factors may play important rules in BA etiology [1, 2, 5-7].

As a promising candidate, the CTLA4 gene has been studied for genetic susceptibility to many autoimmune diseases. Cytotoxic T lymphocyte-associated antigen-4 (CTLA4) is a protein receptor that plays an important role in immune regulation by down-regulating human T lymphocyte immune response [8]. Constitutively expressed on a subset of regulatory T cells (Treg), CTLA4 plays a distinct role in Treg generation, function and homeostasis [9-11]. In BA, deficits in Treg quantity and/or function would disrupt immunologic balance and deteriorate bile duct injury [5]. These findings suggest that CTLA4 gene may be implicated in the pathogenesis of BA.

**Table 1.** Three SNPs selected for the study

| SNP | Common name | Allele major/minor | Gene location | *P* value for HWE |
|---|---|---|---|---|
| rs231725 | - | A/G | 3'UTR | 0.935 |
| rs231775 | 49AG | G/A | Exon 1 | 0.886 |
| rs3087243 | CT60 | G/A | 3'UTR | 0.999 |

3'UTR, 3'untranslated region.

**Table 2.** Oligonucleotide sequences used for genotyping

| SNP | Primers | Sequences |
|---|---|---|
| rs231725 | First | 5'-ACGTTGGATGTCCCAAATTTTGCCTCCACC-3' |
| | Second | 5'-ACGTTGGATGGAGGTGAAACCAAGTATAGC-3' |
| | Extension | 5'-AACAAAAGAGGAGAAATCCA-3' |
| rs231775 | First | 5'-ACGTTGGATGATTTCAGCGGCACAAGGCTC-3' |
| | Second | 5'-ACGTTGGATGCACTCACCTTTGCAGAAGAC-3' |
| | Extension | 5'-GGAGCAGCTGAACCTGGCT-3' |
| rs3087243 | First | 5'-ACGTTGGATGTACCTGCAAGTCATTCTTGG-3' |
| | Second | 5'-ACGTTGGATGCCTGTGTTAAACAGCATGCC-3' |
| | Extension | 5'-CCACCACTATTTGGGATATAAC-3' |

Therefore, we established a case-control study to investigate the possible association between CTLA4 SNPs and BA susceptibility.

## Subjects and methods

### *Subjects*

This study was approved by the ethics committee of the Children's Hospital of Fudan University. Written informed consent was obtained from all subjects' parents or legal guardians before blood samples collection.

BA patients. For this case-control study, we analyzed a total of 113 unrelated Chinese children with BA (70 boys and 43 girls) from the Children's Hospital of Fudan University (Shanghai, China). These BA children were diagnosed by exploratory laparotomy with operative cholangiography between August 2014 and July 2015. The age (mean ± standard deviation) at the time of the operation of these patients was 68.1 ± 20.7 days (range 23-163 days).

Healthy controls. 133 unrelated healthy Chinese children (85 boys and 48 girls) from the Department of Pediatrics were recruited randomly as the healthy controls. All these healthy children were of similar age with the case group and did not show any signs of BA or other liver disease.

### *Genotyping*

Genomic DNA from patients and controls was extracted from whole blood using the TIANamp Blood DNA Kit (Tiangen, Beijing, China), according to the manufacturer's protocol. The three observed SNPs (rs231725, rs231775 and rs3087243) were selected based on previous reports [9, 10], with minor allele frequencies >5% according to the National Center for Biotechnology Information SNP Database (dbSNP) (http://www.ncbi.nlm.nih.bov/SNP/). These SNPs were all located in the CTLA4 gene, SNPs rs231725 and rs3087243 in the 3'-untranslated region (3'-UTR), and SNP rs231775 in exon 1 (**Table 1**). Primers for PCR and single-base extension were designed by the Assay Designers software, version 3.0 (Sequenom, San Diego, CA, USA), and synthesized by Benegene Biotech (Shanghai, China; **Table 2**). All genotyping was performed using the MassARRAY on a matrix-assisted laser desorption ionization-time of flight mass spectrometry platform and analyzed using the MassARRAY Typer software, version 3.4 (Sequenom). The concentration of DNA samples should be more than 10 ng/ul, and OD 260/280 should between 1.6-2.0 before the analysis. Negative and positive control samples were used as quality controls to monitor and control all the process.

### *Statistical analysis*

The Hardy-Weinberg equilibrium (HWE) test was done for each SNP for the case and control groups. The significance in allele and genotype frequencies between BA children and healthy controls was assessed using the $\chi^2$ test. A $p$ value of less than 0.05 was considered to be statistically significant. The odds ratio (OR) and 95% confidence intervals (CI) were also calculated. Those statistical analysis were performed by the SPSS 18.0 program (SPSS Inc., Chicago, IL, USA). The haplotype frequencies of CTLA4 were estimated using the Haploview 4.2 program (http://www.broad.mit.edu/mpg/haploview/).

**Table 3.** Allele frequencies of the three SNPs in the CTLA4 gene of patients with BA and control group

| Allele | | Case, n (%) | Control, n (%) | *P* value | OR (95% CI) |
|---|---|---|---|---|---|
| rs231725 | A | 137 (0.606) | 174 (0.654) | 0.2718 | 0.814 (0.564-1.175) |
| | G | 89 (0.394) | 92 (0.346) | | |
| rs231775 | A | 77 (0.341) | 75 (0.282) | 0.1599 | 1.316 (0.897-1.931) |
| | G | 149 (0.659) | 191 (0.718) | | |
| rs3087243 | A | 46 (0.204) | 37 (0.139) | 0.0572 | 1.582 (0.984-2.543) |
| | G | 180 (0.796) | 229 (0.861) | | |

OR, odds ratio; CI, confidence interval.

**Table 4.** Genotype frequencies of the three SNPs in the CTLA4 gene of patients with BA and control group

| Genotype | | Case, n (%) | Control, n (%) | *P* value | OR (95% CI) |
|---|---|---|---|---|---|
| rs231725 | AG | 59 (0.522) | 56 (0.421) | | |
| | AA | 39 (0.345) | 59 (0.444) | | |
| | GG | 15 (0.133) | 18 (0.135) | 0.2435 | |
| | AA+AG | 98 (0.867) | 115 (0.865) | 0.9525 | 1.023 (0.490-2.135) |
| rs231775 | AG | 55 (0.487) | 51 (0.383) | | |
| | AA | 11 (0.097) | 12 (0.090) | | |
| | GG | 47 (0.416) | 70 (0.526) | 0.2112 | |
| | AG+GG | 102 (0.903) | 121 (0.910) | 0.8484 | 0.92 (0.389-2.172) |
| rs3087243 | AG | 38 (0.336) | 31 (0.233) | | |
| | AA | 4 (0.035) | 3 (0.023) | | |
| | GG | 71 (0.628) | 99 (0.744) | 0.1448 | |
| | AG+GG | 109 (0.965) | 130 (0.977) | 0.5461 | 0.629 (0.138-2.871) |

OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium.

**Table 5.** CTLA4 haplotypes in patients with BA and control group

| Haplotype | Case, n (%) | Control, n (%) | *P* value |
|---|---|---|---|
| GGA | 137 (60.6) | 174 (65.4) | 0.271 |
| AAG | 46 (20.4) | 37 (13.9) | 0.057 |
| AGG | 31 (13.7) | 38 (14.3) | 0.856 |
| GGG | 12 (5.3) | 17 (6.4) | 0.611 |

## Results

In total, three SNPs in the CTLA4 gene of 246 subjects (113 BA patients and 133 healthy controls) were genotyped. The three examined SNPs in both groups were in HWE, as showed in **Table 1**. And the minor allele frequencies of all SNPs were greater than 5%. The allele and genotype frequencies of the three SNPs in the CTLA4 gene are listed in **Tables 3** and **4**. We found no significant differences between BA patients and healthy controls (rs231725, P = 0.2718, OR = 0.814, 95% CI = 0.564-1.175; rs231775, P = 0.1599, OR = 1.316, 95% CI = 0.897-1.931; rs3087243, P = 0.0572, OR = 1.582, 95% CI = 0.984-2.543). Neither when we compare the combination of the homozygous and heterozygous genotypes of the major allele with other genotypes in each of the three SNPs. Distributions of haplotypes were also observed for the three SNPs (**Table 5**), which showed no significant difference between BA patients and healthy controls, either.

## Discussion

On the basis of former findings, we proposed the hypothesis that CTLA4 polymorphisms may be associated with BA susceptibility. However, the three CTLA4 SNPs analyzed in our case-control study demonstrated no significant association with BA.

The etiology of BA is multifactorial and unclear. Among those possible explanations, it is widely acknowledged that genetic variation and immune dysregulation are likely to play strong roles [3, 5]. In recent years, studies investigating genetic variants as susceptibility factors for BA are garnering interest. Some studies discovered a significant association between BA and

SNP rs17095355 on chromosome 10q24, located between XPNPEP1 and ADD3 genes, suggesting that SNPs in XPNPEP1 and ADD3 may serve as a BA susceptibility factor or disease modifier [2, 3, 5-7]. Meanwhile, other variations such as adiponectin SNP (+276G/T, rs1501299), ITGB2 3'-UTR+145C/A and the deletion on chromosome 2q37.3 are also conferred increased susceptibility to BA [1, 12, 13]. More significant, some of these BA-susceptible genes are involved in immune regulations, which indicates that genetic polymorphisms may be associated with the development of inflammation and fibrosis in the affected liver and lead to BA susceptibility [1].

Evidenced by the infiltration of inflammatory cells in liver and the injury of bile duct, the immune dysregulation is also a central pathogenesis of BA [1, 3, 5, 14-19]. Cholangiocytes initially respond to cellular injury through proinflammatory factors and develop fibrosis progression and biliary tree obliteration when the patient's immune system is imbalanced, resulting in liver fibrosis and extrahepatic bile ducts atresia [2]. Treg is an important factor in balancing the immune response and can prevent activation of autoreactive T cells. Deficiency of Treg quantity and/or function would weaken inflammation or autoimmunity inhibition and result in an exaggerated inflammatory response leading to bile duct injury [5, 19]. Previous studies have shown that dysregulation of Tregs is present in murine BA and diminished Treg function may be implicated in the BA etiology [20-22].

CTLA4 and CD28 are two potential targets for Treg manipulation [10]. Encoded by CTLA4 gene located on human chromosome 2q33, CTLA4 is a glycoprotein receptor expressed on the surface of a subset of Treg. It is a homologous molecule of CD28 with an opposing role. The CD28 promotes T cell activation, while CTLA4 competitively binds to B7 ligands (CD80 and CD86) on the surface of antigen-presenting cells (APCs) and delivers inhibitory signals [23, 24]. Tregs capture B7 ligands from APCs, which requires the help of CTLA4 on its surface [25, 26]. In vitro and in vivo studies have shown CTLA4 mutations are associated with Treg frequency and function [11, 26, 27]. Therefore, CTLA4 is a key factor in Treg homeostasis and immune tolerance, while the abnormal expression of CTLA4 can induce autoimmune diseases [11].

Furthermore, the three CTLA4 SNPs selected in our study have been demonstrated to have an association with liver damage and some autoimmune diseases [8, 23, 24, 28-30]. The rs231725 was proved to be significantly associated with primary biliary cirrhosis (PBC) [28, 31-34]. The rs231775 (+49A/G) is related to threonine or alanine dimorphism, which can reduce the inhibitory function of CTLA4 and contributes to autoimmune diseases such as Grave's disease (GD), type 1 diabetes, PBC and systemic lupus erythematous disease [24, 28, 29, 35]. The rs3087243 (CT60) has been reported to influence the production of the soluble isoform of CTLA4 (sCTLA4, secreted by resting T cells and can suppress T-cell activation) [8, 29, 35] and significantly correlated with GD and Hashimoto's thyroiditis [23, 30, 35]. Therefore, we proposed a potential association between the three SNPs with BA susceptibility.

However, our study have some limitations. First, the small sample size may be not enough to discover positive results. Then, multiple comparisons between different subgroups such as gender and geographical regions have not been investigated. Moreover, the limited selection of SNPs in our study may be not widely cover the susceptible genes.

In conclusion, our case-control study is the first one to indicate that there is no significant association between the CTLA4 SNPs (rs231725, rs231775 and rs3087243) and BA susceptibility in Chinese children. Elucidating the role of genetic polymorphisms may provide a better understanding of the etiology and develop new therapeutic strategies both in prevention and treatment. We advocate for further studies with bigger populations, and wider range of CTLA4 SNPs on the associations between BA and CTLA4.

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## Disclosure of conflict of interest

None.

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