IVYSPRING INTERNATIONAL PUBLISHER

*International Journal of Medical Sciences*

2013; 10(3):250-254. doi: 10.7150/ijms.5511

Research Paper

# Recipient Cytotoxic T Lymphocyte Antigen 4 +49 Single-Nucleotide Polymorphism Is Not Associated with Acute Rejection after Liver Transplantation in Chinese Population

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Received: 2012.11.09; Accepted: 2013.01.17; Published: 2013.01.22

## Abstract

**Objective:** Single-nucleotide polymorphisms (SNPs) in Cytotoxic T lymphocyte antigen 4 (CTLA-4) gene have been detected and proved to associate with the incidence of rejection after transplantation. However, previous studies gained inconsistent results about the association between CTLA-4 +49 single-nucleotide polymorphism and susceptibility of allograft rejection. Therefore we sought to clarify whether CTLA-4 +49 SNP influences the incidence of acute rejection after liver transplantation in Chinese population. **Methods:** Genomic DNA from 335 liver transplant recipients was genotyped for CTLA-4 +49 SNP by DNA sequencing. Acute rejection was confirmed by pathologic evidences. The association between CTLA-4 +49 SNP and incidence of acute rejection was then analyzed by dominant, recessive, codominant and overdominant models. **Results:** The incidence of acute rejection within the first 3 months was 11.9%. In acute rejectors, the frequency was 45% for G/G, 10% for A/A and 45% for A/G respectively, compared with 47.5% for G/G, 10.8% for A/A and 41.7% for A/G in non-acute rejectors. And no significant difference of allele distribution between these 2 groups was detected. **Conclusions:** This study suggests that CTLA-4 +49 SNP is not associated with acute rejection after liver transplantation in Chinese population.

Key words: Cytotoxic T lymphocyte antigen 4, liver transplantation, Single Nucleotide Polymorphism.

## Introduction

T cell activation plays a central role in initiating the cascade of rejection after liver transplantation. Two separate signals are required for T cell activation: the antigen-specific signals through T-cell receptor (TCR) and the costimulatory signals from the interaction between CD28 and B7-1, B7-2 [1]. In recent years, costimulatory pathway has gained increasing interest in transplant immunology. Many reports indicated that blocking of costimulatory pathway can prevent transplant rejection and even induce long-term allograft tolerance [2-4]. CTLA-4, which shares the same ligands with CD28, is expressed on activated T cells and provides an inhibitory signal in immune responses [5, 6]. And it is believed to play a key role in inducing peripheral tolerance [7, 8]. Several mechanisms are demonstrated to explain the T cell inhibi-

tion caused by engagement of CTLA-4: the competitive antagonist of CD28 for B7 as well as the direct inhibitory signals mediated by CTLA-4 cytoplasmatic tail [9]. Moreover, CTLA-4 is also expressed on $CD4^+CD25^+$ regulatory T cell, and is believed to play a key role in controlling regulatory T cell activation and therefore contribute to the development of transplant tolerance [10, 11]. Recently CTLA-4Ig has been developed to competitively inhibit the interaction between CD28 and B7 molecules and is proved to prolong the allograft survival, which not only highlights the importance of CTLA-4 in transplant immunology but also offers the potential for more immune-selective immunosuppressive agents [12-14].

CTLA-4 +49 is one of the most well-studied polymorphisms in CTLA-4 gene and is generally considered to play an important role in the individual difference of immune intensity mediated by costimulatory pathway [15]. The A to G variation at position +49 in exon 1 leads to a threonine to alanine substation at position 17 of the signal peptide of the CTLA-4 molecule. CTLA-4 +49 G homozygote leads to decreased CTLA-4 surface expression [16-18] and is believed to reduce the inhibitory function of CTLA-4 on T cell proliferation [18, 19]. The G allele is reported to associate with many autoimmune diseases, as type 1 autoimmune hepatitis, primary biliary cirrhosis [20], autoimmune thyroid disease [21], insulin-dependent diabetes mellitus [22], and rheumatoid arthritis [23]. Moreover, recent studies also reported that CTLA-4 +49 SNP is associated with the outcome after kidney transplantation [24]. However, the available reports about association between CTLA-4 +49 SNP and the susceptibility of allograft rejection are inconsistent [25-28]. The association between CTLA-4 +49 SNP and acute rejection after liver transplantation in Han Chinese population are rarely studied. Therefore, in the view of the importance of costimulatory pathway in the transplant immunology as well as the limited and inconclusive results about the role of CTLA-4 +49 SNP in acute allograft rejection in China, we examined and tried to clarify the possible involvement of CTLA-4 +49 SNP in acute rejection after liver transplantation in Han Chinese population.

## Patients and methods

### Patients enrollment

This study included 335 patients who underwent orthotopic liver transplantation in the Division of Hepatobiliary Pancreatic Surgery, Department of Surgery, First Affiliated Hospital, School of Medicine, Zhejiang University, from 2005 to 2010. The age of patients ranges from 13 to 71 years old. There were 290 male and 45 female involved. And all patients included survived longer than 6 months after transplantation. Written informed consent was obtained from all participants or their guardians and the study protocol was approved by the Ethics Committee of our hospital. The protocol conforms with the ethical guidelines of the 1975 Helsinki Declaration. Routine immunosuppression regimen consisted of tacrolimus or Rapamycin or Cyclosporine with mycophenolate mofetil and steroids. Patients showing clinical symptoms of acute rejection within the first 6 months after liver transplantation underwent liver biopsy. And acute rejection was confirmed by pathologic evidences according to Banff criteria. Treatments of prednisone were given to patients having acute rejection. Those did not have any rejection episode were considered as nonrejecors.

### CTLA-4 genotyping

DNA was isolated from peripheral blood mononuclear cell of the recipients using the QIAamp DNA blood mini kit (Qiagen Inc. Hilden, Germany). Recipient CTLA-4 +49 SNP was typed by means of DNA sequencing. PCR reaction system (10 μl ) included 1x GC buffer (TAKARA, China), 3.0mM $Mg^{2+}$, 0.3mM dNTP, 1 U HotStarTaq polymerase (Qiagen Inc. Hilden, Germany), 1μl templates DNA and 1μl PCR primers: rs231775F:CCATGGCTTGCCTTGGATTTC, rs231775R: CCAGCCAAGCCAGATTGGAGTT. The cycling program was as follows: denaturation at 95°C for 2 min followed by 11 cycles x (94°C 20s, 65°C-0.5°C /cycle 40s, 72°C 1min 30s); 24 cycles x (94°C 20s, 59°C 30s, 72°C 1.5min); 72°C 2 min. Thereafter, 1U SAP and 1U Exonuclease I were added to 10μL of PCR product for purification. The mixture was incubated at 37°C for 60min, followed by incubation at 75°C for 15min. Then, 2μL purified PCR product was mixed with 5μl SNaPshot Multiplex Kit (ABI, USA), 2μl Ultrapure water and 1μl sequencing primer, which is rs231775SR: TTTTTTTTTTTTTTTGTGCAGGGCCA GGTCCTGG, for further reaction. The cycling program was as follows: 96°C 1min; 28 x (96°C 10s, 50°C 5s, 60°C 30s). Then, 1U SAP was added to 10μL of PCR product for purification. The mixture was incubated at 37°C for 60min, followed by incubation at 75°C for 15 min. Then, 0.5μL purified PCR product was mixed with 0.5μl Liz120 SIZE STANDARD and 9μl Hi-Di, and then incubated at 95 °C for 5 min before loaded onto an ABI3730XL Genetic Analyzer (ABI, USA). And the data was analyzed using GeneMapper 4.0 (AppliedBiosystems, USA).

### Statistical analysis

The SNP data were evaluated for Har-

dy-Weinberg equilibrium (bioinfo.iconcologia.net/snpstats/start.htm). Differences in the characteristics of recipients between the rejection group and the nonrejection group were analyzed by chi-square test, using SPSS software for Windows (Statistical Product and Service Solutions, version 14.0, SSPS Inc, Chicago, IL, USA). CTLA-4 genotypes were analyzed by SNPstats (bioinfo.iconcologia.net/snpstats/start.htm). $P<0.05$ was considered statistical significance.

## Results

The incidence of acute rejection within the first 6 months was 40 of 335 (11.9%). The primary diseases in this study were hepatocellular carcinoma (HCC) (37.3%), fulminant hepatitis (17.0%), alcoholic cirrhosis (4.8%) and others (40.9%). There was no significant association between primary diseases and incidence of acute rejection ($P > 0.05$). Neither the recipient age, gender nor blood type was significantly different between rejectors and nonrejectors($P > 0.05$).

We genotyped CTLA-4 +49 SNP in this study and the results were showed in Table 1. The CTLA-4 +49 SNP was in Hardy-Weinberg equilibrium in the cohort. The frequency of the G allele was 68.2% among all patients. In acute rejectors, the frequency was 45% for G/G, 10% for A/A and 45% for A/G respectively, compared with 47.5% for G/G, 10.8% for A/A and 41.7% for A/G in non rejectors. And none of the dominant, recessive, codominant or overdominant models revealed any significant association between CTLA-4 +49 SNP and incidence of acute rejection ($P > 0.05$).

**Table 1.** CTLA-4 +49 snp in liver transplantation recipents in relation to acute rejection.

| Model | Genotype | NAR (n=295) | AR (n=40) | OR(95% CI) | P-value |
|---|---|---|---|---|---|
| Codominant | G/G | 140 (47.5%) | 18(45%) | 1.00 | 0.92 |
| | G/A | 123 (41.7%) | 18(45%) | 1.10(0.55-2.22) | |
| | A/A | 32 (10.8%) | 4(10%) | 0.96(0.30-3.03) | |
| Dominant | G/G | 140 (47.5%) | 18(45%) | 1.00 | 0.77 |
| | G/A-A/A | 155 (52.5%) | 22(55%) | 1.07(0.55-2.09) | |
| Recessive | G/G-A/A | 263 (89.2%) | 36(90%) | 1.00 | 0.87 |
| | A/A | 32 (10.8%) | 4(10%) | 0.91(0.31-2.73) | |
| Overdominant | G/G-A/A | 172 (58.3%) | 22(55%) | 1.00 | 0.69 |
| | G/A | 123 (41.7%) | 18(45%) | 1.11(0.57-2.16) | |

AR, acute rejectors; NAR, non-acute rejectors.

## Discussion

CTLA-4, one of the most extensively investigated costimulatory molecule, acts as a negative regulator of peripheral T cell function. The importance of CTLA-4 in autoimmune diseases, antitumor immunology and transplant immunology has been demonstrated and emphasized [2, 9, 29]. Polymorphisms in CTLA-4 influence the expression, transcription and translation of CTLA-4 and therefore affect its function. Particular polymorphisms such as SNPs in position +49, +106 and -1722 have been reported to influence the susceptibility to autoimmune diseases as Graves' disease, autoimmune hypothyroidism, multiple sclerosis and systemic lupus erythematosus [16, 19, 30, 31]. And high incidence of CTLA-4 AA(CT60) polymorphism has been observed in renal cell cancer [32]. CTLA-4 +49 SNP was also reported to play an important role in non-Hodgkin's lymphoma and oral squamous cell carcinoma [33, 34]. Moreover, the DNA variants of CTLA-4, such as SNPs in +49, -318 and the dinucleotide (AT)n repeat polymorphism, are involved in the outcome of solid organ transplantation [24, 28, 35].

CTLA-4 gene encodes for 2 isoforms of CTLA-4: a membrane bound CTLA-4 (mCTLA-4) and a soluble CTLA-4 (sCTLA-4) [36]. The CTLA-4 +49 A/G polymorphism leads to a threonine to alanine change in amino acid 17 of CTLA-4, which decreases the cell surface expression of mCTLA-4. As a result, CTLA-4 +49 G/G exerts diminished inhibitory effects on T cell proliferation [18, 19]. The protective effect of CTLA-4 +49 A allele in autoimmune diseases has been identified by previous studies. In our study, we enrolled 335 patients who underwent liver transplantation and used dominant, recessive, codominant and overdominant models to evaluate the association between the A/G polymorphism and acute allograft rejection. We found that the allele frequencies in recipients were consistent with normal Chinese population [37]. No significant association between CTLA-4 +49 SNP and incidence of acute rejection has been detected, which was consistent with the results of Slavcheva et al [28].

The continuous use of immunosuppression might be a reasonable explanation for the different effects CTLA-4 +49 A allele has in acute rejection and autoimmune diseases. mCTLA-4 is expressed only after T cell activation. Both CD28 and IL-2 play important roles in the up-regulation of CTLA-4 expression. The surface expression of CTLA-4 decreases significantly when IL-2 production is blocked using cycloporin A [38]. Therefore it is conceivable that immunosuppressive agencies as tacrolimus can inhibit the expression of mCTLA-4 by inhibiting the activation of T cells and the production of IL-2. As a result, CTLA-4 +49 A allele has decreased or even no protective effect against acute rejection under the immunosuppressive therapies.

The studies of Marder et al showed that recipients with CTLA4 +49 G/G genotype had decreased graft survival time compared with A/A and A/G [27]. However the main cause of graft failure is the recurrence of HCV rather than acute rejection. Thus the results of Marder and colleagues are not contradicted with ours. Kwekkeboom and colleagues found that recipient CTLA-4 +49 G/G genotype is associated with reduced acute rejection after liver transplantation [25]. Similarly, Karimi et al also found increased A allele prevalence in rejectors after liver transplantation in Iranian patients [39]. Such results seemed to be unexplainable by previous studies. But the further studies of Kwekkeboom et al soon offered a possible explanation: the CTLA-4 +49 A/+6230 G haplotype is a co-dominant risk allele for acute rejection after liver transplantation. The CTLA-4 +6230 SNP was reported to influence the sCTLA-4 production [26]. The sCTLA-4 is produced by nonactivated T cells and could either inhibit or exacerbate the immune response: during the initiation, sCTLA-4 can block the interaction of CD28-B7; and at latter stages, it blocks the CTLA-4-B7 interaction [36]. Therefore, The CTLA-4 +6230 SNP may influence the incidence of acute rejection by sCTLA-4 secretion. However, the reason why the risk allele is +49A+6230G haplotype, rather than +49G+6230G remains unclear. One possible explanation is that another unknown single allele is linked to the +49A+6230G haplotype and inhibits CTLA-4 function. Moreover, previous research showed that, several SNPs in CTLA-4 gene are related to the outcome of transplantation. It is possible that multiple alleles or genes contribute the susceptibility to acute rejection after liver transplantation and CTLA-4 +49 SNP does not act as primary susceptibility polymorphism. And the inconsistent results may be attributed to different genetic background of different ethnicities, small study number and baseline statistical significance. More studies are needed to demonstrate these hypotheses.

In conclusion, our study reveals that CTLA-4 +49 SNP has no significant association with acute rejection after liver transplantation in Chinese population. Until now, CTLA4 +49 SNP alone is not a promising predictor for the incidence of acute rejection after liver transplantation.

## Acknowledgments

This study was supported by the Foundation for Innovative Research Groups of the National Natural Science Foundation of China (No.81121002) and Zhejiang Provincial Natural Science Foundation of China (No.Y 2100498).

## Competing Interests

The authors have declared that no competing interest exists.

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