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# Association Between Cytokines and Their Receptor Antagonist Gene Polymorphisms and Clinical Risk Factors and Acute Rejection Following Renal Transplantation

Authors' Contribution:  
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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
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**Background:** Acute rejection (AR) after renal transplantation affects both patient and graft survival. There is growing evidence of the genetic association between cytokine or its receptor antagonist and AR in solid organ transplantation. The objectives of this study were to investigate the role of recipient TNF  $\beta$ , IL-10, IL-1 $\beta$ , and IL-1 receptor antagonist (ra) gene polymorphism, as well as traditional clinical variables such as panel-reactive antibody (PRA) levels, donor type, and HLA mismatches in AR following renal transplantation.

**Material/Methods:** TNF  $\beta$  (+252A/G), IL-10 (-592A/C), IL-1 $\beta$  (-511C/T) and IL-1ra (86 bp VNTR) gene polymorphisms were determined in 195 renal allograft recipients with and without AR, using PCR. Both these genotypic variants and clinical risk factors were investigated for correlation with AR within the first year after renal transplantation.

**Results:** Patients with increased pre-transplant PRA levels ( $P<0.001$ ) and donor type ( $P=0.012$ ) were prone to the development of AR. After adjusting for all variables of  $P<0.2$ , a PRA level  $>10\%$  (OR=4.515, 95% confidence intervals=1.738–11.727,  $P=0.002$ ) and the receipt of a graft from a donation after cardiac death (DCD) donor (OR=2.437, 95% confidence intervals=1.047–5.673,  $P=0.039$ ) remained significantly associated with AR in a multivariate logistic regression analysis. No correlation could be found between recipients with an episode and absence of acute rejection and the gene polymorphisms of these cytokines investigated in the present study.

**Conclusions:** This study shows that the presence of increased pre-transplant levels of PRA and the receipt of a graft from DCD donor other than cytokine gene polymorphisms are significant risk factors for AR in renal transplantation. To reduce the occurrence of AR, clinicians should take necessary measures to lower the PRA levels and pay more attention to patients who received a graft from a DCD donor.

**MeSH Keywords:** **Graft Rejection • Kidney Transplantation • Polymorphism, Genetic • Receptors, Cytokine**

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## Background

Acute rejection (AR) after renal transplantation is still a major clinical problem affecting both patient and graft survival. There is growing evidence of the association between certain cytokines or their receptor antagonist gene polymorphisms and AR after renal transplantation. Some studies have revealed the genetic association of IL-1 $\beta$ , IL-1 receptor antagonist (IL-1ra), or TNF  $\beta$  with acute renal graft rejection. However, the polymorphism effect on AR after renal transplantation has not been well studied. [1–3] Panel-reactive antibodies (PRA) have been associated with acute kidney graft rejection, and the complement system has an essential role in PRA-related kidney rejection by the classical C1q-dependent pathway [4–6]. Pre-transplant PRA levels were only investigated in limited studies that explored the association of gene polymorphism with acute renal graft rejection [7–11]. Thus, in the present study we aimed to investigate whether cytokines or their receptor antagonist gene polymorphisms and clinical variables had impacts on the incidence of acute renal graft rejection.

## Material and Methods

### Study population, study design, and data collection

We enrolled 217 recipients who underwent kidney transplantation at the Third Xiangya Hospital of Central South University (Changsha, China) from January 2008 through June 2010 with a follow-up time of 12 months. Twenty-two recipients were excluded because they underwent other procedures in addition to a kidney transplant, had simultaneous transplantations, or were lost to follow-up. Inclusion criteria for the remaining 195 were complement-dependent cytotoxicity lymphocyte cross-match and donor-specific antibody-negative prior to transplantation. Of the 195 available recipients, 49 suffered at least 1 rejection episode, and 28 were biopsy-proven, with the remaining 21 clinically proven. Twenty of 28 patients having biopsy-proven AR had humoral rejection, and 13 of 21 patients with clinically proven AR had antithymocyte globulin-requiring AR. All subjects were classified into either the AR group (n=49) or the non-AR group (n=146). Donor information (age, sex, and donor type); demographic and clinicopathological characteristics of the recipients (age, sex, primary disease, pre-transplant PRA levels, AR episodes, initial immunosuppression (Cyclosporin/Tacrolimus); use of antilymphocytic agents prior to AR; and transplant characteristics (cold ischemia time and HLA no. of 0 mismatches) were analyzed. Informed consent was received from each of the patients and the study was approved by the Ethics Committee of our hospital. AR was diagnosed based on clinical or biopsy findings according to Banff criteria [12].

### Complement-dependent cytotoxicity lymphocyte cross-match and PRA screening and identification tests

Recipient serum and donor T or B lymphocytes were mixed in a Terasaki plate before addition of rabbit complement. If there are antibodies against donor lymphocytes, the sample turns red (positive); if there are no antibodies, the sample turns green (negative) [13]. PRA screening and identification tests were performed using ELISA method (LAT1240; One Lambda, Canoga Park, CA) according to the manufacturer's protocol. In all subjects, the PRA screening test was performed and then screening-positive samples were further tested for classifying these human leukocyte antigen (HLA) antibodies detected into class I and/or class II.

### Genotyping of TNF $\beta$ (+252A/G), IL-10 (-592A/C), IL-1 $\beta$ (-511C/T), and IL-1ra (86 bp VNTR) polymorphism

The genomic DNA was purified from 200  $\mu$ l of peripheral blood samples. TNF $\beta$ , IL-10, IL-1 $\beta$ , and IL-1ra were genotyped by PCR using previously reported primers [14–16]. A volume of 25  $\mu$ l PCR reactions included around 100 ng genomic DNA, 12.5  $\mu$ l 2 $\times$ HSTM Mix (Dongsheng Biological Technology Co., Ltd., Guangzhou, China), 10  $\mu$ M of each primer (Huada Gene Science and Technology Co., Ltd., Wuhan, China), and double-distilled water. The parameters for thermocycling were as follows: denaturation at 94 $^{\circ}$ C for 4 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 56 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C for 30 s, and final extension at 72 $^{\circ}$ C for 5 min.

Eight  $\mu$ l of the amplified products containing TNF $\beta$ , IL-10 gene, and IL-1 $\beta$  gene were digested with 10 units of *Nco*I, *Rsa*I, and *Ava*I restriction enzyme (Fermentas), respectively, at 37 $^{\circ}$ C for 3 h. Amplification of polymorphic regions of IL-1 ra gene containing variable numbers of a tandem repeat (VNTR) of 86 base pairs was also performed using PCR. The PCR fragments and restriction fragments were eventually analyzed by electrophoresis on 2% polyacrylamide gel.

### Statistical analysis

The genotype and allele frequencies of TNF $\beta$ , IL-10, IL-1 $\beta$ , and IL-1 ra gene polymorphisms were calculated in the AR and non-AR groups. Continuous and categorical variables were analyzed using the *t* test, Fisher exact test, or chi-square test, respectively. For multivariate logistic regression models, all variables with  $P < 0.20$  in the univariate analysis were included to identify independent risk factors for AR. The level of significance for statistical testing was defined as  $P < 0.05$  (2-sided). Associations are given as odds ratios (ORs) with a confidence interval (CI) established at 95%. SPSS (version 17.0, SPSS, Inc., Chicago, IL) was used to perform statistical analyses.

**Table 1.** Donor, recipient and transplant characteristics of all recipients with and without AR.

Variables	AR groups (n=49)	Non-AR groups (n=146)	P
Univariate analysis			
Donor characteristics			
Age (yr; mean $\pm$ SD)	35.33 $\pm$ 6.52	36.18 $\pm$ 7.29	0.738
Gender no. (%)			0.904
Male	38 (77.6)	112 (76.7)	
Female	11 (22.4)	34 (23.3)	
Donor type no. (%)			0.012
Cadaveric	17 (34.7)	74 (50.7)	
Living relative	19 (38.8)	55 (37.7)	
DCD	13 (26.5)	17 (11.6)	
Recipient characteristics			
Age (yr; mean $\pm$ SD)	37.37 $\pm$ 8.81	37.97 $\pm$ 10.18	0.721
Gender no. (%)			0.613
Male	35 (71.4)	98 (67.1)	
Female	14 (28.6)	48 (32.9)	
Primary kidney disease no. (%)			0.133
Chronic glomerulonephritis	38 (77.6)	130 (89.0)	
Adult polycystic disease	5 (10.2)	6 (4.1)	
Diabetes	3 (6.1)	3 (2.1)	
Other	3 (6.1)	7 (4.8)	
Pre-transplant PRA levels >10%	12 (24.5)	9 (6.2)	<0.001
Initial immunosuppression no. (%)			0.098
Cyclosporin	15 (30.6)	66 (45.2)	
Tacrolimus	34 (69.4)	80 (54.8)	
Use of antilymphocytic agents prior to AR	6 (12.2)	26 (17.8)	0.381
Transplant characteristics			
Cold ischemia time	4.12 $\pm$ 1.25	3.97 $\pm$ 1.69	0.631
HLA no. of 0 mismatches (%)	16 (32.7)	79 (54.1)	0.146

AR – acute rejection; SD – standard deviation; DCD – donation after cardiac death; PRA – panel-reactive antibodies; HLA – human leukocyte antigen.

## Results

A total of 195 recipients, including 49 AR recipients and 146 non-AR recipients, were finally analyzed. The overall mean AR recipient age was 37.37 $\pm$ 8.81 years (range, 23–61 years). Fourteen AR recipients were female. Ninety-one grafts were harvested from cadaveric donors and 74 patients underwent

living related kidney donor procedures. Seven subjects had PRA class I (+), 8 had class II (+), and 6 had class I plus class II (+).

The traditional risk factors for AR, including demographic and clinical characteristics of both donors and recipients, and transplant characteristics are shown in Table 1. Table 2 presents the genotype and allele frequencies of TNF $\beta$ , IL-10, IL-1 $\beta$ , and IL-1ra

**Table 2.** TNF  $\beta$ , IL-10, IL-1 $\beta$  and IL-1ra genotypic frequencies in recipients with and without AR.

Genotype	AR groups (n=49)	Non-AR groups (n=146)	P
TNF $\beta$ (+252A/G)			0.376
A/A	9 (18.4)	35 (24.0)	
A/G	25 (51.0)	73 (50.0)	
G/G	15 (30.6)	38 (26.0)	
IL-10 (-592A/C)			0.373
A/A	22 (44.9)	77 (52.7)	
A/C	21 (42.9)	53 (36.3)	
C/C	6 (12.2)	16 (11.0)	
IL-1 $\beta$ (-511C/T)			0.664
C/C	9 (18.4)	34 (23.3)	
C/T	29 (59.2)	79 (54.1)	
T/T	11 (22.4)	33 (22.6)	
IL-1ra (86 bp VNTR)			0.901
1/1	39 (79.6)	117 (80.1)	
1/2	7 (14.3)	27 (18.5)	
1/4	3 (6.1)	2 (1.6)	

AR – acute rejection; TNF – tumor necrosis factor; IL – interleukin; ra – receptor antagonist; AR – acute rejection; VNTR – variable numbers of a tandem repeat.

**Table 3.** Logistic regression of potential risk factors for AR.

	OR (CI: 5–95)	P
Initial immunosuppression (Cyclosporin vs. Tacrolimus)	0.800 (0.374–1.711)	0.566
Primary kidney disease (Glomerulonephritis vs. the others)	1.384 (0.922–2.077)	0.117
HLA mismatches (0 mismatches vs. the others)	1.436 (0.749–2.134)	0.186
Donor type (DCD donor vs. the others)	2.437 (1.047–5.673)	0.039
Pre-transplant PRA levels (>10% vs. $\leq$ 10%)	4.515 (1.738–11.727)	0.002

AR – acute rejection; OR – odds ratio; CI – confident interval; HLA – human leukocyte antigen; DCD – donation after cardiac death; PRA – panel-reactive antibodies.

variants in all recipients. Table 1 also showed that in univariate analysis, higher AR incidence was found to be significantly associated with PRA ( $P<0.001$ ) and donor type ( $P=0.012$ ).

Table 3 showed that after adjusting for all variables of  $P<0.2$  in the univariate analysis, a PRA level  $>10\%$  (OR=4.515, 95% CI=1.738–11.727,  $P=0.002$ ) and the receipt of a graft from a donation after cardiac death (DCD) donor (OR=2.437, 95% CI=1.047–5.673,  $P=0.039$ ) remained significant risk factors in a multivariate logistic regression analysis. No significant

difference was found between recipients with an episode of AR or absence of AR regarding TNF  $\beta$ , IL-10, IL-1 $\beta$ , and IL-1ra gene polymorphisms, as well as the other clinical variables.

## Discussion

AR is a major clinical complication affecting both patient and graft survival following renal transplantation. Many studies have investigated the association between cytokine gene

polymorphisms and acute renal graft rejection, with different studies reporting different results. [1,3,5,17–25] The variable clinical diagnostic processes, different PRA, HLA match status, immunosuppressive protocols, small sample size, and ethnicity may be the main reasons for the inconsistent results of different studies [26,27].

We evaluated the effect of genetic variants of TNF $\beta$ , IL-1 $\beta$ , and IL-10, and IL-1ra genes, which are 2 important pro-inflammatory cytokines and 2 important anti-inflammatory factors, respectively, on AR because cytokines and their receptor antagonists play pivotal roles in the immune response. Furthermore, few studies have associated both gene polymorphism of cytokines and pre-transplant PRA levels with AR following renal transplantation [7–11]. In view of that, we aimed to ascertain whether clinical variables such as PRA levels had significant effects on AR.

Our present results agree with other studies suggesting no association of cytokines and receptor antagonist gene polymorphisms with AR in renal transplantation [1,3,17,28–30]. However, the present study revealed that a PRA level >10% had significant influence on AR. The possible explanation was that our subjects mainly developed humoral rejection. Theoretically, cytokines or their receptor antagonists mainly lead to acute cellular rejection, while PRA facilitates acute humoral rejection. It is crucial for clinicians to take necessary measures to

overcome sensitization using novel immunosuppressive protocols, including therapeutic plasma exchange, immunoadsorption, intravenous immunoglobulin, rituximab, bortezomib, and basiliximab [13,31–33].

Our other finding is that the receipt of a graft from a DCD donor constitutes a risk factor for AR after renal transplantation. However, the precise mechanism by which the type of DCD donor influences AR remains unclear. Patients who received a graft from a DCD donor should be followed carefully.

## Conclusions

We revealed the association between the elevated pre-transplant PRA levels and the receipt of a graft from a DCD donor and AR following renal transplantation. Our conclusion should be interpreted with caution due to the relatively small sample sizes. Further investigations with large sample sizes and better study designs are needed to evaluate the actual role of clinical risk factors and cytokines or their receptor antagonist gene polymorphisms in acute cellular and humoral rejection after renal transplantation.

## Conflict of interest

None declared.

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