

# A New Index for Acute Rejection After Renal Transplant: Notch Receptor-1

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## Abstract

**Objectives:** This study aimed at investigating the relation between expression of Notch receptor-1 (Notch 1) in peripheral blood mononuclear cells and acute rejection after renal transplant.

**Materials and Methods:** Ninety-seven patients receiving a renal transplant were randomly selected. Peripheral blood samples before transplant and days 1, 3, 5, 7, 10, 14, 21, and 30 after transplant were retrospectively observed. Expression of Notch 1 was detected by flow cytometry and real-time quantitative polymerase chain reaction.

**Results:** Expression of Notch 1 was correlated with acute rejection and long-term renal function after transplant (as detected by the level of serum creatinine 6 months after transplant). Expression of Notch 1 in peripheral blood mononuclear cells increased before serum creatinine increased. Expression of Notch 1 can reveal the immune state of recipients after transplant, and Notch 1 expression at early time points after transplant can predict long-term renal function.

**Conclusions:** Notch 1 can serve as an important index for acute rejection and long-term renal function after transplant.

**Key words:** *Receptor, Notch 1, Acute rejection, Kidney transplant, Expression*

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## Introduction

Recent years have seen an increasing incidence in the rate of kidney failure (uremia) owing to factors such as bad diet, environmental pollution, and an aging population. Although organ transplant is preferable when treating end-stage kidney failure, rejection caused by transplant remains the biggest obstacle to a successful transplant.<sup>1</sup>

The Notch gene was originally discovered in fruit flies in 1919, so named for the discovery that there were notches at the edges of wings when there was a partial loss of functions played by this gene.<sup>2</sup> The Notch signaling pathway exists extensively and is highly conserved in invertebrates and vertebrates. It is composed of Notch receptors, Notch ligands, and CBF1/RBP-J $\kappa$ /Suppressor of Hairless/LAG-1 binding proteins. Until now, 4 Notch receptors (Notch 1 to 4) and 5 Notch ligands (Deltalike 1, 3, 4, and Jagged 1 and 2) have been identified in vertebrates.<sup>3,4</sup> Notch genes play various functions in mammals. They play roles in regulating differentiation of many tissues, and participate in important physiological processes such as hemopoiesis,<sup>5</sup> development of T cells,<sup>6</sup> and vascularization.<sup>7</sup> Meanwhile, they are closely correlated with tumorigenesis<sup>8</sup> and some nervous system diseases.<sup>9</sup>

In the immune system, Notch signals are associated with differentiation of T cells and B cells, the differentiation and maturation of thymus cells, and the renewal and differentiation of bone marrow hematopoietic progenitor cells.<sup>10</sup> Recent studies have suggested that Notch signals exert roles in the differentiation and regulation of the peripheral immune system. Notch receptors and ligands are expressed on the surface of mature lymphocytes and antigen presenting cells (APCs), and different Notch ligands have different biological effects on T cells.

Jagged 1-Notch signaling pathway can improve T-cell viability while  $\delta$ -Notch signaling pathway deprives it. The interaction between Notch ligands on the surface of APCs and Notch receptors expressed in T cells can prevent T cells from activation-induced cell death.<sup>11</sup> Hoyne and associates discovered that antigens presented by rat APCs in which the human gene *Serrate* was over-expressed could induce transformation of peripheral juvenile CD4<sup>+</sup> T cells into regulatory T cells, and these transformed cells not only could inhibit primary and acquired immunologic reactions, but also transfer tolerance of antigenic specificity into recipient rats.<sup>12</sup>

In addition,  $\delta$ -Notch<sub>1</sub> can inhibit differentiation of mononuclear cells into macrophages, but allow their differentiation into dendritic cells.<sup>13</sup> Wong and associates found that the activation of Notch signals on spleen CD8<sup>+</sup> cells could result in dramatic reduction of IFN- $\gamma$  and increase of IL-10, suggesting that Notch signals could alter the differentiation potential of CD8<sup>+</sup>.<sup>14</sup> Notch signaling pathways also regulate development and maturation of B cells, and a recent study displayed that signals transduced by delta-like proteins could inhibit differentiation of B cells during differentiation of lymphocytes.<sup>15</sup> All these results indicate that Notch signals may regulate the fate of lymphocytes via their effect on cell proliferation, differentiation, and apoptosis.

Acute rejection (AR) remains one of the toughest problems influencing the prognosis after kidney transplant. The key to this lies in early diagnosis of AR in the clinic, based on whether physicians can adjust the dosage of immunosuppressive drugs before functional damage of the allograft, thus, extending longevity of the allograft. Until now, a sensitive and specific detection index for AR after renal transplant has not been found clinically.<sup>16</sup> A recent study exhibited that Notch receptors and ligands were extensively expressed in central and peripheral immunologic systems.<sup>17</sup> They not only played important roles in the processes of immunocyte differentiation and proliferation, but they also participated in the process of activation of peripheral T lymphocytes, indicating that Notch signals might be closely correlated with immune reaction in the allograft. In this study, the level of Notch 1 in peripheral blood mononuclear cells was dynamically detected, and its correlation with rejection after renal transplant was preliminarily explored.

## Materials and Methods

### Data

A total of 97 patients (39 women, 58 men; mean age, 34.3 y; range, 19-61 y) were randomly selected in this study. Patients underwent a renal transplant in our hospital between January 2009 and January 2010. All protocols were approved by the ethics committee of Fuzhou General Hospital before the study began, and the protocols conformed with the ethical guidelines of the 1975 Helsinki Declaration. Written, informed consent was obtained from all patients. Causes of end-stage renal disease included the following: 67 had chronic glomerulonephritis, 7 had polycystic kidney, 2 had diabetic nephropathy, 1 had solitary kidney complicated with renal tuberculosis, 2 had gouty nephropathy, 1 had chronic pyelonephritis, 5 had hypertensive nephropathy, and 12 had unknown reasons. All patients received a renal transplant from a deceased donor with the same ABO blood type, and HLA-A, B, and DR antigens were matched at the 3 to 6 loci. Detection by panel reactive antibodies (PRA) showed that hypersensitivity was found in 2 cases (> 50%), moderate sensitization in 3 cases (range, 20%-49%), low sensitivity in 9 cases (range, 10%-19%), and negativity in others (< 10%). Results by lymphocyte toxic test of pretransplant crossmatch were negative (< 10%).

### Immunosuppressive regimen

All recipients received cyclosporine/tacrolimus + mycophenolate mofetil + prednisone triple immunosuppressive regimen. The initial dosage of cyclosporine was 6 to 8 mg/kg/d. Trough levels of cyclosporine in the blood were maintained between 200 and 300 ng/mL within the first month after transplant, and 2-hour peak values were maintained between 800 and 1200 ng/mL. The initial dosage of tacrolimus was 0.10 to 0.15 mg/kg/d, and its trough level in the blood was maintained between 6 and 10 ng/mL within the first month after the operation. The initial dosage of mycophenolate mofetil was 1000 to 2000 mg/d. The initial dose of methylprednisolone was 6 to 8 mg/kg/d, then its dosage was reduced sequentially. When the effect of methylprednisolone was dropped to 40 mg/d, oral administration of prednisone (20 mg/d) was taken instead. Methylprednisolone 500 mg/d was given for AR for 3 consecutive days. For patients resistant

to hormones, extra anti-thymocyte globulin was given for 7 to 10 days (range, 2.5-5 mg/kg/d via venous drip).

### Reverse-transcription polymerase chain reaction

Total RNA was extracted from PBMCs according to the instructions of a TRIzol Plus RNA Purification Kit (Life Technologies, Grand Island, NY, USA), concentration was measured by ultraviolet spectrophotometer, and cDNA templates were obtained by reverse transcription at 37°C. Reverse-transcription polymerase chain reaction was conducted using the Eva Green I method. The reaction system was composed of 1 µL reverse transcription products, 2.5 µL 10 × buffer solution, 2 mmol/L MgCl<sub>2</sub>, 0.8 mmol/L dNTP, 2.5 U Taq DNA polymerase, 0.5 µmol/L upstream primer, 0.5 µmol/L downstream primer, and 1 µL 20 × Eva Green I. A final volume of 25 µL was obtained by adding tridistilled water.

### Flow cytometry

The same morning of the needle biopsy of the transplanted kidney, 10 mL of venous blood was taken from an empty stomach and anticoagulated by EDTA. The same volume of hydroxypropyl methylcellulose was added, centrifuged at 2000 rpm for 20 minutes at room temperature, and then PBMCs were collected. Cell concentration was adjusted to 1 × 10<sup>6</sup>/mL by adding RPMI-1640 containing 10% fetal bovine serum. Fifty-µL cell suspensions were taken, Notch 1 monoclonal antibodies (Fuzhou Maixin Biotechnology Development Co., Fuzhou, Fujian, China) was added, and then the mixture was incubated at 4°C for 30 minutes and washed twice. FITC-labeled goat-anti-mouse IgG (Lianke Corp., Hangzhou, China) was added for 30-minute incubation at 4°C. Detection was conducted by flow cytometry. Fluorescent histogram was stored in a computer, and the mean fluorescence intensity of the cells was automatically analyzed.

### Statistical analyses

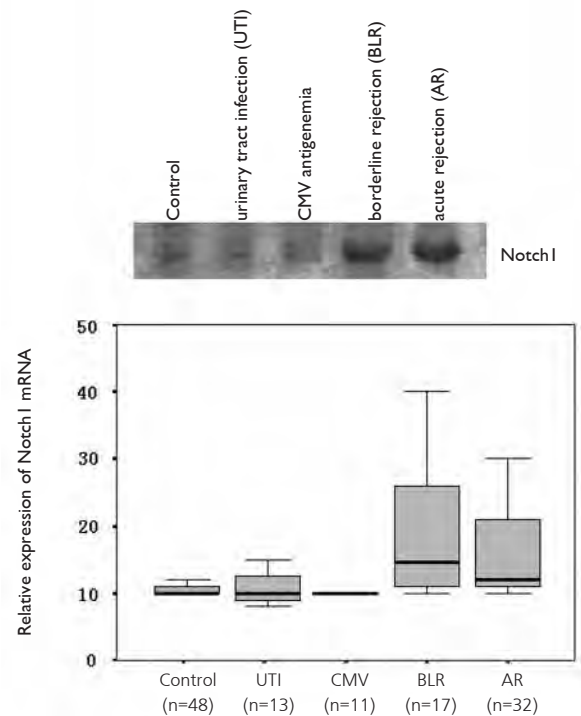
Statistical analyses were performed with SPSS software (SPSS: An IBM Company, version 10.0, IBM Corporation, Armonk, New York, USA). The *t* test was carried out for pairwise comparison between groups, and 1-factor analysis of variance was used for comparison among groups. *P* < .05 was considered statistically significant.

## Results

### Expression level of Notch 1 mRNA in peripheral blood mononuclear cells

Total RNA was extracted from PBMCs. Notch 1 was detected by RT-PCR. The CT method was adopted, taking rRNA as the internal standard for relative quantitation. The expression level of Notch 1 mRNA in the AR group (n=32) and the borderline rejection group (n=17) was notably higher than it was in the stable allograft group (n=48) (*P* < .01). There was no significant difference among the stable allograft, the urinary tract infection, and the *cytomegalovirus* infection groups (*P* > .05) (Figure 1).

Figure 1. Expression of Notch 1 mRNA in PBMCs in Kidney Recipients



Abbreviations: AR, acute rejection; BLR, borderline rejection; CMV, cytomegalovirus; UTI, urinary tract infection

### Expression level of Notch 1 protein by flow cytometry

Mononuclear cells were extracted from peripheral blood, labeled by anti-Notch 1 monoclonal antibodies, and then detected by flow cytometry. The expression level of Notch 1 protein in the AR group (n=32) and the borderline rejection group (n=17) was notably higher than it was in the stable allograft group (n=48) (*P* < .001). There were no significant differences in the stable allograft, urinary tract

infection, and *cytomegalovirus* infection groups ( $P > .05$ ) (Figure 2).

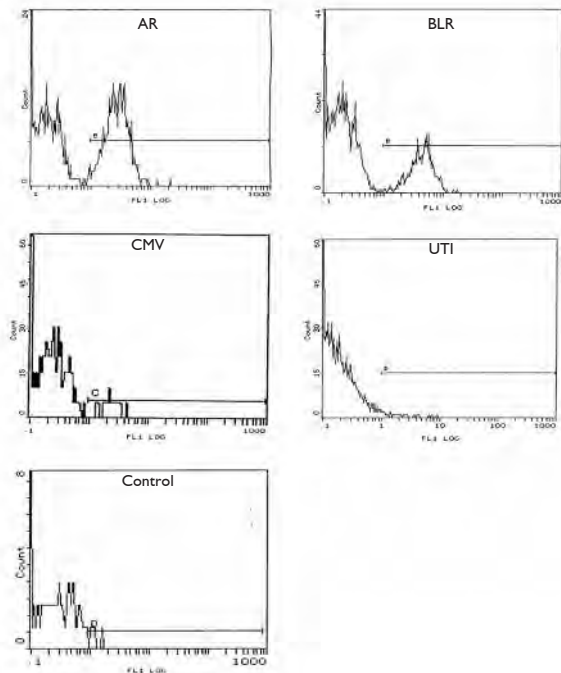
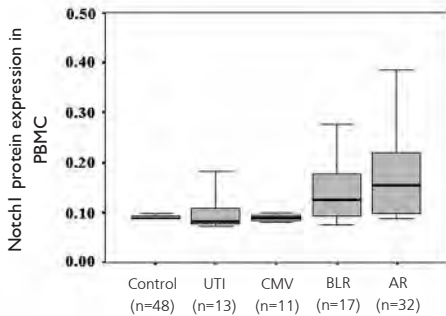
**Dynamic detection of serum creatinine and Notch 1 protein**

Expression of Notch 1 protein was detected at 4 different time points before biopsy (7 days, 5 days, 3 days, and 1 day before biopsy). Results showed that the expression of Notch 1 protein began to increase on the fifth day before AR (n=49) (Figure 3).

**Dynamic detection of Notch 1 expression in peripheral blood mononuclear cells**

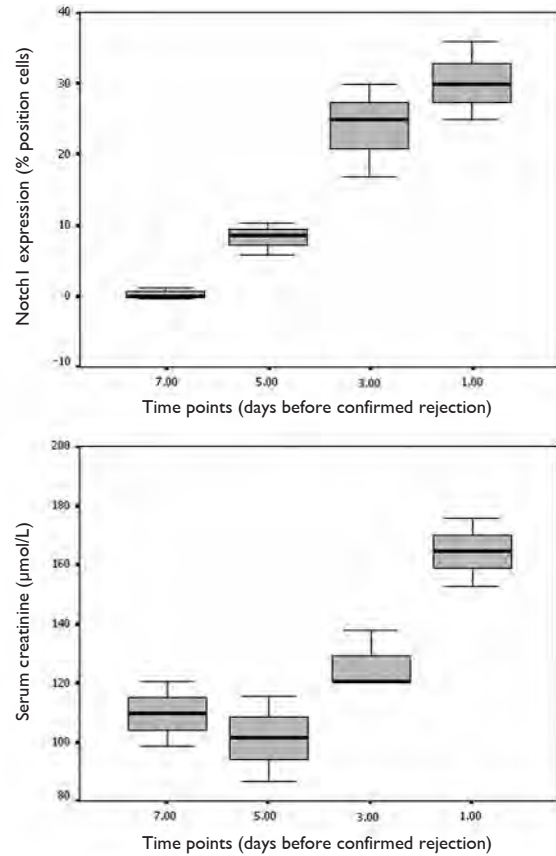
Expression of Notch 1 protein in 1 case was dynamically detected in this study. In Figure 4, columns represent levels of Notch 1 protein, and the curve shows changes of the levels of serum

**Figure 2.** Expression of Notch 1 Protein in PBMCs in Kidney Recipients (By Flow Cytometry)



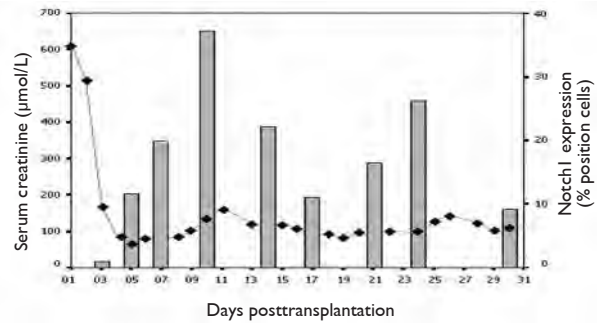
**Abbreviations:** AR, acute rejection; BLR, borderline rejection; CMV, cytomegalovirus; UTI, urinary tract infection

**Figure 3.** Dynamic Detection of Serum Creatinine and Notch 1 Protein



(A) Expression level of Notch 1 protein in PBMCs; (B) the level of serum creatinine.

**Figure 4.** Dynamic Detection of Notch 1 in PBMCs



Columns represent expression of Notch 1, and the curve shows the changes of serum creatinine.

creatinine. The patient was diagnosed with AR (Banff II) by biopsy on ninth day after transplant, and the level of Notch 1 protein increased on third day, the fifth day, and the seventh day after transplant. After the 3-day effect of methylprednisolone, no improvement was found. Antirejection therapy was adopted on the ninth day using antithymocyte globulin. Although the level of serum creatinine showed an obvious decrease on the 13th day,

expression of the Notch 1 protein could still be detected on the seventeenth day after transplant, suggesting that intragraft immune reactions still existed. The level of serum creatinine re-increased on the 25th day after transplant, and amelioration was achieved after treatment with methylprednisolone based on the diagnosis of rejection according to clinical symptoms. But Notch 1 protein still was over-expressed 30 days after transplant.

### Correlation between expression of Notch 1 and long-term renal function after transplant

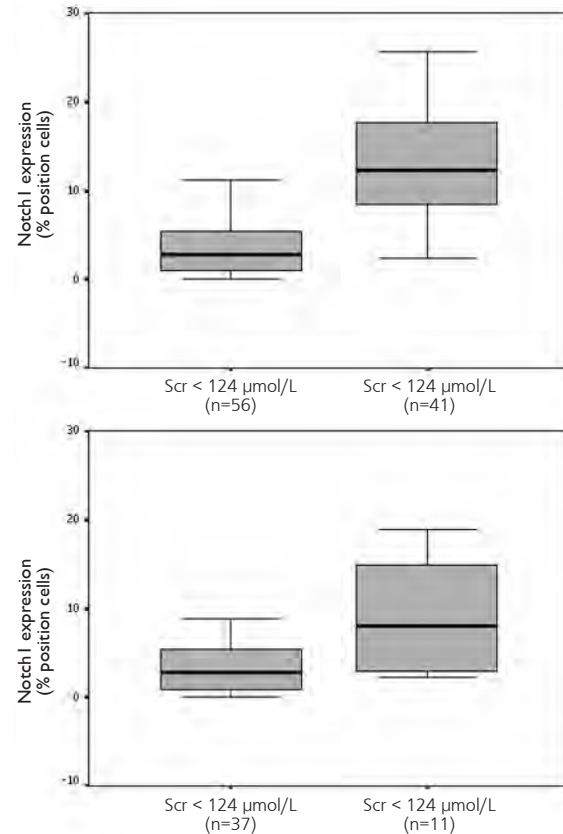
In this study, the level of serum creatinine 6 months after transplant was detected to reflect the long-term renal function after transplant. The recipients were divided into 2 groups based on the level of serum creatinine 6 months after transplant: The serum creatinine > 124  $\mu\text{mol/L}$  group and serum creatinine < 124  $\mu\text{mol/L}$  group. Results show that the expression level of Notch 1 (1 month after transplant) in the former group was notably higher than it was in the latter group ( $P < .05$ ). After AR and borderline rejection recipients were excluded, the rest of the 48 recipients were divided into 2 groups based on the watershed of 124  $\mu\text{mol/L}$  of serum creatinine. Results show that expression of Notch 1 in the serum creatinine > 124  $\mu\text{mol/L}$  group was significantly higher than it was in the serum creatinine < 124  $\mu\text{mol/L}$  group ( $P < .05$ ) (Figure 5).

### Discussion

With wide application of immunosuppressive agents (eg, tacrolimus and mycophenolate mofetil), it has become common that AR after renal transplant loses its typical clinical manifestation, which not only leads to difficulties in clinical diagnosis of AR, but also delays its diagnosis and treatment in some patients. Some scholars have attempted to diagnose AR by detecting its related factors in urine and blood, such as LFA1, TNF- $\alpha$ , IL-2, and IL-6.<sup>18</sup> However, owing to the facts that these indexes lack specificity and susceptibility to AR—even worse, they fail to differentiate AR from acute tubular necrosis sometimes—their application has a great limitation.

Notch signaling pathway is a highly conservative signaling pathway in the process of evolution, and recent studies have discovered that this pathway plays critical roles in the differentiation and

**Figure 5.** Correlation Between Expression of Notch 1 and Long-Term Renal Function After Transplant



(A) Correlation between expression of Notch 1 and long-term renal function in recipients ( $n=97$ ) ( $P < .05$ ); (B) correlation between expression of Notch 1 and long-term renal function in nonrejection recipients ( $n=48$ ) ( $P < .05$ ).

**Abbreviations:** Scr, serum creatinine

regulation of the peripheral immune system. Notch receptors and ligands are expressed on the surface of mature lymphocytes and APCs.

In our previous research, we detected expression of Notch 1 in peripheral blood of 40 patients who had received a kidney transplant by using flow cytometry, and results showed that expression of Notch 1 increased during AR; meanwhile, it showed an obvious synchronic correlation with the level of creatinine. Based on previous research, expression of Notch 1 in peripheral blood samples of 97 kidney recipients were detected in this study. Results showed that the mean fluorescence intensity of Notch 1 in AR group was significantly higher than it was in the infection, control, or stable renal function groups, suggesting that expression of Notch 1 can better reflect the intragraft AR and may serve as a good diagnostic index for AR. In this study, AR happened twice within 1 month after kidney transplant in 1 case, and meanwhile, expression of

Notch 1 showed good correlation with expression of serum creatinine in this case, suggesting that Notch 1 also may serve as an evaluation index for AR treatment effectiveness.

Rejection after kidney transplant can be divided into 3 stages: recognition, proliferation, and reaction. Most studies on AR focus on detection of cytokines, that is, the stage of reaction. Current research has proved that Notch signals and T-cell receptor signals mediate antigen recognition jointly, and play important roles in the process of regulating proliferation of immunocytes.<sup>19,20</sup> Based on findings in the literature, the reaction of Notch signals happen in the stage of signal recognition, which is obviously earlier than the reaction stage in which expression of cytokines begins to increase. Thus, it can predict the early coming AR and provide the possibility of prophylactic treatment of AR before the increase of cytokines.

Our study showed that the expression of Notch 1 changed significantly in the early phase before rejection, the rejection phase, and the stable phase. Expression of Notch 1 began to increase 3 to 5 days before rejection, reached a peak during the rejection phase, then dropped after improvement of rejection. Detection of Notch 1 expression may be helpful for detecting AR as well as realizing early diagnosis and treatment. In addition, our study showed that expression of Notch 1 was correlated with expression of creatinine 6 months after a kidney transplant, which suggests that expression of Notch 1 can indirectly reflect the long-term renal function after transplant.

Until now, most studies on Notch signaling from the perspective of transplant immunology are done in vitro and in animal models. To our knowledge, reports on its clinical study have not been found. This study has made an initial exploration. Our results showed that expression Notch 1 can well reflect the immune state of allografts and exhibit a promising outlook of clinical application in early diagnosis and detection of AR.

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