Association of Forkhead Box P3 Gene Polymorphisms With Allograft Rejection Episodes in Kidney Transplant Patients

A Study From Kashmir, North India

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Introduction. The forkhead box P3 (*FOXP3*) gene is important for regulation and development of T cells, which are mediators of kidney allograft rejection. The *FOXP3* gene polymorphism may be associated with the rejection of kidney transplants. This study was designed to determine the association of *FOXP3* polymorphism with kidney transplant rejection.

Materials and Methods. A total of 118 kidney transplant patients were included in this study and were grouped into rejection (n = 31) and nonrejection (n = 87) groups. The *FOXP3* rs3761548 gene was genotyped by polymerase chain reaction-restriction fragment length polymorphism using the taqman probe technique. Gene polymorphism at rs3761548 of the *FOXP3* gene was analyzed for association with rejection episodes and graft outcome of kidney transplants.

Results. The CC genotype of rs3761548 was not present neither of the study nor the control group. The AA genotype was association with a higher risk of rejection compared to the C/A genotype (odds ratio, 2.329; 95% confidence interval, 1.041 to 5.210). The C/A genotype was also associated with a better response to treatment for rejection (odds ratio, 6.667; 95% confidence interval, 1.319 to 33.707) and better posttransplant graft function (odd ratio, 5.833; 95% confidence interval, 1.727 to 19.704).

Conclusions. Our findings suggested an association between rejection episodes, posttransplant graft function, and the *FOXP3* rs 3761648 polymorphism. Determination of *FOXP3* rs 3761648 C/A genotype might be helpful for the identification of recipients with a lower risk of rejection and better graft survival.

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INTRODUCTION

Kidney transplantation is currently the standard treatment of most cases of end-stage kidney disease.[1] With the introduction of novel immunosuppressive regimens, the outcome of the kidney transplant has improved significantly. While short-term outcome improved significantly, 1-year survival of graft around 80% to 95%,[2] long-term outcome is a concern. A significant number of grafts are lost within 10 years,[3] mostly due to a combination of chronic rejection and side effects of immunosuppressive drugs. Also, acute rejection (AR) occurs in around 20% to 40% of the patients. Acute rejection is an important cause of chronic rejection.[4] Thus, there is an important need for markers associated with or predictive of acute or chronic rejection to improve

the clinical outcome of the kidney transplant.

Development of kidney transplant tolerance is the ultimate goal to decrease the need of immunosuppression and its associated side effects.[5] One of the important cells for development of tolerance is CD4+CD25+ regulatory T cell (Tregs). It is a subset of CD4+ cells and regulates graft rejection and graft-versus-host disease.[6,7] CD4+CD25+ constitute around 5% to 10% of peripheral CD4+ cells.[8] These CD4+CD25+ regulatory T cells have an important role in immune homeostasis and decreasing unwanted inflammatory response to self-antigens.[9] Forkhead box P3 (FOXP3) is a member of forkhead/winged helix protein family of transcription factors. It is expressed in naturally-arising regulatory cells. The *FOXP3* is master regulator gene for the development and function of regulatory T cells and can convert naive T cells to regulatory T cells phenotype.[10]

In humans, the *FOXP3* gene is located on chromosome Xp11.23-q13.3 and is composed of 11 exons.[11] This gene is polymorphic, resulting in a change in FOXP3 functionally or qualitatively, causing an alteration in immune status. The *FOXP3* gene expression is controlled by DNA methylation in the regulatory T cell-specific demethylated region of the *FOXP3* locus.[12] Mutation of *FOXP3* in humans results in severe organ-specific autoimmune disease, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome.[13-15] Variants in the *FOXP3* gene has been reported to contribute to the susceptibility of various autoimmune diseases, like systemic lupus erythematosus, autoimmune thyroid diseases, type 1 diabetes mellitus, primary biliary cirrhosis, vitiligo, and psoriasis.[16-21]

Since *FOXP3* determines the tolerance, its role in kidney transplantation can be important. Recent studies have pointed out the role of *FOXP3* in the rejection of human kidney transplants. In a study by Schaier and colleagues, high levels of FOXP3 regulatory T cells were associated with kidney allograft survival and function.[22] In a kidney biopsy study by Bestard and colleagues, the presence of infiltrating FOXP3 regulatory T cells in kidney biopsy specimen was associated with significantly better graft function, as measured by both serum creatinine and estimated glomerular filtration rate.[23] However, in another study by Bunnag and coworkers, FOXP3 mRNA was higher in kidney transplant biopsies with rejection than without rejection.[24] Muthukumar and colleagues found higher FOXP3 mRNA in urine was associated with AR in kidney transplants.[25] Although conflicting, FOXP3 as a marker of regulatory T cells is found to be increased in kidneys with rejection, which may be due to protective response to effector cells or due to the expression of FOXP3 on effector cells after activation. The *FOXP3* gene polymorphism has been seen to be associated with various autoimmune diseases. In a study of the Chinese population, Qiu and colleagues found an association between the *FOXP3* gene polymorphism and allograft rejection.[26] Engela and coworkers identified a beneficial effect of the *FOXP3* genetic variants on graft survival in kidney transplant patients.[27] This study was done to investigate the role of the *FOXP3* gene polymorphism in kidney transplant rejection in our population.

MATERIALS AND METHODS

Study Population

This retrospective study was carried in the Departments of Internal Medicine, Nephrology and Immunology & Molecular Medicine, Sher-i-Kashmir Institute of Medical Sciences, Srinagar. The study population included 118 live kidney transplantation cases carried from the past 5 years (2011 to 2015). Age- and sex-matched controls were selected from the healthy population not having any kidney abnormality. The inclusion criteria were an age greater than 18 years, receiving a kidney allograft with the minimum follow-up of 1 year, and DNA samples available. The patients were ABO blood group compatible with a maximum of 3/6 mismatch. The exclusion criteria were transplant rejection or failure due to reasons other than immunological causes such as technical problems, graft failure due to any drug or dye intake, urinary tract obstruction, infection, surgical failure, recurrence of original disease in transplanted kidney, and cyclosporine or tacrolimus toxicity. Patients who died with a functioning graft during the study period were not included in the study.

Clinical information of the patients regarding transplantation, age, sex, body weight, immunosuppressive therapy, cyclosporine or tacrolimus blood concentration, the source of graft, rejection episode, and time to first rejection were obtained from the clinical records for hospitalized inpatients or patients who visited the outpatient clinic.

This study was performed as per declaration of Helsinki and its amendments and was approved by an institutional ethics committee under ethical clearance no SIMS1 131/IEC-SKIMS/2016 dated 23th January 2014. Informed consent in written form was taken from the patients.

Immunosuppressive Treatment

All of the patients received triple immunosuppression consisting of calcineurin inhibitors (cyclosporine or tacrolimus), mycophenolate mofetil, and prednisolone as steroid. Out of 118 patients, 35 were on cyclosporine-based regimen and 83 were on tacrolimus regimen. The daily dosage of cyclosporine and tacrolimus was adjusted according to blood cyclosporine C2 levels (cyclosporine level at 2 hours of intake), C0 levels (cyclosporine level at predose), and tacrolimus T0 levels (tacrolimus level at predose). The target cyclosporine C2 level was 1200 ng/mL to 1500 ng/mL during months zero to 1, which was subsequently reduced to 1000 ng/mL to 1200 ng/mL during months 1 to 3, and 900 ng/mL to 1100 ng/mL after month 3 posttransplantation. Similarly, the oral tacrolimus dose was adjusted to a target level in the range of 10 ng/mL to 15 ng/mL during months zero to 3, 8 ng/mL to 10 ng/mL during months 3 to 12, and 6 ng/mL to 8 ng/mL after month 12 posttransplantation. Mycophenolate mofetil was given as an initial dose of 1.5 g/d to 2.0 g/d, and was then reduced to 0.75 g/d to 1.0 g/d, 1 month after transplantation. The steroid regimen consisted of intravenously administered methylprednisolone, 500 mg, at the time of surgery, followed by intravenously administered methylprednisolone, 1 g/d, for the next 3 days, at which time the medication regimen was changed to oral prednisolone, 30 mg/d, which was progressively tapered to 15 mg/d to 20 mg/d by the end of the first postsurgical month. Patients prescribed any other triple immunosuppressive therapy during the study period were excluded.

Definitions of Rejection

The rejection was confirmed by the allograft kidney biopsy and classified according to Banff 2007 working classification into acute antibody-mediated rejection, acute T-cellular mediated rejection, chronic active antibody-mediated rejection, and chronic active T-cellular mediated rejection.

There were 31 patients who experienced a rejection episode. Among these 31 patients, 27 cases were acute T cell-mediated rejections, 3 cases were acute antibody-mediated rejections, and 1 was chronic active T cell-mediated rejection. In the 27 acute T-cellular-mediated rejections, 15 were type Ia, 4 were type Ib, 5 were type IIa, 2 were type IIb, and 1 was type III.

Methodology

Peripheral blood was collected from the patient in 5-mL ethylenediaminetetraacetic acid tube after transplantation and stored at -20°**C** till further use. High-molecular-weight DNA was isolated by using proteinase K and phenol method. Polymerase chain reaction amplification of the desired region was done using a specific primer (forward sequence: 5′GACTTAACCAGACAGCGTAG-3′ and reverse sequence: 5′CTGGTGTGCCTTTGGTCTs-3′) for single nucleotide polymorphism rs 3761548 of Foxp3 gene polymerase chain reaction amplification amplified the promoter region of *FOXP3* gene containing the -3279 C/A polymorphic sites. The amplicon size was 594 bp. The representative *FOXP3* polymerase chain reaction amplification gel picture is presented in Figure 1.

Genotypic analysis of *FOXP3* -3279 C/A polymorphisms by restriction fragment length polymorphism method by enzyme digestion method. The 594-bp polymerase chain reaction products were digested by enzyme PstI (Fermentas). The enzyme *PstI* resulted in digestion into 3 bands of 594 bp, 374 bp, and 190 bp for the heterozygous *CA* genotype, while a single band of 594 bp for the wild *AA* genotype (Figure 2). The *CC* genotype was not found in any of the cases or control samples. Digestion products were checked on 2.5% agarose

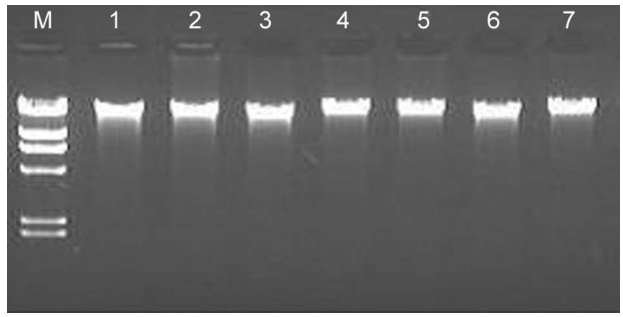

Figure 1. Agarose gel electrophoresis of DNA isolated from blood samples of patients and healthy controls. Lane M indicates lambda DNA Eco RI and Hind III digest; lanes 1, 2, 3, and 6, DNA from patient blood samples; and lanes 4, 5, and 7, DNA from control blood samples.

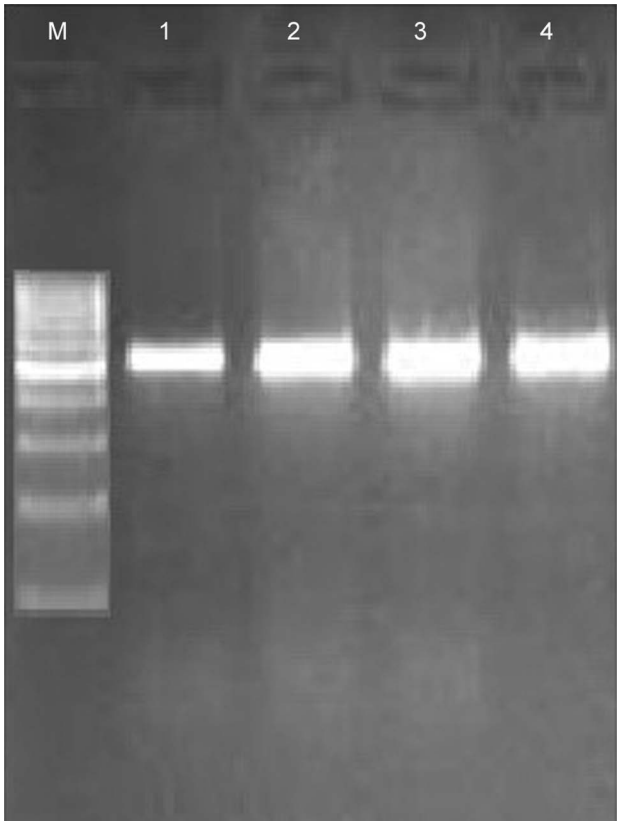

Figure 2. Representative gel picture showing *FOXP3*-3279 C/A amplicon. Lane M contains 100-bp DNA molecular weight marker and lanes 1 to 4 represent 594-bp amplicon of *FOXP3*-3279 single nucleotide polymorphism.

gel by electrophoresis (Figure 3).

Patients were followed for detection of rejection or graft dysfunction for minimum 2 years.

Statistical Analyses

All the continuous variables of the study were shown in terms of descriptive statistics and categorical variables in terms of frequency and percentages. The standard statistical tests like the chi-square test and the Fischer Exact test was used

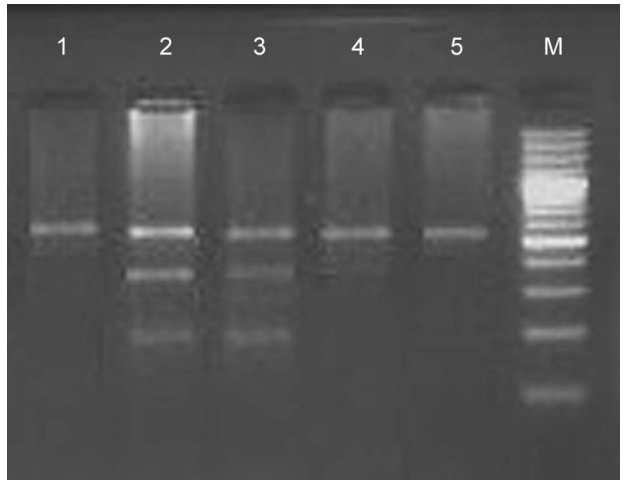

Figure 3. Representative gel picture showing polymerase chain reaction-restriction fragment length polymorphism analysis of *FOXP3*-3279 C/A polymerase chain reaction product. Lane M is the 100-bp DNA molecular size marker; lanes 1, 4, and 5 show AA homozygous (594 bp); and lanes 2 and 3 show CA heterozygous (594-bp+374bp+ 190-bp).

to analyze the data of categorical pattern. Moreover, the odds ratios along with 95% confidence intervals was calculated to interpret the results. The SPSS software (Statistical Package for the Social Sciences, version 20.0, IBM Corp, New York, NY, USA) was used for the statistical evaluation of the data. A *P* value less than .05 was considered significant.

RESULTS

Clinical and Demographic Characteristics

We categorized kidney transplant patients into the rejection group (n = 31) and nonrejection group (n = 83). Table 1 shows the clinical and demographic characteristics of the patients included in our study. There was no significant difference in the clinical and demographic features between rejection and nonrejection groups. There was no age-related difference in rejection episodes. Association of sex

Table 1. Association of Clinical and Demographic Characteristics With Rejection Episodes

Parameter	Rejection	No Rejection	*P*
Age at time of transplant, y	36.5 ± 8.7	37.5 ± 9.5	.70
Male-female ratio	21:6	66:25	.80
Donor age, y	44.0 ± 5.3	43.3 ± 5.2	.43
Body weight, kg	57.3 ± 9.0	56.9 ± 8.9	.45
Height, cm	171.0 ± 10.3	170.0 ± 11.0	.64
Immunosuppression regimen			
Cyclosprine based	9	26	
Tacrolimus based	22	61	.92
Cold ischemia time, min	25.4 ± 10.5	24.6 ± 10.7	.53
Induction antibody therapy	3	9	.91

was also studied with rejection of allograft, which was not significant.

Polymorphism and Outcomes

Analysis of *FOXP3* rs3761548 C/A polymorphism revealed that there were higher rates of rejection episodes in patients with *FOXP3* rs3761548 AA genotype than in patients with *FOXP3* rs3761548 CA genotype (odds ratio, 2.329; 95% confidence interval, 1.041 to 5.210) as shown in Table 2 (P = .02). There was no *FOXP3* rs3761548 CC polymorphism in our population, neither in 118 patients nor in 150 control subjects.

The *FOXP3* rs3761548 CA polymorphism was also associated with a better response to treatment for rejection (odds ratio, 6.667; 95% confidence interval, 1.319 to 33.707; P = .01) and better posttransplant graft function (odds ratio, 5.833; 95% confidence interval, 1.727 to 19.704; P = .005).

DISCUSSION

In the past decade, the influence of gene polymorphism has been studied in the autoimmune diseases and solid organ transplant patients. The role of regulatory T cells has been studied in kidney diseases and kidney transplant patients. In lupus nephritis, both reduced regulatory T cell number and function have been reported.[28-31] In patients with IgA nephropathy, there is a low level of $CD45RA^-$ $FOXP3^+$ and a decreased ratio of regulatory T cells to helper T17 cells.[32]

Kidney transplant outcome has improved significantly with the development of newer immunosuppressive drugs. However, acute rejection is an important cause of transplant injury and treatment cost. Regulatory T cells can be involved in improving kidney allograft survival.[33] The *FOXP3* is a master regulator gene for the development and function of regulatory T cell cells and its significance in allograft rejection and long-term allograft survival has recently become the subject of interest and extensive research. High levels of $FOXP3^+$ regulatory T cells, specifically $CXCR3^+$ regulatory T cells and HLA-DR high$^+$ CD45RA- regulatory T cells in peripheral blood that produce high levels of interferon-γ are associated with kidney allograft survival and function.[33,34]

In our retrospective study, a total of 118 patients with kidney allograft transplant were taken who had undergone transplant after proper immunologic evaluation. More than 22% of our study population were females, which shows an obvious bias in our population towards female sex, a fact which has also been seen in other studies from our part of the world.[31,35] About 26% of our transplant patients developed rejection episodes. There was no significant difference in the clinical and demographic features between rejection and nonrejection groups.

We studied *FOXP3* rs3761548 C/A gene polymorphism in the kidney transplant recipients and the association of this polymorphism with allograft rejection. Out of 118 patients, 74 had CA genotype and 44 had AA genotype. There was no CC genotype in our population, neither in 118 patients nor in 90 control subjects. This is, in contrast, to study by Qiu and colleagues' study, in which CC phenotype was the predominantly present.[26] We need larger population studies to see whether our population have a different genotype which might contribute to increased risk of kidney diseases. We also found that the patients with CA genotype were more frequent among recipients with fewer rejection episodes. Analysis of *FOXP3* rs3761548 C/A polymorphism revealed that there was a rejection rate of 17.6% in patients with *FOXP3* rs3761548 CA genotype and 40.9% in patients with *FOXP3* rs3761548 AA genotype. The difference

Table 2. Association of *FOXP3* Polymorphism With Rejection Episodes, Response to Treatment, and Posttransplant Graft Function

Outcome	Genotype		Odds Ratio (95% Confidence Interval)	*P*
	FOXP3 rs3761548 CA	FOXP3 rs3761548 AA		
Rejection episodes	13	18	2.329 (1.041 – 5.210)	.04
Response to treatment				
Good	10	6		
Poor	3	12	6.667 (1.319 - 33.71)	.03
2-year graft function				
Good	70	33		
Poor	4	11	5.833 (1.727 - 19.704)	.05

was significant. A similar result was seen by Qiu and colleagues.[26] The risk for allograft rejection in rs3761548 AA genotype patients was about 4-fold greater than in CC genotype patients. The rejection rate in patients with CA genotype was 39.1% (9 out of 23) compared to 66.7% (10 out of 15) rejection rate in AA genotype, but the difference was not significant.[26] Gunesacar and colleagues studied the association of *VEGF* 936 C/T gene polymorphism with graft outcome and found T allele associated with good graft outcome.[36]

The *FOXP3* rs3761548 CA polymorphism was also associated with a better response to treatment for rejection and better overall graft function. In a study by Schaier and colleagues, $FOXP3^{+}$ regulatory T cell was increased in patients with stable graft function.[34] In another study by Volker and coworkers, high CD4+CD25+ FOXP3+ was associated with good graft function.[37] Our study is the first study from our part of the world to report an association between *FOXP3* gene polymorphism and risk of transplant rejection risk. However, we need larger prospective studies to prove the association. This association also gives the scope of using regulatory T cells for preventing allograft rejection. Currently, FOXP3+ regulatory T cell therapy is being tested in a multicenter phase I/II study living-donor kidney transplantation.[38]

CONCLUSIONS

Our findings suggested an association between rejection episodes, posttransplant graft function, and *FOXP3* rs 3761648 polymorphism. Determination of *FOXP3* rs 3761648 C/A genotype might be helpful for the identification of recipients with less rejection chance and good graft survival.

CONFLICT OF INTEREST

None declared.

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