Association of *FoxP3* polymorphism with allograft outcome in renal transplantation

FoxP3 유전자다형성과 신이식 성적의 연관성 분석

2017년 7월

서울대학교 대학원

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박 혜 원

A thesis of the Degree of Doctor of Philosophy

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2017 년 5 월

서울대학교 대학원

의학과 검사의학 전공

박 혜 원

박혜원의 의학박사 학위논문을 인준함

2017 년 6 월

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Association of *FoxP3* polymorphism with allograft outcome in renal transplantation

by

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A thesis submitted to the Department of Laboratory Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine at Seoul National University College of Medicine

June 2017

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ABSTRACT

Association of *FoxP3* polymorphism

with allograft outcome in renal transplantation

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Background. FoxP3 is the most reliable marker for regulatory T cells which play an important role in maintaining tolerance of renal allograft. Recently, *FoxP3* gene polymorphisms have been reported to be associated with graft survival in renal transplantation.

Methods. We analyzed the association of *FoxP3* polymorphisms (rs3761548A/C, rs2280883C/T, rs5902434del/ATT, and rs2232365A/G) and graft outcome by polymerase chain reaction with sequence-specific primers (PCR-SSP) on 231 adult renal transplantation recipients performed during the period of 1996-2004 in Seoul National University Hospital.

Results. Patients with rs2280883 TT genotype showed lower acute rejection rate compared to CC or CT genotype (26.9% vs 53.3%, P = 0.038). Patients with rs3761548 CC genotype showed better graft survival compared to AC or AA genotype (log rank test, P = 0.03). Patients with rs2280883 TT genotype showed better graft survival compared to CT or CC genotype (P = 0.02).

Patients with rs3761548 CC genotype showed lower rate or recurrence of underlying glomerular disease compared to AC or AA genotype (P = 0.01).

Conclusion. *FoxP3* polymorphism rs3761548 CC and rs2280883 TT genotypes were associated with superior graft outcome of renal transplantation in Koreans. Further studies are needed in larger number of patients.

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keywords : FoxP3, single nucleotide polymorphism,
           renal transplantation, graft survival
Student Number : 2013-30544

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LIST OF ABBREVIATIONS

FoxP3 forkhead box P3

SNP single nucleotide polymorphisms

AT annealing temperature

F forward

R reverse

PCR-SSP polymerase chain reaction with sequence-specific primers

I inosine

M male

F female

DN diabetic nephropathy

NDN nondiabetic nephropathy

LD living donor

CD cadaveric donor

OS overall survival

RFS rejection-free survival

RcFS recurrence-free survival

1. INTRODUCTION

Improved pre-transplantation evaluation and the development of post-transplantation immunosuppressive therapy have led to a marked improvement in short-term graft survival in renal transplantation. However, long-term graft survival remains unsatisfactory [1].

Immunologic responses of patients play pivotal roles in graft rejection or recurrence of underlying renal disease. Regulatory T cells (Tregs) promote a state of antigen specific peripheral tolerance by suppressing activation and expansion of T effector cells, as reported in experimental models [2, 3]. Therefore they play an important role in maintaining self-tolerance and in regulating graft rejection and graft-versus-host disease [4, 5].

Foxkhead box P3 (FoxP3) is a member of Forkhead box protein, a family of transcription factors that play important roles in regulating the expression of genes [6]. FoxP3 involves in immune system reponses, appearing as a master regulator of the regulatory pathway in the development and function of regulatory T cells [7, 8].

FoxP3 is more specific for Treg cells than CD25 or CD45RB, although it is not completely exclusive. CD4+ effector T cells without suppressive activity may still upregulate FoxP3 expression upon activation. Therefore, FoxP3 cannot be considered as a unique symbol of human Treg cells [9]. However, Treg cells that express FoxP3 are critical in the transfer of immune tolerance, especially self-tolerance. Constitutive expression of FoxP3 is the decisive factor driving the immunosuppressive function of mouse and human Treg cells [10].

Therefore, FoxP3 remains the most reliable marker for Treg [11].

FoxP3 gene polymorphisms, which could affect the function and quantity of FoxP3 molecule, and thus result in the Treg function defects, have been associated with various autoimmune diseases [11, 12].

For renal transplantation, the impact of FoxP3+ Tregs on graft outcomes seems conflicting in previous reports [13-19]. In some study, the presence of intragraft Tregs have been associated with favorable renal allograft outcome [13, 14]. The FoxP3+ Treg/CD3+ T cell ratio positively correlated with graft function at 2 years after transplantation [13]. These cells could direct a FoxP3-induced immune response toward suppression of T effector cells, promoting renal graft acceptance with improved function. Lower level of intragraft FoxP3 mRNA predicts progression in renal transplants with borderline change [15]. The mRNA levels of *FoxP3* in peripheral blood were higher in patients with operational tolerance or stable kidney graft function compared to patients with chronic rejection [16, 17]. However, other groups reported that mRNA for *FoxP3* in the urine of recipients with acute rejection was higher than recipients with normal biopsy [18] and association of higher density of FoxP3+cells with worse graft outcome in recipients with acute cellular rejection [19].

Recently, an association between *FoxP3* gene polymorphisms and graft outcome has been reported also with conflicting results [20-22]. Therefore, we analyzed the association of four *FoxP3* single nucleotide polymorphisms (SNPs) (rs3761548 A/C, rs2280883 C/T, rs5902434

del/ATT, and rs2232365 A/G) with graft outcome in renal transplantation.

2. MATERIALS AND METHODS

2.1. Subjects

This study included 231 renal transplantation cases performed between January 1996 and December 2004 at the Seoul National University Hospital. The baseline characteristics of the 231 kidney transplant recipients are shown in Table 2. Residual DNA samples were collected after routine preoperative tests for HLA genotype. DNA samples from 195 healthy Korean studied in our previous cohort were used [23].

Samples were preserved at $-70℃$ prior to the experiments performed for this study. The following characteristics were collected: age and gender of recipient; age and gender of donor; type of donor (living vs cadaveric donor); primary renal disease causing end-stage renal disease; number of HLA mismatches; number of HLA-DR mismatches; crossmatch result at the time of transplantation; duration of hemodialysis; type of immunosuppression; time of transplantation; occurrence and time point of biopsy-proven acute rejection; recurrence of primary renal disease; 1-, 3-, 5-, 10-year creatinine levels post-transplantation; occurrence and time of graft failure, defined as graft nephrectomy or return to hemodialysis. The study protocol was the Declaration of Helsinki and approved by the institutional review board of Seoul National University Hospital (IRB No. 1306-121-501).

2.2. Analysis of *FoxP3* Gene SNPs

A total of 426 DNA samples were extracted from the peripheral blood of patients and controls by using the LaboPass Genomic DNA Extraction Kit (COSMO, Seoul, Korea) or QuickGene DNA whole blood kit (Fujifilm, Tokyo, Japan) and maintained at −80℃ prior to being used for these analyses. Four FoxP3 polymorphisms (rs3761548 A/C, rs2280883 C/T, rs5902434 del/ATT, and rs2232365 A/G) were analyzed by polymerase chain reaction with sequence-specific primers (PCR-SSP) with some modification [24] (Table 1). Modification includes division of existing primer sequences, slight shift of position, and application of inosine hinges to improve specificity of target polymorphisms. PCR was performed by 40 μL reaction mixture containing 40 ng DNA, 0.2 mM of each primer, 0.8 μL of 10 mM dNTP, 2.0 mM MgCl$_2$, 1.0 U Taq DNA polymerase (Roche applied science, Basel, Switzerland), and 4 μL of 10× reaction buffer. The PCR protocol consisted of an initial denaturation step at 95℃ for 5 min; 35 cycles of denaturation at 95℃ for 30 sec, annealing (temperatures detailed in Table 1) for 30 sec, and extension at 72℃ for 30 sec, and a final extension step at 72℃ for 5 min.

Table 1. Sequence specific primers of *FoxP3* polymorphisms

SNP		AT (℃)		Sequence (5' → 3')
rs3761548	C	59	F	CTGGCTCTCTCCCCAACTGA
			R	ACAGAGCCCATCATCAGACTCTCTA
	A		F	CTGGCTCTCTCCCCAACTGC
			R	ACAGAGCCCATCATCAGACTCTCTA
rs2280883	C	64	F	GATCAAATGGGTGTTACAAGGIIIIITTGGGIAC
			R	CAAGTTCCACAACATGCGACIIIIITTCACCTA
	T		F	GATGATGATTGCAGTGAGGCTIIIIITCAGGATG
			R	TATGTCAATACACCCCCAACTGIIIIICATTCICA
rs5902434	Del	62	F	GAGAAAGAGAGGCAGAGAAACATIIIIAAGAGCAAG
			R	AGGTCTTTAAAAAAATAATAGAATAAAIIIIIGAAGACTT
	ATT		F	GCCATTTATTCTATTATTATTTTTTTIIIIIACCTTACC
			R	GTGGTGAGGGGAAGAAATCATIIIIITCAGATGA
rs2232365	A	67	F	CTTCTACAGGCCCCAGCTCIIIIIACICCATC
			R	AGTGACTAGGCATGGACTCAAAIIIIICATCTGGC
	G		F	CAGCATGGCAAGTGACAGAGAIIIIIAGAGACGG
			R	CCAGCATGGCAAGTGACAGAIIIIIGGAGATAC

Abbreviations: SNP, single nucleotide polymorphism; AT, annealing temperature; F, forward; R, reverse; del, deletion; I, inosine.

2.3. Processing reference sequence data

 The Korean Reference Genome database (KRGDB) were used as reference population [25]. KRGDB is a database analyzed 622 Korean individuals by whole genome sequencing using Illumina Hiseq2000 sequencer. We searched for corresponding rs number in our study and drew up major and minor allele frequency of three SNVs (rs3761548, rs2280883 and rs2232365) and one insertion/deletion variant (rs5902434). Genotype frequencies of these SNPs or indel of *FoxP3* were calculated by a simple allele counting method.

2.4. Predicting the effect of intron variant

For the possibility of epigenetic alteration of intronic variant, rs2280883, the likely consequences of the splice site mutation on splicing efficiency were evaluated using the Netgene2 program (http://www.cbs.dtu.dk/services/NetGene2) [26, 27].

2.5. Statistical analysis

Differences of allele frequency and genotype frequency were compared using a 2-sided Chi-square test or Fisher's exact test, as appropriate. The logistic regression analysis was performed to find the independent association between presence/absence of alleles and disease while adjusting for the covariates. The odds ratio (OR) was calculated using a 95% confidence interval. Univariate and multivariate Cox proportional hazard regression models were used to estimate the crude and adjusted hazard ratios (HRs) and their 95% CIs. Multivariate analysis was performed to confirm the association between FoxP3 polymorphism and graft outcome (acute rejection or all rejection) after considering confounding factors by univariate analysis. Variables with $P < 0.25$ from univariate logistic regression analyses were included in multivariate analysis, which performed by backward stepwise selection. Death-censored graft survival was analyzed using the Kaplan-Meier method and the log-rank test. SPSS for Windows version 18.0 (SPSS, Chicago, IL, USA) was used for statistical analysis.

Allelic and Genotypic frequencies of SNPs of *FoxP3* were calculated by a simple allele/genotype counting method. Allelic distribution in cases and controls [23] was compared by odds ratio statistics using MedCalc Statistical Software version 16.4.3 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2016). The alleles and corresponding homozygous genotypes with major frequency in the control group have been selected as reference (OR = 1). A *P* value

of < 0.05 was considered statistically significant. Linkage disequilibrium (LD), expected heterozygosity were performed using Arlequin software ver.3.5.2.2. [28]. Significance of difference between groups was analysed using Chi square test.

The variance in the haplotypes and Hardy-Weinberg equilibrium exact test and MAF were analyzed using Haploview version 4.2, (http://www.broadinstitute.org/mpg/haploview) [29].

3. RESULTS

3.1. Characteristics of the study population

The baseline characteristics of the 231 kidney transplant recipients are shown in Table 2 and Table 3. Rs3761548 CC and rs2280883 TT genotypes showed significantly better survival (P = 0.038 and P = 0.032, respectively).

Diabetic nephropathy (DN) composes only 5.2% (10/194) causes of renal transplantation. Sixty percent (6/10) of DN recipients and 26.5% (50/189) of non-diabetic nephropathy (NDN) recipients experienced acute rejection during follow up. Sixty percent (6/10) of DN recipients and 36.0% (68/189) of NDN recipients underwent any kinds of rejection episodes. Acute rejection and all rejection hazard ratio for DN is 5.05 and 2.53 times that of NDN (P = 0.001 and 0.039, respectively) (data not shown). Consequently DN recipients experienced shorter graft survival (P = 0.001).

Of all recipients, 28.6% went through acute rejection, and their graft survival was also significantly shorter (P < 0.001). Otherwise, no significant differences in age, primary disease, human leukocyte antigen mismatches, renal transplantation, Anti-HLA immunisation, post-transplantation serum creatinine level or immunosuppressant regimen were found between patients of either SNP groups.

We performed mutivariate analysis on four variates above, however no significant factor was observed (Table 4).

Table 2. Characteristics of the study population and univariate Cox proportional hazards regression analysis with regard to graft survival

Characteristics	Study population (n = 231)	P value*
Recipient		
Median age (IQR) [years]	38 (30-46)	0.329
Gender [M/F]	142/89	0.967
Graft failure [GF-/GF+]	208/23	n/a
FoxP3 polymorphism		
rs3761548 [AC or AA/CC]	209/22	0.038
rs2280883 [CT or CC/TT]	216/15	0.032
rs5902434 [ATT/ATT, del/del or del/ATT]	173/58	0.254
rs2232365 [AG or GG/AA]	173/58	0.254
Primary diseases [DN/NDN][†]	10/165	0.001
Induction therapy [-/+]	199/32	0.445
Donor		
Median age (IQR) [years]	37 (27-48)	0.935
Gender [M/F]	224/7	0.086
Transplant		
Graft origin [LD/CD]	203/28	0.904
Number of HLA-mismatches	2.6 ± 1.5	0.231
Acute rejection [AR-/AR+]	165/66	<0.001
Crossmatch [+/-][‡]	2/229	0.757
Anti-HLA immunization (PRA-positive)[§]		
Class I	36 (26.9%)	0.230
Class II	47 (35.1%)	0.130
Serum creatinine (mg/dL)		
1-year	1.4±1.0	0.554
3-year	1.5±1.3	
5-year	1.6±1.3	
10-year	1.6±1.3	

∗ univariate Cox regression analysis

† 56 (24.1%) cases could not be defined as either primary disease category.

‡ All 231 cases were negative for cytotoxic crossmatch and two cases were positive only for T-cell flowcytometric crossmatch.

§ 98 (42.2%) cases do not have PRA results at the time of transplantation.

Abbreviations: IQR, interquartile range; M, male; F, female; DN, diabetic nephropathy; NDN, nondiabetic nephropathy; n/a, not available; LD, living donor; CD, cadaveric donor.

Table 3. Immunosuppressive regimen of the 231 kidney transplant recipients.

Characteristics	rs3761548		P	rs2280883		P	rs5902434		P	rs2232365		P
	CC	AC or AA		TT	CC or CT		del/del or del/ATT	ATT/ATT		AA	AG or GG	
Immunosuppressive regimen			0.173			0.293			0.908			0.908
CsA+ Pd	23	5		25	3		23	5		23	5	
	(10.4%)	(2.3%)		(11.3%)	(1.4%)		(10.4%)	(2.3%)		(10.4%)	(2.3%)	
CsA+MMF+Pd	47	4		48	3		37	14		37	14	
	(21.2%)	(1.8%)		(21.6%)	(1.4%)		(16.7%)	(6.4%)		(16.7%)	(6.4%)	
CsA+Pd	26	6		29	3		24	8		24	8	
+FK506	(11.7%)	(2.7%)		(13.1%)	(1.4%)		(10.8%)	(3.6%)		(10.8%)	(3.6%)	
CsA+MMF+Pd	45	3		47	1		38	10		38	10	
	(20.3%)	(1.4%)		(21.2%)	(0.5%)		(17.1%)	(4.5%)		(17.1%)	(4.5%)	
CsA+MMF	34	1		34	1		26	9		26	9	
+Pd+FK506	(15.3%)	(0.5%)		(15.3%)	(0.5%)		(11.7%)	(4.1%)		(11.7%)	(4.1%)	

Abbreviations: CsA, cyclosporin A; Pd, prednisolone; MMF, mycophenolate

Table 4. Multivariate analysis of factors related with graft survival in renal transplantation.

Variable	OS		RFS		RcFS	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
rs3761548 [AC or AA/CC]	2.45 (0.38−15.66)	0.343	1.79 (0.27−11.92)	0.546	3.62 (0.73−17.95)	0.115
rs2280883 [CT or CC/TT]	0.56 (0.07−4.35)	0.577	1.21 (0.12−12.72)	0.872	1.46 (0.21−10.41)	0.704
Primary diseases [DN/NDN]	3.37 (0.68−16.80)	0.139	0.71 (0.07−7.30)	0.776	3.29 (0.73−14.75)	0.120
Acute rejection [AR−/AR+]	4.98 (1.70−14.57)	0.003	NA	NA	1.93 (0.75−5.00)	0.174

Abbreviations: OS, overall survival; RFS, rejection free survival; RcFS, recurrence free survival; HR, hazard ratio; CI, confidence interval; DN, diabetic nephropathy; NDN, non-diabetic nephropathy

3.2. Comparison of gene frequency with Korean population database

For the frequencies of alternative alleles of rs3761548 and rs5902434 were found to be significantly high in kidney recipient group as compared to controls indicating genetic predisposition of impaired renal function (P < 0.01 and 0.02; OR = 1.55 and 1.29; 95% CI = 1.12–2.13 and 1.03–1.62, respectively; Table 5).

For rs2280883 and rs2232365, the frequencies of alternative alleles were significantly low in kidney recipient group as compared to controls (P < 0.01 and 0.03; OR = 0.48 and 0.78; 95% CI = 0.34–0.69 and 0.62–0.98, respectively; Table 5).

Table 5. Comparison of allelic frequency of *FoxP3* polymorphisms in recipients with Korean cohort.

FoxP3 polymorphisms	Reference Allele	Alternative Allele	Control Alternative AF (%)	Recipient Alternative AF (%)	OR (95% CI)	*P*
rs3761548	C	A	1022 (82.4)	392 (87.9)	1.55 (1.12–2.13)	< 0.01
rs2280883	T	C	208 (16.8)	41 (8.9)	0.48 (0.34–0.69)	< 0.01
rs5902434	ATT	del	750 (60.5)	307 (66.5)	1.29 (1.03–1.62)	0.02
rs2232365	T	C	484 (39.0)	154 (33.3)	0.78 (0.62–0.98)	0.03

Abbreviations: AF, allelic frequency; OR, odds ratio.

3.3. Linkage disequilibrium and haplotype-based association analysis

LD analysis revealed strong linkage between rs5902434, rs2232365 and rs3761548 in the *FoxP3* gene (r^2 = 0.98, D'= 1.00). Weak correlation was revealed between rs2280883 and rs5902434 in the *FoxP3* gene (r^2 = 0.20, D' = 1.00). Therefore two representative SNP rs2280883 and rs5902434 was included in subsequent genetic analyses. Expected heterozygosity is 0.16 for rs2280883, reflecting an excess of homozygotes. Expected heterozygosity for rs5902434 and rs2232365 is 0.45 and 0.45, respectively. Theta(H) under the infinite-allele model is 0.19 for rs2280883, 0.81 for rs5902434 and 0.80 for rs2232365 (Figure 1).

LD analysis for *FoxP3* was duplicated using both Arlequin 3.5.2.2. and Haploview 4.2. Haplotype blocks were used to measure LD. Samples from kidney allograft recipients exhibited substantial LD amongst themselves (Figure 2). We detected one LD block within *FoxP3*. As shown in Figure 2, the number and the color of the square indicates D' and the gray square denotes D' = 1. The gradient colors demonstrate the strengths of the LDs of the tag SNPs. The LD block 1 haplotype was formed by three tag SNPs (rs2232365:T, rs3761548:A and rs5902434:del), which had nominally significantly different frequencies in the two groups (*P* value <0.001)(Table 6).

Other haplotypes were not significantly associated between kidney allografts and recipients. All SNPs studied but rs2280883 participated in forming haplotypes (Figure 2 and Table 6).

(A)

rs2280883   rs5902434   rs2232365

Expected heterozygosity

(B)

rs2280883   rs5902434   rs2232365

θ_H at different loci
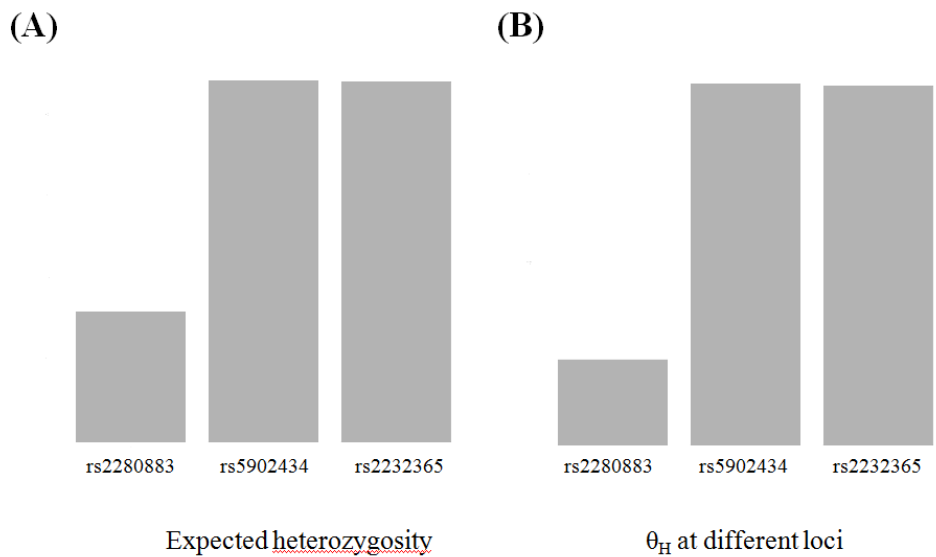
Figure 1. Expected heterozygosity （A） and theta （H） （B） at rs2280883, rs5902434 and rs2232365. rs2280883 shows low genetic diversity and polymorphism. rs5902434 and rs2232365 show rich and even genetic diversity. Values are similar because the segregation of two locus （figure not shown）.
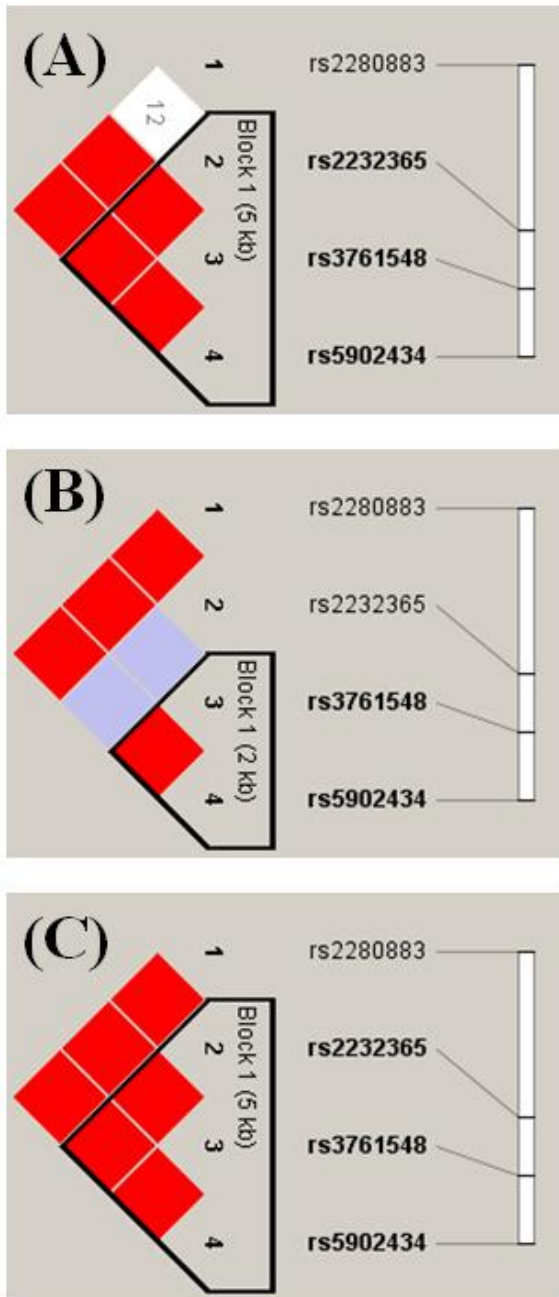
Figure 2. Linkage disequilibrium of *FoxP3* polymorphisms (A) All subjects, (B) kidney transplant recipients, and (C) controls.

Table 6. Association of haplotype frequencies of *FoxP3* polymorphisms with kidney recipients.

Haplotype	Allele frequency		Chi-square	*P*
	Case	Control		
T-A-del	0.061	0.227	15.314	< 0.001
G-A-del	0.312	0.212	3.627	0.25
G-G-ATT	0.620	0.561	1.241	0.50

Abbreviation: del, deletion

3.4. Association of *FoxP3* polymorphisms with graft rejection

The frequency of acute rejection (AR) in rs3761548 AC or AA genotype showed a tendency of increase compared to CC genotype (45.5% vs. 26.8%, *P* = 0.082) (Table 7). The frequency of acute rejection (AR) in rs2280883 CT or CC genotype showed significantly increase compared to TT genotype (53.3% vs. 26.9%, *P* = 0.038) (Table 7). In univariate analysis, rs2280883 CC or CT genotype was a risk factor for acute rejection compared to TT genotype (*P* = 0.04). Other polymorphisms showed no association with acute rejection. Chronic or all rejection is not associated with any *FoxP3* polymorphisms analyzed in this study.

Of the four SNPs analyzed, rs3761548 and rs5902434 were reconstructed a block combined of two haplotypes (G-ATT and A-del). Association between haplotypes and rejection episodes (acute or chronic) was not observed (*P* = 0.933 and *P* = 0.979, respectively).

Table 7. Association of *FoxP3* polymorphisms with graft rejection

FoxP3 SNP		AR (%)		P	CR (%)		P	All rejection		P
		(−)	(+)		(−)	(+)		(−)	(+)	
rs3761548 C/A				0.082			0.260			0.167
	CC	153	56		172	37		131	78	
		(73.2)	(26.8)		(82.3)	(17.7)		(62.7)	(37.3)	
	AC or	12	10		16	6		10	12	
	AA	(54.5)	(45.5)		(72.7)	(27.3)		(45.5)	(54.5)	
rs2280883 C/T				0.038			0.489			0.103
	TT	158	58		177	39		135	81	
		(73.1)	(26.9)		(81.9)	(18.1)		(62.5)	(37.5)	
	CC or	7	8		11	4		6	9	
	CT	(46.7)	(53.3)		(73.3)	(26.7)		(40.0)	(60.0)	
rs5902434 del/ATT				0.407			0.562			0.878
	del/del or del/ATT	126	47		139	34		105	68	
		(72.8)	(27.2)		(80.3)	(19.7)		(60.7)	(39.3)	
	ATT/ ATT	39	19		49	9		36	22	
		(67.2)	(32.8)		(84.5)	(15.5)		(62.1)	(37.9)	
rs2232365 A/G				0.407			0.562			0.878
	AA	126	47		139	34		105	68	
		(72.8)	(27.2)		(80.3)	(19.7)		(60.7)	(39.3)	
	AG or	39	19		49	9		36	22	
	GG	(67.2)	(32.8)		(84.5)	(15.5)		(62.1)	(37.9)	

Abbreviations: SNP, single nucleotide polymorphism; AR, acute rejection; CR, chronic rejection

Table 8. Association of *FoxP3* haplotypes of rs3761548 and rs5902434 with acute or chronic graft rejection

	Haplotype	HF (%)	rejection event (%)		chi-square	*P*-value
			(+)	(−)		
Acute rejection						
	G-ATT	60.8	61 (61.0)	39 (39.0)	0.007	0.933
	A-del	39.2	39 (39.0)	61 (61.0)	0.007	0.933
Chronic rejection						
	G-ATT	60.8	62 (61.1)	40 (38.9)	0.001	0.979
	A-del	39.2	40 (38.9)	62 (61.1)	0.001	0.979

Abbreviations: HF, haplotype frequency; del, deletion

3.5. Graft survival and *FoxP3* polymorphism

Kaplan-Meier analysis was used to examine the relationships between *FoxP3* SNPs and graft survival. Patients with rs3761548 CC genotype showed better graft survival compared to AC or AA genotype (log rank test, *P* = 0.03). Patients with rs2280883 TT genotype showed better graft survival compared to CT or CC genotype (*P* = 0.02) (Figure 3A and B). The mean and 95% CI of time to graft failure for the rs3761548 CC and AC or AA groups were 174.9 ± 3.7 (95% CI: 167.8–182.1) months and 152.0 ± 14.8 (95% CI: 123.0–181.0) months, respectively; For the rs2280883 TT and CT or CC groups were 174.1 ± 3.6 (95% CI: 167.0–181.3) months and 141.3 ± 16.0 (95% CI: 110.0–172.6) months, respectively.

Two haplotypes reconstructed from rs3761548 and rs5902434 shows no association with graft failure (*P* = 0.763).

Figure 3. Kaplan-Meier survival analysis of graft survival and *FoxP3* polymorphism (A) rs3761548 A/C, (B) rs2280883 C/T, (C) rs5902434, and (D) rs2232365. (A) Patients with rs3761548 CC genotype (n = 209) showed better graft survival than those with AC or AA genotype (n = 22) (log rank test, P = 0.03). (B) Patients with rs2280883 TT genotype (n = 216) showed better graft survival than those with CT or CC genotype (n=15) (P = 0.02).
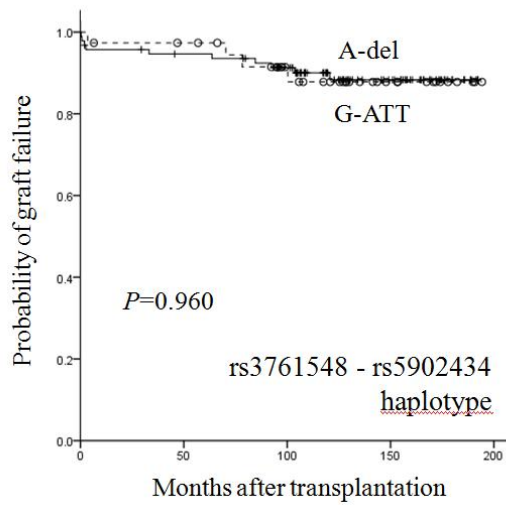
Figure 4. Kaplan–Meier survival analysis of graft survival and *FoxP3* haplotypes of rs3761548 and rs5902434. Neither haplotype showed association with graft survival (log rank test, *P* = 0.960). Ambiguous heterozygotes were excluded from analysis (n = 30).

3.6. Recurrence of underlying glomerular disease posttransplant and *FoxP3* polymorphism

Relationship between *FoxP3* polymorphism and recurrence of underlying glomerular disease was also analyzed by Kaplan–Meier survival analysis. Patients with rs3761548 CC genotype showed lower rate of recurrence of underlying glomerular disease compared to AC or AA genotype (P = 0.01) (Figure 4). The mean and 95% CI of time to recurrence of underlying glomerular disease for the rs3761548 CC and AC or AA groups were 180.9 ± 3.0 (95% CI: 175.1–186.8) months and 140.5 ± 15.3 (95% CI: 110.4–170.5) months, respectively. Two haplotypes reconstructed from rs3761548 and rs5902434 shows no association with recurrence of underlying disease (P = 0.308).
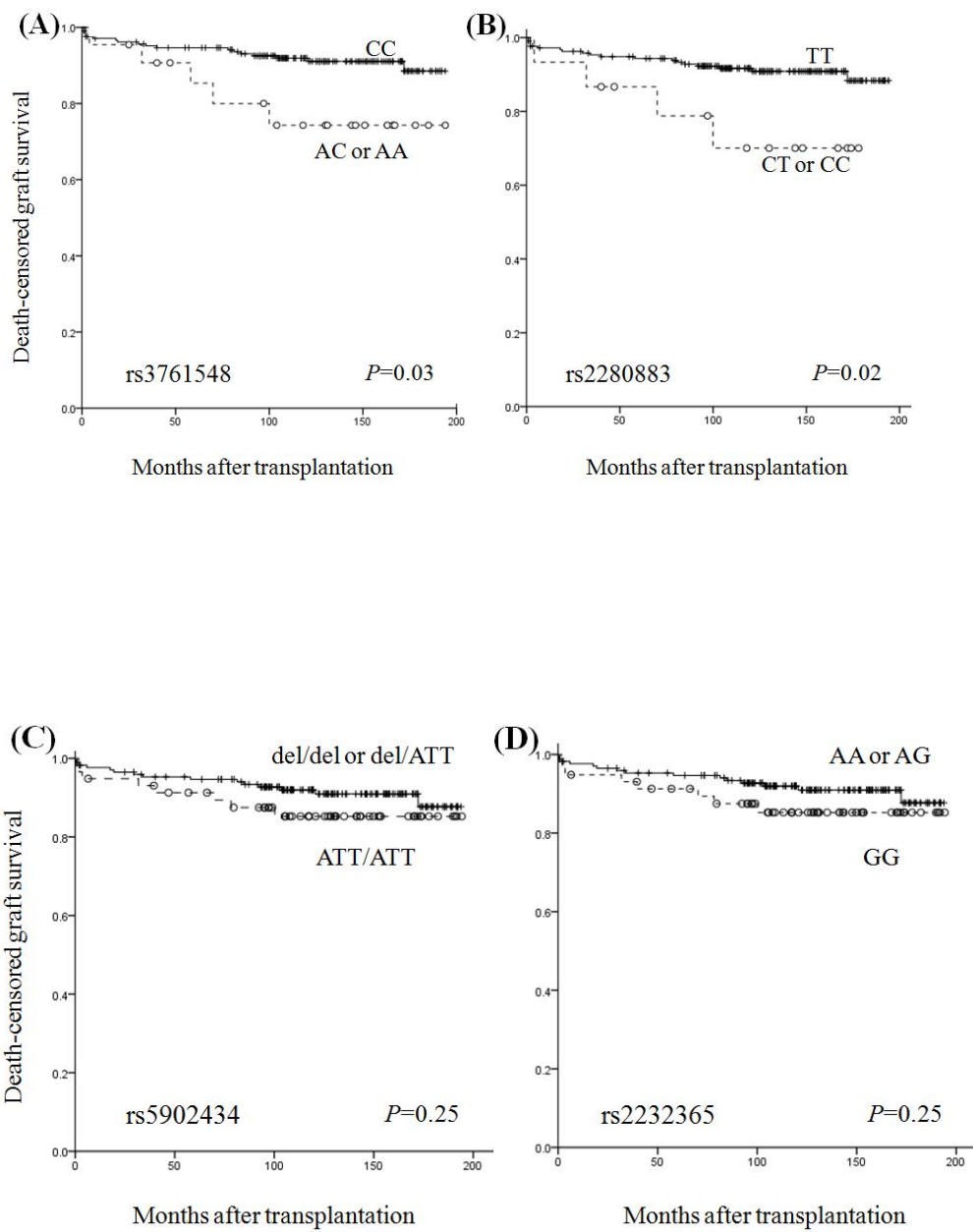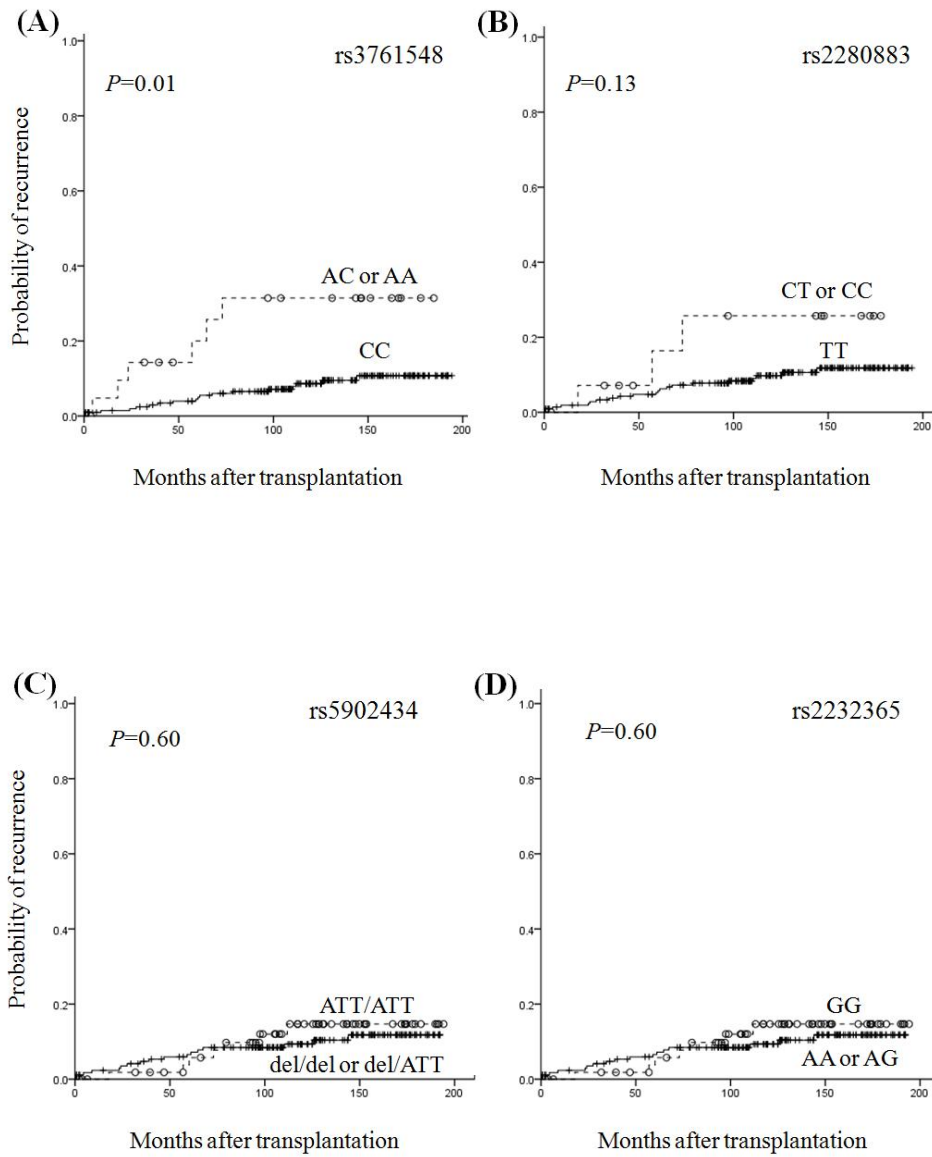
Figure 5. Kaplan-Meier survival analysis of recurrence of underlying glomerular disease posttransplant and *FoxP3* polymorphism (A) rs3761548 A/C, (B) rs2280883 C/T, (C) rs5902434, and (D) rs2232365.

Longer recurrence-free period was observed for genotype with CC homozygote as compared to genotypes with A allele for rs3761548 (log rank test, P = 0.01).

Table 9. Association of *FoxP3* haplotypes of rs3761548 and rs5902434 with the recurrence of underlying disease.

Haplotype	HF (%)	Recurrence (%)		chi-square	*P*-value
		(+)	(−)		
G-ATT	71.2	85 (90.4)	9 (9.6)	1.038	0.31
A-del	28.8	32 (84.2)	6 (15.8)		

Abbreviations: HF, haplotype frequency; del, deletion

3.7. Posttransplant infection and *FoxP3* polymorphism

For rs5902434, recipients with null allele showed marginal significance of higher posttransplant infection, regardless of pathogen (n = 35, 15.2%) than recipients without null allele (n = 5, 2.2%) (OR = 2.69, 95% CI = 1.00–7.23, *P* = 0.05). Recipients with rs2232365 A allele also showed marginal significance of higher posttransplant bacterial infection (n = 35, 15.2%) than recipients without A allele (n = 5, 2.2%) (OR = 2.69, 95% CI = 1.00–7.23, *P* = 0.05) (Table 10). Same finding was observed with all posttransplant bacterial infection for rs 5902434 and rs2232365 (Table 10).

No association between any *FoxP3* polymorphisms and posttransplant infection by other kinds of pathogen (virus, mycobacteria or fungus) was found (Table 12, 13, 14).

Table 10. Patients with posttransplant infection according to *FoxP3* polymorphism.

FoxP3 polymorphism		All infection (%)		OR (95% CI)	*P*
		(+)	(−)		
rs3761548C/T	CC	90 (43.1)	119 (56.9)	1.10	0.83
	AC or AA	10 (45.5)	12 (54.5)	(0.46–2.66)	
rs2280883C/T	TT	6 (40.0)	9 (60.0)	1.16	1.00
	CC or CT	94 (43.5)	122 (56.5)	(0.40–3.37)	
rs5902434 del/ATT	del/del or del/ATT	35 (15.2)	138 (59.7)	2.69	0.05
	ATT/ATT	5 (2.2)	53 (22.5)	(1.00–7.23)	
rs2232365A/G	GG	5 (2.2)	53 (22.5)	2.69	0.05
	GA or AA	35 (15.2)	138 (59.7)	(1.00–7.23)	

Abbreviations: OR, odds ratio; del, deletion

Table 11. Patients with posttransplant bacterial infection according to *FoxP3* polymorphism.

FoxP3 polymorphism		Bacterial infection (%)		OR (95% CI)	*P*
		(+)	(−)		
rs3761548C/T	CC	37 (17.7)	172 (82.3)	0.73	0.77
	AC or AA	3 (13.6)	19 (86.4)	(0.21–2.61)	
rs2280883C/T	TT	1 (6.7)	14 (93.3)	3.09	0.48
	CC or CT	39 (18.1)	177 (81.9)	(0.39–24.16)	
rs5902434 del/ATT	del/del or del/ATT	35 (20.2)	138 (79.8)	2.69	0.05
	ATT/ATT	5 (8.6)	53 (91.4)	(1.00–7.23)	
rs2232365A/G	GG	5 (8.6)	53 (91.4)	2.69	0.05
	GA or AA	35 (20.2)	138 (79.8)	(1.00–7.23)	

Abbreviations: OR, odds ratio; del, deletion

Table 12. Patients with posttransplant viral infection according to *FoxP3* polymorphism.

FoxP3 polymorphism		Viral infection (%)		OR (95% CI)	*P*
		(+)	(−)		
rs3761548C/T	CC	40 (19.1)	169 (80.9)	1.24	0.78
	AC or AA	5 (22.7)	17 (77.3)	(0.43–3.57)	
rs2280883C/T	TT	3 (20.0)	12 (80.0)	0.97	1.00
	CC or CT	42 (19.4)	174 (80.6)	(0.26–3.58)	
rs5902434 del/ATT	del/del or del/ATT	33 (19.1)	140 (80.9)	1.11	0.85
	ATT/ATT	12 (20.7)	46 (79.3)	(0.53–2.32)	
rs2232365A/G	GG	12 (20.7)	46 (79.3)	1.11	0.85
	GA or AA	33 (19.1)	140 (80.9)	(0.53–2.32)	

Abbreviations: OR, odds ratio; del, deletion

Table 13. Patients with posttransplant mycobacterial infection according to *FoxP3* polymorphism.

FoxP3 polymorphism		Mycobacterial infection (%)		OR (95% CI)	*P*
		(+)	(−)		
rs3761548C/T	CC	5 (2.4)	204 (97.6)	NA	1.00
	AC or AA	0 (0.0)	22 (100.0)		
rs2280883C/T	TT	0 (0.0)	15 (100.0)	NA	1.00
	CC or CT	5 (2.3)	211 (97.7)		
rs5902434 del/ATT	del/del or del/ATT	5 (2.9)	168 (97.1)	NA	0.33
	ATT/ATT	0 (0.0)	58 (100.0)		
rs2232365A/G	GG	0 (0.0)	58 (100.0)	NA	0.33
	GA or AA	5 (2.9)	168 (97.1)		

Abbreviations: OR, odds ratio; del, deletion

Table 14. Patients with posttransplant fungal infection according to *FoxP3* polymorphism.

FoxP3 polymorphism		Fungal infection (%)		OR (95% CI)	*P* value
		(+)	(−)		
rs3761548C/T	CC	2 (1.0)	207 (99.0)	4.93	0.26
	AC or AA	1 (4.5)	21 (95.5)	(0.43–56.66)	
rs2280883C/T	TT	0 (0.0)	15 (100.0)	NA	1.00
	CC or CT	3 (1.4)	213 (98.6)		
rs5902434 del/ATT	del/del or del/ATT	2 (1.2)	171 (98.8)	1.50	1.00
	ATT/ATT	1 (1.7)	57 (98.3)	(0.13–16.85)	
rs2232365A/G	GG	1 (1.7)	57 (98.3)	1.50	1.00
	GA or AA	2 (1.2)	171 (98.8)	(0.13–16.85)	
rs3060515 del/ATA/TAATA	del/del or del/TAATA	2 (1.2)	171 (98.8)	1.50	1.00
	TAATA/TAATA	1 (1.7)	57 (98.3)	(0.13–16.85)	

Abbreviations: OR, odds ratio; del, deletion

3.8. In-silico analysis of the intron variant on splicing efficiency of *FoxP3* polymorphism

The Netgene2 splice site prediction program comparing the wild-type FoxP3 sequence, NC_000023.11:g.49252667C, with the NC_000023.11:g.49252667T allele scored the likelihood of the canonical splice site as being active abour 95% and the variant site with the substituted T was abour 95%, similarly (Figure 5).

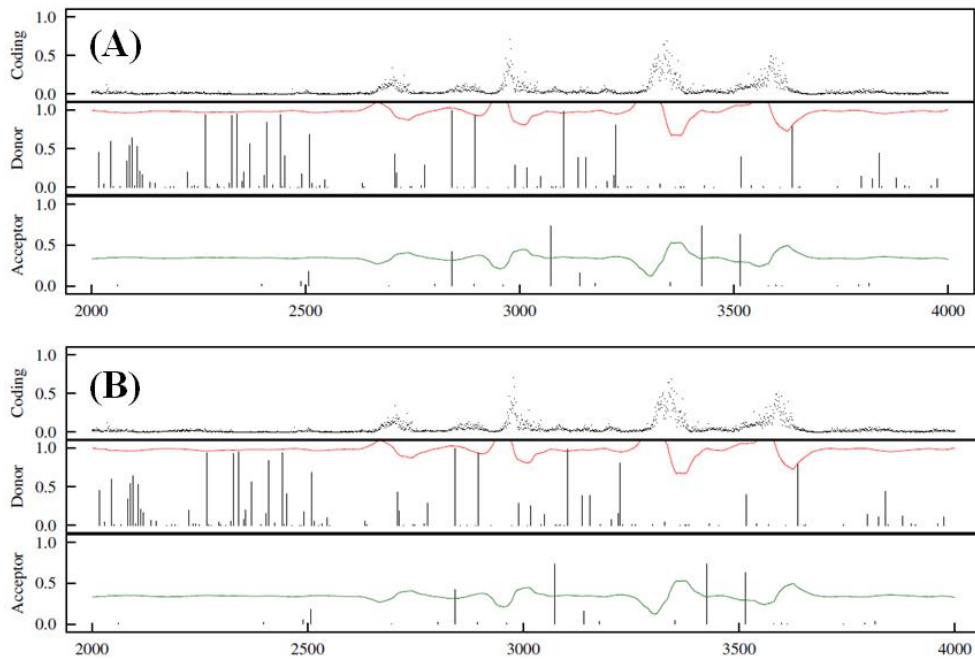**Figure 6.** NetGene2 graphics output of rs2280883 intron variant prediction with (A) C allele, (B) T allele; the top part of ″coding″ is the activity of an ensemble of coding predicting networks, a cyan impulse is a prediction that has been discarded during the refinement, and a magenta colored impulse is a prediction that has been changed by the rule based system. Both graphs shows similar pattern and score (data not shown).

4. DISCUSSION

In our study, rs3761548 AA genotype was associated with inferior graft survival and recurrence of primary renal disorders. Rs3761548 AA genotype was associated with psoriasis [30], unexplained recurrent spontaneous abortion in Chinese [31], and intractability of Graves' disease in Japanese [32]. Recently, association of AA genotype with allograft rejection was reported in renal transplantation in Chinese [20] and in Indian [22], which are somewhat similar to our findings. Polymorphisms of *FoxP3* gene promoter may alter the binding specificity of transcription factors and are relevant to initiating transcription, therefore, might affect the function or quantity of Treg [33]. Oda et al. [12] indicted that rs3761548 AA genotype leads to a loss in binding with E47 and c-Myb, leading to defective transcription of FoxP3. Qiu et al. [20] proved that patients with AA genotype were more prone to allograft rejection in renal transplantation and the function of Treg in patients with AA genotype is weaker than that of CC genotype.

In our study, rs2280883 with C allele was associated with higher acute rejection event; rs2280883 CC genotype was also associated with inferior graft survival. Although there is no article elucidating an association of rs2280883 and renal disease so far, several studies which elicited clinical effects of rs2280883 polymorphisms has been carried out. Analysis of the rs2280883 CC genotype was increased in infertile women with idiopathic infertility in Brazil [34] and Graves's disease in China [35]. The rs2280883 variant was associated with

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susceptibility to systemic sclerosis in Italia [36], and its genotypic frequency exhibited significant differences in patients with primary biliary cirrhosis [37]. The mutant TT genotype was found to be more frequent among patients with hepatitis B-related hepatocellular carcinoma [38]. In our study, rs2280883 polymorphism was not related with chronic rejection. There is consistent study result that also suggest the association of *FoxP3* polymophism and acute rejection [22]. But these are somewhat different finding with previous studies because FoxP3+Treg cells are more involved in chronic rejection regardless subtle or obvious [39].

Polymorphisms of other genes which are relevant with host immune responses such as FasL or IL-17 have been reported [40, 41]. Further studies are needed in larger number of patients and in other ethnic groups to confirm the association of rs2280883 CC genotype with clinical outcome of renal allograft.

Although there have been a few studies regarding the effects of *FoxP3* polymorphisms on infection, it is sufficiently inferable by mechanism FoxP3 works on immune system. Piao et al. suggested that *FoxP3* polymorphism at rs3761548 A allele may be associated with lower postplant CMV infection in allo-HSCT recipients [42]. This finding is consistent with our results, excluding the pathogen.

Of our SNPs studied, rs2280883 is the only intron variant. which is located in intron2 (NC_000023.11:g.49252667T>C). However it shows statistically significant association with graft survival. To elucidate this, we performed in-silico analysis. The variant is at the 902th base

pair of the splice junction, it is theoretically unlike that T>C change may cause splicing defect. However, we presented an evidence that rs2280883C>T is associated with graft outcome. Therefore rs2280883 may not be a pathogenic variant but in linkage disequilibrium with another pathogenic variant. However, this result is merely computational prediction, and additional studies are necessary to confirm the FoxP3 expression level, rather than simple splicing prediction. RT-PCR of transcripts from peripheral Treg cells can be the method.

Expression level of FoxP3 is largely determined by epigenetic regulation [43]. Treg cells possess specific epigenetic features. For example, DNA hypomethylation is specifically observed at Treg signature gene loci, such as *FoxP3*, *Ctla4*, *Ikzf4*, and *Ikzf2* [44] and permissive histone marks are specifically present in Treg cells at the *FoxP3* promoter region[45]. In addition, DNA hypomethylation at *FoxP3* CNS2 (conserved noncoding region 2), an enhancer region, is important for Treg-cell lineage specification, as it enhances FoxP3 transcription ay allowing the binding of transcription factors [46]. These epigenetic control on FoxP3 expression cannot be determined by splicing analysis alone.

In transplant field, in which self tolerance is crucial for successful engraftment, it can be deduced that genetic variants or protein expression level of FoxP3 not only affects graft outcome, but also have potential as a therapeutic option. Based on this deduction, data is accumulated in transplant field, mainly limited to hematopoietic

stem cell transplantation in type 1 diabetes mellitus or IPEX syndrome patients. For kidney transplant, accumulating data suggest that Treg cells might induce graft tolerance in tertiary lymphoid organ in graft, therefore slow down the kinetics of chronic rejection [47]. In animal studies, mice and pigs show systemic tolerance to kidney, skin and heart allografts from the same donor strain, initially dependent on FoxP3+ cells [48, 49]. To lesser extent, the presence of intragraft Tregs has been suggested as a positive predictor of favorable transplant outcome in stable patients, especially with subclinical signs of rejection [50]. However it is still controversial. Xu X et al. conducted an experiment with chronic rejected kidney allografts, showing it could be an epiphenomenon of the inflammatory process [51]. In our study, no association was found between chronic rejection and four SNPs studied, probably because we investigate genetic polymorphism of *Foxp3* only rather than afterward transcription processes. Other regulating genes such as *NFAT1* which can affect the expression of Foxp3 and function of Tregs can play some roles in graft tolerance.

Diabetic nephropathy (DN) composes only 5.2% (10/194) causes of renal transplantation, which is much less compared to recent literature [52, 53]. However, Han et al. reported that diabetes mellitus occupied only 7.1% and 11.7% in 1995-1999 and 2000-2004 period, respectively, and rushed after 2005 as causative disease of kidney transplant-requiring ESRD patients [54]. Therefore our finding is compatible in previous data in Korea taking account of the period of

specimen collection. Recipients with primary DN experienced more acute or any kinds of rejection episodes. However, small number of DN recipients involved in this study makes this finding unreliable.

Boucek P et al. compared kidney transplant outcome between type2 DN (DN2) and non-type2 DN (non-DN) kidney recipients [55]. Although they found no significant difference in Kidney graft survival between type 2 diabetic patients and non-diabetic controls (P = 0.19), further investigation of the data suggests DN2 recipients show far more rejection than non-DN recipients (8% vs. 3%, P value was not available).

This study had some limitations. First, we did not performed the expression level of FoxP3 or epigenetic change in *FoxP3* neither in blood or graft tissue. Second, three SNPs excluding rs2280883 showed strong linkage disequilibrium so that we could not model SNP-SNP interaction.

In conclusion, in our study we revealed the associations of rs3761548AA genotype and rs2280883 CC genotype with inferior graft survival in renal transplantation in Koreans. These findings may help to elucidate the role of Tregs in renal transplantation and predict the clinical outcome of renal allograft.

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국문 초록

서론: FoxP3은 동종이식편 관용유도에 중요한 조절T세포의 가장 신뢰할 수 있는 표지자이다. *FoxP3* 유전자다형성이 신이식의 이식편생존과 연관되었다는 보고가 있어 이를 확인하고자 한다.

방법: 1996년부터 2004년까지 서울대학교병원에서 신이식을 시행한 성인 환자 231례를 대상으로 4부위의 *FoxP3* 유전자다형성 (rs3761548 A/C, rs2280883C/T, rs5902434del/ATT, rs2232365A/G)을 염기서열특이 시발체를 이용한 중합효소연쇄반응(polymerase chain reaction with sequence specific primers, PCR-SSP)를 이용하여 분석하였다.

결과: rs2280883 TT 유전형을 가진 환자들은 CC 혹은 CT 유전형을 가진 환자들에 비하여 유의하게 낮은 급성거부반응 발생을 보였다 (26.9% vs 53.3%, *P* = 0.038). rs3761548CC 유전형을 가진 환자들은 AC 혹은 AA 유전형을 가진 환자들에 비해 우수한 이식편생존을 나타냈다(log rank test, *P* = 0.03). rs2280883 TT 유전형을 가진 환자들은 CC 혹은 CT 유전형을 가진 환자들에 비하여 우수한 이식편생존을 나타냈다(*P* = 0.02). rs3761548CC 유전형을 가진 환자들은 AC 혹은 AA 유전형을 가진 환자들에 비해 낮은 원(原)사구체질환 재발율을 나타냈다(*P* = 0.01).

결론: 한국인에서 *FoxP3* 유전자의 rs3761548 CC 유전자형과 rs2280883 TT 유전자형은 신이식의 우수한 임상성적과 상관관계가 있었다.

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주요어 : FoxP3, 단일염기다형성, 신이식, 이식편생존
학   번 : 2013-30544