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의학석사 학위논문

Association of complement 5 genetic polymorphism with renal allograft outcomes

혈청보체인자 5 의 유전적 다형성과 신장이식 후 이식신 성적의 연관성

2013 년 2 월

서울대학교 대학원
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정 종 철

혈청보체인자 5 의 유전적 다형성과 신장이식 후 이식신 성적의 연관성

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Association of complement 5 genetic polymorphism with renal allograft outcomes

by

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(Directed by Curie Ahn, M.D., Ph.D.)

A Thesis Submitted to the Department of Immunology in Partial Fulfillment of the Requirement of the Degree of Master of Philosophy in Immunology at Seoul National University College of Medicine

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Approved by Thesis Committee:

Professor _____ **Chairman**

Professor _____ **Vice chairman**

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Abstract

Association of complement 5 genetic polymorphism with renal allograft outcomes

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Background: Complements play important roles in both rejection and ischemia-reperfusion injury after transplantation. Complement 5 (C5) is a pivotal complement, which initiates the assembly of the membrane attack

complex, and mediates chemotaxis of various immune cells. I investigated the impacts of genetic variations in C5 and its receptor (C5aR) of both recipients and donors on renal allograft outcomes.

Materials and Methods: Seven single nucleotide polymorphisms (SNPs) in C5 (rs12237774; rs2159776; rs17611; rs25681; rs2241004; rs10985126; rs10818500) and one SNP (rs10404456) in the C5aR gene were genotyped in 191 recipient-donor pairs. The association of the polymorphisms with allograft outcomes was determined.

Results: Three C5 SNPs (rs2159776; rs17611; rs25681) in recipients had a tendency toward a reduced glomerular filtration rate (eGFR) at one year after transplantation. There were four haplotypes in the H2 linkage disequilibrium block, which was formed by four SNPs (rs2159776; rs17611; rs25681; rs2241004). The GGCG haplotype in both recipients and donors was associated with lower GFR at one year (60.9±15.9 vs. 66.4±15.5 ml/minute/1.73m2, P=0.020; 60.6±15.3 vs. 66.2±15.8 ml/minute/1.73m2, P=0.017). The association was sustained over 7 years after transplantation (P=0.015 in recipients; P=0.039 in donors). The presence of the GGCG haplotype in recipients was associated with poorer graft survival (log-rank test, P=0.024). However, C5 polymorphisms were not correlated with serum

C5 level. C5aR polymorphism had no significant impact on the allograft outcomes.

Conclusions: The GGCG haplotype of complement 5 in both recipients and donors was associated with lower renal allograft function.

Keywords: complement, kidney transplantation, polymorphism, transplantation outcome

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LIST OF ABBREVIATIONS

C3	complement 3
C5	complement 5
C5aR	cognate receptor of complement 5
CI	confidence interval
eGFR	estimated glomerular filtration rate
EMT	epithelial to mesenchymal transition
ESRD	end stage renal disease
GEE	generalized estimating equations
GFR	glomerular filtration rate
HLA	human leukocyte antigen
htSNP	haplotype tagging single nucleotide polymorphism
IRI	ischemia reperfusion injury
LD	linkage disequilibrium
MAF	minor allele frequency
MDRD	modification of diet in renal disease
SNP	single nucleotide polymorphism

Introduction

When the complement system was first identified, it was named after the trait that 'complemented' the effects of specific antibody in the lysis of bacteria and red blood cells. Now, the complement is known as a system of more than 30 serum proteins and cell surface receptors, kind of triggered enzyme cascades analogous to the coagulation, fibrinolysis and kinin pathways [1]. The roles of complement system are summarized as host defense activity against infection, interface between innate and adaptive immunity, and clearance of waste product from body [2]. The association of complements with renal disease was reported in atypical membranoproliferative glomerulonephritis and idiopathic hemolytic uremic syndrome with factor H deficiency, several anti-complement autoantibodies with glomerulonephritis, and acute humoral rejection and chronic rejection in renal transplant [3].

Complements are important humoral effectors in innate immunity and they can also play the role of a link between innate and adaptive immune responses [1]. Therefore, they can contribute to tissue injury such as ischemia reperfusion injury (IRI) [4-6], humoral rejection, or acute cellular rejection [7,8] in

transplantation. Ischemia lowers the resistance of endothelial cells to complement attack and exposes the activating surface to complements. Beyond IRI, complements act as effectors in hyperacute, acute, and chronic humoral rejection, and C4d deposition is included in the diagnostic criteria for humoral rejection [9]. Both complement 3 (C3) and complement 5 (C5) can provide costimulation in the interaction between dendritic cells and T cells, and thereby contribute to acute cellular rejection [8,10].

Three complement activation pathways, the classical, alternative and mannose-binding lectin pathways, covers in the cleavage of C3. C3b participates in the formation of C5 convertase, which cleaves C5 into C5a and C5b. C5b forms the membrane-attack complex (C5b-C9), a humoral effector. C5a mediates the immune reaction via chemotaxis and cell activation through interaction with its cognate receptor (C5aR) [2,11,12]. C5aR is present on neutrophils, T cells, B cells, epithelial cells and endothelial cells [13]. C5a-C5aR interaction in antigen presenting cells and T cells can provide costimulatory and survival signals to naïve CD4 T cells, which induce up-regulation of antigen-specific T cell responses [14]. The CD8+ T cell response to influenza type A virus was impaired when mice were treated with C5aR antagonist [15]. C5 also regulates adaptive immunity by the

modulation of dendritic cell function [10]. There is increasing evidence from animal experiments, where antagonists of C5 or C5aR showed protective effects against IRI [5,6,16,17]. Moreover, anti-C5 monoclonal antibodies have been developed as therapeutic antibodies and have performed promisingly in clinical trials for paroxysmal nocturnal hemoglobinuria [18] and transplantation [19-22]. In recent study, Eculizumab, anti-C5 monoclonal antibody showed its clinical efficacy via reducing antibody mediated rejection rate and transplant glomerulopathy [23,24].

Consistent with the importance of complements in transplantation, recent studies have reported the association between polymorphisms of complement genes and clinical outcomes of kidney allograft recipients [25-27]. Among more than 30 proteins in the complement system, the C3 had received focus, because it is in the central part of the complement cascades and its allotypic variants (C3F and C3S) are well-known. However, its minor allele frequency (MAF) is less than 1% in Asian populations [28]. Therefore, we focused on C5, another important complement immediately downstream of C3. Herein, we investigated the association of C5/C5aR gene polymorphisms with renal allograft outcomes.

Materials and Methods

1. Study population

Three hundred eighty-two adult patients (recipient age>18 years) underwent living donor kidney transplantation at the Seoul National University Hospital (SNUH) from January 1996 to February 2007. Among them, DNA samples were available with informed consent for genetic analysis in 191 donor-recipient pairs. There was no significant difference in baseline clinical characteristics between the 191 participants and the non-participants, except the choice of initial calcineurin inhibitor (data not shown). The study protocols were approved by the Institutional Review Board of SNUH (H-0802-059-235). All study processes were conducted under the Declaration of Helsinki.

2. Genotyping

Linkage disequilibrium (LD) blocks of the C5 gene were constructed based on SNPs genotyped in the HapMap Asian JPT+CHB samples (www.hapmap.org). A total of 75 SNPs were genotyped in about 110-kb

region including C5 gene (9q34.1) and its 5' upstream. SNPs with a minor allele frequency of less than 5% were excluded. We predicted 4 LD blocks using the confidence interval (CI) method in Haploview 4.1 [29] (Figure 1). Within each haplotype block, haplotype tagging SNPs (htSNPs) (rs12237774, rs2159776, rs2241004, rs10985126, and rs10818500) were chosen so that any marker in the LD blocks was presumed to be correlated with htSNPs with $r^2 > 0.8$. In addition, two SNPs (rs17611 and rs25681) were added because they were significantly associated with asthma in Japanese [30]. One SNP (rs10404456) was selected in the C5aR gene (19q13.3) because it was completely linked to the other SNPs.

Genotyping was performed using TaqMan SNP Genotyping Assays, according to the manufacturer's instructions.

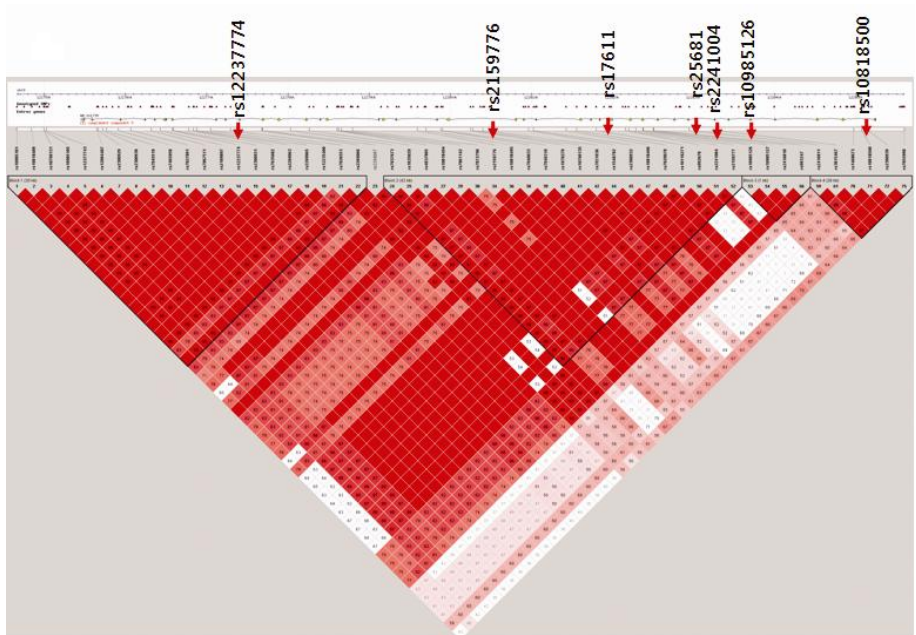


Figure 1. Linkage disequilibrium map of complement 5 gene. Seven single nucleotide polymorphisms genotyped in the present study were depicted by arrows with rs numbers

3. Clinical data analysis

Clinical information was retrieved from the SNUH transplantation database, which includes demographic data on donor and recipient, number of HLA mismatches, initial immunosuppressant regimen, cause of end-stage renal disease (ESRD), comorbid status of diabetes mellitus, presence of biopsy-proven acute rejection, serum creatinine levels and transplantation outcomes. Estimated glomerular filtration rate (eGFR) was calculated using an abbreviated Modification of Diet in Renal Disease (MDRD) formula: $eGFR=186\times([\text{serum creatinine}/88.4]^{-1.154})\times(\text{age})^{-0.203}$(x0.742 for female). The association of genotype/haplotype with clinical outcomes such as acute rejection, graft function, and graft survival was analyzed. Graft loss was defined as death-censored graft loss.

4. Determination of serum C5 level

In order to assess the functional significance of C5 haplotypes, we determined serum C5 levels and genotypes in 100 healthy subjects. Serum C5 concentration was measured using a commercial radial immunodiffusion kit (The Binding Site, Birmingham, UK). In brief, 5 μL of healthy control serum was applied to the pre-cut wells in a radial immunodiffusion kit which

contains anti-C5 antibodies in it. After incubating 72hr at room temperature (approximately 20 ~ 24'c) for fixed time period or until rings are complete (minimum 72 hours), the values were read by measuring the diameter of immunoprecipitation rings and comparing the results with the RID reference table. The mean age of subjects was 43.1±7.2 years and male-to-female ratio was 1.3 (M:F = 56:44).

5. Statistical analysis

Calculated power of our study for common SNPs with MAF of higher than 40% (rs2159776, rs17611, rs25681 and rs10404456) exceeded 80% to detect a difference of 5 ml/min/1.73 m^2 in eGFR between genotype groups. For less common SNPs with 25% MAF (rs2241004) or 15% MAF (rs1223774, rs10985126 and rs10818500), powers were 73% and 65%, respectively. In haplotype analysis using H2 locus, the statistical power of our study was 88% to detect a difference of eGFR. One-way ANOVA or the *t* test was used for the analysis of continuous variables, and the chi-square test or Fisher's exact test was performed for the categorical variables, as appropriate. Association of the C5/C5aR genotype/haplotype with one-year graft function was analyzed using multiple linear regression analysis

(backward stepwise method). Generalized estimating equations (GEEs) were used for analyzing the association of C5 haplotypes with graft function during the 7 years after transplantation [31]. Genotypes or haplotypes that were significantly associated with graft function, were tested in the model with an exchangeable working correlation matrix. Covariates were retained if Wald's P-value was less than 0.05. Association of C5 haplotypes with graft survival was analyzed using the log-rank test. Cox proportional hazard regression was used for multivariate analysis. Statistical analyses were performed using the SPSS statistical package version 17.0 (SPSS Inc, Chicago, IL, USA) and PLINK version 1.05 (http://pngu.mgh.harvard.edu/purcell/plink/) [32].

Results

Baseline clinical characteristics of study population

The mean age of recipients at the time of transplantation was 40±11.8 years, and that of donors was 38±11.1 years. The proportions of male gender in recipients and donors were 63.4% and 50.8%, respectively. The mean number of HLA mismatches was 2.8±1.6. The proportion of living unrelated donors was 25.7%. Preemptive transplantation was performed in 15.7% of cases. Four cases (2.1%) had history of previous renal transplantation. Two cases of transplantation were performed under a desensitization protocol, because of positive T-cell flow cytometric crossmatch results. The standard immunosuppressive regimen consisted of prednisolone, calcineurin inhibitors and antimetabolites. Tacrolimus was used as initial immunosuppressant in 30.9% of patients. Azathioprine was replaced by mycophenolate mofetil beginning in 2000. Divided by antimetabolites era, 33% of transplantation had been performed before 2000. The most common cause of ESRD was glomerulonephritis (40.3%), followed by hypertension (13.1%), diabetes mellitus (7.3%), and unknown causes (31.4%).

Genotyping and construction of LD blocks

All tested SNPs of C5 and C5aR were in Hardy-Weinberg equilibrium. Genotyping failure rates were less than 4% in all SNPs (Table 1). Four LD blocks were constructed in the C5 gene and were designated as H1, H2, H3 and H4. The H2 block consisted of 4 SNPs (rs2159776, rs17611, rs25681 and rs2241004) (Figure 1).

Table 1. Genotyping results and failure rates.

Gene	C5	C5	C5	C5
SNP	Intron 1	V802I	Y544Y	A1422A
rs Number	rs10818500	rs17611	rs25681	rs12237774
Major Allele	A	A	T	G
Minor Allele	G	G	C	A
Major Allele Frequency	0.758	0.507	0.509	0.797
Minor Allele Frequency	0.242	0.493	0.491	0.203
Failed number	6	7	8	8
Failure rate (%)	1.6	1.8	2.1	2.1
Gene	C5	C5	C5	C5AR1
SNP	G385G	Intron 22	IVS12+116G/A	-253C/T
rs Number	rs10985126	rs2159776	rs2241004	rs10404456
Major Allele	A	A	A	C
Minor Allele	G	G	G	T
Major Allele Frequency	0.753	0.537	0.745	0.541
Minor Allele Frequency	0.247	0.463	0.255	0.459
Failed number	6	6	8	14
Failure rate (%)	1.6	1.6	2.1	3.7

Association of C5/C5aR genotypes of recipients/donors with acute rejection

Acute rejection occurred in 44 cases (23.0%) during the follow-up period (mean, 87.7±41.8 months). Most of acute rejection (93.2%) was cellular rejection, except 3 cases of acute humoral rejection. The genotype distributions of C5/C5aR in both recipients and donors were analyzed according to the occurrence of acute rejection (Table 2). Two C5 SNPs (rs10985126 and rs10818500) of the recipients showed an association with acute rejection (P=0.034 for rs10985126 and P=0.009 for rs10818500); however, their significance was lost after Bonferroni's correction for multiple comparison. C5aR genotypes of recipients were not associated with acute rejection. There was also no statistically significant association between donor C5/C5aR genotypes and acute rejection (Table 2).

Association of C5/C5aR genotypes/haplotypes of recipients/donors with graft function

Estimated GFR at one year after transplantation was compared according to the C5 and C5aR genotypes of the recipients and donors (Table

3). Three C5 SNPs of the recipients were associated with graft function (P=0.012 for rs17611; P=0.010 for rs25681; P=0.012 for rs2241004). However, their significance was lost after Bonferroni's correction. Because the three SNPs belong to the H2 block, we analyzed the association of H2 haplotypes with graft function (Table 4). The haplotype frequencies in the H2 block were as follows: 0.516, 0.269, 0.180, and 0.035 for AATA, GGCG, GGCA, and rare haplotypes of which frequencies were less than 0.05 (AGCA, GATA and AGTA), respectively. The GGCG haplotype was significantly associated with lower one-year eGFR (Table 4).

Table 2. Genotype distributions of C5 and C5a receptor SNPs according to occurrence of acute rejection

Gene rs number (Haplotype block)	Genotype	Recipient		P-value[a]	Donor		P-value[a]
		No rejection number (%)	Acute rejection number (%)		No rejection, number (%)	Acute rejection number (%)	
C5 rs12237774 (H1)	GG	97 (67.8)	32 (74.4)	0.277	81 (55.9)	28 (65.1)	0.280
	GA	41 (28.7)	8 (18.6)		56 (38.6)	15 (34.9)	
	AA	5 (3.5)	3 (7.0)		8 (6.2)	0 (0)	
	Total	143 (100)	43 (100)		145 (100)	43 (100)	
C5 rs2159776 (H2)	AA	45 (31.0)	12 (27.9)	0.728	39 (27.1)	15 (34.1)	0.577
	AG	71 (49.0)	20 (46.5)		70 (48.6)	21 (47.7)	
	GG	29 (20.0)	11 (25.6)		35 (24.3)	8 (18.2)	
	Total	145 (100)	43 (100)		144 (100)	44 (100)	
C5 rs17611 (H2)	AA	43 (29.7)	9 (20.9)	0.226	35 (24.1)	13 (31.0)	0.665
	AG	72 (49.7)	20 (46.5)		70 (48.3)	18 (42.9)	
	GG	30 (20.7)	14 (32.6)		40 (27.6)	11 (26.2)	
	Total	145 (100)	43 (100)		145 (100)	42 (100)	
C5 rs25681 (H2)	TT	45 (31.3)	9 (20.9)	0.205	36 (25.2)	13 (29.5)	0.814
	TC	69 (47.9)	20 (46.5)		66 (46.2)	20 (45.5)	
	CC	30 (20.8)	14 (32.6)		41 (28.7)	11 (25.0)	

	Total	144 (100)	43 (100)		143 (100)	44 (100)	
C5	AA	82 (56.2)	23 (53.5)	0.675	81 (57.0)	25 (58.1)	0.590
rs2241004	AG	53 (36.3)	15 (34.9)		53 (37.3)	14 (32.6)	
(H2)	GG	11 (7.5)	5 (11.6)		8 (5.6)	4 (9.3)	
	Total	146 (100)	43 (100)		142 (100)	43 (100)	
C5	AA	93 (64.6)	21 (47.7)	0.034	76 (52.8)	25 (56.8)	0.926
rs10985126	AG	45 (31.3)	17 (38.6)		58 (40.3)	16 (36.4)	
(H3)	GG	6 (4.2)	6 (13.6)		10 (6.9)	3 (6.8)	
	Total	144 (100)	44 (100)		144 (100)	44 (100)	
C5	AA	95 (66.0)	21 (47.7)	0.009	75 (52.1)	26 (59.1)	0.303
rs10818500	AG	45 (31.3)	17 (38.6)		59 (41.0)	15 (34.1)	
(H4)	GG	4 (2.8)	6 (13.6)		10 (6.9)	3 (6.8)	
	Total	144 (100)	44 (100)		144 (100)	44 (100)	
C5aR	CC	45 (31.7)	12 (36.4)	0.672	38 (27.1)	12 (28.6)	0.873
rs10404456	CT	71 (48.6)	20 (40.9)		70 (50.0)	19 (45.2)	
	TT	29 (19.7)	11 (22.7)		32 (22.9)	11 (26.2)	
	Total	144 (100)	44 (100)		140 (100)	42 (100)	

[a] P-value assessed by Chi-square test or Fisher's exact test, as appropriate.

Table 3. Estimated glomerular filtration rate (eGFR) at one year after transplantation according to genotypes of C5 and C5a receptor

Gene rs number (Haplotype block)	Genotype	Recipient			P-value[b]	Donor			P-value[b]
		number	Mean eGFR[a]	SD[a]		number	Mean eGFR[a]	SD[a]	
C5	GG	124	64.1	15.9	0.554	104	62.6	15.4	0.437
rs12237774	GA	47	62.0	15.0		68	65.2	15.8	
(H1)	AA	7	68.3	22.5		8	67.6	13.8	
	Total	178				181			
C5	AA	56	66.6	16.8	0.105	51	64.8	16.7	0.454
rs2159776	AG	85	64.2	14.1		87	64.7	15.0	
(H2)	GG	39	60.0	17.6		42	61.2	16.6	
	Total	180				180			
C5	AA	51	68.0	16.6	0.012[c]	45	66.3	16.0	0.133
rs17611	AG	86	64.1	13.8		84	65.1	15.1	
(H2)	GG	43	58.3	17.7		50	60.4	16.0	
	Total	180				179			
C5	TT	53	68.1	16.3	0.010[d]	46	66.2	15.8	0.197
rs25681	TC	83	64.1	13.8		82	64.9	15.7	
(H2)	CC	43	58.3	17.7		51	60.8	16.1	
	Total	179				179			
C5	AA	100	66.3	15.4	0.012[e]	102	66.4	16.1	0.050[f]
rs2241004	AG	65	62.7	16.4		64	60.7	14.9	
(H2)	GG	16	54.1	12.4		12	59.3	18.4	

	Total	181				181			
C5	AA	110	65.4	15.6	0.328	96	64.7	15.3	0.764
rs10985126	AG	59	61.6	14.3		71	62.9	17.0	
(H3)	GG	11	62.5	24.4		13	62.7	15.2	
	Total	180				180			
C5	AA	112	65.2	15.6	0.185	96	64.0	15.2	0.948
rs10818500	AG	59	60.8	14.5		71	63.4	16.4	
(H4)	GG	9	66.9	24.8		13	62.7	15.2	
	Total	180				180			
C5aR	CC	58	61.7	15.4	0.410	47	67.6	18.0	0.120
rs10404456	CT	84	65.4	16.8		84	61.6	16.4	
	TT	36	63.7	14.5		43	64.7	12.5	
	Total	178				174			

eGFR, estimated glomerular filtration rate; C5aR, complement 5a receptor; SD, standard deviation.

Total numbers of subjects were lower than 191, because grafts were lost within one year or genotyping failure occurred in several patients.

[a] ml/minute/1.73m^2

[b] P-value assessed by ANOVA.

[c,d,e,f] P-values after Bonferroni's correction for multiple comparison were as followings : b=0.192, c=0.16, d=0.192, e=0.800.

Table 4. Estimated glomerular filtration rate (eGFR) at one year after transplantation according to haplotype in H2 locus of C5 gene

Haplotype	Diplotype	Recipient			P-value	Donor			P-value
		number	Mean eGFR[a]	SD[a]		number	Mean eGFR[a]	SD[a]	
AATA	-/-	43	58.3	17.7	0.009[b]	53	60.7	15.8	0.185
	AATA/-	87	64.2	13.7		85	64.6	15.6	
	AATA/AATA	50	68.3	16.6		45	66.3	16.0	
	Total	180				183			
GGCG	-/-	98	66.4	15.5	0.010[b]	106	66.3	15.8	0.055
	GGCG/-	66	62.5	16.4		65	60.9	14.8	
	GGCG/GGCG	16	54.1	12.4		12	59.3	18.4	
	Total	180				183			
GGCA	-/-	123	63.4	16.1	0.691[b]	108	63.2	16.2	0.325
	GGCA/-	50	64.5	14.3		66	64.0	14.7	
	GGCA/GGCA	7	68.3	22.5		9	71.4	18.9	
	Total	180				183			
Rare	-/-	169	64.8	15.7	0.002[c]	167	64.5	15.8	0.408[c]
	Rare/-	11	50.1	11.6		14	57.8	16.0	
	Rare/rare	0				2	56.6	9.1	
	Total	180				183			

eGFR, estimated glomerular filtration rate; SD, standard deviation.

Total numbers of subjects were lower than 191, because grafts were lost within one year or genotyping failure occurred in several patients.

Rare denotes haplotypes, of which frequencies were less than 0.05, including AGCA (frequency=0.027), GATA (frequency=0.0035), and AGTA (frequency=0.0035).

[a] ml/minute/1.73m^2

[b] P-value assessed by ANOVA.

[c] P-value assessed by Mann-Whitney U test or Kruskal-Wallis test

The GGCG haplotype of recipients was associated with eGFR under the dominant model (60.9±15.9 vs. 66.4±15.5 ml/minute/1.73m^2, P=0.020, Table 5). Furthermore, this haplotype of donors was also significantly associated with graft function under the dominant model (60.6±15.3 vs. 66.2±15.8 ml/minute/1.73m^2, P=0.017, Table 5). When recipients were paired with donors according to the GGCG haplotype, 4 pairs were generated (D+/R+, D+/R-, D-/R+, D-/R-). Other clinical variables were tested for the association with eGFR, which are presented in Table 6. Variables with P-value <0.2 were considered as possible covariates, which included recipient age, donor age, donor gender, diabetes, choice of initial calcineurin inhibitor, and acute rejection within one year after transplantation. Multiple linear regression analysis was performed with covariates. Pairs with the GGCG haplotype in both donor and recipient (D+/R+) were independently associated with reduced eGFR compared with pairs with the GGCG haplotype in neither the recipient nor donor (D-/R-) (P=0.012, Table 7). However, neither recipients' nor donors' C5aR SNP was associated with graft function.

Next, eGFR during the 7 years after transplantation was depicted according to the presence of the C5 GGCG haplotype in recipients or donors

(Figure 2A and 2B). The C5 risk haplotype of both recipients and donors was associated with a lower eGFR (R+ vs. R-, P=0.015; D+ vs. D-, P=0.039; multivariate GEE, Table 8). The presence of the C5 GGCG haplotype in donor-recipient pairs (D+/R+ vs. D-/R-) was significantly associated with reduced eGFR independently (P=0.004), along with acute rejection (P<0.001), and donor age (P=0.01) (Figure 2C, Table 9). Taken together, these data demonstrated that C5 polymorphisms in both donor and recipient had a significant impact on renal allograft function.

Table 5. Estimated glomerular filtration rate (eGFR) at one year after transplantation according to C5 GGCG haplotypes

Diplotype	Number	Mean eGFR (±SD)[b]	P-value[c]
Recipient GGCG haplotype (-)[a]	98	66.4 (±15.5)	0.020
Recipient GGCG haplotype (+)[a]	82	60.9 (±15.9)	
Donor GGCG haplotype (-)[a]	106	66.2 (±15.8)	0.017
Donor GGCG haplotype (+)[a]	77	60.6 (±15.3)	

SD, standard deviation; eGFR, estimated glomerular filtration rate.

[a]GGCG haplotype (+)" denotes subjects with at least one GGCG haplotype in the H2 locus (dominant model).

[b] ml/minute/1.73m^2

[c] P-value assessed by *t*-test.

Table 6. Univariate analysis of risk factors for allograft function (eGFR) at one year after transplantation

Parameter	Beta	SE	95% CI	P-value[b]
Female recipient	0.052	2.433	-4.749, 4.853	0.983
Recipient age	-0.141	0.098	-0.335, 0.053	0.152
Female donor	-3.295	2.332	-7.897, 1.307	0.159
Donor age	-0.292	0.103	-0.496, -0.088	0.005
Living unrelated donor (vs. related donor)	1.391	2.681	-3.899, 6.681	0.605
Transplantation period (2000-2007 vs. 1996-1999[a])	-0.440	2.519	-5.411, 4.530	0.861
Number of HLA mismatch	0.451	0.780	-1.089, 1.991	0.564
Preemptive transplant	0.749	3.209	-5.584, 7.081	0.816
Re-transplantation	3.135	8.013	-12.676, 18.946	0.696
Donor/Recipient BMI ratio	-2.280	6.808	-15.716, 11.156	0.738
Diabetes	-5.559	4.017	-13.484, 2.367	0.168
Tacrolimus use (vs. cyclosporine A)	4.535	2.497	-0.392, 9.461	0.071
Acute rejection within one year after transplantation	-9.713	3.225	-16.077, -3.349	0.003

eGFR, estimated glomerular filtration rate; SE, standard error; CI, confidence interval; BMI, body mass index

[a] Comparison of donor-recipient pairs that had received transplantation since 2000 against those who had transplantation during 1996 to 1999.

[b] P-value for univariate linear regression analysis

Table 7. Association of C5 GGCG haplotype in donor-recipient pairs with allograft function (eGFR) at one year after transplantation

Parameter	Beta	SE	95% CI	P-value[b]
Donor age	-0.289	0.101	-0.489, -0.089	0.005
Tacrolimus use (vs. cyclosporine A)	4.853	2.388	0.139, 9.566	0.044
Acute rejection within one year after transplantation	-8.500	3.190	-14.796, -2.205	0.008
D+/R+ vs D-/R-[a]	-6.318	2.480	-11.212, -1.425	0.012

eGFR, estimated glomerular filtration rate; SE, standard error; CI, confidence interval

[a] Comparison of donor-recipient pairs that had the GGCG haplotype in both donor and recipient (D+/R+) against those that had the GGCG haplotype in neither donor nor recipient (D-/R-).

[b] P-value for multiple linear regression analysis.

Table 8. Association of C5 GGCG haplotype of recipients or donors with allograft function (eGFR) during 7 years after transplantation

Parameter	β	SE	95% CI	P-value[a]
Recipient GGCG haplotype (+) vs. (-)	-4.05	1.661	-7.31, -0.78	0.015
Acute rejection	-7.78	2.084	-11.87, -3.70	<0.001
Donor age	-0.22	0.076	-0.37, -0.07	0.004
Female donor	-1.72	1.612	-4.88, 1.45	0.288
Donor GGCG haplotype (+) vs. (-)	-3.54	1.72	-6.90, -0.18	0.039
Acute rejection	-7.83	2.10	-11.94, -3.71	<0.001
Donor age	-0.21	0.08	-0.36, -0.06	0.007
Female donor	-1.78	1.64	-5.00, 1.43	0.277

SE, standard error; CI, confidence interval

[a] P-value for generalized estimating equation

Table 9. Association of C5 GGCG haplotype in donor-recipient pairs with allograft function (eGFR) during 7 years after transplantation

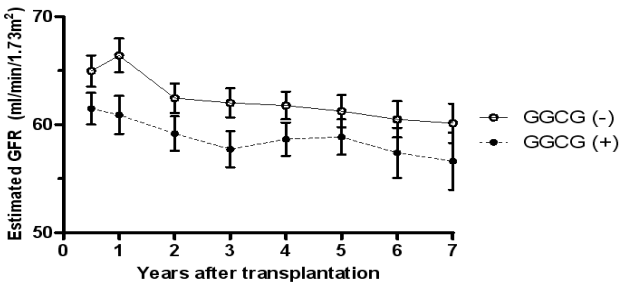
Parameter	β	SE	95% CI	P-value[b]
D-/R+ vs. D-/R-[a]	-3.21	2.53	-8.17, 1.74	0.204
D+/R- vs. D-/R-[a]	-2.07	2.57	-7.11, 2.97	0.421
D+/R+ vs. D-/R-[a]	-5.79	2.02	-9.75, -1.83	0.004
Acute rejection	-7.82	2.08	-11.90, -3.75	<0.001
Donor age	-0.20	0.08	-0.36, -0. 05	0.010
Female donor	-1.89	1.62	-5.06, 1.28	0.242

SE, standard error; CI, confidence interval

[a] Donor/Recipient C5 GGCG haplotype pairs; D-, Donor GGCG haplotype -; D+, Donor GGCG haplotype +; R-, Recipient GGCG haplotype -; R+, Recipient GGCG haplotype +.

[b] P-value for generalized estimating equation.

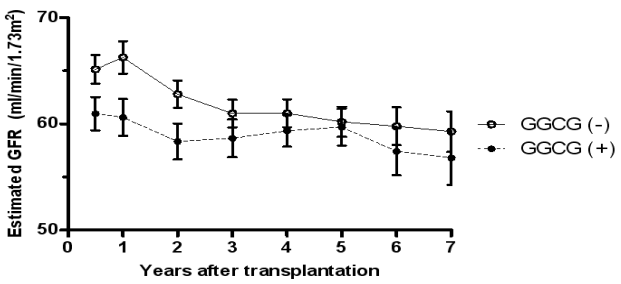
A.



Numbers of subjects at each time point

R(-)	103	100	98	98	98	98	78	60	53
R(+)	85	81	82	80	81	77	66	53	41

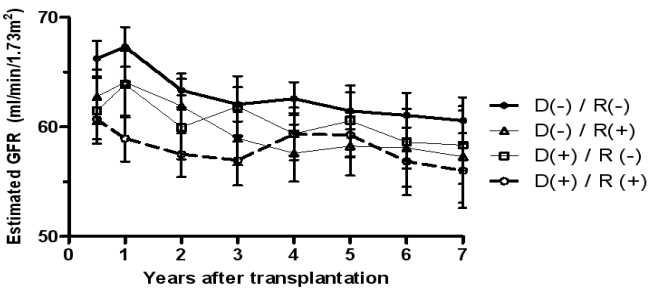
B.



Numbers of subjects at each time point

D(-)	111	107	106	105	106	106	89	70	63
D(+)	80	77	77	76	76	71	57	45	33

C.



Numbers of subjects at each time point

D(-)/R(-)	76	73	72	72	72	73	59	46	42
D(-)/R(+)	32	31	31	30	31	31	28	22	19
D(+)/R(-)	27	27	26	26	26	25	19	14	11
D(+)R(+)	53	50	51	50	50	46	38	31	22

Figure 2. Allograft function according to the GGCG haplotype of C5 gene. Estimated glomerular filtration rate (eGFR) (mean±SE) during 7 years after transplantation according to the presence of the C5 GGCG haplotype of recipients (A), donors (B) and donor-recipient pairs (C). GFR was estimated by means of an abbreviated Modification of Diet in Renal Disease equation. The numbers below the figure denote the number of subjects at risk in each group according to the GGCG haplotype during the 7 years after transplantation.

Association of C5/C5aR haplotypes of recipients/donors with graft survival

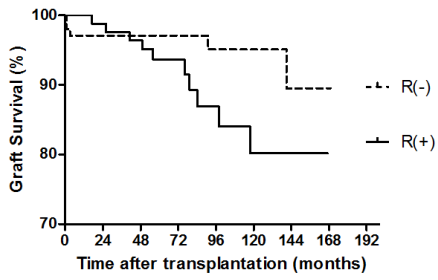
A total of 15 patients (7.9%) lost their allografts. Death occurred in 6 patients. Overall, the 10-year cumulative death-censored graft and patient survival rate were 96.1% and 89.8%, respectively.

Recipients with the C5 GGCG haplotype had poorer graft survival under the dominant model (log rank test, P=0.024, Figure 3A). However, donor GGCG haplotype did not show any association with graft survival (P=0.092, Figure 3B). When we compared graft survival by the combination of recipient and donor GGCG haplotype, D+/R+ donor-recipient pairs had a poorer graft survival compared with D-/R- pairs (HR 3.581, 95% CI 1.100–11.659, P=0.034, Figure 3C). When multivariate analysis was performed by Cox regression, the HR of the C5 GGCG haplotype (D+/R+ vs. D-/R-) was 2.941 (95% CI 0.890–9.719, P=0.077) after adjusting for acute rejection (HR 8.602, 95% CI 2.980–24.831, P<0.001), and the use of tacrolimus as an initial immunosuppression regimen (HR 0.137, 95% CI 0.018–1.039, P=0.055). C5aR polymorphism was not associated with graft survival.

Association of C5/C5aR haplotypes with serum C5 level

The mean serum C5 concentration in 100 healthy volunteers was 188.3±38.5 mg/L (range, 83.0 – 283), which was not correlated with either age or gender. When the association between serum C5 level and H2 haplotype was analyzed, there was no significant association (data not shown).

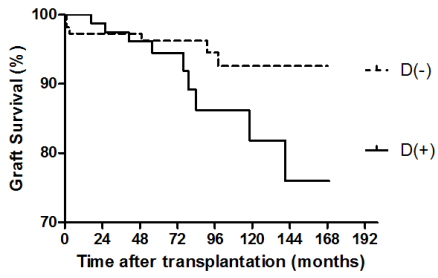
A.



Numbers of subjects at each time point

R(-)	103	100	88	59	42	28	17	2
R(+)	85	83	76	48	32	21	15	1

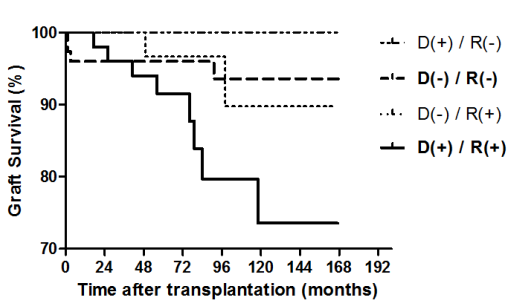
B.



Numbers of subjects at each time point

D(-)	111	108	97	67	49	31	20	1
D(+)	80	79	69	40	27	20	14	3

C.



D(-)/R(-)	76	73	65	46	33	21	14	1
D(-)/R(+)	32	31	31	22	15	9	6	1
D(+)/R(-)	27	24	22	12	9	7	5	2
D(+)R(+)	53	50	45	28	18	13	10	1

Figure 3. Kaplan-Meier estimates of graft survival according to the C5 GGCG haplotype of recipients (A), donors (B) and donor-recipient pairs (C). The presence of recipient GGCG haplotype was associated with a poorer graft survival rate (log rank test, P=0.024). However, the GGCG haplotype of donors showed no association with graft survival rate (P=0.092). D+/R+ donor-recipient pairs had a poorer graft survival compared with D-/R- pairs (HR 3.581, 95% confidence interval 1.100–11.659, P=0.034). The median duration of follow-up was 79 months. The numbers below the figure denote the number of subjects at risk in each group according to the GGCG haplotype during 7 years.

Discussion

C5 plays roles in both humoral and cellular rejection through membrane attack complex formation, chemotaxis and costimulation. Consistent with the importance of C5 in transplantation, we demonstrated for the first time that C5 genetic polymorphisms in kidney transplantation were significantly associated with allograft function. Patients with the risk C5 haplotype (GGCG) had lower graft function during the 7 years after transplantation. However, there was no significant association between this haplotype and acute rejection. Complement production in the kidney allograft seems to continue even without overt histologic injury including acute rejection. Complement gene expression increased in allograft biopsies obtained 3 to 24 months after kidney transplantation compared to that in biopsies at the time of implantation [33]. These data suggest that complements might influence long-term graft outcomes. Two recent animal experiments have shown that complements can mediate renal tubulointerstitial fibrosis. Tubular epithelial cells exposed to C3a, expressed phenotypic and functional characteristics of epithelial-to-mesenchymal transition (EMT) [34]. Treatment with C3a receptor antagonist prevented C3a-induced EMT. The evidence for EMT, including

the deposition of interstitial type I collagen and accumulation of myofibroblasts, was significantly lower in the C3aR-deficient mice [34]. Furthermore, recent study reported that serum C3 level is a reliable marker of renal arteriolosclerosis and components of metabolic syndrome were also associated with the serum C3 level [35]. C5 knockout mice also showed ameliorated renal fibrosis in an experimental nonproteinuric renal damage model [36]. C5a receptor antagonist treatment to wild type mice reduced renal fibrosis, which suggested that the interaction of C5a with C5aR seemed to be a key mediator of renal fibrosis [36]. C5 was also reported to modify liver fibrogenesis [37]. Therefore, the association of C5 with allograft function might be explained by its impacts on renal fibrosis regardless of overt acute rejection. However, we could not compare degree of interstitial fibrosis or tubular atrophy according to the C5 haplotypes for lack of protocol biopsy data, and there had been no clinical report regarding C5 haplotype and its association with CKD progression or CKD itself. On the other hand, we cannot exclude the possibility that C5 might influence repeated subclinical rejection, because we did not perform protocol biopsy [38]. Further studies using the protocol biopsy in a deceased donor kidney transplantation setting could be helpful for clearer interpretation, because complement activation

could have a greater impact on acute rejection in this setting with marked IRI and a larger number of HLA-mismatch.

Although complements are primarily produced in the liver, the kidney also produces complements [12,39]. C3 can be produced by glomerular and tubular epithelial cells, mesangial cells, and endothelial cells. Therefore, C3 derived from donor kidney cells contributes to allograft rejection [8,39,40]. Furthermore, C3 polymorphism of donors was associated with allograft survival in human kidney transplantation [26]. However, impact of the C3 polymorphism on graft outcomes is still controversial, because another large-scale study failed to confirm the association of C3 fast-slow polymorphism with long-term graft outcome [27]. Despite that local synthesis of C5 from renal epithelial cells has not been reported to date, antigen presenting cells and T cells on activation can produce C5 in a paracrine manner [14,41]. Therefore, local concentration of C5 might be elevated in renal allograft tissue, and high C5 activity could contribute to rejection or renal fibrosis [9-16,34]. In parallel, our data showed that C5 polymorphisms of donors as well as those of recipients were significantly associated with allograft function. D+/R+ donor-recipient pairs had lower graft function over 7 years compared with D-/R- pairs. The importance of donor polymorphisms suggests that

donor-derived C5 in addition to recipient-derived C5 might play a role in allograft injury.

Patients with the risk haplotypes had lower graft survival, although multivariate analysis did not support independent influence of the risk haplotypes on graft survival after adjustment for other significant factors such as acute rejection. The survival difference between D+/R+ and D-/R- pairs was not statistically significant. The discrepancy between results on the graft survival and allograft function might be explained by the relatively short follow-up duration and low occurrence rate of graft loss in our study for living donor kidney transplantation.

The C5 gene is 100kb long and located on chromosome 9. The risk polymorphisms associated with allograft outcomes in our study reside in the H2 locus of the C5 gene, which encompasses exon 12 to exon 28. They correspond to a part of the C5 beta chain, C5a, and a part of the C5 alpha chain (Figure 4). Among 4 SNPs in the H2 locus, rs17611 is a nonsynonymous SNP (Ile802Val); however, it is a hydrophobic residue and located in the inner side of C5. Therefore, substitution of Ile802 by Valine does not seem to induce critical structural changes in C5 (by PolyPhen; http://genetics.bwh.harvard.edu/pph/). Therefore, we postulated that the risk

polymorphisms might be linked with another polymorphism that can affect the transcriptional level of the C5 gene. A previous study reported that when both the C5 level and haplotype-tagging polymorphisms of the C5 gene were determined in healthy subjects, C5 levels were significantly higher in individuals with risk C5 genotypes (A allele of rs17611) [37]. However, our study failed to replicate the difference in C5 levels according to genotype/haplotype, possibly because of method of C5 measurement, or the difference in genotyped SNPs. On the other hand, serum C5 concentration might not reflect the local concentration of C5 in the allograft, which could be more important in allograft outcomes. Further studies are needed to determine the functional significance of the risk polymorphisms.

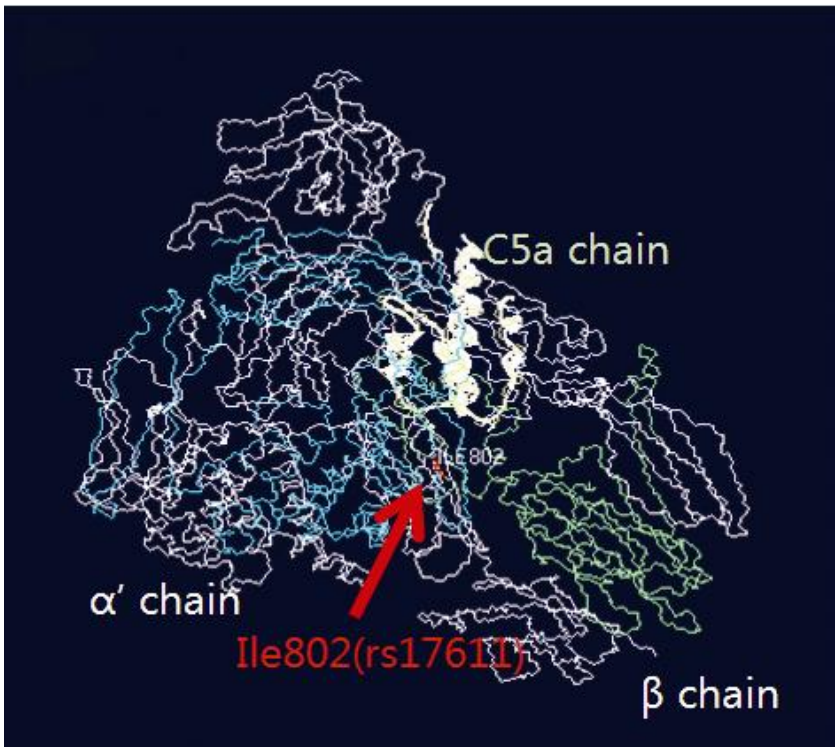


Figure 4. Structure of C5 protein (B) The C5 protein is composed of an alpha and beta chain, and C5a. The H2 locus of the C5 gene corresponds to a part of the alpha chain (blue line), C5a, and a part of the beta chain (green line). Note that Ile802Val (rs17611) is a hydrophobic residue and located in the inner side of the protein.

Conclusion

In conclusion, C5 polymorphism in both recipients and donors was significantly associated with allograft function over 7 years after kidney transplantation, whereas C5aR polymorphism had no significant impact on the allograft outcomes.

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국문 초록

서론: 보체는 신장 이식 후 허혈-재관류 손상과 거부 반응에 있어서 주요한 역할을 담당한다. 보체 인자 5 는 막공격복합체의 형성을 개시하는 중요한 역할을 하며, 다양한 면역세포의 화학 주성을 매개한다. 이에 보체 인자 5 와 수용체 (C5a 수용체)의 유전적 다양성을 신장 이식 공여자와 수여자에서 살펴보았고, 신이식 성적과의 관계를 연구하였다.

방법: 서울대 병원에서 행해진 신이식 중 191 례의 공여자와 수여자의 DNA 를 분석하였다. 보체 인자 5 의 유전자 중 단일 염기 변이 중 7 종의 단일 염기 변이 (rs12237774; rs21597776; rs17611; rs25681; rs2241004; rs10985126; rs10818500)를 분석하였고, C5a 수용체 유전자 중 하나의 단일 염기 변이 (rs10404456)를 분석하였다. 유전적 다형성과 이식신의 성적과의 상관관계를 분석하였다.

결과: 3 종의 보체 인자 5 의 단일 염기 변이 (rs2159776; rs17611; rs25681)가 1 년 후 이식 신 성적이 저하된 것과 상관관계가 있었다. 4 종의 단일 염기 변이 (rs2159776, rs17611, rs25681, rs2241004) 로 구성된 4 종의 하플로타입이 H2 연관 불평형 구역내에 존재하였고, 공여자와 수여자에서 GGCG 하플로타입이 존재하는 경우에 1 년째 MDRD 사구체여과율로 평가한 이식신의 성적이 저하되어 있었다. (60.9±15.9 vs. 66.4±15.5 ml/minute/1.73m2, P=0.020; 60.6±15.3 vs. 66.2±15.8 ml/minute/1.73m2, P=0.017) 이러한 상관관계는 이식 후 7 년째까지의 사구체여과율을 통해 비교해보아도 유지되었다. (P=0.015 수여자 하플로타입의 경우; P=0.039 공여자 하플로타입의 경우) GGCG 하플로타입이 수여자에게서 존재하는 경우에 이식신의 수명이 유의하게 낮았다. (로그랭크 검정, P=0.024) 그러나 보체 인자 5 의 유전적 다형성이 혈중 보체 인자 5 의 농도와는 상관관계가 없었다. C5a 수용체의 유전적 다형성은 이식신의 성적과 상관관계가 없었다.

결론: 공여자와 수여자에서 보체 인자 5 의 GGCG 하플로타입이 존재하는 것은 신기능의 감소와 상관관계가 있었다.

주요어 : 보체, 신장 이식, 유전적 다형성, 이식신 성적
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