Association of CCR5-59029 A/G and CCR2-V64I Variants with Renal Allograft Survival

Mir Davood Omrani[1]*, Mohamad Reza Mokhtari[2], Ali Tagizadae[2], Morteza Bagheri[1], Pedram Ahmad-Poor[2]

[1]Department of Genetics, [2]Department of Urology and Nephrology, Uromia University of Medical Science, Uromia, Iran

ABSTRACT

Background: Despite advances in the medical care of renal transplant recipients which have led to an improvement in allograft survival, renal allograft rejection is still a major obstacle to successful organ transplantation. Understanding the mechanisms contributing to allograft rejection will be of great importance for the development of efficient antirejection strategies. **Objective:** The aim of current investigation was to study the impact of polymorphisms of CCR5Δ32, CCR5- 59029 A/G and CCR2-V64I on renal allograft survival. **Methods:** Using PCR and PCR-RFLP methods in 84 renal transplant recipients, the influence of CCR5Δ32, CCR5- 59029 A/G and CCR2-V64I polymorphisms on renal allograft survival in two rejector and non-rejector groups were examined. Rejector group was defined as having rejection before 1 year and non-rejector group had stable graft function at least for 5 years. **Results:** Significant reductions were found in the risk of renal transplant rejection in recipients possessing the CCR2-64I (A) allele (p=0.03) or 59029-A allele (p=0.03) compared to non-rejector group. There were no significant differences in the frequency of CCR5Δ32 polymorphism in rejector group compared to non-rejector group (p>0.05). **Conclusion:** It was possible to conclude that the chemokine receptors CCR2-V64I (A) and CCR5- 59029 A alleles may influence renal allograft survival.

Keywords: Chemokine Receptors, Genetic Polymorphism, Genotyping, Graft Rejections, Graft Survival, Renal Transplantation

INTRODUCTION

Chemokines, as a diverse group of small chemoattractant mediators, are low molecular mass proteins (1). Chemokines recruit leukocytes from the bloodstream into an allograft; lead to transplant rejection and human inflammatory response of immune system by changing the ratio of Th1/Th2 cytokines (2,3). Single nucleotide variations (polymorphisms) within the promoter or other regulatory sequences of

*Corresponding author: Dr. Mir Davood Omrani, Department of Genetics, Uromia University of Medical Sciences, Uromia, Iran. Tel: (+) 98 4412240166, Fax: (+) 98 441 2234125, e-mail: davood_omrani@umsu.ac.ir

chemokine genes are associated with variations in gene transcription and protein production level. The results of recent investigations have shown that ethnicity can alter the level of production and secretion of chemokines, too. These changes in cytokine expression could play an important role in disease predisposition and give an explanation to different responses in allograft survival (4, 5). Three major chemokines that are known as good candidates in the survival and stability of kidney transplantation are CCR5Δ32, CCR5- 59029 A/G and CCR2-V64I. Chemokine receptor 5 (CCR5) is mapped on chromosome 3p21 and acts as a co-receptor for entry of human immunodeficiency virus type 1. Polymorphic site of CCR5 gene is located in the open reading frame sequence. A 32 base pair deletion in CCR5 gene leads to a premature stop codon which results in the production of a defective protein that fails to be expressed on the surface of target cells (6). Chemokine receptor 2 (CCR2) gene is mapped on chromosome 3p21. CCR2 is a part of human immunodeficiency virus-1 (HIV-1) co-receptor (7). A conservative amino acid at position 64 (Valine) is substituted by a point mutation to Isoleucine (CCR2-64I) (8). CCR5 59029-A allele exists on the same chromosome as CCR2–64I allele (3p21). The presence of A allele at CCR5 59029 is associated with a high activity of the promoter and the presence of G allele at CCR5 59029 causes normal expression of the gene (9). Based on these findings, we tried to evaluate the effect of the polymorphic sites of CCR5Δ32, CCR5- 59029 A/G and CCR2-V64I on renal allograft survival in rejecting and non-rejecting groups.

MATERIALS AND METHODS

Using PCR and PCR-RFLP, the influence of human chemokine receptor genetic variants, CCR5Δ32, CCR5- 59029 A/G and CCR2-V64I on renal allograft survival in 84 renal transplant recipients was examined. For all participants, demographic information, as well as type of donor, number of rejection episodes, and serum creatinine levels were recorded. Rejector group was defined either as histologically proven acute rejection or an acute rise in serum creatinine of more than 20% responding to anti-rejection therapy in those patients in whom biopsy was contraindicated. Since chronic allograft nephropathy has multiple etiologies such as immunologic insults, hypertension, hyperlipidemia, cyclosporine, BK virus, etc it is impossible to define the significance of each insult, therefore inclusion criteria for selecting non-rejector group in this study was the length of allograft survival. Minimum allograft survival of 5 years was set as a threshold for the selection of this group.

DNA Extraction. Genomic DNA was extracted from 3-5 ml of whole blood collected in tubes containing EDTA as anticoagulant and using the salting out method (10).

CCR5Δ32 Genotyping. A polymerase chain reaction (PCR) was carried out for the detection of CCR5Δ32 genotype using primers described by Abdi et al (11). Each PCR reaction was performed in a 25 µl total volume containing 50-100 ng DNA, 10 pmol of each primer, 175 µmol dNTPs, 1.5 mM MgCl2, 1x PCR buffer, and 0.3 U Taq DNA polymerase enzyme (Cinnagen, Tehran, Iran). Thermocycling program (Eppendorf Mastercycler, Germany) consisted of initial denaturation at 94°C for 4 min; 35 cycles at 94°C for 30 sec, 52°C for 45 sec, 72°C for 1 min and a final extension at 72°C for 5 min. Presence or absence of PCR products were monitored by electrophoresis in 2% agarose gel containing ethidium bromide and visualization by U.V. illumination. The PCR reaction yielded a 233-bp amplicon for the wild type and a 201-bp amplicon for the mutant product.

CCR5-59029 Genotyping. For the detection of CCR5- 59029 genotype, PCR-RFLP was performed using the primers described by Abdi et al (11). The PCR conditions were identical to those described above except that the annealing temperature was set at 65°C. The reaction resulted in the formation of a 268 bp amplicon. 10 µl of PCR products were digested by 10 units of Bsp1286I enzyme (New England BioLabs, Beverly, USA) according to the recommendations of the manufacture. When a G allele is present in CCR5-59029, the Bsp1286I enzyme will hydrolyse the amplicon and yield a 130bp fragment on the agarose gel. When an A allele exists in this locus, the amplicon will remain intact (258bp).

CCR2-V64I Genotyping. PCR-RFLP was performed for detection of CCR2-V64I genotype using the primers reported by Abdi et al (11). The PCR procedure was the same as to those for CCR5Δ32, except that an annealing temperature of 65 °C was used for the PCR reactions. PCR product size was about 173 bp. 10 µl of PCR product was digested by 8 unit of BsaBI enzyme (New England BioLabs, Beverly, MA) for 3h as recommended. When an A allele exists in the CCR2-V64I, BsaBI enzyme will digest the amplicon and yield a 149 and a 24 bp fragments confirming the presence of an A allele at position 190 that will lead to encoding isoleucine instead of valine. But if the amplicon remains intact (173bp) in the presence of the enzyme, it means that a G allele exists in this locus. Primer sequences and expected PCR products are summarized in Table 1.

Table 1. Primer sequences and expected PCR products of the chemokines studied

Position	Primer Sequences	Restriction Enzyme	Amplicons
CCR5-del 32 F	5"TGTTTGCGTCTCTCCCAG3"	-	233 ، 201
CCR5-del 32 R	5"CACAGCCCTGTGCCTCTT3"		
CCR5-59029 F	5"CCCGTGAGCCCATAGTTAAAACTC3"	Bsp1286I	268, 130 (G allele), 258 (A allele)
CCR5-59029 R	5"TCACAGGGCTTTTCAACAGTAAGG"		
CCR2-V64 F	5"TTGGTTTTGTGGGCAACATGATGG"	BsaBI	173 (G allele), (149, 24) (A allele)
CCR2-V64 R	5"CATTGCATTCCCAAAGACCCACTC"		

Statistical Analysis. The genotypic and allelic frequencies were obtained by direct counting and division by the number of subjects in each group. The results were analyzed for their fit to Hardy–Weinberg equilibrium using a chi square test. The expected frequencies of each genotype were calculated and compared with the observed results.

RESULTS

From 84 transplant recipients, 52 had good early function and after at least five years they did not require post-transplant dialysis (non-rejectors). In this group, 32 recipients were males and 20 were females. The mean age was 44.7 years. Four (7.7%) of these patients had their graft from a related living donors (RLD), one (1.9%) from cadaver and 47 (90.38%) from unrelated living donors (URLD). The graft survival time of this group ranged from 6-18 year. Eighteen (33.3%) of the cases were Kurdish and 34 (65.4%) were Azari. In CCR5Δ32 genotypes, only one patient (1.9%) was a heterozygous carrier for this mutation and the rest (98.1%) had normal genotypes. When patient genotypes were checked for CCR5-59029 chemokine, 42 (80.77%) had A/A genotype, 9 (17.3%) had A/G genotype and 1 (1.9%) had G/G genotype. In patients with genotypes for CCR2

chemokine, 44 (84.6%) had A/A and 8 (15.4%) had A/G genotypes. Thirty two recipients had a history of acute rejection. Ten of these recipients responded to medical therapy (reversed rejection) and 22 required dialysis (complete rejection). Mean age of the rejection group was 35.4 years. This group consisted of 22 males and 10 females. In this group, 31 patients (96.9 %) had grafts from unrelated donors and only 1 (3.1%) from a cadaver. In CCR5Δ32 genotype, only one patient (3.1%) was a heterozygous carrier for this mutation and the rest (96.9%) had a normal genotype. When patient genotype was checked for the CCR5- 59029 chemokine, 19 (59.4%) had A/A, 12 (37.5%) had A/G and 1 (3.1%) had G/G genotypes. In patients with genotypes for CCR2 chemokine, 20 (62.5%) had A/A and 12 (37.5%) had A/G genotypes. The major reasons for transplantation in these groups were diabetes, glumoronephritis, kidney stone, anatomical disorders, high blood pressure and polycystic kidney. About 98 % of the allografts were from related or unrelated living donors and only 2 % of the allografts were from cadavers. The genotype distributions of all groups were in Hardy–Weinberg equilibrium.

There was no significant difference in the frequency of CCR5Δ32 polymorphism in rejector patients compared to non-rejector patients (p=0.6). However, significant reductions were observed in the risk of renal transplant rejection in recipients possessing CCR2-64I A allele (p=0.02). In addition the group which possessed the 59029-A allele had a significantly lower risk for rejection (p=0.03). Allele and genotype frequencies of both groups, as well as odd ratios (OR), confidence intervals and P-values were calculated and summarized in Table 2.

Table 2. Allele and genotype frequencies of CCR5Δ32, CCR5-59029-A/G and CCR2-V64I polymorphisms in studied groups

Allele and Genotype frequencies	Rejectors F (%) n=32	Non-rejectors F (%) n=52	Odds ratio (95% CI)	P-Value
CCR5 alleles				
+	63 (98.437)	103(99.03)	0.61(0.03-9.95)	0.72
Δ32	1(1.562)	1(0.96)	1.63(0.1-26.60)	0.72
CCR5 genotypes				
+/+	31(96.875)	51(98.07692)	0.6(0.03-10.07)	0.6
+/Δ32	1(3.125)	1(1.923077)	1.64(0.09-27.25)	0.6
Δ32/Δ32	0(0)	0(0)		
CCR5-59029 alleles				
A	50(78.13)	93(89.42)	0.42(0.17-0.99)	0.04
G	14(21.88)	11(10.57)	2.36(1.00-5.60)	0.04
CCR5-59029 genotypes				
A/A	19(59.375)	42(80.769)	0.34(0.13-0.93)	0.03
A/G	12(37.5)	9(17.3076)	2.86(1.04-7.90)	0.03
G/G	1(3.125)	1(1.923)	1.64(0.09-27.25)	0.6
CCR2-V64I alleles				
A	52(81.25)	96(92.30)	0.36(0.13-0.93)	0.03
G	12(18.75)	8(7.69)	2.76(1.06-7.20)	0.03
CCR2-V64I genotypes				
A/A	20(62.5)	44(84.6153)	0.30(0.10-0.85)	0.02
A/G	12(37.5)	8(15.3846)	3.30(1.16-9.32)	0.02
G/G	0(0)	0(0)		

Confidence interval (CI)

DISCUSSION

The study of Fischereder et al. indicated that individuals homozygous for CCR5Δ32 had significantly more stable graft functions in comparison to those heterozygous for CCR5 Δ32 mutation (12). In the present study, the frequency of homozygous CCR5Δ32 genotypes was zero. Since we did not observe significant differences in the frequency of

CCR5Δ32 polymorphism in rejector patients compared to non-rejector patients ($p > 0.05$), therefore our results do not support those of Fischereder et al. However, our findings are in agreement with those of Gharagozloo et al. (13) with the exception that in our study, CCR5Δ32 allele frequency was approximately 0.98% and lower than that of Gharagozloo et al. (1.4%) reported for southern Iranian normal population. This may be due to geographic or ethnic differences (13). Our results also showed that patients with CCR2–64I allele and those homozygous and heterozygous for the CCR5- 59029 A promoter allele had less rejection episodes. Even in a haplotype study, the frequency of these two alleles was higher in patients with stable graft (non-rejector) compared to patients with early rejection (less than one year). Such a result emphasizes the protective effects of these alleles in allograph transplantation procedures. These findings are also in agreement with Abdi's report (11). McDermott et al. (9) and Martin et al. (14) have concluded that CCR5- 59029 A/A and CCR2 64I/64I polymorphisms are in complete linkage disequilibrium. However, it is not clear whether CCR2 or CCR5 plays an important role in disease severity. Maybe one can conclude that both CCR2 and CCR5 demonstrate an additive effect in this regard (8, 9, 14). McDermott et al. also reported that CCR5 59029 A allele corresponds to a high activity of the promoter (9). Wu et al. (15) and Paxton et al. (16) reported that mutations of CCR5 promoter mainly affect the rate of the cell surface expression of CCR5 which may vary significantly among populations with the wild type alleles of CCR5. Also McDermott et al. (9) and Shieh et al. (17) reported that CCR5 59029 A allele is associated with a high promoter activity, therefore CCR5 59029 A/A genotype will correspond to a high level of production and expression of CCR5 antigens on the surface of T CD4+ cells (9,17). Other studies also emphasized the important role of chemokine receptors in allograft outcomes. For example, treatment with an antagonist of chemokine receptors improved functional strategies against the rejection of organ transplants. RANTES has a critical role in acute rejection pathogenesis (18-20). Met-RANTES, as an antagonist of the chemokine receptor, blocks inflammatory leukocyte trafficking into renal allografts and results in a reduction of the proinflammatory cytokine expression (21). Renal transplant rejection is accompanied with a high level of proinflammatory cytokine production and an inflammatory process (22). In a model of renal transplant, Met-RANTES is related to survival and long function of the renal allograft (23). Grone et al. reported that Met-RANTES suppresses the inflammatory alloresponses in acute rejection of rat kidney transplants (23). Particularly, Met-RANTES treatment leads to down regulation of the levels of CCR5 expression and activation (23). In our study, CCR5Δ32 heterozygosity had no significant influence on renal allograft rejection. MCP-1 (monocyte chemoattractant protein-1) and CCR2 have a role in pathogenesis of kidney transplantation rejection. On the other hand, monocyte trafficking into kidney allograft rejection is mediated by MCP-1 (24). Interestingly, CCR2 -/- mouse models have significant defects in delayed alloresponses, inflammatory cytokine production (25), and extravagation of monocytes (26) leading to less inflammation (27). In agreement with these findings, the results of our study showed that an association with CCR2–64I may indicate low levels of alloresponses and linkage disequilibrium would correspond to the presence of association with CCR5- 59029 A.

Based on the finding of this study, it may be concluded that the chemokine receptors CCR2-V64I and CCR5- 59029 A alleles may increase renal allograft survival.

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