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Short Communication

# The effect of chemokine receptor gene polymorphisms (CCR2V64I, CCR5-59029G>A and CCR5Δ32) on renal allograft survival in Pakistani transplant patients

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ABSTRACT

*Background:* Gene polymorphisms of the chemokine receptors *CCR2* and *CCR5* (CCR2V64I, CCR5-59029G>A and CCR5Δ32) have been shown to be associated with renal allograft rejection. The aim of this study was to investigate the association of these polymorphisms with allograft rejection among Pakistani transplant patients.
*Method:* A total of 606 renal transplant patients and an equal number of their donors were included in this study. DNA samples were used to amplify polymorphic regions of CCR2V64I, CCR5-59029G>A and CCR5Δ32 by polymerase chain reaction using sequence specific primers. The amplified products of CCRV64I and CCR5-59029G>A were digested with restriction enzymes (BsaB1 and Bsp12861) respectively. The CCR5Δ32 genotypes were determined by sizing the PCR amplicons. The association of these polymorphisms with the biopsy proven rejection and other clinical parameters was evaluated using the statistical software SPSS v.17.
*Results:* In this study, the G/G genotype of *CCR2*V64I was associated with a high frequency of allograft rejection ($p=0.009$; OR = 2.14; 95% CI = 1.2–3.7). Rejection episode(s) in the GA + AA genotypes were found to be significantly lower as compared to the GG genotype ($p=0.009$; OR = 0.4; 95% CI = 0.2–0.8). The Kaplan–Meier curve also indicated a reduced overall allograft survival for patients with the G/G genotype of *CCR2*V64I (59.2 ± 1.4 weeks, log $p=0.008$). There was a significant association with rejection by female donors possessing the CCR2 GG genotype ($p=0.02$; OR = 2.6; CI = 1.1–6.3) and male donors with the CCR5-59029 GG genotype ($p=0.004$; OR = 1.7; CI = 1.03–3.01).
*Conclusion:* This study shows an association of the CCR2V64I (G/G) genotype with renal allograft rejection. However, no such association was found for the *CCR5* gene polymorphisms. Therapeutic interventions such as blocking the CCR2 receptor (especially G polymorphism) may yield better survival of renal allograft in this patient group. Further, chemokine receptors may be added to the spectrum of the immunogenetic factors that are known to be associated with renal allograft rejection.



## 1. Introduction

Recent advances in understanding the role of the immune system in kidney allograft tolerance has markedly improved the short term graft survival rate (<15% acute rejection/year; Nankivell and Alexander, 2010). However, long term graft survival is still a major problem. Events that have been suggested to influence the short and long term graft survival include acute rejection, chronic rejection, interstitial fibrosis and tubular atrophy (IFTA) and delayed graft function. Several immune-regulatory molecules have been suggested to play important roles leading to allograft rejection. Variations in the genes that encode these molecules have been shown to influence their expression and function. Chemokines and their receptors are implicated in kidney transplant rejection (Alexander et al., 2010; Bedognetti, 2011; Fischereder and Schroppel, 2009). On the basis of the presence of the first two amino terminal cystine residues in the primary amino acid sequence, they are classified into four kinds (CL, CCL, CXCL and CX3CL). Their biological actions are mediated through their respective G protein-coupled receptors (C, CC, CXC and CX3C respectively). The genes for the CC motif receptors, *CCR2* and *CCR5* are mapped on chromosome 3p21. The V64I polymorphism in the *CCR2* and −59029G>A and Δ32 in the *CCR5*

*Abbreviations:* CCR2, Chemokine Receptor 2; CCR5, Chemokine Receptor 5; HLA, Human Leukocyte Antigen; PCR, Polymerase Chain Reaction; SSP, Sequence Specific Primer; SPSS, Statistical Package for the Social Sciences; KM, Kaplan–Meier; IFTA, Interstitial Fibrosis and Tubular Atrophy; AR, Acute Rejection; MCP1, Monocyte Chemotactic Protein-1; RANTES, Regulated on Activation Normal T-cell Expressed and Secreted protein; MIP-1, Macrophage Inflammatory Protein-1; EDTA, Ethylenediaminetetraacetic acid; HWE, Hardy–Weinberg Equilibrium.

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genes have been widely studied due to their association with kidney allograft rejection (Abdi et al., 2002; Omrani et al., 2008).

Chemokine receptor 2 (CCR2) is specific for the monocyte chemotactic protein-1 (MCP-1). It is expressed on the surface of monocytes, activated memory T cells, B cells and basophils. It plays an important role in attracting the monocytes and T-cells to the site of injury. A point mutation (G to A) in the *CCR2* gene at codon position 64 leads to an amino acid change from valine to isoleucine (V64I) in the trans-membrane region of the protein. This polymorphism has been shown to be correlated with the reduction of CCR2 function in the antiallograft immune response and to be involved in the down modulation of CCR5 expression which plays an important role in graft rejection (Nakayama et al., 2004; Segerer et al., 2001). CCR2+/64I (heterozygous for minor allele) and CCR2-64I/64I (homozygous for minor allele) have been found to be associated with the reduced incidence of rejection episodes (Hancock, 2002; Singh et al., 2008; Yigit et al., 2007). Contradictory results have been reported by Gorgi et al. (2011), who have concluded that the CCR2-64I allele is involved in an increased risk of acute rejection in Tunisian allograft recipients.

The pro-inflammatory chemokines, RANTES (regulated on activation normal T-cell expressed and secreted protein) and MIP-1 (macrophage inflammatory protein-1) interact specifically with the CCR5 receptor. This receptor is expressed on activated memory T cells, natural killer cells, immature dendritic cells and monocytes/macrophages. It is involved in the inflammatory response, immune cell trafficking, angiogenesis and metastasis. Polymorphisms of the *CCR5* gene have been shown to be associated with renal allograft rejection (Krensky and Clayberger, 2011). A promoter region polymorphism (59029G>A) of *CCR5* increases the expression level and subsequently its activity by attracting the monocytes or T-cells. Therefore, the allograft recipients carrying the A/A genotype of CCR5-59029G>A polymorphism had poor graft survival or repeated rejection episodes (Ran-hui et al., 2009). Conflicting results have also been reported in different populations that show a reduction of the incidence of AR in the A/A and G/A genotypes (Abdi et al., 2002; Omrani et al., 2008; Yigit et al., 2007). The other mutant form of CCR5 gene is CCR5Δ32, in which the deletion of a 32 base-pair sequence results in the synthesis of a nonfunctional receptor (Omrani et al., 2008). Fischereder et al. (2001) have shown that the mutant receptor significantly influences renal allograft survival. These observations suggest that the same genetic variation may play different roles in different ethnic groups in the context of allograft outcome.

This study was carried out to determine the role of polymorphisms of the *CCR2* and *CCR5* genes in long term graft survival among kidney allograft recipients in our population. The effects of the donor's genetic variations on the allograft outcome were also evaluated.

## 2. Material and methods

This study was conducted on patients that received kidney transplants between June 2009 and February 2011 at the Sindh Institute of Urology and Transplantation (SIUT). A total of one thousand two hundred and twelve (1212) individuals of various ethnicities from different regions of Pakistan have been included in this study. The cohort consists of six hundred and six (N = 606; 481 males and 125 females; mean age 30.13 ± 10.73 years) renal transplant recipients and an equal number of relatives who were living donors (349 were male and 257 were female; mean age 34.89 ± 10.15).

Complete demographic data including age, gender, ethnicity and relationship of donors with the recipients are summarized in Table 1. Pre-operative data include the number of HLA mismatches while post operative data include cold ischemia time, induction type and immunesuppression therapy (Table 2). Biopsy proven rejection based on Banff 97 classification was used to categorize samples of rejection cases (Solez et al., 1993). The research protocol was approved by the Institutional Review Board and conformed to the Tenets of the Declaration of Helsinki. Written informed consent was obtained from all the subjects.

### 2.1. Genotyping of CCR2V64I, CCR5-59029G>A and CCR5Δ32 polymorphism

Blood samples (4 ml) of each recipient and donor were collected in EDTA containing vacutainers before renal transplantation. DNA was purified by the standard phenol/chloroform extraction method and was stored at 4 °C.

The genotyping of CCR2V64I, CCR5-59029G>A and CCR5Δ32 polymorphisms was performed with some minor modifications using specific primers as described by Abdi et al. (2002). The primer sequences for CCR2V64I polymorphism were 5′TTGGTTTTGTGGGCAACATGATGG-3′ and 5′-CATTGCATTCCCAAAGACCCACTC-3′. For genotyping, polymerase chain reaction (PCR) was performed in a thermal cycler (ABI 9700). Briefly, an annealing temperature of 65 °C was used to amplify the 173 bp region. The product was digested overnight with 0.2 U BseJI restriction enzyme (BsaBI: Fermentas) at 65 °C and electrophoresed on a 3% agarose gel. In the presence of the A allele the digestion occurs at 190 nucleotide position giving two fragments of 149 bp and 24 bp. In the presence of G the 173 bp amplicon remains uncut. In the heterozygotes, bands of 173 bp, 149 bp and 24 bp were seen.

For genotyping of the CCR5-59029G>A polymorphism, a region of 268 bp was amplified by PCR at 65 °C (annealing temperature), followed by digestion with 0.2 U SduI (Bsp12861: Fermentas) at 37 °C overnight. The sense primer for polymorphism was 5′-CCCGTGAGCCCATAGTTAA AACTC-3′ and the antisense primer was 5′-TCACAGGGCTTTTCAACAG TAAGG-3′. The digested products were resolved in 3% agarose gel. Bsp12861 digests the amplicon in the presence of the G allele, resulting in a band of 130 bp for the G homozygotes. In the presence of homozygous A allele a single band of 258 bp, while in heterozygote G/A 2 bands of 130 bp and 258 bp were observed.

The *CCR5Δ32* genotype was determined by sizing the PCR amplicons. The fragment was amplified using the sense primer 5′-TGTTTGCGTCTCTC CCAG-3′ and the antisense primer 5′-CACAGCCCTGTGCCTCTT-3′ at 57 °C that result in a 233 bp product for wild type amplicon and a 201 bp deletion fragment.

### 2.2. Statistical analysis

The clinical and genotyping data for the patients and controls were analyzed using the Statistical Package for Social Sciences software (SPSS version 17; SPSS Inc., Chicago, IL, USA). The $\chi^2$ goodness of fit test was used to check the Hardy–Weinberg Equilibrium (HWE). The association of CCR2V64I, CCR5-59029G>A and *CCR5Δ32* with clinical features was analyzed using the chi-squared test of independence with the appropriate degree of freedom. The strength of the association was measured by odds ratio (OR) with a 95% confidence interval (CI). The p-values less than 0.05 were considered to be significant. The cumulative survival time of renal allograft was calculated by Kaplan–Meier (KM) curve and the Log rank test was used to calculate the

**Table 1**
Description of recipients and donors.

| | Donor | Recipient |
|---|---|---|
| Total number (N) | 606 | 606 |
| Gender | | |
| Female | 257 (42.4%) | 125 (20.6%) |
| Male | 349 (57.6%) | 481 (79.4%) |
| Mean age | 34.89 ± 10.15 | 30.13 ± 10.73 |
| Relation of recipient with donor | | |
| Sibling | 332 (54.8%) | |
| Parents | 156 (25.7%) | |
| Offspring | 42 (7%) | |
| Wife[a] | 58 (9.6%) | |
| Husband[a] | 8 (1.3%) | |
| Other blood relatives[b] | 10 (1.6%) | |

[a] Consanguineous.
[b] Cousins.