ABSTRACT

End-stage renal disease is associated with the inflammatory state characterized by infiltrating macrophages/lymphocytes, a major source of chemokines. The aim of this study was to determine the association of CCR2 G190A polymorphism in patients with chronic renal failure (CRF) requiring hemodialysis. Seventy CRF patients and thirty healthy controls were enrolled in the current study; PCR-RFLP technique was used to assess the gene frequencies of CCR2 G190A. The results of the present study showed that there was a significant difference in the genotype and allele frequency distribution of CCR2 G190A in CRF patients and control subjects. A significant association was found between CCR2 G190A and CRF risk, especially, the AA genotype (OR= 2.5, 95% CI=0.26-23.66) and A allele (OR=2.9, 95% CI=1.14-7.3). The polymorphism was significantly associated with the presence of diabetes mellitus. From this study, it could be concluded that, a significant association was found between the AA genotype of CCR2 G190A polymorphism and CRF. Other chemokine polymorphisms in renal pathologies have to be further investigated with larger population based studies.

Key words: CCR2, polymorphism, PCR-RFLP, CRF.

INTRODUCTION

Inflammation is a key component of the immune response to infection, irritation or injury. Inflammation involves series of cellular events that rely on chemical messengers known as chemokines which send out signals to attract inflammatory cells, or leukocytes, to the site
Chemokines can be classified into four main subfamilies: CXC, CC, CX3C and XC according to their amino acid composition (Sezgin et al., 2011). Chemokines exert their biological effects by interacting with G protein-linked transmembrane receptors called chemokine receptors. Chemokine receptors contain 7 transmembrane domains that are found mainly on the surface of leukocytes (Murphy, 2002). After interaction with their specific chemokine ligands, chemokine receptors trigger flux of the intracellular calcium ions. This cause the onset of the chemotaxis process to traffic the cells to a desired location within the body (Mudroch and Finn, 2000). 

Chemokines receptor 2 (CCR2) is the receptor for monocyte chemoattractant protein-1 MCP or CCL2 which induce monocytes to leave the blood stream and enter the surrounding tissue to become tissue macrophages (Villeda et al., 2011). The polymorphism, CCR2-V641 (CCR2 G190A) is a transition mutation where valine 64 of CCR2 in changed to Isoleucine. CCR2 mutations have been associated with many diseases such as insulin dependent diabetes mellitus and reduced risk for sever coronary artery disease (Sezgin et al., 2011).

The pathogenic mechanisms that lead to chronic kidney diseases (CKD) converge on a common pathway that results in progressive interstitial fibrosis, peritubular capillary loss with hypoxia, and destruction of functioning nephrons because of tubular atrophy (Blanche and Rulan, 2005). Locally released chemokines contributes to the renal damage in CKD by releasing inflammatory and profibrotic factors (Anders et al., 2003). Stopping the chemokine signal is vital to resolve the inflammatory process (Anders et al., 2001).

The aim of this study was to determine the association of CCR2 G190A polymorphism in patients with chronic renal failure (CRF) requiring hemodialysis.

**MATERIALS AND METHODS**

**Subjects:**

This study was carried out at the Medical Biochemistry and Internal medicine departments, Faculty of Medicine, Menoufia University, it included 70 subjects admitted to nephrology unit and was undergoing hemodialysis for approximately 5.1±2.1 years and the mean hemodialysis duration was 5.2±1.4 hours per week. Thirty age
and sex-matched healthy individuals were selected as a control group. The study was approved by ethical committee of Faculty of Medicine, Menoufia University. A written informed consent was obtained from all subjects included in the study.

**Methods:**

Six ml of venous blood was withdrawn, after 10 hours overnight fasting; 2ml blood was transferred into plain tube and 4ml into EDTA (ethylene diamine tetra acetic acid) containing tube. Colorimetric kinetic determination of serum creatinine (Fawcett and Scott, 1960)

Total genomic DNA was extracted from peripheral blood with QIAamp DNA minikit (Qiagen Hilden, Germany) and stored at -20°C. CCR2 G190A genotype was determined by polymerase chain reaction/ restriction fragment length polymorphism (PCR-RFLP) analysis. The PCR mixture in a 25 µL final volume consisted of 12.5 µL PCR mastermix (Fermentas, St, leon- Rot, Germany), 9.5 µL H₂O, 1 µL of each primer, and 1 µL DNA. The following primers were used for amplification: forward 5´-CATTGCAATCCCAAAGACCCACTC-3´ and reverse 5´-TTGGTT TTGTGGGCAACATGATGG-3´.

Initial denaturation at 94°C for 5 minutes was followed by 33 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds. Final extension step was at 72°C for 5 minutes. Then the amplification products were separated by electrophoresis through 2% agarose gel stained with ethidium bromide, 50 bp Ladder was used. One band was observed (173 bp) (figure 1).

PCR product (5 µL) was digested for 2 hours at 65°C (with 2.5u of BsaBI restriction endonuclease (Fermentas). Digestion products were analyzed by electrophoresis on 2% agarose. 50 bp Ladder was used. GG genotype was identified with a single 173 bp band. AA genotype was identified with two bands, 149 bp and 24 bp and those with three bands 173 bp, 149 bp and 24 bp as GA heterozygotes (figure 2).

**Statistical analysis:**

Statistical analysis was performed using the SPSS 20 software package. Chi-square test is used to study association between two qualitative variables. The difference between 2 groups was performed by student’s t-test for parametric variables. Odds ratio, describe the probability that people who are exposed to a certain factor will have a
disease compared to people who are not exposed to this factor. A P-value of < 0.05 was considered statistically significant.

RESULTS

The results of the present study showed no significant difference was found between control and CRF patient group regarding age and gender distribution (P>0.05). CRF patients had statistically significant decreased GFR, while significant increased serum creatinine level, compared with controls (P<0.05) (table 1). There was a significant difference in the genotype and allele frequency distribution of CCR2-641 in CRF patients and control subjects. The frequency of CCR2-641 genotypes GG, AG and AA in patient group was 57.1%, 37.1% and 5.7% in comparison with 83.3%, 13.3% and 3.3% in control group, respectively. A significant association was found between CCR2-641 and chronic renal failure risk, specifically, the AG genotype (OR= 2.8, 95% CI= 1.40-5.51), combined AG and GG genotypes (OR= 4.1, 95% CI=1.27-13.03) and A allele (OR=2.9, 95% CI=1.14-7.3) (table 2). The polymorphism was significantly associated with the presence of diabetes mellitus (P=0.025), however, the polymorphism was not significantly associated with the presence of hypertension (P=0.214) (table 3).

Figure (1): The agarose gel electrophoresis for CCR2 G190A gene polymorphism before digestion, represented by fragments of 173bp (lanes 2-9), with lane 1 represents DNA ladder (50 bp).
Figure (2): The agarose gel electrophoresis for CCR2 G190A gene polymorphism after digestion by BsaBI restriction enzyme, Lane 1 DNA ladder (50 bp), lanes 2, 3, 4, 5 and 8 represent GG genotype (single 173 bp band), lanes 6, 7 and 11 represent AA genotype (two bands, 149 bp and 24 bp), lanes 9, 10, 12 and 13 represent AG genotype (three bands 173 bp, 149 bp and 24 bp).

Table (1): Demographic and clinical characteristics of studied groups

<table>
<thead>
<tr>
<th></th>
<th>Patients (n= 70) Mean ±SD</th>
<th>Controls (n= 30) Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>60.2±9.4</td>
<td>58.9±10.7</td>
</tr>
<tr>
<td>Gender (n, %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>43 (61.4)</td>
<td>19 (63.3)</td>
</tr>
<tr>
<td>Female</td>
<td>27 (38.6)</td>
<td>11 (36.7)</td>
</tr>
<tr>
<td>Hemodialysis duration (years)</td>
<td>5.1±2.1</td>
<td>-</td>
</tr>
<tr>
<td>Hypertension (n, %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>51 (72.9)</td>
<td>-</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>DM (n, %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>24 (34.3)</td>
<td>-</td>
</tr>
<tr>
<td>Negative</td>
<td>46 (65.7)</td>
<td>-</td>
</tr>
<tr>
<td>GFR (mL/min/1.73 m²)</td>
<td>3.4±0.6</td>
<td>0.8±0.3*</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P< 0.001 significant, #t test
Table (2): Genotype and allele frequencies of CCR2-641 polymorphism among the two studied groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (n=70) n (%)</th>
<th>Controls (n=30) n (%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2-641 Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-GG</td>
<td>40 (57.1)</td>
<td>25 (83.3)*</td>
<td>1.00</td>
</tr>
<tr>
<td>-AG</td>
<td>26 (37.1)</td>
<td>4 (13.3)</td>
<td>4.1 (1.27-13.03)</td>
</tr>
<tr>
<td>-AA</td>
<td>4 (5.7)</td>
<td>1 (3.3)</td>
<td>2.5 (0.26-23.66)</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-G</td>
<td>106 (75.7)</td>
<td>54 (90.0)*</td>
<td>1.00</td>
</tr>
<tr>
<td>-A</td>
<td>34 (24.3)</td>
<td>6 (10.0)*</td>
<td>2.9 (1.14-7.30)</td>
</tr>
</tbody>
</table>

*P<0.05 significant, OR: odd's ratio

Table (3): Genotype distribution of CCR2-641 polymorphism in patients regarding presence of hypertension and DM

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>GG (n=40)</th>
<th>AG (n=26)</th>
<th>AA (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>1 (25)</td>
<td>4 (15.4)</td>
<td>14 (35)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1 (25)</td>
<td>4 (15.4)</td>
<td>19 (47.5)*</td>
</tr>
</tbody>
</table>

*P<0.05 significant * Chi square test

DISCUSSION

Inflammatory diseases of the kidney that progress to ESRD are characterized by accumulation of interstitial leukocytes, which are a major source of proinflammatory and profibrotic cytokines and are therefore critical in mediating fibroblast proliferation, differentiation into myofibroblasts, matrix production, and tubular damage (Eddy, 2000).

The aim of this study was to determine the association of CCR2 G190A polymorphism in patients with chronic renal failure (CRF) requiring hemodialysis.

Human CCR2 binds CCL2, CCL7, CCL8, CCL13 and CCL16 chemokines39. CCL2, an important ligand of CCR2, is expressed by tubular epithelial cells and infiltrating leukocytes in animal models of
progressive nephropathies and renal fibrosis (Vielhauer et al., 2001). Human renal biopsy studies have confirmed such an expression pattern for CCL2 in various human kidney diseases (Anders et al., 2003). Although diabetic and vascular nephropathies are two major causes of progressive renal disease, only a few studies have been published on the role of chemokines in these disorders. An increased expression of CCL2 was reported in human diabetic nephropathy that correlated with the degree of tubulointerstitial damage and macrophage infiltration (Banba et al., 2000). In angiotensin II-dependent rat models of hypertensive nephrosclerosis, CCL2 expression was increased and was temporally and spatially related to macrophage infiltration (Okada et al., 2000).

Human studies in patients with lupus nephritis also showed that urinary CCL2 levels correlated with the extent of renal disease activity and macrophage infiltration (Wada et al., 1996). In mice lacking the CCR2 receptor, renal pathology after nephrotoxic serum was worse despite reduced glomerular macrophage infiltration, indicating that lack of CCR2 may influence other immune mechanisms besides the local cell infiltration (Bird et al., 2000). After ischemia-reperfusion renal injury the number of interstitial infiltrated macrophages was markedly smaller and the area of tubular necrosis was significantly lower in CCR2-deficient mice than that of wild-type mice (Furuichi et al., 2003).

Blocking CCR2 effectively impairs renal macrophage recruitment, which reduces renal injury in various disease models. Human renal biopsy studies that examined the spatial expression of CCR2 found similar CCR2 expression to that found in rodent disease (Segerer et al., 2000).

We have evaluated the relationship between CCR2 genotypes and disease subgroups which cause CRF in patients. No significant association were found between CCR2 genotypes and hypertension, but significantly associated with diabetes mellitus which may be an evidence of role of CCR2 genotypes in diabetic nephropathy.

Immunological and inflammatory mechanisms play a significant role in the development and progression of diabetic nephropathy (DN) (Navarro-Gonzalez et al., 2009). Monocytes and/or macrophages and their adherence to endothelial cells, and overexpression of proinflammatory cytokines and chemokines, contribute to the pathogenesis of DN (Nguyen et al., 2006). Deletion or blockade of C-C chemokine ligand-2 (CCL2/MCP-1) results in diminished macrophage infiltration and reduced renal injury in both type 1 and type 2 diabetes in mice (Ninichuk et al., 2008). Furthermore in humans, macrophage accumulation occurs in DN and correlates strongly with the progression of renal impairment.
Infiltrating macrophages release lysosomal enzymes, nitric oxide, reactive oxygen species, transforming growth factor-β, vascular endothelial growth factor, and cytokines such as TNF-α, interleukin-1, and IFN-γ (Tesch, 2007), which could play a pivotal role in the development and progression of DN. The renal-protective effect of CCR2 antagonists correlates with a significant reduction of kidney macrophage infiltration. This suggests that macrophages expressing CCR2 are pivotal during the pathogenesis of DN. These results are consistent with recent reports that CCR2 blockade preserved renal function with an associated reduction in kidney macrophage infiltration in type 2 diabetic mice (Kang et al., 2010). However, Kelly and Dominguez (2010), did not find any association between CCR2 genotypes and diabetic nephropathy.

Conclusion:
From this study, it could be concluded that, a possible significant association was found between the AA genotype of CCR2 G190A polymorphism and CRF. In order to reveal the role of chemokines in renal pathogenesis further investigations should be carried out using other chemokines and chemokine receptors in different renal diseases.

REFERENCES


