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Original Article

Association of CCL5 rs2107538, and CCL2 rs3760396 Gene Polymorphisms with the Risk of Cardiovascular Disease

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Abstract

Background: Chemokines are proinflammatory cytokines that play key roles in development of cardiovascular diseases (CVD). Chemokine-induced recruitment of peripheral leucocytes to tissues is a crucial step in the CVD progression. CC chemokines ligand 5, 2 (CCL5 and CCL2), have been characterized as emerging inflammatory biomarkers of atherosclerotic CVD. The aim of this study was to find out whether genetic polymorphisms of CCL5 -403 G>A (rs2107538) and CCL2 –927 G>C, (rs3760396) were associated with the risk of CVD.

Methods: In this case-control study, 500 Iranian individuals including 250 CVD patients and 250 healthy subjects as the control group participated in 2017. Genotyping of CCL5 -403 G>A and CCL2 –927 G>C polymorphisms were executed using Tetra-ARMS PCR method.

Results: At genotypic level both CCL5 -403 G>A and CCL2 –927 G>C polymorphisms were not associated with the risk of CVD (P>0.05), even after adjustment by age, sex, race, and history of hypertension, DM and smoking. However, the CCL2 –927 C allele was associated with an increased risk of CVD (OR=1.42, P=0.050) with a higher prevalence in CVD patient than in controls (17% vs. 12%). Moreover, the haplotype analysis revealed that CCL5/CCL2 haplotype (G/C) was a risk factor for CVD (OR=2.13, P=0.001), and that carriers of this haplotype were at 2.13-fold higher risk of CVD than subjects with G/G haplotype.

Conclusion: CCL2 –927 C variant and CCL5/CCL2 haplotype (G/C) were associated with susceptibility to CVD, and were risk factors for CVD in our population but more studies with large sample size are recommended.

Keywords: CC chemokines ligand 5, 2; Cardiovascular disease; Genetic polymorphism

Introduction

Cardiovascular diseases (CVD) still are one of the major causes of disability and death in many countries, mostly in developed countries (1,2). Convincing evidence suggests that as heart disease progresses the inflammatory cytokine response is activated, causing continuation of dele-

terious effects on the heart and vasculature, leading to progression of LV (left ventricular) dysfunction and heart failure. Chemokine-induced recruitment of peripheral leucocytes to tissues is a critical step in the development of inflammatory responses. Chemokines are small molecular weight proteins that cause chemoattraction and activation of leukocytes, and they play an important role in immune reactivity and pathology of CVD and atherosclerosis (3,4). One of the best-studied CC chemokines CC chemokines ligand 2 (CCL2) or MCP-1 (monocyte chemotactic protein-1) has been characterized as an emerging inflammatory biomarker of atherosclerotic CVD, and is a key ligand for CC chemokine receptor 2 (CCR2). In vitro studies and animal models shows elevated levels of circulating CCL2 have been positively correlated with atherosclerosis, myocardial infarction size, and incidence of coronary heart disease (CHD) (5,6). Another chemokine that plays a critical role in development of CVD is CCL5 or RANTES (regulation upon activation normal T cell expressed and secreted), which belongs to the CC chemokines family and secreted from CD8+ T cells, epithelial cells, fibroblasts and platelets . The CCL5-mediated cell migration is facilitated through its interaction with chemokine receptors CCR1-3 and CCR5 (7,8). Increased serum concentrations of CCL5 are associated with obesity, type 2 diabetes (T2D), coronary atherosclerosis and other cardiovascular risk factors including atherosclerosis (9-11).

Gene polymorphisms that modify expression and/or bioavailability of chemokines and their cellular receptors may affect leucocyte trafficking in inflammatory diseases, including arteriosclerosis and CVD. The CCL5 polymorphism -403G/A; rs2107538 has been shown to increase the promoter activity, result in increased expression of CCL5 and affect the CCL5 mediated inflammatory diseases. The CCL5 -403G/A variation has been found a risk factor for coronary arteriosclerosis (12), coronary artery disease (CAD) and atherothrombotic cerebral infarction (ACI) (11).

The CCL2 –927 G>C, (rs3760396) polymorphism has been associated with an increased transcriptional activity of CCL2 protein in vitro (13). Although a very few studies examined the possible impact of this variation on susceptibility to Carotid intima-media thickness (IMT) (14), MI (15) and ischemic stroke (IS) (16), but to the best of our knowledge there is no study assessing the this polymorphism in CVD patients.

In the current study we aimed to assess the possible relationships between two functional polymorphisms in the CCL5 -403G/A (rs2107538) and CCL2 -927 G/C (rs3760396) and the risk of CVD in an Iranian population.

Materials and Methods

Patients and clinical data collection

According to the case study model in 2017, 500 individuals including 250 CVD patients and 250 healthy subjects were included for the genotyping of CCL5 and CCL2 polymorphisms. The patients were from different parts of Sistan and Baluchistan and Kurdistan provinces of Iran.

Criteria for sample selection: At the time of enrollment, participants completed questionnaires on race/ethnic status, demographics and history of cigarette smoking, hypertension and diabetes mellitus (DM) (Table 1). Hypertension determined as systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg or therapy for hypertension. DM diagnosed as a fasting blood glucose level >7 mmol/L or current use of hypoglycemic agents (16). The CVD patients were identified as the presence of recognized myocardial infarction, coronary insufficiency (unstable angina with demonstrated ischemic electrocardiographic changes), death due to CHD, or atherothrombotic stroke. Blood samples were collected after a 12-hour overnight quickly before cardiovascular procedures. The study was certified by the institutional review board of cardiovascular institute, and the participating hospitals.

The local Ethics Committee of the Zahedan University of Medical Sciences approved the project,

and written informed consent was taken from all participants.

Table 1: Clinical and biochemical data in CVD case and control subjects

Variable	*CVD patients*	*Controls*	*P-value*
NO.	250	250	
Age (yr)	59.7±11.2	64.2±10.1	<0.001
Men n (%)	147 (58.8)	97 (38.8)	<0.001
Ethnicity			0.002
Fars	159 (63.6)	159 (63.6)	
Balouch	63 (25.2)	82(32.8)	
Others	28 (11.2)	9 (3.6)	
Current smoking n (%)	79 (31.6)	61 (24.4)	0.045
History of hypertension n (%)	106 (42.4)	61 (24.4)	<0.001
History of Diabetes Mellitus n (%)	106 (42.4)	37 (14.8)	<0.001

DNA extraction and PCR-based allele genotyping

Blood samples were taken in EDTA-containing tubes and genomic DNA was extracted from peripheral blood leukocytes using salting-out method. Genotyping of CCL5 -403G/A and CCL2-927 G/C were carried out using Tetra amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) method. The T-ARMS-PCR method uses two external primers, forward outer (FO) and reverse outer (RO) creating a control band and two allele specific inner primers (RI and FI). This method simultaneously amplifies both alleles in one single-PCR tube. All primer sequences and fragments size are listed in Table 2.

Table 2: Primers sequence for detection of CCL5 rs2107538, and CCL2 rs3760396 gene polymorphisms

SNPs	*Primer sequence (5' to 3')*	*Amplicons Size*
CCL5 rs2107538 G>A		
CCL5-FO	TCATCAGTTTCCTCTTTGACC	338 bp
CCL5-RO	CTGCCTCAATTTACAGTGTG	
CCL5-FI (G allele)	CTGCTTATTCATTACAGATCTGAC	139 bp
CCL5-RI (A allele)	ATGGATGAGGGAAAGGTGA	241 bp
CCL2 rs3760396 G>C		
CCL2-FO	TAGCTGCCATAACCAGGGATG	409 bp
CCL2-RO	AGAGTGCTTACTCTGCCAGG	
CCL2-FI (C allele)	CAGAGAGAGGACCCAAGGAG	267 bp
CCL2-RI (G allele)	ACAAGTCCTCCAACTAGTTCCG	183 bp

PCR was performed by commercially available Prime Taq premix (Genetbio, South Korea) based on the manufacturer recommended protocol. Into a 0.2-mL PCR tube with a final volume of 20µL, 1µL of template DNA (~100 ng/µL), 1 µL of each primer (10 µM), 10 µL Taq premix and 6 µL DNase-free water were added. The Cycling conditions for CCL5 -403G/A were an initial denaturation at 94 °C for 5 min followed by 30 cycles of 40 sec at 94 °C, annealing temperature for 45 sec at 54 °C and 45` sec at 72 °C, with a final extension of 5 min at 72 °C. The PCR conditions for CCL2-927 G/C polymorphism were 5 min at 94 °C followed by 30 cycles of

94°C for 40 sec, 60 °C for 45 sec, 72 °C for 45 sec followed by a final extension step for 5 min at 72 °C. PCR products were segregated using standard electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and images were taken under UV light. The amplicons size for CCL5 -403G/A were 241 bp for A allele and 139 bp for G allele, while the product size for the two outer primers (control band) was 338 bp (Fig. 1). The amplicons size for the CCL2-927 G/C were 183 bp for the G allele, 267 bp for the C allele and 409 bp for the control outer band (Fig. 2).

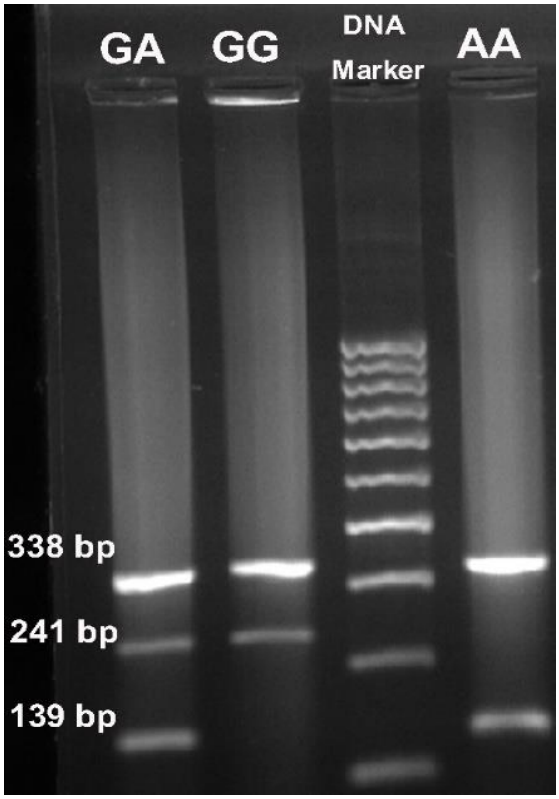

Fig. 1: Electrophoresis Photograph of PCR products of CCL5 -403 G>A polymorphism using Tetra-ARMS-PCR, sample 1: GA; sample 2: GG; sample 3: AA

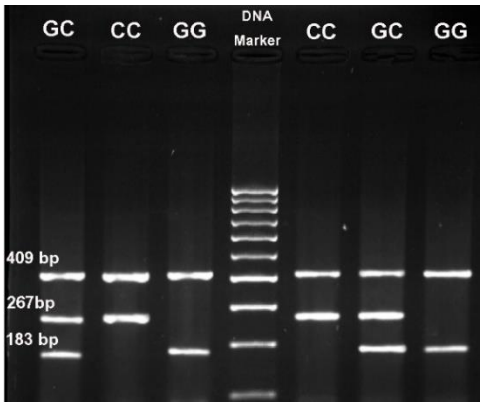

Fig. 2: Electrophoresis Photograph of PCR product of CCL2 -927 G/C polymorphism using Tetra-ARMS-PCR, sample 1,5: GC; sample 2,4: CC; sample 3,6: GG

Statistical analysis

Statistical analyses were performed using SPSS for Windows ver. 18.0 (SPSS, Chicago, IL, USA). Statistical analyses of continuous variables of demographic data were evaluated by Student's t-test. Genotypes and allele frequencies were calculated using the X^2 or Fisher probability test statistic. ORs and 95% CIs were computed with the use of binary logistic regression analyses. Adjusted ORs were stratified by age, sex, ethnicity, history of CVD, hypertension, DM, and current smoking. Frequencies of haplotypes in the controls and patients were estimated using the HAPSTAT-3.0 software (17). A two-sided *P*-value less than 0.05 was considered statistically significant.

Results

The clinical characteristics of subjects including age, sex, ethnicity, history of CVD, hypertension, DM, and smoking status were statistically different between CVD patients and healthy controls (Table1). The frequency of the CCL5 rs2107538, and CCL2 rs3760396 genotypes were compared between 250 CVD cases and 250 controls (Table 3). Although the CCL5 rs2107538 GA genotype was more prevalent in the control group than in CVD patients (41% vs. 38%), the difference remained statistically non-significant (OR=0.21,

95% CI=0.04-1.21, *P*=0.081). Furthermore, the allelic frequency of this polymorphism was similarly distributed between two groups with the frequency of 20% and 23% in cases and controls, respectively and this difference did not reached statistical significance (OR=0.84, 95% CI=0.61-1.15, *P*=0.281).

Table 3: The genotype and allele frequencies of CCL5 rs2107538, and CCL2 rs3760396 variations between CVD patients and controls

Polymorphism	*CVD Patients n (%)*	*Controls n (%)*	**Odd Ratio (95%CI)*	**P-value*
CCL5 rs2107538 G/A				
GG	152 (60.8)	141 (56.4)	1.00	-
GA	96 (38.4)	103 (41.2)	0.21 (0.04-1.21)	0.081
AA	2 (0.8)	6 (2.4)	0.87 (0.58-1.31)	0.510
Alleles				
G	400 (80.0)	385 (77.0)	1.00	-
A	100 (20.0)	115 (23.0)	0.84 (0.61-1.15)	0.281
CCL2 rs3760396 G/C				
GG	170 (68.0)	187 (74.8)	1.00	-
GC	75(30.0)	63 (25.2)	1.23 (0.78-1.92)	0.365
CC	5 (2.0)	0 (0.0)	-	0.999
Alleles				
G	415 (83.0)	437 (87.4)	1.00	-
C	85 (17.0)	63 (12.6)	1.42 (1.00-2.02)	0.050

*ORs and 95% CIs were computed with the use of binary logistic regression analyses. * Adjusted ORs were stratified by age, sex, race, and history of hypertension, DM and smoking.

With respect to CCL2 rs3760396 G/C polymorphism, our study failed to find any significant association between CCL2 and CVD risk at genotypic level (*P*>0.05). The difference of CCL2 genotype frequencies between two groups remained non-significant after adjustment for age, sex, race, and history of hypertension, DM and smoking (Table 3). However, at allelic level, the frequency of CCL2 variant allele (C allele) was relatively higher in CVD patients than in control subjects (17% vs. 12%), and the difference reached the borderline of statistical significance (OR=1.42, 95% CI=1.00-2.02, *P*=0.050).

Table 4 represents analysis of demographic characteristics of subjects according to CCL5 and CCL2 genotypes. The mean age of CCL2 -927 GC+CC genotype carriers was relatively higher than those patients with CCL2 -927 GG genotypes (*P*=0.037). Additionally, the CCL2 GC+CC genotype was more frequent in male subjects than in women and the difference was statistically significant (*P*=0.036). However, no association among other risk factors of CVD and distribution of CCL2 and CCL5 genotypes was found (*P*>0.05).

Haplotype Analysis of CCL2 and CCL5 polymorphisms

Four haplotypes of the *CCL2 and CCL5* loci are shown in Table 5. The haplotypes analysis revealed that the CCL5/CCL2haplotype, G/C, was associated with the risk of CVD and was a risk factor for CVD (OR=2.13, 95% CI=1.39-3.29, *P*=0.001). Moreover, we analyzed the association of *CCL2 and CCL5* genotypes combination and CVD (Table 5), but no significant association was found (*P*>0.05).

Table 4: Demographic characteristics of CVD patients according to CCL5 -403 G/A and CCL2 -927 G/C genotypes

Variable	*CCL5 rs2107538 G/A*		*P-value*	*CCL2 rs3760396 G/C*		*P-value*
	GG	GA+AA		GG	GC+CC	
Age (yr)	59.4	60.3	0.540	58.7	61.9	**0.037**
Sex: Male n (%)	88 (59.9)	59 (40.1)	0.793	93 (63.3)	54(36.7)	**0.036**
Female n (%)	64 (62.1)	39 (37.9)		77 (74.8)	26 (25.2)	
Ethnicity	54.0	46.0	0.134	65.1	34.9	0.505
Balouch %						
Fars %	65.4	34.6	0.134	70.4	29.6	0.505
Others %	50.0	50.0	0.134	60.7	39.3	0.505
Current smoking n (%)	47 (59.5)	32 (40.5)	0.782	53 (65.8)	27 (34.2)	0.663
History of hypertension n (%)	68 (64.2)	38 (35.8)	0.362	75 (70.8)	31 (29.2)	0.493
History of Diabetes n (%)	71 (67.0)	35 (33.0)	0.090	68 (64.2)	38 (35.8)	0.163

Table 5: Prevalence of haplotypes of CCL5 -403 G/A and CCL2 -927 G/C polymorphisms between CVD patients and control subjects

Haplotypes	*Patients (%)*	*Controls (%)*	*P-value*	*OR*	*95% CI*
CCL5 /CCL2					
G/G	0.664	0.701	-	1	-
A/C	0.034	0.057	0.075	0.577	0.31-1.07
A/G	0.166	0.173	0.783	1.40	0.68-1.32
G/C	0. 136	0.069	0.001	2.13	1.39-3.29

Discussion

In the current study we found significant association of CVD patients with -927 G/C, rs3760396 polymorphism in CCL2 gene. The C allele of -927 G/C polymorphism was a risk factor for CVD, and that carriers of the C allele were at 1.4-fold increased risk of CVD than subjects with the G allele. Additionally, the haplotype analysis revealed that CCL5/CCL2 haplotype, G/C, was more frequent in CVD patients than in control group (0.136 vs. 0.069) and was associated with increased risk of CVD. Carriers of G/C haplotype were at 2.13-fold elevated risk of CVD than those with the G/G haplotype. With respect to another SNP, our study showed that the CCL5 -403 G/A polymorphism was not associated with the risk of CVD at both allelic and genotype levels. Thus far, the CCL5 -403 G/A polymorphism has been extensively examined in a huge spectrum of inflammatory diseases but the results have been inconsistent and contradictory. In agreement with our findings, possible association of CCL2 G-927C had examined with the risk of carotid intima-media thickness (IMT) in stroke patients, and found the CCL2 variation a risk factor for common carotid artery (CCA) IMT in additive model (15). However, in other studies this polymorphism was a non-significant factor for myocardial infarction (MI) (15) and ischemic stroke (IS) (16).

Chemokines are inflammatory cytokines characterized by their ability to cause directed migration of leukocytes into inflamed tissue. Several other leukocyte/macrophage responses such as cell proliferation, enzyme secretion, induction of re-

active oxygen species, and promotion of foam cell formation have been observed in vitro after chemokine stimulation. *CCL2gene* is mapped on long arm of chromosome 17 in cluster of other chemokine genes, and includes 3 exons expanding over 2000 bp. CCL2 gene possesses both distal and proximal regulatory elements essential for cytokine-induced and constitutive activity, respectively (16). The CCL2 –927; guanine (G)/cytosine (C) (rs3760396) is part of a cluster of SNPs that is present in the proximal promoter region of the CCL2 gene, characterized as the 12-O-tetradecanoylphorbol 13-acetate (TPA) response region (TRE) and has been associated with intimal medial thickness (IMT). The TRE is a regulatory region that triggers CCL2 transcription in response to increased mechanical shearing from hypertension (13). The CCL2 –927 C SNP is associated with an increased transcriptional activity of CCL2 protein in vitro (13).

Increased levels of CCL2 have been correlated with markers of the metabolic syndrome, including obesity, insulin resistance, T2D and hypertension (18), as well as with higher risk of atherosclerosis (6). CCL2 is produced by cardiovascular cells, including ECs, VSMCs (vascular SMCs) and cardiac myocytes. Mice deficient in either the CCL2 or CCR2 genes showed reduced arterial lesion formation (12), decreased ventricular dilation and preserved cardiac as well as less post-infarct myocardial remodeling (18).

Our results are in accordance with studies on different cardiovascular disorders including CHD (meta-analysis) (19), MI (20), CAD (meta-analysis) (11), and diabetic nephropathy in T2D subjects. In a meta-analysis on CHD, the CCL5 -403 G/A was a non-significant factor for CVD (19). Likewise, in the meta-analysis (11), no evidence of significant association was found between G-403A polymorphism and CAD risk in any genetic model, however, the stratified analysis by ethnic group contradictorily revealed that CCL5 G-403A polymorphism was a protective factor for CAD in Asians whereas its role as a risk factor was observed in Caucasian population, as other studies did on CAD (12) and cardiac mortality in T2DM hemodialysis patients (8), respectively.

Elevated levels of serum CCL5 have been observed in several cardiovascular diseases including CHD (4), CAD (11, 21), acute MI (22, 23) and atherosclerosis (24). The *CCL5* −403A allele was shown to be associated with a lower serum level of RANTES (8, 25) and was a protective factor for T1D and CAD (24). Two independent studies, the -403A allele is associated with lower serum CCL5 concentrations and was correlated with protection from T1D and CAD, respectively (26,27). In our study although *CCL5* −403 G/A SNP was a non-significant factor, but the GA genotype as well as A allele (low CCL5 producers) were more frequent in controls than in cases. The lower levels of serum CCL5 in subjects carrying the AA genotype (mainly controls) compared with those with the GG genotype (CVD patients) could potentially corroborate the clear CVD protection associated with the *CCL5*. CCL5 protein has also been detected in human carotid (27), aorta (28), vein, atherosclerotic plaque and SMC (29). Blocking RANTES-mediated signalling in vivo led to impaired T-cell and monocyte recruitment to inflammatory sites, and reduced progress of atherosclerosis or early myocardial reperfusion (27,30).

The main limitation of our study, which should be consider in the interpretation of our results, is statistically Signiant in some variables such as age, gender and ethnic.

Conclusion

For the first time, we reported that probably the CCL2 G-927C polymorphism a risk factor for CVD. Additionally, the CCL5/CCL2 haplotype, G/C, was associated with an increased risk of CVD on our population. Further studies on larger populations to find out are warranted to validate our results.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or fal-

sification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Disclosure Statement

No competing financial interests exist.

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