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The *CXCR2* Gene Polymorphism Is Associated with Stroke in Patients with Essential Hypertension

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Ischemic stroke · Chemokines · *CXCR2* · Essential hypertension

Abstract
Hypertension is the major risk factor for stroke, and genetic factors contribute to its development. Inflammation has been hypothesized to be the key link between blood pressure elevation and stroke. We performed an analysis of the association between inflammatory mediator gene polymorphisms and the incidence of stroke in patients with essential hypertension (EH). The study group consisted of 625 individuals (296 patients with noncomplicated EH, 71 hypertensive patients with ischemic stroke, and 258 control subjects). Both patients and controls were ethnic Tatars originating from the Republic of Bashkortostan (Russian Federation). The analysis has shown that the risk of ischemic stroke was associated with the *CXCR2* rs1126579 polymorphism. Our results indicate that among patients with EH, the heterozygous genotype carriers had a higher risk of stroke (OR = 1.72, 95% CI 1.01–2.92), whereas the *CXCR2**C/C genotype was protective against stroke (OR = 0.32, 95% CI 0.12–0.83). As shown by the gene-gene interaction analysis, the *CXCR2* rs1126579 polymorphism was also present in all genotype/allele combinations associated with the risk of stroke. Genetic patterns associated with stroke also included polymorphisms in the *CCL2, CCL18, CX3CR1, CCR5*, and *CXCL8 (IL8)* genes, although no association between these loci and stroke was detected by individual analysis.

Introduction

Arterial hypertension is an established risk factor for stroke, although the exact mechanisms of the development of cerebrovascular complications of hypertension remain unclear. Inflammation plays an important role in cardiovascular system remodeling that leads both to elevated blood pressure and stroke.

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Chemokines are small proinflammatory mediators that act as chemoattractants and recruit leukocytes to the site of inflammation. Chemokines constitute the largest family of cytokines, consisting of approximately 50 ligands, 20 signaling and 5 nonsignaling receptors [1]. Chemokines act synergistically with common risk factors promoting atherosclerosis development and mediate cell communications during cerebral ischemia.

In the present paper, we studied the association between chemokines and their receptor gene polymorphisms and the risk of stroke in patients with essential hypertension (EH).

Materials and Methods

The study group consisted of 296 patients with EH without cardiovascular complication (mean age 49.32 ± 9.37 years), 71 patients with EH who suffered from ischemic stroke (mean age 52.55 ± 6.31 years), and 258 healthy individuals (mean age 43.41 ± 7.05 years) without clinical symptoms of cardiovascular or cerebrovascular disease. Both patients and controls were men of the Tatar ethnic group from the Republic of Bashkortostan (Russia). Patients enrolled in the study were treated at the Republic Centre of Cardiology and the Ufa City Emergency Hospital (Ufa, Republic of Bashkortostan, Russia). Controls were recruited at the Republic Centre of Blood Transfusion (Ufa, Republic of Bashkortostan, Russia). The Clinical characteristics of the study groups are provided in table 1.

DNA was isolated from 8 ml of whole venous blood using standard phenol-chloroform extraction. Genotyping was performed using PCR-RFLP or PCR with the allele-specific primers method with primers designed using PrimerSelect 5.05 software (DNAStar Inc., Madison, Wisc., USA). For the *CCL2* rs1024611 (–2518 A>G) polymorphism, the primer sequences were as follows: F 5′-cagcgggggagggcatct-3′, R 5′-acagggaaggtgaagggtat-3′; restriction enzyme *Pvu*II; fragment lengths: Aallele 105 bp, G-allele 51 bp, and 54 bp. For the *CCL18* rs8073066 polymorphism, F 5′-tgtgatctgtgctgctcccat-3′ and R 5′-cctgcttatcaagccaaaggtc-3′ as well as the *Bsr*I restriction enzyme were used; fragment lengths: C-allele 144 and 91 bp, and T-allele 235 bp. For the *CCR2* rs1799864 [190 A>G (Val64Ile)] polymorphism, the primers were F 5′-tgcggtgtttgtgttgtgtggtca-3′ and R 5′-agatggccaggttgagcaggt-3′; restriction endonuclease *Fok*I; fragment lengths: A(I)-allele 198, 84, and 74 bp, and G(V)-allele 282 and 74 bp. For the *CX3CR1* rs3732378 [848 A>G (Thr280Met)] polymorphism, the primers were F 5′-ggactgagcgcccacacagg-3′ and R 5′-aggctggccctcagtgtgact-3′; restriction enzyme *Alw*26I; fragment lengths: A-allele 148 bp, G-allele 128 and 20 bp. For detection of the *CCR5* rs333 (Δ32 I/D) polymorphism, we used the following primers: F 5′-tgccgcccagtgggactttg-3′ and R 5′-cggcaggaccagccccaag-3′; fragment lengths: I-allele 350 bp, D-allele 318 bp. The *CXCR2, CXCL1,* and *CXCL8 (IL8)* polymorphisms were genotyped using allele-specific primers. For the *CXCR2* rs1126579 (+1235 T>C) polymorphism, the primer sequences were: F 5′-ggcacacttccactactctcta-3′, R 5′-gcagagctccagcaaatga-3′, C-allele 5′-cccattgtggtcacaggaag-3′, and T-allele 5′-cccattgtggtcacaggaagt-3′. The positive control fragment length was 253 bp, and the amplified allele fragment was 124 bp. For the *CXCL1* rs4074 (57 A>G) polymorphism, the primer sequences were F 5′-gccttcatttgaggcccagt-3′, R 5′-aatgggtgccctgagtaggt-3′, G-allele 5′-ctggggaaactgcattcggag-3′, and A-allele 5′-ctggggaaactgcattcggaa-3′; they produced positive control of 277 bp and allele of 102 bp. For the *CXCL8 (IL8)* rs4073 (–251 A>T) polymorphism, we used the following primers: F 5′-tgattggctggcttatcttca-3′, R 5′-tcagggcaaacctgagtcatc-3′, A-allele 5′-tccacaatttggtgaattatgaat-3′, and T-allele 5′-tccacaatttggtgaattatgaaa-3′. The positive control length was 316 bp, and the allele length was 196 bp.

Amplification was performed in a T100™ thermal cycler (BioRad, Berkeley, Calif., USA) programmed for an initial denaturation step (95°C for 1 min) followed by 28 cycles of amplification (denaturation at 95°C for 20 s, primer annealing at a specific temperature for 30 s,

Table 1. Clinical characteristics of the study groups

Parameter	Control	EH	EH + stroke
Subjects	258	296	71
Age, years	43.41±7.05	49.32±9.37	52.55±6.31
Sex	male	male	male
Age at onset, years	n.a.	43.16±8.32	42.19±8.89
Risk factors			
Family history			
Hypertension	20 (7.75)	57 (19.26)	16 (22.54)
Cerebrovascular disease	7 (2.71)	44 (14.86)	14 (19.72)
Hypertension + cerebrovascular disease	3 (1.16)	16 (5.41)	3 (4.23)
Hypertension (SBP ≥140 mm Hg and/or DBP ≥90 mm Hg)	0%	100%	80%
SBP	121.97±4.51	158.66±15.02	150.00±11.55
DBP	80.20±2.13	95.13±8.64	96.25±2.13
Smoking	140 (54.26)	184 (62.16)	45 (63.38)
Physical inactivity	166 (64.34)	189 (63.85)	36 (50.7)
Unhealthy diet	17 (6.59)	42 (14.19)	20 (28.17)
Glucose	n.a.	5.02±1.94	4.23±0.68
Cholesterol	n.a.	5.16±1.31	5.40±1.39
LDL	n.a.	3.30±1.13	3.61±1.16
Obesity	19 (7.36)	46 (15.54)	20 (28.17)
Overweight	164 (63.57)	195 (65.88)	41 (57.5)
BMI	25.66±3.48	27.37±3.75	29.56±4.97

Values are shown as means ± standard deviation or n (%), unless otherwise indicated. SBP = Systolic blood pressure; DBP = diastolic blood pressure; LDL = low-density lipoprotein; BMI = body mass index; n.a. = not applicable.

elongation at 72°C for 30 s) and a final extension (72°C for 4 min). PCR and restriction products were separated by the electrophoresis on 2% agarose gel and identified using Mega-Bioprint 1100 gel documentation system (Vilber Lourmat, Collégien, France).

The study data were stored and managed using IBM SPSS Statistics for Windows, version 22.0. For each SNP, compliance with Hardy-Weinberg expectations was tested using Arlequin 3.0 software. Fisher's two-tailed exact test was applied to estimate the differences between allele and genotype frequency distribution in the study groups. The association between allele and/or genotype combinations and the risk of stroke was analyzed by APSampler 3.6.0; the program and its description are available at https://code.google.com/p/apsampler (common algorithm has been described elsewhere [2]). To adjust for multiple testing, we used the standard permutation test.

Statement of Ethics

The study protocol was approved by the Ethics Committee of the Institute of Biochemistry and Genetics, USC RAS, Ufa, Russia, and written informed consent was obtained from each participant.

Results

The observed genotype and allele frequency distribution of the studied loci are shown in table 2. Genotype frequencies for all SNPs were in accordance with the Hardy-Weinberg equilibrium. Distribution of the *CXCR2* rs1126579 genotype frequencies differed significantly

Table 2. Genotype and allele frequency distribution in the study groups

Genotype/allele	Control $p \pm s_p$ (CI)	EH $p \pm s_p$ (CI)	Stroke $p \pm s_p$ (CI)	EH p OR (CI_{OR})	Stroke p OR (CI_{OR})
CXCL1					
G/G	26.47±2.52 (21.61–31.79)	34.85±3.07 (28.85–41.24)	26.76±5.25 (16.94–38.59)	**0.039** 1.49 (1.03–2.15)	1.000
A/G	53.92±2.85 (48.16–59.61)	46.47±3.21 (40.05–52.99)	56.34±5.89 (44.05–68.09)	0.086	0.792
A/A	19.61±2.27 (15.31–24.51)	18.67±2.51 (13.96–24.18)	16.9±4.45 (9.05–27.66)	0.827	0.738
G	53.43±2.02 (49.39–57.44)	58.09±2.25 (53.54–62.54)	54.93±4.18 (46.36–63.28)	0.126	0.780
A	46.57±2.02 (42.56–50.61)	41.91±2.25 (37.46–46.46)	45.07±4.18 (36.72–53.64)	0.126	0.780
CXCR2					
T/T	34.1±2.71 (28.79–39.72)	30.47±3.02 (24.63–36.82)	32.86±5.61 (22.09–45.12)	0.404	0.889
T/C	46.56±2.86 (40.85–52.33)	51.5±3.27 (44.89–58.08)	60±5.86 (47.59–71.53)	0.259	**0.047** 1.72 (1.01–2.92)
C/C	19.34±2.26 (15.06–24.23)	18.03±2.52 (13.31–23.57)	7.14±3.08 (2.36–15.89)	0.73	**0.013** 0.32 (0.12–0.83)
T	57.38±2 (53.34–61.34)	56.22±2.3 (51.58–60.78)	63.64±4.19 (54.82–71.83)	0.710	0.206
C	42.62±2 (38.66–46.66)	43.78±2.3 (39.22–48.42)	36.36±4.19 (28.17–45.18)	0.710	0.206
CCL18					
T/T	18.61±2.35 (14.18–23.74)	33.8±3.22 (27.52–40.53)	24.24±5.27 (14.54–36.36)	**0.0002** 2.23 (1.47–3.38)	0.305
T/C	53.65±3.01 (47.55–59.67)	44.44±3.38 (37.7–51.34)	51.52±6.15 (38.88–64.01)	**0.046** 0.67 (0.47–0.96)	0.785
C/C	27.74±2.7 (22.52–33.44)	21.76±2.81 (16.45–27.86)	24.24±5.27 (14.54–36.36)	0.142	0.645
T	45.44±2.13 (41.21–49.71)	56.02±2.39 (51.19–60.76)	50±4.35 (41.18–58.82)	**0.001** 1.53 (1.19–1.97)	0.382
C	54.56±2.13 (50.29–58.79)	43.98±2.39 (39.24–48.81)	50±4.35 (41.18–58.82)	**0.001** 0.65 (0.5–0.84)	0.382
CCR2					
V/V	74.84±2.48 (69.58–79.6)	66.52±3.13 (59.97–72.63)	75.38±5.34 (63.13–85.23)	**0.042** 0. 67(0.46–0.98)	1.000
V/I	23.86±2.44 (19.19–29.04)	31.28±3.08 (25.31–37.75)	23.08±5.23 (13.53–35.19)	0.061	1.000
I/I	1.31±0.65 (0.36–3.31)	2.2±0.97 (0.72–5.07)	1.54±1.53 (0.04–8.28)	0.506	1.000
V	86.76±1.37 (83.82–89.35)	82.16±1.8 (78.32–85.57)	86.92±2.96 (79.89–92.19)	**0.047** 0.7 (0.5–0.98)	1.000
I	13.24±1.37 (10.65–16.18)	17.84±1.8 (14.43–21.68)	13.08±2.96 (7.81–20.11)	**0.047** 1.42 (1.02–1.99)	1.000

$p \pm s_p$ = Genotype/allele frequency ± standard error; p = level of significance ($p < 0.05$ are given in bold).

Table 3. Allele and genotype combinations most significantly associated with stroke

CXCR2 rs1126579	*CCR5* rs333	*CCL2* rs1024611	*IL8* rs4073	*CX3CR1* rs3732378	*CCL18* rs8073066	Control, %	Stroke, %	Fisher's p value	P_{perm}	OR	CI_{OR}
T	I/I					63.92	82.09	0.0028	0.016	2.59	1.32–5.08
C/C		A				6.15	19.61	0.005	0.026	0.27	0.09–0.77
T	I					92.54	79.22	0.006	0.032	3.25	1.25–8.50
C/C			C			4.76	17.33	0.0067	0.032	0.24	0.07–0.80
C/C				T		6.25	19.05	0.0075	0.035	0.28	0.10–0.82
T		G			A	43.75	27.06	0.0082	0.035	2.1	1.19–3.69

P_{perm} = Standard permutation p value.

between patients with stroke and the control group, while the frequencies of the *CXCL1* rs4074, *CCL18* rs8073066, and *CCR2* rs1799864 genotypes were significantly different in the group of patients with noncomplicated EH compared to the control group.

We observed a statistically significant increase in *CXCR2**T/C genotype frequency in the group of patients with stroke (60 vs. 46.56% in the control group, p = 0.047) and a reduction of the number of the *CXCR2**C allele homozygotes in the same group (7.14 vs. 19.34%, p = 0.013). The analysis of the association has shown that the carriers of the *CXCR2**T/C genotype had a higher risk of stroke (OR = 1.72, 95% CI 1.01–2.92). The *CXCR2**C/C genotype was associated with a decreased risk of stroke (OR = 0.32, 95% CI 0.12–0.83).

The group of patients with noncomplicated EH was characterized by overrepresentation of the *CXCL1**G/G genotype (34.85 vs. 26.47% in the control group, p = 0.039, OR = 1.49, 95% CI 1.03–2.15). *CCL18**T/T genotype frequency was also significantly increased in patients with noncomplicated EH (33.8 vs. 18.61%, p = 0.0002, OR = 2.23, 95% CI 1.47–3.38). The *CCL18**T/C genotype and C allele were associated with a lower risk of EH (OR = 0.67, 95% CI 0.47–0.96, p = 0.046, and OR = 0.65, 95% CI 0.5–0.84, p = 0.001, respectively). Comparing the *CCR2* rs1799864 polymorphism genotype and allele frequency distribution in patients with noncomplicated EH with the control group, we found a reduced number of *CCR2**V/V genotype carriers (66.52 vs. 77.84% in the control group, p = 0.042, OR = 0.67, 95% CI 0.46–0.98). The *CCR2**I allele was associated with an increased risk of EH (OR = 1.42, 95% CI 1.02–1.99, p = 0.047).

Using the APSampler algorithm, we obtained 2,587 genotype/allele combinations of the studied polymorphic variants associated with stroke. Combinations that remained significantly associated with stroke after the permutation test had been applied are presented in table 3. A total of 6 genotype/allele combinations corresponded to the aforementioned criteria. *CXCR2* rs1126579 was present in all combinations, *CCR5* rs333 and *CCL2* rs1024611 were featured in two combinations each, *IL8* rs4073, *CX3CR1* rs3732378, and *CCL18* rs8073066 made an appearance once. Three patterns were associated with an increased risk of stroke, and three were found to be protective against it.

Discussion

In our study, we have shown that the risk of stroke in EH patients is associated with the *CXCR2* rs1126579 polymorphism. CXCR2 is involved in the control of immune cell trafficking between bone marrow, blood, and peripheral tissues during inflammation. CXCR2-mediated signals promote the release of neutrophils into blood and into tissues, promote monocyte

adhesion to the endothelium, and stimulate mast cell migration to peripheral tissues [1]. The rs1126579 polymorphism (T-to-C substitution at position 1235 in the 3′-UTR of the *CXCR2* gene) is reportedly located in the microRNA-binding site and affects CXCR2 protein expression [3]. This polymorphism has been linked with different types of cancer, and the interaction between rs1126579-T and high serum levels of IL8, its endogenous ligand, has been demonstrated to play an important role in the protection from cancer [3].

There was no association observed between stroke and other polymorphisms when analyzed individually. However, the analysis of gene-gene interactions has revealed three combinations associated with stroke (table 2). It is worth noting that all combinations associated with stroke in hypertensive patients included the *CXCR2* rs1126579 polymorphism, and the *CXCR2**T allele was part of all three combinations predisposing to stroke. Protective combinations included, in addition to the *CXCR2**C/C genotype, *CCL2**A, *IL8**C, or the *CX3CR1**T allele.

While the importance of the interaction between CXCR2 and its ligand IL8 has already been established, the CXCR2 interplay with other chemokines is less clear. The *CCR5*Δ32 (rs333) polymorphism consists of a 32-bp deletion and results in frameshift mutation that produces defective protein-lacking regions responsible for signal transduction [4]. The implication of this polymorphism in the susceptibility to HIV infection has been extensively investigated [4]. The *CCR5**D allele was shown to be protective against cerebrovascular events in patients with rheumatoid arthritis [5]. An association was detected between *CCR5*Δ32 deletion and increased plasma high-density lipoprotein cholesterol and decreased plasma triglyceride levels [6]. Balistreri et al. [7] reported an association between the *CCR5**I/I genotype and myocardial infarction in the Sicilian population. This genotype was also reported to negatively affect the chances of longevity. However, no association with myocardial infarction was detected in the Russian population [8]. In our study, we discovered that the *CCR5**I/I genotype and *CCR5**I allele are included in the patterns associated with stroke. The lack of association with *CCR5**D may be due to the small sample size.

The *CCL2* rs1024611*G allele was previously found to be associated with increased *CCL2* expression, higher CCL2 serum levels, and enhanced leukocyte recruitment to the tissues [9, 10]. There is abundant evidence of the implication of this polymorphism in different disease phenotypes, but the results are often inconsistent, which may reflect the linkage between rs1024611 and another *CCL2* polymorphism, rs13900 [11]. No association has been found between the CCL2 plasma level and major adverse cardiovascular and cerebrovascular events [12]. Other genes may also have an impact on rs1024611 effects, as shown in our study.

The rs3732378 polymorphism in the exon 2 of the *CX3CR1* gene results in threonine-to-methionine substitution at position 280 and results in a reduced affinity to fractalkine [13, 14]. An association was reported between the *CX3CR1**M allele and a decreased risk of carotid atherosclerosis [15]. However, the *CX3CR1**M/M genotype has been shown to increase the risk of ischemic cerebrovascular disease [16]. This observation is in line with our finding that the *CX3CR1**T allele is a part of a combination protective against stroke.

A triallelic pattern associated with an increased stroke risk included the rs8073066 polymorphism located near the *CCL18* gene. This SNP is virtually unexplored, and there is no data on its functionality or implication for disease development. All the more interesting is the fact that in our study, this polymorphism was found to be associated with the risk of EH without cardiovascular or cerebrovascular complications. It happens to be the only cross-link between the observed patterns of associations for stroke and noncomplicated EH, since the risk of the latter was found to be associated with the only two of the studied gene markers that were absent in the genotype/allele combinations associated with stroke: *CCR2* rs1799864 and *CXCL1* rs4074.

The results of our study show that the *CXCR2* rs1126579 polymorphism is significantly associated with ischemic stroke, both individually and in combination with the genotype and/or alleles of other chemokine genes. Further investigation is needed in order to elucidate the exact role of the CXCR2 chemokine receptor in the pathogenesis of stroke, but selective antagonism of the interaction between this receptor and its many ligands may be a promising strategy for stroke therapy. CXCR2 antagonists have already been studied in the treatment of respiratory diseases such as chronic obstructive pulmonary disease and asthma and were shown to reduce neutrophil recruitment into the lung and diminish underlying inflammation [17–20]. The noncompetitive allosteric antagonist of the CXCR1 and CXCR2 chemokine receptors was found to be protective against brain damage in the murine models of middle cerebral artery occlusion and reperfusion [21, 22]. Treatment with the CXCR2 ligand-binding protein Evasin-3 was associated with a reduction in ischemic brain neutrophil infiltration at early stages of ischemic brain injury in mice; however, it did not show further beneficial effects on stroke outcome, which may reflect the complexity of the nonselective ligand-receptor interaction [23].

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