

Original Article

Polymorphisms of Fractalkine Receptor CX3CR1 Gene in Patients with Symptomatic and Asymptomatic Carotid Artery Stenosis

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Aim: The chemokine fractalkine is expressed in vascular endothelium, exerting a pro-atherogenic effect. Two single-nucleotide polymorphisms of the *CX3CR1* gene (T280M and V249I) affect fractalkine receptor expression and function. We aimed to assess the prevalence of *CX3CR1* polymorphisms and the association with ischemic cerebrovascular attacks in a cohort of carotid atheromatous disease patients and age-matched controls.

Methods: Using PCR-RFLP, we analyzed allelotypes for T280M and V249I in 150 patients with and 151 controls without carotid atherosclerosis assessed using carotid duplex ultrasound; the subjects were patients admitted for any reason to a tertiary hospital. Genotype data were compared with modifiable risk factors for cerebrovascular disease and the reason for admission, using ischemic stroke as an endpoint. Stroke types associated with carotid atherosclerosis were analysed separately.

Results: The M280 allelic frequency was lower among carotid atherosclerosis patients than controls (0.15 versus 0.23, adjusted OR 0.47, 95% CI 0.30–0.74). Absence of M280 allele was an independent factor associated with carotid atherosclerosis (OR 3.70, 95% CI 1.92–7.14), stronger than hypertension, dyslipidemia, diabetes and cigarette smoking. The I249 allele was also under-represented in carotid atherosclerosis; this was not statistically significant. T280M and V249I genotypes were not associated with admission due to ischemic stroke of the large vessel subtype (TOAST classification, 73 episodes), whereas carotid atherosclerosis, previous ischemic event, age, hypertension, diabetes, hyperlipidemia and cigarette smoking were all independently associated.

Conclusions: The M280 fractalkine receptor gene allele is associated with a lower risk of carotid atheromatous disease, independent from the modifiable cerebrovascular risk factors.

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Key words; Atherosclerosis, Stroke, Transient ischemic attack, Cytokine

Introduction

Cerebrovascular attacks (CVAs), a leading cause of mortality and morbidity in the developed world, consist of ischemic events in more than 80% of cases¹. Atherosclerosis plays a cardinal role, either by producing an embolus from a ruptured plaque or by

occlusion of cerebral vessels from plaque formation *in situ*². Atherosclerosis is an inflammatory process that entails interactive participation of inflammatory cells with vascular endothelium, smooth muscle cells, and fibroblasts³. Chemokines mediate the accumulation of inflammatory cells in atherosclerotic plaque⁴ and their effect has been associated with atherosclerosis progression in both functional and genetic studies^{5,6}.

Fractalkine is a chemokine expressed in both soluble and transmembrane forms⁷. Soluble fractalkine is a potent chemoattractant for monocytes and T cells and the transmembrane form is expressed on endothelial cells under stimulation by proinflammatory molecules⁸. Detection of fractalkine in human atheroscle-

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rotic lesions and its absence in normal arteries supported its role in atherosclerosis⁹. Two single-nucleotide polymorphisms (SNPs) are in linkage disequilibrium within the coding region of the *CX3CR1* gene (which encodes for the fractalike receptor) resulting in the substitution of valine by isoleukine (V249I) and threonine by methionine (T280M), respectively¹⁰. The 280M minor allele has been independently associated with a decreased risk of coronary artery disease (CAD)¹¹⁻¹³. Data on the role of the 249I minor polymorphic allele are contradictory: both negative^{14, 15} and positive¹² associations with CAD risk have been reported, whereas other studies did not reveal any association^{11, 13}.

Data on the role of *CX3CR1* polymorphisms in cerebrovascular disease are more limited and conflicting. The 280M allele has been independently associated with a reduced risk of internal carotid artery (ICA) occlusive stenosis, whereas the 249I allele has been found more commonly among people with hard carotid atheromatous plaques (which are considered more stable than the soft plaque)¹⁶. In another study, homozygosity for the 280M allele, but not the 249I, was associated with decreased intima-media thickness of the common carotid artery¹⁷. In contrast, the rare homozygosity of the 280M and, less so, the 249I allele has been independently associated with an increased risk of brain infarction in a large case-control study¹⁸, whereas lack of any association between *CX3CR1* SNPs and cerebrovascular disease has also been reported in other population settings¹⁹.

To further elucidate the role of *CX3CR1* gene polymorphisms in ischemic cerebrovascular disease, we performed genotype analysis in a cohort of patients with known carotid atheromatous disease and age-matched controls without clinically detectable carotid atherosclerosis. We also compared genotype data against the history of cerebrovascular attacks (strokes and/or TIAs) and the conventional epidemiologic risk factors for cerebrovascular disease among study subjects.

Materials and Methods

The study population consisted of all adults admitted, for any reason, to a general medicine ward of a teaching hospital in Athens during a 1-year period (September 2003–August 2004), who underwent a carotid duplex ultrasound or had one available for the 6 months prior to hospitalization. For patients admitted with stroke, carotid ultrasound was performed as part of the routine work-up during hospitalization. All other patients were offered the test, subject to their consent. Carotid artery stenosis was evaluated using

previously defined criteria²⁰. In detail, peak systolic velocity (PSV), end-diastolic velocity, and carotid index (peak internal carotid artery velocity ÷ common carotid artery velocity) were calculated, with PSV >130 cm/s serving as criterion for ICA stenosis >50% and PSV >200 cm/s for stenosis >70%, respectively, according to previously validated criteria²¹. Stenosis >50% in the presence of symptoms or >70% in asymptomatic patients was considered clinically significant^{22, 23}. Patients were excluded from the study if they had a history of cancer, atrial fibrillation (or evidence of intermittent atrial fibrillation), hemorrhagic stroke, or if they had undergone carotid endarterectomy. Patients with significant carotid stenosis (cases) were matched to those without significant disease (controls) in terms of age. Controls therefore consisted of unselected consecutive adults admitted, for any reason, to our ward and for which carotid ultrasound (performed for a medical indication or upon their consent) did not reveal significant carotid artery disease, as previously specified. Demographic characteristics, major modifiable risk factors for cerebrovascular disease (smoking, hypertension, diabetes, dyslipidemia), history of cerebrovascular disease (ischemic strokes and/or transient ischemic attacks, TIA), cardiovascular disease and other comorbidities, as well as the use of aspirin or any other anti-thrombotic therapy were recorded for both cases and controls. Ischemic cerebrovascular disease was assessed using clinical and radiologic (computerized tomography and/or magnetic resonance imaging) data, as appropriate and patients were classified according to the TOAST diagnostic system²⁴. Risk factors for cerebrovascular disease were assessed according to standard definitions²⁵. Main characteristics of cases and controls are shown in **Table 1**.

Peripheral blood was collected in EDTA anticoagulated tubes. Genomic DNA was processed with proteinase K, followed by phenol extraction and ethanol precipitation, and was resuspended in 50 μ L TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA purity was assessed measuring the A_{260}/A_{280} ratio with a UV/VIS spectrophotometer.

Screening for allelic polymorphisms was performed using a previously described polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) approach¹⁵. Briefly, a 588-base pair DNA fragment containing both polymorphic sites (T280M and V249I) was initially amplified using the primers and PCR conditions shown in **Table 2**. Assays were performed by adding 100 ng genomic DNA to a reaction mixture containing 1X PCR buffer, 250 μ M dNTPs, 2.0 mM MgCl₂, 0.2 mM of each primer and

Table 1. Main characteristics of the study population

	Carotid atherosclerosis <i>n</i> = 150	Controls <i>n</i> = 151	<i>p</i>
Mean age (SD)	76.4 (9.4)	76.7 (9.6)	NS
Male sex (%)	74 (49.3)	59 (39.1)	NS
Smokers (%)	84 (56)	58 (38.4)	0.002
Hypertension (%)	99 (66)	63 (41.7)	<0.001
Diabetes (%)	58 (38.7)	38 (25.2)	0.012
Dyslipidemia [†] (%)	99 (66)	66 (43.7)	<0.001
Statin use	70 (46.7)	46 (30.5)	0.004
Aspirin use	43 (28.7)	33 (21.9)	NS
Admission due to stroke/TIA	105 (70)	80 (53)	0.002
Stroke Classification [§]			
Large vessel disease	64 (61)	9 (11.3)	<0.001
Small vessel disease	20 (19)	34 (42.5)	
TIA	9 (8.6)	18 (22.5)	
Undetermined etiology	12 (11.4)	19 (23.8)	
Previous ischemic events			0.029
None	89 (59.3)	104 (68.9)	
One	23 (15.4)	27 (17.9)	
Multiple	38 (25.3)	20 (13.2)	

[†] Presence of one of the following: pharmacological treatment, total serum cholesterol >6.2 mmol/L, LDL-cholesterol >4.1 mmol/L.

[§] According to the TOAST classification (24). Cardioembolic and strokes of other determined etiology were excluded from the study and TIA were analysed separately.

NS = Not significant

Table 2. Parameters of PCR-RFLP analysis for the detection of polymorphisms of the *CX3CR1* fractalkine receptor gene

PCR		Primer sequence	Conditions	Product size (bp)	
		F-CCGAGGTCCTTCAGGAAATCT	94°C 3 min	588	
		R-TCAGCATCAGGTTTCAGGAACTC	94°C 30 sec	35 cycles	
			52°C 40 sec		
			72°C 55 sec		
			72°C 10 min		
RFLP	Polymorphism	Endonuclease		Wild-type	Mutant
	T280M	BsmBI	55°C for 3 hours	75, 216, 297	216, 372
	V249I	AclI	37°C for 3 hours	383, 205	588

0.2 U *Taq* DNA polymerase (Life Technologies Ltd., UK) to a 25 μ L total reaction volume. The PCR products underwent RFLP analysis with restriction endonucleases, conditions and bands (obtained following electrophoresis on 2% agarose gel) shown in **Table 2**.

PCR-RFLP results were confirmed by DNA sequencing of randomly selected samples (1 out of 5) from all variants (homozygous for each genotype and heterozygous) with an ABI 377 automated sequencer (PE Applied Biosystems, Warrington, UK) using the

same primers and fluorescent DNA capillary electrophoresis.

Data were analyzed using SPSS for Windows, release 11.0.1 (SPSS, Chicago, IL, USA). To determine whether the distribution of each polymorphic genotype fulfilled the Hardy-Weinberg equilibrium, the χ^2 test was used to compare the observed number of subjects with the expected number.

Defining the homozygous phenotype for the dominant allele as the reference group, the odds ratio (with 95% confidence interval) for atheromatous dis-

Table 3. Genotype combinations among the 301 subjects of the study

V249I status	T280M status			<i>p</i> <0.001
	280T/T	280T/M	280M/M	
249V/V	139	14	2	
249V/I	58	64	1	
249I/I	2	11	10	

ease was calculated for each polymorphism. Using logistic regression analysis, the association of each polymorphism with atheromatous disease was adjusted for age, gender, smoking status, diabetes mellitus, hypertension, dyslipidemia, and use of statins and aspirin. To assess the independent role of 280M and 249I mutants in stroke, we constructed a separate logistic regression model which included the parameters listed above, as well as the presence of atheromatous disease, stroke type²⁴ and number of previous cerebrovascular events (ischemic stroke and/or TIA). Using genotype prevalence data from a similar population¹³ we estimated that our sample gave 80% power to detect at least a 2-fold increase of OR with a probability of Type I error $\alpha=0.05$. Continuous variables (i.e. age) were examined using one-way analysis of variance. Variables distributed in non-parametric fashion were examined using the Mann-Whitney U or the Kruskal-Wallis test, as appropriate. Normality was tested using the Kolmogorov-Smirnov test.

Approval to conduct this research was obtained from the Ethics Committee of Sotiria Hospital.

Results

We examined the prevalence of T280M and V249I polymorphisms of the *CX3CR1* fractalkine receptor gene in 150 patients with clinically significant carotid atheromatous disease and 151 age-matched controls. Genotype distributions of both polymorphisms were compatible with the Hardy-Weinberg hypothesis (Table 3). The allelic frequency of the M280 mutant was lower among patients with clinically significant carotid atheromatous disease than in age-matched controls (0.15 versus 0.23, adjusted odds ratio 0.47, 95% confidence interval 0.30–0.74; $p=0.008$). Allelic and genotypic frequencies are shown in Table 4. The odds ratio for carotid atherosclerosis among carriers of the M280 allele was 0.34 (95% CI 0.20–0.60; $p<0.001$), after adjusting for age, gender, smoking status, diabetes mellitus, hypertension, dyslipidemia, and use of statins and aspirin. The I249 mutant allele was also under-represented among

patients with carotid atherosclerosis (compared with the control group) but this difference did not reach statistical significance (Table 4). In multivariate analysis, absence of the M280 allele was the strongest factor independently associated with carotid atheromatous disease (OR 3.70, 95% CI 1.92–7.14; $p=0.001$), its effect being stronger than that of hypertension, dyslipidemia, diabetes and cigarette smoking, which were all also independent risk factors for atheromatous disease (Table 5).

One hundred and eighty-five study patients were admitted to the hospital due to an ischemic cerebrovascular attack (stroke or TIA, Table 1). In multivariate analysis, T280M and V249I polymorphisms were not associated with ischemic cerebrovascular attack, whereas carotid atheromatous disease (OR 1.42, 95% CI 1.30–1.56), a history of previous ischemic event (OR 6.27, 95% CI 3.3–11.92), age (OR 1.04, 95% CI 1.01–1.07), hypertension (OR 1.39, 95% CI 1.29–1.50), diabetes (OR 1.1, 95% CI 1.02–1.21), hyperlipidemia (OR 2.21, 95% CI 1.24–3.93) and cigarette smoking (OR 1.31, 95% CI 1.22–1.37) were all independently associated with admission due to stroke/TIA. When the 73 episodes of the stroke subtype (large vessel disease) associated with carotid artery atherosclerosis²⁴ were analyzed separately, similar results were obtained; neither T280M nor V249I polymorphisms were independently associated with admission due to this type of ischemic stroke (data not shown).

Discussion

In this study, we found a significantly lower prevalence of the M280 mutant allele of the *CX3CR1* gene among patients with carotid atheromatous disease than in age-matched controls without clinically significant carotid atherosclerosis. The association between the M280 allele and carotid artery atherosclerosis was independent of the “conventional” risk factors for vascular disease. Furthermore, in multivariate analysis, this polymorphism exerted the strongest effect on the prevalence of carotid atherosclerosis in our cohort: absence of M280 was associated with an almost 4-fold risk for carotid atherosclerosis, which was higher than the risk individually conferred by any of the “conventional” vascular risk factors (Table 5). In contrast, the other known polymorphism on the *CX3CR1* gene (V249I) examined in this study did not correlate with the presence of carotid atheromatous disease.

Our results are in agreement with those from limited existing studies, concurring with an inverse association between the 280M allele and carotid artery atheromatous disease¹⁶, perhaps due to decreased

Table 4. Allelic and genotypic prevalence for *CX3CR1* gene polymorphisms

Polymorphism	Carotid atherosclerosis <i>n</i> = 150 (%)	Controls <i>n</i> = 151 (%)	Odds ratio (95% CI)	<i>p</i> value
T280M				
T/T	112 (74.7)	87 (57.6)	1	
T/M	31 (20.7)	58 (38.4)	0.42 (0.25-0.70)	<i>p</i> < 0.001
M/M	7 (4.6)	6 (4)	0.91 (0.29-2.79)	
M280 carriers	38 (25.3)	64 (42.4)	0.34 (0.20-0.60) [†]	<i>p</i> = 0.002
T allele frequency	0.85	0.77	1	
M allele frequency	0.15	0.23	0.47 (0.30-0.74) [†]	<i>p</i> = 0.01
V249I				
V/V	82 (54.7)	73 (48.3)	1	
V/I	59 (39.3)	64 (42.4)	0.82 (0.51-1.32)	NS
I/I	9 (6)	14 (9.3)	0.57 (0.23-1.4)	
I249 carriers	68 (45.3)	78 (51.7)	0.64 (0.35-1.18) [†]	NS
V allele frequency	0.74	0.7	1	
I allele frequency	0.26	0.3	0.71 (0.58-1.09) [†]	NS

[†] adjusted for age, gender, smoking status, diabetes mellitus, hypertension, dyslipidemia, and use of statins and aspirin.
CI = Confidence interval; NS = Not significant.

Table 5. Multivariate analysis of factors associated with carotid atheromatous disease

	Correlation Coefficient (95% CI)
Age	1.02 (0.99-1.04)
Male sex	1.58 (0.93-2.70)
Cigarette smoking	2.29 (1.34-3.92)
Hypertension	2.94 (1.76-4.92)
Diabetes	2.08 (1.20-3.63)
Dyslipidemia	2.77 (1.65-4.66)
Absence of M280 allele	3.70 (1.92-7.14)
I249 allele	0.69 (0.32-1.21)

intima-media thickness¹⁷). Although the exact functional role of the fractalkine receptor has not been fully elucidated, it has been proposed that the presence of the 280M allele impairs the ability of the molecule to participate in the inflammatory process underlying the development of atherosclerosis; this is supported by the reduced intima-media thickness and the decreased adhesive function, signaling and chemotaxis of leukocytes from subjects homozygous for the 280M allele^{11, 17}). The role of the V249I polymorphism, although in linkage disequilibrium with T280M, is less clear: we could not find any association between this polymorphism and carotid atheromatous disease, which is also consistent with previous reports^{16, 17}), although in one of those studies the 249I allele was associated with the consistency of the plaques, but not

with the overall prevalence of carotid disease¹⁶).

Despite the protective role of the 280M allele against carotid atherosclerosis, a negative association between this allele and ischemic cerebrovascular disease has not been detected: one case-control study found a positive risk of brain infarction among homozygotes for the 280M allele¹⁸), whereas another study did not detect any association¹⁹). Our study was not designed to specifically address this issue, but we note that, in contrast with carotid atherosclerosis and the major vascular risk factors, *CX3CR1* genotypes were not independently associated with admission due to an ischemic cerebrovascular event. This was also the case when the stroke category (large vessel disease) associated with carotid artery occlusive disease was analyzed separately; no such analysis has been performed in previous relevant studies.

The discrepancy of 280M allelotype associations between carotid atherosclerosis and ischemic strokes is difficult to explain in the absence of prospective studies. There is good experimental evidence of the role of the fractalkine receptor during the atherogenetic process^{8, 26}) and that the rare homozygosity for 280M/249I confers defective adhesive function and chemotaxis¹¹). Notably, however, vascular atherosclerosis leading to ischemic stroke is a multifactorial process in which several parameters and risk factors are implicated²). This can mask the effect of a rare condition, such as 280M/249I homozygosity, in multivariate analysis. In this regard, it is interesting that the risk of brain infarction previously associated with homozy-

gosity for rare *CX3CR1* alleles was more marked in patients with no previous cardiovascular events¹⁸), although that analysis was also restricted by the small number of homozygous patients. On the other hand, recent data suggests that *CX3CR1* deficiency has a favorable effect on ischemic damage and inflammation²⁷), which is in contrast with the results from genetic case-control studies^{18, 19}). In our study, we noted an observed number of 280M homozygosity slightly higher than expected among carotid atherosclerosis patients, given the described protective association of the 280M allele presence (**Table 4**). This was likely due to the very small number of homozygous cases, which did not allow for safe conclusions; however, the role of the rare 280M homozygosity needs further clarification at both functional and epidemiologic levels. Given that polymorphisms on other genes, such as *MMP* and *TIMP*, have recently also been implicated in atherosclerosis via regulation of carotid artery intima-media thickness²⁸), it is apparent that the exact role of the *CX3CR1* gene and its polymorphisms in carotid atherosclerosis and progression to ischemic strokes has not yet been elucidated.

There are several limitations of our study. Its cross-sectional, non-randomised nature allows association, but not necessarily causation to be assumed. Selection bias is also inherent: patients who were not admitted due to stroke underwent carotid examination voluntarily in contrast with stroke sufferers for whom the test was part of the work-up. Related to this, the rate of admissions due to stroke was (expectedly) particularly high among patients with carotid atherosclerosis. Efforts to minimize bias included matching cases to controls in terms of age, exclusion of patients with hemorrhagic or cardioembolic strokes and examining the role of *CX3CR1* polymorphisms separately in the stroke subtype associated with carotid atherosclerosis. Data from CT-angiogram or surgery were not included in the analysis since they were not available for all patients and could not be obviously performed in controls. Carotid disease was assessed with Doppler ultrasound. This test is subject to inter-operator variations, the effect of which seems to have been minimized with the development of newer techniques, machines and criteria^{21, 29}). Finally, unidentified parameters might have interfered with our results: transcranial Doppler was not used and the effect of intracranial atherosclerosis could not be estimated, similar to high-sensitivity C-reactive protein, to reflect the degree of inflammation in atheromatous plaques.

Despite the above limitations, our study provides further evidence that the 280M allele has a strong protective effect against carotid atherosclerosis, which is

independent of the major “conventional” risk factors for cerebrovascular disease. Whether this is translated to an effect on the prevalence of ischemic cerebrovascular attacks needs to be addressed with a prospective, massive-scale population study. Validation of such hypothesis and elucidation of its mechanism will have obvious benefits, offering therapeutic perspectives for genetic modification, as well as incorporating the evaluation of genetic parameters into risk assessment for ischemic stroke, to guide therapeutic decisions appropriately.

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