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RANK/RANKL/OPG pathway: genetic association with history of ischemic stroke in Italian population

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Abstract. – OBJECTIVE: RANKL is a member of the TNF superfamily that stimulates chemokine release, monocyte/macrophage matrix migration and matrix metalloproteinase activity and plays an important role in atherosclerosis. In our study, we have evaluated whether RANKL gene polymorphisms are involved in ischemic stroke in Italian subjects.

PATIENTS AND METHODS: In a retrospective study we have included 487 patients (242 males, 245 females) with history of ischemic stroke and 543 control subjects without history of ischemic stroke (277 males, 276 females). The rs9533156, and rs2277438 gene polymorphisms of the RANKL gene were analyzed by PCR and restriction fragment length polymorphism.

RESULTS: We found that the rs9533156 gene polymorphism of the RANKL gene was significantly (55.0% versus 36.5%, $p < 0.0001$) and independently (adjusted OR 6.28 [2.34-4.21]) associated with history of ischemic stroke. No statistically significant difference was found between the two groups in our population for the rs2277438 gene polymorphism ($p = 439$). Furthermore, we have confirmed that rs 3134069, rs 2073617 and rs 2073618 polymorphisms of the OPG gene were significantly and independently associated with cerebrovascular disorders.

CONCLUSIONS: The present study identifies, for the first time, the genetic variant of RANKL as an independent risk factor for ischemic stroke.

Key Words:
History of ischemic stroke, OPG gene polymorphisms, RANKL gene polymorphisms.

Introduction

Acute ischemic cerebrovascular disease, despite relevant progress in prevention and treatment, remains a very important pathology, representing the first cause of disability[1], the second cause of dementia[2] and the third cause of mortality[3]. Every year, in Italy, there are about 196,000 new cases of stroke: among these about 20% die in the following month and about 30% survive with disabling consequences[4,5]. The observations on the racial disparities existing in clinical outcomes after stroke have resulted in genetic studies focusing on specific polymorphisms.

Receptor activator of nuclear factor-κB ligand (RANKL), its receptor RANK and osteoprotegerin (OPG) are members of tumor necrosis factor (TNF) superfamily and they form a key cytokine triad involved in bone metabolism, specifically osteoclastogenesis[6,7]. The role of the OPG/RANKL/RANK system on bone metabolism[8] and vascular calcification[9] is known. Immune cells[10] express these molecules and this system could be associated with the regulation of immune and inflammatory responses[11,12].

Several studies showed that RANKL is up-regulated in vulnerable plaque prone to rupture and contributes to the transition from a stable to an unstable plaque phenotype in both murine and human atherosclerosis[13]. RANKL stimulates chemokine release, monocyte/macrophage matrix migration and matrix metalloproteinase ac-

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tivity, improves angioneogenesis and endothelial permeability and could promote vascular calcification[14-16]. Hanada et al[17] showed that the RANK protein was expressed in astrocytes and neurons in the medial septal nucleus and the preoptic area, and RANKL mRNA was expressed in the lateral septal nucleus. RANKL is expressed in macrophages and CD4+ T cells[18]. OPG is expressed in macrophages[19] and mature B cells[18].

Elevated serum OPG levels have been found to be associated with the severity[20], subtype[21], poor functional outcome and mortality of ischemic stroke[22] and with unstable angina[23], acute myocardial infarction[24] and vulnerable carotid plaques[25].

Several genetic polymorphisms have been identified in the OPG and RANKL genes. The clinical relevance of these SNPs is based on the fact that plasma levels and/or functional activity may be strongly influenced by these gene variants.

The aim of the present case-control study were to determine whether the rs 3134069, rs 2073617 and rs 2073618 polymorphisms of the OPG gene and the rs9533156, and rs2277438 gene polymorphisms of the RANKL gene play an important role in ischemic cerebrovascular disease in an Italian population with a history of ischemic stroke (HIS).

Patients and Methods

Patients

Patients and controls were recruited among subjects consecutively admitted to the Department of Medicine of the A. Gemelli University Hospital of Rome, Italy and to the Department of Medicine of the St. M. Goretti Hospital, Latina (Italy), from February 1, 2011, to May 31, 2016. Patients who had an ischemic stroke in the past and had survived this event were enlisted, in the group of patients with a history of ischemic stroke (HIS). The cerebral ischemic event had been documented by computerized tomography scan (CT scan) or magnetic resonance imaging (MRI) of the brain. Exclusion criteria from the study were cerebral hemorrhage, history of cranial trauma, atrial fibrillation, other major sources of cardio-embolism, tumors, coagulation disorders, autoimmune diseases and chronic inflammatory diseases. After exclusion of these cases, 487 subjects were enrolled. Five hundred and forty three individuals, with the same exclusion criteria, matched for age and gender, and without clinical or radiological evidence of cerebrovascular disease, were recruited as controls. Brain imaging evaluation was performed in both patients and controls by CT scan and/or MRI. Individuals without a history of ischemic stroke (WHIS) had no family history of stroke. All subjects were of European descent and were from central and southern Italy. Diabetes mellitus was determined by the presence of an existing diagnosis, fasting blood glucose > 126 mg/dL, glycohemoglobin A1c > 5.8%, or by use of antidiabetic medication or insulin.

A complete medical history was collected for all individuals enrolled in the study, included smoking habits, coronary artery disease (CAD), peripheral arterial occlusive disease (PAOD) and drug treatment. Hypertension was defined as a systolic blood pressure > 140 mm Hg, a diastolic blood pressure > 90 mm Hg and > 130 mm Hg, a diastolic blood pressure > 85 mm Hg for the diabetic patients, or current treatment with an antihypertensive drug.

Hypercholesterolemia was defined as either a need for hypolipidemic drugs or total plasma cholesterol level > 5.18 mmol/L. Approval for this study was provided by the Ethics Committees of A. Gemelli University Hospital of Rome and St M. Goretti Hospital, Latina (Italy). Informed consent was obtained from participating patients.

Genetic Testing

Samples of DNA were extracted from peripheral blood by standard procedures and assayed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) for the detection of OPG rs 3134069, rs 2073617 and rs 2073618 gene polymorphisms and of RANKL rs9533156, and rs2277438 gene polymorphisms, as previously described[26,27].

Statistical Analysis

Demographic and clinical data between groups were compared by chi-squared test and by *t*-test.

Genotype and allele frequencies were compared by χ^2-test. To estimate the association between genotype and HIS presence, a logistic regression model was used. Hardy-Weinberg equilibrium was assessed by a 2-test or Fisher's exact test as appropriate. Linkage disequilibrium calculation was performed using software Haploview 4.1 for all pairwise SNP combinations. Odds ratios were calculated with 95% confidence interval and in all cases were adjusted for

age, sex and presence of hypertension, hypercholesterolemia, diabetes mellitus, coronary artery disease, peripheral arterial occlusive disease and smoking. All analyses were performed with the use of Intercooled STATA 6.0 for Windows (Statistics/Data Analysis, Stata Corporation). Statistical significance was established at $p < 0.05$.

Results

Table I shows the demographic and clinical data of patients with and without HIS. In univariate correlations, there were no significant differences between the groups in terms of age ($p = 0.373$), sex ($p = 0.427$) and former smoking ($p = 0.763$). In contrast, hypertension ($p = 0.022$), hypercholesterolemia ($p = 0.012$) coronary artery disease ($p = 0.001$), peripheral arterial occlusive disease ($p = 0.001$), diabetes ($p = 0.001$) and current smoking ($p = 0.001$) were significantly more frequent in patients with HIS than in subjects WHIS.

Table II shows the genotypic distribution of the rs 3134069, rs 2073617 and rs 2073618-gene polymorphisms. Genetic distribution of all SNPs was in Hardy-Weinberg equilibrium. These SNPs are not in linkage disequilibrium. Of the 487 patients with HIS, the genotype distribution of the rs 3134069 gene polymorphism was 159 *GG*, 219 *TG,* and 109 *TT*, which was significantly different to that observed in the 543 subjects WHIS (51 *GG*, 263 *TG,* and 229 *TT*). The frequency of the *GG* genotype in patients with HIS (32.6%) was significantly higher than in those WHIS (9.4%; $p < 0.0001$). Similarly, the genotype distribution of the rs 2073617 polymorphism was 139 *CC*, 221 *TC,* and 127 *TT* in patients with cerebrovascular disease, which was significantly different to that observed in the patients WHIS (54 *CC*, 267 *TC,* and 227 *TT*) and the frequency of the *CC* genotype in patients with HIS (28.5%) was significantly higher than in those WHIS (9.0%; $p < 0.0001$). In addition, the genotype distribution of the rs2073618 polymorphism was 135 *CC*, 221 *GC,* and 131 *GG* in patients with HIS, which was significantly different to that observed in the subjects WHIS (73 *CC*, 223 *GC,* and 247 *GG*). The frequency of the *CC* genotype in patients with HIS (27.7%) was significantly higher than in those WHIS (13.4%; $p < 0.0001$; Table II).

Following these observations, we used a logistic regression analysis to evaluate whether these gene variations were independent variables associated with HIS and we found, after adjusting for relevant confounding variables (age, sex, hypertension, hypercholesterolemia, coronary artery disease, peripheral arterial occlusive disease, diabetes and smoking) that the *GG*, *CC,* and *CC* genotypes of the rs 3134069, rs 2073617, and rs 2073618 gene polymorphisms were independently associated with HIS (adjusted OR 3.67 [2.12-4.46], OR 4.43 [3.25-5.01], and OR 4.68 [2.43-4.06], respectively, Table II).

Table III. shows the genotypic distribution of the rs9533156, and rs2277438 gene polymorphisms of the RANKL gene. Genetic distribution of all SNPs was in Hardy-Weinberg equilibrium and these SNPs are not in linkage disequilibrium.

Of the 487 patients with HIS, the genotype distribution of the rs9533156 gene polymorphism was 268 *TT*, 167 *CT,* and 52 *CC*, which was significantly different to that observed in the 543 subjects WHIS (198 *TT*, 208 *CT,* and 137 *CC*). The frequency of the *TT* genotype in patients

Table I. Demographic and clinical data in subjects with HIS and in subjects WHIS.

	HIS (n = 487)	WHIS (n = 543)	p
Age (years ± SD)	71.8 ± 4.1	71.1 ± 4.2	0.373*
Male: Female ratio	242:245	277:266	0.427#
Hypertension, n (%)	261 (53.6%)	203 (37.4%)	0.022#
Hypercholesterolemia, n (%)	278 (57.1%)	233 (42.9%)	0.012#
CAD, n (%)	198 (40.6%)	125 (23.0%)	0.001#
PAOD, n (%)	154 (31.6%)	101 (18.6%)	0.001#
Diabetes mellitus	281 (57.7%)	163 (30.0%)	0.001#
Smoking (current), n (%)	205 (42.1%)	152 (28.0%)	0.001#
Smoking (former), n (%)	95 (19.5%)	111 (20.4%)	0.763#

SD, standard deviation; CAD, coronary artery diseases; PAOD, peripheral arterial occlusive disease. *Statistical test was performed with Student's *t*-test. #χ^2-test for categorical values.

Table II. Genotype distribution in patients with HIS and WHIS.

		HIS (n = 487)	WHIS (n = 543)	p	Adjusted OR (95% CI)
OPG Genotypes					
SNPs of T245G (rs 3134069)	GG	159 (32.6%)	51 (9.4%)	< 0.0001*	3.67 (2.12-4.46)#
	TG	219 (45.0%)	263 (48.4%)		
	TT	109 (22.4%)	229 (42.2%)		
SNPs of T950C (rs 2073617)	CC	139 (28.5%)	54 (9.0%)	< 0.0001*	4.43 (3.25-5.01)#
	TC	221 (43.4%)	267 (49.2%)		
	TT	127 (26.1%)	227 (41.8%)		
SNPs of G1181C (rs 2073618)	CC	135 (27.7%)	73 (13.4%)	< 0.0001*	4.68 (2.43-4.06)#
	GC	221 (45.4%)	223 (41.1%)		
	GG	131 (26.9%)	247 (45.5%)		

*Chi-square test for categorical values; #OR (odds ratio) adjusted for age, sex, hypertension, hypercholesterolemia, CAD, PAOD, diabetes mellitus and smoking.

with HIS (55.0%) was significantly higher than in those WHIS (36.5%; $p < 0.0001$). The distribution of rs2277438 genotypes was 35 *GG*, 125 *GA*, 327 *AA* in the HIS patients and 35 *GG*, 91 *GA*, 417 *AA* in the control subjects. No statistically significant difference was found between the two groups in our population ($p = 439$).

Finally, we used a logistic regression analysis and we found, after adjusting for relevant confounding variables (age, sex, hypertension, hypercholesterolemia, coronary artery disease, peripheral arterial occlusive disease, diabetes mellitus and smoking) that the *TT* genotype of the rs9533156 gene polymorphism was independently associated with HIS (adjusted OR 6.28 [2.34-4.21]; Table III).

Discussion

Our study is the first report showing that rs9533156 gene polymorphism of the RANKL gene is significantly and independently associated with the increased risk of ischemic stroke in an Italian population. In particular, we found that the genotype distribution of the rs9533156 gene polymorphism of the RANKL gene was significantly higher in patients with HIS than in subjects WHIS (55.0% *versus* 36.5%, $p < 0.0001$; Table III). In our population, the occurrence of ischemic stroke was 6.28-fold higher in patients homozygous for the *T* allele, of the rs9533156 gene polymorphism compared with other control individuals. No statistically significant difference was found in the genotypes distribution of rs2277438 gene polymorphism of the RANKL gene between the two groups in our population ($p = 439$).

The RANK/RANKL/OPG pathway play an important role in production and activation of osteoclasts, and therefore in the regulation of bone re-absorption. Thus, most studies of the RANKL gene have focused on the link between RANKL genetic variation and bone diseases such as os-

Table III. Genotype distribution in patients with HIS and WHIS.

		HIS (n = 487)	WHIS (n = 543)	p	Adjusted OR (95% CI)
RANKL genotypes					
SNPs of rs9533156	TT	268 (55.0%)	198 (36.5%)	< 0.0001*	6.28 (2.34-4.21)#
	CT	67 (34.3%)	208 (38.3%)		
	CC	52 (10.7%)	137 (25.2%)		
SNPs of rs2277438	GG	35 (7.2%)	35 (6.4%)	0.439*	
	GA	125 (25.7%)	91 (16.8%)		
	AA	327 (67.1%)	417 (76.8%)		

*Chi-square test for categorical values; #OR (odds ratio) adjusted for age, sex, hypertension, hypercholesterolemia, CAD, PAOD, diabetes mellitus and smoking.

teoporosis and fracture[28,29]. Some experimental studies have shown preliminary evidences for a role of RANKL in plaque destabilization in acute vascular diseases[15,23,30,31]. Kiechl et al[32] showed that baseline serum level of RANKL was an important predictor of acute cardiovascular events such as myocardial infarction and ischemic stroke. How RANKL is involved in these vascular events is still unknown. It was demonstrated that RANKL enhances matrix metalloproteinase activity in vascular smooth muscle cells and chemokine (MCP-1) release from peripheral blood mononuclear cells[15,23,30,31]. In the final chain of events causing plaque destabilization, the key processes are monocyte/macrophage matrix migration and matrix degeneration. On the other hand, RANKL could stimulate osteogenic differentiation and calcification of vascular smooth muscle cells[14,15,30,31]. Calcium deposits in the intimal and medial layers could amplify wall shear stresses and attenuate plaque stability[33]. Up-regulation of RANKL is triggered by pro-inflammatory cytokines like interleukin-1 alpha, tumor necrosis factor-alpha and interleukin-6 and may be viewed as part of the immune-inflammatory milieu seen in advanced plaques[4,15,30,31]. All evidence supports the possibility that genetic variation of RANKL may also have an important correlation with plaque stability and the development of cerebrovascular events. Shimamura et al[34] demonstrated that the stimulation of RANKL/RANK signaling through the deletion of OPG or exogenous RANKL addition prevented the further exacerbation of infarct volume and cerebral edema by inhibiting the production of inflammatory cytokines.

In our current work, we confirmed our previous report in which rs3134069, rs2073617, and rs2073618 variant genotypes of the OPG gene were significantly and independently associated with the increased risk of history of ischemic stroke in Italian diabetic patients[35]. In this study, the occurrence of ischemic stroke was 3.67-, 4.43-, and 4.68-fold higher in patients homozygous for the G, C and C alleles, respectively, of the rs3134069, rs2073617, and rs2073618 gene polymorphisms compared with the controls.

It was demonstrated that these gene polymorphisms are functionally important. Patients carrying the aforementioned high-risk genotypes showed a median protein concentration statistically higher than in control subjects[25].

Several lines of evidence support the concept that OPG is a marker rather than a mediator of cardiovascular disease. Atherosclerosis is a chronic inflammatory condition[36]; pro-inflammatory cytokines such as interleukin-1β and TNF-α are known to induce OPG expression in human vascular smooth muscle cells[37]. It was shown increased levels of OPG in severe coronary artery disease (CAD)[38] and in unstable carotid plaque in patients underwent carotid endarterectomy[39]. Serum OPG levels is an important marker of bone homeostasis, vascular calcification and inflammation; serum OPG high concentrations may promote matrix degradation potentially contributing to plaque destabilization and future cardiovascular events[40]. Plasma OPG is an independent risk factor for long-term mortality following acute ischemic stroke[22] and for progressive atherosclerosis and cardiovascular diseases[41].

This report has some potential limitations. It was a case-control study; therefore a recruitment and survival bias cannot be excluded. Our data were obtained from a cohort of European descents and include subjects with other cardiovascular diseases; therefore, comorbidity might represent a confounding factor and the generalization of our findings regarding other age groups or ethnicities are unclear. The size of the studied population is relatively small and could lead to false positive; then, our findings need to be confirmed in larger samples, and should also be tested in groups of different ethnic origins. Some of the genes investigated in this study present more than one single nucleotide polymorphisms and it might be interesting to evaluate whether other genetic haplotypes play a role in subjects with HIS. We did not perform a detailed experiment on the functional activity of rs9533156 RANKL SNP. The exact function or its influence on RANKL protein expression remains unclear.

Conclusions

The present work identifies genetic variant of RANKL as an independent risk factor for ischemic stroke. These data further suggest a role for RANKL as a reliable biomarker cerebrovascular disease. The associations between RANKL and HIS demonstrated in this study support further investigation to clarify a possible role of RANKL as a biomarker to identify patients with, or at risk of, cerebrovascular events.

Conflict of Interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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