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Association of RANK/RANKL/OPG gene polymorphisms with risk of peripheral arterial disease (PAD) and critical limb ischemia in the general Italian population

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ABSTRACT

Background: Peripheral arterial disease (PAD) is an important determinant of the excessive morbidity and mortality in atherosclerotic patients, especially in patients with critical limb ischemia (CLI). Several studies recently conducted have demonstrated that the RANK/RANKL/OPG system plays an important role in the metabolism of the bone and in vascular pathology, including atherogenesis and arterial calcification and is involved in plaque instability and rupture by inducing plaque calcification.
Aim and methods: The aim of the present study to evaluate whether the rs3134069, the rs2073617, and the rs2073618 polymorphisms of the OPG gene, the rs9533156 and the rs2277438 gene variants of the RANKL gene and the rs1805034 gene polymorphism of the RANK gene are associated with presence and severity of PAD in general Italian population. Our study included 1221 patients (523 with PAD and 698 controls without PAD).
Results: We found that the rs3134069, the rs2073617, and the rs2073618 polymorphisms of the OPG gene, the rs9533156 gene variants of the RANKL gene and the rs1805034 gene polymorphism of the RANK gene were significantly and independently associated with PAD. We also found that these five polymorphisms act synergistically in patients with PAD and are associated with different levels of risk for PAD and CLI, depending on the number of high-risk genotypes carried concomitantly by a given individual.
Discussion: Genetic variations of rs3134069, rs2073617, and rs2073618 on the OPG gene, rs9533156 on the RANKL gene and rs1805034 on the RANK gene are associated with the risk to develop PAD and these five gene polymorphisms act synergistically in patients with PAD and are associated with different risk for PAD.
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1. Introduction

Atherosclerosis, the primary cause of cardiovascular diseases, is a complex chronic inflammatory disorder in the walls of medium and large arteries, is characterized by the interaction between genetic and environmental factors, as well as between chronic inflammation and altered immune function (Lim et al., 2014; Marinkovic et al., 2013) and its clinical consequences, such as cerebrovascular disease, coronary artery disease (CAD), and peripheral arterial disease (PAD), are potentially life-threatening (Munger and Hawkins, 2004). Peripheral arterial disease (PAD) represents a local manifestation of a systemic atherosclerotic disorder, increases 2–6–fold in both cerebrovascular and cardiovascular events and is associated with an annual mortality rate of 4%–6% (Malyar et al., 2016). In addition to causing lifestyle-limiting claudication symptoms, uncontrolled disease can progress on to critical limb ischemia (CLI) that represents the end stage of PAD with a high rate of limb loss along and patient mortality (Norgren et al., 2007). In a recent study, a higher prevalence of PAD was observed in Greece (28.0%) and Italy (22.9%), showing a greater risk of PAD in an Italian population (Sanna et al., 2011).

Several basic and clinical science studies recently conducted have demonstrated that the RANK/RANKL/OPG system plays an active role in the metabolism of the bone tissue, in vascular pathology, including atherogenesis and arterial calcification (Kiechl et al., 2004; Ziegler et al., 2005). It has been previously demonstrated in clinical and experimental study that heart failure is associated with increased expression of the OPG/RANKL/RANK axis and that increased serum levels of RANKL and OPG are significantly correlated with hemodynamic,

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functional, and neurohormonal parameters for disease severity (Ueland et al., 2005). In addition, it has been shown, in a rat model of post-infarction heart failure, an increased gene expression of RANK, OPG, and RANKL in the ischemic part of the left ventricle. OPG is an important soluble secreted protein that lacks trans-membrane and cytoplasmic domains. It binds to a receptor activator of nuclear factor-κB ligand (RANKL) (Simonet et al., 1997), whose receptor is the receptor activator of NF-κB (RANK), and inhibits RANKL/RANK signaling. Serum OPG levels were significantly correlated with coronary artery calcification in asymptomatic Korean patients with Type 2 diabetes (Jung et al., 2010), with heart failure (Ueland et al., 2005) and severity of peripheral artery disease (Ziegler et al., 2005), with acute myocardial infarction (Crisafulli et al., 2005), and vulnerable carotid plaques (Straface et al., 2011), plays an important role in inflammatory bone diseases (Turk et al., 2009), and in the formation of osteoporosis (Dai and Shen, 2007). Several studies have shown that OPG/RANK/RANKL axis is involved in plaque instability and rupture by inducing plaque calcification (Panizo et al., 2009) and recent reports have demonstrated, in a prospective large population based study, that RANKL serum levels is associated with future cardiovascular diseases such as ischemic stroke and myocardial infarction (Kiechl et al., 2007). RANKL increases total MMP activity in human fibroblasts and in vascular smooth muscle cells (MMP-9 and MMP-2), and may play an important role in plaque stability (Sandberg et al., 2006; Ueland et al., 2005). Moreover, it was demonstrated that RANKL enhances chemokine (MCP-1) release from peripheral blood mononuclear cells (Collin-Osdoby, 2004; Hofbauer and Schoppet, 2004; Min et al., 2005) and it is involved in the final chain of events causing plaque destabilization, the key processes characterized by monocyte/macrophage matrix migration and matrix degeneration. On the other hand, RANKL could stimulate osteogenic differentiation and calcification of vascular smooth muscle cells (Collin-Osdoby, 2004; Hofbauer and Schoppet, 2004; Min et al., 2005), and calcium deposits in the intimal and medial layers could amplify wall shear stresses and attenuate plaque stability (Huang et al., 2001). 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 a part of the immune-inflammatory milieu seen in advanced atherosclerotic plaques (Collin-Osdoby, 2004; Hofbauer and Schoppet, 2004; Min et al., 2005).

On the basis of these reports, we evaluated the gene polymorphisms identified in the OPG, RANKL and RANK genes: the clinical relevance of these SNPs is based on the fact that functional activity and/or plasma levels may be strongly influenced by these gene variants. We already demonstrated that the rs3134069, rs2073617and rs2073618 gene polymorphisms of the OPG gene were associated with a median OPG protein concentration that was statistically higher in patients with internal carotid artery stenosis than in control patients (Straface et al., 2011).

Therefore, the aim of the this study were to determine whether the rs3134069, the rs2073617, and the rs2073618 polymorphisms of the OPG gene, the rs9533156 and the rs2277438 gene variants of the RANKL gene and the rs1805034 gene polymorphism of the RANK gene are associated with presence and severity of PAD in general Italian population.

2. Materials and methods

2.1. Subjects

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 May 1, 2012, to June 30, 2016. In the group of patients with PAD were enlisted, in a retrospective study, subjects who had a history of critical limb ischemia. Inclusion criteria for the PAD group were Caucasian race and presence of PAD at Fontaine's stage II, III, or IV. Diagnosis of PAD was performed in accordance with the criteria established by the Ad Hoc Committee on Reporting Standards of the Society for Vascular Surgery and the International Society for Cardiovascular Surgery (Rutherford et al., 1986). All patients had an ankle-brachial-index (ABI) lower than 0.8 and underwent bilateral high-resolution B-mode ultrasonography evaluation (EcocolorDoppler Acuson 128XP/10, Acuson, Mountain View, CA, USA, with an 4 MHz trans- ducer). Severity of PAD was defined according to the Fontaine's staging system: patients were considered affected by stage II when they presented intermittens claudicatio, by stage III when they presented rest pain, and by stage IV when ischemic trophic lesions of the lower limbs were present. According with recommendations of the Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II), patients with ulcers, or gangrene, ischemic rest pain, attributable to objectively proven PAD were considered affected by critical limb ischemia (CLI) (Norgren et al., 2007).

Six-hundred and ninety eight subjects, matched for age and gender and with an ABI ≥ 1 and normal findings at bilateral high-resolution B-mode ultrasonography evaluation, were included as controls. Subjects without peripheral arterial disease (WPAD) had no family history of PAD.

Exclusion criteria from the study were coagulation disorders, atrial fibrillation, other major sources of cardio-embolism, chronic inflammatory diseases, cancer (current or previous), infectious diseases, and autoimmune diseases. All subjects were of European descent, coming from central and southern Italy. For all subjects enrolled in the study, a complete medical history was collected and included coronary artery disease (CAD), smoking habits, diabetes, lipid profile, hypertension, history of ischemic stroke (HIS), body mass index (BMI) and drug treatment. 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. Hypertension was defined as a systolic blood pressure > 140 mmHg, a diastolic blood pressure > 90 mmHg, and >130 mmHg, a diastolic blood pressure > 85 mmHg for the diabetic subjects, 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 the A. Gemelli University Hospital of Rome and St M. Goretti Hospital, Latina (Italy). Informed consent was obtained from participating patients.

2.2. SNP genotyping

Samples of DNA were extracted from peripheral blood by standard procedures and assayed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (Hinf I, Hinc II, Xsp I and Hha I) for the detection of OPG rs3134069, rs2073617 and rs2073618 gene polymorphisms and of RANK rs1805034 gene polymorphism as previously described (Biscetti et al., 2014; Pereira et al., 2014).

Genotyping of the rs9533156 polymorphism of the RANKL gene was performed by amplification of a 552-bp fragment with oligonucleotide primers 5′-TGGTCAGCAACTTCCTTCTG-3′ (sense) and 5′-GACAT TCCTCCTGCATCCAT-3′ (antisense); PCR amplifications were carried out in a total volume of 20 μl with a reaction mixture containing 10 ng genomic DNA, 30 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0,1% Igepal®-CA360, 200 μM of each deoxy-NTP, 1 pmol/μl of each primer, and 1.25 U Taq DNA polymerase (5 PRIME MasterMix). The PCR amplification conditions included an initial denaturation at 94 °C for 5 min, followed by 30 cycles with denaturation at 94 °C for 30 s, annealing at 46 °C for 30 s, and extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 7 min. After PCR amplification, 5 μl was digested by restriction endonuclease with 3 U TspR I (New England Biolabs, Frankfurt, Germany) for 2 h at 65 °C and resolution by electrophoresis on a 2.0% agarose gel. The 552-bp PCR product is cleaved into 303- and 250-bp fragments only in the presence of a T nucleotide.

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