



Association between osteoprotegerin gene polymorphisms and cardiovascular disease in type 2 diabetic patients

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Abstract

Osteoprotegerin (*OPG*) gene polymorphisms (T245G, T950C and G1181C) have been associated with osteoporosis and early predictors of cardiovascular disease. The aim of this study was to evaluate whether these polymorphisms contribute to cardiovascular disease (CVD) in type 2 diabetic patients. We performed a case-control study with 178 CVD subjects with diabetes and 312 diabetic patients without CVD to assess the impact of variants of the *OPG* gene on the risk of CVD. The *OPG* gene polymorphisms were analyzed by using the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). There was no significant association between the T245G and G1181C polymorphisms and CVD in the additive genetic model (OR = 0.96, 95% CI 0.64-1.45, $p = 0.79$; OR = 1.06, 95% CI 0.81-1.39, $p = 0.65$, respectively). However, the C allele of the T950C polymorphism was independently associated with a risk of CVD in type 2 diabetic patients in this genetic model (OR = 1.38, 95% CI 1.07-1.80, $p = 0.01$). This study provides evidence that the C allele of the T950C polymorphism is associated with increased risk of CVD in diabetic patients. However, well-designed prospective studies with a larger sample size are needed to validate these results.

Keywords: cardiovascular disease, osteoprotegerin, polymorphism.

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Introduction

Mortality due to cardiovascular disease (CVD) has declined worldwide during the past few decades (Wijeyesundera *et al.*, 2010). However, during the same time period, the number of CVD deaths attributable to diabetes has increased (Puri *et al.*, 2012). Certain factors need to be considered when explaining these divergent trends. First, although the prevalence of other risk factors, such as smoking, hypertension and hypercholesterolemia, has been reduced by prevention programs, the incidence of diabetes has been steadily rising (Chen *et al.*, 2011). Second, there has been no significant decline in the increased cardiovascular risk experienced by diabetic subjects. Clearly, there is an urgent need to curb the current epidemic of diabetes and to prevent CVD in diabetics. Although little is known about the factors underlying the increased cardiovascular risk in diabetic patients, studies in diabetic and nondiabetic subjects suggest that the risk of CVD is influenced by genetic factors (Lusis *et al.*, 2004).

Osteoprotegerin (*OPG*), also known as osteoclastogenesis inhibitory factor, is a member of the tumor necrosis

factor receptor superfamily of cytokines (Simonet *et al.*, 1997). Clinically, *OPG* may play a role in the development of osteoporosis, inflammatory bone diseases, multiple myeloma and malignant bone resorption (Goranova-Marinova *et al.*, 2007; Turk *et al.*, 2009). In addition, *OPG* has been associated with the presence and severity of cardiovascular events: elevated serum *OPG* concentrations have been found to correlate with the severity of peripheral artery disease and heart failure, symptomatic carotid stenosis, unstable angina, vulnerable carotid plaques and acute myocardial infarction (Golledge *et al.*, 2004; Crisafulli *et al.*, 2005; Ueland *et al.*, 2005; Ziegler *et al.*, 2005; Sandberg *et al.*, 2006; Kadoglou *et al.*, 2008; Song *et al.*, 2012).

The gene encoding *OPG* is affected by common, functionally important genetic polymorphisms that have been associated with osteoporosis and neuroarthropathy and are considered early predictors of cardiovascular disease (Collin-Osdoby, 2004; Soufi *et al.*, 2004; Stykarsdottir *et al.*, 2008; Pitocco *et al.*, 2009). Some recent studies have shown that the T245G, T950C and G1181C polymorphisms of the *OPG* gene are associated with the vulnerability of carotid plaques and risk of stroke (Straface *et al.*, 2011; Biscetti *et al.*, 2012).

However, the extent to which these genetic markers predispose to increased cardiovascular complications in type 2 diabetes remains uncertain. The purpose of the pres-

ent case-control study was therefore to determine whether the T245G (rs3134069), T950C (rs2073617) and G1181C (rs2073618) polymorphisms of the *OPG* gene play a role in CVD in type 2 diabetic patients.

Materials and Methods

Study population

All participants were randomly recruited from the Department of Internal Medicine of The First Affiliated Hospital of Xinxiang Medical University in Weihui (Henan Province, People's Republic of China), from September 12, 2007, to July 20, 2012. Type 2 diabetes was diagnosed according to WHO criteria (Alberti and Zimmet, 1998). Age at diagnosis of type 2 diabetes was > 40 years in 98% of the subjects and the minimum age at onset was 37 years. Type 1 diabetes was carefully excluded on clinical grounds based on a review of medical records, on the fasting C-peptide levels and on the absence of islet-related auto-antibodies. For the purpose of this study, CVD was defined as the occurrence of a fatal or nonfatal myocardial infarction or coronary artery bypass grafting. Subjects diagnosed with CVD before the diagnosis of type 2 diabetes were excluded, as were those diagnosed with stroke and/or angina. After these exclusions, 181 women (117 CVD case subjects and 64 control subjects) and 309 men (195 CVD case subjects and 114 control subjects) were enrolled in the study. Hypertension was diagnosed as a systolic blood pressure (SBP) \geq 140 mmHg and/or a diastolic blood pressure (DBP) \geq 90 mmHg, or the current use of antihypertensive medication. Hypercholesterolemia was diagnosed as total cholesterol \geq 5.2 mmol/L, and/or triglyceride \geq 1.7 mmol/L and/or low density lipoprotein cholesterol \geq 3.1 mmol/L. HbA1c was measured on the same day that the samples were taken using a standard assay and a Bio-Rad Variant HPLC II system (Bio-Rad Laboratories, Hemel Hempstead, UK), in accordance with the Diabetes Control and Complication Trial recommendations (Mitka, 2009).

All subjects were of Han Chinese origin from central China and belonged to independent pedigrees. Informed consent was obtained from all participants and the study was approved by the institutional review board of Xinxiang Hospital. Experiments were done according to the principles expressed in the Declaration of Helsinki.

Single nucleotide polymorphism genotyping

DNA was extracted from peripheral blood by standard procedures and screened for the *OPG* gene polymorphisms T950C, T245G and G1181C using the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), as previously described (Soufi *et al.*, 2004; Pitocco *et al.*, 2009; Biscetti *et al.*, 2012). A 570-bp fragment around the T950C polymorphism was amplified with the primer pairs: 5'-TGCGTCCGGATCTTGCTGATCGG-3' and 5'-GGGCGCGGCGGGCGCGCCAG

GGACTTACCACGAGCGCGCAGCACAGCAA-3'. The reaction was done in a final volume of 25 μ L that included 1 ng of genomic DNA, 200 μ mol of dNTP mixture/L, 0.2 μ mol of each primer/L, 2 GC buffer and 1 U of TaKaRa LA *Taq* DNA polymerase (TaKaRa Biomedicals, Dalian, Liaoning, China). The PCR was done in a 2720 thermocycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation at 95 $^{\circ}$ C for 5 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 62 $^{\circ}$ C for 30 s and extension at 72 $^{\circ}$ C for 30 s, with a final extension at 72 $^{\circ}$ C for 5 min. The PCR products were digested with *HincII* restriction endonuclease (Fermentas, Burlington, ON, Canada) at 37 $^{\circ}$ C for 16 h and digestion products were separated on 2.5% agarose gels containing ethidium bromide. The 570 bp PCR product is cleaved into 288 bp and 282 bp fragments only in the presence of a C nucleotide at position 950. The PCR primers for T245G (271 bp fragment) were 5'-CGAACCCTAGAGCAAAGTGC-3' and 5'-TGTCTGATTGGCCCTAAAGC-3'. PCR was done as described above except that the annealing temperature was 56 $^{\circ}$ C. The PCR products were digested with *HinfI* restriction endonuclease (Fermentas) and separated on 3% agarose gels containing ethidium bromide. In the presence of a T nucleotide at position 245, the 271 bp PCR product was cleaved into 202 bp and 69 bp fragments. Genotyping of the G1181C *OPG* exon 1 polymorphism was done using a mis-matched oligonucleotide approach. A 570 bp fragment was amplified with the primers 5'-TGCGTCCGGA TCTTGCTGGATCGG-3' and 5'-GGGCGCGGCGGG CGCGCCAGGGACTTACCACGAGCGCGCAGCAC AGCTA-3', the latter containing a T instead of an A nucleotide two bases before the 3' end; this position corresponds to the third base of codon 3 that encodes lysine in exon 1 of the *OPG* gene and the substitution introduces an artificial *XspI* restriction site in the presence of the mutant allele. PCR was done as described above and the PCR products were digested with *XspI* restriction endonuclease (Fermentas) for 16 h and subsequently separated on 3% agarose gels containing ethidium bromide. In the presence of a C nucleotide at position 1,181, the 570 bp PCR product was cleaved into 522 bp and 48 bp fragments. To independently validate the PCR-RFLP approach, we analyzed 49 randomly selected DNA samples (10% of the total samples) by direct sequencing and by PCR-RFLP; the results obtained with these two approaches were identical, indicating that the PCR-RFLP method was reliable.

Statistical analysis

Chi-square tests and *t*-tests were used to compare proportions and means of baseline characteristics between CVD and control subjects. Logistic regression was used to estimate the odd ratios (ORs) and 95% CIs for CVD risk, after adjusting for age, sex, body mass index (BMI), hypercholesterolemia, smoking and hypertension. Hardy-Weinberg equilibrium was assessed with the Chi-square test or

Fisher’s exact test, as appropriate. Linkage disequilibrium was calculated using the software Haploview 4.1 for all pairwise single nucleotide polymorphism (SNP) combinations. All data analyses were done with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft Corp., Redmond, WA, USA). A value of $p < 0.05$ indicated significance and all statistical tests were two sided.

Results

The demographic and clinical data for the type 2 diabetic subjects with and without CVD are shown in Table 1. Univariate comparisons revealed no significant differences between the groups in terms of sex, age, BMI, HbA1c, hypertension and hypercholesterolemia. In contrast, there were significantly more ($p = 0.02$) smokers among diabetic patients with CVD than among those without CVD.

Table 2 shows the genotypic distribution of the T245C, T950C and G1181C gene polymorphisms. The genotype frequencies for all three polymorphisms were in Hardy-Weinberg equilibrium ($p > 0.05$) in the CVD and control subjects. Analysis with Haploview 4.0 revealed no linkage disequilibrium for any pair-wise combination among the three selected SNPs. Of the 178 patients with

CVD, the genotype distributions of the T245G and G1181C gene polymorphisms were not significantly different from those observed in the 312 subjects without CVD. Similarly, the minor allelic frequency (MAF) of the T245G and G1181C gene polymorphisms were 0.11 and 0.34 in patients with CVD, which was not significantly different from that in subjects without CVD ($p = 0.88$ and $p = 0.73$, respectively). In contrast, there was a positive association between the T950C gene polymorphism and CVD in type 2 diabetic patients ($p = 0.04$ for genotype and $p = 0.02$ for allele, respectively). Based on this finding, we used unconditional logistic regression analysis to evaluate whether these gene variations were independent variables associated with CVD in type 2 diabetic patients. After adjusting for relevant confounding variables (age, sex, BMI, hypercholesterolemia, smoking and hypertension), the C allele of the T950C polymorphism was found to be independently associated with the risk of CVD in type 2 diabetic patients in the additive model (OR = 1.38, 95% CI 1.07-1.80, $p = 0.01$). However, there was no significant association for the T245G and G1181C gene polymorphisms in the additive model (OR = 0.96, 95% CI 0.64-1.45, $p = 0.79$ and OR = 1.06, 95% CI 0.81-1.39, $p = 0.65$, respectively). After adjusting for relevant confounding variables, none of the three poly-

Table 1 - Demographic data for type 2 diabetic patients with and without CVD.

Variables	Control subjects (n = 312)	CVD subjects (n = 178)	p value
Male (%)	195 (62.5)	114 (64.0)	0.87
Age (years)	57.2 + 9.5	58.3 + 9.8	0.83
BMI (kg/m ²)	24.9 + 3.5	25.2 + 3.1	0.96
HbA1c (%)	6.6 + 1.6	6.8 + 1.8	0.88
Diabetes > 10 years n (%)	194 (62.2)	109 (61.2)	0.92
Diabetes < 10 years n (%)	118 (37.8)	69 (39.8)	0.89
Smoking n (%)	119 (38.1)	100 (56.2)	0.02
Hypertension n (%)	177 (56.7)	122 (68.5)	0.21
Hypercholesterolemia n (%)	130 (41.7)	98 (55.1)	0.09

Data are the mean + standard deviation or % unless otherwise indicated; BMI, body mass index; HbA1c, hemoglobin A1c.

Table 2 - Association between variants of the OPG gene and CVD in patients with type 2 diabetes.

SNPs (M > m)	Population	MM	Mm	mm	$P_{genotype}$	MAF	P_{allele}	Additive model	
		N (%)	N (%)	N (%)				OR (95%CI)	P
T245G (T > G) (rs3134069)	Control	247 (79.2)	58 (18.6)	7 (2.2)	0.98	0.11	0.88	0.96 (0.64 1.45)	0.79
	CVD	142 (79.7)	32 (18.0)	4 (2.3)					
T950C (T > C) (rs2073617)	Control	108 (34.6)	152 (48.7)	52 (16.7)	0.04	0.49	0.02	1.38 (1.07 1.80)	0.01
	CVD	49 (27.5)	84 (47.2)	45 (25.3)					
G1181C (G > C) (rs2073618)	Control	141 (45.2)	135 (43.3)	36 (11.5)	0.67	0.34	0.73	1.06 (0.81 1.39)	0.65
	CVD	81 (45.5)	72 (40.4)	25 (14.1)					

$P_{genotype}$ and P_{allele} were calculated using the two-tailed Chi-square test or Fisher’s exact test. Odds ratios (OR, with 95% confidence interval) were computed using additive genetic models with multivariate unconditional logistic regression analysis adjusted for covariance. M, major allele; m, minor allele; MAF, minor allele frequency; SNP, single-nucleotide polymorphism.

morphisms was associated with CVD in type 2 diabetic patients in the recessive or dominant model (all *p* values > 0.05).

Discussion

Osteoprotegerin (*OPG*), a key factor in bone remodeling, is a member of the tumor necrosis factor (TNF) receptor family and a decoy receptor for the receptor activator of nuclear factor- κ B ligand (RANKL) and TNF-related apoptosis inducing ligand (TRAIL). In addition to its role in the skeletal system, *OPG* may have a role in vascular disease and has been implicated in human atherogenesis (Simonet *et al.*, 1997; Emery *et al.*, 1998; Browner *et al.*, 2001). Previous studies had found serum *OPG* levels to be positively correlated with the presence and severity of CVD (Jono *et al.*, 2002; Schoppet *et al.*, 2003). Clinical studies have shown that high serum *OPG* levels are an independent risk factor for progressive atherosclerosis and cardiovascular diseases (Kiechl *et al.*, 2004; Oh *et al.*, 2005). For type 2 diabetic patients in particular, Avignon *et al.* (2005) reported an independent association between *OPG* levels and asymptomatic coronary artery disease. *OPG* gene polymorphisms have been associated with osteoporosis and vascular impairment (Hofbauer and Schoppet, 2002). Furthermore, subjects with a C allele in the promoter region at position 950 (TC and CC) have significantly higher circulating *OPG* serum levels, and genetic variations in the *OPG* gene confer an increased risk of CVD and carotid plaque vulnerability in Caucasians (Soufi *et al.*, 2004; Ohmori *et al.*, 2006; Straface *et al.*, 2011). However, Rhee *et al.* (2006) observed that polymorphisms in the promoter region of the *OPG* gene were not associated with aortic calcification or coronary artery disease in Koreans. Given this background, the aim of this study was to investigate the possible association between genetic variations in the *OPG* gene and the risk of CVD in type 2 diabetic patients.

This study is the first to show that the C allele of the T950C (rs2073617) polymorphism in the *OPG* gene is significantly and independently associated with an increased risk of CVD in type 2 diabetic patients. This finding agrees with previous reports (Soufi *et al.*, 2004; Ohmori *et al.*, 2006). The T950C polymorphism is located 129 bp upstream from the TATA box, 13 bp downstream from an activating protein 2 binding site and 32 bp upstream from a specificity protein 1 binding site. The biological significance of the association observed here is related to the fact that gene variants are functionally important. Previous studies have shown that the C allele of the T950C (rs2073617) polymorphism in the *OPG* gene is significantly and independently associated with increased serum osteoprotegerin levels (Hofbauer and Schoppet, 2002; Straface *et al.*, 2011). Interestingly, Biscetti *et al.* (2012) also showed that polymorphisms in the *OPG* gene were associ-

ated with increased risk of ischemic stroke in diabetic patients. These findings further support that conclusion that osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease not only in the general population but also in type 2 diabetic patients. This conclusion is consistent with the concept that the individual chances of presenting an atherosclerotic-related disease may be affected by a susceptibility profile that is genetically defined (Brito *et al.*, 2009; Ding and Kullo, 2009; Mollsten *et al.*, 2009). These data further suggest a role for *OPG* as a reliable biomarker in cardiac and vascular disease. Although the mechanisms linking *OPG* and vascular disease require further study, the association between *OPG* and CVD in type 2 diabetic patients shown here requires further investigation to clarify the possible role of *OPG* as a biomarker for identifying patients with, or at risk of, cardiovascular events.

This study has several limitations. First, the sample size in this study was relatively small and could yield a false positive result. In addition, the relatively small sample size and rare frequency of the T245G polymorphism may have resulted in insufficient statistical power to detect a positive association. Our findings need to be confirmed in larger samples and in ethnically different groups. Second, this was a case-control study. Consequently, recruitment and survival bias cannot be excluded, particularly in the control population in which the possibility that some of the controls might develop cardiovascular disease in the future could not be eliminated. Third, this study was restricted to Han Chinese in order to avoid the possible confounding effect of race. For other ethnic groups or races, other genetic markers may be more effective in detecting the predisposing effect of the loci described here. The choice of appropriate markers requires knowledge of the differences in linkage disequilibrium patterns among races.

In summary, osteoprotegerin gene polymorphisms associated with CVD in the general population were also associated with CVD in diabetic subjects. Although diabetic and non-diabetic individuals showed similarities in their genetic susceptibility to CVD, diabetic patients had certain peculiarities in their genetic architecture that influenced their susceptibility to CVD.

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