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Original Article

Association of OPG gene polymorphisms with the incidence and radiographic stage of knee osteoarthritis

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Abstract: Background: We investigated two osteoprotegerin (*OPG*) single nucleotide polymorphisms (SNPs), T950C and A163G, for their association with knee osteoarthritis (KOA) disease progression. Methods: A total of 120 KOA patients and 120 control subjects were recruited for this study. Genotype frequencies and allele frequencies of the *OPG* gene polymorphisms were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Serum OPG levels were determined by enzyme-linked immunoassay (ELISA). Results: The T950C CC genotype and C allele frequencies were significantly higher in KOA patients, compared to controls (all $P < 0.05$). The C allele carriers (TC+CC) were associated with significantly increased risk of KOA than those carrying the TT genotype. Within the T950C genotypes, serum OPG levels of TC and CC genotypes were significantly higher than the TT genotype ($P < 0.05$), but no difference was observed between the three genotypes in control group (all $P > 0.05$). Importantly, radiographic analysis showed that the highest frequency of late stage KOA occurred in patients with the *OPG* T950C CC genotype, while TC genotype was more frequently associated with middle stages and the TT genotype with early stage in KOA patients. Logistic regression analysis showed that body mass index (BMI) (OR = 1.744, 95 CI% = 1.630-2.879, $P < 0.001$), T950C (OR = 6.616, 95 CI% = 2.476-17.682, $P < 0.001$) and serum OPG levels (OR = 2.860, 95 CI% = 2.152-3.802, $P < 0.001$) were independent risk factors for KOA. Conclusion: Based on our results, we propose that *OPG* T950C genetic polymorphism is associated with the development of KOA, and the C allele increased the risk and severity of KOA. However, the *OPG* A163G polymorphism did not correlate with the radiographic stage in KOA patients.

Keywords: Osteoprotegerin, genetic polymorphisms, knee joint osteoarthritis, incidence, radiographic stage, polymerase chain reaction-restriction fragment length polymorphism, enzyme-linked immunoassay, genotypes and alleles

Introduction

Osteoarthritis (OA) is a skeletal disease caused by effusion and synovitis, with serious impact on the quality of life, especially in middle age and older patient populations [1]. The knee is the most frequently affected joint in OA. Knee osteoarthritis (KOA) is a degenerative arthritis in the knee characterized by mechanical abnormalities due to joint degradation, including articular cartilage and subchondral bone damage [2]. Longitudinal study in US and UK has shown that the onset of KOA is associated with advancing age, and additionally, obese individuals are at highest risk of KOA [3]. On a global scale the disease has stunning statistics, and an estimated 250 million people suffer from KOA, which represents nearly 3.6% of the total world population [4]. The prevalence of KOA varies from < 10% to > 90% depending on several factors, including geographical location [5]. In particular, women have a higher risk of KOA than men, with female to male ratios ranging from 1.5:1 to 4:1 [6]. The pathogenesis of KOA involves both intrinsic and extrinsic factors such as age, weight, gender, trauma to the knee, muscle weakness, and genetic factors [7]. Unfortunately, current therapeutic methods for OA treatment are ineffective in preventing OA progression, and therefore, study of genetic factors contributing to the disease progression may help in understanding the mechanisms of KOA, with the ultimate goal of designing effective clinical interventions for KOA [8].

Osteoprotegerin (OPG) is also known as osteoclastogenesis inhibitory factor or tumor necrosis factor receptor superfamily member 11B,

and is a glycoprotein composed of 401 amino acid residues [9]. OPG functions as a decoy receptor and binds strongly to receptor activator for nuclear factor κB ligand (RANKL), and the RANK/RANKL/OPG system has important functions in inflammation, skeletal remodeling and the vascular system [10]. In the context of disease states, down-regulation of OPG levels correlated with altered fat distribution, and appeared to decrease cardiovascular risk [11]. The OPG/RANKL ratios are critical to maintain a proper between balance bone resorption and bone formation, and increased OPG/RANKL ratio may prompt osteoblast proliferation and differentiation via suppressing osteoclast survival and formation [12]. Increased level of serum OPG is also an indicator of chronic kidney disease, and OPG effects on bone resorption and bone loss in this disease setting may be a secondary complication [13]. Considering the role of OPG in regulating bone turnover and bone formation, genetic alteration in the *OPG* gene may influence bone mass, increase risk of fractures, and play a role in OA [14]. Alterations in OPG serum levels are associated with genetic variations, including T950C, A163G and G1181C polymorphisms in *OPG* gene, and premenopausal women with *OPG* variants were shown to have a distinct bone mineral density (BMD) compared to normal individuals [15, 16]. Measurements of BMD using radiographic absorptiometry showed a strong correlation between *OPG* gene polymorphisms and BMD in postmenopausal women [17]. Accordingly, our study used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay for assessing the association between the distribution of two osteoprotegerin (*OPG*) single nucleotide polymorphisms (SNPs) (T950C and A163G) and the development of KOA, and further confirmed by testing serum OPG levels.

Materials and methods

Ethics statement

The protocol of the study was reviewed and approved by the commitment of Dongying People's Hospital. All subjects provided written informed consent prior to diagnostic and therapeutic procedure during their hospitalization. All procedures were conducted according to the Declaration of Helsinki [18].

Subjects

A total of 120 patients, diagnosed with primary KOA, were selected for participation in this study conducted from March 2013 to May 2014 at the Department of Orthopaedics of the Affiliated Sixth People's Hospital of Shanghai Jiao tong University. Among 120 KOA patients, there are 65 male and 55 female (37~87 years old) with the mean age of 58.05 ± 18.83. All the patients were diagnosed according to the American College of Rheumatology (ARHP, Revised Edition), 1995 [19]. All the subjects were not diagnosed with other disorders, such as rheumatism, rheumatoid arthritis, autoimmune joint disease, traumatic arthritis, and skeletal dysplasia. Radiographic films of lateral plain of both knees and axial view patella of all patients were taken. Based on radiographic examinations, KOA patients were divided into early (50 cases), middle (45 cases) and late (25 cases) stages as it related to KOA disease progression in the patient group. In early stage patients, osteosclerosis or osteophyma was observed, but no joint space narrowing was detected. In middle stage patients, joint space were narrowed or disappeared, and in late stage patients, wearing and defection of weight-bearing surface of the tibia was clearly detected. The 120 healthy volunteers were selected from the Medical Examination Center at the same hospital within the same study period. There were 70 male and 50 female of age 32 to 86 in the control group, with the mean age of 56.21 ± 11.14 years. Blood urine test and radiographic examinations of the lateral plain of both knees were performed on healthy volunteers, and no abnormality were detected, including joint inflammation, joint pain, swelling, skin tenderness or limitation of movement. Cardiopulmonary diseases, diabetes, hypertension and other chronic diseases were also excluded. Informed consent was obtained from all the experimental subjects before the study was performed.

Blood samples collection

A volume of 5 ml venous blood was collected into tubes containing EDTA as anticoagulant from all study subjects in the early morning. Within 30 minutes of collection, blood samples were centrifuged at 3000 rpm for 10 min. The supernatants, representing the serum, was

Table 1. Comparison of baseline characteristics between the case and control groups

	Case group	Control group	*P*
Age	58.05 ± 18.83	56.21 ± 11.14	0.358
Gender			0.603
Male	65 (54.17)	70 (58.33)	
Female	55 (45.83)	50 (41.67)	
BMI (kg/m^2)	25.64 ± 3.41	24.52 ± 3.26	0.01
Course of disease (months)	74.88 ± 9.67	-	
Combined with hypertension	22 (18.33)	-	
Combined with diabetes	12 (10.00)	-	
Family history	21 (17.50)	-	
X-ray staging		-	
Initial stage	50 (41.67)		
Middle stage	45 (37.50)		
Late stage	25 (20.83)		
Single knee	87 (72.50)	-	
Both knees	33 (27.50)		

Note: BMI, body mass index.

transferred into clean 1.5 ml Eppendorf (EP) tubes, which were labeled and stored at -70°C freezer until further use. These samples were used to measure serum OPG concentrations. The blood cells precipitated at the bottom were transferred into 1.5 ml EP tubes, labeled and stored at -70°C freezer as well. These samples were used for genomic DNA extraction.

SNP genotyping

Genomic DNA was extracted with TIANamp DNA extraction Kit (Qiagen Company, USA). The A260 and A280 of the isolated DNA were measured to obtain A260/A280 (optical density, OD) ratio, which was between 1.8 and 2.0 for all the samples, indicating DNA of high purity. The T950C region was amplified by PCR using 5'-GTTCCTCAGCCCGGTGGCTTTT-3' (sense) and 5'-TGTGGTCCCCGGAAACCTCAGG-3' (antisense) primers, and A163G was amplified using 5'-CCATCATCAAAGGGC TATTGG-3' (sense) and 5'-CTG GAG ACA TAT AAC TTG AAC-3' (antisense) primers. All primers were synthesized by Sangon Biotech (Shanghai, China). The polymerase chain reaction (PCR) reactions were carried out in a 25 μl reaction mixture containing 1 μl of genomic DNA template, 12.5 μl of 2 × Taq Master Mix, 1 μl of each primer, and 9.5 μl of ddH_2O. The PCR amplifications cycles were performed as follow: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 57°C for 40 s, and extension at 72°C for 60 s. Final extension step was performed at 72°C for 10 min. The PCR products were confirmed by 1.5% agarose gel electrophoresis. PCR products was digested with Ase I (A163G) and HinC II (T950C) at 37°C for 4 h, and the digested product was separated on 2% agarose gels (45 min with the voltage of 300 V). Genotyping test and analysis were performed based on the DNA pattern observed by gel electrophoresis, and the results were recorded.

Measurement of OPG level in serum

To each microtiter plate well, 50 μl volumes of standard, blank, or samples were added per well. The blank well contained sample diluents. To each well, 50 μL of biotin labeled antibody was added and the plate was covered with sealer and incubated for 1 h at 37°C. Residual liquid of each well was discarded and the wells were washed with wash buffer. A volume of 80 μL of streptavidin-HRP was added to the wells and incubated for 30 min at 37°C. Following similar washing steps, 50 μL of substrate A and B were added to each well and mix gently, incubating for 10min at 37°C, avoiding exposure to light. The reaction was terminated by adding 50 μL of Stop Solution to each well. The results were read by immediately measuring the OD value of each well at 450 nm in a microplate reader. The standard curve was drawn by setting the concentration of standard as the vertical axis and the OD value as horizontal axis. The concentrations of each sample were determined using the standard curve.

Statistical analysis

The data was analyzed with SPSS17.0 software. χ^2 test was used to compare the data and if the genotype distribution conformed to Hardy-Weinberg equilibrium. Results were expressed as mean ± standard deviation (Mean ± SD). The *t* test was applied to compare patient group and control group. Logistic regression analysis was performed to calculate the odds

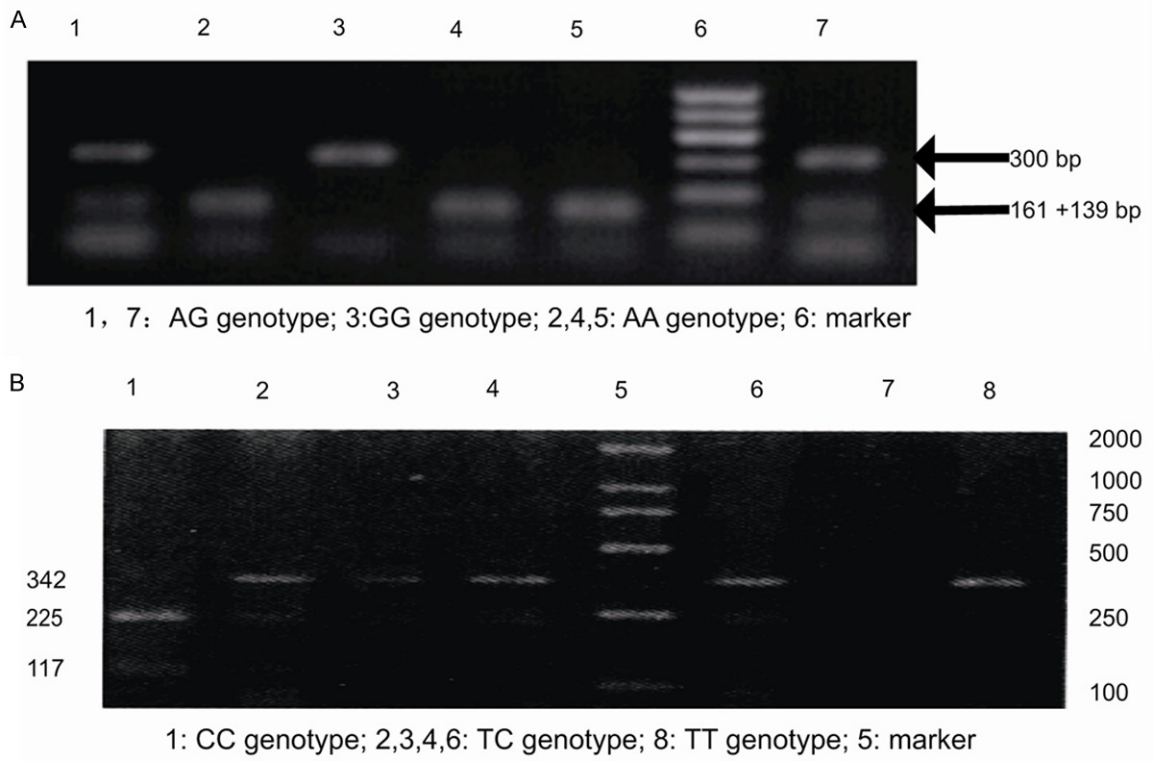

Figure 1. Electropherogram of A163G and T950C genetic polymorphisms in the *OPG* gene after specific restriction enzyme digestion (A: Electropherogram of A163G genetic polymorphisms in the *OPG* gene; B: Electropherogram of T950C genetic polymorphisms in the *OPG* gene).

ratio (OR) of different genotypes and 95% confidence interval (CI) represented relative risk. A value of $P < 0.05$ was considered as statistically significant.

Results

Baseline characteristics

As shown in **Table 1**, no significant difference was found in comparison of gender and age between the case and control groups (both $P > 0.05$). Significant difference was observed in comparison of BMI between the case (BMI: 25.64 ± 3.41) and control (24.52 ± 3.26) groups ($P = 0.010$). The mean course of disease in the case group was 74.88 ± 9.67 months with 22 cases of KOA combined with hypertension, 12 cases of diabetes mellitus, 21 cases of family history, 87 cases of single knee, and 33 cases of both knees.

PCR products after enzyme digestion

In agarose gel electrophoresis of the PCR-RFLP samples, the AA homozygote of A163G loci of *OPG* gene, containing the Ase I restriction site, showed two bands of 161 bp and 139 bp. When the Ase I restriction site was absent, in GG homozygote, a 300 bp band is observed as expected. The AG heterozygote showed 3 bands of 161 bp, 139 bp, and 300 bp in size (**Figure 1A**). In T950C loci, the TT homozygote showed a band with 342 bp, and the CC homozygote showed 2 bands of 177 and 225 bp, while the TC heterozygote showed 3 bands of 342 bp, 225 bp and 117 bp (**Figure 1B**). All the observed bands were according to the expected sizes.

The frequencies of genotypes and alleles

Both in patient and control groups, the distribution frequencies of the genotypes and alleles of A163G and T950C were according to Hardy-Weinberg equilibrium, indicating that the frequency of each gene has reached genetic equilibrium and the selected samples were representative of the population. As shown in **Table 2**, distribution frequencies of the three genotypes (TT, TC and CC) in polymorphic site T950C were 58.3%, 34.2%, and 7.5%, respectively in

Table 2. Genotype and allele frequency distribution of A163G and T950C genetic polymorphisms in the *OPG* gene between the case group and the control group

		Control group	Case group	χ^2	*P*	OR (95% CI)
T950C	TT	70 (58.3%)	51 (42.5%)			1
	TC	41 (34.2%)	40 (33.3%)			1.34 (0.76-2.34)
	CC	9 (7.5%)	29 (24.2%)	14.370	< 0.001	4.42 (1.93-10.15)
	TC+CC	50 (41.7%)	(57.5%)			1.89 (1.13-3.16)
	T	181 (75.4%)	142 (59.2%)			1
	C	59 (24.6%)	98 (40.8%)	14.40	< 0.001	2.12 (1.43-3.13)
A163G	AA	68 (56.7%)	69 (57.5%)			1
	AG	33 (27.5%)	32 (26.7%)			1.05 (0.58-1.89)
	GG	19 (15.8%)	19 (15.8%)	0.002	0.968	1.02 (0.49-2.08)
	AG+GG	52 (43.3%)	51 (42.5%)	0.020	0.967	0.97 (0.58-1.61)
	A	169 (70.4%)	170 (70.8%)			1
	G	71 (29.6%)	70 (29.2%)	0.010	0.920	1.02 (0.69-1.51)

Note: OR, odd ratio; CI, confidence interval.

Table 3. Comparison of serum OPG levels in the genetic polymorphisms of T950C (TT, TC and CC genotypes) and A163G (AA, AG and GG genotypes) in *OPG* gene

		Control group (ng/ml)	Case group (ng/ml)	*P*
	TT	118.74 ± 2.11	121.25 ± 2.12	< 0.001
T950C	TC	119.56 ± 2.25	124.96 ± 2.23#	< 0.001
	CC	119.6 ± 2.03	125.35 ± 2.34#	< 0.001
A163G	AA	119.16 ± 2.13	122.16 ± 2.31	< 0.001
	AG	119.21 ± 2.26	122.51 ± 2.16	< 0.001
	GG	120.10 ± 2.32	123.14 ± 2.52	< 0.001

Note: #, compared with the wild homozygous genotypes, $P < 0.05$.

Table 4. Association of the genetic polymorphisms of T950C (TT, TC and CC genotypes) and A163G (AA, AG and GG genotypes) in *OPG* gene with the radiographic stage (early, middle, and late stages)

		Radiographic stage		
		Early (n = 50)	Middle (n = 45)	Late (n = 25)
	TT	40 (80.0%)	8 (17.8%)	3 (12.0%)
T950C	TC	5 (10.0%)	32 (71.1%)	3 (12.0%)
	CC	5 (10.0%)	5 (11.1%)	19 (76.0%)
	χ^2	20.070	19.000	25.140
	P	< 0.001	< 0.001	< 0.001
A163G	AA	35 (70.0%)	24 (53.3%)	10 (40.0%)
	AG	10 (20.0%)	15 (33.3%)	7 (28.0%)
	GG	5 (10.0%)	6 (13.3%)	8 (32.0%)
	χ^2	2.390	0.746	4.081
	P	0.303	0.689	0.130

KOA patients, and the frequencies of T and C alleles in this group were 75.4% and 24.6%, respectively. In the control group in polymorphic site T950C, the frequencies of the three genotypes (TT, TC and CC) were 42.5%, 33.3%, and 24.2%, and frequencies of T and C alleles were 59.2% and 40.8%, respectively. The CC genotype and the C allele were significantly higher in patient group than the control group and there was statistical significance in the differences (all $P < 0.05$). The C allele carrying genotypes was associated with an increased risk of KOA, compared to the TT genotype (OR = 1.89, 95% CI = 1.13-3.16). However, distribution frequencies of genotypes and alleles in A163G loci showed no statistical differences between the cases and controls (all $P > 0.05$).

OPG levels in serum among different genotypes

As shown in **Table 3**, for any genotypes (TT, TC or CC) in T950C loci, serum levels of OPG in KOA patients were significantly higher than the controls (all $P < 0.001$). Moreover, patients carrying CC or TC genotype had relatively higher OPG levels than those with TT, and there were statistically significant differences ($P < 0.001$). In the control group, however, no difference in serum OPN levels was observed among the three genotypes (all $P > 0.05$). For any genotypes (AA, AG or GG) in A163G loci, serum OPG levels in patients is significantly higher than that in the controls, with statistical significance (all $P < 0.001$), yet no significant differences were found within the three genotypes (all $P > 0.05$).

Table 5. Logistic regression analysis

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I. for EXP(B)	
							Lower	Upper
Gender	-.383	.384	.993	1	0.319	.682	.321	1.448
Age	.019	.013	2.086	1	0.149	1.019	.993	1.046
BMI	-.296	.085	12.136	1	< 0.001	1.744	1.630	2.879
T950C	1.890	.502	14.193	1	< 0.001	6.616	2.476	17.682
A163G	.311	.381	.668	1	0.414	1.365	.647	2.880
OPG	1.051	.145	52.348	1	< 0.001	2.860	2.152	3.802

Note: BMI, body mass index; B, partial regression coefficient; S.E., standard error; df, degree of freedom; Sig., significance; Exp(B) [exponent function (partial regression coefficient)]; 95% CI, 95% confidence interval.

Associations of OPG gene polymorphisms with the radiographic stage

Based on the radiographic stage, 120 KOA patients were divided into early stage (50 patients), middle stage (45 patients) and late stage (25 patients). As shown in **Table 4**, for the T950C polymorphism, TT genotype was most frequent in the 50 early stage patients (80.0%) compared to TT or CC genotype (80.0% vs. 10.0% vs. 10.0%, both $P < 0.001$). The TC genotype was most frequent in the 45 middle stage patients (70.0%) compared to TT or CC genotype (70.0% vs. 20.0% vs. 10.0%, $P < 0.001$), while in the 25 late stage patients, CC genotype was most frequent (76.0%) and was higher than TT or TC (76.0% vs. 12.0% vs. 12.0%, $P < 0.001$). However, for the polymorphic site A163G, there were no obvious correlation between frequencies of genotypes in A163G and the stages of KOA based on radiographic examinations (all $P > 0.05$).

Logistic regression analysis

Patients with KOA or not was regarded as dependent variable, and age, gender, BMI, serum OPG levels, T950C, and A163G were enrolled in regression model. The result showed that no correlation was found between the susceptibility of KOA and gender, age, A163G ($P > 0.05$), while, BMI (OR = 1.744, 95 CI% = 1.630-2.879, $P < 0.001$), T950C (OR = 6.616, 95 CI% = 2.476-17.682, $P < 0.001$) and serum OPG levels (OR = 2.860, 95 CI% = 2.152-3.802, $P < 0.001$) were independent risk factors for KOA (**Table 5**).

Discussion

Our study provides preliminary evidence that *OPG* T950C polymorphism may be associated with the incidence of KOA, and the frequencies of C allele and C carrier of *OPG* T950C polymorphism were higher in KOA patients compared to healthy controls, but the *OPG* A163G polymorphism may not have an effect on the incidence of KOA, suggesting that specific polymorphisms in *OPG* gene may serve as indicators of the risk of developing KOA. Previous studies based on pathophysiologic analyses have revealed that several disorders of mineral metabolism, including OA, involve alteration in the RANK/RANKL/OPG system that disrupt osteoclast formation and differentiation, and stimulating osteoblast activities, suggesting that OPG is a crucial player involved in the balance between bone resorption and bone formation [20, 21]. Specifically, RANK expressed in osteoclasts is a mediator of bone remodeling and bone metabolism, and its interaction with RANKL promotes the formation and activation of osteoclasts, leading to bone loss. OPG binds to RANKL and, by competitively inhibiting RANKL interactions with its receptor RANK, OPG suppresses bone resorption and attenuate osteoclastogenesis [9]. Consistent with this, a T950C (rs3102735) mutant in the *OPG* locus was implicated in BMD and bone turnover related with bone loss, resulting in increased risk of KOA [22, 23]. On the other hand, similar to our observations, other studies also concluded that A163G polymorphism located in the *OPG* promoter region is not associated with OA risk [15, 24].

We also show that the frequency of T950C CC genotype was highest in KOA patients with advanced stages, based on radiographic examinations, compared to other KOA patients, which emphasizes the contribution of the CC genotype in the risk of KOA. Multiple studies have suggested that *OPG* genetic polymorphism may have a negative effect on bone resorption and thus may serve as potential regulator of BMD, causing further deterioration of the disease in patients with KOA [25, 26]. BMD is a standard measurement in OA using radiographic absorptiometry, and accounts for over 70% bone strength functions that are associated with the conditions of bone structural deterioration and bone mass. Interesting-

ly, radiographic measurements in this study directly observed the correlations with BMD and bone strength of the genetic variants in *OPG* gene, contributing to a more serious deterioration in patients with KOA [27, 28]. A previous study showed that although the T950C polymorphism is not differentially distributed among OA patients, patients with CC genotype showed decreased BMD compared with TT genotype due to enhanced OPG [14]. The CC genotype of T950C variant was identified and the polymorphism was found to alter OPG function, and was associated with up-regulated serum level of OPG, suggesting that OPG expression levels are important for maintaining bone health in KOA patients with advanced stage [29].

Importantly, patients with advanced disease were linked to the CC genotype and higher level of serum OPG, suggesting that C allele may be a major risk factor for development of KOA, influenced by increased serum OPG levels. Several studies have indicated that *OPG* genetic polymorphism was closely associated with the susceptibility to KOA [30, 31]. In this study, OPG was measured in serum using ELISA, and all genotypes (TT, TC or CC) in T950C, showed significantly higher OPG levels in serum in KOA patients compared to controls. The potential association between serum OPG levels and bone mineral density, osteoporotic fractures or biochemical bone turnover markers have been investigated [32, 33], and the mechanisms involve OPG activity on RANKL to inhibit the production and survival of osteoclasts [22]. Based on our results and previous supporting studies, we postulated that KOA patients at early stage may have slightly elevated serum OPG, compared to controls, owning to the TT genotype in T950C, that may impair bone metabolism and bone health, but in patients with advanced-stage KOA a significantly higher level of OPG in serum inhibits bone resorption and bone mass. In support of this, Straface Giuseppe found that CC variant of T950C gene polymorphism was closely correlated with circulating OPG serum levels, and CC variant genotype may stimulate the production of OPG from osteoblasts for in an attempt to regain balance of the disrupted bone metabolism, and thus the C allele may predict the higher OPG serum level and KOA progression [34]. In the present study, the A163G polymorphism in *OPG* gene showed no significant relationship with serum OPG concentrations or radiographic stages.

In summary, based on our results, *OPG* T950C polymorphism is associated with the increased incidence and more aggressive radiographic stage of KOA, and the C allele and C carrier may be the risk factor for the development of KOA, along with detecting increased OPG serum level. However, there was no correlation between *OPG* A163G genetic polymorphism and KOA.

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Disclosure of conflict of interest

None.

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