RESEARCH ARTICLE

Osteoprotegerin polymorphisms are associated with alcohol-induced osteonecrosis of femoral head in Chinese Han population from Henan province

YIZHOU LI[1], YONGCHANG GUO[2], QUANJIAN WANG[2], YONGRI OUYANG[3], YUJU CAO[2], TIANBO JIN[3,4] and JIANZHONG WANG[1]*

[1]*Inner Mongolia Medical University, Hohhot, Inner Mongolia 010050, People's Republic of China*
[2]*Zhengzhou TCM Traumatology Hospital, Zhengzhou, Henan 450016, People's Republic of China*
[3]*National Engineering Research Center for Miniaturized Detection System, Xi'an, Shanxi 710069, People's Republic of China*
[4]*The College of Life Sciences, Northwest University, Xi'an, 710069, People's Republic of China*

Abstract

Alcohol-induced osteonecrosis of femoral head (ONFH) is one of the most important pathogenesis of nontraumatic ONFH. However, its pathogenesis mechanism is still unknown. Osteoprotegerin (OPG) has been implicated in multiple functions including blocking osteoclast maturation, controlling vascular calcifications, promoting tumour growth and metastasis. This study is focussed on *OPG* gene polymorphisms associated with alcohol-induced ONFH. A total of 509 participants (209 patients and 300 normal individuals) were recruited, and we selected 13 single-nucleotide polymorphisms (SNPs) to evaluate the association between genetic susceptibility variants and alcohol-induced ONFH by using the χ^2 test and genetic model analysis. Overall, *OPG* SNPs (rs1485286, rs1032128 and rs11573828) were confirmed the strongest increasing risks on alcohol-induced osteoporosis of femoral head in recessive model (rs1485286: OR, 1.71; 95% CI, 1.07–2.73; $P = 0.025$ for T/T); (rs1032128: OR, 1.73; 95% CI, 1.08–2.77; $P = 0.022$ for G/G); (rs11573828: OR, 3.89; 95% CI, 1.02–14.85; $P = 0.033$ for T/T). SNP rs11573856 was considered as a protective effect to the occurrence of alcohol-induced ONFH, while adjusted for age and gender in dominant and log-additive models (rs11573856: adjusted OR, 0.60; 95% CI, 0.37–0.96; $P = 0.033$ for G/A–A/A); (rs11573856: adjusted OR, 0.63; 95% CI, 0.41–0.96; $P = 0.042$). We conclude that *OPG* gene polymorphisms were associated with the occurrence of alcohol-induced ONFH.

[Li Y., Guo Y., Wang Q., Ouyang Y., Cao Y., Jin T. and Wang J. 2016 Osteoprotegerin polymorphisms are associated with alcohol-induced osteonecrosis of femoral head in Chinese Han population from Henan province. *J. Genet.* **95**, 983–989]

Introduction

Osteonecrosis of the femoral head (ONFH) is death of the cellular portion of femoral head with subsequent bone structure changes and collapse of femoral head, leading to bone fracture and dysfunction of hip joint (Kim *et al.* 2007). Multiple risk and pathogenic factors have been implicated in the development of nontraumatic ONFH (ONF). However, the concrete pathogenesis of ONF is still widely unknown, although some macroscopic risk factors have been identified the association of it including corticosteroid usage, alcohol intake, infections, marrow in filtrating diseases and coagulation defects (Hong *et al.* 2010).

Previous studies have supported a view point that alcoholism is a potential main effect on proliferation and activity of osteoblasts and osteoclasts, which induces bone formation decrease and bone resorption increase (Maurel *et al.* 2011). It was also suggested that alcohol-induced ONFH was due to intraosseous hypertension caused by adipose cell hypertrophy and proliferation, which may result in decreasing the blood supply of femoral head (Youm *et al.* 2010). However, the main causes of alcohol-induced ONFH were unbalanced on osteoclasts–osteoblasts and osteocyte apoptosis. Three relative molecules, osteoprotegerin (OPG), receptor activator of nuclear factor kappa B (RANK), and its ligand (RANKL) are involved in the regulation of osteoblasts and osteoclasts, and OPG/RANK/RANKL pathway also has been proposed an impairment of angiogenesis as a mechanism of ONF

*For correspondence. E-mail: wangjianzhongWJ@126.com.

Keywords. *OPG* gene; polymorphism; single-nucleotide polymorphism; alcohol-induced osteonecrosis of femoral head.

(Pillai 2010; Rushbrook and Pennington 2013; Samara *et al.* 2014).

OPG, expressed by bone marrow-derived mesenchymal stem cell is a member of the tumour necrosis factor (TNF) receptor family and a soluble receptor that inhibits osteoclast activation and bone resorption by binding to RANKL (Jiang *et al.* 2010; Rushbrook and Pennington 2013; Samara *et al.* 2014). OPG was the first molecule identified in the OPG/RANK/RANKL pathway and initially cloned as a potential inhibitor of osteoclastogenesis. Based on the findings, we suggested that ethanol consumption can increase the risk of ONFH occurrence, and balanced on osteoclasts–osteoblasts and osteocyte apoptosis caused by OPG low expression may be the most important mediator to alcohol-induced ONFH.

Genetic variations that are considered biologically normal and may influence protein transcription, expression of related factors, immunoreaction and contribute towards individual susceptibilities to certain pathological diseases (Zhou *et al.* 2015). Over the last decades, variable candidate genes have been conducted and linked to ONF such as *OPG*, *RANK* and *RANKL* regulating the balance between osteoclasts–osteoblasts (Hadjigeorgiou *et al.* 2008; Shang *et al.* 2013). Owing to the special role of *OPG* gene in osteoclastogenesis and remodelling, previous studies have also identified *OPG* gene polymorphisms associated with multiple cancers, vertebral fractures and bone mineral density (BMD) (Bonfa *et al.* 2015; Omar *et al.* 2015).

In summary, these findings led us to investigate the association between *OPG* polymorphisms and alcohol-induced ONFH.

Materials and methods

Study population

All participants were members of the Chinese Han population. ONFH cases were recruited between January 2013 and May 2015 from Zhengzhou TCM Traumatology Hospital in Zhengzhou. The control participants were enrolled from Zhengzhou Medical Center in Henan. This protocol was approved by the Clinical Research Ethics Committee of Inner Mongolia Medical University. Written informed consent was obtained from all participants before their participation in the study. All of the participants signed an informed consent agreement. Zhengzhou TCM Traumatology Hospital approved the use of human tissue in this study.

Inclusion and exclusion criteria

Diagnosis was made according to the following criteria proposed by the Research Committee (Fukushima *et al.* 2013): (i) collapse of the femoral head without joint space narrowing or acetabular abnormality on radiographs, including the crescent sign; (ii) demarcating sclerosis in the femoral head without joint space narrowing or acetabular abnormality; (iii) 'cold in hot' on bone scans; (iv) low-intensity band on T1-weighted MRI (band-like pattern); and (v) trabecular and marrow necrosis on histology. Nontraumatic femoral head osteonecrosis was diagnosed patients who met two or more of the five criteria. Those who followed the below conditions were excluded (Asano *et al.* 2003): (i) Those who diagnosed ONFH before alcohol intake. (ii) Those who showed nontypical MR images that did not satisfy the diagnostic criteria, for instance, low band-like signals in the femoral head in T1-weighed images. (iii) Those who suffered from a hip joint disease and direct trauma during the alcohol-intake period. (iv) People who did not agree to be enrolled in this study.

The control subjects were defined by the following criteria: those who did not have hip pain and anteroposterior and frog-leg lateral pelvic radiographs and were not showing any lesions. All participants who were related to patients were excluded from the control group.

Clinical data and demographic information

We used a standard epidemiological questionnaire and in-person interview to collect personal data, including the residential regions; age; gender; education status; histories of medication use (including oral corticosteroids); alcohol consumption and osteopathic diseases; underlying medical conditions (hyperlipidaemia). Regarding alcohol consumption, we collected information about age at starting and cessation, frequency of drinking and volume of alcohol intake by beverage type. The case information was collected from physicians who were treating the patients or from a medical chart review.

Selection of SNPs and methods of genotyping

The majority of SNPs selected were not reported earlier, some SNPs were identified the association with other diseases, such as BMD, rheumatoid arthritis (RA) and Paget's disease of bone. The minor allele frequencies of all the SNPs were $>5\%$ in the Hap Map of the Chinese Han Beijing (CHB) population. Extraction of DNA from whole blood samples was performed using the Gold Mag-Mini whole blood genomic DNA purification kits (Gold Mag, Hainan, China), and the DNA concentration was measured using a Nano Drop 2000 spectrophotometer.

We designed primers for amplification and extension reactions using Sequenom Mass ARRAY Assay Design 3.0 software (Sequenom, San Diego, USA) (Gabriel *et al.* 2009) (table 1). Genotyping was performed using the Sequenom Mass ARRAY RS1000 system and the standard protocol recommended by the manufacturer. After the experimentation progress mentioned above, data management and analysis were conducted using Sequenom Typer 4.0 software (Thomas *et al.* 2007; Gabriel *et al.* 2009).

Statistical analysis

We used Microsoft Excel and SPSS 18.0 statistical package (SPSS, Chicago, IL, USA) to perform statistical analyses. In our study, we achieved $P \leq 0.05$ among all the P values as the

Table 1. Formation of primers in *OPG* gene.

SNPs	2P seq.	1P seq.	UEP seq.
rs3134053	ACGTTGGATGGACTTTGTTCTGGGCACTTG	ACGTTGGATGTGGATTAACTCCATAAGGGC	TCTGGGCACTTGGGATACAA
rs11573896	ACGTTGGATGATCTAGTGTCAACCCACTCC	ACGTTGGATGTCCCTTGACCTGAGAGTTTC	ggttAGACAATTAAAAGCCCCCAGGAA
rs1485286	ACGTTGGATGTGGCACATGCTCTAAGTTTG	ACGTTGGATGTCATTACGTCTCCACCAAC	ccccAACAAGTTAATTACCCTGACTC
rs3102725	ACGTTGGATGGAAGATGCATACACCAGTAG	ACGTTGGATGGCTTTTGTCCAAAGCTACTG	ACCAGTAGAATTCTCTAGTCAAAT
rs1905786	ACGTTGGATGCACTGCAAAACAATTGGAGG	ACGTTGGATGCCCTCACAATTTCCAACCTG	ggaggAAGAGCAAGAAAAGACTCAA
rs1032128	ACGTTGGATGGAATAGGTATGACGTGTTGG	ACGTTGGATGGTTCATGGATGTGCTCTTCG	gagtTATGACGTGTTGGCTGGTT
rs3134056	ACGTTGGATGGCAAAGGACATGAGCTTATC	ACGTTGGATGGATAATTTTAAAAGGGTGC	TGCATAGTATTCCATAGTGTATA
rs3134058	ACGTTGGATGGCACATATTTTGGCTCACAG	ACGTTGGATGTTGGCTTTCAAACCTGATGC	CTTCCTTTGGGCTTCTGTTA
rs11573856	ACGTTGGATGGAAACCGAATCTCAAAGAAG	ACGTTGGATGCTTAGGTTCAGACGGGATAG	ggAAAGGTGTCAGAGCTAG
rs11573849	ACGTTGGATGAGCCAGTTAGATGGTTGAAG	ACGTTGGATGTAACTGGGATTTGGCTACTG	cccGTTTATTGGCATGACATAAGAC
rs3102731	ACGTTGGATGGAAAAGATACACATAACACCC	ACGTTGGATGGGGCTTTTGTGAAGGTATTG	ACATAACACCCTACCCA
rs11573828	ACGTTGGATGGGAGTTCTGATCTAGACTCA	ACGTTGGATGAGAATAATCCCACGTCCCAC	atATTTCTTATATCAGTGGTCAATAG

threshold of statistical significance. The validation of each SNP frequency in control subjects was tested for departure from Hardy–Weinberg equilibrium (HWE) using an exact test. The method of χ^2 test was used to calculate the genotype frequencies of case and control individuals (Adamec 1964). To make the information more valuable, unconditional logistic regression analysis with adjustment for age and gender was performed to test odds ratios (ORs) and 95% confidence intervals (CIs) (Bland and Altman 2000). The possibility of gender difference as a source of population substructure was evaluated by a genotype test for each SNP in male and female controls, and the number of significant results at the 5% level was compared with the number expected by χ^2 test. The association of certain SNPs with the risk of ONFH was tested in five genetic models (allele, codominant, dominant, recessive and log-additive models); unconditional logistic regression analysis with adjustment for age and gender was used to calculate the ORs and 95% CIs (Bland and Altman 2000).

Results

We conducted a case–control study, 209 patients and 300 normal individuals enrolled, to identify association between *OPG* polymorphisms and alcohol-induced ONFH. Thirteen SNPs in *OPG* gene were in accordance with 5% HWE *P* level. The associations between SNP genotypes and the susceptibility of alleles to alcohol-induced ONFH were performed by χ^2 analysis. The results are shown in table 2.

We assumed that the minor allele of each SNP was a risk factor compared with the wild-type allele. Four genetic analysis models, codominant, dominant, recessive and log-additive were applied to analyse the associations between SNPs and alcohol-induced ONFH risks using the logistic test. Three SNPs (rs1485286, rs1032128 and rs11573828) were discovered to be associated with an increased risk of alcohol-induced ONFH. Additionally, SNP rs11573856 was considered as a protective effect for the occurrence of alcohol-induced ONFH. We discovered a notable associations regarding increased risks among three SNPs (rs1485286, rs1032128 and rs11573828) and alcohol-induced ONFH with different genotype distributions in recessive model (rs1485286: OR, 1.71; 95% CI, 1.07–2.73; $P = 0.025$ for T/T); (rs1032128: OR, 1.73; 95% CI, 1.08–2.77; $P = 0.022$ for G/G); (rs11573828: OR, 3.89; 95% CI, 1.02–14.85; $P = 0.033$ for T/T). Further, the results mentioned above are still remarkable when calculated using the unconditional logistic regression analyses adjusted for age and gender (rs1485286: adjusted OR, 1.78; 95% CI, 1.02–3.12; $P = 0.041$ for T/T); (rs1032128: adjusted OR, 1.75; 95% CI, 1.00–3.15; $P = 0.048$ for G/G); (rs11573828: adjusted OR, 7.07; 95% CI, 1.09–54.44; $P = 0.013$ for T/T). SNP rs11573828 in *OPG* gene was not only the notable risk factor in recessive model, but also in codominant model after adjusted for confounding factors (rs11573828: adjusted OR, 7.49; 95% CI, 1.06–53.13; $P = 0.042$ for T/T).

SNP rs11573856 was considered as a protective effect for the occurrence of alcohol-induced ONFH, while adjusted for age and gender in dominant and log-additive models (rs11573856: adjusted OR, 0.60; 95% CI, 0.37–0.96; $P = 0.033$ for G/A–A/A); (rs11573856: adjusted OR, 0.63; 95% CI, 0.41–0.96; $P = 0.042$) (table 3).

Pairwise linkage disequilibrium (LD) analysis was performed for the *OPG* gene using the polymorphisms detected

Table 2. Basic information of SNPs in *OPG* gene.

SNP no.	Chromosome	Location	Allele	MAF in case group	MAF in control group	HWE *P*	*P* value
rs3134053	8q	Intron of OPG	T<C	0.322	0.318	0.791	0.899
rs11573896	8q	Intron of OPG	A<T	0.141	0.130	0.799	0.623
rs1485286	8q	Intron of OPG	T<C	0.430	0.378	0.714	0.099
rs3102725	8q	Intron of OPG	A<G	0.159	0.168	0.681	0.682
rs1905786	8q	Intron of OPG	T<C	0.289	0.243	0.529	0.099
rs1032128	8q	Intron of OPG	G<A	0.428	0.380	0.463	0.122
rs3134056	8q	Intron of OPG	G<A	0.385	0.422	0.813	0.244
rs3134058	8q	Intron of OPG	G<A	0.452	0.423	0.724	0.362
rs11573856	8q	Intron of OPG	A<G	0.130	0.162	0.834	0.154
rs11573849	8q	Intron of OPG	T<G	0.161	0.155	0.077	0.794
rs3102731	8q	Intron of OPG	A<G	0.158	0.168	0.681	0.658
rs11573828	8q	Intron of OPG	T<C	0.161	0.149	0.164	0.606
rs1564861	8q	Promoter	C<A	0.392	0.430	1	0.231

SNPs are excluded at 5% HWE *P* level. HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency.

Table 3. Logistic regression analysis of the association between SNPs and risks of alcohol-induced ONFH.

SNP	Model	Genotype	OR[a] (95% CI)	P^a	OR[b] (95% CI)	P^b
rs1485286	Codominant	C/C	1		1	
		T/C	0.97 (0.65–1.43)	0.08	0.96 (0.60–1.54)	0.12
		T/T	1.68 (1.00–2.81)		1.74 (0.94–3.25)	
	Dominant	C/C	1		1	
		T/C–T/T	1.12 (0.78–1.62)	0.54	1.13 (0.73–1.76)	0.59
	Recessive	C/C–T/C	1		1	
		T/T	**1.71 (1.07–2.73)**	**0.025**	**1.78 (1.02–3.12)**	**0.041**
	Log-additive	–	1.23 (0.96–1.58)	0.11	1.25 (0.93–1.69)	0.14
rs1032128	Codominant	A/A	1		1	
		A/G	0.93 (0.63–1.38)	0.068	0.97 (0.60–1.54)	0.14
		G/G	1.66 (0.99–2.80)		1.71 (0.92–3.19)	
	Dominant	A/A	1		1	
		A/G–G/G	1.09 (0.75–1.57)	0.66	1.13 (0.72–1.75)	0.6
	Recessive	A/A–A/G	1		1	
		G/G	**1.73 (1.08–2.77)**	**0.022**	**1.75 (1.00–3.05)**	**0.048**
	Log-additive	–	1.22 (0.95–1.57)	0.13	1.24 (0.92–1.68)	0.15
rs11573856	Codominant	G/G	1		1	
		G/A	0.73 (0.48–1.11)	0.32	0.61 (0.37–1.00)	
		A/A	0.76 (0.22–2.63)		0.44 (0.11–1.84)	
	Dominant	G/G	1		1	
		G/A–A/A	0.73 (0.49–1.10)	0.13	**0.60 (0.37–0.96)**	**0.033**
	Recessive	G/G–G/A	1		1	
		A/A	0.82 (0.24–2.83)	0.75	0.51 (0.12–2.09)	0.35
	Log-additive	–	0.77 (0.54–1.10)	0.15	**0.63 (0.41–0.96)**	**0.031**
rs11573828	Codominant	C/C	1		1	
		T/C	0.88 (0.58–1.32)	0.084	0.89 (0.55–1.46)	**0.042**
		T/T	3.76 (0.98–14.40)		**7.49 (1.06–53.13)**	
	Dominant	C/C	1		1	
		T/C–T/T	0.98 (0.66–1.45)	0.91	1.05 (0.65–1.69)	0.84
	Recessive	C/C–T/C	1		1	
		T/T	**3.89 (1.02–14.85)**	**0.033**	**7.70 (1.09–54.44)**	**0.013**
	Log-additive	–	1.10 (0.77–1.55)	0.6	1.21 (0.80–1.83)	0.37

OR[a] and P^a without adjustment. OR[b] and P^b adjusted for gender and age. $P \leq 0.05$ are in bold which indicates statistical significance.

in this study. The pattern of LD was analysed using two parameters, r^2 and D'. Two main linkage blocks were observed across the locus (figure 1). Block 1 was constituted six closely-linked SNPs: rs3134053, rs11573896, rs1485286, rs3102725, rs1905786 and rs1032128. Then, the association between inferred haplotypes and alcohol-induced ONFH risk among the individuals was analysed. We found the risk haplotype 'CTTGTG' between the six SNPs in block 1 associated with the risk of alcohol-induced ONFH (OR, 1.41; 95% CI, 1.00–1.98; $P = 0.048$) without adjustment for gender and age. Block 2 was also constituted six closely-linked SNPs: rs3134056, rs3134058, rs11573856, rs11573849, rs3102731 and rs11573828. A protective factor was confirmed between the haplotype 'CTTGTG' of six SNPs in block 2 and alcohol-induced ONFH after adjusted for gender and age (OR, 0.61; 95% CI, 0.37–0.99; $P = 0.046$) (table 4).

Discussion

Chronic alcohol consumption can induce the occurrence of alcohol-induced ONFH, which is one of the most important risk factors. Additionally, several genetic polymorphisms are associated with susceptibility to alcohol-induced ONFH have been identified (Kim *et al.* 2008). Whereas each polymorphism may contribute to only a small relative risk of alcohol-induced ONFH, a combination of several responsible polymorphisms and other risk factors may be more important. To our knowledge, this is the first study to identify the association between alcohol-induced ONFH and *OPG* polymorphisms. In this study, we detected 13 SNPs in *OPG* gene and we concluded that *OPG* gene polymorphisms (rs1485286, rs1032128 and rs11573828) may increase the risk of alcohol-induced ONFH. SNP (rs11573856) may be a protective effect. Further, haplotype analysis suggested that alcohol-induced ONFH risk was substantially elevated among individuals with specific haplotypes. 'CTTGTG' was found to increase the risk of alcohol-induced ONFH, which indicated the complexity of *OPG* gene in the development of alcohol-induced ONFH. We also conclude that 'GAAGGC' was identified as a protective factor for the development of alcohol-induced ONFH, adjusted by confounding factors (table 5).

The associations between *OPG* polymorphisms with alcohol-induced ONFH found in the present study can be explained by its possible roles in OPG/RANK/RANKL pathway. First of all, human *OPG* is located on chromosome 8q, belongs to OPG/RANK/RANKL pathway. Secondly, OPG plays an important role in bone formation and inhibition of osteoclast activation. Additionally, recent

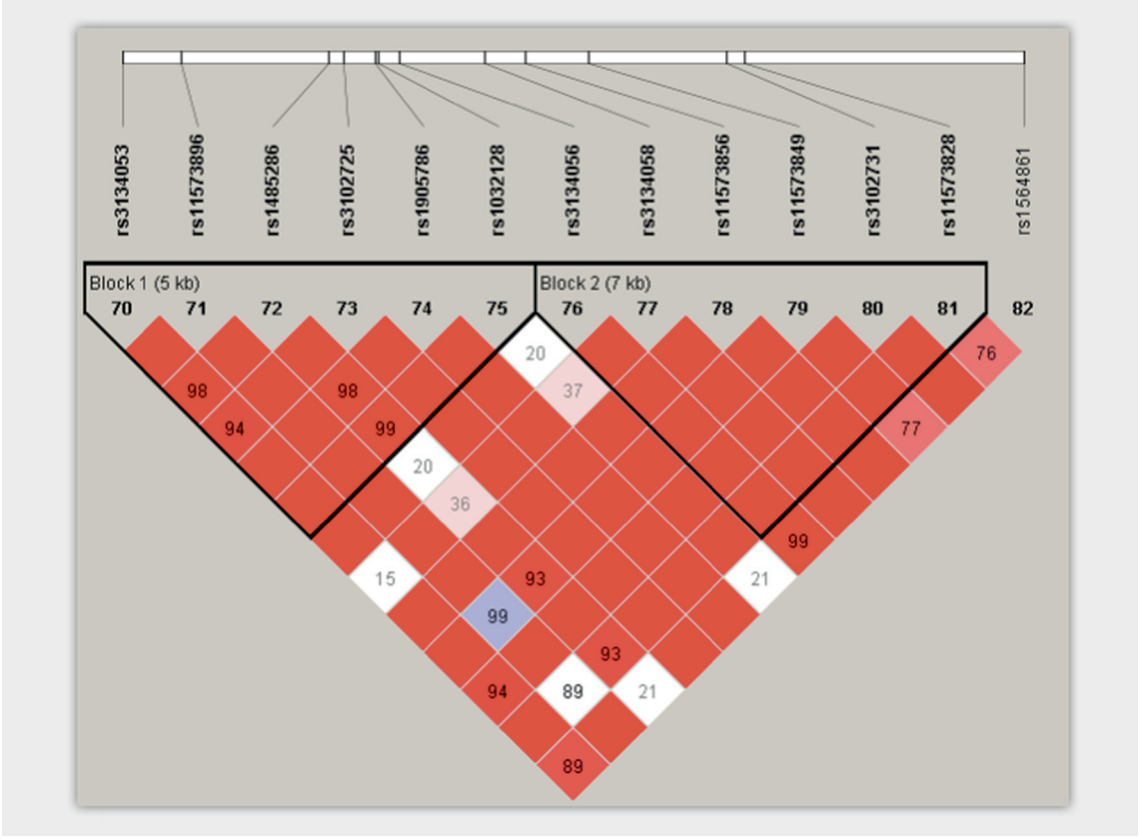

Figure 1. Linkage disequilibrium of polymorphic sites in the *OPG* on chromosomee 8. Block 1 includes rs3134053, rs11573896, rs1485286, rs3102725, rs1905786 and rs1032128. Block 2 includes rs3134056, rs3134058, rs11573856, rs11573849, rs3102731 and rs11573828. The LD between two SNPs is standardized D' (red scheme).

Table 4. Multiple genes haplotype frequencies and the association with the risk of alcohol-induced ONFH.

Gene	Block	Haplotype	Freq.	OR[a] (95% CI)	P^a value	OR[b] (95% CI)	P^b value
OPG	1	CTCGCA	0.278	1	–	1	–
		CTTGTG	0.262	**1.41 (1.00–1.98)**	**0.048**	1.43 (0.96–2.14)	0.08
		TTCACA	0.163	1.19 (0.79–1.78)	0.41	1.22 (0.76–1.96)	0.41
		TTCGCA	0.158	1.24 (0.83–1.86)	0.29	1.42 (0.88–2.29)	0.15
		CATGCG	0.134	1.28 (0.83–1.96)	0.26	1.52 (0.91–2.54)	0.11
OPG	2	AGGGGC	0.271	1	–	1	–
		GAGGGC	0.258	0.86 (0.62–1.21)	0.39	0.98 (0.66–1.47)	0.93
		AGGGAC	0.164	0.83 (0.57–1.22)	0.35	0.85 (0.54–1.32)	0.46
		AAGTGT	0.156	0.90 (0.60–1.34)	0.59	0.97 (0.60–1.57)	0.9
		GAAGGC	0.149	0.70 (0.47–1.06)	0.093	**0.61 (0.37–0.99)**	**0.046**

OR[a] and P^a without adjustment. OR[b] and P^b adjusted for gender and age. $P \leq 0.05$ are in bold which indicates statistical significance.

Table 5. Level of alcohol consumption in case and control individuals.

Characteristic	Control ($n = 300$)	Case ($n = 209$)	P value
Alcohol consumption (dose/mg per week) (mean ± SD)	0	528.92 ± 71.52	0

*$P > 0.05$ indicates not statistically significant.

experimental and epidemiological data supported that OPG secreted by impairment of endothelial cells, underlies a possible link between the osseous and vascular systems (Benslimane-Ahmim *et al.* 2011). Alcohol has an inhibitory effect on the differentiation of mesenchymal stromal cells (MSCs) into osteoblasts and OPG, so that alcohol consumption may decrease OPG expression. In this way, alcohol may indirectly bring down the function of negatively regulating osteoclast differentiation and blocking pathophysiological induction of bone resorption. Moreover, alcohol consumption may influence the osseous blood supply for femoral head by inducing vascular endothelial cell necrosis and make femoral head blood more hypercoagulable. At the same time, OPG was low-expressed because of vascular endothelial cell necrosis decrease. In summary, low expression of OPG may bring down its function of inhibiting osteolysis progress, inducing bone formation and destroying the OPG/RANKL ratio, which results in alcohol-induced ONFH gradually.

The comparison of our data with those focussed in other literature on one SNP (rs1032128, G<A). A previous study supported that this SNP (rs1032128) located in the untranslated region of the *OPG* gene was associated with volumetric BMD and bone geometry, and the minor allele frequency (MAF) (A<G) was turned out to be a risk effect on femoral neck and lumbar spine BMD (Roshandel *et al.* 2011). In our study, for SNP (rs1032128, G<A), we found allele G, a risk effect on alcohol-induced ONFH, and we also found genotype G/G, a risk factor in recessive model. Additionally, SNP (rs1485286) *OPG* gene polymorphism has turned out to be a strong candidate for regulating susceptibility to Paget's disease of bone (PDB) because of its mutation increasing the bone turnover, and this association turned out to be driven in female, while subsequently analysing male and female separately (Beyens *et al.* 2007; Chung and Van Hul 2012). However, some SNPs were not captured including rs11573828 (Beyens *et al.* 2007). Although recent studies have not identified SNP (rs11573828) as a risk factor or a protective one in ONFH, we found this SNP (T<C) allele T, a risk effect on alcohol-induced ONFH and genotype T/T, a risk factor in recessive model. Further, a recent study has reported that SNP (rs11573856) in *OPG* gene is not related to BMD and osteoporotic fracture (Jurado *et al.* 2010). We also concluded that this SNP (rs11573856) was not associated with increasing the risk factor of alcohol-induced ONFH, however, it maybe a protective factor.

Despite the current study possessing enough energy, some limitations were inherent in the case–control study. Firstly, because our sample size (209 patients and 300 normal individuals) was not relatively large among alcohol-induced ONFH association studies published to date, we did not conduct population stratification of alcohol consumption amount, and we have not confirmed that this locus is significant in alcohol consumer (table 5). The negative results of major SNPs in this study may convert into positive ones when we increase the sample size of alcohol-induced ONFH, which may drive more powerful conclusion. Secondly, the heterogeneity in alcohol consumption and comorbidities were not evaluated in this study, and evaluation of heterogeneity in drinking behaviours has contributed to the progress in elucidating the pathogenesis of alcohol-induced ONFH in other studies. Thirdly, in this study, we selected alcohol-induced ONFH patients and matched controls were used in the same hospital to avoid selection bias. However, this bias was unlikely to be of significance because the patient groups did not differ in the distributions of demographic variables and genotype frequencies. Finally, alcohol-induced ONFH can be classified into different clinical stages, so that we can make subgroup analysis for the further study, however, it may increase the difficulty to collect samples.

In conclusion, we provided new evidence for the association between SNPs and haplotypes of the *OPG* gene and alcohol-induced ONFH. These findings indicate that genetic

variants of the *OPG* gene play an intricate role in the development of alcohol-induced ONFH, and interactions among loci in the *OPG* gene may be more important than a single locus. This study offers important insights into the aetiology of alcohol-induced ONFH.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (nos. 81160228 and 81260284). It is our great honour to express heartfelt thanks to all the patients and individuals for their participation. We are also grateful for the clinicians and other hospital staff who contributed to the blood sample and data collection for this study.

References

Adamec C. 1964 Example of the use of the nonparametric test. Test X2 for comparison of 2 independent examples. *Cesk. Zdrav.* **12**, 613–619.

Asano T., Takahashi K. A., Fujioka M., Inoue S., Satomi Y., Nishimo H. *et al.* 2003 Genetic analysis of steroid-induced osteonecrosis of the femoral head. *J. Orthop. Sci.* **8**, 329–333.

Benslimane-Ahmim Z., Heymann D., Dizier B., Lokajczyk A., Brion R., Laurendeau I. *et al.* 2011 Osteoprotegerin, a new actor in vasculogenesis, stimulates endothelial colony-forming cells properties. *J. Thromb. Haemost.* **9**, 834–843.

Beyens G., Daroszewska A., de Freitas F., Fransen E., Vanhoenacker F., Zmierczak H. G. *et al.* 2007 Identification of sex-specific associations between polymorphisms of the osteoprotegerin gene, TNFRSF11B, and Paget's disease of bone. *J. Bone Miner. Res.* **22**, 1062–1071.

Bland J. M. and Altman D. G. 2000 Statistics notes. The odds ratio. *BMJ* **320**, 1468.

Bonfa A. C., Seguro L. P., Caparbo V., Bonfa E. and Pereira R. M. 2015 RANKL and OPG gene polymorphisms: associations with vertebral fractures and bone mineral density in premenopausal systemic lupus erythematosus. *Osteoporos. Int.* **26**, 1563–1571.

Chung P. Y. and Van Hul W. 2012 Paget's disease of bone: evidence for complex pathogenetic interactions. *Semin. Arthritis Rheum.* **41**, 619–641.

Fukushima W., Yamamoto T., Takahashi S., Sakaguchi M., Kubo T., Iwamoto Y. *et al.* 2013 The effect of alcohol intake and the use of oral corticosteroids on the risk of idiopathic osteonecrosis of the femoral head: a case–control study in Japan. *Bone Joint J.* **95-b**, 320–325.

Gabriel S., Ziaugra L. and Tabbaa D. 2009 SNP genotyping using the Sequenom MassARRAY iPLEX platform. *Curr. Protoc. Hum. Genet.*, 2.12.1–2.12.16.

Hadjigeorgiou G., Dardiotis E., Dardioti M., Karantanas A., Dimitroulias A. and Malizos K. 2008 Genetic association studies in osteonecrosis of the femoral head: mini review of the literature. *Skeletal Radiol.* **37**, 1–7.

Hong J. M., Kim T. H., Kim H. J., Park E. K., Yang E. K. and Kim S. Y. 2010 Genetic association of angiogenesis- and hypoxia-related gene polymorphisms with osteonecrosis of the femoral head. *Exp. Mol. Med.* **42**, 376–385.

Jiang X. Y., Chen Y., Xu L., Li X., Cao F. F., Li L. *et al.* 2010 Association of LPR5 polymorphism with bone mass density and cholesterol level in population of Chinese Han. *Exp. Clin. Endocrinol. Diabetes* **118**, 388–391.

Jurado S., Nogues X., Agueda L., Garcia-Giralt N., Urreizti R., Yoskovitz G. *et al.* 2010 Polymorphisms and haplotypes across the osteoprotegerin gene associated with bone mineral density and osteoporotic fractures. *Osteoporos. Int.* **21**, 287–296.

Kim T. H., Baek J. I., Hong J. M., Choi S. J., Lee H. J., Cho H. J. *et al.* 2008 Significant association of SREBP-2 genetic polymorphisms with avascular necrosis in the Korean population. *BMC Med. Genet.* **9**, 94.

Kim T. H., Hong J. M., Park E. K. and Kim S. Y. 2007 Peroxisome proliferator-activated receptor-gamma gene polymorphisms are not associated with osteonecrosis of the femoral head in the Korean population. *Mol. Cells* **24**, 388–393.

Maurel D. B., Jaffre C., Rochefort G. Y., Aveline P. C., Boisseau N., Uzbekov R. *et al.* 2011 Low bone accrual is associated with osteocyte apoptosis in alcohol-induced osteopenia. *Bone* **49**, 543–552.

Omar H. S., Shaker O. G., Nassar Y. H., Marzouk S. A. and ElMarzouky M. S. 2015 The association between RANKL and osteoprotegerin gene polymorphisms with breast cancer. *Mol. Cell Biochem.* **403**, 219–229.

Pillai L. 2010 Genistein aglycone demonstrates a protective and reversible effect on the development of steroid-induced secondary osteoporosis and increases bone breaking strength *in vivo*. *J. Clin. Densitom.* **13**, 215–222.

Roshandel D., Holliday K. L., Pye S. R., Ward K. A., Boonen S., Vanderschueren D. *et al.* 2011 Influence of polymorphisms in the RANKL/RANK/OPG signaling pathway on volumetric bone mineral density and bone geometry at the forearm in men. *Calcif. Tissue Int.* **89**, 446–455.

Rushbrook J. and Pennington N. 2013 The effects of alcohol in orthopaedic patients. *Orthop. Trauma* **27**, 104–170.

Samara S., Dailiana Z., Chassanidis C., Koromila T., Papatheodorou L., Malizos K. N. *et al.* 2014 Expression profile of osteoprotegerin, RANK and RANKL genes in the femoral head of patients with avascular necrosis. *Exp. Mol. Pathol.* **96**, 9–14.

Shang M., Lin L. and Cui H. 2013 Association of genetic polymorphisms of RANK, RANKL and OPG with bone mineral density in Chinese peri- and post-menopausal women. *Clin. Biochem.* **46**, 1493–1501.

Thomas R. K., Baker A. C., Debiasi R. M., Winckler W., Laframboise T., Lin W. M. *et al.* 2007 High-throughput oncogene mutation profiling in human cancer. *Nat. Genet.* **39**, 347–351.

Youm Y. S., Lee S. Y. and Lee S. H. 2010 Apoptosis in the osteonecrosis of the femoral head. *Clin. Orthop. Surg.* **2**, 250–255.

Zhou X., Yishake M., Li J., Jiang L., Wu L., Liu R. *et al.* 2015 Genetic susceptibility to prosthetic joint infection following total joint arthroplasty: a systematic review. *Gene* **563**, 76–82.

Received 12 January 2016, in revised form 23 April 2016; accepted 4 May 2016
Unedited version published online: 5 May 2016
Final version published online: 1 December 2016

Corresponding editor: ARUN KUMAR