EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 40, No. 4, 418-426, August 2008

Association of polymorphisms in the Interleukin 23 receptor gene with osteonecrosis of femoral head in Korean population

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DOI 10.3858/emm.2008.40.4.418

Accepted 24 April 2008

Abbreviations: CI, confidence interval; IL23R, interleukin 23 receptor; LD, linkage disequilibrium; ON, osteonecrosis; ONFH, osteonecrosis of the femoral head; OR, odd ratio; SNP, single nucleotide polymorphism

Abstract

Osteonecrosis of the femoral head (ONFH) is known as death of the cellular portion of the femoral head due to an interruption in the vascular supply. The underlying pathophysiology regarding bone cell death remains uncertain. Recently, several studies have shown that autoimmune disorders were related to the development of osteonecrosis. This study investigated the genetic effects of Interleukin 23 receptor (*IL23R*) polymorphisms regarding the risk of ONFH. Ten SNPs were selected and genotyped in 443 ONFH patients and 273 control subjects in order to perform the genetic association analysis. It was found that polymorphisms of the *IL23R* gene (rs4655686, rs1569922 and rs7539625) were significantly associated with an increased risk of ONFH (*P* values; 0.0198-0.0447, OR; 1.30-1.49). Particularly, a stratified analysis based on etiology (alcohol, steroid or idiopathic) showed that the associations between these polymorphisms and ONFH were most significant in idiopathic ONFH patients (*P* values; 0.0001-0.0150, OR; 1.45-2.17). These results suggest that *IL23R* polymorphisms may play an important role in the development of ONFH.

Keywords: femur head; interleukin-23; osteonecrosis; polymorphism, single nucleotide

Introduction

Osteonecrosis of the femoral head (ONFH) is a devastating disease that frequently leads to the progressive collapse of the femoral head, followed by degenerative arthritis of the hip joint. Non-traumatic ONFH has been associated with various factors such as corticosteroid usage, alcoholism, infections, marrow infiltrating diseases, coagulation defects, and some autoimmune diseases (Mont *et al*., 1998; Jones, 1999; Glueck *et al*., 2003; Boss *et al*., 2004; Childs, 2005). Among several confounding pathogenic mechanisms regarding ONFH, a vascular hypothesis appears to be the most persuasive, presuming that a decrease in the local blood flow in the femoral head plays a pivotal role in the pathogenesis of ONFH (de Camargo *et al*., 1984; Kerachian *et al*., 2006).

Particularly, some autoimmune disorders, including systemic lupus erythematosus (SLE), polymyalgia rheumatica (PMR), rheumatoid arthritis (RA), and inflammatory bowel disease (IBD), are related to the development of osteonecrosis (Migliaresi *et al*., 1994; Abu-Shakra *et al*., 2003; Boss *et al*., 2004; Tektonidou and Moutsopoulos, 2004; Lane, 2006). These diseases cause enough vasculature defects to cause a loss of microvascular flow to intraosseous tissue (Tektonidou and Moutsopoulos, 2004; Childs, 2005). Several studies reported that immunologic factors such as interleukins and TNFs might influence the development of osteonecrosis (Mont *et al*., 1998; Boss *et al*., 2004; Tektonidou and Moutsopoulos, 2004; Weitzmann and Pacifici, 2005; Sato and Takayanagi, 2006).

IL23 is a proinflammatory cytokine and consists of a p19 subunit and a p40 subunit of IL12. IL23R pairs with IL12R and both are necessary for IL23

signal pathway. IL23 regulates the activity of an immune response and promotes inflammation through the engagement of IL23R. These cytokines are primarily expressed in immune system cells such as a T cell, macrophage, and dendritic cell (Oppmann *et al*., 2000; Parham *et al*., 2002). Recently, it has been reported that IL23 deficient mice were resistant to collagen-induced arthritis (Murphy *et al*., 2003; Sanchez *et al*., 2007) and IL23 also inhibited osteoclastogenesis by RANKL (Receptor Activator for Nuclear Factor κB Ligand) through T cell activation (Kamiya *et al*., 2007).

Recently, many studies have reported that *IL23R* was related to inflammatory disorders. Several variations of the *IL23R* gene have been reported to influence the risk of a developing skin disorder called psoriasis (Capon *et al*., 2007; Cargill *et al*., 2007). Also, polymorphisms of the *IL23R* gene were associated with susceptibility to IBD and Crohn's disease (CD) in the genome wide association studies previously published (Cardon, 2006; Duerr *et al*., 2006; Raelson *et al*., 2007). In order to determine whether polymorphisms of the *IL23R* gene are associated with the susceptibility of ONFH, the genotype and allele frequencies between ONFH patients and controls were compared in this study.

Materials and Methods

Subjects

The study population was comprised of 443 unrelated patients (366 men, 77 women; mean age: 49.7 ± 13.3) with ONFH and 273 (206 men, 67 women; mean age: 52.1 ± 10.6) unrelated control subjects consecutively enrolled at the Kyungpook National University Hospital (Daegu, Korea) from 2002 to 2006. Patients were diagnosed and subgrouped by criteria which were described previously (Kim *et al*., 2007). Briefly, patients were subgrouped, according to etiological factors, into osteonecrosis, idiopathic (181 cases), steroid-induced (56 cases), and alcohol-induced (206 cases) groups. The control subjects were defined by the following criteria: those having no hip pain and others whose anteroposterior and frog leg lateral pelvic radiographs did not show any lesions, or with a sclerotic margin or subchondral collapse consistent with ONFH. We excluded all persons related to patients from our control group. This study was approved by the Institutional Review Board of Kyungpook National University Hospital, and all subjects gave written informed consent.

Selection of SNP and genotyping

Genomic DNA was isolated from peripheral blood of each individual by using a FlexiGene DNA Kit (QIAGEN, Valencia, CA). A total of 10 polymorphic sites regarding *IL23R* were selected by considering their location, allele frequencies and relevance to diseases based on public databases (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/, HAPMAP; http:// www.hapmap.org/index.html.en). The genotype identification was performed by using a Taqman probe (Applied Biosystems, Foster city,

Table 1. Frequencies of IL23R gene polymorphisms in ONFH patients and normal controls.

dbSNP ID	Position	Genotype				Frequency[a]	Heterozygosity	HWE[b]	
								case	control
rs4655686	Intron 3	TT	AT	AA	N				
		284	340	76	700	0.351	0.456	0.304	0.158
rs1569922	Intron 4	CC	CT	TT	N				
		216	368	107	691	0.421	0.488	0.030	0.246
rs7539625	Intron 6	AA	AG	GG	N				
		185	372	148	705	0.474	0.499	0.290	0.257
rs7518660	Intron 7	GG	GA	AA	N				
		370	283	58	711	0.281	0.404	0.490	0.879
rs10789229	Intron 8	TT	CT	CC	N				
		605	105	5	715	0.080	0.148	1.000	0.664
rs1008193	Intron 9	CC	CG	GG	N				
		625	85	4	714	0.065	0.122	1.000	0.256
rs6693831	Intron 9	CC	CT	TT	N				
		425	240	45	710	0.232	0.357	0.697	0.078

[a]Frequencies of rare alleles. [b]*P* values of deviation from Hardy-Weinberg equilibrium among ONFH patients and control.

Table 2. Association analyses of IL23R gene polymorphisms with the risk of ONFH.

dbSNP	Location	Genotype	ONFH patients	Normal controls	Co-dominants	Dominant	Recessive	Allele
					OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
					P	*P*	*P*	*P*
rs4655686	Intron 3	TT	163 (38.08)	121 (44.49)				
		TA	211 (49.3)	129 (47.43)	**1.32 (1.04-1.68**)	1.32 (0.97-1.81)	1.67 (0.99-2.84)	**1.29(1.02-1.62)**
		AA	54 (12.62)	22 (8.09)	**0.0254**	0.0793	0.0570	**0.0314**
rs1569922	Intron 4	CC	121 (28.47)	95 (35.71)				
		CT	232 (54.59)	136 (51.13)	**1.30 (1.03-1.65)**	**1.41 (1.01-1.96)**	1.38 (0.88-2.14)	**1.27 (1.01-1.58)**
		TT	72 (16.94)	35 (13.16)	**0.0298**	**0.0442**	0.1571	**0.0386**
rs7539625	Intron 6	AA	105 (23.97)	80 (29.96)				
		AG	231 (52.74)	141 (52.81)	**1.31 (1.04-1.64)**	1.37 (0.97-1.93)	**1.49 (1.01-2.21)**	**1.29 (1.04-1.60)**
		GG	102 (23.29)	46 (17.23)	**0.0198**	0.0767	**0.0447**	**0.0235**
rs7518660	Intron 7	GG	224 (50.56)	146 (54.48)				
		GA	178 (40.18)	105 (39.18)	1.20 (0.94-1.52)	1.17 (0.86-1.59)	1.58 (0.87-2.87)	1.20 (0.94-1.53)
		AA	41 (9.26)	17 (6.34)	0.1496	0.3115	0.1308	0.1469
rs10789229	Intron 8	TT	373 (84.2)	232 (85.29)				
		TC	67 (15.12)	38 (13.97)	1.13 (0.75-1.68)	1.15 (0.75-1.77)	0.87 (0.14-5.35)	1.13 (0.76-1.68)
		CC	3 (0.68)	2 (0.74)	0.5608	0.5117	0.8804	0.5603
rs1008193	Intron 9	CC	384 (86.88)	241 (88.6)				
		CG	56 (12.67)	29 (10.66)	1.17 (0.75-1.82)	1.23 (0.77-1.97)	0.59 (0.08-4.33)	1.17 (0.75-1.83)
		GG	2 (0.45)	2 (0.74)	0.4899	0.3928	0.6023	0.4863
rs6693831	Intron 9	CC	260 (58.69)	165 (61.8)				
		CT	157 (35.44)	83 (31.09)	1.07 (0.83-1.37)	1.14 (0.83-1.55)	0.90 (0.48-1.67)	1.07 (0.83-1.38)
		TT	26 (5.87)	19 (7.12)	0.6188	0.4292	0.7380	0.6089

Genotype distributions, odds ratio (OR; 95% confidence interval) and corresponding *P* values for logistic analyses of three alternative models and alleles are shown. Logistic regressions were applied in codominant (homozygote for major allele vs heterozygote vs homozygote for minor allele), dominant (homozygote for major allele vs heterozygote + homozygote for minor allele), recessive (homozygote for major allele + heterozygote vs homozygote for minor allele), and allele (major vs minor) models.

Table 3. Association of IL23R gene polymorphisms between idiopathic ONFH patients and controls.

dbSNP	Location	Genotype	ONFH patients	Normal controls	Co-dominants	Dominant	Recessive	Allele
					OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
					P	*P*	*P*	*P*
rs4655686	Intron 3	TT	58 (33.33)	121 (44.49)				
		TA	87 (50)	129 (47.43)	**1.53 (1.14-2.06)**	**1.54 (1.03-2.29)**	**2.17 (1.19-3.94)**	**1.48 (1.12-1.96)**
		AA	29 (16.67)	22 (8.09)	**0.0049**	**0.0353**	**0.0112**	**0.0064**
rs1569922	Intron 4	CC	36 (20.81)	95 (35.71)				
		CT	96 (55.49)	136 (51.13)	**1.73 (1.29-2.32)**	**2.03 (1.29-3.17)**	**2.03 (1.22-3.35)**	**1.64 (1.24-2.16)**
		TT	41 (23.7)	35 (13.16)	**0.0003**	**0.0020**	**0.0060**	**0.0005**
rs7539625	Intron 6	AA	30 (16.67)	80 (29.96)				
		AG	96 (53.33)	141 (52.81)	**1.77 (1.33-2.36)**	**2.14 (1.33-3.43)**	**2.07 (1.31-3.25)**	**1.69 (1.29-2.22)**
		GG	54 (30)	46 (17.23)	**0.0001**	**0.0017**	**0.0017**	**0.0002**
rs7518660	Intron 7	GG	79 (43.65)	146 (54.48)				
		GA	82 (45.3)	105 (39.18)	**1.45 (1.08-1.96)**	**1.52 (1.04-2.23)**	**1.84 (0.93-3.66)**	**1.44 (1.07-1.93)**
		AA	20 (11.05)	17 (6.34)	**0.0150**	**0.0318**	**0.0808**	**0.0154**
rs10789229	Intron 8	TT	146 (80.66)	232 (85.29)				
		TC	34 (18.78)	38 (13.97)	1.32 (0.82-2.12)	1.40 (0.84-2.31)	0.60 (0.05-6.85)	1.31 (0.82-2.10)
		CC	1 (0.55)	2 (0.74)	0.2581	0.1953	0.6821	0.2601
rs1008193	Intron 9	CC	152 (84.44)	241 (88.6)				
		CG	27 (15)	29 (10.66)	1.31 (0.78-2.20)	1.40 (0.80-2.45)	0.61 (0.05-6.92)	1.32 (0.78-2.22)
		GG	1 (0.56)	2 (0.74)	0.3126	0.2345	0.6877	0.3057
rs6693831	Intron 9	CC	96 (53.04)	165 (61.8)				
		CT	71 (39.23)	83 (31.09)	1.28 (0.95-1.72)	1.44 (0.98-2.12)	1.12 (0.54-2.33)	1.30 (0.95-1.77)
		TT	14 (7.73)	19 (7.12)	0.1116	0.0619	0.7539	0.0995

CA), according to the manufacturer's instructions. Primer Express (Applied Biosystems) was used in order to design both the PCR primers and the MGB TaqMan probes. One allelic probe was labeled with the FAM dye and the other was labeled with fluorescent VIC dye. Detailed procedures regarding the PCR reaction for Taqman assay have been described previously (Kim *et al*., 2006). Fluorescence data files from each plate were collected and analyzed by using automated allele-calling software (SDS 2.2, Applied Biosystems).

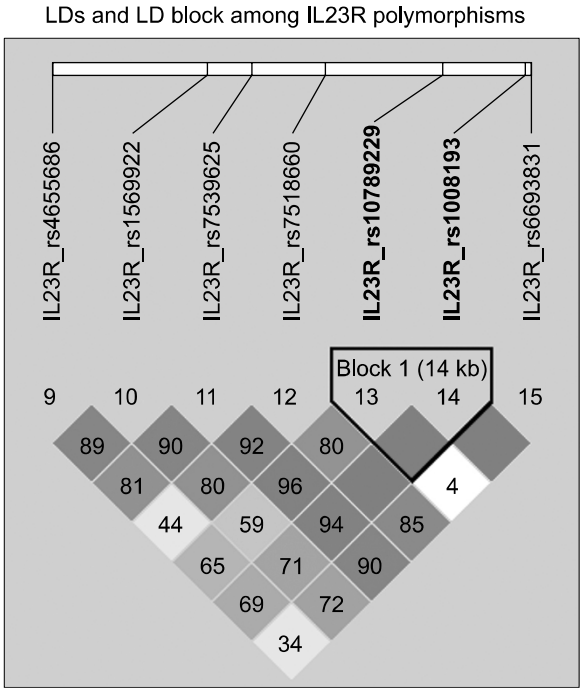
Statistical analyses

Chi-square tests were used in order to determine whether individual variants were in intra-locus equilibrium in the samples (Hardy-Weinberg equilibrium). Logistic regression analyses were used to calculate the odds ratios (OR), 95% confidence intervals (CI) and corresponding *P* values of each SNP and haplotypes controlling for age and sex as covariates with three alternative models (codominant, dominant and recessive). The linkage disequilibrium (LD) between loci was measured by using the absolute value of Lewontin's D' (|D'|) (Hedrick, 1987). Haplotype structures and their frequencies were estimated from genotyped data within the LD block by using the Haploview 3.32 (http://www.broad.mit.edu/mpg/haploview/), which estimates haplotype by an accelerated EM algorithm similar to the partition ligation method (Qin *et al*., 2002). All analyses were two-tailed and a *P*-value of < 0.05 was considered to be statistically significant. Statistical analyses were performed by using SAS 9.1 (SAS Institute Inc., Cary, NC).

Results

In order to investigate the association of *IL23R* gene polymorphisms with respect to ONFH, ten polymorphic sites in the *IL23R* gene were genotyped regarding 443 ONFH patients and 273 control subjects. Among the 10 SNPs genotyped, three SNP sites did not fulfill our criteria in a call rate (CR) > 0.90, minor allele frequency (MAF) > 0.05 and Hardy-Weinberg equilibrium (HWE) > 0.05. Seven SNP sites were in the HWE ($P > 0.05$) (Table 1). The *P* values of each polymorphism were obtained using logistic regression analyses by comparison between ONFH patients and the controls. It was found that rs4655686, rs1569922 and rs7539625 polymorphisms of the *IL23R* gene were significantly associated with the risk of ONFH in an alternative analysis model (*P* values; 0.0198-0.0447, OR; 1.30-1.49) (Table 2). These results suggested that the minor allele of rs4655686, rs1569922 and rs7539625 (A, T and G, respectively) contributes to an increase in the risk of ONFH.

A



B

Haplotypes in IL23R

Haplotypes	rs10789229	rs1008193	Frequency
IL23R-ht1	T	C	0.918
IL23R-ht2	C	G	0.065
IL23R-ht3	C	C	0.016

Figure 1. Linkage disequilibrium coefficients and haplotypes of IL23R (A) Linkage disequilibrium coefficients (|D'|) and LD block among IL23R polymorphisms. (B) Haplotypes of IL23R

Further analysis based on pathological etiology (alcohol-, steroid- or idiopathic) showed that the genotypes of rs4655686, rs1569922, rs7539625 and rs7518660 of *IL23R* gene were specifically associated with the risk of ONFH in the idiopathic ONFH subgroup in all alternative models (*P* values; 0.0001-0.0150, OR; 1.45-2.17) (Table 3). These results suggested that the *IL23R* polymorphisms were possibly important risk factors in the idiopathic ONFH. However, there was no significant association with regard to alcohol- and steroid-induced subgroups (data not shown).

Since the LD pattern has been hypothesized to be highly structured as conserved blocks of sequence separated by hotspots of recombination, the final function of a conserved haplotype may be the result of interaction among polymorphisms within the block. LD coefficients (|D'|) between all SNP pairs were calculated, and complete LD (i.e. D' = 1) were found between the rs10789229 and rs1008193 (Figure 1A). Additionally, we calculated the haplotype frequencies with two loci (rs10789229, rs1008193) within the LD block, and tested for a haplotype association between the controls and ONFH patients. Two major haplotypes among three haplotypes explained more than 98% of the variation (Figure 1B). As shown in Table 4, haplotypes were not associated with susceptibility to ONFH. In addition, haplotype analysis in subgroup patients also showed no association with the risk of ONFH (data not shown).

Discussion

ONFH is a devastating disease that frequently leads to a progressive collapse of the femoral head, followed by degenerative arthritis of the hip joint. The precise pathophysiology of ONFH is not known, but it has been suggested that a common pathogenesis of ONFH involves an interruption of the circulation of blood to the anterior-superior-lateral region of the femoral head (Atsumi and Kuroki, 1992). A previous study reported that idiopathic ONFH coincided with an increased release of a VEGF and proinflammatory factors (Boss *et al*., 2004). These factors include the recruitment of endothelial progenitor cells, macrophages, osteoclasts, fibroblasts, and osteoblasts, which, pervading throughout the necrotic areas, initiate the reparative processes. The bone effect resulting from systemic inflammation may lead to a variety of bone diseases including RA, osteoporosis and osteonecrosis (Migliaresi *et al*., 1994; Abu-Shakra *et al*., 2003; Lane, 2006). According to a previous report (Tektonidou and Moutsopoulos, 2004),

Table 4. Association of IL23R gene haplotypes with ONFH patients and normal controls.

Haplotype[a]	Genotype	Controls	Patients	Co-dominants		Dominant		Recessive		Allele	
				OR (95% CI)[b]	P[b]	OR (95% CI)[b]	P[b]	OR (95% CI)[b]	P[b]	OR(95% CI)[b]	P[b]
IL23R-ht1	-/-	2 (0.74)	3 (0.68)								
(T-C)	ht1/-	38 (13.97)	67 (15.16)	0.88 (0.59-1.32)	0.5484	1.14 (0.19-7.03)	0.8858	0.86 (0.56-1.32)	0.5001	0.88 (0.59-1.32)	0.5479
	ht1/ht1	232 (85.29)	372 (84.16)								
IL23R-ht2	-/-	241 (88.6)	384 (86.88)								
(C-G)	ht2/-	29 (10.66)	56 (12.67)	1.17 (0.75-1.82)	0.4899	1.23 (0.77-1.97)	0.3928	0.59 (0.08-4.33)	0.6023	1.17 (0.75-1.83)	0.4863
	ht2/ht2	2 (0.74)	2 (0.45)								

Haplotypes: rs10789229 - rs1008193

immunologic factors may be related with pathogenesis of osteonecrosis.

Cytokines are recognized as being involved in the bone metabolism process. IL3, IL6, and IL11 may act to increase the proliferation as well as the differentiation of osteoclast precursors (Goldring and Goldring, 1996; Mont *et al*., 1998). A previous study had suggested that IL23, an IL6/IL12 cytokine member, was related to collagen-induced arthritis in mouse models and also inhibited osteoclast formation (Murphy *et al*., 2003; Kamiya *et al*., 2007). IL4 and IFN-γ, induced by IL23, have been shown to block osteoclastogenesis by means of inhibition of the transcription factors NFATc1 and c-Fos, and induction of TNF receptor-associated factor 6 (TRAF6) (Mundy, 1996; Takayanagi *et al*., 2002; Moreno *et al*., 2003; Kamel Mohamed *et al*., 2005; Gao *et al*., 2007). Therefore, these cytokines may represent a major target for the prevention of inflammation related bone diseases and promote bone metabolism. A genome wide association study has shown that the *IL23R* gene was associated with autoimmune diseases, including IBD, CD and psoriasis (Cardon, 2006; Duerr *et al*., 2006; Raelson *et al*., 2007). The association between *IL23R* and these diseases has been replicated in many other studies with different populations (Cargill *et al*., 2007; Cummings *et al*., 2007; Dubinsky *et al*., 2007; Glas *et al*., 2007; Rioux *et al*., 2007; Roberts *et al*., 2007). The recent findings of the association studies suggested that the IL-23/IL-23R cytokine receptor system is also involved in the pathogenesis of several diseases.

In this study, we have tried to determine the contribution of *IL23R* gene polymorphisms in regards to ONFH. We found that polymorphisms of the *IL23R* gene were significantly associated with ONFH in the Korean population. In particular, the genotypes of rs4655686, rs1569922, rs7539625 and rs7518660 of the *IL23R* gene were specifically associated with the risk of ONFH in the idiopathic subgroup (Table 3). These results suggested that inflammatory factors might be one of the important risk factors in regards to idiopathic ONFH. However, there were no significant associations shown in both alcohol- and steroid-induced subgroups. No association with ONFH in these subgroups could be attributable to different etiological characteristics of subgroup patients. Corticosteroid therapy and alcoholism, which can change fat metabolism, and accumulating cell stress, which causes occlusion of the blood flow, were major risk factors with respect to osteonecrosis (Gold and Cangemi, 1979; Asano *et al*., 2003). In addition, alcohol and steroids promote the generation of reactive oxygen species (ROS), resulting in oxidative stress. Therefore, it was generally thought that the effect of alcohol abuse or excessive steroid administration was stronger than genetic factors in regards to alcohol- and steroid-induced ONFH patients. On the other hand, genetic factors may be more strongly related to ONFH among idiopathic patients due to no obvious reasons having been identified.

Our study had several concerns. Firstly, since our hospital is a tertiary institution covering the Daegu-Kyungpook region, more severely ill patients are usually referred to our hospital, thus possibly resulting in selection bias. Secondly, patients in the early stage of osteonecrosis might have been included in the control group since only patients were identified by a MRI and we did not check the MRI results of the control group. The numbers of controls were less than numbers of cases since the age and sex-matched normal controls were difficult to obtain. Thirdly, the incidence of ONFH in Korea is relatively higher and sex-biased (male: female = 4:1) in comparison to other populations. The majority of osteonecrosis in Korea is either idiopathic or related to excessive alcohol use. Most male patients represent alcohol-induced cases, whereas female patients represent idiopathic or steroid-induced cases. These facts suggested that the etiology of ONFH may be quite complex and possibly attributable to gender and ethnic differences.

ONFH is one of the most common diseases of the hip in Korea, responsible for more than half of the underlying causes of total hip arthroplasty. Therefore, early diagnosis of the disease using several noted genetic markers will provide beneficial information regarding individual susceptibility to ONFH and may also help high-risk individuals to take precautions against further aggravating symptoms. In conclusion, the present study suggests, for the first time, to our knowledge, that the polymorphisms of the *IL23R* gene are likely to be associated with the risk of ONFH, at least in the Korean population. Further replication studies in a large cohort would be needed to confirm the suggested association.

Acknowledgements

This work was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (Project No.: A010252), and intramural grants from the Korea National Institute of Health, Korea Center for Disease Control, Republic of Korea.

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