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Genetic association of angiogenesis- and hypoxia-related gene polymorphisms with osteonecrosis of the femoral head

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Abbreviations: HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; MAF, minor allele frequency; ONFH, osteonecrosis of the femoral head; SNP, single nucleotide polymorphism

Abstract

Multiple factors have been implicated in the development of osteonecrosis of the femoral head (ONFH). In particular, non-traumatic ONFH is directly or indirectly related to injury of the vascular supply to the femoral head. Thus, hypoxia in the femoral head caused by impaired blood flow may be an important risk factor for ONFH. In this study, we investigated whether genetic variations of angiogenesis- and hypoxia-related genes contribute to an increased risk for the development of ONFH. Candidate genes were selected based on known hypoxia and angiogenesis pathways. An association study was performed using an Affymetrix Targeted Genotyping 3K Chip array with 460 ONFH patients and 300 control subjects. We showed that single nucleotide polymorphisms (SNPs) in the genes *TF*, *VEGFC*, *IGFBP3*, and *ACE* were associated with an increased risk of ONFH. On the other hand, SNPs in the *KDR* and *NRP1* genes were associated with protection against ONFH. The most important finding was that one SNP (rs2453839) in the *IGFBP3* gene was significantly associated with a higher risk of ONFH (P = 0.0061, OR 7.74). In subgroup analysis, most candidate gene variations that were associated with ONFH occurred in the idiopathic subgroup. Among other SNPs, *ACE* SNPs were associated with steroid-induced ONFH (P = 0.0018-0.0037, OR > 3). Collectively, our findings suggest that genetic variations in angiogenesis- and hypoxia-related genes may help to identify susceptibility factors for the development of ONFH in the Korean population.

Keywords: anoxia; Asian continental ancestry group; femur head; neovascularization, physiologic; osteonecrosis; polymorphism, single nucleotide

Introduction

Osteonecrosis of the femoral head (ONFH) is a bone disorder that usually affects middle-aged men 30-50 yr of age (Jones, 1999; Glueck *et al*., 2003). Various factors have been implicated in the development of ONFH. However, the exact pathogenesis of non-traumatic ONFH is largely unknown, although it has been associated with corticosteroid usage, alcoholism, infections, marrow infiltrating diseases, and coagulation defects (Jones, 1999; Glueck *et al*., 2003; Childs, 2005). Most of these risk factors are closely related to direct or indirect injury to the vascular supply of the femoral head.

Genetic associations between ONFH and hypofibrinolysis or thrombophilia along with coagulation have been reported (Glueck *et al*., 2001; Bjorkman *et al*., 2004). Coagulation-related factors including plasminogen-activating inhibitor-1 (PAI-1), Factor V (F5), prothrombin, and methylenetetrahydrofolate reductase (MTHFR) are associated with ONFH development. Therefore, many studies have proposed that a pathogenetic mechanism, such as coagulation and angiogenesis, also seems to be major risk factor for osteonecrosis (ON).

Angiogenesis is a physiological process involving the growth of new blood vessels for the blood supply. Also, angiogenesis is essential for development, wound healing of most tissues, and bone remodeling (Colnot *et al*., 2003; Fong *et al*., 2003).

Vascular endothelial growth factor (VEGF), an essential regulator for angiogenesis, plays a key role in vascular endothelial cell migration, proliferation, and permeability. In particular, VEGF is important for bone formation processes such as morphogenesis of a normal growth plate, including blood vessel invasion and cartilage remodeling (Harper and Klagsbrun, 1999; Komatsu and Hadjiargyrou, 2004). In addition, VEGF is highly expressed in the edematous zone of ON adjacent to the necrotic area, indicating that it might be an important factor for bone tissue repair (Radke *et al*., 2006). Another angiogenic factor, PAI-1, is also highly expressed in dysbaric osteonecrosis (Miyanishi *et al*., 2002). Many studies have shown that VEGF enhances blood flow in necrotic bone or avascular bone (Suzuki *et al*., 2004; Ma *et al*., 2007). These studies have suggested that the genetic variations in angiogenesis-related gene may affect the development of ONFH (Kerachian *et al*., 2006).

A hypoxic condition is the first stimulus for angiogenesis (Arnett *et al*., 2003; Lin *et al*., 2004). Disruption of the blood supply to damaged bone often causes hypoxia (Glowacki, 1998; Lewis *et al*., 1999) and, thus, a loss of blood supply (Arnett *et al*., 2003; Childs, 2005). Consequently, hypoxia is known to induce both apoptosis and necrosis of cells and is associated with various vascular diseases including cardiovascular disease, cerebral ischemia, pulmonary hypertension, and cancer (Semenza, 2000; Lattimore *et al*., 2005). In particular, hypoxia accelerates atherosclerosis by promoting the accumulation of lipid loading in the arterial wall (Lattimore *et al*., 2005). Many studies have shown that hypoxia activates a number of cell regulatory processes including angiogenesis, erythropoiesis, energy metabolism, cell survival, and iron homeostasis (Semenza, 2001; Hickey and Simon, 2006). During the bone regeneration typically seen in fracture repair or distraction osteogenesis, the expression of hypoxia-inducible factor-1α (HIF-1α) and its target genes, such as VEGF, was directly correlated with cellular survival, and blood vessel and callus formation (Komatsu and Hadjiargyrou, 2004). In a previous study, HIF-1 also was shown to bind to the VEGF promoter and form a complex that activates transcription of the VEGF gene (Stein *et al*., 1998). Hypoxia leads to enhanced bone regeneration by inhibiting chondrocyte and osteoblast apoptosis (Komatsu *et al*., 2007). Hypoxia also develops in bone fractures and in the joints of patients with rheumatoid arthritis (RA), leading to necrosis (Lewis *et al*., 1999). In fact, we previously have shown that a polymorphism in the HIF-1α gene is closely associated with ONFH (Hong *et al*., 2007).

A number of studies have reported that hypoxia and angiogenesis are related to the biological pathways of bone diseases, including ONFH (Lewis *et al*., 1999; Semenza, 2000; Radke *et al*., 2006). However, the genetic link between hypoxia and angiogenesis and ONFH is not yet known. In this study, we attempted to determine whether allelic variations in angiogenesis- and hypoxia-related genes are associated with the progression and pathogenesis of ONFH.

Results

To identify genetic factors involved in susceptibility to ONFH, we conducted an association study using SNP chip array data in 460 ONFH patients and 300 controls. Candidate genes were selected from previous microarray reports (Pacicca *et al*., 2003; Wieczorek *et al*., 2003; Salim *et al*., 2004; Tardif *et al*., 2004; Fang *et al*., 2005; Hopwood *et al*., 2005; Zhou *et al*., 2005). SNPs in these genes were selected based on a call rate (CR) >0.90, minor allele frequency (MAF) >0.05, and Hardy-Weinberg equilibrium (HWE) >0.05 using a public database (http://www.ncbi.nlm.nih.gov/SNP/). Total numbers of SNPs found in genes related to angiogenesis (31 genes) and the SNPs selected for genotyping (212 SNPs) are listed in Supplemental data Table S1.

Among them, our results reveal that 6 candidate genes (TF, KDR, VEGFC, IGFBP3, NRP1 and ACE) are significantly associated with ONFH (Supplemental data Table S2). SNP IDs, MAF, and HWE of the genotyped SNPs are presented in Supplemental data Table S2. The genotype distributions between ONFH patients and control groups were compared and applied to the Hardy-Weinberg equilibrium (Supplemental data Table S2). Each genotype was also analyzed with the use of codominant, dominant, recessive, and allele genetic models.

We found significant associations of genotypic and allelic frequencies for several SNPs of candidate genes with ONFH (Table 1). Three SNPs (rs1880669, rs2692695, rs2718806) of the Transferrin (TF) gene showed association with ONFH in the codominant and dominant models (P = 0.0049-0.0448, OR 1.27-1.69). Two SNPs (rs1485766, rs3775203) of Vascular endothelial growth factor C (VEGFC) were also associated with increased risk of ONFH (P = 0.0042-0.0107, OR 1.33-1.67). A significant association for three SNPs (rs4309, rs4344, rs4461142) of Angiotensin I converting enzyme (ACE) was also found in the

Table 1. Association between candidate gene polymorphisms and ONFH among ONFH cases and controls.

Gene	SNP rs ID	Position	Genotype	ONF patients	Normal controls	Codominant OR (95% CI)[a] *P*[a]	Dominant OR (95% CI)[a] *P*[a]	Recessive OR (95% CI)[a] *P*[a]
TF	**rs1880669**	Intron10	TT	103 (23.41)	83 (30.86)	1.23 (0.99-1.52)	**1.50 (1.06-2.11)**	1.14 (0.80-1.63)
			TC	224 (50.91)	124 (46.1)	0.0635	**0.0208**	0.4780
			CC	113 (25.68)	62 (23.05)			
	rs2692695	Intron12	GG	82 (19.52)	75 (28.3)	**1.27 (1.02-1.59)**	**1.69 (1.17-2.43)**	1.13 (0.79-1.61)
			GA	225 (53.57)	125 (47.17)	**0.0345**	**0.0049**	0.5044
			AA	113 (26.9)	65 (24.53)			
	rs2718806	Intron12	GG	366 (83.18)	242 (88.64)	**1.57 (1.01-2.43)**	**1.62 (1.03-2.55)**	1.20 (0.11-13.35)
			GA	72 (16.36)	30 (10.99)	**0.0448**	**0.0384**	0.8826
			AA	2 (0.45)	1 (0.37)			
	rs1525889	Intron14	AA	139 (32.1)	99 (36.94)	1.10 (0.87-1.37)	1.26 (0.91-1.74)	0.93 (0.61-1.41)
			AC	225 (51.96)	125 (46.64)	0.4274	0.1591	0.7333
			CC	69 (15.94)	44 (16.42)			
KDR	**rs6837735**	Intron2	CC	128 (29.16)	69 (25.37)	0.80 (0.64-1.00)	0.82 (0.58-1.16)	**0.67 (0.47-0.97)**
			CT	230 (52.39)	135 (49.63)	0.0521	0.2713	**0.0357**
			TT	81 (18.45)	68 (25)			
	rs1870377	Exon11	TT	140 (32.48)	95 (34.8)	0.94 (0.75-1.17)	1.13 (0.82-1.56)	**0.67 (0.45-0.99)**
			TA	229 (53.13)	122 (44.69)	0.5732	0.4547	**0.0488**
			AA	62 (14.39)	56 (20.51)			
VEGFC	**rs1485766**	Intron4	TT	90 (20.32)	80 (29.3)	**1.37 (1.10-1.71)**	**1.67 (1.18-2.38)**	1.37 (0.96-1.97)
			TG	233 (52.6)	134 (49.08)	**0.0050**	**0.0042**	0.0861
			GG	120 (27.09)	59 (21.61)			
	rs2333496	Intron4	TT	185 (42.24)	108 (40.3)	0.82 (0.66-1.03)	0.92 (0.67-1.25)	**0.55 (0.35-0.86)**
			TC	208 (47.49)	114 (42.54)	0.0897	0.5872	**0.0087**
			CC	45 (10.27)	46 (17.16)			
	rs3775203	Intron4	TT	100 (22.83)	84 (31)	**1.33 (1.07-1.65)**	**1.57 (1.11-2.21)**	1.36 (0.94-1.95)
			TG	222 (50.68)	129 (47.6)	**0.0103**	**0.0107**	0.1016
			GG	116 (26.48)	58 (21.4)			
IGFBP3	**rs3110697**	Intron3 Intron4	GG	237 (54.48)	139 (52.65)	1.07 (0.83-1.38)	0.95 (0.69-1.29)	**2.085 (1.03-4.22)**
			GA	164 (37.7)	114 (43.18)	0.5874	0.7203	**0.0412**
			AA	34 (7.82)	11 (4.17)			
	rs2453839		TT	293 (66.14)	191 (70.48)	**1.37 (1.02-1.82)**	1.23 (0.88-1.71)	**7.74 (1.79-33.41)**
			TC	128 (28.89)	78 (28.78)	**0.0351**	0.2184	**0.0061**
			CC	22 (4.97)	2 (0.74)			
NRP1	**rs12573218**	Intron2	CC	290 (73.6)	153 (62.96)	**0.60 (0.43-0.83)**	**0.59 (0.42-0.84)**	0.32 (0.07-1.36)
			CT	101 (25.63)	85 (34.98)	**0.0019**	**0.0033**	0.1218
			TT	3 (0.76)	5 (2.06)			
	rs12358370	Intron2	CC	399 (90.27)	231 (84.62)	**0.56 (0.36-0.87)**	**0.55(0.35-0.87)**	0.31 (0.03-3.57)
			CG	42 (9.5)	40 (14.65)	**0.0096**	**0.0109**	0.3485
			GG	1 (0.23)	2 (0.73)			
	rs2269091	Intron6	GG	304 (69.89)	164 (62.84)	**0.74 (0.55-0.98)**	0.72 (0.52-1.01)	0.55 (0.21-1.46)
			GT	123 (28.28)	88 (33.72)	**0.0382**	0.0532	0.2308
			TT	8 (1.84)	9 (3.45)			
ACE	**rs4309**	Exon8	TT	121 (27.44)	100 (36.76)	1.24 (0.99-1.54)	**1.50 (1.08-2.08)**	1.09 (0.73-1.62)
			TC	239 (54.2)	124 (45.59)	0.0627	**0.0147**	0.6833
			CC	81 (18.37)	48 (17.65)			
	rs4344	Intron17	AA	129 (29.72)	98 (37.26)	1.17 (0.93-1.47)	**1.42 (1.02-1.96)**	0.98 (0.65-1.48)
			AG	235 (54.15)	120 (45.63)	0.1709	**0.0367**	0.9050
			GG	70 (16.13)	45 (17.11)			
	rs4461142	Intron26	CC	107 (24.6)	94 (34.69)	**1.34 (1.07-1.68)**	**1.63 (1.16-2.28)**	1.26 (0.84-1.87)
			CT	241 (55.4)	131 (48.34)	**0.0113**	**0.0044**	0.2596
			TT	87 (20)	46 (16.97)			

[a]Logistic regression analyses were used for calculating the OR (95% CI) and *P*-value.

dominant model (P = 0.0044-0.0367, OR 1.34-1.63). The most significant association with risk of ONFH was identified in a single SNP (rs2453839) of the Insulin-like growth factor binding protein 3 (IGFBP3) gene in the recessive model (P = 0.0061, OR 7.74) (Table 1).

On the other hand, several SNPs in the Kinase insert domain receptor (KDR), VEGFC, and Neuropilin 1 (NRP1) genes showed a protective effect against ONFH. Two SNPs (rs6837735, rs1870377) of KDR were associated with a protective effect against ONFH in the recessive model (P = 0.0357, 0.0488, OR 0.67, 0.67, respectively). SNP rs2333496 of VEGFC (P = 0.0087, OR 0.55) and three SNPs (rs12573218, rs12358370, rs2269091) of NRP1 (P = 0.0019-0.0423, OR 0.55-0.75) were associated with a reduced risk of ONFH. There were no statistically significant interactions between any other SNPs and ONFH (Table 1).

For further analysis, we classified ONFH cases on the basis of pathological etiology into three subgroups (alcohol-induced, idiopathic, and steroid-induced, Table 2). We found that a significant association was found between SNPs in the *TF* gene and the idiopathic subgroup (P = 0.0008-0.0486, OR 1.38-2.37). This result suggests that *TF* gene variations are risk factors for increased susceptibility to idiopathic ONFH. Two SNPs (rs1485766, rs3775203) in the VEGFC gene were also associated with the risk of developing ONFH in the alcohol and idiopathic subgroups (P = 0.0259-0.0347, OR 1.34-1.59 and P = 0.0010-0.0456, OR 1.38-2.26, respectively). In addition, three SNPs (rs4309, rs4344, rs4461142) in *ACE* were associated with susceptibility to ONFH in the steroid subgroup (P = 0.0018-0.0037, OR 3.26-3.68 in the dominant model). However, one SNP (rs6837735) of KDR (P = 0.0011-0.0107, OR 0.42-0.70) and three SNPs (rs12573218, rs12358370, rs2269091) of NRP1 (P = 0.01120.0446, OR 0.49-0.68) had protective effects in the idiopathic subgroup. The most significant association was found in the *IGFBP3* gene. SNP (rs2453839) was significantly associated with alcohol-induced and idiopathic ONFH in the recessive model. The odds ratio of the susceptibility allele was 6.15 (P = 0.0194) and 8.67 (P = 0.0066) in the two subgroups, respectively. This result suggests that subjects carrying the minor homozygous allele (CC) of rs2453839 tend to have a higher risk of developing ONFH (Table 2).

We also constructed an LD and performed haplotype analyses based on genotype data of SNPs in the candidate genes. The haplotypes with a MAF $\geq$ 5% were observed within an LD block. There is one LD block in the TF gene, three LD blocks in the *KDR* gene, one LD block in the *IGFBP3* gene, five LD blocks in the *NRP1* gene, and two LD blocks in the *ACE* gene (Supplemental data Figure S1A-F). Several haplotypes were found to be associated with ONFH (Table 3). Subjects carrying the KDR-ht6 allele (42%) had a higher risk of ONFH than those not carrying the KDR-ht6 allele (35%) (P = 0.146, OR 1.32). However, TF-ht2 (P = 0.0151, OR 0.72) and KDR-ht10 (P = 0.0026, OR 0.52) were associated with a reduced risk of ONFH, which was not predicted by individual SNP analyses. NRP1-ht16 was also related to a decreased ONFH risk (P = 0.0118, OR 0.67). There were no significant differences in candidate genes between the other haplotypes and ONFH (Table 3).

Discussion

Many studies have shown that the correlation between hypoxia and the regulation of angiogenesis is a significant component of homeostatic mechanisms (Semenza, 2000; Semenza, 2001). Hypoxia is a primary stimulus for angiogenesis and is associated with various vascular diseases (Semenza, 2000, 2001), including osteonecrosis (Glowacki, 1998).

ONFH is caused by the temporary or permanent loss of blood supply to the femoral head (Jones, 1999; Glueck *et al*., 2003; Childs, 2005). Therefore, it is hypothesized that variation in genes involved in angiogenesis by hypoxia might influence the development of ONFH. Previously, we reported that polymorphisms of *VEGF* and *HIF-1*α, major target genes for angiogenesis and hypoxia, were associated with increased susceptibility to ONFH (Hong *et al*., 2007; Kim *et al*., 2008). These results suggested a genetic association between polymorphisms in genes related to angiogenesis and hypoxia and the development of ONFH. However, the genetic effects of many other genes involved in angiogenesis and hypoxia pathways on ONFH development have not been fully demonstrated. To further understand the genetic effects of angiogenesis- and hypoxia-related genes on ONFH, we selected candidate genes showing enhanced expression in hypoxic conditions using previously reported microarray data and SNPs from the dbSNP.

The candidate genes in this study, including *TF*, *KDR*, *VEGFC*, *IGFBP3*, *NRP1*, and *ACE*, are regulated by angiogenesis and hypoxia (Garcea *et al*., 2006), and have been implicated in various vascular diseases (Beckman *et al*., 1998; Padro *et al*., 2002). Transferrin (TF) is essential for iron

Table 2. Subgroup association between candidate gene polymorphisms with ONFH among ONFH cases and controls.

Subgroup	Gene	SNP rs ID	Codominant		Dominant		Recessive		Allele	
			OR (95% CI)[a]	*P*[a]	OR (95% CI)[a]	*P*[a]	OR (95% CI)[a]	*P*[a]	OR (95% CI)[a]	*P*[a]
Alcohol	TF	rs1525889	1.02 (0.78-1.34)	0.8876	1.14 (0.77-1.69)	0.5252	0.86 (0.51-1.45)	0.5747	1.02 (0.77-1.35)	0.8854
		rs1880669	1.17 (0.90-1.53)	0.2304	1.38 (0.91-2.11)	0.1326	1.11 (0.71-1.73)	0.6531	1.19 (0.91-1.56)	0.2131
		rs2692695	1.27 (0.96-1.67)	0.0920	**1.64 (1.04-2.60)**	**0.0332**	1.16 (0.74-1.81)	0.5250	1.28 (0.97-1.68)	0.0865
		rs2718806	1.64 (0.98-2.74)	0.0626	1.69 (0.97-2.92)	0.0633	2.15 (0.19-23.93)	0.5349	1.65 (0.98-2.76)	0.0595
	KDR	rs6837735	0.95 (0.73-1.25)	0.7222	0.96 (0.63-1.47)	0.8505	0.91 (0.58-1.44)	0.6885	0.95 (0.73-1.25)	0.7192
		rs1870377	0.90 (0.69-1.18)	0.4528	1.07 (0.72-1.60)	0.7458	0.62 (0.37-1.04)	0.0713	0.90 (0.68-1.19)	0.4468
	VEGFC	**rs1485766**	**1.35 (1.02-1.78)**	**0.0345**	1.52 (0.98-2.36)	0.0636	1.45 (0.92-2.28)	0.1140	**1.34 (1.02-1.75)**	**0.0365**
		rs2333496	0.91 (0.69-1.20)	0.5061	1.03 (0.69-1.52)	0.9036	0.67 (0.39-1.15)	0.1457	0.91 (0.69-1.20)	0.4979
		rs3775203	**1.37 (1.04-1.80)**	**0.0259**	**1.59 (1.03-2.46)**	**0.0347**	1.43 (0.90-2.28)	0.1348	**1.36 (1.04-1.79)**	**0.0265**
	IGFBP3	rs3110697	1.18 (0.85-1.64)	0.3097	1.12 (0.76-1.64)	0.5792	1.98 (0.80-4.92)	0.1398	1.168 (0.86-1.60)	0.3300
		rs2453839	1.30 (0.90-1.87)	0.1598	1.15 (0.75-1.75)	0.5227	**6.15 (1.34-28.22)**	**0.0194**	1.31 (0.91-1.89)	0.1525
	NRP1	rs12573218	0.68 (0.45-1.03)	0.0654	0.68 (0.44-1.06)	0.0881	0.31 (0.03-2.87)	0.3002	0.70 (0.47-1.04)	0.0805
		rs12358370	0.59 (0.34-1.03)	0.0643	0.56 (0.31-1.01)	0.0534	0.81 (0.06-11.72)	0.8775	0.59 (0.34-1.03)	0.0632
		rs2269091	0.86 (0.60-1.24)	0.4241	0.89 (0.59-1.34)	0.5661	0.56 (0.17-1.88)	0.3459	0.87 (0.61-1.24)	0.4290
	ACE	**rs4309**	1.22 (0.92-1.61)	0.1711	**1.66 (1.09-2.51)**	**0.0178**	0.89 (0.53-1.48)	0.6499	1.20 (0.92-1.58)	0.1825
		rs4344	1.21 (0.92-1.60)	0.1746	**1.62 (1.08-2.45)**	**0.0204**	0.89 (0.53-1.49)	0.6579	1.21 (0.92-1.59)	0.1791
		rs4461142	1.17 (0.88-1.54)	0.2731	1.16 (0.77-1.76)	0.4861	1.33 (0.81-2.20)	0.2590	1.16 (0.88-1.53)	0.2783
Idiopathic	TF	**rs1525889**	1.21 (0.91-1.61)	0.1805	**1.59 (1.04-2.41)**	**0.0309**	0.93 (0.55-1.56)	0.7705	1.20 (0.91-1.58)	0.1915
		rs1880669	**1.38 (1.05-1.81)**	**0.0204**	**2.06 (1.29-3.27)**	**0.0023**	1.17 (0.76-1.82)	0.4801	**1.38 (1.05-1.80)**	**0.0201**
		rs2692695	**1.41 (1.07-1.87)**	**0.0160**	**2.37 (1.43-3.92)**	**0.0008**	1.14 (0.73-1.76)	0.5676	**1.39 (1.06-1.83)**	**0.0175**
		rs2718806	1.64 (0.97-2.77)	0.0665	**1.72 (1.00-2.95)**	**0.0486**	0 (0,∞)	0.9868	1.60 (0.96-2.67)	0.0727
	KDR	**rs6837735**	**0.68 (0.51-0.91)**	**0.0085**	0.79 (0.52-1.22)	0.2886	**0.42 (0.25-0.70)**	**0.0011**	**0.70 (0.54-0.92)**	**0.0107**
		rs1870377	0.95 (0.73-1.25)	0.7372	1.20 (0.80-1.81)	0.3786	0.64 (0.38-1.08)	0.0939	0.95 (0.73-1.25)	0.7373
	VEGFC	**rs1485766**	**1.59 (1.20-2.1)**	**0.0011**	**2.26 (1.39-3.66)**	**0.0010**	**1.55 (1.01-2.39)**	**0.0456**	**1.56 (1.19-2.04)**	**0.0012**
		rs2333496	**0.70 (0.53-0.93)**	**0.0152**	0.78 (0.53-1.15)	0.2032	**0.36 (0.18-0.70)**	**0.0027**	**0.70 (0.52-0.93)**	**0.0136**
		rs3775203	**1.38 (1.05-1.80)**	**0.0197**	1.53 (0.99-2.38)	0.0577	1.54 (1.00-2.39)	0.0521	**1.39 (1.06-1.83)**	**0.0168**
	IGFBP3	**rs3110697**	0.97 (0.70-1.32)	0.8226	0.77 (0.52-1.14)	0.1941	**2.28 (1.03-5.05)**	**0.0416**	0.97 (0.71-1.32)	0.8246
		rs2453839	**1.46 (1.02-2.09)**	**0.0375**	1.30 (0.87-1.96)	0.1919	**8.67 (1.83-41.18)**	**0.0066**	**1.44 (1.01-2.04)**	**0.0414**
	NRP1	rs12573218	**0.58 (0.38-0.88)**	**0.0114**	**0.55 (0.35-0.87)**	**0.0112**	0.51 (0.10-2.77)	0.4378	**0.61 (0.41-0.91)**	**0.0163**
		rs12358370	**0.49 (0.27-0.90)**	**0.0211**	**0.49 (0.26-0.92)**	**0.0270**	0 (0,∞)	0.9875	**0.49 (0.27-0.90)**	**0.0207**
		rs2269091	**0.68 (0.47-0.99)**	**0.0419**	**0.64 (0.42-0.97)**	**0.0355**	0.68 (0.20-2.26)	0.5271	**0.69 (0.48-0.99)**	**0.0446**
	ACE	rs4309	1.17 (0.90-1.52)	0.2507	1.17 (0.78-1.74)	0.4454	1.33 (0.83-2.15)	0.2383	1.18 (0.90-1.55)	0.2344
		rs4344	1.06 (0.80-1.39)	0.6926	1.08 (0.72-1.62)	0.6990	1.07 (0.64-1.78)	0.8053	1.06 (0.80-1.40)	0.6874
		rs4461142	**1.50 (1.14-2.00)**	**0.0045**	**1.99 (1.28-3.09)**	**0.0022**	1.42 (0.88-2.29)	0.1552	1.47 (1.12-1.93)	0.0053
Steroid	TF	rs1525889	1.14 (0.74-1.76)	0.5410	1.24 (0.65-2.36)	0.5239	1.15 (0.52-2.54)	0.7388	1.15 (0.74-1.78)	0.5350
		rs1880669	1.06 (0.70-1.61)	0.7798	1.00 (0.52-1.94)	0.9991	1.19 (0.59-2.40)	0.6307	1.07 (0.69-1.64)	0.7729
		rs2692695	1.03 (0.68-1.58)	0.8771	0.96 (0.49-1.89)	0.9078	1.15 (0.57-2.32)	0.7019	1.04 (0.67-1.60)	0.8742
		rs2718806	0.90 (0.34-2.35)	0.8223	0.91 (0.34-2.41)	0.8427	0 (0,∞)	0.9914	0.90 (0.35-2.32)	0.8255
	KDR	rs6837735	0.85 (0.55-1.31)	0.4500	0.70 (0.35-1.38)	0.2960	0.93 (0.49-1.91)	0.8503	0.85 (0.55-1.31)	0.4514
		rs1870377	1.12 (0.74-1.72)	0.5890	1.44 (0.74-2.82)	0.2868	0.89 (0.41-1.94)	0.7662	1.13 (0.73-1.74)	0.5814
	VEGFC	rs1485766	0.96 (0.63-1.48)	0.8677	0.99 (0.51-1.92)	0.9656	0.91 (0.43-1.92)	0.8110	0.96 (0.626-1.483)	0.8662
		rs2333496	0.90 (0.58-1.40)	0.6473	0.99 (0.53-1.85)	0.9667	0.69 (0.28-1.70)	0.4133	0.90 (0.575-1.407)	0.6422
		rs3775203	1.31 (0.85-2.02)	0.2224	1.74 (0.84-3.62)	0.1369	1.18 (0.57-2.44)	0.6520	1.31 (0.85-2.03)	0.2194
	IGFBP3	rs3110697	1.17 (0.70-1.98)	0.5491	1.10 (0.59-2.04)	0.7685	1.87 (0.51-6.84)	0.3445	1.15 (0.71-1.85)	0.5811
		rs2453839	1.58 (0.88-2.82)	0.1240	1.50 (0.80-2.84)	0.2090	4.94 (0.61-40.06)	0.1349	1.50 (0.87-2.58)	0.1436
	NRP1	rs12573218	0.51 (0.25-1.04)	0.0641	0.54 (0.26-1.13)	0.1041	0 (0,∞)	0.9831	0.58 (0.31-1.09)	0.0920
		rs12358370	0.75 (0.32-1.78)	0.5148	0.78 (0.32-1.91)	0.5808	0 (0,∞)	0.9908	0.75 (0.32-1.78)	0.5147
		rs2269091	0.70 (0.38-1.30)	0.2574	0.74 (0.38-1.42)	0.3637	0 (0,∞)	0.9806	0.73 (0.41-1.30)	0.2871
	ACE	**rs4309**	**1.61 (1.03-2.51)**	**0.0374**	**3.68 (1.63-8.33)**	**0.0018**	0.90 (0.40-2.06)	0.8085	**1.57 (1.02-2.42)**	**0.0397**
		rs4344	1.54 (0.97-2.43)	0.0664	**3.33 (1.51-7.37)**	**0.0029**	0.76 (0.31-1.89)	0.5618	1.49 (0.96-2.32)	0.0741
		rs4461142	1.56 (0.98-2.48)	0.0600	**3.26 (1.47-7.25)**	**0.0037**	0.83 (0.34-2.02)	0.6733	1.49 (0.96-2.30)	0.0729

[a]Logistic regression analyses were used for calculating OR (95% CI) and P value. Dominant analysis: homozygotes for the major allele vs. heterozygotes vs. homozygotes for the minor allele. Recessive analysis: homozygotes for the minor allele vs. heterozygotes vs. homozygotes for the major allele.

Table 3. Haplotype association between candidate gene polymorphisms and ONFH.

Haplo-type[a]	Chromo-some	Gene	Haplotype	SNP markers	Frequency	Haplotype OR[a]	*P*[b]
ht1	3	TF	C-G-G-C-C	rs1880669-rs2692695-rs2718806-rs1525889-rs1049296	0.411	1.10 (0.87-1.39)	0.4366
ht2			**T-A-G-A-T**		0.252	**0.72 (0.55-0.94)**	**0.0151**
ht3			T-A-G-A-C		0.247	0.99 (0.76-1.29)	0.9483
ht4			C-G-A-A-C		0.080	1.48 (0.95-2.30)	0.0832
ht5	4	KDR	C-T	rs2305949-rs6837735	0.465	0.81 (0.65-1.01)	0.0553
ht6			**C-C**		0.391	**1.32 (1.06-1.65)**	**0.0146**
ht7			T-C		0.144	0.90 (0.66-1.22)	0.4910
ht8			T-C-A	rs12502008-rs7667298-rs2071559	0.608	1.01 (0.81-1.27)	0.9059
ht9			G-T-G		0.322	1.20 (0.94-1.52)	0.1386
ht10			**G-C-A**		0.066	**0.52 (0.34-0.80)**	**0.0026**
ht11	7	IGFBP3	G-G-T	rs3110697-rs6953668-rs9282734	0.742	0.95 (0.74-1.22)	0.6854
ht12			A-G-T		0.170	1.03 (0.77-1.38)	0.8395
ht13			A-A-G		0.088	1.05 (0.71-1.53)	0.8235
ht14	10	NRP1	C-G-G	rs12573218-rs1331324-rs11009340	0.526	1.04 (0.82-1.30)	0.7690
ht15			C-C-T		0.271	1.31 (1.00-1.72)	0.0505
ht16			**T-C-G**		0.142	**0.67 (0.49-0.92)**	**0.0118**
ht17			C-C-G		0.054	1.12 (0.68-1.84)	0.6572

[a]Values were constructed by the EM algorithm with genotyped SNP, [b]Logistic regression analysis was used for calculating the OR (95% CI) and *P*-value.

regulation involved in the hypoxia and angiogenesis pathway. TF promotes migration and invasion of endothelial cells and thus is an essential angiogenesis factor produced by hypertrophic cartilage during endochondral bone formation (Carlevaro *et al*., 1997; Li *et al*., 2003). VEGFC is another essential factor for angiogenesis and endothelial cell growth, and participates in the progression and development of angiogenic diseases (Cao *et al*., 1998). Angiotensin I converting enzyme (ACE) plays a key role in the rennin-angiotensin system and has been associated with various diseases, including coronary artery disease, type 2 diabetes, hypertension, stroke, and lupus (Saeed *et al*., 2005; Tsai *et al*., 2007; Yamagishi *et al*., 2007). In addition, ACE has been identified as a representative candidate gene in cardiovascular diseases (Niu *et al*., 2002). Kinase insert domain receptor (KDR) and neuropilin 1 (NRP1), which are VEGF receptors, are expressed in most vascular endothelial cells and regulate angiogenesis and cell migration, survival, and proliferation. Furthermore, KDR is related to the degree of bone marrow angiogenesis and is required for tumor neovascularization (Padro *et al*., 2002). KDR polymorphisms have been associated with a risk of coronary heart disease and cancer (Forsti *et al*., 2007; Wang *et al*., 2007).

In this study, we found that genetic variations in *TF, VEGFC*, *IGFBP3*, and *ACE* were associated with an increased risk of ONFH. Interestingly, however, *KDR* and *NRP1* and one SNP in *VEGF* (rs2333496) had a protective effect against ONFH development. It is not clear why the SNPs in *VEGF* and its receptors that play critical roles in angiogenesis exert a protective effect on ONFH. However, this effect may be related to the expression of VEGF in the edematous region of ON and its role in repairing ON (Radke *et al*., 2006).

ONFH patients were classified based on their etiology (alcohol-induced, idiopathic, and steroid-induced groups), and the SNPs that were associated with total ONFH showed differential association with these groups. SNPs of *TF* and *VEGFC* in idiopathic ONFH and SNPs of *ACE* in the steroid subgroup were also found to be susceptibility factors for ONFH. Several SNPs of *KDR* and *NRP1* showed a protective effect against developing ONFH, particularly idiopathic ONFH. However, the sample size in steroid-induced groups is not enough to get power calculation. Further study is required to identify replications in large sample size of cases.

The most important finding in our report is that one SNP (rs2453839) of *IGFBP3* is significantly

associated with susceptibility to ONFH, especially alcohol-induced and idiopathic ONFH. Individuals bearing the minor allele genotype of rs2453839 showed a higher risk for ONFH than those bearing the common allele. IGFBP3 plays a role in promoting hypoxia-induced angiogenesis (Granata *et al*., 2007; Li *et al*., 2007). Among IGFBP members, IGFBP3 is most abundant in the circulatory system and is associated with atherosclerosis (Jones and Clemmons, 1995). Several polymorphisms in the IGFBP3 gene are also associated with cancer risk (Granata *et al*., 2007; Li *et al*., 2007). However, this SNP was associated only with ONFH, suggesting that *IGFBP3* rs2453839 is relatively specific for ONFH.

In addition, as shown in the association analysis of SNPs (Tables 1 and 2), the KDR-ht10 and NRP-ht16 haplotypes were associated with decreased risk of ONFH. Particularly, the minor allele (T) of rs12573218 in the *NRP1* gene contributed to the protective effect against ONFH. However, KDR-ht6 was associated with an increased risk of ONFH. This result suggests that the major allele (C) of rs6837735 in the *KDR* gene contributes to the risk of ONFH. TF-ht2 also showed a protective association against ONFH, which is different from the individual SNP analyses. Therefore, these data suggest that ONFH is significantly associated with a specific candidate gene haplotype.

In conclusion, we investigated the genetic association of candidate genes related to angiogenesis and hypoxia with ONFH. The strongest association with ONFH correlated with a single SNP (rs2453839) in the *IGFBP3* gene, and this SNP may act as a diagnostic marker for ONFH. In addition, other SNPs also can be considered for the prediction of susceptibility or resistance to ONFH.

Methods

Subjects

A total of 460 unrelated patients with ONFH (377 men, 83 women; age: 49.7 $\pm$ 13.3 yr) and 300 unrelated control subjects (210 men, 90 women; age: 52.1 $\pm$ 10.6 yr) were consecutively enrolled at the Kyungpook National University Hospital (Daegu, Korea) from 2002 to 2006. ONFH diagnoses were established by evidence of osteonecrosis through magnetic resonance imaging (MRI) in Stage 1 of the Association Research Circulation Osseous (ARCO) classification system and plain radiographs in Stages 2, 3, and 4. Control subjects were defined in the following way: they had no hip pain, and anteroposterior and frog leg lateral pelvic radiographs did not show any lesions with a sclerotic margin or subchondral collapse consistent with ONFH. According to etiological factors, ONFH cases were classified into one of the following subgroups: alcohol-induced (215 cases), idiopathic (186 cases), and steroid-induced osteonecrosis (59 cases). Cases with a demonstrable history of direct trauma or with the possibility of a combination of causes were excluded. Steroid-induced osteonecrosis was defined by a history of taking prednisolone (1,800 mg) or an equivalent over 4 weeks with nephritic syndrome, systemic lupus erythematosus, rheumatoid arthritis, allergic asthma, or organ transplantation. Alcohol-induced osteonecrosis was diagnosed by the consumption of more than 400 ml of pure ethanol per week or alcohol-induced fatty liver and liver cirrhosis. All individuals provided informed consent for their participation in the study, and this project was approved by the Institutional Review Board.

Candidate gene and SNP selection

To identify genetic factors associated with the risk of ONFH, we examined previous microarray data from bone marrow cells in hypoxic conditions. Normal and human mesenchymal stem cells (hMSC) were prepared from femoral tissue from osteoarthritis, osteoarthritic human chondrocytes, and the femur fracture in rat. Human and mouse bone marrow-derived MSCs from normoxic and hypoxic conditions were also used (Pacicca *et al*., 2003; Wieczorek *et al*., 2003; Salim *et al*., 2004; Tardif *et al*., 2004; Fang *et al*., 2005; Hopwood *et al*., 2005; Zhou *et al*., 2005).

For this study, we chose candidate genes associated with angiogenesis, lipid metabolism, oxidative stress, and hypoxia, the major pathogenic conditions of ONFH, on the basis of their known biological function and supportive literature. Approximately 3,000 SNPs for genotyping candidate genes were obtained from public databases (dbSNP, KSNP, HapMap) for verification. We primarily tested for SNPs in candidate genes known to regulate angiogenesis by hypoxia (Supplemental data Table S1).

Genotyping

DNA was extracted from samples using the FlexiGene DNA Kit (QIAGEN) and was quantified using PicoGreen (Invitrogen). The genotyping of DNA samples was performed using an Affymetrix Targeted Genotyping 3K Chip array according to the manufacturer's protocols. The Targeted Genotyping (TG) chip using molecular inversion probe technology with Gene chip universal microarrays provides a method that is capable of analyzing thousands of variants in a single reaction. In brief, a mixture of 2 μg of genomic DNA and molecular inversion probes is heat denatured and brought to annealing temperature. MIP probes have two specific homology sequences that leave a 1-bp gap when hybridized to the genome. They also contain specific tag sequences that are ultimately read on the microarray. In addition to these elements that are specific to each probe, there are two PCR primers that are common to all probes. These primers face away from each other and therefore cannot facilitate the amplification. After the probes are hybridized, the reaction is split into four tubes, with one of the four nucleotides added to each tube.

When the gap is filled in with the appropriate nucleotide, a unimolecular ligation event is catalyzed. After eliminating the linear probes with exonucleases, PCR, using the common primers that now face each other, is performed in the four tubes. In addition to signal amplification, a fluorescent label is introduced by a PCR primer into each of the four tubes. The four reactions are then mixed and hybridized onto a tag array. The arrays are washed and loaded onto a GeneChip Scanner 3000 7G (Affymetrix). Images are then analyzed using GCOS software (Affymetrix). Finally, the TG analysis software measures the data quality and generates genotypes for arrays that have met a specific set of quality control criteria.

Statistical and haplotype analyses

Permutation tests were used to determine whether individual variants were in equilibrium at each locus in the population (Hardy-Weinberg equilibrium). Logistical 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. We consider the genetic models of dominant, recessive, and codominant SNPs and haplotypes. Genotypes were given codes of 0, 1, and 2; 0, 0, and 1; or 0, 0, and 1 in the codominant, dominant, and recessive models, respectively. We examined Lewontin's D' (|D'|) and the linkage disequilibrium (LD) coefficient r^2 between all pairs of biallelic loci (Hedrick, 1987). Haplotype structures and their frequencies were estimated from genotyped data for the eight SNPs within the linkage disequilibrium block using Haploview version 3.32 (http://www.broad.mit.edu/mpg/haploview/), which estimates the haplotype by an accelerated EM algorithm similar to the partition ligation method (Qin *et al*., 2002). The Haploview program was also used to calculate pairwise linkage disequilibrium (D' and r^2) for each mark pair. All analyses were two-tailed, and a *P*-value of < 0.05 was considered statistically significant. Statistics were performed using SAS 9.1 (SAS Institute Inc., Cary, NC).

Supplemental data

Supplemental Data include two tables and can be found with this article online at http://e-emm.or.kr/article/article_files/ SP-42-5-06.pdf.

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