Supplemental Data

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

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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 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).

Methods

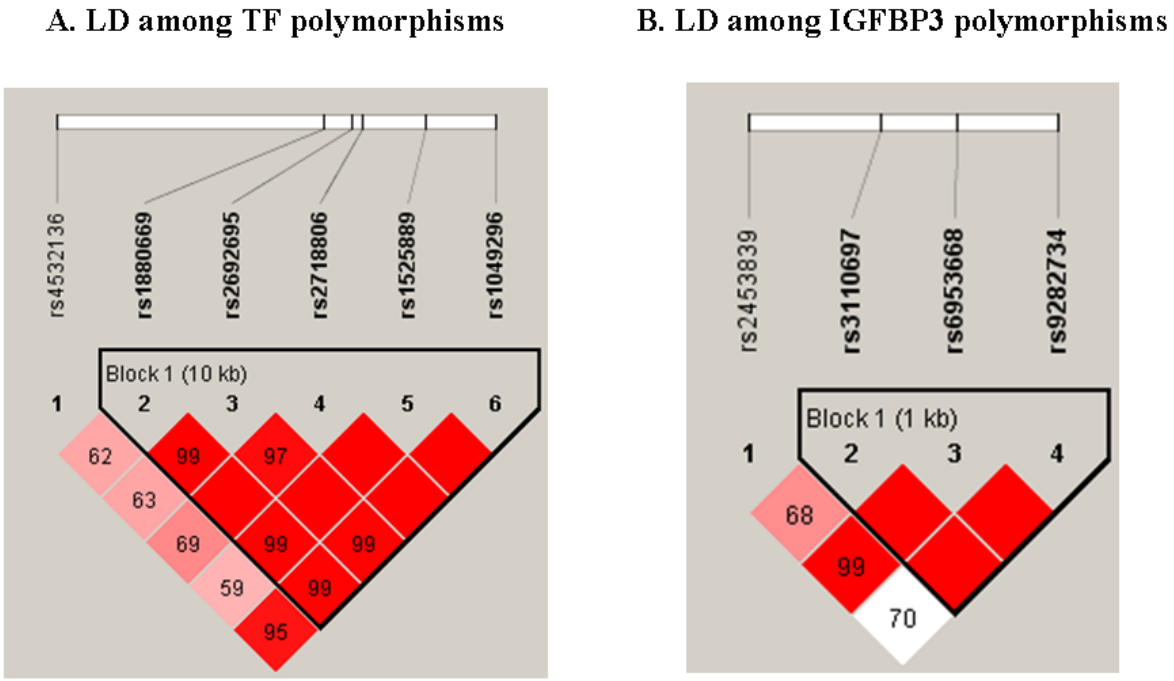
Candidate gene and SNP selection

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).

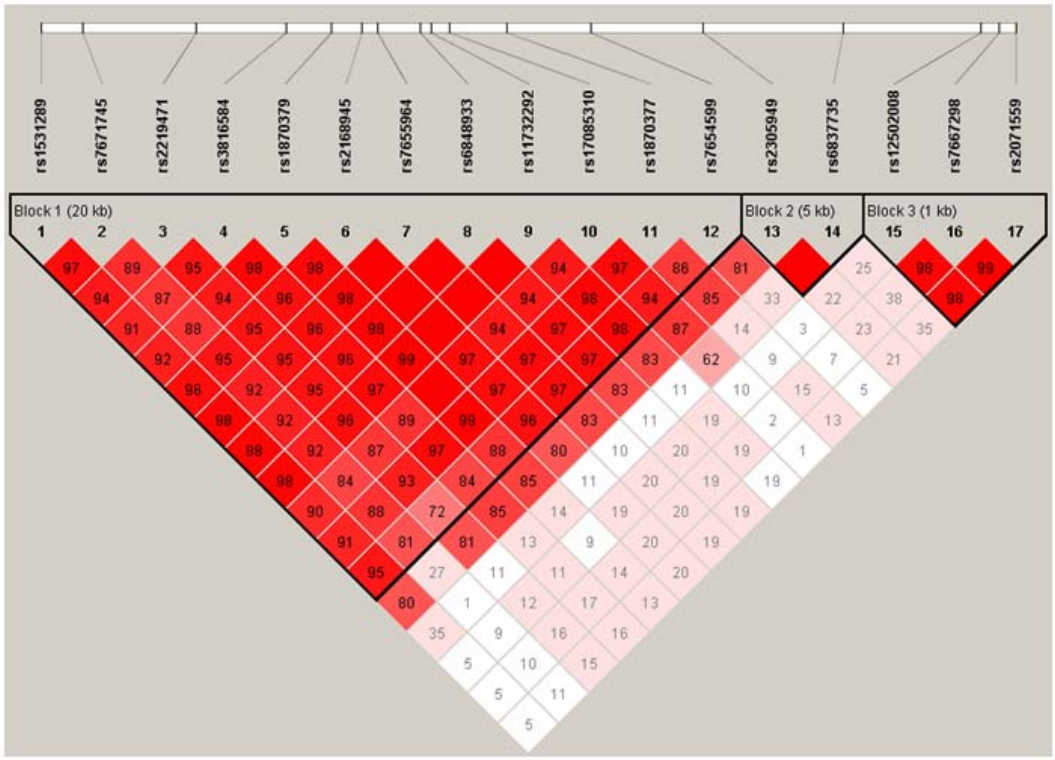
For this study, we chose candidate genes associated with angiogenesis, lipid metabolism, oxidative stress, and hypoxia, the major pathogenic conditions of ONFH, on the basis of their known biological function and supportive literature. Approximately 3,000 SNPs for genotyping candidate genes were obtained 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).

Figure S1. Linkage disequilibrium (D') among SNPs in candidate gene.

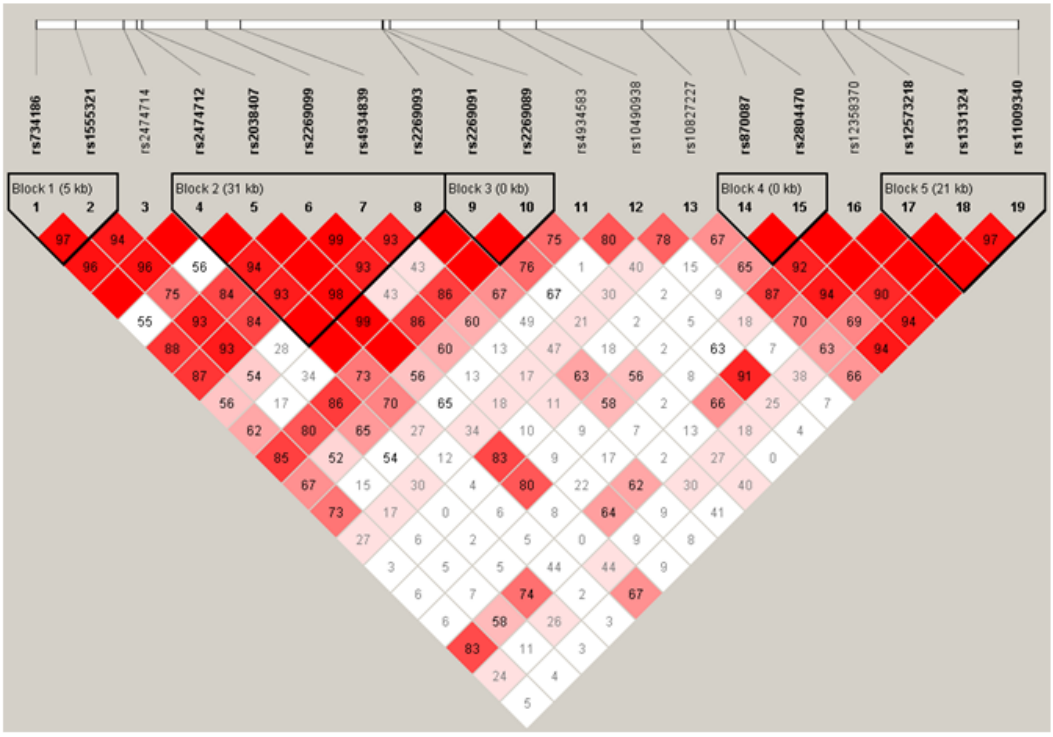
Pairwise LD diagrams between SNP markers are shown, as calculated by Haploview software (http://www.broad.mit.edu/mpg/haploview/). Red denotes complete LD (r^2=1), Pink denotes intermediated LD ($0<r^2<1$), white denotes no LD (r^2=0). LD Blocks represent a region of strong LD between polymorphisms. A-E. LD among TF, IGFBP3, KDR, NRP1 and ACE SNPs.



C. LD among KDR SNPs



D. LD among NRP1 SNPs



E. LD among ACE SNPs

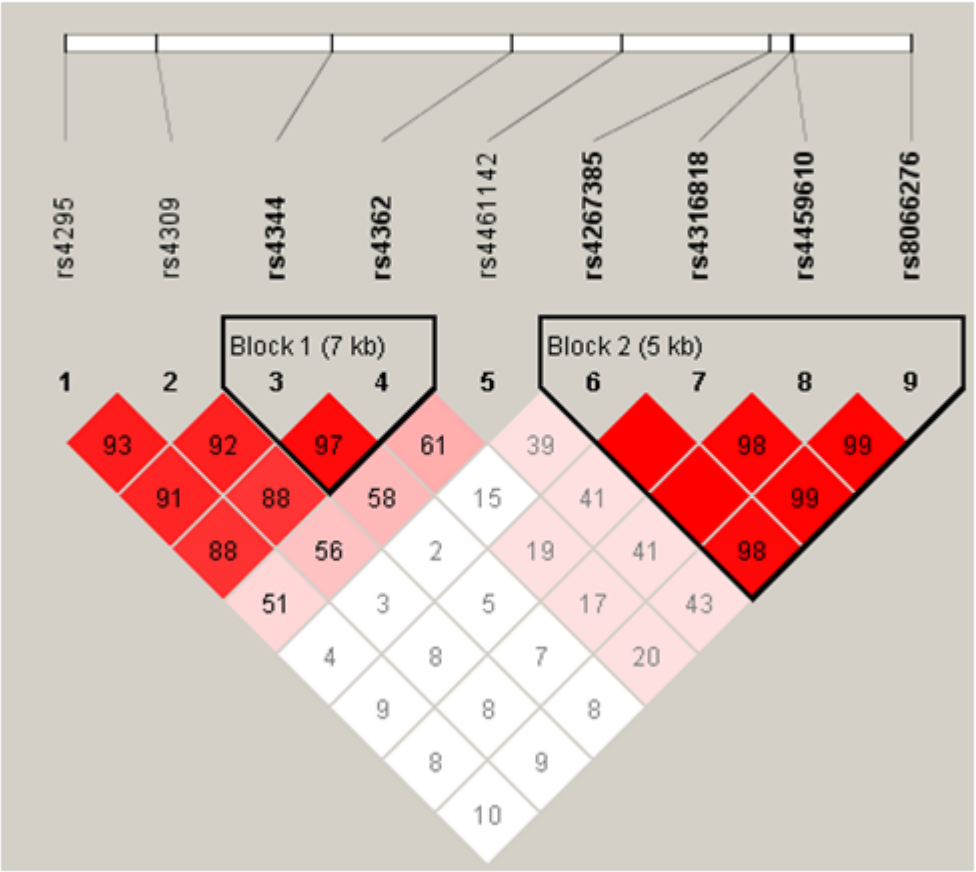


Table S1. List of candidate genes and SNPs.

Chromosome	Gene name	Putative function	SNPs	Selected SNPs
1	UTS2	Blood pressure regulation	4	4
	MTHFR	Angiogenesis	15	11
	SELP	Platelet activation/Degranulation	9	5
3	ITGB5	Angiogenesis	17	12
	TF	Iron metabolism	12	6
	PTX3	Thrombogenesis/Immune signaling	3	3
4	KDR	Angiogenesis	24	17
	FGF5	Growth factor/Cytokine	9	7
	ANXA5	Blood coagulation	15	11
	FGF2	Growth factor/Cytokine	6	6
	FGB	Platelet activation	8	2
	VEGFC	Growth factor/Cytokine	6	5
5	FGF1	Growth factor/Cytokine	4	4
6	EDN1	Growth factor/Cytokine	2	1
	VEGF	Angiogenesis	19	17
	PLG	Blood coagulation	1	1
7	IGFBP3	Growth factor/cytokine	4	4
	EPO	Erythropoiesis	1	0
10	ITGB1	Angiogenesis	13	13
	NRP1	Angiogenesis	24	19
	FGFR2	Growth factor/Cytokine	2	1
11	ADM	Growth factor/Cytokine	2	0
13	FLT1	Angiogenesis	26	23
15	THBS1	Angiogenesis	8	6
16	VKORC1	Angiogenesis	4	4
17	ALOX12	Iron metabolism	4	3
	VTN	Atherogenesis/Thrombus formation	2	1
	IGFBP4	Growth factor/Cytokine	3	3
	ITGB3	Angiogenesis	12	11
	ACE	Blood pressure regulation	15	9
	PECAM1	Adhesion	7	3

Table S2. Selected candidate gene SNP and allele frequency.

Chr	Gene	SNP rs ID	Position	Major/ Minor allele	Allelic Frequency[a]		HWE[b]
					Case	Control	
3	TF	rs4532136	Intron1	G/A	0.329	0.224	0.481
	TF	rs1880669	Intron10	T/C	0.829	0.461	0.262
	TF	rs2692695	Intron12	A/G	0.898	0.481	0.388
	TF	rs2718806	Intron12	G/A	0.094	0.059	1.000
	TF	rs1525889	Intron14	A/C	0.635	0.397	0.704
	TF	rs1049296	Exon15	C/T	0.299	0.278	0.542
4	KDR	rs2071559	Promoter	A/G	0.480	0.307	1.000
	KDR	rs7667298	Exon1	C/T	0.439	0.305	0.889
	KDR	rs12502008	Intron1	T/G	0.552	0.397	1.000
	KDR	rs6837735	Intron2	C/T	0.691	0.498	0.898
	KDR	rs2305949	Intron5	C/T	0.155	0.149	0.814
	KDR	rs7654599	Intron9	T/C	0.286	0.230	0.390
	KDR	rs1870377	Exon11	T/A	0.618	0.429	0.173
	KDR	rs17085310	Intron13	G/A	0.126	0.144	0.622
	KDR	rs11732292	Intron13	A/C	0.541	0.349	0.591
	KDR	rs6848933	Intron13	C/G	0.545	0.352	0.508
	KDR	rs7655964	Intron15	A/C	0.547	0.354	0.504
	KDR	rs2168945	Intron15	A/C	0.545	0.348	0.282
	KDR	rs1870379	Intron15	A/C	0.592	0.417	0.212
	KDR	rs3816584	Intron16	A/G	0.604	0.419	0.137
	KDR	rs2219471	Intron20	T/C	0.621	0.421	0.264
	KDR	rs7671745	Intron22	G/A	0.333	0.285	1.000
	KDR	rs1531289	Intron25	C/T	0.223	0.192	0.246
	VEGFC	rs2333496	Intron4	T/C	0.478	0.384	0.096
	VEGFC	rs1485766	Intron4	G/T	0.887	0.462	0.904
	VEGFC	rs3775203	Intron4	T/G	0.844	0.452	0.541
	VEGFC	rs3775202	Intron4	T/C	0.642	0.398	0.892
	VEGFC	rs3775194	Intron4	G/C	0.147	0.124	0.777
7	IGFBP3	rs9282734	Exon2	T/G	0.103	0.092	1.000
	IGFBP3	rs3110697	Intron3	G/A	0.345	0.258	0.053
	IGFBP3	rs6953668	Intron3	G/A	0.098	0.092	1.000
	IGFBP3	rs2453839	Intron4	T/C	0.234	0.151	0.055
10	NRP1	rs11009340	Intron2	G/T	0.359	0.248	0.139
	NRP1	rs1331324	Intron2	G/C	0.702	0.470	0.388

	NRP1	rs12573218	Intron2	C/T	0.156	0.195	0.097
	NRP1	rs12358370	Intron2	C/G	0.052	0.081	0.682
	NRP1	rs2804470	Intron2	A/G	0.384	0.262	0.209
	NRP1	rs870087	Intron2	T/C	0.449	0.288	0.369
	NRP1	rs10827227	Intron2	T/C	0.744	0.441	0.899
	NRP1	rs10490938	Intron4	C/T	0.115	0.112	0.547
	NRP1	rs4934583	Intron5	A/G	0.297	0.215	0.370
	NRP1	rs2269089	Intron6	G/C	0.779	0.483	0.112
	NRP1	rs2269091	Intron6	G/T	0.188	0.203	0.576
	NRP1	rs2269093	Intron6	A/G	0.135	0.148	1.000
	NRP1	rs2229934	Exon8	A/G	0.532	0.339	0.419
	NRP1	rs2269099	Intron8	C/T	0.537	0.338	0.341
	NRP1	rs2038407	Intron9	A/T	0.099	0.115	0.769
	NRP1	rs2474712	Intron9	T/C	0.177	0.130	0.096
	NRP1	rs2474714	Intron10	G/A	0.271	0.231	0.870
	NRP1	rs1555321	Intron11	A/G	0.578	0.375	0.596
	NRP1	rs734186	3'UTR	A/G	0.252	0.207	0.355
	ACE	rs4295	Intron2	C/G	0.698	0.410	0.315
	ACE	rs4309	Exon8	T/C	0.714	0.404	0.391
	ACE	rs4344	Intron17	A/G	0.666	0.399	0.442
	ACE	rs4362	Exon23	C/T	0.730	0.421	0.704
17	ACE	rs4461142	Intron26	C/T	0.766	0.411	1.000
	ACE	rs4267385	Intron26	C/T	0.265	0.242	1.000
	ACE	rs4316818	Exon26	T/C	0.285	0.249	1.000
	ACE	rs4459610	Exon26	A/T	0.293	0.254	1.000
	ACE	rs8066276	Intron35	T/C	0.282	0.240	0.873

[a] Frequencies of minor alleles.

[b] *p*-values of deviation from Hardy-Weinberg equilibrium among ONFH cases and controls.