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Polymorphisms of 3-hydroxy-3-methylglutaryl Coenzyme A Reductase Gene Are Not Associated with the Osteonecrosis of Femoral Head in Korean

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Osteonecrosis of the femoral head (ONFH) is a multifactorial disease and certain individuals are more at risk or may be predisposed to it. An altered lipid metabolism is one of the major risk factors for osteonecrosis, especially corticosteroid therapy and alcoholism. 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statin used as lipid-clearing agent, have been known to decrease the risk of osteonecrosis in patients receiving steroids and affect coagulation and fibrinolysis. Therefore we evaluated the association of HMG-CoA reductase gene polymorphisms and haplotypes between osteonecrosis patients and normal controls. We directly sequenced the HMG-CoA reductase gene in 24 Korean individuals, and identified five sequence variants. Four SNPs (-6933C>T, -6045T>G, +12673G>A, and +18128C>T) were selected and genotyped in 349 male ONFH patients and 300 male control subjects. The genotypes, allele frequencies, and haplotypes of the polymorphisms in the total patients as well as in the subgroup by etiology were not significantly different from those in the control group. In addition, no significant differences between each genotype of the polymorphisms and plasma lipid level could be found in the control group. These results suggest that the polymorphisms and haplotypes of HMG-CoA reductase gene are unlikely to be associated with a susceptibility to ONFH.

Key words : 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), osteonecrosis, coagulation, polymorphism

Introduction

Osteonecrosis (ON) is a pathologic process in which cell death in the components of bone occurs due to an interruption in the vascular supply of blood or a decreased blood flow. While osteonecrosis can occur in many anatomical locations, the femoral head is most commonly affected. This is probably because of restricted perfusion [1,21]. As osteonecrosis of the femoral head (ONFH) is a devastating disease that mainly affects middle-aged men, it creates a substantial socioeconomic cost as well as a burden for patients and their families [2].

The precise pathophysiology of ONFH is not known, but it has been suggested that several mechanisms have been implicated in the pathogenesis of osteonecrosis, including vascular occlusion, coagulopathy including thrombophilia and hypofibrinolysis, altered lipid metabolism, fat emboli, and increased bone-marrow pressure [2,13,35].

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR) is the rate-limiting enzyme of the mevalonate pathway, through which cells synthesize cholesterol from acetate moieties [10]. The HMGCR pathway is the metabolic pathway for a number of essential cellular compounds. Drugs inhibiting cholesterol biosynthesis affect bone metabolism through inhibition of the mevalonate pathway resulting in the inhibition of protein prenylation required for osteoclast activity [24,28] and many cellular processes. Recent studies have demonstrated that HMGCR inhibitors, statin as a lipid clearing agent, decrease the risk of osteonecrosis in patients receiving steroids [25]. It also affect the coagulation and fibrinolysis processes by modulating the expression of plasminogen activator inhibitor type-1 (PAI-1) and tissue type PA (t-PA), which may be

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implicated in osteonecrosis [17,27,31]. Therefore, it is presumed that the inhibitory effect of statins on the mevalonate pathway is involved in the regulation of some key steps of the coagulation and fibrinolysis processes. Recent studies have also suggested that statins influence bone turnover by stimulating bone formation through increased expression of the bone morphogenetic protein-2 (BMP-2) [23], which has been shown to play an important role in fracture and bone regeneration [32].

Therefore, we hypothesized that the mevalonate pathway may play an important role in development of osteonecrosis, and polymorphisms of HMGCR gene might be associated with the susceptibility of ONFH. To address these issues, we performed direct sequencing to detect polymorphisms and statistical analyses to examine the genetic association of the HMGCR gene polymorphisms with ONFH in the Korean population.

Materials and Methods

Subject

We studied 349 unrelated male patients with ONFH (age: 53.6±9.0 years, range: 19-78) and 300 non-ONFH male control subjects (age: 46.8±13.5 years, range: 39-69). Control subjects were recruited from spouses of the patients, and the general population of a cohort study in Ansan city and Ansung town performed by the Korean National Institute of Health.

The diagnosis was established by evidence of an osteonecrosis on a magnetic resonance imaging (MRI) in stage 1 of an ARCO (Association Research Circulation Osseous) classification system and plain radiographs in stages 2, 3 and 4 [5]. The control subjects were defined by if they had no hip pain and if 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, patients were subgrouped into osteonecrosis groups, idiopathic (91 cases), steroid-induced (59 cases), and alcohol-induced (199 cases). Patients with a demonstrable history of direct trauma or with possible combined 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, and organ transplantation [15]. 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. The characteristics of the patients are summarized in Table 1. There were no significant differences between patients and control subjects except for mean age. All individuals gave their informed consent for study participation, and the study was approved by the Institutional Review Board.

Sequencing analysis of the HMGCR gene

Genomic DNA was extracted from peripheral blood leukocytes using the FlexiGene DNA Kit (Qiagen, Valencia, CA, USA). To discover the sequence variants in HMGCR gene, promoter regions (~1.5 kb), all exons and their flanking regions were amplified by PCR, and then sequenced using a MegaBase 1000 DNA analyzer (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA) in 24 randomly selected DNA samples. Twenty-seven sets of primers for HMGCR gene amplification and sequencing analysis were designed based on GenBank sequences (Ref. seq. for HMGCR mRNA: NM_0008592 and contig NT_006713) (Table Ad1). Sequence variants were verified by chromatograms.

Genotyping with fluorescence polarization detection

For genotyping of the polymorphic sites, amplifying primers and probes were designed for TaqMan assay [19]. Primer Express (Applied Biosystems, Foster City, CA, USA) was used to design both the PCR primers and the MGB TaqMan probes. One allelic probe was labeled with the FAM dye and the other with the fluorescent VIC dye. PCRs were run in the TaqMan Universal Master mix without UNG (Applied Biosystems), with PCR primer concentrations of 900 nM and TaqMan MGB-probe concentrations of 200 nM. Reactions were performed in a 96-well format in a total reaction volume of 10 ul using 20 ng of genomic DNA. The plates were then placed in a thermal cycler (7500HT, Applied Biosystems) and heated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for

Table 1. Clinical profiles of the study subjects

	Normal controls	ONFH patients
Number of subjects	300	349
Age [mean (range)]*	53.6 (39-69)	46.8 (19-78)
Total cholesterol (mg/dl)	170.6±29.9	169.79±34.5
HDL-cholesterol (mg/dl)	43.8±10.2	43.5±13.1
Body mass index (kg/m^2)	22.95±3.03	23.21±2.85

*p<0.05 for difference between total patients and controls

15 sec and 60°C for 1 min, and the fluorescence intensity in each well of the plate was read. Fluorescence data files from each plate were analyzed using automated allele-calling software (SDS 2.1, Applied Biosystems). Genotyping quality control was performed in 10% of samples by duplicate checking (rate of concordance in duplicates>99%)

Statistics

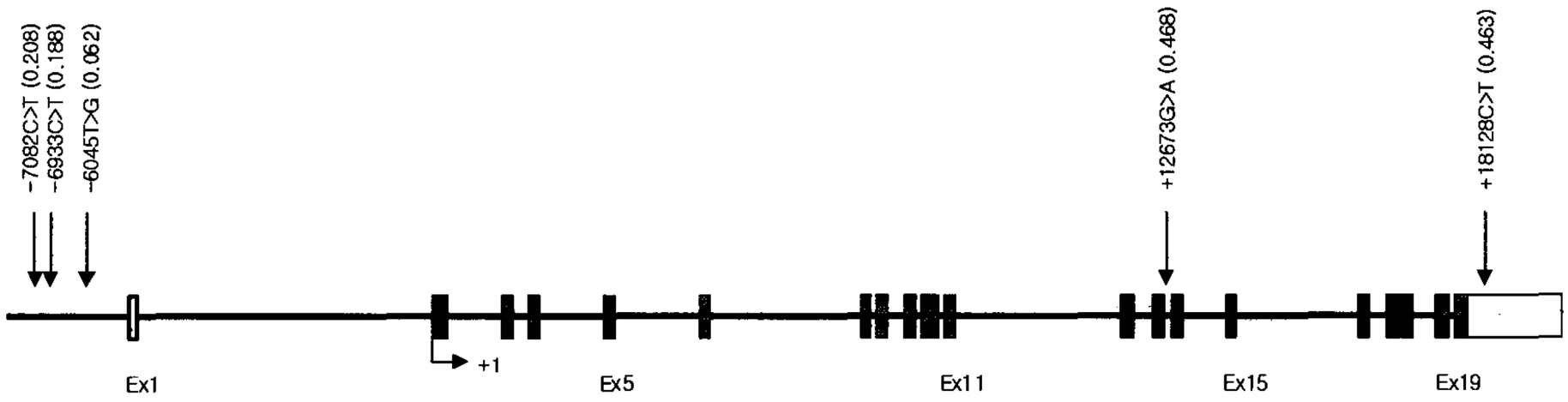
We used $\chi 2$ tests to determine whether individual variants were in Hardy-Weinberg equilibrium at each locus in the population. Logistical regression analyses were used to calculate the odds ratios (OR), and their 95% confidence interval (CI) for single nucleotide polymorphism (SNP) sites. The *p*-values of codominant, dominant and recessive models are given. We employed a widely used measure of linkage disequilibrium (LD) between all pairs of biallelic loci, D′, and r^2 [11]. Haplotypes of each individual and their frequencies were inferred using software based on the partition ligation- expectation maximization (PL-EM) algorithm [26] and permutation test was performed, with genotyped SNPs. A Fisher's exact test or $\chi 2$ test was applied to compare the frequency of discrete variables between controls and patients. Continuous variables were compared by Student's t-test or ANOVA. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

To detect genetic polymorphisms in HMGCR gene, we directly sequenced all exons, their intron boundaries, and ~1.5kb of the 5′ flanking regions from 24 individuals. Five SNPs, three (-7082C>T, -6933C>T, -6045T>G) in the promoter region, one (+12673G>A) in intron, and one (+18128C>T) in 3′-UTR (defined by the nucleotide position from the translational start site), were identified in HMGCR gene (Fig. 1A).

To examine the relationship between HMGCR gene polymorphisms and ONFH in men, we genotyped the 4 SNPs for association studies (n=649). Genotype distributions in all loci were in Hardy-Weinberg equilibrium ($p>0.05$), and the frequencies of each SNP are shown in Table Ad2. Linkage disequilibrium (|d′|) was estimated as Lewontin's D′-value for the possible pairs of the SNPs (Fig. 1B). We compared the genotype and allele frequencies of all genotyped SNPs between the ONFH patients and the controls. There was no significant association between individual HMGCR SNPs and ONFH (Table 2). We also compared each genotype of these polymorphisms, in subgroups based on etiological

A. Map of *HMGCR* (3-hydroxy-3-methylglutaryl-Coenzyme A reductase) on chromosome 5q13.3-q14 (27 kb)



B. LDs among *HMGCR* SNPs

		|D′|			
		-6933C>T	-6045T>G	+12673G>A	+18128C>T
r^2	-6933C>T		1	0.972	0.986
	-6045T>G	0.015		0.641	0.676
	+12673G>A	0.190	0.023		0.993
	+18128C>T	0.191	0.025	0.971	

Fig. 1. Gene maps and haplotypes of *HMGCR*. Coding exons are marked by black blocks, and 5′ and 3′ UTRs by white blocks. The first base of the translation start site is denoted as nucleotide +1. The frequencies of polymorphisms not subjected to larger scale genotyping were based on sequence data (n=24). A. Polymorphisms identified in *HMGCR* on chromosome 5q13.3-q14 (Ref. seq. for *HMGCR* mRNA: NM_0008592 and contig NT_006713) B. Linkage disequilibrium coefficients (|D′| and r^2) among *HMGCR* polymorphisms.

Table 2. Genotypes and allelic frequencies of the HMGCR gene polymorphisms between ONFH patients and controls

Position[a]	Genotype	Control	ONFH	Odds ratio[b]	P
		n (%)	n (%)	(95%CI)	
-6933C>T	CC	202 (67.6)	219 (64.2)	1.00	0.641
	CT	87 (29.1)	111 (32.6)	1.18 (0.84-1.65)	
	TT	10 (3.3)	11 (3.2)	1.02 (0.42-2.44)	
	CT+TT[C]	97 (32.4)	122 (35.8)	1.16 (0.84-1.61)	0.375
	C	491 (82.1)	549 (80.5)	1.00	
	T	107 (17.9)	133 (19.5)	1.11 (0.84-1.47)	0.462
-6045T>G	TT	263 (88.6)	303 (87.8)	1.00	0.710
	TG	32 (10.8)	41 (11.9)	1.11 (0.68-1.82)	
	GG	2 (0.7)	1 (0.3)	0.43 (0.04-4.81)	
	TG+GG[C]	34 (11.5)	42 (12.2)	1.07 (0.66-1.74)	0.777
	T	558 (93.9)	647 (93.8)	1.00	
	G	36 (6.1)	43 (6.2)	1.03 (0.65-1.63)	0.899
+12673G>A	GG	88 (29.6)	87 (25.7)	1.00	0.249
	GA	142 (47.8)	184 (54.4)	1.31 (0.91-1.90)	
	AA	67 (22.6)	67 (19.8)	1.01 (0.65-1.59)	
	GA+AA[C]	209 (70.4)	251 (74.2)	1.22 (0.86-1.72)	0.274
	G	318 (53.5)	358 (53.0)	1.00	
	A	276 (46.5)	318 (47.0)	1.02 (0.82-1.28)	0.837
+18128C>T	CC	86 (29.9)	96 (28.0)	1.00	0.484
	CT	136 (47.2)	178 (51.9)	1.17 (0.81-1.69)	
	TT	66 (22.9)	69 (20.1)	0.94 (0.60-1.46)	
	CT+TT[C]	202 (70.1)	247 (72.0)	1.10 (0.78-1.55)	0.605
	C	308 (53.5)	370 (53.9)	1.00	
	T	268 (46.5)	316 (46.1)	0.98 (0.79-1.23)	0.869

[a]Calculated from the translation start site
[b]Logistic regression analyses were used for calculating OR (95% CI: confidence interval)
[c]Dominant analysis: homozygotes for the major allele vs. heterozygotes+homozygotes for the minor allele

factors. Although the genotypes and allele frequencies of -6933C>T in alcohol subgroup were somewhat different from those of controls, none of polymorphisms was statistically significant (Table 3).

Genetic sequence association with ONFH was further investigated by comparing haplotype frequencies between patients and controls. As shown in Table 4, there was no significant difference between patients and controls, even when two-locus haplotypes, blocked by the Four Gamete method, were compared (Table 4).

To determine whether the HMGCR polymorphism contributes to the level of cholesterol, the association between genotype and the values of lipids was determined. However, there was no significant association between genotypes and total cholesterol level (Table 5).

Discussion

Although the pathogenesis of osteonecrosis has not been fully elucidated, it is commonly accepted that the final common pathway for the development ONFH involves a interruption of the circulation of blood to anterior-superior-lateral part of the femoral head, thus leading to ischemic insult and bone collapse [3]. Conditions associated with osteonecrosis include corticosteroid administration, alcohol abuse, fat emboli, hemoglobinopathy, and systemic lupus erythematosus [2].

Some studies have suggested that genetic mutations leading to thrombosis may be related to osteoncrosis. Intravascular coagulopathy including thrombotic and fibrinolytic abnormality may play an etiologic role in ONFH and some researchers investigated the associations between osteonecrosis and genes related to the coagulation and fibrinolytic system [1,4,6,20]. Altered fat metabolism and fat emboli have also been proposed as the underlying etiological event that leads to disruption of the vascular supply. Wang *et al.* have reported that steroids administered to growing and adults rabbits caused fat emboli as well as

Table 3. Association of genotypes and alleles between the ONFH subgroup patients and controls

Position[a]	Subjects	Genotypes n (%)			*P*	Allele frequency n (%)		Odds ratio[b]	*P*
		CC	CR	RR		C	R	(95%CI)	
-6933C>T	Controls (n=299)	202 (67.6)	87 (29.1)	10 (3.3)		491 (82.1)	107 (17.9)		
	Alcohol (n=192)	116 (60.4)	70 (36.5)	6 (3.1)	0.232	302 (78.7)	82 (21.3)	1.25 (0.90-1.72)	0.179
	Idiopathy (n=91)	62 (68.1)	26 (28.6)	3 (3.3)	0.995	150 (82.4)	32 (17.6)	0.98 (0.63-1.51)	0.924
	Steroid (n=58)	41 (70.7)	15 (25.9)	2 (3.4)	0.883	97 (83.6)	19 (16.4)	0.90 (0.53-1.53)	0.696
-6045T>G	Controls (n=297)	263 (88.6)	32 (10.8)	2 (0.7)		558 (93.9)	36 (6.1)		
	Alcohol (n=195)	170 (87.2)	24 (12.3)	1 (0.5)	0.853	364 (93.3)	26 (6.7)	1.11 (0.66-1.87)	0.702
	Idiopathy (n=91)	82 (90.1)	9 (9.9)	0 (0.0)	0.711	173 (95.1)	9 (4.9)	0.81 (0.38-1.71)	0.573
	Steroid (n=59)	51 (86.4)	8 (13.6)	0 (0.0)	0.683	110 (93.2)	8 (6.8)	1.13 (0.51-2.49)	0.767
+12673G>A	Controls (n=297)	88 (29.6)	142 (47.8)	67 (22.6)		318 (53.5)	276 (46.5)		
	Alcohol (n=192)	50 (26.0)	110 (57.3)	32 (16.7)	0.102	210 (54.7)	174 (45.3)	0.96 (0.74-1.24)	0.724
	Idiopathy (n=90)	22 (24.4)	49 (54.4)	19 (21.1)	0.513	93 (51.7)	87 (48.3)	1.08 (0.77-1.51)	0.660
	Steroid (n=56)	15 (26.8)	25 (44.6)	16 (28.6)	0.620	57 (50.9)	55 (49.1)	1.19 (0.80-1.79)	0.389
+18128C>T	Controls (n=288)	86 (29.9)	136 (47.2)	66 (22.9)		308 (53.5)	268 (46.5)		
	Alcohol (n=193)	51 (26.4)	108 (56.0)	34 (17.6)	0.151	210 (54.4)	176 (45.6)	0.96 (0.74-1.25)	0.776
	Idiopathy (n=91)	22 (24.2)	50 (54.9)	19 (20.9)	0.416	94 (51.7)	88 (48.3)	1.08 (0.77-1.50)	0.667
	Steroid (n=59)	23 (39.0)	20 (33.9)	16 (27.1)	0.166	66 (55.9)	52 (44.1)	0.91 (0.61-1.35)	0.625

[a]Calculated from the translation start site
[b]Logistic regression analyses were used for calculating OR (95% CI: confidence interval)
*CC: Major homozygote, CR: Heterozygote, CR: Minor homozygote, C: Major allele, R: Minor allele

Table 4. Estimated haplotype frequencies of selected SNPS in ONFH patients and controls

Haplotype				Frequency[a]		OR (95% CI)	*P*[b]
-6933C>T	-6045T>G	+12673G>A	+18128C>T	Control	Case		
C	T	A	T	0.45	0.45	1.02 (0.81-1.27)	0.882
C	T	G	C	0.31	0.28	0.86 (0.68-1.10)	0.236
T	T	G	C	0.18	0.19	1.07 (0.80-1.42)	0.646
C	G	G	C	0.05	0.06	1.36 (0.83-2.23)	0.224
C	T			0.76	0.75	0.93 (0.72-1.19)	0.434
T	T			0.18	0.19	1.09 (0.82-1.45)	0.420
C	G			0.06	0.06	1.03 (0.65-1.62)	0.923
		G	C	0.54	0.53	0.99 (0.79-1.23)	0.732
		A	T	0.46	0.46	0.96 (0.77-1.20)	0.894

[a]Values were constructed by EM algorithm with genotyped SNPs
[b]Values were analyzed by permutation test

25% increase in fat content of adipocytes in the femoral heads, and also suggested that hypercholesterolemia may play a role in the development of osteonecrosis. That is, steroid-treated animals developed increased serum cholesterol levels with a fatty metamorphosis of their livers and fat emboli that partially obliterated the microcirculation of their femoral heads [29].

Cholesterol reducing drugs, statins, lower the intraosseous pressure in femoral heads of steroid-treated rabbits [30] and decreases the risk of osteonecrosis in patients receiving steroids [25]. In addition, statins not only inhibit the cellular production of cholesterol but also the biosynthesis isoprenoid intermediates of the mevalonate pathway. Isoprenoids are essential for the post-translational modification of several proteins involved in intracellular signaling pathways [10].

In our study, we attempted to find out whether polymorphisms and haplotypes of HMGCR were associated with ONFH or plasma level of cholesterol. The genotypes, allele frequencies, and haplotypes of polymorphisms in the total male patients, as well as in the subgroup patients stratified by etiology, were not significantly different from those in the control group (Table 2, 3). In addition, there were no significant differences between each genotype of

Table 5. Plasma level of total cholesterol, HDL-cholesterol according to the genotypes of HMGCR polymorphisms in the control group

Locus	Genotype	No. (%)	Age	Total cholesterol	HDL*-cholesterol
Rs3761739	CC	202 (67.6)	55.2±8.4	177.8±31.6	43.3±9.8
	CT	87 (29.1)	53.3±9.1	178.8±29.3	44.1±10.7
	TT	10 (3.34)	53.7±9.8	185.0±22.6	49.1±12.6
	P		0.266	0.819	0.312
Rs3761741	TT	263 (88.6)	54.8±8.7	177.5±31.1	44.1±10.4
	GT	32 (10.8)	52.1±7.9	183.4±27.3	41.7±7.6
	GG	2 (0.01)	49.5±9.2	196.0±21.2	34±0.0
	P		0.255	0.496	0.235
Rs3846662	GG	88 (29.6)	53.8±8.2	178.4±30.5	45.1±10.3
	AG	142 (47.8)	54.7±9.0	179.7±29.8	44.1±10.3
	AA	67 (22.6)	55.3±8.9	175.4±33.3	41.0±9.2
			0.606	0.700	0.064
Rs12916	CC	86 (29.9)	54.0±8.2	178.0±30.5	45.2±10.3
	CT	136 (47.2)	54.8±9.0	179.0±29.6	44.2±10.4
	TT	66 (22.9)	55.3±8.9	175.4±33.3	41.0±9.2
	P		0.669	0.778	0.054

* High density lipoprotein

the polymorphisms and plasma cholesterol level in control group (Table 5). These results suggest that the polymorphisms analyzed in this study are unlikely to be associated with susceptibility to ONFH. The present study has some limitations. Epidemiologic study has shown that idiopathic (36%) and alcohol-induced ONFH (35%) are more prevalent in Korea than steroid-induced (14%). Therefore, the association will be false negative/or false positive due to the small sample size (steroid; 59) and may not be statistically relevant. Moskal *et al.* have reported that 60 to 85% of patients with osteonecrosis have elevated level of serum cholesterol and triglycerides [22]. We hypothesized that hypercholesterolemia may play an important role in development of osteonecrosis. Therefore, we performed the association study of SNPs and haplotypes of HMG-CoA reductase gene. However, our study showed no difference in mean serum cholesterol level between ON patients and control group. The lack of association might be due to an ethnic difference or other genetic factors that are involved. Thrombophillia and hypofibrinolysis are considered as important pathogenesis of nontraumatic osteonecrosis in Caucasians [7,9]. However, they were not associated in Korean people [18]. In Caucasians, the G1691A mutation of factor V (factor V Leiden) [34], the G20210A mutation of prothrombin [34], the PAI-1 4G/5G polymorphism [4,8], and the methylene tetrahydrofolate reductase C677T allele [33] have been associated with osteonecrosis. These gene mutations were not investigated completely in Korean people in association with osteonecrosis. However, we could not find factor V Leiden gene mutation in Korean patients with osteonecrosis [14]. Other gene polymorphisms studies relating vascular ischemia or hypoxia have been done recently in Korea. Koo *et al.* reported that microsatellite polymorphism in intron 4 of endothelial nitric oxide synthase gene might be a genetic risk factor of ONFH [16]. We could find the association of hypoxia inducible factor-α gene polymorphism with osteonecrosis in a Korean population [12].

In conclusion, we performed direct sequencing to detect polymorphisms and case-control association analysis between ONFH patients and normal controls with 4 selected SNPs of HMGCR gene. We did not find any evidence supporting an association of HMGCR genetic polymorphisms and haplotypes for ONFH and, we also did not find hypercholesterolemia to be associated with ONFH in a Korean population.

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초록 : 한국인에서 HMG-CoA reductase 유전자다형성과 대퇴골두무혈성괴사증과의 연관성 분석

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대퇴골두무혈성괴사증은 다원적인 질병으로 특정 집단의 경우 더 많은 위험성을 내포하고 있다. 특히 스테로이드의 과용과 알코올 남용 등으로 인한 지질대사의 변화는 골괴사증의 주요 원인 중 하나이다. 본 연구는 골괴사 환자와 대조군 사이에서 HMG-CoA reductase 유전자의 다형성과 질환발생과의 연관성에 대해 알아보았다. 24명의 한국인을 대상으로 HMG-CoA reductase 유전자를 시퀀싱하여 5곳의 유전자 다형성을 확인하였다. 349명의 남성 환자와 300명의 남성 대조군을 대상으로 네 곳(-6933C>T, -6045T>G, +12673G>A, +18128C>T)의 유전자 다형성의 빈도를 비교하였다. 그 결과 HMG-CoA reductase 유전자의 다형성과 질환발생 및 혈장 지질농도와는 어떠한 상관관계도 보이지 않았다.