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Molecules and Cells

Lack of Association of *MTHFR* Gene Polymorphisms with the Risk of Osteonecrosis of the Femoral Head in a Korean Population

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Some studies have suggested that coagulation disorders may be implicated in osteonecrosis of the femoral head (ONFH). The C677T polymorphism of the 5, 10-methylenetetrahydrofolate reductase (*MTHFR*) gene has been postulated to be a genetic risk factor for venous thromboembolism and osteonecrosis in Caucasians, but this relationship has not been established in other populations. In this study, we conducted case-control analysis of whether *MTHFR* polymorphisms are associated with ONFH in Korean patients. Fifteen single nucleotide polymorphisms (SNPs) were selected and genotyped in 443 ONFH patients and 273 control subjects using the TaqMan 5′ allelic discrimination assay. Comparison of ONFH and control subjects using logistic regression models revealed no statistically significant differences in the frequencies of the *MTHFR* polymorphisms and haplotypes. Further analysis stratified by etiology also showed no association. These results suggest that *MTHFR* polymorphisms play no significant role in susceptibility to ONFH in the Korean population.

INTRODUCTION

Osteonecrosis (ON) of the femoral head (ONFH) is a debilitating bone disease characterized by necrosis of the bone tissue, resulting in the collapse of the joint cartilage and femoral head and a subsequent loss of joint function (Assouline-Dayan et al., 2002). Although the pathophysiology of ONFH has not been fully elucidated, several possible causes have been suggested, including thrombophilia and hypofibrinolysis (Glueck et al., 1997), microvessel destruction (Matsui et al., 1992), fat embolism due to altered lipid metabolism, and increased bone-marrow pressure with fat cell enlargement (Kawai et al., 1985; Wang et al., 1977).

Recently, an increased tendency for intravascular coagulation has been proposed as a pathogenic mechanism of ONFH (Glueck et al., 1997; Jones, 1992; Zalavras et al., 2004). In support of this hypothesis, protein C and S deficiency, elevated levels of lipoprotein-a, the factor V Leiden mutation, and the prothrombin G20210A mutation have all been identified as genetic risk factors for hypercoagulability and ON (Bjorkman et al., 2004; Wermes et al., 1999; Zalavras et al., 2002b). Hyperhomocysteinemia has also been identified as an independent risk factor for thrombotic events and osteonecrosis (Glueck et al., 2001). Homocysteine promotes the formation of thromboembolic lesions by damaging vascular endothelial cells and enhancing coagulation activity on vascular walls (Lentz et al., 2002). The C677T (alanine to valine) polymorphism in the gene encoding 5, 10-methylenetetrahydrofolate reductase (*MTHFR*), an enzyme that plays a role in the remethylation of homocysteine, has been identified as a common cause of MTHFR enzyme deficiency (Kang et al., 1991) and has also been postulated to be a genetic risk factor for osteonecrosis (Glueck et al., 2001).

Almost all of the epidemiological evidence supporting associations between genetic polymorphisms and ON has been observed in Caucasian populations. However, there are obvious racial differences in the prevalence of these polymorphisms, and information on their relationship with ON is limited and inconclusive (Hessner et al., 1999; Hsu et al., 2001; Lu et al., 2002). Therefore, the purpose of this study was to investigate whether genetic variants of the *MTHFR* gene, including the C677T polymorphism, are associated with ONFH in a Korean population.

MATERIALS AND METHODS

Subjects

This study was approved by the Institutional Review Board of Kyungpook National University Hospital, and all individuals participating in the study gave their informed consent. A total of 443 unrelated patients with ONFH (366 men, 77 women; mean age: 49.7 ± 13.3) and 273 control subjects (206 men, 67 women; mean age: 52.1 ± 10.6) were consecutively enrolled at the Kyungpook National University Hospital (Korea) from 2002 to 2006. Patients were diagnosed and subgrouped by criteria that have been described in a previous study (Kim et

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Table 1. Clinical profiles of the ONFH patients and control subjects in this study

	Control (n = 273)	Total (n = 443)	Idiopathic (n = 181)	Alcohol-Induced (n = 206)	Steroid-induced (n = 56)
Age (mean ± SD)*	52.1 ± 10.6	49.7 ± 13.3	49.3 ± 14.2	52.0 ± 11.9	42.5 ± 13.2
Sex (male/female)*	206/67	366/77	131/50	201/5	34/22
Affected (Uni/Bilateral)		187/256	89/92	77/129	21/35
BMI (kg/m^2) (mean ± SD)	23.4 ± 3.01	23.2 ± 2.81	23.2 ± 2.70	23.3 ± 2.85	22.6 ± 3.04

*$P < 0.05$, for differences between patients and controls
SD, standard deviation

al., 2008). Briefly, patients were subgrouped according to etiological factors, into alcohol-induced (206 patients), steroid-induced (56 patients), and idiopathic (181 patients) osteonecrosis groups. Eleven patients were excluded from the study on the basis of a demonstrable history of femur fracture (9 patients) or multiple myeloma (2 patients). Control subjects were defined by a lack of hip pain and the absence of any lesions with a sclerotic margin or subchondral collapse consistent with ONFH in anteroposterior and frog-leg lateral pelvic radiographs. All persons related to patients were excluded from the control group. The clinical characteristics of controls and patients are summarized in Table 1.

Genotyping

Genomic DNA was isolated from peripheral blood leukocytes collected from each study participant using the FlexiGene DNA Kit (Qiagen, USA). Using public databases (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/, HAPMAP; http://www.Hapmap.org/index.html.en), a total of 15 single nucleotide polymorphism (SNP) sites on the *MTHFR* gene were selected on the basis of their location, allele frequencies, and disease relevance. Genotypes were determined using a TaqMan™ fluorogenic 5′-nuclease assay with predesigned TaqMan Probes (Applied Biosystems, USA). All reactions were carried out following the manufacturer's protocol. Detailed procedures regarding the PCR reaction and Taqman assay have been described previously (Lee et al., 2009). Primer Express (Applied Biosystems) 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 labeled with fluorescent VIC dye. The fluorescence data files from each plate were collected and analyzed using automated allele-calling software (SDS 2.2, Applied Biosystems). Genotyping quality control was performed on 10% of the samples by conducting duplicate analyses. The rate of concordance between duplicate samples was greater than 99%.

Statistical analyses

For subsequent analysis, SNP sites were required to meet the minimum criteria of a call rate (CR) > 95.0, a minor allele frequency (MAF) > 0.05, and Hardy-Weinberg equilibrium (HWE) > 0.05. Significant deviations from HWE in the genotype frequency of each SNP were evaluated using the χ^2 test. Statistical significance was determined by the *P* values obtained from logistical regression analyses, controlling for age and sex as covariates with three alternative models (codominant, dominant, and recessive). To assess the risk of phenotypes, the odds ratios (ORs) and 95% confidence intervals (CIs), were also estimated using a logistic regression procedure. The linkage disequilibrium (LD) between loci was measured by using the absolute value of Lewontin's D′ (|D′|) (Hedrick, 1987). Haploview 3.32 (http://www.broad.mit.edu/mpg/haploview/), which uses an accelerated expectation-maximization algorithm for haplotype analysis (Barrett et al., 2005), was used to estimate haplotype structures and their frequencies within LD blocks from the genotype data. Fisher's exact test or χ^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. All analyses were two-tailed, and *P* values < 0.05 were considered to be statistically significant. Power calculation was performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) (Purcell et al., 2003). Power was estimated with an α of 0.05, assuming the disease prevalence to be 1% and the risk allele frequencies to be the values observed in control samples.

RESULTS

In order to investigate the association between *MTHFR* gene polymorphisms and ONFH, fifteen polymorphic sites were genotyped in 443 ONFH patients and 273 control subjects. Among the 15 SNPs genotyped, six SNP sites did not fulfill our criteria of a CR > 95.0, MAF > 0.05, and HWE > 0.05 (Table 2). The *P* values of each polymorphism that met the criteria were compared between ONFH patients and controls using logistic analysis. As shown in Table 3, there were no significant differences in allele and genotype frequencies between the ONFH patients and control subjects. When patients were subdivided by etiology and an association analysis was conducted, none of the polymorphisms were associated with the risk of ONFH in any subgroup (data not shown).

Haplotype blocks were constructed using LD coefficients (|D′|) between all SNP pairs, and two blocks were identified (Fig. 1A). Based on LD coefficients, haplotypes were reconstructed (Fig. 1B), and haplotype frequencies were compared between normal controls and ONFH patients. As shown in Table 4, no significant differences were observed between patients and controls. Power analysis indicated that more than 80% power in detecting an association with ONFH was obtained when the genotype relative risk (GRR) was set at 1.35-1.63 under a multiplicative model of inheritance.

DISCUSSION

A number of genetic association studies have been conducted to link specific genes to the pathogenesis of ON. The majority of these studies have concentrated on gene polymorphisms affecting the coagulation and fibrinolytic systems (Ferrari et al., 2002; Zalavras et al., 2002a; 2002b). Several studies have reported that the factor V Leiden mutation (G1691A, Arg506Gln) increases the risk of primary ON (Bjorkman et al., 2004; 2005; Zalavras et al., 2004), but other studies have failed to observe

Table 2. SNP markers of the *MTHFR* gene genotyped in this case-control study

rs No.	Position	Amino acid substitution	Genotype				MAF	CR	Heterozygosity	HWE*
			C/C	C/R	R/R					
rs17367504	*Intron 2*		AA	AG	GG	N				
			566	138	11	715	0.112	99.9	0.199	0.238
rs9651118	*Intron 2*		TT	CT	CC	N				
			266	291	93	650	0.367	90.8	0.465	0.396
rs17037396	*Intron 2*		CC	CT	TT	N				
			549	141	12	702	0.118	98.1	0.207	0.236
rs2066461	*Exon 3*	Thr115Thr	GG	GT	TT	N				
			661	0	1	662	0.001	92.5	.	.
rs2066466	*Exon 3*	Thr139Thr	CC	CT	TT	N				
			708	0	0	708	0.000	98.9	.	.
rs4846052	*Intron 4*		CC	CT	TT	N				
			471	209	32	712	0.192	99.5	0.310	0.057
rs1801133	*Exon 5*	Ala222Val	GG	AG	AA	N				
		(C677T)	245	332	130	707	0.419	98.7	0.487	0.902
rs6541003	*Intron 5*		AA	AG	GG	N				
			477	206	32	715	0.189	99.9		0.029
rs1994798	*Intron 7 (boundary)*		AA	AG	GG	N				
			448	227	37	712	0.211	99.4	0.333	0.204
rs12121543	*Intron 7 (boundary)*		CC	AC	AA	N				
			450	204	28	682	0.191	95.3	0.309	0.222
rs1476413	*Intron 10 (boundary)*		CC	CT	TT	N				
			436	210	7	653	0.172	91.2	0.284	0.103
rs3818762	*Intron 11 (boundary)*		GG	CG	CC	N				
			472	211	32	715	0.192	99.9	0.311	0.079
rs2077360	*Exon 12 (3'-UTR)*		GG	AG	AA	N				
			610	0	0	610	0.000	85.2	-	-
rs11559040	*Exon 12 (3'-UTR)*		GG	AG	AA	N				
			598	108	8	714	0.087	99.7	0.159	0.423
rs1537514	*Exon 12 (3'-UTR)*		GG	CG	CC	N				
			583	120	10	713	0.098	99.6	0.177	0.177

MAF, minor allele frequency in cases and controls; CR, call rate (%); Heterozygosity, frequency of heterozygotes (two different alleles) for that locus.
*HWE: *P* values of deviation from Hardy-Weinberg Equilibrium in controls

these associations (Asano et al., 2004; Glueck et al., 1999). Significantly, Lee et al. (2003) reported finding no relationship between ONFH and factors associated with increased thrombosis or impaired fibrinolysis in Korean patients with ONFH. A possible explanation for these discrepancies may lie in geographic and ethnic differences in the prevalence of disease-associated mutations. The factor V Leiden and 20210A mutations were not observed in previous studies of Korean populations (Chang et al., 2008; Hessner et al., 1999; Kim et al., 2003), nor did they occur in our study population. Their absence suggests that neither mutation is a genetic risk factor for ONFH in Korean patients.

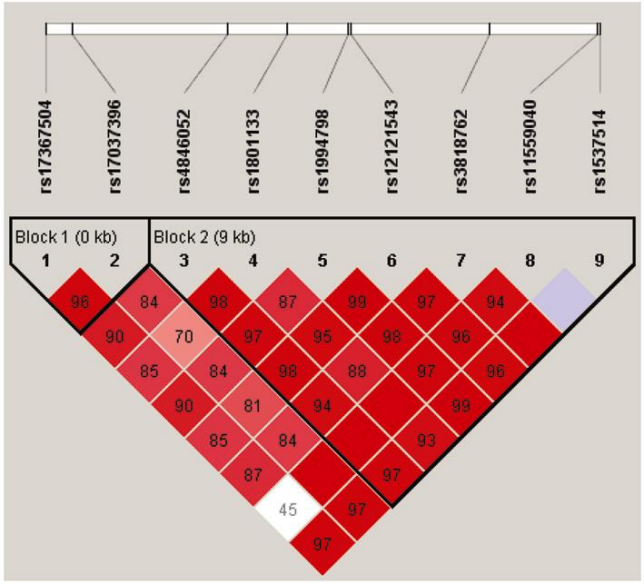
Several case-control studies have examined the association between the C677T polymorphism in *MTHFR* and ONFH. However, reports concerning the role of this polymorphism in the pathogenesis ONFH have been inconsistent (Asano et al., 2004; Glueck et al., 1999; Zalavras et al., 2002a). Glueck et al. (2001) reported a statistically significant association between the incidence of osteonecrosis and the *MTHFR* C677T gene mutation in 36 patients. Zalavras et al. (2002a) demonstrated a similar result in 66 patients. Chang et al. (2008) suggested that the C677T polymorphism plays a role in the pathogenesis of ON in Korean populations. In contrast, Asano et al. (2004) reported finding no association between the *MTHFR* C677T gene polymorphism and the development of osteonecrosis in a Japanese cohort. The data presented in this study do not support previous reports of a positive relationship between the *MTHFR* C677T variant and ONFH, particularly in Korean populations (Chang et al., 2008). The lack of consistency across these studies may be the result of the geographic and ethnic variability of populations or the probability of a type II error resulting from small sample sizes.

The prevalence of *MTHFR* C677T homozygosity (TT) appears to differ across ethnic groups. Frosst et al. (1995) reported the rate of homozygosity in French-Canadian subjects to be 12%. Other studies have reported rates of approximately 5% in a Caucasian population, 12-15% in populations of European, Middle Eastern, and Japanese origin, 5.4% in a Finnish population, and 1.4% in an African-American population (Motulsky, 1996). How-

Table 3. Analyses of association between *MTHFR* gene polymorphisms and the risk of ONFH

rs No.	Position	Geno-type	Frequencies (%)		Co-dominant		Dominant		Recessive	
			Cases	Controls	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
rs17367504	Intron 2	AA	352 (79.64)	214 (78.39)						
		AG	85 (19.23)	53 (19.41)	0.89 (0.64-1.25)	0.503	0.92 (0.64-1.34)	0.678	0.52 (0.16-1.73)	0.285
		GG	5 (1.13)	6 (2.20)						
rs17037396	Intron 2	CC	343 (78.13)	206 (78.33)						
		CT	90 (20.50)	51 (19.39)	0.95 (0.69-1.33)	0.781	1.00 (0.69-1.45)	0.995	0.57 (0.18-1.80)	0.335
		TT	6 (1.37)	6 (2.28)						
rs4846052	Intron 4	CC	292 (66.21)	179 (66.05)						
		CT	133 (30.16)	76 (28.04)	0.93 (0.71-1.21)	0.570	0.97 (0.71-1.34)	0.870	0.65 (0.32-1.33)	0.236
		TT	16 (3.63)	16 (5.90)						
rs1801133 (C677T)	Exon 5	GG	160 (36.53)	85 (31.60)						
		AG	201 (45.89)	131 (48.70)	0.86 (0.70-1.07)	0.185	0.80 (0.57-1.10)	0.168	0.86 (0.58-1.28)	0.462
		AA	77 (17.58)	53 (19.70)						
rs1994798	Intron 7	AA	275 (62.64)	173 (63.37)						
		AG	143 (32.57)	84 (30.77)	0.99 (0.76-1.28)	0.931	1.02 (0.74-1.40)	0.919	0.86 (0.44-1.68)	0.653
		GG	21 (4.78)	16 (5.86)						
rs12121543	Intron 7	CC	275 (65.17)	175 (67.31)						
		AC	131 (31.04)	73 (28.08)	1.05 (0.79-1.38)	0.750	1.10 (0.79-1.52)	0.588	0.86 (0.40-1.86)	0.700
		AA	16 (3.79)	12 (4.62)						
rs3818762	Intron 11	GG	288 (65.01)	184 (67.65)						
		CG	137 (30.93)	74 (27.21)	1.04 (0.80-1.36)	0.753	1.10 (0.80-1.52)	0.560	0.84 (0.41-1.74)	0.647
		CC	18 (4.06)	14 (5.15)						
rs11559040	Exon 12 (3′-UTR)	GG	368 (83.45)	230 (84.25)						
		AG	68 (15.42)	40 (14.65)	1.06 (0.73-1.54)	0.776	1.06 (0.70-1.60)	0.797	1.16 (0.27-4.96)	0.841
		AA	5 (1.13)	3 (1.10)						
rs1537514	Exon 12 (3′-UTR)	GG	362 (82.09)	221 (81.25)						
		CG	74 (16.78)	46 (16.91)	0.92 (0.65-1.31)	0.653	0.95 (0.64-1.40)	0.787	0.62 (0.175,2.171)	0.451
		CC	5 (1.13)	5 (1.84)						

A



B

		Haplotypes	Frequency
Block1	*ht1*	AC	0.881
	ht2	GT	0.109
Block2	*ht1*	CAACGGG	0.403
	ht2	CGACGGG	0.378
	ht3	TGGACGC	0.094
	ht4	TGGACAG	0.083

Fig. 1. Linkage disequilibrium coefficients of *MTHFR* polymorphisms and haplotypes of the *MTHFR* gene (A). Linkage disequilibrium coefficients (|D′|) and LD blocks among *MTHFR* polymorphisms (B). *MTHFR* haplotypes.

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

Loci	Genotype	Controls	Patients	Co-dominants		Dominant		Recessive	
				OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Block1-ht1 A-C	-/-	7 (2.66)	6 (1.37)	1.11 (0.80-1.54)	0.520	2.02 (0.67-6.13)	0.213	1.06 (0.73-1.53)	0.754
	ht1/-	53 (20.15)	90 (20.55)						
	ht1/ht1	203 (77.19)	342 (78.08)						
Block1-ht2 G-T	-/-	207 (78.71)	349 (79.68)	0.91 (0.65-1.28)	0.593	0.94 (0.64-1.36)	0.727	0.59 (0.17-2.09)	0.416
	ht2/-	51 (19.39)	84 (19.18)						
	ht2/ht2	5 (1.90)	5 (1.14)						
Block2-ht1 C-A-A-C-G-G-G	-/-	85 (33.60)	161 (38.61)	0.86 (0.69-1.08)	0.188	0.80 (0.58-1.12)	0.192	0.84 (0.56-1.28)	0.430
	ht1/-	122 (48.22)	190 (45.56)						
	ht1/ht1	46 (18.18)	66 (15.83)						
Block2-ht2 C-G-A-C-G-G-G	-/-	114 (45.06)	162 (38.85)	1.22 (0.97-1.51)	0.083	1.29 (0.94-1.78)	0.115	1.33 (0.86-2.06)	0.206
	ht2/-	104 (41.11)	181 (43.41)						
	ht2/ht2	35 (13.83)	74 (17.75)						
Block2-ht3 T-G-G-A-C-G-C	-/-	205 (81.03)	343 (82.25)	0.89 (0.62-1.28)	0.520	0.92 (0.62-1.38)	0.698	0.48 (0.13-1.83)	0.284
	ht3/-	43 (17.0)	70 (16.79)						
	ht3/ht3	5 (1.98)	4 (0.96)						
Block2-ht4 T-G-G-A-C-A-G	-/-	216 (85.38)	345 (82.73)	1.23 (0.82-1.85)	0.320	1.21 (0.78-1.86)	0.396	2.73 (0.30-24.8)	0.372
	ht4/-	36 (14.23)	68 (16.31)						
	ht4/ht4	1 (0.4)	4 (0.96)						

Block1: rs17367504 (A > G) / rs17037396 (C > T), Block2: rs4846052 (C > T) / rs1801133 (G > A) / rs1994798 (A > G) / rs12121543 (C > A) / rs3818762 (G > C) / rs11559040 (G > A) / rs1537514 (G > C)

ever, no clear association between the homozygous mutation and thrombosis could be demonstrated in patients with venous thromboembolism. In our study, the frequency of homozygosity in the control group was substantially higher (19.7%) than that reported in Caucasians and African-Americans. The majority of osteonecrosis in Korea is either idiopathic or related to excessive alcohol use (Kim and Rubash, 2006), and ONFH is one of the most common diseases of the hip in Korea. This incidence is relatively high compared with that in other countries. ONFH is the underlying cause of more than half of the total hip arthroplasty procedures conducted in Korea, whereas it is relatively rare in the Unites States. Thus, ethnic differences may play a significant role in the discrepancies observed across studies.

Another possible explanation for these conflicting results is the difference in sample size. The previous association studies between C677T and ONFH were carried out on small study populations (Glueck et al., 2001; Zalavras et al., 2002a), and this may have led to the generation of false positive or negative signals. Chang et al. (2008) reported an association between ONFH and the *MTHFR* C677T gene mutation in 71 patients. However, the association with ONFH shown by the C/T heterozygote in that study is difficult to explain in light of the additional finding that the T/T homozygote is not associated with ONFH. Our analysis of the effect of *MTHFR* variants on ONFH involved a relatively large patient population compared to previous studies. Power analyses indicated that, when genotype relative risk was set at 1.35-1.63 under a multiplicative model of inheritance, we would have 80% power in detecting any possible association. Typically, larger studies report weaker associations or no association at all, in comparison with smaller studies which more frequently report strong associations (Ioannidis et al., 2003). Sampling biases could also be responsible for conflicting results. For example, associations may only be found in extreme samples, or publication bias may lead to the preferential publication of significant results or preliminary findings over negative results.

In conclusion, our finding of no difference in the frequencies of *MTHFR* polymorphisms among ONFH patients and controls suggests there is no association between ONFH and *MTHFR* polymorphisms in Korean patients. Further studies, with larger sample sizes and detailed coagulation profiling, are necessary to fully assess the significance of this gene in ONFH.

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