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Methionine synthase reductase polymorphisms are associated with serum osteocalcin levels in postmenopausal women

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Abbreviations: BMD, bone mineral density; BMI, body mass index; Hcy, homocysteine; ht, haplotype; MTRR, methionine synthase reductase; YSM, years since menopause

Abstract

Homocysteine (Hcy) is thought to play an important role in the development of osteoporosis and fracture. Methionine synthase reductase (MTRR) is an enzyme involved in the conversion of Hcy to methionine. We hypothesized that certain genetic polymorphisms of *MTRR* leading to reduced enzyme activity may cause hyperhomocysteinemia and affect bone metabolism. We therefore examined the associations of the A66G and C524T polymorphisms of the *MTRR* gene with bone mineral density (BMD) and serum osteocalcin levels in postmenopausal women. Although we did not detect any significant associations between *MTRR* polymorphisms and BMD or serum osteocalcin levels, we found that the 66G/524C haplotype, which has reduced enzyme activity, was significantly associated with serum osteocalcin levels in a gene-dose dependent manner ($P = 0.002$). That is, the highest osteocalcin levels (34.5 ± 16.8 ng/ml) were observed in subjects bearing two copies, intermediate osteocalcin levels (32.6 ± 14.4 ng/ml) were observed in subjects bearing one copy, and the lowest levels of osteocalcin (28.8 ± 10.9 ng/ml) were observed in subjects bearing no copies. These results suggest that the 66G/524C haplotype of the *MTRR* gene affect bone turn over rate.

Keywords: bone density; methionine synthase reductase; osteocalcin; polymorphism; postmenopause

Introduction

Hyperhomocysteinemia has been reported to be an independent risk factor for osteoporotic fractures, cardiovascular disease and cancer (Song *et al.,* 2001; Wald *et al.,* 2002; Gilfix, 2003; Lee *et al.,* 2004; McLean *et al.,* 2004; van Meurs *et al.*, 2004; Sato *et al.,* 2005). Treatment with vitamin B12 and folate, which lower Hcy levels, has been found to markedly decrease the risk of hip fracture in stroke patients (Sato *et al.,* 2005), indicating that Hcy may play an important role in the development of osteoporosis. Although the precise mechanisms of Hcy-induced osteoporosis has not been determined, interfering with collagen cross-linking (Lubec *et al.,* 1996), increased osteoclastic bone resorption (Herrmann *et al.,* 2005) and decreased osteoblastic bone formation (Kim *et al.,* 2006), have been reported to contribute to the development of Hcy- induced osteoporosis.

Hcy, an intermediate metabolite of methionine, is reconverted to methionine by the transfer of a methyl group from methylenetetrahydrofolate, a reduction catalyzed by the enzyme methionine synthase (Dimitrova *et al.,* 2002). This remethylation process requires adequate levels of the cofactor, activated cobalamin, which are maintained by the enzyme *MTRR*. Thus reduced activity of *MTRR* should disturb the remethylation of Hcy resulting in hyperhomocysteinemia. Indeed, a genetic defect in *MTRR* has been detected in patients with homocystinuria,

an inherited disorder of Hcy metabolism characterized by severe hyperhomocysteinemia and early onset of atherosclerosis and osteoporosis (Leclerc *et al.,* 1998). Although the A66G and C524T genetic polymorphisms of *MTRR* have been reported to be associated with enzyme activity (Olteanu *et al.,* 2002) and plasma Hcy concentration (Botto *et al.,* 2003; Vaughn *et al.,* 2004), the association of these polymorphisms with bone metabolism has not been determined. We therefore investigated the relationships between the A66G and C524T polymorphisms of *MTRR* gene and bone mineral density and serum osteocalcin levels in postmenopausal women.

Materials and Methods

Subjects

The study population consisted of 560 apparently healthy, postmenopausal women who had visited Asan Medical Center (Seoul, Korea) (Kim *et al.,* 2005). In brief, menopause was defined as the absence of menstruation for at least 6 months, which was confirmed by a serum FSH concentration > 30 IU/l. Women who were prematurely menopausal (under 40 years of age) were excluded. Subjects were also excluded if they had taken drugs, such as bisphosphonates, estrogen and thyroid hormones, that might affect bone metabolism for more than 6 months or within the previous 12 months, or if they had suffered from any disease, such as thyroid diseases, hyperparathyroidism and renal failure, that might affect bone metabolism. Women were also excluded if they had osteophytic formation above the fourth grade of the Nathan classification (Nathan *et al.,* 1962), and/or severe facet joint osteoarthritis in the lumbar spine diagnosed using conventional spine radiographs. The study was approved by the Institutional Review of Board of Asan Medical Center, and written informed consent was obtained from each participant.

BMD measurement

BMD at the lumbar spine (L2-L4) and femoral neck was measured using dual-energy X-ray absorptiometry (Lunar, Expert XL, Madison, WI) in 431 women. In the remaining 129 women, BMD was measured using Hologic equipment (QDR 4500-A, Waltham, MA). The coefficients of variation (CV) for the Lunar and Hologic equipment were 0.82% and 0.85%, respectively, for the lumbar spine and 1.12% and 1.20%, respectively, for the femoral neck.

Table 1. PCR primers and probes used for *MTRR* genotyping.

Locus	Primer sequence (5'-3')	
A66G	Forward	AGCAGGGACAGGCAAAGG
	Reverse	GCAGAAAATCCATGTACCACAGCTT
	Probe-1 (VIC)	ATCGCAGAAGAAATATGTGA
	Probe-2 (FAM)	ATCGCAGAAGAAATGTGTGA
C524T	Forward	ACTCCCGGTGGCATCAC
	Reverse	ATGTGTAGCAGCTCTGACTTCAC
	Probe-1 (VIC)	CTGCATCCTCGAGGAC
	Probe-2 (FAM)	CTGCATCCTTGAGGAC

Measurement of serum osteocalcin levels

Fasting venous blood samples were obtained between 8 and 10 A.M. and centrifuged, and the sera were stored at -80°C until assayed. Serum osteocalcin concentrations were determined using an immunoradiometric assay kit (OSTEO-RIACT, CIS bio international, France). The mean inter-assay CV was 2.8%, and the mean intra-assay CV was 5.2%.

Genotyping analysis

Genomic DNA was extracted from peripheral blood leukocytes using a Wizard Genomic DNA purification kit (Promega, Madison, WI). For genotyping of the *MTRR* gene (Ref. Seq. of *MTRR* mRNA: NM_002454 and contig: NT_006576), PCR primers and probes were designed by Primer Express (Applied Biosystems, Foster City, CA) (Table 1). One allelic probe was labeled with the FAM dye and the other with the fluorescent VIC dye. PCR reaction were performed in a 384-well format in TaqMan Universal Master mix without UNG (Applied Biosystems), in a total reaction volume of 5 μl using 20ng of genomic DNA, PCR primer concentrations of 900 nM and TaqMan MGB-probe concentrations of 200 nM. The plates were placed in a PE 977 thermal cycler (Applied Biosystems) and heated at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The TaqMan assay plates were transferred to a Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity in each well of the plate was read. Fluorescence data files from each plate were analyzed using automated software (SDS 2.1; Applied Biosystems).

Statistics

χ^2 tests were used to determine whether individual variants at each locus were in Hardy-Weinberg equilibrium in the population. We exmamined Le-

wontin's D'(|D'|) and the linkage disequlibrium coefficient, r2, between the biallelic loci (Hedrick, 1987; Hedrick *et al.,* 2001).

Haplotypes (ht) of each individual were inferred using the algorithm (PHASE; Stephens *et al.*, 2001) which uses a Bayesian approach incorporating *a priori* expectations of haplotypic structure based on population genetics and coalescent theory. Phase probabilities of all polymorphic sites for haplotypes were calculated for each individual using this software. Individuals with phase probabilities of less than 97% were excluded in analysis. The genetic effects of inferred haplotypes were analyzed in the same way as polymorphisms. Multiple regression analyses were performed for BMD controlling for age (continuous variable), years since menopause (YSM; continuous variable), weight, height, and type of bone densitometer as covariates, and osteocalcin controlling for age (continuous variable), YSM (continuous variable), weight and height. A value of $P < 0.05$ was considered statistically significant.

Results

The mean age, height, weight, body mass index (BMI) and YSM of the subjects were 59.4 ± 7.2 years (range 46-83 years), 154.8 ± 5.3 cm, 56.5 ± 7.4 kg, 23.6 ± 2.9 kg/m^2 and 10.4 ± 8.2 years (range 1-35 years), respectively. As expected, the age, YSM, weight, and height were each significantly correlated with BMD at both the lumbar spine and femoral neck (Table 2). BMD measured by Lunar equipment (0.870 ± 0.182 g/cm^2 at the lumbar spine and 0.723 ± 0.128 g/cm^2 at the femoral neck) was significantly higher than BMD measured by Hologic equipment (0.764 ± 0.116 g/cm^2 at the lumbar spine and 0.606 ± 0.098 g/cm^2 at the femoral neck. $P < 0.0001$ for each). Therefore, we added the type of the densitometry as a covariate during statistical analysis (Kim *et al.,* 2005). As previously described (Cifuentes *et al.,* 2003) body weight was found to be negatively correlated with serum osteocalcin levels (Table 2) and was included as a covariate for statistical analysis. Regression analysis revealed that *MTRR* polymorphisms showed no association with BMI.

The frequencies of variant alleles for the A66G and C524T polymorphisms were 0.29 and 0.13, respectively. The polymorphisms were in Hardy-Weinberg equilibrium. Two sites were not in linkage disequilibrium ($r^2 = 0.156$, linkage disequilibrium coefficient = 0.637). The frequency of the 66G allele was significantly lower than observed in Caucasian subjects (0.29 vs. 0.5; Wilson *et al.,* 1999).

We analyzed the associations between BMD and these polymorphisms after adjustment for age, YSM, weight, height and type of bone densitometer (Table 3). We found, however, that neither polymorphism was associated with BMD at the lumbar spine and femoral neck. Although we observed weak associations between the A66G and C524T alleles and serum osteocalcin concentrations, these associations were not significant (Table 4).

Since the haplotype, including 66G/524C alleles (frequency 0.19), was reported to be associated with

Table 2. Clinical profiles and multiple regression analyses of BMD

Clinical profiles		Lumbar spine BMD			Femoral neck BMD			Osteocalcin		
Variables	Mean ± SD	β	SE	*P*	β	SE	*P*	β	SE	*P*
Age (years)	59.4 ± 7.2	-0.004	0.002	0.01	-0.003	0.001	0.005	-0.310	0.172	0.07
Weight (kg)	56.5 ± 7.4	0.006	0.001	< 0.0001	0.003	0.001	< 0.001	-0.243	0.087	0.006
Height (cm)	154.8 ± 5.3	0.003	0.001	0.013	0.002	0.001	0.09	0.203	0.131	0.12
YSM (years)	10.4 ± 8.2	-0.003	0.002	0.02	-0.005	0.001	< 0.001	0.265	0.152	0.08
Densitometer	-	-0.125	0.015	< 0.001	-0.135	0.010	< 0.001			
Spine BMD (g/cm^2)										
Lunar (431)[a]	0.870 ± 0.182									
Hologic (129)[a]	0.764 ± 0.116									
Femoral neck BMD (g/cm^2)										
Lunar	0.723 ± 0.128									
Hologic	0.606 ± 0.098	$R^2 = 0.31$			$R^2 = 0.45$			$R^2 = 0.028$		

[a]Number of subjects who received BMD examination by each densitometer.

Table 3. Lumbar spine and femoral neck BMD (g/cm^2) relative to *MTRR* genotype.

Locus	Lumbar spine BMD (g/cm^2)				Femoral neck BMD (g/cm^2)			
	C/C*	C/R	R/R	*P*	C/C	C/R	R/R	*P*
A66G	0.85 ± 0.17 (281)	0.85 ± 0.18 (232)	0.84 ± 0.20 (42)	NS	0.70 ± 0.13	0.7 ± 0.13	0.69 ± 0.14	NS
C524T	0.85 ± 0.16 (413)	0.84 ± 0.20 (135)	0.84 ± 0.17 (7)	NS	0.69 ± 0.13	0.7 ± 0.14	0.73 ± 0.15	NS
66G/524C	0.85 ± 0.17 (362)	0.85 ± 0.17 (177)	0.84 ± 0.2 (16)	NS	0.70 ± 0.13	0.70 ± 0.13	0.66 ± 0.16	NS

Data adjusted for age, years since menopause, weight, height, and densitometry. All results presented as mean ± SD (number of subjects). *C/C, C/R, and R/R represent homozygotes for the common allele, heterozygotes and homozygotes for the rare allele, respectively.

Table 4. Serum osteocalcin levels (ng/ml) relative to *MTRR* genotype.

Locus	Genotype			*P* value
	C/C*	C/R	R/R	
A66G	29.3 ± 11.4 (220)	30.9 ± 13.3 (168)	32.1 ± 14.3 (31)	NS
C524T	30.6 ± 13.0 (315)	29.1 ± 10.4 (98)	24.5 ± 6.5 (6)	NS
66G/524C	28.8 ± 10.9 (282)	32.6 ± 14.4 (122)	34.5 ± 16.8 (15)	0.002†

Data adjusted for weight. All results presented as mean ± SD (number of subjects). *C/C, C/R, and R/R represent homozygotes for the common allele, heterozygotes and homozygotes for the rare allele, respectively. † *P* value for both codominant and dominant model.

reduced enzyme activity (Olteanu *et al.,* 2002), we examined the association of this haplotype with BMD and osteocalcin concentration. Although we observed no difference in BMD at the lumbar spine and femoral neck according to the haplotype (Table 3), we observed an association between this haplotype and serum osteocalcin levels ($P = 0.002$ in both codominant and dominant models) in a gene-dose dependent manner. That is, the highest osteocalcin concentrations (34.5 ± 16.8 ng/ml) were detected in individuals bearing two copies of this haplotype, intermediate levels (32.6 ± 14.4 ng/ml) were detected in subjects bearing one copy, and the lowest levels (28.8 ± 10.9 ng/ml) were observed in subjects bearing no copies (Table 4).

Discussion

In relation to Hcy-induced osteoporosis, some studies (Miyao *et al.,* 2000; Abrahamsen *et al.,* 2003; Bathum *et al.,* 2004; Villadsen *et al.,* 2005) have reported that the C677T polymorphism of the gene encoding methylenetetrahydrofolate reductase, which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, the substrate for Hcy methylation, was associated with osteoporosis and increased fracture risk. However, to our knowledge, an association between the polymorphisms of *MTRR* gene and bone metabolism has not been reported.

We have shown here that the *MTRR* haplotype (66G/524C) is associated with serum osteocalcin concentrations in postmenopausal women. Although neither polymorphism was significantly associated with BMD and serum osteocalcin levels, we observed trends between these *MTRR* polymorphisms and osteocalcin concentrations. Specifically, subjects with the 66G and 524C alleles had higher serum osteocalcin levels than those with the 66A and 524T alleles, respectively. The positive association of the 66G/ 524C haplotype with osteocalcin levels may result from the sum of the modest effects of each individual allele on bone metabolism.

MTRR is a dual flavoprotein that catalyses the conversion of cob(II)alamin into methyl-cob(III)alamin, the cofactor of methionine synthase (Olteanu *et al.,* 2001; 2002). FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide) facilitate electron transfer from NADPH to cob(II)alamin during reductive methylation. This reductive methylation of cob(II)alamin may serve to maintain methionine synthase in its active form. The A66G and C524T sequence variants change the coding sequence from isoleucine to methionine (Ile22Met) and from serine to leucine (Ser175Leu), respectively. The A66G variant is located in the FMN domain and may therefore interact with methionine synthase (Hall *et al.,* 2000; 2001). Although we did not measure plasma Hcy levels, the 66G/524C haplotype was found to have 4-fold lower enzyme activity than wild type (Olteanu *et al.,* 2002) and the 66G allele has

been reported to be associated with higher plasma Hcy concentrations (Botto *et al.,* 2003; Vaughn *et al.,* 2004). Our finding, demonstrating that the 66G/524C haplotype was associated with higher osteocalcin concentrations, further supports that hyperhomocysteinemia is associated with higher osteocalcin levels (Dhonukshe-Rutten *et al.,* 2005).

Osteocalcin is a product of osteoblasts that is considered a marker of bone formation (Brown *et al.,* 1984). However, osteocalcin is also released from bone matrix into blood during bone resorption, suggesting that osteocalcin is also a marker of bone turnover (Delmas *et al.,* 1990; Page *et al.,* 1993; Ivaska *et al.,* 2004). Thus the higher serum osteocalcin levels observed in individuals with the 66G/524C haplotype may reflect increased bone turnover rather than simply increased bone formation and thus may be associated with an increased risk of fracture (Garnero *et al.,* 1996).

Osteoporosis is a skeletal disorder characterized by compromised bone strength, which predisposes individuals to an increased risk of fracture (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, 2001). In patients with homocystinuria, severe hyperhomocysteinemia is associated with very low bone mass (Parrot *et al.,* 2000). In the general population, mild to moderate hyperhomocysteinemia is associated with an increased risk of fracture (McLean *et al.,* 2004; van Meurs *et al.,* 2004; Sato *et al.,* 2005), but not with BMD, suggesting that mild to moderate increases in Hcy concentration may compromise bone strength by reducing bone quality rather than bone density. This may explain, at least in part, the lack of association between *MTRR* polymorphisms and BMD.

This study has some limitations. Since serum Hcy concentration was not measured in our study subjects, we could not document a direct association between the *MTRR* 66G/524H haplotype and serum homocysteine concentration. However, previous studies, demonstrating an association of *MTRR* 66G/524H haplotype with low enzyme activity and thus high plasma Hcy level, suggest that this haplotype has a functional relevance in regulation of Hcy levels in plasma. We did not measure the concentration of other biochemical markers of bone turnover. Measurement of other specific bone turnover markers would support the relationship between *MTRR* haplotype and bone metabolism. In addition, we did not measure the serum concentrations of vitamin B12 and folate concentration. Since the effect of the A66G polymorphism of *MTRR* would be more pronounced in subjects with low cobalamin levels (Wilson *et al.,* 1999), our results assume no difference in cobalamin and folate concentrations.

In conclusion, we found that the 66G/524C haplotype of the *MTRR* gene was associated with serum osteocalcin concentrations in postmenopausal women, suggesting that this haplotype may affect bone turnover rate.

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