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Association of the Methylenetetrahydrofolate reductase C677T polymorphism and fracture risk in Chinese postmenopausal women*

Xiumei Hong^{a,b,d}, Yi-Hsiang Hsu^c, Henry Terwedow^c, Genfu Tang^d, Xue Liu^d, Shanqun Jiang^d, Xin Xu^c, and Xiping Xu^{a,b,d}

a School of Life Science, University of Science and Technology of China, Huangshan Road, Hefei City, Anhui Province, China

b Center for Population Genetics, Division of Epidemiology and Biostatistics, School of Public Health, University of Illinois at Chicago, Chicago, IL

c Program for Population Genetics, Harvard School of Public Health, Boston, MA

d Anhui Medical University, Institute of Medicine, Anhui, China

Abstract

Osteoporotic fractures are a leading cause of disability and, indirectly, of death in the elderly population. Previous studies have shown that homocysteine level and the C677T polymorphism in the gene encoding methylenetetrahydrofolate reductase (MTHFR) may be involved in the development of osteoporosis and its related fracture in European populations. The aim of this study was to verify the association of this polymorphism with bone mineral density (BMD) and fractures in our 1899 Chinese postmenopausal women. The C677T T-allele frequency in this population was 39.2%. The distribution of the MTHFR genotypes followed the Hardy-Weinberg equilibrium. BMD at total body, total hip or femoral neck did not significantly vary with MTHFR C677T genotype. The T-allele carrier tended to have higher risk of having osteoporosis or osteopenia, but the difference was statistically insignificant. However, Poisson regression analysis revealed that the T-allele carriers had an increased risk of fractures (RR=1.7, 95%CI=1.1–2.7, $p=0.01$) which occurred before or after menopause. As far as fracture incidence after menopause was concerned, the CT or TT genotype had more than twice the risk of the CC genotype (RR=2.5, 95%CI=1.2–4.9, $P=0.009$). This association was independent of age, physical activity, occupation, passive smoking, height, weight, years since menopause, and total hip BMD.

Our data show that the MTHFR C677T polymorphism is an independent predictor of fracture risk, although it only had a weak effect on BMD. Further study on the mechanistic role that this polymorphism plays in the development of fractures may lead to better understanding of the etiology of osteoporotic fracture.

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Correspondence: Xiping Xu, M.D., Ph.D., Professor, Director, Center for Population Genetics, Division of Epidemiology and Biostatistics, School of Public Health M/C 923, University of Illinois at Chicago, 1603 W Taylor, Rm 978B, Chicago, IL 60612, TEL: 312-413-3667 FAX: 312-996-0064, Email: xipingxu@uic.edu.

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Keywords

Methylenetetrahydrofolate Reductase Gene; Fracture; Osteoporosis; Genetics; Postmenopausal Women

Introduction

Osteoporosis is a common metabolic bone disorder characterized by reduced bone mass, increased skeletal fragility and microarchitectural deterioration, and, as a consequence, increased bone fracture [1]. Osteoporotic fracture is a leading cause of disability and, indirectly, death in the elderly [2]. 30% of women and 12% of men are affected at some point during life and, thus, imposing a major economic burden on society.

Homocysteine is a thiol-containing amino acid formed during the metabolism of methionine. Recently, several lines of evidence indicate that high homocysteine levels are involved in the pathology of osteoporosis and bone fractures. First, homocysteinuria is a rare autosomal recessive disease characterized by increased circulating homocysteine levels and by early-onset osteoporosis and fractures [3,4]. Second, in animal studies, chicks fed a homocysteine-supplemented diet had altered bone growth, bone matrix, and bone composition when compared with control chicks[5]. Third, recent data from two cohort studies show that total homocysteine plasma level is positively related with osteoporotic fractures[6,7]. Moreover, in the NHANES III, men and women with homocysteine levels $\geq 20\mu\text{mol/L}$ had significantly lower hip bone mineral density (BMD) than individuals with homocysteine levels $< 10\mu\text{mol/L}$ [8].

It is believed that several enzymes, including 5,10 Methylenetetrahydrofolate reductase (MTHFR), play a key role in the metabolism of homocysteine. A point mutation in the MTHFR gene (C677T), which induces a substitution of valine for alanine, is a common variant, which is associated with reduced enzyme activity and with increased homocysteine levels. Some studies reported this polymorphism to be associated with either an increased risk of osteoporotic fractures[9,10] or lower BMD [11–14]. However, other studies fail to confirm this association. For example, Jorgensen et al. reported a reduced risk of osteoporotic fractures in postmenopausal women heterozygous or homozygous for the polymorphism T677 in MTHFR [14]; Conversely, Golbahar et al found no association between this polymorphism and femoral neck or lumbar spine BMD in Iranian women [15]. This variation among studies may partly be due to the differences in study populations, such as ethnicity, age, environment, and genetic make-up.

In this investigation, we studied a subset of postmenopausal women taken from a large community-based osteoporosis study conducted in Anhui Province, China in year 2003–2005. In this subset, we evaluated the effect of the MTHFR C677T polymorphism on BMD at total hip, total body and femoral neck and on the risk of bone fractures.

Material and Methods

Study population

This study is part of a large community-based osteoporosis study initiated in 2003, which has been previously described[16]. In brief, among residents of Anhui Province, China, men and women with (1) aged 25–64 years and (2) a minimum of three participating siblings were recruited. Participants with a history of the following conditions were excluded, including diabetes mellitus-Type 1, renal failure, chronic infections such as TB or other diseases, malignancy, or rickets or other metabolic bone diseases. Women, self-reported without

menstrual cycle for more than one year and not due to surgery (hysterectomy or oophorectomy), pregnancy, or lactation, were considered as postmenopausal women. Only postmenopausal women without a history of cigarette smoking were genotyped and analyzed in current study. We also excluded 7 postmenopausal women with a history of calcium and/or multiple-vitamins supplements for 6 or more cumulated-months, and 5 postmenopausal women with a history of estrogen treatment after menopause. Finally, 2245 postmenopausal women were available for this study. The study protocol was approved by the Human Subjects Committee (the institutional review board, IRB) of the Harvard School of Public Health and by the Ethics Committee of Anhui Medical University. Written informed consent was explained to, read and signed by each participant.

BMD measurement and fracture identification

Dual X-ray absorptiometry (DXA, GE-Lunar Prodigy, Madison, WI) was used to measure bone mineral content (BMC) and BMD (g/cm^2) through whole-body and total hip scans. We defined osteoporosis as a total hip BMD of more than 2.5 standard deviations (SDs) below the average peak BMD of young healthy Chinese in the same study area ($T\text{-score} < -2.5$). Osteopenia was defined as a total hip BMD between 1–2.5 SDs below the peak BMD ($-2.5 < T\text{-score} < -1$). Bone fracture information was collected with a questionnaire. Fracture frequency, fracture site, and the age(s) at which the participants had their first and last fractures were recorded. In this investigation, only first bone fracture events were analyzed. To increase statistical power, the first fracture in any skeletal location was documented as an outcome measure. However, hand, foot, skull and face fractures thought to be due to nonosteoporotic events, were excluded.

Potential confounders

Height was measured without shoes to the nearest 0.1 cm on a portable stadiometer and weight was measured in light indoor clothing without shoes to the nearest 0.1 kg. Body mass index (BMI) was calculated as $\text{weight}/\text{height}^2$ (kg/m^2). Information on participants' gender, date of birth, disease history, occupation, physical activity level, and menstrual and reproductive history, including age at menarche, date of last menstrual period, history of HRT, were collected by standardized questionnaire. A diet questionnaire was used to collect each participant's consumption of local food items/groups, such as staple food (mainly rice), milk, seafood, preserved food, meat (mainly pork), fruit and vegetables.

SNP genotyping

Fasting venous blood samples were obtained from the study participants and genomic DNA was extracted from blood lymphocytes by a standard salting-out procedure. The MTHFR C677T genotyping was performed by polymerase chain reaction with the primers 5'-CAA AGG CCA CCC CGA AGC -3' (sense) and 5'-AGG ACG GTG CGG TGA GAG TG-3' (anti-sense). Samples were amplified for 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 59 °C for 45 s, and extension at 68 °C for 45 s; and then followed by a final extension step at 68 °C of 7 min. Since the c-to-t transition at nucleotide 677 produces a Hinf I digestion site, the endonuclease can cleave the amplified product derived from the mutant restriction site into 175-bp and 70-bp fragments, and leaves the wild-type gene unaffected. The digested products were separated electrophoretically on 3% agarose gels. After genotyping, 1899 subjects with available DNA samples and complete genotyping information were in the final analysis. There is no significant difference in the principal characteristics of subjects with complete genotyping information and subjects with missing genotyping information.

Statistical methods

The main outcomes of this study are osteoporosis/osteopenia status and fracture incidence. Multivariate linear GEE regression model adjusted for age, height, weight, physical activity, passive smoking, years since menopause was used to test the association of BMD at total body, total hip, femoral neck with MTHFR C677T genotyping. Multivariate logistic regression models adjusted for age, physical activity, occupation, passive smoking, height, weight, years since menopause were used to estimate the independent risk of this polymorphism on osteoporosis and osteopenia.

We used two outcome variables to calculate the incidence of first bone fracture. The first variable was the incidence of overall first bone fracture events occurring before or after menopause, and which was expressed as the accumulation of person-years at risk starting from date of birth until the date of first fracture or until the data collection date. The second variable was the incidence of first bone fracture occurring after menopause, and which was expressed as the accumulation of person-years at risk starting from the age at menopause until the date of first fracture or until the data collection date. When the latter variable was analyzed, 57 subjects, whose first bone fracture occurred during pre-menopause, were excluded.

Poisson regression was used to calculate the risk ratios and the 95 percent confidence intervals of the incidence of bone fracture by MTHFR C677T genotypic classes, with adjustment for age, height, weight, physical activity, occupation, passive smoking, years since menopause, and total hip BMD.

We further used the dietary variables (such as weekly consumption of seafood, weekly consumption of fruits and weekly consumption of meats) as surrogate markers for vitamin B complex/folate intakes and then tested the interactive effect between each dietary variable and MTHFR genotypes for BMDs and fractures by adding a product term to the regression model.

Results

Among 1899 postmenopausal subjects, the C677T allele frequencies were in Hardy-Weinberg equilibrium ($\chi^2=3.7$, $p=0.06$). The distribution of the genotypes: CC, CT and TT were 35.9%, 49.8%, and 14.3%, respectively. The population characteristics of each genotypic group are showed in Table 1. Differences in these characteristics among the genotypic classes were compared by an ANOVA test for continuous variables and by a χ^2 -test for categorical variables. None of these characteristics varied significantly among the genotypic classes. Regarding diet information, we found that on a weekly basis, 98.1% of the subjects consumed more than 1500g of vegetables, 99.1% subjects drank less than 250g of milk, 77.4% consumed less than 250g of fish, 59.1% subjects consumed more than 250g of preserved food, 82.3% subjects consumed less than 250g of fruits and 68.3% consumed less than 250g of meat. The distribution of the food frequency across genotyping groups was comparable by a χ^2 -test ($p>0.05$).

MTHFR genotyping, bone mineral density and osteoporosis

As shown in Figure 1, the average total body, total hip and femoral neck BMDs did not vary significantly with different MTHFR C677T genotypes. After adjustment for age, physical activity, occupation, passive smoking, height, weight, years since menopause in the multivariate regression model, the MTHFR C677T polymorphism was still insignificantly related to BMD at each bone site (data not shown).

As shown in Table 2, T-allele carriers tended to have a higher risk of having osteoporosis and have higher risk of having osteopenia/osteoporosis, *but the associations were statistically insignificant* [$P>0.05$].

MTHFR genotyping and bone fracture

109 subjects had a self-reported bone fracture in hip, vertebra, wrist, arm, leg, ankle or pelvis and were included in this analysis. The average age at first fracture was 41.7 ± 13.2 years old. Fifty-two of these fracture events occurred after menopause. The distribution of fracture types stratified by genotypes is shown in Table 3. Generally, the major type of fracture was wrist fracture in each genotype. The CT or TT genotype tended to be related with a higher incidence rate of fractures or fractures after menopause.

Adjusting for age, physical activity, occupation, passive smoking, height, weight, years since menopause, and total hip BMD, compared to subjects with CC genotype, the relative risk (RR) of having bone fracture was 1.7 (95% CI=1.1–2.7, $p=0.02$) and 1.9 (95% CI=1.1–3.3, $p=0.02$), respectively for the subjects with CT genotype and with TT genotype. When combined the TT, CT genotypes, the RR of having bone fracture was 1.7 (95% CI=1.1–2.7, $p=0.01$) for subjects carrying the T-allele, and the proportion of bone fractures attributable to TT or CT genotype. Regarding the incidence of first bone fracture occurring after menopause, the MTHFR C677T genotype also presented a significant, T-allele dominant effect. Fracture incidence after menopause in the T-allele carriers was increased by a factor of 2.5 (95% CI=1.2–4.9, $P=0.009$), compared to subjects carrying the CC genotype. [Table 4].

Further, we used the available dietary variables as surrogate markers for vitamin B complex/folate intakes and then tested the interactive effects between the dietary variables and MTHFR genotypes for BMDs and fractures, but no significant interaction was detected [data not shown].

Discussion

Our data demonstrate a significant and independent impact of the MTHFR C677T genotype on the risk of fractures in postmenopausal Chinese women. The CT or TT genotypes had an increased risk of fracture occurring before or after menopause. The incidence rate of fracture after menopause was increased by a factor of 2.5 in T-allele carriers, compared to subjects carrying the CC genotype.

The frequency of the MTHFR 677T allele varies substantially in different regions of the world and among ethnic groups. The allele frequency is 0.09 in west Africans[17], and 0.06 in Canadian Inuits[18], while in Caucasian, Japanese, and Chinese populations, the allele frequency is much higher [19]. A north-to-south increase of allele 677T prevalence has been observed in Europe [20]. In contrast, a north-to-south decrease of TT genotype was reported in China [21]. More interestingly, the distribution of the MTHFR genotype may modify its effect on the plasma homocysteine[17]. All these findings strengthen the importance of studying the influence of this polymorphism in different populations. In this investigation, subjects were enrolled from south-central China. The frequency of the TT genotype and the T-allele are 14.3% and 39.2%, respectively, which is comparable to a previous study from this region in China[21]. The high prevalence of the T-allele and the TT genotype in our Chinese population indicates that population attributable risk due to the MTHFR C677T polymorphism may be relatively greater than that seen in many previously reported studies.

A significant association between the MTHFR TT genotype and low lumbar spine and total body BMD has been shown in postmenopausal Japanese women[13]. This result could not be confirmed in a previous Chinese study [22], or in the present study, although we found that the TT genotype tended to have higher risk of osteopenia or osteoporosis, which is statistically insignificant. An explanation for this discrepancy may lie in differences in the intake of nutritional factors, such as riboflavin, B6, B12, and folate, which are necessary to homocysteine metabolism [23,24]. A recent study[25] in a Scottish female cohort suggests that BMD is decreased in the TT genotype only if riboflavin intake is low. Accordingly, nutritional factors

could be confounders in the relationship between the MTHFR polymorphism and BMD. In our study, self-reported medication history (oral contraception, Hormone Replacement Therapy (HRT), calcium, vitamin D and multiple vitamins supplements) was collected. Only 7 subjects had a history of calcium and/or multiple-vitamins supplements for 6 or more cumulated-months and were excluded. Leafy green vegetables (like spinach and turnip greens), fruits (like citrus fruits and juices), and dried beans and peas are all natural sources of folate. The B-group vitamins, although present in many foods, are delicate and easily destroyed, particularly by alcohol and cooking. Food processing also removes B vitamins, making white flours, breads and rice less nutritious than their whole grain counterparts. We collected participants' consumption of local food items, such as milk, seafood, preserved food, meat, vegetables, and fruits based on a simple questionnaire [26], but, due to the nature of the questionnaire, we can not quantify the intake of vitamin B complex intake or folate from daily diet. However, the main source of food in our study cohort is limited and the participant dietary intake was relatively homogenous. The distribution of dietary variables in this investigation was comparable across genotyping groups. We also used these dietary variables as surrogate markers for vitamin B complex/folate intakes and estimated their interactive effects with MTHFR genotypes to BMDs and fractures, but no significant interactions were found.

A Danish case-control study of 237 cases and 200 age-matched controls described an odd ratio of 1.93 for osteoporotic vertebral fracture in the TT genotype when compared to the combined CC/CT group [10]. Another Danish prospective study followed 1748 postmenopausal women. In this latter study, 42 women in the control group and 10 women in the HRT treatment group who sustained fractures were included in the analysis and the fracture incidence in the first five years after menopause was increased more than 2-fold in the control group with the TT genotype (RR=2.6) [12]. Consistent with these two studies, we found that our subjects with the TT genotype had a 2.3-fold higher risk of fracture incidence after menopause. Such associations are independent of age, years since menopause, physical activity, occupation, passive smoking, currently alcohol consumption, and total hip BMD. However, we also found that the effect of the CT genotype on the risk of fractures was similar in magnitude to that of TT genotype, and, thus, indicate that the MTHFR C677T polymorphism represents a T-allele dominant model on the risk of fracture. Based on these findings, the MTHFR C677T genotype may play an independent role in the development of osteoporotic fracture.

The mechanism underlying the association between the MTHFR C677T polymorphism and the risk of fracture is not clear. However, we speculate that the increased risk of fracture in T-allele carriers is not mainly mediated through BMD. It is well known that the MTHFR C677T polymorphism is associated with an increased homocysteine level. And high homocysteine level may play a role in the risk of fracture. As shown in a Dutch study [6], the risk of non-vertebral fracture increases by a factor of two for subjects in the highest quartile of homocysteine levels when compared to subjects in the other three quartiles. The similar magnitude of association was reported in the Framingham study between increased homocysteine levels and the incidence of hip fracture in women [7]. Although the mechanism by which high homocysteine level may lead to fracture has not been determined, some data indicate that it can interfere with collagen cross-linking [27] and result in an altered matrix and more fragile bone. We hypothesize that the MTHFR C677T polymorphism can interfere with the development of the microarchitecture of bone via the elevated homocysteine level, which independently of the amount of mineral in the bone.

The possibility exists that the MTHFR C677T polymorphism plays an indirect effect on bone via altered DNA methylation. DNA methylation is a critical epigenetic mechanism, which involves the initiation of chromatin remodeling and gene expression regulation [28]. Aberrant DNA methylation is believed to play an important role in promotion of a number of diseases [29–31]. Recently, several reports have shown that individuals carrying the MTHFR 677TT

genotype experience significantly decreased DNA methylation [32,33]. This process may be involved in homocysteine-related pathology in vascular diseases[30] and tumors[33]. Further studies are necessary to determine whether DNA hypomethylation resulting from the MTHFR C677T polymorphism can affect the incidence of bone fracture.

Another explanation for the observed association is that: it is not the MTHFR C677T polymorphism in the MTHFR gene itself that increases the risk of fracture, but variants in nearby genes which are in linkage disequilibrium with this polymorphism. As reported[34], a mutation in the lysyl hydrolase 1 (LH1) gene, which is located 150 kb from the MTHFR gene on Chromosome 1, is associated with decreased LH 1 activity and results in weakened collagen crosslinkage.

This investigation has several limitations. First, it is a retrospective study and bias due to memory errors can not be ruled out; Second, the fracture events studied here are self-reported and diagnosed by the local physicians. No further verification was done and we cannot fully discriminate osteoporotic fractures from fractures caused by high-energy trauma. Third, due to the limitation of our diet information, we still could not completely rule out the possibility that the effect of the MTHFR C677T polymorphism could interact with vitamin B complex and/or folate. Because folate and vitamins B6 and B12 are major determinants of homocysteine concentrations, the inadequacy of one or more of these vitamins may modify the effect of homocysteine on bone phenotype. Further study is necessary to address this issue

In conclusion, we found that the MTHFR C677T polymorphism plays an independent role in predicting the risk of fracture. Further studies are necessary to elucidate the underlying mechanism of the MTHFR C677T polymorphism involvement in the development of bone fracture. Identification of this mechanism may help to better understand the etiology of osteoporosis and osteoporotic fracture in the future and may lead to improved strategies for early prevention, identification, and treatment.

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Fig 1. Mean and standard deviation of bone mineral density (BMD) at total hip, total body and femoral neck, when stratified by MTHFR C677T genotypic class

Table 1
Principal characteristics of the study population stratified by MTHFR C677T genotype.

	CC genotype (N=682)	CT genotype (N=945)	TT genotype (N=272)
	Mean (SD)	Mean (SD)	Mean (SD)
Age (yrs)	53.2±4.3	53.4±4.6	54.0±4.6
Weight (kg)	51.4±7.7	50.5±7.3	51.3±7.5
Height (cm)	152.7±5.1	152.3±5.0	152.8±5.2
BMI (kg/m ²)	22.0±3.0	21.7±2.9	21.9±2.8
Age at menarche (yrs)	15.9±1.8	15.8±1.8	15.9±1.9
Age at menopause (yrs)	46.4±4.4	46.6±4.45	46.9±4.3
Years since menopause (yrs)	6.7±4.8	6.9±5.1	7.1±5.3
Age at first fracture occurred (yrs)	42.4±11.2	42.2±14.1	38.8±13.2
	Number (%)	Number (%)	Number (%)
Passive smoking ^a	516 (75.7)	701 (74.2)	202 (74.3)
Alcohol consumption ^b	10(1.5)	22(2.3)	2 (0.7)
Physical activity ^c			
Slight	83 (12.2)	125 (13.3)	44 (16.2)
Moderate	432 (63.4)	566 (59.9)	176 (64.7)
Heavy	167 (24.5)	254 (26.9)	52 (19.1)
Occupation			
Farmer	628 (92.1)	852 (90.2)	245(90.1)
Osteoporosis status			
Normal	339 (49.7)	423(44.8)	121 (44.5)
Osteopenia	305 (44.7)	457(48.4)	131 (48.2)
Osteoporosis	38 (5.6)	65 (6.9)	20 (7.3)

^a Non-cigarette smokers whose spouse or other close family members are current smokers.

^b Current alcohol consumption of more than one drink per week.

^c $p < 0.05$. The difference of these variables among different genotyping groups was compared by ANOVA test for continuous variable and χ^2 -test for categorical variable.

Table 2

The adjusted ORs of having osteopenia or osteoporosis with the MTHFR C677T polymorphism

MTHFR	Control group	Osteoporosis ^b		Osteopenia ^c or osteoporosis	
	N (%)	Case (%)	OR (95%) ^a	Case (%)	OR (95%) ^a
Co-dominant model					
CC	339 (38.4)	38 (30.9)	1.0	343 (33.7)	1.0
CT	423 (47.9)	65 (52.8)	1.2 (0.7–2.0)	522 (51.4)	1.2 (0.9–1.4)
TT	121 (13.7)	20 (16.3)	1.5 (0.7–3.0)	151 (14.9)	1.2 (0.9–1.6)
T-allele dominant model					
CC	339 (38.4)	38 (30.9)	1.0	343 (33.8)	1.0
CT+TT	544 (61.6)	85 (69.1)	1.3 (0.8–2.0)	673 (66.2)	1.2 (0.9–1.4)

^a Age, physical activity, occupation, passive smoking, height, weight, years since menopause and current alcohol consumption were adjusted in the multivariate logistic model.

^b Osteoporosis was defined as a total hip BMD of more than 2.5 standard deviations (SDs) below the average peak BMD of young healthy Chinese women from the same population

^c Osteopenia was defined as a total hip BMD between one SD and 2.5 SDs below the average peak BMD of young healthy Chinese women from the same population

Table 3

The distribution of different types of incident fracture according to MTHFR C677T genotypic classes

Type of bone fracture	Bone fracture ever occurring ^a				Bone fracture occurring after menopause			
	CC ^b	CT	TT	Total	CC	CT	TT	Total
Hip	0	6 (9.7)	2 (10.0)	8 (7.3)	0	5 (15.1)	1 (11.1)	6 (11.5)
Wrist	10 (37.0)	33 (53.2)	6 (30.0)	49 (45.0)	3 (30.0)	18 (54.6)	2 (22.2)	23 (44.2)
Vertebrae	4 (14.8)	6 (9.7)	3 (15.0)	13 (11.9)	3 (30.0)	4 (12.1)	1 (11.1)	8 (15.4)
Extremities ^c	12 (44.5)	16 (25.8)	7 (35.0)	35 (32.1)	4 (40.0)	6 (18.2)	3 (33.3)	13 (25.0)
Pelvis	1 (3.7)	1 (1.6)	2 (10.0)	4 (3.7)	0	0	2 (22.2)	2 (3.9)

^a Included fractures occurring before or after menopause^b Expressed as: Number (%)^c Extremities include the arm, leg and ankle

Table 4The adjusted^a relative risk of incident fracture by the MTHFR C677T genotypic class

MTFHR	Bone fracture event ever occurring		Bone fracture event occurring after menopause	
	No. of case/total subjects (%)	RR(95%CI)	No. of case/total subjects (%)	RR(95%CI)
Co-dominant model				
CC	27/682 (4.0)	1.0	10/665 (1.5)	1.0
CT	62/945 (6.6)	1.7(1.1–2.7) ^b	33/916 (3.6)	2.5(1.2–5.1) ^b
TT	20/272 (7.6)	1.9 (1.1–3.3) ^b	9/261 (3.5)	2.3 (1.0–5.6)
T-allele dominant model				
CC	27/682 (4.0)	1.0	10/665 (1.5)	1.0
CT+TT	82/1217 (6.7)	1.7 (1.1–2.7) ^b	42/1,177 (3.6)	2.5 (1.2–4.9) ^c

^a Models adjusted by age, years since menopause, passive smoking status, current alcohol consumption, occupation, physical activity, weight, height, and total hip BMD.

^b p<0.05

^c p<0.01