Relation of Polymorphism in the Promotor Region for the Human Osteocalcin Gene to Bone Mineral Density and Occurrence of Osteoporosis in Postmenopausal Chinese Women in Taiwan

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Osteoporosis is a common disorder with a strong genetic component. Our aim was to evaluate the correlation of the *HindIII* osteocalcin gene polymorphism to bone mineral density (BMD) and their relationship to osteoporosis. We determined the *HindIII* osteocalcin gene polymorphism using polymerase chain reaction (PCR)-based restriction analysis in postmenopausal Chinese women in Taiwan. The osteocalcin gene polymorphism was detected by the restriction enzyme *HindIII*, where the H allele indicated the absence of the cuttable site and the h allele indicated its presence. We then related the genotypes to BMD and occurrence of osteoporosis in these women. The allelic frequencies for postmenopausal Chinese women in Taiwan were 64% for h and 36% for H in *HindIII* restriction fragment length poly-

morphisms. The prevalence of each genotype in the study population was 37.7% hh, 52.6% Hh, and 9.7% HH. The subjects with genotype hh had the greatest BMD at the lumbar spine and the femoral neck, and those with HH had the smallest BMD at the femoral neck, but these differences did not reach statistical significance. The *HindIII* osteocalcin genotype showed a significant effect on the prevalence of osteoporosis in the subjects at the femoral neck, that is, women with genotype HH had a 6.4 times greater risk for osteoporosis ($P < 0.05$), and those with genotype Hh had a 1.2 times greater risk than women with genotype hh. In conclusion, the *HindIII* osteocalcin gene polymorphism is associated with reduced BMD and predisposes women to osteoporosis at the femoral neck. J. Clin. Lab. Anal. 15:251–255, 2001. © 2001 Wiley-Liss, Inc.

Key words: polymerase chain reaction-based restriction analysis; lumbar spine; femoral neck

INTRODUCTION

Osteoporosis is a common disabling, age-related disease characterized by reduced bone mineral density (BMD), disorganization of skeletal integrity and microarchitecture, and increased risk of fragility fracture (1). BMD is strongly related to genetic control: as much as 60% of the variance in BMD has been attributed to genetic factors (2). The results of at least one twin study have further suggested that there are genetic influences on age-related bone loss (3).

Osteocalcin (also known as bone Gla protein (BGP)) is one of the major noncollagenous proteins of uncertain func-

tion synthesized exclusively by osteoblasts (4). Because osteocalcin biosynthesis is a marker of differentiated mature osteoblasts, clinical assay of serum osteocalcin is widely used for in vivo monitoring of bone formation rates (5,6). Recently, single nucleotide polymorphisms (SNPs) were used as a tool

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for mapping the disease gene (7), and Dohi et al. (8) found an association between this novel polymorphism in the promoter region for the human osteocalcin gene with BMD in postmenopausal Japanese women, thereby making it possible to search the candidate genes for the genetic determinants of BMD.

To our knowledge, no other study specifically testing the skeletal effects of polymorphism in the promoter region for the human osteocalcin gene in postmenopausal women has been conducted. An exploration of the relationship between osteocalcin genotypes and the occurrence of osteoporosis in different ethnic groups was therefore thought to be of interest. In this respect, we studied the correlation between the polymorphism in the promoter region for the human osteocalcin gene and BMD, and their relationship to the development of osteoporosis in postmenopausal Chinese women in Taiwan.

SUBJECTS AND METHODS

Subjects

One hundred seventy-five postmenopausal women 45–74 years old (mean $\pm$ standard deviation: 54.25 ± 5.90 years) were randomly enrolled among patients who underwent risk evaluation for osteoporosis at the Department of Obstetrics and Gynecology, China Medical College. Women were excluded from the study if they: 1) had undergone bilateral ovariectomies or had natural menopause occurring before the age of 40; 2) had taken drugs or had a history of taking drugs that affect bone metabolism, such as glucocorticoids, thyroxin, antiepileptics, bisphosphonates, calcitonin, or hormone replacement therapy for more than 4 months; 3) weighed more than 100 kilograms; and 4) had disease, such as primary hyperparathyroidism, hyperthyroidism, diabetes, cirrhosis, and kidney failure. For each woman, a detailed medical history was obtained and her dietary calcium intake was assessed using a sequential questionnaire that included foods accounting for the majority of dietary calcium (9). This study was approved by the medical ethics committee of China Medical College Hospital, and written informed consent was obtained from all the women who participated in this study.

Polymerase Chain Reaction (PCR)

PCRs were carried out in a total volume of 50 μ l, containing genomic DNA (30 ng), 2–6 pmole of each primer, 1 $\times$ Taq polymerase buffer (1.5 mM $MgCl_2$), and 0.25 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). The genomic DNA was prepared from peripheral blood using a DNA Extractor WB kit (Wako, Japan). The primers for the *HindIII* osteocalcin gene polymorphism were forward (5'-CCGCAGCTCCCAACCACAATAAGCT-3') and backward (5'-CAATAGGGCGAGGAGT-3') according to the report of Dohi et al. (8). PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA). The cycling condition for *HindIII* osteocalcin gene polymorphism was set as

follows: one cycle at 94°C for 8 min, 30 cycles at 94°C for 30 sec, 59°C for 30 sec, and 72°C for 60 sec, and one final cycle of extension at 72°C for 10 min.

PCR-Based Restriction Analysis

The 253 bp PCR products (10 μ l) were mixed with two units of *HindIII* (New England BioLabs, Beverly, MA) and the reaction buffer was mixed according to the manufacture's instructions. The reaction was incubated for 3 hr at 37°C. Then, 10 μ l of the products were loaded into 3% Agarose gel containing ethidium bromide for electrophoresis. The restriction site was designed to be located at position nt 298 (C to T transition) of the human osteocalcin promoter or position nt –152 numbered from the putative transcription start site. An uncuttable site is "C". The genotype of the *HindIII* osteocalcin gene polymorphism was determined by observing the number of bands on the digest: homozygotes lacking the *HindIII* site (HH) produced an unexcisable fragment (253 bp) at lane 3; homozygotes containing the *HindIII* site (hh) produced an excisable fragment (232 bp) at lane 1; and heterozygotes (Hh) produced two band sizes at lane 2 (Fig. 1).

Assessments of BMD

BMD of the lumbar spine (L2–L4) and the right femoral neck were measured using dual-energy X-ray absorptiometry (DPX-L densitometer, Lunar, Madison, WI). Osteoporosis was

Fig. 1. The genotype of the *HindIII* osteocalcin gene polymorphism was determined by observing the number of bands on the digest: homozygotes containing the *HindIII* site (hh) produced an excisable fragment (232 bp) at lane 1; heterozygotes (Hh) produced two band sizes at lane 2; and homozygotes lacking the *HindIII* site (HH) produced an unexcisable fragment (253 bp) at lane 3. M: marker, 100-bp ladder.

defined according to the classifications of the World Health Organization (10). The diagnosis of osteoporosis was made if a patient's BMD value was 2.5 standard deviations or >2.5 standard deviations below the mean of premenopausal Chinese women in Taiwan, at the lumbar spine or the right femoral neck (11).

Statistical Analysis

BMD and other relevant clinical variables of the three genotype groups were compared by analysis of variance. Multiple linear regression was used to adjust BMD values for confounding factors such as age, anthropometric variables, and calcium intake. A multivariate linear regression model was designed to test for the changing effects of *HindIII* osteocalcin genotype on BMD with age. For this analysis, the subjects were classified into 6-year age groups (45–50, 51–56, 57–62, 63–68, and 69–74 years). To test whether the allelic distribution of the *HindIII* osteocalcin genotypes satisfied the Hardy-Weinberg equilibrium, the chi-square test was used to compare the observed and expected frequencies of the various alleles. Odds ratios (with 95% confidence intervals) for the development of osteoporosis were calculated for the subjects with different *HindIII* osteocalcin genotypes using the multivariate logistic regression model. All statistical tests were two-sided. A *P* value less than 0.05 was considered statistically significant. All the calculations were performed by the Statistical Package for Social Sciences (SPSS for Windows, release 8.0, SPSS Inc, Chicago, IL).

RESULTS

The prevalence of each genotype in the study population was 37.7% hh, 52.6% Hh, and 9.7% HH. The allelic frequencies for postmenopausal Chinese women in Taiwan were 64% for h and 36% for H in *HindIII* RFLPs. The allelic frequency satisfied the Hardy-Weinberg equilibrium. Table 1 shows

anthropometric, dietary, and BMD according to *HindIII* osteocalcin genotype. The three genotypic groups did not differ significantly among factors such as age, height, weight, years since menopause, dietary calcium intake, and BMD (unadjusted or age-adjusted).

When the study subjects were divided into 6-year age categories, the subjects with genotype hh had the greatest BMD among women ages 45–50, 57–62, 63–68, and 69–74 at the lumbar spine, and those with HH had the smallest BMD among women ages 45–50, 51–56, and 63–68 at the femoral neck, but these differences did not reach statistical significance.

As shown in Fig. 2, BMD of the lumbar spine and the femoral neck for 175 postmenopausal women was represented as the least square mean adjusted for age, height, and weight. The subjects with genotype hh had the greatest BMD among subjects at the lumbar spine and the femoral neck, and those with HH had the smallest BMD at the femoral neck, but these differences did not reach statistical significance.

After adjustment for potential confounding factors, including age, height, and weight in the multivariate logistic regression model, the *HindIII* osteocalcin genotype showed a significant effect on the prevalence of osteoporosis in the subjects at the femoral neck; that is, women with genotype HH had a 6.4 times greater risk for osteoporosis (*P* < 0.05), and those with genotype Hh had a 1.2 times greater risk than women with genotype hh, although the latter odds ratio did not reach statistical significance (Table 2).

DISCUSSION

The allelic frequencies for postmenopausal Chinese women in Taiwan were 64% for h and 36% for H in *HindIII* RFLPs. This result showed that postmenopausal Chinese women in Taiwan had a higher frequency of the H allele when compared with postmenopausal Japanese women (about 22.8%)

TABLE 1. Characteristics of postmenopausal women according to *HindIII* osteocalcin genotype^a

Characteristic	<i>HindIII</i> osteocalcin genotype			<i>P</i> value
	hh n = 66	Hh n = 92	HH n = 17	
Age (yr)	54.64 ± 5.88	54.01 ± 5.95	54.00 ± 5.93	0.794
Height (cm)	155.15 ± 5.69	155.95 ± 5.50	157.31 ± 4.59	0.348
Weight (kg)	55.96 ± 7.93	56.65 ± 8.28	60.34 ± 8.50	0.161
Years since menopause	5.19 ± 4.72	6.21 ± 5.24	5.06 ± 4.90	0.475
Daily calcium intake (mg)	537 ± 146	524 ± 145	577 ± 180	0.406
Bone mineral density (g/cm ²)				
Lumbar spine				
Unadjusted	1.01 ± 0.17	0.98 ± 0.16	1.01 ± 0.14	0.491
Age-adjusted	1.01 ± 0.02	0.97 ± 0.02	1.01 ± 0.04	0.272
Femoral neck				
Unadjusted	0.79 ± 0.12	0.80 ± 0.10	0.79 ± 0.11	0.752
Age-adjusted	0.79 ± 0.01	0.80 ± 0.01	0.79 ± 0.03	0.853

^aValues are a mean ± SD. ANOVA denotes analysis of variance.

A

B

Fig. 2. Adjusted (A) lumbar and (B) femoral neck BMD values according to *HindIII* osteocalcin genotype. Values are adjusted for age, height, and weight. The solid bar represents the upper 95% confidence limit. Analysis of variance did not show any significant differences in mean adjusted BMD at the lumbar spine ($P = 0.136$) and the femoral neck ($P = 0.603$).

(8). The prevalence of each genotype in the study population was 37.7% hh, 52.6% Hh, and 9.7% HH. In comparison, the hh genotype frequency is much higher among postmenopausal Japanese women (about 61.9%) (8).

Dohi et al. (8) reported that there was an association between the *HindIII* osteocalcin gene polymorphism and lumbar BMD in postmenopausal Japanese women. However, our

results found the correlation between the *HindIII* osteocalcin gene polymorphism and BMD, and their relationship to the development of osteoporosis at the femoral neck. This result is similar to Gustavsson et al.'s report (12).

There are a number of possible explanations for the discrepancy between the results of our study and those of Dohi et al. (8). First, there is allelic heterogeneity between different populations, which is exemplified by the lower frequency of the hh genotype in the Chinese in Taiwan as compared to the Japanese. Second, there is histological heterogeneity between different populations, which is exemplified by the lower BMD of the HH genotype only at the femoral neck (not at the lumbar spine) in the Chinese in Taiwan as compared to the Japanese. The lumbar spine is predominantly trabecular bone.

Finally, the present study used a method of PCR-based restriction analysis to approach the SNPs. SNPs may provide a method of defining patients at risk of certain diseases and their prognoses, thereby enabling the physician to select a specific therapy based on specific genetic variations (13). Osteoporosis is a complex disease, and it is likely that several genes influence bone density. Multiple candidate genes have been identified in young adults, including vitamin D receptor, estrogen receptor α , interleukin 6, and transforming growth factor $\beta 1$ (12). Osteocalcin (BGP) is an abundant bone extracellular matrix protein in the family of mineral-binding Gla proteins, which are determinants of bone formation (14). Therefore, the osteocalcin gene is expected to be an important genetic component for the formation of bone mass. The influence of the *HindIII* osteocalcin gene polymorphism on the activity of the osteocalcin promoter remains to be determined (8). The present study shows that the *HindIII* osteocalcin gene polymorphism is associated with reduced BMD and predisposes women to osteoporosis at the femoral neck. We believe that the role of the *HindIII* osteocalcin gene polymorphism is likely to contribute to the pathogenesis of osteoporosis at the femoral neck in our population. Further study of the promoter in the regulation of osteocalcin gene expression is important to elucidate the pathogenesis of osteoporosis.

In conclusion, the results of our study suggest that the *HindIII* osteocalcin gene polymorphism may be one of the candidate risk gene markers responsible for osteoporosis in postmenopausal Chinese women in Taiwan. Further studies of other SNPs with regard to osteoporosis using additional

TABLE 2. Odds ratio of different *HindIII* osteocalcin genotypes on the development of osteoporosis in 175 postmenopausal women

Genotype	Osteoporosis at lumbar spine Odds ratio (95% CI) ^a		Osteoporosis at femoral neck Odds ratio (95% CI) ^a	
	Age-adjusted	Multivariate	Age-adjusted	Multivariate
hh	1.0	1.0	1.0	1.0
Hh	1.4 (0.36–3.6)	1.7 (0.6–5.1)	0.8 (0.2–2.6)	1.2 (0.3–5.0)
HH	0.3 (0.1–3.1)	0.6 (0.1–6.4)	2.6 (0.5–12.9)	6.4* (1.0–39.9)
Per copy of the H allele	0.9 (0.5–1.8)	1.2 (0.5–2.5)	1.4 (0.6–3.2)	2.3 (0.8–6.0)

^aCI denotes confidence interval. Odds ratios have been adjusted for age, height, and weight. * $P < 0.05$.

genetic markers might provide new information about the genetic background underlying BMD in humans.

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