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Osteocalcin HindIII gene polymorphism not associated with bone mineral density—A study in North Indian postmenopausal osteoporotic women

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Osteoporosis is an important health problem in India owing to the prevalence of vitamin D deficiency across all ages, low level of awareness and higher risk of complications. This disease is characterized by decreased bone mass, bone strength and higher risk of bone fracture. Here, we investigated association between osteocalcin HindIII gene polymorphism and bone mineral density (BMD) in postmenopausal osteoporotic and postmenopausal healthy North Indian women, possibly the first study of this kind in the aforesaid population. We investigated Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) of osteocalcin HindIII in 254 postmenopausal osteoporotic (56.12±7.004 years) and 254 postmenopausal healthy (55.27±5.93 years) North Indian women. BMD at lumbar spine (L_1-L_4), femoral neck, hip and forearm was measured by dual energy X-ray absorptiometry (DEXA). The results showed no significant correlation between osteocalcin HindIII gene polymorphism and BMD and we conclude that osteocalcin HindIII gene polymorphism may not have major effects on BMD variation in postmenopausal North Indian women.

Key words: BMD, BMI, Osteoporosis, Restriction fragment length polymorphism

Osteoporosis is a multifactorial skeletal disease characterized by low bone mineral density (BMD) and increased fracture risk and it is a growing health problem[1]. The International Osteoporosis Foundation (IOF), Switzerland has projected that factors such as ageing populations, sedentary and urban lifestyles in the Asian countries will lead to a situation that by 2050 these countries will account for more than half of the world's hip fractures[2,3]. Currently, there are 125 million people suffering from osteoporosis in India, Japan, USA and Europe alone. With approximately 80% of its urban population under vitamin D deficiency, India alone is reported to have 50 million people as either osteoporic or having low bone mass[3]. Worldwide, people of working age appears to suffer more (40%) with osteoporotic fractures[2]. . Reduced bone mineral density (BMD) is a major determinant for risk of osteoporosis[4,5], which is under strong genetic control. It is estimated that 46-62% of BMD variation can be attributed to genetic factors, including variations in expressions of various candidate genes encoding proteins such as calciotropic hormones, cytokines, bone matrix protein, growth factors and its receptor which all contribute in bone metabolism and in the pathogenesis of osteoporosis[6,7].

Osteocalcin, a member of non-collagenous protein family, also known as bone gamma caroboxyglutamic acid (BGP) is found in bone. It is exclusively produced by osteoblasts under transcriptional regulation through a vitamin D response element and is well known as a marker for differentiated mature osteoblasts and a determinant of the bone calcification process[8-11]. Osteocalcin may also be involved in the recruitment and differentiation of osteoclasts precursors at the bone surface, suggesting its possible role in bone resorption and bone remodelling[12,13]. The human osteocalcin gene is located in 1q25-31 chromosomal region and genetic evidence suggests that osteocalcin is a candidate gene. In genome wide linkage analysis quantitative trait loci (QTLs) contributing to normal variation in BMD phenotype identified with a maximum logarithm (base 10) of

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Abbreviations: BMD, Bone mineral density; BMI, Body mass index; PCR, Polymerase Chain Reaction; RFLP, Restriction Fragment Length Polymorphism

odds (LOD) score of 3.1 at 1q21-23 chromosomal region[14].

Recently, Dohi *et al*.[15] have identified a polymorphism in osteocalcin gene with the HindIII restriction enzyme known as HindIII polymorphism. A study on healthy adolescents from Sweden found association between HindIII polymorphism and BMD of humerus[16]. On the contrary, other studies did not find any association between BMD and osteocalcin polymorphism in different populations[17-19]. Studies specifically associating the promoter region of the human osteocalcin gene polymorphism with BMD in Indian postmenopausal women are lacking. In the present study, we investigated the association of osteocalcin gene polymorphism with BMD in North Indian postmenopausal women.

Material and Methods

We recruited 254 postmenopausal osteoporotic women (aged 56.124 ± 7.004) and 254 age-matched postmenopausal healthy women (aged 55.270 ± 5.939) who were attending the OPD at Department of Rheumatology, King George's Medical University (KGMU), Lucknow. All of these women had not been experiencing spontaneous menses for at least 1 year. Before entering this study, a questionnaire concerning health condition and medical history was taken with informed consent from every subject. In this study, exclusion criteria included hormone replacement therapy, secondary osteoporosis, endocrinal disorders, inflammatory arthritis, tuberculosis, cancer, liver or kidney disease and use of vitamin, minerals and antioxidants. The study protocol was approved by the ethics committee of KGMU, Lucknow, India

BMD measurements

The BMD (g/cm^2) was measured by dual energy X-ray absorptiometry (Lunar Prodigy, Madison, WI, USA) at lumbar spine L_1-L_4, femoral neck, hip forearm left skeletal site. Percent coefficient of variance ranged from 0.5% to 1.1% depending on the measurement site. According to World Health Organization (WHO) criteria, the normal bone mass defined as BMD measurements at or above - 1 standard deviation (S.D.) from the optimal peak bone density (T-score) of healthy young adult of the same sex. BMD measurement at or below -2.5 S.D. from the optimal peak bone density of healthy young adult of the same sex was considered osteoporotic[20].

DNA Isolation and Genotyping

Blood for genotyping was drawn in the ethylene diamine tetra acetic acid (EDTA) vials. DNA was extracted from peripheral venous blood samples by using the salting-out method. The DNA samples were amplified by polymerase chain reaction (PCR). Detection of the promoter region of BGP gene was performed by PCR amplification of a region carrying primers originating in promoter region of BGP gene (Forward primer) 5′-CCG CAG CTC CCA ACC ACA ATA AGCT-3′ (Reverse primer)- 5′-CAA TAG GGC GAG GAG T-3′ producing an 253 basepair (bp) fragment. To PCR products were generated in the final volume of 25 µL containing 1.2 µL genomic DNA, 0.4 µL of forward and reverse primers (10 pmole conc.), 0.5 µL of 10 mM of each dNTP's and 5 units of Taq polymerase (Applied Biosystems, Foster City, USA) was used to obtain the PCR product. The PCR protocol was: 94°C for 5 min followed by 35 cycles at 94°C for 35 s, 60°C for 35 s 72°C for 35 s and final extension at 72°C for 7 min. After amplification, the PCR products were digested with restriction endonuclease HindIII (New England Biolabs Inc, Ipswich, MA, USA) at37°C for 1 h followed by electrophoresis with 3.5% agarose gel stained with ethidium bromide. The osteocalcin gene allele was designated as (H) in the absence or as (h) in the presence of HindIII site

Statistical analysis

Statistical analysis was done using SPSS version 16.0 (Chicago, IL, USA). All categorical variables were expressed as percentages. All continuous variables were expressed as means ± standard deviation. Chi-square goodness of fit test was used to look for Hardy-Weinberg equilibrium of genotypes. Genotype and allele frequency amongst cases and controls were compared using chi-square test. Means between two groups were compared using independent sample t test; means between more than two groups were compared using one way ANOVA. Binary logistic regression was done to calculate odds ratio and 95% CI of genetic, demographic and anthropometric factors associated with osteoporosis. For binary logistic regression case status was taken as dependent variable. Age, BMI, year since menopause and genotype were taken as independent variable. All p values less than 0.05 were taken as significant.

Results

In the present case control study, it was found that BMD of all skeletal sites was significantly decreased while BMI, year since menopause and weight was significantly higher in postmenopausal osteoporotic women when compared with postmenopausal healthy women. The genotype frequencies were 36.6% (93 subjects) for hh, 51.6% (131 subjects) for Hh, and 11.8% (30 subjects) for HH in the postmenopausal osteoporotic women, and 29.9% (76 subjects) for hh, 59.8% (152 subjects) for Hh, and 10.2% (26 subjects) for HH in the postmenopausal healthy women. The allele frequencies for h were 59.8 and 62.4%, and for H, 40.2 and 37.6 % in the postmenopausal healthy and osteoporotic women, respectively. The osteocalcin genotype and allele frequency distributions in the postmenopausal healthy and postmenopausal osteoporotic women had no statistically significant difference (P=0.169 for the genotype distribution, and P=0.408 for allele frequency distribution) (Table 1)

The independent predictor of osteoporosis was investigated in the whole group (postmenopausal healthy and osteoporotic women) by using binary logistic regression. Higher age, higher BMI and year since menopause (*P* value=0.005, 0.015, 0.001, respectively) were found to be independent significant predictor of osteoporosis. Furthermore, genotypes Hh (odds ratio=1.159, 95% CI; 0.622-2.161, *P* value=0.642) and HH was not found independently associated with osteoporosis (odds ratio=0.788, 95% CI; 0.437-1.421, *P* value=0.642) (Table 2).

When comparing the demographical, anthropometric and BMD of osteoporotic patients with osteocalcin gene genotypes, no significant differences were observed in genotypes of osteocalcin gene with respect to BMI (hh=24.95±3.65, Hh=24.86±3.56, HH=25.16±3.98 kg/m2, p=0.92) and years since menopause (hh=10.9±7.3, Hh=11.8±11.2, HH=10.8±8.3 years, p=0.76). Comparing with different sites of BMD in g/cm2; spine (hh=0.651±0.147, Hh=0.659±0.165, HH=0.686±0.139, p=0.56), hip (hh=0.553±0.172. Hh=0.575±0.14, H=0.590±0.156, p=0.41), forearm (hh=0.610±0.133, Hh=0.612±0.151, HH=0.628±0.158, p=0.82) and femoral neck (hh=0.658±0.096, Hh=0.659±0.129, HH=0.672±0.120, p=0.84) and genotypes of osteocalcin gene, no significant difference was observed. Furthermore, no significant differences were found with respect to age weight and height (Table 3).

Table 1—Comparison of clinical, anthropometric, BMD (g/cm^2) and genetic characteristics of cases and controls

Variable	Osteoporotic women (N=254)	Controls (N=254)	*P* value
Age years (mean ± SD)	56.124 ±7.004	55.270 ±5.939	0.139
Sex Female (%)	254 (100%)	254 (100%)	-
Weight Kg (mean ± SD)	57.835 ±9.150	56.055 ±6.937	0.014*
Height cm (mean ± SD)	151.504 ±8.695	152.535 ±6.391	0.128
Years since menopause (mean ± SD)	11.408 ±9.702	9.401 ±6.1	0.005*
BMI Kg/m^2 (mean ± SD)	24.929 ±3.629	24.059 ±2.989	0.003*
BMD Lumbar spine (L_1 –L_4) (mean±SD)	0.660 ±0.155	0.902 ±0.121	<0.001*
BMD Hip (mean ± SD)	0.569 ±0.156	0.841 ±0.117	<0.001*
BMD forearm (mean ± SD)	0.613 ±0.145	0.819 ±0.109	<0.001*
BMD femoral neck (mean ± SD)	0.660 ±0.117	0.794 ±0.095	<0.001*
Genotypes			
hh	93 (36.6%)	76 (29.9%)	0.169
Hh	131 (51.6%)	152 (59.8%)	
HH	30 (11.8%)	26 (10.2%)	
Allele			
H	317/508 (62.4%)	304/508 (59.8%)	0.408
H	191/508 (37.6%)	204/508 (40.2%)	

All data are shown as mean ± SD
*P <0.05 is considered statistically significant

Table 2—Binary logistic regression showing odds ratios of demographic, anthropometric and genetic characteristics of developing osteoporosis

Variables	*P* value	Odds ratio	95% CI
Age	0.005*	0.862	0.777 - 0.956
BMI	0.015*	1.071	1.014 - 1.131
Years since menopause	0.001*	1.188	1.072 - 1.316
Genotypes			
hh	-	1	1
Hh	0.642	1.159	0.622 - 2.161
HH	0.429	0.788	0.437 - 1.421

*Significance (P <0.05)

Table 3—Comparison of demographic, anthropometric and BMD of osteoporotic patients according to osteocalcin gene genotypes

Variables	Hh (N=93)	Hh (N=131)	HH (N=30)	*P* value
Age years (mean±SD)	56.070 ±7.370	56.189 ±6.566	56.003 ±7.899	0.987
Weight Kg (mean±SD)	57.656 ±8.7408	57.527 ±8.941	59.733 ±11.200	0.480
Height cm (mean±SD)	151.710 ±5.874	151.725 ±5.840	149.900 ±19.853	0.562
Years since menopause (Mean±SD)	10.981 ±7.285	11.842 ±11.387	10.837 ±8.354	0.762
BMI Kg/m^2 (mean±SD)	24.947 ±3.646	24.863 ±3.560	25.158 ±3.978	0.921
BMD Lumbar spine (L_1- L_4) (mean±SD)	0.651 ±0.147	0.659 ±0.165	0.686 ±0.139	0.563
BMD Hip (mean±SD)	0.553 ±0.172	0.575 ±0.143	0.590 ±0.156	0.414
BMD forearm (mean ± SD)	0.610 ±0.133	0.612 ±0.151	0.628 ±0.158	0.825
BMD femoral neck (mean±SD)	0.658 ±0.096	0.659 ±0.129	0.672 ±0.120	0.844

P <0.05 is considered statistically significant & Parenthesis indicates the number of subjects in each group

Discussion

Osteocalcin is a member of non-collagenous protein family and involved in bone metabolism, resorption and bone remodelling[8-11]. Animal studies whereby targeted deletion of the osteocalcin gene in mice results a phenotype with high bone mass and improved functional quality of bones[21].

Therefore, the osteocalcin gene is expected to be an important genetic factor for the formation of bone mass. Bone remodelling is a physiological process that happens throughout the lifespan and peak bone mass is attained in the second decade of life after which progressive loss of bone occurs[22]. Peak bone mass is the most important predictor of BMD and identification of genetic factor influencing peak bone mass. This parameter might be useful in early detection and prevention of osteoporosis[1]. There are multiple genes involved in regulating bone metabolism and these gene and their variants may differ with ethnicity[23,24].

In the present study, we investigated the association of osteocalcin HindIII gene polymorphism with BMD in North Indian postmenopausal women with and without osteoporosis. We did not observe any significant association between the osteocalcin HindIII gene variant and BMD variations. Yamada *et al*.[25] noticed significant association between the BMD of lumbar spine/hip and the osteocalcin HindIII gene polymorphism in postmenopausal Japanese women but not in pre-menopausal women. However, significant association between the osteocalcin HindIII gene polymorphism and variation of BMD has been reported in Caucasians population. In osteocalcin HindIII gene polymorphism, allele h was associated with higher hip BMD in 630 Caucasian subjects from 53 pedigrees[26]. Another study on the adolescent females of Caucasian population, showed lower BMD at the humerus and femoral neck was associated in the presence for H allele[16]. Our results are consistent with some earlier studies in different Asian populations. Dohi *et al*.[15] did not find any significant association with BMD of lumbar spine and osteocalcin HindIII gene polymorphism in Japanese postmenopausal women. Similar to our observation, no association was found between osteocalcin HindIII gene polymorphism and BMD in the white American pre- and peri-menopausal women[19]. A study on postmenopausal Chinese women in Taiwan also failed to find any significant association between the osteocalcin gene HindIII polymorphism and BMD[27]. Other studies carried out in Mainland China also did not find any significant association between BMD and osteocalcin gene HindIII polymorphism in pre- and post- menopausal Chinese women[17,18]. Their data along with our present findings did not support the role of osteocalcin gene variant QTL for BMD variation in postmenopausal women and even pre-menopausal women in Indian and Chinese ethnicity.

In the present study, we have shown that the osteocalcin HindIII gene polymorphism, in postmenopausal women with osteoporosis, is not associated with BMD. The BMD at lumbar spine for North Indian population was found to vary according to rank order of genotypes (HH>Hh>hh) i.e. it was the highest for H allele and the lowest for h allele.

In summary, our investigation on the association between the osteocalcin HindIII gene polymorphism and BMD in postmenopausal osteoporotic and postmenopausal healthy North Indian women, possibly the first study of this kind in this population, suggested that there was no such significant association. In other words, the osteocalcin HindIII gene polymorphism do not have any significant association with the bone mineral density in postmenopausal osteoporotic and postmenopausal

healthy North Indian women. Hence, it can be concluded that osteocalcin HindIII gene polymorphism may not have major effects on BMD variation in postmenopausal North Indian women.

The present research, however, has some limitations as it was carried out in only ethnically homogenous North Indian women and women who visited Rheumatology clinic for bone mass examination. Therefore, further studies are needed for postmenopausal women from the general population and other ethnic groups of India.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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