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 (L1-L4), 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 problem1. 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 fractures2,3. 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 mass3. Worldwide, people of working age appears to suffer more (40%) with osteoporotic fractures2. Reduced bone mineral density (BMD) is a major determinant for risk of osteoporosis4,5, 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 calcitropic hormones, cytokines, bone matrix protein, growth factors and its receptor which all contribute in bone metabolism and in the pathogenesis of osteoporosis6,7.

Osteocalcin, a member of non-collagenous protein family, also known as bone gamma carboxyglutamic 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 process8-11. 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 remodelling12,13. 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\textsuperscript{14}. Recently, Dohi \textit{et al.}\textsuperscript{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\textsuperscript{16}. On the contrary, other studies did not find any association between BMD and osteocalcin polymorphism in different populations\textsuperscript{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\textsuperscript{2}) was measured by dual energy X-ray absorptiometry (Lunar Prodigy, Madison, WI, USA) at lumbar spine L\textsubscript{1}-L\textsubscript{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\textsuperscript{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’-CGC CAG CTC CCA ACC ACA ATA AGCT-3’ (Reverse primer)- 5’-CAA TAG GCC 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 and 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 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=0.76). 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>
<thead>
<tr>
<th>Variable</th>
<th>Osteoporotic women (N=254)</th>
<th>Controls (N=254)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age years (mean ± SD)</td>
<td>56.124 ± 7.004</td>
<td>55.270 ± 5.939</td>
<td>0.139</td>
</tr>
<tr>
<td>Sex Female (%)</td>
<td>254 (100%)</td>
<td>254 (100%)</td>
<td>-</td>
</tr>
<tr>
<td>Weight Kg (mean ± SD)</td>
<td>57.835 ± 9.150</td>
<td>56.055 ± 6.937</td>
<td>0.014*</td>
</tr>
<tr>
<td>Height cm (mean ± SD)</td>
<td>151.504 ± 8.695</td>
<td>152.535 ± 6.391</td>
<td>0.128</td>
</tr>
<tr>
<td>Years since menopause (mean ± SD)</td>
<td>11.408 ± 9.702</td>
<td>9.401 ± 6.1</td>
<td>0.005*</td>
</tr>
<tr>
<td>BMI Kg/m² (mean ± SD)</td>
<td>24.929 ± 3.629</td>
<td>24.059 ± 2.989</td>
<td>0.003*</td>
</tr>
<tr>
<td>BMD Lumbar spine (L₁–L₄) (mean±SD)</td>
<td>0.660 ± 0.155</td>
<td>0.902 ± 0.121</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BMD Hip (mean ± SD)</td>
<td>0.569 ± 0.156</td>
<td>0.841 ± 0.117</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BMD forearm (mean ± SD)</td>
<td>0.613 ± 0.145</td>
<td>0.819 ± 0.109</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BMD femoral neck (mean ± SD)</td>
<td>0.660 ± 0.117</td>
<td>0.794 ± 0.095</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Table 1—Comparison of clinical, anthropometric, BMD (g/cm²) and genetic characteristics of cases and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>hh (36.6%)</th>
<th>Hh (51.6%)</th>
<th>HH (11.8%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele H</td>
<td>317/508 (62.4%)</td>
<td>304/508 (59.8%)</td>
<td>204/508 (40.2%)</td>
</tr>
</tbody>
</table>

| Allele H | 317/508 (62.4%) | 304/508 (59.8%) | 204/508 (40.2%) |

All data are shown as mean ± SD

*P < 0.05 is considered statistically significant

<table>
<thead>
<tr>
<th>Variables</th>
<th>P value</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.005</td>
<td>0.862</td>
<td>0.777 - 0.956</td>
</tr>
<tr>
<td>BMI</td>
<td>0.015</td>
<td>1.071</td>
<td>1.041 - 1.131</td>
</tr>
<tr>
<td>Years since menopause</td>
<td>0.001*</td>
<td>1.188</td>
<td>1.072 - 1.316</td>
</tr>
</tbody>
</table>

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

*Significance (P < 0.05)
any significant association between the osteocalcin
with and without osteoporosis. We did not observe
differ with ethnicity
metabolism and these gene and their variants may
multiple genes involved in regulating bone
mass. This parameter might be useful in early
identification of genetic factor influencing peak bone
mass is the most important predictor of BMD and
which progressive loss of bone occurs
22
with BMD in North Indian postmenopausal women
mass is attained in the second decade of life after
association of osteocalcin HindIII gene polymorphism
that happens throughout the lifespan and peak bone
mass. Bone remodelling is a physiological process
improved functional quality of bones
mice results a phenotype with high bone mass and
8-11
protein family and involved in bone metabolism,
Discussion
P
BMD
BMD Hip
BMD Lumbar
BMI Kg/m
BMD femoral
BMD forearm
BMD (mean ± SD)
BMI (mean ± SD)
BMD Spine (L1, L4)
Years since menopause
Height cm
Weight Kg
Age years
variables
of osteoporotic patients according to osteocalcin gene genotypes

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hh (N=93)</th>
<th>Hh (N=131)</th>
<th>HH (N=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age years (mean±SD)</td>
<td>56.070 ±6.370</td>
<td>56.189 ±6.566</td>
<td>56.003 ±7.899</td>
<td>0.987</td>
</tr>
<tr>
<td>Weight Kg (mean±SD)</td>
<td>57.656 ±8.7408</td>
<td>57.527 ±8.941</td>
<td>59.733 ±11.200</td>
<td>0.480</td>
</tr>
<tr>
<td>Height cm (mean±SD)</td>
<td>151.710 ±5.874</td>
<td>151.725 ±5.840</td>
<td>149.900 ±19.853</td>
<td>0.562</td>
</tr>
<tr>
<td>Years since menopause (mean±SD)</td>
<td>10.981 ±7.285</td>
<td>11.842 ±11.387</td>
<td>10.837 ±8.354</td>
<td>0.762</td>
</tr>
<tr>
<td>BMI Kg/m² (mean±SD)</td>
<td>24.947 ±3.646</td>
<td>24.863 ±3.560</td>
<td>25.158 ±3.978</td>
<td>0.921</td>
</tr>
<tr>
<td>BMD Lumbar spine (L1, L4) (mean±SD)</td>
<td>0.651 ±0.147</td>
<td>0.659 ±0.165</td>
<td>0.686 ±0.139</td>
<td>0.563</td>
</tr>
<tr>
<td>BMD Hip (mean±SD)</td>
<td>0.553 ±0.172</td>
<td>0.575 ±0.143</td>
<td>0.590 ±0.156</td>
<td>0.414</td>
</tr>
<tr>
<td>BMD forearm (mean ± SD)</td>
<td>0.610 ±0.133</td>
<td>0.612 ±0.151</td>
<td>0.628 ±0.158</td>
<td>0.825</td>
</tr>
<tr>
<td>BMD femoral neck (mean±SD)</td>
<td>0.658 ±0.096</td>
<td>0.659 ±0.129</td>
<td>0.672 ±0.120</td>
<td>0.844</td>
</tr>
</tbody>
</table>

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 remodelling8-11. Animal studies whereby targeted deletion of the osteocalcin gene in mice results a phenotype with high bone mass and improved functional quality of bones21.

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 occurs22. 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 osteoporosis1. There are multiple genes involved in regulating bone metabolism and these gene and their variants may differ with ethnicity23,24.

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 pedigrees26. 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. 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 women19. A study on postmenopausal Chinese women in Taiwan also failed to find any significant association between the osteocalcin gene HindIII polymorphism and BMD27.

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 women17,18. 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.

Acknowledgement

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Conflict of Interest

The authors declare that they have no conflict of interest.

References


