Association of Methylene Tetrahydrofolate Reductase Polymorphism with BMD and Homocysteine in Premenopausal North Indian Women

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ABSTRACT
Background and Aim: Osteoporosis (OP) is a common nutrigenomic disease associated with various genetic components. Observational studies have indicated that mildly elevated homocysteine was a strong risk factor for osteoporotic fractures. Yet there is no clear biologic mechanism for an effect of homocysteine on bone. The aim of this study was to investigate the association of MTHFR C677T and A1298C polymorphisms, and to verify the association of these polymorphisms with bone mineral density and homocysteine in premenopausal women of northern India.

Material and Methods: We included 402 north Indian patients with altered BMD, both Osteopenic (OPN) and Osteoporosis, and normal controls. Genotype identification for MTHFR C677T and A1298C polymorphisms were analyzed by PCR-RFLP method, correlated with Bone Mineral Density (BMD), Homocysteine (Hcy), Folate and Vitamin B12.

Results: The study groups did not differ in terms of age, weight and body mass indices. Prevalence of Genotype frequencies (GFs) for MTHFRC677T OP were (n: 402): CC 361 (89.8%), CT 25 (6.22%), TT 16 (3.98%) and that for MTHFR A1298C were (n: 402) AA 353(87.81%), AC 29(7.21%), CC 20(4.98%). Folate was significantly lower in the OP group than those in both the other groups, while there was no significant difference in Hcy in the OP group relative to OPN, as compared to controls.

Conclusion: The GFs for MTHFR C677T and A1298C polymorphisms were not different between both groups. In conclusion, polymorphism of the MTHFR 677T is associated with small differences in BMD with folate levels. Further, more investigations should be done in larger studies for other epigenetic pathways, that may increase the risk of Osteoporosis.

INTRODUCTION
Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and increased fracture risk [1]. Osteoporosis is characterized by pathologically low bone mass and an increased risk for bone fractures [1], which is a serious problem in an increasingly aging population. Bone Mineral Density (BMD) is the primary factor affecting susceptibility to fractures [2]. Studies done on families [3,4], and twins [5,6] have suggested that maximal bone mass and the rate of bone loss associated with aging are determined by genetic as well as by environmental factors. Several factors are known to affect bone metabolism, and to increase the risk of bone fractures. Recently, it has been suggested that an increased plasma homocysteine (Hcy) levels caused by polymorphisms of the gene encoding methylenetetrahydrofolate reductase (MTHFR), are associated with bone fractures [1]. Even mild elevation of Hcy has been reported to be responsible for bone fractures [2]. However, confusing data exist, regarding the relation between Hcy and bone loss in post-menopausal women, because of heterogeneity of studies [3-7]. Also, the mechanism for increased fracture risk and Hcy is not clear [8-11]. Levels of Hcy are inversely related to the folates and possibly, vitamin B12 [12]. While some studies reported that folate deficiency, but not Hcy and vitamin B12, had an important role in the vertebral bone mineral density (BMD) decline of post-menopausal women, others showed that vitamin B12 was an independent risk factor for osteoporosis and bone fractures [12,13]. In addition, recent studies done on twins have described the contribution of gene-environment interactions to BMD [7]. Therefore, identifying genes or gene products associated with BMD would be useful for predicting bone mass and for clarifying the mechanism of bone loss in osteoporosis. Moreover, the use of genetic markers would allow an earlier intervention among those who are at an increased risk for osteoporosis, and pave the way for development of a targeted therapy. Early-onset osteoporosis associated with homocystinuria is thought to be caused by the effect of homocysteine or other metabolites interfering with the cross-linking of collagen [14]. The plasma homocysteine concentrations are higher in the post-menopausal than in the premenopausal women [15]. Based on these findings, we speculated that homocysteine participates in the pathophysiological process of osteoporosis in the general population. This study investigated the association of the genotype of MTHFR with BMD, to determine whether this polymorphism could serve as a useful genetic marker of osteoporosis among premenopausal women.

MATERIAL AND METHODS
The study design was a prospective observational study that started in April 2007 and continued till March 2010. The study population comprised 402 women with altered BMD, both Osteopaenic (150) and Osteoporosis (44) and normal controls (207). The study subjects were from government hospitals and all concerned ethical clearances and consents were taken from respective authorities. The inclusion and exclusion criteria in short sentences and to the point were as such: Subjects with hormone replacement therapy, receiving medications affecting bone mineralization, Hcy level, folates and vitamin B12, liver or renal disease, endocrine or metabolic abnormalities excluded. Evaluation of dietary habits, addictions, milk or milk product usage and physical activity was well considered.

Bone Mineral Density (BMD) Measurements
Axial BMD (lumbar spine BMD, LBMD) was measured by dual-energy X-ray absorptiometry.
Biochemical Indices

Non-fasting serum, plasma, and urine samples were collected as baseline data at the time of enrollment. Routine biochemical examinations, including those of serum and urinary levels of calcium and creatinine, serum levels of total protein, alkaline phosphatase (Al-P) activity, were done immediately. Plasma levels of total homocysteine were measured by using a high performance liquid chromatography (HPLC) system [16]. Vitamin B12 and folate were measured using chemiluminescence assay kits for vitamin B12 and folate (INMAS, New Delhi), India.

Genomic DNA Analysis

Analysis of MTHFR Gene Polymorphism: C677T and A1298C were analyzed by polymerase chain reaction (PCR) by using specific primers and protocols [17]. The PCR-amplified fragments were digested with endonucleases, HinfI and MboII and they were analyzed by doing electrophoresis on 6% and 8% polyacrylamide gels, respectively.

STATISTICAL ANALYSIS

We classified the subjects into three groups according to their genotypes [Table/Fig-1]. Comparisons of BMD and biochemical markers among the three genotypes were performed using analysis of variance (ANOVA). When the differences in the BMD and biochemical markers were tested between two groups, statistical analysis was performed using non parametrical analysis (Student's t-test). A p value of less than 0.05 was considered to be statistically significant. Quantitative data were expressed as mean ± standard deviation (Mean ± SD).

RESULTS

The study population comprised 402 women with altered BMD, both Osteopenic and Osteoporosis and normal controls. The genotype distributions among the 402 individuals were as follows for 677T, 361 (89.8%) CC, 25 (6.22%), CT, and 16 (3.98%) TT genotypes, and for 1298C, which showed 353 (87.81%) AA, 29(7.21%)AC, 20(4.98%) CC respectively [Table/Fig-1].

The groups did not differ in terms of age, weight and body mass indices [Table/Fig-2]. The variables distributed between the groups, which ranked according to BMD, were summarized [Table/Fig-2-4]. Though there was a trend of increasing frequency of the MTHFR TT genotype across the groups, this was not significant (p=0.601). Folate was significantly lower in the OP group than in both the other groups, while there was no significant Hcy difference in the OP group relative to OPN. The groups did not differ in vitamin B6 or vitamin B12 statuses. The associations of BMD in the population as a whole, have been shown in partial correlation analysis, after being corrected for age, weight and height, which confirmed the significant association of folate with BMD (spine, femur and forearm) [Table/Fig-2-4].

The lumbar spine BMD values of 677T were 0.81 ± 1.42, 1.08 ± 1.01, -2.51 ± 1.27 g/cm² for CC, CT, TT respectively. and those for 1298C were -0.91 ± 1.45, -0.91 ± 1.33, -0.61 ± 1.22 g/cm² for AA, AC and CC respectively. The lumbar spine BMD in the subjects with TT genotype were significantly lower than in those with CC genotype and it showed a significant negative correlation with the presence of the T allele (p<0.05), while lumbar spine BMD in the subjects with 1298C genotype showed no correlation with the presence of the C allele (p=0.09) [Table/Fig-5 and 6]. The Forearm BMD values of 677T were 0.85 ± 1.36, -0.92 ± 0.98, -1.5 ± 1.29/cm² for CC, CT, TT, and those of 1298C were -0.88 ± 1.3, -0.76 ± 1.18, -1.03 ± 1.47/cm² for AA, AC and CC respectively. The forearm BMD in the subjects with TT genotype were significantly lower than in those with CC genotype and it showed a significant negative correlation with the presence of the T allele (p<0.05), while lumbar spine BMD in the subjects with 1298C genotype showed no correlation with the presence of the C allele (p=0.06) [Table/Fig-5 and 6].

Though there was a trend of decreasing folate across the groups, this was not significant, and there was no difference in BMD between the genotypes. There was a lack of association of BMD with MTHFR genotype. This was the effect of interaction of vitamin status with MTHFR genotype on BMD. In those with the CT genotype, those at the lowest quintiles of folate and vitamin B12 concentrations had
significantly lower tHcy concentrations, which in case of folate, was associated with a significant reduction in BMD.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal (n=207)</th>
<th>Osteopoenic (n=150)</th>
<th>Osteoporosis (n=44)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>43.4±3.1</td>
<td>44.4±3.41</td>
<td>47.1±3.03</td>
<td>&lt;0.005**</td>
</tr>
<tr>
<td>Weight</td>
<td>67.29±11.7</td>
<td>60.49±11.0</td>
<td>53.5±12.38</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>BMI</td>
<td>28.47±4.8</td>
<td>26.18±4.5</td>
<td>23.88±5.2</td>
<td>&lt;0.005***</td>
</tr>
<tr>
<td>S. Ca</td>
<td>9.44±1.18</td>
<td>9.48±0.53</td>
<td>9.66±0.42</td>
<td>NS</td>
</tr>
<tr>
<td>Ionic Ca</td>
<td>1.13±0.05</td>
<td>1.13±0.09</td>
<td>1.12±0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphate</td>
<td>3.69±0.57</td>
<td>3.64±0.48</td>
<td>3.64±0.37</td>
<td>NS</td>
</tr>
<tr>
<td>ALP</td>
<td>223.76±73.9</td>
<td>234.38±68.14</td>
<td>248.03±95.29</td>
<td>NS</td>
</tr>
<tr>
<td>Folate</td>
<td>11.48±12.9</td>
<td>9.71±6.5</td>
<td>10.65±7.78</td>
<td>0.497</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>15.30±18.05</td>
<td>13.42±6.12</td>
<td>9.39±3.6</td>
<td>0.554</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>337.06±287.7</td>
<td>288.22±170.78</td>
<td>355.64±272.82</td>
<td>0.553</td>
</tr>
<tr>
<td>Frequency of MTHFR 1298C CC genotype</td>
<td>188 (91.26%)</td>
<td>162 (90.7%)</td>
<td>8 (72.3%)</td>
<td>&lt;0.05***</td>
</tr>
<tr>
<td>Frequency of MTHFR 677T CT genotype</td>
<td>15 (7.28%)</td>
<td>105 (46%)</td>
<td>0 (0%)</td>
<td>&lt;0.05***</td>
</tr>
<tr>
<td>Frequency of MTHFR 677T TT genotype</td>
<td>3 (1.46%)</td>
<td>105 (46%)</td>
<td>3 (27.27%)</td>
<td>&lt;0.05***</td>
</tr>
<tr>
<td>Frequency of MTHFR 1298C AA genotype</td>
<td>172 (83.49%)</td>
<td>168 (91.8%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Frequency of MTHFR 1298C AC genotype</td>
<td>20 (9.7%)</td>
<td>9 (4.91%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Frequency of MTHFR 1298C CC genotype</td>
<td>14 (6.7%)</td>
<td>6 (3.27%)</td>
<td>11 (100%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The high prevalence of osteoporosis, which is accompanied by homocystinuria and increased plasma homocysteine levels in premenopausal women, prompted us to study the relationship between homocysteine and bone metabolism. We therefore investigated the association between bone metabolism and polymorphism of the MTHFR gene, which regulates plasma homocysteine concentration, Folate and Vitamin B12. The present study demonstrated that the TT genotype of the MTHFR gene was significantly associated with low BMD. The prevalence of TT genotype in this study was 3.98%. These values were lower than those in control group subjects in reported studies such as those done in Canada (12%, 0.38) [18], Boston (14.4%, 0.33) [19], and Tokyo (10.2% 0.33) [20]. The frequency of the TT genotype in the normal group was in line with those of other studies which had been reported previously.

Genetic insufficiency of folate results from the inefficiency of the enzymes in the folate metabolism cycle, the most important one being Methylene tetrahydrofolate reductase or MTHFR. Two common polymorphisms are seen in MTHFR gene namely; C677T and A1298C [8]. The C677T (Ala-to-Val) transition produces thermostability and a somewhat reduced enzyme activity in vitro [9]. Individuals homozygous for the C677T mutation have moderately increased concentrations of fasting plasma homocysteine, especially in the presence of low (<15.4 mol/L) plasma folate levels [10]. The second prevalent polymorphism (A1298C) is associated with a decreased enzyme activity in vitro. This genetic variant consists of an A6C transversion at nucleotide 1298, which produces a Glu-to-Ala substitution [11]. We conducted this study to analyze the influence of polymorphisms of the MTHFR gene, which were observed at the 677 and 1298 nucleotides in the gene sequence, among a study population of 402 subjects. We observed that polymorphic variants of the MTHFR gene were more predominant among cases than among controls, which were substantiated by a significant p-value of 0.05. Our study showed a negative correlation with T allele, while 1298C showed no significant correlation with the C allele.

Cagnacci et al. [21] found that folate and not tHcy was independently related to BMD of lumbar spine in Italian women, whereas Golbahar et al. [22] found a correlation between tHcy and BMDs of both femoral neck and lumbar spine in 271 post-menopausal Iranian women. These differences may therefore be caused by genetic differences in the populations studied, or at the sites of the BMD measurement.

Reported associations of MTHFR genotype and BMD have also been
inconsistent. Positive associations were found in post-menopausal Japanese women [23], and in post-menopausal Danish women [24], but not in post-menopausal Iranian women [25] or in post-menopausal or elderly Chinese women [26]. Villadsen et al., [27] found an association of MTHFR genotype with osteoporotic vertebral fractures in 338 Danish osteoporotic patients as compared to those in controls, but only a weak association with lumbar spine BMD. McLean et al., [28], using data from 1632 male and female subjects in the Framingham Osteoporosis Study, did find an association between MTHFR polymorphism and BMD, but they suggested that this was dependent upon folate status. In our study, there was a significant correlation of BMD at spine and femur, with presence of T allele at position c677T, while there was no correlation in our study at position 1298, as regards BMD at any site. Our data lend support to this view; among those with the CT genotype, those in the lowest quintile for folate (less than 5.0 μg/L), had significantly lower BMDs.

Several recent studies have shown that plasma concentrations of folate, vitamins B6, and B12, were negatively correlated and that increased intake of these vitamins could reduce plasma homocysteine levels in patients with coronary artery disease [29]. Those who had TT genotype of MTHFR could be more susceptible to the hyperhomocysteinaemic effects of a poor folate intake and they could also be more responsive to subsequent folate therapy [21]. In our study, no correlation could be elicited between BMD and Homocysteine and B12. Folate however, did not affect the BMD [21]. In our study, no correlation could be elicited between BMD and Homocysteine and B12. Folate however, did not affect the BMD [21].

CONCLUSION

Dietary folate insufficiency and variants of MTHFR can contribute to a reduced 5-MTHF pool, leading to hyperhomocysteinemia. Additionally, 5-MTHF deficiency can lead to a reduced methylation of DNA and consequent reduction of bone synthesis and turnover. Subjects with lower quintiles of folate, in the presence of the T allele, demonstrate hypomethylation and therefore, they may have a bearing on BMD, independent of hyperhomocysteinemia, or a direct effect via other mechanisms. Other known or unknown candidate genes may be needed to be examined further.

REFERENCES