An SP1-binding site polymorphism in the COLIAI gene: may be a strong predictor for low bone density in thalassemia major.

Research Article

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Summary

Background: β-Thalassemia major is an inherited blood disorder with a high prevalence in the Mediterranean region. Osteoporosis represents an important cause of morbidity in β-thalassemia major and its pathogenesis has not been completely elucidated. Genetic factors play an important role in the pathogenesis of osteoporosis and several candidate gene polymorphisms have been implicated in the regulation of this process. A G→T polymorphism in the regulatory region of the collagen type I alpha 1 (COL1A1) gene at a recognition site for transcription factor Sp1 has been strongly associated with osteoporosis.

Aim: To determine the distribution of COLIAI polymorphism and its relationship with bone mineral density (BMD) in thalassemia major patients of North Indian.

Material and Methods: G→T polymorphism was detected in 150 β-thalassemia major patients by PCR-RFLP and their Bone mineral density (BMD) was measured by Dual Energy X ray Densitometry (DXA). Biochemical levels were estimated by ELISA.

Results: The study indicated 19.8% of β - thalassemia patients as homozygous for wild type G/G (SS), 35.8% as heterozygous G/T (Ss) and 43.4% as homozygous mutant T/T (ss) alleles. A significant association of Sp1 polymorphism with Z-score of BMD at hip (p = 0.047) and at lumbar spine (p=0.001) region was observed.

Conclusion: Our results raise the possibility that genotyping at the Sp1 site can be used to identify the osteoporosis susceptibility in thalassemia major patients in order to provide primary prevention and better management to them.
I. Introduction:

Thalassemia major is associated with a number of morbidities related to bone including bone pain, deformities, delayed bone age, growth failure, rickets, spinal deformities, secondary nerve compression, pathologic fractures, osteopenia and osteoporosis (1, 2). Osteoporosis is characterized by low bone mass and disruption of bone architecture, resulting in reduced bone strength and increased risk of fractures (3). With optimal blood transfusions and chelating therapy, there has been substantial improvement in life expectancy and quality of life of these patients (2, 4). Thus, as thalassemic patients age, osteoporosis is emerging as an important cause of morbidity (5).

Osteoporosis or fracture risk is predicted by Bone mineral density (BMD) scores at different sites of interest (6, 7). Dual energy X-ray absorptiometry (DEXA) is an excellent non-invasive choice for repeated measurements of any temporal changes of BMD because of 1% precision rate and low radiation exposure (7).

Several genes are also thought to be involved in the pathogenesis of osteoporosis. Collagen type I alpha 1 (COLIA1) is one of the most prominent candidate genes, whose polymorphic variants have been consistently found to be associated with osteoporosis in different populations (8–10). COLIA1 encodes the alpha 1 chain of collagen type I, which is the most abundant structural protein in the bone matrix. Grant et al. (11) have identified a common guanine to thymidine (G→T) polymorphism in the first intron of COLIA1. This polymorphism affects one of the binding sites of the transcription factor Sp1 and results in increased expression of collagen type I alpha 1 in bone matrix in T allele carriers. This leads to imbalance in the amount of alpha chain production for helical formation of the collagen proteins affecting the bone density and quality and predisposing to fractures (9).

Previous studies have shown that the T allele is associated with osteoporosis, lower BMD (9, 11), and increased fracture risk (8, 10, 11, 12). Moreover, in a very large prospective meta-analysis, it has been observed that the Sp1 polymorphism in the COLIA1 gene is associated with reduced BMD and incident vertebral fractures independent of BMD (13).

The biomechanical properties of bone with respect to its organic and inorganic content too have been found to be affected with Sp1 polymorphism carrier status (9). This polymorphism has been associated with low BMD and an increased risk of osteoporotic fracture in β-thalassemia major patients in different populations (3). Therefore, the aim of the present study is to examine the distribution of COLIA1 polymorphism and its relationship with BMD in thalassemia major patients from north India.

II. Material and Methods

2.1 Subjects:

One hundred and fifty beta thalassemia major patients attending Department of Genetics, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow were recruited for the study. All patients were maintained on a regular transfusion program according to a monthly regimen with the aim of maintaining pre-transfusional hemoglobin levels above 9 g/dl. Serum ferritin levels were acceptable in all patients. The study protocol was approved by the ethics committee of the SGPGIMS. Written informed consent was obtained from parents or legal guardian as age below 18 years. After enrolment, the subject’s medical history was documented by a review of previous medical records. The subject interview questionnaire included items on demographics, medical and surgical history (e.g. splenectomy), and medication usage was filled by subjects. A medical record review was also done by the research coordinator from Hospital Information System and
by interviewing patient’s, which included documentation of transfusion and chelating history and recent endocrine laboratory values.

2.2 Biochemical analysis:
Serum calcium, phosphorous, alkaline phosphatase, albumin, and creatinine were measured by automated analyzer (Randox). Serum calcium was corrected if serum albumin was lower than 4gm/dl. Vitamin D was measured by enzyme linked immunosorbant assay (IDS-UK). Serum 25OHD3 and iPTH (intact parathyroid hormone), TSH (thyroid stimulating, hormone) were measured by commercially available kits. Hypovitaminosis D was defined as per Lipps’ criteria: sufficiency >30ng/ml, insufficiency 20-30, deficiency 10-20, severe deficiency <10ng/ml.

2.3 Anthropological Measurement:
Subjects were weighed using a digital scale with light clothing without shoes (accuracy 0.1 kg). Height was measured in an upright position without shoes by a stadiometer (accuracy 0.1 cm). Body mass index (BMI, kg/m²) was calculated by dividing body weight (kg) by the height squared (m²).

2.4 Bone mineral density (BMD):
Bone mineral density (BMD) of the lumbar spine (L1±L4) and hips were determined on dual X-ray absorptiometry (DEXA, HOLOGIC, USA) in patients greater than 12 years of age. Pediatric software was used for children with a weight below 30 kg. During measurement of the lumbar spine, the child was supine, and the physiological lumbar lordosis was flattened by elevation of the knees. The Z-score of BMD at hip, lumbar spine and forearm was categorized in three subgroups (a) normal: Z between >-1 and above (b) Low bone mass: Z between ≤ -1 and -2 (c) Severely low bone mass: Z score below ≤ -2.

2.4 Genotype analysis:
DNA was extracted from the peripheral blood by phenol choloroform method described by Poungcz et al 1982. (15)

2.5 Sp1 polymorphism:
A modified PCR-based screening method (Grant et al, 1996) was performed for detection of Sp1 polymorphism (11).
A mismatched primer was used which introduces a restriction site for the Fnu4HI in polymorphic alleles with the T substitution. The sequence of the primers for detection of Sp1 polymorphism were 5'-TACCTTCTGGACTATTTTTGG-3' (Forward) and 5' - GTCCAGCCCTCATCCTGGCC-3'(Reverse).The conditions for PCR amplification were as follows: 200 ng genomic DNA, 10pmol of each primer, 20μM each deoxynucleotidetriphosphate, 1U Taq DNA polymerase (Banglore Genei, India) were constituted in a 50μL reaction. The initial denaturation was performed at 94°C for 3 min, followed by 40 cycles at 94°C for 50 s, 62°C for 10 s, 72°C for 15 min, and a final extension at 72°C for 5 min. The amplicon was digested at 37°C with Fnu4HI, restriction enzyme (New England Biolabs (UK) Ltd. UK) as per manufacturer’s instructions. The digested products were separated and documented on 12% Polyacrylamide gel electrophoresis.

Figure 1: Depiction of frequency pattern of 713-8 delC sequence variation of TgFβ in thalassemia cases.
The different alleles were assessed according to the digestion pattern detected with the restriction enzyme as shown in Figure. 1. The resulting alleles S and s reflect the presence of guanine and thymidine, respectively. Hence, the absence of the recognition site on both alleles (no digestion) results in the SS genotype, whereas the presence of the site on both alleles is characterized by the ss genotype. The SS genotype showed bands of 180bp, 54 bp, 23bp. The ss genotype generated two fragments of 180 and 77 bp. The heterozygotes displayed four fragments of 180, 77, 54 and 23 bp, designated as Ss.

2.6 Statistical Analysis:
An analysis of relationships between bone mineral density and clinical parameters, i.e. age, body mass and height was performed with the use of the Spearman’s rank correlation. Student’s t-test was used to compare the means of the two samples. The difference in the mean value of the various biochemical and BMD indicators in subjects with 3 different genotype groups related to Sp1 gene polymorphism was analyzed by using ANOVA followed by Bonferoni’s test for posthoc analysis if the ANOVA was significant. A P value <0.05 was considered significant.

2.7 Result:
The anthropometric, haematological and densitometry data are shown in Table I. The mean age of thalassemia patients enrolled in our study was 16.66 ± 5.45 years. Reduced growth pattern was observed with mean height of girls being 137.71 ± 16.96 cms and that of boys was 137.6±18.45. While 54.7% boys and 28.7% of girls were of short stature (as height<5 percentile), 41.6% boys and 33.3% of girls were found to be underweight.

BMD of the examined subjects was expressed as the Z-score according to the WHO classification (World Health Organization, 1994). A large majority of the patients showed bone mass below one standard deviation from the mean value of the healthy subjects. In the lumbar spine, 20 % had normal BMD whereas 37.5% cases had Z score between -1.0 to -2.5 SD suggesting low BMD (osteopenic). An analysis of Spearman’s rank correlation coefficient indicated a significant influence of serum vitamin D on Z score of BMD at lumbar spine in the studied group of patients (r = 0.422, P value = 0.031) . Z score was found below -2.5 SD in 42.5% cases indicating osteoporosis at lumbar spine. Z score of BMD at hips was found as normal, osteopenic and osteoporotic in 12.5%, 55% and 32.5% respectively. The osteopenia /osteoporosis were more prevalent in the spine but there was no significant difference between sexes. Biochemical hypocalcemia (s. total calcium <8.5mg/dl) was present in 27.5% subjects. Hyperphosphatemia (s. Ip >5.5mg/dl) was present in 42.5% subjects .Serum creatinine and serum albumin was found normal in all cases. Vitamin D deficiency (s.25OHD3 = 10-20ng/ml) and severe deficiency (<10ng/ml) was present in 80.6% and 12.9% subjects respectively.

In our study, distribution of COLIAI genotypes in the β-thalassemia major patients observed was 19.8% SS (homozygous for G/G), 35.8% Ss (G/T heterozygote) and 43.4% ss (homozygous for T/T) (Figure. 2) with 37.7% S, 61.3 % s allele frequencies in thalassemia major patients.
The correlation between mean Z-score values of lumbar spine BMD, COLIA1 Sp1 polymorphism and the various anthropometric and biochemical variables is provided in Table 1. The subjects with `Ss' and `ss' genotypes had a lower bone mass than those with `SS' genotype those with homozygous `s' allele had the lowest bone mass. Sp1 polymorphism was found significantly associated with Z score of BMD at lumbar spine (P value = 0.001) and also at Z score of BMD at hips (P value = 0.047). BMD values were still lower in `ss' subjects, the difference between men and women was not significant because of the small number of homozygotes. Vitamin D is significantly associated with Sp1 polymorphism (P value=0.05).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SS</th>
<th>Ss</th>
<th>ss</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Weight(kg)</td>
<td>27.6±6.9</td>
<td>29.8±9.23</td>
<td>36.2±8.36</td>
<td>0.004</td>
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<tr>
<td>Height(cm)</td>
<td>124±19.3</td>
<td>120.87±17.7</td>
<td>137.22±8.24</td>
<td>0.007</td>
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<tr>
<td>BMI(㎏/㎡)</td>
<td>19.58±3.5</td>
<td>20.7±4.4</td>
<td>20.1±3.04</td>
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<tr>
<td>S.Ca(mg/dl)</td>
<td>8.45±1.26</td>
<td>8.59±1.11</td>
<td>8.7±1.1</td>
<td>0.667</td>
</tr>
<tr>
<td>S.P.(mg/dl)</td>
<td>5.17±1.15</td>
<td>5.23±0.980</td>
<td>5.3±1.24</td>
<td>0.918</td>
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<tr>
<td>S. Al(PO4)(mg/dl)</td>
<td>312.7±188</td>
<td>287±119.9</td>
<td>315.1±148.6</td>
<td>0.750</td>
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<tr>
<td>S. 25(OH) Vit. D (nmol/l)</td>
<td>7.99±8.6</td>
<td>11.4±8.83</td>
<td>9.59±8.5</td>
<td>0.050</td>
</tr>
<tr>
<td>Z Score lumbar spine</td>
<td>-1.42±1.01</td>
<td>-2.1±1.86</td>
<td>-2.70±1.03</td>
<td>0.001</td>
</tr>
<tr>
<td>Z Score hips</td>
<td>-1.66±1.67</td>
<td>-2.1±1.18</td>
<td>-2.19±1.02</td>
<td>0.047</td>
</tr>
</tbody>
</table>

**Table 1**: Anthropological, Biochemical and Bone mineral density characteristics of thalassemia patients related with genotypes
III. Discussion:

In thalassemia, bone disease in the form of low bone mass remains a frequent, debilitating and poorly understood problem, even among well transfused and chelated pre-pubertal and adult patients (16). The pathogenesis of osteoporosis in β-thalassemia major is complex. Several genetic and acquired factors act in concert to produce bone disease. The acquired causes include factors such as defective GH-IGF-I axis, which primarily interferes with bone neoformation (17), iron deposition in bone (18), vitamin D deficiency (19), deferrioxamine bone toxicity (20) and increased resorption due hypogonadism and delayed puberty (21). Also, the primary disease itself may cause a mechanical interruption of bone formation through bone marrow expansion, leading to cortical thinning, increased distortion and fragility of the bones (25).

Evidence from family and twin studies has clearly shown that genetic factors play an important role in the pathogenesis of osteoporosis and the inheritance of bone mass is under polygenic control (23). Recently, great interest has been aroused regarding the relationship of polymorphisms in the genes of COL1A1, vitamin D receptor, estrogen receptor and BMD. However, results have been controversial which emphasize the need for further investigations (3, 24). Various studies have verified the association between polymorphism of the binding site of the transcription factor Sp1 in the first intron of the COL1A1 gene and bone mass in several populations (6, 25-27). Our study was designed to investigate the distribution of this COL1A1 polymorphism in North Indian β-thalassemia major patients and its relationship with bone mass.

In the present study 19.4% had the Ss (G/T) genotype, 80.6% had the SS (G/G) genotype and the remaining 43.4% had ss genotype (T/T) type. The frequency of the s allele was found to be higher than S allele in low bone mass thalassemic patients. Our results were in accordance with previous studies showing that the Ss and ss genotypes were associated lower bone mass than SS genotype. The homozygous patients for s allele had the lowest bone mass (3). In the present study, thalassemia patients showed significantly lower BMD values both at the lumbar spine and total hip. There was no significant difference between sexes regarding BMD which was in accordance with previous studies (28, 29).

Several previous studies have shown that the COL1A1 genotype predicts osteoporotic fracture by mechanisms which are partly independent of BMD. Jin et al. (30) reported increased risk of fracture in correlation to SP1 polymorphism in spite of modest correlation with BMD and Gaudio et al. (31) as he reported absence of SP1/BMD correlation and that thalassemia major patients benefit from osteoporosis therapy, indicating that the polymorphism may act as a marker for bone quality as well as bone density (8, 32, 33). The association between gene polymorphisms and bone mass in thalassemia major needs to be considered, bearing in mind that the disease is a well documented cause of secondary osteoporosis with multiple negative influences on bone metabolism that could appear to be a result of the effect of the polymorphism. In fact, many factors in thalassemia major can lead to unbalanced bone remodelling and increased bone resorption.

In order to secure normal health in β thalassemia patients, optimal supportive care with regular transfusions and chelation along with early management of possible endocrine complications is warranted. Our results raise the possibility that genotyping at SP1 site could be of clinical value in identifying the thalassemic patients at risk of developing osteoporosis and fractures and hence can play a role in better management of osteoporosis in
thalassemic patients [30]. Early detection of mutation at the Sp1-binding site on the COLIA1 gene should be in regular practice to initiate preventive therapy before fractures occur in children with β-thalassemia major.

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**References**


