Osteoporosis in β-Thalassemia Major Patients: Role of COLIA1 Gene G-T Polymorphism

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Abstract

Background: Growth impairment and osteoporosis are serious causes of morbidity in patients with β-thalassemia major (β-TM). Desferoxamine (DFO) toxicity and iron overload have been proposed as the main underlying reasons. G-T polymorphism in regulatory region of COLIA1 gene has recently been associated with reduced bone mass and osteoporotic fractures in postmenopausal women.

Objectives: To detect the possible implication of COLIA1 gene polymorphism in pathogenesis of osteoporosis in β-TM.

Study Design: Twenty five patients with β-TM and 20 healthy controls were investigated for the G-T polymorphism of COLIA1 gene using restriction enzyme analysis. Bone mineral density (BMD), growth parameters, serum ferritin level and duration of chelation therapy were also assessed.

Results: We detected a heterozygous polymorphism of COLIA1 gene in 12% of β-TM patients and 25% of the control group. Thalassemic patients had significant lower BMD than normal controls (p<0.01). Significant correlation was observed between low BMD and both duration of DFO intake and high ferritin level. Within the control group: Subjects with G/T genotype had significantly lower femoral and lumber BMD than those with G/G genotype. In thalassemic patients: No significant difference was found in BMD between the two COLIA1 genotypes.

Conclusion: We cannot detect evident role for COLIA1 gene polymorphism in the pathogenesis of osteoporosis in β-TM patients although this role has been detected in the control group. Further studies that include higher number of patients and more than one genetic polymorphism are needed in order to evaluate the role of genetic factors in the pathogenesis of osteoporosis in thalassemic patients.

Key Words: COLIA1 gene – Osteoporosis – Thalassaemia major.

Introduction

BETA thalassemia is a group of autosomal recessive hereditary haemoglobinopathy characterized by a deficiency or absence of β globin chain of adult hemoglobin [1]. β-thalassemia major (β-TM) is a severe clinical phenotype that manifests in early infancy [2]. Conventional management of β-TM requires regular blood transfusion and chelation therapy to prevent iron overload. In this way the survival time of these patients can be significantly prolonged, however, new complications appear. Osteoporosis is one of them and it is responsible for serious morbidity in adult patients with TM. Osteoporosis is characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and significantly higher frequency of fractures [3]. Multiple factors are involved in the pathogenesis of osteoporosis in TM, they include the primary disease itself causing bone marrow expansion [4], iron overload [5], chelating agents toxicity [6], hormonal deficiencies [7], calcium, zinc and vitamin D deficiencies and decreased physical activity [8].

In the last few years, many studies have suggested an important role of genetic factors in the regulation of bone mineral density (BMD) [9,10,11]. The gene encoding for one of the bone matrix proteins: Collagen type I (COL1) plays a role in the determination of BMD [12]. A polymorphism in the binding motif of the nuclear transcription factor Sp1 in the promoter region of COLIA1 gene was associated with reduced BMD and predisposed men and women to osteoporotic fractures [12,13]. Susceptibility to osteoporotic fracture is found to be determined by allelic variation specifically at the Sp1 site, rather than other polymorphic sites at the COLIA1 gene [14]. This polymorphism results from single base exchange from G (guanine-allele S) to T (thymine-allele s). The ss genotype was
found to have the lowest BMD than Ss and SS genotypes [12]. We designed this study to investigate the possible association between BMD, COLIA1 gene G-T polymorphism and treatment dependent factors in π-TM patients.

**Patients and Methods**

Twenty-five patients with π-TM (12 males and 13 females), were enrolled in this study. They have selected from patients referred from New Children Hospital, Cairo University to Human Genetics Department in the National Research Center, Cairo, Egypt. They are classified as π-TM according to the age of onset and frequency of blood transfusion. The study also included 20 unrelated normal persons of matched age and sex with no family history of thalassemia as a control group.

All cases were subjected to the following:

- Complete history taking: Frequency of blood transfusion, the age of starting chelation therapy and duration of chelation were recorded.

- Anthropometric measures: Patients’ weight, standing and sitting heights were recorded, then they were expressed as a standard deviation score (SDS).

- Serum ferritin level measurement: By sandwich immunoassay on Elecsys 2010 analyzer using reagents from Roche Diagnostics GmbH, Mannheim, Germany.

- Dual energy absorbiometry (DXA):

  DXA (Norland XR 46 Rev.3.9.6/2.3.1) was done to all cases on two sites (lumber spines and femoral neck) to measure the bone density in these sites.

  BMD is expressed as g/cm$^2$. Z-scores were calculated and the patients were divided into normal (z score between zero and -1), osteopenic (z score between -1 and -2.5) and osteoporotic (z score below -2.5) [18].

- Detection of G-T polymorphism in COLIA1 gene using restriction enzyme analysis:

  - Principle:

    PCR was used to amplify DNA fragment (264bp) from the COLIA1 gene enclosing the first intron of the gene which is the binding site for the nuclear transcription factor Sp 1. Then Bal1 enzyme was used to digest the amplified fragments in order to detect the presence of G-T polymorphism at the Sp1 binding site [16]. If polymorphism is present, the enzyme will cut the amplified fragment (264bp) into two fragments (246bp and 18bp), this restriction pattern will be designated (T/T) and the genotype will be (ss). If there is no polymorphism, the enzyme will not cut the 264 bp fragment then it will be designated as (G/G) and the genotype will be (SS). If the patient has the polymorphic site on only one chromosome, the restriction pattern will be designated as (G/T) and the genotype will be (Ss) which can be detected by the presence of both 246bp and 264bp fragments.

  - Steps:

    DNA extraction:

    Briefly, blood samples were collected on Na$\text{2}$EDTA as an anticoagulant, genomic DNA was extracted from peripheral blood leucocytes by phenol/chloroform technique as mentioned before [17].

    Polymerase chain reaction (PCR):

    Reagents and primers were provided by Qiagen, Valencia, USA, reaction mixture was performed with a final volume of 50$\gamma$l as follows:

    - 2$\gamma$g of genomic DNA (2$\gamma$1), 20pmol of each primer (10$\gamma$1), 2 U of Taq DNA polymerase (0.4$\gamma$1), 0.25 mM dNTPs (5$\gamma$1), 10x PCR reaction buffer (5$\gamma$1), Q solution (10$\gamma$1) and dd H$_2$O (17.6$\gamma$1).

    The sequence of PCR primers used was:

    - Sp1 forward primers: 5’-GTCCAG CCCTCATC-CTGGCC-3’ and

    - Sp1 reverse primers: 5’-TAACTTCTGGACTTTGGT-3’

    The PCR was performed under the following conditions:

    Initial denaturations at 94°C for 5 minutes, a total of 35 cycles were done including: Denaturation at 94°C for 1min, primer annealing at 65°C for 1 min, primer extension at 72°C for 1 min. and a final extension at 72°C for 5min using a thermal cycler (Perkin Elmer).

    To be sure that the amplification had occurred correctly, about 8$\gamma$l of each amplified product were submitted to electrophoresis on 2% agarose gel treated with ethidium bromide (1mg/ml) to view the 264bp band of the amplified fragment of COLIA1 gene.

    Enzyme digestion:

    The amplified products were digested with the Bal1 restriction enzyme (Promega, Madison, USA) according to the manufacturer instructions. The digest was performed with a final volume of 25$\gamma$l
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as follows: 15 µl amplified products, 0.5 µl Bal1 enzyme (15 U), 2.5 µl bovine serum albumin (0.1mg/ml), 2.5 µl buffer G and 4.5 µl ddH2O. The digest was mixed well, centrifuged for few seconds and incubated overnight at 37ºC.

Electrophoresis:

The digested products were submitted to electrophoresis on 8% polyacrylamide gel and analyzed under ultraviolet light.

The PCR product containing allele s, which identifies the G-T substitution, resulting in a band of 246bp compared with the non-cleaved S allele that shows a band of 264bp.

Statistical analysis:

Data were computerized and analyzed by EPI Info version 6.2 produced through the collaboration between CDC/WHO and by SPSS PC+, version 9. Data were presented by means ± SD and percentages. The following tests of significance were used: t test between percentages, regression analysis and correlation. A level of significance was set so as p≤0.05 was considered significant, p≤0.01 was considered highly significant and p>0.05 was considered insignificant.

Results

Clinical and hematological data:

The study included 25 patients with β-TM, their mean age was 18.12 ± 5 years, age of onset ranged between 3 months and 2 years, frequency of blood transfusion ranged between 8 and 24 times/year, age starting DFO ranged between 1 and 15 years old, duration of DFO intake ranged between 1 and 20 years except for 3 cases who showed irregular or discontinued intake after 1 year, mean value of serum ferritin level was 2861.4 ±750ng/ml.

Anthropometric data:

Three out of 25 patients (12%) were less than 2 SD below the mean of normal weight. Short stature was found in 36% of patients who were less than 2 SD below the mean of normal standing height and 20% of patients were less than 3 SD. Truncal shortening was found in 48% of patients who were less than 2 SD below the mean of normal sitting height.

Densitometric data:

Assessment of BMD at the neck of femur showed that 11 out of 25 patients (44%) had osteopenia and 4 patients (16%) had osteoporosis, when assessing BMD at the lumber spine, it was found that 12 patients (48%) had osteopenia and 4 patients (16%) had osteoporosis.

Regarding the control group, their mean age was 23±2.3 years, their weight and height were within the normal range for adults and assessment of BMD shows that 6 persons (30%) had osteopenia at the neck of femur and 2 persons (10%) had osteopenia at the lumber spine.

COLIA1 genotyping:

The result of PCR amplification of the 264bp fragment of the COLIA1 gene that encloses the binding site of the nuclear transcription factor Sp1 was shown in (Fig. 1).

After enzyme digestion of the amplified products, it was found that 22 out of 25 β-TM (88%) were G/G (SS) and 3 patients (12%) were G/T (Ss). In the control group 15 cases (75%) were G/G (SS) and 5 cases (25%) were G/T (Ss). (Fig. 2) & (Table 1).

Table 1: Distribution of COLIA1 genotype between thalassemic patients and controls.

<table>
<thead>
<tr>
<th>COLIA1 genotype</th>
<th>Patients (Number=25)</th>
<th>Controls (Number=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>22 (88%)</td>
<td>15 (75%)</td>
</tr>
<tr>
<td>Ss</td>
<td>3 (12%)</td>
<td>5 (25%)</td>
</tr>
<tr>
<td>ss</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Data analysis:

By univariate analysis of variables it was found that:

Thalassemic patients had significant lower femoral and lumber BMD compared to normal controls (p<0.01).

Table 2: Comparison between thalassemic patients and controls regarding BMD.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients Mean ± SD</th>
<th>Controls Mean ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z score of femur</td>
<td>-1.29±1.02</td>
<td>-0.11±0.83</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Z score of lumber spine</td>
<td>-1.42±0.85</td>
<td>-0.37±0.92</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Within the control group: Subjects with G/T genotype had significantly lower femoral (p<0.05) and lumber (p<0.01) BMD than those with G/G genotype.

Table 3: Comparison of BMD between the two COLIA1 genotypes within the control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>G/G Mean ± SD</th>
<th>G/T Mean ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z score of femur</td>
<td>-0.11±0.87</td>
<td>-1.16±0.59</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Z score of lumber spine</td>
<td>0.14±0.71</td>
<td>-0.86±0.78</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
**In thalassemic patients:** No significant difference was found in BMD of femur and lumber spines between the two COLIA1 genotypes.

Table (4): Comparison of BMD between the two COLIA1 genotypes in β-TM patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>G/G Mean ± SD</th>
<th>G/T Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z score of femur</td>
<td>–0.87±1.15</td>
<td>–0.93±0.58</td>
<td>p=0.835</td>
</tr>
<tr>
<td>Z score of lumber spine</td>
<td>–0.79±1.09</td>
<td>–1.03±0.93</td>
<td>p=0.532</td>
</tr>
</tbody>
</table>

Regression analysis of all variables was done and showed that:

Significant positive correlation was observed between early age of starting DFO and decrease sitting height in male β-TM patients (p<0.01).

Significant negative correlation was observed between long duration of DFO intake and low BMD at the neck of femur (p<0.01) and lumber spine (p<0.05) in β-TM patients.

Significant negative correlation was observed between high ferritin level and low lumber BMD in female β-TM patients (p<0.05).

In this study, significant positive correlation was observed between early age of starting DFO and truncal shortening in male β-TM patients (p<0.01), also the lowest sitting height SDS detected in this study (5 SD below the mean) was for a male patient who started DFO intake at the age of 1 year.

Other investigators [20] detected truncal shortening in all of their studied patients in whom DFO therapy was started at a mean age of 3 years and found that height disproportion was more severe in boys. It was concluded that DFO toxicity is responsible for the finding that growth rate of thalassemic patients who started chelation in early childhood is significantly slower than that of patients who started chelation in late childhood or early adolescence [21].

In our study, significant negative correlation was observed between long duration of DFO intake and low BMD at the neck of femur (p<0.01) and lumber spine (p<0.05). In another study [22], BMD was investigated in 203 β-TM patients and it was found that age when iron chelation was started and duration of chelation therapy were associated with low BMD.

In this study, significant negative correlation was observed between long duration of DFO intake and low BMD in female β-TM patients (p<0.05).

In another study [23], the mean serum ferritin level in thalassemic patients with a final short stature was significantly higher than in those with a normal final height and a mean ferritin level of 3000ng/ml was found to be the cut-off for final short stature. Iron deposition in bone impairs osteoid maturation and inhibits mineralization locally, resulting in focal osteomalacia with subsequent reduction of BMD and bone tensile strength [24].

**Discussion**

As survival improves in patients with β-TM - due to introduction of regular transfusion regimens together with effective iron chelation - a new range of problems emerges particularly in relation to their growth and development [18]. Growth problems in β-TM can be divided into two categories: Problems related to physical growth (weight, actual height and disproportion) and those related to bone mineralization and development. In this study we investigated the anthropometric parameters and BMD in 25 patients with transfusion dependent β-TM and studied the possible association between these parameters and treatment dependent factors (such as iron overload and DFO toxicity) and with genetic polymorphism of COLIA1 gene which has been shown to be significantly related to bone mass and osteoporotic fractures [19].
During the last decade, many investigators observed that low BMD, osteopenia and osteoporosis remain serious complications, even in well-transfused and iron-chelated patients with a frequency of approximately 40-50% in the studied populations suggesting the presence of other factors [25,26,27].

Genetic factors play an important role in the pathogenesis of osteoporosis by complex mechanisms involving variation in several genes that regulate BMD and bone geometry and quality [28]. One of the most important candidate genes for predisposition to osteoporosis is the COL1A1 gene. G-T polymorphism in the binding motif of the nuclear transcription factor Sp 1 in the promoter region of COL1A1 gene was associated with reduced BMD and an increased risk of osteoporotic fracture in several studies [13,19,29]. But the role of COL1A1 gene polymorphism in pathogenesis of osteoporosis in thalassemia is still controversial [3].

In our study the distribution of COL1A1 genotype among the control group was SS (75%), Ss (25%). By univariate analysis, subjects with Ss genotype had significantly lower femoral \((p<0.05)\) and lumbar \((p<0.01)\) BMD than those with SS genotype. These results were in concordance with other investigators.

In the Netherlands, the relation of the COL1A1 polymorphism to bone density is studied in 1778 postmenopausal women (age from 55-80 years) and BMD in the Ss group was significantly lower than that in the SS group at both femoral neck \((p=0.003)\) and lumbar spine \((p=0.02)\). In the ss group, BMD was significantly lower than that in the SS group \((p=0.05)\) for the femoral neck and \(p=0.005\) for the lumbar spine) [30].

In another study on 715 Italian peri and postmenopausal women (age from 47-85 years), COL1A1 genotype was significantly associated with hip BMD, with the highest values in the SS group and the lowest in the ss group \((p<0.05)\) [29].

However, other investigators did not detect this association. In a study on postmenopausal danish women (age from 45-54 years), no association was found between COL1A1 genotypes and BMD [31]. Also, in another study on 220 healthy Brazilian women (age from 20-46 years), no association was found between Sp1 binding site polymorphism and lumber or femoral BMD [16].

These data taken together may indicate that there may be population-dependent [32] and age-dependent [31] variations in the effect of the COL1A1 genotype on BMD as these studies were conducted on different populations of different age groups.

In this study, the distribution of COL1A1 genotypes in thalassemic patients was SS (88%) and Ss (12%).

In a study conducted in Italy, they investigated 135 \(P\)-TM patients and the frequency of the genotype was SS 55%, Ss 41% and ss 4% [33].

In another study the genotype distribution among British thalassemic patients was SS genotype 66%, Ss genotype 30% and ss genotype 4% [34].

The frequency of COL1A1 genotype in a group of Turkish \(P\)-TM patients was SS 35.7% and Ss 64.3% [35].

The low frequency of the "s" allele in thalassemic patients in our study may be attributed to small sample size or different study population.

In this study we found no association between COL1A1 polymorphisms and BMD at the lumber spines and femoral neck in the thalassemic patients nor did we detect an over-representation of the "s" allele in patients with osteoporosis. Thus, our data do not confirm other studies; in a study on 135 Italian patients with \(P\)-TM (mean age=24 years), patients with Ss and ss genotypes had a significantly lower BMD than patients with SS genotype \((p=0.014)\) and the homozygous patients for s allele had the lowest bone mass [33]. In Turkey, thalassemic patients who are heterozygotes (Ss) at the polymorphic Sp1 site have a significantly lower BMD than homozygotes (SS) \((p=0.01)\) [35].

Our finding is in consistent with a study conducted in Italy and investigated 238 \(P\)-TM patients with a mean age of 24 years and found no association between BMD and polymorphisms in COL1A1 gene [3].

Lack of association between COL1A1 polymorphism and BMD in our group of thalassemic patients may be explained by low frequency of the "s" allele in our study population. Also, many genes are implicated in the pathogenesis of osteoporosis in TM like collagen type I gene, vitamin D, estrogen and calcitonin receptors genes [36] together with multiple acquired factors like diet, lifestyle and drugs [27]. Contradictory results regarding the association between COL1A1 gene polymorphism and BMD in \(P\)-TM patients were shown in the two studies conducted in Italy [3,33] although they
studied a similar group of patients in the same age group. This may indicate that environmental factors may conceal weaker genetic associations.

**In Conclusion:** We found no evident role for COLIA1 gene polymorphisms in the pathogenesis of osteoporosis in this group of β-TM patients although this role has been detected in the control group. Further studies that involve a larger group of patients and include more than one genetic polymorphism are needed in order to evaluate how the COLIA1 Sp1 polymorphism in combination with other genetic markers could contribute to the prediction of osteoporosis and fracture risk in thalassemic patients.

**References**


