Thalassaemia Patients with Polymorphism of *COL1A1* Sp1 are at Greater Risk of Spine Degenerative Changes

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ABSTRACT

Objective: To explore the association of the *COL1A1* Sp1 polymorphism with decreased bone density and spinal changes in β-thalassaemia patients.

Study Design: Cross-sectional, comparative study.

Place and Duration of the Study: University of Health Sciences, Lahore, from May 2020 to June 2021.

Methodology: A total of 110 participants (55patients and 55 controls) of either gender, with ages ranging from 18-40 years were enrolled in the study. Bone density parameters including T-score, Z-score, bone-transmission time (BTT), and amplitude-dependent speed of sound (ADSOS) were assessed by ultrasound bone profiler. Lumbar spine radiographs were collected from patients and assessed for spine changes. Genotype analysis was done by HRM-PCR. Data were analysed using SPSS version 23.

Results: All bone density parameters were significantly lower in β-thalassaemai patients ($p<0.001$). Spine degenerative changes were more obvious in patients with age <25 years ($p=0.04$). Loss of lumbar lordosis was seen in 74.5% of the patients. The frequency of mutant allele (ss) was 7.3% while heterozygous (Ss) frequency was found to be 33.6%. The polymorphism showed significant association with T-scores ($p=0.03$) and ADSOS ($p=0.02$) in patients. Radiographic grades were higher in osteopenic and osteoporotic patients ($p=0.04$).

Conclusion: The association of polymorphism with decreased bone density in β-thalassaemia patients revealed the potential role of genetics in bone changes and related disorders.

Key Words: *Bone Density, COL1A1 Polymorphism, Osteoporosis, Thalassaemia.*

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INTRODUCTION

Osteoporosis is a common observation in β-thalassaemia major (TM) patients predisposing them to increased risk of fractures. Even after regular transfusion and chelation therapy thalassaemia patients have an imbalance between bone formation and resorption. The consequence of imbalance is decreased bone mineral density (BMD) which is more obvious in the axial skeleton.[1] Bones are affected due to bone marrow expansion which causes mechanical disruption in these patients leading to cortical thinning and coarse trabecular pattern. The pathogenesis of decreased bone mass in TM is complex, involving both environmental and genetic factors.

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Among the numerous genetic factors related to osteoporosis, polymorphism at the Sp1 site of the collagen type 1A1 (*COL1A1*) gene is considered a major causative factor and has been linked to low BMD and high osteoporotic fractures. *COL1A1* gene encodes the α1 protein chain of type I collagen, a major protein of bones.[2] Type I collagen is composed of two α1 and one α2 chains, encoded by *COL1A1* and *COL1A2* genes, respectively. The gene leading to the formation of α1chain of type I collagen is located on 17q21.33, and is the most studied gene in the family of collagenes genes.[3]

Polymorphism in *COL1A1* has also been linked to other diseases like osteogenesis imperfecta type 1, Ehlers-Danlos syndrome, Caffey disease and idiopathic osteoporosis.[4] Previous studies on different populations established significant association between mutant allele 's' of *COL1A1* and reduced bone density in thalassaemia patients leading to the conclusion that *COL1A1* Sp1 polymorphism is associated with low BMD and increased fracture risk in these patients.[5] Since thalassaemia patients often present with spine changes, it is crucial to understand whether there is a link between this polymorphism and actual spine changes. The current study was designed in consideration of this gap in knowledge. In addition, thalassaemia being

widely prevalent in Pakistan with an estimated frequency of 5,000-9,000 births per year and a carrier rate of 9.8 million,[6] molecular and genetic association is still unknown. So far, no data is available on the association and genetic background of bone disease in thalassaemia patients in Pakistani population.

This study aimed to find out the frequency of mutant allele of *COL1A1* in Pakistani population and role of this polymorphism in osteoporosis along with the spine changes in β-thalassaemia patients.

METHODOLOGY

This comparative, cross-sectional study was done at the Department of Anatomy and Allied Health Sciences, University of Health Sciences, Lahore, from May 2020 to June 2021. The study included a total of 110 participants with 55 adult thalassaemia patients of both genders and 55 age and sex matched healthy controls. A sample size of 110 (55 patients and 55 controls) was estimated by using 5% level of significance, 80% power of test using the WHO calculator for sample size calculation according to the proportions of mutant genotype (ss) of 13% in thalassaemic patients and 0% in healthy controls. The sampling technique employed was non-probability convenient sampling technique. The subjects were enrolled in the study after written informed consent from either patients or parents and approval by Ethical Review Committee of the institution (UHS/REG-18/ERC/2433).

Patients enrolled in the study had pre-transfusion haemoglobin (Hb) above 9g/dl and serum ferritin <2000ng/dl to avoid low Hb and high serum ferritin-induced bone disease. The age range was 18 to 40 years.

The patients with short stature, any endocrine disorder (hypogonadism and hypoparathyroidism) and those taking regular calcium and vitamin D supplements were excluded from the study as all these factors could affect the bone turnover.

Full demographic profile (age, gender, weight, height, etc.), medical history (bone pains, spontaneous fractures, etc.) and surgical history (splenectomy) were recorded. Drug history including the type of the chelating agent and frequency of taking chelation therapy was obtained from the patients.

The lateral spine radiographs of patients were assessed for disc space narrowing and anterior osteophytes. Decrease in disc space height was graded according to the standard criteria. Three grades were given from grade 0 (no height loss), grade 1 (mild height loss) to grade 2 (moderate to severe height loss).

Presence of bony outgrowth or projection of the vertebral body arising from the superior and inferior anterior borders was defined as anterior osteophyte. Their presence was recorded as grade 1 (small osteophyte) and grade 2 (moderate to large osteophyte).

The total scoring on the basis of disc space narrowing and anterior osteophytes was established for each of the lumbar segments and the highest grade was considered as final.[7] Loss of lumbar lordosis was also assessed in these radiographs. Lumbar lordosis angle (LLA) was measured as the angle of the line intersecting the upper margin of the L1 vertebra and the upper margin of the S1 vertebra.[8,9]

Ultrasound bone density measurement of all the study participants was done using the bone densitometer Sonic Bone Profiler (IGEA, Capri, Italy, and Model BP01). T-scores (bone density that is two and half standard deviations below the mean with reference to a thirty-year-old male/female) and Z-scores (bone density that is two and half standard deviations below the mean with reference to a person of the same age and gender) were recorded that indicated the general bone conditions either as normal (T-score >-1), osteopenic (T-score-1 to -3.2) or osteoporotic (T-score <-3.2).[10] The amplitude-dependent speed of sound (ADSOS) and bone transmission time (BTT) were also recorded.

Approximately 5-10 ml of blood sample was withdrawn from each of the study participant for the assessment of Sp1 polymorphism (rs1800012). The genomic DNA was isolated using the Gene JET Genomic DNA Purification kit (Thermo Fisher, USA) according to the manufacturer's protocols. Primer pair for the amplification of first intron of the *COL1A1* gene harboring the polymorphism of Sp1 binding site was designed using the Primer-3 software. All the samples including patients and controls were assessed for SNP genotyping using HRM-PCR. Genotypes were analysed based on thermal denaturation of amplified double stranded DNA by performing high resolution melting curve analysis on Precision Melt Analysis software (Bio-Rad, USA). Results of HRM-PCR analysis were further confirmed by Sanger sequencing.

The data were entered and analysed using SPSS 23.0. Mean ± SD was calculated for quantitative variables. Frequencies and percentages were calculated for qualitative variables. Independent sample t-test was applied to determine the difference of means (age, BMI, and bone density scores) between two groups and One-way ANOVA was used to compare the means of BMD scores (T-score, Z-score, ADSOS, and BTT) among genotypes. Pearson's Chi-square and Fisher's exact test were used to compare the qualitative parameters (bone density categories and radiographic grades) between the groups. Regression analysis was done to find out the risk related to the presence of genotypes with reference to a normal genotype. A p-value ≤0.05 was considered as significant.

RESULTS

In this study of 110 subjects, 55 participants were patients of thalassaemia, and 55 were healthy controls. In the group of thalassaemia, 32 (58.2%) were males and 23 (41.8%) were females with mean age of 24.89 ± 4.4 years while in the control group, 36 (65.5%) were males and 19 (34.5%) were females with mean age of 30.96 ± 4.8 years (p<0.001). There was no significant difference in gender distribution between the two groups. The BMI was significantly higher in controls as compared to the patient group (p<0.001).

Table I: Comparison of densitometry data of patients and controls.

Variables	Group (55)	Mean ± SD	Range	p-value
T-score	Thalassaemia	-3.9±1.8	-7.1 - 0.5	0.001*
	Control	-2.7±1.8	-6.5 - 1.1	
Z-score	Thalassaemia	-3.9±2.3	-11.0 - 1.2	0.001*
	Control	-2.5±1.8	-6.4 - 1.1	
ADSOS	Thalassaemia	1846.3±127.4	1624.0 - 2160.0	<0.001*
	Control	1935.7±128.7	1671.0 - 2198.0	
BTT	Thalassaemia	1.2±0.4	0.0 - 1.9	<0.001*
	Control	1.6±0.4	0.0 - 2.3	

*T-score, Z-score, ADSOS: Amplitude dependent speed of sound, BTT: Bone transmission time. *Independent sample t-test.*

Table II: Percentage distribution of bone density categories.

Group	Bone Density Categories			Total	p-value
	Normal	Osteopenic	Osteoporotic		
Thalassaemic patients	4 (7.3%)	15 (27.3%)	36 (65.5%)	55	0.01*
Control	11 (20%)	22 (40%)	22 (40%)	55	
Total	15 (13.6%)	37 (33.6%)	58 (52.7%)	110	

**Chi-square test.*

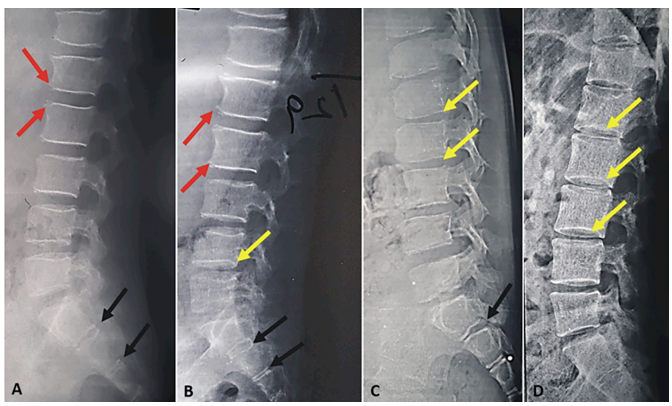

Figure 1: Lateral radiographs showing anterior osteophytes (red arrows) disc height loss (yellow arrows) and non-fusion of sacral vertebrae (black arrows) in (A) a 24-year-old male (B) a 31-year-old male (C) a 20-year-old male and (D) a 21-year-old female. Note the loss of lumbar lordosis in C & D.

All patients were on high transfusion scheme with mean pre transfusion Hb of 9.4 ± 0.5g/dl. Their mean serum ferritin was 1411.5 ± 529.8 ng/dl, and they were taking chelation therapy regularly. Out of 55 patients in the thalassaemia group, 11 (20%) were having bone pain and 9.1% had history of fracture after moderate to severe trauma. None of them had spontaneous fracture.

The radiographic assessments revealed that majority of the patients were having mild to moderate disc space narrowing with small anterior osteophytes (Figure 1). There were 62% of the patients with radiographic grade 1. Normal grade (grade 0) and grade 2 were found in 30.9% and 7.3% individuals, respectively. No disc calcification was evident in any radiograph.

The radiographic grades were not significantly different between males and females, but considering the age, these grades were significantly higher in patients with age of less than 25 years (p=0.04). Almost half of the patients above 25 years had normal radiographic grades.

Loss of lumbar lordosis (<20°) was found in the majority (74.5%) of patients while 23.6% had angle within the normal range (20 to 40°). Only, 1.8% showed hyper-lordosis (>40°) as shown in Figure 1. No significant effect of age and gender were seen on loss of lordosis.

An incidental finding of non-fusion of sacral segments (Figure 1) was evident in 29 (52.7%) of thalassaemia patients, and was not correlated with age, gender, or any other demographic or clinical feature.

All the bone density parameters were significantly lower in the patient group as compared to the control group (p<0.001). The mean ± SD of T-score and Z-score, ADSOS, and BTT were significantly reduced in the patient group (p=0.001) (Table I).

Majority of the patients (65.5%) were osteoporotic while in the control group, this percentage was 40%; the difference was statistically significant (p=0.01) as seen in Table II.

In the total sample population, the frequency of normal homozygous dominant wild type (SS) genotype was found in 59.1%, heterozygous (Ss) was found in 33.6%, and homozygous recessive/mutant (ss) genotype in 7.3% of the individuals. Among patients, wild type was found in 69.1% as compared to 49.1% in controls. While the mutant genotype was 5.5% in patients as compared to 9.1% in controls. No significant difference was observed in genotype distribution (wild type and mutant) between the two groups. Distribution of mutant allele was also not found to be gender-dependent.

In the control group, the frequencies of C and A alleles were 60.8% and 39.1%, in the osteopenic group was 60.7% and 39.2%, and in the osteoporotic group was 78.0% and 21.9%. Logistic regression analysis was used adjusting age, gender, and Sp1. No significant differences between (*COL1A1* Sp1) rs1800012 alleles and genotypes among the different study groups were observed (Table III).

Table III: Logistic regression analysis of the *COL1A1* Sp1 polymorphisms.

Allele/ Genotype	Normal (n=15)	Osteopenic (n=37)			Osteoporotic (n=58)			Osteopenic (n=37)		Osteoporotic (n=58)	
		Univariate logistic regression						Multivariate logistic regression			
	n (%)	n (%)	OR (95% CI)	p	n (%)	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
C	14 (60.8)	31 (60.7)	1 (reference)		57 (78.0)	1 (reference)		NA	NA	NA	NA
A	9 (39.1)	20 (39.2)	1.0036 (0.3660-2.7516)	0.9945	16 (21.9)	2.290 (0.838-6.252)	0.100				
CC	6 (40.0)	17 (45.9)	1 (reference)		42 (72.4)	1 (reference)		1 (reference)		1 (reference)	
CA	8 (53.3)	14 (37.8)	0.6176 (0.1729-2.2062)	0.4582	15 (25.9)	0.2679 (0.0797-0.8997)	0.0331	0.975 (0.232-4.101)	0.975	0.305 (0.086-1.078)	0.065
AA	1 (6.7)	6 (16.2)	2.1176 (0.2097-21.3896)	0.5248	1 (1.7)	0.1429 (0.0079-2.5985)	0.1886	2.482 (0.198-31.199)	0.481	0.106 (0.005-2.114)	0.142

**OR: Odds ratio; CI: Confidence interval.*

Among the thalassaemia patients, significant association was seen between bone density parameters and genotype, with 73.7% of wild type being found in osteoporosis. It was also seen that patients carrying mutant alleles had a higher percentage of osteopaenia and osteoporosis, and this relationship was found to be significant (p=0.02). Thalassaemia patients group showed a significant association between the polymorphism of the *COL1A1* gene and T-scores (p=0.03) and ADSOS (p=0.02). The mean of these parameters of bone density was significantly lower in individuals with recessive homozygous traits (ss) as compared to dominant wild type (SS) and heterozygous (Ss). The Z-scores were seen to be reduced in recessive homozygous traits (ss) but significance could not be achieved. However, no significant association was found between the category of bone density and genotype in healthy controls.

Radiographic grades of spine changes were not significantly associated with polymorphism in thalassaemia patients, however, the trend of disc space narrowing and increased anterior osteophytes were found in patients with heterozygous and mutant alleles. All three patients with mutant genotype (ss) had lumbar lordosis angle of less than 20°.

BMD parameters showed significant association with spine radiographic changes in thalassaemia patients. Osteopenic and osteoporotic individuals were having significantly higher radiographic grades. Patients with sacral non-fusion had significantly reduced BTT (p=0.04).

DISCUSSION

In the current study, bone density parameters were found to be significantly lower in thalassaemia patients as compared to the controls. Most of the patients showed spine degeneration and these changes were more obvious in patients with age of less than 25 years. Loss of lumbar lordosis was seen in almost 2/3rd of the patients. Radiographic grades were higher in osteopenic and osteoporotic thalassaemia patients. The frequency of the mutant alleles (ss) determining polymorphism was only 7.3% in the total sample population, however, heterozygous frequency was found in 33.6% of the participants. No significant difference in polymorphism occurrence was found between thalassaemia patients and control groups. The polymorphism showed a significant association with T-score, ADSOS, and radiographic grades in only the thalassaemia patients group.

The significant difference in bone density scores between thalassaemia and control groups was consistent with many previous studies done on different populations. Low bone mass with reduced T- and Z-scores were reported to be present in thalassaemia patients despite transfusion and optimum chelation.[11] Low BMD and osteoporosis were common problems in adolescent age group of thalassaemia patients, even in the treated patients leading to back pain and increased risk of osteoporotic fractures. The current study reported that majority (61.8%) of the thalassaemia patients had grade 1 radiographic changes in the lumbar spine. The finding was favoured by previous studies as spine changes were commonly reported in the thalassaemia patients and were linked with osteoporosis.[12,13] Loss of lumbar lordosis was an important determinant of back pain in thalassaemia patients and was studied as a phenomenon secondary to osteoporotic changes in spine and resultant postural compensation.[8] The reduced-bone density in the current sample population of thalassaemia patients and their significant association with spine changes like the previous reports on other populations suggested that reduced-bone density is the primary factor contributing to spine changes and reduced LLA ultimately leading to back pain.[12,14]

An incidental finding in the study group was the presence of non-fused sacral vertebrae in almost half of the thalassaemia patients which was in significant correlation with BTT. BTT strictly reflected the bone properties regardless of the confounding effects of soft tissues[15] and is believed to be the parameter mostly related to cortical thickness. This suggested that patients with delayed ossification also have reduced cortical thickness. According to the authors' knowledge, this sacral non-fusion had never been reported in thalassaemia patients; however, this feature determined the delayed skeletal maturity and may be associated with the administration of desferrioxamine in these patients which is reported to interfere with the spinal growth-plate development.[16] This finding must be further explored as it can be highly associated with pain-related symptoms.

The finding regarding the distribution of alleles related to Sp1 polymorphism in the study's population was found different from other populations. In Indian population, *COL1A1* polymorphism with mutant genotype (ss) was reported in 43% of the thalassaemia patients, compared to 5.5% in the current study.[17] A study done on a Turkish popu-

lation revealed that there was no "ss" genotype in control[18] contrary to this study's findings where "ss" genotype was also found in control group, and no significant difference was found in the distribution of mutant allele between thalassaemia and control groups. The difference in the polymorphism of *COL1A1* in this population might be due to different ethnicity, however, a large-scale study to see the distribution of Sp1 polymorphism in the general population and in thalassaemia patients is highly advocated.

Despite the lack of significant difference of Sp1 polymorphism between controls and thalassaemia patients, a significant association was seen between *COL1A1* gene polymorphism and low BMD scores (T-score and ADSOS) only in the patient group. Many studies on thalassaemia patients in different populations showed that *COL1A1* was an important determinant of low BMD;[19,20] however, no such study had been reported in this population. The strange association of *COL1A1* polymorphism with bone density scores only in thalassaemia patients showed the probable role of epigenetic factors in triggering bone disease in thalassaemia. Epigenetic processes are thought to influence the gene expression.[21] The different internal body environment of thalassaemia patients can have an impact on triggering epigenetic changes by switching the genes on or off, thus, leading to bone density changes. The strong association of *COL1A1* polymorphism with bone density scores which in turn links to spine degenerative changes in thalassaemia patients suggested that there might be a genetic predisposition in these patients to get severe form of spine changes leading to back pain.

The current study reported the pattern of polymorphism of *COL1A1* Sp1 gene in thalassaemia patients and healthy controls in the Pakistani population for the first time. The interesting finding of non-fused sacral vertebrae in adult patients of thalassaemia was reported for the first time in the literature indicating it to be another major reason contributing to back pain. However, a comparatively small sample size was a limitation of the study. Due to restricted time and resources, a large cohort could not be incorporated. Radiographs of controls in this study could have been added but it was avoided due to unnecessary exposure to radiation.

CONCLUSION

The low bone density score of thalassaemia patients is associated with *COL1A1* Sp1 polymorphism that can cause spine changes leading to low back pain. The findings revealed the potential role of genetics in bone changes and related disorders in the local Pakistani population.

ETHICAL APPROVAL:
The study was approved by the Ethical Review Committee of the University of Health Sciences, Lahore, Pakistan (approval number: UHS/REG-18/ERC/2433). All procedures were in accordance with the ethical standards of the institutional review committee and with the declaration of Helsinki ethical principles for the medical research involving human subjects.

PATIENTS' CONSENT:
The subjects were enrolled in the study after obtaining written informed consent from either patients or parents of the patient.

COMPETING INTEREST:
The authors had no competing interest and nothing to disclose.

AUTHORS' CONTRIBUTION:
SH, UZ: Conceived the study, recruited the study population, and wrote the first draft of the manuscript.
SH: Collected the data and samples, conducted the experimental work.
OS, BI, SM: Helped and supported the genetic analysis and interpretation.
TF: Assisted in sampling and publication.
UZ: Supervised the work and provided the intellectual and administrative support along with the analyses, interpretation of the data and finalising the manuscript.
All authors read and agreed on the final draft of the manuscript.

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