Vitamin D Receptor (VDR) Gene Polymorphisms (*FokI*, *BsmI*) and their Relation to Vitamin D Status in Pediatrics β Thalassemia Major

Shereen Mohamed Elhoseiny¹ · Dalia Saber Morgan² · Asmaa Mohamed Rabie³ · Samer Tharwat Bishay³

Received: 1 March 2015 / Accepted: 6 May 2015 / Published online: 19 May 2015
© Indian Society of Haematology & Transfusion Medicine 2015

Abstract Vitamin D is critical for calcium, phosphate homeostasis and for mineralization of the skeleton, especially during periods of rapid growth. Vitamin D Deficiency leads to rickets (in children) and osteomalacia (in adults). Expression and activation of the vitamin D receptor (VDR) are necessary for the effects of vitamin D, in which several single nucleotide polymorphisms have been identified especially (*FokI*, *BsmI*). In this study serum 25 (OH) vitamin D3 levels were estimated by Enzyme Linked Immunosorbent Assay [ELISA], VDR (*FokI*, *BsmI*) gene polymorphisms were analyzed by polymerase chain reaction-restriction fragment length polymorphism assay [PCR–RFLP]. Serum levels of calcium, phosphorus, alkaline phosphatase and ferritin were determined in 50 Pediatrics beta thalassemia major patients and 60 controls. Patients had significantly lower serum calcium ($p < 0.001$) lower serum vitamin D3 ($p < 0.001$) with elevated levels of phosphorus ($p < 0.001$) and alkaline phosphatase than controls ($p = 0.04$). Of the patients studied, 60 % had vitamin D deficiency (<20 ng/ml), 20 % had vitamin D insufficiency (21–30 ng/ml) and 20 % had sufficient vitamin D status (>30 ng/ml). Patients harboring mutant (Ff,ff) and wild (BB) genotypes were associated with lower serum calcium ($p = 0.08, 0.02$) respectively, lower vitamin D3

levels ($p < 0.001, 0.01$) respectively. They were also suffering from more bony complications although the difference was not statistically significant ($p > 0.05$). In conclusion, these results suggest that the VDR (*FokI*, *BsmI*) gene polymorphisms influence vitamin D status, (Ff,ff), BB genotypes had lower vitamin D levels, so they might influence risk of development of bone diseases in beta thalassemia major.

Keywords VDR polymorphism · *FokI* · *BsmI* · 25 (OH) vitamin D3 · Thalassemia · PCR–RFLP

Introduction

Beta-thalassemia, an inherited blood disorder, mainly affects people from the Mediterranean region. This life threatening anemia is so severe that regular blood transfusions and iron-chelation therapy is obligatory throughout life [1].

Bone disease comprising of low bone mineral density (BMD), bone pain, and fractures is a characteristic feature of thalassemia [2]. The etiology of bone disease in thalassemia is multifactorial and is still under investigation. Factors such as hormonal deficiency, especially gonadal failure, bone marrow (BM) expansion, increased iron stores, desferrioxamine toxicity, calcium and vitamin D deficiency have been implicated to have a serious impact on the impaired bone metabolism [3].

Vitamin D is one of the important factors required for bone development and maintenance of bone mass as well as the principal factor for normal calcium and phosphate homeostasis [4, 5]. Besides the essential role in calcium homeostasis and bone metabolism, research in the last two decades has shown that cell differentiation, inhibition of

✉ Shereen Mohamed Elhoseiny
shelhoseny@windowslive.com

¹ Department of Clinical and Chemical Pathology, Beni Suef Teaching Hospital, Beni Suef University, El Beah st. no.9057, Mokattam, Cairo, Egypt

² Department of Pediatrics, Beni Suef Teaching Hospital, Beni Suef University, Beni Suef, Egypt

³ Department of Pediatrics, Beni Suef General Hospital, Beni Suef, Egypt

cell growth, immunomodulation, and control of other hormonal systems are also biological processes where the vitamin D endocrine system plays an important role [6]. Vitamin D deficiency is increasingly identified among patients with beta-thalassemia major, especially during the winter period and with ageing [7, 8].

Most of the biological actions of vitamin D are mediated by a high-affinity intracellular receptor vitamin D receptor (VDR) that acts as a nuclear transcription factor, regulating the synthesis of proteins involved in bone mineral homeostasis and cell proliferation [9]. The VDR gene is located on chromosome 12 (q12–q14), is 75 kb in size, and is composed of 11 exons. Exons 2 and 3 encode the amino acids involved in DNA binding, and exons 7, 8, and 9 are implicated in vitamin D binding. The VDR gene contains many single nucleotide polymorphisms (SNPs) that could potentially modify the expression and activation of VDR. *FokI* T > C (rs2228570), *BsmI* G > A (rs1544410), *ApaI* G > T (rs7975232), and *TaqI* T > C (rs731236) are the most widely SNPs studied of this gene [10]. The *FokI* polymorphism is a T/C transition at the translation initiation site of exon 2 in the 5' coding region of the VDR gene. This change creates a new start codon (ATG to ACG) resulting in a peptide that is shorter by 3 amino acids (427 compared with 424), which has higher transcriptional activity compared with the full-length vitamin D receptor protein [11, 12].

The aim of this study was to characterize the expression pattern of the vitamin D receptor gene polymorphisms (*FokI*, *BsmI*) in Pediatrics patients with beta thalassemia major (using polymerase chain reaction-restriction fragment length polymorphism assay [PCR–RFLP] technique) in relation to clinical features and laboratory findings at diagnosis, and its correlation with vitamin D status, 25 (OH) D3 by Enzyme Linked Immunosorbent Assay [ELISA] and bone diseases.

Patients and Methods

Patients

The present study was conducted on 50 Pediatrics patients with Beta Thalassemia major. They were 29 males (58 %) and 21 females (42 %) with age ranging from 2 to 15 years old. Sixty unrelated healthy individuals were served as control group. Both patients and controls were recruited from Faculty of Medicine, Beni Suef University Hospital. Data confidentiality was preserved according to the Revised Helsinki Declaration of Bioethics (2008) [13]. Informed consent was obtained from all the patients or parents of children who agreed and participated in this study.

Patients were subjected to (1) Full history taking including: onset and duration of thalassemia, frequency of blood transfusion, Iron chelation therapy: type and compliance, presence of complications of thalassemia e.g. cardiac disease, liver disease, diabetes, presence of complications that could be attributed to vitamin D deficiency e.g. pathological fractures, family history of thalassemia and other nutritional deficiency. (2) Thorough clinical examination: This includes: (a) General examination: Vital signs, anthropometric measurements including weight, height & body mass index (BMI). Signs of heart disease e.g. pallor, dyspnea, and cyanosis, mongoloid features, skin colour and jaundice. (b) Abdominal examination: to detect hepatomegaly, tender liver, and splenomegaly. (c) Musculoskeletal system: to detect bone pain, tenderness, deformity, pathological fractures, muscle weakness. (3) Laboratory investigations including: Complete blood count (CBC), retics, hemoglobin electrophoresis, ferritin, serum calcium, phosphorus & alkaline phosphatase.

Methods

Sampling

Venous blood was obtained by a sterile venipuncture and divided into 2 parts. One part was delivered into an ethylene diamine tetra-acetic acid (EDTA) tube (used for separation of cell pellet which was stored for DNA extraction and subsequent VDR gene polymorphism) while the other part was centrifuged and sera were obtained and stored at -20°C for assay of:

II-Serum 25 Hydroxy Vitamin D3 level by Enzyme-Linked Immunosorbent Assay [ELISA] using (*DRG 25-OH vitamin D (total) ELISA EIA-5396*, *DRG Instruments GmbH, Germany*). Serum 25 hydroxy vitamin D level was measured according to the manufacturer's instructions.

Currently accepted standards for defining vitamin D status in children and adolescents are:

- Vitamin D sufficiency: $25(\text{OH})\text{D} \geq 30 \text{ ng/mL}$
- Vitamin D insufficiency: $25(\text{OH})\text{D}$ between 21 and 29 ng/mL
- Vitamin D deficiency: $25(\text{OH})\text{D} \leq 20 \text{ ng/mL}$ [14].

III-DNA isolation and Vitamin D receptor (VDR) (*FokI*, *BsmI*) gene polymorphisms genotype analysis by polymerase chain reaction-restriction fragment length polymorphism assay [PCR–RFLP]:

Mononuclear cells (MNCs) were isolated from peripheral blood at diagnosis by Ficoll density gradient centrifugation. Genomic DNA was extracted using *Thermo Scientific GeneJET whole blood Genomic DNA purification kit* (Cat. #K0781, #K0782, *Fermentas Life Sciences*) according to the manufacturer's instructions.

VDR (*FokI*, *BsmI*) gene polymorphisms were determined with a polymerase chain reaction-restriction fragment length polymorphism assay [PCR–RFLP]. The PCR primers were:

FokI—Forward: 5′GAT GCC AGC TGG CCC TGG CAC TG3′, Reverse: 5′ATG GAA ACA CCT TGC TTC TTC TCC CTC 3′

BsmI—Forward: 5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′, Reverse: 5′-AAC CAG CGG AAG AGG TCA AGG G-3′ [15].

PCR assay was performed for each sample in a final reaction volume of 25 μ L, using 5 μ L genomic DNA, 12.5 μ L universal master mix, 1 μ L forward primer, 1 μ L reverse primer, together with 5.5 μ L distilled water (DW).

PCR conditions were as follows: denaturation at 94 °C for 5 min, followed by 40 cycles of PCR: denaturation at 94 °C (30 s), annealing at 59.5 °C (*FokI*) and 63.5 °C (*BsmI*) for (30 s), and extension at 72 °C (30 s) [15]. All reactions were done using the thermal cycler Applied Biosystems (Perkin Elmer 9600).

Following PCR, aliquots of the amplified PCR products was digested with *FokI* restriction enzyme (Fermentas, Fast Digest® *FokI* Cat. No.: #FD2144) and *BsmI* restriction enzyme (Fermentas,® *BsmI* Cat. No.: # ER0961) respectively according to the manufacturer's protocols.

The presence or absence of the enzyme recognition site was identified by ethidium bromide staining of fragments separated in a 2 % agarose gel electrophoresis, then visualized using UV transilluminator. DNA molecular weight markers (QIAGEN GelPilot) 50–500 bp Ladder {cat no. 239025} and 100–1000 bp Ladder {cat no. 239045} were used to assess the size of the PCR–RFLP products (*FokI*, *BsmI*) respectively.

Genotypes were assigned as FF, Ff and ff for the VDR-*FokI* polymorphism and BB, Bb, bb for the *BsmI* polymorphism. For the *FokI* cleaved PCR products, the single resultant fragment was 273 bp (FF), whereas the two resultant fragments digested with *FokI* enzyme were 198 and 75 bp (ff), and the heterozygous gave rise to 3 fragments 273, 198 and 75 bp (Fig. 1). For the *BsmI* cleaved PCR products, the single resultant fragment was 823 bp (BB), whereas the two resultant fragments were 648 and 175 bp (bb), and the heterozygous gave rise to 3 fragments 823, 648 and 175 bp (Fig. 2). For quality control, genotyping of 10 % of the samples was repeated and interpreted blindly by two different observers and proved to be identical to the initial results.

Statistical Methods

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data of scores were expressed as mean and standard deviation or

median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann–Whitney test (non parametric *t* test). Comparison between 3 groups was done using Kruskal–Wallis test (non-parametric ANOVA) then post-Hoc “Scheffe test” on rank of variables was used for pair-wise comparison. Odds ratio (OR) with it 95 % confidence interval (CI) were used for risk estimation. A *p* value <0.05 was considered significant [16]

Results

The present study was conducted on 50 Pediatrics patients with Beta Thalassemia major, 29 males (58 %) and 21 females (42 %) with age ranging from 2 to 15 years. Sixty healthy unrelated individuals served as control group. They were 33 males (55 %) and 27 females (45 %) with age ranging from 2.5 to 15 years.

Results of VDR polymorphism (*FokI*, *BsmI*) gene polymorphisms among patients and controls are shown in Table 1. There were no statistically significant difference between patients and controls (*p* > 0.05).

Biochemical parameters among Thalassemic patients and Controls Table 2:

Vitamin D levels were significantly lower in patients (20.3 ± 14.7) than controls (46.2 ± 16.3) ng/ml (*p* < 0.001). Of the patients studied, 60 % had vitamin D deficiency (< 20 ng/ml), 20 % had vitamin D insufficiency (21–30 ng/ml) and 20 % had sufficient vitamin D status (>30 ng/ml). There was a highly statistically significant difference between patients and controls as regards serum calcium level, phosphorus level (*p* < 0.001) in both and alkaline phosphatase level (*p* = 0.04). Thalassemic patients had lower calcium and higher phosphorus and alkaline phosphatase levels than controls.

Clinical and Hematological findings in Thalassemic patients in relation to VDR (*FokI*) gene polymorphism Table 3.

There was a statistically significant difference between wild type (FF) and mutant types (Ff or ff) as regards age, duration of illness (*p* = 0.03) and hepatomegaly (*p* < 0.001). Patients with mutant types were older in age, had longer duration of illness and had hepatomegaly more than wild type. Although mutant types were shorter than wild type; yet this difference was near statistically significance (*p* = 0.06). Although bony pain, tenderness, deformity and pathological fractures were more frequently represented among mutant types compared to wild type, yet the difference was not statistical significance (*p* > 0.05).

Fig. 1 PCR-RFLP analysis of VDR (*FokI*) gene polymorphism using *FokI* restriction enzyme. M: DNA molecular weight marker: 50–500 bp. Lane 1, 2, 4, 5 & 7: Homozygous wild type (FF): 1 band at 273 bp. Lane 3, 6 & 8: Heterozygous mutant (Ff): 3 bands at 273, 198 & 75 bp. Lane 9: Homozygous mutant (ff) 2 bands at 198 & 75 bps

Fig. 2 PCR-RFLP analysis of VDR (*BsmI*) gene polymorphism using *BsmI* restriction enzyme. M: DNA molecular weight marker: 100–1000 bp. Lane 1, 3, 4, 7 & 9: Homozygous wild type (BB): 1 band at 823 bp. Lane 2, 6 & 8: Heterozygous mutant (Bb): 3 bands at 175, 648 & 823 bps. Lane 5: Homozygous mutant (bb): 2 bands 175 & 648 bps

Clinical and Hematological findings in Thalassemic patients in relation to VDR (*BsmI*) gene polymorphism Table 4.

The only statistically significant difference between wild type (BB) and mutant types (Bb or bb) was in serum calcium level, being lower in wild type than mutant ones ($p = 0.02$). Although bone tenderness, deformity and pathological fractures were more frequently represented among wild type compared to mutant types, yet the difference was not statistical significance ($p > 0.05$).

Impact of VDR (*FokI*, *BsmI*) genotypes on vitamin D status in Thalassemic patients Table 5:

Regarding *FokI* gene; vitamin D levels were significantly lower in patients harboring mutant (Ff,ff) genotypes (14.4 ± 13) than wild FF genotype (26.3 ± 14) ng/ml ($p < 0.001$). Vitamin D deficiency were more frequently represented among mutant (Ff,ff) genotypes (66.7 %) than wild FF genotype (33.3 %) ($p = 0.004$, odds ratio 6.000 (95 % CI: 1.693–21.262). Regarding *BsmI* gene; wild genotype BB had lower vitamin D levels (15.4 ± 11.8) than mutant genotypes (Bb,bb) (26.6 ± 15.8) ng/ml ($p = 0.01$). Although vitamin D deficiency were more

frequently represented among wild (BB) genotype (66.7 %) than mutant (Bb,bb) genotypes (33.3 %), yet this difference was near statistically significant ($p = 0.06$, odds ratio 0.103 (95 % CI: 0.333–1.077).

FokI, *BsmI* alleles in relation to clinical and laboratory data in Thalassemic patients Table 6.

FokI patients harboring f allele were older in age ($p = 0.01$), had longer duration of illness ($p = 0.008$), lower vitamin D levels ($p < 0.001$), had lower serum calcium levels ($p = 0.04$), had lesser weight ($p = 0.01$), lower height ($p = 0.01$) and more frequent hepatomegaly ($p < 0.001$) than F allele.

BsmI patients harboring B allele had longer duration of illness ($p = 0.005$), lower vitamin D levels ($p = 0.002$), had lower serum calcium levels ($p = 0.01$), had lesser weight ($p = 0.03$), lower BMI ($p = 0.05$) than b allele.

VDR (*FokI*, *BsmI*) alleles and its relation to vitamin D deficiency Table 7.

FokI Vitamin D deficiency were more frequently represented among f allele (56.7 %) than F allele (43.3 %) ($p = 0.001$, odds ratio 5.353 (95 % CI: 1.841–15.561).

Table 1 Vitamin D receptors (FokI, BsmI) genotype frequency among thalassemic patients versus controls

	Group		P value	Odds ratio	(95 % confidence interval)
	Thalassemic patient N = 50	Controls N = 60			
<i>FokI</i> gene					
Wild genotype “FF”	25 (50 %)	36 (60 %)	Reference		
Mutant heterozygous Genotype”Ff”	19 (38 %)	18 (30 %)	0.4 ^a	1.520 ^a	(0.483–4.787) ^a
Mutant homozygous Genotype “ff”	6 (12 %)	6 (10 %)	0.6 ^b	1.440 ^b	(0.252–8.221) ^b
All mutant genotypes “Ff, ff”	25 (50 %)	24 (40 %)	0.4 ^c	1.500 ^c	(0.524–4.297) ^c
<i>BsmI</i> gene					
Wild genotype “BB”	28 (56 %)	30 (50 %)	Reference		
Mutant heterozygous Genotype “Bb”	17 (34 %)	21 (35 %)	0.8 ^d	0.867 ^d	(0.278–2.708) ^d
Mutant homozygous Genotype “bb”	5 (10 %)	9 (15 %)	0.5 ^e	0.595 ^e	(0.120–2.958) ^e
All mutant genotypes (Bb & bb)	22 (44 %)	30 (50 %)	0.6 ^f	0.786 ^f	(0.278–2.221) ^f

P < 0.05 Sig; P < 0.001 HS; P > 0.05 NS. Sig significant, HS highly significant, NS not significant

^a Comparison between heterozygous mutant Ff genotype versus wild FF genotype among Thalassemic patients versus controls

^b Comparison between homozygous mutant ff genotype versus wild FF genotype among Thalassemic patients versus controls

^c Comparison between all mutant (Ff, ff) genotypes versus wild FF genotype among Thalassemic patients versus controls

^d Comparison between heterozygous mutant Bb genotype versus wild BB genotype among Thalassemic patients versus controls

^e Comparison between homozygous mutant bb genotype versus wild BB genotype among Thalassemic patients versus controls

^f Comparison between all mutant (Bb, bb) genotypes versus wild BB genotype among Thalassemic patients versus controls

Table 2 Biochemical parameters among thalassemic patients and controls

Parameters	Thalassemic patients (50 cases)	Control (60 cases)	P value
Vitamin D levels ng/ml	20.3 ± 14.7 (2.3–55)* 17.8**	46.2 ± 16.3 (21.8–73.1) 44.5	<0.001
Serum calcium (mg/dl)	8.6 ± 0.8 (7–10.3) 8.5	9.4 ± 0.5 (8.6–10.3) 9.4	<0.001
Serum phosphorus (mg/dl)	5 ± 0.8 (3–6.5) 5.1	3.8 ± 0.6 (2.9–4.5) 4	<0.001
Alkaline phosphatase (U/L)	354.9 ± 153.4 (125–785) 348	274.8 ± 75 (160–430) 260	0.04

Bold values are statistically significant

P < 0.05 Sig; P < 0.001 HS; P > 0.05 NS. Sig significant, HS highly significant, NS not significant

* Mean ± SD (range)

** Median

BsmI Vitamin D deficiency were more frequently represented among B allele (81.7 %) than b allele (18.3 %) (p = 0.01, odds ratio 0.337 (95 % CI: 0.136–0.836).

Discussion

The survival of patients with thalassemia major has progressively improved with advances in therapy; however, osteoporosis and cardiac dysfunction remain frequent complications. Adequate circulating levels of vitamin D are essential for optimal skeletal health and reducing

fracture risk [17]. Most of the biological actions of vitamin D are mediated by an intracellular receptor (VDR) in which several single nucleotide gene polymorphisms have been identified. Vitamin D deficiency is increasingly identified among thalassemic patients [18].

Thus, in an attempt to increase our understanding of the interaction between vitamin D status, the risk of development of bone diseases and the genetic polymorphism of VDR, we studied the genetic polymorphisms of two members of the VDR (*FokI* and *BsmI*), in a case–control study conducted on a cohort of Egyptian Beta Thalassemic major patients.

Table 3 Clinical and Hematological findings in 50 Thalassemic patients in relation to VDR (*FokI*) gene polymorphism

Parameter	FokI gene		P value
	Wild FF N = 25	Mutant Ff, ff N = 25	
Gender: Male: No (%)	13 (44.8%)	16 (55.2%)	0.3
Female: No (%)	12 (57.1%)	9 (42.9%)	
Age “years”	7.4 ± 3.5 (2–14)*	9.8 ± 3.8 (2.5–15)	0.03
	7**	10	
Duration of illness “years”	6.6 ± 3.6 (1–13.5)	9.8 ± 3.8 (2–15)	0.03
	5	9	
Weight (kg)	27.1 ± 9.6 (9–46)	22.1 ± 7.3 (10–38)	0.08
	27	22	
Height (cm)	124.8 ± 17.4 (90–151)	115.3 ± 18.1 (87–150)	0.06
	125	110	
Body mass index (kg/m ²)	17.1 ± 2.9 (11–21.4)	16.5 ± 2.9 (11.6–24.7)	0.3
	17	15.8	
Serum calcium (mg/dl)	8.8 ± 0.8 (7–10.2)	8.5 ± 0.8 (7–10.3)	0.08
	8.9	8.5	
Serum phosphorus (mg/dl)	4.9 ± 0.8 (3–6.5)	5.1 ± 0.8 (3.1–6.5)	0.1
	5	5.2	
Alkaline phosphatase (U/L)	371.4 ± 157.8 (186–750)	338 ± 150.2 (125–785)	0.4
	369	300	
Serum ferritin (ng/ml)	1478.8 ± 1315.1 (209– 4821)	1665 ± 1721.7 (109.4–7280.1)	0.9
	821.8	811	
Bone aches (32 cases)	15 (46.9 %)	17 (53.1 %)	0.5
No (%)			
Tenderness (8 cases)	3 (37.5 %)	5 (62.5 %)	0.4
No (%)			
Deformity (12 cases)	4 (33.3 %)	8 (66.7 %)	0.1
No (%)			
Pathological fracture (3 cases) No (%)	1 (33.3 %)	2 (66.7 %)	1.0
Hepatomegaly (22 cases)	4 (18.2 %)	18 (81.8 %)	<0.001
No (%)			

Bold values are statistically significant

P < 0.05 Sig; P < 0.001 HS; P > 0.05 NS. Sig significant, HS highly significant, NS not significant

* Mean ± SD (range)

** Median

In the current study, genotype frequencies of *FokI* were similar to those previously reported for other populations; 50 % of the patients were homozygotes for F allele, 38 % were heterozygotes and 12 % were homozygotes for the f allele [18, 19].

Low serum calcium levels with elevated levels of serum inorganic phosphorus and alkaline phosphatase were found in our thalassemic patients. Our results are in accordance with De Sanctis et al. [20] who reported hypocalcemia as a late complication of iron overload in cases of beta-thalassemia. Tantawy et al. [21] found that 75 % of their beta-thalassemic patients had a low calcium level. The low calcium level was probably caused by a combination of

hypoparathyroidism and osteomalacia evidenced by elevated bone alkaline phosphatase presumably resulting from deficient calcium intake. Impaired calcium homeostasis is thought to be a consequence of iron overload seen in β -thalassemic transfused patients [22]. Other mechanisms leading to disturbed calcium and phosphorus homeostasis include decrease intake, impaired absorption, and reduced synthesis of vitamin D [23].

In the current study, mutant *FokI* genotypes (Ff,ff) were associated with lower serum calcium levels than wild type; but the difference did not reach statistical significance ($p = 0.08$). While wild *BsmI* genotype BB was the one with lower serum calcium levels than mutant types (Bb,bb), the

Table 4 Clinical and Hematological findings in 50 Thalassemic patients in relation to VDR (*BsmI*) gene polymorphism

Parameter	BsmI gene		P value
	Wild BB No = 28	Mutant Bb, bb No = 22	
Gender:			
Male: No (%)	18 (62.1%)	11 (37.9 %)	0.3
Female: No (%)	10 (47.6%)	11 (52.4 %)	
Age “years”	9 ± 3.5 (3–15)* 9**	8.1 ± 4.3 (2–15) 7.5	0.3
Duration of illness “years”	8.2 ± 3.5 (2.5–15) 8.3	7.2 ± 4.3 (1–14.5) 6.3	0.3
Weight (kg)	23 ± 9 (9–41) 22	25.8 ± 8.7 (12 – 46) 25	0.2
Height (cm)	118.1 ± 20.4 (87–150) 117.5	121.6 ± 16.5 (90–151) 121	0.5
Body mass index (kg/m ²)	16.2 ± 2.9 (11–21.6) 16.2	17.2 ± 2.8 (12.5–24.7) 16.7	0.2
Serum Calcium (mg/dl)	8.4 ± 0.9 (7–10.2) 8.4	8.9 ± 0.6 (8–10.3) 9.1	0.02
Serum Phosphorus (mg/dl)	5 ± 1 (3–6.5) 5.1	5.1 ± 0.4 (4–5.9) 5.1	0.9
Alkaline phosphatase (U/L)	362.3 ± 179.5 (125–785) 305	345.5 ± 115.4 (186–586) 364.5	0.7
Serum ferritin (ng/ml)	1774.6 ± 1780 (109.4–7280.1) 816.4	1313.9 ± 1089.3 (186–3434.8) 939.5	0.5
Bone aches (32 cases) No (%)	16 (50 %)	16 (50 %)	0.2
Tenderness (8 cases) No (%)	5 (62.5 %)	3 (37.5 %)	0.2
Deformity (12 cases) No (%)	8 (66.7 %)	4 (33.3 %)	0.07
Pathological fracture (3 cases) No (%)	2 (66.7 %)	1 (33.3 %)	0.5
Hepatomegaly (22 cases) No (%)	12 (54.5 %)	10 (45.5 %)	0.8

Bold values are statistically significant

P < 0.05 Sig; P < 0.001 HS; P > 0.05 NS. Sig significant, HS highly significant, NS not significant

* Mean ± SD (range)

** Median

difference was statistically significant ($p = 0.02$). FokI and other polymorphisms of VDR gene have been associated with altered calcium homeostasis and impaired bone metabolism [18]. But Singh et al. [2] did not find any association between serum calcium, phosphorus or alkaline phosphatase with any of the VDR gene polymorphisms (*FokI*, *BsmI* and *TaqI*).

In the current study, thalassemic patients had lower levels of serum 25 (OH) vitamin D3 than controls, the difference was highly statistically significant ($p < 0.001$). Of the patients studied, 60 % had vitamin D deficiency (<20 ng/ml), 20 % had vitamin D insufficiency

(21–30 ng/ml) and 20 % had sufficient vitamin D status (>30 ng/ml). Our results are in accordance with Dimitriadou et al. [18] who recorded low levels of serum 25 (OH) vitamin D3 in patients than controls, also 59.4 % of their thalassemic patients had vitamin D deficiency. Again Singh et al. [2] results are in accordance with our results and they reported that about 80.6 % of their patients had vitamin D deficiency. In contrary to our results, El-Edel et al. [24] found normal levels of 25 (OH) D3 in their thalassemic patients, however, it was lower among older patients compared to children. In agreement with our

Table 5 Impact of VDR (*FokI*, *BsmI*) genotypes on vitamin D status in Thalassemic patients

Vitamin D	FokI gene		P value	Odds ratio	(95 % confidence interval)
	Wild Type FF (25 cases)	Mutant Types Ff, ff (25 cases)			
Vitamin D levels ng/ml	26.3 ± 14 (6.9–55)* 24.7**	14.4 ± 13 (2.3–49.3) 9.7	<0.001		
Vitamin D deficiency ≤20 ng/ml No (%)	10 (33.3 %)	20 (66.7 %)	0.004	6.000	1.693–21.262
Vitamin D non deficiency ≥20 ng/ml No (%)	15 (75 %)	5 (25 %)			
BsmI gene					
	Wild Type BB (28 cases)	Mutant Types Bb, bb (22 cases)			
Vitamin D levels ng/ml	15.4 ± 11.8 (2.3–47.5) 13.1	26.6 ± 15.8 (5.3–55) 23.6	0.01		
Vitamin D deficiency ≤20 ng/ml No (%)	20 (66.7 %)	10 (33.3 %)	0.06	0.103	0.333–1.077
Vitamin D non deficiency ≥20 ng/ml No (%)	8 (40 %)	12 (60 %)			

Bold values are statistically significant

P < 0.05 Sig; P < 0.001 HS; P > 0.05 NS. Sig significant, HS highly significant, NS not significant

*Mean ± SD (range)

** Median

findings, low levels of vitamin D in patients with beta thalassemia major have been frequently described previously [25, 26].

This could be explained by some studies who demonstrated that vitamin D deficiency, osteomalacia and rickets in thalassemic patients were attributed to defective 25 hydroxylation of vitamin D in the liver due to iron overload and subsequent liver dysfunction [27]. Other mechanisms include decreased intake, impaired absorption, or reduced synthesis of vitamin D [23].

In the current study, mutant *FokI* genotypes (Ff,ff) were associated with lower 25 (OH) D3 levels than wild type; the difference was highly statistically significant ($p = <0.001$). Vitamin D deficiency were more frequently represented among mutant (Ff,ff) genotypes (66.7 %) than wild FF genotype (33.3 %) ($p = 0.004$). In agreement with our results; Dimitriadou et al. [18] found that thalassemic patients harboring the ff genotype was associated with lower 25 (OH) D3 concentrations. Ff genotypes was associated with lower 25 (OH)D3 concentrations also in patients with multiple sclerosis [28].

In the ff variant, initiation of translation occurs at the first ATG site, giving a long version of VDR protein comprised 427 amino acids. Conversely, in the FF variant, translation begins at the second ATG site instead of the first, resulting in a protein shortened by three amino acids

[29]. It has been demonstrated that a length of the VDR, affected by the presence of the polymorphisms, could lead a lower activation of target cells, since a longer VDR protein appears to have a decreased transcriptional activity [30]. Thus, the f allele leads to the production of a 3 amino acid longer and less functional VDR protein, compared to the product of the F allele [31].

While in *BsmI* gene polymorphism; wild genotype BB was the one with lower vitamin D levels than mutant genotypes (Bb,bb), the difference was statistically significant ($p = 0.01$). Although vitamin D deficiency were more frequently represented among wild (BB) genotype (66.7 %) than mutant (Bb,bb) genotypes (33.3 %), yet this difference was near statistically significant ($p = 0.06$).

In our study, when bone diseases were considered, bony pain, tenderness, deformity and pathological fractures were more frequently represented among mutant *FokI* (Ff,ff) genotypes and wild *BsmI* BB genotype, yet the difference was not statistically significance ($p > 0.05$).

Singh et al. (2011) [2] found lower bone mineral density (BMD) of lumbar spine with FF and BB genotypes when studying 40 beta thalassemic patients.

El-Edel et al. [24] when analyzing *BsmI* VDR gene polymorphism revealed that β -thalassaemia patients with the BB genotype had significantly lower bone mineral density (BMD) compared to those with bb or Bb

Table 6 Impact of (*FokI*, *BsmI*) alleles on clinical and laboratory data in Thalassemic patients

Parameters	FokI- allele		P value	BsmI allele		P value
	F allele	f allele		B allele	b allele	
Age “years”	7.9 ± 3.7 (2–15)* 8**	10 ± 3.7 (2.5–15) 10	0.01	9 ± 3.6 (2–15) 9	7.4 ± 4.2 (2–15) 6	0.07
Gender:	67.2 %	32.8 %	0.6	77.6%	22.4 %	0.2
Male: (%) Female: (%)	71.4 %	28.6 %		66.7%	33.3 %	
Duration of illness “years”	7.1 ± 3.7 (1–14.5) 7.5	9.2 ± 3.7 (2–15) 9	0.008	8.2 ± 3.6 (1–15) 8.5	6.6 ± 4.2 (1 – 14.5) 5	0.05
Vitamin D level (ng/ml)	23.8 ± 14.3 (3.2–55) 23.3	12.6 ± 12.3 (2.3–49.3) 8.2	<0.001	17.6 ± 13 (2.3–51.5) 15.2	27.8 ± 16.2 (5.3–55) 23.9	0.002
Serum calcium (mg/ml)	8.7 ± 0.8 (7–10.3) 8.7	8.4 ± 0.8 (7– 10.3) 8.3	0.04	8.5 ± 0.8 (7–10.3) 8.5	9 ± 0.6 (8–10.3) 9.1	0.01
Serum phosphorus (mg/ml)	5 ± 0.8 (3–6.5) 5	5.1 ± 0.7 (3.1–6.5) 5.2	0.4	5 ± 9 (3–6.5) 5.1	5.1 ± 4 (4–5.9) 5	0.5
Alkaline phosphatase (U/L)	365.5 ± 155 (186–785) 360	331.3 ± 146.9 (125–785) 300	0.3	356.5 ± 164.4 (125–785) 310	350.6 ± 117.7 (186–586) 360	0.8
Weight (kg)	27.7 ± 9.4 (9–46) 27	23.2 ± 8.2 (9–46) 22	0.01	21.6 ± 8.8 (9–42) 21	25.7 ± 8.6 (9–46) 25	0.03
Height (cm)	127 ± 16.5 (90–151) 129	116.9 ± 18.1 (87–151) 115	0.01	115.2 ± 19.9 (87–150) 110	121.9 ± 17.3 (87–151) 122	0.1
Body mass index (kg/m ²)	16.9 ± 2.9 (11–21.4) 16.6	16.7 ± 2.8 (11–24.7) 16.6	0.7	15.9 ± 2.8 (11–21.6) 15.7	17.1 ± 2.8 (11–24.7) 16.8	0.05
Hepatomegaly (%)	47.7 %	52.3 %	<0.001	72.7 %	27.3 %	0.9

Bold values are statistically significant

P < 0.05 Sig; P < 0.001 HS; P > 0.05 NS. Sig significant, HS highly significant, NS not significant

*Mean ± SD (range)

** Median

genotypes. This finding agrees with that reported by other studies who found that *BsmI* VDR gene polymorphism was associated with osteopenia [23, 32, 33].

El-Edel et al. [24] added that patients with BB genotype had lower height standard deviation (HSD) compared to those with either bb or Bb genotype. Shorter height and lower BMD were reported in patients with BB VDR genotype and VDR gene polymorphism was associated with adult stature and bone size indicating a significant role of it in skeletal growth [32, 34]. They concluded that *BsmI*

VDR polymorphism may affect the severity of osteoporosis by influencing vitamin D activity and BB VDR genotype can be considered as a risk factor for the occurrence of osteoporosis in β -thalassaemia.

Low bone mass in thalassemia is attributed to bone marrow expansion and consequent reduction of trabecular bone tissue and cortical thinning due to increased but ineffective hematopoiesis. Advances in transfusion management and chelation therapy have achieved an improvement in skeletal development and cosmetic bone appearance.

Table 7 Classification of Thalassemic patients according to vitamin D deficiency in relation to VDR (FokI, BsmI) alleles

Vitamin D groups	FokI allele		P-value	Odds ratio	95 % confidence interval
	F allele	f allele			
Vitamin D deficiency ≤ 20 ng/ml	43.3 %	56.7 %	0.001	5.353	1.841–15.561
Vitamin D non deficiency > 20 ng/ml	87.5 %	12.5 %			
	BsmI allele				
	B allele	b allele			
Vitamin D deficiency ≤ 20 ng/ml	81.7 %	18.3 %	0.01	0.337	0.136–0.836
Vitamin D non deficiency > 20 ng/ml	60 %s	40 %			

Bold values are statistically significant

P < 0.05 Sig; P < 0.001 HS; P > 0.05 NS. Sig significant, HS highly significant, NS not significant

However, despite optimal conventional treatment and decline in endocrine complications, low bone density is still reported in thalassemic patients [35].

However, some limitations of the current study should be acknowledged. Due to financial limitations, we did not perform bone mineral density (BMD) to our patients, which could be a good indicator for osteoporosis and osteoporotic fracture risk. Polymorphisms of other VDR genotypes, i.e., (*TaqI*, *ApaI*, and *Cdx-2*), and their possible interactions with *FokI*, *BsmI* variants were not evaluated.

In conclusion, we found that vitamin D deficiency is common in beta thalassemia major patients. This deficiency coupled with low calcium levels leads to the development of bone diseases. In addition, genetic factors may also influence vitamin D status as reflected by low vitamin D in subjects with BB and (Ff, ff) genotypes, whom also suffers from more bony complications. So, adequate calcium intake and vitamin D administration during skeletal development can increase bone mass and decrease bone loss in adult life. So, better understanding of the functional consequences of VDR (*FokI*, *BsmI*) gene polymorphisms would provide a basis for future studies of the role of this polymorphism in vitamin D status and bone diseases in beta thalassemia major.

Conflict of Interests Author Shereen Mohamed Elhoseiny, author Dalia Saber Morgan, author Asmaa Mohamed Rabie and author Samer Tharwat Bishay declare that they have no conflicts of interests. The authors alone are responsible for the content and writing of the paper.

Funds No funds was received from any source.

Human/Animal Rights and Informed Consent This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all

individual participants included in the study. All patients, parents of children patients or healthy controls included in this study were informed and approved upon participating in this study. Neither patients' names nor photos were included in this study. As for the study, it is an original work on Egyptian Pediatrics Beta Thalassemia Major patients. It is an original contribution not previously published and is not under consideration for publication elsewhere. Each author has reviewed the final version of the work, believes it represents valid work, and approves it for publication in your journal.

References

- Gaudio A, Morabito N, Xourafa A, Macri I, Meo A, Morgante S, Trifiletti A, Lasco A, Frisina N (2008) Bisphosphonates in the treatment of thalassemia associated osteoporosis. *J Endocrinol Invest* 31(2):181–184
- Singh K, Kumar R, Shukla A, Phadke SR, Agarwal S (2012) Status of 25-hydroxyvitamin D deficiency and effect of vitamin D receptor gene polymorphisms on bone mineral density in thalassemia patients of North India. *Hematology* 17(5):291–296
- Voskaridou E, Terpos E (2004) New insights into the pathophysiology and management of osteoporosis in patients with beta thalassemia. *Br J Haematol* 127:127–139
- Bischoff-Ferrari HA, Dietrich T, Orav EJ, Dawson-Hughes B (2004) Positive association between 25-hydroxy vitamin D levels and bone mineral density: a population-based study of younger and older adults. *Am J Med* 116:634–639
- Meier C, Woitge HW, Witte K, Lemmer B, Seibel MJ (2004) Supplementation with oral vitamin D3 and calcium during winter prevents seasonal bone loss: a randomized controlled open-label prospective trial. *J Bone Miner Res* 19:1221–1230
- Dusso AS, Brown AJ, Slatopolsky E (2005) Vitamin D. *Am J Physiol Renal Physiol* 289:F8–F28
- Moulas A, Challa N, Chaliasos N, Lapatsanis PD (1997) Vitamin D metabolites (25-hydroxyvitamin D, 24,25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D) and osteocalcin in β -thalassaemia. *Acta Paediatr* 86:594–599
- Vogiatzi MG, Macklin EA, Trachtenberg FL, Fung EB, Cheung AM, Vichinsky E et al (2009) Differences in the prevalence of growth, endocrine and vitamin D abnormalities among the various thalassemia syndromes in North America. *Br J Haematol* 146:546–556

9. Borgna-Pignatti C, Rugolotto S, De Stefano P, Zhao H, Cappellini MD, Del Vecchio GC, Romeo MA et al (2004) Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. *Haematologica* 89:1187–1193
10. Zintzaras E, Rodopoulou P, Koukoulis GN (2006) *BsmI*, *TaqI*, *ApaI* and *FokI* polymorphisms in the vitamin D receptor (VDR) gene and the risk of osteoporosis: a meta-analysis. *Dis Markers* 22:317–326
11. Alimirah F, Peng X, Murillo G, Mehta RG (2011) Functional significance of vitamin D receptor *FokI* polymorphism in human breast cancer cells. *PLoS ONE* 6:e16024
12. OMIM (2012) Online Mendelian Inheritance in Man. Johns Hopkins University, Baltimore, MD. MIM Number: {601769}. Available at [<http://www.ncbi.nlm.nih.gov/omim/>]
13. World Medical Association. Declaration of Helsinki (2008) Ethical principles for medical research involving human subjects, The 59th WMA General Assembly, Seoul
14. Bowden SA, Robinson RF, Carr R, Mahan JD (2006) Prevalence of vitamin D deficiency and insufficiency in children with osteopenia or osteoporosis referred to a paediatric metabolic bone clinic. *Pediatrics* 121:1585–1590
15. Mishra DK, Wu Y, Sarkissyan M, Sarkissyan S, Chen Z et al (2013) Vitamin D receptor gene polymorphisms and prognosis of breast cancer among African-American and hispanic women. *PLoS ONE* 8(3):e57967
16. Dawson B, Trapp RG (2001) Basic and clinical biostatistics, 3rd edn. McGraw-Hill Inc, New York
17. Soliman A, De Sanctis V, Yassin M (2013) Vitamin D status in thalassemia major: an update. *Mediterr J Hematol Infect Dis* 5(1):e2013057
18. Dimitriadou M, Christoforidis A, Fidani L, Economou M, Perifanis V, Tsatra L, Katzos G, Metaxa M (2011) *FokI* gene polymorphism of vitamin D receptor in patients with beta-thalassemia major and its effect on vitamin D status. *Hematology* 16(1):54–58
19. Dimitriadou M, Christoforidis A, Economou M, Teli A, Printza N, Tzimouli V, Tsatra I, Fidani L, Papachristou F, Athanassiou-Metaxa M (2011) *FokI* polymorphism of vitamin D receptor gene and the presence of renal dysfunction in patients with β -thalassemia major. *Pediatr Hematol Oncol* 28(6):509–516
20. De Sanctis V, Borsari G, Brachi S, Gubellini E, Gamberini MR, Carandina G (2008) A rare cause of heart failure in iron-overload thalassaemic patients-primary hypoparathyroidism. *Georgian Med News* 156:111–113
21. Tantawy AA, El Kholy M, Moustafa T, Elsedfy HH (2008) Bone mineral density and calcium metabolism in adolescents with beta-thalassemia major. *Pediatr Endocrinol Rev* 6(1):132–135
22. Karimi M, Habibzadeh F, De Sanctis V (2003) Hypoparathyroidism with extensive intracranial calcification in patients with β -thalassemia major. *J Pediatr Endocrinol Metab* 16:883–886
23. Dresner Pollack R, Rachmilewitz E, Blumenfeld A, Idel-son M, Goldfarb AW (2000) Bone mineral metabolism in adult with Beta-thalassaemia major and intermedia. *Br J Haematol* 111:902–907
24. El-Edel RH, Ghonaim MM, Abo-Salem OM, El-Nemr FM (2010) Bone mineral density and vitamin D receptor polymorphism in beta-thalassemia major. *Pak J Pharm Sci.* 23(1):89–96
25. Napoli N, Carmina E, Bucchieri S, Sferrazza C, Rini GB, Di Fede G (2006) Low serum levels of 25-hydroxy vitamin D in adults affected by thalassemia major or intermedia. *Bone* 38(6):888–892
26. Wood JC, Claster S, Carson S, Menteer JD, Hofstra T, Khanna R et al (2008) Vitamin D deficiency, cardiac iron and cardiac function in thalassemia major. *Br J Haematol* 141:891–894
27. Chatterton BE, Thomas CM, Schultz CG (2003) Liver density measured by DEXA correlates with serum ferritin in patients with beta-Thalassemia Major. *J. Clin Densitom* 6(3):283–288
28. Orton SM, Morris AP, Herrera BM, Ramagopalan SV, Lincoln MR, Chao MJ et al (2008) Evidence for genetic regulation of vitamin D status in twins with multiple sclerosis. *Am J Clin Nutr* 88:441–447
29. Palomer X, González-Clemente JM, Blanco-Vaca F, Mauricio D (2008) Role of vitamin D in the pathogenesis of type 2 diabetes mellitus. *Diabetes Obes Metab* 10:185–197
30. Deng HW, Shen H, Xu FH et al (2002) Tests of linkage and/or association of genes for vitamin D receptor, osteocalcin, and parathyroid hormone with bone mineral density. *J Bone Miner Res* 17:678–686
31. Whitfield GK, Remus LS, Jurutka PW, Ziter H, Ak Oza, Ht Dang et al (2001) Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene. *Mol Cell Endocrinol* 177:145–159
32. Ferrara M, Matarese SM, Francese M, Borrelli B, Coppola A, Coppola L, Esposito L (2002) Effect of VDR polymorphisms on growth and bone mineral density in homozygous beta thalassaemia. *Br J Haematol* 117(2):436–440
33. Garnero P, Munoz F, Borel O, Sornay-Rendu E, Delmas PD (2005) Vitamin D receptor gene polymorphisms are associated with the risk of fractures in postmenopausal women, independently of bone mineral density. *J Endocrinol Invest* 28(4):312–321
34. Lorentzon M, Lorentzon R, Nordstrom P (2000) Vitamin D receptor gene polymorphism is associated with birth height, growth to adolescence, and adult stature in healthy Caucasian men: a cross-sectional and longitudinal study. *J Clin Endo Metab* 85:1666–1671
35. Vogiatzi MG, Autio KA, Schneider R, Giardina PJ (2004) Low bone mass in prepubertal children with thalassemia major; insights into the pathogenesis of low bone mass in thalassemia. *J Pediatr Endocrinol Metab* 17:1415–1421