BONE MINERAL DENSITY AND VITAMIN D RECEPTOR POLYMORPHISM IN β-THALASSEMIA MAJOR

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ABSTRACT

Osteoporosis is the most prevalent bone complication in β-thalassemic patients despite regular transfusions and iron chelation therapy. Although its etiology is multi-factorial, genetic factors play an important role in pathogenesis. These factors have not yet been clearly defined, however, osteoporosis may be related to vitamin D receptor gene *BsmI* polymorphism. In this study, *BsmI* vitamin D receptor gene polymorphism was analyzed using polymerase chain reaction and *BsmI* restriction fragment length polymorphism in 42 regularly treated- β-thalassemic patients of different ages. Bone mineral density was measured by peripheral quantitative ultrasound at the heel of the foot. Serum levels of alkaline phosphatase, calcium, phosphorus, ferritin and 25-hydroxyvitamin D_3 were determined. Patients were divided into two groups according to pubertal signs: group I (22 children), and group II (20 adolescents and adults). The Z-scores of bone mineral density in both groups were -1.32 ± -0.9 and -2.30 ± -1.02 respectively, with a significant difference between the two groups. The height standard deviation and 25-hydroxyvitamin D_3 were significantly decreased in group II compared to group I. Moreover, significantly lower bone mineral density and height standard deviation were detected among patients with BB vitamin D receptor genotype. Therefore, this genotype may be considered as a risk factor for osteoporosis in β-thalassemic patients.

Keywords: β-thalassemia, vitamin D receptor polymorphism, bone mineral density, osteoporosis.

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. Commonly occurring complications, especially in adult patients, are osteopenia and osteoporosis, the etiology of which is multi-factorial (Arisal *et al.,* 2002 and Gaudio *et al.*, 2008).

Bone abnormality in thalassemic patients may be attributed to bone marrow (BM) expansion, toxic effect of desferrioxamine (DFO) on osteoclasts and osteoblasts (Voskaridou *et al.,* 2003), chelation of micronutrients such as zinc which play critical roles in bone metabolism (Bekheirnia *et al.,* 2004), iron deposition in bones and endocrine dysfunction (Domrongkitchaiporn *et al.,* 2003).

Bone mineral density (BMD) is the major determinant of osteoporotic fracture risk. A strong genetic component has been suggested and several candidate gene polymorphisms have been implicated in the regulation of this process; collagen type I alpha 1, vitamin D receptor (VDR), estrogen receptor- alpha and calcitonin receptor genes (Bandres *et al.,* 2005).

VDR belongs to the superfamily of steroid receptors and has a nearly ubiquitous tissue distribution, including cells of the human hematolymphopoietic tissue (Soldati *et al.,* 2004). It is a candidate gene, however, its significance has not been clearly defined (Lo and Singer, 2002). Biological actions of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] are mediated by interaction with its specific nuclear receptor VDR in target cells. Through transcriptional or posttranscriptional patterns of activity, the $1,25(OH)2D_3$–VDR complex can modulate the effects of vitamin D (Soldati *et al.,* 2004).

The aim of the present study was to evaluate the role of *BsmI* polymorphism on the development of osteoporosis in thalassemic patients.

Subjects and methods

I- Subjects

Forty two thalassemic patients (23 females and 19 males) with age ranging from 4.3 to 28.3 years were involved in our study. All cases were recruited from the Hematology Clinic at Menoufiya University Hospital. All patients were under DFO chelation therapy, given subcutaneously or less commonly intravenously. Cases were divided into two groups on the basis of the pubertal stage present at the time of the study. Group I, prepubertal stage (cases without any pubertal signs), included 22 children ranging in age from 4.3 to 12.4 years. Group II included 20 adolescents and adults ranging in age from 13.1 to 28.3 years. Patients who had other medical conditions (heart failure, renal failure or hypoparathyroidism) and those using medications which affect bone mineral metabolism

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(as glucocorticoids or anticonvulsant drugs) were excluded from the study.

Complete history was obtained with special emphasis on age, sexual development, bone pain, fractures, chelation therapy and hormonal therapy. Thorough physical examination was performed for all the studied subjects with special stress on weight, height and secondary sexual characters. Height standard deviation (HSD) was calculated.

II- Bone mineral density measurement

Measurement of BMD was performed for all the studied patients on the heel of the foot using peripheral quantitative ultrasound densitometer (Achillis Express, Lunar Ultrasound Corp., USA) and the result was expressed as the *Z*-score. Patients were considered to be osteopenic (Z-score < -1 and > -2.5) or osteoporotic (Z-score ≤ -2.5) based on WHO definitions (Zajickova and Zokova, 2003).

III- Laboratory methods

1- Sampling

From each patient, venous blood was obtained by a sterile venipuncture and divided into 2 parts. One part was delivered into an EDTA tube (used for separation of cell pellet which was stored for DNA extraction and subsequent VDR gene polymorphism) while the other part was used for measurement of biochemical parameters.

2- Biochemical tests

Serum calcium, phosphorus and alkaline phosphatase (ALP) levels were determined by Synchron CX5 (Beckman Inc, USA), 25-hydroxyvitamin D_3 ($25OHD_3$) was measured by competitive enzyme-immunoassay (EIA) (Roche Diagnostics) and ferritin was measured by EIA (Diamed Eurogen, Belgium).

3- Vitamin D receptor gene polymorphism analysis by PCR & RFLP

DNA was isolated from venous blood as described by Davis *et al* (1986). *BsmI* VDR gene polymorphism was studied using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).

Two primers,5`-CAACCAAGACTCAAGTACCGCGTCAGTGA-3` and 5`-AACCAGC GGAAGAGGTCAAGGG-3`, were used to amplify the 825 base-pair (bp) fragment of VDR gene including intron 8 *BsmI* restriction site (Morrison *et al.*,

Table 1: Descriptive data of prepubertal patients with β-thalassemia (group I)

Patient number	Age (years)	Gender	HSD	BMD Z-score	Serum 25(OH) D_3 (ng/ml)	VDR genotype
1	7.1	Female	-0.85	-0.4	6.6	bb
2	4.3	Female	0.6	0.5	17.1	bb
3	6.5	Female	-0.7	-0.8	12.3	bb
4	5.2	Female	-0.9	-0.9	18.1	bb
5	9.7	Female	-1.2	-1.8#	20.2	Bb
6	8.3	Female	-1.1	-1.6#	14.5	Bb
7	8.3	Female	-0.5	-0.8	16.5	Bb
8	6.3	Female	-1.8	-0.9	18.3	Bb
9	9.2	Female	-2.1	-1.9#	12.3	BB
10	8.6	Female	-1.4	-1.6#	13.2	BB
11	11.6	Female	-1.7	-1.3#	9.1	BB
12	8.3	Female	-1.7	-2.1#	7.9	BB
13	8.8	Male	0.8	-0.6	7.7	bb
14	6.1	Male	0.6	-1.8#	8.5	Bb
15	11.7	Male	0.1	0.6	22.1	bb
16	12.3	Male	-0.2	-2.6*	10.5	bb
17	5.1	Male	0.5	-0.6	8.9	Bb
18	9.4	Male	-0.3	-1.3#	9.2	Bb
19	12.4	Male	-0.7	-2.1#	9.3	Bb
20	10.3	Male	-1.8	-2.3#	13.2	BB
21	10.6	Male	-1.2	-2.8*	5.2	BB
22	11.2	Male	-1.5	-1.9#	15.7	BB
Mean±SD	8.70±2.42		-1.01±0.30	-1.32±0.90	12.56±4.71	

B= common allele, b= infrequent allele. BMD Z-score from < -1 to > -2.5 indicates patients with osteopenia and < -2.5 indicates patients with osteoporosis. Data indicate that 11 patients had osteopenia (#) (6 females and 5 males) and 2 males had osteoporosis (*).

Table 2: Descriptive data of adolescent and adult patients with β-thalassemia (group II)

Patient number	Age (years)	*HSD*	Gender	BMD (Z-score)	Serum 25(OH) D_3 (ng/ml)	VDR genotype
1	15.3	-1.8	Female	-2.4#	9.9	BB
2	18.9	-1.9	Female	-2.8*†	9.4	BB
3	21.3	-1.8	Female	-2.8*‡	9.4	Bb
4	18.1	-2.1	Female	-2.7*†	10.1	Bb
5	28.3	-1.9	Female	-2.3#	8.3	Bb
6	18.1	-1	Female	-1.9#	13.3	bb
7	13.1	-1.1	Female	-0.8	10.5	Bb
8	14.4	-0.3	Female	0.3	12.3	Bb
9	22.3	-1.5	Female	-2.3#	9.4	Bb
10	17.9	-1.2	Female	-1	10.3	bb
11	25.6	-2.2	Female	-3.6*†	8.4	BB
12	13.6	-1.7	Male	-1.9#	6.3	bb
13	19.5	-2.1	Male	-3.3*†	9.4	BB
14	16.2	-2.2	Male	-2.6*†	10.2	BB
15	17.1	-0.5	Male	-1.3#	10.1	bb
16	20.3	-1.7	Male	-4.0*†	9.8	BB
17	18.9	-2.7	Male	-3.2*†	10.5	BB
18	17.2	-1.5	Male	-3.1*†	9.3	BB
19	22.6	-1.6	Male	-2.1#	10.2	bb
20	14.6	-1.5	Male	-2.1#†	8.3	bb
Mean±SD	18.67±3.94	-1.62±0.58		-2.30±1.02	9.77±1.45	

BMD Z-score from < -1 to > -2.5 indicates patients with osteopenia and < -2.5 indicates patients with osteoporosis. Data indicate that 8 patients had osteopenia (#) (4 females and 4 males) and 9 had osteoporosis (*) (4 females and 5 males). †Patients with hypogonadism. ‡Patient with DM and hypogonadism.

1994). Twenty five microliter reaction Mix containing standard PCR buffer with 1.5 mM MgCl, 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCL, 0.4 umol/L of dNTPs, 0.3 umol/L of each primer, 100 ng of DNA and 2 units of *Taq* polymerase (finenzymes) was prepared. The PCR included an initial denaturation step at 94°C for 4 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 63°C for 1 minute, and extension at 72°C for 1 minute and finally 5 minutes extension at 72°C. The amplified 825 bp product was digested with *BsmI* restriction enzyme at 37°C overnight (Roche Molecular Biochemicals) according to the Manufacturer's protocol. Digested products were electrophoretically separated on 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. Absence of *BsmI* restriction site (825 bp) was assigned as common allele *B* and the genotype was considered homozygous (*BB*). Presence of restriction site resulting in 650 bp and 175 bp fragments was assigned as infrequent mutant allele *b* and the genotype was considered homozygous (*bb*). Presence of 825, 650, and 175 bp fragments indicated heterozygosity (*Bb*).

IV- Statistical analysis

Data were collected, tabulated and statistically analyzed by SPSS (9.1) statistical package (SPSS incorporation, Chicago, Ilinois, USA) on IBM compatible personal computer. Student's t-test and chi-square (X^2) tests were used for quantitative and qualitative data respectively. Linear regression analysis was used to study the relationship between variables.

Results

BMD Z score < -1 was found among 71.4% of the studied patients (11 cases had osteoporosis and 19 had osteopenia).

1- Clinical and laboratory findings of the studied children (group I)

Table 1 shows that HSD was -1.01± 0.3 and BMD Z-score was -1.32 ± 0.9 [11 of them had osteopenia (BMD Z-score < -1 and > -2.5) and 2 had osteoporosis (BMD Z-score < -2.5)].

2- Clinical and laboratory findings of the studied adolescents and adults (group II)

Hypogonadism was found among 9 cases and diabetes mellitus (DM) was diagnosed in one case among this group. The mean BMD Z-score of the nine patients with hypogonadism was –2.73 ± 1.6 (being significantly lower compared to those without hypogonadism of the same age group). The single case diagnosed as DM was osteoporotic with BMD Z-score of -2.8 (table 2).

3- Comparison of the clinical and laboratory findings among the two studied groups

There was no significant difference between both groups regarding serum levels of calcium, phosphorus and ALP. However, the HSD, BMD Z-score and serum $25(OH)D_3$ were lower among adults compared to children (table 3). However, no significant difference was found between males and females regarding various parameters except HSD which was significantly lower among females compared to males in group I (table 4).

Table 3: BMD, VDR genotype and biochemical parameters of the studied groups of patients with β-thalassemia major.

The studied parameters	Group I (n =22)	Group II (n =20)
Age (years)	8.695±2.422	18.67±3.94***
Gender M/F	10/12	9/11
HSD	-1.01± 0.30	-1.62±0.58***
Pretransfusion Hb (gm/dl)	7.2±0.9	6.9±0.7
Serum ferretin (ng/ml)	1370±512	1490±680
$25(OH)D_3$ (ng/ml)	12.56± 4.71	9.77±1.45*
ALP (IU/L)	200±25	190±15
Calcium (mg/dl)	10.1±0.9	9.7±0.4
Phosphorus (mg/dl)	3.5±0.9	3.2±0.8
BMD (Z-score)	-1.32±0.90	- 2.30±1.02**
VDR *Bsm1* BB% Bb% bb%	 7 (32%) 7 (32%) 8 (36%)	 8 (40%) 6 (30%) 6 (30%)

Data are expressed as means ± SD. Group I included 22 children without any pubertal signs (age from 4.3 to 12.4 years), Group II included 20 adolescents and adults (age from 13.1 to 28.3 years). Student's t- and X^2 tests were used for comparison of quantitative and qualitative data respectively. *P < 0.05, **P < 0.01, ***P < 0.001.

Table 4: HSD, $25(OH)D_3$, BMD Z-score in relation to gender of patients with β-thalassemia major among the studied groups.

The studied parameters	Males	Females
Group I:	(n = 10)	(n =12)
Age (years)	9.79+2.50	7.78+2.02
HSD	-0.77+0.10	-1.11+0.736*
$25(OH)D_3$ (ng/ml)	11.03+4.85	13.84+4.39
BMD Z-score	-1.54 +1.06	-1.13+0.74
Group II:	(n = 9)	(n =11)
Age (years)	17.8 + 2.84	19.39+4.7
HSD	-1.722+0.606	-1.53+0.573
$25(OH)D_3$ (ng/ml)	9.34+1.32	10.11+1.51
BMD Z-score	-2.63+0.84	-2.03+1.11

Data are expressed as means + SD. A significant difference in HSD was found only between males and females in group I using Student's t-test, P < 0.05.

4- The relation between VDR gene polymorphism and the studied variables in β-thalassemic patients

Both the HSD and the BMD Z-score of both groups were significantly lower among patients with BB genotype compared to those with Bb or bb genotype. However, the age and serum level of $25(OH)D_3$ were not significantly different (tables 5 and 6).

Table 5: Relation between VDR genotype and BMD and the other studied variables in β-thalassemia patients (group I)

The studied parameters	VDR genotype		
	BB (n =7)	bb (n =8)	Bb (n =7)
Age in years	9.97 ± 1.29	8.5 ± 2.38	7.75 ± 2.94
HSD	-1.63 ± 0.29[a]	-0.82 ± 0.40	-0.74 ± 0.10
$25(OH)D_3$ ng/ml)	10.9 ± 3.7	13.8 ± 4.7	12.9 ± 5.6
BMD Z-score	-1.99 ± 0.48*[a]	-1.3 ± 0.56	-1.08 ± 0.75

Data are expressed as means ± SD, P < 0.05. Patients with BB genotype had significantly lower HSD (compared to both [a]bb and [a]Bb genotype) and BMD Z-score (compared to both *bb and [a] Bb genotype).
*Using Student's t-test; [a]Using one way ANOVA followed by Tukey-Kramer test.

Table 6: Relation between VDR genotype and BMD and the other studied variables in β-thalassemia patients (group II)

The studied parameters	VDR genotype		
	BB (n =8)	bb (n =6)	Bb (n=6)
Age in years	18.98 ± 3.17	18.50 ± 3.50	18.38 ± 5.70
HSD	-2.01 ± 0.37*	-1.33± 0.54	-1.37 ± 0.61
$25(OH)D_3$ (ng/ml)	9.61 ± 0.64	10.1 ± 0.37	9.65 ± 2.7
BMD Z-score	-3.1 ± 0.5[a]	-1.7 ± 0.8	-1.8 ± 1.07

Values are expressed as means ± SD, P < 0.05. Patients with BB genotype had significantly lower HSD and BMD Z-score in comparison to those with both bb and Bb genotype.
*Student's t-test and [a] one way ANOVA followed by Tukey-Kramer test were used

5- Correlation between BMD and the other studied variables

Among the 2 studied groups, BMD Z-score was negatively correlated with age and positively correlated

with HSD. However, no relationship was detected between BMD and 25(OH)D_3 (table 7).

Table 7: Correlation between BMD and the other studied variables among groups I and II β-thalassemia patients.

The studied variables	BMD (Group I)	BMD (Group II)
	r value	r value
Age	-0.49*	- 0.47*
25(OH)D_3	0.41	0.37
HSD	0.51*	0.80**

Correlation coefficient test was used to correlate BMD with the studied variables. * P < 0.05, ** P < 0.01.

6- Polymorphism of the VDR gene

Fig. 1 shows *BsmI* digestion of the amplified products from intron 8 of the VDR gene. Absence of *BsmI* restriction site (825 bp) was assigned as the common allele *B* and the genotype was considered accordingly as homozygous (*BB*) as in lane 6. The presence of *BsmI* restriction site (resulting in 650 bp and 175 bp fragments) was assigned as mutant allele *b* and the genotype was considered as homozygous (*bb*) as illustrated in lanes 1, 4 and 5. Presence of 825, 650 and 175 bp fragments indicated that the genotype is heterozygous (*Bb*) as indicated in lanes 2 and 3.

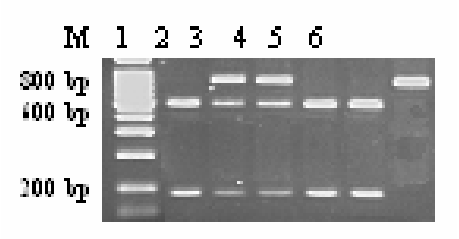

Fig. 1: *BsmI* digestion of PCR products from intron 8 of VDR gene.

M = 100 bp size marker. Lanes 1, 4 and 5: patients with homozygous bb genotype. Lanes 2 and 3: patients with heterozygous Bb genotype. Lane 6: a patient with homozygous BB genotype.

DISCUSSION

Osteoporosis is a complex multi-factorial disease where environment, diet and genetics play a role in determining susceptibility. It represents an important cause of morbidity in patients with β-thalassemia major (Morrison *et al.,* 2006 and Angelopoulos *et al.,* 2007). In this study, BMD was reduced in 30 out of the studied 42 (71.4%) thalassemic patients (45.2% had oesteopenia and 26.2% had osteoporosis), a finding which was similarly reported by Benigno *et al* (2003) and Ladis *et al* (2004). BMD is a good index of bone status in these patients (Molyvda-Athanasopoulou *et al.,* 1999). In thalassemia, increased marrow erythropoiesis and extensive iron deposition occurs resulting in expansion of BM cavities and reduced trabecular bone volume leading to decreased bone tissues and osteoporosis (Domrongkitchaiporn *et al.,* 2003 and Mahachoklertwattana *et al.,* 2006). Moreover, growth hormone (GH)/insulin-like growth factor (IGF)-I /insulin-like growth factor-binding protein (IGFBP)-3 axis and hypothalomo-pituitary-gonadal axis dysfunction may be other contributing factors (Dundar *et al.,* 2007). In spite of the associated severe bone destruction, bone formation remains intact and thereby a more intensive treatment comprising hormonal replacement is required (Voskaridou *et al.,* 2001).

This study showed that BMD in adolescents and adults was significantly lower compared to that of prepubertal children. Moreover, a significant negative correlation was found between BMD and age in both groups indicating that BMD is affected by age. A lower bone mass was reported in adults as compared to children and a negative correlation between age and BMD Z-score was demonstrated in thalassemia (Lo and Singer, 2002). In the present study, growth retardation was evident in thalassemic patients (especially in group II) and HSD was positively correlated with BMD Z-score among both groups. These findings are similarly reported by Filosa *et al* (1997) and Mahacholertwattana *et al* (2003) and indicate that short stature and osteoporosis are significant problems in thalassemic patients. In these patients, short stature and reduced growth velocity were attributed to dysfunction of the GH-IGF-1 axis because improvement of growth velocity was demonstrated by recombinant GH treatment (Sartorio *et al.,* 2000 and La Rosa *et al.,* 2005).

In this study, hypogonadism and/or delayed puberty were found among 9 out of the studied 42 patients (all of them were in group II). These patients also had significantly lower BMD Z-score compared to other patients of the same group. This finding may indicate that hypogonadism may be one of the factors responsible for osteoporosis. Hypogonadism and delayed puberty were reported in 30 - 50% of β-thalassemia cases and were attributed to iron deposition and oxidative damage induced by free radicals affecting the anterior pituitary, ovarian follicles and testis (Soliman *et al.,* 2000 and Skordis *et al.,* 2006). Another contributing factor to osteoporosis may be DM which was reported to be another complication emerging in adolescent thalassemic patients (Lo and Singer, 2002). The only one diabetic case in the current study had osteoporosis with BMD Z-score of -2.8. These results may indicate that decreased BMD is associated with the presence of endocrine disorders. Low BMD in thalassaemia was also associated with cardiomyopathy, DM and chronic hepatitis (Origa *et al.,* 2005).

In this study, there was no significant difference of BMD Z-score between males and females in the 2 studied groups, a finding which was reported by Ferrara *et al.,* (2002), however, Dressner *et al* (2000) showed that BMD

Z-score of the spine and distal radius were significantly lower in men than women. Serum calcium, inorganic phosphate and ALP levels were found to be within normal in both groups of the studied patients. Similar results were reported by Rioja *et al* (1990). However, De Sanctis *et al* (2008) reported that hypocalcaemia is a late complication of iron-overloaded patients with β-thalassaemia major. Some studies demonstrated that vitamin D deficiency, oesteomalcia and rickets in thalassemic patients were attributed to defective 25 hydroxylation of vitamin D in the liver due to iron overload (Chatterton *et al.,* 2003). Other mechanisms include decreased intake, impaired absorption, or reduced skin production (Dressner *et al.,* 2000). Some studies reported normal vitamin D levels with the use of effective chelating agents (Filosa *et al.,* 1997). In the present study, serum 25-OH-D_3 concentration was normal in the 2 studied groups, however, it was lower among older patients compared to children, a result which was reported by Napoli *et al* (2006). Nakamura *et al* (2005) reported that low serum vitamin D and calcium levels were important predictors of low BMD. Chen and chow (2001) demonstrated that oral administration of vitamin D plus calcium improved BMD. In the current study, analysis of *BsmI* VDR gene polymorphism revealed that β-thalassaemia patients with the BB genotype had significantly lower BMD compared to those with bb or Bb genotypes. This finding agrees with that reported by Dressner *et al* (2000), Ferrara *et al.,* (2002) and Garnero *et al* (2005) who found that *BsmI* VDR gene polymorphism was associated with osteopenia. However, Wu *et al* (2007) showed that VDR genotype was not related to BMD and indicated that VDR polymorphism could not be used as a genetic marker for predicting the risk of osteoporosis. The relationship between *BsmI* VDR polymorphism and BMD was reported by several investigators (Morrison *et al.,* 1994 and Arabi *et al.,* 2006). In addition, patients with BB genotype in the current study had lower 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 (Lorentzon *et al.,* 2000 and Ferrara *et al.,* 2002). In conclusion, *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.

ABBREVIATIONS

ALP, alkaline phosphatase; BM, bone marrow; BMD, bone mineral density; DM, diabetes mellitus; DFO, desferrioxamine; GH, growth hormone; HSD, height standard deviation; IGF, insulin-like growth factor; 25(OH)D_3, 25-hydroxyvitamin D_3; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor. EIA, enzyme immunoassay; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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