Association of Serum Vitamin D Level with its Receptor Gene Polymorphism Bsml in Beta Thalassemia Major Patients from East India

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ABSTRACT

Background

Vitamin D deficiency is commonly identified in beta thalassemia major patients, related to iron accumulation.Vitamin D mediates its action upon binding to vitamin D receptor (VDR), a classical nuclear receptor. Several single nucleotide gene polymorphisms has been identified in VDR gene among which Bsml is commonly studied for its association with bone mineralisation and osteoporosis.

Objective

To explore the association between the Vitamin D Receptor Polymorphism (BsmI) and serum levels of Vitamin D, ionised Calcium, alkaline phosphatase in patients with beta thalassemia major.

Method

VDR gene was studied for Bsml polymorphisms from purified DNA in thirty six beta thalassemic patients (cases) - fourteen male and twenty two females, and thirty three controls after amplification by PCR followed by restriction digestion using appropriate restriction enzymes. Allelic differences between two groups were assessed by chi square and odds ratio test. Any potential link between the polymorphic variations and vitamin D status were assessed by post hoc ANOVA with bonferroni correction among the three genotypes.

Result

The distribution of BB genotype was significantly higher among the case groups (thalassemic group, χ^2= 9.77, p= 0.008). The odds ratio for the allele B was significantly higher in thalassemia group for a range of 1.97 to 5.94 for 95 percent cofidence interval (χ^2=10.4, p=0.0013). Serum Vitamin D, ionised Calcium were significantly low ($p < 0.001$) and Alkaline phosphatase ($p < 0.001$), was significantly high in thalassemics (cases). The genotype BB group had significantly low Vitamin D (p=0.001) and ionised Calcium ($p < .001$) compared to Bb and bb. The bb genotype had the highest levels of Vitamin D and ionised Calcium among the three genotypes.

Conclusion

The thalassemic patients are prone to Vitamin D deficiency and the superimposed predominance of BB genotype in them may be a risk factor for osteoporosis and cardiac dysfunction. Moreover, the study indicated genotype bb to have a probable protective role against Vitamin D deficiency in beta thalassemic patients

KEY WORDS

Beta thalassemia, Vitamin D receptor , Vitamin D receptor gene

INTRODUCTION

Thalassemia syndromes are endemic in the Mediterranean basin, tropical Africa, the Middle East and the Indian subcontinent and in aggregate are among the most common inherited disorders of humans.[1] Beta thalassemia are caused by mutations that diminish the synthesis of β-globin chains. Etiologically, thalassemia is multifactorial disease but genetics play an important role in its pathogenicity.[2] Mineralisation and growth of bones is under the polygenic control of Collagen genes, vitamin D receptor (VDR) and oestrogen receptor (OesR) genes.[3] Vitamin D deficiency is commonly identified in beta thalassemia patients.[4] Bone diseases associated with vitamin D deficiency comprise an important cause of morbidity in β-thalassemia.[4] Furthermore, iron overload in these transfused patients leads to impaired Calcium homeostasis.[5] Other possible mechanisms leading to this impairment includes reduced intake, impaired absorption, and decreased synthesis of vitamin D due to defective 25 hydroxylation of vitamin D in the liver caused by iron overload as well as reduced skin production.[6,7] Vitamin D mediates its action upon binding to classical nuclear receptor called vitamin D receptor (VDR), a ligand activated transcription factor that is widely distributed throughout the body.[8] Until now around 200 different types of single nucleotide gene polymorphisms have been identified for the Vitamin D receptor gene, among which Fok1, ApaI, BsmI and Taq1 are most commonly studied and are considered as a self-marker in various disorders.[9]

Although, both the prevalence of beta thalassemia and vitamin D deficiency are quite alarming in the Eastern India, but we could scarcely find any report regarding the association of vitamin D deficiency with VDR polymorphism among these patients in this part of the country. Based on these lacunae we hypothesized that there is an association of VDR polymorphism with vitamin D deficiency in the Eastern Indian population suffering from beta thalassemia and hence outlined the present study aiming to analyze it.

Our objective was to determine Serum Vitamin D, ionised Calcium, alkaline phosphatase in patients with beta thalassemia major as well as in control subjects measured by the ELISA technique, ion selective electrode and standard spectrophotometric technique respectively and Vitamin D Receptor Polymorphism (BsmI) in patients with beta thalassemia major as well as in control subjects. Finally, we aimed to determine the association between the Vitamin D Receptor Polymorphism (BsmI) and status of the analytes

METHODS

It was a hospital based observational study. The present study was an observational, non interfering, cross sectional hospital based study. The study population included paediatric thalassemic patients. The control group were selected from the healthy relatives of the patients who will accompany them being age matched.

Cases were taken from the thalassemia unit of the institution as per the following criteria:

***Inclusion criteria**

a) The age of the β-thalassemia major subjects were between the ages of 2 to 15 yrs.

b) They had already received multiple blood transfusions or iron therapy.

***Exclusion criteria**

a) The newly diagnosed patients were excluded.

b) The subject who were diabetic, had other medical conditions (heart failure, renal failure) or any known diseases.

c) Subject who had active infection and inflammation.

d) Subjects on medications which affect bone mineral metabolism (glucocorticoids or anticonvulsant drugs)

Controls were selected from healthy age matched individuals haematological diseases, chronic or active infection and inflammation.

The study was done from April 2016 to March 2017. Selection of cases were done from the thalassemia unit of the institution. Biochemical investigations and result analysis were performed in the department. of biochemistry. Following inclusion and exclusion criteria thirty six thalassemic patients (cases) - fourteen male and twenty two females and thirty three controls were selected by the method of convenience.

After selection, proper history was taken regarding age of onset of thalassemia, blood transfusion frequency, iron chelation therapy, splenectomy, family history, any medical complication. Vital signs, anthropometric measurements, abdominal examination for detection of hepatomegaly, tender liver and splenomegaly clinically and USG in indeterminate cases, musculoskeletal system for detection of bone tenderness, pain, any deformity, pathological fractures and muscle weakness by examining the muscle strength by clinical grading. Following this, 5 ml of venous blood was drawn aseptically after informed consent and divided into two aliquots- one in EDTA vial for DNA isolation and one clotted sample for serum ferritin, vitamin D, ionised Calcium, alkaline phosphatase estimation.

Study was done following all guidelines of the Helsinki declaration, 1975, revised in 2000, and was initiated after obtaining the approval of institutional ethical committee.

Assay of study parameters

i) Serum 25-OH vitamin D was measured by the ELISA technique (Epitope Diagnostics, Inc) in the Dept. of Biochemistry and the biological reference value was 30-50 ng/ml.

ii) Serum ionised calcium, alkaline phosphatase: These analytes were assayed by ion selective electrode (Roche) and standard spectrophotometric technique (Coral Biosystems) respectively. The biological reference value were 1.33 to 1.93 milimole/l and 94-498 U/L respectively

iii) DNA isolation: DNA was isolated from the EDTA blood by the methodof Blin and Stafford.[10] 1.5 ml of EDTA blood was lysed with lysing buffer in presence of proteinase K for 3 hours at 56° C. The supernatant obtained was treated with 1 ml of Tris saturated phenol, mixed well and was centrifuged at 8000 rpm at 4° C for 10 minutes. The supernatant obtained was treated with chloroform isoamyl alcohol (99:1), mixed well and centrifuged at 8000 rpm at 40 C for 4 minutes. The supernatant obtained in previous step was treated with 1.5 ml of ice cold absolute alcohol and 200 µl of 3 M sodium acetate. Precipitated DNA was put in 70% ethanol to remove water molecules followed by their preservation in Tris EDTA buffer, pH 7.6 at 40 C for a day and then at -200 C indefinitely.

iv) PCR amplification of the separated DNA: PCR master mix containing all four dNTPs, Taq polymerase, MgCl2 and the requisite buffer at optimum concentration was obtained from the Thermo Fisher Scientific (USA). For a total volume of 25 µl PCR mixture, 12.5 ml of master mix, 0.8 µl each of forward primer (5'AGTGTGCAGGCGATTCGTAG3') and reverse primer (5'ATAGGCAGAACCATCTCTCAG3'), 1 µl of template DNA (1 µg) and 9.9 µl of nuclease free sterile water was mixed. PCR was performed for 35 cycles with the following steps: denaturation at 94˚C for 4 minutes; 35 cycles of denaturing at 94˚C for 30 seconds; annealing at 58.5˚C for 30 seconds; and extension at 72˚C for 30 seconds with a final extension of 5 minutes at 72˚C. The PCR products were run against 100 bp ladder (Thermofischer Scientific, USA) in a 1.2% Agarose gel with ethidium bromide staining. The length of theproduct was confirmed to be 191 bp (fig. 1).

v) Restriction digestion of the PCR Products: After amplification of the target DNA, 15 µl of each PCR product was digested with 1 µl of BsmI restriction enzyme (rs 1544410 - Biolab, Cut Smart) with 5 µl buffer and 29 µl water at 65˚C for about 20 minutes. The digested products were subjected to electrophoresis and separated on 2% agarose gel containing 0.5 µg/ml ethidium bromide. BsmI cuts the GAATG^C sequence between the G and C, as shown here by the "^" symbol. Absence of BsmI restriction site in the191bp VDR gene fragment was assigned as common allele B and the genotype was considered homozygous (BB) resulting a single band in gel (191 bp). Presence of restriction site resulting in 115 bp and 76 bp fragments was assigned as mutant allele b and the genotype was considered homozygous (bb). Presence of 191, 115 and 76 bp fragments indicated heterozygosity (Bb). (fig. 2)

Statistical analysis: The significance of difference between the distribution of genotype frequency and allelic frequency were analyzed by chi square test among the case and control groups and the odds ratio for 95% confidence interval was noted for allelic frequency. Results of difference in means of serum vitamin D, ionised Calcium and alkaline phosphatase values were compared between the case and control groups were analysed by student's t test. The difference in means of the distribution of these analytes was compared between homozygotes for both alleles and heterozygotes by one way ANOVA followed by bon ferroni correction. All the statistical analysis were carried in the SPSS 16 software (SPSS Inc. Chicago, USA), considering p value < 0.05 to be statistically significant.

RESULTS

This table showing the result of Student t test done to compare the mean values of serum analytes between cases and controls revealed serum vitamin D and ionised calcium were significantly low in beta thalassemia children compared to the control population (p<0.001). On the contrary, the level of alkaline phosphatase was significantly high in the case group (p<0.001).

Table 1. Comparison of mean values of serum analytes between cases, n=36 (paediatric thalassemic) and controls, n=33 (non thalassemics)

Analyte Tested	Mean±SD (Case)	Mean±SD (Control)	p
25 OH Vitamin D(ng/ml)	25.94 ± 8.84	57 ± 14.30	<0.001*
i Ca(mmol/l)	0.83 ± 0.38	1.87 ± 0.44	<0.001*
ALP(U/ml)	788.72 ± 152.64	449.36 ± 158.49	<0.001*

* p value < 0.05 considered to be statistically significant, i Ca- ionised calcium

Table 2. Chi square test to compare genotype distribution between cases and controls

	Homozygote(AA)	Heterozygote(AG)	Homozygote(GG)
Case	16	15	5
Control	6	12	15

χ^2=9.77, p value=0.008 (p value <0.05 to be statistically significant)

This table showing the result of Chisquare test to compare genotype distribution in cases and controls showed Homozygote(BB) to be significantly predominant in beta thalassemia children(chisquare=9.77, pvalue=0.008)

Table 3. Chi square test to compare allelic distribution in cases and controls

	B allele	b allele
Case	47	25
Control	24	42

χ^2=10.4, P value=0.0013, Odds ratio=1.97 to 5.94 for 95 percent cofidence interval (p value < 0.05 to be statistically significant).

This table showing the result of Chi square test to compare allelic distribution in cases and controls revealed presence of B allele to be significantly high in beta thalassemia children (chisquare=10.44, p value=0.0013, oddsratio=1.97 to 5.94 for 95 percent confidence interval).

This table showing the result of one way anova to compare analyte means between the three genotype groups showed vitamin D and ionised calcium were significantly lowest in BB homozygotes and highest in bb homozygotes (p=0.001, p<0.001 respectively). On the other hand, alkaline phosphatase levels comparison revealed significant result (p=0.09).

Table 4. One way anova to compare analyte means between the three genotype groups,

Mean±SD of analytes of the groups	Vitamin D (ng/ml)	Ionised Calcium (mmol/l)	ALP (U/ml)
Group 1(BB)	22.81 ± 7.5	0.55 ±0.12	848±104.24
Group 2(Bb)	25.64±7.66	0.935±0.28	730.38±185.25
Group 3(bb)	30.2±13.61	1.5±0.34	785±94.86
P value	.001*	<.001*	.09

*p value <0.05 considered to be statistically significant.

In this table showing the result of Post hoc anova with Bonferroni correction for comparing intergroup analyte means there was significant diference in vitaminD means between BB and bb (p=0.001) as well as between Bb and bb (p=0.005), but not between BB and Bb in beta thalassemia children which signified the cardinal role of bb homozygote in protecting against vitamin D deficiency. There was significant difference in between all three genotype groups for serum ionized calcium levels.

Table 5. Post hoc anova with Bonferroni correction for comparing inter group analyte means, showing the p values in the table.

Groups		Vitamin D	Ionised Calcium	ALP
1	2	.875	<0.001*	.088
	3	.001*	<0.001*	1.000
2	1	.875	<0.001*	.088
	3	.005*	<0.001*	1.000
3	1	.001*	<0.001*	1.000
	2	.005*	<0.001*	1.000

*p value <0.05 considered to be statistically significant.

DISCUSSION

The Vitamin D Receptor (VDR), a member of the intracellular steroid receptor superfamily mediates the biological actions of Vitamin D (VD) by acting as a nuclear transcription factor and regulating the synthesis of proteins of cell proliferation and bone,mineral homeostasis.[11] VDR gene, located on chromosome no. 11, consists of 11 exons.[12] The exons involved in synthesis of DNA binding domain of the VDR protein are exons 2, 3 and those involved in VD binding domain are 7, 8 and 9.[12] It is a candidate gene with many single nucleotide polymorphisms (SNPs) that might potentially modify the expression of VDR.[13] Bsm I restriction site is generated by SNP in intron 8 causing A to G substituition giving rise to three potential genotypes which can be designated as BB, Bb and bb.[12] This affects

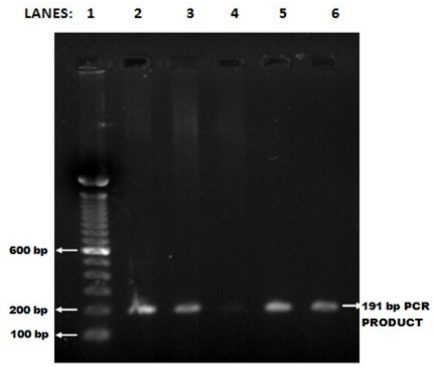

Figure 1. Amplication of VDR gene fragment PCR products (191 bp) are shown on 1.2% Agarose gel. The marker is a 100bp ladder.

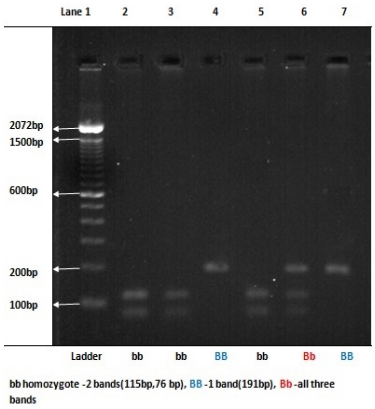

Figure 2. MRestriction digestion of PCR product. Lane 1 is a 100bp DNA ladder, lane 2,3,5 shows 2 bands of bb (mutant) homozygote, lane 4, 7 shows a single band of BB (wild) homozygote and lane 6 shows all three bands of Bb heterozygote.

the VDR gene expression which may be due to to the mRNA instability.[14,15] It has been hypothesised that the BsmI polymorphism site located in close proximity to the 3' untranslated area could influence the stability of its mRNA transcript.[12] Some studies revealed that an association of VDR gene polymorphisms with obesity alter the circulation of vitamin D by sequestering it into adipose tissues.[16]

The frequencies of BB, Bb and bb genotypes differ in different population.[17] In Chinese women the frequencies were 2.3, 18.1 and 79.6%, while in Caucasian population it was 15.4, 47.4, and 37.2% for the same genotypes.[18,19] The inconsistencies among these studies can be due to factors which could affect gene regulation in different genotypes such as the sample size, age, ethnic ancestry and lifestyle factors.[20,21] In the current study, the distribution of genotype BB is significantly higher among the case groups (thalassemic group) (table 2). The allele B is also significantly high in the thalassemic patients. The odds ratio for the allele B is significantly higher in thalassemia group for a range of 1.97 to 5.94 for 95 percent confidence interval (table 3). In our study, we have found change of A to G base in the intron no. 8 that resulted in the mutant allele b showing significantly higher values of both vitamin D and calcium in blood of the bb genotype in the case (thalassemic) group. (table 4). We found that

the b mutant allele protects against vitamin D deficiency in beta thalassemia patients. These observations are further strengthened by the results of post hoc ANOVA (table 5) where only the mutant genotype for both alleles (bb) was found to provide significant protection against vitamin D deficiency. Elhoseiny et al. reported in their study that in BsmI VDR polymorphism., BB genotype had significantly lower Vitamin D level and lower bone mineral density (BMD) than genotype (Bb,bb).[12] In a study by Singh et al. lower bone mineral density (BMD) of lumbar spine was found in BB genotype among 40 beta thalassemics.[22] The same findings were reported by other studies who found that BsmI VDR gene polymorphism was associated with osteopenia.[6,23] All these studies indicated a significant role of VDR polymorphism in skeletal growth and concluded that BB VDR genotype can be considered as a risk factor for the development of osteoporosis in b-thalassaemia. A meta analysis concluded that recessive allele b of BsmI VDR gene polymorphism is a preventive factor of osteoporosis which strengenths the same results of the current study.[19] It is necessary to compare the entire genome of the healthy people and beta thalassemia patient of the same genotype, analyze the genomic organization of the VDR locus.[17] Identification of relationship between the genes in the same chromosomal area and their interactions may be required to analyze the association of the polymorphism with osteoporosis. Identification of possible gene-gene interactions throughout the whole genome would be necessary to analyze the predominance of allele B in thalassemia.

Low serum ionised calcium levels with elevated levels of serum inorganic phosphorus and alkaline phosphatase were found in the thalassemic patients of current study (table 1). The result is in accordance with De Sanctis et al. who found hypocalcaemia to be a complication of iron overload in beta-thalassemia.[24] Tantawy et al. reported that 75% of their betat halassemic patients had low calcium level probably caused by a combination of hypoparathyroidism and osteomalacia evidenced by elevated bone alkaline phosphatase.[25] Vitamin D deficiency (<20 ng/ml) and insufficiency (20-30 ng/ml) is reported to be high in thalassemic patients in many countries inspite of presence of good sunshine.[26] Osteoporosis and cardiac dysfunction are frequent complications in beta thalassemia which are linked to vitamin D deficiency.[27]

Our study had certain limitations. Larger sample size was required to improve the statistical importance of this study. VDR gene polymorphisms other than BsmI were not dealt with in the current study. The Bone mineral density assay which could not be afforded, if done would have provided more valuable, relevant data about incidences of osteoporosis in the thalassemic children in the current setup.Thus thalassemics are prone to Vitamin D deficiency and the superimposed predominance of BB genotype in them may be a risk factor for osteoporosis and cardiac dysfunction.

CONCLUSION

A significant association between the BsmI gene polymorphism and osteoporosis related to VDR gene could be a valuable biological tool not only for the early diagnosis, but also for the prediction of susceptibility to osteoporosis and early initiation of oral or parental use of vitamin D to significantly improve bone mineralisation and in beta thalassemia major. Further, better understanding of functional consequences of VDR gene Polymorphisms in future studies would strengthen the association of this polymorphism in vitamin D status and and its biological role in reproduction, growth, and other body systems.

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