

Analysis of VDR Gene Polymorphism in Beta Thalassemia Major (Beta Thalassemia Major / Vitamin D / Calcium / 825T/T Vitamin D Receptor Gene)

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ABSTRACT

Vitamin D Receptor (VDR) Gene has an important role in calcium and bone metabolism because is required in mineralization of normal bone, calcium absorption, organizing calcium and phosphate homeostasis, and regulating the secretion of parathyroid hormone. Genetics research found that *BsmI* polymorphism region in VDR gene relates to osteoporosis, other bone diseases, and cartilage metabolism in particular allele. The aim of this research is to analyze polymorphism of VDR (825G/T) gene in the eighth intron of beta thalassemia major on Sundanese population. In this case-control study, SNP 825G/T of 66 beta thalassemia major patients (case group) and 15 controls (control group) were evaluated using PCR-RFLP with restriction of enzyme *BsmI*. Vitamin D and calcium level were analyzed using ELISA. Genotype distributions and allele frequencies were analyzed chi-squared test. This study found that the vitamin D level was significantly lower on case group than on control group (vitamin D 56.1 pmol/L 80.30%; $p < 0.001$), and calcium level was significantly higher

on case group than on control group (> 8.5 mg/dL; $p = 0.025$). Genotype frequency 825 VDR on case group was compared with control group, and there was no BB genotype in control group. There is only one case on case group (OR, 2.63; 95%CI, 0.73-9.64). B allele is significant in both case and control groups. There was no significant difference between case and control groups (OR, 2, 95%CI, 0.71-5.92). There was a significant difference in level of vitamin D and calcium, and also in allele frequency between on case and control groups.

Keywords: Beta thalassemia major, Vitamin D, Calcium, 825G/T Vitamin D receptor gene

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INTRODUCTION

Thalassemia is an inherited blood disorder caused by a reduction of one or more globin chain synthesis. This quantitative abnormality causes instability in globin chain synthesis, partial hemoglobin production, and a decrease in red blood cell age.¹

The disease was initially found by Cooley and Lee in 1925 and has clinical manifestations in the form of face appearance characteristics, bone changes, and splenomegaly. According to Whipple and Bradford, children with this disease have genetic origin from Italy and Greece, located in the Mediterranean Sea. Therefore, the term *Thalassemia* is from “*The sea (Mediterranean) in blood*”. The term is from Greek language *thalassa* which means sea, and *haima* which means blood.^{2,3,4,5}

Thalassemia gene spreads widely. The distribution area includes the border area of the Mediterranean Sea, most of Africa, the Middle East, India, and Southeast Asia. Some 40% of the population has one or more thalassemia genes in the regions of Southeast Asia. The highest concentration of thalassemia gene distribution area is in Southeast Asia and in the population from the west coast of Africa. In Thailand, there are 223,000 people diagnosed with alpha thalassemia.^{3,6,7,8,9}

Beta thalassemia gene is widely spread in the Mediterranean area such as Italy and Greece, northern Africa, the Middle East area, South India, Sri Lanka to Southeast Asia region, and Indonesia. The regions are known as the thalassemia basin. The highest frequency of beta thalassemia was found in Maeowo Islands, in the southwest of Pacific, Vanuatu. About 20% of the population has beta thalassemia-characteristic genes and more than 50% are thalassemia trait

of alpha thalassemia. There is no evidence of gene frequency in Indonesia. The estimation is about 3-5%, similar to Malaysia and Singapore. There are 20 new cases of beta thalassemia in Dr. Cipto Mangunkusumo Hospital Jakarta annually.^{10,11,12,13}

Hemoglobin [Hb] is formed from heme and globin that consists of 4 chains of polypeptide (tetramer). Normal people will form HbA with raising level of about 95% of all hemoglobin. The rest consists of HbA₂ and the level is less than 2%, and HbF level is less than 4% in normal condition. HbA globin tetramer consists of two alpha chains and two beta chains. The HbA₂ consists of two alpha chains and two delta chains. HbF consists of two alpha chains and two gamma chains.^{14,15,16}

Thalassemia indication is a disorder in one or more globin chain. Beta thalassemia has a disorder in beta chain production that causes a decrease in HbA level. On the other hand, HbA₂ and HbF production is not interrupted because it does not need beta chain and can definitely produce higher in normal condition. Excessive HbA₂ and HbF formation may support to resolve the problem of oxygen transportation deficiency until specific boundary.¹⁷ The gene that regulates alpha chain synthesis is located in chromosome 16, while beta, gamma, and delta chain synthesis is in chromosome 11.¹⁷

VDR gene polymorphism is located in the eighth intron and is detected by *BsmI* restriction enzyme.²¹ Meta-analysis result explained that *BsmI* bb genotype frequency are 2% in Asians, 5% in African Americans, and 17% in Caucasians.^{18,19,20, 21, 22} Aim of this research is to analyze polymorphism of Vitamin D Receptor (825G/T) gene in the eighth intron of BTM patient on Sundanese population.

MATERIAL AND RESEARCH METHOD

Research Subject

The subject of this research was BTM outpatient in Dr. Hasan Sadikin General Hospital Bandung and control group of healthy children. Case group inclusion criteria were: boys and girls aged 11-13 years with BTM who would have blood transfusion in the Thalassemia Clinic of Dr. Hasan Sadikin General Hospital Bandung. Control group inclusion criteria were: boys and girls aged 11-13 years with healthy condition, diagnosed by pediatrician. The reason on age qualification is because at the age between 11-13 years, bone density will achieve its maximum.

Ethic approval was granted by the Medical Research Ethics Committee, Faculty of Medicine of Padjadjaran University with register number 132/FKUP-RSHS/KEPK/Kep/EC/2007. Selection on subject and sample collection was done only after informed consent had been signed by parents. All of these activities are appropriate with The Code of Ethics of The World Medical Association (Declaration of Helsinki) for research involving human.

Examination of Vitamin D (25 Dihydroxy Vitamin D) and Calcium Level

The examination of vitamin D level was applied using ELISA method. The total of Vitamin D (25(OH)D) level was examined using ELISA method on the blood serum by competitive-binding principal as mentioned on the kit instruction. Sample with extra EDTA or heparin was injected into the polypropylene tube and was stored in temperature of 20°C. Each of the tubes was categorized into control, sample, and calibrator. Biotin solution 1 mL 25-D was included in all tubes and was then centrifuged for 10 seconds. From every tubes, 200 µL of the solution was transferred to the well at antibody coated-microplate, covered by plastic, and was then incubated in temperature between 18-25°C for 2 hours. After incubation, every well was washed 3 times with wash solution. An extra 200 µL of conjugated enzymes were added in every well, and was then covered by plastic. The next process was incubation in 18-25°C temperature for 30 minutes. 200 µL of Tetra Methyl Benzidine (TMB) was added to all wells, covered with plastic, and was incubated again between 18-25°C for 30 minutes. After an extra 100 µL of stop solution, in the next 30 minutes the specimen was ready for absorption analysis with microplate reader.

DNA Isolation

The DNA was isolated from the blood by DNA Isolation Kit method from Pharmacia, then 200 ng DNA was used as template for PCR. The DNA isolation steps were: 300 µL EDTA was added to whole blood back and forth for 10 minutes, 900 µL of RBC lysis solution was added and it was centrifuged for 20 seconds, and the supernatant was removed by micropipette. A 300 µL cell lysis solution was poured and vortexed for 20 seconds until it became homogeneous, and then it was centrifuged for 3 minutes, and afterwards, the supernatant was removed. With a new kit, 600 µL propranolol solution was poured. It was shaken back and forth and then centrifuged for 1 minute,

afterwards, the supernatant was removed. Next, 600 µL of 70% cold alcohol was poured. Then, it was shaken until it became clear and was then centrifuged for 1 minute. The supernatant was removed, and then it was put into Eppendorf tube for 7 minutes. A 50µ TE buffer was given, and it was mixed with filler pipette. Afterwards, it was incubated in 37°C for 1 hour.

Polymerase Chain Reaction (PCR)

PCR compound (with a total volume of 50 µl) consists of: 200 ng DNA template in TE 1x. buffer of 0.6 µL primer-pairing (40 pmol), 1 µL dNTP (10 mM) (consists of dATP, dCTP, .GTP, dTTP), 5 µL buffer solution PCR (0.25 M KCl; 0.05 M Tris HCl pH 8.4; 1.5 mM MgCl and 0.0005% gelatin), 0.25 µL Taq Polymerase (5 unit/µL), and 50 µL mineral oil (Sigma) above PCR compound to prevent evaporation.

Agarose Gel Electrophoresis

Agarose plate of 1% was used as DNA electrophoresis place. First, 0.3% g agarose and 30 mL TAE buffer were put into small Erlen Mayer tube that contained magnet bar. Then, this mixture was stored on the thermocycler in 60°C temperature until the agarose solution was clear. If the solution is clear, it indicates homogeneous solution formation. After that, 3 µL ethidium bromide 10 mg/mL was put into the tube back and forth skillfully. This mixture was then poured into jelly template which contained comb. After it became a solid gel, the wells would form as a place to accommodate DNA. Agarose solution was left until it froze, and afterwards, the comb was removed carefully to avoid the agarose from tearing apart.

Electrophoresis procedure was applied after the gel is cold and solid (about 30 minutes). The comb was removed slowly in straight up position, then the gel along with the buffer were removed from the template and were put inside the electrophoresis tank. Next, the electrophoresis buffer 1x was put inside the electrophoresis tank until the whole top surface of the gel was submerged (about 3-5 mL). The bottom part of amplified DNA sample was collected slowly with Gilson pipette by the tip of the pipette to be placed into the tube foundation to completely collect all of the paraffin. DNA sample was then put inside the Eppendorf. The sample was applied in the gel holes at 20 µL with glison pipette assistance. Loading had to be done very carefully and slowly to avoid the sample from spilling outside the target hole. Every gel for electrophoresis always had one hole as a marker 0 x 174 Hae III-digested (Perkin Elmer Cetus) and one hole was left blank. DNA sample was applied, alternating between the mutant and normal for every detected DNA area. After the loading was complete, the tank was closed and was connected by the power supply, and then it was run for 1 hour with stable voltage of 100 volts. After electrophoresis was complete, the gel was removed from the buffer and was visually observed in ultraviolet lamp and was photographed as documentation.

Examination of VDR Gene Polymorphism

Genotyping was carried out as described in the previous report. For VDR gene polymorphism, it was at 8 intron. Forward primer was 5'-CAACCAAGACTACAAGTACCGCGTCAGTGACGTGA3' and reverse primer was 3'AACCAGCGGAAGAGGTCAAGGG5²⁰.

The PCR process was performed with the following condition: initial denaturation in 95°C for 3 minutes, denaturation in 94°C for 30 seconds, annealing in 62°C for 30 seconds, extension in 72°C for 30 seconds as many as 39 cycles, and then the final extension was in 72°C for 5 minutes. RFLP analysis was performed using *Bsm I* enzyme. The procedure was similar with the previous enzyme. Polymorphism genotype analysis of VDR gene was performed using *Bsm I* enzyme. There was polymorphism G → T in intron and exon VIII and IX. PCR product was 825 pb, digested with *Bsm I* restriction enzyme. GTATGC region was cut using *Bsm I* restriction enzyme. The result was electrophoresised inside a 2% agarose that contained ethidium bromide for 50 minutes in 90V. B → allele was a common allele (wild type), and there was no cutting region. Therefore, the DNA fragment was visible, which was 825 pb. b → allele was an infrequent allele (mutant allele), and there

was a cutting region because of the existence of polymorphism (G → T). Thus, the DNA fragment was visible, which were 175 pb and 650 pb.

Statistical Analysis

The differences of genotype, dominant model and recessive, and allele frequency between BTM case and control group were analyzed by chi-squared test.

RESULTS

Characteristics of Research Subjects

Qualified subject number with inclusion and exclusion criteria were 66 patients and 15 controls. The research result was examined in order to test the hypothesis whether patients of genotype analysis and control and risk factor were correlated with vitamin D and calcium level. Another analysis was a research on subject characteristics data to know the difference between patient and control group. Research subject characteristics examination was performed by anamnesis that was given to patient and control group. The research subject characteristics data are in sex and age.

Table 1: Characteristics of Research Subjects

Characteristics	Group		Significance
	Patient n = 66	Control n = 15	
1. Sex:			
- Male	37 (56.1)	3 (20)	$\chi^2 = 6.358$ $p = 0.012$
- Female	29 (43.9)	12 (80)	
2. Age:			
- 11 years	30 (45.45)	2 (13.33)	$Z_{MW} = 5.453$ $p < 0.001$
- 12 years	27 (40.91)	2 (13.33)	
- 13 years	9 (13.64)	11 (73.34)	

The result of subject characteristics is presented in Table 1. The statistical analysis result in Table 1 shows that there is a significant difference in the characteristics between patient and control group.

Patient group predominantly consisted of male subjects (56.1%), whereas control group predominantly consisted of female subjects (80%). According to the statistics, there is a significant difference between patient and control group, with a value of $p=0.012$.

According to age, patient group was mostly from 11 years (45.45%), whereas control group was 13 years old (73.34%). Statistically, there is a significant difference between the two groups, with a value of $p<0.001$.

Vitamin D and Calcium Level

Table 1 explains the cut point of vitamin D level (56.1) with 80.3% sensitivity and 86.7% specificity. In patient group, 13

subjects (19.7%) are above the cut point and 53 subjects (80.30%) are below the cut point. In control group, 13 subjects (86.7%) are above the cut point and 2 subjects (13.3%) are below the cut point. As a result, the accuracy is high ($66/81 = 81.5\%$).

Table 2 describes the cut point of calcium level (8.5 mg/dL) with 36.4% sensitivity and 93.3% specificity. In patient group, 24 subjects (36.37%) are below the cut point and 43 subjects (63.63%) are above the cut point. In control group, All of the subjects are above the cut point. Consequently, the accuracy is high ($38/81 = 47\%$). Since the sample number is not big, then this result can be taken as an initial result for future research.

The description of the number of patient and control group is based on vitamin D and calcium cut point level in blood, and the accuracy is shown in 2x2 table (Table 2).

Table 2: Vitamin D and Calcium Level Based on Cut Point and Accuracy Value

Variable	Group		Sensitivity (%)	Specificity (%)	Accuracy (%)
	Patient n=66	Control n=15			

1. Vit. D Level

≤ 56.1 pmol/L	53 (80.30)	2 (13.3)	80.3	86.7	8.5
>56.1 pmol/L	13 (19.7)	13 (86.7)			
2. Ca Level					
≤ 8.5 mg/dL	24 (36.37)	0	36.4	93.3	47
>8.5 mg/dL	42 (63.63)	15 (100)			

Note: Level of Vit D $\chi^2 = 25.15$; $p < 0.001$
 Calcium $\chi^2 = 5.051$; $p = 0.025$

Table 2 shows that 53 subjects (80.30%) of BTM patient have ≤ 56.1 pmol/L vitamin D level, whereas there are only 2 subjects (13.3%) having the same level of vitamin D in control group. It also shows that 13 subjects (19.7%) of BTM patient have > 56.1 pmol/L vitamin D level, and there are also 13 subjects (86.7%) having the same level of vitamin D in control group. The differences are significant, with a value of $p < 0.001$.

Table 2 also shows that 24 subjects (36.37%) of BTM patient have ≤ 8.5 mg/dL calcium level, whereas there is none in control group. It also shows that 42 subjects (63.63%) of BTM patient have > 8.5 mg/dL calcium level, whereas there are 15 subjects (100%) having the same level of calcium in control group. The differences are significant, with a value of $p = 0.025$.

Analysis of SNP G825T VDR with vitamin D and Calcium Level

Table 3: Examination of B Allele and b Frequency in 825 VDR Genotype in CaseGroup

	Genotype			Total
	BB	Bb	Bb	
Individual Number	1	42	23	66
Number of B Allele	0	42	46	88
Number of b Allele	2	42	0	44
Allele Total Number	2	84	46	132

B Allele frequency in population = $44/132 = 0.333$
 b Allele frequency in population = $88/132 = 0.667$

PCR result with primers was conducted to obtain polymorphisms alkaline target G825T VDR gene, as shown in Table 3. Electrophoresis description of PCR product in the form of DNA band is 825 pb. Afterwards, the PCR product was digested with *BsmI* restriction enzyme. If it is cut, electrophoresis description

shows that the DNA band are 650 pb and 175 pb, which explains the 825T allele existences. If it is not cut, it only shows the PCR product of 825 pb, which explains the 825G allele existences (normal) (Table 4).

Table 4: Frequency of BB, Bb and bb Allele in 825 VDR Genotype in Control Group

	Genotype			Total
	BB	Bb	Bb	
Individual Number	0	6	9	15
Number of B Allele	0	6	18	24
Number of b Allele	0	6	0	6
Allele Total Number	0	12	18	30

B allele frequency in population = $6/30 = 0.2$
 b allele frequency in population = $24/30 = 0.8$
 Frequency of b allele (mutant) is 24 and G allele (normal) is 6.

The number of Bb genotype in this research is the total of B allele and b allele. Therefore, the total number is two times from every allele. Homozygous 825 VDR genotype is bb

genotype, with no B allele. On the contrary, there is no b allele with BB genotype.

Table 5: G825T VDR Genotype Frequency in Beta Thalassemia Major Case

Compared with Control Case

Genotype (%)	Case n=66	Control n=15	χ^2	P value	Odds ratio	CI 95%
Bb	23 (35)	9 (60)	1.682	0.046	2.63	(0.73-9.64)
bb/BB	42 (65)	6 (40)				

Note:*)=based on Fisher's Exact test

Examination result on allele frequency was retrieved, and then it was continually being examined for its genotype frequency. Statistics in genotype did not show significant differences between genotype frequency in patient group (23BB, 42 Bb, 1bb) and control group (9BB, 6 Bb, 0bb).

There is no BB genotype examination in control group. Research with larger control sample could be the reason to ensure this condition. However, because there is only 1 case (1.5%) in BTM, there is no significant differences occurred (Table 5).

Table 6: Individual Number with 825GT Genotype Allele *TGF B1* in Beta Thalassemia

Major and Control Case

Allele (%)	Case	Control	χ^2	p value	Odds ratio	CI 95%
B	44	6	2.04	0.13	2	(0.71-5.92)
B	88	24				

Note: *) =based on Fisher's Exact test

Table 6 shows individual number with B and b allele in 825 VDR genotype. B allele is not only found in 6 control subjects (40%), but it is also found in beta thalassemia major case, with 43 subjects (65.1%).

There are 65 subjects (98.4%) of b allele in beta thalassemia mayor case and 15 subjects (100%) in control group. B allele is higher in BTM group, whereas b allele is higher in control group. There is no significant difference between BTM and control groups.

DISCUSSION

Table 1 shows a distribution based on sex and age group in patient and control group. The data result shows differences with significant result. BTM in outpatient clinic has various ages. The sampling collection method is consecutive. Consequently, all existence and qualified sample participate in this research.

ROC curve result of vitamin D level shows that the cut point is in 56.1 pmol/L value (Table 1). The normal value boundary of vitamin D level for BTM is in that value. As an active metabolism, vitamin D is an important regulator for calcium homeostasis and has an important role in growth process. BTM patients will have hormone disorder, and as a result, synthesis process of vitamin D will be disturbed. Additionally, there are more BTM patients with deficiency of vitamin D level compared with control group.

The result of vitamin D cut point in BTM explains that 53 subjects level is below the cut point. Transformation of provitamin D into vitamin D is processed in the skin. Consequently, the skin color has a strong influence to vitamin D production where melanin pigment is a good keeper from sunshine and to reduce vitamin D photosynthesis process. Vitamin D deficiency in BTM is perhaps caused by the activity of hormonal imbalance.^{24,25}

This research found that more Bb genotype heterozygote appears in beta thalassemia mayor. This finding confirms the previous statement that osteoblastogenesis process is strongly related to the transformation of provitamin D into vitamin D with the help of sunshine.²⁶ Hormone disorder in BTM will influence this process. Vitamin D acts as anabolic hormone in the bone by direct effect on osteoblasts. The effect result strongly depends on osteoblasts cell maturation degree. Osteoblasts differentiates in a series from mesenchymal cell, osteoblast precursors which are known as progenitor cell and preosteoblast, and osteoblasts an early stadium, vitamin D will stimulate osteoblast differentiation

of progenitor cell, and will then be followed by osteoblast proliferation stimulation. The next stadium will become a modulator production of extra matrix.²⁷

Another finding shows that BB homozygous genotype appears in 23 patients. This result is relevant with the statement that the gene in vitamin D expression part is Vitamin D Receptor (VDR) gene. VDR gene is a ligand activated transcription factor located in monocyte cell and active T and B lymphocyte. The activity and effect of VDR gene determine the vitamin D level. VDR gene polymorphism can influence VDR gene activity and is an important factor in the transformation of provitamin D into vitamin D disorder. Some polymorphisms of VDR gene that have been identified are *FokI* in exons II, *TaqI* in exons IX, and *BsmI* and *ApaI* that are located in intron and exons VII and IX. The transformation of VDR gene mRNA expression is identified by genotype variance from VDR gene.²⁸

There are more BTM in ROC curve for calcium level above the cut point, and all of control group is above the cut point (8.5 mg/dL) (Table 4). This result is coherent with the calcium absorption theory which is highly influenced by vitamin D. More activity in vitamin D synthesis causes more calcium level in the blood.

Calcium metabolism process is greatly influenced by parathormone hormone. A minimum quantity in hormone production is caused by parathyroid glands. In relevance to **Rund's viewpoint, minimum hormone is probably one of main factors of bone and tooth growth inhibitors**, as most of structure formations depend on hormone activity. Parathyroid glands disorders in BTM children are correlated with child growth and development. This theory is consistent with the statement that healthy bone formation is supported with diet with sufficient protein, calcium, phosphate, vitamin C and D, and it also depends on sufficient thyroid hormone supply. Parathormone production from parathyroid gland influences the rate of bone dynamic. If parathyroid gland insufficiency occurs in the period of tooth formation, there will be enamel hypoplasia, malformation of root canal, and incomplete bone and tooth formation.^{25,29}

The prevalence of every vitamin D receptor gene polymorphism in every race would have differences. The reason is unknown. Moreover, population based on specific phenotype has different prevalence, as shown in *BsmI* Genotype distribution on Table 6.

B allele in BTM has enough sufficient number. B allele has the function in osteogenesis process. The osteogenesis is also regulated in chromosome 12 with specific molecule structure and has an affinity bond with vitamin D as ligand inside the osteoblasts cell core. The structure formed by amino acid cysteine located in one series of amino acid that forms tetrahedral complex with zinc (Zn) by producing loop is known as finger amino acid with Zn complex on the foundation. Zn finger proximal provides specificity in VDRE, while the second Zn finger regulates the binding of $1\alpha 25(\text{OH})_2\text{D}_3$.^{28,30} One of the controversial aspects in human VDR is human VDR polymorphism and the relationship with the bone mineral density (BMD), pathophysiology, osteoporosis, hyperparathyroidism, breast cancer, and prostate cancer. Morrison firstly reported that, VDR allele may be used to predict BMD by evaluating BsmI border in intron that is separated by exons VIII and IX that correlate with lumbar spine. On the contrary, there is no BsmI in VDR, correlated with the lower BMD. Morrison's research in Australia twin population of Irish gene found that the VDR genotype number is up to 75% genetic component of BMD, although the latest report underwent some revision.^{26,31}

CONCLUSION

This study found that vitamin D level is significantly lower in BTM, whereas calcium level is significantly higher in BTM. Also, mutant genotype BB and allele B is significantly found in BTM.

AUTHOR CONTRIBUTION

Eriska Riyanti as a researcher and author for writing manuscript, Ani Melani Maskoen as a researcher and author for writing manuscript, Roosje Rosita Oewen a researcher and author for writing manuscript, N. B. Pratidina a researcher and fourth author for writing manuscript, Harun Achmad a researcher, author for writing manuscript and as a correspondence author, Yunita Feby Ramadhany as a author for this manuscript.

CONFLICT OF INTEREST

We have no conflict of interest to declare.

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