

ASSOCIATION OF EXON 2 VITAMIN D RECEPTOR (*FokI*) GENE POLYMORPHISM AMONG THALASSEMIC PATIENTS IN KARACHI

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ABSTRACT

The vitamin D receptor (VDR) gene serves as a candidate gene for susceptibility to several diseases. The gene has a critical role in the modulation of bone mineral density in thalassemia patients. VDR gene polymorphisms are reported to be associated with an increased risk of thalassemia associated bone mineral density complications. The aim of this study was to assess the distribution of hVDR *FokI* polymorphism among thalassemia patient in Karachi. Thirty nine (39) thalassemia patients and 78 healthy controls were recruited for this study. Genomic DNA was extracted using kit method followed by amplification with VDR *FokI* specific primer and subjected to Restriction fragment length polymorphism (RFLP). Genotype distribution was assessed through Hardy Weinberg equilibrium while genotype association and risk was evaluated by Pearson chi square and odds ratio with 95% CI respectively. Our results showed that the genotype distribution of *FokI* VDR polymorphism was in HWE ($\chi^2=0.392$, $P> 0.05$) along with a non-significant association between cases and controls ($X^2=0.484$, $P> 0.05$). However, FF genotype and F allele seems to have a significantly greater risk of developing thalassemia associated bone mineral density complications.

Keywords: Thalassemia, Vitamin D receptor gene (VDR), *FokI* polymorphism,

INTRODUCTION

Thalassemia are the heterogeneous group of inherited disorders in α and β globin genes of haemoglobin (Hb) which results in life-threatening anemia and requires regular blood transfusion for survival. To date alpha and beta-thalassemias are the most common inherited known single-gene disorders (Kremastinos *et al.*, 2007) with an estimate of 5000-9000 children born every year with β -thalassemia and a carrier rate of 5-7% (9.8 million) in total population. Etiologically thalassemia is multifactorial disease but genetics play an important in its pathogenicity. In Pakistan the most common globin gene mutations that have been identified in Thalassemia patients includes: IVS 1-5, Fr 8-9, IVS 1-1, Cd-30, CT and Del 619bp (Ansari *et al.*, 2011) these mutations have also been reported in other populations around the world (Saleh-Gohari and Bazrafshani, 2010; Bashyam *et al.*, 2004). Along with these globin gene mutations some other genetic modifiers such as XmnI (Winichagoon *et al.*, 2000), *COLIA 1*, *COLIA2*, *OesR* & VDR gene polymorphisms have also been reported (Thein, 2004) which play an important role in the disease prognosis. Bone thickness and growth is under the polygenic control of *COLIA 1*, *COLIA 2*, vitamin D receptor (VDR) and oestrogen receptor (*OesR*) genes (Perrotta *et al.*, 2000). Vitamin D deficiency is more prominent in thalassemia patients and most of the recent researches relate it with iron accumulation in myocardial patients (Dimitriadou *et al.*, 2011).

Vitamin D mediates its action upon binding to classical nuclear receptor called vitamin D receptor (VDR) which is a ligand activated transcription factor (Mizwicki and Norman, 2009; Awad *et al.*, 2012) that is widely distributed throughout the body and mediates its genomic and non-genomic actions (Lehmann and Meurer, 2010; Norman, 2006). Until now around 200 different types of single nucleotide gene polymorphisms have also been identified so far among which *FokI*, *Apal*, *BsmI* and *TaqI* are most commonly studied and are considered as a self-marker in various disorders (Swapna *et al.*, 2011; Yan *et al.*, 2005). VDR *FokI* is a thymine/cytosine (T/C) polymorphism located in the first of two potential start (ATG) codons on VDR that leads to different translational initiation sites resulting in long (FF) and short (ff) variants of VDR. The ff genotype is more susceptible to pathogenesis (Mail *et al.*, 2007; Bid *et al.*, 2005) as compare to FF or Ff whereas some studies reveal that Ff or FF genotypes are associated with severe cancer progression than ff genotype (Xu *et al.*, 2003). A study has been done on North Indian population which showed a significant association of *FokI* and *Bsm I* polymorphism with BMD of lumbar spine in Thalassemia patients (Singh *et al.*, 2012). A higher incidence of FF genotype was also be detected along with vitamin D deficiency ($P=0.03$) in patients with β thalassemia major in Greece population (Dimitriadou *et al.*, 2011).

To date there is no data present in Pakistan that shows association between VDR polymorphism and Thalassemia mutations. Therefore, we attempt to investigate the association of *Fok1* VDR gene polymorphism at 5' region of exon 2 in patients of Thalassemia.

MATERIALS AND METHOD

Subjects: Present investigation is a retrospective cohort case control study being carried out on thalassemia subjects. They were recruited during the period of January 2012 to December 2012 from a tertiary hospital located in Karachi. Thirty nine (39) clinically diagnosed thalassemia patients (mean age = 9.64 ± 6.62 yrs) and 78 healthy subjects (mean age = 22 ± 1.9) both males and females were recruited for the present investigation in a ratio of 1:2 (case: control). Individuals diagnosed with any other kind of hemoglobinopathy, anemia, infections, liver and renal abnormalities were excluded. Peripheral blood was drawn from antecubital vein of subjects after obtaining informed consent and structured questionnaire. Complete blood profile of the patients was also carried out using COULTER® Ac·T diff™ Analyzer, USA.

DNA extraction: Genomic DNA was extracted from peripheral blood using a commercial kit method (Gene JET Genomic DNA Purification Kit, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania). In brief, 200µl of whole blood was mixed with 400µl of lysis solution and 20µl of Proteinase K solution. The sample was incubated at 56°C for 10 minutes with occasional vortexing. Later 200µl of 96% ethanol was added and whole lysate applied to DNA purification column and centrifuged at 6000 x g for 1min. The column was washed with 500 µl of wash buffer I and II with centrifugation at 8000 x g and 12000 x g for 1 and 3 minutes respectively. Later 200 µl of elution buffer was applied to the column and genomic DNA eluted in 1.5 ml sterile micro centrifuge tube and stored at -86°C for future investigation.

Amplification of genome: Amplification of *Fok1* VDR gene was carried out using VDR gene specific primers F5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' and R5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3' (Morrison *et al.*, 1994). PCR was carried out using Kapa Taq DNA polymerase master mix (Kapa Biosystem, USA) as per manufacturer instruction. In brief, a reaction volume of 20µl containing 10µl 2x Kapa Taq DNA polymerase master mix, 25 nm forward and reverse primers and 5µl of genomic DNA were subjected to thermal cycling profile (Initial denaturation for 2 mins. at 95°C, 30 cycles for 30sec at 95°C, 58°C for 30sec, 68°C for 30sec and 1 cycle of final extension for 7min at 68°C) on an automated thermal cycler (Veriti, Applied Biosystem, USA). The PCR products were electrophoresed on 1.5% agarose gel (0.5µg/ml ethidium bromide) and visualized using gel documentation system (GelDoc-It² imager, UK).

RFLP analysis: RFLP of the amplified samples (265 bp) was performed in a reaction volume of 20 µl (1µl *FokI* endonuclease, 5µl PCR products, 2µl buffer and 2µl water) at 37°C for 10 mins followed by gel electrophoresis (2% agarose gel, 0.5µg/ml ethidium bromide) and visualized using Vision works LS software on ChemiDoc-It² imager.

Statistical analysis: Hardy-Weinberg equilibrium and Pearson Chi square tests were used to compare the genetic distribution between cases and controls. Crude odd ratios and 95% confidence interval were also calculated to estimate the risk. All calculations were done at significance level 0.05. Other variables were articulated as means and percentages.

RESULTS

During the course of study, a sample of 39 cases and 16 controls with or without having gene mutations was selected. It was noted that 20.51% Punjabi, 25.64% Sindhi, 23.08% Balochi, 17.94% Urdu speaking and 5.13% remaining have thalassemia mainly due to following custom traditional marriages and having BRADARI system. It was also observed that BTM (41% in males, 28% in females) and BTI (8% in males, 10% in females) were found to be the most common type among patients in Karachi. Upon comparison no major differences have been observed among male and female patients of thalassemia on the basis types of thalassemia as mentioned in **Fig. 1**.

Peripheral blood was taken in EDTA tubes to check the blood profile of patients which showed decrease blood hemoglobin levels, low Hematocrit and high total leukocyte counts (**Fig. 2**).

DNA of all 117 subjects was extracted using kit method and extracted DNA was amplified using PCR specific for *Fok1* VDR gene resulted in 265 bp product (**Fig. 3**).

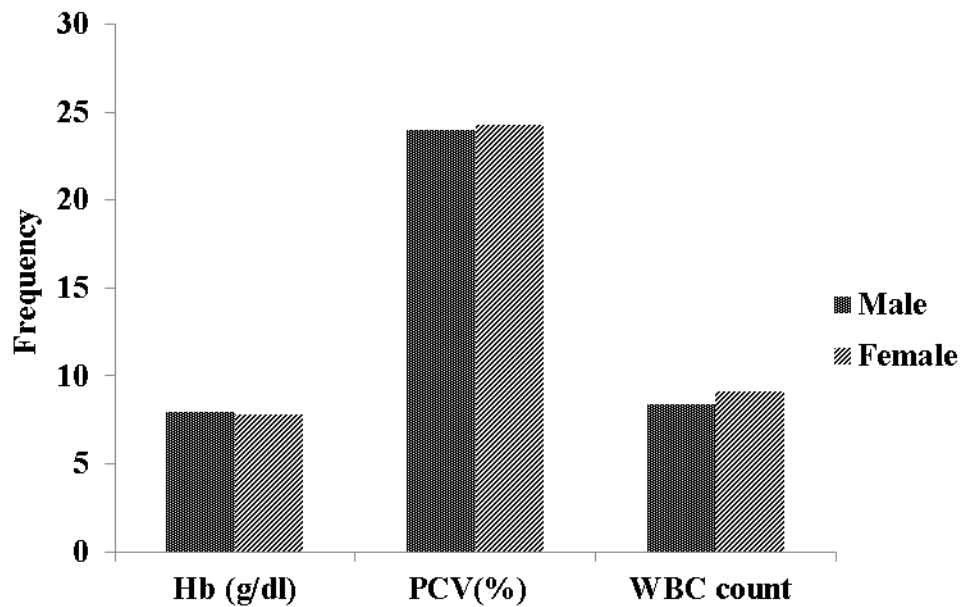


Fig 1. Distribution of Thalassemia types in male and female subjects. Hb=Hemoglobin, PCV=Packed cell volume, WBC=white blood cells

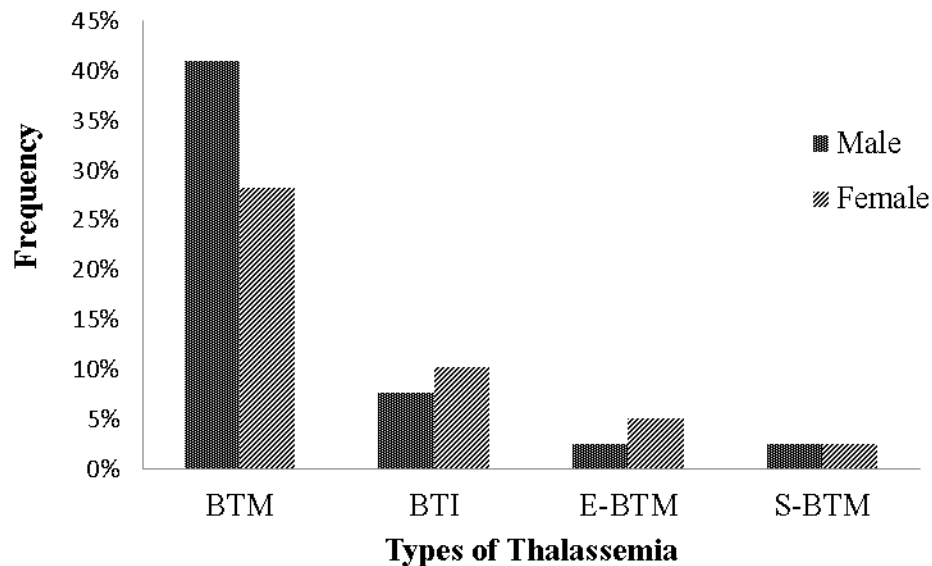


Fig 2. Blood profile of Thalassemic patients.

BTM = Beta Thalassemia major, BTI = Beta Thalassemia intermedia, E-BTM = Hemoglobin E Beta Thalassemia major, S-BTM = Hemoglobin S Beta Thalassemia major

RFLP analysis of PCR product after digestion with *FokI* restriction endonuclease showed one type of homozygous dominant undigested product FF (265 bp) and two types of digested products heterozygous Ff and homozygous recessive ff (265,196, 69 and 196, 69) as shown in **Fig. 4**.

The genotype distribution in control group was in HWE ($p > 0.05$) for the VDR-*FokI* polymorphism (Table 1).

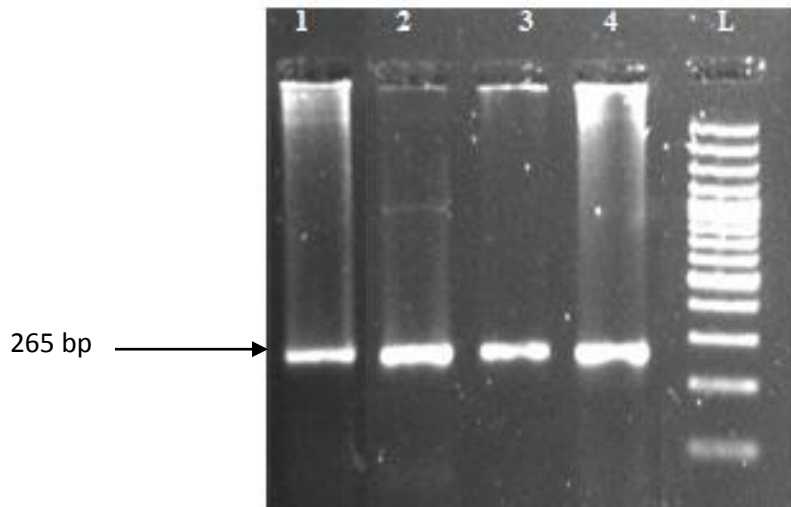


Fig 3. Gel electrophoresis of 265 bp fragment from Vitamin D receptor gene.
Key: Lane L: Marker (100 bp, Fermentas, Germany), Lane 1- 4: PCR products

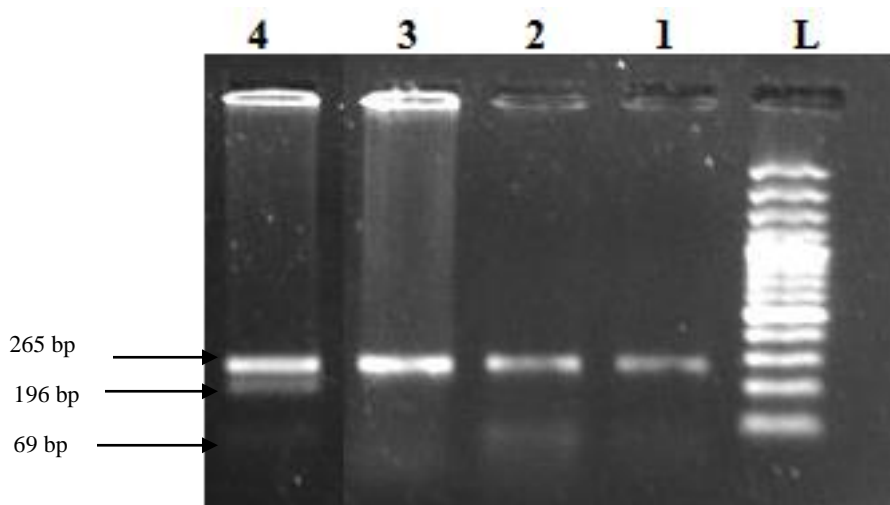


Fig 4. RFLP of VDR gene treated with FokI Restriction endonuclease.
Key: Lane L: Marker (100 bp, Fermentas, Germany)
Lane1-4: PCR-RFLP products digested with *FokI* restriction enzyme

Table 1. Genotype distribution in Hardy Weinberg Equilibrium.

Genotype	Observed Value	Predictive Value	HWE (X^2)	P-value
FF	56	56.70	0.392	0.531
Ff	21	19.61		
ff	1	1.70		
Total	78	78.00		

Our results showed that no significant association ($P > 0.05$, $X^2=0.484$) between VDR *FokI* gene polymorphism and thalassemia in cases and controls. However, a tendency exists toward FF (T/C) genotype (O.R=1.273, 95% C.I=0.556-2.914) showed no significant association at 0.05 alpha level (Table 2).

Table 2. Association of VDR gene (*FokI*) polymorphism in Thalassemia patients.

Genotype	Cases n	Control n	Pearson X ²	P- value	Crude Odds ratio	(95% C.I)	Chi X ²	P-value
FF	26	56	0.484	0.785	1.273	0.556-2.914	0.326	0.586
Ff	12	21			0.829	0.356-1.928	0.19	0.663
ff	1	1			0.494	0.030-8.107	0.254	0.614
Total	39	78						

It was also noted that individuals with F allele (O.R= 1.265, 95% C.I=0.611-2.620) may be more prone towards developing bone mineral density associated complications as compare to f allele.

Table 3. Allelic Probabilities of cases and controls.

Alleles	Allelic Frequencies		Allelic Probabilities		Crude Odds ratio	(95% C.I)	Chi X ²	P-value
	Cases (n)	Control (n)	Cases	Control				
F	64	133	0.821	0.853	1.265	0.611-2.620	0.401	0.526
f	14	23	0.179	0.147	0.791	0.382-1.638		

It was evaluated that homozygous FF genotype was found most common with frequency 38.46% in male and 30.77 % in female patients while heterozygous Ff genotype was found 15.28% in male and 15.38% in female patients with varied HSD i.e. (1.33 ± 0.20) found higher among heterozygous as compared to homozygous (1.12 ± 0.24) Whereas ethnically Punjabis (12.82 %) are found more susceptible towards altered allele with Ff while Urdu (7.69%), Sindhi (7.69 %) and Pashto (2.56 %) showed little susceptibility towards it. The blood profile illustrates that Hb conc. and PCV are slightly higher among heterozygous Ff genotype as compared to homozygous dominant FF genotype while WBC count are higher in dominant FF gene.

It has been observed that Ff genotype is common among subjects having Fr type genetic mutations including (Fr 8-9, Fr 16, Fr 41-42) whereas other types of globin chain mutation in thalassemia patients and its correlation with VDR gene polymorphism is demonstrated in Table 4.

Table 4. Correlation between VDR genotype, genetic mutations and other studied polymorphisms in patients of thalassemia.

Studied parameters	Mean \pm SD or %	VDR genotype		
		FF (n=26)	Ff (n=12)	ff (n=1)
Age (years)	9.64 \pm 6.62	7.81 \pm 5.62	13.8 \pm 7.1	1
Genetic mutations				
IVSI (-1 & -5)	13 (33.33%)	9 (23.08%)	4 (10.26%)	0%
Fr(8-9, 16, 41-42)	11 (28.21%)	10 (25.64%)	1(2.56%)	0%
Cd (-5, -15 & -30)	6 (15.38%)	3 (7.69%)	3 (7.69%)	0%
Cap ⁺¹	2 (5.13%)	0 (0%)	2 (5.13%)	0%
Others	2(5.13%)	2(5.13%)	0(0%)	0(0%)
No mutation	12 (28.21%)	7 (17.95%)	4 (10.26%)	1(2.5%)
XmnI polymorphism				
Homozygous dominant(+/+)	6 (15.38%)	3 (7.69%)	3 (7.69%)	0%
Homozygous recessive (-/-)	12 (30.77%)	8 (20.51%)	4 (10.26%)	0%
Unidentified	21 (53.85%)	16 (41.03%)	5 (12.82%)	0%

Data is expressed in terms of means \pm SD or frequency %.

DISCUSSION

Thalassemia is a complex familial and genetic hemoglobinopathy and is defined as a defect in globin chain synthesis of hemoglobin which is characterized by severe anemia. Apart from global distribution the most dominant type of thalassemia occurring in *Karachiites* is beta thalassemia major (BTM) as shown in **Fig. 1**. It is a homozygous blood disorder resulting in severe transfusion dependent hemolytic anemia. The main cause of high rate of BTM is non-screening prior to marriages in the world which usually results in 25% probability of the progeny having BTM. It was noted that the patients with BTM has low PCV, high WBC's and low hemoglobin levels (**Fig. 2**) which is in accordance with the previous studies. We carried out a study on vitamin D receptor (*FokI*) genetic profile in young adults having thalassaemia with the hope of gaining new insight into establishing risk predictors of the disease.

Osteoporosis is the most common conditions arise from beta thalassemia (Angelopoulos *et al.*, 2007) whereas infectious complications and immune abnormalities are also considered to be second most common cause, which are the major reason of morbidity (Farmakis *et al.*, 2003). Thalassaemic osteopathy is a multifactorial disorder and about 80% of alteration in bone mineral density (BMD) is determined by the effects of many genes (Carbonell *et al.*, 2005, Stewart and Ralston, 2000) including the vitamin D receptor (VDR) and COL1A1 genes which are auspicious genetic determinants of bone mass (Giguère and Rousseau, 2000). The association of Vitamin D receptor gene polymorphisms with thalassaemic osteopathy is highly debatable. The VDR gene locus has been located at the short arm of chromosome 12q on the region 13–14 and composed of about 9 exons. It has been documented that the polymorphisms in VDR gene start codon and 3' end region may play a role in modulation of BMD (Tektite *et al.*, 2002).

Present investigation is the first of its kind in Karachi population in order to determine VDR *FokI* gene polymorphism in thalassemia. Our investigation revealed that no significant association ($P > 0.05$, $X^2=0.484$) between cases and controls. According to our results VDR *FokI* gene was in equilibrium by Hardy Weinberg principle (chi square = 0.392, P-value > 0.05). Similar study was done on Egyptian prepubertal thalassaemic individuals that suggested VDR gene polymorphisms of *FokI* and *BsmI* polymorphisms may be important determinants of bone mass in prepubertal males having thalassemsias (Tantawy *et al.*, 2010).

The frequency of gene distribution of homozygous dominant (FF), heterozygous (Ff) and homozygous recessive (ff) was 66.66%, 30.7% and 2.5% in cases and 71.79%, 26.92% and 1.28% in controls respectively. However it was observed that a risk tendency exists toward FF genotype (O.R=1.273, 95% C.I=0.556-2.914). Results demonstrated that subjects carrying F allele (O.R= 1.265, 95% C.I=0.611-2.620) may be more prone towards BMD associated complications with thalassemia as compare to f allele. Previously a study has also been done on Greece population to establish the association of *FokI* polymorphism in relation with renal dysfunction in thalassemia patients which reported that the frequencies of genotype distribution were found about 47.06% FF, 41.18% Ff, and 11.76% ff genotype. This previous study also revealed that f allele is more prone to have impaired renal function in patients with thalassemia with increased cystatin C levels in serum (Dimitriadou *et al.*, 2011).

VDR *FokI* receptor gene polymorphism has been related with various diseases such as cancers, urolithiasis, inflammatory bowel disease and osteoporosis. It was documented that FF genotype was prevalent in Finish, Australians and Black Pennsylvanias whereas ff genotype was significantly different in all populations except North Indians and Black Pennsylvanias (Bid and Mittal, 2003). Swapna *et al.*, (2011) demonstrated the association of VDR *FokI* polymorphism on Hypertension and showed that the genotype distribution and allele frequencies of *FokI* (T/C) VDR polymorphism were highly significant between cases and controls. FF genotype and allele F were at risk for developing hypertension in both sexes with positive family history and habit of smoking.

Limitations of this investigation were small sample size and estimation of BMD in Thalassemia and polymorphisms in COL1A1 genes in cases and controls. Present investigation strongly recommends that a more extensive study must be carried out to determine the role of VDR polymorphisms in thalassemia patients along with serum vitamin D levels.

Conclusion

It may be concluded that F allele carrier are at risk of developing thalassemia associated complications.

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