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Exon 2 Vitamin D Receptor (Fok-I) Gene Polymorphism and the Evaluation of its Correlation With Renal Dysfunction in Patients With β-Thalassemia Major in East Azerbaijan Province of Iran

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Abstract

Objectives: Several studies have shown that major beta-thalassemia patients suffer from renal dysfunction. Genetic is one of the crucial factors in this phenomenon. Accordingly, this study aimed to evaluate the correlation between renal dysfunction and Fok-I polymorphism in the vitamin D receptor (VDR) gene of major beta-thalassemia patients.

Materials and Methods: Sixty thalassemic patients and sixty healthy individuals were involved in this case-control study. Robust renal and urine analyzes were done in terms of performance evaluation. Finally, genotype assessment for Fok-I polymorphism was performed via the polymerase chain reaction-restriction fragment length polymorphism method.

Results: In general, renal dysfunction embracing proteinuria and hyperfilteration were observed in the thalassemic group. As regards patients' genotype frequencies, 51.6%, 36.6%, and 11.6% were homozygous for F allele (FF), heterozygous (Ff), and homozygous for f allele (ff), respectively. Eventually, the frequencies of FF and Ff alleles were 49.1% and 50.8%, respectively, in normal group I.

Conclusions: Our data suggested that there is no correlation between renal dysfunction and Fok-I polymorphism in major beta-thalassemic patients. Thus, further studies are needed about plausible pathways involved in renal dysfunction, to demonstrate the motives of renal dysfunction in major beta-thalassemia patients.

Keywords: Vitamin D receptor, Fok-I polymorphism, Thalassemia, Renal insufficiency

Introduction

It is approved that thalassemia is one of the most prominent single-gene abnormalities. This disorder leads to a kind of microcytic-hypochromic anemia which is originated from the inherited impairment of hemoglobin synthesis and thus lowers the erythrocyte life span (1). In addition, beta-thalassemia is more common than alpha-thalassemia in Iran (2). Currently, the quality of life for thalassemic patients is increasingly improved with the use of newborn screening, regular blood transfusion, and effective treatment. However, there are a quite number of difficulties (2,3), one of which is the iron overload that is the most fundamental issue (4). Renal dysfunctions associated with beta-thalassemia major was first reported in 1975 (5). Several renal abnormalities in beta-thalassemic patients were illuminated comprising the up-regulation of the stream of renal plasma, urine concentration impairment, renal tubular acidosis, and tubulopathies (3,4,6). Many studies have approved renal disorders in beta-thalassemia although the exact mechanisms of this phenomenon are not clarified completely (7). Vitamin D in active species with parathyroid hormone rises the reabsorption of calcium (Ca) from the kidney. Previous research on thalassemia patients showed that vitamin D deficiency occurred in these patients (8). Most of the physiological actions of vitamin D is done via connecting to specific intracellular receptors. Further, the occurrence of single nucleotide polymorphism) in vitamin D receptor (VDR) could potentially modify the transcriptional activities of the target genes (9,10). Furthermore, VDR polymorphisms can occur in the 5' promoter, 3' untranslated region, and exonic sequences. The restriction fragment length polymorphism (RFLP) method, which is done on the VDR gene, has shown a variety of

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polymorphisms including Tru91, TaqI, BsmI, EcoRV, ApaI between exon 8 and 9, as well as Cdx2 in the promoter region and Fok-I in exon 2 (10). Fok-I polymorphism is one of the most common polymorphisms in VDR that is associated with two allelic variants including F and f. f allele leads to the generation of the longer variant of the VDR protein which is less functional compared to the F variant (11). One of the target genes of vitamin D is located in the epithelial Ca channel in the proximal intestine and distal nephrons (12). Previous studies have represented that Fok-I polymorphism sensitizes individuals to renal abnormalities (13-15). Considering the above-mentioned explanations, this study assessed the plausible relevance of renal performance with the existence of Fok-I polymorphism in beta-thalassemic patients and the control group.

Materials and Methods

Patient Recruitment and Design

Sixty patients (38 males and 22 females) with major beta-thalassemia were enrolled in this study after referring to the Children Hospital of Tabriz and 60 healthy individuals were also selected as a control group. First, the researcher thoroughly described the aim of the study to the patients and the healthy group who were involved in our scrutiny, and then they were asked to read the informed consent letter and sign it if they were willing to participate in the study. It should be mentioned that most of the involved patients were in a comparable stage of the disease. The demographic and clinical information including age (years), gender (n%), weight (kg), height (cm), diagnosis age, blood pressure (mm Hg), body temperature (˚C), and the history of splenectomy, renal, and heart failure, and vitamin D (tab per day) consumption was collected via interviewing and reviewing the hospital reports. Patients were excluded from the study if they exhibited characteristics such as iron deficiency, minor beta-thalassemia, minor beta-thalassemia combined with iron deficiency, and intermedia beta-thalassemia. Moreover, other exclusion criteria included other hemoglobinopathies, recurrent urinary tract infection, ongoing treatment with nephrotoxic drugs, and other major anomalies.

DNA Extraction and Genotyping

In this regard, 2 mL of peripheral blood were obtained in the ethylenediamine tetraacetic acid by means of a vacutainer blood collection tube and then stored at 4°C for further steps. Additionally, genomic DNA was extracted via the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacture's instruction. The primers conducted for Fok-I polymorphism extension were as follows:

Forward: 5AGCTGGCCCTGGCACTGACTCTGGCTCT,
Reverse: 5ATGGAAACACCTT GCTTCTTCTCCCTC (16).

In addition, the polymerase chain reaction (PCR) consisted of first denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62.5°C for 30 seconds, and extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. SensoQuest PCR thermal cycler (Sensquest, German, SensoQuest GmbH) was used For performing PCR. Furthermore, the Fok-I restriction enzyme (Fermentas & Biolab) was used for digesting amplification products, and the generated fragments for F allele were 272 bp and 198 bp and that of the f allele was 74 bp. Finally, these products were resolved in a 2% agarose gel and then stained with safe stain (Cinagene, Iran) and visualized by a UV light (Biometra, German).

Statistical Analysis

The outcomes were presented as the mean ± standard deviation. The Statistical Package for Social Sciences, version 16 was used for statistical analyses. The *t* test and the chi-square test were selected to compare quantitative and qualitative variables, respectively. Moreover, Kruskal-Wallis and ANOVA tests were used to compare the variables among the genotypes. A *P* value of less than 0.05 was considered statistically significant.

Results

Overall, 60 patients including 38 males (63.3) and 22 females (36.7) with major beta-thalassemia, as well as sixty healthy people as the control group encompassing 25 males (41.7%) and 35 females (58.3%) were enrolled in this study. Table 1 presents the evaluated demographic and anthropometric parameters including age (year), weight (kg), height (cm), body mass index (kg/m²), blood pressure (mm Hg), and body temperature (˚C). Patients were within the age range of 3 to 34 years old and the average age of the patient group was 16 years old (median =17). Additionally, the mean period of disease diagnosis age was 20 months and the mean of blood pressure was 100/65 mm Hg. Similarly, none of the patients had a history of taking renal function or vitamin D status interfering medications. On the other hand, seven patients had splenectomy and 5 of them had a family history of heart and liver diseases. In terms of vitamin D supplementation, 33 patients had vitamin D intake (1 tab/day: 200 mg/d). Table 2 summarizes data related to calciuria, proteinuria, and glomerular filtration rate (GFR), namely, modification of diet in renal disease (MDRD) and Cockcroft-Gault equation (CG), which were extracted from renal damages in patients with major-β-thalassemia. PCR and RFLP techniques were carried out for all patients (Figures 1 and 2). Based on the results, 31 (51.6%) and 22 (36.6%) patients showed FF and Ff genotypes, respectively, and only 7 (11.6%) patients had ff genotype. When patients were classified according to the Fok-I genotype, no statistical significance was observed regarding calciuria (*P* value: FF-Ff 0.6, FF-ff 0.9, and Ff-

Table 1. The Weight, Height, and BMI Values Based on Gender and Age

Parameters	Patient	Control
Gender (n %)	Male 38 (63.3), female 22 (36.7)	Male 25 (41.7), female 35 (58.3)
Age (y) (lowest-highest)	16.97±7.14 (3-32)	23.3±1.05 (4-40)
Weight (kg) (lowest-highest)	44.13±15.81 (15-76)	58.75±15.48 (28-90)
Height (cm) (lowest-highest)	142.17±30.8 (50-191)	148.92±29.52 (68-181)
BMI* (kg/m²) (lowest- highest)	21.07± 2.72 (17.7-29.6)	24.7 3.15± (19.5-32.3)
Body temperature	36.7± 0.21 (36.3-37)	36.98 0.6± (36-37.8)

Note. Data are presented as the mean ± SD. *BMI: Body mass index. To prevent errors in BMI calculation, 36 patients and 20 controls (persons under 19) were omitted from the study.

Table 2. Biochemical Tests and Urine Analyses Between Patients and the Control Group

	Patient	*P*	Control
GFR (MDRD) (mL/min/1.73 m²)	154±66	0.000	101±31
GFR (CG) (mL/min/1.73 m²)	151±64	0.001	121±29
Calciuria (mg/mg)	0.67±0.07	0.8	0.69±0.05
Proteinuria (mg/mg)	0.05±0.06	0.000	0.02±0.01

Note. Data are provided as the mean ± SD. A *P* value < 0.05 was considered as statistically significant.
GFR: Glomerular filtration rate; MDRD: Modification of diet in renal disease; CG: Cockcroft-Gault equation.

ff 0.5), proteinuria (*P* value: FF-Ff 0.8, FF-ff 0.7, and Ff-ff 0.9) and GFR (*P*-value: CG [FF-Ff of 0.9, 0.7, and 0.8] and MDRD [FF-Ff of 0.8, 0.9, and 0.9]), the details of which are shown in Table 3. Based on the data in Table 4, all these parameters were normal in the control group (*P* value > 0.05). All results were retrieved from the thesis conducted in Tabriz University of Medical Sciences.

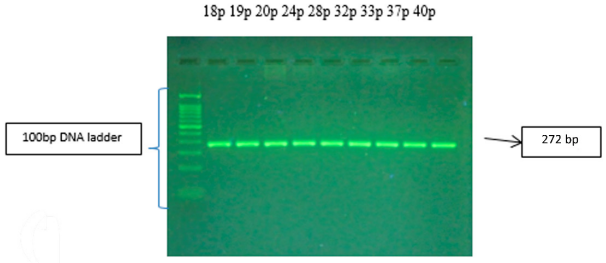

Figure 1. Polymerase Chain Reaction Products of Fok-I Polymorphism (p: patient).

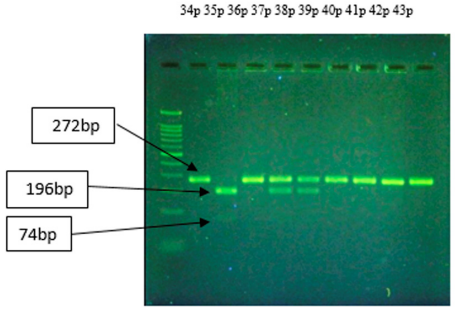

Figure 2. Restriction Fragment Length Polymorphism Products After Enzyme Digestion (patients 34-43).

Discussion

Dysfunction in the organs of the body such as heart, liver, kidney, and endocrine glands is one of the prominent side effects of blood transfusion in patients with major beta-thalassemia (4). It is approved that one of the plausible impairments in patients with beta-thalassemia is renal insufficiency and the early detection of this abnormality has precious values (3,4,6). In addition, one of the most fundamental issues in these patients is iron deposition in renal tubules. Hence, a variety of tests expands to evaluate the tubular and glomerular functions (16,17). GFR is an appropriate parameter for the evaluation of glomerular performance. The study by Mastrangelo et al as cited in Trabert et al was the first one to focus on GFR in 1975(18), which demonstrated that beta-thalassemia patients had normal GFR during the ten years of follow-up. Malaki et al (19) confirmed that hyperfilteration (GFR>130) would occur in these patients. Moreover, irregular transfusion and age raised as the cause of abnormal GFR ranges. In terms of the tubular function, Smolkin et al (20) reported that proteinuria, calciuria, and the excretion of β2 microglobulin, N acetyl beta D-glucosaminidase, and phosphate, along with the increased amount of cystatin C, creatinine, and albumin were observed in beta-thalassemic patients.

The presence of renal damages in patients with major beta-thalassemia opens new perspectives for assessing genetic factors as involved influential parameters. This study aimed to scrutinize the possibility of the correlation between the Fok-I polymorphism on the VDR gene and renal insufficiencies in major beta-thalassemia patients in East Azerbaijan province of Iran.

Fok-I is one of the most common polymorphisms of the

Table 3. Biochemical and Urine Parameters Stratified According to Fok-I Genotype in the Patient Group

Thalassemia n/Total	FF 31/60	Ff 22/60	ff 7/60	*P* Value Between Groups
GFR (MDRD) (mL/min/1.73 m²)	151±63	159±77	153±59	FF-Ff 0.9, FF-ff 0.8, Ff-ff 0.9
GFR (CG) (mL/min/1.73 m²)	148±57	157±80	141±50	FF-Ff 0.8, FF-ff 0.9, Ff-ff 0.7
Calciuria (mg/mg)	0.07±0.08	0.05±0.05	0.08±0.06	FF-Ff 0.6, FF-ff 0.9, Ff-ff 0.5
Proteinuria (mg/mg)	0.06±0.08	0.05±0.04	0.04±0.02	FF-Ff 0.8, FF-ff 0.7, Ff-ff 0.9

Note. GFR: Glomerular filtration rate; MDRD: Modification of diet in renal disease; FF: F allele; Ff: Heterozygous; CG: Cockcroft-Gault equation. A *P* value < 0.05 was considered statistically significant.

Table 4. Biochemical and Urine Parameters Stratified According to Fok-I Genotype in Healthy Control Group

Healthy Group n/Total	FF 30/60	Ff29/	*P* Value
GFR (MDRD) (mL/min/1.73 m²)	95±25	104±34	0.2
GFR (CG) (mL/min/1.73 m²)	115±23	126±32	0.1
Calciuria (mg/mg)	0.07±0.05	0.06±0.04	0.7
Proteinuria (mg/mg)	0.02±0.02	0.02±0.01	0.5

Note. GFR: Glomerular filtration rate; MDRD: Modification of diet in renal disease; GC: ; FF: F allele; Ff: Heterozygous; CG: Cockcroft-Gault equation. Data were shown as mean ± SD. A *P* value < 0.05 was considered as significant statistically.

VDR gene which leads to the down-regulated transcription of this gene in host organs (9,10). The presence of Fok-I and other VDR polymorphisms was approved in bone disease, diabetes, cancer, Alzheimer's, hypertension, breast cancer, cardio-renal syndromes, and renal disease (18,21-23). Further, Vigo Gago et al (24) showed that the F phenotype is associated with an increase in parathyroid hormone (PTH) levels. In kidney damage, 1, 25(OH) 2D3 and PTH decrease and increase, respectively. Thus, the F phenotype is a candidate as an inducible factor on the PTH increase in kidney damages. This reverse effect on vitamin D can arise from the higher sensitivity of F allele to 1, 25(OH) 2D3. In contrast to another study, the f allele was more common in patients with 1,25(OH)2D3 deficiency, but not in the decreased amount of 25(OH)D3 (11) cases. Dimitriadou et al (13) found that patients who are homozygous for the f allele (ff) had increased levels of cystatin C. Although our genotype frequency resembled that of the above-mentioned study, and renal dysfunction was observed in the patient group, no significant correlation was found between renal dysfunction and Fok-I polymorphism in the present study. It seems that β2 microglobulin can help us to justify this discrepancy by checking early factors like cystatin C. Furthermore, Dimitriadou et al reported different results about the Fok-I polymorphism (13), which contradicts the results of this study. Probably, different countries have distinguished programs such as newborn screening, regular blood transfusion, and effective treatment with iron chelators for handling thalassemia patients which could affect the results of different studies. Likewise, although our cases showed renal dysfunction, most of them showed an early-stage instead of the end-stage renal disease. Maybe they needed more time to show any correlation.

According to our data, hyperfiltration and proteinuria as the features of renal dysfunction were observed in the patient group although there was no significant relevance between Fok-I polymorphism and renal insufficiency. Hence, further studies in gene polymorphisms are needed to illuminate the possible mechanisms which are involved in the renal dysfunction in major beta-thalassemic patients. Having a better perception toward renal deficiencies could deeply lower the difficulties of thalassemia patients' routine life.

Conflict of Interests

The authors declare no conflict of interests.

Ethical Issues

This study was approved by the Ethics Committee of Tabriz University of Medical Sciences (Ethical code: #5.4.849).

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