Iran. J. Ichthyol. (Special Issue 1, 2022): 98-103

P-ISSN: 2383-1561; E-ISSN: 2383-0964
http://www.ijichthyol.org

Research Article

Correlation between vitamin D deficiency and its receptor (*FokI*-rs2228570) gene polymorphisms in anemic men

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Abstract
Vitamin D is a fat-soluble vitamin that has a unique feature as it is considered the only vitamin that is synthesized in the body. Gene polymorphism of the vitamin D receptor (*FokI*-rs2228570) gene has been proposed as the major cause of anemia. The goal of this research was to examine the link between vitamin D insufficiency and the gene polymorphism for vitamin D receptors in anemic patients. A case-control study including 120 men anemic patients without any kidney disorders have been compared with 60 healthy men as a control. One single nucleotide polymorphism (SNP) *FokI*- rs2228570 was detected by PCR and PCR-RFLP techniques. Serum vitamin D, erythropoietin levels, and some biochemical parameters were measured by ELISA. The mutant homozygous genotype ff was more frequent in anemic patients (45%) than control (15%). Also, the f allele frequency was a common allele in the patients (0.62%) with a significant decrement of vitamin D and hemoglobin levels, i.e. the presence of mutant allele represents the risk factor for developing anemia compared with genetic patterns FF and Ff. The genetic frequencies also affect vitamin D conditions as indicated by low levels in mutant patterns (Ff, ff) in which the patients suffer from severe anemia.

Keywords: Calciferol, Anemia, Vitamin D receptor, Gene polymorphism.

Citation: Mohamed, A.H.; Alkhafaji, R.S. & Al-Saadi, A.H. 2022. Correlation between vitamin D deficiency and its receptor (*FokI*-rs2228570) gene polymorphisms in anemic men. Iranian Journal of Ichthyology 9(ICAB Special issue 2022): 98-103.

Introduction

Calciferol is a fat-soluble vitamin that has a unique feature as it is considered as the only vitamin that is synthesized in the body, primarily by exposition to UV-light from the sun and then, producing 25(OH)D in the liver and finally to its vital form of 1,25-dihydroxy D by the kidney (Bischoff-Ferrari et al. 2004). It has an important role in keeping calcium and phosphorus levels as required for bone growth (Meier et al. 2004). Several studies have been proved the biological activities of vitamin D in inducing and inhibiting cell growth and proliferating, also it has an essential role in the body defense system as an anti-inflammatory factor (Tian et al. 2007). Other works have been revealed the fundamental roles of vitamin D in metabolic processing, and endothelial tissues functions (Lee 2011) and its important role in erythropoiesis and its correlation with anemia (Kersey et al. 2011).

The deficiency of this vitamin D means its low level in the blood causing several health problems, and the most important one is associated with anemia and low hemoglobin levels. There is a link between vitamin D deficiency and anemia, where the active pattern calciferol binds to vitamin D receptor (VDR) i.e. the VDR has a role in decreasing hemoglobin

levels in anemic patients (Swamy et al. 2000; Dimitriadou et al. 2011).

Anemia is a well-known health problem and WHO was defined anemia like a status by which the number of red blood cells and hemoglobin levels insufficient to meet body physiological requirements (McLean et al. 2009). There are a wide variety of reasons that stimulate the low ability of red blood cells to carry the oxygen and therefore cause anemia such as iron deficiency, inadequate globin synthesis, kidney failure, inflammation, last long bleeding, and malabsorption (Weiss & Goodnough 2005). Several investigations have been conducted on the relationship between vitamin D receptor gene polymorphisms and hemoglobin concentration in anemic individuals who do not have chronic kidney disease have been condensed (Wang et al. 2014).

The vast majority of vitamin D biological actions were carried out by binding to its high-affinity receptors. The VDR gene exists on chromosome 12(q12-14) with a size of 75kb with "11 exons", where 2 and 3 exons express for amino acids implicated in DNA bounding, while 7, 8, and 9 exons are involved in vitamin D bounding. Single nucleotide polymorphisms SNPs (*FokI*-rs2228570, *TaqI*-rs731236, *ApaI*-rs7975232, and *BsmI*-rs1544 410) are the best-known SNPs of VDR gene polymorphism (Alimirah et al. 2011). The FokI SNP includes the transition of thymine (T) to cytosine (C) in the exon 2 resulting in a novel start codon ATG being changed to ACG, resulting in a three-amino-acid shorter protein molecule with higher transcriptional activity than the original (Singh et al. 2012). The current study aimed to investigate the relationship between polymorphisms at *FokI* site of VDR gene and anemia in patients without any renal diseases.

Materials and Methods

Venous blood samples were taken using 5ml disposable syringes from 120 men patients with anemia without renal diseases and 60 healthy men as the control group. 2ml of the blood samples was deposited in EDTA tubes, gently mixed for 3min, and then separated into two parts: the first part was used for serum tests, and the second part was stored at –20ºC for later use in genetic analysis. Serum albumin, iron, ferritin, transferrin, and Hb levels were estimated for each specimen. Vitamin D and erythropoietin levels in the blood were determined using an ELISA kit.

DNA was extracted from the blood samples using the extraction kit according to the manufacturer's protocol (Geneaid, Korea). Using a Nano-droop instrument, the purity of isolated DNA was assessed. PCR-RFLP was used to identify polymorphism in the vitamin D receptor (FokI-rs2228570) gene by forward (5'-GATGCCAGCTGGCCCTGGCACTG-3') and reverse (5'-ATGGAAACACCTTGCTTCTT CTCCCTC-3') primers (Mishra et al. 2013).

In a 25µl total reaction volume, the PCR reaction mixture included 250ng/l template DNA, 400M of each dNTP, 12.5µl buffer with 1U Go Taq DNA polymerase (Promega), 10M of each primer, and 3mM $MgCl_2$. The GTC Series thermocycler (Cleaver Scientific/UK) was used to perform the amplification processes. Initial denaturation at 96ºC for 5min, followed by 40 cycles (denaturation at 94°C for 45s, primer annealing at 59.5ºC for 30 s, and template elongation at 72ºC for 10min). After PCR, the product has been digested by the FokI-restriction enzyme at 37ºC overnight. In order to detect the presence or absence of VDR-*FokI*-rs2228570 gene polymorphism, the ethidium bromide staining was used in a 3% agarose gel electrophoresis visualized using UV-trans illuminator, the size of *FokI* gene size has been detected with 100-1000bp DNA marker.

Statistical analysis: SPSS version 23 was used for the statistical analysis. Means±SD was used to depict continuous variables. To compare the means of two groups, a student t-test was performed.

Results

DNA isolated from blood samples had a molecular weight of 50-200ng/l and purity of 1.7-2.2 (Fig. 1).

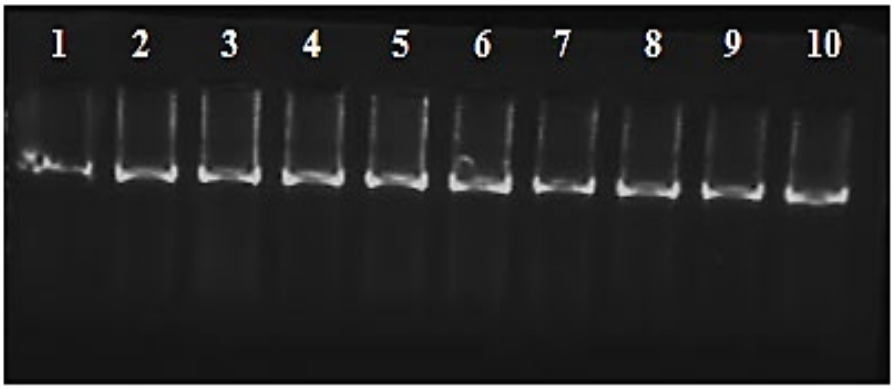

Fig.1. Electrophoresis of DNA extracted from blood of the patients and control groups. [Lane: 1-6 DNA from patients, lane: 7-10 from control; 1% agarose, 75V, 20Am for 1h].

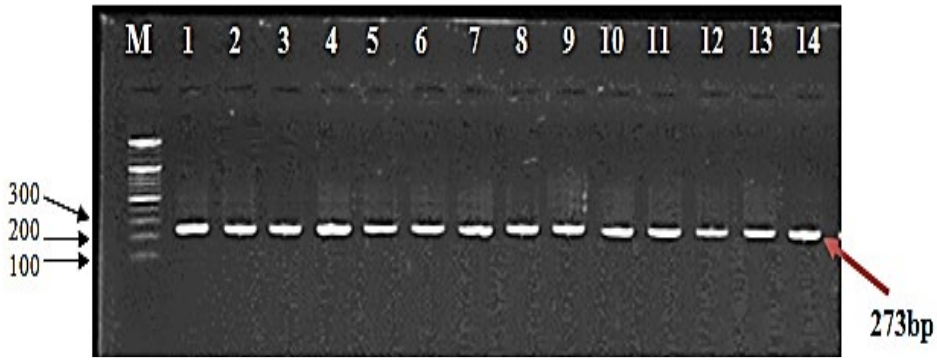

Fig.2. Electrophoresis of PCR product of *VDR-FokI*-rs2228570 gene amplification. [M: DNA marker (100bp), lane 1-7 from anemic patients, lane 8-14 from control; 1% agarose, 75V, 20mA for 1h].

Table 1. Genotypes and allele frequencies of the vitamin D receptor-*FokI*-rs2228570 allele in anemic patients and control groups.

Genotype Pattern	Genotype Frequency (%)				
	Anemic patients No:120	Control No: 60	*P*-Value	Odds Ratio	95% CI
FF	27 (23%)	40 (67%)		Reference	
Ff	39 (32%)	11 (18%)	0.03*	2.448	1.0057-4.574
ff	54 (45%)	9 (15%)	0.0001**	4.6364	2.0944-10.2637
Allele Frequency%	F (0.38)	F (0.75)			
	f (0.62)	f (0.25)			

**P*-value≤0.05, ** *P*-value≤0.001

The results of VDR-*FokI*-rs2228570 gene genotyping revealed one band of approximately 273bp for both anemia patients and control groups (Fig. 2). The results of PCR-RFLP of the *FokI*-rs2228570 gene in men with anemia and control groups using the FokI-restriction enzyme revealed that the homozygous FF pattern has one band around 273bp, the homozygous FF pattern has two bands around 75 and 198bp, and the heterozygous Ff pattern has three bands around 75, 198, and 273bp (Fig. 3). The results also revealed that the frequency of mutant homozygote pattern ff is greater in anemic patients (45%) than those of healthy (15%) and that the mutant allele frequency f was highest in the patients (0.62%) compared to the control (0.25%). This suggests that the mutant homozygote pattern was linked to anemia (Table 1).

The levels of vitamin D and hemoglobin in anemic patients were lower ($P \leq 0.01$), whereas the levels of erythropoietin were higher ($P \leq 0.01$) in anemic

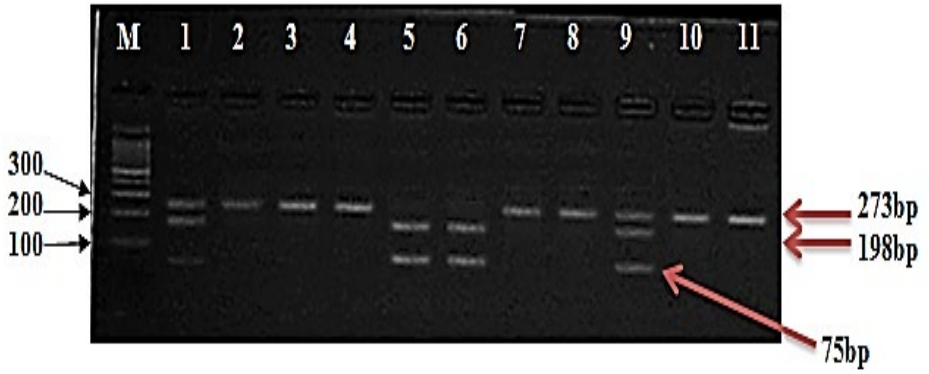

Fig.3. Electrophoresis of PCR-RFLP for PCR product (273bp) with restriction enzyme FokI, 3% agarose, 75V, 20mA for 2h. [Lane M: DNA marker100bp, Lane: 2, 3,4,7 ,8,10,11 showing homozygote type (FF) genotype, Lane:1,9 heterozygote mutant type (Ff) genotype, Lane:5,6 showing homozygote mutant type (ff)].

Table 2. Some of physiological parameters in anemic patients and control groups.

Indices	Control (Mean±S.D.)	Anemic Patients (Mean±S.D.)	P-Value
Age (Year)	47.61±7.91	48.26±8.47	0.73
Albumin (g/dl)	5.66±1.93	5.18±1.88	0.27
Serum Iron (mg/dl)	83.46±5.63	81.44±4.76	0.09
Ferritin (ng/ml)	30.23±5.12	29.86±5.49	0.76
Transferrin (ng/ml)	30.00±6.22	30.60±5.34	0.64
Vitamin D (ng/ml)	21.38±2.24	13.39±4.20	0.0001**
Erythropoietin (mu/ml)	5.97±2.65	22.57±5.11	0.0001**
Hb (mg/dl)	16.53±2.44	9.28±2.62	0.0001**

S.D.: standard deviation; * Signification at *P*-value≤0.01

patients. The results showed no significant differences in albumin, iron, ferritin, and transferrin levels in both study groups (Table 2).

Effect of *FokI*-rs2228570 gene polymorphisms: In the anemic patients, it was revealed significant differences between non-mutant wild-type-FF and mutant-type-Ff and ff. The levels of vitamin D and hemoglobin were reduced ($P \leq 0.01$) in the mutant type, whereas the levels of erythropoietin were significantly raised ($P \leq 0.01$) (Table 3).

Discussion

This study investigated the role of vitamin D (*FokI*-rs2228570) receptor gene polymorphisms in patient's men with anemia in Babylon Province, Iraq to elucidate the link between vitamin D insufficiency and genetic variation of vitamin D receptor on the development of anemia. There are assumptions that vitamin D receptor gene polymorphism could be convoluted with the progression of anemia because the vital part of vitamin D, calciferol plays an important role in erythropoiesis (Sim et al. 2010). Therefore, this study tried to examine the idea that the gene polymorphism of the FokI receptor may portend the erythropoietin and hemoglobin levels, which are needed in anemic patients (Mohsen et al. 2013).

In this study, the *FokI*-rs2228570 variations [Ff] and ff were significantly associated with low vitamin D and hemoglobin levels than wild type [FF], improving that *FokI* gene polymorphism has been associated with vitamin D deficiency and altered red blood cell formation (Dimitriadou et al. 2011). However, Singh et al. (2012) pointed out no any significant association between vitamin D deficiency and Hb levels with the *FokI* gene polymorphism in anemic patients.

Our results showed the lower vitamin D levels in

Table 3. *VDR-FokI-*rs2228570 gene polymorphisms effect in Vitamin D, erythropoietin and Hb levels in anemic patients**.**

Indices	VDR-FokI-rs2228570 gene		P
	Wild type-FF (Mean ± S.D.)	Mutant type-Ff, ff (Mean ± S.D.)	
Vitamin D (ng/ml)	16.75±2.05	9.25±2.30	0.001*
Erythropoietin (mu/ml)	16.50±2.74	27.33±2.64	0.001*
Hb (mg/dl)	12.00±1.85	7.75±1.91	0.001*

S.D.: standard deviation; * Signification at *P* value ≤ 0.01

anemic patients than control, because these disorders are caused by a failure of 25(OH) D in the liver result in increased iron concentration and consequently a liver problem (Zaidan & Mohamed 2014; El-Edel et al. 2017).

In the mutant type [ff], translation starts at the first [ATG] site, resulting in a long vitamin D receptor protein of 427 amino acids, whereas in the wild type [FF], translation starts at the second [ATG] site rather than the first. This results in a shortened protein chain of 424 amino acids (Palomer et al. 2007; Israa et al. 2012). This change in amino acid length of vitamin D receptor is significantly affected by the occurrence of polymorphism (Deng et al. 2002; Maysaa et al. 2016). Consequently, when compared to VDR of the F allele, the f allele results in the creation of VDR with three more amino acids and decreased biological activity (Zaidan et al. 2015).

As conclusion, the current research showed a correlation between *VDR FokI* gene polymorphisms and anemia in men and there is a relationship between vitamin D deficiency and anemia in men.

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