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ASSOCIATION OF *FOK*I RESTRICTION FRAGMENT LENGTH POLYMORPHISM GENOTYPES WITH AUTOIMMUNE THYROID DISEASES

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ABSTRACT

Vitamin D, besides its function in regulating the metabolism of calcium-phosphorus, turned out to play an important immunomodulating role. VDR gene polymorphisms have been investigated for a presumed association with autoimmune thyroid diseases with conflicting results. The current study was conducted in Duhok Central Public Health laboratory and the Scientific Research Center/College of Science/University of Duhok. It included thirty-five autoimmune thyroid patients and thirty-two healthy control subjects. Thyroid hormones and antithyroid antibody serum levels were assayed. PCR-Restriction fragment length polymorphism analysis was performed for genotyping and detecting mutations in VDR gene of both patients and control. The results indicated that no statistically significant difference was found when mean serum concentrations of thyroid hormones and TSH of patients and controls were compared. While, significant difference ($p<0.05$) emerged when the mean serum concentrations of anti-TPO antibodies were compared. Furthermore, RFLP genotyping analysis by utilizing *Fok* I restriction enzyme showed that 54.3 % of the patients belonged to FF genotype, 31.4 % stood for Ff heterozygote genotype, and the last genotype (ff) involved 14.3 % of the patients. In the control group, 27 (84.4 %) represented FF genotype, the remaining 5 subjects stood for Ff genotype correspondent to 15.6 % of the controls while ff genotype had not been detected (0 %). In conclusion, different RFLP genotypes of the VDR receptor revealed various levels of association with AITDs.

INTRODUCTION

Vitamin D, which is also known as "sunshine vitamin", has a significant function in both bones as well as non-bone tissues (Annweiler *et al*., 2013). The autocrine and paracrine biological functions of vitamin D are exerted by interacting with its intracellular receptor known as vitamin D receptor (VDR) (Heine *et al*., 2008). The ubiquitous expression of VDR in various tissues, including the heart, kidney, breast, muscle, prostate, colon, brain and immune cells, renders this as a conventional target of modulation in the pathogenesis of many diseases including variety of metabolic syndrome, cancers, dermal disorders, and renal transplant (Huang *et al*., 2013).

Vitamin D3 is physiologically converted into an active form named 1α, 25dihydroxyvitamin D3 [1α, 25(OH)2D3 or calcitriol] which is responsible for VDR binding and exerting its intended functions (Sakharkar *et al*., 2017). On chromosome 12q the VDR gene is located which consists of eight proteins coding exons (exons 2 9) and one untranslated exon (Buttigliero *et al*., 2011).

Vitamin D receptor is a potent transcription factor, but is ligand-dependent. It has two zinc finger arrangements with a distinct DNA binding domain and a carboxy terminal ligand-binding domain (Imtiaz *et al*., 2012). VDR dimerizes with the retinoid X receptor (RXR) after binding to its ligand, calcitriol (1,25 (OH)2D); this binding in turn leads to a conformational change, that stimulate the translocation of the heterodimer into the nucleus, where it interacts with vitamin D response elements (VDRE) in the promoter regions which ultimately results in regulation of the target gene at the transcriptional level (Institute of Medicine, 2011).

Interaction of 1,25(OH)2D with the intracellular VDR regulates more than nine hundred genes involved in a variety of physiological activities, including both adaptive and innate immunity (Khammissa *et al*., 2018). Vitamin D receptor polymorphisms have been

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speculated to be significant environmental risk factors for the development of autoimmune disease (Bizzaro *et al*., 2017). Some reports proposed an influence of vitamin D receptor polymorphisms on the development of autoimmune thyroid disease (AITD) (Yazici *et al*., 2013). However, other studies failed to demonstrate a firm correlation and showed how deficiency of serum vitamin D is not related to the early stages of thyroid autoimmunity (Effraimidis *et al*., 2012), on the other hand, only a weak inverse correlation has been obtained between serum 25(OH)D values and anti-thyroid peroxidase antibody titers in a previous survey which targeted an Asian Indian community (Chen *et al*., 2007).

MATERIALS AND METHODS

Patients and sampling

Thirty-five thyroid patients were selected for the present study, their ages ranged between 25-61 years old. Thirty-two apparently healthy subjects were also included as controls. Clinical assessment was also followed to denote preliminary abnormal thyroid function. Samples were collected during the period from May to September 2018 from individuals who attended Duhok central public health laboratory. Three milliliters of venous blood were aspirated from each subject into a gel tube, left for 10 minutes to dry, and centrifuged at 5000 rpm for 10 minutes to obtain the serum component of blood. Two additional milliliters of blood were transferred to EDTA tube to be used for extracting DNA.

Measurements of thyroid hormones and anti-TPO antibody serum levels

In Duhok central health laboratory, serum levels of tri-iodothyronine (T3), thyroxine (T4), and thyroid stimulating hormone (TSH) were assayed by Cobas e411 Analyser (Roche, USA) using TSH, T3, and T4 measurement kits (Elecsys®, USA). To measure anti-TPO antibody serum level ELISA method at wavelength 450 nm was utilized (Anti-TPO measurement kit (AESKULISA®, Germany)).

Extraction of DNA

In Duhok central health laboratory, extraction of DNA was performed by using Extra-Gene I kit manufactured by BAG Health care® (Germany). The concentration and purity of the resultant DNA were estimated by using Nanodrop.

PCR amplification of vitamin D receptor gene

In the scientific research center, polymerase chain reaction was adopted to amplify the gene responsible for encoding vitamin D receptor by using specific primers (F:5' AGCTGGCCCTGGCACTGACTCTGGCT3';R:5' ATGGAAACACCTTGCTTCTTCTCCCTC) (Kostner *et al*., 2009); the amplification conditions were illustrated in Table 1. PCR products were electrophoresed and visualized by UV light according to Sambrook *et al*., (1989).

Table (1): Amplification conditions of vitamin D receptor gene

Step	Temperature	Time	Number of cycles	Product size (bp)
Initial denaturation	95°C	5 minutes		
Denaturation	94°C	45 seconds	35 cycles	265
Annealing temperature	60°C	45 seconds		
Extension	72°C	45 seconds		
Final extension	72°C	10 minutes		
Hold	4°C			

Restriction fragment length polymorphism analysis

Amplification bands (265 bp) were then digested by incubation with *Fok* I (Biolab®) restriction enzyme at 37^{o}C for 60 minutes (Al-Temmemy, 2008). Thereafter, five microliters of the digested product were loaded into 3 % agarose

gel for second run electrophoresis and visualized by UV-illumination.

All procedures followed the manufacturers' instructions, assays and molecular approaches were conducted at Duhok central public health laboratory and scientific research center/university of Duhok. Data were statistically analyzed by the SPSS (IBM Corporation, New York, NY, USA) statistical package (Version 20.0).

RESULTS

Serum TSH, T3, T4 and anti-TPO Levels

The results of the current work showed mean TSH serum levels of 13.2±22 mIU/ml for the patients,which exceeded of the control group 2.01±1.11 mIU/ml but the two means were statistically non significantly different. Furthermore, T3 and T4 mean serum levels were also non significantly different and represented by 2.2±0.9 nmol/L, 1.67±0.38 nmol/L; and 100.3±47.0 nmol/L, 93.48±23.55 nmol/L for patients and control groups, respectively (Table 2).

Table (2): The mean serum concentration of thyroid hormones for the patient and control

Hormones	Category	Frequency	Mean serum concentration ±SD	Units
TSH	**Patients**	35	13.2±22	mIU/ml
	Control	32	2.01±1.11	
T3	**Patients**	35	2.2±0.9	nmol/L
	Control	32	1.67±0.38	
T4	**Patients**	35	100.3±47.0	nmol/L
	Control	32	93.48±23.55	

However, mean serum concentration measurements of anti-TPO were 47.2±40.3 IU/ml for the patients' group while it was 8.46±5.32 IU/ml for the control group and they were statistically significantly different (P< 0.05) (Table 3).

Table (3): Mean serum concentration of anti-TPO for patients and control groups

Factor	Category	Number	Mean Anti-TPO serum concentration (IU/ml) ±SD
Anti-TPO	Patients	35	47.2±40.3*
	Control	32	8.46±5.32

*Significant at p<0.05

Extraction of DNA, visualization and measurement of purity and concentration

The results of the present work showed that DNA was successfully extracted, electrophoresed and visualized by UV illumination as indicated in Figure 1. The results also indicated that the mean DNA concentration was 440 ng/µl for the patients while it was 395 ng/µl for the controls and the purity of the extracted DNA was 1.8 and 1.64 For patients and controls, respectively. The final concentration of DNA was then adjusted to 100 ng/µl for later use in PCR experiments.

PCR amplification of vitamin D receptor gene

The results of the current study revealed that vitamin D receptor gene was amplified in both patients and control group as indicated by PCR product bands of 265 bp (Figure 1).

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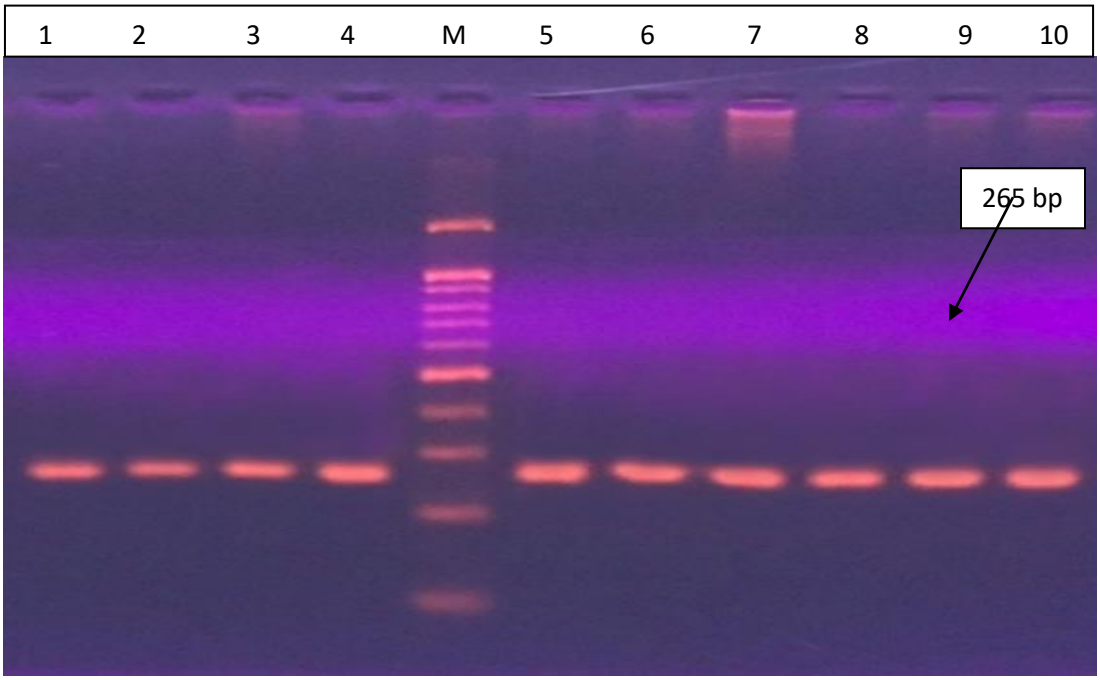

Fig. (1): PCR amplification of vitamin D receptor gene. Amplicon size was 265 bp. Run on 1.75 % agarose gel, 5v/cm, for 60 minutes. Lanes one through ten show bands of amplification. Lane M represents molecular weight marker (1500-100bp).

Restriction fragment length polymorphism (RFLP) analysis

Figure 2 demonstrated RFLP genotyping analysis by utilizing *Fok* I restriction enzyme, it is shown that 19 subjects (54.3 %) of the patient group were FF genotype represented by a single undigested band of 265 bp; 11 individuals (31.4) % of patients stood for Ff heterozygote genotypes with three band of 265 bp, 196 bp and 69 bp; the last genotype was symbolled ff, involved 5 (14.3 %) of the patients group, and referred to by two bands of 196 bp and 69 bp. In the control group, 27 (84.4 %) stood for FF genotype, Ff genotype represented by 5 (15.6 %) of the group while ff genotype had not been detected (0 %).

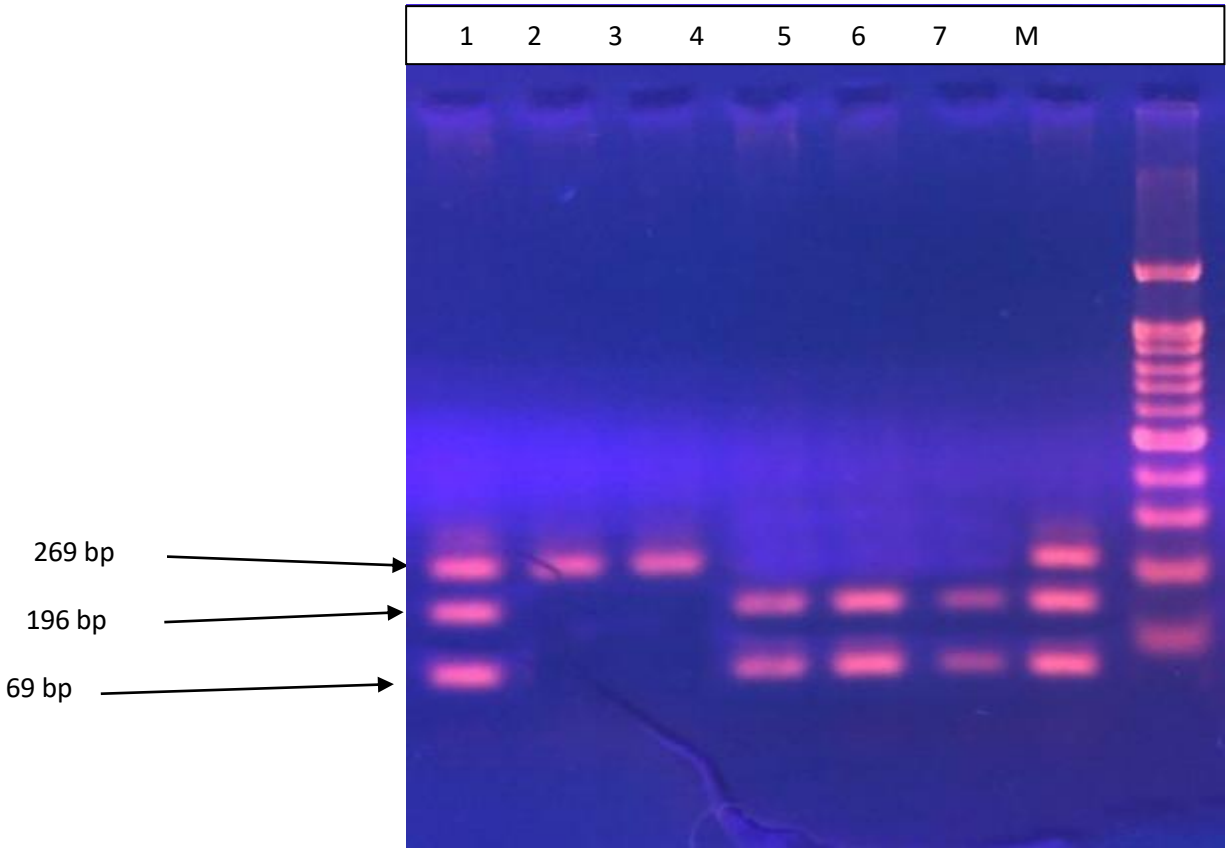

Fig. (2): Restriction fragment length polymorphism analysis. PCR amplification bands were digested with *Fok* I restriction enzyme, the resulting bands were run on 3 % agarose gel for 45 minutes. Lane 2 and 3 represent homozygote FF genotypes, lanes 1 and 7 represent Ff genotypes, lanes 4, 5, and 6 represent ff genotypes. Lane M is the molecular size marker (1500-100 bp).

DISCUSSION

In the past decade, more interest has been directed towards the non-calcemic effect of vitamin D (Kim, 2017). Many observational approaches and meta-analyses revealed correlation between vitamin D serum levels and outcome of many wide spread diseases, including chronic diseases, endocrine diseases, development of cancer, and autoimmune disorders (Dutta *et al*., 2014). Especially, cells of the immune system (B lymphocytes, T lymphocytes, and antigen presenting cells), because of the expression of 1α-hydroxylase (CYP27B1), are capable of synthesizing the biologically active form of vitamin D, which is characterized by immunomodulatory features. Furthermore, the expression of VDR in these cells implies a local effect of vitamin D in the immune response (Altieri *et al*., 2017).

The factors that contribute to the development of autoimmune disorders are a mixture of environmental factors, hormonal effects and genetic susceptibility. Low vitamin D level and VDR polymorphism have been considered as critical environmental risk attributes for the establishment of autoimmune diseases. Data are gathering to support that VDR polymorphism (especially *Bsm*I, *Apa*I, *Taq*I, and *Fok*I polymorphism genotypes) are associated with an increased incidence of autoimmune diseases, and it has been demonstrated that the binding of VDR and its ligand initiates an anti-inflammatory response on innate immunity and an immunosuppressive and regulatory effects on adaptive immunity (Bizzaro *et al*., 2017).

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One meta-analysis investigated the association between vitamin D level and autoimmune thyroid disease (AITD) through an accurate and detailed systematic literature review (Wang *et al.*, 2015). concluded that serum 25(OH)D was lower in AITD individuals compared to healthy controls. Other studies failed to establish a firm correlation. Investigators showed how vitamin D deficiency is not associated with early stages of thyroid autoimmunity (Effraimidis *et al*., 2012). , while an Asian Indian community-based survey found only a weak inverse correlation between serum 25(OH) D values and TPO-Ab titers (Goswami *et al*., 2009).

Many common allelic forms have also been detected in the VDR gene and are the focus of the multiple previous investigations. The existence of a T/C transition polymorphism (ATG to ACG) at the first of two possible translation initiation sites in exon II (Baker *et al*., 1988). has been verified using the *Fok* I restriction endonuclease (Gross *et al*., 1996). Individuals with the C allele (designated F) initiate translation at the second ATG site and lack the three NH2~ terminal amino acids of the full-length VDR protein (Arai *et al*., 1997). Conversely, subjects with the T allele (designated f) initiate translation at the first ATG site and synthesize the full-length (427 amino acids) VDR protein (Eccleshall *et al*., 1998). The ff genotype frequency was 4 percent among African Americans and 13-18 percent among Asians and Caucasians in one report (Morrison *et al*., 1992).

Vitamin D receptor polymorphisms was investigated in one hundred and nine Hashimoto's thyroiditis (HT) victims in China, their obtained results contradicted those of the present study as they claimed FF homozygous of the VDR *Fok* I polymorphism had a higher risk for Hashimoto's thyroiditis (Lin *et al*., 2006).

The results of the present study were also inconsistent with those of others who studied the association between Hashimoto's thyroiditis and *Fok*I, *Apa*I and *Taq*I RFLP polymorphisms in VDR gene, their data suggested that female individualpopulation who carry the FF homozygous of *Fok*I polymorphism in the VDR gene may have a higher risk of developing HT (Djurovic *et al*., 2015). However, in another study, *Fok*I, FF genotype of HT patient as 44.9% and 58% in the control group were observed (Guleryuz *et al*., 2016), the results of the present work mimic the later mentioned results which indicated a higher percentage of the FF genotype in the controls compared to the patients and thus implying no association between this genotype and AITDs.

Accumulated Data support the notion of an association between an elevated incidence of AITDs and the VDR polymorphisms of the VDR. One separate study conducted in Turkey, involved 111 Turkish patients with HT as well as 159 controls, investigated the distribution of VDR variants. It revealed a link between VDR gene *Fok*I FF genotypes and an increased risk of HT (Yazici *et al*., 2013).

In another study, a proposed functional VDR polymorphisms (*Apa*I rs7975232, *Fok*I rs2228570, *Taq*I rs731236, and *Bsm*I rs1544410) are involved in the pathogenesis of autoimmune thyroid diseases (Inoue *et al*., 2014). Concerning HT, the frequency of the CC genotype for the *Fok*I polymorphism was higher in patients than in controls. Contradictory outcomes were observed in a meta-analysis of eight studies, in which the findings indicate both *Taq*I and *Bsm*I polymorphisms are significantly associated with AITD risk, but not the *Fok*I or *Apa*I polymorphisms (Feng *et al*., 2013). The later outcome was supported by the results of the current study.

The results of the present study disagreed with those of others who suggested an association between *Fok*I FF genotype and the susceptibility to AITDs from the one hand, but they came in line with the same group results which indicated a correlation between Ff genotype and AITDs on the other hand (Zarrin *et al*., 2018).

Furthermore, other two separate studies conducted by Inoue *et al*., (2014) and Meng *et al*., (2015) on Japanese and Chinese populations, respectively, supported the findings of the current work, as it was demonstrated that *Fok*I genotypes and distribution of alleles among patients with autoimmune thyroid diseases and control groups were not statistically different.

However, even if vitamin D receptor locus does not seem to be related to the conditioning of the genetic predisposition to HT, vitamin D deficiency may likely participate in the initiation and progression of the disease, acting as an environmental trigger (Huang, 2013). Thus,

controversial point of views on the vitamin D role in AITD onset is stated by the scientific community. Several cofactors may have an impact on the results of epidemiologic surveys, such as obesity, sun exposure, sedentary life (Altieri *et al*., 2017).

CONCLUSION

Vitamin D receptor gene polymorphism genotypes can be an influential factor that may be implied for an association with autoimmune thyroid diseases.

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