Evaluating the role of vitamin D receptor polymorphisms on susceptibility to tuberculosis among Iranian patients: a case-control study

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ÖZET

İranlı hastalarda tüberküloza yatkınlıkta vitamin D polimorfizmlerinin rolünün değerlendirilmesi: Olgu-kontrol çalışması

Yakın zamanda birçok genetik çalışma, HLA, VDR, NRAMP1, MBL, TNF-α ve bunların akciğer tüberkülozu gibi hastalıklara yatkınlık ile ilişkilerine odaklandı. Bazı çalışmalar akciğer tüberkülozunda VDR polimorfizmlerinin yatkınlık ve koruyucu rollerinin olduğunu gösterdi. Olgu-kontrol çalışması ile tüberkülozlu olgu (n= 164) ve kontrol (n= 50) gruplarından kan örnekleri alındı. Her bir polimorfizm için özel primerler ve enzimler kullanılarak PCR-RFLP tekniğiyle lökositlerden DNA ekstrakte edildi. Apa I, Bsm I, Fok I ve Taq I olarak bilinen VDR polimorfizmleri her iki grupta değerlendirildi. Bu çalışmada sadece kombine genotipler olan AbfT ve AabbFfTT, kişileri akciğer tüberkülozuna karşı koruyucu olan istatistiksel olarak anlamlı faktörlerdi. Belirtilen her iki genotip tüberküloza karşı koruyucu faktörlerdi ve bu çalışmada tüberküloza yatkınlığa neden olan bir genotip bulunmadı. Bu konuda daha fazla çalışma yapılması gereklidir.

Anahtar Kelimeler: *TB, VDR, polimorfizmler.*

SUMMARY

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Many genetic studies recently have focused on HLA, VDR, NRAMP1, MBL, TNF-α, and their relationships with susceptibility to diseases such as pulmonary tuberculosis. Some studies showed predisposing and protective roles for VDR polymorphisms in pulmonary tuberculosis. Through a case-control study, blood samples were taken from tuberculosis case (n= 164) and control (n= 50) groups. DNA was extracted from white blood cells by PCR-RFLP technique using special primers and enzymes for each polymorphism. VDR polymorphisms are known as ApaI, BsmI, FokI, and Taq I which were evaluated within the two mentioned groups. Combined genotypes AbfT and AabbFfTT were the only statistically significant factors which protected people against pulmonary TB in this study. Two mentioned genotypes were protective factors against TB and this study could not find any predisposing genotype to TB. More study is requested on this matter.

Key Words: *TB, VDR, polymorphisms.*

One third of the world population are infected by *Mycobacterium tuberculosis* and among these, one is getting the active disease (TB) per second (1-4).

The majority of tuberculosis cases are latent TB. One out of ten latent TB cases, in average, gets active TB and this is led by individual differences among peoples' susceptibility to it (3).

During TB vaccination in 1926 in Lubeck Germany, children were inoculated by liquid containing live *M. tuberculosis* instead of *Mycobacterium bovis* as BCG vaccine, accidentally. Not all the inoculated people got active TB, and this situation was not reasonable (5,6).

On the other hand, studies on monozygotic twins in relation to TB showed higher co-occurrence of TB between them like many other diseases (5,7,8).

The above mentioned examples remind the theory of natural selection and lead us to observe genetic differences among human races, one of which could be single nucleotide polymorphisms (SNPs) (5).

Since the ancient age it was thought that vitamin D had a great protective role against *M. tuberculosis* bacilli. It was used to treat skin TB before the relative chemotherapy was applied (9). Through a study in England, the same pattern was found out about vitamin D deficiency, and active TB among Asian immigrants, and this fact empowered the theory that vegetarian diet is a major risk factor to get active TB (10).

Vitamin D effects are mediated by vitamin D receptor (VDR), the coding gene of which includes polymorphisms such as *ApaI, BsmI, FokI,* and *TaqI*. VDR has a 9-exon coding gene on chromosome 12q. *FokI* is within exon II, *BsmI* in exon IX, and both *ApaI* and *TaqI* polymorphisms are placed within the exons VIII and IX.

MATERIALS and METHODS

Through a case-control study, 214 participants entered this research, and were divided into two groups including case (164 patients) and control (50 healthy people). Participants were either symptomatic people who referred to Masih Daneshvari Hospital, Tehran, Iran from March 2006 to March 2007, healthy people who worked in exposure to *M. tuberculosis* in the hospital, or patients' families. They were examined by physicians. Chest X-ray and three serial sputum smear tests were done for them as well. In case of existence of active TB, they attended this study as cases and if not, as controls.

All the people attended this work gave inform consents according to the information given by researchers about the objectives, methods, and outcome of the study.

The process of genetic tests followed as below:

- **Extracting WBCs from whole blood:** Hemoglobin can disturb the polymerase chain reaction (PCR) procedure and it has to be separated from the blood to permit DNA extraction from WBCs and amplification. So, WBCs were separated from venous whole blood by centrifuge using RBC lysis buffer and PBSLX.

- **DNA extraction from WBCs:** Phenol-chloroform protocol was used to extract DNA in this study. The process was done using SE buffer,

10% SDS, 20 mg/mL proteinase K, saturated phenol, and chloroform isoethyl alcohol. SE buffer makes WBCs lysed by 3 M (molars) NaCl and 0.5 M EDTA in 60°C which is also the optimal temperature for proteinase K to act. Tris EDTA (TE) was used as DNA solvent.

• **VDR genotyping:** VDR has 25 various polymorphisms from which 4 are more important and have been studied related to tuberculosis. The above obtained DNA underwent PCR to be amplified and evaluated through restriction-fragment-length polymorphism (RFLP) in contact with a different special enzyme for each of them.

One primer was used for each polymorphism of VDR including *BsmI*, *FokI*; while according to the joint place of the two, one primer was allocated for *ApaI* and *TaqI* together at the same time. These primers and their products sizes are summarized in Table 1 and the PCR protocols are described in Table 2.

Each of the PCR products was kept with special enzyme for a night (3 hours for *BsmI*) in optimal temperature. The optimal temperature and products sizes of RFLP could be found in Table 3. The products were electrophoresed on 0.5-2% agarose gels.

Outcome Measures

The main purpose of this study was to find a relationship between VDR polymorphism genotypes and susceptibility to active pulmonary TB. In order to achieve this aim, we evaluated the frequencies of VDR genotypes mentioned in Table 3.

After electrophoresis, each part of PCR products broken by RFLP, made a band based on its weight which was visible in UV after staining by ethidium bromide. Figure 1 illustrates the electrophoresis pattern of each genotype of polymorphisms. The positions of bands and their accompaniment pattern showed the kind of genotype which the person had. We analyzed the results using SPSS16.

RESULTS

All the people who referred to the Iranian research institute of TB and lung disease (NRITLD) and underwent diagnostic procedures including laboratory tests and chest radiographies meeting the inclusion criteria in the period of the study were 214 and were divided into 164 cases and 50 controls based on the diagnosis.

The mean age was not different between the groups and they were matched for gender. Out of controls 22 (44%) and 28 (56%) were male and female, respectively. Cases had 79 (48.2%) females and 85 (51.8%) males.

Table 4 illustrates the frequencies of haploid and diploid VDR genotypes in two groups. Polymorphisms *ApaI* and *TaqI* were in the same rate. Although having remarkable odds ratios for both polymorphisms *BsmI* and *FokI*, confidence intervals did not confirm their significance. So, no difference was detected between cases and controls in VDR polymorphisms individually.

From 164 cases, 50 were sampled randomly (equal to controls) to compare single and combined polymorphisms in a right case-control design. No difference was found among single

Table 1. Primers and products' size for each VDR polymorphism.

Polymorphism	Primer	Product size
ApaI and *TaqI*	5′-GGG ACG ATG AGG GAT GGA CAG AGC-3′ 5′-GGA AAG GGG TTA GGT TTG ACA GGA-3′	2000 bp
BsmI	5′-CAA CAA AGA CTA CAA GTA CCG CGT CAG TGA-3′ 5′-AAC CAG CGG GAA GAG GTC AAG GG-3′	825 bp
FokI	5′-AGCTGG CCC TGG CACTGA CTC TGC TCC-3′ 5′-ATGGAA ACA CCT TGC TTC TTC TTC CTC-3′	265 bp

VDR: Vitamin D receptor.

Table 2. PCR protocols for each VDR polymorphism.

Polymorphism	PCR protocol
ApaI and *TaqI*	96°C for 1 min 30 cycles at: 94°C for 1 min 55°C for 1 min 72°C for 1 min
BsmI	94°C for 4 min 35 cycles at: 94°C for 30 s 63°C for 30 s 72°C for 60 s 72°C for 2 min
FokI	96°C for 1 min 30 cycles at: 94°C for 45 s 60°C for 45 s 72°C for 45 s

PCR: Polymerase chain reaction, VDR: Vitamin D receptor.

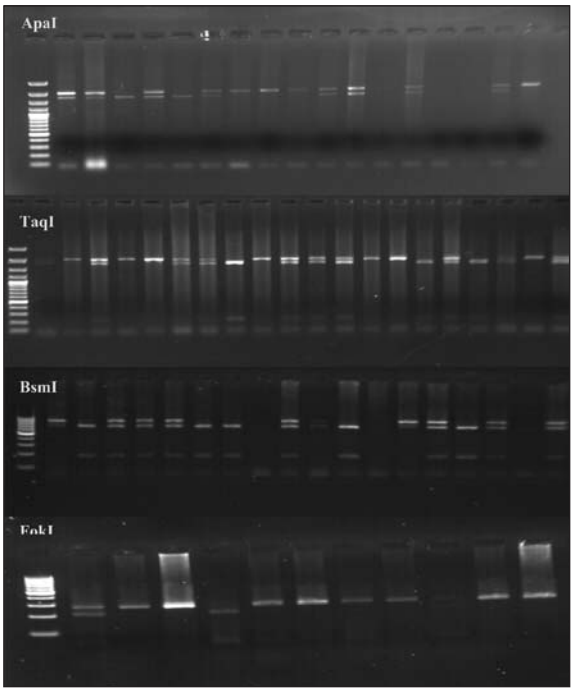

Figure 1. The pattern of VDR polymorphisms after electrophoresis.

polymorphisms. Haploid and diploid combinations of the four mentioned polymorphisms of VDR were compared between 50 cases and 50 controls for their rates (Figure 2).

Genotypes AbfT (haploid) and AAbbFfTT (diploid) were detected as different factors in this step. Odds ratio for AbfT was 0.24 with confidence interval (CI) equal to 0.11-0.56 (95%) and for AAbbFfTT was 0.2 with CI of 0.04-0.9 (95%). These proportions are seen in diagram 2 and Table 5.

DISCUSSION

In deed, pulmonary TB is resulted by pathogen-host-environment interaction (4,9). Individual diversities such as genotypes in host and pathogen have created several patterns of disease transmission.

Many studies made VDR suspected in TB involvement. Some of them found a predisposing role for *FokI* FF genotype related to TB (9-11). Some others introduced allel T of *TaqI* polymorphism as a mediator to get TB more (11).

On one hand, Wilbur says allel F of *FokI* is a protective factor against TB and recessive allel of *TaqI* (t) is a motivator of cell-mediated immune response to TB infection (11). Also Bellamy confirmed that *TaqI* tt genotype is lower among TB cases (12).

Table 3. Temperature and product size for VDR polymorphisms in RFLP.

Polymorphism	Temperature (°C)	Product size (bp)
ApaI	55	AA (2000), Aa (2000, 1700, 300), aa (1700, 300)
TaqI	65	TT (2000), Tt (2000, 1800, 200), tt (1800, 200)
BsmI	65	BB (825), Bb (825, 650, 175), bb (650, 175)
FokI	37	FF (265), Ff (265, 196, 69), ff (196, 69)

VDR: Vitamin D receptor.
RFLP: Restriction-fragment-length polymorphism.

Table 4. Frequencies of VDR polymorphisms genotype in the groups of the study.

Polymorphism		Group			Sig
		Control	Case	Total	
ApaI	AA	32 (64%)	93 (56.7%)	125 (58.4%)	0.46
	Aa	11 (22%)	51 (31.1%)	62 (29%)	
	Aa	7 (14%)	20 (12.2%)	27 (12.6%)	
	A	75 (75%)	237 (72.3%)	312 (72.9%)	
	a	25 (25%)	91 (27.7%)	116 (27.1%)	
BsmI	BB	0	23 (14%)	23 (10.7%)	0.04
	Bb	29 (58%)	86 (52.4%)	115 (53.7%)	
	Bb	21 (42%)	55 (33.5%)	76 (35.5%)	
	B	29 (29%)	132 (40.2%)	161 (37.6%)	
	b	71 (71%)	196 (59.8%)	267 (62.4%)	
FokI	FF	15 (30%)	97 (59.1%)	112 (52.3%)	0.001
	Ff	30 (60%)	57 (34.8%)	87 (40.7%)	
	Ff	5 (10%)	10 (6.1%)	15 (7%)	
	F	60 (60%)	251 (76.5%)	311 (72.7%)	
	f	40 (40%)	77 (23.5%)	117 (27.3%)	
TaqI	TT	26 (52%)	63 (38.4%)	89 (41.6%)	0.97
	Tt	24 (48%)	93 (56.7%)	117 (54.7%)	
	Tt	0	8 (4.9%)	8 (3.7%)	
	T	76 (76%)	219 (66.8%)	295 (68.9%)	
	t	24 (24%)	109 (33.2%)	133 (31.1%)	

VDR: Vitamin D receptor.

On the other hand, Bornman found no relationship between VDR polymorphisms occurrence and TB distribution through her study across three West African countries (13). Bornman concludes that gene-gene and gene-environment interactions play a great role in adjusting the effects of VDR polymorphisms on TB susceptibility.

A meta-analysis by Lewis says all the studies on VDR in relation to TB are weak, small, and unreliable (14). Lewis accuses wrong selection of controls and not to consider some factors such as serum vitamin D level in failure of the studies. He believes that 2000 people are needed to participate in case and control groups in order to obtain an odds ratio of 1.4 with 80% power and type I error of 0.01.

According to the findings of this study, no relationship was defined between single polymorphisms of VDR gene and TB susceptibility which might be resulted from ignoring participants occupation, diet and disposed illnesses like immune compromised cases.

Combination of VDR polymorphisms showed significant difference of two groups for a haploid and a diploid genotype probably because of synergistic effects of them. This type of evaluation on combined gene polymorphisms is a new aspect of genetic study on VDR which empower this research. Two genotypes of AbfT and AAbbFfTT were defined as protective factors against TB through current study.

It is undeniable that more powerful studies with more participants is needed to obtain more reli-

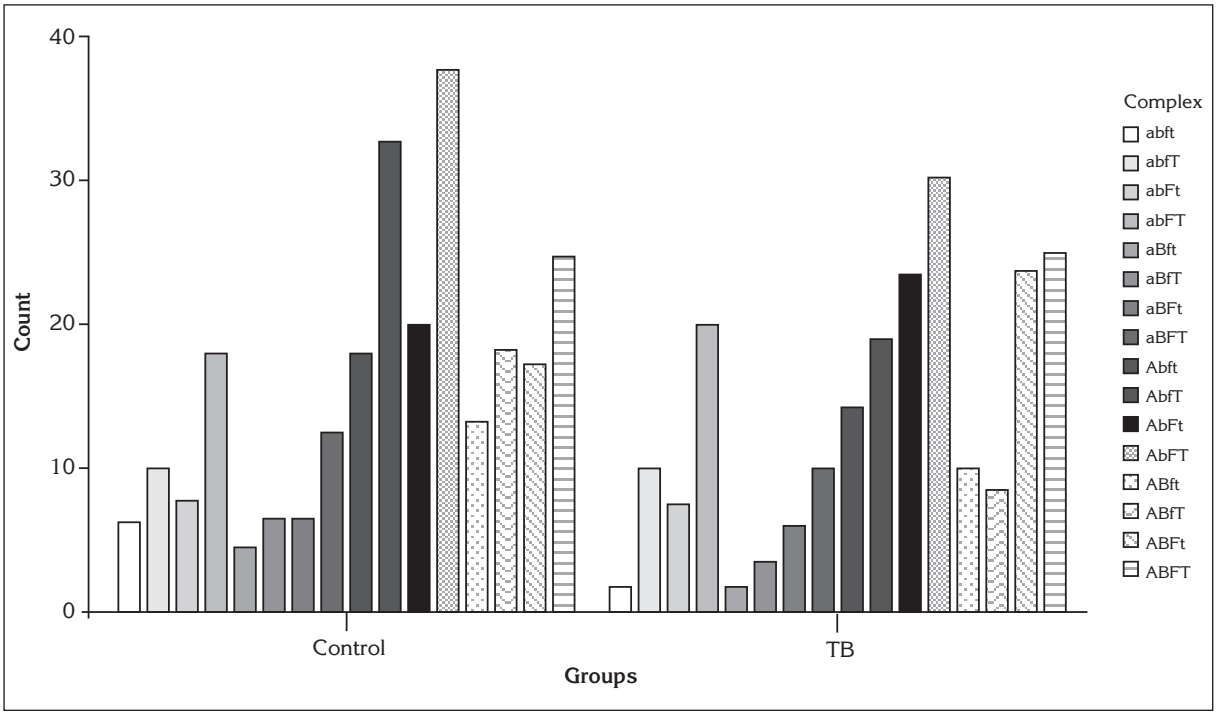

Figure 2. The distribution of haploid combined genotypes of VDR polymorphisms among cases and controls.

Table 5. Distribution of significantly different genotypes of combined VDR polymorphisms between case and control groups in haploid and diploid pattern.

Frequency / Genotype	Control	Case	Odds ratio	CI (95%)
AbfT	33	16	0.242	0.105-0.558
AAbbFfTT	9	2	0.190	0.039-0.929

VDR: Vitamin D receptor.

able findings and generalize them to the society of Iran.

It would be very important to consider the interactions of confounding factors with known classical variables like cell-mediated immunity, history of having closed contacts to TB cases, nutritional diet, etc. in this case, accompany of VDR polymorphisms and immunologic markers such as cytokines and some suspected genetic factors like NRAMP1 is strongly advised to be challenged on.

Study Limitations

This study had some limitations among which the most important was limit control participants because of low number of people in closed contact to *M. tuberculosis*. This situation pushed us to disobey case-control model somewhere. Therefore, some studies showed that genetic studies in special situation like gene-gene or gene-environment interactions can use case-only design. We are going to expand the study as a multi-central study with bigger control group to have more power to find the mentioned relationships.

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