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Association of Vitamin D Receptor Polymorphisms to Pulmonary Tuberculosis in Turkish Patients: An Up-to-date Meta-Analysis and A Case-Control Study

Türk Hastalarda Vitamin D Reseptörü Polimorfizmleri ve Pulmoner Tüberküloz Ilişkisi: Güncel Meta Analizi ve Vaka-Kontrol Çalışması

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ABSTRACT

Introduction: *Tuberculosis is a major global health issue, threatening millions of lives every year. To understand the interactions between host and the pathogenic factors, many association studies are being held in different populations and most of the time results are controversial. Vitamin D receptor is one of the immunomodulatory molecules that may have an effect on susceptibility to tuberculosis. Up to now, there was no positive association reported with the tuberculosis and Fok I or Taq I polymorphisms of VDR gene in tuberculosis patients of Turkish origin. The aim of this study was to make an updated meta-analysis and a case-control study in our group of patients for figuring out the association between Fok I and Taq I polymorphisms of VDR and TB.*

Materials and Methods: *In the present study, association of pulmonary tuberculosis and VDR gene's Fok I (rs 2228570) and Taq I (rs20731236) polymorphisms were investigated in our patient group from Malatya, and a comparison was made by a meta-analysis with the mentioned polymorphisms. Ninety-four healthy controls and 80 patients are subjects of case control study. The samples are genotyped for Taq I and Fok I polymorphisms by using TaqMan SNP genotyping kits. The allelic and genotypic distributions were analyzed by exact significance of the Pearson's test or Fisher's exact tests. Meta-analyses for each SNP were conducted under four different genetic models. The statistical significance of the pooled ORs was determined by a Z test and publication bias was evaluated by Egger's test.*

Results: *Our case control study Taq I polymorphism showed no significant association to TB which also matched with our meta-analysis. On the other hand, for Fok I polymorphism, genotypes and allele frequencies were significantly different in our focus group (p 0.044). Sex based analysis gave significantly different results in women with TB as well even though our meta-analysis showed no association to that polymorphism.*

Conclusion: *The reasons underlying the susceptibility to TB is still not clear. The immune response process is so complex and many molecules are taking part in these reactions. So, there are a lot of candidate molecules to be checked in the association studies. VDR is still one of those molecules and may be other than by checking single SNPs, haplotypes could be under investigation with bigger populations.*

Key Words: *Tuberculosis; VDR polymorphisms; Genotyping; Vitamin D; Association study*

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ÖZ

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Giriş: *Tüberküloz küresel bir halk sağlığı problemidir ve her yıl milyonlarca insanın hayatını tehdit etmektedir. Konak ve patojenik faktörler arasındaki etkileşimlerin anlaşılabilmesi için farklı popülasyonlarda birçok bağlantı çalışması yürütülmekte ancak bu çalışmalar çoğu zaman tartışmalı bir şekilde sonuçlanmaktadır. Vitamin D reseptörü (VDR) tüberküloza yatkınlıkta ya da dirençte etkisi olduğu düşünülen immünmodülatör faktörlerden bir tanesidir. Türk popülasyonunda VDR polimorfizmleri ile tüberküloza yatkınlık arasında bağlantı olduğunu rapor eden bir çalışma bulunmamaktadır. Bu nedenle hasta grubumuzda tüberküloz ve VDR geninin Fok I ve Taq I polimorfizmleri arasındaki bağlantıyı incelemek için bir vaka-kontrol çalışması ve meta -analizi yapmayı amaçladık. Sunduğumuz bu çalışma ülkemiz popülasyonunda Fok I polimorfizmi ile tüberküloza yatkınlık arasında istatistiksel olarak anlamlı bağlantı gösteren ilk çalışmadır.*

Materyal ve Metod: *Sunduğumuz bu çalışmada Malatya ili ve çevresinden oluşturulmuş hasta grubumuzda VDR'nin Fok I (rs2228570) ve Taq I (rs20731236) polimorfizmleri ile pulmoner tüberküloz bağlantısını taranmış ve sözü edilen polimorfizmlere yönelik bir meta analizi ile karşılaştırılmıştır. Bu doğrultuda vaka kontrol çalışmasına 94 sağlıklı birey ve 80 hasta dahil edilmiştir. Örnekler TaqMan SNP genotipleme kitleri aracılığı ile Taq I ve Fok I polimorfizmleri için genotiplendirilmiştir. Alelik ve genotipik dağılımlar, Pearson kesin ki-kare ya da Fisher kesin ki-kare testleri ile incelenmiştir. Meta analizleri her polimorfizm için dört ayrı genetik model altında yürütülmüştür. Ortak odds oranlarının anlamlılığı Z testi ile değerlendirilmiştir. Yayın yanlılığının belirlenmesi için Egger testi kullanılmıştır.*

Bulgular: *Vaka-kontrol çalışmamız Taq I polimorfizmi ile tüberküloz arasında anlamlı bir ilişkiye ulaşamamıştır ve bu bulgu meta analizi çalışması ile örtüşmektedir. Diğer yandan Fok I polimorfizmi genotipleri ve alel frekanslarının hedef hasta grubumuzda istatistiksel olarak anlamlı farklılığa sahip olduğu bulunmuştur (p= 0.044). Cinsiyete bağlı analizler de benzer şekilde istatistiksel olarak anlamlı bir sonuca ulaşmışken meta analizinde cinsiyetle ilişkili bir bağlantı bulunamamıştır.*

Sonuç: *Tüberküloza yatkınlığın altında yatan nedenler hala açık değildir. İnfeksiyonlara karşı immün yanıt süreci karmaşık bir süreçtir ve bu reaksiyonlarda birçok molekül görev almaktadır. Vitamin D reseptörü bu moleküllerden sadece bir tanesidir ve tüberküloza yatkınlık için bu aday moleküllerin polimorfizmlerinin daha kapsamlı çalışmalarla incelenmesine ihtiyaç duyulmaktadır.*

Anahtar Kelimeler: *Tüberküloz; VDR polimorfizmleri; Genotipleme; D vitamini; Bağlantı çalışması*

INTRODUCTION

Tuberculosis (TB) is an important cause of morbidity and mortality worldwide. 1/3 of the population is infected with *Mycobacterium tuberculosis* (MTB). According to World Health Organization 2019 Global Tuberculosis Report, 10 million people were infected with the disease and 1.2 million lives were lost in 2018[1]. Tuberculosis accounts for 2.5% of all diseases worldwide and 26% of preventable deaths[2]. It can remain in the latent phase for a very long period of time after infecting the individuals. Although some of the infected individuals show symptoms, the disease never develops in some, even ~ 90% of these individual's situations are improved by the immune system's response. As seen in many infectious diseases, the differences between the numbers of infected and sick people are caused by differences in balance between host defense and the virulence of the organism. The mechanisms that prevent the disease phenotype in some infected individuals cannot be understood exactly. The susceptibility to active disease can be related with genetic factors[2].

Evidence from epidemiological studies shows a link between vitamin-D deficiency and tuberculosis. In the UK and Indonesia, levels of serum $25(OH)D_3$ have been found to be lower in TB patients, which is also related with disease severity. $1.25(OH)D_3$ which is the active metabolite

of vitamin D -an immunomodulatory molecule. $1,25(OH)D_3$ is converted from 25 hydroxyvitamin D3 by 1α-hydroxylase enzyme. When *M. tuberculosis* infection activates the receptors' innate immune system, the following signaling events upregulates the expression of 1α-hydroxylase and vitamin D receptor (VDR) in monocytes and macrophages and this leads to increased potential binding of $1,25(OH)D_3$ to VDR. Antimicrobial peptides like cathelicidins are involved in the first line of defense against infections and LL-37 is one of the members of cathelicidin family which is the only member of this family identified in humans, its production may be enhanced by $1,25(OH)D_3$. This peptide modulates the immune system by attracting monocytes, T cells and neutrophils to the infection region. LL-37 production is upregulated in neutrophils and macrophages with the presence of $1,25(OH)D_3$[3].

The nuances found on VDR gene could affect the cellular functions of $1,25(OH)D_3$ and could be link to disease susceptibility. In many populations, several association studies have been held related with VDR polymorphisms and susceptibility to TB but the results are inconsistent.

We aimed to investigate the association of Fok I (rs2228570) and Taq I (rs20731236) polymorphisms of VDR gene and susceptibility to pulmonary tuberculosis (PTB) in a meta-analysis and also in a case-control study with a group of Turkish patients.

MATERIALS and METHODS

Subjects

Turkish pulmonary tuberculosis patients and age and sex-matched healthy control individuals were the subjects of this study. Patients were recruited from the Malatya Provincial Health Directorate and molecular study was performed in Department of Moleculer Biology and Genetics. The study was performed in accordance with the guidelines of the Declaration of Helsinki and was approved by local ethics committee (Protocol #2016/150). All subjects were informed about the procedures, and consent was taken before the start of the study. Ninety-four control subjects and 80 patient subjects were included in the analysis for Fok I (rs2228570) genotyping and 93 control subjects and 78 patient subjects were included in the analysis for Taq I (rs20731236) genotyping depending on the reaction quality.

Genotyping

Genomic DNA was extracted from peripheral blood using a commercial kit (PureLink® Genomic DNA Mini Kit; Invitrogen, Carlsbad, CA, USA), according to the manufacturers' protocol. All samples were coded to ensure anonymity. The quantity of DNA was measured by Qubit assays (Thermo Fisher Scientific Inc., USA). DNA samples were stored at -20°C until analysis. All samples were genotyped using the TaqMan® Single-nucleotide polymorphism (SNP) Assays (Applied Biosystems, Foster City, CA, USA): C_12060045_20 for rs2228570 and C_2404008_10 for rs20731236. All assays were performed in total volume of 10 µL, using TaqMan Genotyping MasterMix on 96-well plates. Negative control and three samples with known genotypes were included in each assay. StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used for genotyping reactions.

Statistical Analysis for Case-Control Study

The data was summarized by count and percent. Hardy-Weinberg equilibrium was tested by a chi-square distribution with 1 degrees of freedom. Differences between the groups due to allelic and genotypic distributions were analyzed by exact significance of the Pearson's test or Fisher's exact tests where appropriate. In comparisons, the significance level was considered as 0.05.

Meta-Analysis

To evaluate the association between PTB and VDR polymorphisms, odds ratio (OR) was used as the effect size. To decide the appropriate meta-analytic method, heterogeneity among studies were interpreted by I2 and Cochran's Q test statistics. The studies considered as heterogeneous if $I2>50\%$ or $p< 0.05$ for Q statistic[4]. Since for all genetic models the heterogeneity was high, the DerSimonian-Laird (DSL) random effects model was used to achieve the pooled effect size. Meta-analyses were conducted under four different genetic models for each SNP. For Taq 1: T allele vs t allele, TT genotype vs t-allele carriers

(Tt+tt), T-allele carriers (TT+Tt) vs tt genotype and TT genotype vs tt genotype. For Fok 1; F allele vs f allele, FF genotype vs f-allele carriers (Ff+ff), F-allele carriers (FF+Ff) vs ff genotype and FF genotype vs ff genotype. The statistical significance of the pooled ORs was determined by a z test and publication bias was evaluated by Egger's test. In all analyses, the significance level was considered as 0.05 and statistical analyses were done by STATA 14.0 software (Stata Corporation, College Station, Texas, USA).

Literature Search

Search words VDR or vitamin D receptor and PTB were used on databases (PubMed, Web of Science, Google Scholar) date up to November 2019. To find other appropriate studies, we also examined the references of the key studies. The selection process of the studies included in the meta-analysis is given by the flow diagram in Figure 1.

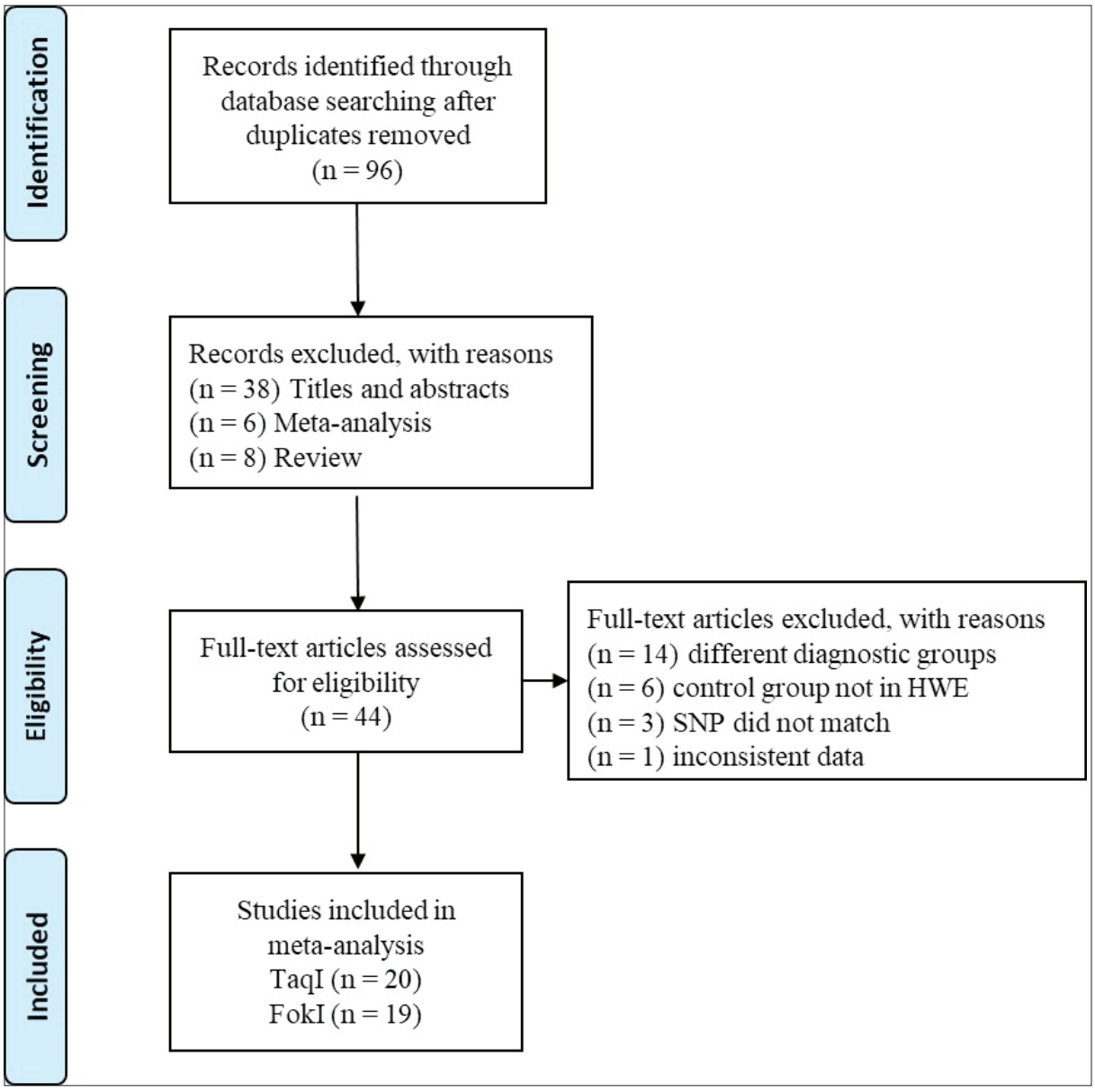

Figure 1. Flow diagram of the selection process of the studies included in meta-analysis.

Inclusion-Exclusion Criteria

Our hypothesis was to investigate the effect of VDR Fok I and Taq I polymorphisms on susceptibility to PTB. To test that hypothesis, our meta-analysis must have met the following criteria: i) the studies which concern the association between VDR SNPs and PTB and also healthy individuals as controls in case-control studies were included in meta-analysis; ii) the studies do not have a genotype distribution of control population in Hardy-Weinberg Equilibrium (HWE); iii) and in which patients diagnosed with different type of TB rather than PTB; iv) the subjects with multiple diseases; v) the publications not in English were excluded. The characteristics of the studies included in meta-analysis resulted from the selection process are given in Table 1 for Taq I and in Table 2 for Fok I polymorphisms.

RESULTS

In the case-control study, we could not find a significant association for Taq I genotype and allele frequencies (In controls: TT-49.5%, Tt-39.8 %, tt-10.7 %, in patients: TT-52.6%, Tt-29.5%, tt-17.9% for the allele frequencies T 69.4% and t 30.6 % in controls and T 67.3% and 32,7% in patients). The sex-based analysis was also performed, and the results were again not significantly different. The results of the case control study of Taq I is given in Table 3 and the sex related analysis of Taq I is given in Table 4. On the contrary, for Fok I polymorphisms the genotypes and allele frequencies were significantly different in our focus group (In controls: FF-63,8%, Ff-28.7%, ff-7.5% in patients: FF-45%, Ff-45%, ff-10% p 0.044 and allele frequencies in controls F-78,2%, f-21,8% and in patients

Table 1. Characteristics of the studies included in meta-analysis for Taq I

	Genotype Distribution						
	Case			Control			
First author, year	TT	Tt	tt	TT	Tt	tt	Ethnicity
Bellamy, 1999	204	177	27	188	177	49	African
Wilkinson, 2000	21	16	2	45	58	13	Asian in West London
Delgado, 2002	325	30	3	96	10	0	Cambodian
Bornman, 2004	174	132	37	331	253	50	West African
Lombard, 2006	50	29	5	47	34	1	Venda of South Africa
Olesen, 2007	150	145	25	161	150	34	West African
Banoei, 2010	8	33	19	33	24	5	Iranian
Ates, 2011	49	65	14	30	39	11	Turkish
Kang, 2011	134	14	1	85	8	1	Korean
Alexandra, 2013	16	52	0	43	48	19	Romanian
Rashedi, 2014	35	34	15	38	41	11	Iranian
Salimi, 2014	52	54	14	67	50	14	Iranian
Mestre, 2015	51	33	2	58	38	1	Venezuelan
Harishankar, 2016	36	39	15	42	39	8	Indian
Jafari, 2016	38	46	12	56	58	8	Iranian
Lee, 2016	186	12	0	149	20	1	Han Taiwanese
Panwar, 2016	66	28	12	90	14	2	Indian
Rizvi, 2016	92	27	11	104	22	4	Indian
Devi, 2018	86	73	10	116	86	25	Indian
Silva-Ramírez, 2019	132	110	15	228	199	30	Mexican
This study, 2019	41	23	14	46	37	10	Turkish

Table 2. Characteristics of the studies included in meta-analysis for Fok I

	Genotype Distribution						
	Case			Control			
First author, year	FF	Ff	ff	FF	Ff	ff	Ethnicity
Wilkinson, 2000	24	14	1	74	39	2	Asian in West London
Bornman, 2004	258	138	20	444	242	32	West African
Liu, 2004	29	63	28	85	120	35	Han Chinese
Lombard, 2006	43	21	2	66	18	2	Venda of South Africa
Olesen, 2007	198	106	16	207	118	19	West African
Banoei, 2010	30	21	9	29	27	6	Iranian
Marashian, 2010	97	57	10	15	30	5	Iranian
Ates, 2011	58	60	10	35	37	8	Turkish
Kang, 2011	30	58	15	41	43	21	Korean
Singh, 2011	55	40	6	96	110	19	Indian
Wu, 2013	72	96	45	101	88	22	Chinese Kazakh
Rashedi, 2014	44	33	7	50	32	8	Iranian
Salimi, 2014	65	44	11	93	31	7	Iranian
Sinaga, 2014	27	42	7	30	34	12	Indonesian
Mestre, 2015	34	47	12	26	60	16	Venezuelan
Wu, 2015	57	70	24	226	181	46	Chinese
Lee, 2016	44	104	50	51	87	32	Han Taiwanese
Devi, 2018	59	106	4	119	90	18	Indian
Silva-Ramírez, 2019	76	119	62	80	218	159	Mexican
This study, 2019	36	36	8	60	27	7	Turkish

Table 3. Genotype and allele frequencies for Taq I

	Genotype n(%)					Allele n(%)		
Group	**TT**	**Tt**	**tt**	**p**	**Hardy-Weinberg**	**T**	**t**	**p**
Control	46 (49.5)	37 (39.8)	10 (10.7)	0.231	0.537	129 (69.4)	57 (30.6)	0.685
Patient	41 (52.6)	23 (29.5)	14 (17.9)		0.003	105 (67.3)	51 (32.7)	

Table 4. Sex related analysis for Taq I

	Genotype n(%)				Allele n(%)		
Women	**TT**	**Tt**	**tt**	**p**	**T**	**t**	**p**
Control	5 (31.3)	8 (50.0)	3 (18.7)	0.262	18 (56.3)	14 (43.7)	0.602
Patient	20 (50.0)	11 (27.5)	9 (22.5)		51 (63.7)	29(36.3)	
Men	**TT**	**Tt**	**tt**	**p**	**T**	**t**	**p**
Control	41 (53.2)	29 (37.7)	7 (9.1)	0.709	111 (72.1)	43 (27.9)	0.995
Patient	21 (55.3)	12 (31.6)	5 (13.1)		54 (71.1)	22 (28.9)	

Table 5. Genotype and allele frequencies for Fok I

	Genotype n(%)					Allele n(%)		
Group	**FF**	**Ff**	**ff**	**p**	**Hardy-Weinberg**	**F**	**f**	**p**
Control	60 (63.8)	27 (28.7)	7 (7.5)	**0.044**	0.126	147 (78.2)	41 (21.8)	**0.025**
Patient	36 (45.0)	36 (45.0)	8 (10.0)		0.818	108 (67.5)	52 (32.5)	

Table 6. Sex related analysis for Fok I

	Genotype n(%)			Allele n(%)			
Women	**FF**	**Ff**	**ff**	**p**	**F**	**f**	**p**
Control	11 (64.7)	3 (17.6)	3 (17.6)	**0.043**	25 (73.5)	9 (26.5)	0.358
Patient	14 (35.0)	22 (55.0)	4 (10.0)		50 (62.5)	30 (37.5)	
Men	**FF**	**Ff**	**ff**		**F**	**f**	**p**
Control	49 (63.6)	24 (31.2)	4 (5.2)	0.514	122 (79.2)	32 (20.8)	0.320
Patient	22 (55.0)	14 (35.0)	4 (10.0)		58 (72.5)	22 (27.5)	

Table 7. The results of the meta-analyses under different genetic models for Taq I

			p		
Genetic model	**OR (95% C.I.)**	**I^2 (%)**	**P_Q**	**P_Z**	**P_E**
T vs t[21]	1.123 (0.960-1.313)	69.8	<0.001	0.147	0.155
TT vs (Tt+tt)[21]	1.135 (0.950-1.355	60.8	<0.001	0.163	0.123
(TT+Tt) vs tt[21]	1.219 (0.869-1.709)	59.0	<0.001	0.252	0.402
TT vs tt[21]	1.304 (0.892-1.905)	64.5	<0.001	0.170	0.294

†the superscript numbers in brackets represent the number of studies included in meta-analysis.
‡P_Q: p for Q test; P_Z: p for z test; P_E: p for Egger's bias test.

Table 8. The results of the meta-analyses under different genetic models for Fok 1

			p		
Genetic model	**OR (95% C.I.)**	**I^2 (%)**	**P_Q**	**P_Z**	**P_E**
F vs f[20]	1.090 (0.926-1.283)	76.4	<0.001	0.300	0.624
FF vs (Ff+ff)[20]	1.142 (0.914-1.426)	76.7	<0.001	0.243	0.771
(FF+Ff) vs ff[20]	1.030 (0.800-1.326)	52.3	0.003	0.818	0.939
FF vs ff[20]	1.070 (0.768-1.491)	67.6	<0.001	0.691	0.980

†the superscript numbers in brackets represent the number of studies included in meta-analysis.
‡P_Q: p for Q test; P_Z: p for z test; P_E: p for Egger's bias test.

F-67.5% and f-32.5% p 0.025) and also sex based analysis gave significantly different results in women with TB. The results of genotyping of Fok I polymorphism and sex related analysis was given in Table 5 and Table 6 respectively.

Literature search resulted in 20 studies for Taq I (rs20731237) and 19 studies for Fok I (rs2228570) that met the inclusion criteria. We also included case-control results of this study in the meta-analysis. In tables 7 and 8, pooled OR

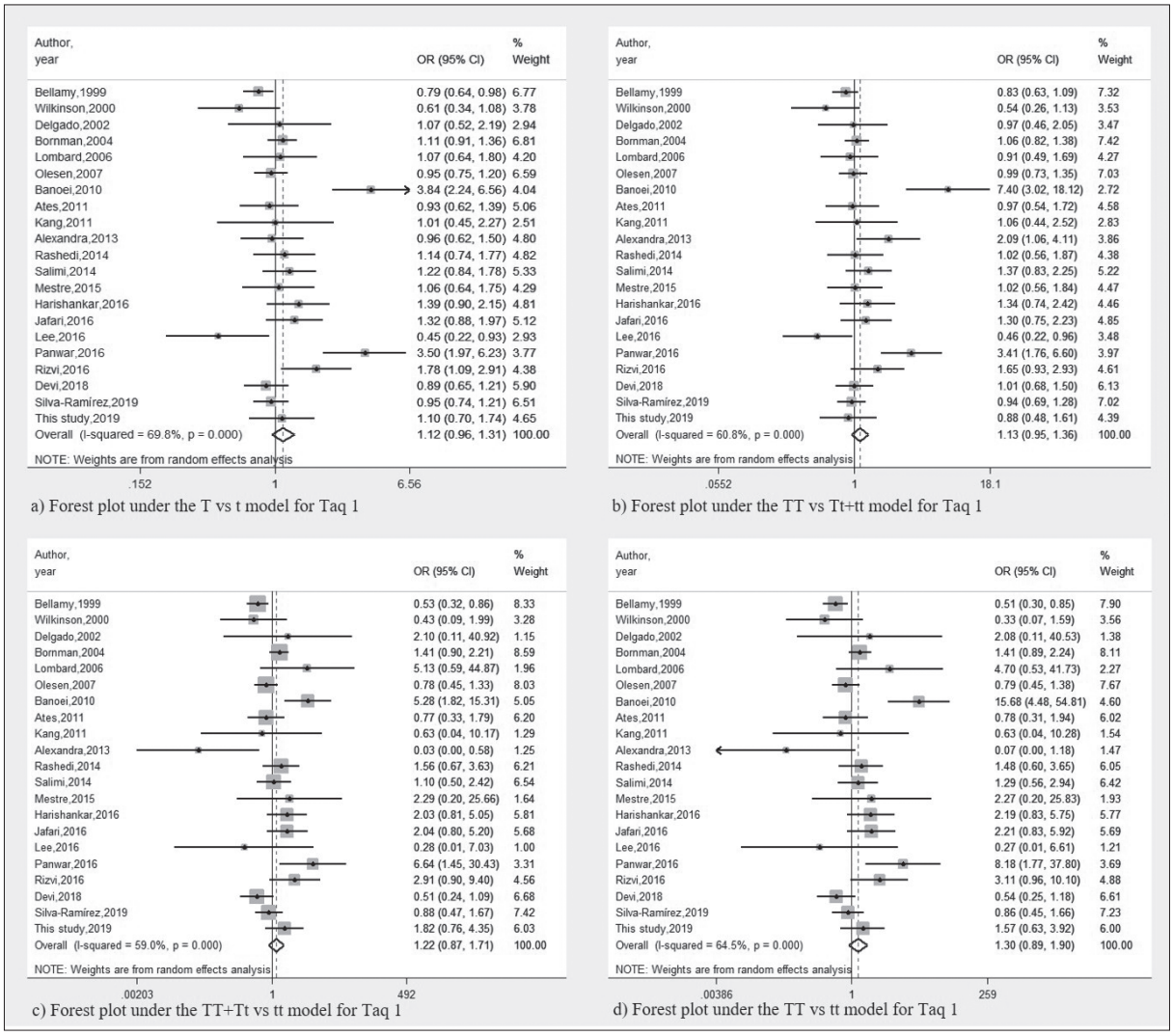

Figure 2. Forest graphs under different genetic models for the association between Taq I and PTB.

estimates and 95% confidence intervals (95% C.I.) under different genetic models and p values; for the Q heterogeneity test (PQ), for the significance of the pooled estimate (PZ) and for the Egger's bias test (PE) are given respectively for Taq I and Fok I polymorphisms. The number of studies included in the meta-analysis under each genetic model is indicated in parentheses by superscripts in the tables. OR estimates, 95% C.I. and the weights of independent studies are presented by forest plots (Figure 2, Figure 3). No publication bias was determined by the Egger's test. We could not find any association between Taq I or Fok I polymorphism and tuberculosis under any genetic model that were analyzed in meta-analysis and are given in Tables 7 and 8.

DISCUSSION

In many studies performed in different populations of the world in different centers, there were inconsistent results related with the VDR polymorphisms and TB susceptibility. The present study aimed to analyze this relation in a meta-analysis and a case control study from our population. We checked the Taq I and Fok I polymorphisms in our population and tried to compare the results with other reports from other regions of the world. In the meta analysis, we

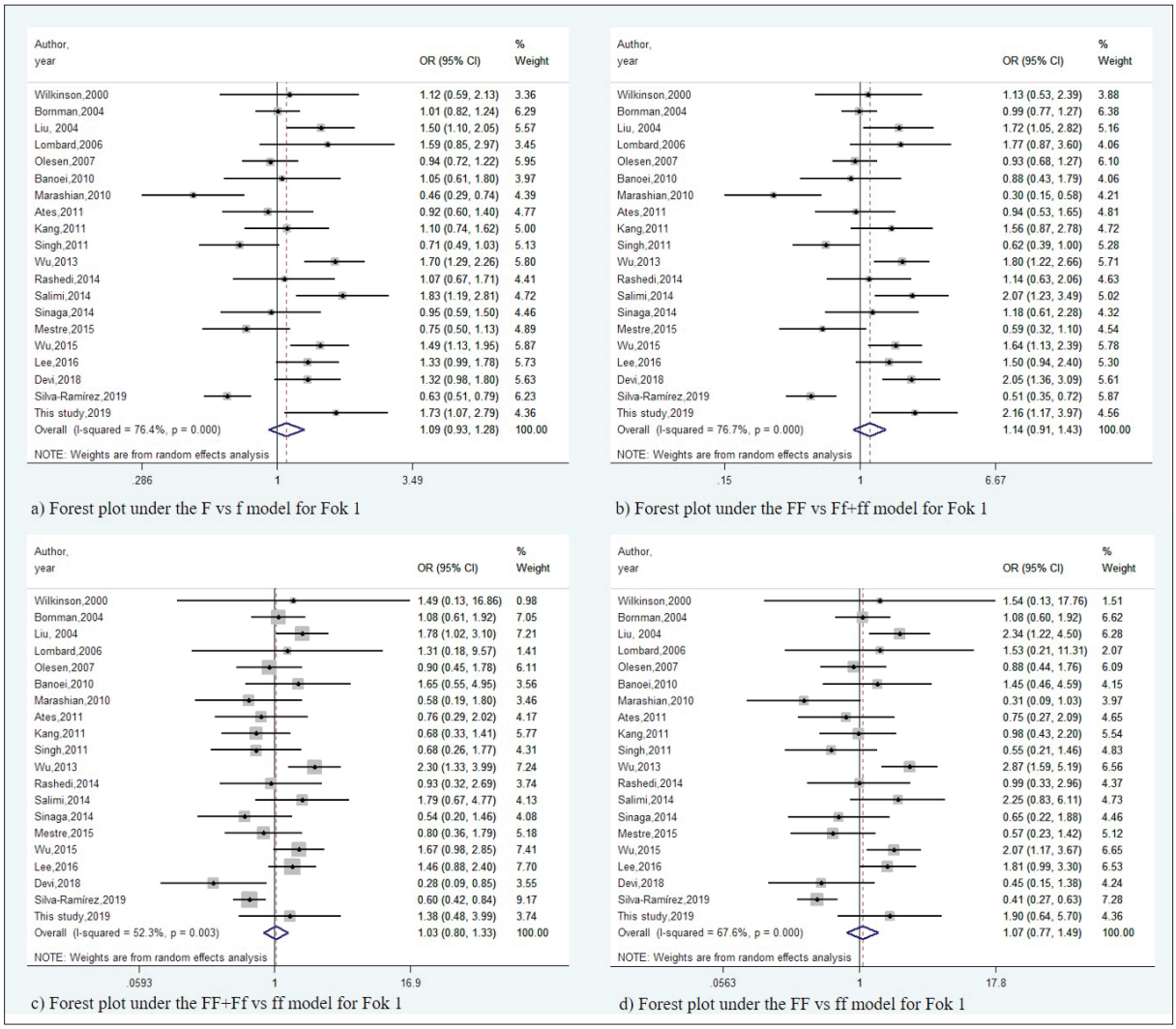

Figure 3. Forest graphs under different genetic models for the association between Fok I and PTB.

could not detect any association between the Fok I and Taq I polymorphisms of VDR gene and pulmonary tuberculosis. Although our case-control study revealed a significant association to Fok I (rs 2228570) polymorphism but on the other side we could not reach a meaningful result with Taq I polymorphisms. According to our results F allele carriers are protected (p 0.025), and also having an FF genotype is a protective characteristic but Ff genotype is susceptible to tuberculosis for Fok I polymorphism (p 0.044). When the analysis run for the sex, we got the significant results for the genotypes as well (p 0.043) just like in all populations the FF genotypes are protective against TB. These results do not match those of the study of Ates et al., in which the percentages of the genotypes of controls and normal subjects were so close to each other which was another study from Turkish population collected from Istanbul region[5]. The study we present here is the only report that was performed with Turkish PTB patients, which resulted in positive association to Fok I polymorphism.

In the meta-analysis for Taq I, it turned out that having t allele[6,7] and having T[8-10] in some populations is a susceptibility factor for tuberculosis, and on the other side, other studies, including our analysis as well, could not find a

significant association[5,11-24]. For other genetic models, the results can be seen in Figure 2.

In several studies, researchers have reported an association to increased risk of TB with Fok I genotypes. On the other side, in many populations, they have found no association between VDR variants and TB patients. For Fok I polymorphism, the protective allele seems to be f in different populations[24,25] and F is protective in other populations[19,26-28,this study]. Whereas the other populations gave negative association results[5,7,8,11,13-16,18-20,23,29]. The other models' results are given in Figure 3.

The population that was included in this case-control study was from the Anatolian region which has a higher incidence rate compared to the other[30] and also compared to Ates et al's report[5]. Although both groups are Turkish, our group of patients came from a more homogenous population in origin, which may explain the inconsistency of the results of ours and theirs.

CONCLUSION

Many different factors play roles in developing active diseases which includes complex molecular interactions in the immune system. In those, both acquired and inherited factors show their effects. The inconsistency of the results of single SNPs maybe explained by these factors, not only one or two SNPs can be used as a biomarker for developing active TB but maybe haplotypes should be considered. It is also possible to think the variations of many genes that take part in the host immune response contribute to develop an active form in the individuals. When the effect of vitamin D is thought, we considered that VDR was an important factor for developing a proper immune response for infectious diseases, so we focused on its polymorphisms, and even though the meta-analysis found no association between the SNPs we investigated and the PTB, we found an association in our group of patients for Fok I polymorphism in our case-control study and Taq polymorphism results showed parallelism with the meta-analysis.

The reason why some people develop active TB but others not is still in question and in order to be protected from *M. tuberculosis*, the puzzle needs to be solved by investigating other genes' polymorphisms with larger number of subjects.

ETHICS COMMITTEE APPROVAL

This study was obtained from İnönü University Faculty of Medicine Clinical Researches Ethical Committee (Date: 27.07.2016, Decision No: 2016/150).

CONFLICT of INTEREST

The authors declare no conflict of interests.

AUTHORSHIP CONTRIBUTIONS

Concept and Design: CA, HGB

Data Collection or Processing: CA, HGB, RDV

Analysis/Interpretation: CA, RDV

Literature Search: CA, HGB

Writing: CA, HGB

Final Approval: CA, HGB, RDV

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