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**ORIGINAL PAPER**

# *Apa* I and *Taq* I polymorphisms of VDR (vitamin D receptor) gene in association with susceptibility to tuberculosis in the Romanian population

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## Abstract

*The negative effect of the Mycobacterium tuberculosis infection in humans depends on the genetic profile of each individual in correlation with the environmental factors (socio-economic status, hygiene levels etc.).*

*Romania has the greatest number of tuberculosis cases among European countries; thus, in order to identify some of the risk factors for this infectious disease a case-control study was conducted. The main goal of this study was the identification of a possible association between ApaI and TaqI polymorphisms of the vitamin D receptor (VDR) gene and susceptibility to TB.*

*The data show small differences on the allele frequencies for both VDR gene polymorphisms between control and TB groups and higher differences when genotype frequencies are analyzed. The TB group is characterized by an excess of heterozygosity for both polymorphisms ($F_{is}$ for TaqI = -0.614, $F_{is}$ for ApaI = -0.558) compared with controls ($F_{is}$ for TaqI = 0.096, $F_{is}$ for ApaI = -0.044).*

*The absence of 'tt' and 'aa' genotypes in patients appears to be associated with resistance to TB development (OR = 0.1651, 95% CI 0.0622-0.4383 for 'tt' and OR = 0.1538, 95% CI 0.0646-0.3663 for 'aa', respectively), whereas 'Tt' and 'Aa' genotypes apparently confer susceptibility to TB (OR = 3.7669, 95% CI 2.0515-6.9165 for 'Tt', and OR = 2.2294, 95% CI 1.2044-4.1266 for 'Aa', respectively).*

*The results of this study are in accordance with those obtained by other authors for West African populations, but in total discrepancy with the results for Indian, Iranian and Chinese populations.*

**Key words:** TB susceptibility, VDR gene, *Apa*I, *Taq*I, Romanian population

## Introduction

Tuberculosis (TB) still remains an important cause of morbidity and mortality worldwide, and Romania occupies the first place among European countries regarding TB incidence [1]. Many studies indicated that in the immune interaction between the host and *Mycobacterium tuberculosis*, the genetic inherited factors play a key role.

Several case-control studies have identified the association between TB and candidate genes, such as the NRAMP1 (natural resistance-associated macrophage protein 1) gene [2-3], the VDR (vitamin D receptor) gene [4-6], the genes for 1 and 10 interleukins (IL-1 and IL-10), the gene for interferon γ and the TNF- α (tumor necrosis factor α) gene [7].

$D_3$ vitamin acts as a modulator activating the monocytes and stimulating the body's immune system [8-9]. The active form of vitamin D ($1,25\text{-}(OH)_2D_3$) is involved in the immune system regulatory activity via its binding with VDR. In the recent years, the VDR gene has drawn the researchers' interest, due to the hypothesized association between VDR genetic variation, immunity, and TB.

The VDR gene consists of a complex intron/exon structure located on the long arm of the human chromosome 12 [10]. It was mapped on 12q13-14 by Szpirer *et al*. [11], but Taymans *et al*., [12] considered it to be more centromeric (12cen-q12). It contains 15 exons (protein-coding exons 2-9 and untranslated exons 1a-1f) and 2 alternate promoter regions [12]. The VDR gene includes multiple allelic variants, some of which may lead to alterations in the VDR function and may contribute to susceptibility to immune mediated disorders. The most common variants are single nucleotide polymorphisms (SNPs), four of these polymorphisms: *Fok*I, *Bsm*I, *Apa*I and *Taq*I being most intensively investigated [13-15].

The *Apa*I SNP, found in intron 8, results in a T→G change (the T allele is designated 'A' while the G allele is designated 'a'). Because *Apa*I is intronic, away from the intron-exon boundaries, it is not known to produce splicing errors and, therefore, it is unlikely to have functional consequences [16].

The *Taq*I polymorphism is located within exon 9 where the T→C (ATT to ATC codon substitution) modification leads to a silent transition in codon 352 [17]. This polymorphism, similarly to *Apa*I, is unlikely to alter VDR function as both codons specify for isoleucine. Investigators have reported that there are no allele specific differences in VDR mRNA or even an association between the 't' allele and the reduced levels of VDR mRNA [15]. However, other studies indicated that the *Taq*I may influence the T helper cells type 1 (TH1) vs. T helper cells type 2 (TH2) balance. Homozygotes for 'tt' tend to produce a TH1-type immune response and 'TT' homozygotes produce a TH2-type response. Individuals homozygous for 'tt' have been found to be resistant to pulmonary TB, indicating that the TH1-type immune response is protective for the individuals with TB [18].

Using a case-control design, the main goal of this study was to establish the genetic profile of control and TB patients using the VDR gene polymorphisms (*Apa*I and *Taq*I) and to identify a possible association between these polymorphisms and sensibility/resistance to TB.

## Material and methods

Subjects

The study was conducted on 68 patients with active pulmonary TB (53 males and 15 females) diagnosed in "Marius Nasta" Hospital from Bucharest. At the same time, a control group of 110 unrelated, healthy individuals (56 males and 54 females) was randomly recruited. All subjects originated from the Southern region of Romania (Table 1) and were previously informed about the content of the research and gave their written consent for participation in this research.

**Table 1** Regional and sex distribution of TB patients and controls

| | TB patients | | Controls | |
|---|---|---|---|---|
| **Region** | **Males** | **Females** | **Males** | **Females** |
| Bucharest | 33 | 7 | 20 | 30 |
| South-West | 14 | 5 | 18 | 13 |
| South-East | 6 | 3 | 18 | 29 |
| **Total** | **53** | **15** | **56** | **54** |

VDR analysis

Genomic DNA was isolated from EDTA-stabilized venous blood using Wizard® Genomics DNA Purification Kit (Promega Corporation, Madison, WI, USA). VDR gene polymorphisms *(Apa*I *and Taq*I*)* were genotyped by the ARMS-PCR method [19], using the

following SSP primers: Apa–A-5'-GTGGGATTGAGCAGTGAGGT-3'; Apa–a-5'-GTGGGATTGAGCAGTGAGGG-3'; Taq–T-5'-CGGTCCTGGATGGCCTCA-3'; Taq–t-5-CGGTCCTGGATGGCCTCG-3'. The PCR reactions were performed in 25µl final volume containing 2.5µl GoTaq Flexi Buffer 5X, 0.5µl dNTP mix, 0,5µl $MgCl_2$, 0,5µl for each primer (A, a, T and t), 0,2µl Taq Polymerase, 1µl DNA and ultra pure water. The amplification parameters were the following: $96^0$C/1min; 5 cycles ($95^0$C/25sec, $61^0$C/40sec, $72^0$C/42sec.); 21 cycles ($96^0$C/25sec, $65^0$C/50sec, $61^0$C/45sec.); 4 cycles ($96^0$C/25sec, $55^0$C/60sec, $72^0$C/120sec.), and a final extension at $72^0$C/5min.

To assure the validity of the ARMS-PCR amplification, random samples of each polymorphic region were sequenced using the BigDye v3.0 sequencing chemistry and ABI PRISM 310 Genetic Analyzer (Applied Biosystems) [19]. No discrepancies were found compared with expected sequences.

Data analysis

The raw data for each locus and each population (allele and genotype frequencies, and $F_{is}$ value for heterozigosity level) were computed using the *GenePop* on the Web software, version 4.0.10 [20]. The genotype frequencies in patients and controls were tested for Hardy-Weinberg equilibrium (HWE) using 3x2 $\chi^2$ test (Fisher's method) with 4 *df*. Because the aim of the study was to identify the association between the VDR polymorphisms and TB susceptibility, exact G test for the genotypic differentiation was performed and $\chi^2$, p-values and S.E. (standard error) were determined. Also, the strength of association was estimated by crude odds ratios (OR), with 95% confidence interval (95% CI). The statistical significance was accepted for $p<0.05$.

## Results

Tables 2 and 3 present the raw data for each polymorphism in both control and TB groups, respectively.

The overall distribution of the *Taq*I allele and the genotype frequencies shows no major differences in and between groups (p=0.902, S.E.=0.00070) (Table 2). In what regards the genotype distribution, the 'TT' homozygotes have a similar occurrence in both sexes in control group (frequency is 0.393 in males, respectively 0.389 in females), which differs from what was recorded in TB patients (overall frequency 0.235 with 0.264 in males and 0.133 in females).

$F_{is}$ value, computed as in Weir & Cockerham (1984), represents a measure of the heterozygosity level; thus, when $F_{is}$ is positive there is a deficit of heterozygotes, and when is negative, an excess. Data analysis regarding *Taq*I polymorphism shows that there is an excess of heterozygosity in the patient group ($F_{is}$= -0.614), which is higher in the TB females ($F_{is}$= -0.750) than in males ($F_{is}$= -0.575).

For the *Apa*I polymorphism, the differences between the two analyzed groups are statistically significant (p=0.006, S.E.=0.00034): the 'a' allele is much better represented in the healthy individuals than in the affected ones ($f_{a\ control}$ = 0.491, $f_{a\ patients}$ = 0.360) (Table 3). Both control and patient groups are characterized by an excess of heterozygosity, but this is much more evident in TB affected individuals ($F_{is}$ = -0.558) than in healthy ones ($F_{is}$= -0.044). Males present a higher degree of heterozygote excess ($F_{is}$= -0.625) than females ($F_{is}$= -0.330), a situation which is conversely comparative with *Taq*I.

**Table 2**. Distribution of VDR-*Taq*I polymorphisms in TB patients and control group

| | **Control** (n=110) | | | **TB patients** (n=68) | | | **OR (95%CI)*** |
|---|---|---|---|---|---|---|---|
| | Males | Females | Overall | Males | Females | Overall | |
| **Allele number/frequencies** | | | | | | | |
| T | 70/0.625 | 64/0.593 | 134/0.64 | 67/0.632 | 17/0.733 | 84/0.618 | |
| t | 42/0.375 | 44/0.407 | 86/0.36 | 39/0.368 | 13/0.267 | 52/0.382 | |
| **Genotype number/frequencies** | | | | | | | |
| TT | 22/0.393 | 21/0.389 | 43/0.391 | 14/0.264 | 2/0.133 | 16/0.235 | 0.50 (0.26-0.94) |
| Tt | 26/0.464 | 22/0.408 | 48/0.436 | 39/0.736 | 13/0.867 | 52/0.765 | 3.77 (2.05-6.92) |
| tt | 8/0.143 | 11/0.203 | 19/0.173 | 0 | 0 | 0 | 0.17 (0.06-0.44) |
| **Fis** | 0.019 | 0.167 | 0.096 | -0.575 | -0.750 | -0.614 | |
| **p** | 0.6149 | | | High. sign. | | | |
| $\chi^2$ | 2.6673 | | | ∞ | | | |

Abbreviations: OR – odds ratio; CI – confidence interval; TT – homozygotes for the absence of the *Taq*I second restriction site on both alleles; Tt – heterozygotes; aa – homozygotes for the presence of the *Taq*I second restriction site on both alleles.
*For a 2x2 Contingency Tables, crude OR calculated from overall data for genotype comparisons: TT vs. Tt+tt , Tt vs. TT+tt, tt vs. TT+Tt

**Table 3.** Distribution of VDR- *Apa*I polymorphisms in TB patients and control group

| | **Control** (n=110) | | | **TB patients** (n=68) | | | **OR (95%CI)*** |
|---|---|---|---|---|---|---|---|
| | Males | Females | Overall | Males | Females | Overall | |
| **Allele number/frequencies** | | | | | | | |
| A | 51/0.454 | 57/0.527 | 108/0.509 | 65/0.613 | 22/0.733 | 87/0.640 | |
| a | 61/0.546 | 51/0.473 | 112/0.491 | 41/0.387 | 8/0.267 | 49/0.360 | |
| **Genotype number/frequencies** | | | | | | | |
| AA | 12 /0.214 | 15/0.259 | 27/0.245 | 12/0.227 | 7/0.467 | 19/0.278 | 1.19 (0.59-2.37) |
| Aa | 29/0.518 | 29/0.555 | 58/0.528 | 41/0.773 | 8/0.533 | 49/0.721 | 2.23 (1.24-4.13) |
| aa | 15/0.268 | 10/0.186 | 25/0.227 | 0 | 0 | 0 | 0.16 (0.06-0.37) |
| **Fis** | -0.029 | -0.108 | -0.044 | -0.625 | -0.333 | -0.558 | |
| **p** | | 0.9754 | | | High. sign. | | |
| $\chi^2$ | | 0.4806 | | | ∞ | | |

Abbreviations: OR – odds ratio; CI – confidence interval; AA – homozygotes for the absence of the *Apa*I restriction site on both alleles; Aa – heterozygotes; aa – homozygotes for the presence of the *Apa*I restriction site on both alleles.
*For a 2x2 Contingency Tables, OR calculated from overall for genotype comparisons: AA/Aa+aa , Aa/AA+aa, aa/AA+Aa

The control group is in Hardy-Weinberg equilibrium for both polymorphisms ($\chi^2$=3.7295, df=8, p=0.8977), but the situation is different for the TB patient group, which is characterized by a significant departure from Hardy-Weinberg equilibrium ($\chi^2$=∞, df=8, p=high.sign.).

The null hypothesis that genotypes have the same distribution in all populations was tested by performing an unbiased estimate of the P-value of a log-likelihood ratio (G) based exact test. This analysis revealed a significant difference concerning the genotype distribution across both loci between TB affected patients and control ($\chi^2$=10.461, p=0.033). Taking into account the genotype combinations for both polymorphisms, in TB patients the prevalence of

the double heterozygote genotype 'TtAa' (48.5%), followed by the heterozygotes for only one polymorphism (TTAa 23.5% and TtAA 28%) was noticed.

In our study, both homozygote genotypes for rare allele, 'aa' and 'tt', seem to be associated with a resistance to the development of active TB (OR=0.1538, 95%CI: 0.0646-0.3663 for 'aa' and OR=0.1651, 95% CI: 0.0622-0.4383 for 'tt'), while the heterozygote genotypes, 'Aa' and 'Tt' are correlated with susceptibility to pulmonary TB development (OR=2.2294, 95% CI: 1.2044-4.1266, respectively OR=3.7669, 95% CI: 2.0515-6.9165)(Tables 2-3).

## Discussion

Several case-control studies, performed in populations with high TB incidence, have studied the correlation between the VDR gene polymorphisms and genetic susceptibility to TB, but the results were inconclusive, especially because the populations were ethnically and geographically different and genetically diverse [4-6, 13, 15, 21-23].

Using the same type of study, we have observed a possible association between biallelic variants of the *Apa*I and *Taq*I VDR gene polymorphisms and susceptibility to develop active TB in the Romanian population from the Southern region of the country. Because tuberculosis is prevalent in males rather than in females, the data were analysed not only for the entire group but also taking into account the gender of the patients. In TB patients there is an increased frequency of heterozygote genotypes 'Aa' and 'Tt', both in males and females, compared with controls, whereas an increased frequency of homozygote genotypes 'AA' and 'TT' was observed in male and female controls rather than in patients. The genotypes 'aa' and 'tt' appear to be associated with resistance to TB development, whereas the genotypes 'Aa' and 'Tt' could be responsible for Romanian population susceptibility to TB.

These data are in accordance with the ones obtained by Bellamy et.al. [4] for the Gambian population and by Wilkinson et al. [6] for the Gujarati Hindus from London, which showed significantly lower frequency of the *Taq*I-tt genotype among TB patients. Thus, the 'tt' genotype may reduce susceptibility to either initial mycobacterial infection or to the progress of latent infection to active disease. However, we cannot conclude that the 'T' allele predisposes to a more aggressive form of TB due to its similar frequencies in both studied groups.

Other case-control studies, carried out on northern Indian [13-15], Iranian [21] and Chinese [5] populations indicated an association of the 'tt' genotype with susceptibility to pulmonary TB, either gender related, or overall. But, Delgado et al. [22], in a similar study, found no association between the VDR gene and TB in Cambodians.

Regarding the *Apa*I polymorphisms of the VDR gene there are a few studies involving this SNP in association with TB susceptibility. A study conducted by Selvaraj et.al. on the Indian population shows that the 'AA' genotype is associated with resistance to pulmonary TB in males, but not in females [15]. The data obtained by us bring into question a possible association of homozygote genotype 'aa' with the decreased risk of active TB development, but we believe that it is important to emphasize that the *Apa*I SNP has no known functional significance, because it is located within intron 8 of the VDR gene. The 'AA' allele frequency in the TB group (0.64) is similar with that recorded for African populations (0.67) [23] which are more "susceptible" to develop TB. More significant associations were recorded in other studies for certain combined haplotypes (i.e. *Fok*I-*Apa*I) and not for *Apa*I alone [15], so it is necessary to extend the research with other type of polymorphisms from the same genomic region.

## Conclusions

Our findings add to the existing data in the literature concerning the associations found between the VDR gene SNPs and active tuberculosis among different populations and geographical areas. One could speculate that the polymorphic variants of the VDR gene, together with other gene and environmental factors, may be responsible for an altered cell-mediated immunity to *M. tuberculosis* in a susceptible or resistant male or female host [14]. The different results obtained in various case-control studies from literature can be explained by the fact that the VDR gene is not the only gene that is involved in the genetic determination of TB susceptibility. Also, gene-environment and gene-gene interactions might modulate the expression of the VDR gene and the action of vitamin D through this receptor. Even if the *Apa*I and *Taq*I restriction sites at 3' untranslated region of the VDR gene are considered nonfunctional SNPs, the data suggests that they are in strong linkage disequilibrium with one or more functional polymorphisms involved in the regulation of VDR expression [4].

In the present study, some limitations should be considered when interpreting the results. The generalisation of the conclusion may be limited because the sex ratio of our affected group was 1:0.28. Also, the assessment of the environmental factors such as diet, tobacco and alcohol consumption was based on self-reported data, and the possibility of inaccurate exposure should therefore be considered. The susceptibility and resistance to pulmonary tuberculosis are the result of the interactions between environment, socio-economic status and host genes and, therefore, further studies are required to elucidate the complex correlations between the VDR gene variants and the immune response against TB. These data will lead to a better understanding of the immunological and genetic pathways in tuberculosis, and will offer new insights into the potential treatment and prophylaxis – all very important aspects that have to be clarified in order to reduce the incidence of this disease in countries such as Romania, with high prevalence and incidence of *M. tuberculosis* infections.

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