

VARIANTS IN *VDR* AND *NRAMP1* GENES AS SUSCEPTIBILITY FACTORS FOR TUBERCULOSIS IN THE POPULATION OF SERBIA

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Tuberculosis (TB) is granulomatous diseases caused by *Mycobacterium tuberculosis* (MTB). TB is a highly **infectious disease** that primarily affects the lungs. One-third of human population is infected with MTB, therefore it is of utmost significance to determine the factors that influence the individual susceptibility to the disease. Host genetic factors have been recognized as essential for susceptibility to TB, since only 5% to 10% of infected individuals develop the disease. A number of candidate genes has been intensively studied, the most of which were connected with the function of macrophages, thus participating in immune response. Here we examined the gene variants of *VDR* (*FokI*) and *NRAMP1* (INT4, D543N, 3'UTR) genes in aim to make the correlation between these genetic factors and risk of TB in Serbian patients. This study included 110 TB patients and 67 healthy controls. Pulmonary TB was diagnosed by clinical symptoms, radiological evidence of TB and bacteriological criteria (Culture- positive/ smear- positive). Genotyping was performed using PCR-RFLP method. Our findings revealed significant prevalence of ff genotype and variant allele f of the *FokI VDR* gene variant in patients compared to control group. Based on the our results the carriers of ff genotype are five times more at risk to tuberculosis than carriers of FF and Ff genotype in our population. The

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results of analyzed SNPs in *NRAMP1* gene showed no statistically significant difference in distribution of the gene variants between patient and control groups. Therefore, we could conclude that the genotype of the *VDR* gene is a factor that strongly contributes to susceptibility to TB in Serbian population.

Key words: Genetics, Pulmonary tuberculosis, Polymorphism

INTRODUCTION

Tuberculosis (TB) is a highly **infectious disease** that primarily affects the lungs, but can also affect other organs. TB is one of the leading public health problems according to WHO global Tuberculosis Report from 2014, where the annual incidence in 2013 was estimated as 9.0 million people and 1.5 million died from the disease (http://www.who.int/tb/publications/global_report/en/). In Serbia the annual incidence in 2013 was 1211 new cases (the incidence rate 17/100000) (<http://www.tbc.zdravlje.gov.rs/eng/>). Considering that approximately one-third of the world's population is infected, but that 5% to 10% of infected individuals develop disease (FRIEDEN *et al.*, 2003), it is of utmost significance to determine the factors which influence the individual susceptibility to the disease. It is well known that environmental and lifestyle factors should be considered, but lately host genetic factors have been recognized as essential for susceptibility to TB (BELLAMY, 2003). In the past two decades, multiple candidate genes have been investigated in relation to TB, showing significant discrepancies mostly related to the ethnicity, but also to the severity of the disease and existence of co-infections, mostly HIV. The most of the target loci were localized in genes participating in immune response. Vitamin D receptor (VDR), along with Natural resistance-associated macrophage protein (NRAMP1), encoded by solute carrier gene *SLC11A1*, are factors shown to be involved in intracellular killing of *Mycobacteria* (BELLAMY *et al.*, 2000, ZMUDA *et al.*, 2000). VDR and NRAMP1 exert their immuno-modulatory effects through activation of monocytes and restriction of growth of MTB in macrophages (LEMIRE *et al.*, 1984).

In recent years, it became apparent that low serum level of vitamin D plays a role in susceptibility to infection (NNOAHAM and CLARKE, 2008, CANNELL *et al.*, 2006, GINDE *et al.*, 2009) and development of autoimmune diseases (CUTOLO, 2008). The effects of active metabolite of vitamin D 1,25-dihydroxycholecalciferol ($1,25(\text{OH})_2\text{D}_3$) are achieved by interaction with the vitamin D receptor (VDR), a ligand-dependent transcription regulatory molecule, belonging to the superfamily of nuclear receptors. The majority of immune cells express the vitamin D receptor, and more than 900 genes are regulated by VDR (KONGSBÄK *et al.*, 2013). The interaction of $1,25(\text{OH})_2\text{D}_3$ with the VDR is able to activate monocytes, stimulate cell mediated immunity and suppress lymphocyte proliferation (TACHI *et al.*, 2003). Many microbes down-regulate the expression of *VDR* gene to weaken the host defense, but the variants in *VDR* gene that modulate the expression of *VDR* gene or causing the change on protein level, also influence the immunological capacity of the host.

The NRAMP1 protein is an integral membrane protein expressed exclusively in the lysosomal compartment of monocytes and macrophages (CANONNE-HERGAUX *et al.*, 1999), where it functions as iron transporter. It influences the phagolysosomal function of macrophages, maintenance of acidity, and production of nitric oxide. NRAMP1 is involved in early and effective granulomatous response, which is critical for the containment of bacterial burdens or the spread of pulmonary MTB infection. The bacterial burden could result in future clinical TB.

Not many studies analyzed gene variants in the *VDR* and *NRAMP1* genes in relation to TB on Caucasians. Moreover, existing studies on Caucasians showed controversial findings to African and Asian populations, which were mostly studied so far, probably due to different genetic background in these populations. Therefore, we performed the case - control study on Serbian TB patients, which is the first time to investigate genetic basis of TB in our population. We analyzed gene variant *FokI* in *VDR* gene rs2228570 along with three variants of *NRAMP1* gene: INT4 (469+14G>C) rs3731865, D543N (codon 543 Asp to Asn) rs17235409 and 3'UTR (1729 + 55del4) rs17235416 in aim to make the correlation between these genetic factors and risk of TB in Serbian patients.

MATERIALS AND METHODS

Patients and controls

Our study was approved by the Ethics Committee of the Clinical Centre of Serbia, Belgrade, Serbia. Written informed consent was obtained for all patients.

The study enrolled 110 patients, 71 men and 39 women, with a mean age of 60.33 (SD-17.82), range 21-86. Patients were underwent first-line **TB treatment** from February 2013 to February 2014 at Clinic of Pulmonology, Clinical Center of Serbia in Belgrade, Serbia. All patients had active pulmonary TB diagnosed by clinical symptoms, radiological evidence of TB and bacteriological criteria (positive sputum sample for acid-fast bacilli and/or positive sputum culture of the MTB strains). All TB patients included in this study were drug sensitive. The control group consisted of 67 healthy volunteers, matched to patient's group by ethnicity, gender and age. All patients and controls were vaccinated with BCG (bacillus Calmette-Guerin).

Blood sampling and DNA preparation

Venous blood was collected into two 4.5-ml sodium citrate anticoagulant tubes (Vacutainer, Becton-Dickinson, Plymouth, UK). Genomic DNA was isolated from whole peripheral blood with QIAampDNA Mini Kit (Qiagen GmbH, Hilden, Germany), and stored at -20°C until analysis.

Genotyping

The detection of gene variants of *NRAMP1* and *VDR* genes were determined using the PCR-RFLP method using published protocols. The gene variant *FokI* rs2228570 of *VDR* was determined using protocol described by Kang *et al.* (KANG *et al.*, 2011). Details are available in Supplement material. Gene variants INT4 (469+14G>C) rs3731865 as well as D543N (codon 543 Asp to Asn) rs17235409 and 3'UTR (1729 + 55del4) rs17235416 of *NRAMP1* gene were genotyped using protocols described in publications (JIN *et al.*, 2009, OUCHI *et al.*, 2003), respectively.

Statistical analysis

Comparison of allele and genotype frequencies between patients and controls were performed by Chi-square test with Yates's correction or Fisher exact test wherever appropriate. Differences were considered to be statistically significant in all cases when p value was less than 0.05. Odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated as measures of association. Haldane correction was used where appropriated. Data was analyzed using the SPSS for Windows 20.0 software (SPSS, Inc, Chicago, IL, USA).

RESULTS

Strong association of FokI VDR polymorphism to TB

The results of the analysis of the *FokI VDR* gene variant in patients with tuberculosis and controls are presented in Table 1. All genotype and allele distributions were in Hardy-Weinberg equilibrium. We found significant association of ff genotype with TB using recessive model ($P=0.01$, $OR=5.0337$, $95\%CI= 1.149-17.598$). The presence of f allele was significantly more frequent in patients compared to control group ($P=0.02$, $OR=1.1753$, $95\%CI=1.111 - 2.767$).

Table 1. Genotype and allele distributions and frequencies in VDR gene among patients with TB and control group

| VDR | Genotype | Patients n=110 | Controls n=67 | OR (95% CI) | P Value |
|-------------|----------|-------------------|------------------|------------------|------------|
| rs2228570 | FF | 37 (34.0%) | 30 (45.0%) | 5.0337 | 0.01 |
| | Ff | 52 (47.0%) | 34 (50.5%) | (1.149 – 17.598) | |
| <i>FokI</i> | ff | 21 (19.0%) | 3 (4.5%) | | |
| Allele | F | 57.0% | 70.1% | 1.1753 | 0.02 |
| frequency | f | 43.0% | 29.9% | (1.111 – 2.767) | |
| FF+Ff vs ff | | | | | |

#Abbreviations: OR- Odds Ratio; CI- Confidence Interval

Table 2. Genotype and allele distributions and frequencies in NRAMP1 gene among patients with tuberculosis and control subjects

| NRAMP1 | Genotype | Patients n=110 | Controls n=67 | OR (95% CI) | P Value |
|---------------|----------|-------------------|------------------|------------------|------------|
| rs3731865 | GG | 63 (57.3%) | 45 (67.2%) | 1.526 | 0.21 |
| | GC | 42 (38.2%) | 20 (29.9%) | (0.809– 2.878) | |
| INT4 | CC | 5 (4.5%) | 2 (2.9%) | | |
| Allele | G | 76.4% | 82.0% | 1.419 | 0.20 |
| frequency | C | 23.6% | 18.0% | (0.827-2.435) | |
| GG vs GC + CC | | | | | |
| rs17235409 | GG | 109 (99.1%) | 67 (100%) | 1.229 | 0.61 |
| | GA | 1 (0.9%) | 0 | (0.041-37.148) | |
| D543N | AA | 0 | 0 | | |
| Allele | G | 99.5% | 100.0% | 1.224 | 0.62 |
| frequency | A | 0.5% | 0 | (0.041– 36.728) | |
| GG+GA vs AA | | | | | |
| rs17235416 | II | 109 (99.1%) | 66 (98.5%) | 0.6091 | 1 |
| | ID | 1 (0.9%) | 1 (1.5%) | (0.012 – 31.061) | |
| 3'UTR | DD | 0 | 0 | | |
| Allele | I | 99.5% | 99.2% | 0.6073 | 1 |
| frequency | D | 0.5% | 0.8% | (0.038 – 9.791) | |
| II + DI vs DD | | | | | |

#Abbreviations: OR- Odds Ratio; CI- Confidence Interval

Lack of association between candidate SNPs in NRAMP1 gene and TB

The results of the analysis of the *NRAMP1* gene variants INT4, D543N and 3'UTR in patients with TB and controls are shown in Table 2. All genotype and allele distributions were in Hardy-Weinberg equilibrium. Recessive model was used for of all genotype comparisons. There was no statistically significant difference in distribution of the allele variants between patient and control groups for all three examined SNPs in *NRAMP1* gene. For two SNPs rs17235409 and rs17235416, we did not find any homozygote carriers either in patients or in control group. Obviously, both polymorphisms were in strong linkage disequilibrium and at a marginally low allele frequency (<0.05) in our population.

DISCUSSION

The main target of MTB is the respiratory tract. Alveolar macrophages are the first line of defense of the host against pathogens. To establish the infection, MTB must first get into the alveolar macrophages through phagocytosis or endocytosis. The capability of MTB to survive inside of macrophages is crucial for its pathogenesis, and a key to MTB virulence is its ability to inhibit the fusion of phagosomes with lysosomes. On the other hand, fusion and normal function of phagolysosomal vesicles is essential for the host defense against MTB. Thus, the genes related to the function of macrophages have been investigated as candidate genes for susceptibility to TB, as well as for transition from latent to active TB. The principal in this respect is the *NRAMP1* gene, which has been studied extensively. In general, the significance of *NRAMP1* for development of TB was demonstrated to be variable in various ethnical groups (VEJBAESYA *et al.*, 2007, RYU *et al.*, 2000, ZHANG *et al.*, 2005, DUBANIEWICZ *et al.*, 2005). Results from recent meta-analysis emphasize the ethnic-specific genetic association to TB (MEILANG *et al.*, 2012). Variations in representation of specific genotype have also been observed in relation to severity of disease and transition from latent to active form of the disease (ZHANG *et al.*, 2005). There are only a few studies on *NRAMP1* gene in Caucasians, showing that European-origin populations are believed to have a high level of resistance to TB because of intensive natural selection for the last 300 years (STEAD, 1992).

Our results showed no correlation of INT4, D543N and 3'UTR polymorphisms with susceptibility to TB in our study group. We have found low frequency of variant alleles of D543N and 3'UTR polymorphisms, as shown in studies in other European populations (DUBANIEWICZ *et al.*, 2005, STAGAS *et al.*, 2011, SOBORG *et al.*, 2002). The distribution of alleles in our population was different from those obtained on Asian and African populations (BELLAMY *et al.*, 1998, RYU *et al.*, 2000, LIAW *et al.*, 2002), showing significant ethnical divergence.

Among four polymorphisms of *VDR* gene mostly analyzed so far (*FokI*, *BsmI*, *ApaI* and *TaqI*), in the context of different diseases, only *FokI* polymorphism alters the structure of *VDR* protein. Namely, *FokI* SNP is located at the translation start site and the T>C transition causes truncation of the protein for three amino acids. Consequently, the two forms of the protein exist: long (presence of nucleotide T-f form) and short (presence of nucleotide C-F form). These two protein forms differ in their transcriptional activity (VAN ETTEN *et al.*, 2007). *VDR FokI* gene variant was shown to be related to susceptibility to tuberculosis in many studies and ethnicities (LIU *et al.*, 2004, RATHORED *et al.*, 2012). Although recent meta-analysis showed that *FokI* polymorphism was associated with a significantly increased risk of tuberculosis in the Chinese population (CHEN *et al.*, 2013), our results showed that the variant allele f is also a significant risk factor for tuberculosis in Serbian population. According to our findings, ff genotype and f

allele were much more frequent in TB patients than in control subjects. Thus, the carriers of ff genotype are five times more at risk to tuberculosis than carriers of FF and Ff genotype. In recent years, the anti-bacteria effects of Vitamin D in the human immune system have been explored extensively (ZASLOFF, 2006). In this respect, personalized approach in prevention and treatment of TB according to genetic profile of *VDR* gene should be introduced.

CONCLUSIONS

This is the first report of genetic basis of tuberculosis in Serbian population. The present study suggests that *FokI* variant of *VDR* gene may be associated with susceptibility to tuberculosis in Serbian patients. Although we studied common polymorphisms in *NRAMP1* and *VDR* genes, further analysis of other factors related to tuberculosis will gain clearer picture of the role of genetic factors to disease susceptibility.

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VARIJANTE U GENIMA *VDR* I *NRAMP1* KAO FAKTORI PREDISPOZICIJE ZA TUBERKULOZU U SRPSKOJ POPULACIJI

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Izvod

Tuberkuloza je granulomatozna bolest koju izaziva bakterija *Mycobacterium tuberculosis* (MTB). Trećina svetske populacije inficirana je MTB, te je stoga veoma značajno odrediti faktore koji dovode do individualne osetljivosti na ovaj bakterija. Obzirom da od ukupnog broja zaraženih osoba samo 5-10% razvije bolest, pretpostavlja se da u osnovi individualne osetljivosti leže genetički faktori. Veliki broj gena kandidata proučavan je u ovom kontekstu, a većina je vezana za funkcionisanje makrofaga, odnosno imunskog sistema. Predmet ovog istraživanja bio je da ispita genske varijante u genima *VDR* (*FokI*) i *NRAMP1* (INT4, D543N, 3'UTR) u cilju povezivanja ovih genetičkih faktora sa predispozicijom za TB u srpskoj populaciji. Studija je obuhvatila 110 pacijenata obolelih od tuberkuloze i 67 zdravih kontrola. Plućna TB dijagnostifikovana je na osnovu kliničkih simptoma, radioloških nalaza i bakterioloških kriterijuma (pozitivan nalaz u kulturi/pozitivan razmaz sputuma). Genske varijante su detektovane koršćenjem metode PCR-RFLP i potvrđene metodom sekvenciranja po Sangeru. Naši rezultati su pokazali statistički značajnu razliku u distribuciji genotipa ff i varijantnog alela f u okviru genske varijante *FokI VDR* kod pacijenata u odnosu na kontrolnu grupu. Na osnovu dobijenih rezultata zaključeno je da nosioci genotipa ff imaju pet puta veći rizik za nastanak tuberkuloze od nosilaca genotipova FF i Ff u našoj populaciji. Analizom genskih varijanti gena *NRAMP1* nisu pronađene statistički značajne razlike u distribuciji genotipova niti alela između ispitivanih grupa. Naši rezultati jasno ukazuju da je gen *VDR* važan faktor koji doprinosi osetljivosti na MTB, a time i razvoju TB u srpskoj populaciji.

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