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Original Article

Impact of the Polymorphism of the *PACRG* and *CD80* Genes on the Development of the Different Stages of Tuberculosis Infection

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What's Known

- After infection by *Mycobacterium tuberculosis,* within 2 years, 5% of individuals develop clinical manifestations of primary tuberculosis. Five to ten percent of those infected during the course of their life develop secondary tuberculosis.
- There is an assumption that different genetic factors affect the risk of primary and secondary tuberculosis, but genetic studies considering these stages of the disease separately are limited.

What's New

- In our study, for the first time, we identified an association between polymorphisms of *PACRG* (rs10945890) and *CD80* (rs1880661) and the development of different stages of tuberculosis infection. These genes have never been studied before regarding tuberculosis.
- We found a novel proof that predisposition to different forms of tuberculosis infection is under the control of different genetic factors of the host.

Abstract

Background: Tuberculosis (TB) is one of the most significant health-care problems worldwide. The host's genetics play an important role in the development of TB in humans. The disease progresses through several stages, each of which can be under the control of different genes. The precise genes influencing the different stages of the disease are not yet identified. The aim of the current study was to determine the associations between primary and secondary TB and the polymorphisms of novel candidate genes for TB susceptibility, namely *CD79A, HCST, CXCR4, CD4, CD80, CP, PACRG,* and *CD69.*

Methods: A total of 357 patients with TB (130 cases with primary TB and 227 cases with secondary TB) from the Siberian region of Russia as well as 445 healthy controls were studied. The study was performed at the Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, Russia, between July 2015 and November 2016. Genotyping was carried out using MALDI-TOF mass spectrometry and PCR-RFLP. The associations between the single-nucleotide polymorphisms and TB were assessed using logistic regression adjusting for covariates (age and gender). Multiple testing was addressed via the experiment-wise permutation approach. The statistical significance threshold was a P value less than 0.05 for the permutation P values. The analyses were done in R 3.2 statistical software.

Results: An association was established between the rs1880661 variant of the *CD80* gene and secondary TB and the rs10945890 variant of the *PACRG* gene and both primary and secondary TB. However, the same allele of *PACRG* appeared to be both a risk factor for reactivation (secondary TB) and a protector against primary infection.

Conclusion: The results suggested that the *CD80* and *PACRG* genes were associated with susceptibility to different forms of TB infection in the Russian population.

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Keywords • Tuberculosis • Polymorphism, genetic • Polymorphism, single nucleotide

Introduction

Tuberculosis (TB) remains one of the most dangerous infectious human diseases. In 2015, there were approximately 10.4 million new TB cases and 1.8 million people (including 0.4 million

people with HIV) died as a result of the disease.[1] Importantly, the infection of a human with *Mycobacterium tuberculosis* (*M. tuberculosis*) is not enough for TB to progress into a clinical disease. Only about 10% of infected cases develop active disease, while the rest remain latently infected or completely get rid of the bacterium. The outcome of the infection is dictated by such factors as the environment, virulence of the bacterial strain, infection load, and host's individual immune system features which are strongly genetically determined.

The involvement of certain regions of the human genome in the susceptibility to TB has been the target of active research over the past decades. Candidate genes studies have revealed many gene-encoding enzymes with immune functions, and some of them have been shown to exert a "major gene" effect on TB susceptibility.[2] Thus far, 10 genome-wide association studies on TB have been published; they have identified more than 20 genes associated with the disease including *ASAP1, AGMO, FOXP1*, which are involved in the functioning of macrophages and dendritic cells.[3]

Further, studies on atypical familial mycobacteriosis have revealed rare mutations in the *IL12B/IFNG* genes, responsible for anti-infectious immunity. The mutations cause the development of severe and usually lethal disease in response to nonpathogenic or mildly pathogenic bacteria such as *M. bovis*, *M. avium*, and *Salmonella enterica*.[4] These studies have played an important role in the identification of genes involved in immune response against mycobacteria.

Despite the remarkable achievements in understanding the pathogenesis of TB, there are substantial unresolved issues in the diagnosis, prophylaxis, and treatment of the disease. Lack of understanding of the mechanisms of the reactivation of latent infections, which creates a huge reservoir of dangerous disease distribution for many years, is especially worrying.[5] The existent diagnostic tools are based on the analysis of the sputum and the X-ray examination of patients with *M. tuberculosis*; however, their disadvantages include a delay in the finding of the bacterium in sputum, which postpones the treatment.[6] Additionally, there is a lack of information on molecular genetic mechanisms whereby susceptibility to TB converts into the disease, resulting in a slow progress in the development of effective treatments and prevention strategies.

More recently, systems biology and bioinformatics approaches have been utilized for the discovery of novel anti-TB drug targets. In particular, bioinformatics strategies are directed toward the studies of host–pathogen interactions[7, 8] because it is known that the success of *M. tuberculosis* is driven by its capability to modify human immune response,[9] with the different strains of the bacterium being able to induce various patterns of the host's immune response.[10]

An approach based on the revelation of functional interactions between genes and proteins involved in gene networks might improve our understanding of the nature of the dynamic reaction to infection and help establish the most important molecular participants in the disease development. Thanks to the network approach describing protein–protein interactions for the genes differentially expressed in patients with TB, a pattern of genes called "common core" for the disease has been discovered including the genes that are important in immune response such as *STAT1*, *PLSCR1*, *C1QB*, *OAS1*, *GBP2*, and *PSMB9*.[11]

In our previous study, we reconstructed an associative network for TB and revealed novel candidate genes including *CD4, CD69, CD79, CD80, MUC16, HCST, ADA, CP, SPP1, CXCR4, AGER,* and *PACRG*.[12] As a follow-up, in the current study, we analyzed regulatory polymorphisms for the genes from the associative network to assess their pathogenetic significance for the different stages of TB infection.

Materials and Methods

The present study was approved by the Ethics Committee of the Research Institute of Medical Genetics of Tomsk NRMC, and signed informed consent was obtained from all the participants. The study was performed at the Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, Russia, between July 2015 and November 2016.

The diagnosis of TB was established on the basis of sputum microscopy data with mandatory X-ray examination of the lungs to determine the form of the disease and the prevalence of a specific process. All the patients with TB were divided into subgroups of primary TB and secondary TB depending on the clinical features. The patients with lymph-node involvement and tuberculous primary complex were assigned to the primary TB subgroup. The subgroup of secondary TB comprised individuals with the pulmonary forms of the disease characterized by changes of a specific character on the X-ray picture. HIV-positive patients were excluded. The control group consisted of healthy individuals without a history of TB. The participants were

predominantly Russians residing in the city of Tomsk or Tomsk Region, West Siberia, Russia. The demographic and clinical data for each patient were collected.

DNA samples were retrieved for 357 TB patients and 445 healthy controls from the DNA Bank of the Research Institute of Medical Genetics of Tomsk NRMC (table 1). The sample of the TB patients comprised 130 cases with primary TB and 227 cases with reactivation. The control group for primary TB was deliberately older than the case group (16.6±15.0 vs. 39.5±17.0) to ensure that the control individuals were not affected by TB up until adulthood.

For genotyping, we chose 14 single-nucleotide polymorphisms (SNPs) in 8 genes in which regulatory capacity was established using data from the Regulome Database (table 2). The database classifies SNPs into classes according to the combined status of overlap with functional categories such as transcription factor-binding sites, DNase I hypersensitivity, and promoters and assigns respective scores from 1 to 6 with a smaller score meaning a higher functional impact of an SNP (http://regulomedb.org/).

Genotyping was carried out using MALDI-TOF mass spectrometry and PCR-RFLP. For MALDI-TOF mass spectrometry, iPLEX GOLD kits (Agena Bioscience) and MassARRAY Analyzer 4 (Seqeunom) were used. Genotype calls were done automatically by MassARRAY Typer 4 software. PCR-RFLP was carried out using custom primers and specific restriction endonucleases (Fermentas and Sibenzyme) (table 3). All the analyses were conducted in the “Medical Genomics” Core Facilities of the Research Institute of Medical Genetics.

The associations between the SNPs and TB were assessed using logistic regression adjusting for covariates (age and gender). Additive, dominant, and recessive genetic models were tested. In the dominant model, rare allele homo- and heterozygotes were tested against common allele homozygotes. In the recessive model,

Table 1: Demographics of the studied individuals.

Group	Number	Mean age±SD (y)	P
Tuberculosis*	357	29.0±17.4	
Females	125	23.0±16.3	<0.001
Males	232	32.3±17.1	
Primary tuberculosis	130	16.6±15.0	
Females	63	14.3±13.0	<0.001
Males	67	18.8±16.3	
Secondary tuberculosis	227	36.1±14.5	
Females	62	31.7±14.6	0.007
Males	165	37.8±14.1	
Healthy controls	445	39.5±17.0	
Females	273	38.3±17.2	-
Males	172	41.5±16.7	

*Primary and secondary tuberculosis together; P value for the Student *t*-test for comparisons between the groups of patients and healthy individuals

Table 2: List of the studied SNPs with localization and the Regulome Database scores

Gene	Location	SNP	MAF	Marker Position	Score in Regulome Database
CXCR4	2q22.1	rs12691874	0.31 (A)	2:136122904	2a
CD80	3q13.33	rs59569688	0.18 (T)	3:119559065	2b
CD80	3q13.33	rs3915165	0.23 (T)	3:119560504	2b
CD80	3q13.33	rs1880661	0.34 (G)	3:119560001	2b
CP	3q23-q25	rs7623663	0.16 (T)	3:149224171	2b
PACRG	6q26	rs12211969	0.13 (G)	6:163312136	2b
PACRG	6q26	rs58627325	0.14 (A)	6:163309605	2a
PACRG	6q26	rs6455894	0.20 (A)	6:163311988	2a
PACRG	6q26	rs10945890	0.30 (C)	6:163308974	2b
CD69	12p13.31	rs75343219	0.076 (G)	12:9761162	2b
CD4	12p13.31	rs2855534	0.47 (G)	12:6789355	2b
CD4	12p13.31	rs7296859	0.25 (C)	12:6784998	1f
CD79A	19q13.2	rs10417985	0.40 (T)	19:41873065	2b
HCST	19q13.12	rs11878547	0.09 (C)	19:35902284	2b

SNP: Single-nucleotide polymorphism; MAF: Minor allele frequency

Table 3: Sequences of the primers and the methods of genotyping

ID SNP	Primer Forward 5’	Primer Reverse 5’	Primer Extension	Methods, Restriction Endonuclease and Fragments
rs10417985	ACGTTGGATGACTTGCCAGATATCCCACAG	ACGTTGGATGTCTTTTCTGAGGCACAGAGC	gggtGAGTGGCTAGGTCCAGG	MALDI-TOF
rs11878547	ACGTTGGATGTCTTCTCAGCGTTTCATGCC	ACGTTGGATGGTAGGGCCAAGAAAATTTGC	ctgtCCAAGAAAATTTGCTGATTAAATG	MALDI-TOF
rs12691874	ACGTTGGATGGGTGACCTCAGACAGCTATA	ACGTTGGATGAAACTTGACAGTCCACAGGG	caggCCACAGGGCTCTAGG	MALDI-TOF
rs2855534	ACGTTGGATGTGCCATCTTTTTCTTGCCGC	ACGTTGGATGGAAAATGCCAAAGTCAAGGG	gggtCTTAACAGTGGCAGTGACA	MALDI-TOF
rs59569688	ACGTTGGATGAAAGAGACTTATTCACCAG	ACGTTGGATGAGACTGTGGTGAGCTATGGT	aAGAATTTGTTTTTCTTAAGATAGAAT	MALDI-TOF
rs7296859	ACGTTGGATGTTGACTTCCAGGCCACAGAC	ACGTTGGATGTTTGCAGATTCCAGACCCGA	ccttaCACAGACTCACAGAGCTG	MALDI-TOF
rs7623663	ACGTTGGATGTTGTGGTAGTTACTCTTCTC	ACGTTGGATGCCCCTCTCCCTCCTATTTAA	GCATGTGGCAGGAAGT	MALDI-TOF
rs12211969	ACGTTGGATGGGGTTTATGCAATGGGCTC	ACGTTGGATGTAGGCATGAAAGAGGTGGAC	TGCAATGGGCTCTGTCCT	MALDI-TOF
rs3915165	ACGTTGGATGGGGTTTATGCAATGGGCTC	ACGTTGGATGTAGGCATGAAAGAGGTGGAC	TGCAATGGGCTCTGTCCT	MALDI-TOF
rs58627325	ACGTTGGATGTTCAAGCTTCCGAAAGCAGG	ACGTTGGATGCTGAGTGAATCAGGAAATGG	aAGGAAATGGTTAAGAGGTGA	MALDI-TOF
rs6455894	ACGTTGGATGTGGTCTATTCAGCTCTTGAC	ACGTTGGATGGTCACTCATAAATGGTGCCT	GTGCCTTTTGTCGGCATAT	MALDI-TOF
rs75343219	ACGTTGGATGAGTGGGATTTTCCCAGACTC	ACGTTGGATGACTTAGATTATGCTGTCTCC	ggtaCTGCCTTAAATTTCTAGAAAAC	MALDI-TOF
rs1880661	AAGATGGTGGGATTCAGAGG	TGTTTCTGTGCTGGTCTCAA	-	*Bme18 TT 160 TC 160+122+38 CC 122+38l
rs10945890	CCAATCAGAAGAAAGCCAGC	TCTCGCTGAAGCAACACTGA	-	*HinfI CC 219 CT 219+122+97 TT 122+97

*PCR-PLRF; SNP: Single-nucleotide polymorphism

common allele homo- and heterozygotes were tested against rare allele homozygotes. The additive model corresponded to a trend test for the genotypes with the genotypes coded as 0, 1, or 2 to reflect the minor allele counts. The best model was chosen using the Akaike Information Criterion. The SNP effects were quantified with odds ratios (ORs) and 95% confidence intervals. Multiple testing was addressed using experiment-wise permutations. Models with a permutation P value less than 0.05 were considered statistically significant. The statistical analyses were carried out in R 3.2 statistical software.

Results

We chose new candidate genes of special interest based on the results of our previous study: *CD4, CD69, CD79, CD80, MUC16, HCST, ADA, CP, SPP1, CXCR4, AGER,* and *PACRG*.[12] To the best of our knowledge, these genes have never been studied with respect to TB. To select SNPs in these genes, we took into account such parameters as localization in the 5' region of the gene, global frequency of the minor allele equal to or greater than 5%, and SNPs with a high confidence of functional consequence in the gene's region using the Regulome Database. Accordingly, for genotyping, we selected 14 SNPs in 8 genes (*CD4, CD69, CD79, CD80, HCST, CP, CXCR4*, and *PACRG*) (table 2).

In the control group as well as in the patient group, all the SNPs met the Hardy–Weinberg equilibrium expectation. The rs75343219 SNP in *CD69* was monomorphic in Russians and was excluded from the subsequent analysis.

An association was established between TB and the rs1880661 polymorphism in the *CD80* gene (table 4). When primary TB was considered separately from secondary TB, an association was found between this polymorphism and secondary TB only. The prevalence of the rs1880661*C allele of the *CD80* gene was 40.9% in the patients with secondary TB, 44.7% in those with primary TB, and 46,8% in the control group.

Moreover, the rs10945890 variant in *PACRG* was associated with both primary TB and secondary TB; nonetheless, there was a recessive effect of the polymorphism for primary TB and a dominant effect for secondary TB. The same allele rs10945890*C of the gene *PACRG* was associated with a decreased risk of primary TB (OR=0.26 [0.04; 0.89]; P=0.03), while it was correlated with an increased risk of reactivation (OR=1.47 [1.02; 2.13]; P=0.04). The frequency of the rs10945890*C allele of the *PACRG* gene was 20.5% in the patients with primary TB, 28.6% in those with secondary TB, and 25.6% in the control group.

Discussion

We carried out an analysis of the association between the different stages of TB infection and potential regulatory SNPs in the *CD4*, *CD69*, *CD79*, *CD80*, *HCST*, *CP*, *CXCR4*, and *PACRG* genes. Most of the proteins encoded by these genes are involved in immune signaling and are responsible for the effectiveness of immune reactions to the invasion of the pathogen. We found that the polymorphisms in the *CD80* and *PACRG* genes were associated with the different stages of TB in Russians.

The *CD80* gene encodes a transmembrane receptor, a co-stimulator for antigen presentation by macrophages and dendritic cells. Its expression is reduced in mycobacterial infection, the mechanism by which mycobacteria suppress the adaptive immune response.[13] According to our previous data, *CD80* is the most promising candidate gene of all the TB associative network.[12] We studied 3 SNPs in this gene (rs59569688, rs3915165, rs1880661) and found that rs1880661 was associated with secondary TB. This polymorphism is an expression quantitative trait locus and influences the expression of *CD80* and *ADPRH* in a tissue-specific manner (figure 1). The SNP is associated with the differential expression of *CD80* in dendritic cells before and after mycobacterial infection.[14] The association between TB and this SNP was established for the first time in the current study; still, there are other polymorphisms in *CD80* associated with immune-mediated diseases such as celiac disease (rs11712165)[15] and primary biliary

Table 4: Statistically significant models for the association between tuberculosis and the polymorphisms of the *PACRG* (rs10945890) and *CD80* (rs1880661) genes

Sample	SNP	Best Model				
		Risk allele	%	Effect	OR [95% CI]	P perm*
Tuberculosis	rs1880661	C	41.6	Dominant	0.68 [0.48;0.96]	0.03
Primary tuberculosis	rs10945890	C	20.5	Recessive	0.26 [0.04;0.89]	0.03
Secondary tuberculosis	rs10945890	C	28.6	Dominant	1.47 [1.02;2.13]	0.04
	rs1880661	C	40.9	Dominant	0.67 [0.46; 0.97]	0.04

*Experiment-wise permutation P value; SNP: Single-nucleotide polymorphism

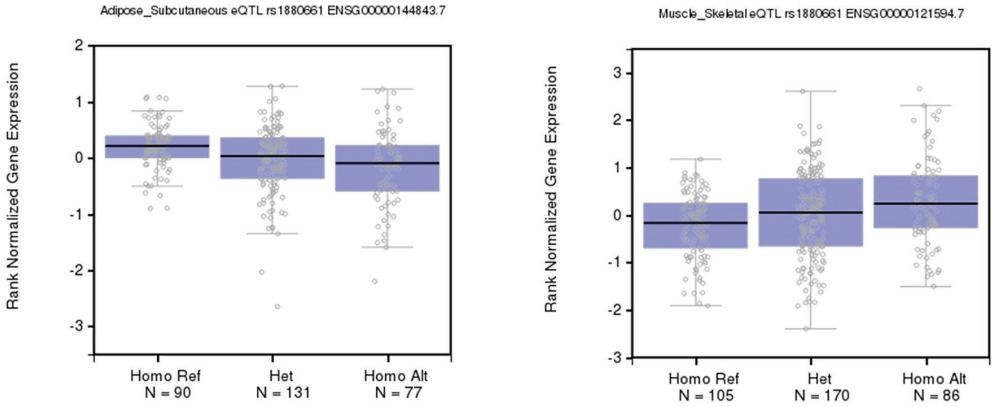

Figure 1: Box plots of the associations between the genotypes of rs1880661 with *CD80* (ENSG00000121594.7) in the muscle tissue and *ADPRH* (ENSG00000144843.7) in the adipose tissue expression (data of the GTEx project portal (http://www.gtexportal.org/home/). Homo Ref, Het, and Homo Alt accord with CC, CT, and TT genotype, respectively.

cirrhosis (rs2293370).[16]

The *PACRG* gene encodes the Parkin co-regulated protein and is located on 6q26, the cluster with the related gene *PARK2.* These genes have a common regulatory region and are involved in ubiquitin-mediated protein degradation. Furthermore, previous research has shown that they are important for susceptibility to diseases caused by *M. ulcerans* and *M. leprae*.[17] The variant of *PACRG* associated with TB in the current study (rs10945890) has never been studied for association with TB or other diseases. It is also challenging to explain why the same allele of the gene is associated with a decreased risk of primary TB (OR=0.26 [0.04; 0.89]; P=0.03), while it increases the risk of reactivation (OR=1.47 [1.02; 2.13]; P=0.04). Functional studies as well as replication in other populations will be required to delineate this.

None of the other studied genes was found to be associated with TB in the current study; nevertheless, their analysis in other populations may still be fruitful given their functional importance in TB pathogenesis.

The *CD69* gene is located on 12p13.31 and encodes type II transmembrane glycoprotein. A previous investigation reported an increased expression of the *CD69* gene in TB patients.[18] Elsewhere, polymorphism rs4763879 in this gene was found to be associated with type I diabetes.[19]

The *CD4* gene (12p13.31) encodes the membrane glycoprotein of T-lymphocytes, which plays an important role in T-helper cell activation. The deficit of CD4+ T cells promotes susceptibility to *M. tuberculosis* infection.[20] With the exception of the current study, the polymorphisms of the *CD4* gene have never been studied in TB susceptibility.

The *CD79A* gene encodes the Ig-α protein expressed in B-lymphocytes. The protein is essential for the immune pathogenesis of TB.[21] The gene is located on 19q13.2 and is associated with cancer.[22] Nonetheless, there is currently a dearth of data on the polymorphisms of the *CD69* gene and susceptibility to TB.

The *HCST* (*DAP10*) gene, located on 19q13.12, encodes a transmembrane signaling adaptor containing the YxxM motif in its cytoplasmic domain. The expression of the *HCST* gene was found repressed during late stages of infection in nonhuman primates infected by *M. tuberculosis*.[23] This gene is of interest for the study of the different stages of TB infection, but the polymorphisms of this gene have never been studied in this respect.

The *CP* gene encodes ceruloplasmin, a metalloprotein which binds up to 95% of the blood cuprum. Copper along with other microelements is important in protecting against pathogenic microorganisms,[24] which underlies the antibacterial function of ceruloplasmin. Ceruloplasmin is an acute-phase protein; its concentration in tandem with the levels of cuprum ions is elevated in lung TB patients.[25] Defects in the *CP* gene can lead to a disruption in the binding and transport function of ceruloplasmin and, as a result, an increase in sensitivity to intracellular pathogens such as mycobacteria. No studies on the association between this gene variants and TB are available.

The gene *CXCR4* (2q22.1) encodes chemokine (CXC motif) receptor 4 and is involved in angiogenesis induced by granuloma.[26] The polymorphism of the gene *CXCR4*, rs2680880, is associated with overall survival from colorectal brain metastases.[27] Another SNP of this gene, rs953387, links with juvenile idiopathic arthritis, which is an autoimmune disease.[28]

Thus, although the above genes are important

for an effective immune response to the invasion of the pathogen, they have not been extensively studied in TB. Our study, therefore, provides the first glance at these genes with regard to TB.

The majority of the genetic studies on TB are focused on establishing associations between the disease per se and genetic variants. Nevertheless, studies considering the clinical forms or stages of the disease are limited. The current study was carried out to reveal genetic factors associated with the various stages of TB.

In contemporary studies of TB, the major question is why immunity is able to control the infection in primary contact, whereas it cannot prevent reactivation. High resistance to primary TB actually predisposes to the development of secondary TB.[29] The majority of immunocompetent individuals would develop delayed hypersensitivity activating T-cells and Th1-immunity, which effectively controls primary TB. In spite of this, this process has little effect on secondary TB and, in addition, neither immunization nor natural infection results in immunity to secondary TB.[30] This means that mycobacteria employ an effective strategy to avoid the host's immune response or even benefit from it.[31]

Recently, it has been noted that the immune response characterized by elevated activity of CD4+T-cells and increased levels of IFN-γ causes the development of secondary TB, thus contradicting with a widely accepted view that impaired immunity leads to the reactivation of latent infection.[32] Even though the risk of TB is increased when immunity is weakened, the disease etiology in individuals with impaired immunity differs from the disease etiology in immunocompetent individuals. The development of the disease in immunocompromised people is caused by an uncontrolled proliferation of the bacterium, while in individuals with a healthy immune system, it is the damage to the lung tissue that causes the development of active disease.[31] These different mechanisms may be the basis for the clinical heterogeneity of TB; consequently, different genes can be involved. Thus, primary and secondary TB can be controlled by different host genes,[33, 34] which is supported by the results of the current study.

The advantage of the current study is its focus on the analysis of novel candidate genes and stratified analyses according to the stages of TB infection. Be that as it may, the study has a limitation in that the control group was significantly older than the case group. This was done, however, to avoid the possible risk of TB in the subsequent life of young individuals, if taken as control. Another limitation is the lack of a replication sample. Hence, our findings require independent validation.

Conclusion

In summary, our data suggested that the studied polymorphisms in the *CD80* and *PACRG* genes affected susceptibility to the different stages of TB infection (primary and secondary TB) in Russian patients. If replicated in independent samples, the mechanisms of the associations are to be disclosed in experimental studies. Nevertheless, given that we analyzed SNPs from the regulatory regions of the genes, the mechanisms are likely related to the modulation of the gene expression.

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Conflict of Interest: None declared.

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