DOI 10.1007/s11055-021-01074-6
Neuroscience and Behavioral Physiology, Vol. 51, No. 3, March, 2021

Analysis of Associations of Genetic Predisposition Markers Identified in Genome-Wide Studies with Multiple Sclerosis

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Translated from Zhurnal Nevrologii i Psikhiatrii imeni S. S. Korsakova, Vol. 120, No. 7, Iss. 2, pp. 54–60, July, 2020. Original article submitted April 2, 2020. Accepted April 7, 2020.

Objectives. To carry out a replicative analysis of the associations with multiple sclerosis of genetic markers of autoimmune diseases identified as a result of genome-wide studies in ethnically homogeneous groups of Russians and Tatars living in the Republic of Bashkortostan. **Materials and methods.** A group of 1724 people (547 patients with multiple sclerosis, 1177 members of the control group) underwent genotyping using allele-specific PCR and PCT with restriction fragment length polymorphism analysis for polymorphic variants rs2069762 of the *IL2* gene, rs759648 of the *PVT1* gene, rs1800682 of the *FAS* gene, and rs12708716 of the *CLEC16A* gene. Associations of these genetic markers with multiple sclerosis were studied using the PLINK program by logistical regression using an additive genetic model with sex as a covariate. **Results.** In the Tatar group we found an association between the rs759648*C of *PVT1* with multiple sclerosis (OR = 1.42, $p = 0.023$). Meta-analysis of the results in the two ethnic groups confirmed the association of the rs759648*C allele of *PVT1* with the disease (a random effects model and a fixed effect model: OR = 1.29, $p = 0.018$). **Conclusions.** These data provide evidence of an association between the polymorphic variant rs759648 of *PT1* and multiple sclerosis in the Russian and Tatar populations living in the Republic of Bashkortostan. No associations with the other polymorphic variants studied were found in these two ethnic groups.

Keywords: multiple sclerosis, genetic polymorphism, analysis of associations, genome-wide association studies.

Multiple sclerosis (MS) is a chronic disseminated demyelinating disease of the CNS, which is accompanied by progressive neuroaxonal degradation. MS is commoner in women than men and the ratio of the number of affected women to the number of men has increased significantly in recent decades (2.3–3.5:1), which reflects an increase in the incidence of MS among women [1]. The prevalence of MS in the Russian population is 50 per 100,000, and the prevalence in the Republic of Bashkortostan is 38 per 100,000 [2, 3].

MS is regarded as an autoimmune disease which develops as a result of a T-cell reaction to CNS autoantigens in individuals genetically predisposed to the disease [4]. Genome-wide association studies (GWAS) have now identified about 400 genetic variants associated with MS, most of which are biomarkers for the immune response and inflammation and are often associated with other autoimmune diseases (https://www.ebi.ac.uk/gwas/efotraits/EFO_0003885). As most genetic markers identified in GWAS have minor effects, their detection requires large cohorts, which are obtained by recruiting people from different ethnic groups. This ethnic heterogeneity can affect the study results because different populations can have different allele frequencies of polymorphic variants and different patterns of linkage disequilibrium [5]. This can produce false positive results due to population stratification, such that validation of GWAS results for independent and preferably homogeneous ethnic groups is needed [6].

The aim of the present work was to carry out a replication analysis of associations with MS of genetic predisposition

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TABLE 1. Nomenclature and Chromosomal Locations of the Polymorphic Loci Studied Here and Primer Sequences, Restriction Enzymes, Nomenclature of Alleles, and Sizes of Amplified Fragments

Gene	Polymorphism	Chromosomal location	Primers and restriction enzymes	Alleles and fragment sizes
IL2	rs2069762 -384G>T	4:123377980 5'-UTR	F 5'-tgaaacaggaaaccaatacact-3' R 5'-cccacacttaggtgatagctc-3' G 5'-cacatgttcagtgtagtttttg-3' T 5'-cacatgttcagtgtagtttttt-3'	IC 239 140
PVT1	rs759648	8:129158945 intron	C 5'-cttcaccacctccaactgc-3' F 5'-atctgccccattgctctgtc-3' R 5'-cctgccccagactctgtttt-3' A 5'-catgtacaaatacccacattttgtt-3'	IC 324 C 169 A 197
FAS	rs1800682 -670A/G	10:90749963 intron	F 5'-tgg cca gga aat aat gag taa cga-3' R 5'- gcc ttg gct aat tgc tgg agt c-3' (MvaI)	A (285) G (184, 101)
CLEC16A	rs12708716	16:11179873 intron	F 5'-tacctgtgggaagtgacttgg-3' R 5'-gccaaggaagccaaagttcc-3' G 5'-tgggcagtagggagaatcatg-3' A 5'-tgggcagtagggagaatcata-3'	IC 211 162

Data from the Genome Reference Consortium Human build 37 (GRCh37.p13); IC – internal control; 5'-UTR – 5'-untranslated region.

markers identified in GWAS in groups of Russians and Tatars living in the Republic of Bashkortostan (Russian Federation).

Materials and Methods. The study group consisted of 1724 people of Russian ($n = 773$) and Tatar ($n = 951$) ethnicity permanently resident in the Republic of Bashkortostan. The group of patients ($n = 547$) consisted of people registered at the Republican Multiple Sclerosis Center (RMSC). Diagnoses of MS were established according to the McDonald criteria (2017) [7]. The severity of neurological deficit was assessed using the Expanded Disability Status Scale (EDSS); increases in neurological deficit were characterized using the rate of progression calculated as the ratio of the level of disability on the EDSS in points to the duration of disease in years. The ratio of women to men in the group of MS patients was two. The mean age of MS patients was 40.46 ± 9.98 (median 41) years.

The control group included 1177 presumptively healthy people with no neurodegenerative or other chronic diseases. Mean age in the control group was 37.76 ± 10.88 (median 38) years. Ethnic group assignments were made on the basis of questionnaires addressing the ethnicities and places of birth of three generations of ancestors.

DNA was extracted from 8 ml of whole venous blood using the standard phenol-chloroform method. Genotyping was run using the polymerase chain reaction (PCR) or PCR followed by restriction fragment length polymorphism (RFLP) analysis using a T100™ thermal cycler (BioRad, USA). Oligonucleotide primers were selected using the program DNAStar v. 5.05 and the https://www.ncbi.nlm.nih.gov/snp/ database. The genetic variants analyzed, primer sequences, restriction enzymes, and amplified fragments are shown in Table 1. Fragments obtained by amplification and restriction were then separated by electrophoresis in 2% agarose gels and identified using the video gel documentation system Mega-Bioprint 1100 (Vilber Lourmat, France).

Studies were performed in compliance with ethical principles for medical research on humans incorporated in the Helsinki Declaration of the World Medical Association (2013). All participants gave written voluntary informed consent to take part in the study.

Results were processed statistically in IBM SPSS Statistics v. 21 and PLINK v1.07 [8]. Correspondence of the observed genotype and allele distributions of the markers of interest with the theoretically expected Hardy–Weinberg law were assessed using Fisher's test. Associations between polymorphic variants of MS were analyzed by logistical regression using an additive genetic model in which sex was a covariate. The additive model proposes that the presence of two copies of an allele predisposing to disease development has a two times greater influence on phenotype than the presence of one copy. Meta-analysis of the results in two ethnic groups was run using a random effects model and a fixed effect model. The relative risk of disease for carriers of minor alleles was calculated as the odds ratio (OR). Differences were regarded as significant at $p < 0.05$.

Results. The clinical characteristics of groups of patients with MS depending on ethnicity are shown in Table 2. The mean duration of disease in the overall group of patients with MS at the moment of the study was 13.26 ± 9.72 years (median 12 years) and the level of disability on the EDSS was 4.48 ± 1.54 points (range 1–8 points), and the rate of progression of neurological deficit was 0.71 ± 0.99 points/year (median 0.38 points/year). In the group of Russians, the secondary progressive type of MS was commonest (52.9%), while the remitting course was commonest in Tatars (43.7%).

The distribution of genotype frequency distributions for the polymorphic markers studied here in the control group, both among Russians and Tatars, corresponded to the distributions theoretically expected on the basis of the

TABLE 2. Clinical Characteristics of MS Patients

Parameters	Russians (n = 283)	Tatars (n = 264)
Age ($M \pm SD$), years	40.6 ± 9.77	40.89 ± 9.75
Sex, % women	66.8	66.7
Age at disease onset ($M \pm SD$), years	27.64 ± 8.9	27.53 ± 8.89
Disease duration, ($M \pm SD$), years	13.17 ± 9.53	13.36 ± 9.93
Type of course, %		
remitting	36.9	43.7
primary progressive	10.2	15.6
secondary progressive	52.9	40.7
Clinical symptoms, %		
sensory disorders	17.1	12
oculomotor disorders	4.9	4.4
motor disorders	35	29.9
impaired coordination	18.6	18.7
combined motor and coordination	4.2	6.4
disorders	2.7	2.8
symptoms of cranial nerve lesions	14.4	19.9
retrobulbar neuritis/other	3	6
EDSS ($M \pm SD$), points	4.41 ± 1.56	4.46 ± 1.77
Rate of progression ($M \pm SD$), points/year	0.73 ± 1.09	0.69 ± 0.89

Hardy–Weinberg law (Tables 3 and 4). Analysis of associations of the genetic markers of interest with MS in the group of Tartars identified an association of the *PVT1* rs759648*C allele with the disease (OR = 1.42, 95% CI 1.05–1.92, $p = 0.023$) (see Table 4). In the group of Russians, no associations between the polymorphic markers of interest and MS were seen. Meta-analysis of the study results in the two ethnic groups confirmed the existence of an association of the rs759648*C allele of *PVT1* with MS (random effects model) and the fixed effect model (OR = 1.29, $p = 0.018$) (Table 5).

Discussion. We conducted a replicative analysis of associations with MS of polymorphic markers of genes predisposing to autoimmune diseases detected in GWAS in independent cohorts of inhabitants of the Republic of Bashkortostan, i.e., ethnic Russians and Tatars. This study confirmed the association with MS of the polymorphic marker rs759648 of the *PVT1* gene, which has previously been demonstrated to be associated with MS in a GWAS involving 41505 people of European origin (14802 patients with MS, 26703 subjects in the control group) [9]. Our results are consistent with GWAS data in relation to the risk allele for this polymorphism, though the effect seen here for the rs759648*C allele of *PVT1* was somewhat higher than found in the GWAS (OR = 1.08, 95% CI_{OR} 1.06–1.11, $P = 5.05 \cdot 10^{-10}$ GWAS; OR = 1.42, P = 0.023 in the Tatars group, OR = 1.29, P = 0.018 in the meta-analysis for the two ethnic groups). The smaller size of the effect in the GWAS is due to the ethnic heterogeneity of the study group, which included the inhabitants of 11 countries, including Belgium, Denmark, Finland, France, Germany, Italy, Norway, Sweden, and the UK, as well as members of the white populations of Australia, New Zealand, and the USA [9].

Previous studies indicated that the rs759648 polymorphism influences the extent of STAT1-mediated phosphorylation in MS patients receiving treatment with interferon β 1b, as indicated by activation of proinflammatory mechanisms [10]. In addition, an association of rs759648 with the microstructural characteristics of the white matter of the brain was found [11]. The polymorphic variant rs759648 is found at the *PVT1* locus in a region containing several other MS-associated polymorphic variants in linkage disequilibrium with this marker. In particular, the rs1861842 polymorphism, for which an association with MS was found in Afro-Americans (OR = 1.30, 95%CI_{OR} 1.14–1.48, $P = 8.5 \cdot 10^{-5}$), is linked with rs759648 in Europeans ($r^2 = 0.4$), while the strength of this linkage in people of

TABLE 3. Results of Analysis of Associations of the Polymorphic Variants Studied Here with MS in the Russian Ethnic Group

Gene, polymorphism	Genotype	Control		MS patients		P_{HWE}	OR (95% CI)	*p*
		n	%	*n*	&			
IL2 rs2069762	T/T	139	58.4	107	45.53	0.117	0.94 (0.721.23)	0.668
	G/T	66	27.73	102	43.4			
	G/G	33	13.87	26	11.06			
PVT1 rs759648	A/A	176	59.86	126	56.76	0.231	1.16 (0.861.58)	0.336
	C/A	108	36.73	83	37.39			
	C/C	10	3.4	13	5.86			
FAS rs1800682	A/A	106	30.99	70	29.79	0.231	1.05 (0.831.32)	0.677
	G/A	159	46.49	108	45.96			
	G/G	77	22.51	57	24.26			
CLEC16A rs12708716	A/A	148	43.53	116	48.54	0.632	0.86 (0.671.10)	0.218
	G/A	149	43.82	99	41.42			
	G/G	43	12.65	24	10.04			

Here and Table 4: *n* – number; *p* – frequency, P_{HWE} – significance of Hardy–Weinberg equilibrium; OR – odds ratio for minor allele; CI95 – 95% confidence interval for odds ratio; P – significance.

TABLE 4. Results of Analysis of Associations of the Polymorphic Variants Studied Here with MS in the Tatar Ethnic Group

Gene, polymorphism	Genotype	Control		MS patients		P_{HWE}	OR (95% CI)	*p*
		n	%	*n*	%			
IL2 rs2069762	T/T	121	44.81	79	37.26	0.68	1.22 (0.92 ± 1.61)	0.160
	G/T	122	45.19	109	51.42			
	G/G	27	10	24	11.32			
PVT1 rs759648	A/A	165	62.5	111	53.62	1	**1.42 (1.05 ± 1.92)**	**0.023***
	C/A	88	33.33	79	38.16			
	C/C	11	4.17	17	8.21			
FAS rs1800682	A/A	72	26.28	51	23.83	1	1.0 (0.77 ± 1.29)	0.980
	G/A	138	50.36	118	55.14			
	G/G	64	23.36	45	21.03			
CLEC16A rs12708716	A/A	145	54.51	112	51.38	0.417	1.21 (0.90 ± 1.62)	0.202
	G/A	107	40.23	87	39.91			
	G/G	14	5.26	19	8.72			

African origin was significantly lower ($r^2 = 0.08$) [12]. This suggests that both genetic markers are tag-SNP (i.e., tightly linked with a number of other polymorphic variants, such that they are inherited together) in Europeans and reflect the existence of a functional polymorphism in this part of the genome linked with disease development. In addition, rs759648 is in linkage disequilibrium ($r^2 = 0.6$) with the polymorphic variant rs2019960 in the *PVT1* gene, which has also been shown to be associated with MS [13, 14].

The *PVT1* gene maps to the long arm of chromosome 8 (8q24) and by means of alternative splicing forms several long noncoding RNA species (lncRNA) as well as six microRNAs: miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p, and miR-1208 (see Fig. 1) [15, 16]. lncRNA

TABLE 5. Meta-Analysis of Study Results in Two Ethnic Groups

Gene, polymorphism	*n* patients with MS/*n* controls	A1/A2	OR (R)	P (R)	OR (F)	P (F)
IL2 rs2069762	447/608	G/T	1.06	0.659	1.06	0.561
PVT1 rs759648	429/558	C/A	**1.29**	**0.018**	**1.29**	**0.018**
FAS rs1800682	449/616	G/A	1.03	0.710	1.03	0.710
CLEC16A rs12708716	457/606	G/A	0.99	0.953	0.98	0.796

A1 – minor allele; A2 – "wild-type" allele; P(R) – significance for the random effects model; P(F) – significance for fixed effect model; OR(R) – odds ratio for random effects model; OR(F) – odds ratio for fixed effect model. The odds ratio was calculated for the minor allele. Results reaching the level of statistical significance ($p < 0.05$) are in bold.

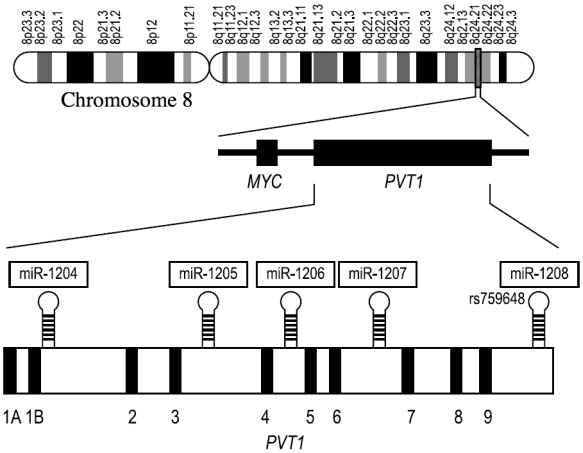

Fig. 1. Graphical representation of the *PVT1* locus.

are transcripts of length greater than 200 bases not carrying any protein structure information and presumptively playing an important role in regulating transcription and mRNA processing, though the concrete biological roles of most lncRNA remain unclear [17].

MicroRNAs are small noncoding RNA molecules (20–22 bases) which are involved in regulating the activity of genes associated with specific nucleotide sequences in the mRNA and affect processes such as cell proliferation and differentiation and apoptosis. MicroRNAs can modulate the operation of the immune system, influencing activation of immunocompetent cells, cytokine secretion, and the development of immunological tolerance [18].

Apart from *PVT1*, chromosomal region 8q24 includes the well-known oncogene MYC, and tumor cells show simultaneous amplification of these genes [19]. Increases in the number of copies of *PCT1* are seen in diseases such as breast cancer, ovarian cancer, acute myeloid leukemia, lymphogranulomatosis, and juvenile malignant astrocytoma; nonetheless, the pathophysiological consequences of impairment to the regulation of the *PVT1* locus continues to remain unclear [20]. In normal cells, in contrast to tumor cells, *PVT1* was found to function as a "trap," acting as a competitive endogenous RNA and decreasing the quantity of microRNA available for binding with mRNA [17]. It has also been shown that *PVT1* induces angiogenesis by activating the STAT3-VEGFA signal pathway, leading to an increase in the permeability of the blood-brain barrier for, among others, immunocompetent cells, which may promote the development of MS [21].

We did not find any associations for any other of the markers studied with MS (see Tables 3 and 4). Previous GWAS identified an association between polymorphic variants of IL2 rs2069762, FAS rs1800682, and CLEC16A rs12708716 with MS and other autoimmune diseases, including type 1 diabetes mellitus, psoriasis, Crohn's disease, nonspecific ulcerative colitis, ankylosing spondylitis, and primary sclerosing cholangitis [22–28]. The IL rs2069762*T allele was also shown to be part of the combination associated with MS in Bashkirs living in the Republic of Bashkortostan [29]. This allele has been shown to be linked with decreased IL-2 production [30, 31]. In addition, the IL2 rs2069762*T allele is linked with the IL2 rs2069772*G allele, which is part of the combination associated with an increased risk of MS in Bashkirs, Russians, and Tartars from the Republic of Bashkortostan [32]. The absence of an association between these polymorphic variants and MS seen in our study may be linked with the influence of intergenic interactions, whose detection requires polygenic analysis.

Conclusions. We found an association between the *PVT1* rs759648 polymorphism and MS in groups of Russians and Tartars living in the Republic of Bashkortostan. These results, along with analysis of published data, suggest that the part of the genome containing the *PVT1* gene is important for regulation of the functioning of the immune system and the development of inflammation in the CNS. Further research is needed in this area to clarify the role of the *PVT1* gene in the pathogenesis of MS.

This study was partially supported by the Russian Foundation for Basic Research (Grant No. 17-44-020735r_a) and NIR No. AAAA-A16-116020350031-4; biological material (DNA) for studies was obtained from the Collection of Human Biological Materials of the Institute of Biochemistry and Genetics, Ufa Research Center, Russian Academy of Sciences, supported by the Bioresource Collections Program

of the Russian Federal Agency for Scientific Organizations; the study was carried out using the equipment of the Biomik Center for collective use and the UNU Kodink (Institute of Biochemistry and Genetics, Ufa Research Center, Russian Academy of Sciences).

The authors have no conflicts of interests.

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