Indian J Med Res 144, December 2016, pp 865-876
DOI: 10.4103/ijmr.IJMR_1233_14

Quick Response Code:

IREB2, CHRNA5, CHRNA3, FAM13A & hedgehog interacting protein genes polymorphisms & risk of chronic obstructive pulmonary disease in Tatar population from Russia

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Received August 17, 2014

***Background & objectives*: Chronic obstructive pulmonary disease (COPD) is a complex chronic inflammatory disease of the respiratory system affecting primarily distal respiratory pathways and lung parenchyma. This study was aimed at investigating the association of COPD with *IREB2, CHRNA5, CHRNA3, FAM13A* and hedgehog interacting protein (*HHIP*) genes in a Tatar population from Russia.**

***Methods*: Six single nucleotide polymorphisms (SNPs) (rs13180, rs16969968, rs1051730, rs6495309, rs7671167, rs13118928) were genotyped by the real-time polymerase chain reaction in this study (511 COPD patients and 508 controls). Logistic regression was used to detect the association of SNPs and haplotypes of linked loci in different models. Linear regression analyses were performed to estimate the relationship between SNPs and lung function parameters and pack-years.**

***Results*: The rs13180 (*IREB2*), rs16969968 (*CHRNA5*) and rs1051730 (*CHRNA3*) were significantly associated with COPD in additive model [P_{adj}=0.00001, odds ratio (OR)=0.64; P_{adj}=0.0001, OR=1.41 and P_{adj}=0.0001, OR=1.47]. The C-G haplotype by rs13180 and rs1051730 was a protective factor for COPD in our population (P_{adj}=0.0005, OR=0.61). These results were confirmed only in smokers. The rs16969968 and rs1051730 were associated with decrease of forced expiratory volume in 1 sec % predicted (P_{adj}=0.005 and P_{adj}=0.0019).**

***Interpretation & conclusions*: Our study showed the association of rs13180, rs16969968 and rs1051730 with COPD and lung function in Tatar population from Russia. Further studies need to be done in other ethnic populations.**

Key words *CHRNA3/5* - chronic obstructive pulmonary disease - *IREB2* - polymorphism

Chronic obstructive pulmonary disease (COPD) is a complex chronic inflammatory disease of the respiratory system affecting primarily distal respiratory pathways and lung parenchyma. It manifests with

partially reversible bronchial obstruction and shows a progressive course with lung emphysema and increasing respiratory failure[1]. Smoking is generally considered as a principal risk factor for COPD: the disease develops in 20-30 per cent of smokers[1]. An important internal risk factor is hereditary predisposition. Genetic mechanisms underlying COPD have been extensively investigated all over the world[2].

Genome-wide association studies (GWAS) have identified several loci associated with COPD, in particular, in chromosomal regions 15q25.1 near cholinergic receptor, nicotinic, alpha 3/5 (*CHRNA3/5*) and iron-responsive element binding protein 2 (*IREB2*), the chromosome 4q24 region near family with sequence similarity 13, member A (*FAM13A*) and chromosome 4q31 region near hedgehog interacting protein (*HHIP*)[3-7]. Single nucleotide polymorphisms (SNPs) of these genes have been found to be associated with COPD, lung function parameters and smoking behaviour in different Caucasian and Mongoloid populations[8-11].

The gene cluster *CHRNA3/A5/B4* encodes nicotinic acetylcholine receptor subunits alpha 3, alpha 5 and beta 4. These genes are expressed in the central nervous system and in bronchial epithelium these play a key role in the formation of nicotine addiction[10,12-15]. *IREB2* is located in the same chromosomal region 15q25.1 and encodes iron-responsive element-binding protein 2, which is involved in maintaining iron balance in lung tissues[11,16]. Intracellular iron concentration plays an important role in oxidative stress[16]. *IREB2* expression is modulated by hypoxia, which commonly accompanies COPD[16]. *FAM13A,* a gene located on 4q22, comprises 25 exons and encodes FAM13A, a protein with a key role in signal transduction[9]. It is known that hypoxia enhances *FAM13A* expression[17]. *HHIP* is located on 4q31.21-4q31.3 and encodes a transmembrane glycoprotein that binds three members of the Hedgehog signalling pathway, which all play an important role in lung development[18]. COPD-associated polymorphisms were identified in the *HHIP* enhancer; substitutions at these sites result in a decrease in the promoter activity[19,20].

The molecular genetic basis of COPD remains largely unclear, data obtained from different populations and ethnic groups often disagree[2,8-11]. These problems are largely related to the complex nature of this disease and to the strong genetic diversity of human populations[21]. The frequency distribution of the *IREB2, CHRNA5, CHRNA3, FAM13A* and *HHIP* polymorphisms and their association with COPD has not yet been investigated in populations of Russia. This study was aimed at investigating the association of COPD with polymorphisms of *IREB2, CHRNA5, CHRNA3, FAM13A* and *HHIP* in a Tatar population from Russia.

Material & Methods

The study protocol was approved by the Local Ethical Committee of Institute of Biochemistry and Genetics of Ufa Scientific Center of Russian Academy of Sciences (IBG USC RAS), Ufa, Russia (Ufa, Protocol No 17, December 7, 2010). Written informed consent was obtained from all participants. The patients and controls were selected from December 2010 to January 2013 from the pulmonary departments of the Ufa City Hospitals №13, №18, №21 (Ufa, The Republic of Bashkortostan, Russia). The laboratory work was conducted in the Genomics Department, IBG USC RAS Ufa, Russia. The blood samples (4 ml) were collected from unrelated patients with COPD (affected group) and unrelated control group (unaffected group) age, sex and ethnically matched. Ethnic origin (up to the third generation) of all the participants was derived by direct interviews with examined persons.

Inclusion and exclusion criteria: The diagnosis of COPD was made according to the International Classification of Diseases tenth revision (ICD 10)[22] and following the recommendations of the GOLD (2011)[1]. For all patients with COPD, the diagnosis was based on the medical histories and the results of general, clinical and special tests (chest X-ray, spirometry measures and fibrobronchoscopy), physical examination and laboratory tests. Patients were excluded from the study if they had diagnosis of asthma and lung cancer. The predicted values for forced vital capacity (FVC), forced expiratory volume in 1 sec (FEV_1) and FEV_1/FVC ratio were generated using previously defined prediction equations as detailed to the European Coal and Steel Community[23,24]. All COPD patients had post-bronchodilator FEV_1/FVC values of <70 per cent. The study group consisted of 511 unrelated COPD patients.

The control group comprised of 508 unrelated age, sex and ethnicity (Tatar population) matched healthy residents of Ufa with no history of chronic diseases such as respiratory system pathology and allergic diseases in the anamnesis. These individuals came to the Ufa City Hospitals №13, №18, №21 (Ufa, Russia) for regular medical examination. All control group individuals had normal lung function (FEV_1/FVC >70%, FEV_1 >80%) (Table I).

Table I. Characteristics of the study and control groups

Parameters	COPD (N=511)	Controls (N=508)
Male (%)	452 (88.54)	457 (89.96)
Female (%)	59 (11.46)	51 (10.04)
Age (±SD) (yr)	62.74±12.7	58.82±7.22
BMI (±SD) (kg/m^2)	25.81±5.92*	27.06±3.84
Pack-years for smokers (±SD)	44.58±25.92*	38.54±23.12
Smoking status (%)		
Current and former smokers (%)	392 (76.71)	375 (73.94)
Non-smokers (%)	119 (23.29)	133 (26.06)
Post-FEV_1% (±SD)	41.68±19.32***	130±52.1
Post-FEV_1/FVC ratio (±SD)	58.66±13.66	87.94±10.69
FVC % (±SD)	44.22±17.88	128.1±32.05
GOLD status (%)		
Stage II	149 (29.16)	
Stage III	139 (27.20)	
Stage IV	223 (43.64)	

P<0.05 ***<0.001 compared to controls. Pack-years PY=Number of cigarettes per day×number of years smoked)/20. BMI, body mass index; SD, standard deviation; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; Post, post-bronchodilator; GOLD, global initiative for chronic obstructive lung disease; COPD, chronic obstructive pulmonary disease

Sample size: The sample size was calculated by Quanto software[25]. The sample size (n=511 for study group and n=508 for control group) was sufficient to detect the association of the examined five candidate genes and COPD with more than 80 per cent power (Power: 95.53%, disease prevalence, 7%, error: 5%). Based on minor allele frequency (MAF) of six candidate SNPs: *IREB2* (rs13180), *CHRNA5* (rs16969968), *CHRNA3* (rs1051730), *CHRNA3* (rs6495309), *FAM13A* (rs7671167), *HHIP* (rs13118928) in Caucasians (HapMapCEU)[21], a power calculation was performed for the study. To detect an odds ratio (OR) of 2.0, assuming a power of 80 per cent, significance level 5 per cent, disease prevalence, 7 per cent, 225 persons were required in each group for *IREB2*, 218 persons for *CHRNA5*, 218 persons for *CHRNA3* (rs1051730), 138 persons for *CHRNA3* (rs6495309), 163 persons for FAM13A, 215 persons for *HHIP*. To account for possible variations in the genotype distribution in small datasets, 511 patients were included in COPD group and 508 individuals in control group.

Genotyping: Genomic DNA was isolated from peripheral blood leucocytes using the standard phenol-chloroform extraction procedure[26]. Six SNPs: rs13180 (*IREB2*), rs16969968 (*CHRNA5*), rs1051730, rs6495309 (*CHRNA3*), rs7671167 (*FAM13A*), rs13118928 (*HHIP*) were examined by real-time PCR, with the use of commercial kits (TaqMan SNP discrimination assays) custom designed by (*http://testgen.ru*, "TestGene" LLC, Ulyanovsk, Russia). Real-time PCR amplified in 20 µl of the reaction mixture containing 1 µl of genomic DNA (concentration 30 ng/µl) and PCR Master Mix containing 200 nM of each dNTP, 67 mM Tris-HCl (pH 8.8 at 25°C), 16.6 mM $(NH_4)_2SO_4$, 0.01 per cent Tween - 20, 2 mM $MgCl_2$, 500 nM primers, 250 nM fluorogenic probes and 1.5 units of *Taq* polymerase (Thermo Fisher Scientific Inc., USA). PCR was performed by initial denaturation for 2 min at 95°C, 40 cycles of denaturation for 10 sec at 94°C, annealing for 1 min at different annealing temperatures (Table II). Accumulation of specific PCR-product by hybridization and cleavage of double-labelled fluorogenic probe during amplification was detected with a BioRad CFX96 instrument (Bio-Rad Laboratories Inc., USA). End-point fluorescence and genotype discrimination were determined according to the BioRad CFX96 protocol, using CFX Manager software. Individuals with each of the three possible genotypes for each SNP were confirmed by sequencing by kit ("TestGene" LLC, Ulyanovsk, Russia) and included on each genotyping tray as control. For quality control, 5 per cent duplicates and blank controls were also taken up along with the samples in each experiment. The genotyping was blind to case or control status of the samples. The data were excluded after examining missingness, reproducibility and inbreeding. All subjects with a genotype call rate of <95 per cent were removed. Subsequently, SNPs were filtered according to their proportion of missing, MAF or deviation from Hardy-Weinberg-Equilibrium (HWE)[27].

Statistical analysis: For the quantitative traits, the mean values and standard deviations were calculated; the group comparison was performed with a non-parametric Mann–Whitney U-test. The frequencies of qualitative traits were compared using the Pearson's chi-square analysis. Statistical analysis was carried out with the Statistica v. 6.0 programme (StatSoft Inc., Tulsa, OK, USA). MAF and the agreement of the genotype distribution to the HWE (χ^2), the association analysis using the basic allele test and the calculation of the OR for the rare allele of each locus and the significance

Table II. Sequences of the amplification primers and fluorogenic probes and polymerase chain reaction conditions

SNP	Sequences of the primers, 5'-3'	Sequences of the fluorogenic probes, 5'-3'	Annealing temperature (°C)
IREB2-rs13180	F- AGAGGGAATAAAATGCACA	FAM- ctgttttggcCgaaagttatga -BHQ-1	60
	R- CTGGAAGGAACTGAAGTG	VIC- ctgttttggcTgaaagttatga -BHQ-2	
HHIP-rs13118928	F- CTAGGCATATCCTATATTC	FAM- aactgaaTgaTagacaatc -BHQ-1	56
	R- CCATGAAAAATTCTTGAA	VIC- aactgaaTgaCagacaatc -BHQ-2	
FAM13A-rs7671167	F- CTCAGAACACACATCAAC	FAM- aggtaaaTtcCaaAacataagg -BHQ-1	58
	R- CTTCCATGTCAACTAACG	VIC- aggtaaaTtcTaaAacataagg -BHQ-2	
CHRNA5-rs16969968	F- AGTGGACCAAAATCTTCTA	FAM- aatagaatCgagcgcagc -BHQ-1	56
	R- CCTTCATGATGTGTCTTG	VIC- aatagaatTgagcgcagc -BHQ-2	
CHRNA3-rs1051730	F- CAAGGACTATTGGGAGAGC	FAM- ccccaggctacaaacacgacatcaa -BHQ-1	60
	R- GGGATGATGAGGTTGATGG	VIC- ccccaggctaTaaacacgacatcaa -BHQ-2	
CHRNA3-rs6495309	F- CGTGGAAGATGTTCAAATCA	FAM- agccatcagggacaCgcaaattaaaacca -BHQ-1	60
	R- TCTGGAGTGGCTATACCA	VIC- agccatcagggacaTgcaaattaaaacca -BHQ-2	

A reporter dye at the 5′ end of each probe - VIC, dye is linked to the 5′ end of the allele 1 probe; FAM, dye is linked to the 5′ end of the allele 2 probe; a non-fluorescent quencher at the 3′ end of each probe BHQ-1 and BHQ-2. SNP, single-nucleotide polymorphism. *Source*: Primers were chosen from the nucleotide sequences deposited in GenBank (*http://www.ncbi.nlm.nih.gov/nuccore/*) using Primer Express Software v2.0 Applications-Based Primer Design Software (*http://home.appliedbiosystems.com/*). Primers and fluorogenic probes were purchased from "TestGene" LLC (Ulyanovsk, Russia)

of inter-group differences in allele and genotype frequencies (Chi-square test for sample heterogeneity and the *P* value) and Cochran-Armitage trend test were performed with PLINK v. 1.07[28]. To control Type I error rate, Bonferroni correction for multiple comparison was performed. Logistic regression was used to detect the association of SNPs and haplotypes of linked loci in different models, accounting for quantitative and binary traits [gender, age, pack-years, smoking status, body mass index (BMI)]. The significance of the obtained model accounting for all variables was verified by the significance of the likelihood ratio test (P_{adj}). The best model was chosen using the Akaike's information criterion (AIC). For each significant locus ($P<0.05$), the model with lowest AIC was chosen. Linear regression analyses were performed to estimate the relationship between SNPs and quantitative phenotypes, such as lung function parameters and pack-years. The regression analysis was performed with PLINK v. 1.07 and SNPStats packages[27,29]. The linkage disequilibrium (LD) structure in the *CHRNA3/5* and *IREB2* region and haplotype frequencies, the standard LD coefficient (D'), the inter-group differences in the haplotype frequencies were calculated with Haploview 4.2[29].

Results

Systematic quality control procedures were performed to obtain a high quality of data. Subsequently, SNPs were filtered according to their proportion of missings, MAF or deviation from HWE within the controls. For the control group, the following results were obtained: *IREB2* (rs13180) (*P*=0.123, MAF=0.410), *CHRNA5* (rs16969968) (*P*=0.457, MAF=0.271), *CHRNA3* (rs1051730) (*P*=0.494, MAF=0.238), *CHRNA3* (rs6495309) (*P*=0.984, MAF=0.204), *FAM13A* (rs7671167) (*P*=0.141, MAF=0.465), *HHIP* (rs13118928) (*P*=0.954, MAF=0.334). The data on the allele and genotype frequency distribution of six SNPs: *IREB2* (rs13180), *CHRNA5* (rs16969968), *CHRNA3* (rs1051730, rs6495309), *FAM13A* (rs7671167), *HHIP* (rs13118928) in COPD and control groups were obtained (Table III).

The COPD and control groups differed significantly in the allele and genotype frequency distributions of *IREB2* (rs13180) (*P*=0.00001, OR=0.67 for allele test and *P*=0.00001, OR=0.64 for Cochran-Armitage test), *CHRNA5* (rs16969968) (*P*=0.0001, OR=1.42 and *P*=0.00034, OR=1.41) and *CHRNA3* (rs1051730) (*P*=0.0002, OR=1.44 and *P*=0.00015, OR=1.47) (Table III). At the next stage, the association was analyzed using various models. By this approach, the *IREB2* (rs13180) showed association with COPD in recessive model (P_{adj}=0.0001, P_{cor}=0.0006, OR=0.61) and additive model (P_{adj}=0.00001, P_{cor}=0.00006, OR=0.64) (Table IV). The COPD risk was higher in homozygous and heterozygous carriers of the rare A

Table III. Allele and genotype frequencies of the *IREB2, CHRNA5, CHRNA3, FAM13A* and *HHIP* polymorphisms in chronic obstructive pulmonary disease (COPD) patients and control subjects

Gene refSNP	Minor allele	Alleles/ Genotypes	HWE (*P*)	COPD, n (%)	Control, n (%)	*P* [a]	*P* [b]	OR (95% CI)
IREB2 rs13180 *C>T*	C	C/T	0.123	325/697 (31.80/68.20)	417/599 (41.04/58.96)	0.00001	-	0.67 (0.56-0.80)
		CC/CT/TT		61/203/247 (11.94/39.73/48.34)	92/233/183 (18.11/45.87/36.02)	0.0001	0.00001	0.64 (0.52-0.79)
CHRNA5 rs16969968 *G>A*	A	A/G	0.457	354/668 (34.68/65.32)	276/740 (27.12/72.88)	0.0001	-	1.42 (1.18-1.72)
		AA/AG/GG		67/222/222 (12.90/43.55/43.55)	38/200/270 (7.46/39.32/53.22)	0.001	0.00034	1.41 (1.12-1.79)
CHRNA3 rs1051730 *G>A*	A	A/G	0.494	318/704 (31.12/68.88)	242/774 (23.79/76.21)	0.0002	-	1.44 (1.19-1.76)
		AA/AG/GG		50/217/244 (9.84/42.55/47.61)	20/201/287 (3.99/39.60/56.41)	0.0001	0.00015	1.47 (1.16-1.86)
CHRNA3 rs6495309 *G>A*	A	A/G	0.984	195/830 (19.08/80.92)	207/809 (20.41/79.59)	0.506	-	0.91 (0.74-1.14)
		AA/AG/GG		23/149/339 (4.50/29.15/66.35)	15/177/316 (2.97/34.86/62.16)	0.087	0.51	0.92 (0.72-1.18)
FAM13A rs7671167 *G>A*	G	G/A	0.141	466/556 (45.61/54.39)	472/544 (46.50/53.50)	0.747	-	0.96 (0.81-1.15)
		GG/GA/AA		119/229/163 (23.23/44.76/32.01)	112/249/147 (22.00/49.00/29.00)	0.393	0.75	0.97 (0.78-1.20)
HHIP rs13118928 *A>G*	G	G/A	0.954	317/705 (30.97/69.03)	339/677 (33.39/66.61)	0.277	-	0.89 (0.74-1.08)
		GG/GA/AA		51/215/245 (9.94/42.05/48.01)	70/198/240 (13.87/39.05/47.08)	0.155	0.59	0.92 (0.68-1.34)

[a]Chi-square test for allele or genotypes frequency difference between COPD and control; [b]Cochran–Armitage trend test, OR with 95% CI for minor allele in basic allele test or Cochran–Armitage trend test. OR, odds ratio; CI, confidence interval; HWE, Hardy–Weinberg equilibrium; SNP, single nucleotide polymorphism

allele of *CHRNA5* (rs16969968) (P_{adj}=0.001, P_{cor}=0.006, OR=1.47 in dominant model). The highest level of significance (P_{adj}=0.0001, P_{cor}=0.0006) and the lowest AIC were obtained for the additive model, the risk of COPD was associated with each copy of the rare A allele (OR=1.41) (Table IV). In the COPD group, the frequency of AA genotype of the *CHRNA3* (rs1051730) two-fold increase (9.8 vs. 4% in control, P_{adj}=0.0002, P_{cor}=0.0012, OR=2.63, in recessive model). The lowest Akaike's information criteria testing the association of rs1051730 was obtained for the additive model with corresponding P_{adj}=0.0001 (P_{cor}=0.0006, OR=1.47) (Table IV).

Haplotype association analysis: Since *IREB2* (rs13180), *CHRNA5* (rs16969968), *CHRNA3* (rs1051730, rs6495309) belong to the same LD block on chromosome 15q25, we analyzed the haplotype frequencies of these polymorphisms in COPD and controls groups. The pairwise LD values (D' - Lewontin's coefficient, r^2- correlation coefficient between the two loci) were calculated for rs13180, rs16969968, rs1051730, rs6495309 in chromosome 15q25. Strong LD between SNPs were found (Figure and Table V). The strong levels of LD between rs16969968 and rs1051730 (D'=0.783, r^2=0.516) were observed. Both SNPs were significantly associated with COPD in the Tatar population with similar ORs.

Based on the results of the haplotype frequency analysis of *CHRNA5* (rs16969968), *CHRNA3* (rs1051730, rs6495309), it was observed that the COPD group differed significantly from control individuals in their haplotype frequency distribution (Table VI). The percentage of the A-A-G haplotype by rs16969968, rs1051730, rs6495309 was higher among COPD patients (25.8% in COPD vs. 20.74% in control, P_{adj}=0.0078, OR=1.44). Haplotype analysis demonstrated significant association of *IREB2* (rs13180) and *CHRNA3* (rs1051730) C-G haplotype

Table IV. Association between *IREB2, CHRNA5, CHRNA* polymorphisms and chronic obstructive pulmonary disease (COPD)

Gene, SNP	Minor allele	Genotype/ model	COPD (N=511), n (%)	Control (N=508), n (%)	OR_{adj} (95% CI)	P_{adj} [a]	P_{cor}
IREB2 rs13180 C>T	C	TT TC CC Codominant	247 (48.5) 203 (39.7) 61 (11.8)	183 (36.02) 233 (45.87) 92 (18.11)	1.00 0.72 (0.52-1.00) 0.38 (0.25-0.60)	0.0001	0.0006
		TT TC+CC Dominant	247 (48.5) 264 (51.5)	183 (36.02) 325 (63.98)	1.00 0.60 (0.44-0.81)	0.0009	0.0054
		TT+TC CC Recessive	450 (88.2) 61 (11.8)	416 (81.89) 92 (18.11)	1.00 0.61 (0.43-0.86)	0.0001	0.0006
		Log-additive	-	-	0.64 (0.52-0.79)	0.00001	0.00006
CHRNA5 rs16969968 G>A	A	GG GA AA Codominant	222 (43.5) 222 (43.5) 67 (12.9)	270 (53.2) 200 (39.3) 38 (7.5)	1.00 1.35 (0.98-1.87) 2.11 (1.22-3.67)	0.001	0.006
		GG GA+AA Dominant	222 (43.5) 289 (56.5)	270 (53.2) 238 (46.8)	1.00 1.47 (1.08-2.00)	0.001	0.006
		GG+GA AA Recessive	444 (87.1) 67 (12.9)	470 (92.5) 38 (7.5)	1.00 1.84 (1.08-3.12)	0.002	0.012
		Log-additive	-	-	1.41 (1.12-1.79)	0.0001	0.0006
CHRNA3 rs1051730 G>A	A	GG GA AA Codominant	244 (47.6) 217 (42.5) 50 (9.8)	287 (56.4) 201 (39.6) 20 (4)	1.00 1.27 (0.94-1.73) 2.92 (1.53-5.59)	0.0001	0.006
		GG GA+AA Dominant	244 (47.6) 267 (52.4)	287 (56.4) 221 (43.6)	1.00 1.42 (1.06-1.91)	0.0018	0.011
		GG+GA AA Recessive	461 (90.2) 50 (9.8)	488 (96) 20 (4)	1.00 2.63 (1.39-4.95)	0.0002	0.0012
		Log-additive	-	-	1.47 (1.16-1.86)	0.0001	0.0006

[a]P_{adj}, significance in the likelihood ratio test for the regression model adjusted for age, sex, BMI, smoking status and pack-years; P_{cor}, significance after the Bonferroni correction for multiple testing; BMI, body mass index; OR_{adj}, adjusted odds ratio; CI, confidence interval; SNP, single-nucleotide polymorphism

Table V. Linkage disequilibrium between the *CHRNA3/5* and *IREB2* genes polymorphic markers

Locus 1	Locus 2	D'	LOD	r^2	CIlow	CIhi	Distance
IREB2 rs13180	*CHRNA5* rs16969968	0.787	25.07	0.136	0.68	0.86	93,437
IREB2 rs13180	*CHRNA3* rs1051730	0.657	13.61	0.08	0.53	0.76	104,851
IREB2 rs13180	*CHRNA3* rs6495309	0.404	12.47	0.088	0.31	0.49	125,757
CHRNA5 rs16969968	*CHRNA3* rs1051730	0.783	87.09	0.516	0.73	0.83	11,414
CHRNA5 rs16969968	*CHRNA3* rs6495309	0.928	18.47	0.101	0.81	0.98	32,320
CHRNA3 rs1051730	*CHRNA3* rs6495309	0.776	10.32	0.059	0.61	0.88	20,906

D', value of normalized linkage disequilibrium coefficient (Lewontin's coefficient) between the two loci; LOD, log of the likelihood OR, a measure of confidence in the value of D'; r^2, correlation coefficient between the two loci; CIlow, 95% confidence lower bound on D'; CIhi, 95% confidence upper bound on D'; Dist, distance (in bases) between the loci; OR, odds ratio

with COPD [P_{adj}=0.0005, OR=0.61 95% confidence interval (CI) 0.47-0.81] (Table VI).

Association of IREB2, CHRNA3/A5, FAM13A and HHIP polymorphisms with COPD stratified by smoking status: No significant gene was observed by environment interactions in the logistic regression analysis of six studied SNPs with smoking status and pack-years of smoking. Genotype and environment interactions were also analyzed by comparing the OR values for studied SNPs obtained for the groups of smokers and non-smokers.

The COPD risk in smokers was associated with *CHRNA5* (rs16969968) (P_{adj}=0.002, P_{cor}=0.012, OR=1.44) and *CHRNA3* (rs1051730) and (P_{adj}=0.00004, P_{cor}=0.00024, OR=2.11) in additive model and *IREB2* (rs13180) in recessive model (P_{adj}=0.00001, P_{cor}=0.00006, OR=0.49) (Table VII). The A-A-G haplotype by rs16969968, rs1051730, rs6495309 (P_{adj}=0.0071, OR=1.56 95% CI 1.13-2.16) and of C-G haplotype by *IREB2* (rs13180) and *CHRNA3* (rs1051730) (P_{adj}=0.0001, OR=0.58 95% CI 0.42-0.80) were significantly associated with COPD in smokers.

Single markers and haplotype association analysis in non-smokers did not demonstrate any significant association with COPD. The groups of COPD patients, stratified by smoking status - smokers (n=392) and non-smokers (n=119) were also compared. The groups of COPD patients did not differ by any of the studied polymorphisms.

Genetic association results between the IREB2, CHRNA3/A5, FAM13A and HHIP polymorphisms and lung function parameters: The relationship between the rs13180 (*IREB2*), rs16969968 (*CHRNA5*), rs1051730, rs6495309 (*CHRNA3*), rs7671167 (*FAM13A*), rs13118928 (*HHIP*) and lung function parameters

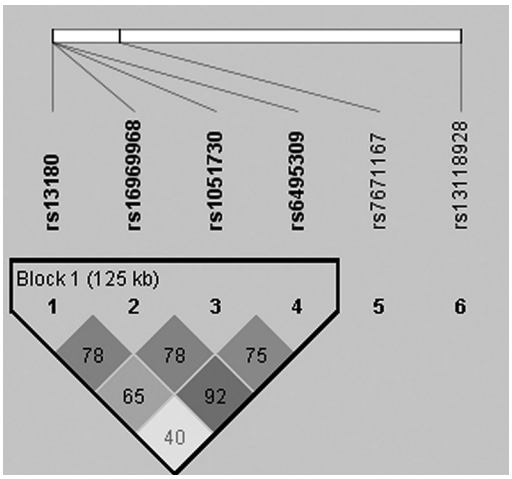

Figure. Visualization of linkage disequilibrium among single-nucleotide polymorphisms on chromosome 15q25 in Tatar population from Russia (linkage disequilibrium values are presented as D' and linkage disequilibrium block, calculated with Haploview 4.2 software).

Table VI. Association of *IREB2*, *CHRNA5* and *CHRNA3* haplotypes with chronic obstructive pulmonary disease (COPD)

Haplotype	Overall frequency	Haplotype association with COPD		
		Frequency in patients/controls	OR (95% CI)	P_{adj} [b]
rs16969968 G>A - rs1051730 G>A - rs6495309 G>A of *CHRNA3/A5* (n=1019)[a]				
G-G-G	0.4601	0.4325/0.491	1.00	-
A-A-G	0.234	0.258/0.2074	1.44 (1.10-1.88)	0.0078
G-G-A	0.1854	0.1709/0.2015	0.96 (0.72-1.28)	0.77
A-G-G	0.0746	0.08/0.0686	1.30 (0.84-2.01)	0.24
G-A-G	0.0335	0.0385/0.0274	1.51 (0.78-2.93)	0.22
Rare	0.0125	0.0201/0.0041	4.98 (0.76-32.65)	0.095
Haplotype association in general (*P*)				0.0082
rs13180 C>T - rs1051730 G>A of *IREB2* and *CHRNA3* (n=1019)				
T-G	0.3955	0.4191/0.3715	1.00	-
C-G	0.3291	0.2697/0.3911	0.61 (0.47-0.81)	0.0005
T-A	0.2313	0.2638/0.1957	1.15 (0.85-1.56)	0.37
C-A	0.044	0.0474/0.0439	1.27 (0.67-2.41)	0.47
Haplotype association in general (*P*)				0.0001

[a]number of individuals included in the regression analysis; [b]P_{adj}, significance in the likelihood ratio test for the regression model adjusted for sex, age, pack-years, smoking status, BMI. BMI, body mass index; OR, odds ratio; CI, confidence interval

Table VII. Association analysis of *IREB2*, *CHRNA5*, *CHRNA3* polymorphisms with chronic obstructive pulmonary disease (COPD) in smokers

Gene, SNP	Minor allele	Test/model	COPD (N=392), n (%)	Control (N=375), n (%)	OR_{adj} (95% CI)	P_{adj} [a]	P_{cor}
IREB2 rs13180 C>T	C	TT TC CC Codominant	187 (47.7) 156 (39.8) 49 (12.5)	121 (32.27) 175 (46.67) 79 (21.07)	1.00 0.65 (0.44-0.97) 0.31 (0.19-0.51)	0.0001	0.0006
		TT TC+CC Dominant	187 (47.7) 205 (52.3)	121 (32.27) 254 (67.73)	1.00 0.52 (0.36-0.74)	0.0003	0.0018
		TT+TC CC Recessive	347 (87.5) 49 (12.5)	296 (78.93) 79 (21.07)	1.00 0.49 (0.25-0.61)	0.0001	0.0006
		Log-additive	-	-	0.57 (0.45-0.73)	0.0001	0.0006
CHRNA5 rs16969968 G>A	A	GG GA AA Codominant	167 (42.7) 176 (45) 49 (12.2)	196 (52.3) 155 (41.3) 24 (6.4)	1.00 1.34 (0.92-1.95) 2.29 (1.16-4.54)	0.005	0.03
		GG GA+AA Dominant	167 (42.7) 225 (57.3)	196 (52.3) 179 (47.7)	1.00 1.47 (1.02-2.10)	0.012	0.072
		GG+GA AA Recessive	343 (87.8) 49 (12.2)	351 (93.5) 24 (6.4)	1.00 2.00 (1.03-3.86)	0.01	0.06
		Log-additive	-	-	1.44 (1.09-1.90)	0.002	0.012
CHRNA3 rs1051730 G>A	A	GG GA AA Codominant	183 (46.7) 174 (44.4) 44 (8.9)	216 (57.6) 147 (39.2) 12 (3.2)	1.00 1.39 (0.97-2.00) 3.50 (1.48-8.32)	0.0001	0.0006
		GG GA+AA Dominant	183 (46.7) 218 (53.3)	216 (57.6) 159 (42.4)	1.00 1.55 (1.09-2.19)	0.0013	0.0078
		GG+GA AA Recessive	357 (91.1) 44 (8.9)	363 (96.9) 12 (3.1)	1.00 3.02 (1.29-7.07)	0.0001	0.0006
		Log-additive	-	-	1.58 (1.18-2.11)	0.00004	0.00024

[a]P_{adj}, significance in the likelihood ratio test for the regression model adjusted for age, sex, pack-years, BMI. P_{cor}, significance after the Bonferroni correction for multiple testing. OR_{adj}, adjusted odds ratio; CI, confidence interval; BMI, body mass index; SNP, single-nucleotide polymorphism

was investigated. The rs16969968 (*CHRNA5*) was associated with $FEV_{1\%}$ predicted in dominant model (b=−3.81, s.e.=1.48, P_{adj}=0.005). The presence of GA genotype for the rs1051730 (*CHRNA3*) was associated with a 4.36 per cent decrease in $FEV_{1\%}$ predicted (b=−4.36, s.e.=1.72, P_{adj}=0.0019) (Table VIII).

There was no significant association between the rs13180 (*IREB2*), rs16969968 (*CHRNA5*), rs1051730, rs6495309 (*CHRNA3*), rs7671167 (*FAM13A*), rs13118928 (*HHIP*) and FEV_1/FVC predicted in the COPD patients.

Discussion

The purpose of our study was to analyze the contribution of *IREB2, CHRNA5, CHRNA3, FAM13A* and *HHIP* polymorphisms to COPD in the ethnically homogenous Tatar population from Russia. It was found that rs13180 (*IREB2*), rs16969968 (*CHRNA5*) and rs1051730 (*CHRNA3*) were associated with COPD in the Tatar population. The minor alleles of both rs16969968 (*CHRNA5*), rs1051730 (*CHRNA3*) and the A-A-G haplotype by rs16969968, rs1051730, rs6495309 of *CHRNA3/A5* were significantly associated with COPD.

Polymorphisms of rs16969968 (*CHRNA5*) and rs1051730 (*CHRNA3*) were significantly associated with lung function ($FEV_{1\%}$ predicted) after adjusting for age, sex, BMI, pack-years and smoking status. Similar results were observed in a Chinese population[30-32]. Our data also corroborated with the results obtained in several Caucasian populations[4-6]. A meta-analysis by Zhang *et al*[14] showed that A allele of rs1051730 (*CHRNA3*) was a COPD susceptibility factor, both for respiratory airway obstruction and for emphysematous destruction of lung parenchyma. Shpagina *et al*[33] did not find any association between rs1051730 (*CHRNA3*) and occupational chronic obstructive lung disease in Russian population from Novosibirsk. Siedlinski *et al*[34] confirmed the existence of direct effects of the *CHRNA3, IREB2, FAM13A* and *HHIP* loci on COPD development. This study indicated that the association of the *CHRNA3* locus with COPD was significantly mediated by smoking status, and *IREB2* associated with COPD independent of smoking. Lococo *et al*[35] demonstrated that the variants on the gene cluster *CHRNA3/A5/B4* were associated with nicotine addiction and antismoking therapy side effects. We did not observe any significant association of these loci with the smoking index. The rs13180 (*IREB2*), rs16969968 (*CHRNA5*) and rs1051730 (*CHRNA3*) were significantly associated with COPD only in smokers, which might be due to the insufficient size of the non-smoker sample in our study.

Table VIII. Association results between, *CHRNA5* and *CHRNA3* polymorphisms and lung function in chronic obstructive pulmonary disease (COPD) patients

Gene, SNP	Model	*P*	$FEV_{1\%}$ predicted, mean (SE)[a]	*b* (95% CI)
CHRNA5 rs16969968 G>A	GG GA+AA Dominant	0.005	41.51 (1.7) 38.32 (1.48)	0.00−3.81 (-6.45 - -1.17)
	AA+GG GA	0.0048	41.13 (1.56) 37.93 (1.56)	0.00−3.82 (-6.45 - -1.19)
CHRNA3 rs1051730 G>A	GG+AA GA	0.0019	40.44 (1.47) 37.91 (1.72)	0.00−4.36 (-7.08 - -1.64)

[a]Linear regression analysis adjusting for age, gender, BMI, pack-years and smoking status. Data presented are beta, mean and SE with two-sided *P* values. FEV_1, forced expiratory volume in 1 sec; SE, standard error; SNP, single-nucleotide polymorphism; BMI, body mass index; CI, confidence interval

Table IX. Minor allele frequencies (%) of the *IREB2, CHRNA5, CHRNA3, FAM13A* and *HHIP* polymorphisms in control Tatar population and in worldwide populations

Gene refSNP	Minor allele	Control Tatar population (N=508)	Caucasian HapMap-CEU[a] (N=226)	Chinese HapMap-HCB (N=86)	Japanese HapMap-JPT (N=172)	Africans HapMap-YRI (N=226)	Indians HapMap-GIH (N=176)
IREB2 rs13180 C>T	C	41.04	36.7	54.7	65.1	80.5	51.1
CHRNA5 rs16969968 G>A	A	27.12	38.5	3.6	1.2	0	21.0
CHRNA3 rs1051730 G>A	A	23.79	38.5	3.5	1.2	9.7	21.0
CHRNA3 rs6495309 G>A	A	20.41	19.2	52.4	48.8	27.9	46.6
FAM13A rs7671167 G>A	G	46.50	48.2	47.7	39.0	42.9	46.0
HHIP rs13118928 A>G	G	33.39	38.9	30.2	34.9	7.1	47.2

[a]*Source*: Ref. 21

HapMap-CEU, Utah residents with ancestry from northern and western Europe (Caucasian); HapMap-HCB, Han Chinese in Beijing, China; HapMap-JPT, Japanese in Tokyo, Japan, HapMap-YRI, Yoruba in Ibadan, Nigeria; HapMap-GIH, Gujarati Indians in Houston, TX, USA; SNP, single-nucleotide polymorphism

Analysis of the allele frequency distribution patterns at the *CHRNA5* (rs16969968) and *CHRNA3* (rs1051730) among the Tatar ethnic groups in comparison with the worldwide populations indicated that significant interethnic differences were found among the Tatar population and Mongoloid and Africans populations, having a minimum frequency of rare alleles of both markers [3.6% in Chinese (HapMap-HCB), 1.2% in Japanese (HapMap-JPT), 0 per cent in Africans (HapMap-YRI) for rs16969968; and 3.5, 1.2 and 9.7% for rs1051730][21] (Table IX). Analysis of previously published data concerning *CHRNA5* (rs16969968) and *CHRNA3* (rs1051730) markers allele frequencies distribution showed that the Tatars ethnic group from Russia differed significantly in terms of these markers from the general population of Caucasians (HapMap-CEU[21]), in which the rs16969968 and rs1051730 rare allele frequency was 38.5 per cent. Tatars and Indians are in an intermediate position, for them the frequency of minor allele does not exceed 28 per cent. Allele frequencies in Tatars were similar in the prevalence of the polymorphic variants of *CHRNA5* (rs16969968) and *CHRNA3* (rs1051730) markers among Indians (HapMap-GIH)[21] (27.12 vs. 21.0% and 23.79 vs. 21.0%).

We also analyzed the potential COPD association with the rs6495309 polymorphism of the *CHRNA3* promoter in Tatar population from Russia. This locus is particularly interesting, since it alters the promoter affinity to the octamer-binding transcription factor (Oct-1), thus affecting *CHRNA3* expression[31]. However, in the population studied, rs6495309 (*CHRNA3*) was not significantly associated with COPD or its progression. It has been shown to be associated with COPD in populations from China and Korea[36,37]. It was also linked with lung cancer risk and prognosis in a Chinese population[37]. Significant associations of rs6495309 with COPD observed in Mongoloid populations may be related to the high frequency of the A allele, which ranges from 48.8 per cent in the Japanese to 52.4 per cent in the Chinese[21]. On the other hand, rs6495309 allele and genotype frequencies in Tatars from Russia are similar to those found in other Caucasian populations[21], where A is the minor allele with frequencies of below 20 per cent (Table IX).

IREB2 polymorphism rs13180 was also associated with COPD in the population studied. The C-G haplotype by rs13180 and rs1051730 SNPs of *IREB2* and *CHRNA3* was a protective factor for COPD in the present study. *IREB2* polymorphisms have been reported to be associated with lung function parameters and with COPD in a nonsmoking Chinese population[30]. In a Polish population, rs13180 was associated with severe COPD[10]. Chappell *et al*[11] confirmed the involvement of *IREB2* polymorphisms in predisposition to COPD in Caucasian populations. The SNPs in the *IREB2* showed associations with COPD in case-control and family-based studies[5,10,30-32]. Two key processes involved in COPD pathogenesis and lung tissue damage, proteolysis and oxidative stress, are related to each other via intracellular iron homeostasis[5,11]. It has been shown that *IREB2* is actively expressed in the lungs, whereas aberrations of iron balance can cause oxidative stress and lung tissue inflammation[5].

Our study did not confirm an association between the rs13118928 (*HHIP*) and COPD in Tatars population from Russia. However, the frequencies of the minor G allele and the GG genotype in the Tatar population were similar to those observed in other Caucasian populations[8,21]. GWASs and subsequent replication studies showed that *HHIP* polymorphisms were associated with COPD and lung function parameters[19,20]. According to a study by Zhou *et al*[8], rs13118928 contributed to the risk of severe COPD in smokers in a Polish population. In another study, rs13118928 was not associated with COPD in the Chinese, but rs12504628 was associated with FEV_1/FVC, while rs10519717 of *HHIP* affected the severity of the disease[38].

In our study, rs7671167 polymorphism of *FAM13A* was not associated with COPD development, pulmonary function parameters and smoking index. rs7671167 was shown to be associated with COPD in former smokers in a Chinese population[9] and with COPD and lung function parameters in a population of Southern India[39]. The G allele of rs7671167 was shown to decrease the risk of COPD and lung cancer[3]. Choo *et al*[40] demonstrated an association between the emphysematous COPD phenotype.

In conclusion, our results showed association of rs13180 (*IREB2*), rs16969968 (*CHRNA5*) and rs1051730 (*CHRNA3*) with COPD and pulmonary function in Tatar population from Russia. Further studies involving other ethnic groups and populations of Russia are required to verify the genetic associations detected in GWASs of COPD.

Acknowledgment

This work was supported by the Russian Foundation for Basic Research grants (№13-04-00287, №14-04-97006, №14-06-97003) and the Russian Scientific Foundation for Humanities grant (13-06-00101).

***Conflicts of Interest*:** None.

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