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Association Between *CHRNA3* and *CHRNA5* Nicotine Receptor Subunit Gene Variants and Nicotine Dependence in an Isolated Populationof Kashubians in Poland

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Background: Genome-wide and allelic association studies have shown the contribution of *CHRNA5-A3-B4* nicotinic receptor subunit gene cluster within chromosome 15 to nicotine dependence (ND). While an association between several single-nucleotide polymorphisms (SNPs) at that locus and smoking quantity (cigarettes per day; CPD) has been well recognized, there are some inconsistencies in demonstrating the influence of these SNPs on other ND phenotypes. This uncertainty motivated us to examine the association of 3 selected SNPs (*CHRNA3* rs1051730, rs6495308, and *CHRNA5* rs55853898) with ND in an isolated population of Kashubians from Poland.

Material/Methods: The study sample consisted of 788 current daily smokers. ND was assessed by CPD, the Fagerstrom Test for Nicotine Dependence (FTND), its brief version - Heavy Smoking Index (HSI), and time to first cigarette after waking (TTF). The correlation between studied SNPs and dichotomized values of ND measures was assessed in the regression analysis. Bonferroni corrected p-value of 0.017 was set for a type 1 error.

Results: We found a robust association between risk allele A of rs1051730 and CPD >10 (odds ratio (OR)=1.77, 95% confidence interval (CI): 1.20-2.59, p=0.004), and a weak association, which did not survive correction for multiple testing, with FTND ≥4. No associations between studied SNPs and HSI or TTF were demonstrated.

Conclusions: Our findings confirm that rs1051730 influences ND phenotype, as defined by CPD.

MeSH Keywords: Polymorphism, Genetic • Receptors, Cholinergic • Smoking

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Background

Tobacco use poses a major health challenge worldwide. This dangerous habit claims the lives of almost 6 million people yearly. According to the World Health Organization, with the increasing cigarette consumption in developing countries, this number may increase to an estimated 8 million by the year 2030 [1]. Studies focused on identifying the factors which influence smoking-related behavior and elucidating the mechanisms that lead to nicotine dependence (ND) could aid in developing more effective interventions, such as smoking prevention and more effective treatment of ND, for controlling this tobacco epidemic [2].

Classical twin studies have yielded strong evidence that both genetic and environmental factors contribute significantly to smoking and ND [3–6]. Recently, newer-generation genetic studies have enabled identification of the genome region within locus 15q24-25.1 that might be involved in ND [7,8]. S ubsequently, several case-control and genome-wide association studies have reported associations between smoking behavior phenotypes and single-nucleotide polymorphisms (SNPs) in genes which encode alfa5, alfa3, and beta4 subunits of the neuronal nicotinic acetylcholine receptor (nAChR) and form a gene cluster (*CHRNA5-CHRNA3*-CHRNB4) within this region [8–24].

Nicotine found in tobacco is a highly addictive substance responsible for maintaining smoking behavior [25]. These properties of nicotine result from a stimulation of dopamine release in the brain and activation of the reward pathway, and are crucial to the development of substance dependence [26–28]. Thus, effects of nicotine in the central nervous system largely depend on nAChRs, and gene variants which alter the function of nACHRs subunits might influence ND.

Variation in the *CHRNA5-A3-B4* gene cluster within chromosome 15 is a source of promising candidates for smoking- and nicotine-dependence-related phenotypes. Two single-nucleotide polymorphisms (SNPs) of particular interest are rs16969968 in *CHRNA5* and rs1051730 in *CHRNA3*, which show a high degree of mutual correlation in samples of European ancestry (linkage disequilibrium [LD] decay [D']=1.0, and LD correlation coefficient [R2]=1.0), and are therefore often treated interchangeably [12–14,17,20–24]. The majority of reports focusing on these variants demonstrated their correlation with nicotine dependence or smoking quantity [12–14,17,20–23]. The consistency in results between different surveys indicates that these genetic findings are robust, regardless of the phenotypic classification systems and analytic methods used. In addition, several meta-analyses confirmed the significant influence of rs1051730-rs16966968 variants on smoking quantity, with stronger association when objective measures of tobacco consumption were used in comparison with self-reported cigarette consumption [29–31]. Despite the perfect linkage disequilibrium between these 2 SNPs, rs1051730 was found to provide an even stronger signal than rs16966968 [30]. While the functional relevance of rs1051730 is unknown, it may be a strong tag SNP for functional haplotypes in the *CHRNA5-A3-B4* gene cluster [30].

Additionally, there is evidence that a number of other SNPs within the *CHRNA5-A3-B4* gene cluster, including rs6495308 and rs55853698, may influence smoking phenotypes [9–11,17,19,20,29,32]. For instance, the intronic SNP at the rs6495308 locus has been reported to influence ND, as defined by the Fagerström Test for Nicotine Dependence (FTND) or smoking quantity; however, the significance of these associations did not always survive correction for multiple testing [9–11,17,19,20,32]. In one report, an association between rs6495308 and habitual smoking, defined as smoking more than 20 CPD, was found [32]. Although most studies were conducted in subjects of European origin, positive results were also replicated in populations of other ethnicities, such as Asians. For instance, significant associations of rs6495308 with smoking quantity at both the individual SNP and the haplotype levels were revealed in Korean males [19]. An additional SNP of interest, rs55853698, which lies within the promoter region of CHRNA5, is highly correlated with rs1051730 in Europeans (r^2>0.96) [29]. This SNP has drawn attention since it was first reported by Liu et al. [29]. For the first time, in a large sample of over 41,000 individuals of European descent, the investigation of this region, by using imputation genotypes for all SNPs across this locus from the 1000 Genomes Project and repeating meta-analysis, a highly significant association was identified between smoking quantity and the rs55853698 SNP. Due to the location of rs55853698 in the 5'-untranslated region of CHRNA5, it is suggested that this SNP affects mRNA transcription. The association of rs55853698 with smoking quantity was even slightly higher than that of rs1051730, an SNP that has been firmly established as influencing smoking [12–14,20–22].

In recent years, the importance of isolated populations for discovering the genetic determinants of behaviors or common diseases has been frequently highlighted. The homogeneity of the shared environmental factors and the limited allelic diversity in a genetically homogenous population makes this a valuable tool for genetic association studies [33,34]. For instance, the genes that influence smoking and ND might be enriched in such a population. Therefore, studies on small isolated populations may serve as a promising strategy for revealing potential genotype-phenotype association [35].

An association between selected SNPs and ND phenotypes has been consistently reported in individuals of European descent; however, the purpose of our study was to replicate these results in a Polish population. It is important to realize that genetic factors are thought to exert different effects on ND across different populations due to variations in the extent of linkage

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disequilibrium. Therefore, it is considered that associations between common variants and complex phenotypes demonstrated in genome-wide association studies must be extensively replicated across populations which may differ in SNP allele frequencies and associations of these SNPs with ND [36]. In addition, as the limited allelic diversity characteristic of isolated populations is known to favor the identification of associations in genetic studies, we focused on the isolated, genetically homogenous population of Kashubians.

Material and Methods

Subjects

Demographic and genealogical data and data related to smoking habits were collected from outpatients attending several health centers located in northern Poland in the region called Kashubia.

The number of indigenous Kashubians who inhabit this region is estimated at nearly 230,000 [37]. The isolation of this relatively small population is assumed to be mainly linguistic, cultural, and geographical, because its genetic structure has not yet been explored in detail. However, high endogamy rates, slow population expansion, and insignificant immigration make Kashubians a genetically isolated population.

Questionnaire-based face-to-face interviews with adult nonrelated current daily smokers who self-identified themselves as Kashubians were conducted by nurses.

Kashubian origin was accepted if the individual's parents and all grandparents were Kashubians who were fluent in the Kashubian dialect. A separate database compiling all living first-degree relatives of participants (parents, adult siblings, and offspring) was created to avoid the inclusion of first-degree relatives in the study sample. This database was searched each time new subjects were recruited, to screen for their potential first-degree relatives who may have already been recruited into the study. If 2 subjects appeared to be first-degree relatives, only 1 was recruited (preferably the younger smoker), because cigarette consumption becomes more frequent and sustained, and nicotine dependence develops, in young adulthood. Self-reporting of daily cigarette smoking for at least 1 year at the time of enrollment into the study was the defining criterion for a current daily smoker.

All subjects gave their written, informed consent for inclusion in the study. The protocol was approved by the Ethics Committee of the Medical University of Gdansk.

Table 1. Demographics and smoking profile in the Kashubian sample of current smokers.

Characteristics	Values: N (%), Mean ±SD
Males	506 (64.2)
Age (years)	43.6±13.8
Age category	
<30 yrs	162 (20.6)
30–45 yrs.	230 (29.2)
46–60 yrs.	320 (40.6)
>60 yrs.	76 (9.6)
Age at starting smoking (years)	18.9±3.8
Smokers who started smoking in the age ≤16 yrs	174 (22.1)
Duration of smoking; years	23.0±12.6
Number of cigarettes smoked daily	17.5±7.7
CPD category	
≤5 CPD	35 (4.4)
6–10 CPD	172 (21.8)
11–15 CPD	35 (16.5)
16–20 CPD	349 (44.3)
>20 CPD	102 (12.9)
FTND	4.2±2.1
HSI	2.8±1.3
TFC ≤5 min	213 (27.0)

A convenience sample of 788 current smokers aged 18–85 (mean age 43.6±13.8), including 282 women aged 20–73 years (mean age 46.5±11.5 years) and 506 males aged 18–85 years (mean age 42.0±14.6 years), was selected.

The mean age at which subjects starting smoking was 18.9±3.8 years, and the mean scores for CPD, FTND, and HSI in the sample were 17.5±7.7, 4.2±2.1, and 2.8±1.3, respectively. The demographic and smoking habit data in the study population is shown in Table 1.

Polymerase chain reaction

PCR was conducted in a total volume of 50 µL, including 2 µL template, 0.2 µL of each sense and antisense primers (Table 2), and 2×Master Mix Plus High GC (A&A Biotechnology; Gdynia, Poland), which contain 0.1 U/µL of DNA polymerase, 4 mM MgCl2, 200 µM dAtP, dCTP, dGTP, dTTP, and stabilizers.

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Table 2. PCR sense- and biotinylated antisense primers for amplifying fragments of the *CHRNA3* and *CHRNA5* genes, and sequencing primers*.

SNP	Primer	Sequence
rs1051730	rs1051730F	5'-AGG GAG AGG AGG ACA GAA A-3'
	biot-rs1051730R	5'-[Btn]AAG GAC TAT TGG GAG AGC G-3'
	rs1051730-B*	5'-TTG TAC TTG ATG TCG TGT TT-3'
rs55853698	rs55853698R-C	5'-TTG GGG AAT GGA GCA CTG AGT-3'
	biot-rs5585369F-C	5'-[Btn]GCT AGG CTG AGG CTG CTG TC-3'
	rs55853698*	5'-AAA CGA GGG CAG ACG CAG C-3'
rs6495308	rs6495308F	5'-TGT GTC CCC AAA CCT CTT CT-3'
	biot-rs6495308R	5'-[Btn]ACC CTT CTC AAC TAC GAC CT-3'
	rs6495308*	5'-CCT CTT CTC TAC TCT CC-3'

PCR was performed using the iCycler system (Bio-Rad). Cycling preceded by an initial denaturation at 94°C for 3 min was conducted with 35 cycles at 94°C for 30 s, at 60°C for 30 s and at 72°C for 30 s.

Pyrosequencing

The 3 selected SNPs within *CHRNA3* and *CHRNA5* genes were detecting by pyrosequencing performed using a PSQ 96MA analyzer (Pyrosequencing AB, Uppsala, Sweden) and PyroMark®GoldQ96 Reagents (Qiagen) in accordance with the manufacturer's instructions. We used 25 µL PCR product prepared with the use of a PyroMark Q96 Vacuum Workstation (Qiagen) for pyrosequencing according to the instructions of the manufacturer. Five pmol of the sequencing primers (Table 2) was applied to detect the polymorphisms.

Nicotine dependence measures

Nicotine dependence was assessed by the Fagerström Test for Nicotine Dependence (FTND), which is a commonly used questionnaire consisting of 6 items derived from the Fagerström Tolerance Questionnaire [38,39]. In scoring the FTND, yes/no items are scored from 0 to 1 and multiple-choice items are scored from 0 to 3. The items are summed to yield a total score of 0–10. A higher total FTND score corresponds to higher smoker nicotine dependence.

The second nicotine dependence phenotype was the Heaviness of Smoking Index (HSI), which is considered a shortened version of the FTQ/FTND and retains the 2 main items of these questionnaires: the number of cigarettes smoked per day (CPD) and the time to first cigarette after waking (TTF). The sum of CPD and TTF categorical measures constitutes the Heaviness of Smoking Index scores, which range from 0 to 6. HSI scores for the number of cigarettes smoked per day are: 0: 0–10 CPD; 1: 11–20 CPD; 2: 21–30 CPD; and 3: CPD >30, and for the time to first cigarette after waking, scores are: 0: TTF >60 min; 1: TTF 31–60 min; 2: TTF 6–30 min; and 3: TTF ≤5 min.

Although there is a consistent relationship between the number of cigarettes smoked per day and craving and urge to smoke (which correspond to the time to first cigarette after waking), there is also evidence to suggest that these 2 factors may not share the same etiology [40,41]. Therefore, we also studied the potential association of the selected SNPs with these single FTND- and HSI-derived items separately.

Statistical analysis

Haploview software (version 4.2) was used for performing the Hardy-Weinberg equilibrium (HWE) test [42]. Allele and genotype frequencies were compared between groups with the use of Yates' chi squared (χ^2) test.

Statistical analysis was performed using the Statistica 10.0 software (StatSoft Inc., USA). Correlations between rs1051730, rs6495308, and rs55853698 and the selected ND phenotypes were estimated using logistic regression analysis. ND phenotypes are presented as dichotomous traits and were incorporated into the analysis with cut-off points ≥4 and ≥3 for FTND and HSI, respectively. CPD and TTF, as proxy ND phenotypes, were dichotomized into low-dose smoking (≤10 CPD) and high-dose smoking (>10 CPD) categories, and into smoking the first cigarette up to 5 min after waking (TTF ≤5 min) and later (TTF >5 min). These cut-offs differentiated highly dependent smokers from less dependent ones in the study sample. Gender and age were included as covariates into all analyses and the results are presented as age- and sex-adjusted odds ratios (ODs) with their 95% confidence intervals (95%

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Table 3. Frequencies of alleles and genotypes in *CHRNA3* rs1051730-, *CHRNA3* rs6495308-, and *CHRNA5* rs55853698-tagging *loci* in the studied population and HapMap and 1000 Genomes CEU reference populations.

Polymorphism	Allele/ genotype	Frequency (%)		*p*-value
		Kashubians (north Poland)	Reference population (HapMap* or 1000 Genomes** CEU)	
rs1051730	A	30.5	38.5*	**0.02**
	G	69.5	61.5*	
	AA	10.6	15.9*	0.10
	AG	39.7	45.1*	0.27
	GG	49.6	38.9*	**0.034**
rs64955308	A	77.2	79.0*	0.55
	G	22.8	21.0*	
	AA	60.3	62.5*	0.65
	AG	33.9	33.0*	0.86
	GG	5.8	4.5*	0.56
rs55853698	A	79.3	60.1**	**0.00**
	C	20.7	39.9**	
	AA	70.3	37.4**	**0.00**
	AC	18.0	45.5**	**0.00**
	CC	11.7	17.2**	0.16

* Allele and genotype counts and frequencies derived from HapMap Data Release 27 Phase II + III, February 2009; ** allele and genotype counts and frequencies derived from 1000 Genomes Project Data Release Phase 3, May 2013. Values in boldface denote a significant association.

CI). We used the Bonferroni correction procedure for multiple testing in the logistic regression analyses, with a significance level of 0.016 (p-value divided by the number of studied SNPs) as a type 1 error.

Results

Genotyping of the 3 selected SNPs covering the *CHRNA5* and *CHRNA3* genes was successfully performed in all subjects, and genotypes were then checked for deviation from Hardy-Weinberg equilibrium (HWE). Distributions of genotypes for rs1051730 and rs6495308 (p=0.82 and p=0.59, respectively) were in HWE, and rs55853698 was found to show a strong deviation from HWE in the studied sample (p=0.000).

Comparison of allele frequencies and genotypes for rs6495308 found in our sample and samples downloaded from the HapMap and 1000 Genomes CEU reference populations (samples of Utah residents with Northern and Western European ancestry [*www.hapmap.org*; *www.1000genomes.org*]) showed no significant differences. However, the minor rs1051730-A and rs55853698-C alleles were significantly more frequent (p=0.02 and p=0.00) in the Kashubian sample. The frequencies of the rs1051730 and rs6495308 alleles in *CHRNA3*, and rs55853698 in *CHRNA5*, and the frequencies of the genotypes in the studied population are shown in Table 3.

The associations of rs1051730, rs64955308, and rs55853698 with ND phenotypes defined by FTND, CPD, his, or TTF are presented in Table 4.

We found a robust association of risk allele A of rs1051730 with CPD >10 (p=0.004), and a weak association that did not withstand correction for multiple testing with FTND ≥4. No associations between the studied SNPs and HSI or TTF were found (Table 4).

The post hoc analysis of ND using a relaxed CPD >5 cut-off, as well as 2 other cut-offs (CPD >15 and CPD >20), revealed significant associations between A-allele of rs1051730 and CPD, but p-value withstood Bonferroni correction only when CPD >15 was assumed as a cut-off (p= 0.05, p=0.012, and p=0.04, respectively) (Table 5).

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Table 4. Logistic regression results for the association between ND defined by FTND, CPD, HSI or TTF and selected polymorphisms.

SNP/genotype	FTND		OR	CPD		OR	HSI		OR	TTF		OR
	No. of subjects		(95% CI)	No. of subjects		(95% CI)	No. of subjects		(95% CI)	No. of subjects		(95% CI)
	<4	≥4	*p*-value	≤10	>10	*p*-value	<3	≥3	*p*-value	≤5 min	>5 min	*p*-value
rs1051730 (*CHRNA3*)												
GG	171	220	1	120	271	1	193	198	1	93	298	1
AA + AG	147	250	1.40 (1.00–1.95) 0.049	87	310	**1.77 (1.20–2.59) 0.004**	174	223	1.18 (0.85–1.64) 0.31	120	277	1.42 (0.99–2.06) 0.059
rs6495308 (*CHRNA3*)												
AA	188	287	1	114	361	1	218	257	1	131	344	1
GG+AG	130	183	0.98 (0.73–1.32) 0.90	93	220	0.81 (0.58–1.13) 0.22	149	164	0.99 (0.73–1.34) 0.94	82	231	0.99 (0.71–1.39) 0.95
rs55853698 (*CHRNA5*)												
AA	226	329	1	146	409	1	267	288	1	145	410	1
CC+CA	92	141	0.88 (0.61–1.27) 0.49	61	172	0.72 (0.47–1.08) 0.11	100	133	1.12 (0.79–1.60) 0.52	68	165	0.96 (0.64–1.41) 0.82

The values in boldface denote a significant association when applying Bonferroni corrected p-value of 0.017. The underlined values denote a significant association when applying significance level p=0.05 non corrected for multiple testing. ORs are adjusted for age, sex and remaining polymorphisms.

Discussion

Numerous studies have explored the influence of the *CHRNA5/CHRNA3/CHRNB4* gene cluster in chromosome 15 on smoking-related behaviors and ND, with FTND and CPD being the most frequently studied ND phenotypes [8–24]. Several studies have also examined the link between SNPs within this genome region and HSI, or a single FTND/HSI-derived item, TTF [20,22]. The association between the *CHRNA5/CHRNA3/CHRNB4* gene cluster and smoking habits has been firmly established through several genome-wide meta-analyses, which have identified SNPs in this locus as strong association signals with cigarettes smoked per day (CPD) as a proxy for ND [29–31,43–45]. These reports support the evidence that smoking quantity/frequency is most consistently associated with SNPs within the 15q25 locus, particularly with rs16966968 and rs1051730.

In concordance with these reports, we found a robust association between *CHRNA3* rs1051730 and CPD. In addition, a nominally significant association of this SNP with FTND was found. However, the insufficient sample size may have hampered the demonstration of a stronger association. Moreover, the lower minor allele frequency (MAF) of rs1051730 in the study population (30.5%) compared to other samples of Caucasian origin may have reduced the statistical power of the study. For example, the MAF values in the CEU reference population derived from HapMap data and in the sample of European Americans analyzed by Saccone et al. were 38.5% and 35.0%, respectively [10].

FTND, which is considered as a standard measure of ND in clinical practice and research, encompasses 2 main aspects of dependence: physiological and psychological. The latter component of dependence contributes to nearly 20% of the total variance in FTND scores [39,46]. The identification of only a single, weak association of FTND with rs1051730 in the group of Kashubians may have, in part, resulted from these properties of the ND measure. It has been suggested that demonstrating the association of FTND with a given genetic variant is especially difficult, because a single SNP can exerts its influence on a particular, narrowly defined physiological or psychological aspect of ND [47].

In addition to FTND and CPD, we used another single FTND-derived item, TTF alone, and the combination of both items, HSI, as measures of ND. Since the average HSI score was 2.8±1.3 in

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Table 5. Association between the number of cigarettes smoked daily and studied polymorphisms.

SNP/genotype	CPD		OR	CPD		OR	CPD		OR
	No. of subjects		(95% CI)	No. of subjects		(95% CI)	No. of subjects		(95% CI)
	≤5	>5	*p*-value	≤15	>15	*p*-value	≤20	>20	*p*-value
rs1051730 (*CHRNA3*)									
GG	23	368	1	182	209	1	349	42	1
AA + AG	12	385	2.35 (1.00–5.53) 0.05	155	242	**1.54 (1.10–2.15) 0.012**	337	60	1.65 (1.02–2.68) 0.04
rs6495308 (*CHRNA3*)									
AA	18	457	1	194	281	1	408	67	1
GG+AG	17	296	0.77 (0.38–1.56) 0.47	143	170	0.87 (0.65–1.18) 0.38	278	35	0.84 (0.53–1.32) 0.45
rs55853698 (*CHRNA5*)									
AA	25	10	1	234	321	1	482	73	1
CC+CA	530	223	0.63 (0.26–1.55) 0.32	103	130	0.73 (0.50–1.04) 0.08	204	29	0.72 (0.43–1.22) 0.23

The values in boldface denote a significant association when applying Bonferroni corrected p-value of 0.017. The underlined values denote a significant association when applying significance level p=0.05 non corrected for multiple testing. ORs are adjusted for age, sex and remaining polymorphisms.

our sample, we used HSI ≥3 as a cut-off point to differentiate smokers with low/medium level of ND from those with high level of ND, as opposed to HSI ≥4, which has been used as a cut-off point by other authors [48]. We found that none of the SNPs investigated in the present study influenced HSI or TTF, although such associations have been reported in the literature. For instance, Marques-Vidal et al. reported that rs1051730 was correlated with HSI ≥5 (OR and 95% CI for each additional allele was 1.31; 1.00–1.71) and with smoking within 5 min after waking up (OR=1.32; 95% CI: 1.05–1.65) [22].

As in another study, CPD >10 was used as a cut-off value for low-dose smoking and high-dose smoking [21]. However, in other reports different definitions of light and heavy smokers were used, with CPD >20 as the most frequently used cut-off value [20]. In our post-hoc analysis, in which this cut-off value was used, the result was also positive, although not significant after correction for multiple testing. A similar result was obtained when comparing smokers who smoked up to 5 CPD with individuals who smoked more CPD. In turn, using CPD >15 as a cut-off value again revealed a significant association between rs1051730 and amount smoked.

The second polymorphism studied in the group of Kashubians was rs6495308. The frequencies of A (major) and G (minor) alleles of rs6495308 in Kashubians were similar to the frequencies observed in populations of European or African ancestry. For instance, MAF in our study population was 0.23. In the CEU reference population, or in samples of European-Americans and African-Americans from other studies, MAF ranged from 0.21 to 0.30 [10,32]. In contrast, in the Korean population, risk A-allele was the minor allele and MAF was 0.26 [19].

Initially, A-allele of rs6495308 was found to be a risk allele since carriers of A-allele of rs6495308 were more likely to report smoking 25 CPD or more in comparison with G-allele carriers [11]. Saccone et al. found that A-allele increased OR for ND defined by FTND ≥4, but this difference was not significant after applying the Bonferroni-corrected p-value, whereas G-allele (minor) may have conferred protection against ND in European-Americans and African-Americans [8,9]. Finally, Li et al. reported that the association of rs6495308 with smoking quantity has been confirmed, at both the individual SNP and at the haplotype level, which indicates that this polymorphism influences ND via an unknown mechanism [19]. In our study, we did not demonstrate an association between this SNP and CPD or FTND, which is consistent with the results obtained

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from a non-representative sample of the general Polish population [49]. Two other ND phenotypes studied in our analyses, HSI and TTF, were also not associated with rs6495308. This finding is consistent with the negative results of another study, although that study reported that carriers of both the rs1051730-AA and rs6495308-TT genotypes had approximately 2-fold greater odds for TTF [20]. We did not perform haplotype analysis, and the sample size and the statistical power of the study may have been insufficient to reveal an association of rs6495308 with ND phenotypes. Therefore, we could not confirm the previous findings of this association and we therefore consider the present study exploratory.

Rs55853898 was the third SNP investigated in the study population. We selected this SNP for our allelic association analysis for 2 reasons: firstly, this SNP seemed to be of interest due to evidence of its strong influence on CPD [29]; secondly, the frequencies of the alleles and genotypes of rs55853898 had not been studied before in Kashubians. We did not find an association between this SNP and any of the ND phenotypes, including CPD. Additionally, the distribution of rs55853698 alleles and genotypes did not appear to be in Hardy-Weinberg equilibrium in the isolated population of Kashubians studied.

To summarize, the association between rs1051730 and CPD as a proxy of ND measure was confirmed in the Kashubian population. However, we failed to replicate the other associations reported in previous studies [8,9,11,14,17,19,20,22,32].

Strengths of the study

The present study has several strengths that should be emphasized. Firstly, as the group of Kashubians we studied could be considered homogenous in genetic and cultural influences, we attempted to obviate possible stratification of the population. Secondly, by including only nonrelated subjects in the study, we attempted to exclude individuals who potentially shared influences of common family environment on ND phenotypes.

Limitations

The present study has several limitations that may have influenced the results obtained. First, it is likely that the sample size and statistical power were too small to replicate some of the previously reported results [50]. In addition, as our study design required convenience sampling, it was likely that our study population was not representative of all Kashubian smokers. Another limitation of the present study arises from the fact that the data on smoking behavior, obtained from the questionnaires, may be subject to phenotypic misclassification. For instance, as smoking quantity was self-reported and not verified by the biochemical investigation of tobacco exposure (i.e., the measurement of serum or plasma cotinine levels), it is likely that smokers underreported the CPD smoked [51]. Finally, Kashubian ancestry (i.e., genetic homogeneity of the studied population, excluding the potential first-degree relatives in the sample) was not verified at the genetic level but was based only on self-reports of the participants.

Despite its potential limitations, we consider the present study a valuable estimate of ND in an isolated population of Kashubians, which contributes to evidence of the role of chromosome 15q24-25.1 in influencing ND phenotypes.

Conclusions

Our findings confirm that *CHRNA5* rs1051730 significantly influences nicotine dependence, as defined by CPD. The continuing search for genes and their polymorphic variants involved in the development of ND, and replication of results across different ethnic populations, is expected to be useful in increasing the efficacy of treatment of nicotine dependence by enabling the implementation of approaches tailored to an individual's genetic background.

Conflicts of interest

All authors certify that they have no competing interests.

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