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Short Communication

Lack of an association between SNPs within the cholinergic receptor genes and smoking behavior in a Czech post-MONICA study

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Abstract

Smoking has a significant heritable component of approximately 30-60%. Recent genome wide association studies have identified single nucleotide polymorphisms (SNPs) within the nicotinic cholinergic receptor subunits 3 (rs578776), 5 (rs16969968) and β3 (rs6474412), which are associated with nicotine dependence in Western European populations. To analyze the association in a Czech population, we genotyped 1,191 males and 1,368 females (post-MONICA study). The WHO protocol was used to examine smoking status and the number of cigarettes smoked per day. There were 32.1% current and 27.6% past smokers among the males and 22.5% current and 13.8% past smokers among the females. We have not confirmed the original results: the SNPs rs16969968 ($p = 0.07$), rs578776 ($p = 0.16$) and rs6474412 ($p = 0.76$) were not associated with smoking status (never-smokers *vs.* ever-smokers) in the entire population, if a codominant model of analysis was used. This result was valid for both the male and female subpopulations if analyzed separately and adjusted for age. Finally, in ever-smokers, the number of cigarettes smoked per day was also independent of different genotypes, regardless of which polymorphism (and gender) was analyzed (the lowest p value was 0.49). The association between the cholinergic receptors–nicotinic subunits (-3, -5 and -ß3), and smoking behavior may be population-dependent.

Keywords: cholinergic receptors, polymorphism, smoking.

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Cigarette smoking is the most common form of tobacco use and is a major preventable cause of cancer, chronic respiratory diseases and cardiovascular disease. Despite the intensive preventive programs to stop smoking, approximately 30% of adults continue to smoke worldwide (Zatonski *et al.*, 2012). The environmental influences on tobacco smoking have been described, but twin studies have confirmed that genes also play an important role in smoking initiation, persistence and ability to stop smoking. The heritable component of smoking is estimated to be approximately 30-60% (Li and Burmeistr, 2009, Treur *et al.*, 2014).

To date, many gene candidates, such as D2 dopamine receptor (Munafò *et al.*, 2009), SLC6A3 dopamine transporter (Vandenbergh *et al.*, 2002), BDNF (Suriyaprom *et al.*, 2013) and HTTPLRF (Suriyaprom *et al.*, 2012), have been analyzed. However, the results are inconclusive, often due to the relatively low numbers of individuals included in the studies.

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Recently, genome-wide association studies (GWAs) have identified single nucleotide polymorphisms (SNPs) within or near the subunits of the cholinergic receptors nicotinic, 3 (OMIM acc. No. 118503, rs578776), 5 (OMIM acc. No. 118505, rs16969968), which is located within the *CHRNA5/A3/B4* gene cluster at 15q25, and β3 (OMIM acc. No. 118508, rs6474412), which is located at 8p11. These SNPs are associated with nicotine dependence and the number of cigarettes smoked per day in Western European populations (Bierut 2009, Thorgeirsson *et al.*, 2010).

Nicotinic cholinergic receptors represent a large group of conserved proteins that code for ligand-gated ion channels. These proteins mediate quick signal transmission at synapses, and because they bind nicotine, they are in a pathway responsible for the physiological responses to smoking. Also the results of mouse knock out models demonstrated the importance of these receptors in nicotine transport (Ware *et al.*, 2012).

Based on the previously published results (Bierut, 2009, Thorgeirsson *et al.*, 2010, Ware *et al.*, 2012), we supposed that the cholinergic receptor SNPs are associated with smoking behavior (ever- *vs.* never-smoking, past smoking, number of cigarettes smoked per day in smokers) in a Czech central European population.

Variants within the cholinergic receptors were analyzed in adults aged 34-73 years at the time of examination in 2000/2001, which included 1,191 males and 1,368 females (post-MONItoring of CArdiovascular disease study) (Cifkova *et al.*, 2010). Written informed consent was acquired from all individuals, and the study was approved by the institutional Ethics Committee and was conducted in agreement with the Helsinki Declaration of 1975.

The WHO protocol (Tunstall-Pedoe *et al.*, 2003) was used to examine smoking status and the number of cigarettes smoked per day in 1997/1998, 2000/2001 and 2007/2008. Information about the current smoking status (current-, past- and never- smoker) and the number of cigarettes smoked per day was collected via self-completed (under supervision of trained nurse) questionnaires (Cifkova *et al.*, 2010). Never-smokers were self-reported individuals who had consumed fewer than five packages during their lifetime. Further forms of tobacco use (chewing and snuffing) were not self-reported within the population. Complete information was available from the first two examinations (conducted in 1997/1978 and 2000/2001) and from 84.4% of individuals examined in 2007/2008.

DNA was isolated using a slightly modified version of the method of Miller *et al.* (1988). Individual genotypes were analyzed by PCR-RFLP using PCR chemicals and restriction enzymes from Fermentas International Inc. (Burlington, Ontario, Canada). PCR incubations were performed using MJ Research DYAD Disciple PCR cyclers. Oligonucleotide sequences (some primers were mismatched to create new restriction sites) and further details are summarized in Table 1. Restriction fragments were separated via horizontal gel electrophoresis on a 10% polyacrylamide gel using the MADGE platform (Day *et al.*, 1996).

Deviations from Hardy Weinberg equilibrium were tested using an online calculator (Court 2005-2008). The differences in the genotype frequencies between the groups were assessed using a Chi-square test (3x3 or 3x2 tables) in dominant, codominant and recessive models for both of the years of examination. Table 2 presents the p values for all three unadjusted models at first examination. The number of cigarettes smoked per day in association with individual genotypes was analyzed by ANOVA in the dominant, codominant and recessive models for all years of examination. Table 3 shows the numbers and p values obtained for the codominant model in the second examination. Due to the large differences between males and females in the number of cigarettes smoked per day, the entire population was not analyzed. The results presented in detail are from the examination conducted in 2000/2001 because there were no significant differences among the three examinations.

There were 32.1% current and 27.6% past smokers in males and 22.5% current and 13.8% past smokers in females in 2000/2001. The mean number of cigarettes smoked per day was 15.7 ± 8.7 in males (min = 1, max = 50) and 11.3 ± 6.4 (min = 1, max = 61) in females.

The call rate was over 93.9% for each analyzed SNP, and the call rates were similar in smokers and non- or past smokers. The genotype distributions of all three analyzed polymorphisms were within Hardy-Weinberg equilibrium ($p = 0.19$ for rs16969968, $p = 0.44$ for rs578776 and $p = 0.79$ for rs6474412) and did not differ significantly between males and females. Minor allele frequencies were similar to the frequencies found in other Caucasian populations (Bierut, 2009, Thorgeirsson *et al.*, 2010).

The SNPs rs16969968 ($p = 0.07$), rs578776 ($p = 0.49$) and rs6474412 ($p = 0.76$) were not associated with smoking status (when never-smokers *vs.* ever-smokers were compared) in the entire population, if the codominant model of analysis was used. Additionally, negative results were observed for both the male and female subpopulations, if analyzed separately (Table 2) and after adjusting for age.

Some borderline differences (non-significant after adjustment for multiple testing) were observed for the rs6474412 SNP only. Here, it appears that CC homozygotes are less likely to be smokers and that, if they do smoke, they have a slightly higher chance to quit smoking.

Finally, in ever-smokers, the number of cigarettes smoked per day was also independent of genotype, regardless of the polymorphism (and gender) analyzed (all p-values were 0.16 or higher; for details see Table 3). However, in agreement with the previous results, a trend for a

Table 1 - Genotyping details for analysis of SNPs of interest.

Polymorphism	Primer sequences	PCR product	Enzyme	Size of restriction fragments (bp)	Allele
Rs16969968	5'atg aag aag tca tgt aga cag gta ctt c 5'tac aca tca cag acc tca cgg aca tc	165 bp	*Tag*I	165 97 + 68	A G
Rs6474412	5'cct ctt ttc ctg tgt cta ttt gat ggc 5'ttc acc ctg caa aga tac tca act ctt cac c	134 bp	*Hae*III	133 109 + 26	T C
Rs578776	5'ttc ttt act ggg tct aaa ggg cta tgc c 5'atc cac cca gtt tat ggt gta cta ag	167 bp	*Nla*III	167 100 + 67	T C

Table 2 - Smoking habits and variants within the cholinergic receptor genes. Data from the survey performed in 2000/2001 are given. The uncorrected p-values for genotype differences between the subgroups in order of dominant/co-dominant/recessive models are shown. After Bonferroni correction, no significant differences were observed.

rs	Total population						Males						Females					
16969968	Smokers		Past smokers		Never smokers		Smokers		Past smokers		Never smokers		Smokers		Past smokers		Never smokers	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
GG	308	45.4	232	45.1	542	40.8	173	46.1	149	46.0	183	38.8	135	44.6	83	43.7	359	41.8
GA	305	45.0	224	43.6	633	47.6	164	43.7	144	44.4	232	49.1	141	46.5	80	42.1	401	46.7
AA	65	9.6	58	11.3	155	11.6	38	10.1	31	9.6	57	12.1	27	8.9	27	14.2	98	11.4
p	0.37/0.17/0.07						0.48/0.17/0.05						0.19/0.39/0.68					
rs	Total population						Males						Females					
578776	Smokers		Past smokers		Never smokers		Smokers		Past smokers		Never smokers		Smokers		Past smokers		Never smokers	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
CC	350	51.8	280	54.9	727	55.4	184	49.3	172	53.6	256	54.9	166	54.8	108	57.1	471	55.6
CT	278	41.1	201	39.4	500	38.1	159	42.6	130	40.5	178	38.2	119	39.3	71	37.6	322	38.0
TT	48	7.1	29	5.7	86	6.5	30	8.0	19	5.9	32	6.9	18	5.9	10	5.3	54	6.4
p	0.62/0.55/0.30						0.55/0.49/0.26						0.84/0.97/0.88					
rs	Total population						Males						Females					
6474412	Smokers		Past smokers		Never smokers		Smokers		Past smokers		Never smokers		Smokers		Past smokers		Never smokers	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
TT	411	61.4	224	63.0	793	60.1	227	61.7	215	66.1	283	60.3	184	61.1	109	57.7	510	60.0
TC	232	34.7	160	31.1	459	34.8	130	35.3	93	28.6	164	35.0	102	33.9	67	35.5	295	34.7
CC	26	3.9	30	5.8	67	5.1	11	3.0	17	5.2	22	4.7	15	5.0	13	6.9	45	5.3
p	0.05/0.05/0.04						0.30/0.17/0.24						0.63/0.88/0.75					

Table 3 - Number of cigarettes smoked per day according the cholinergic receptor gene genotypes. Uncorrected p-values for genotype differences between the subgroups are given (all p = n.s.). Data from the survey performed in 2000/2001 are presented.

	Males		Females	
	N	N of cigarettes	N	N of cigarettes
rs16969968				
GG	173	15.7 ± 9.1	135	11.4 ± 6.6
GA	164	16.2 ± 8.1	141	10.7 ± 6.5
AA	38	18.3 ± 9.7	27	12.6 ± 10.9
p	0.17	0.16		
rs578776				
CC	184	16.7 ± 8.4	166	11.2 ± 7.3
CT	159	15.9 ± 9.2	119	11.3 ± 6.9
TT	30	15.1 ± 8.6	18	11.6 ± 6.0
p	0.79	0.84		
rs6474412				
TT	227	15.9 ± 8.9	184	11.4 ± 7.1
TC	130	16.8 ± 9.0	102	10.8 ± 6.5
CC	11	16.3 ± 8.0	15	11.8 ± 8.8
p	0.95		0.97	

higher number of cigarettes smoked per day in the minor AA genotype (rs16969968) carriers was detected.

Similar and negative results were also obtained when the results from examinations conducted in 1997/1998 and 2007/2008 were examined. These results are not shown in detail.

In our study, we did not confirm the original finding that variants within the three nicotinic cholinergic receptors are associated with smoking behavior. This finding was observed in both males and females regardless of the year of examination.

Our data contrast previously published papers that investigated the rs16969968 polymorphism. This variant represents the amino acid change Asp398Asn (1192G > A) at a highly conserved position between species and is, thus, most likely to be of biological significance. Most (Bierut, 2009, Ware *et al.*, 2011, Breetvelt *et al.*, 2012, Conlon and Bewick, 2011, Chen *et al.*, 2012) but not all (Verde *et al.*, 2011) studies have presented an association between the minor Asn (A) allele and higher nicotine dependence (numbers of cigarettes smoked per day or different genotype frequencies between smokers and nonsmokers). An additional study detected that individuals with at least one rs16969968 allele have a higher risk of being heavy smokers in adulthood if they started to smoke during adolescence (Hartz *et al.*, 2012).

Our data are in agreement with a recent meta-analysis by Ware *et al.* (2011), who detected a significant effect on the number of cigarettes smoked per day; one risk-allele (A) was associated with approximately one additional cigarette per day. In our study, we detected a similar effect on the number of cigarettes smoked per day. However, likely due to the large range in the number of cigarettes smoked per day (1-61), the difference was not significant.

The second variant, which was previously described to be associated with nicotine dependency (for a review, see Bierut *et al.*, 2008), rs578776, is located at the same locus as the neighboring gene, but it is not in linkage disequilibrium with rs16969968. This variant (546C > T) does not change the amino acid, and the mechanism of the potential biological significance is unknown.

The last analyzed SNP (rs6474412) is located at a different region but was also originally associated with the number of cigarettes smoked per day (Thorgeirsson *et al.*, 2010). However in this case, the effect appears to be much lower (one risky T allele is associated with only an approximately 0.3 cigarette per day increase), and our study did not have sufficient power to detect such a tiny effect.

In our previous work (Hubacek *et al.*, 2012), we were not able to confirm the association between smoking behavior and another GWAs-recognized gene: the *FTO* gene (Bierut *et al.*, 2007). Due to the geographical location factors like population stratification or founder effect are very unlikely to cause these non-replications. There could be several another reasons for the non-replication(s) on our study.

For example, socioeconomic status may play an important role in smoking behavior because the original GWAs was performed primarily in West European populations. The prevalence of smoking is higher in post-communistic countries, and this fact could mask the real effect of the genes. The fact that most current smokers have or had parents who smoke could reflect not only genetic predisposition but also negative environmental influence.

Secondly, the WHO protocol that we used to select individuals did not necessarily focus on smoking itself, but on smoking as a risk factor of cardiovascular disease. Tests of nicotine dependence that could better reflect nicotine dependence, such as the Fagerström Test for Nicotine Dependence, were not available. Conversely, the number of cigarettes smoked per day represents a simple but representative marker of nicotine dependence (Chen *et al.*, 2012).

Additionally, the numbers of examined individuals within our study are relatively high, and importantly, almost all of the individuals were examined three times within ten years. This frequency decreases the risk of self-reported misinterpretations. Finally, the accuracy of the obtained data is supported by the fact that, in contrast to the alcohol consumption, where clear underestimations, especially in females, are common (Roche and Deehan, 2002), smoking is still not viewed as a “social stigma”. The fact that our results were not significant at all three examinations over ten years lowered the risk of false negative results.

Interestingly, in smokers there was a (not-significant) decrease in the numbers of cigarettes smoked per day over the three examinations. This decrease could reflect the economic situation, rather than genetic predisposition or social factors. With the decreasing number of cigarettes smoked, even slight differences between genotypes and cigarettes smoked per day disappeared.

In summary, the association of SNPs of interest with smoking status (never-, ever-, or past-smokers) was not confirmed in all studies (which could be caused by the low power of some published studies to detect small effects in single variants). Additionally, the commonly analyzed value is the effect of the number of cigarettes smoked per day. Here, the largest studies detected consistent but only very slight effects: one high risk allele is weakly associated with 0.3-1.0 additional cigarette smoked per day. Such a small difference has hardly any clinical significance, especially between the smokers who commonly smoke one or more packages per day. Despite the relatively high number of individuals included, our study may have had insufficient power to detect such small differences.

We observed that the association between the subunits of nicotinic cholinergic receptors (-3, -5 and -β3) and smoking behavior may be population-dependent and is likely not valid in the Czech (Slavonic) central European population. Different genetic backgrounds or environmental conditions could modify the effect of the analyzed polymorphisms.

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