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A Variant Associated with Nicotine Dependence, Lung Cancer and Peripheral Arterial Disease

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Abstract

Smoking is a leading cause of preventable death, causing approximately five million premature deaths world-wide each year[1, 2]. Evidence for genetic influence on smoking behaviour and nicotine dependence (ND)[3-8] has prompted a search for susceptibility genes. Furthermore, assessing the impact of sequence variants on smoking-related diseases is important for public health reasons[9, 10]. Smoking is the major risk factor for lung cancer (LC)[11-14], and one of the main risk factors for peripheral arterial disease (PAD)[15-17]. We have identified a common variant in the nicotinic acetylcholine receptor gene cluster on chromosome 15q24 with an effect on smoking quantity, ND and the risk of two smoking-related diseases in populations of European descent. The variant has an effect on the number of cigarettes smoked per day in 15,771 smokers ($P=6\times10^{-20}$). The same variant associated with ND in a previous genome-wide association study using low quantity smokers as controls (OR=1.3, $P=1\times10^{-3}$)[18, 19], and with a similar approach we observe a highly significant association with ND (OR =1.40, $P=7\times10^{-15}$). Comparison of LC (N=1,024) and PAD (N= 2,738) cases with about 30,000 population controls each showed that the variant confers risk of LC (OR=1.31, $P=1.5\times10^{-8}$) and PAD (OR=1.19, $P=1.4\times10^{-7}$). The findings highlight the role of nicotine addiction in the pathogenesis of other serious diseases and provide a case study of the role of active gene-environment correlation[20] in the pathogenesis of disease.

To perform a genome-wide association (GWA) study of smoking quantity (SQ), we utilised questionnaire data limited to basic questions on smoking behaviour that were available for a large number of lifetime smokers. The GWA scan comprises 10,995 Icelandic smokers who

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Author Contributions: T.E.T., F.G., P.S., and T.R. contributed equally to this work. T.E.T., F.G., P.S, T.R., A.W., D.F.G., A.K, and K.S. wrote the first draft of the paper. Ha.S., H.J.I, T.G., and S. J., recruited and diagnosed the Icelandic lung cancer patients. S.E.M. recruited and diagnosed the Icelandic peripheral arterial disease patients. T.B., H.K., J.G.S., I.H, V.R., H.O., T.T., and S. J. recruited and diagnosed nicotine addiction subjects. K.K.H.A., F.dV., P.F.A.M., and L.A.K. recruited and diagnosed the subjects from the Netherlands. D.I., M.J.V., L.A., B.S., L.M, and J.I.M. recruited and diagnosed the Spanish subjects. G.T.J. and A.M.vR. recruited and diagnosed the subjects from New Zealand. T.M., B.P. and M.H. recruited and diagnosed subjects from Austria. A.G. and B.L. recruited and diagnosed subjects from Sweden. A.F. and R.P. recruited and diagnosed subjects from Italy. A.W., A.I., S.N.S, J.T.B., S.T., J.G., M.J., J.S., O.O., S.N.S. performed genotyping and experimental work. L.J.G., G.B, and K.K incorporated phenotypic data into a database and analysed it. T.E.T., F.G., P.S., T.R., A.W., K.P.M., A.M., G.T., D.F.G., and A.K. analysed the data. T.E.T., F.G., P.S., T.R., K.P.M., Hr.S., T.J., J.I.M., L.K., H.O., T.T., J.R.G., S.J., D.G:, U.T., A.K., and K.S. planned, supervised and coordinated the work. All authors contributed to the current version of the paper.

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had been assayed with Infinium HumanHap300 SNP chips (Illumina). A set of 306,207 single nucleotide polymorphisms (SNPs), fulfilling our quality criteria, was tested. We focussed on cigarette smoking, with SQ reported as cigarettes per day (cpd). All SQ data were clustered into categories (See Supplementary Information) and we refer to them as "SQ levels", the SQ levels are: 0 (1-10 cpd), 1 (11-20 cpd), 2 (21-30 cpd), and 3 (31+ cpd). Each increment represents an increase in SQ of 10 cpd. Allele T of rs1051730 was most strongly associated with SQ, and the association was highly significant ($P=5\times10^{-16}$). The SNP is within the *CHRNA3* gene in a linkage disequilibrium block also containing two other nicotinic acetylcholine receptor (nAChR) genes, *CHRNA5* and *CHRNB4*[18]. Six other SNPs on chromosome 15q24 passed the threshold of genome-wide significance ($P<2\times10^{-7}$), but they are all correlated with rs1051730 (r^2 = 0.14-0.93). After correction for rs1051730 none of these six SNPs showed a p-value below 1×10^{-3} (See Supplementary Table 1). A Quantile-Quantile plot for the GWA scan (See Supplementary Figure 1a) shows the observed excess of signals, whereas a Quantile-Quantile plot after removing 182 markers located within 1 Mb of rs1051730 is consistent with noise (see Supplementary Figure 1b), illustrating that all of the strongest signals standing out in the first plot are located on chromosome 15q24. An additional 2,950 smokers from Iceland were genotyped for rs1051730 giving a total of 13,945 smokers (Table 2) with mean variant frequency of 34.7%, which is not significantly different from the frequency of 34.4% observed in 4,203 individuals who were genotyped and reported never having smoked (OR=1.01, 95% CI: 0.96-1.07, P=0.60). Indeed, the frequency of the variant in the 3,627 low quantity smokers (≤ 10 cpd), is significantly less than the frequency in those who do not smoke (OR=0.83, 95%CI:0.78-0.90, $P=4.5\times10^{-7}$). The increase in frequency between levels varies, and the largest increase (4.5%) is observed between the lowest levels (0 and 1), whereas the increase between the highest levels (2 and 3) is just 1.1%. In the context of a case-control LC study, an additional 523 smokers from Spain and 1,375 smokers from the Netherlands were genotyped. Multiple regression analyses of SQ data from the three countries with adjustment for sex and year of birth were performed (Table 3). Results from Spain and the Netherlands combined gave an estimated increase of 0.074 SQ units (P = 0.012) for each copy of the variant, which is not significantly different (P = 0.45) from the estimate of 0.098 SQ units ($P=1\times10^{-18}$) based on the Icelandic data. Combining all results, each copy of the variant was estimated to increase SQ level by 0.095 units ($P=6\times10^{-20}$), which corresponds to approximately one cpd. A recent GWA study reported association between SQ and rs6495308 ($P=6.9\times10^{-5}$, r^2=0.18 to rs1051730 in the HapMap project) for about 7,500 individuals from two study groups, as well as association between SQ and rs1317286 ($P=2.6\times10^{-6}$, r^2=0.90 to rs1051730 in the HapMap project) in a candidate gene study based on 1,740 heavy smokers (>25cpd) and 6,200 low quantity smokers (<5 cpd)[21].

Sex and year of birth are also strongly associated with SQ (Table 3). However, neither the interaction of the variant and sex, nor the variant and year of birth, are significant, indicating that the effect of the variant is similar for both sexes and is robust to population-wide changes in smoking habits over time. The phenotypic variance explained by the variant was highest in Iceland, amounting to 0.7%.

Association of the same variant with ND was previously reported in a candidate gene study involving 3,713 SNPs[18]. We assessed the association with ND, defined as a score of 4 or higher on the Fagerstrom Test for Nicotine Dependence (FTND)[22] or endorsement of at least 3 of the 7 DSM-IV criteria (See Supplementary Information). The variant is associated with ND in Iceland in a subset of 2,394 smokers from the SQ study tested both against 28,455 population controls (OR=1.17, 95%CI:1.10-1.25, P=3.3×10^{-6}), and 3,506 low-quantity smokers (OR=1.40, 95%CI:1.29-1.52, P=7×10^{-15}). The latter OR of 1.40 is comparable to the results of the candicate gene study[18]. They used non-ND smokers as controls (i.e. individuals who had smoked but have an FTND score of 0), and reported association with rs1051730 (OR = 1.3, P=1×10^{-3}). Rs1051730 is in strong linkage disequilibrium with rs16969968 (r^2=0.90 in the HapMap project) which was highlighted in the previous study with a similar result (OR = 1.3, P=6×10^{-4}) [18]. Dependence on nicotine drives repeated self-administration of nicotine[23-25] and high SQ is a strong sign of ND and one of the criteria for a diagnosis of ND (SQ is included in the FTND scale). This and the fact that the ND subjects are part of the SQ study means that the associations with ND and SQ cannot be considered independent results. Both the FTND and the DSM-IV scales include many items that are not based on SQ and their total scores are measures of ND severity. In our ND group, positive scores on most items in both scales show a trend toward higher frequency of the variant, as does the total score on both the FTND and DSM-IV scales. Thus the frequency of the variant increases with addiction severity, and is 46.8% and 43.8% for the highest decile of FTND, and DSM-IV, respectively (see Supplementary Table 2a/b). ND is believed to be the main reason for continued smoking. To explore the frequency of the variant in the context of the ability to quit smoking, we investigated differences between 6,388 current and 6,687 past smokers from the SQ analysis by a logistic regression model adjusting for sex and year of birth. The variant was associated with current smoking with an OR of 1.07 (95%CI:1.01-1.13, P=0.015) (See Supplementary Table 3) and the effect is similar when corrected for SQ (OR=1.06, 95% CI:1.00–1.12, P=0.036), indicating that carriers of the variant are less likely to quit smoking.

Smoking is a major risk factor for many diseases and we decided to study the effect of the variant on LC and PAD risk directly. The LC study was based on 1,024 cases and 32,244 controls from Iceland, Spain and the Netherlands (Table 1) and the PAD study was based on 2,738 cases and 29,964 controls from five Caucasian populations (Iceland, New Zealand, Austria, Sweden, and Italy) (Table 1). The results for LC and PAD (Table 4) represent the overall effect on LC and PAD including indirect effects through SQ and ND. Significant association was observed with LC for both the Icelandic data (OR = 1.27, P = 4.1×10^{-5}) and for Spain and the Netherlands combined (OR = 1.39, P = 6.6×10^{-5}). These two estimates are not significantly different from each other (P = 0.34), and combining results from all three groups gave an OR of 1.31 (95%CI:1.19-1.44, P=1.5×10^{-8}). There is no significant difference in frequency of the variant between histological types of LC, which is not surprising given the small number of cases per group (see Supplementary Table 4). Association with PAD was found in both the Icelandic data (OR = 1.18, P = 5.3 ×10^{-5}) and in the foreign populations combined (OR = 1.23, P = 5.9 × 10^{-4}). These two estimates are not significantly different from each other (P = 0.57), and combining results from all five groups gave an OR of 1.19 (95%CI:1.12-1.27, P=1.4×10^{-7}).

Genotypic ORs for LC, PAD and ND did not deviate significantly from those obtained for the multiplicative model (see Supplementary Table 5), and no significant differences in the ORs between sexes were observed (See Supplementary Table 6).

According to our estimates for Icelandic LC patients, the correlation between SQ and LC is consistent with numbers reported in other studies[26, 27]. Combining these estimates with our estimate of the association of the variant with SQ, the expected OR between the variant and LC is only about 1.05 in Iceland (See Supplementary Information), which is well below the direct OR estimate for LC of 1.27 (95%CI: 1.13-1.43). A similar indirect estimate for PAD is 1.04, which again is substantially lower than the observed direct estimate of 1.18 (95%CI: 1.09-1.27). It is not surprising that the odds ratios for LC and PAD cannot be explained by the effect of the variant on SQ alone, as the involvement of both SQ and the duration of smoking in LC and PAD has been established in previous studies[15, 28]. The SQ data for most individuals were derived from a single point in time and cannot be expected to cover all aspects of smoking behaviour affected by the variant and relevant to LC and PAD. An effect on other aspects of smoking behaviour, smoking duration in particular, is likely to account for the observed difference between the indirect and direct estimates of the LC and PAD risks. An alternative possibility is that the variant directly confers risk of LC and PAD, *e.g.* by increasing the vulnerability to tobacco smoke.

Overall, we have unequivocally demonstrated a correlation between a sequence variant in the cluster of nAChR genes on chromosome 15 and SQ and ND. The variant does not influence smoking initiation, but among smokers, carriers of the variant smoke more than non-carriers and have higher rates of ND. This variant was reported in a previous study of 1,050 ND cases and 879 controls who smoked and had an FTND score of 0[18, 19] and the authors concluded that the variant contributes to ND[18]. This conclusion is put on firm ground by the highly significant OR of 1.40 ($P=7\times10^{-15}$) for ND compared to low-quantity smokers (≤ 10 cpd). The direct measurement of the risk of LC and PAD revealed genome-wide significant associations with allelic ORs of 1.31 and 1.19, respectively. This demonstrates that a sequence variant associated with ND, a brain disorder, confers risk of lung and cardiovascular diseases through an effect on behaviour, providing a case study of active gene-environment correlation[20] in the pathogenesis of disease. A calculation of the population attributable risk (PAR) for the variant gives 18% for LC and 10% for PAD. While these PARs are at best ballpark figures given the complex interplay between the variant, smoking, and smoking-related diseases, it is likely that the variant accounts for a substantial fraction of PAD and LC cases and the associated morbidity and mortality.

The results of the study described here show that it is important to keep in mind while attempting to shed light on the role of nature versus nurture in the pathogenesis of common/complex disease that not only do variants in the sequence of our genome influence how we respond to our environment but also our tendency to seek or avoid environment. Hence the line between nature and nurture is sometimes conspicuously absent.

METHODS

Icelandic Subjects

For all studies involving Icelandic subjects, the study protocols were approved by the National Bioethics Committee (NBC) and the Data Protection Authority (DPA) of Iceland. The DPA encrypted all personal identifiers associated with information or blood samples using the third-party encryption system[31]. Overall the Icelandic study involves 10,995 subjects with information on SQ available in the GWA, an additional 2,950 subjects with information on SQ, and 4,203 never-smokers. In the studies of LC and PAD, 665 and 1,503 patients, respectively, and 28,752 population controls were used (for details see Table 1).

Smoking

All Icelandic subjects in the study of smoking-related phenotypes, including Icelandic population controls, were originally recruited for different genetic studies conducted over eleven years (1996-2007) at deCODE genetics and information on the number of cigarettes smoked per day (cpd) was available from questionnaires. The cpd information was categorised into SQ level and used as a quantitative variable. Detailed information on SQ was also available for the foreign LC populations (Supplementary Information), but not for the foreign PAD populations.

Nicotine Dependence

For a subset of the Icelandic smokers, information on the criteria used to diagnose ND was available from ongoing studies of ND and Anxiety/Depression[32]. We excluded individuals with diagnoses of other substance dependence or abuse giving a total of 2,394 ND subjects. A score of 4 or higher on the FTND[22], or endorsement of three or more DSM criteria were used to assign affected status for ND. Additional information on the Icelandic smoking and ND study group is available in the Supplementary Information.

Lung Cancer

Iceland—Recruitment was initiated in the year 1998 using a nationwide list from the Icelandic Cancer Registry (ICR). Approximately 1,265 LC patients were alive during the period of recruitment and 665 participated in the project. Information in the ICR includes year and age at diagnosis, year of death, SNOMED code and ICD-10 classification. Histological and cytological verification was available for 647 cases; the remaining 18 cases were diagnosed clinically.

The Netherlands—The 90 patients and 2,018 controls were identified retrospectively through three different ongoing studies on genetic risk factors of disease. All three study protocols were approved by the Institutional Review Board of the Radboud University Nijmegen Medical Centre.

Spain—Patients were recruited from the Oncology Department of Zaragoza Hospital, from June 2006 to June 2007, and of 330 patients that were invited to participate, 292 enrolled (88%). Clinical information including age at onset and histology were collected from

medical records. The 1,474 control individuals were approached at the Zaragoza University Hospital (ZUH). Study protocols were approved by the Institutional Review Board of ZUH.

Peripheral Arterial Disease

Iceland—Patients have been recruited over the past nine years, as part of a genetic study at deCODE, from a registry of individuals diagnosed with PAD at the major hospital in Reykjavik, the Landspitali University Hospital, during the years 1983 to 2006. Diagnosis was confirmed by vascular imaging or segmental pressure measurements.

Austria—Patients and controls were recruited through the Linz Peripheral Arterial Disease (LIPAD) study during 2000 to 2002, at the St. John of God Hospital, Department of Surgery. Of the patients admitted for evaluation of suspected or definite PAD, all patients with chronic atherosclerotic occlusive disease of the lower extremities associated with typical symptoms—such as claudication or leg pain on exertion, rest pain, or minor or major tissue loss—were included on the basis of the final clinical diagnosis established by attending vascular surgeons. The diagnosis was verified by interview, physical examination, noninvasive techniques, and angiography[33]. All control subjects were patients at the same hospital and fulfilled the following criteria: no clinical indication of PAD by history and physical examination; systolic brachial blood pressure equal to or less than the blood pressure in each of the right and left anterior tibial and posterior tibial arteries (ie, ABI ≥1.0)[33]. Smoking status was assessed according to Rutherford et al., 1997[34].

Sweden—Patients and controls were recruited at the Department of Vascular Diseases at Malmö University Hospital, a single referral centre for all patients with critical limb ischemia in the three southernmost health-care districts in Sweden (723,750 inhabitants in 2001). The diagnosis of critical limb ischemia was made in accordance with Trans-Atlantic Inter-Society Consensus scientific criteria[35] of ulceration, gangrene, or rest pain caused by PAD proven by ankle pressure (<50 to 70 mm Hg), reduced toe pressure (<30 to 50 mm Hg), or reduced transcutaneous oxygen tension. Diagnosis was confirmed by an experienced vascular surgery consultant and toe pressure measurements in patients with arteries in the affected leg that were non-compressible and the ankle pressure was >50 to 70 mm Hg. The control group consisted of healthy individuals without symptomatic PAD included in a health screening programme for a preventive medicine project[36].

Italy—Patients and controls were recruited among subjects admitted to the Department of Internal Medicine and Angiology of the A. Gemelli University Hospital of Rome, from 2000 to 2001. Inclusion criteria for the PAD group were Caucasian origin and presence of PAD, diagnosed in accordance with established criteria[37]. All patients had an ankle/arm pressure index lower than 0.8 and were at Fontaine's stage II, with intermittent claudication and no rest pain or trophic lesions. Inclusion criteria for the control group were Caucasian origin, absence of PAD and CAD and no relationship with cases. Additional exclusion criteria from the study were tumours, chronic inflammatory diseases, and autoimmune diseases[38].

New Zealand—Patients were recruited from the Otago-Southland region, and PAD was confirmed by an ankle brachial index<0.7, pulse volume recordings and angiography/

ultrasound imaging. The control group consisted of elderly individuals with no history of vascular disease from the same geographical region. Controls were asymptomatic for PAD and had ankle brachial indexes >1. An abdominal ultrasound scan excluded concurrent abdominal aortic aneurysm from both the PAD and control groups and Anglo-European ancestry was required for inclusion.

Genotyping

All 10,995 samples in the GWA study of SQ were genotyped using genotyping systems and specialised software from Illumina (Human Hap300 and Human Hap300-duo+ Bead Arrays, Illumina)[39]. Rs1051730 was genotyped using a Centaurus assay (Nanogen) for 8,566 Icelandic samples and all samples in the foreign study groups. Information on the genotyping and quality control is in the Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Demographics, Age and Phenotype Breakdown.

Study Group	Cases			Controls		
	N	male / female	Age (SD)	N	male / female	Age (SD)
Smoking phenotypes						
Cigarettes per day available						
Iceland	13,945	6,134 / 7,811	58.7 (17.8)			
Spain	523	354 / 169	54.0 (16.3)			
Netherlands	1,375	762 / 613	61.5 (10.2)			
Nicotine Dependence (Iceland)	2,394	800 / 1,594	48.1 (11.0)	28,455	12,600 / 15,855	58.7 (21.8)
Never smokers (Iceland)	4,203	1,273 / 2,930	55.4 (21.6)			
Lung Cancer						
Iceland	665	346 / 319	69.7 (11.1)	28,752	12,174 / 16,578	56.8 (21.5)
Spain	269	238 / 31	64.9 (11.6)			
Netherlands	90	71 / 19	68.5 (9.5)			
Peripheral Arteial Disease						
Iceland	1,503	926 / 577	74.2 (10.6)	28,752	12,174 / 16,578	56.8 (21.5)
New Zealand	441	251 / 189	70.6 (9.6)	435	248 / 187	68.2 (6.4)
Austria	457	322 / 135	68.4 (11.0)	403	284 / 119	67.3 (10.7)
Sweden	172	92 / 80	77.5 (9.9)	140	64 / 76	67.9 (1.5)
Italy	165	111 / 54	73.0 (9.3)	234	162 / 72	72.6 (6.4)

Table 2

Genotype Status and Smoking Quantity (SQ) Level of 13,945 Icelandic Smokers.

Cigarettes per day (SQ level)	Genotype of rs1051730			Total	Frequency of T allele
	GG	GT	TT	n (Freq.)	
1 to 10 (0)	1,743	1,558	326	3,627 (0.260)	0.305
11 to 20 (1)	2,727	2,865	810	6,402 (0.459)	0.350
21 to 30 (2)	1,145	1,416	427	2,988 (0.214)	0.380
31 and more (3)	341	448	139	928 (0.067)	0.391
All levels (Frequency)	5,956 (0.427)	6,287 (0.451)	1,702 (0.122)	13,945 (1.000)	0.347
Mean SQ level (SD)	1.01 (0.85)	1.12 (0.86)	1.22 (0.85)	1.09 (0.86)	

Table 3

Multiple Regression of Smoking Quantity Level as a Function of rs1051730 Genotype, Sex and Year of Birth

							Interactions
		Copies of T allele		Sex (male)		YOB (categorical)	Allele × sex allele × age
Study Group	**n**	**estimate (95% CI)**	***P***	**estimate (95% CI)**	***P***	***P***	***P***
Iceland	13,945	0.098 (0.076 - 0.120)	1×10^{-18}	0.411 (0.383 - 0.438)	$< 10^{-16}$	$< 10^{-16}$	0.53 0.85
Spain	523	0.061 (−0.059 - 0.180)	0.32	0.504 (0.290 - 0.718)	$< 10^{-5}$	0.006	0.80 0.76
The Netherlands	1,375	0.078 (0.012 - 0.145)	0.021	0.326 (0.225 - 0.427)	$< 10^{-9}$	$< 10^{-4}$	0.68 0.27
Foreign combined	1,898	0.074 (0.016 - 0.132)	0.012	NA	-	-	-
All combined	15,771	0.095 (0.075 - 0.115)	6×10^{-20}	NA	-	-	-

Multiple regression of SQ level on allele T, sex and year of birth (yob), giving adjusted values for each explanatory variable adjusting for the others. For the tests of interaction, the interaction terms involving the variant were individually added to the initial model

Table 4

Association of rs1051730 allele T with Lung Cancer and PAD

	Controls		Cases				
Study Group	**n**	**freq**	**n**	**freq**	**OR**	**(95% CI)**	***P***
Lung Cancer							
Iceland	28,752	0.342	665	0.398	1.27	(1.13 - 1.43)	4.1×10^{-5}
Spain	1,474	0.390	269	0.483	1.46	(1.22 - 1.76)	5.4×10^{-5}
The Netherlands	2,018	0.314	90	0.350	1.18	(0.86 - 1.61)	0.31
Foreign combined	3,492	-	359	-	1.38	(1.18 - 1.62)	6.6×10^{-5}
All combined	32,244	-	1,024	-	1.31	(1.19 - 1.44)	1.5×10^{-8}
PAD							
Iceland	28,752	0.342	1,503	0.379	1.18	(1.09 - 1.27)	5.3×10^{-5}
New Zealand	435	0.274	441	0.337	1.35	(1.10 - 1.65)	0.0041
Austria	403	0.352	457	0.395	1.20	(0.99 - 1.46)	0.068
Sweden	140	0.304	172	0.331	1.14	(0.81 - 1.60)	0.46
Italy	234	0.378	165	0.412	1.15	(0.86 - 1.54)	0.33
Foreign combined	1,212	-	1,235	-	1.23	(1.09 - 1.39)	5.9×10^{-4}
All combined	29,964	-	2,738	-	1.19	(1.12 - 1.27)	1.4×10^{-7}