The *CHRNA5-A3* Region on Chromosome 15q24-25.1 Is a Risk Factor Both for Nicotine Dependence and for Lung Cancer

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Common variants in the nicotinic acetylcholine receptor gene cluster on chromosome 15q24-25.1 were associated with lung cancer risk in three recently published independently conducted genome-wide association studies, with no consensus as to the relative impact of the variants on the propensity to smoke vs a direct carcinogenic effect. To further explore our hypothesis that these variants are indeed associated with both cancer causation and nicotine dependence, we performed a more detailed analysis of the association of these putative risk genotypes with smoking phenotype, as well as in lifetime never smokers, and in other smoking-related cancers. We demonstrate a statistically significant association of the variants with both nicotine dependence, as well as lung cancer phenotypes, including earlier age at lung cancer onset. The variants were associated with higher risks of lung cancer in lower smoking-exposed strata, and in individuals with a strong family history of lung or smoking-related cancers. In contrast, we found no evidence that the variants were associated with elevated risks in 547 lifetime never-smoking lung cancer case subjects, nor in other smoking-related cancers (bladder and renal). Thus, we conclude that the variants are implicated both in smoking behavior and more directly in lung cancer risk.

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We recently completed a genome-wide association (GWA) analysis of 315 450 tagging single nucleotide polymorphisms (SNPs) in 1154 lung cancer patients of European ancestry who were current or former smokers and 1137 control subjects who were frequency matched to the lung cancer patients by age, sex, race, and smoking status (1). Two SNPs, rs1051730 and rs803419, that mapped to a region of strong linkage disequilibrium within 15q25.1 were strongly associated with risk of lung cancer, with an odds ratio (OR) for rs1051730 of 1.31 ($P = 9.84 \times 10^{-6}$). This finding was replicated in an additional 711 case subjects and 632 control subjects from Texas ($P = .00042$) and in 2013 case subjects and 3062 control subjects from the United Kingdom ($P = 2.33 \times 10^{-10}$). The overall P value was $<7.00 \times 10^{-18}$. The region of interest encompasses the nicotinic acetylcholine receptor subunit genes *CHRNA3* and *CHRNA5* (as well as *CHRNB4*), which have a defined role in nicotine dependence (2,3) and have been hypothesized to have a direct role in downstream signaling pathways that promote carcinogenesis (4,5). Other genes encompassed by this region include the *IREB2* iron-sensing response element, *PSMA4* (proteasome alpha 4 subunit isoform 1), and *LOC123688*, a gene of unknown function.

Thorgeirsson et al. (6) detected a statistically significant association of these variants with measures of nicotine dependence and concluded that this explained the elevated risk with lung cancer that they also observed. In contrast, in their analysis of 847 smoking control subjects identified from their genome-wide study, Hung et al. (7) did not observe an association of these variants with any of the measures of nicotine dependence based on the Fagerstrom tolerance questionnaire. This instrument includes a series of questions designed to assess the physiological level of addiction to nicotine, and includes such criteria as time to first cigarette in the morning and number of cigarettes smoked per day, both strong predictors of nicotine dependence. Hung et al. did, however, detect an increased risk of lung cancer in both ever and never smokers who carried the variants, and argued for a direct association between the variants and lung carcinogenesis. Our analysis (1) suggested a weak association with nicotine dependence (based on number of cigarettes smoked per day and pack-years of exposure) but a stronger direct association with lung cancer risk per se (1). However, in the small number of lifetime never smokers (n = 125) analyzed in our GWA study, we did not detect an association of SNPs in this region of chromosome 15 with lung cancer risk (1).

In an accompanying editorial pointing out these conflicting conclusions, Chanock and Hunter (8) recommended detailed analysis of smoking intensity and of lung cancer risk in a larger series of nonsmokers. To further explore our hypothesis that these variants are associated with both cancer causation and nicotine dependence, we performed a more detailed analysis of the association of these putative risk genotypes with smoking phenotype, family history, and other epidemiological and clinical covariates, using the combined discovery and replication sets from Texas (1858 ever smoking case subjects and 1763 control subjects, all non-Hispanic whites). We additionally genotyped 547 lung cancer case subjects and 649 control subjects who were all self-reported lifetime never smokers (having smoked fewer than 100 cigarettes), derived from the same source as the GWA population (ie, case accrual from The University of Texas M. D. Anderson Cancer Center and control subjects selected from enrollees in a multispecialty physician practice). Of these never smokers, 78.5% were

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Caucasians, 12.5% were African Americans, 8.5% were Hispanics, and 0.5% were of other ethnicities. Finally, we genotyped 790 ever smoking patients with bladder or renal cancer and 587 control subjects from two ongoing smoking-related case–control studies, all of whom were Caucasian.

We comprehensively evaluated smoking phenotypes according to genotypes in our combined discovery and replication GWA datasets (1). We used the Kruskal–Wallis test for comparing smoking dependence variables among genotypes, separately in case subjects and controls, and by current and former smoking status. We also used the Cochran–Armitage trend for allelic tests of association with strata of nicotine dependence measures. ORs were estimated using logistic regression analysis.

Because rs1051370 is in strong linkage disequilibrium with rs8034191 (R^2 = 0.85), we report the results for rs1051370 only. The overall prevalence of the homozygous variant rs1051370 genotype was 16.3% for ever smoking case subjects and 11.1% for ever smoking controls. The variant genotype was exhibited by 17% of current smoking case subjects and 12% of controls, compared with 16% and 11%, respectively, of former smoking case subjects and controls. Thus, the genotype differences were larger by case–control status than by current vs former smoking status, with case subjects enriched for the variant genotype.

Carriers of the variant AA (adverse) genotype reported the highest number of cigarettes smoked per day in both case subjects and controls (P = .004 and P < .001, respectively) (Table 1). Similar patterns were evident when the data were further dichotomized by former vs current smokers (data not shown). There was no association between genotype and duration of smoking for either current or former smokers (data not shown). Carriers of the adverse AA genotype of rs1051730 exhibited the highest Fagerstrom Test for Nicotine Dependence (FTND) scores (Table 1) of 5.13 and 5.10 for case subjects and controls, P = .012 and P < .001, respectively. These trends also achieved statistical significance separately for current and former smoking controls (P = .01 and .002, respectively), with borderline significance for case former smokers (P = .068, data not shown).

To compare the genotype frequencies with those observed by Thorgeirsson et al. (6) we also clustered daily smoking quantity (SQ) data (again based on our combined discovery and replication GWA datasets) into four levels (1–10, 11–20, 21–30, and ≥31 cigarettes per day, Table 2). The mean SQ for case subjects was 1.62 for the wild-type of rs1051730, 1.66 for heterozygotes, and 1.84 for homozygotes (P for trend = .004, data not shown). For control subjects, the respective SQ values were 1.48, 1.55, and 1.84, P for trend <.001. The prevalences of the A allele (Table 2) were 0.28, 0.32, 0.36, and 0.37 by stratum of increasing number of cigarettes smoked per day for the controls (P < .001) compared with 0.35, 0.37, 0.44, and 0.41 for the case subjects, P = .004 from the Cochran–Armitage test. The largest increase in allele prevalence in controls was between the lowest two SQ levels (4%), with the smallest increase (1%) between the two highest SQ levels, a pattern identical to that noted by Thorgeirsson et al. (6) (Table 2). The prevalence of the A allele was 0.30, 0.35, and 0.33 by tertiles of decreasing length of smoking cessation for controls and 0.37, 0.39, and 0.40, respectively, for case subjects (Table 2). Thus, although the trends were not statistically significant, the adverse A allele was lowest in subjects who reported the longest duration of cessation, suggesting an impact of this genotype on nicotine dependence and failure to achieve sustained smoking cessation. There was no association between allele prevalence and duration of smoking.

We also evaluated trends in A allele prevalence by time to first cigarette, one of the integral FTND measures (Table 2). Among both current and former smoking controls, the highest prevalence of the adverse A allele was in smokers who reported smoking their first cigarette within 5 minutes of waking up, with trend P values of .011 and .006, respectively. However, no such pattern was evident in case subjects who were current or former smokers (P = 1 and .935, respectively).

Schlaepfer et al. (9) noted that a 3′UTR SNP (rs1948) in the same gene cluster (that is in linkage disequilibrium with rs1051730 with D' = 0.99) was statistically significantly associated with earlier age of smoking initiation. We were able to replicate this statistically significant pattern among our currently smoking controls, for whom the mean age at initiation was 18.1 years for carriers of the wild-type genotype and 17.4 years for the adverse genotype, P for trend = .043, vs 17.0 and 17.1 years, respectively, for case smokers, data not shown.

In stratified analysis, the lung cancer risk associated with the homozygous variant

CONTEXT AND CAVEATS

Prior knowledge

Recently, genetic variations in a region of chromosome 15 that encompasses a gene implicated in nicotine dependence had been linked to the risk of lung cancer in genome-wide association studies, but data were not definitive as to whether the variants were linked to lung cancer per se or to nicotine dependence.

Study design

The associations of one of the genetic variants with a number of measures of nicotine dependence were studied using a set of case and control subjects from the initial studies. The risk of lung cancer conferred by the genetic variants was also evaluated in a group of case and control subjects who reported that they had never smoked.

Contribution

The results of this study suggested that the variant allele was associated with a number of measures of increased nicotine dependence, including higher scores on the Fagerstrom Test of Nicotine Dependence. No association of the genetic variant with lung cancer risk observed in never smokers.

Implications

The increased risk of lung cancer conferred by the genetic variants might be explained by an increased likelihood of nicotine dependence, although some of the results of this study and a previous study of the variant in subjects who reported that they had never smoked suggests that the variants may also have a direct role in lung carcinogenesis.

Limitations

The many tests of association performed in the present study raise the possibility that some of the reported associations may be due to chance alone.

From the Editors

Table 1. Smoking intensity and dependence by rs1051730 genotype*

	Case subjects						Control subjects					
Genotype	**n**	**Cig/d**	***P* value†**	**n**	**FTND score**	***P* value†**	**n**	**Cig/d**	***P* value†**	**n**	**FTND score**	***P* value†**
GG	685	27.16		583	4.68		764	25.06		729	4.13	
AG	869	27.09		733	4.63		770	25.99		733	4.27	
AA	300	29.80	.004	259	5.13	.012	193	29.36	<.001	187	5.10	<.001

* N = number of case or control subjects used to analyze a given smoking behavior; Cig = cigarettes; FTND = Fagerstrom Test for Nicotine Dependence.

† From Kruskall–Wallis test.

genotype was highest in lightest smokers (adjusted OR = 2.14 for smokers of <20 cigarettes per day, 1.93 for the middle tertile [20–29] and 1.54 for the highest smoking category [30+], Table 3). This finding argues for a role for genetic susceptibility in the lesser exposed group, and against a sole effect on lung cancer risk of this locus through its effects on nicotine dependence. Risk estimates for the variant genotype were slightly higher for younger patients (median age of <61 years), adjusted for sex, pack-years, and genotyping center, OR = 1.79 (95% confidence interval [CI] = [1.32 to 2.43]) than for older patients, OR = 1.69 (95% CI = [1.25 to 2.28]) and substantially higher for women (OR = 2.53, 95% CI = [1.82 to 3.53]), compared with men (OR = 1.37, 95% CI = [1.03 to 1.81], Table 3). Younger patients and women would be predicted to have lighter smoking histories and these findings are thus consistent with our observation of the strongest association of rs1051370 with lung cancer in the lightest smokers.

Because it has recently been demonstrated that the effect size of the risk allele in familial lung cancer was larger than that observed in sporadic lung cancer (10), we also stratified our data by family history of lung cancer and smoking-related cancers in the case probands. The ORs of lung cancer for homozygous carriers of the variant genotypes were 1.85 (1.27–2.71) for case probands with one first-degree family member with lung cancer and 2.44 (1.04–5.73) for probands with two or more relatives with lung cancer (Table 3). For family history of smoking-related cancers (including lung, head and neck, bladder, renal, pancreas), these respective ORs for probands with 1 and more than 1 affected first-degree relatives were 1.88(1.34–2.62) and 2.53 (1.36–4.70) (data not shown). These patterns could be explained either by shared genetic profiles or by shared heavy smoking histories within the families, or by a combination thereof.

For never-smoking case subjects, the genotype distributions deviated from that expected by the Hardy–Weinberg equilibrium (P = .014), but not in the never-smoking control subjects (P = .299). This is not a surprising finding given the select nature of the case patients and their ethnic heterogeneity (the P value for white case subjects alone was .057). We observed no association of the variants with lung cancer risk among these newly genotyped 547 never-smoking lung cancer case subjects and 649 never-smoking control subjects (Table 3). The frequency of the homozygous variant was 10.1% and 7.9% for the case subjects and control subjects, respectively (OR = 1.06 [0.65–1.73]). The homozygous variant genotype prevalence in never-smoking control subjects is identical to that we noted in the longest duration control quitters (7.9%). The heterozygous genotype was associated with a statistically significantly reduced risk of lung cancer (OR = 0.62, 95% CI = [0.46 to 0.82]). There was no difference in these estimates when we stratified by self-reported exposure to environment tobacco smoke.

Age at onset of lung cancer was modified by genotype. Carriers of the homozygous variant genotype had a median age at onset of 61 years compared with 64 and 66 years for the heterozygous and wild-type genotypes, respectively (P = .001), data not shown. This finding could be explained by either of our two etiologic hypotheses—risk mediated through nicotine dependence or a direct effect. Histological-specific risks associated with the variant genotype in ever smokers were similar; OR for squamous carcinoma = 1.55 (95% CI = 1.06 to 2.25) and OR for

Table 2. Prevalence of A allele of rs1051730 by select measures of nicotine dependence

	Case subjects			Control Subjects		
Smoking measure	**n**	**Allele prevalence**	***P****	**n**	**Allele prevalence**	***P****
No. of cigarettes/d†						
1–10	133	0.35		139	0.28	
11–20	546	0.37		457	0.32	
21–30	367	0.44		234	0.36	
31+	423	0.41	.004	326	0.37	<.001
Duration of cessation (y)						
23+	175	0.37		145	0.30	
13–22	211	0.39		179	0.35	
<13	620	0.40	.197	317	0.33	.430
Time to first cigarette (min)						
Former smokers						
>1 h	154	0.39		138	0.29	
31–60	191	0.39		119	0.33	
6–30	258	0.40		167	0.31	
≤5	251	0.39	.935	188	0.38	.006
Current smokers						
>1 h	44	0.39		85	0.30	
31–60	84	0.44		119	0.32	
6–30	166	0.40		180	0.36	
≤5	129	0.41	1.000	130	0.39	.011

* *P* values calculated using Cochran–Armitage test.

† Thorgeirsson et al. (6) reported allele prevalences in 13945 Icelandic smokers by number of cigarettes per day to be 0.31, 0.35, 0.38, and 0.39, respectively.

adenocarcinoma = 1.63 (95% CI = [1.22 to 2.17]). We also examined the association of the variants with prior physician-diagnosed emphysema, a well-established risk factor for lung cancer in both former and current smokers (11). We observed elevated risks associated with the variant genotype in both case subjects (OR = 1.80, [1.27 to 2.53]) and controls who were current smokers (OR = 1.51 [0.90 to 2.53]) (data not shown). No such risk was observed in former smokers.

Finally, we analyzed the prevalence of the genotypes in a case–control set that included 653 bladder cancer case subjects and 439 controls and 137 renal cancer case subjects and 148 controls matched according to age, sex, and ethnicity (Table 3). The adjusted ORs for the variant heterozygous and homozygous genotypes were 1.15 (0.9, 1.45) and 0.87 (0.69, 1.26), respectively, Since the association of pack-years with the disease outcomes was strong ($P < .001$), but there was no impact of the variants on cancer risk, the data argue for a specific effect in the bronchial epithelium.

This analysis has several limitations. Many subgroup analyses that we have performed contributed to a multiple comparison problem. While a major finding of our study was no association with lung cancer risk in lifetime never smokers, Hung et al. (7) reported statistically significantly increased ORs for the homozygous variant rs8034191 genotype (OR = 1.63) and in the codominant model (OR = 1.25, $P = .013$) although they studied a smaller group of never-smoking case subjects (354) than ours (547).

Nevertheless, our data do provide further evidence for the role of the *CHRNA3/A5/B4* gene cluster variants in liability for nicotine dependence, based on our findings of a strong association of the risk genotype with FTND score and SQ indices, and in control subjects, with evidence for earlier age at smoking initiation and time to first cigarette. Also, although the trends were not statistically significant, the adverse allele prevalence was lowest in former smokers who had quit smoking the earliest, highlighting a potential role of the variant in the ability to quit. Recently, Bierut et al. (12) in an independent sample of families affected with alcoholism replicated their nicotine dependence GWA findings that the nonsynonymous coding SNP of the *CHRNA5* gene, rs16969968 (which is in complete linkage disequilibrium with rs1051370), is strongly predictive of habitual smoking and further demonstrated that the variant form of the A5 subunit altered receptor function, but not receptor expression.

Table 3. Risk estimates for rs1051730 genotype by select variables in ever smokers*

Genotype	Case subjects No. (%)	Control subjects No. (%)	Adjusted OR (95% CI)	*P* value
Smokes <20 cigarettes/d†				
GG	123 (42.27)	195 (48.63)	1.0	
AG	130 (44.67)	173 (43.14)	1.31 (0.93 to 1.83)	.119
AA	38 (13.06)	33 (8.23)	2.14 (1.24 to 3.7)‡	.006
Smokes between 20 and 30 cigarettes/d†				
GG	258 (37.55)	289 (45.37)	1.0	
AG	334 (48.62)	290 (45.53)	1.27 (1 to 1.6)	.04
AA	95 (13.83)	58 (9.110)	1.93 (1.33 to 2.8)§	<.001
Smokes more than 30 cigarettes/d†				
GG	304 (34.70)	280 (40.64)	1.0	
AG	405 (46.23)	307 (44.56)	1.24 (0.99 to 1.55)	.058
AA	167 (19.06)	102 (14.80)	1.54 (1.14 to 2.07)‡	.004
Age <61 y‖				
GG	233 (32.86)	359 (43.73)		
AG	335 (47.25)	350 (42.63)	1.47 (1.17 to 1.84)	.001
AA	141 (19.89)	112 (13.64)	1.79 (1.32 to 2.43)	<.001
Age ≥61 y‖				
GG	453 (39.29)	427 (45.09)		
AG	537 (46.57)	435 (45.93)	1.14 (0.95 to 1.38)	.157
AA	163 (14.14)	85 (8.98)	1.69 (1.25 to 2.28)	.0006
Male‖				
GG	402 (38.07)	430 (43.43)		
AG	490 (46.40)	437 (44.14)	1.20 (0.99 to 1.45)	.070
AA	164 (15.53)	123 (12.42)	1.37 (1.03 to 1.81)	.028
Female‖				
GG	284 (35.24)	356 (45.76)		
AG	382 (47.39)	348 (44.73)	1.41 (1.13 to 1.75)	.002
AA	140 (17.37)	74 (9.51)	2.53 (1.82 to 3.53)	<.001
One first-degree relative with lung cancer‖				
GG	106 (35.45)	786 (44.46)	1.0	
AG	144 (48.16)	785 (44.40)	1.36 (1.03 to 1.78)	.030
AA	49 (16.39)	197 (11.14)	1.85 (1.27 to 2.71)	.002
2 + affected first-degree relatives‖				
GG	15 (32.61)	786 (44.46)	1.0	
AG	22 (47.83)	785 (44.45)	1.46 (0.75 to 2.85)	.265
AA	9 (19.56)	197 (11.14)	2.44 (1.04 to 5.73)	.041
Never smokers¶				
GG	294 (53.75)	317 (48.84)	1.0	
AG	198 (36.20)	281 (43.30)	0.62 (0.46 to 0.82)	.001
AA	55 (10.05)	51 (7.86)	1.06 (0.65 to 1.73)	.807
Bladder and renal cancers£				
GG	339 (42.91)	280 (47.70)	1.0	
GA	364 (46.08)	240 (40.89)	1.15 (0.91 to 1.45)	.247
AA	87 (11.01)	67 (11.41)	0.87 (0.69 to 1.26)	.461

* Current and former smokers combined. OR = odds ratio; CI = confidence interval.

† Adjusted for age, sex, and genotyping center.

‡ *P* for trend <.01.

§ *P* for trend <.001.

‖ Adjusted (according to stratum) for age, sex, pack-years, and genotyping center.

¶ Adjusted for age, sex, ethnicity, and secondhand smoke exposure.

£ Adjusted for age, sex, pack-years, study, and study by pack-year interaction.

Our data also argue against a sole effect of nicotine dependence and for a parallel and direct role of SNPs in the *CHRNA3/A5* region in genetic susceptibility to tobacco carcinogenesis. Specifically, higher risks for the variants were noted in lightest smokers, as well as in younger patients and women, who would be expected to report lower levels of tobacco exposure. Conversely, we noted the lowest risk in the heaviest smoking category, negating the hypothesis that variations in the *CHRNA3/A5/B4* region solely modulate lung cancer risk through their effect on smoking intensity. Further, there was no association of the variants with other smoking-associated cancers. Hung et al. (7) likewise detected no association between the variants and risk of upper aerodigestive tract and esophageal cancers (n = 2262 case subjects), suggesting that the increased risk of lung cancer associated with the presence of the variants may be specific to the bronchial epithelium and not other epithelial smoking-related cancers, although we recognize that the smoking-associated risks for bladder and renal cancer are of a far lower magnitude than they are for lung cancer. The earlier age at lung cancer onset and the higher risk for the variants in probands with multiple first-degree relatives with lung cancer are consistent with either a direct or an indirect role of the variants in lung carcinogenesis.

Bronchial epithelial cells selectively express both alpha 3 and 5 subunits, and both nicotine and the tobacco-specific nitrosamine, NNK, are agonists for the receptors and their affinity is reported to be greater than that for the physiological agonist, acetylcholine (13). Nicotine and its metabolites may therefore play a more direct role in lung cancer induction through activation of autocrine-proliferative signaling networks. Our finding that there was no association in never smokers suggests, however, that present or past tobacco exposure is a necessary component in this carcinogenic pathway. This is consistent with recent data showing that chronic nicotine exposure results in increased cholinergic signaling (with increased levels of receptors and ligands and decreased levels of receptor inhibitors) in squamous cell lung cancer (14). The multifaceted stimulation of the intrinsic cholinergic signaling system by smoking results in receptor-mediated cellular proliferative effects and may provide potential targets for new therapies for lung cancer and for smoking cessation interventions.

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