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Research Article

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Abstract

Backgrounds: Multiple polymorphisms affecting smoking behavior have been identified through genome-wide association studies. Circulating levels of the nicotine metabolite cotinine is a marker of recent smoking exposure. Hence, genetic variants influencing smoking behavior are expected to be associated with cotinine levels.

Methods: We conducted an analysis in a lung cancer case–control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. We investigated the effects of single-nucleotide polymorphisms (SNP) previously associated with smoking behavior on (i) circulating cotinine and (ii) lung cancer risk. A total of 894 cases and 1,805 controls were analyzed for cotinine and genotyped for 10 polymorphisms on 7p14, 8p11, 10q23, 15q25, and 19q13.

Results: Two variants in the nicotinic acetylcholine receptor subunit genes *CHRNA5* and *CHRNA3* on 15q25, rs16969968 and rs578776, were associated with cotinine ($P = 0.001$ and 0.03, respectively) in current smokers and with lung cancer risk ($P < 0.001$ and $P = 0.001$, respectively). Two 19q13 variants, rs7937 and rs4105144, were associated with increased cotinine ($P = 0.003$ and $P < 0.001$, respectively) but decreased lung cancer risk ($P = 0.01$ for both, after adjusting for cotinine). Variants in 7p14, 8p11, and 10q23 were not associated with cotinine or lung cancer risk.

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Conclusions: 15q25 and 19q13 SNPs were associated with circulating cotinine. The directions of association for 15q25 variants with cotinine were in accordance with that expected of lung cancer risk, whereas SNPs on 19q13 displayed contrasting associations of cotinine and lung cancer that require further investigation.

Impact: This study is the largest to date investigating the effects of polymorphisms affecting smoking behavior on lung cancer risk using circulating cotinine measures as proxies for recent smoking behavior. *Cancer Epidemiol Biomarkers Prev; 20(10); 2250–61. ©2011 AACR.*

Introduction

Smoking is the main risk factor for lung cancer, accounting for nearly 85% of cases in men and 50% of cases in women worldwide (1, 2). Smoking exposure is usually assessed through questionnaires [e.g., cigarettes smoked per day (CPD) and duration of smoking], measures that have several limitations (3). Circulating levels of cotinine, the nicotine metabolite, provide an accurate measure of recent tobacco smoke exposure and are able to account to some degree for individual differences in smoking practices, such as depth of inhalation and how completely each cigarette is smoked (4, 5). Cotinine has a half-life of approximately 17 hours and reflects active and second-hand smoking and smoking intensity over the last 1 to 2 days (4, 5).

Several genome-wide association studies (GWAS) on smoking behavior have identified multiple loci associated with CPD and other measures of tobacco addiction (6–13). Genetic variants influencing CPD would be expected to have a more prominent effect on cotinine levels in current smokers, and such variants are also expected to influence the risk of lung cancer.

The 15q25 locus encodes the nicotinic acetylcholine receptor (nAChR) subunit α5, α3, and β4, members of the family of ligand-gated ion channels, which play an important role in the development of nicotine addiction (14, 15). Nicotine binds to the nAChR causing its activation and the release of neurotransmitters. Variants on the 15q25 locus are associated with increased vulnerability to tobacco addiction and changed smoking behavior including increasing CPD (7, 8, 12, 16), and were also identified as the main susceptibility locus in several lung cancer GWAS (13, 17, 18). Some other loci associated with CPD identified through GWAS, including the *CHRNB3–CHRNA6* locus on 8p11, the *CYP2A6–CYP2B6* locus on 19q13, and the 7p14 locus, have also been found to be associated with a small increase in lung cancer risk (8).

The effects of these loci on lung cancer risk might be mediated by their effect on smoking behavior. However, in the case of the 15q25 locus, adjusting for self-reported smoking (smoking status, pack-years, and CPD) only partially attenuates the risk effect (18, 19), and the remaining approximately 30% increase in risk observed per risk allele seems to be in excess of that expected from the increase in CPD conferred by the missense variant. Nevertheless, as CPD is a crude measure of how 15q25 variants influence propensity to smoke, additional aspects of smoking such as differences in inhalation may explain this association. Using cotinine measurements together with self-reported information might increase the reliability of smoking exposure data and allow for a more thorough (although by no means complete) adjustment for recent smoking behavior.

In order to investigate how loci modifying smoking behavior influence circulating cotinine levels and lung cancer risk, we conducted an analysis within a nested lung cancer case–control study from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. Circulating cotinine level measured in serum or plasma was included as a proxy of prediagnosis smoking behavior, together with traditional questionnaire-based smoking measures.

Materials and Methods

Study description

This case–control study was nested within the EPIC cohort, which is an ongoing multicenter prospective study that recruited more than 520,000 healthy individuals between 1992 and 2000. Baseline nondietary and dietary questionnaires were completed at enrolment, as well as anthropometric measurements and blood samples which were collected during an enrolment examination at a study center. Detailed study descriptions of recruitment, follow-up, and collection of questionnaire data and blood samples in EPIC have been provided elsewhere (20).

This EPIC lung study, including selection criteria, has also been described in detail previously (18, 21, 22). The study included lung cancer cases diagnosed for on average of 62 months, and a minimum of 1 month, after blood collection together with matching controls from 8 of the 10 participating countries: France, Italy, Spain, United Kingdom, The Netherlands, Greece, Germany, and Sweden (excluding the Malmö center). Wherever possible, 2 controls were matched to each case by study center, gender, date of blood collection (±1 month, relaxed to ±5 months for sets without available controls), and date of birth (±1 year, relaxed to ±5 years for sets without available controls). Overall, 894 cases and 1,805 controls were included in the analysis (Table 1). Information on tobacco consumption was collected in a nondietary questionnaire as a part of the recruitment procedure in the EPIC cohort. Study participants were classified as never, current, or ex-smokers. Duration of

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Table 1. Distribution of selected demographic variables by case–control status in the EPIC lung cancer study

	All cases and controls			
	Controls ($N = 1,805$)		Cases ($N = 894$)	
	n	%	*n*	%
Smoking status[a]				
Never smokers	705	39.8	96	10.9
Former smokers	659	37.2	258	29.3
Current smokers	409	23.0	526	59.8
Gender				
Men	1,117	61.9	556	62.2
Women	688	38.1	338	37.8
Age[b], y				
<40	37	2.1	19	2.1
40–49	276	15.3	133	14.8
50–59	722	40	355	39.7
60–69	610	33.8	307	34.3
>70	160	8.9	80	9.0
Country				
France	48	2.7	24	2.7
Italy	278	15.4	139	15.6
Spain	259	14.4	130	14.5
United Kingdom	355	19.7	175	19.6
The Netherlands	241	13.4	121	13.5
Greece	186	10.3	90	10.1
Germany	312	17.3	157	17.6
Sweden	126	7.0	58	6.5
Histology				
SCLC			108	12.1
Adenocarcinoma			270	30.2
LCLC			50	5.6
SCC			199	22.3
Other[c]			267	29.9

Abbreviations: LCLC, large cell lung carcinoma; SCC, squamous cell carcinoma; SCLC, small-cell lung carcinoma.
[a]Information on smoking status was missing for 32 controls and 14 cases.
[b]At the date of blood collection.
[c]Including missing histology for French study.

smoking was calculated on the basis of the collected information on age of smoking initiation and age at recruitment for current smokers or age at smoking cessation for ex-smokers. In addition, information on the number of cigarettes currently smoked and smoked at ages 20, 30, 40, and 50 was collected. On the basis of this information, the average number of CPD was calculated. Information on smoking interruptions was available only for 4 coordinating centers in EPIC and therefore not taken into account.

All participants gave written informed consent to participate in the study, which was approved by the local ethics committees in the participating countries and the Institutional Review Board of the International Agency for Research on Cancer (IARC).

Genotyping methods and biochemical analysis

Biochemical measurement of cotinine was done at Bevital A/S, Bergen, Norway. Cotinine levels were measured in serum by liquid chromatography/tandem mass spectrometry (23). For the Swedish cohort cotinine levels were measured in plasma. The laboratory coefficients of variations were 2% to 3% for repeated analyses within the same day and were approximately 6% between days. Cases and controls were analyzed in a random order, and laboratory personnel were blinded to case–control status.

We reviewed the literature and identified several GWAS investigating smoking behavior, as assessed by CPD, as an outcome (6–10, 12, 13). Overall, 10 single-nucleotide polymorphisms (SNP) from 5 loci which were found to be genome-wide significant for CPD—7p14, 8p11, 10q23, 15q25, and 19q13—were selected for genotyping (Table 2). Genotyping was carried out by the 5′ exonuclease assay (TaqMan) at IARC. PCR primers and TaqMan probes were synthesized by Applied Biosystems. Highly correlated proxies ($r^2 = 1.0$) were genotyped in place of assays that were unable to be designed as TaqMan assays. Only one SNP of any correlated group of variants ($r^2 > 0.5$) was genotyped.

Cases and controls were randomly mixed when genotyped and laboratory staff were blinded to case–control status. A random selection of 5% of the study subjects was genotyped twice for quality control. Genotyping success rate per SNP in this study ranged between 93% and 100%. Internal duplicate concordance was more than 98.7% for all variants. All variants showed genotype distributions consistent with that expected under Hardy–Weinberg equilibrium, using a P threshold of 0.005 (Bonferroni correction for 10 tests).

Statistical methods

The distribution of cotinine levels between smoking cases and controls were compared by the Kruskal–Walis test. The associations between SNPs and cotinine levels or CPD were investigated in current smokers by using multivariate linear regression models with smoking variables (CPD or cotinine) as outcomes, adjusting for study center, gender, and case–control status. The mean cotinine levels adjusted for study center, gender, and case–control status were calculated for each genotype.

Risk analysis was carried out by using conditional logistic regression by estimating ORs and their 95% CIs. Risk effects of smoking measured as cotinine and CPD on lung cancer risk were analyzed for 10 categories of increasing cotinine levels (76–200 nmol/L, 201–400 nmol/L, 401–600 nmol/L, etc.), with subjects showing cotinine levels below 75 nmol/L as a reference category

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Table 2. Characteristics of SNPs selected for genotyping

SNPs	Locus	Gene	Minor allele	MAF	Previously observed effects of SNPs on CPD (8, 6) or CPD levels (1–10 CPD, 11–20, 21–30, and ≥31; ref. 7		
					β	*P*	References
rs215614[a]	7p14	*PDE1C*	G	0.36	0.22	2×10^{-7}	8
rs13273442[b]	8p11	*CHNB3*	A	0.23	−0.29	4×10^{-8}	8
rs1329650	10q23	*LOC100188947*	T	0.29	−0.37	6×10^{-10}	6
rs1028936	10q23	*LOC100188947*	C	0.19	−0.45	1×10^{-9}	6
rs16969968	15q25	*CHRNA5*	A	0.39	1.00	6×10^{-72}	6
					0.08	4×10^{-65}	7
rs578776	15q25	*CHRNA3*	A	0.27	−0.06	7×10^{-37}	7
rs4105144	19q13	2 kb 5′ *CYP2A6*	T	0.35	−0.39	2×10^{-12}	8
rs3733829	19q13	*EGLN*	G	0.36	0.33	1×10^{-8}	6
rs7937	19q13	UTR *RAB4B*	C	0.46	−0.24	2×10^{-9}	8
rs7260329	19q13	Intron, *CYP2B6*	T	0.32	−0.20	5×10^{-6}	8

Abbreviations: MAF, minor allele frequency; UTR, untranslated region.

[a]Genotyped instead of proxy rs215605 ($r^2 = 1.0$; $D' = 1.0$).

[b]Genotyped instead of proxy SNPs rs6474412 ($r^2 = 1.0$; $D' = 1$, effect on CPD $\beta = 0.30$, SE = 0.05, $P = 1.7 \times 10^{-8}$; ref. 8) and rs13280604 ($r^2 = 1.0$; $D' = 1.0$, effect on CPD $\beta = 0.31$, SE = 0.05, $P = 1.3 \times 10^{-8}$; ref. 8).

equivalent to never smokers (24), and also for deciles of CPD defined by control individuals, using never smokers as a reference category. ORs for SNPs were calculated by using the per rare allele log-additive model as overall significance test (*P*). We subsequently adjusted for various smoking variables, including cotinine levels, average CPD, and duration of smoking. We conducted exploratory analysis by using 2 models: (i) adjusted by quartiles of smoking variables defined by the distribution among corresponding controls and (ii) adjusted by continuous smoking variables. The results for both models are presented. Furthermore, unconditional logistic regression models were used to allow stratification by smoking status (current, former, and never smokers), adjusting for matching variables (gender, date of birth, date of blood collection, and country). To investigate if the risk effect of genotypes is constant across different levels of smoking exposure, we tested for multiplicative interaction of genotype with quartiles of cotinine levels/CPD. Likelihood ratio tests, comparing the models with and without the interaction terms, were used to evaluate statistical significance.

The nominal and reported significance level for this study was set up to $\alpha = 0.05$.

Regression calibration was used to correct for some of the dilution effects due to day-to-day variation in cotinine levels. We obtained repeat measurements 1 and 3 years apart for 502 individuals, including 96 current smokers who had not changed their smoking status, from the placebo arm of a randomized trial from Norway (WENBIT; ref. 25). The samples were analyzed in the same laboratory and using the same protocol as the EPIC samples. We used these measurements to estimate the within-individual variance of cotinine, assuming that the long-term average was the ideal predictor of lung cancer. This allowed us to calculate regression dilution ratios (RDR) and obtain the adjusted ORs for the effect of cotinine on lung cancer risk by multiplying the observed regression coefficients with the RDR, as described by Clarke and colleagues (26). To account for the effect of regression dilution in the adjustment of the SNPs ORs for lung cancer, we applied the method described by Rosner and colleagues (27), modified to the fact that the genotype data were not available for the participants with repeated cotinine measurements. Further details are provided in the Supplementary Methods.

All statistical analyses were conducted by SAS version 9.2. Power calculations were done by QUANTO version 1.2 for the main effect of gene and log-additive model of inheritance (28).

Results

Genetic variation, circulating cotinine, and cigarettes per day

The effect of SNPs on cotinine levels and CPD was investigated among current smokers only ($n = 935$). We did not observe a significant association of any of the investigated SNPs with CPD (Table 3). In contrast, increased cotinine levels were associated with the minor alleles of rs578776 and rs16969968 on 15q25 (P_{trend} = 0.03 and 0.001, respectively), as well as rs4105144 and rs7937 on 19q13 ($P_{trend} = 0.0001$ and 0.003, respectively; Table 3). The direction of effects of 15q25 variants on

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Table 3. Cotinine level and CPD per allele in current smokers in the EPIC lung cancer study

SNP	Expected effect on CPD and cotinine (7,8)	CPD (n)		Cotinine level (nmol/L)	
		N	Mean (95% CI)	*N*	Mean (95% CI)
rs215614 (7p14, *PDE1C*)					
AA	Low level	280	16.8 (15.7–17.9)	340	1,317.1 (1,205.3–1,429)
AG		352	15.7 (14.7–16.7)	410	1,268.4 (1,159.6–1,377.3)
GG	High level	88	15.9 (14–17.7)	102	1,269.9 (1,120.5–1,419.3)
	Trend test[a]:	$\beta = -0.7$, SE = 0.5, $P = 0.15$		$\beta = -31.7$, SE = 32.2, $P = 0.33$	
rs13273442 (8p11, *CHRNB3*)					
GG	High level	447	16.3 (15.4–17.2)	536	1,295.2 (1,190.9–1,399.5)
GA		227	16.2 (15–17.4)	273	1,268.5 (1,156.2–1,380.7)
AA	Low level	42	17.2 (14.7–19.7)	44	1,328.5 (1,127.5–1,529.4)
	Trend test[a]:	$\beta = 0.2$, SE = 0.5, $P = 0.72$		$\beta = -6.4$, SE = 35.6, $P = 0.86$	
rs1329650 (10q23)					
GG	High level	383	16.1 (15.1–17)	445	1,292 (1,186.1–1,397.8)
GT		255	16.4 (15.3–17.5)	313	1,291.1 (1,179.6–1,402.6)
TT	Low level	64	17.1 (15.1–19.2)	76	1,302.4 (1,134.1–1,470.7)
	Trend test[a]:	$\beta = 0.5$, SE = 0.5, $P = 0.32$		$\beta = 2.8$, SE = 32.5, $P = 0.93$	
rs1028936 (10q23)					
AA	High level	495	16 (15.1–16.9)	588	1,298.4 (1,194.9–1,401.9)
AC		195	16.3 (15–17.5)	234	1,254.4 (1,135.1–1,373.7)
CC	Low level	31	17.3 (14.5–20.2)	35	1,215.6 (990.7–1,440.5)
	Trend test[a]:	$\beta = 0.4$, SE = 0.5, $P = 0.42$		$\beta = -42.9$, SE = 37.9, $P = 0.26$	
rs16969968 (15q25, *CHRNA5*)					
GG	Low level	280	15.9 (14.8–17)	331	1,176.7 (1,063.9–1,289.4)
GA		348	16.4 (15.4–17.3)	417	1,301 (1,195–1,406.9)
AA	High level	131	16.6 (15.2–18.1)	161	1,357.1 (1,231–1,483.2)
	Trend test[a]:	$\beta = 0.4$, SE = 0.4, $P = 0.35$		$\beta = 96$, SE = 28.8, $P = 0.001$	
rs578776 (15q25, *CHRNA3*)					
GG	High level	394	16.7 (15.8–17.7)	481	1,339 (1,231.6–1,446.4)
GA		281	15.6 (14.5–16.6)	324	1,229.7 (1,113.5–1,346)
AA	Low level	32	15.3 (12.5–18)	39	1,276 (1,058.8–1,493.1)
	Trend test[a]:	$\beta = -1$, SE = 0.5, $P = 0.06$		$\beta = -77.4$, SE = 36.2, $P = 0.03$	
rs4105144 (19q13, *CYP2A6*)					
CC	High level	335	16.1 (15.1–17.1)	394	1,193.2 (1,086.3–1,300.2)
CT		287	16.5 (15.4–17.6)	343	1,330.5 (1,218.6–1,442.3)
TT	Low level	81	16.4 (14.5–18.3)	108	1,413.6 (1,277.2–1,550.1)
	Trend test[a]:	$\beta = 0.2$, SE = 0.5, $P = 0.65$		$\beta = 118.3$, SE = 30.2, $P = 0.0001$	
rs3733829 (19q13)					
AA	Low level	299	16.2 (15.1–17.2)	356	1,312.1 (1,201.5–1,422.6)
AG		314	16.5 (15.4–17.5)	383	1,283.1 (1,172.9–1,393.3)
GG	High level	98	15.9 (14.3–17.6)	107	1,168.4 (1,019.7–1,317)
	Trend test[a]:	$\beta = -0.02$, SE = 0.4, $P = 0.97$		$\beta = -59.5$, SE = 31.1, $P = 0.06$	
rs7937 (19q13, *RAB4B*)					
TT	High level	227	16.3 (15.2–17.5)	264	1,176.1 (1,059.9–1,292.2)
TC		339	16.3 (15.3–17.3)	409	1,340.1 (1,231.2–1,448.9)
CC	Low level	153	16.3 (14.9–17.7)	180	1,332.1 (1,211.8–1,452.3)
	Trend test*:	$\beta = -0.01$, SE = 0.4, $P = 0.98$		$\beta = 86.3$, SE = 29.3, $P = 0.003$	
rs7260329 (19q13, *CYP2B6*)					
CC	High level	383	16.2 (15.2–17.1)	443	1,275.2 (1,168.3–1,382)
CT		273	16.7 (15.6–17.8)	331	1,304.6 (1,194.1–1,415.2)
TT	Low level	66	15.6 (13.5–17.6)	78	1,320.1 (1,156.6–1,483.6)
	Trend test[a]:	$\beta = 0.02$, SE = 0.5, $P = 0.97$		$\beta = 25.2$, SE = 32.2, $P = 0.43$	

[a]Linear trends in CPD and cotinine levels were assessed by linear regression models adjusted for center, gender, and case–control status.

R^2 between the SNPs are less than 0.50.

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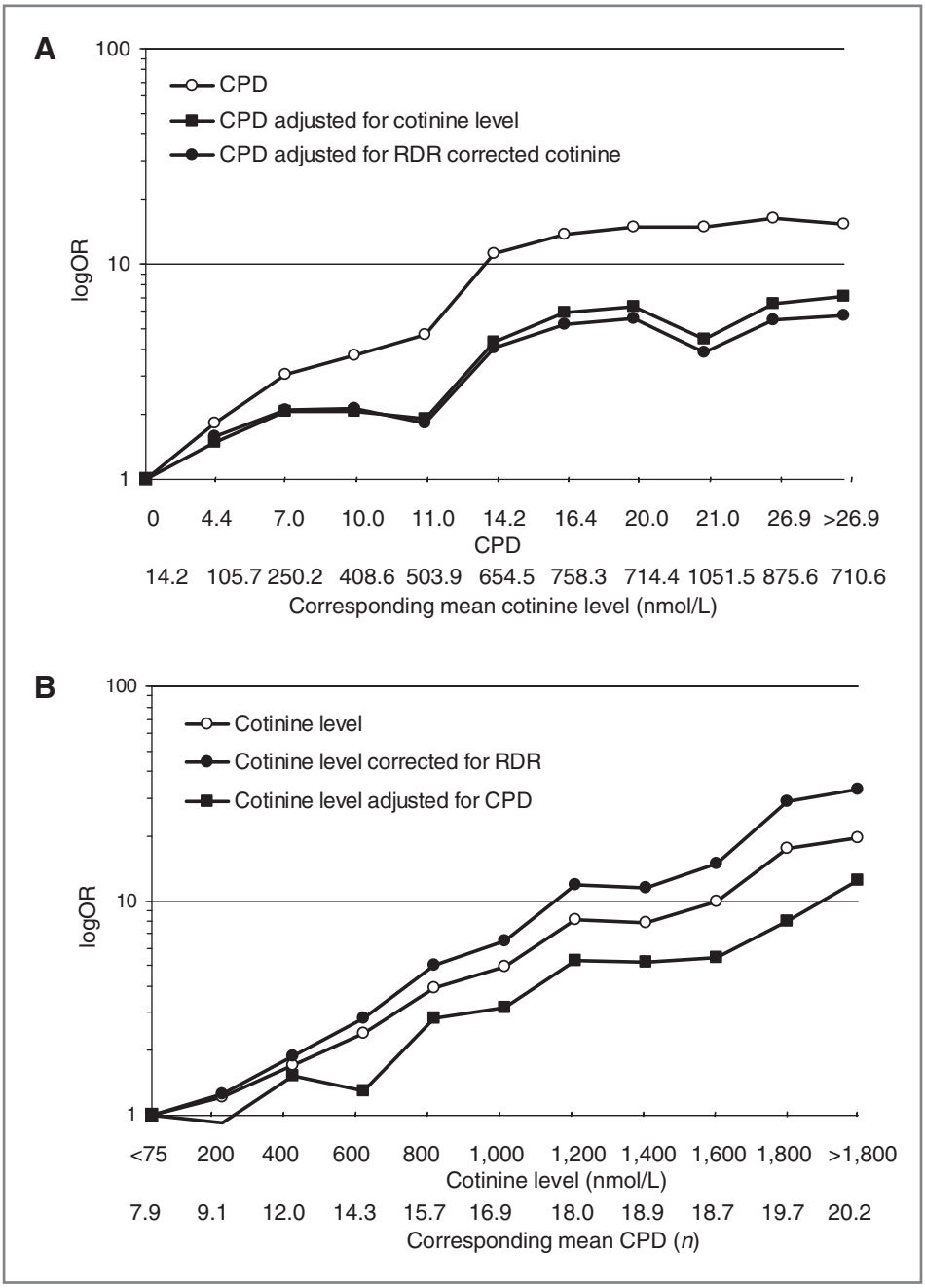

Figure 1. ORs for the risk of lung cancer by CPD (A) and cotinine level (B). A, ORs for the risk of lung cancer for deciles of CPD are presented before adjustment ($P_{trend} = 2 \times 10^{-53}$) and after adjustment for cotinine level ($P_{trend} = 4.5 \times 10^{-20}$). Corresponding mean cotinine level for each percentile of CPD is given. Nonsmokers were used as the reference group. Corresponding ORs and 95% CIs are presented in the Supplementary Table S1. The analysis includes only individuals with available cotinine and CPD measurements. B, ORs for the risk of lung cancer for 200 nmol/L intervals of cotinine level before $P_{trend} = 3 \times 10^{-73}$) and after correction for RDR and adjustment for CPD ($P_{trend} = 1.5 \times 10^{-29}$) are presented. Reference group for the cotinine level—individuals with less then 75 nmol/L. Corresponding ORs and 95% CIs are presented in the Supplementary Table S2.

circulating cotinine levels were consistent with that expected on the basis of previously published results for CPD (Table 2). In contrast, the 19q13 variants showed opposite effects on circulating cotinine level compared with those reported for CPD previously (Table 2). No other SNPs were significantly associated with cotinine levels.

Circulating cotinine levels, cigarettes per day, and lung cancer risk

In risk analysis, both cotinine levels and CPD were positively associated with lung cancer risk (OR = 1.34, 95% CI: 1.29–1.39, $P_{trend} = 2 \times 10^{-53}$ per decile of CPD; OR = 1.36, 95% CI: 1.3–1.4; $P_{trend} = 3 \times 10^{-73}$ per 200 nmol/L of cotinine; Fig. 1; Supplementary Tables S1 and S2). The risk increased monotonically with increasing cotinine levels and reached an OR of 19.6 (95% CI: 12.5–30.8) for subjects having cotinine levels higher than 1,800 nmol/L. The estimated RDR taking into account the within-person variation was 0.86 and correction for regression dilution resulted in notably higher ORs than those from uncorrected measurements (Fig. 1). In contrast, the risk increase associated with CPD deciles reached a plateau at 20 to 21 CPD and the maximum

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observed OR was 16.4 (95% CI: 10.3–26.1) for CPD levels between 21 and 26.9 (Fig. 1; Supplementary Table S1). Mutual adjustments of cotinine and CPD attenuated the maximum risk association for CPD from OR = 16.4 to OR = 6.5 which is a considerably greater attenuation than seen for the maximum OR for cotinine (OR = 19.6 to OR = 12.4, Fig. 1).

Genetic variation and lung cancer risk

Both rs16969968 (OR = 1.31, 95% CI: 1.16–1.48, $P < 0.001$; ref. 18) and rs578776 on 15q25 (OR = 0.79, 95% CI: 0.69–0.91, $P = 0.001$) were associated with risk (Table 4), as well as rs7937 on 19q13 (OR = 0.88, 95% CI: 0.77–1.00, $P = 0.05$; Table 4). No other SNP showed evidence of association with lung cancer risk.

Adjusting for cotinine and CPD separately attenuated these associations to varying degrees (maximum attenuation 29% for adjustment of rs578776 for cotinine), as did adjustments for both cotinine and duration of smoking (maximum attenuation a substantial 43% for adjustment of rs578776 for as-measured cotinine and duration of smoking; Fig. 2). Adjustment for regression dilution bias–corrected cotinine led to attenuation from OR = 1.31 to OR = 1.23 for rs16969968 and from OR = 0.79 to OR = 0.87 for rs578776 (Table 4).

After stratifying by smoking status (current, former, and never smokers), the associations of rs16969968 and rs578776 on 15q25 with risk were present only in current (OR = 1.39, 95% CI: 1.15–1.68, $P < 0.001$, and OR = 0.68, 95% CI: 0.54–0.86, $P = 0.001$, respectively) and former smokers (OR = 1.31, 95% CI: 1.05–1.63, $P = 0.01$ for rs16969968 only; Fig. 2), but not in never smokers (OR = 1.18, 95% CI: 0.84–1.65, $P = 0.32$ for rs16969968; OR = 1.07, 95% CI: 0.76–1.51, $P = 0.70$ for rs578776). The risk effect in smokers seemed to be constant among different levels of smoking exposure measured as CPD ($P_{interaction} = 0.55$ and 0.11 for rs16969968 and rs578776, respectively) and cotinine ($P_{interaction} = 0.92$ and 0.36 for rs16969968 and rs578776, respectively). rs4105144 and rs7937 on 19q13 were robustly associated with lung cancer risk in current smokers only after adjusting for smoking (cotinine level, CPD, duration of smoking; Table 4; Fig. 2). Similar to SNPs on 15q25, no interaction between these 2 SNPs and levels of CPD ($P_{interaction} = 0.94$ and 0.88 for rs4105144 and rs7937, respectively) and cotinine ($P_{interaction} = 0.90$ and 0.20 for rs4105144 and rs7937, respectively) was detected in smokers in this study. Among current smokers, adjustment for as-measured cotinine led to attenuations of estimated effects by 44% for rs16969968 and 18% for rs578776; adjustment for regression dilution bias–corrected cotinine led to further attenuation of the estimated effect of the 15q25 locus (OR = 1.06, 95% CI: 0.77–1.46; OR = 0.84, 95% CI: 0.6–1.18). Inversely, adjustment for cotinine and regression dilution bias–corrected cotinine enhanced the apparent effect of 19q13 SNPs (Table 4).

Discussion

In this study, we investigated whether SNPs on 7p14, 8p11, 10q23, 15q25, and 19q13, previously found to be associated with CPD in GWAS, are related to circulating cotinine, a biomarker of recent smoking exposure, as measured in a prospective case–control study nested within EPIC. Only SNPs 15q25 and 19q13 loci had measurable effects on circulating cotinine levels, but showed no association with CPD. As previously shown (18), variants on 15q25 were also associated with lung cancer risk. Smoking exposure measures, both self-reported (CPD) and circulating cotinine levels, could only partly account for the risk associations of 15q25 variants. However, adjustment for regression dilution bias–corrected cotinine led to substantial attenuation of these estimates. An association with lung cancer risk opposite to that predicted by the association with circulating cotinine levels was detected for the 19q13 locus (rs7937 and rs4105144).

Cotinine and CPD as lung cancer risk predictors

CPD and other self-reported variables reflecting smoking behavior have been used extensively as measures of tobacco exposure in epidemiologic studies of lung cancer, including studies on genetic factors. As tobacco smoking is the major risk factor for lung cancer (29), accurate measures of tobacco exposures are essential. However, it is known that assessing smoking exposure using questionnaires will be subject to misclassification (3, 30). Studies on the relationship between questionnaire measures of tobacco exposure (e.g., CPD) and biomarkers of tobacco exposure (e.g., cotinine; refs. 4, 31–36) have reported a nonlinear relationship, particularly among heavy smokers, suggesting misclassification at high CPD or differences in inhalation and other smoking styles between heavy and light smokers (37–39). Accordingly, in epidemiologic studies lung cancer risk has been shown to steadily increase up to 20 to 30 CPD, but plateau for subjects reporting CPD more than 20 to 30 (38). Consistently, the excess ORs of lung cancer risk for each pack-year of exposure was shown to increase with increasing intensity of smoking only for subjects who smoke up to 20 CPD (33). We observed similar results in this study, where little excess in risk was noted for those reporting more than 20 CPD (Fig. 1). As expected, we also observed that cases reporting similar tobacco consumption levels had higher cotinine levels than controls, even after accounting for number of cigarettes smoked over the last 24 hours (mean cotinine level in controls adjusted for number of cigarettes smoked in last 24 hours = 1,113 nmol/L vs. mean cotinine level in cases = 1,433 nmol/L; $P < 0.001$). In contrast with CPD, the relationship between cotinine and risk increased monotonically, consistent with previous observations reported by Boffetta and colleagues (37). Similarly, Yuan and colleagues (40) observed an association of cotinine with lung cancer risk among smokers with comparable smoking history, but no

Table 4. ORs and 95% CI for the risk of lung cancer for all cases and controls and for current smokers in the EPIC lung cancer study

SNPs	Expected effect on lung cancer (8, 13, 17, 18)	Effect allele	Cases	Controls	Unadjusted model		Model adjusted for cotinine[a]		Model adjusted for regression dilution bias–corrected cotinine[a]	
					OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
All cases and controls										
rs215614 (7p14, *PDE1C*)	↑	G	819	1,643	1.03 (0.91–1.18)	0.61	1.06 (0.91–1.23)	0.47	1.07 (0.91–1.24)	0.42
rs13273442 (8p11, *CHRNB3*)	↓	A	820	1,646	0.98 (0.85–1.14)	0.83	1.03 (0.87–1.22	0.73	1.03 (0.87–1.23)	0.72
rs1329650 (10q23)	n/a	T	801	1,568	1.07 (0.93–1.24)	0.34	1.06 (0.89–1.25)	0.53	1.05 (0.89–1.25)	0.55
rs1028936 (10q23)	n/a	C	819	1,649	1.02 (0.87–1.19)	0.85	1.04 (0.86–1.26)	0.67	1.05 (0.87–1.27)	0.62
rs16969968 (15q25, *CHRNA5*)	↑	A	868	1,749	1.31 (1.16–1.48)	<0.001	1.26 (1.09–1.45)	0.002	1.23 (1.06–1.42)	0.01
rs578776 (15q25, *CHRNA3*)	↓	A	812	1,658	0.79 (0.69–0.91)	0.001	0.85 (0.72–1)	0.06	0.87 (0.74–1.03)	0.10
rs4105144 (19q13, *CYP2A6*)	↓	T	810	1,635	0.93 (0.82–1.06)	0.29	0.87 (0.75–1.02)	0.09	0.86 (0.74–1.01)	0.06
rs3733829 (19q13)	No effect	G	811	1,647	1.05 (0.92–1.19)	0.46	1.14 (0.98–1.33)	0.09	1.15 (0.99–1.34)	0.07
rs7937 (19q13, *RAB4B*)	↓	C	811	1,638	0.88 (0.77–1.00)	0.05	0.84 (0.72–0.98)	0.02	0.83 (0.71–0.97)	0.02
rs7260329 (19q13, *CYP2B6*)	↓	T	821	1,640	0.90 (0.79–1.03)	0.12	0.98 (0.84–1.14)	0.76	0.99 (0.84–1.15)	0.86
Current smokers[b]										
rs215614 (7p14, *PDE1C*)	↑	G	484	368	0.95 (0.77–1.17)	0.62	0.99 (0.79–1.23)	0.92	1.03 (0.81–1.31)	0.81
rs13273442 (8p11, *CHRNB3*)	↓	A	480	373	0.98 (0.78–1.24)	0.90	1.00 (0.77–1.28)	0.98	1.01 (0.78–1.30)	0.96
rs1329650 (10q23)	n/a	T	470	364	1.16 (0.94–1.44)	0.18	1.14 (0.90–1.43)	0.28	1.08 (0.85–1.38)	0.53
rs1028936 (10q23)	n/a	C	482	375	1.05 (0.82–1.34)	0.72	1.07 (0.82–1.4)	0.62	1.11 (0.85–1.46)	0.44
rs16969968 (15q25, *CHRNA5*)	↑	A	511	398	1.39 (1.15–1.69)	<0.001	1.22 (0.99–1.5)	0.06	1.06 (0.77–1.46)	0.72
rs578776 (15q25, *CHRNA3*)	↓	A	469	375	0.68 (0.53–0.86)	0.001	0.74 (0.57–0.95)	0.02	0.84 (0.60–1.18)	0.32
rs4105144 (19q13, *CYP2A6*)	↓	T	480	365	0.85 (0.70–1.04)	0.12	0.74 (0.59–0.92)	0.01	0.65 (0.48–0.88)	0.005
rs3733829 (19q13)	No effect	G	475	371	1.13 (0.92–1.39)	0.23	1.23 (0.99–1.54)	0.07	1.31 (1.03–1.68)	0.03
rs7937 (19q13, *RAB4B*)	↓	C	482	371	0.85 (0.70–1.03)	0.10	0.76 (0.62–0.94)	0.01	0.71 (0.56–0.90)	0.005
rs7260329 (19q13, *CYP2B6*)	↓	T	486	366	0.85 (0.69–1.05)	0.14	0.8 (0.65–1.03)	0.09	0.81 (0.64–1.02)	0.07

NOTE: ORs and 95% CIs for the SNPs were calculated by using conditional to matching variables logistic regression.

Abbreviation: n/a, no available information on effect of the SNP on lung cancer risk.

[a]Model was adjusted by continuous cotinine variable.

[b]Unconditional logistic regression adjusted for matching variables (year of birth, year of blood donation, gender, and country).

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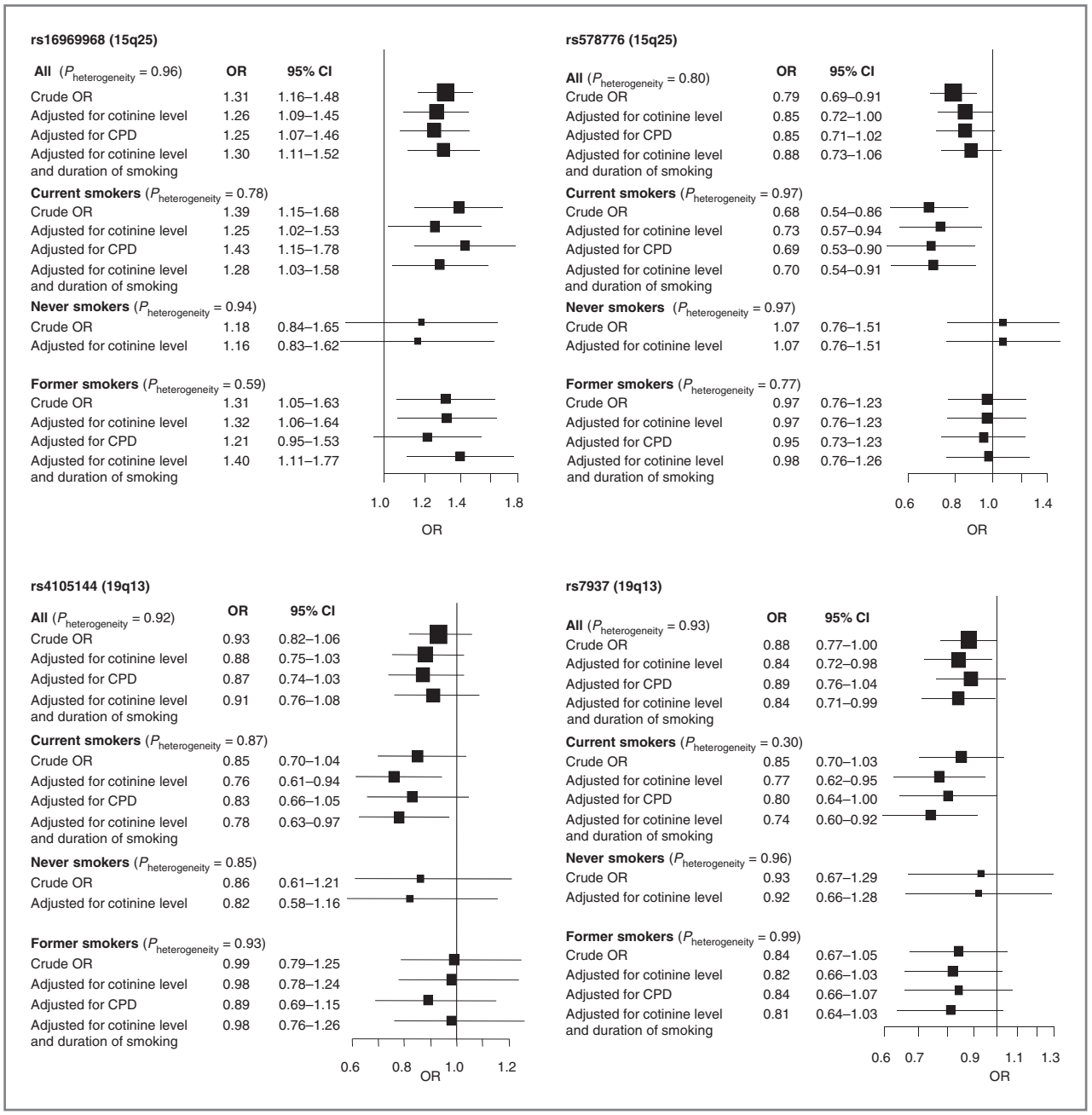

Figure 2. Effect of 15q25 locus (rs16969968 and rs57876) and 19q13 locus (rs7937 and rs4105144) on the risk of lung cancer. ORs and 95% CIs for the risk of lung cancer are calculated applying conditional logistic regression adjusted for quintiles of cotinine levels/CPD/duration of smoking. Effect of SNPs in smoking strata (current, former, and never smokers) was calculated by using unconditional logistic regression adjusted for matching variables (date of birth, country, date of blood collection, and gender).

association was detected by Church and colleagues (41) in current smokers. In mutually adjusted analysis of cotinine and CPD, the association of cotinine with risk was considerably less attenuated than that of CPD. This is consistent with the notion that circulating cotinine captures other aspects of smoking behavior and dose than does CPD, such as inhalation depth and the degree to which each cigarette is smoked. However, the association of CPD remains substantial, suggesting that unlike circulating cotinine it has value for capturing past smoking behavior.

As with all biochemical measurements, cotinine levels are subject to both measurement error and normal day-to-day variations. In regression analysis, these variations lead to regression dilution bias and subsequent underestimation of ORs (26, 42). To correct for this bias, we

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estimated RDRs by use of repeat samples. The RDR-corrected ORs of cotinine were, as expected, notably higher than the corresponding uncorrected values, indicating that the underlying risk associated with cotinine is substantially underestimated (Fig. 1).

Effect of the studied loci on CPD and circulating cotinine levels and lung cancer risk

Polymorphisms on 7p14, 8p11, 10q23, 15q25, and 19q13 have been associated with smoking behavior in GWAS, typically measured as CPD (6–8, 12, 13). However, in this study, we did not detect any associations between previously implicated SNPs and CPD (Table 3), possibly because of the limited sample size. Indeed, the statistical power to detect the expected effect of SNPs on CPD ranged from 10% to 60% for the variants studied. Conversely, SNPs on 15q25 were clearly associated with cotinine levels, as well as lung cancer risk, consistent with the expected direction of association noted in the original GWAS. Similarly, an association with cotinine levels and other nicotine metabolites was previously described for the 15q25 locus (43) with the effect being stronger for cotinine than CPD (36).

This association of SNPs on 15q25 with lung cancer risk has been suggested to be mediated through changes in propensity to smoke tobacco (13, 18). In the current study, the estimated risk effect of 15q25 SNPs was attenuated to varying degrees when controlling for various smoking variables, including CPD, duration of smoking, and cotinine levels. In this study, adjustment for regression dilution bias–corrected cotinine in current smokers led to attenuation of the rs16969968 OR, thus supporting the hypothesis of the 15q25 association with lung cancer risk being mediated by smoking behavior. However, the regression dilution method is not perfect and relies on several assumptions that may not hold. First, the correction is estimated using measurements taken 3 years apart and assuming a constant mean rate. The issue is further complicated by our incomplete understanding of the relation between life-course smoking and lung cancer risk: a lifetime mean may not be the ideal predictor even if we were able to estimate it with precision. In addition, our estimates of regression dilution were obtained from a distinct population, geographically unrepresentative of the EPIC lung study, for which no genotype data were available. Our method also assumes that the extent of day-to-day variation in smoking is independent of genotype, which may not be correct. Taking these limitations together, our regression dilution corrections may be either an undercorrection or an overcorrection, and the result should be interpreted with caution. Naturally, similar concerns of regression dilution apply to self-reported CPD, in this case, we had repeated estimates from 5 time points. We used these to calculate an average CPD and this was the variable used in the analysis. Nevertheless, this analysis represents a first attempt to circumvent the limitation inherent in most observational studies using a single measurement.

SNPs on 19q13 were also associated with cotinine, but the directions of the observed associations were opposite to those originally observed with CPD. Thus, the rs7937 SNP (*T* allele) on 19q13 was associated with decreased lung cancer risk, consistent with the previous study showing an association with lower CPD (8), but increasing levels of cotinine. Consequently, estimates of its effect on lung cancer risk were augmented by correction for regression dilution.

The 19q13 locus contains several *CYP2* genes, including *CYP2A6*—the major enzyme involved in the metabolism of nicotine. CYP2A6 catalyzes *C*-oxidation of nicotine to cotinine, which is in turn metabolized to trans-3′-hydroxycotinine (44, 45). It would seem plausible that genetic variants in this gene may induce slower nicotine metabolism (12, 46) and accumulation of circulating cotinine, and subsequently, a reduction in smoking intensity with a lower lung cancer risk as consequence. Although this hypothesis would explain the opposing effects of 19q13 SNPs on cotinine and CPD, circulating measurements of the ratio of 3′-hydroxycotinine to cotinine would be required to further elucidate these complex associations. Overall, these observations highlight the disparate mechanisms of variants on 15q25 and 19q13 in their effects on smoking behavior and subsequent lung cancer risk.

To our knowledge, this study is the largest to date investigating the effects of SNPs on 7p14, 8p11, 10q23, 15q25, and 19q13 on lung cancer risk, which also uses circulating cotinine measures as proxies for recent smoking behavior. The study further benefits from several important characteristics, including the prospective study design and detailed information on tobacco exposure. The study was, however, not adequately powered to detect the small risk effects expected of some of the studied SNPs (OR ranging from 1.05 to 1.12). It would have also been desirable to measure alternative nicotine metabolites to better describe the opposing associations of SNPs on 19q13.

In conclusion, this study confirms previous associations of SNPs on 15q25 with cotinine levels. The study also indicates that circulating cotinine levels may provide more refined information on recent smoking exposure than CPD as assessed by questionnaires. The intriguing associations of SNPs on 19q13 with cotinine levels, opposite to that of CPD and lung cancer risk, should be studied further by measuring additional nicotine metabolites. Finally, this study indicates that the degree to which the established effects of 15q25 SNPs on lung cancer risk are mediated by smoking may be underestimated by use of crude measures of smoking such as CPD. Further studies with a range of objective smoking measures covering a greater period of lifetime smoking are required to further elucidate this issue.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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