Original Article

Variation in the α5 nicotinic acetylcholine receptor subunit gene predicts cigarette smoking intensity as a function of nicotine content

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A single-nucleotide polymorphism (SNP) in the α5 nicotinic acetylcholine receptor subunit gene, rs16969968, has been repeatedly associated with both smoking and respiratory health phenotypes. However, there remains considerable debate as to whether associations with lung cancer are mediated through effects on smoking behavior. Preclinical studies suggest that α5 receptor subunit expression and function may have a direct role in nicotine titration during self administration. The present study investigated the association of CHRNA5 polymorphisms and smoking topography in 66 smokers asked to smoke four nicotine-containing (nicotine yield = 0.60 mg) and four placebo (nicotine yield < 0.05 mg) cigarettes, during separate experimental sessions. Genotype at rs16969968 predicted nicotine titration, with homozygotes for the major allele (G:G) displaying significantly reduced puff volume in response to nicotine, whereas minor allele carriers (A:G or A:A) produced equivalent puff volumes for placebo and nicotine cigarettes. The present results suggest that puff volume may be a more powerful objective phenotype of smoking behavior than self-reported cigarettes per day and nicotine dependence. Further, these results suggest that the association between rs16969968 and lung cancer may be mediated by the quantity of smoke inhaled.

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Introduction

Genome-wide association studies of tobacco smoking have consistently identified strong signals from polymorphisms in the long arm of chromosome 15.1 Most notably, polymorphisms in a cluster of genes coding for the α5, α3 and β4 nicotinic acetylcholine receptor subunits are associated with a variety of smoking-related phenotypes and health outcomes.2 The first genome-wide association studies specific to nicotine dependence identified a strong association with a single-nucleotide polymorphism (SNP) in the α5 receptor subunit gene at rs16969968.3 Homozygotes for the minor allele (that is, A:A) were nearly twice as likely to be nicotine dependent as heterozygotes (A:G) or those without a minor allele (G:G). This SNP has since received considerable attention because of its documented relationship between rs16969968 and smoking, it is not surprising that this SNP is also linked to respiratory health problems such as lung cancer2,8,13–17 and COPD.8,14 However, it has been argued that the association between this variant and lung cancer risk is not substantially mediated by differences in smoking intensity.18 The majority of this work has used broad and subjective measures to define smoking behavior (for example, cpd and pack years smoked).2,19 Measures that rely solely on self report may not be as reliable, or sensitive to genetic effects, as objective measures of smoking. In addition, such measures do not account for variation between individuals regarding nicotine and carcinogen exposure from each cigarette.20,21 Consequently, more objective measures of smoking behavior are needed to better estimate variation in health risk as a function of rs16969968 genotype.2,19

Recent work has incorporated an examination of smoker’s exposure to toxicants as objective measures of tobacco use. Following a single cigarette, higher levels of plasma nicotine and a tobacco-specific carcinogen are observed among carriers of a minor allele at rs16969968, relative to noncarriers.22 In another study, higher levels of cotinine, a nicotine metabolite, were observed amongst rs16969968 minor allele carriers (or rs1051730; a proxy SNP for rs16969968 in Caucasian populations), even when controlling for cpd. As expected, this SNP was also more strongly associated with cotinine levels than with self-reported cpd.19 These studies demonstrate that rs16969968 predicts aspects of smoking not accounted for by more global measures (for example, cpd). Rather, more proximal and objective measures of smoking behavior (that is, endophenotypes) may help to clarify the association of this SNP with lung cancer. Yet, additional work is needed to determine the mechanism that accounts for differences observed in toxicant exposure between genotypes.

Given evidence that smokers can adjust the nicotine dose delivered from a cigarette by altering their smoking pattern (for example, by puffing longer or deeper),23 it is plausible that
smokers with a risk genotype inhale more toxicants by smoking each cigarette more intensively than noncarriers. This idea converges with preclinical work demonstrating α5 receptor involvement in nicotine self-administration. For example, mice with a null mutation of the α5 receptor gene (Chrna5) self-administered more doses than wild-type controls when nicotine was delivered in moderate to high concentrations, but not for low or placebo concentrations. Unlike wild-type controls, knockouts failed to reduce rates of nicotine administration when nicotine dose concentration was increased beyond moderate levels. Thus, polymorphisms that interfere with the function of the α5 subunit in smokers may similarly alter self-administration of nicotine delivered via cigarette smoking. A precise measure of nicotine self-administration in humans is smoking topography: puff number, volume, duration and inter-puff-interval per cigarette. Compared with self-reported cpd, smokers’ puff topography better predicts exposure to toxicants such as nicotine, carbon monoxide and carcinogens, and thus may serve as an endophenotype for smoking behavior and respiratory health.

Using data from our previously published work, the present study sought to examine the influence of α5 receptor gene SNPs on smokers’ puff topography. For this study, the topography outcome measure of interest was total puff volume per cigarette. It was hypothesized that minor allele carriers at rs16969968 would smoke nicotine-containing cigarettes more intensively (larger total puff volumes) than noncarriers, as is suggested by prior studies, which have demonstrated the association of rs16969968 with nicotine and carcinogen exposure. Consistent with the nicotine self-administration data provided from preclinical genetic studies, we expected no relationship between genotype and puff volume in response to placebo cigarettes. In addition, we explored the association of several other noncoding SNPs in CHRNAS (rs11637635, rs17408276, rs3829787, rs4275821, rs588765, rs569207, and rs684513) with smokers’ total puff volume per cigarette. Although the functional effects of these SNPs are not currently understood, each has been shown to predict smoking and/or risk of respiratory disease.

MATERIALS AND METHODS

Participants

Eighty-three current cigarette smokers were recruited from the Tampa Bay area for a study investigating the effects of nicotine dose on neural indices of attention (the results of this primary study are not reported here). Eligible participants were required to be between the ages of 18–70 years and to have smoked 15 or more cpd for the past 2 years (biochemically verified by expired air carbon monoxide levels ≥10 p.p.m. and urinary cotinine level ≥100 ng ml⁻¹). Participants were excluded from the study if they reported using nicotine-containing products other than cigarettes within the past 3 months; were currently attempting to quit smoking (including use of smoking cessation medications); tested positive for psychoactive drug use or pregnancy; met criteria for a DSM-IV Axis I disorder (that is, psychosis, major depressive episode, manic/hypomanic episode, panic disorder, current alcohol or substance abuse) as assessed by the Structured Clinical Interview for DSM disorders; reported any past head injury or loss of consciousness; reported any serious medical conditions such as cancer or cardiopulmonary disease; or were unable to read and understand the consent forms or questionnaires. This sample has been used previously to describe the influence of cigarette nicotine content on smoking topography. Data were collected during a period from January 2009 to May 2012.

Procedure

An initial screening session was required to complete the informed consent and establish eligibility status. During this session, participants provided demographic data and self-report measures related to smoking behavior, including the Fagerström Test for Nicotine Dependence (FTND). Participants were then scheduled to attend two 2.5-h experimental sessions, each of which was preceded by overnight (that is, 12 h) abstinence from use of nicotine/tobacco (CO level ≤10 ppm or no greater than half of their CO level at the initial screening session) and alcohol (blood alcohol level <0.001%). During each double-blind and counterbalanced session, participants were required to smoke either nicotine-containing (Quest 1, 8.9 mg) or placebo (Quest 3, 1.0 mg) cigarettes (Vector Tobacco Inc, Research Triangle Park, NC, USA). Four of the condition-assigned cigarettes were smoked ad libitum through a mouthpiece that was connected to a smoking topography device. Initiation of each cigarette was spaced ~40 min apart, and followed by the completion of the Modified Cigarette Evaluation Questionnaire. The participant was fitted with an electroencephalogram cap as part of the primary study between smoking bouts 1 and 2 and was required to undergo tasks of attention and working-memory between smoking bouts 2 and 3 and bouts 3 and 4. This study was approved by the Moffitt Scientific Review Committee and the institutional review board of the University of South Florida. As such, it was conducted in accordance with the standards outlined in the 1964 Declaration of Helsinki.

Measures

Genetics. Buccal cells were collected for genotyping. Participants were required to rinse their mouths with water, use a tongue depressor to gently scrape the inside of their cheeks and tongue and then rinse their mouth with saline solution.

Smoking topography. Cigarettes were smoked through a mouthpiece connected to a pressure transducer via the Clinical Research Support System (Borgwaldt, KC, Richmond VA, USA). Inhalation-induced pressure changes were amplified, digitized and sampled at a rate of 1000 Hz, and software converted signals to air flow (ml s⁻¹) for data integration. This device is effective for quantifying smoke exposure and has negligible effects on smoking behavior.

Data analyses

Genotyping. Genomic DNA was extracted from buccal cells using the Gentra Puregene tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s protocol. DNA samples were genotyped using the Illumina GoldenGate assay (Illumina, San Diego, CA, USA) at the Moffitt Cancer Center’s Molecular Genomic Core and genotypes were called using the BeadStudio algorithm.

Statistical analysis

The primary analysis investigated the effects of minor allele carrier status at rs16969968 and cigarette nicotine content on total puff volume. Secondary analyses tested this same effect for polymorphisms at the following noncoding SNPs: rs11637635, rs17408276, rs3829787, rs4275821, rs588765, rs569207 and rs684513. As our sample contained relatively few individuals homozygous for the minor allele with regard to several of our SNPs (that is, 4.50% for rs16969968), genotype was dichotomized to increase the statistical power. That is, minor allele carriers (heterozygotes and minor homozygotes) were compared with noncarriers (major homozygous).

To examine the SNP effects and potential interactions with cigarette nicotine content, we used mixed-model repeated measures analyses with a scaled identity covariance structure. Specifically, models included fixed effects for genotype, nicotine content (nicotine vs placebo) and the interaction of these two factors, with cigarette trial as a covariate and random effect. Bonferroni-corrected planned comparisons were then conducted to further characterize interactive effects that included genotype (that is, genotype or genotype × nicotine content). All models were also re-examined while controlling for other significant predictors of puff topography (for example, FTND, race, and ethnicity), and are reported below.

RESULTS

Sample characteristics

Seventeen participants were excluded from the analysis due to either procedural errors in the smoking topography equipment (n = 16) or missing genotype data (n = 1). The remaining 66 participants (50 males) self-identified their race as Caucasian (n = 52), African American (n = 12) or American Indian or Alaskan native (n = 1). One participant did not identify a racial background. Seven participants self-identified their ethnicity as Hispanic,

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whereas the remainder identified as non-Hispanic (n = 58), or did not report (n = 1). Participants had an average age of 39.6 (s.d. = 12.1) years, smoked 22.5 (s.d. = 6.9) cpd and had a moderate nicotine-dependence score of 5.77 (s.d. = 1.87) on the FTND. Table 1 presents the frequencies of carrier status across all SNPs. Generally, there were no carrier status differences in self-reported smoking measures. However, minor allele carriers at rs11637635 (t = 2.06, P = 0.04) and rs17408276 (t = 2.57, P = 0.01) showed lower levels of nicotine dependence as assessed by the FTND. Minor carriers at rs11637635 (t = 2.08, P = 0.04), rs17408276 (t = 2.41, P = 0.02) and rs588765 (t = 2.11, P = 0.04) also reported smoking fewer cpd. In addition, Caucasians were more likely to carry a minor allele at rs17408276 (χ2 (1, N = 65) = 11.29, P = 0.004), rs3829787 (χ2 (1, N = 65) = 16.25, P < 0.001), and rs4275821 (χ2 (1, N = 65) = 12.57, P = 0.002). Minor allele frequencies for each SNP are presented in Table 2.

Predictors of total puff volume
Ethnicity, race, FTND, cpd, number of quit attempts over the past year predicted total puff volume (Ps < 0.05). On average, total puff volumes were lower amongst Caucasians when compared with participants identifying with a different racial background (12 African Americans and 1 native American/Alaskan). Hispanic ethnicity was associated with reduced puff volumes, and FTND was positively associated with total puff volume. Puff volumes for racial and ethnic subgroups are illustrated in Figure 1. Puff volume was not predicted by age, gender, age of first cigarette, age of regular smoking, age of daily smoking, highest number of cpd or cessation confidence. To control for the general effects of race, ethnicity and nicotine dependence on total puff volume, these variables were included as covariates in subsequent analyses. Two participants who did not report on either race or ethnicity were excluded from these analyses (final n = 64). FTND was chosen as a covariate because it is one of the best validated and widely used indices of nicotine dependence. Cigarettes per day and number of quit attempts were not included as covariates, as they partially determine and are highly correlated with FTND.

### Table 1. Results for total puff volume across all SNPs with nicotine content and genotype effects, controlling for cigarette trial and nicotine dependence (FTND)

| SNP          | Minor allele | Frequency (%) | Nicotine | Placebo | Overall | Cigarette Type |
|--------------|--------------|---------------|----------|---------|---------|----------------|
|              |              |               | M (s.e.) | M (s.e.) | M (s.e.) |                |
| rs16969968   | Non carrier  | 59.4          | 467.86   | 21.03   | 531.75  | 21.10          |
|              | Carrier      | 40.6          | 536.36   | 25.38   | 577.42  | 25.71          |
|              | Carrier      | 71.9          | 477.41   | 22.82   | 510.42  | 22.75          |
| rs17408276   | Non carrier  | 31.2          | 498.44   | 24.52   | 546.52  | 25.13          |
|              | Carrier      | 68.8          | 482.38   | 24.16   | 516.04  | 24.08          |
| rs3829787    | Non carrier  | 29.7          | 513.51   | 24.25   | 562.63  | 24.88          |
|              | Carrier      | 70.3          | 464.26   | 24.03   | 498.03  | 23.97          |
| rs4275821    | Non carrier  | 28.1          | 520.99   | 24.37   | 580.53  | 25.07          |
|              | Carrier      | 71.9          | 458.48   | 22.99   | 488.87  | 22.93          |
| rs588765     | Non carrier  | 23.4          | 515.30   | 26.79   | 552.55  | 27.72          |
|              | Carrier      | 76.6          | 475.90   | 22.62   | 514.45  | 22.56          |
| rs637137     | Non carrier  | 65.6          | 497.00   | 22.05   | 525.22  | 22.41          |
|              | Carrier      | 34.4          | 481.42   | 25.08   | 538.46  | 24.66          |
| rs684513     | Non carrier  | 73.4          | 493.85   | 21.45   | 524.59  | 21.75          |
|              | Carrier      | 26.6          | 484.25   | 27.47   | 542.88  | 26.79           |

Abbreviation: M, mean, s.e., standard error; SNP, single-nucleotide polymorphism. Carriers are defined as individuals with at least one copy of the minor allele. Gene, and Gene X Nicotine effects which met traditional significance (Ps < 0.05) are presented in bold.

As depicted in Table 1, a significant nicotine effect (P = 0.006) and a significant genotype by nicotine content interaction was observed for rs16969968 (P = 0.008). Planned comparisons revealed that participants who did not carry a minor allele produced significantly reduced puff volumes when smoking nicotine-containing cigarettes relative to placebo cigarettes (12.01%, P < 0.001; see Figure 2 and Table 1). Total puff volume did not differ by nicotine content amongst carriers (P > 0.05). Ethnicity was a significant predictor in the model (P = 0.018), and both race and FTND trended toward significance (P = 0.081 and 0.086, respectively). To further examine the possibility that the observed interaction effects resulted from combining participants with different racial and ethnic backgrounds, separate analyses were also conducted on racial and ethnic subgroups. The effects observed in the combined sample were also observed in the Caucasian (n = 52) and non-Hispanic (n = 57) subgroups (see Figure 3). Analyses in both groups yielded significant genotype by nicotine interactions (P = 0.017 and P = 0.014, respectively).

**DISCUSSION**

Recent studies have demonstrated the importance of using proximal and objective measures of smoking behavior to clarify...
the relationship between rs16969968, cigarette use, and respiratory diseases such as lung cancer.\textsuperscript{2,19} In keeping with this idea, the proposed study examined smokers’ puff topography as a potential mechanism by which rs16969968 may influence toxicant exposure. However, several variables were associated with puff volume in our sample, most notably race, ethnicity, and nicotine dependence (FTND). Prior studies have generally not observed differences in smoking topography measures across racial groups\textsuperscript{45–47} but see ref. \textsuperscript{48}. Although, race differences might well be expected given that risk alleles for smoking intensity are not equally distributed across racial groups\textsuperscript{49–51} but see ref. \textsuperscript{48}. The present study may have been more sensitive to subtle race effects given that multiple measurements of smoking topography were obtained from each participant and all participants were required to smoke the same cigarette brand. Prior studies also have not observed a relationship between smoking topography and subjective measures of nicotine dependence;\textsuperscript{47,48} However, smoking topography has been shown to predict other smoking phenotypes such as the number of cpd, number of past quit attempts,\textsuperscript{49} and smoking cessation success.\textsuperscript{50,51}

Table 2. Minor allele frequency, puff volume and demographic characteristics by racial and ethnic group

| Minor allele | Race | Ethnicity |
|-------------|------|-----------|
|             | Caucasian | African American | American Indian or Alaskan native | Non-Hispanic | Hispanic |
| N (% of sample) | 52 (78.79) | 12 (18.18) | 1 (1.52) | 58 (87.88) | 7 (10.61) |
| rs16969968 | A (A/G) | 0.25 | 0.08 | 0.50 | 0.22 | 0.21 |
| rs11637635 | A (A/G) | 0.48 | 0.25 | 0.50 | 0.45 | 0.43 |
| rs17408276 | C (C/T) | 0.48 | 0.13 | 0.50 | 0.42 | 0.43 |
| rs3829787 | A (A/G) | 0.48 | 0.08 | 0.50 | 0.42 | 0.43 |
| rs4275821 | C (C/T) | 0.48 | 0.17 | 0.50 | 0.44 | 0.43 |
| rs569207 | A (A/G) | 0.20 | 0.25 | 0.00 | 0.19 | 0.29 |
| rs588765 | T (T/C) | 0.54 | 0.29 | 0.50 | 0.51 | 0.43 |
| rs637137 | A (A/T) | 0.20 | 0.25 | 0.00 | 0.19 | 0.29 |
| rs684513 | G (G/C) | 0.16 | 0.13 | 0.00 | 0.14 | 0.29 |

Demographic

| Age | 39.40 (12.15) | 42.42 (12.34) | 32.00 | 39.67 (11.90) | 38.29 (15.11) |
| CPD | 22.17 (6.35) | 24.42 (8.53) | 25.00 | 22.48 (6.67) | 22.71 (8.56) |
| FTND | 5.48 (1.80) | 6.92 (1.62) | 9.00 | 5.83 (1.83) | 5.29 (2.43) |

Puff volume

| Nicotine | 500.69 (167.46) | 522.61 (106.37) | 638.14 | 516.53 (160.09) | 413.02 (106.19) |
| Placebo | 530.881 (118.70) | 639.96 (124.60) | 590.31 | 555.83 (119.87) | 483.11 (149.06) |
| All | 520.36 (116.66) | 560.59 (126.44) | 614.22 | 538.20 (115.17) | 447.17 (126.57) |

Abbreviations: CPD, cigarettes smoked per day; FTND, Fagerström Test for Nicotine Dependence; MAF, minor allele frequencies. Means are presented for puff volumes and demographic values with s.d. expressed in parentheses.

Figure 1. Mean ± s.e.m. total puff volumes by racial and ethnic subgroup.

Figure 2. Mean ± s.e.m. total puff volumes for nicotine (grey bars) versus placebo (black bars) cigarettes by dichotomized genotype at rs16969968 (*P < 0.05; **P < 0.01; ***P < 0.001).
cigarettes amongst minor allele carriers (A/G or A/A), but were significantly reduced for nicotine-containing relative to placebo cigarettes (12% reduction) amongst noncarriers (G/G). None of the self-report measures of smoking behavior (for example, cpd, age of first cigarette, age of daily smoking initiation) or nicotine dependence (FTND) were significantly predicted by genotype at rs16969968.

The genotype × nicotine interaction observed is consistent with preclinical work; α5 knock-out mice do not reduce self-administration rates in response to increasing nicotine dose concentrations as is observed in wild-type controls. Of course, in order to make a more meaningful comparison with animal models, smokers’ puff topography must be assessed across a wide range of nicotine doses. Until recently, research cigarettes were not readily available for this purpose. A new line of cigarettes (22nd Century Group, Inc. Clarence, NY, USA), now available from the National Institute on Drug Abuse, might be used in future work to readily available for this purpose. A new line of cigarettes (22nd Century Group, Inc. Clarence, NY, USA), now available from the

Figure 3. Mean ± s.e.m. total puff volumes for nicotine (grey bars) versus placebo (black bars) cigarettes by dichotomized genotype at rs16969968 amongst the majority racial and ethnic subsamples (*P < 0.05; **P < 0.01; ***P < 0.001).

Another important consideration is that the α5 subunit plays a role in nicotine self-administration, such mechanisms may serve as targets for the development of α5 modulators.

As a secondary analysis, the present study was limited by a modest sample size. Larger scale replications will be necessary to determine the generalizability of these findings. In addition, biological markers of smoke exposure (for example, expired air carbon monoxide, plasma nicotine concentration) were not collected in the present study, preventing a direct comparison between puff volume and toxicant exposure. Finally, while we present an association between smoking behavior and the noncoding regions of CHRNA5 could shed light on regulatory mechanisms related to α5 subunit expression. To the extent that expression and function of this subunit plays a role in nicotinic self-administration, such mechanisms may serve as targets for the development of allosteric α5 modulators.

In conclusion, we report that a coding SNP in CHRNA5 (rs16969968) is associated with total puff volumes produced when smoking nicotine-containing cigarettes. Specifically, minor-
allele carriers do not appear to reduce the volume of their puffs in response to increased nicotine content as was observed with noncarriers. In contrast to the measure of smoking topography, self-report measures of smoking and nicotine dependence were not significantly associated with rs16969968. Moreover, genotype remained predictive of puff volume even after controlling for nicotine dependence, ethnicity and race. Thus, as a proximal and objective measure of smoking behavior, puff topography measures may serve as an endophenotype for exploring the relationship between genetic variation, smoking and subsequent health consequences. In addition, topography measures may be useful for testing hypotheses developed from preclinical investigations regarding the functional effects of candidate SNPs. As preclinical investigations continue to explore the function of candidate genes identified from genome-wide association studies, human experimental investigations of gene x drug/environment interactions will become increasingly necessary to develop and assess novel treatments for tobacco dependence.36

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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