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ORIGINAL ARTICLE

The functional mu opioid receptor (OPRM1) Asn40Asp variant predicts short-term response to nicotine replacement therapy in a clinical trial

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ABSTRACT
To determine whether the functional mu-opioid receptor (OPRM1) Asn40Asp variant predicts the comparative efficacy of different forms of NRT, we conducted a clinical trial of transdermal nicotine (TN) *vs* nicotine nasal spray (NS) in 320 smokers of European ancestry. Smokers carrying the OPRM1 Asp40 variant ($n=82$) were significantly more likely than those homozygous for the Asn40 variant ($n=238$) to be abstinent at the end of treatment, and reported less mood disturbance and weight gain. The genotype effect on treatment outcome was most pronounced among smokers receiving TN, particularly during the 21 mg dose phase. Smokers who carry the OPRM1 Asp40 variant are likely to have a favorable response to TN and may benefit from extended therapy with the 21 mg dose.
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INTRODUCTION

Nicotine replacement therapies (NRTs), including transdermal nicotine, nicotine gum, and nicotine nasal spray, are effective treatments for tobacco dependence.[1] However, there is substantial variability in treatment response and up to 95% of smokers utilizing NRT relapse to their former smoking patterns.[2,3] In the absence of empirical data to identify the likely responders to different forms of NRT,[1] smokers and their practitioners must resort to a one-size-fits-all model of tobacco dependence treatment.

To suggest factors that may be useful to individualize treatment, we examined the role of the functional mu-opioid receptor (OPRM1) gene in response to alternate forms of NRT. The mu-opioid receptor is the primary site of action for the rewarding effects of the endogenous opioid peptide beta-endorphin,[4] which is released following acute and short-term nicotine administration.[5,6] Exon 1 of the human OPRM1 gene includes a common Asn40Asp (A118G) mis-sense single-nucleotide polymorphism (SNP). The Asp40 variant increases the binding affinity of beta-endorphin for this receptor by three-fold, relative to the wild-type Asn40 OPRM1.[7] The Asp40 variant in OPRM1 is found in about 25–30% of individuals of European ancestry,[8,9] and is therefore sufficiently common to explain the clinically significant differences in response to different forms of NRT.

The present study examined whether genetic variation in OPRM1 predicts the comparative efficacy of transdermal nicotine (TN) *vs* nicotine nasal spray (NS) in

a randomized clinical trial of 320 smokers of European ancestry. These two alternate forms of NRT were selected based on their different pharmacokinetic properties. Specifically, TN provides gradual and stable delivery of nicotine, resulting in higher levels of overall replacement.[10–12] By comparison, smokers deliver NS intermittently, resulting in lower and more variable venous nicotine levels.[11,13,14] We predicted that smokers with at least one copy of the OPRM1 Asp40 variant would benefit more than those with the wild-type genotype (Asn40 homozygotes) from nicotine delivery via TN, based on the following logic. Nicotine increases release of beta-endorphin,[5,6] which would provide greater hedonic value for those with the Asp40 allele, due to the potential for greater mu receptor occupancy by beta-endorphin.[7] This effect should be stronger for smokers treated with TN compared with NS, due to higher and more stable levels of nicotine replacement. A corollary hypothesis was that the OPRM1 Asp40 variant would not protect individuals from relapse after cessation of NRT.

Our hypotheses follow from a recent observation that the OPRM1 Asn40Asp variant may influence therapeutic response to naltrexone therapy for alcohol dependence.[15] In that study, the Asp40 variant was associated with a decreased risk for relapse to heavy drinking during 12-week placebo-controlled trials of naltrexone therapy among alcohol-dependent individuals.

RESULTS

In all, 56% of participants were female, 53% were college graduates, and the average age was 46 (SD = 11.4) years. On average, participants smoked 24 cigarettes/day (SD = 9.4) and had baseline cotinine levels of 243.3 ng/ml (SD = 115.9). A total of 238 (74.4%) participants were homozygous for the Asn40 variant (Asn40/Asn40), 77 (24.0%) were heterozygous (Asn40/Asp40), and five (1.6%) were homozygous for the Asp40 variant (Asp40/Asp40). Thus, the prevalence of the two common genotypes was 238 (74%) for Asn40 homozygotes and 82 (26%) for carriers of the Asp40 variant (Asn40/Asp40 or Asp40/Asp40). In all, 28% of participants in the TN group had at least one copy of the Asp variant, compared with 24% in the NS group ($P = 0.44$). There were no significant differences in baseline variables by OPRM1 genotype or treatment group. In total, 95% of participants completed the 6-month follow-up assessment; participants retained did not differ from those lost in terms of group assignment or baseline characteristics (all P's > 0.10) (Figure 1).

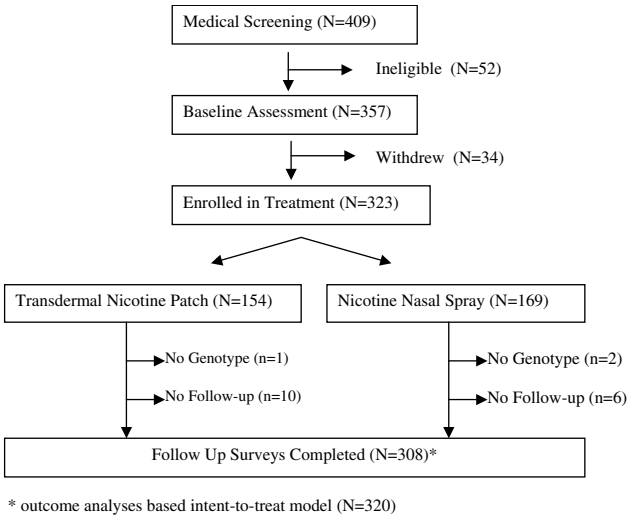

Figure 1 Flow diagram of trial participation.

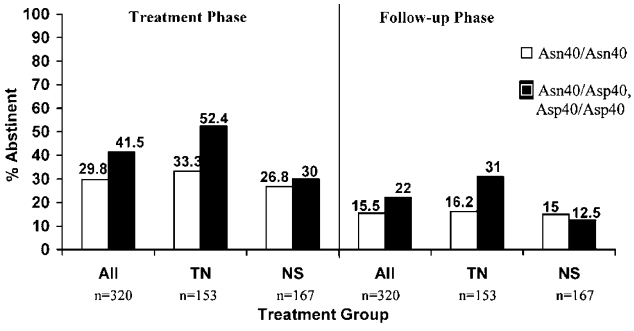

Figure 2 Effect of OPRM1 Asn40Asp variant on abstinence by treatment. TN = transdermal nicotine, NS = nasal spray. Effects of genotype on abstinence are significant overall and for the TN group during the treatment phase only.

Treatment Outcome

As shown in Figure 2, at the end of the NRT treatment phase, 41% of carriers of the Asp40 variant were abstinent at the end of the treatment phase compared with 30% of Asn40 homozygotes. There was a large effect of genotype on abstinence in the TN group (52 *vs* 33%), but not in the NS group (30 *vs* 27%). By contrast, significant effects of genotype on abstinence did not persist after treatment was discontinued (Figure 2). Overall abstinence rates were comparable to those achieved in previous studies.[16–18]

The logistic regression models for the treatment and follow-up phase are shown in Table 1. Smokers carrying the OPRM1 Asp40 variant were significantly more likely to be abstinent at the end of the treatment phase (OR = 1.79, 1.05–3.06, $P = 0.03$). The genotype effect on abstinence was significant for TN treatment (OR = 2.4, 1.14–5.06, $P = 0.02$) and nonsignificant for NS treatment (OR = 1.28, 0.58–2.84). The effects of genotype on abstinence after treatment was discontinued (6-month follow-up) were not significant. Consistent with previous research,[19] females were significantly less likely than males to be abstinent at both time points. The model was unchanged when a term for study site was included. Two-way interactions between sex, genotype, and treatment, and the three-way interaction, were tested and not statistically significant in either model.

Although the present study did not include a placebo control group, we were able to conduct a *post hoc* analysis to

Table 1 Logistic regression models of abstinence during the treatment and follow-up phases

Variable	*Levels*	*Treatment phase*		*Follow-up phase OR (CI)*	
		OR (CI)	P	*OR (CI)*	P
Sex	Male = 0 Female = 1	0.53 (0.33–0.87)	0.01	0.52 (0.28–0.97)	0.04
Treatment	TN = 0 NS = 1	0.66 (0.41–1.06)	0.09	0.72 (0.40–1.30)	0.28
Baseline cotinine	Continuous	0.99 (0.99–1.00)	0.40	0.99 (1.00–1.01)	0.55
OPRM1	Asn/Asn = 0 Asn/Asp, Asp/Asp = 1	1.79 (1.05–3.06)	0.03	1.63 (0.86–3.09)	0.14

test for an effect of the OPRM1 Asn40Asp variant on abstinence among 190 smokers enrolled in a placebo-controlled bupropion smoking cessation trial conducted by our study team. As described in more detail elsewhere,[20] the ascertainment, screening, and treatment procedures were the same as those in the present trial, with the exception of the pharmacotherapy. In this group of placebo-treated smokers, there was no significant effect of OPRM1 genotype (OR = 0.75, CI = 0.33–1.72, $P = 0.50$), and smokers with the Asp40 OPRM1 genotype were *less* likely to be abstinent than those with the Asn40 variant (13 *vs* 21%, respectively). We tested the OPRM1 gene by treatment group interactions using design variables to code for the interaction effects in a logistic regression, with the placebo group as the base category. The effect of OPRM1 genotype on abstinence in the TN and placebo group was significantly different ($OR_{interaction} = 3.18$, CI = 1.05–9.63, $P = 0.04$). However, the difference in genotype effects in the TN and NS groups was not ($P = 0.40$).

Post-hoc Analysis of Lapse and Recovery Events

In order to understand the origin of the genotype effect on relapse at the end of treatment in the TN group, we examined the series of transition events that accounted for the differential treatment outcomes. For those currently smoke-free, time-to-lapse events did not differ significantly by OPRM1 genotype during the first phase of treatment (0–27 days) (HR = 1.21, log-rank $\chi^2(1) = 1.84$, $P = 0.18$), or during the second phase of treatment (HR = 0.97, log-rank $\chi^2(1) = 0.04$, $P = 0.85$). However, for those who had lapsed (ie, smoked at least a puff of a cigarette), recovery events occurred more quickly for smokers with the Asp40 variant compared to those homozygous for the Asn40 variant during the first phase of 21 mg treatment (HR = 1.52, log-rank $\chi^2(1) = 10.70$, $P = 0.0011$) (Figure 3). There was no significant genotype effect on recovery during the 14 and 7 mg TN treatment phase (HR = 1.17, log-rank $\chi^2(1) = 0.77$, $P = 0.38$), and no effects of genotype on lapse or recovery events in the NS treatment condition.

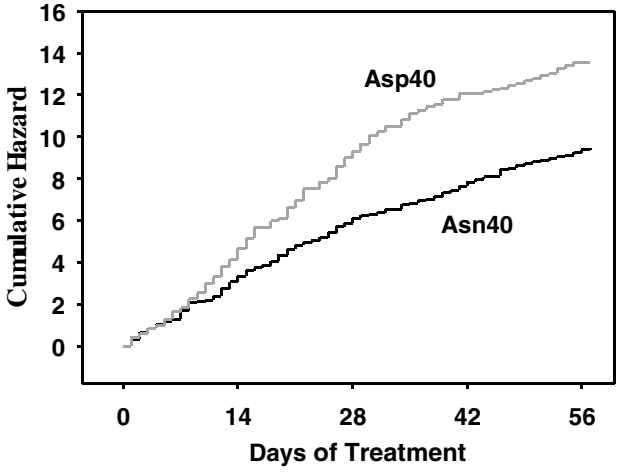

Figure 3 Cumulative hazard for recovery events in the TN group, by OPRM1 genotype. The cumulative hazard represents the summation of the per-capita rate of transition from smoking to smoke-free. It is interpreted like a survival curve, so that divergence indicates an effect, while parallel changes indicate no effect. In the TN group, the cumulative hazard is significantly different by genotype during the first 28 days of 21 mg treatment.

Abstinence-induced Weight Gain

In a linear regression model of weight gain from baseline to the end of treatment among participants who were abstinent at the end of treatment ($n = 105$), OPRM1 genotype was a significant predictor of weight gain (beta = −3.86, $P < 0.05$), controlling for baseline cotinine, baseline body mass index, sex, and treatment group. Smokers with at least one copy of the Asp40 variant gained about one-third as much weight as smokers homozygous for the Asn40 variant during the treatment phase (1.7 *vs* 5.3 lbs, respectively) (Figure 4). There was no effect of genotype on weight gain from the end of treatment to the 6-month follow-up (0.9 *vs* 0.7 lbs for Asp40 carriers *vs* Asn40

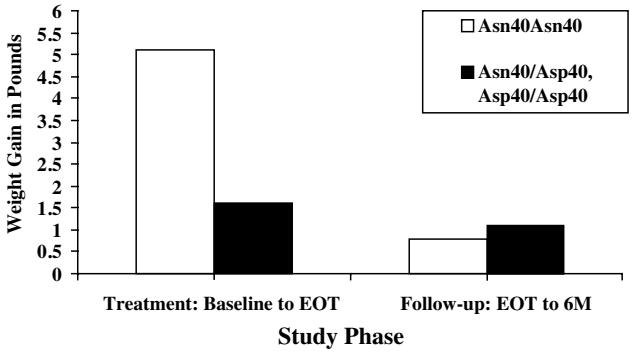

Figure 4 Changes in weight gain from baseline to end of treatment (EOT) and EOT to 6-month follow-up by OPRM1 genotype. EOT = end of treatment, 6M = 6-month follow-up. The effect of OPMR1 genotype is significant only for the treatment phase, and not for the follow-up phase or total study period.

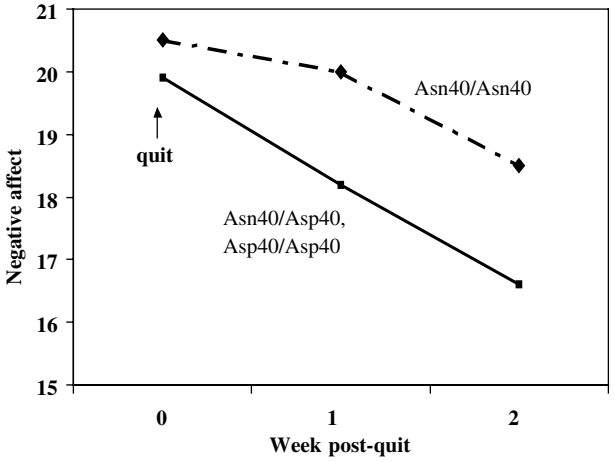

Figure 5 Changes in negative mood symptoms during the first 2 weeks of abstinence.

homozygotes respectively, $P = 0.29$), or total weight change from baseline to 6 months ($P = 0.58$).

Abstinence-induced Mood Disturbance

Effects of genotype on abstinence-related mood symptoms focused on the first 2 weeks of abstinence, and included participants who had confirmed abstinence at this time-point ($n = 216$). In linear regression models, there was a significant effect of OPRM1 genotype on change in negative mood symptoms during this period (beta = −2.06, $P = 0.02$), controlling for sex and treatment group. As shown in Figure 5, smokers with the Asp40 variant reported a significant decline in negative mood symptoms during the first 2 weeks of abstinence. Further, reductions in negative affect during this period strongly predicted abstinence in a logistic regression model at the end of treatment, controlling for baseline cotinine, sex, and treatment (OR = 0.92, CI = 0.89–0.96, $P < 0.001$).

Treatment Use and Percent Cotinine Replacement

During the first 2 weeks of treatment, the average number of daily doses of NS was 9.5 (SD = 1.0), and the average number of days per week of TN usage was 6.5 (SD = 1.3). There were no significant differences in compliance by genotype (all P's > 0.10). At baseline, the average cotinine levels were 234 ng/ml (SD = 104) for the TN group and 251 ng/ml (SD = 125) for the NS group ($P = 0.17$). After 1 week of treatment, average cotinine levels (among participants with CO-verified abstinence and cotinine samples, $n = 228$) were 206 ng/ml (SD = 92) for TN and 94 ng/ml (SD = 94) for NS participants ($t = 9.0$, $P < 0.001$). Average percent cotinine replacement differed in the TN and NS groups ($t = 2.8$, $P = 0.006$). Specifically, among participants with CO-verified abstinence at session 4 who received TN, percent replacement was 116% (SD = 106; median = 95%, range = 1.5–723%), compared with 50% for those receiving NS (SD = 71; median = 27%, range = 2.1–428%). There were no differences in percent cotinine replacement or in post-treatment nicotine or cotinine levels by genotype (all P's > 0.10).

Haplotype Analysis

The four OPRM1 SNPs exhibited high levels of linkage disequilibrium ($D' = 0.88$–0.99). Haplotypes were estimated based on three of the four markers (5′UTR, A118G (Asn40Asp), and Exon X), and the results revealed four unique haplotypes with frequencies >10%. Haplotype frequencies for abstainers and relapsers were not significantly different. This finding supports the hypothesis that the A118G SNP is the only functional variant for this phenotype, because there is extensive LD across the gene.

Analysis of Population Stratification

As with all association studies, confounding due to population stratification is a concern. Population stratification occurs when both of two conditions are met.[21] First, allele frequencies under investigation must vary among subpopulations. Second, disease prevalence or mean phenotypic value must vary among subpopulations. If researchers are unaware of the subpopulations, subjects can be unknowingly selected from differing subpopulations and a spurious association between genotype and phenotype can be created. To determine if unrecognized subpopulations existed within the study, 41 random markers measured upon participants of the study were utilized within a statistical test developed by Pritchard and Rosenberg.[22] The hypothesis test failed to reject the null hypothesis of one homogeneous study population ($\chi^2 = 40.58$, df = 41, $P = 0.49$).

DISCUSSION

This clinical trial provides the first evidence that genetic variation in the mu-opioid receptor influences response to NRT. Consistent with a pharmacogenetic hypothesis, smo-

kers carrying the less common OPRM1 Asp40 variant were significantly more likely than those homozygous for the wild-type Asn40 variant to be abstinent at the end of the nicotine replacement phase. A subgroup analysis revealed that this effect was only significant in the TN group, with 52% of smokers with at least one copy of the Asp40 variant abstinent at the end of treatment, compared with 33% of those homozygous for the Asn40 variant. Further, a more fine-grained analysis of recurrent lapse and recovery events during TN treatment indicated that smokers with at least one copy of the Asp40 variant exhibited a significantly higher rate of recovery from short smoking lapses (ie slips) than those homozygous for the Asn40 variant, particularly during the 21 mg TN treatment phase. Consistent with an enhanced response to NRT among Asp40 carriers, these smokers had attenuated weight gain and fewer symptoms of mood disturbance. The magnitude of differences in NRT outcomes between these subgroups defined by OPRM1 genotype is also clinically significant, in that it is comparable to the differences in abstinence rates[17,23] weight gain[24] and abstinence symptoms[25] achieved when comparing active pharmacotherapies with placebo. Consistent with previous research,[19] males had a more favorable response to NRT (independent of treatment type) than females.

While a few recent studies have reported evidence for the effects of inherited genetic variation on smoking cessation and response to tobacco dependence treatment,[26–28] the present study is the first to examine the role of the endogenous opioid system. There is a substantial body of evidence from preclinical animal and human studies implicating the endogenous opioid system, and the mu-opioid receptor in particular, in the reinforcing effects of nicotine administration. For example, acute and short-term repeated exposure to nicotine increases release of enkephalins in the rat brain[29,30] and release of beta-endorphin in fetal hypothalamic cells.[5] Once released, these peptides bind to mu opioid receptors, producing direct reinforcing effects of nicotine, as well as indirect effects via dopamine release.[6] Although the effects of nicotine on the opioid system in humans are more equivocal,[31,32] nicotine administration via cigarette smoking has been shown to produce dose-related increases in plasma beta-endorphin.[33]

Based on previous data and the results of the present study, possible mechanisms for the effect of OPRM1 genetic variation on response to NRT can be considered. Nicotine increases central level of beta-endorphin,[6] and the Asp40 OPRM1 variant has a three-fold greater binding affinity of beta-endorphin by this receptor.[7] This may enhance the hedonic effects of nicotine treatment and decrease aversive abstinence effects in smokers who carry the Asp40 variant. Assuming that the OPRM1 genotype effect on response to NRT is mediated by increased beta-endorphin occupancy at the mu receptor, the higher levels of nicotine delivered by TN should have greater beneficial effects for this group, compared with the lower levels of nicotine from NS. In other words, the Asp40 variant may functionally boost the effect of nicotine from TN to a more clinically effective threshold. Our data showing that smokers with at least one copy of the Asp40 variant reported significantly less negative mood symptoms during the first 2 weeks of abstinence support the hypothesis of different hedonic effects of NRT in the two genotype groups. These effects may result from direct stimulation of the mu receptor by beta-endorphin, or from effects of beta-endorphin on dopamine release.[34]

Our data also provide evidence that smokers with at least one copy of the Asp40 variant gain significantly less weight during NRT treatment than those homozygous for the Asn40 variant. Consistent with a pharmacogenetic hypothesis, these differences were no longer present at follow-up. This result may also be explained by increased beta-endorphin occupancy at the mu opioid receptor during NRT treatment among smokers with the Asp40 variant. Male knockout mice deficient in beta-endorphin exhibit hyperfagia, as well as increased body weight,[35,36] suggesting that increased central levels of beta-endorphin released by nicotine may actually suppress feeding. Other data support an association between central beta-endorphin and novelty-induced locomotion,[37] suggesting that effects of beta-endorphin on body weight may be mediated, in part, by increased physical activity.

While additional validation of the present findings in future clinical trials is warranted, this line of research may ultimately have an impact on treatment decision-making for tobacco-dependent smokers. For example, based on the protective effect of TN among smokers with at least one copy of the OPRM1 Asp40 variant, the higher rate of recovery from lapses during the 21 mg dose phase, and the absence of a genotype effect after medication was discontinued, we speculate that these smokers would benefit from extended 21 mg TN therapy. Although previous studies have not supported a sustained benefit of higher dose (44 mg) TN[38] or of longer duration of treatment,[39,40] subgroups of smokers defined by OPRM1 genotype may benefit from a longer 21 mg dose phase, or even maintenance TN therapy as an alternative to cigarette smoking. Although maintenance therapy for other addictions remains controversial, and poses both ethical and practical concerns,[41] the reduction in harm associated with long-term NRT *vs* continued smoking supports the need for continued investigation in this area.

Our study has some limitations. First, we used an open-label design to provide a direct comparison of alternate NRTs with different delivery profiles. Although this design more closely simulates the usual clinical regimen (compared to a double placebo approach), it limits our ability to draw conclusions regarding the absolute efficacy of TN and NS in smokers with different OPRM1 genotypes. However, data from a separate double-blind clinical trial of bupropion[25] did not reveal an OPRM1 effect on abstinence among placebo-treated subjects (21% of the Asn40 homozygotes *vs* 13% of carriers of the Asp40 variant were abstinent) and the magnitude of effects in the TN *vs* placebo groups was significantly different. A second limitation of the study is that it was not possible to examine different minority groups, given the very low frequency of the Asp40 variant in

same groups (eg 2–9% of African Americans[8]). One could speculate that the lower prevalence of the Asp40 allele in African Americans relative to persons of European or Chinese ancestry might translate into differences in the efficacy of NRT in these ethnic groups. However, very little is known about the efficacy of NRT in minority groups,[42,43] and treatment outcomes would also be influenced by ethnic variation in nicotine metabolizing enzymes.[44] Third, since the prevalence of OPRM1 Asp40 homozygotes is very low, it was not possible to examine this group separately. Fourth, it should be noted that smokers who volunteer for smoking cessation research programs may not be representative of smokers in the general population; however, they would be comparable to treatment-seeking smokers, the target population for future clinical integration of pharmacogenetics.

Despite these limitations, the present study provides a first step toward identifying genetic subgroups of smokers who may achieve greater benefit from NRT. Larger clinical trials are needed to validate the present findings, and to explore whether smokers who carry the OPRM1 Asp40 variant benefit from extended or maintenance treatment with TN or other NRTs. The results of these investigations could potentially enable practitioners to select the optimal type, dose, and length of NRT for individual smokers. Given the substantial tobacco-related morbidity and mortality in the US and abroad, the clinical and public health implications of such research could be significant.

MATERIALS AND METHODS

Smokers responding to local media advertisements for free smoking cessation treatment and to physician referrals were screened for eligibility and recruited in Washington DC and Philadelphia, Pennsylvania from February 2000 through April 2003. Eligible individuals were current cigarette smokers of European ancestry who were ages $\geq$18 years and smoked at least 10 cigarettes per day for the prior 12 months. Exclusion criteria included: pregnancy or lactation, uncontrolled hypertension, unstable angina, heart attack or stroke within the past 6 months, current treatment or recent diagnosis of cancer, drug or alcohol dependence, current diagnosis or history of a DSM-IV psychiatric disorder, and current use of bupropion or nicotine-containing products other than cigarettes.

Of the 357 smokers who met the above eligibility criteria, 323 provided written informed consent and attended the first treatment session (90.5%). These participants were included in the intent-to-treat analysis. Genotyping could not be performed on three of the 323 consenting participants (0.6%), and these participants are excluded from all analyses. Thus, the final sample included 320 smokers: 153 were randomized to receive TN and 167 were randomized to receive nicotine NS. A flow diagram of trial participation is shown in Figure 1.

Study Design and Treatment Procedures

The trial was an open-label randomized clinical trial of TN *vs* NS for smoking cessation, using a block randomization scheme generated by a senior data manager. An open-label trial design was selected for the following reasons: (a) the comparable efficacy of these NRTs relative to placebo has been established;[1,18,45–47] (b) the use of a double placebo design would increase participant burden and potentially reduce compliance and retention, thereby biasing the study outcomes; and (c) an open-label trial more closely simulates the usual clinical regimen.

The University of Pennsylvania and Georgetown University Institutional Review Boards approved all research procedures, and all participants provided written informed consent. Lifetime prevalence of psychosis, major depression, bipolar disorder, and substance abuse was determined using the SCID-NP (non-patient) for DSM-IV. All eligible participants provided a blood sample for genotyping and cotinine analysis.

Nicotine nasal spray (NS; Nicotrol®; Pharmacia, Helsingborg, Sweden) was initiated on the target quite date (TQD) (week 3) and provided to participants over an 8-week period. At the second counseling session (week 2), participants were shown how to deliver a 1.0 mg dose (0.5 mg spray in each nostril), and instructed to use NS 8–40 times per day (with a maximum of 5 doses per hour) beginning on the TQD. After 4 weeks of use, participants were instructed to taper their NS dose by one-third for a 2-week period, and then by another third for the final 2 weeks of treatment.

TN (Nicoderm® CQ; Glaxo Smith Kline, Research Triangle Park, NC, USA) was used by participants over an 8-week treatment period, beginning with the morning of the TQD (week 3). A 24-h tapered-dose formulation was used: 4 weeks of 21 mg, 2 weeks of 14 mg, and 2 weeks of 7 mg. All participants received seven sessions of standardized behavioral group counseling over an 11-week period, including instruction in the management of smoking triggers, relapse prevention, and stress management techniques.[25]

To assess smoking status, telephone interviews were conducted at the end of treatment (EOT, 8 weeks following the TQD) and at 6-month follow-up (6 months following the TQD) using a standard timeline follow-back method.[48] Participants who reported complete abstinence (not even a puff of a cigarette) for at least the 7 days prior to the assessment were asked to complete an in-person visit for biochemical verification of abstinence.

Outcome Variables

Smoking status

Sustained abstinence, at the end of the treatment phase (EOT) and 6-month follow-up, was the primary outcome measure.[49] Relapse was defined as seven consecutive days of smoking at any time during the follow-up period. This is the definition recommended by the National Heart Lung and Blood Institute, and is used commonly in smoking cessation trials.[50] Self-reported abstinence was confirmed weekly during treatment and at each endpoint by a carbon monoxide (CO) reading $<$10 ppm.[51] Participants who discontinued participation or were otherwise lost (5% at EOT and at 6-month follow-up) were presumed to have relapsed to smoking and coded as such in outcome analyses (ie intent to treat analysis).[51]

Daily smoking status

Time-line follow-back methods were used to determine the daily smoking status (smoked on a given day (=1) or not (=0)), and times of transition events such as smoking lapses (smoking at least a puff of a cigarette) (0 to 1) and recoveries from lapses (resuming abstinence after a lapse, rather than experiencing a full-blown 7-day relapse) (1 to 0). Cohorts were constructed of those currently at risk for a particular transition; that is, those currently smoking and those currently smoke-free. Representation of the population at risk for recurrent event analysis followed Hosmer & Lemeshow's[52] counting process representation.

Weight

Weight was measured at baseline using a balance beam scale that was calibrated daily. Weights at the end of treatment and 6-month follow-up were based on adjusted self-reported weight. Self-reported weight and measured weight at baseline were highly correlated ($r=0.97$, $P<0.00001$), suggesting that any self-report bias would be minimal.

Treatment Variables

Compliance

Participants recorded their daily use of NRT. Those assigned to TN recorded their daily application of patches, and those assigned to NS recorded the number of doses per day. Since use of NRT may be confounded with smoking status (ie participants may discontinue NRT if they relapse), we focused on the average use of TN or NS during the first 2 weeks of treatment.

Cotinine replacement

Percent cotinine replacement from treatment was calculated by dividing participants' plasma cotinine levels following 1 week of treatment by their pretreatment plasma cotinine levels ($n=274$).[53] These time points were selected to coincide with a period of regular smoking and after steady-state cotinine levels from treatment were achieved.[53] Cotinine levels were analyzed by gas chromatography with nitrogen phosphorus detection, modified for analysis using a capillary GC column.[54]

Abstinence-related mood symptoms

The positive and negative affect scale (PANAS) was administered during the treatment phase, with a 7-day timeframe, to assess abstinence-induced changes in positive (eg enthusiastic, strong) and negative mood (eg distressed, upset) symptoms.[55] Changes in abstinence-related mood symptoms from week 3 (quit date) to week 5 (two weeks post-quit) were calculated.

OPRM1 genotype

The primary genotype of interest for this pharmacogenetic analysis was the functional OPRM1 Asn40Asp variant. As reported previously,[15] participants were classified according to the presence or absence of the Asp40 variant (Asn40/Asp40 or Asp40/Asp40 *vs* Asn40/Asn40). Genotyping for the variant was completed using the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR was performed using 2.25 ng of DNA, 2.5 μl of ABI Taqman® Universal Mastermix (Foster City, CA, USA), 0.125 μl of water, and 0.125 μl of 40 × Assay by Design SNP Assay for the OPRM1 Asn40Asp variant listed as rs1799971 in Table 2 (ABI, Foster City, CA, USA). The 5 μl reactions were performed in a 384-well plate (ABI, Foster City, CA, USA). Thermal cycling was completed in an MJ Research Tetrad (MJ Research, Waltham, MA, USA) using the following conditions: (1) 2 min hold at 50°C to activate the AmpErase® Uracil N-glycosylase (UNG), an enzyme in the Universal Mastermix used to prevent PCR contamination, (2) 10 min denaturation step at 95°C, (3) 50 cycles of 95°C for 15 s and 60°C for 1 min, and (4) 4°C hold. The plates were scanned utilizing the Allelic Discrimination End-Point Analysis on the ABI Prism® 7900HT Sequence Detection System. The Allelic Discrimination data were analyzed by the AutoCalling algorithm of the SDS v2.1 Software (ABI, Foster City, CA, USA). The OPRM1 Asn40Asp assay was validated using control DNAs from the Coriell Institute for Medical Research. The controls were 40 samples from the Caucasian Human Variation Panel of 50 (HD50CAU), 40 samples from the African American Human Variation Panel (HD50AA), and 15 samples from the CEPH/UTAH Pedigree 1333. The Human Variation Panel samples yielded high-quality PCR amplification and genotypes could be deter-

Table 2 OPRM1 SNP's selected for analysis

SNP	*Polymorphism*	*Position on Chr 6*	*Primer sequences*	*Probe sequences*
rs1799971	A/G	154315928	CGGTTCCTGGGTCAACTTGT GTTCGGACCGCATGGGT	ATGGCAACCTGTCC ATGGCGACCTGTC
rs510769	A/G	154317150	TCTTCTTTAACAAAAACAGATATATGGCATTTCAC GATATTGATTGTGTTGGTGTTGATGTGT	ATTCACATGTAATATTTG ATTCACATGTAGTATTTG
rs540825	A/T	154369577	GGTCCAGGGTACACAACCAA ACCTATACCTTCCCTGTCTTGCT	TCTAGAGCAAGGCTGC TCTAGAGCATGGCTGC
rs13203041	T/C	154394311	GTAAGAAAAGAGGACTGTGGAAACAGA CAAACATCTCCTTTTCTCCTTAGCAGTA	ATGATAGCAACAATTATT ATAGCAGCAATTATT

mined with a high degree of certainty for all 95 samples. The CEPH family genotypes were analyzed using PedCheck (http://watson.hgen.pitt.edu/register/soft_doc.html) and no Mendelian inheritance errors were found. Using the same protocols, we genotyped three other SNPs in OPRM1 (rs510769 in the 5′ intron region, rs540825 in the Exon X region (Genbank Accession number AY036622, human MOR-1R), and rs13203041 in the 3′ intron region) (Table 2).

Demographic factors

Gender, education, marital status, age, and ethnic ancestry of grandparents were assessed by self-report during the pretreatment assessment visit.

Statistical Analysis

T-tests and χ^2 tests of association were used to identify factors associated with smoking outcome. All statistical tests were two-sided. Variables with significant ($P<0.10$) associations with smoking status, and those of *a priori* significance (eg treatment group, pretreatment cotinine levels) were entered into logistic regression models. Consistent with a pharmacogenetic hypothesis, separate models of sustained abstinence were generated for the treatment phase and the post-treatment follow-up phase, because the pharmacotherapy was only present for the treatment phase. The main effects of OPRM1 genotype and covariates were tested in a first step, and interaction terms were included in a second step. A similar approach was used for linear regression models for continuous mid-treatment measures of mood symptoms and weight change. Analyses examining the effect of genotype were conducted on time-to-transition data, by applying log-rank tests to either the smoking or smoke-free cohorts. These tests were applied separately for days 0–27 (the 21 mg phase of TN) and days 27–56 (the remainder of the treatment phase).

As a secondary analysis, we calculated the extent of linkage disequilibrium among all the four OPRM1 SNPs using 2LD. (2LD is a DOS executable disequilibrium calculator, written by JH Zhao, j.zhao@iop.bpmf.ac.uk, based on the estimation maximization (EM) algorithm described by Long and colleagues.)[56] Haplotypes were generated and those occurring at a frequency $\geq 10\%$ were evaluated for association with smoking outcomes using an EM algorithm-based estimation of haplotype frequencies in conjunction with a log-linear model of haplotype and outcome frequencies.[57] In addition, to determine whether unrecognized subpopulations exist within the study population (ie population stratification), 41 random markers were genotyped and tested using the model developed by Pritchard and Rosenberg.[22]

DUALITY OF INTEREST

None declared

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