Original investigation

Response to Transdermal Selegiline Smoking Cessation Therapy and Markers in the 15q24 Chromosomal Region

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Abstract

Introduction: Current treatments for smoking cessation have limited efficacy. A potential pharmaceutical treatment for smoking cessation is selegiline, a selective and irreversible monoamine oxidase B inhibitor. A few clinical trials have been carried out using selegiline but the results have been mixed. We sought to determine if genetic markers in cholinergic loci in the 15q24 chromosomal region predict response to smoking cessation therapy with selegiline.

Methods: We performed an 8-week double-blind, placebo-controlled clinical trial of the selegiline transdermal system in heavy smokers, with follow-up at weeks 25 and 52. Eight single nucleotide polymorphisms (SNPs) in the 15q24 region, which contains the genes for the nicotinic acetylcholine receptor subunits CHRNA5, CHRNB4, and CHRNA3, were investigated for association with treatment response.

Results: The CHRNA5 promoter SNP rs3813567 was associated with both point prevalence abstinence and post-quit craving. Carriers of the minor C allele treated with selegiline showed lower rates of abstinence and higher levels of craving than selegiline-treated non-carriers, indicating that the rs3813567 C allele adversely affects abstinence in selegiline-treated smokers. This effect was not present among placebo-treated smokers. Selegiline-treated smokers with the CHRNA5 rs680244 GG genotype had lower post-quit craving, and unlike placebo-treated GG-carrying smokers, did not experience a post-quit increase in depressive symptoms.

Conclusions: Variants in genes encoding cholinergic receptors affect abstinence, craving and mood in selegiline-treated smokers. Selegiline primarily affects dopamine levels in the brain, but cholinergic input affects nicotine-induced dopaminergic activity. These markers may have value in identifying those likely to respond to selegiline for smoking cessation.

Introduction

Pharmacologic treatments for smoking cessation, which include nicotine replacement therapy (NRT), bupropion, and varenicline, are only partially efficacious. Even in combination therapy, relapse rates

often exceed 50%.¹ There is need for more effective smoking cessation therapies as the Centers for Disease Control and Prevention reported that 19.3% of adults over the age of 18 were smokers in 2010,² which leads to hundreds of thousands of deaths due to lung cancer, heart disease, and chronic obstructive pulmonary disease.³

A potential aid to smoking cessation is the drug selegiline, a selective and irreversible inhibitor of monoamine oxidase.⁴ Chronic treatment with selegiline results in increased dopamine release in animals.⁵ Selegiline might help with smoking cessation because nicotine activates the brain dopaminergic system in humans, which is key in the reinforcing effects of smoking.⁶

Only a few clinical trials of selegiline for smoking cessation have been carried out so far. One trial reported a three-fold increase in point prevalence abstinence (PPA) at week 8, the study end point, but the study included only 40 smokers.⁷ A placebo counterbalanced trial of 15 smokers reported that participants showed decreased craving, especially during abstinence, and reduced smoking quantity.⁸ A randomized double-blind placebo-controlled trial of oral selegiline plus NRT in 109 smokers reported an association between selegiline and reduced craving.⁹ In contrast, another study of comparable design and similar size showed no effect of oral selegiline on craving or abstinence.¹⁰ We recently performed the first trial of transdermal selegiline for smoking cessation.¹¹ The transdermal system is Food and Drug Administration (FDA) approved for the treatment of depression. In our clinical trial, overall transdermal selegiline was no better than placebo for smoking cessation. A subsequent study of transdermal selegiline for smoking cessation also yielded no positive results.¹² Despite the apparent lack of efficacy for selegiline overall, we hypothesized that there could be variation in response to selegiline that is due to genetic factors.

The 15q24 chromosomal region (Entrez Gene nomenclature) contains the genes encoding the nicotinic cholinergic receptor subunits $\alpha 5$, $\alpha 3$, and $\beta 4$ that are important in nicotine addiction and smoking cessation.¹³ Nicotine up-regulates CHRNA5 expression,¹⁴ and CHRNA5 contributes to the $\alpha 4\beta 2\alpha 5$ nicotinic receptor, which is involved in nicotine-stimulated dopamine release in the striatum.¹⁵ The striatum is involved in brain reward pathways and crucial to the development of substance dependence.^{16,17} Also, variants in the CHRNA5-CHRNA3-CHRNA4 gene cluster on chromosome 15 have been implicated in multiple aspects of nicotine addiction and smoking cessation.^{18–26} Although selegiline is thought to primarily affect dopamine metabolism, there is strong evidence for an interaction between the cholinergic and dopaminergic systems in nicotine addiction.^{27–29}

Materials and Methods

Study Design and Subjects

Participants were recruited as part of a clinical trial approved by the Stanford University Panel for the Protection of Human Subjects in Medical Research. A full description of the recruitment and treatment protocols has been published.¹¹ All participants were adult smokers (18–65 years of age) and smoked 10 or more cigarettes a day. Participants were screened for current mental health and other medical issues that would preclude their inclusion in the trial. As part of a double-blind protocol the 243 participants were split into two groups with the males and females being randomized separately to obtain a balance between the genders, as there is evidence that gender may affect some smoking cessation therapies.^{30–32} One half of the participants received 8 weeks of treatment with the selegiline transdermal system at a dose of 6 mg per 24 hr and the other half an identical placebo patch. Cognitive Behavioral Therapy was provided at weekly clinic visits during the acute phase of treatment. Patients were assessed at baseline, 24 hr post-quit and then weekly during the 8-week acute phase and at the week 25 and 52 follow-up visits.

Expired-air carbon monoxide was measured at each clinic visit to confirm abstinence. Twelve of the 243 smokers participating in the trial opted not to provide a DNA sample. The self-reported ethnic distribution of the 231 remaining participants with genetic data was 77.1% Caucasian, 9.1% Hispanic, 3.9% Asian, 1.3% Black, 0.4% Other, and 8.2% Mixed Ancestry.

Genotyping and Single Nucleotide Polymorphism Selection

Genomic DNA was extracted from 10 ml of EDTA-treated frozen whole blood using the Gentra PureGene kit (Qiagen). Genotyping was carried out using Taqman real-time polymerase chain reaction assays (Applied Biosystems) under standard conditions. The eight single nucleotide polymorphisms (SNPs) were selected on the basis of either their potential to impact gene function or expression levels, their association with smoking related behaviors in the current literature or their potential for imputing other nearby SNPs in the HapMap CEU population. Further, we have previously demonstrated the association of these markers with baseline smoking and smoking cessation.³³ Genotype and allele frequencies are shown in [Table 1](#). [Supplementary Table S1](#) shows genotype and allele frequencies for the Caucasian cohort. Linkage disequilibrium (LD) was assessed using the standard setting for r^2 in Haploview 4.2.³⁴ [Figure 1](#) shows the LD map for the full cohort, whereas LD for the Caucasian cohort is shown in [Supplementary Figure S1](#).

Population Substructure

To test for population stratification, principal component analysis (PCA) was carried out using data for 68 ancestral informative markers (AIMs) collected from 802 smokers in our combined studies cohort and 494 individuals selected from the HapMap populations that best matched our sample of smokers, which is predominantly self-reported Caucasian but shows evidence of diverse ethnic ancestry. The combined studies cohort consisted of patients from the selegiline study and from two additional smoking cessation clinical trials (MT1 and MT2), which were recruited from the same geographic region using the same recruitment and selection methods as the selegiline cohort.^{33,35,36} The HapMap populations were: ASW (African-Americans from the South West of USA), CEU (UTAH resident of North Western European ancestry), MEX (Mexican-Americans living in Los Angeles), CHD (Chinese living in Metropolitan Denver, Colorado), and TSI (Tuscans, Italy).

Principal components (PCs) were calculated using the AIMs data derived from the combined studies cohort without the HapMap samples. The first three AIM PCs accounted for 29% (PC1 = 14.9%, PC2 = 9.9%, and PC3 = 4%) of the observed variance. When patients in the combined studies cohort were graphed against representative HapMap populations using the first three PCs created from the 68 AIMs, only one individual in the present selegiline study obviously failed to cluster with their self-reported ethnic group, as identified by the HapMap ethnicity cohorts. This individual was reclassified as undetermined.

Demographics and Baseline Clinical Measures

Comparisons of age, ethnicity, gender distribution, years of formal education, marital status, baseline body mass index (BMI), baseline smoking quantity and baseline modified Fagerstrom Questionnaire score^{37,38} were carried out among genotype groups using either a one-way analysis of variance (ANOVA) or Pearson's chi-square test.

Table 1. Position, Allelic Distribution in the Full Selegiline Study Cohort and Role of all Genotyped Single Nucleotide Polymorphisms (SNPs)

rs#	Gene	Position	Major homozygote	Heterozygote	Minor homozygote	Minor allele (major allele)	MAF (Chr. #)	HWP	Role
rs680244	CHRNA5	78871288	59	111	56	A (g)	49.3% (452)	.792	Intron 1
rs2229961	CHRNA5	78880752	223	4	0	A (g)	0.9% (348)	.893	Ile > Val at codon 134 (exon 4)
rs16969968	CHRNA5	78882925	91	101	34	A (g)	37.4% (452)	.494	Asn > Asp at codon 398 (exon 5)
rs578776	CHRNA3	78888400	120	89	14	T (c)	26.2% (446)	.641	3' UTR (exon 6)
rs1051730	CHRNA3	78894339	91	99	32	A (g)	36.7% (444)	.548	Tyr > Tyr at codon 215 (exon 5)
rs8192475	CHRNA3	78911230	209	18	0	A (g)	4.0% (454)	.534	His > Arg at codon 37 (exon 2)
rs12914008	CHRNA4	78923505	213	13	0	A (g)	2.9% (452)	.656	Ile > Thr at codon 91 (exon 2)
rs3813567	CHRNA4	78934551	146	79	4	C (t)	19.0% (458)	.067	Promoter region

Chr. = chromosome; HWP = Hardy-Weinberg *p* value; MAF = minor allele frequency; UTR = untranslated region. Relative positions on chromosome 15 were taken from dbSNP build 131.

Figure 1. Linkage disequilibrium (LD) map of the 15q24 region showing the position of the genotyped single nucleotide polymorphisms. LD is expressed as r^2 values. Map is based on the full sample.

These factors have previously been shown to affect successful smoking cessation.^{35,39} The same analyses were carried out to compare those who provided DNA and those who did not.

Pharmacogenetic Analysis

All analyses were carried out separately for the selegiline and placebo treatment groups. The primary clinical outcome measure for the study was PPA. PPA was defined as a report of nonsmoking (not even a puff) for seven consecutive days prior to contact and an expired-air carbon monoxide level of <10 ppm. Week 25 PPA was the primary end point for the study, although week 8 and week 52 were also considered. Logistic regression was carried out with PPA as the outcome measure and genotype as the predictor. Gender was also included in the model. Craving, withdrawal, and mood were assessed 24 hr post-quit and at each weekly clinic visit during the 8-week acute phase of the study. Craving was measured at baseline and at all clinic phone appointments by averaging the responses to the following two questions: "Have you felt cravings for a cigarette?" and "Have you felt strong urges to smoke?," with participants rating their level of discomfort on an ascending 0–6 scale.⁴⁰ Withdrawal symptoms were rated using the Minnesota Nicotine Withdrawal Scale.⁴¹ Mood was assessed at all clinic visits during treatment with the 20-item Center for Epidemiological Studies Depression Scale (CES-D).⁴² Longitudinal analyses were performed for these measures during the acute phase of the study using a general estimating equation model with the SAS GENMOD routine. Data were included from the following time points: 24 hr post-quit and weeks 1–8. Effects in the model were genotype, treatment week, and genotype by week interaction. In the analysis of CES-D, baseline (week 0) was also included to correct for some moderately significant differences in mean CES-D score among genotype groups observed at baseline.

The percent change in BMI between the beginning of the study and the end of the acute phase (week 8), compliance with the treatment protocol, and adverse events scores were investigated using a one-way ANOVA. Compliance and adverse event scores were calculated as previously described.³³ When the minor allele frequency (MAF) was less than 5%, the minor homozygote was grouped with

the heterozygotes for analysis. Due to the significant differences in MAF between the self-reported ethnic groups, we performed all analyses in both the full cohort and the Caucasian-only cohort.

We also tested for haplotype associations with PPA. In Haploview we defined a custom block of 5 SNPs encompassing a region previously associated with nicotine dependence and poor response to smoking cessation.^{18,43} Then, chi-square tests for associations of haplotypes with PPA were performed using Haploview.

Multiple Testing

A Bonferroni correction for multiple statistical comparisons was used. Two of the SNPs genotyped (rs16969968 and rs1051730) were in near complete LD ($r^2 = 0.98$). The CHRNA5 synonymous SNP rs2229961 was excluded from pharmacogenetic analyses due to low MAF. Therefore, the threshold for significance was set to $p < .0083$ ($=.05/6$).

Results

Pharmacogenetic Analysis

Analysis of PPA showed that at week 8, there was a trend toward increased PPA among selegiline-treated patients without the minor C allele of the CHRNA4 promoter SNP rs3813567 (logistic regression, $p < .036$ in full cohort; in Caucasians $p < .02$), but this result failed to pass the correction for multiple testing. However, at week 25 in the full cohort rs3813567 significantly predicted PPA among selegiline treated patients (logistic regression; $p = .005$). This effect can be visualized by comparing the numbers of rs3813567 minor allele carriers (CT and CC genotypes) in the abstinent and non-abstinent groups at week 25 (Table 2). Minor allele carriers were far more frequent among non-abstinent selegiline-treated patients than among abstinent patients (χ^2 , $p = .003$; odds ratio [OR] = 6.16, 95% confidence interval [CI] = 1.67–22.69). There were no significant genetic effects among placebo-treated subjects ($p > .05$; OR = 1.44, 95% CI = 0.63–3.27). Among Caucasians, there was a trend toward a similar effect at week 25 (logistic regression; $p < .009$), although it did not meet our criterion for multiple correction of $p = .0083$. However, a contingency table analysis (Supplementary Table S2) showed a significant

association of minor allele carriers (CT and CC genotypes) with lack of abstinence among selegiline-treated Caucasians (χ^2 , $p < .001$; OR = 15.83; 95% CI = 1.96–127.2), but not among placebo-treated smokers ($p > .05$; OR = 1.09, 95% CI = 0.35–3.40). There were

Table 2. Week 25 Abstinence and rs3813567 Genotype

		TT	CT/CC
Week 25 selegiline	Not abstinent	37	38
	Abstinent	18	3
		TT	CT/CC
Week 25 placebo	Not abstinent	42	24
	Abstinent	17	6

no significant genetic effects on abstinence at week 52. No other SNP showed a significant effect on PPA at any time point. Step-wise regression analysis of the top 10 AIMs PCs showed no associations with PPA in the present study, further indicating that the PPA results were independent of ethnicity.

Because others have found haplotype associations with smoking in the rs680244 to rs1696998 region,^{18,43} we tested a custom haplotype block encompassing rs680244, rs2229961, rs16969968, rs578776, and rs1051730 for associations with PPA. Six haplotypes were identified in this block, but none were associated with PPA. The frequencies of all haplotypes are given in [Supplementary Table S3](#), with the top panel showing results for the full sample, and the lower panel showing results for the Caucasians.

Longitudinal analysis of craving over the 8-week acute treatment period showed that the CHRNA4 promoter polymorphism rs3813567 had a significant effect on craving in the full selegiline treatment group after correction for multiple testing ($p = .008$). Minor C allele carriers (CT and CC genotypes) showed significantly higher levels of craving ([Figure 2](#)). There was no significant SNP by

Figure 2. Effect of rs3813567 and rs680244 genotype on mean scores for craving (top, middle panels) and of rs680244 genotype on Center for Epidemiological Studies Depression Scale (CES-D) (bottom panels) over the 8-week treatment period. Results for selegiline and placebo are presented separately. Lines represent scores for individual genotype groups. The first point in each graph represents baseline assessment, whereas the second point represents 24 hr after quitting. Subsequent points represent weekly assessments. Full sample.

time interaction, suggesting that the difference at 24 hr post-quit was maintained thereafter. Among placebo treated patients, rs3813567 had no effect on craving. Among Caucasians, there was a similar trend toward increased craving in rs3813567 minor allele carriers, but this effect did not survive correction for multiple testing ($p < .02$) (Supplementary Figure S2). In addition, the CHRNA5 intron polymorphism rs680244 showed an association with craving ($p = .003$) among selegiline-treated patients in the full cohort that survived Bonferroni correction, but not in the Caucasian selegiline group ($p = .05$). In the full cohort, selegiline-treated carriers of the rs680244 GG genotype showed significantly lower levels of post-quit craving, and in particular did not report the post-quit increase in craving experienced by non-carriers. There were no effects of rs680244 genotype on craving in placebo-treated patients.

For CES-D in the placebo-treated subjects, there was a significant SNP by time interaction ($p = .004$) that survived correction for multiple testing, with rs680244 GG carriers showing high levels of depressive symptoms post-quit that decreased by day 28 (Figure 2). For the Caucasian cohort, the p value was .002 (Supplementary Figure S2). There was no significant rs680244 SNP by time interaction effect on CES-D in selegiline-treated subjects, and specifically no initial increase in CES-D among GG carriers.

None of the genetic markers had effects on compliance, withdrawal, change in BMI, or adverse event score that survived correction for multiple testing.

Demographics and Baseline Clinical Measures

Analysis by genotype of the demographic and baseline clinical measures in the full cohort, which included age, gender, years of formal education, marital status, baseline BMI, baseline smoking quantity and baseline modified Fagerstrom score, revealed no significant differences among genotype groups for rs3813567 or rs680244 (Supplementary Table S4). Similar results were obtained with the Caucasian group (Supplementary Table S5).

No significant divergence between the baseline measures for those participants who provided DNA and those who did not was observed with the exception of ethnicity, which was significant ($p < .0001$) with all but 1 of the 12 individuals declining to provide DNA reporting non-Caucasian ancestry.

A comparison of genotype frequencies between Caucasians and non-Caucasians showed significant associations between ethnicity and genotype for all SNPs except for rs680244 and for rs2229961. In addition, rs12914008 and rs8192475 were only observed in the Caucasian population. Further analysis revealed differences in MAFs between the two major ethnic groups, Caucasian ($n = 177$) and Hispanic ($n = 22$), similar to those previously reported by this group for the MT1 and MT2 cohorts that we recruited from the same geographic region.³³ These differences were significant for rs16969968 and rs1051730, which are in near complete LD ($r^2 = 0.99$) (Figure 1), and for rs680244.

Discussion

Previous studies of selegiline for smoking cessation therapy have produced mixed results.^{7-10,12} In our selegiline clinical trial, overall the drug had no effect on any of the primary outcome measures.¹¹ However, the present pharmacogenetic analysis of the same clinical dataset revealed several significant results.

The most interesting pharmacogenetic findings were with the CHRNA4 SNP rs3813567, which showed significant associations

with PPA and craving. Carriers of the minor C allele at rs3813567 (CT and CC genotypes) treated with selegiline showed higher craving than non-carriers throughout the 8-week treatment period and were less likely to be abstinent at week 25. In contrast, in the placebo group, where there were no significant differences between the genotype groups in craving or PPA. The lack of a genetic effect on PPA at the week 8 time point among selegiline-treated patients could be because cognitive behavioral therapy mitigated the effects of higher craving among minor allele carriers. By week 52 the difference between selegiline and placebo-treated carriers was absent, indicating the effect is reduced by time.

The rs3813567 result is of particular interest because CHRNA4 knockout mice have been shown to have decreased withdrawal symptoms.⁴⁴ Further, rs3813567 is located within the CHRNA4 promoter, which contains key regulatory elements, and is important to the temporal and tissue specific regulation of CHRNA4.^{45,46} Regulation of the 15q24 region is complex with CHRNA5, CHRNA3 and CHRNA4 often being co-expressed. CHRNA4 contains a 3' enhancer element involved in this combined regulation.^{47,48} The rs3813567 major T allele was reported to be associated with risk for smoking,⁴⁹ but those results were not based on a selegiline clinical trial. Our results indicate that during selegiline treatment it was the C minor allele that was over-represented among those not abstinent.

The CHRNA5 intron polymorphism rs680244 also showed an association with craving in the full cohort of selegiline-treated patients, with GG carriers not experiencing an increase in craving in the early weeks of the trial. The rs680244 SNP was also a predictor for post-quit mood change (CES-D score) in the full and in the Caucasian placebo cohorts. Carriers of the GG genotype at rs680244 showed high mean CES-D scores post-quit, but scores eventually decreased by about 28 days. The absence of this effect in the selegiline cohort suggests that transdermal selegiline, which is FDA approved as an antidepressant, could mitigate genetic vulnerability to depressive symptoms after quitting. The rs680244 G allele has previously been linked to greater nicotine dependence in women.⁵⁰ In one smoking cessation study, the G allele influenced abstinence in smokers treated with placebo, but this effect depended on the haplotype context.⁴³

Haplotype associations with smoking have been reported in the 15q24 region.^{18,43} We found no haplotypes associations with PPA in our study. Thus, in our sample combinations of SNPs on a single chromosome are not better predictors than individual markers. There are additional SNPs in 15q24 that we did not genotype. These might yield haplotypes that predict selegiline response.

Although our study involved more patients than prior studies of selegiline for smoking cessation, statistical power to detect pharmacogenetic effects was limited for SNPs with rare minor alleles. In general our pharmacogenetic findings were strongest in the full cohort due to increased statistical power. However, in the full selegiline study cohort a comparison of the allele frequencies between the self-reported ethnicities showed that for all SNPs except rs680244 MAFs were significantly different between Caucasians and others. Fortunately, the pharmacogenetic results in the Caucasian group were not markedly different from those in the full cohort. Further, PCs derived from the AIM scores, which are composite measures of ethnicity, showed no association with PPA.

Finally, selegiline is thought to act primarily by increasing dopamine release in the brain.⁵ However, there is ample evidence that cholinergic input modulates activity in brain dopaminergic

nuclei.^{15,27–29,51} This may explain why variation at cholinergic loci affects selegiline treatment outcomes in smokers.

In conclusion, our results show that polymorphisms in the 15q24 region have significant pharmacogenetic effects on transdermal selegiline treatment outcomes including abstinence, craving, and mood. Smokers carrying the minor allele C of the CHRNA5 SNP rs3813567 (CT and CC genotypes) may have impaired ability to quit when treated with selegiline, possibly due to higher levels of craving. The CHRNA5 intron polymorphism rs680244 showed associations with craving and mood after quitting. For medications such as selegiline with marginal or inconsistent overall efficacy, pharmacogenetic analysis may be useful in identifying patient subgroups suited or ill-suited to the drug.

Supplementary Material

Supplementary Tables S1–S5 and Figures S1 and S2 can be found online at <http://www.ntr.oxfordjournals.org>

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Declaration of Interests

LCL: named as an inventor on a patent application on genetic markers for antidepressant response filed by Stanford University. AFS: past 3 years: Consultant for BrainCells, CeNeRx, Corcept, Eli Lilly, Forest Labs, Jones/Day, Merck, NeuroNetics, Novartis, Pathway Diagnostics, Pfizer, PharmaNeuroBoost, Sanofi-Aventis, Sunovion, Synosis, Xhale, Xytis, Wyeth. Equity in BrainCells, CeNeRx, Corcept (co-founder), Forest, Merck, Neurocrine, NovaDel, Pfizer, PharmaNeuroBoost, Somaxon, Synosis, Xhale. Intellectual Property, named inventor on pharmacogenetic use patents on prediction of antidepressant response. Speaking, Merck, Sanofi-Aventis. GMM: named as an inventor on a patent application on genetic markers for antidepressant response filed by Stanford University. Consultant for Brain Resource, Ltd.

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