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Significant Association of Catechol-*O*-Methyltransferase (*COMT*) Haplotypes with Nicotine Dependence in Male and Female Smokers of Two Ethnic Populations

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The catechol-*O*-methyltransferase (*COMT*) gene plays a prominent role in dopaminergic circuits central to drug reward. Allelic variants within the *COMT* gene are therefore potential candidates for examining interindividual differences in vulnerability to nicotine dependence (ND). We analyzed five single nucleotide polymorphisms (SNPs), including the Val/Met variant (rs4680), which results in a three- to four-fold difference in enzyme activity within *COMT*, for association with the three ND measures, SQ, HSI, and FTND, in 602 nuclear families of African-American (AA) or European-American (EA) origin. The Val/Met variant showed a significant association with the three ND measures in the pooled and EA samples and with FTND in the AA sample. Haplotype analysis revealed a major protective A-G-T haplotype (frequency 23.6%) for rs740603-rs4680-rs174699 in the AA sample (minimum $Z = -3.35$; $P = 0.0005$ for FTND), a major protective T-G-T haplotype (frequency 15.2%; minimum $Z = -2.92$; $P = 0.003$ for FTND) in the EA sample, and a high-risk C-A-T haplotype (frequency 16.9%; minimum $Z = 3.16$; $P = 0.002$ for SQ) in the AA sample for rs933271-rs4680-rs174699. Furthermore, we found that the significant haplotypes within *COMT* were gender-specific and the significantly associated A-G-T is protective in AA females only, whereas T-G-T is protective in EA males only. Moreover, we found a major high-risk T-A-T haplotype (frequency 56.7%) that showed significant association with the three ND measures in EA males. Further examination of two protective haplotypes, A-G-T in AAs and T-G-T in EAs, indicated that the low *COMT* enzyme activity Met allele is protective to become nicotine dependent. In summary, our results provide evidence for a role of *COMT* in the susceptibility to ND and further confirm that its effect is ethnic and gender specific. *Neuropsychopharmacology* (2006) **31,** 675–684. doi:10.1038/sj.npp.1300997; published online 14 December 2005

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INTRODUCTION

According to a report from the World Health Organization, 1.3 billion people worldwide smoke tobacco (WHO, 2005). With 5 million tobacco-related deaths per year, tobacco use is the leading cause of preventable death in the world today. Although nearly 70% of smokers in the U.S. report that they are interested in quitting, <5% of those who try quitting remain tobacco-free for 12 months (US Department of Health and Human Services, 1988). There is substantial evidence for a genetic component in the vulnerability to nicotine dependence (ND) based on twin and family segregation studies (Sullivan and Kendler, 1999; Li *et al*, 2003) as well as data from studies with inbred mouse strains (Hatchell and Collins, 1980; Robinson *et al*, 1996). However, only limited association studies on candidate genes and/or linkage analyses for susceptibility loci have consistently produced positive findings (for a review, see Li *et al*, 2004).

Nicotine has been shown to be the primary addictive component of tobacco smoking, stimulating the release of dopamine from neurons in the ventral tegmental area, an action thought to underlie its rewarding effects (Nisell *et al*, 1994; Pontieri *et al*, 1996). ND is a complex disorder for which the combined effects of multiple interactive genes, each with a small effect, likely confer additive genetic contributions. The influence of genes on regulatory processes in the human brain is particularly difficult to resolve, given that a functional genetic variant may affect not only the protein coded by the gene in question, but may also have downstream effects, contributing to the overall system response.

In this study, we focus on the catechol-*O*-methyltransferase (*COMT*) gene, first cloned by Lundstrom *et al* (1991). The protein encoded by this gene catalyzes the transfer of a methyl group from S-adenosyl-methionine (SAM) to a hydroxyl group of catecholamines, including the

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neurotransmitters dopamine, epinephrine and norepinephrine, or of catechol estrogen resulting in the degradation of catecholamines (Weinshilboum *et al*, 1999). *COMT* is encoded by a single gene with six exons that has been mapped to chromosome 22q11.21 (Grossman *et al*, 1992). The *COMT* protein exists in two isoforms resulting from two mRNA transcripts from two promoters (P1 and P2): a soluble form found in the cell cytoplasm (S-*COMT*; 221 aa) and a longer, membrane-bound form (MB-*COMT*; 271 aa) (Tenhunen *et al*, 1994). The MB-*COMT* is predominantly expressed in the brain, while S-*COMT* is predominantly expressed in blood and in other tissues, such as liver and kidney (Tenhunen *et al*, 1994; Lundstrom *et al*, 1995). Although *COMT* is expressed widely throughout the brain, its enzymatic activity appears to be particularly important in the prefrontal cortex, where it inactivates dopamine (Garris *et al*, 1993; Gogos *et al*, 1998; Matsumoto *et al*, 2003).

The important role of *COMT* in the dopamine pathway has further been demonstrated by genetic and pharmacological studies in rodents. For example, *COMT*-deficient mice exhibit sexually dimorphic and region-specific changes in dopamine levels, notably in the prefrontal cortex (Gogos *et al*, 1998; Akil *et al*, 2003). In addition, *COMT* knockout mice show a number of behavioral and neurological effects, including alterations in anxiety-like behavior (Holmes, 2001). Heterozygous *COMT*-deficient male mice further exhibit increased aggressive behavior (Gogos *et al*, 1998), and the more aggressive inbred strains had lower *COMT* expression levels in the hippocampus relative to the less aggressive strains (Fernandes *et al*, 2004). Furthermore, the *COMT* inhibitor tolcapone has been found to yield improvements in lesion- or drug-induced deficits of memory in rats (Khromova *et al*, 1995, 1997).

A common valine–methionine (Val/Met) substitution at codons 108 and 158 in the S-*COMT* and MB-*COMT* transcripts, respectively, has been reported to account for $>95\%$ of the variations of this enzyme activity in humans. The Val108 allele has three- to four-fold higher enzyme activity than the Met108 allele and heterozygotes have intermediate enzyme activity (Lotta *et al*, 1995; Lachman *et al*, 1996; Weinshilboum *et al*, 1999). The Val158Met (*rs4680*) variant has been shown to significantly affect protein abundance and enzyme activity but not mRNA expression, suggesting that differences in protein integrity account for the difference in enzyme activity between alleles (Chen *et al*, 2004). Therefore, the Val108/158Met genetic polymorphism has been the subject of intense molecular epidemiologic study and has been reported to be associated with risk for a number of diseases including schizophrenia, obsessive-compulsive disorder, disordered cognitive abilities (for a review, see Bilder *et al*, 2004), anorexia nervosa (Frisch *et al*, 2001), anxiety (Enoch *et al*, 2003; McGrath *et al*, 2004), and drug abuse and alcoholism (Vandenbergh *et al*, 1997; Tiihonen *et al*, 1999; Horowitz *et al*, 2000; Kauhanen *et al*, 2000; Wang *et al*, 2001). However, many of these studies are controversial.

To date, only three studies have investigated a possible association between genetic variant(s) within *COMT* and smoking behavior. Two did not identify an association between *COMT* and smoking initiation, persistence or cessation (David *et al*, 2002), or tobacco consumption (McKinney *et al*, 2000). Colilla and co-workers recently reported on an association between the *COMT* Val/Met variant with exsmoker status in EA females, but not in AA females (Colilla *et al*, 2005). Given the role of *COMT* in dopamine metabolism, the role of the dopamine reward pathway in ND, and the evidence that nicotine stimulates the release of dopamine (Nisell *et al*, 1995; Pontieri *et al*, 1996), it makes this gene an attractive candidate gene for an association study with the vulnerability to ND, as reported herein.

MATERIALS AND METHODS

Participants and Smoking Phenotypes

Participants were of either African-American (AA) or European-American (EA) origin from the Mid-South Tobacco Family (MSTF) cohort, recruited primarily from the states of Tennessee, Mississippi, and Arkansas in the U.S. during 1999–2004. Proband smokers were required to be at least 18 years of age, to have smoked tobacco for at least the last 5 years, and have consumed an average of 20 cigarettes per day for the last 12 months. Siblings and parents of a smoking proband were recruited whenever possible, regardless of their smoking status. Extensive data were collected on each participant, including demographics (e.g., sex, age, race, biological relationships, weight, height, years of education, and marital status), medical history, smoking history and current smoking behavior, and personality traits, utilizing the various questionnaires available at the NIDA Genetics Consortium Website (http://zork.wustl.edu/nida). All participants provided informed consent. The study protocol and forms/procedures were approved by all participating Institutional Review Boards.

The degree of ND of each smoker was ascertained by the three most commonly used measures: Smoking Quantity (SQ; defined as the number of cigarettes smoked per day), the Heaviness of Smoking Index (HSI; 0–6 scale), and the Fagerstrõm Test for ND score (FTND; 0–10 scale) (Heatherton *et al*, 1991). Our primary reasons for examining all three measures were: (a) the current lack of consensus as to the best approach to assess ND as a phenotype, and (b) to permit maximum cross-reference with previous studies of ND. The SQ provides a simple, quantified index of the amount of consumption (using a 0–3 point compressed format), whereas HSI includes one item addressing quantity (SQ) and another item assessing urgency, that is, 'How soon after you wake up do you smoke your first cigarette?' The FTND score includes the HSI plus other indicators of behavioral propensity to smoke under various circumstances. The FTND has been accepted as a standard in both clinical and research settings, although recent evidence suggests ND is a broader and more complex construct than previously considered (Swan, 2003). Given the presence of overlap in the content of the three ND measures, there exist fairly robust correlations among them ($r = 0.88$–0.94). Of the 2037 participants, the average age was 39.4 ± 14.4 (SD) years for the AAs and 40.5 ± 15.5 years for EAs. The average nuclear family size was 3.14 ± 0.75 for AAs and 3.17 ± 0.69 for EAs. The average HSI and FTND scores of smokers were 3.7 ± 1.4 and

6.26 ± 2.15 for AAs and 3.9 ± 1.4 and 6.33 ± 2.22 for EAs, respectively. The average number of cigarettes smoked per day was 19.4 ± 13.3 for AA smokers and 19.5 ± 13.4 for EA smokers. A detailed description of demographic and clinical characteristics for the participants in the study is presented in Table 1.

DNA Extraction, SNP Selection, and SNP Genotyping

DNA was extracted from peripheral blood samples using an extraction kit purchased from Qiagen Inc. (Valencia, CA). SNPs selected for our investigation include the commonly used Val/Met missense SNP (rs4680) and a SNP (rs4633) within exon 3 of the MB-*COMT* transcript or exon 1 of the S-*COMT* transcript that defines a synonymous C→T (His/His) change. To have a better coverage of the 27 kb genomic region, we also selected three intronic SNPs (Figure 1). All five SNPs investigated in this study were selected from the NCBI SNP-database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = snp), and had a minimum allele frequency (MAF) of 0.15 or greater according to the frequency from the database. Information regarding these SNPs, including their location within the gene, chromosomal position, allelic variants, and primer/probe sequences, is listed in Table 2.

Table 1 Clinical Characteristics for the Pooled, EA and AA Samples

Characteristic	African-American	European-American	Pooled
No. of nuclear families	402	200	602
Avg. members/family	3.14 ± 0.75	3.17 ± 0.69	3.15 ± 0.73
No. of subjects	1366	671	2037
Gender (% female)	66.1	69.5	67.2
Age (years)	39.4 ± 14.4	40.5 ± 15.5	39.7 ± 14.8
No. of smokers	1053	515	1568
Age of smoking onset (years)	17.3 ± 4.7	15.5 ± 4.4	16.7 ± 4.7
Years smoked	20.4 ± 12.5	23.2 ± 13.5	21.3 ± 12.9
No. cigarettes/day	19.4 ± 13.3	19.5 ± 13.4	19.5 ± 13.3
HSI score	3.7 ± 1.4	3.9 ± 1.4	3.8 ± 1.4
FTND score	6.26 ± 2.15	6.33 ± 2.22	6.29 ± 2.17

The SNPs were genotyped using the *Taq*Man assay in a 384-well microplate format (Applied Biosystems Inc., Foster City, CA). Briefly, 15 ng of DNA was amplified in a total volume of 7 µl containing an MGB probe and 2.5 µl of *Taq*Man universal PCR master mix. The amplification conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 95°C for 25 s and 60°C for 1 min. Allelic discrimination analysis was performed on the ABI Prism 9700 Sequence Detection System. To ensure the quality of the genotyping, consistent results were required for eight control samples added to each 384-well reaction plate.

Statistical and Association Analyses

Pair-wise LD between all SNP markers was assessed using the program Haploview (Barrett *et al*, 2005) with the option of determining haplotype blocks according to the criteria defined by Gabriel *et al* (2002). The PedCheck program (O'Connell and Weeks, 1998) was used to identify any inconsistent Mendelian inheritance, nonpaternity, or typing errors. A total of 136 genotyping inconsistencies were detected in the AA sample and 66 in the EA sample of approximately 10 000 assays and were excluded from all subsequent statistical analyses.

Associations between a single SNP and the three ND measures were determined by the PBAT program using generalized estimating equations (Lange *et al*, 2003). Since the current version of PBAT (v. 1.2) cannot perform haplotype-based association analysis, the FBAT program was used to determine associations between each ND measure and haplotypes from multiple SNP combinations with the option of computing *P*-values of the *Z* statistic using Monte Carlo sampling under the null distribution of no linkage and no association (Horvath *et al*, 2004). In the present analysis, we tested all consecutive three and four multilocus combinations, the five SNP haplotype combinations, as well as all other possible three and four SNP combinations. Gene × ethnicity interactions for the pooled sample and gene × gender interactions for the pooled, AA, and EA samples were determined by FBAT interaction statistics, an option provided within PBAT (for details, see http://www.biostat.harvard.edu/~clange/pbat3/default.htm). Three genetic models (additive, dominant, and recessive) were tested for both individual and multi-locus SNPs. For all FBAT and PBAT association tests, we applied sex and age

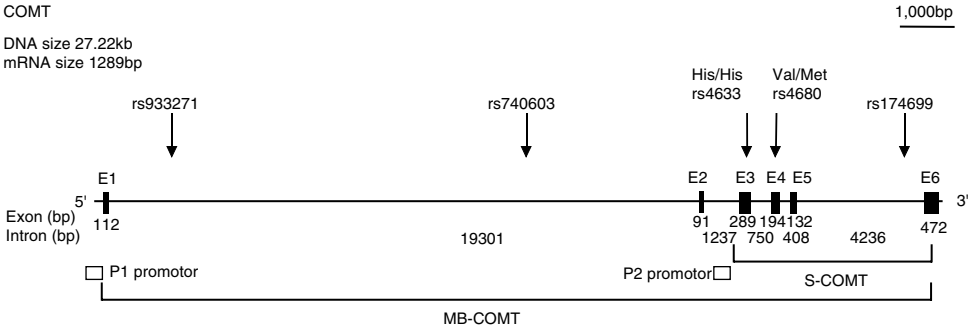

Figure 1 *COMT* gene structure and location of the five SNPs used in the present investigation. The two exonic SNPs, rs4633 and rs4680, cause a synonymous (His/His) and nonsynonymous (Val/Met) amino acid change, respectively. The two promoters (P1 and P2) are indicated as well as the two isoforms, MB-*COMT* and S-*COMT*, resulting from transcription from P1 and P2, respectively.

Table 2 Positions, Nucleotide Variation, Minor Allele Frequency and Primer/Probe Sequences of 5 SNPs Within *COMT*

dbSNP ID	SNP localization	Chromosome position	Alleles	Amino acid	Forward (F) and reverse (R) primer and probe (P) sequences
rs933271	Intron1	18305961	T/C		F: CAGTTGTGGTTACTTTCTGGAGAGA R: GGCCGCCCAGGAAGAC P: TCCTGCATGCCG/ACATG
rs740603	Intron1	18319731	A/G		F: CACTGTGAGGCACTGAGGAT R: CCACATGCACGCCACAT P: CCCTCACA/GCGTGCAT
rs4633	Exon3	18324789	C/T	His→His	F: CTGCTCATGGGTGACACCAA R: GCTCGCAGTAGGTGTCAATGG P: TGAACCAT/CGTGCTGCA
rs4680	Exon4	18325825	G/A	Val→Met	F: GACTGTGCCGCCATCAC R: CAGGCATGCACACCTTGTC P: TTTCGCTGGCG/ATGAAG
rs174699	Intron5	18329012	T/C		F: ACCTGCTCCTCTGACACTGT R: GAGAACTGGCTAAACATGCATCAG P: AATCTAATGCCG/ATGGAGAA

as covariates when analyzing the AA and EA samples separately; sex, age, and ethnicity covariates were employed in the pooled sample, and age as a covariate in the AA and EA samples for each gender. We used these covariates because they have been previously shown to influence ND (Edwards *et al*, 1995; Perez-Stable *et al*, 1998; Benowitz *et al*, 1999; Heath *et al*, 1999; Madden *et al*, 1999; Li *et al*, 2003). The three ND measures, SQ, HSI, and FTND, were analyzed individually. All associations found to be significant were corrected for multiple testing according to the SNP spectral decomposition (SNPSpD) approach (Nyholt, 2004) for individual SNP analysis, and using Bonferroni correction by dividing the significance level by the number of major haplotypes (frequency $>5.0\%$) for haplotype-based association analysis.

RESULTS

Association Analysis of Individual SNPs

Individual SNP analysis using PBAT-GEE revealed a significant association for the Val/Met polymorphism (rs4680) with all three adjusted ND measures in the pooled samples; this association remained significant after correction for multiple testing for FTND under both the dominant ($P=0.006$) and recessive ($P=0.007$) models (adjusted *P*-value at the 0.05 significance level is 0.013; Table 3). Given the potential genetic differences in ND across racial groups (Benowitz *et al*, 1999), we used FBAT interaction statistics to determine the presence of heterogeneity in our samples and found a significant marker-ethnicity interaction for all three ND measures in the pooled samples for the Val/Met variant (*P*-value ranged between 0.003 and 0.01 for all three ND measures; data not shown). Moreover, we compared the allele frequencies of the five SNPs within *COMT* in the pooled, AA and EA samples and noticed differences between the two ethnic samples for several SNPs (based on calculations of the allele frequency by directly counting the numbers of each allele from the progenitors of our samples; Table 4). This suggests an ethnic-specific allele distribution in our AA and EA samples, which is consistent with results reported by other researchers for the Val/Met variant (Palmatier *et al*, 1999; DeMille *et al*, 2002).

As a result of the significant gene and ethnicity interaction present in the pooled samples and the different distribution of the allele frequencies across ethnic samples, we analyzed the AA and EA samples separately to minimize this source of heterogeneity. Individual SNP analysis for each ethnic group resulted in a significant association for SNP rs4680 with all three age- and sex-adjusted ND measures under different genetic models in the EA sample ($P=0.009$–0.02; Table 3). This association remained significant for the adjusted SQ after correcting for multiple testing. In the AA sample, we also found a significant association of rs4680 with the FTND score ($P=0.04$). However, this association was no longer significant after correction for multiple testing (Table 3).

In addition to examining an ethnic-specific effect of *COMT* on ND, we also investigated gender specificity by analyzing the males and females of each ethnicity separately. Such an effort was motivated by: (1) our finding of a significant gender-gene interaction for rs4680 in the pooled and EA samples ($P=0.006$–0.02) for the three ND measures (data not shown); and (2) reports of sex-specific enzyme activity and sexually dimorphic effects for *COMT* on genetic susceptibility to several psychiatric disorders (Boudikova

Table 3 *P*-Values for Association of Single SNPs Within *COMT* with Three ND Measures in the Pooled, AA and EA Samples

	Pooled sample			AA sample			EA sample		
SNP ID	**SQ**	**HSI**	**FTND**	**SQ**	**HSI**	**FTND**	**SQ**	**HSI**	**FTND**
rs933271	0.13	0.17	0.16	0.06	0.15	0.15	0.83	0.55	0.43
rs740603	0.74	0.55	0.59	0.20	0.21	0.21	0.26	0.49	0.44
rs4633	0.52	0.80	0.58	0.55	0.66	0.45	0.26	0.71	0.80
rs4680	0.02^{d}	$0.02^{d,r}$	**0.006^{d}**	0.16	0.09	$0.04^{d,r}$	**$0.01^{a,r}$**	$0.02^{a,d,r}$	$0.02^{a,d,r}$
	0.03^{r}		**0.007^{r}**				**0.009^{d}**		
rs174699	0.32	0.32	0.32	0.17	0.22	0.30	0.32	0.32	0.32

The adjusted *P*-value after correction for multiple testing at the 0.05 significance level is 0.01 (bold).
Superscripts indicate the genetic models used for analysis; a = additive, d = dominant, and r = recessive model.
For the pooled samples, the three ND measures were adjusted for age, sex, and ethnicity; for each ethnic-specific sample group only age and sex were used as covariates for the ND measures.

Table 4 Observed *Versus* Reported Allele Frequency of Five SNPs Within *COMT*

dbSNP ID	Major/minor alleles	Corresponding minor allele frequency in pooled samples	Corresponding minor allele frequency in AA samples	Corresponding minor allele frequency in EA samples
rs933271	T/C	0.33	0.39	0.26
rs740603	A/G	0.47	0.42	0.53
rs4633	C/T	0.41	0.32	0.52
rs4680	G/A	0.59	0.48	0.73
rs174699	T/C	0.03	0.02	0.54

et al, 1990; Gogos *et al*, 1998; Weinshilboum *et al*, 1999; Chen *et al*, 2004). We found a nominally significant association of rs4680 with all three ND measures in the EA males ($P = 0.03$–0.049) and with SQ in the AA females ($P = 0.03$–0.04); however, these associations were no longer significant after correction for multiple testing.

The pair-wise D' values of the five SNPs within *COMT* were generally low except for rs4633 and rs4680, indicating that these two SNPs are highly linked (Figure 2). According to the criteria for block definition from Gabriel and colleagues (Gabriel *et al*, 2002), a single haplotype block of 1.0 kb encompassing rs4633 and rs4680 could be found in the EA sample, but it was not present in the AA sample.

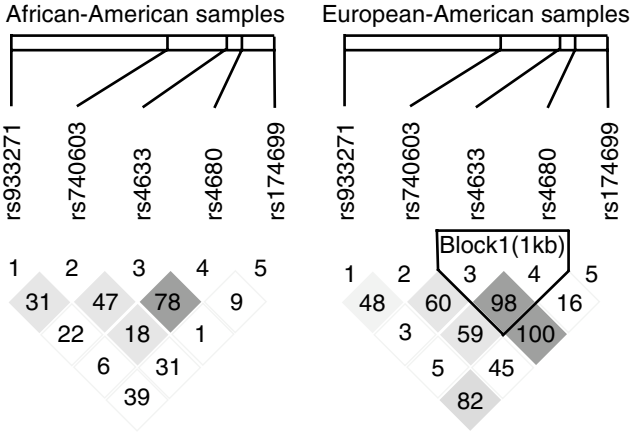

Figure 2 Haploview-generated LD map of five SNPs within *COMT* in the AA and EA samples. Regions of high LD (D' = 1 and LOD > 2) are shown in dark gray. Markers with lower LD (0.21 < D < 1 and LOD > 2) are shown in tints of gray, with the intensity decreasing with decreased D' value. Regions of low LD and low LOD scores (LOD < 2) are shown in white. Haplotype blocks were defined by the Haploview program with the option of using the haplotype block definition proposed by Gabriel *et al* (2002).

Haplotype Analysis of Multiple SNPs

Haplotype-based association analysis was performed for all possible three- and four-SNP combinations (including consecutive and nonconsecutive SNPs), and the five-SNP combination within *COMT*. Based on differences found across the ethnic samples for the single SNP analysis, we performed separate haplotype analyses on the AA and EA samples. In the AA sample, we found a major haplotype A-G-T (frequency of 23.6%) formed by the consecutive SNPs rs740603-rs4680-rs174699 (spanning 9.3 kb) that revealed a significant inverse association with all three adjusted ND measures (minimum $Z = -3.35$; $P = 0.0005$ for the FTND score; Table 5A). These associations remained significant after Bonferroni correction for testing of four major haplotypes for HSI and FTND under the dominant model. In the EA sample, no significantly-associated major haplotypes for this three-SNP combination could be found.

Haplotype analysis of the nonconsecutive SNP combination rs933271-rs4680-rs174699 (spanning 23 kb), revealed a major T-G-T haplotype at a frequency of 15.2% in the EA

Table 5 Z and Permutation *P*-Values for the Significant Associated *COMT* Haplotypes for rs740603-rs4680-rs174699 (A) and rs933271-rs4680-rs174699 (B) with Three ND Measures in the AA and EA Samples

	AA sample				EA sample			
Haplotype	**%**	**SQ**	**HSI**	**FTND**	**%**	**SQ**	**HSI**	**FTND**
(A) rs740603-rs4680-rs174699								
A-G-T	23.6		−2.31[a]	**−2.46[a]**	3.6			
		−2.54[d]	(0.02)	**(0.01)**		−0.59	−0.76	−1.06
		(0.01)	***−3.07[d]***	***−3.35[d]***		(0.63)	(0.5)	(0.35)
			(0.002)	***(0.0005)***				
(B) rs933271-rs4680-rs174699								
T-G-T	30.1	−0.3	−1.22	−1.52	15.2	−2.41[a]	**−2.53[a]**	**−2.73[a]**
		(0.77)	(0.22)	(0.13)		(0.02)	**(0.008)**	**(0.004)**
						−2.48[d]	***−2.85[d]***	***−2.92[d]***
						(0.01)	***(0.003)***	***(0.003)***
C-A-T	16.9	***3.16[a]***	**2.61[a]**	**2.44[a]**	18.7			
		(0.002)	**(0.009)**	**(0.01)**		−1.21	−0.81	−2.92
		2.79[d]	2.37[d]	2.26[d]		(0.22)	(0.43)	(0.34)
		(0.005)	(0.02)	(0.02)				
		2.06[r]						
		(0.04)						

The adjusted *P*-value at the 0.05 significance level after Bonferroni correction for four major haplotypes in the AA sample is 0.0125 (bold) and at the 0.01 significance level is 0.0025 (bold and italics); for three major haplotypes in the EA sample are 0.017 (bold) and 0.003 (bold and italics), respectively.
Superscripts indicate the genetic models used in the analysis; a = additive and d = dominant model.
The ND measures used in the analysis were corrected for age and sex.

sample that showed a significant negative association after Bonferroni correction with all three ND measures (minimum $Z = -2.92$; $P = 0.003$ for the FTND score; Table 5B). In contrast, for the AA sample, we found a significant positive association for a different haplotype C-A-T (present in 16.9%) for this three-SNP combination with all three ND measures (maximum $Z = 3.16$; $P = 0.002$ for SQ). By examining the allele combination of each significant haplotype, we found that the high activity Val allele (underlined G in the haplotypes) is associated with protective haplotypes, whereas the low-activity Met allele (underlined A in the haplotype) is present in the high-risk haplotypes.

Similar to the analytical strategy used for individual SNPs, we examined the association between haplotypes and ND measures in males and females separately. Haplotype analysis of rs740603-rs4680-rs174699 revealed a major A-G-T haplotype (frequency of 23.6%) in the AA females that showed a significant inverse association after Bonferroni correction with HSI and FTND (minimum $Z = -3.10$; $P = 0.002$; Table 6A). In addition, two major haplotypes, formed by SNPs 933271-rs4680-rs174699, showed significant associations with three ND measures in the EA male sample subgroup. The first major haplotype T-A-T with a frequency of 56.7%, not found to be significant in the previous analysis collapsing across gender (Table 6B), now revealed a significant positive association after Bonferroni correction with all three ND measures (maximum $Z = 3.11$; $P = 0.001$ for SQ). A second major haplotype, T-G-T (15.2%), which was previously found to be significant in overall analyses, now also showed a significant negative association with all three ND measures for EA males (minimum $Z = -3.59$; $P = 0.00002$). Finally, we found a different haplotype, C-A-T (16.9%), formed by the same rs933271-rs4680-rs174699 SNP combination that was significantly associated with SQ in AA females ($Z = 2.44$, $P = 0.01$). Consistent with our previous analyses, the high-activity Val allele (underlined G in the haplotypes) is associated with protective haplotypes whereas the low-activity Met allele (underlined A in the haplotype) is present in the high-risk haplotypes in these gender-specific findings.

DISCUSSION

Dopaminergic pathways play an important role in the pathogenesis of ND. This suggests the potential importance of *COMT*, which has been shown to moderate dopamine metabolism via inhibitory mechanisms. We tested this hypothesis in the 602 nuclear families comprised of smokers and nonsmokers of either AA or EA origin in the MSTF cohort to determine whether behavioral associations with *COMT* polymorphisms exist. Two exonic SNPs, including the nonsynonymous Val/Met variant (rs4680) that has been extensively reported in the literature, and three intronic SNPs within *COMT* were analyzed for association with three adjusted ND measures. We

Table 6 *Z* and Permutation *P*-Values for the Significant Associated *COMT* Haplotypes rs740603-rs4680-rs174699 (A) and rs933271-rs4680-rs174699 (B) with Three ND Measures in Males and Females of AA and EA Samples

	AA sample				EA sample			
Gender/Haplotype	**%**	**SQ**	**HSI**	**FTND**	**%**	**SQ**	**HSI**	**FTND**
(A) rs740603-rs4680-rs174699								
Female								
A-G-T	23.6		−2.75[a]	−2.82[a]	3.6	−0.27	−0.31	−0.48
		−2.28[d]	(0.007)	−0.004		(0.8)	(0.76)	(0.62)
		(0.02)	−2.99[d]	−3.10[d]				
			(0.003)	***−0.002***				
(B) rs933271-rs4680-rs174699								
Male								
T-A-T					56.7	***3.11*[a]**	***2.91*[a]**	***2.83*[a]**
						(0.001)	***(0.002)***	***(0.003)***
	31	0.6	0.66	0.9		**2.52[d]**	2.36[d]	2.09[d]
		(0.56)	(0.5)	(0.38)		**(0.009)**	(0.02)	(0.04)
						2.48[r]	**2.39[r]**	**2.49[r]**
						(0.01)	**(0.01)**	**(0.01)**
T-G-T	30.1	−1.24	−1.38	−1.49	15.2	***−3.47*[a]**	***−3.50*[a]**	***−3.59*[a]**
		(0.2)	(0.16)	(0.13)		***(0.001)***	***(0.0002)***	**(0.00002)**
						***−3.11*[d]**	***−3.45*[d]**	***−3.47*[d]**
						(0.001)	**(0.00008)**	***(0.0002)***

The adjusted *P*-value at the 0.05 significance level after Bonferroni correction for four major haplotypes in the AA sample is 0.0125 (bold) and at the 0.01 significance level is 0.0025 (bold and italics); for three major haplotypes in the EA sample are 0.017 (bold) and 0.003 (bold and italics), respectively.
Superscripts indicate the genetic models used in the analysis; a = additive, d = dominant, and r = recessive model.
The ND measures were corrected for age.

performed association analysis on the pooled sample and the two ethnic groups separately. The reasons for this are three-fold. First, the frequencies of the Val and Met alleles are substantially different among several ethnic populations. The low-activity allele (Met) is less common in individuals of African origin (McLeod *et al*, 1994; Palmatier *et al*, 1999; Ameyaw *et al*, 2000; DeMille *et al*, 2002), and multimarker haplotypes in *COMT* show marked differences across populations (Palmatier *et al*, 2004). These findings are consistent with the findings of the current study in which the A (Met) allele was present in 48% in the AA sample and 73% in the EA sample. Further, our ethnic samples showed a different haplotype block structure within *COMT*. Second, ethnic differences in nicotine metabolism (Perez-Stable *et al*, 1998; Benowitz *et al*, 1999) and gender differences, both in the response to smoking (Perkins *et al*, 1999) and in the genetic influences on ND (Li *et al*, 2003) have been reported. Although it remains to be established (Pearce *et al*, 2004) evidence suggests that there are genetic differences among ethnic groups (Burchard *et al*, 2003; Bamshad *et al*, 2004). And third, we found a significant marker and ethnicity interaction for rs4680 in our pooled sample.

After correction for multiple testing, significant associations of the Val/Met variant (rs4680) within *COMT* were found in the pooled and EA samples for the FTND score and SQ, respectively. Haplotype analysis of three-SNP combinations with ND, including the Val/Met variant, revealed a major A-G-T protective haplotype for rs740603-rs4680-rs174699 in the AA sample and a major T-G-T protective haplotype in the EA sample for rs933271-rs4680-rs174699. This latter three-SNP combination also showed a different major C-A-T haplotype in the AA sample that was positively associated with the three ND measures. Our results indicate that an ethnic-specific haplotype within the *COMT* gene is associated with ND, a finding consistent with the ethnic differences in allele frequencies of some of the SNPs examined, and with the LD/block structure differences between the two ethnic groups. The protective haplotypes have the Val (G in the haplotype) allele whereas the high-risk haplotypes contain the Met (A in the haplotype) allele in all three associated haplotypes. This suggests that in our sample, the low enzyme activity Met allele correlates with a higher vulnerability to develop ND. Recently, Colilla *et al* (2005) found overtransmission of the Met allele in female exsmokers suggesting that the Met allele is correlated with successful quitting whereas in our results the Met allele is associated with ND. Our results are in line with findings from both animal and human studies which show that reward processes are mediated by dopaminergic pathways from the ventral tegmental area to nucleus accumbens and frontal cortex (Kalivas, 1993), and that nicotine stimulates the release of dopamine from neurons (Corrigall *et al*, 1992; Nisell *et al*, 1994; Pontieri *et al*, 1996; Balfour *et al*, 2000; Smolka *et al*, 2004). Therefore, individuals with low-activity *COMT* haplotypes may experience a longer-lasting and

more effective dopamine release in the brain, thereby increasing the magnitude and/or duration of reward derived from smoking and the risk of becoming nicotine dependent.

Several lines of evidence suggest that *COMT* activity differs between men and women, most likely due to the effects of estrogen metabolism. It has been shown that *COMT* expression is regulated by estrogen through the estrogen-responsive elements in its promoter region (Xie *et al*, 1999). Women generally have 20–30% lower *COMT* activity levels than men (Boudikova *et al*, 1990). In addition, sexually dimorphic effects of *COMT* have been shown in *COMT*-mutant mice (Gogos *et al*, 1998), and in genetic susceptibility to affective disorder, obsessive-compulsive disorder, anxiety and panic disorder, and schizophrenia (Karayiorgou *et al*, 1997; Shifman *et al*, 2002; Enoch *et al*, 2003; Lee *et al*, 2005). Our findings suggest a sex-specific genetic component of ND; in particular, identification of the protective A-G-T haplotype of rs740603-rs4680-rs174699 in AA females, and the protective T-G-T haplotype of rs933271-rs4680-rs174699 in EA males.

It has been suggested that the effect of the Val/Met polymorphism on dopamine-mediated frontal cortical function may be the neurobiological mechanism underlying the clinical association with psychological disorders (Weinberger *et al*, 2001). This polymorphism has been linked to various diseases/disorders, including schizophrenia, Parkinson's disease, obsessive-compulsive disorder, drug abuse and alcoholism, albeit the associations are generally weak and replication has been inconsistent. Thus, it is difficult to conclude unequivocally whether the risk of any of these disorders is influenced by *COMT*. Some of the inconsistency could be attributable to: (1) population-substructure differences between different study samples; (2) definition of the complex traits analyzed; and (3) lack of power to detect an association due to small sample sizes or SNP selection. In this study, we considered these shortcomings and addressed them to the degree possible. Since our sample is family-based, we minimized or excluded potential confounding effects of population stratification. Furthermore, we used a large sample set of extensively phenotyped smokers and nonsmokers of two ethnicities, which provides increased power to detect associations between genetic variants and ND. The three measures analyzed are those most commonly used in genetic research and clinical settings to assess ND, permitting the opportunity for cross-study replication by others.

We included additional SNPs in our analysis besides the Val/Met variant and performed both single-SNP and haplotype analyses. We observed additional significant results for haplotype analysis as compared with single SNP analysis. These data suggest that if the *COMT* gene is indeed involved in susceptibility to ND, its influence cannot be fully accounted for by the Val/Met polymorphism, and other functional variants close to or in strong LD with these SNPs are likely to exist. In that case, *COMT* may be a modifier of the smoking phenotype, rather than a direct susceptibility allele. Moreover, since the LD between the analyzed SNPs was generally low, we plan to analyze more SNPs within *COMT*, as well as adjacent genes. Although the Val/Met variant of the *COMT* gene has been extensively studied, the underlying mechanism remains unknown (Goodman *et al*, 2002) and we can only speculate on the nature of the protective effect of the Val allele in our smoker sample.

In summary, we provide strong evidence for a relationship between allelic variants of *COMT* and three ND measures in EA and AA smokers. We found ethnic-specific protective haplotypes within *COMT* against the expression of ND, as well as gender differences of *COMT* haplotypes in both AA and EA populations. Finally, regardless of ethnicity and gender, the high enzyme activity Val allele is always present in the protective haplotypes, whereas the low activity Met allele is associated with high-risk haplotypes for ND.

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