

ORIGINAL ARTICLE

# Linkage and association studies in African- and Caucasian-American populations demonstrate that *SHC3* is a novel susceptibility locus for nicotine dependence

MD Li<sup>1</sup>, D Sun<sup>1,2</sup>, X-Y Lou<sup>1</sup>, J Beuten<sup>1</sup>, TJ Payne<sup>3</sup> and JZ Ma<sup>4</sup>

<sup>1</sup>Department of Psychiatry and Neurobehavioral Sciences, University of Virginia, Charlottesville, VA, USA; <sup>2</sup>Department of Animal Genetics and Breeding and Key Laboratory of Animal Genetics and Breeding of the Ministry of Agriculture, China Agricultural University, Beijing, PR China; <sup>3</sup>ACT Center for Tobacco Treatment, Education and Research, University of Mississippi Medical Center, Jackson, MS, USA and <sup>4</sup>Department of Public Health Sciences, University of Virginia, Charlottesville, VA, USA

Our previous linkage study demonstrated that the 9q22–q23 chromosome region showed a 'suggestive' linkage to nicotine dependence (ND) in the Framingham Heart Study population. In this study, we provide further evidence for the linkage of this region to ND in an independent sample. Within this region, the gene encoding Src homology 2 domain-containing transforming protein C3 (*SHC3*) represents a plausible candidate for association with ND, assessed by smoking quantity (SQ), the Heaviness of Smoking Index (HSI) and the Fagerström Test for ND (FTND). We utilized 11 single-nucleotide polymorphisms within *SHC3* to examine the association with ND in 602 nuclear families of either African-American (AA) or European-American (EA) origin. Individual SNP-based analysis indicated three SNPs for AAs and one for EAs were significantly associated with at least one ND measure. Haplotype analysis revealed that the haplotypes A-C-T-A-T-A of rs12519–rs3750399–rs4877042–rs2297313–rs1547696–rs1331188, with a frequency of 27.8 and 17.6%, and C-T-A-G-T of rs3750399–rs4877042–rs2297313–rs3818668–rs1547696, at a frequency of 44.7 and 30.6% in the AA and Combined samples, respectively, were significantly inversely associated with the ND measures. In the EA sample, another haplotype with a frequency of 10.6%, A-G-T-G of rs1331188–rs1556384–rs4534195–rs1411836, showed a significant inverse association with ND measures. These associations remained significant after Bonferroni correction. We further demonstrated the *SHC3* contributed 40.1–59.2% (depending on the ND measures) of the linkage signals detected on chromosome 9. As further support, we found that nicotine administered through infusion increased the *Shc3* mRNA level by 60% in the rat striatum, and decreased it by 22% in the nucleus accumbens (NA). At the protein level, *Shc3* was decreased by 38.0% in the NA and showed no change in the striatum. Together, these findings strongly implicate *SHC3* in the etiology of ND, which represents an important biological candidate for further investigation. *Molecular Psychiatry* advance online publication, 19 December 2006; doi:10.1038/sj.mp.4001933

**Keywords:** nicotine; linkage analysis; tobacco dependence; rat brain; *SHC3*; expression

## Introduction

Nicotine is the primary substance in tobacco that maintains its continued use and results in addiction. Like many other substance dependencies, nicotine dependence (ND) is a complex quantitative trait influenced by both genetic and environmental factors (for recent reviews, see Sullivan and Kendler<sup>1</sup> and Li *et al.*<sup>2</sup>). Our previous meta-analysis of the genetic

parameter for ND with 17 twin studies indicated that the weighted mean heritability for ND is 0.56 for adult smokers.<sup>2</sup> Further, we identified that several chromosomal regions are likely to harbor susceptibility loci for ND in the 313 extended Framingham Heart Study (FHS) families.<sup>3</sup> Of these regions, an ~13-cM interval on chromosome 9q22 showed suggestive linkage to ND. The linkage of this region to smoking behaviors at a nominally significant level has been supported by three independent studies.<sup>4–6</sup> Very recently, we determined that the GABA<sub>B</sub> receptor subunit 2 (*GABAB2*) and neurotrophic tyrosine kinase receptor, type 2 (*NTRK2*; or called *TrkB*) genes, located within this region, were significantly associated with ND in our Mid-South Tobacco Family (MSTF) sample.<sup>7,8</sup>

Correspondence: Dr MD Li, Department of Psychiatry and Neurobehavioral Sciences, Section of Neurobiology, University of Virginia, 1670 Discovery Drive, Suite 101, Charlottesville, VA 22911, USA.

E-mail: ml2km@virginia.edu

Received 5 June 2006; revised 24 October 2006; accepted 29 October 2006

The Src homology 2 domain-containing transforming protein C3 (*SHC3*; also named *Rai*, *N-Shc* or *ShcC*) is encoded by a single gene with 12 exons that has been mapped to the 9q21.3–q22.2 region.<sup>9</sup> *SHC3* is a more recently identified neuron-specific member of the Shc-like adaptor protein family,<sup>10–12</sup> which plays a critical role during the transition of proliferating neural stem cells to postmitotic neurons.<sup>13,14</sup> All of the Shc family members link a number of tyrosine kinase receptors with multiple intracellular signaling cascades. *SHC3* is expressed predominantly in the mature neurons of the central nervous system (CNS) and transmits neurotrophin signals from the TrkB receptor to the Ras/mitogen-activated protein kinase (MAPK) pathway,<sup>15,16</sup> which has been implicated in both the reinforcing effects of drugs of abuse and the neural plasticity associated with various addictive drugs.<sup>17–20</sup> With *ShcC*-null mice, it was found that the gene not only mediates TrkB-Ras/MAPK signaling but also is involved in the regulation of *N*-methyl-D-aspartate receptor (NMDAR) function.<sup>21</sup> Furthermore, a recent study revealed a novel function of *Shc3* in the regulation of the neuronal adaptive response to environmental stress, suggesting that *Shc3* functions as a stress-response gene that increases phosphatidylinositol 3-kinase activation and Akt phosphorylation after hypoxic or oxidation insults.<sup>22</sup> As a consequence, *SHC3* expression might protect neuronal cells from stress-induced apoptosis.

As it is located within a suggestive linkage region for ND in the FHS cohort,<sup>3</sup> a result which we now report as replicated in an independent cohort, and being an important adapter protein in several neuronal signaling cascades underlying substance dependencies, *SHC3* represents a plausible candidate for involvement in ND and other substance abuse. No studies of *SHC3* gene in the involvement of ND or other substance abuse have been reported. Thus, this study represents the first designed to determine: (1) if there exists a significant association between *SHC3* and ND; (2) if *SHC3* contributes to the detected linkage signal to ND on chromosome 9; and (3) if nicotine has any regulatory effects on the expression of *Shc3* in the rat brain.

## Materials and methods

### Human study population

The subjects used in this study are of either African-American (AA) or European-American (EA) origin and were recruited primarily from the Mid-South states of Tennessee, Mississippi and Arkansas in the US during 1999–2004. Proband smokers were required to be at least 21 years of age, smoked 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, ND, and personality traits assessed by various questionnaires, available at 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.

In this study, ND was ascertained by the three measures most commonly used in the literature: smoking quantity (SQ: defined as the number of cigarettes smoked per day), the Heaviness of Smoking Index (HSI: 0–6 scale), which includes SQ and smoking urgency (i.e., how soon after waking up does the subject smoke the first cigarette?), and the Fagerström Test for ND (FTND: 0–10 scale).<sup>23</sup> Given the presence of overlap in the contents of the three ND measures, there exists fairly robust correlations among them in the AA ( $r=0.88\sim 0.97$ ), EA ( $r=0.91\sim 0.97$ ) and combined ( $r=0.89\sim 0.97$ ) samples. A detailed description of the demographic and clinical characteristics of the MSTF sample is presented in Table 1.

### DNA extraction, single-nucleotide polymorphisms selection and genotyping

The DNA was extracted from peripheral blood samples of each participant using a kit from Qiagen

Table 1 Characteristics of African-American, European-American and combined samples

Characteristic	African-American	European-American	Combined
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
SQ (# cigarette/day)	19.4 ± 13.3	19.5 ± 13.4	19.5 ± 13.3
HSI	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

Abbreviations: FTND, Fagerström Test for nicotine dependence; HSI, Heaviness of Smoking Index; SQ, smoking quantity.

Inc. (Valencia, CA, USA). Genotyping for microsatellite markers was carried out at the Center for Inherited Disease Research (CIDR). The CIDR marker set used in the study was composed primarily of trinucleotide and tetranucleotide repeats and consists of 404 primer pairs (385 on autosomal chromosomes, 16 on chromosome X and 3 on chromosome Y) with an average spacing of 8.6 cM throughout the genome. There are no gaps in the map larger than 18 cM and the average marker heterozygosity is 0.76.

On the basis of the high heterozygosity (minor allele frequency  $\geq 0.05$ ) and coverage of the gene that was as uniform as possible, 11 single-nucleotide polymorphisms (SNPs) within the intronic, coding and 3 untranslated region regions of *SHC3* were selected from the National Center for Biotechnology Information (NCBI) database; however, none of them leads to change in amino-acid residues of the protein sequence. Table 2 provides detailed information on these SNPs. All SNPs were genotyped using the TaqMan SNP Genotyping Assay in a 384-well micro-

plate format (Applied Biosystems, Foster City, CA, USA). Briefly, 15 ng of DNA was amplified in a total volume of 7  $\mu$ l containing a minor groove binder (MGB) probe and 2.5  $\mu$ l of TaqMan universal polymerase chain reaction (PCR) master mix. Allelic discrimination analysis was performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). To ensure the quality of the genotyping, SNP-specific control samples were added to each 384-well plate.

#### Linkage analysis

Extensive quality checks have been carried out to verify the consistency of marker genotyping and of stated pedigree relationships. The PedCheck program was used to identify any inconsistent Mendelian inheritance, non-paternity or typing errors.<sup>24</sup> The errors identified in PedCheck were assumed to have occurred in the genotyping process and the associated markers were set as missing for the appropriate pedigree. Also, we used all markers to check pedigree

Table 2 Positions, nucleotide variation, minor allele frequency and primer/probe sequences of 11 SNPs within *SHC3*

dbSNP ID	SNP location	Chrom. pos.	Alleles	MAF	Forward (F) and reverse (R) primer and probe (P) sequence (5'-3') probe (P) sequences
rs12519	Exon 12 3' UTR	88857636	A/G	0.39	F: CTGTACAAATTATCCCTCTATATTTATATTTTTT AAGACTAAGAAAGAT R: TGCCTGGACAACACTACAGTTAAACTTT P: TTCTGTGCTGTA/GTGTTC
rs4877041	Intron 10	88883303	G/A	0.42	F: AGAGAAAGCTGACGGGTGTTG R: TTAAGGGAAGGTGGAGCTCCTA P: CCTCTGTGA/GTGACTTGC
rs3750399	Exon 10	88886517	C/T	0.31	F: ACAGAAAACCCACTCATGTCAAAGAG R: TGCGGTCAGCAGTGCT P: AGCCCG/AAGGAAAG
rs4877042	Intron 9	88889712	T/C	0.26	F: GGGCATGAGGCAGTGACT R: CAACAGCAAAAACCCATCTCAAAA P: ACCAATCAGG/ATAGTGCC
rs2297313	Intron 6	88898916	A/G	0.43	F: GCTTTGGTCTGAGGATTCCTTCTAT R: CCAGAGAGACAAAGTCCAGGTTTAA P: TTCCAGAAACTCA/GCTCCAA
rs3818668	Intron 5	88910085	G/C	0.28	F: AACATAGTCAGTTGTGTCTGTTAAAGAA R: CCTGTGTCCCTGACTGTGT P: CTCTCACTTGTG/CATTAGT
rs1547696	Intron 2	88923674	C/T	0.33	F: GCAAGAAACAAATGTTTTTGTATGTTTATGCT R: TTTAGATGATACGTGATTGGTCACTTTGA P: CACAGTTG/AGAATATT
rs1331188	Intron 2	88954941	A/C	0.30	C_8791194_10 <sup>a</sup>
rs1556384	Intron 1	88993681	G/A	0.12	F: GGAGGAGAAGGAGCCCTAGTG R: ACACTGGTGTGGTAAGGATTTTCAA P: TGGTGCAA/GTTTTA
rs4534195	Intron 1	88998202	T/C	0.40	F: CACCTCAGCAGAAGAAATTTCACT R: CCTCGCCTTTAGTCATATTTCTTTATTCA P: CTCCAGCCG/ATGTGTC
rs1411836	Intron 1	89005435	A/G	0.45	F: GTGCCGTGATCAACAGCAACTG R: TCCCAGCAGTATACAAATAGGCT P: TCCAGAAGGTA/GACCTG

Abbreviations: MAF, minor allele frequency for each SNP provided in NCBI SNP database; SNP, single-nucleotide polymorphism; UTR, untranslated region.

<sup>a</sup>The primer and probe sequences were unable to provide because we purchased the assay for this SNP directly from the ABI.

errors by means of the RelCheck program.<sup>25</sup> To avoid bias, individuals with a false consanguineous relationship were excluded from further analysis.

Genotypes from all family members were used to calculate multipoint identity by descent (IBD) allele sharing distributions using the GENIBD program of the S.A.G.E. (v. 5.0) package. SIBPAL, a model-free S.A.G.E. linkage program, was then used to perform the linkage analysis based on all possible sib-pairs. Evidence for linkage was assessed by a Haseman–Elston regression with dependent variable options W3 and W4, which transforms the sib-pair's trait values to a weighted combination of the squared trait difference and squared-mean corrected trait sum, allowing for the non-independence of sib-pairs.<sup>26</sup> In SIBPAL, default options were used for all parameters in the trait regression method except that the options W3 (i.e., the weighted combination of squared trait difference and squared mean-corrected trait sum adjusting for the non-independence of sib-pairs) and W4 (i.e., the non-independence of squared trait sums and differences) were examined. Both options yielded essentially the same results on the three ND measures (SQ, HSI and FTND). Sex and age were entered as covariates in all analyses. The S-Plus (v. 6.1, Seattle, WA, USA) and SAS (v. 8.2, Cary, NC, USA) packages were used to prepare the data in the required format for using linkage programs and to analyze the data generated by the linkage analysis programs.

To assess the contribution of *SHC3* to the detected linkage signal, we repeated the linkage analysis, modeling all 11 SNPs of *SHC3* as covariates in the Haseman–Elston regression. Such an approach is almost identical to that of Iyenger *et al.*<sup>27</sup> and can be implemented using SIBPAL. In theory, including SNPs for *SHC3* as covariates during the linkage analysis effectively eliminates the contribution of the gene to the linkage signal. By comparing the linkage profiles before and after adjustment for *SHC3*, we can determine whether *SHC3* can fully or partially account for the detected linkage signal on chromosome 9.

#### Family-based association analysis

Similarly, we used the PedCheck program to determine genotyping consistency for Mendelian inheritance of all *SHC3* SNPs. To avoid bias, a total of 62 and 15 inconsistencies in the AA and EA samples for *SHC3*, respectively, that is, 0.28 and 0.07% of the 22,352 assays, were treated as missing in further statistical analysis. To verify the quality of our genotyping, we also checked the SNP data for any significant departure from Hardy–Weinberg equilibrium (HWE). The HWE at each locus was assessed by the  $\chi^2$ -test. The allele frequencies for each genetic marker were calculated using the FREQ program of S.A.G.E. (v. 5.0). Pair-wise linkage disequilibrium (LD) among all SNP markers was assessed using Haploview,<sup>28</sup> with the determination of haplotype blocks based on block definitions proposed by Gabriel *et al.*<sup>29</sup>

Associations between individual SNPs and the ND measures were determined by the PBAT program (v. 3.0) using generalized estimating equations.<sup>30</sup> Associations between each ND measure and haplotypes from multiple SNP combinations were examined using the FBAT program (v. 1.55), with the computation of *P*-values for the *Z* statistic based on the Monte Carlo sampling option under the null distribution of no linkage and no association.<sup>31</sup> Three genetic models (additive, dominant and recessive) were tested, with gender and age entered as covariates in the AA and EA samples; gender, age and ethnicity served as covariates for the combined sample. Relationships with the three ND measures were examined individually. All associations found to be significant were corrected for multiple testing according to the SNP spectral decomposition (SNPSpD) approach<sup>32</sup> for individual SNP analysis and using Bonferroni correction (dividing the significance level by the number of major haplotypes with a frequency >5.0%) for haplotype-based analysis.

#### Animals, nicotine administration and brain punches

Adult male Holtzman rats (250–350 g; HSD, Madison, WI, USA) were randomly assigned to nicotine-treated and control groups. Nicotine bitartrate was administered via osmotic minipumps (Model 2ML1, Azlet Corp., Palo Alto, CA, USA) at a daily dose of 3.15 mg/kg (calculated as the free base) in saline (pH 7.4) for 7 days. Rats in the saline-administered control group were otherwise handled and treated identically. All rats were housed at 22°C on a 12-h light/dark cycle with standard laboratory rat chow and water freely available.

After 7 days of nicotine treatment, rats were anesthetized with isoflurane, and the brains were removed immediately for sectioning. Coronal 2-mm sections were prepared using a Stoelting tissue slicer (Chicago, IL, USA). Punches were excised from the prefrontal cortex (PFC), NA, striatum, amygdala, hippocampus (HP), medial basal hypothalamus (MBH) and ventral tegmental area (VTA) using a brain punch tissue set from myNeuroLab.com (St Louis, MO, USA) employing coordinates from Paxinos and Watson.<sup>33</sup> All animal-related experimental procedures were approved by the Institutional Animal Use and Care Committee.

#### RNA isolation, reverse transcription and real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse transcribed with the random hexamer primers according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The TaqMan MGB probe and primers were designed according to the cDNA sequence of rat *Shc3* (GenBank Accession No. XM\_341498), and were synthesized by Applied Biosystems (Foster, CA, USA). The forward and reverse primer sequences were 5-GCCCCGATCA CCCGTACTA-3' and 5'-TCAATCGAGCATCGAGAAA CC-3' with an expected PCR product size of 85 bp.

The MGB probe was 5'-AACAGCGTTCCCAACAA-3'. The PCR amplification of 2  $\mu$ l of cDNA was carried out in a total volume of 20  $\mu$ l according to the instruction manual for TaqMan Gene Expression Assays (Applied Biosystems, Foster, CA, USA). The mRNA level of each sample was determined using a standard calibration method<sup>34</sup> and normalized to the level of 18S rRNA in each sample.

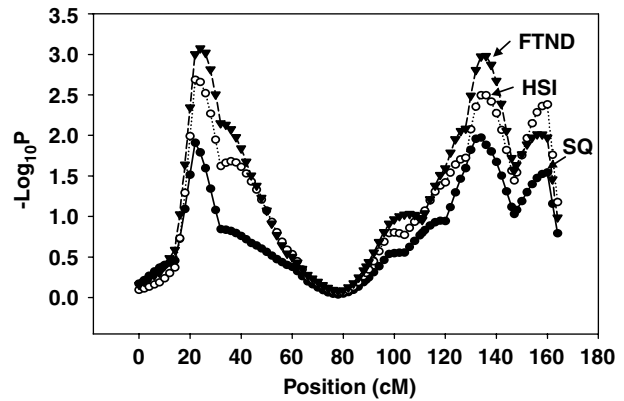
#### Western blotting analysis

Total protein was extracted from individual frozen brain punches by homogenization with a sonicator in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodiumdodecylsulfate (SDS)), and the protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Thirty micrograms of total protein were separated by 8% SDS-polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membranes (0.45  $\mu$ m) at 80 V for 1.5 h. Then the membrane was hybridized overnight at 4°C with mouse anti-Shc3 antibody (dilution 1:5000; BD Biosciences, San Jose, CA, USA). After exposure to horseradish peroxidase-conjugated secondary antibodies at 4°C for 3 h, the membranes were exposed to X-ray films. The membranes were then stripped and re-probed with antibody to  $\beta$ -actin (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was used for normalization of the protein content in each sample. Finally, the films were scanned for quantitative analysis with ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA, USA). The significance of differences between the nicotine-treated and control groups was analyzed by Student's *t*-test, and *P*-values less than 0.05 were considered statistically significant.

## Results

#### Independent replication of 'suggestive' linkage of chromosome 9q22-q23 to ND

Previously, we identified a region on chromosome 9 that showed a 'suggestive' linkage to ND, as assessed by the number of cigarettes smoked per day, in the FHS sample.<sup>3</sup> To determine if such linkage could be replicated in an independent cohort, we performed linkage analysis on the MSTF sample. As shown in Figure 1, we found a 'suggestive' linkage of FTND to chromosome 9 ( $P=0.00105$  at 136 cM) based on the threshold suggested by Nyholt.<sup>35</sup> In comparing the linkage regions on chromosome 9 identified in both the FHS and MSTF samples, we found these regions overlapped substantially, thus providing an independent replication of the association for this region with ND. Although the *P*-values for linkage of this region to SQ ( $P=0.0107$  at 134 cM) and HSI ( $P=0.0032$  at 136 cM) failed to meet the threshold required for a claim of a suggestive linkage, peaks for both these ND measures were identified within this region (see Figure 1). Additionally, another peak on chromosome 9 at the range of 10–50 cM showed a 'suggestive'



**Figure 1** Linkage analysis results for SQ, HSI and FTND on chromosome 9 in the Mid-South Tobacco Family (MSTF) cohort, using the SIBPAL program of S.A.G.E. (v. 5.0).

linkage to the FTND ( $P=0.000096$  at 26 cM). A detailed genomewide linkage analysis of the MSTF cohort is planned for a separate report. On the basis of our linkage analysis results from both the FHS and MSTF cohorts, we initiated a search for candidate genes for ND within this region near the peak at 136 cM on chromosome 9. Within this region, *SHC3* is of considerable interest on the basis of its biological functions.

#### Association between individual SNPs of SHC3 and ND

Based on the results from  $\chi^2$  tests, we found that all 11 SNPs for *SHC3* were in HWE in the AA, EA and combined samples, respectively, indicating no genotyping errors existed in our data set. Individual SNP analysis using the PBAT-GEE program indicated nominally significant associations for 2 of 11 SNPs for *SHC3*, rs3818668 and rs1547696, with two age-, gender- and ethnicity-adjusted ND measures in the combined sample. These associations were no longer significant after correction for multiple testing by the SNPSPD approach.<sup>32</sup> We also analyzed the 11 SNPs for each ethnic group separately. Three SNPs (rs12519, rs2297313 and rs1547696) in the AA sample and one SNP (rs1556384) in the EA sample yielded significant associations with at least one ND measure under different genetic models. Once again, none were significant after correction for multiple testing (Table 3).

#### Association between haplotypes of SHC3 and ND

Using the FBAT program, haplotype-based association analysis was performed for different combinations of the 11 SNPs within *SHC3*. Figure 2 shows the pair-wise  $|D'|$  values and predicted haplotype blocks for the 11 SNPs within *SHC3* in the combined, AA and EA samples. In the AA and combined samples, we found a major haplotype, A-C-T-A-T-A, formed by SNPs rs12519, rs3750399, rs4877042, rs2297313, rs1547696 and rs1331188, at a frequency of 27.8 and 17.6%, respectively, which demonstrated a significant inverse association with all three adjusted ND

Table 3 Minor allelic frequencies and *P*-values for associations of individual *SHC3* SNPs with three ND measures in the AA, EA and combined samples

SNP ID	African-American sample				European-American sample				Combined sample			
	Frequency*	SQ	HSI	FTND	Frequency*	SQ	HSI	FTND	Frequency*	SQ	HSI	FTND
rs12519	0.2630	<b>0.04<sup>d</sup></b>	0.09 <sup>d</sup>	0.07 <sup>r</sup>	0.4989	0.79 <sup>d</sup>	0.68 <sup>r</sup>	0.29 <sup>r</sup>	0.338	0.06 <sup>d</sup>	0.13 <sup>d</sup>	0.10 <sup>r</sup>
rs4877041	0.1923	0.35 <sup>r</sup>	0.38 <sup>r</sup>	0.31 <sup>r</sup>	0.4358	0.33 <sup>d</sup>	0.20 <sup>r</sup>	0.32 <sup>r</sup>	0.268	0.93 <sup>r</sup>	0.62 <sup>r</sup>	0.64 <sup>r</sup>
rs3050399	0.2196	0.72 <sup>r</sup>	0.43 <sup>d</sup>	0.45 <sup>r</sup>	0.3533	0.65 <sup>a</sup>	0.44 <sup>a</sup>	0.71 <sup>a</sup>	0.261	0.63 <sup>a</sup>	0.92 <sup>d</sup>	0.68 <sup>d</sup>
rs4877042	0.4505	0.15 <sup>r</sup>	0.12 <sup>d</sup>	0.13 <sup>d</sup>	0.1980	0.65 <sup>a</sup>	0.69 <sup>d</sup>	0.59 <sup>r</sup>	0.440	0.17 <sup>r</sup>	0.11 <sup>d</sup>	0.11 <sup>r</sup>
rs2297313	0.2232	0.10 <sup>d</sup>	<b>0.05<sup>d</sup></b>	<b>0.05<sup>d,r</sup></b>	0.4616	0.35 <sup>d</sup>	0.33 <sup>r</sup>	0.49 <sup>r</sup>	0.297	0.31 <sup>d</sup>	0.28 <sup>d</sup>	0.19 <sup>r</sup>
rs3818668	0.3276	0.06 <sup>a</sup>	0.06 <sup>a</sup>	0.10 <sup>a</sup>	0.2416	0.20 <sup>a</sup>	0.15 <sup>d</sup>	0.28 <sup>d</sup>	0.462	<b>0.03<sup>a</sup></b>	<b>0.03<sup>a</sup></b>	0.06 <sup>a</sup>
rs1547696	0.3520	<b>0.02<sup>d,r</sup></b>	<b>0.03<sup>d,r</sup></b>	<b>0.05<sup>d</sup></b>	0.3153	0.36 <sup>d</sup>	0.31 <sup>d</sup>	0.24 <sup>d</sup>	0.456	<b>0.009<sup>d,r</sup></b>	<b>0.02<sup>d,r</sup></b>	<b>0.03<sup>d,r</sup></b>
rs1331188	0.2777	0.35 <sup>d</sup>	0.29 <sup>d</sup>	0.35 <sup>d</sup>	0.1869	0.51 <sup>d</sup>	0.43 <sup>d</sup>	0.31 <sup>d</sup>	0.249	0.26 <sup>d</sup>	0.22 <sup>d</sup>	0.26 <sup>d</sup>
rs1556384	0.0312	0.78 <sup>a</sup>	0.71 <sup>a</sup>	0.74 <sup>a</sup>	0.1182	<b>0.02<sup>a</sup>,0.04<sup>d,r*</sup></b>	0.09 <sup>a</sup>	0.06 <sup>a</sup>	0.058	0.18 <sup>a</sup>	0.15 <sup>r</sup>	0.16 <sup>r</sup>
rs4534195	0.3572	0.35 <sup>r</sup>	0.38 <sup>r</sup>	0.30 <sup>r</sup>	0.3597	0.30 <sup>d</sup>	0.37 <sup>d</sup>	0.26 <sup>d</sup>	0.358	0.24 <sup>d</sup>	0.24 <sup>r</sup>	0.15 <sup>r</sup>
rs1411836	0.0150	0.32 <sup>a</sup>	0.23 <sup>a</sup>	0.12 <sup>a</sup>	0.1376	0.07 <sup>d</sup>	0.08 <sup>r</sup>	0.09 <sup>d</sup>	0.052	0.08 <sup>d</sup>	0.08 <sup>r</sup>	0.09 <sup>d</sup>

\*Indicates minor allele frequency for each SNP.

Significant associations at the 0.05 significance level are bold.

Superscripts indicate genetic models used for analysis: a = additive, d = dominant and r = recessive.

For Combined sample, the three ND measures were adjusted for age, gender and ethnicity; for each ethnic-specific sample, only age and gender were used as covariates.

measures in the AA sample ( $Z = -2.74$  to  $-2.99$ ; permutation  $P$ -values = 0.0061–0.0028) and the combined sample ( $Z = -2.60$  to  $-3.03$ ; permutation  $P$ -values = 0.0093–0.0025; with 135 and 35 families contributing to the analysis for the additive and recessive models, respectively; Table 4). These associations remained significant after Bonferroni correction for testing eight major haplotypes in the AA sample and six in the combined sample with all three ND measures (adjusted significance levels are 0.0063 and 0.0083, respectively, for eight and six major haplotypes).

We identified another major haplotype, C-T-A-G-T (with a frequency of 44.7% in the AA sample and 30.6% in the combined sample), formed by SNPs rs3750399, rs4877042, rs2297313, rs3818668 and rs1547696, that revealed a significant negative association with all three adjusted ND measures under the recessive model for five major haplotypes in the AA sample ( $Z = -2.39$  to  $-2.55$ ; permutation  $P$ -values = 0.0170–0.0106; with 92 families contributing to the analysis; Table 5) and the combined sample ( $Z = -2.43$  to  $-2.62$ ; permutation  $P$ -values = 0.0153–0.0087; with 93 families contributing to the analysis; Table 5). This haplotype remained significantly associated only with SQ and HSI after Bonferroni correction (adjusted significance level is 0.01; Table 5).

For the EA sample, the haplotype A-G-T-G of rs1331188–rs1556384–rs4534195–rs1411836, at a frequency of 10.6%, showed a significant inverse association with the three ND measures ( $Z = -2.13$  to  $-2.74$ ; permutation  $P$ -values = 0.0355–0.0062; with 33 families involved in the analysis; Table 5) under the additive and dominant models. These associations remained significant for SQ and FTND after Bonfer-

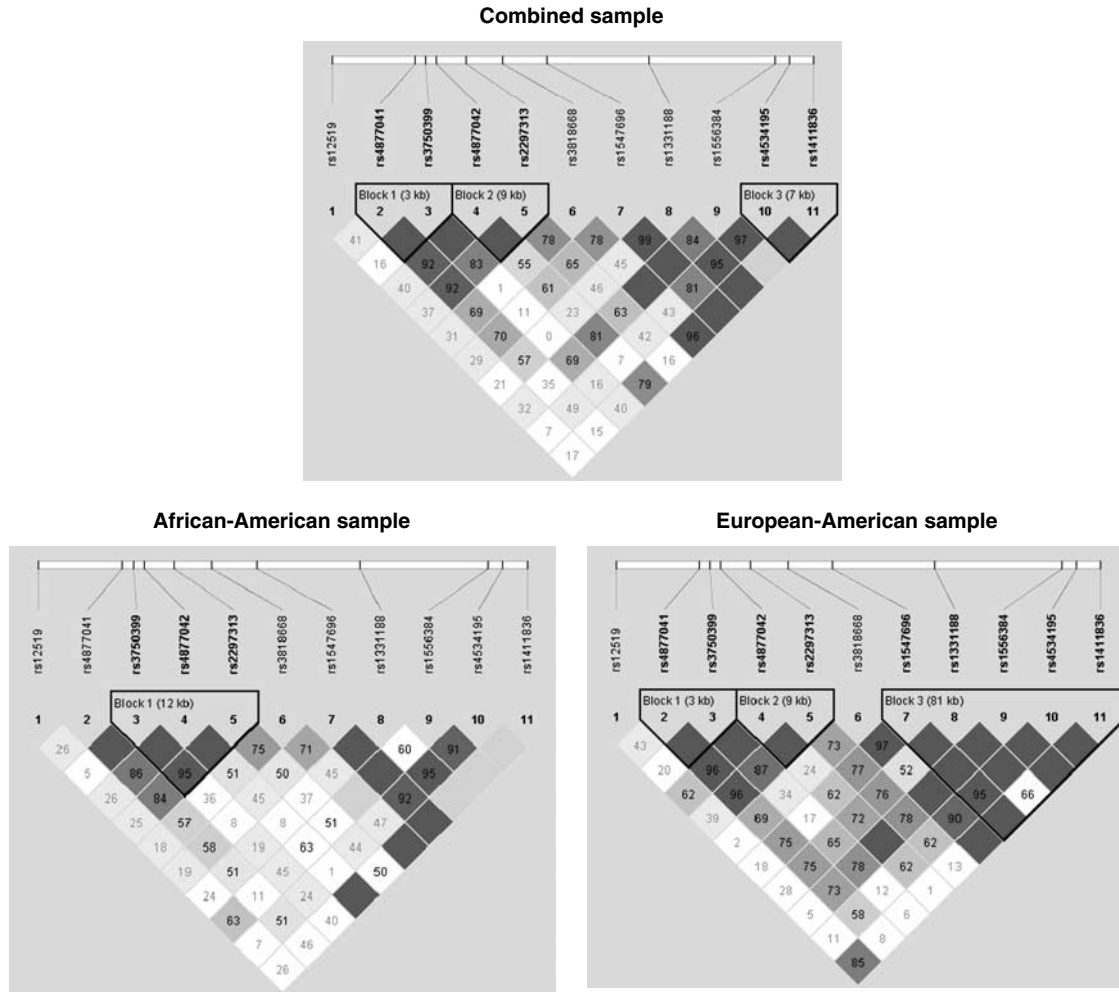
roni correction for testing of five major haplotypes (adjusted significance level is 0.01).

#### Determination of contribution of *SHC3* to linkage signal on chromosome 9

To determine whether *SHC3* accounts for the linkage signal detected on chromosome 9, we performed another linkage analysis by including all 11 SNPs for *SHC3* as covariates to eliminate the contribution of the gene to the linkage signal. By comparing the linkage analysis results before and after correction for *SHC3*, we found that inclusion of SNPs for *SHC3* as covariates reduced, but did not completely eliminate, the linkage signal (see Figure 3). The inclusion of *SHC3* SNPs decreased the detected linkage signal on chromosome 9 by 40.1, 46.4 and 59.2% for SQ, HSI and FTND score, respectively. These results indicate that *SHC3* does indeed contribute to the linkage signal detected on chromosome 9 for ND.

#### Regulation of *Shc3* mRNA by nicotine in rat brain

To identify the regulatory effects of chronic nicotine treatment on expression of *Shc3* (an orthologue of the human *SHC3* gene) mRNA in rat brain, a real-time RT-PCR assay was employed. Figure 4 presents the statistical analysis results of *Shc3* mRNA in seven brain regions in nicotine-treated and control rats. After normalization by the corresponding 18S rRNA level of each sample, we found that the mRNA level of *Shc3* was upregulated by 60% in the striatum ( $P < 0.01$ ) and downregulated by 22% in the NA ( $P < 0.01$ ) of the nicotine-treated rats compared with saline-treated controls. No significant differences in the mRNA levels of *Shc3* were detected in the PFC, amygdala, MBH, HP or VTA between the two experimental groups.



**Figure 2** Haploview-generated LD map of the 11 SNPs within *SHC3* in the combined, AA and EA samples. Regions of high LD ( $D' = 1$  and  $\text{LOD} > 2$ ) are shown in dark gray. Markers with lower LD ( $0.21 < D' < 1$  and  $\text{LOD} > 2$ ) are shown in dark through light gray, with the color intensity decreasing with decreasing  $D'$ -value. Regions of low LD and low LOD scores ( $\text{LOD} < 2$ ) are shown in white. The number within each box indicates the  $D'$  statistic value between the corresponding two SNPs. Haplotype blocks in the three samples were produced by the Haploview program with the option of using block definitions proposed by Gabriel *et al.*<sup>29</sup>

#### Protein expression changes of *Shc3* in rat brain

To determine if the protein level of *Shc3* is also altered by nicotine, we performed Western blotting analysis of the seven brain regions, in which two bands corresponding to isoforms p54 and p65 of *Shc3* could be detected for each sample (Figure 5). Because the expression of p65 isoform is less abundant or undetectable in most regions, only the p54 isoform was examined. We also conducted Western blotting analysis for  $\beta$ -actin on the same samples/gels to check the loading efficiency and protein concentration of each sample. After normalization, we found that the level of *Shc3* p54 isoform was reduced by 38.0% in the NA ( $P < 0.05$ ) of the nicotine-treated rats compared with saline-treated controls. No expression differences at the protein level were detected in the other six regions. By comparing expression trends of *Shc3* in response to nicotine, we found a parallel

pattern between the mRNA and p54 isoform expression profiles in the NA. The difference was in the striatum, in which no significant difference was obtained at the protein level, but a significant difference was found at the mRNA level (60%;  $P < 0.01$ ).

#### Discussion

Previously, we reported identification of an approximately 13-cM region on chromosome 9q22–q23 that showed a 'suggestive' linkage to SQ in the FHS sample.<sup>3</sup> In this study, we demonstrated an independent replication of this linkage in the MSTF cohort. By searching this linked region on chromosome 9, we identified several plausible candidate genes for ND and provided strong evidence that two of them, *GABAB2* and *NTRK2*, are significantly associated

Table 4 Z- and permutation P-values for associations of significant and major SHC3 haplotypes formed by rs12519-rs3750399-rs4877042-rs2297313-rs1547696-rs1331188 with three ND measures

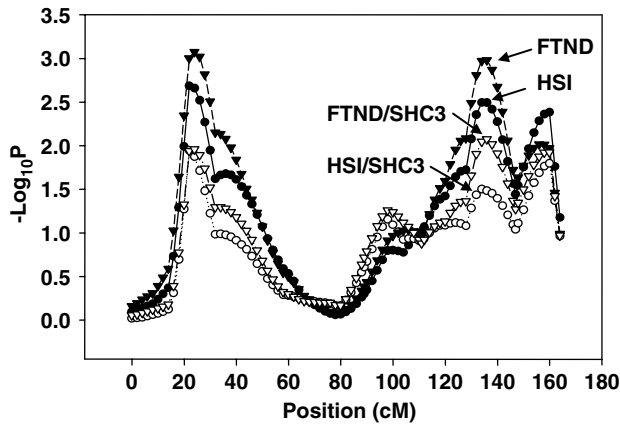
Haplotype	African-American sample				European-American sample				Combined sample			
	%	SQ	HSI	FTND	%	SQ	HSI	FTND	%	SQ	HSI	FTND
A-C-T-A-T-A	27.8	-2.99 <sup>a</sup> (0.0028)	-2.80 <sup>a</sup> (0.0052)	-2.78 <sup>a</sup> (0.0055)	0.5	DNS	DNS	DNS	17.6	-3.03 <sup>a</sup> (0.0025)	-2.86 <sup>a</sup> (0.0043)	-2.82 <sup>a</sup> (0.0048)
		-2.74 <sup>a</sup> (0.0061)	-2.60 <sup>a</sup> (0.0094)	-2.55 <sup>a</sup> (0.0108)						-2.78 <sup>a</sup> (0.0054)	-2.65 <sup>a</sup> (0.0082)	-2.60 <sup>a</sup> (0.0093)
G-C-C-G-C-A	7.5	2.04 <sup>d</sup> (0.0417)	2.25 <sup>d</sup> (0.0243)	2.30 <sup>d</sup> (0.0213)	30.2	-0.68 <sup>d</sup> (0.4986)	-0.86 <sup>d</sup> (0.3882)	-0.51 <sup>d</sup> (0.6112)	15.8	1.12 <sup>d</sup> (0.2616)	0.96 <sup>d</sup> (0.3395)	1.22 <sup>d</sup> (0.2223)
A-C-C-G-C-A	6.5	0.97 <sup>d</sup> (0.3335)	1.17 <sup>d</sup> (0.2407)	1.40 <sup>d</sup> (0.1621)	13.8	1.73 <sup>a</sup> (0.0839)	1.29 <sup>a</sup> (0.1971)	1.48 <sup>a</sup> (0.1400)	9.2	1.85 <sup>d</sup> (0.0640)	1.85 <sup>a</sup> (0.0644)	2.20 <sup>a</sup> (0.0277)
Global P-value		0.4386 <sup>a</sup> 0.0228 <sup>a</sup>	0.4235 <sup>a</sup> 0.0342 <sup>a</sup>	0.4016 <sup>a</sup> 0.0387 <sup>a</sup>		0.5905 <sup>a</sup> 0.7541 <sup>d</sup>	0.7231 <sup>a</sup> 0.9057 <sup>d</sup>	0.8431 <sup>a</sup> 0.9250 <sup>d</sup>		0.6576 <sup>a</sup> 0.0205 <sup>a</sup>	0.7745 <sup>a</sup> 0.0302 <sup>a</sup>	0.6532 <sup>a</sup> 0.0339 <sup>a</sup>

Abbreviations: DNS, data not shown; FTND, Fagerström Test for nicotine dependence; HSI, Heaviness of Smoking Index; SQ, smoking quantity. Adjusted P-values at the 0.05 significance levels after Bonferroni correction for eight major haplotypes in the AA sample are 0.0063 and for six major haplotypes in the combined samples are 0.0083, respectively. Associations that remained significant after correction for multiple testing are bold. Superscripts indicate genetic models used in the analysis: a = additive, d = dominant and r = recessive. The ND measures used in the analysis were corrected for age, gender and ethnicity in the combined samples and for age and gender in each individual ethnic sample. DNS (haplotype frequency < 5%).

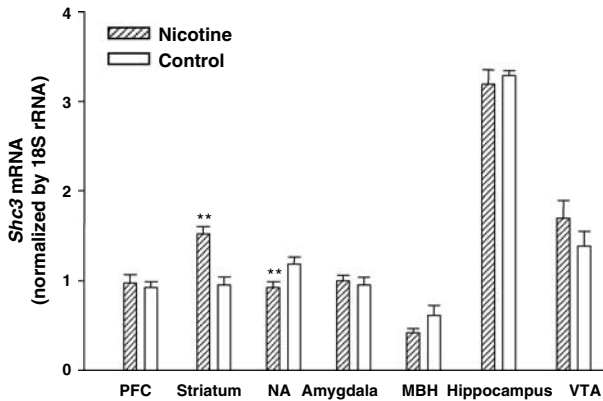
Table 5 Z- and permutation P-values for associations of significant and major haplotypes formed by a five- or four-SNP combination within SHC3 for with three ND measures

No. of SNPs and haplotype	African-American sample				European-American sample				Combined sample			
	%	SQ	HSI	FTND	%	SQ	HSI	FTND	%	SQ	HSI	FTND
Four-SNP* A-C-T-G	2.9	0.52 <sup>a</sup> (0.6015)	0.56 <sup>a</sup> (0.5750)	0.53 <sup>a</sup> (0.5943)	10.6	-2.74 <sup>a</sup> (0.0062)	-2.34 <sup>a</sup> (0.0191)	-2.73 <sup>a</sup> (0.0064)	5.4	-1.66 <sup>a</sup> (0.0974)	-1.26 <sup>a</sup> (0.2073)	-1.56 <sup>a</sup> (0.1194)
Global P-value		0.4204 <sup>a</sup>	0.4549 <sup>a</sup>	0.4203 <sup>a</sup>		-2.71 <sup>d</sup> (0.0068)	-2.13 <sup>d</sup> (0.0335)	-2.55 <sup>d</sup> (0.0107)		0.1238 <sup>a</sup> 0.2427 <sup>a</sup>	0.1550 <sup>a</sup>	0.3251 <sup>a</sup>
Five-SNP+ C-T-A-G-T	44.7	-2.55 <sup>a</sup> (0.0106)	-2.52 <sup>a</sup> (0.0118)	-2.39 <sup>a</sup> (0.0170)	6.0	-0.68 <sup>a</sup> (0.4987)	-0.95 <sup>a</sup> (0.3426)	-0.69 <sup>a</sup> (0.4876)	30.6	-2.62 <sup>a</sup> (0.0087)	-2.56 <sup>a</sup> (0.0104)	-2.43 <sup>a</sup> (0.0153)
C-C-G-C-C	12.9	2.11 <sup>d</sup> (0.0347)	2.43 <sup>d</sup> (0.0150)	2.62 <sup>d</sup> (0.0087)	43.0	1.66 <sup>a</sup> (0.0973)	0.97 <sup>a</sup> (0.3304)	1.00 <sup>a</sup> (0.3168)	24.1	1.38 <sup>a</sup> (0.1689)	1.25 <sup>a</sup> (0.2122)	1.61 <sup>a</sup> (0.1076)
Global P-value		0.0312 <sup>a</sup> 0.4272 <sup>d</sup>	0.0411 <sup>a</sup> 0.2846 <sup>d</sup>	0.0556 <sup>a</sup> 0.2312 <sup>d</sup>		0.9989 <sup>a</sup> 0.2417 <sup>a</sup>	0.8452 <sup>a</sup> 0.5350 <sup>a</sup>	0.8283 <sup>a</sup> 0.5982 <sup>d</sup>		0.0709 <sup>a</sup> 0.7061 <sup>a</sup>	0.0459 <sup>a</sup> 0.8031 <sup>a</sup>	0.0624 <sup>a</sup> 0.7098 <sup>a</sup>

Abbreviations: FTND, Fagerström Test for ND; HSI, Heaviness of Smoking Index; SHC3, Src homology 2 domain-containing transforming protein C3; SNP, single-nucleotide polymorphisms; SQ, smoking quantity. Adjusted P-values at the 0.05 significance levels after Bonferroni correction for five major haplotypes in the AA and combined samples are 0.01. Associations that remained significant after correction for multiple testing are bold. Superscripts indicate the genetic models used in the analysis: a = additive, d = dominant, and r = recessive. The ND measures used in the analysis were corrected for age, gender and ethnicity for the Combined sample and for age and gender in each ethnicity-specific sample. \*rs1331188-rs1556384-rs4534195-rs1411836; +rs3750399-rs4877042-rs2297313-rs3818668-rs1547696.



**Figure 3** Determination of contribution of *SHC3* variants to the detected linkage signal for HSI and FTND. The approach used in linkage analysis was the same as used to produce the data in Figure 1 except that all SNPs within *SHC3* were included as covariates in these linkage analyses.



**Figure 4** Comparison of mRNA levels of *Shc3* in nicotine-treated and control groups in seven rat brain regions after 7 days of nicotine administration. Nicotine increased *Shc3* mRNA by 60% in the striatum and decreased it by 22% in the NA. Values are given in mean  $\pm$  s.e.m. (\*\* $P < 0.01$ ;  $n = 5-8$ /group).

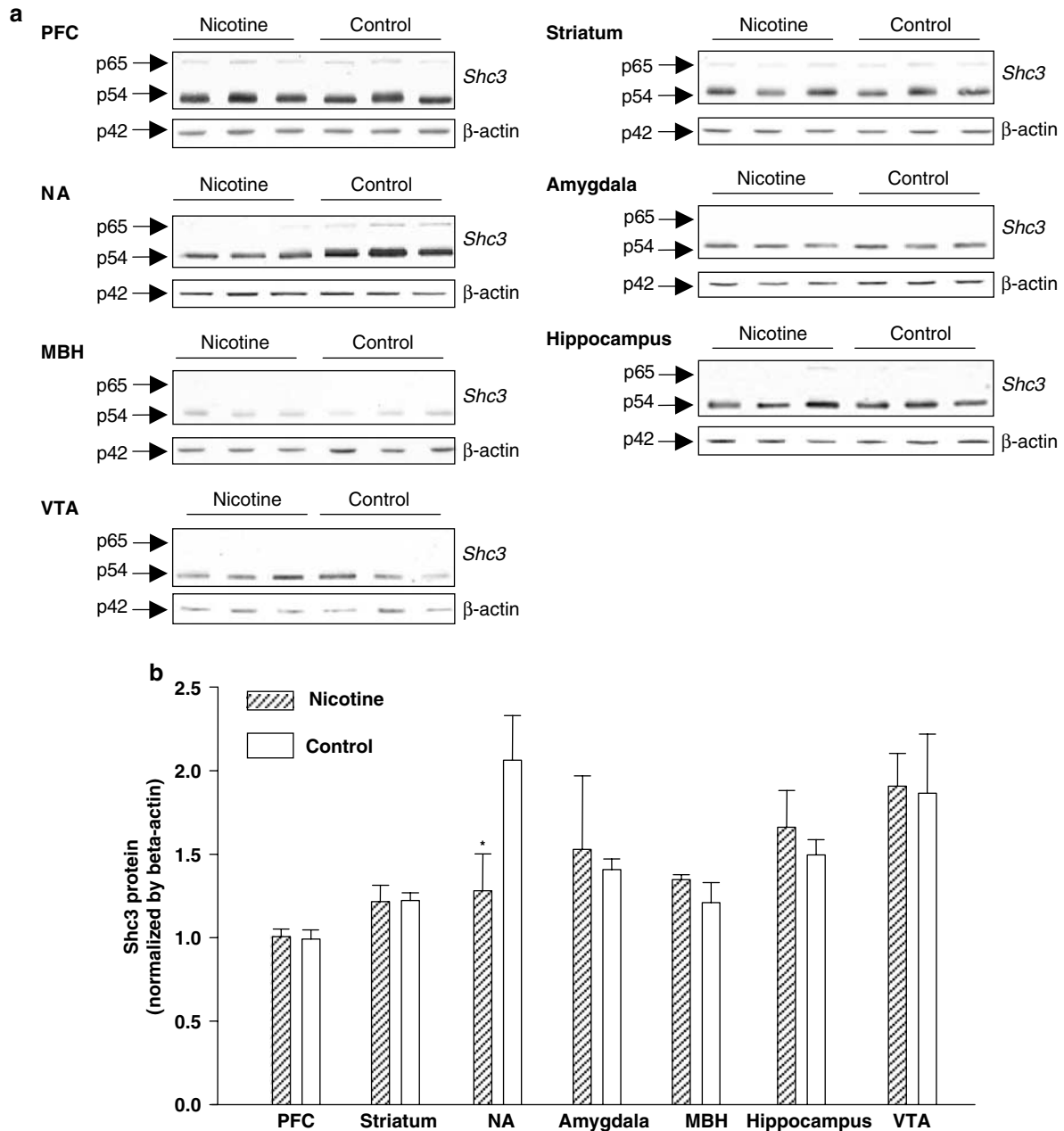
with ND in the MSTF cohort.<sup>7,8</sup> Subsequently, we demonstrated that the mRNA and protein levels of *Gpr51* and *Ntrk2* (orthologues of human *GABAB2* or *GPR51* and human *NTRK2*, respectively) are significantly modulated by nicotine in multiple rat brain regions.<sup>36</sup> This provides further experimental evidence that these genes play a significant role in ND. *SHC3* is another candidate gene within the linked region based on its biological functions in activating the PI3K-PKB and MAPK pathways,<sup>14,37</sup> in regulating NMDA receptor function,<sup>21</sup> and the neuronal adaptive response to environmental stress.<sup>22</sup>

In this study, 11 SNPs within *SHC3*, as well as the haplotypes of various combinations of these SNPs, were analyzed for associations with three adjusted ND measures in 602 nuclear families of either AA or EA origin. In light of the ethnic-specific character-

istics of the SNPs among different ethnic groups<sup>29,38</sup> as well as known ethnic differences in ND and nicotine metabolism,<sup>39,40</sup> we not only performed association analysis on the combined sample (to increase power) but also on ethnicity-specific samples separately. We identified two major haplotypes, A-C-T-A-T-A at a frequency of 27.8 and 17.6%, respectively, and C-T-A-G-T at 44.7 and 30.6%, respectively, in the AA and combined samples. Another major haplotype, A-G-T-G, at a frequency of 10.6% in the EA sample, exhibited significant inverse association with at least two ND measures after Bonferroni correction. Thus, these three haplotypes appear to have a protective role in preventing the development of ND. Moreover, the detection of different and significant haplotypes between the two ethnic groups are reinforced by the LD analysis results in which only one block (i.e., rs3750399–rs4877042–rs2297313) was identified in the AA sample whereas three blocks (i.e., rs4877041–rs3750399, rs4877042–2297313 and rs1547696–rs1331188–rs1556384–rs4534195–rs1411836) were identified in the EA sample (Figure 2). These findings indicate haplotype specificity exists across ethnic populations, with differences in the associations between AA and EA samples most likely attributable to the ethnic differences in the distributions of SNP and haplotype frequencies.

Our genetic study has several unique strengths, including a large family-based sample of extensively phenotyped smokers and nonsmokers. Our sample is significantly larger than those used in most previous studies of ND (for reviews see *Batra et al.*<sup>41</sup> and *Noble*<sup>42</sup>), and thus provides greater power to detect potential associations of genetic variants with ND. Furthermore, our sample represents two major ethnic groups in the US, permitting an examination of genetic differences in ND across these populations. Finally, we used a family-based approach for association analysis, which has the advantage over unconditional analysis (e.g., case-control) in that it minimizes the potential for the confounding effects of population stratification.

On the basis of the significant association between *SHC3* and ND and further demonstration of 40.1–59.2% contribution by *SHC3* variants (depending on the ND measure) to the detected linkage peak on chromosome 9, we subsequently investigated the regulatory effect of nicotine on *Shc3* in seven rat brain regions. Our molecular studies demonstrated that chronic nicotine administration significantly modulated the mRNA level of *Shc3* in the striatum and NA, and the protein level in the NA. The difference noticed between mRNA and protein for *Shc3* in the striatum (60% increase in mRNA vs no change in protein) may be due to the regulation by nicotine at the translation level in these regions or protein degradation after translation. Given that *Shc3* is a critical adaptor protein in mediating the TrkB-Ras/MAPK signaling pathway and in the regulation of NMDAR function, we suspect *SHC3* to be a major



**Figure 5** Western blotting analysis of Shc3 expression in seven rat brain regions. **(a)** Western blotting images for Shc3 and  $\beta$ -actin in seven rat brain regions. **(b)** Statistical analysis results of the Shc3 protein level normalized by the corresponding  $\beta$ -actin level, which indicated that nicotine decreases Shc3 protein by 38% in the NA. Values are given in mean  $\pm$  s.e.m. (\* $P < 0.05$ ;  $n = 3$ /group).

factor in regulating the physiological effects of addictive drugs on the brain. This is primarily because both the MAPK pathway and NMDARs have been demonstrated to play a crucial role in neural development, synaptic plasticity, learning and memory, and are regulated by various addictive drugs including nicotine, ethanol, cocaine, morphine and tetrahydrocannabinol.<sup>18,43–47</sup> Further, many recent reports have shown that activation of the extracellular signal-regulated kinase pathway (ERK), an important member of the MAPK pathway, depends on both

dopamine receptors (e.g., D1) and NMDARs in the regions involving reward circuitry innervated by dopamine neurons.<sup>20,46,48,49</sup> It is likely that the mechanism underlying regulation of Shc3 by nicotine is the specific modulation of the phosphorylation state of ERK by stimulation of dopamine secretion and expression of dopamine or glutamate receptors in the dopaminergic areas. This activity subsequently changes the downstream intracellular cascade signaling transductions related to drug addiction. As the adaptor between ERK and the downstream signaling

pathway, expression of *Shc3* is changed along with the state of ERK phosphorylation.

Another interesting finding is that the three significant and major haplotypes identified are all negatively associated with ND in both ethnic populations. This implies that the *SHC3* gene functions to protect against the development of ND. Such a protective effect might be explained by the stress-ameliorating function of *SHC3*.<sup>22</sup> For example, Troglio et al.<sup>22</sup> reported that *ShcC*-null mice had significantly higher mortality, more severe neurological deficits, and increased apoptosis and size of the infarct area, compared with wild-type mice. Several epidemiological studies have documented strong associations between depression and smoking;<sup>50,51</sup> however, the presence of a causal relation between depression and smoking is unknown. Findings from two studies suggest that the relation may not be causal, but rather entirely the result of genetic factors that predispose to both disorders.<sup>52,53</sup> Thus, in view of the findings from this study and the known biological function of *SHC3*, it is reasonable to hypothesize that *SHC3* functions in a protective role for depression as well.

In summary, we provided a replication of 'suggestive' linkage of chromosome 9q22–q23 to ND in an independent cohort. Further, we demonstrated that *SHC3* is significantly associated with ND in both AA and EA samples and contributes 40.1–59.2% of the linkage signals detected on chromosome 9 for the three ND measures. Our family-based association analysis revealed three major haplotypes that were protective against the development of ND. Animal experimentation demonstrated that the expression of *Shc3* was regulated by nicotine in the NA at both the mRNA and protein levels and in the striatum at the mRNA level. These findings indicate that *SHC3* is an important gene in the etiology of ND, implicating its importance as a biological candidate in future investigations of ND.

## Acknowledgments

This project is funded by NIH Grants DA-12844 and DA-13783 to MDL. We thank the Center for Inherited Disease Research (CIDR) for performing the MSTF genome scan. Detailed information on laboratory methods and markers can be found at <http://www.cidr.jhmi.edu>. Some of the results of this paper were obtained using the program S.A.G.E. (v. 5.0), which is supported by a US Public Health Service Resource Grant (RR03655) from the National Center for Research Resources. We also thank the Rutgers University Cell and DNA Repository, the contractor for the NIDA Center for Genetic Studies, co-directed by Dr Jay Tischfield and Dr John Rice.

## References

1 Sullivan PF, Kendler KS. The genetic epidemiology of smoking. *Nicotine Tob Res* 1999; **1**(Suppl 2): S51–S57; discussion S69–S70.

- 2 Li MD, Cheng R, Ma JZ, Swan GE. A meta-analysis of estimated genetic and environmental effects on smoking behavior in male and female adult twins. *Addiction* 2003; **98**: 23–31.
- 3 Li MD, Ma JZ, Cheng R, Dupont R, Williams KC, Payne TJ et al. A genome-wide scan to identify loci for smoking rate in the Framingham Heart Study populations. *BMC Genet* 2003; **4**(Suppl 1): S103.
- 4 Bergen AW, Korczak JF, Weissbecker KA, Goldstein AM. A genome-wide search for loci contributing to smoking and alcoholism. *Genet Epidemiol* 1999; **17**(Suppl 1): S55–S60.
- 5 Bierut LJ, Rice JP, Goate A, Hinrichs AL, Saccone NL, Foroud T et al. A genomic scan for habitual smoking in families of alcoholics: common and specific genetic factors in substance dependence. *Am J Med Genet* 2004; **124A**: 19–27.
- 6 Gelernter J, Liu X, Hesselbrock V, Page GP, Goddard A, Zhang H. Results of a genomewide linkage scan: support for chromosomes 9 and 11 loci increasing risk for cigarette smoking. *Am J Med Genet* 2004; **128B**: 94–101.
- 7 Beuten J, Ma JZ, Payne TJ, Dupont RT, Crews KM, Somes G et al. Single- and multilocus allelic variants within the GABAB receptor subunit 2 (GABAB2) gene are significantly associated with nicotine dependence. *Am J Hum Genet* 2005; **76**: 859–864.
- 8 Beuten J, Ma JZ, Payne TJ, Dupont RT, Lou XY, Crews KM et al. Association of specific haplotypes of neurotropic tyrosine kinase receptor 2 (NTRK2) gene with vulnerability to nicotine dependence in African-Americans and European-Americans. *Biol Psychiatry* (Advance online publication, 19 May 2006; doi:10.1016/j.biopsych.2006.02.023).
- 9 Yamaguchi F, Yamaguchi K, Tokuda M, Brenner S. Molecular cloning of EDG-3 and N-Shc genes from the puffer fish, Fugu rubripes, and conservation of synteny with the human genome. *FEBS Lett* 1999; **459**: 105–110.
- 10 Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G et al. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* 1992; **70**: 93–104.
- 11 Pelicci G, Dente L, De Giuseppe A, Verducci-Galletti B, Giuli S, Mele S et al. A family of Shc related proteins with conserved PTB, CH1 and SH2 regions. *Oncogene* 1996; **13**: 633–641.
- 12 O'Bryan JP, Songyang Z, Cantley L, Der CJ, Pawson T. A mammalian adaptor protein with conserved Src homology 2 and phosphotyrosine-binding domains is related to Shc and is specifically expressed in the brain. *Proc Natl Acad Sci USA* 1996; **93**: 2729–2734.
- 13 Cattaneo E, Pelicci PG. Emerging roles for SH2/PTB-containing Shc adaptor proteins in the developing mammalian brain. *Trends Neurosci* 1998; **21**: 476–481.
- 14 Conti L, Sipione S, Magrassi L, Bonfanti L, Rigamonti D, Pettirossi V et al. Shc signaling in differentiating neural progenitor cells. *Nat Neurosci* 2001; **4**: 579–586.
- 15 Nakamura T, Muraoka S, Sanokawa R, Mori N. N-Shc and Sck, two neuronally expressed Shc adapter homologs. Their differential regional expression in the brain and roles in neurotrophin and Src signaling. *J Biol Chem* 1998; **273**: 6960–6967.
- 16 Liu HY, Meakin SO. ShcB and ShcC activation by the Trk family of receptor tyrosine kinases. *J Biol Chem* 2002; **277**: 26046–26056.
- 17 Nestler EJ. Molecular neurobiology of addiction. *Am J Addict* 2001; **10**: 201–217.
- 18 Brunzell DH, Russell DS, Picciotto MR. *In vivo* nicotine treatment regulates mesocorticolimbic CREB and ERK signaling in C57Bl/6J mice. *J Neurochem* 2003; **84**: 1431–1441.
- 19 Thomas GM, Hagan RL. MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci* 2004; **5**: 173–183.
- 20 Valjent E, Pages C, Herve D, Girault JA, Caboche J. Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. *Eur J Neurosci* 2004; **19**: 1826–1836.
- 21 Miyamoto Y, Chen L, Sato M, Sokabe M, Nabeshima T, Pawson T et al. Hippocampal synaptic modulation by the phosphotyrosine adapter protein ShcC/N-Shc via interaction with the NMDA receptor. *J Neurosci* 2005; **25**: 1826–1835.
- 22 Troglio F, Echart C, Gobbi A, Pawson T, Pelicci PG, De Simoni MG et al. The Rai (Shc C) adaptor protein regulates the neuronal stress response and protects against cerebral ischemia. *Proc Natl Acad Sci USA* 2004; **101**: 15476–15481.

- 23 Heatherton TF, Kozlowski LT, Frecker RC, Fagerstrom KO. The Fagerstrom test for nicotine dependence: a revision of the Fagerstrom Tolerance Questionnaire. *Br J Addict* 1991; **86**: 1119–1127.
- 24 O'Connell JR, Weeks DE. PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet* 1998; **63**: 259–266.
- 25 Boehnke M, Cox NJ. Accurate inference of relationships in sib-pair linkage studies. *Am J Hum Genet* 1997; **61**: 423–429.
- 26 Shete S, Jacobs KB, Elston RC. Adding further power to the Haseman and Elston method for detecting linkage in larger sibships: weighting sums and differences. *Hum Hered* 2003; **55**: 79–85.
- 27 Iyengar SK, Song D, Klein BE, Klein R, Schick JH, Humphrey J et al. Dissection of genomewide-scan data in extended families reveals a major locus and oligogenic susceptibility for age-related macular degeneration. *Am J Hum Genet* 2004; **74**: 20–39.
- 28 Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**: 263–265.
- 29 Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B et al. The structure of haplotype blocks in the human genome. *Science* 2002; **296**: 2225–2229.
- 30 Lange C, Silverman EK, Xu X, Weiss ST, Laird NM. A multivariate family-based association test using generalized estimating equations: FBAT-GEE. *Biostatistics* 2003; **4**: 195–206.
- 31 Horvath S, Xu X, Lake SL, Silverman EK, Weiss ST, Laird NM. Family-based tests for associating haplotypes with general phenotype data: application to asthma genetics. *Genet Epidemiol* 2004; **26**: 61–69.
- 32 Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *Am J Hum Genet* 2004; **74**: 765–769.
- 33 Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. Academic Press: Sydney, 1986.
- 34 Winer J, Jung CK, Shackel I, Williams PM. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes *in vitro*. *Anal Biochem* 1999; **270**: 41–49.
- 35 Nyholt DR. All LODs are not created equal. *Am J Hum Genet* 2000; **67**: 282–288.
- 36 Sun D, Huang W, Hwang YY, Zhang Y, Zhang Q, Li MD. Regulation by nicotine of Gpr51 and Ntrk2 expression in various rat brain regions. *Neuropharmacology* (Advance online publication, 21 June 2006; doi:10.1038/sj.npp.1301134).
- 37 Pelicci G, Troglio F, Bodini A, Melillo RM, Pettirossi V, Coda L et al. The neuron-specific Rai (ShcC) adaptor protein inhibits apoptosis by coupling Ret to the phosphatidylinositol 3-kinase/Akt signaling pathway. *Mol Cell Biol* 2002; **22**: 7351–7363.
- 38 Wall JD, Pritchard JK. Haplotype blocks and linkage disequilibrium in the human genome. *Nat Rev Genet* 2003; **4**: 587–597.
- 39 Perez-Stable EJ, Herrera B, Jacob III P, Benowitz NL. Nicotine metabolism and intake in black and white smokers. *JAMA* 1998; **280**: 152–156.
- 40 Benowitz NL, Perez-Stable EJ, Fong I, Modin G, Herrera B, Jacob III P. Ethnic differences in N-glucuronidation of nicotine and cotinine. *J Pharmacol Exp Ther* 1999; **291**: 1196–1203.
- 41 Batra V, Patkar AA, Berrettini WH, Weinstein SP, Leone FT. The genetic determinants of smoking. *Chest* 2003; **123**: 1730–1739.
- 42 Noble EP. D2 dopamine receptor gene in psychiatric and neurologic disorders and its phenotypes. *Am J Med Genet* 2003; **116B**: 103–125.
- 43 Lu L, Hope BT, Dempsey J, Liu SY, Bossert JM, Shaham Y. Central amygdala ERK signaling pathway is critical to incubation of cocaine craving. *Nat Neurosci* 2005; **8**: 212–219.
- 44 Tang K, Wu H, Mahata SK, O'Connor DT. A crucial role for the mitogen-activated protein kinase pathway in nicotinic cholinergic signaling to secretory protein transcription in pheochromocytoma cells. *Mol Pharmacol* 1998; **54**: 59–69.
- 45 Mazzucchelli C, Vantaggiato C, Ciamei A, Fasano S, Pakhotin P, Krezel W et al. Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. *Neuron* 2002; **34**: 807–820.
- 46 Sanna PP, Simpson C, Lutjens R, Koob G. ERK regulation in chronic ethanol exposure and withdrawal. *Brain Res* 2002; **948**: 186–191.
- 47 Konu O, Kane JK, Barrett T, Vawter MP, Chang R, Ma JZ et al. Region-specific transcriptional response to chronic nicotine in rat brain. *Brain Res* 2001; **909**: 194–203.
- 48 Chen J, Sidhu A. The role of D1 dopamine receptors and phospho-ERK in mediating cytotoxicity (Commentary). *Neurotox Res* 2005; **7**: 179–181.
- 49 Wang C, Buck DC, Yang R, Macey TA, Neve KA. Dopamine D2 receptor stimulation of mitogen-activated protein kinases mediated by cell type-dependent transactivation of receptor tyrosine kinases. *J Neurochem* 2005; **93**: 899–909.
- 50 Breslau N, Peterson EL, Schultz LR, Chilcoat HD, Andreski P. Major depression and stages of smoking. A longitudinal investigation. *Arch Gen Psychiatry* 1998; **55**: 161–166.
- 51 Murphy JM, Horton NJ, Monson RR, Laird NM, Sobol AM, Leighton AH. Cigarette smoking in relation to depression: historical trends from the Stirling County Study. *Am J Psychiatry* 2003; **160**: 1663–1669.
- 52 Kendler KS, Neale MC, MacLean CJ, Heath AC, Eaves LJ, Kessler RC. Smoking and major depression. A causal analysis. *Arch Gen Psychiatry* 1993; **50**: 36–43.
- 53 Dierker LC, Avenevoli S, Stolar M, Merikangas KR. Smoking and depression: an examination of mechanisms of comorbidity. *Am J Psychiatry* 2002; **159**: 947–953.