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Significant Association of *ANKK1* and Detection of a Functional Polymorphism with Nicotine Dependence in an African-American Sample

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The dopaminergic system in the brain plays a critical role in nicotine addiction. Genetic variants in the dopaminergic system, including those in dopamine receptor genes, represent plausible candidates for the genetic study of nicotine dependence (ND). We investigated various polymorphisms in the dopamine D_2 receptor gene (*DRD2*) and its neighboring ankyrin repeats and kinase domain containing 1 gene (*ANKK1*) to determine whether they were associated with ND. We examined 16 single nucleotide polymorphisms (SNPs) at *DRD2* and 7 SNPs at *ANKK1* in our Mid-South Tobacco Family cohort, which consisted of 2037 participants representing two distinct American populations. Several SNPs (rs7131056, rs4274224, rs4648318, and rs6278) in *DRD2*, along with the *Taq* IA polymorphism (rs1800497) in *ANKK1*, revealed initial significant associations with ND in European Americans, but not after correction for multiple testing, indicating a weak association of *DRD2* with ND. In contrast, associations for *ANKK1* with ND in the African-American and pooled samples, specifically for SNP rs2734849, remained significant after correction. With a non-synonymous G to A transition, rs2734849 produces an amino-acid change (arginine to histidine) in C-terminal ankyrin repeat domain of ANKK1. Using the luciferase reporter assay, we further demonstrated that the variant alters expression level of NF-κB-regulated genes. Since *DRD2* expression is regulated by transcription factor NF-κB, we suspect that rs2734849 may indirectly affect dopamine D_2 receptor density. We conclude that *ANKK1* is associated with ND and polymorphism rs2734849 in *ANKK1* represents a functional causative variant for ND in African-American smokers.

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INTRODUCTION

Tobacco use is the leading cause of preventable death in developed countries. According to the 2005 Centers for Disease Control and Prevention estimates, smoking annually causes 18% of total deaths, or approximately 440 000 people, in the United States alone, with more than US$168 billion in direct and indirect medical costs. The addictive potential of tobacco is exemplified by the difficulty in quitting. Nearly 35 million smokers make a serious attempt to quit each year, but less than 7% who try to quit on their own remain tobacco free more than a year. Of the roughly 4000 ingredients in cigarette smoke, nicotine is the primary addictive component. Studies have demonstrated that individual vulnerability to develop nicotine dependence (ND) is influenced by a combination of genetic and environmental factors, with an estimated heritability of at least 50% (Li *et al*, 2003; Sullivan and Kendler, 1999).

The addictive effects of nicotine operate through the dopaminergic system in the brain. Exposure to nicotine increases neurotransmitter dopamine release (Nisell *et al*, 1994; Pontieri *et al*, 1996). In particular, the dopaminergic mesocorticolimbic reward pathways have been frequently implicated in the etiology of nicotine and other drug addictions, as well as non-drug addictive behaviors. Consequently, a great deal of attention has been devoted to determining whether variations in genes within a dopaminergic system could account for the heritable

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factors in susceptibility to ND (Ho and Tyndale, 2007; Li, 2006; Li *et al*, 2004). Since dopamine receptors mediate effects of dopamine, the five G-protein-coupled receptors, classified into D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4) groups, are considered viable candidates for the study of the genetic bases of ND. Of the five dopamine receptors, D_1 and D_2 emerge as two key components of the dopaminergic system. They are largely expressed in the nucleus accumbens and ventral caudate-putamen, two reward-related brain regions, and their concurrent absence in the early postnatal period is lethal (Kobayashi *et al*, 2004). Previously, we have reported the significant association of *DRD1* with ND in an independent study (Huang *et al*, 2008). In this study, we will focus on determination of a possible association of dopamine D_2 receptor encoding gene *DRD2* with ND.

DRD2 has been extensively investigated regarding its association with various psychiatric disorders, including nicotine addiction, with the majority focusing on a polymorphism known as *Taq* IA (rs1800497 in this study and in the NCBI dbSNP database). On the basis of a large number of studies, conflicting results have been documented with respect to smoking behavior. Some reported positive findings (Anokhin *et al*, 1999; Cinciripini *et al*, 2004; Comings *et al*, 1996; Erblich *et al*, 2004), but not all (Bierut *et al*, 2000; Johnstone *et al*, 2004; Singleton *et al*, 1998). A similar pattern of conflicting results exists regarding the association of *DRD2* with alcoholism (Munafo *et al*, 2007). Although our previous meta-analysis revealed a significant association of *Taq* IA polymorphism with smoking behavior (Li *et al*, 2004), which was verified by another meta-analysis (Munafo *et al*, 2004), significant heterogeneity was noted across studies. When a random-effects model was applied to account for between-study differences, no significant association remained (Munafo *et al*, 2004).

The *Taq* IA polymorphism is located 9.5 kb downstream from *DRD2*. Although the *DRD2 Taq* IA polymorphism notation has been well established in the literature, a recent report indicated that it actually resides in the neighboring gene *ANKK1* (ankyrin repeats and kinase domain containing 1 gene) (Neville *et al*, 2004). The encoded protein ANKK1, previously named as SgK288, belongs to a family of serine/threonine kinases in a branch of human kinome, which share a highly homologous amino- (N-) terminal kinase domain (see Supplementary Figure 1 for details) (Manning *et al*, 2002). Although their carboxyl (C) termini differ, this family of proteins including receptor-interacting protein kinases (RIPKs) 1–4 have been shown to be involved primarily in activation of transcription factor NF-κB (Meylan and Tschopp, 2005). ANKK1 is highly similar to RIPK4, sharing not only the N-terminal kinase domain, but also the C-terminal ankyrin repeat domain. The 'C' to 'T' transition of *Taq* IA polymorphism causes an amino-acid change (Glu713Lys) in the C-terminal ankyrin repeat domain, which has been suggested to mediate protein–protein interaction (Michaely *et al*, 2002; Neville *et al*, 2004).

Interestingly, a recent family-based association study revealed that *ANKK1* is significantly associated with ND in both African-American (AA) and European-American (EA) populations, while *DRD2* showed a relative weaker association (Gelernter *et al*, 2006). Little evidence was found for association of the *Taq* IA polymorphism with ND. Although the *Taq* IA polymorphism is a non-synonymous base substitution, it was suggested to be potentially attributable to linkage disequilibrium (LD) with a more centromeric genetic variant within *DRD2* or *ANKK1* (Gelernter *et al*, 2006).

Since a region near *DRD2* on human chromosome 11q23 has demonstrated significant linkage with cigarette consumption and ND in genome-wide scans (Gelernter *et al*, 2007; Morley *et al*, 2006) and *DRD2* has an established close relationship with its neighboring *ANKK1* in genetic studies, we evaluated possible associations of both *DRD2* and *ANKK1* with ND in our Mid-South Tobacco Family (MSTF) cohort. Our results not only reveal a stronger association of *ANKK1* than that of *DRD2* with ND in both AA and pooled samples, but also identify a possible causative single nucleotide polymorphism (SNP) rs2734849. Moreover, we demonstrate that the rs2734849 polymorphism is functional in the ANKK1 negative regulation of transcription factor NF-κB.

MATERIALS AND METHODS

Study Participants

The subjects in this study were of either AA or EA origin and were recruited from the mid-south states in the United States during 1999–2004, designated as the 'MSTF' cohort (Li, 2006; Li *et al*, 2005). Proband cigarette smokers were required to be at least 21 years old, to have smoked for at least the last 5 years, and to 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. The cohort included 2037 subjects in 602 nuclear families, with 671 subjects in 200 EA families and 1366 subjects in 402 AA families. Extensive clinical data were collected from each participant, including demographics (eg, sex, age, race, biological relationships, weight, height, years of education, and marital status), medical history, smoking history, current smoking behavior, ND, and personality traits, assessed by various questionnaires, available at National Institute on Drug Abuse Genetics Consortium website (http://zork.wustl.edu/nida). Individuals exhibiting substance abuse other than for alcohol were excluded. All participants provided informed consent. The study protocol and forms/procedures were approved by all participating Institutional Review Boards.

For each smoker, the degree of ND 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 point scale), which includes SQ and smoking urgency (ie, how soon after waking up does the participant smoke the first cigarette); and the Fagerström test for ND score (FTND, 0–10 point scale) (Heatherton *et al*, 1991). All three measures have been used consistently in our previous genetic studies on ND; please refer to these papers for detailed demographic and clinical characteristics of the MSTF cohort (eg, Beuten *et al*, 2006, 2007; Li *et al*, 2005, 2007).

DNA Extraction, SNP Selection, and Genotyping

Genomic DNA samples were prepared from peripheral blood tissue provided by each participant using the Maxi blood DNA extraction kit from Qiagen (Valencia, CA). In total, 7 SNPs for *ANKK1* and 16 SNPs for *DRD2* were selected from the NCBI dbSNP database to cover the entire genomic region of the *ANKK1* and *DRD2* genes. Prior published reports addressing SNPs within *DRD2* and *ANKK1*, minor allele frequency, functional potential, and validation evidence were considered in determining final SNP selection. Information regarding these SNPs, including their IDs, allelic variants, contig positions, heterozygosities, and site functions is shown in Table 1. The TaqMan primer–probe set for each SNP was purchased from Applied Biosystems (Foster City, CA) and their sequences are provided in Supplementary Table 1. PCR reactions for genotyping were performed in a 384-well microplate format, using TaqMan universal PCR master mix. A standard PCR amplification protocol with 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 25 s at 95°C and 1 min at 60°C was applied. The following allelic discrimination analysis was carried out using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

SNP- and Haplotype-Based Association Analysis

The PedCheck program (O'Connell and Weeks, 1999) was used to identify any inconsistent Mendelian inheritance, non-paternity, and/or genotyping errors. The detected inconsistencies were excluded from subsequent association analyses. To verify data quality, we also checked the genotyping results for any significant departure from Hardy–Weinberg equilibrium (HWE). Pairwise LD between all SNPs was evaluated using the Haploview program (Barrett *et al*, 2005) with the option of determining haplotype blocks according to the criteria defined by Gabriel *et al* (2002).

Individual SNP association was determined using the Pedigree-Based Association Test (PBAT) program employing generalized estimating equations (Lange *et al*, 2004). Haplotype-based association was determined using the Family-Based Association Test (FBAT) program with the option of computing *P*-values of the *Z* statistic based on Monte Carlo sampling (Horvath *et al*, 2004). The AA and EA samples were analyzed separately, with gender and age included as covariates in both PBAT and FBAT analyses. Ethnicity was employed as a covariate for analyses of the pooled sample. All three ND measures (SQ, HSI, and FTND)

Table 1 Information Regarding SNPs Selected from NCBI dbSNP Database and their Allele Frequencies in AA, EA, and Pooled Samples

						Allele frequency		
dbSNP ID	**Allele**	**Heterozygosity**	**Contig position (NT_033899)**	**SNP location**	**SNP function**	**AA**	**EA**	**Pooled**
ANKK1								
rs10891545	T/C	0.496	16820481	Promoter		0.42/0.58	0.82/0.18	0.55/0.45
rs7945132	T/A	0.470	16824889	Intron 1		0.77/0.23	0.65/0.35	0.73/0.27
rs4938013	C/A	0.454	16826886	Exon 2	Ile151Ile	0.77/0.23	0.65/0.35	0.73/0.27
rs7118900	G/A	0.457	16829237	Exon 5	Thr239Ala	0.69/0.31	0.78/0.22	0.72/0.28
rs11604671	G/A	0.300	16830475	Exon 6	Arg318Gly	0.89/0.11	0.55/0.45	0.79/0.21
rs2734849	A/G	0.288	16832576	Exon 8	Arg490His	0.84/0.16	0.55/0.45	0.75/0.25
rs1800497	C/T	0.432	16833244	Exon 8	Glu713Lys	0.65/0.35	0.76/0.24	0.68/0.32
DRD2								
rs6278	G/T	0.327	16843140	Exon 8	3′ UTR	0.94/0.06	0.81/0.19	0.90/0.10
rs6279	C/G	0.500	16843489	Exon 8	3′ UTR	0.65/0.35	0.33/0.67	0.55/0.45
rs1079594	T/G	0.373	16845228	Intron 7		0.90/0.10	0.82/0.18	0.87/0.13
rs6275	T/C	0.500	16845893	Exon 7	His313His	0.64/0.36	0.31/0.69	0.54/0.46
rs2075654	G/A	0.365	16851482	Intron 2		0.95/0.05	0.81/0.19	0.91/0.09
rs2587548	C/G	0.387	16854628	Intron 2		0.80/0.20	0.46/0.54	0.70/0.30
rs2075652	C/T	0.279	16857314	Intron 2		0.96/0.04	0.99/0.01	0.97/0.03
rs1079596	G/A	0.397	16859035	Intron 0		0.81/0.19	0.81/0.19	0.81/0.19
rs4586205	G/T	0.498	16869545	Intron 0		0.67/0.33	0.25/0.75	0.54/0.46
rs7125415	C/T	0.243	16873097	Intron 0		0.82/0.18	0.88/0.12	0.84/0.16
rs4648318	A/G	0.489	16875805	Intron 0		0.52/0.48	0.74/0.26	0.59/0.41
rs4274224	G/A	0.499	16881868	Intron 0		0.67/0.33	0.49/0.51	0.62/0.38
rs7131056	A/C	0.499	16892190	Intron 0		0.63/0.37	0.42/0.58	0.57/0.43
rs4648317	C/T	0.342	16893948	Intron 0		0.85/0.15	0.86/0.14	0.86/0.14
rs4350392	C/A	0.334	16898133	Intron 0		0.87/0.13	0.85/0.15	0.86/0.14
rs6589377	A/G	0.265	16918152	5′ end		0.83/0.17	0.63/0.37	0.76/0.24

were analyzed under the additive and dominant models. 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.

Vector Construct

The full-length cDNA of *ANKK1* was fused with a FLAG tag at N terminus, cloned into pcDNA 3.1 expression vector (Invitrogen, Carlsbad, CA), and sequenced to ensure it contained a G-allele for SNP rs2734849 (encoding arginine, R490). The A-allele of rs2734849 (for arginine to histidine mutation, R490H) was obtained by a site-directed mutation using QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA) and the following designed primers: 5′-gacccc aacctgcatgaggctgagggc-3′ (forward) and 5′-gccctcagcctcatg caggttggggtc-3′ (reverse). The lysine to arginine mutation (K51R) at ANKK1 kinase domain was also realized with a site-directed mutation using the following designed primers: 5′-gagtacgccatcaggtgcgccccctgc-3′ (forward) and 5′-gcagggggcgcacctgatggcgtactc-3′ (reverse). All constructs were confirmed by DNA sequencing.

Cell Transfection and Luciferase Assay

Human neuroblastoma SH-SY5Y cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in a 1 : 1 mixture of minimum essential medium and F12 medium with fetal bovine serum added to a final concentration of 10%. The transfections were performed with Lipofectamine 2000 reagent (Invitrogen), in accordance with the manufacturer's protocol. Each *ANKK1* expression vector (R490, R490H, or K51R) was co-transfected with the PathDetect pNF-κB-Luc *cis*-reporter plasmid (Stratagene) with a 3 : 1 ratio, using pcDNA 3.1 vector as a mock control. The luciferase activities in SH-SY5Y cells after 48 h cell transfection were analyzed using the Luciferase Assay System with 20/20^n luminometer method (Promega, Madison, WI). In each experiment, quadruplicate transfections were made for each plasmid and their luciferase activities were averaged. Three independent experiments were conducted to assure findings were replicable.

RESULTS

Analysis of SNPs with HWE and Pairwise LD Tests

We genotyped a total of 23 SNPs, 7 within *ANKK1* and 16 within *DRD2*. Tests for HWE indicated that no SNP deviated significantly from HWE in either the AA or EA sample (minimum $P=0.164$ and 0.475 for the AA and EA samples, respectively), confirming the high quality of the genotyping data. The allele frequencies for each SNP in the AA, EA, and pooled samples are shown in Table 1. In light of ethnic-specific characteristics of SNPs (Gabriel *et al*, 2002; Wall and Pritchard, 2003) and known ethnic differences in ND and nicotine metabolism (Benowitz *et al*, 1999; Perez-Stable *et al*, 1998), we performed a separate association analysis on each ethnic sample. Figure 1 demonstrates the pairwise LD structures among the 23 SNPs in the AA and EA samples. The r^2 values were calculated using the Haploview program (Barrett *et al*, 2005) and haplotype blocks were determined according to the criteria specified by Gabriel *et al* (2002). We observed two LD blocks of *ANKK1* in AAs, which were different from those in EAs. In addition, three LD blocks of *DRD2* observed in the EA sample were not found in the AA sample. Notably, the rs1800497 polymorphism of *ANKK1* was not in a LD block of *ANKK1* in AAs, but was in a LD block crossing *ANKK1* and *DRD2* in EAs (Figure 1).

Association Analysis of Individual SNPs

Program PBAT-GEE (Lange *et al*, 2004) was employed for individual SNP analysis. For *DRD2*, we found that rs7131056 and rs4274224 were significantly associated with all three ND measures (SQ, HSI, and FTND) ($P=\sim$0.036–0.048), rs4648318 with FTND ($P=0.041$), and rs6278 with both HSI ($P=0.040$) and FTND ($P=0.039$) in the EA sample. In AAs, only SNP rs6589377 yielded a significant association with FTND ($P=0.049$). However, all these associations were nonsignificant after correction for multiple testing on the basis of the SNPSpD approach (Nyholt, 2004). No SNP was found significantly associated with any ND measure in the pooled sample (Table 2).

For *ANKK1*, three SNPs (rs1800497, rs2734849, and rs11604671) were significantly associated with ND. Of these, rs1800497 showed significant associations with HSI under the dominant model in both EA and pooled samples ($P=0.038$ and 0.042, respectively) and with FTND in EAs ($P=0.043$). Also, SNP rs11604671 showed significant associations under the dominant model with SQ ($P=0.028$) and HSI ($P=0.047$) in AAs and under the additive model with SQ ($P=0.023$), HSI ($P=0.0091$), and FTND ($P=0.050$) in the pooled sample. All these associations, except rs11604671 with HSI, were rendered nonsignificant after correction for multiple testing. In contrast, rs2734849 had significant associations with all three ND measures in both AA and pooled samples under the dominant model even after correction for multiple testing ($P<0.010$). Under the additive model, rs2734849 yielded significant associations with HSI in AAs ($P=0.023$); it was also associated with SQ ($P=0.023$), HSI ($P=0.0064$), and FTND ($P=0.027$) in the pooled sample (Table 2).

Haplotype-Based Association Analysis

We employed the FBAT program (Horvath *et al*, 2004) to assess haplotype-based associations for all five contiguous SNPs using a sliding window approach (Lin *et al*, 2004). For haplotypes formed by rs2075654–rs2587548–rs2075652–rs1079596–rs4586205 in *DRD2*, haplotype G-G-C-G-T had significant associations under the dominant model with FTND in EAs ($Z=-2.13$, $P=0.033$, at a frequency of 56%) and with all three ND measures in AAs (frequency = 11%). However, only the association with HSI in AAs remained significant after Bonferroni correction ($Z=2.67$, $P=0.0075$) (Table 3).

In *ANKK1*, haplotype T-T-C-G-A, formed by rs10891545–rs7945132–rs4938013–rs7118900–rs11604671, yielded significant

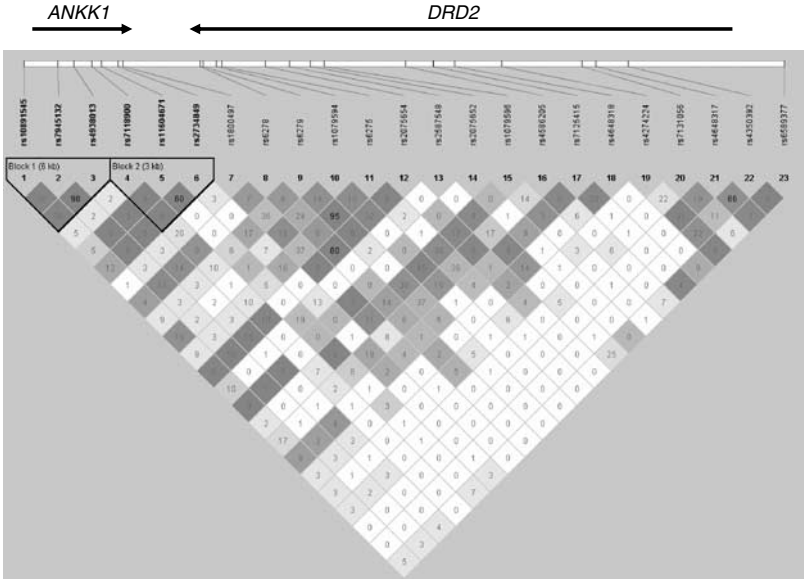

Figure 1 Haploview-generated LD patterns for 23 SNPs within *ANKK1* and *DRD2* in the AA and EA samples. Pairwise LD between all SNPs was evaluated using the Haploview program (Barrett *et al*, 2005) with the option of determining haplotype blocks according to the criteria defined by Gabriel *et al* (2002). The number in each box represents the r^2 value for each SNP pair.

associations under the additive model with all three ND measures in EAs (frequency = 47%) and in the pooled sample (frequency = 20%). Significant associations were identified under the dominant model for SQ in AAs (frequency = 6%) and with all three ND measures in the pooled sample as well. After Bonferroni correction, the significant associations remained for SQ ($Z = 2.70$, $P = 0.0071$) and HSI ($Z = 2.72$, $P = 0.0066$) in the pooled sample under the additive model (Table 4). Another haplotype C-G-A-G-G, formed by rs4938013–rs7118900–rs11604671–rs2734849–rs1800497, was significantly associated with all three ND measures under both additive and dominant models in both AA (frequency = 5%) and pooled (frequency = 19%) samples. Most of these associations remained significant after Bonferroni correction ($Z > 2.64$, $P \leqslant 0.0083$) (Table 5).

Function of ANKK1 in NF-κB-Regulated Gene Expression

Sequence analysis revealed that ANKK1 has apparent similarity to the NF-κB-activating RIPKs with a highly homologous N-terminal kinase domain (see Supplementary Figure 1 for details). In sharing the C-terminal ankyrin repeat domain (Figure 2a), ANKK1 has overall identity of 37% and similarity of 52% with RIPK4. In view of RIPK's function in regulating transcription activity of NF-κB, we investigated the capacity of ANKK1 to activate

Table 2 Association *P*-Values of Each SNP with Three ND Measures under the Additive (Top Line) and Dominant (Bottom Line) Models in AA, EA, and Pooled Samples

	AA			EA			Pooled		
dbSNP ID	**SQ**	**HSI**	**FTND**	**SQ**	**HSI**	**FTND**	**SQ**	**HSI**	**FTND**
ANKK1									
rs10891545	0.41	0.55	0.52	0.34	0.25	0.35	0.25	0.30	0.33
	0.22	0.33	0.29	0.32	0.22	0.40	0.16	0.24	0.24
rs7945132	0.59	0.27	0.34	0.35	0.53	0.67	0.32	0.21	0.32
	0.47	0.25	0.33	0.35	0.41	0.42	0.35	0.26	0.40
rs4938013	0.62	0.27	0.37	0.38	0.54	0.65	0.34	0.21	0.33
	0.38	0.18	0.28	0.33	0.40	0.40	0.31	0.20	0.35
rs7118900	0.31	0.33	0.43	0.12	0.070	0.10	0.11	0.094	0.15
	0.16	0.14	0.14	0.10	0.062	0.075	0.17	0.14	0.16
rs11604671	0.14	0.058	0.17	0.094	0.076	0.16	0.023	**0.0091**	0.050
	0.028	0.047	0.061	0.080	0.076	0.11	0.051	0.015	0.068
rs2734849	0.10	0.023	0.068	0.16	0.13	0.20	0.023	**0.0064**	0.027
	0.0063	**0.00053**	**0.0026**	0.074	0.075	0.092	**0.010**	**0.0014**	**0.0080**
rs1800497	0.43	0.68	0.64	0.18	0.083	0.12	0.18	0.24	0.26
	0.27	0.26	0.29	0.085	0.038	0.043	0.066	0.042	0.054
DRD2									
rs6278	0.36	0.67	0.72	0.31	0.13	0.17	0.90	0.44	0.45
	0.16	0.22	0.23	0.17	0.040	0.039	0.75	0.29	0.27
rs6279	0.88	0.83	0.90	0.81	0.96	0.95	0.96	0.80	0.92
	0.34	0.27	0.47	0.93	0.82	0.71	0.35	0.43	0.73
rs1079594	0.62	0.62	0.80	0.43	0.22	0.24	0.89	0.70	0.60
	0.55	0.55	0.66	0.24	0.095	0.080	0.78	0.61	0.50
rs6275	0.88	0.88	0.80	0.50	0.73	0.96	0.78	0.73	0.84
	0.33	0.29	0.52	0.84	0.67	0.71	0.24	0.37	0.74
rs2075654	0.31	0.66	0.70	0.59	0.32	0.39	0.81	0.67	0.70
	0.16	0.22	0.23	0.27	0.091	0.079	0.18	0.17	0.11
rs2587548	0.52	0.18	0.34	0.24	0.22	0.45	0.23	0.068	0.21
	0.29	0.10	0.21	0.16	0.18	0.25	0.27	0.11	0.29
rs2075652	0.54	0.39	0.33	0.72	0.60	0.49	0.55	0.36	0.31
	0.61	0.39	0.32	0.94	0.72	0.70	0.62	0.36	0.29
rs1079596	0.83	0.83	0.94	0.67	0.40	0.44	0.91	0.80	0.64
	0.72	0.86	0.52	0.29	0.14	0.11	0.74	0.60	0.51
rs4586205	0.12	0.082	0.092	0.74	0.47	0.30	0.22	0.23	0.32
	0.083	0.071	0.12	0.16	0.23	0.12	0.19	0.16	0.33
rs7125415	0.69	0.81	0.92	0.55	0.57	0.44	0.57	0.68	0.82
	0.61	0.60	0.81	0.51	0.52	0.44	0.80	0.39	0.34
rs4648318	0.53	0.38	0.42	0.68	0.24	0.12	0.72	0.76	0.93
	0.19	0.12	0.13	0.25	0.10	0.041	0.21	0.25	0.32
rs4274224	0.49	0.32	0.17	0.34	0.44	0.35	0.95	0.72	0.55
	0.37	0.23	0.11	0.039	0.040	0.047	0.79	0.60	0.65
rs7131056	0.82	0.98	0.90	0.24	0.19	0.22	0.60	0.46	0.56
	0.86	0.63	0.76	0.044	0.036	0.048	0.19	0.30	0.26
rs4648317	0.73	0.50	0.49	0.45	0.44	0.37	0.82	0.97	0.94
	0.57	0.39	0.37	0.51	0.44	0.39	0.90	0.60	0.64
rs4350392	0.56	0.31	0.49	0.95	0.96	0.92	0.66	0.43	0.66
	0.61	0.34	0.57	0.53	0.76	0.82	0.62	0.38	0.61
rs6589377	0.50	0.061	0.049	0.50	0.56	0.31	0.94	0.26	0.34
	0.29	0.12	0.16	0.45	0.47	0.25	0.55	0.28	0.40

Significant *P*-values after correction for multiple testing are provided in bold.
The adjusted *P*-value at the 0.05 significance level for *ANKK1* is 0.013; for *DRD2* is 0.0051.
Within each cell, the top line represents *P*-values for the additive model; the bottom line for the dominant model.
Age and sex were included as covariates for ethnic-specific analyses. Ethnicity was controlled for in the pooled sample.

Table 3 *Z*-Scores and Permutation *P*-Values for Two Major Haplotypes of rs2075654–rs2587548–rs2075652–rs1079596–rs4586205 in *DRD2* with Three ND Measures under the Additive (Top Line) and Dominant (Bottom Line) Models in AA, EA, and Pooled Samples

			SQ			HSI			FTND		
Sample	**Haplotype**	**%**	**No. of family**	***Z* (*P*)-value**	**Global *P*-value**	**No. of family**	***Z* (*P*)-value**	**Global *P*-value**	**No. of family**	***Z* (*P*)-value**	**Global *P*-value**
AA	G-C-C-G-G	52	200	−0.67 (0.50)	0.39	204	−0.59 (0.56)	0.11	204	0.01 (0.99)	0.041
			134	−0.59 (0.55)	0.23	140	−0.80 (0.43)	0.037	141	−0.80 (0.42)	0.012
	G-G-C-G-T	11	92	1.52 (0.13)	0.39	93	1.89 (0.058)	0.11	94	1.69 (0.095)	0.041
			90	2.37 (0.018)	0.23	91	**2.67 (0.0075)**	0.037	92	2.46 (0.014)	0.012
EA	G-C-C-G-G	19	57	0.78 (0.44)	0.73	57	1.30 (0.20)	0.68	57	1.64 (0.10)	0.56
			48	0.12 (0.91)	0.20	49	0.53 (0.60)	0.086	49	0.69 (0.49)	0.030
	G-G-C-G-T	56	65	0.19 (0.85)	0.73	66	−0.33 (0.74)	0.68	66	−0.66 (0.51)	0.56
			30	−1.16 (0.25)	0.20	31	−1.77 (0.077)	0.086	31	−2.13 (0.033)	0.030
Pooled	G-C-C-G-G	41	252	−0.21 (0.83)	0.48	258	0.07 (0.94)	0.23	263	0.78 (0.44)	0.098
			179	−0.45 (0.65)	0.35	186	−0.40 (0.69)	0.10	194	−0.32 (0.75)	0.033
	G-G-C-G-T	26	154	1.26 (0.21)	0.48	158	1.21 (0.23)	0.23	161	0.79 (0.43)	0.098
			117	1.55 (0.12)	0.35	123	1.51 (0.13)	0.10	130	1.08 (0.28)	0.033

The significant *Z*-scores and permuted *P*-values are shown in bold.
The adjusted permutation *P*-values at the 0.05 significance level after Bonferroni correction for three (in EAs), five (in the pooled), and six (in AAs) major haplotypes are 0.017, 0.010, and 0.0083, respectively.
Age and sex were included as covariates for ethnic-specific analyses. Ethnicity was controlled for in the pooled sample.
% = haplotype frequency; no. of family = number of informative families; global *P*-value = permutation-based global haplotypic *P*-value reported by FBAT.

an NF-κB-luciferase reporter. Surprisingly, we found that an overexpression of ANKK1 (R490) in human neuroblastoma SH-SY5Y cells resulted in a weak suppression ($\sim$5%) of NF-κB-reporter gene expression (Figure 2b), which differed from RIPK4 activation of NF-κB (Meylan *et al*, 2002). Compared with the critical role of RIPK4 N-terminal kinase domain in activation of NF-κB (Meylan *et al*, 2002), we found that the kinase domain of ANKK1 was not involved in suppression of NF-κB activity. We demonstrated that a lysine-to-arginine mutation of ANKK1 (K51R), predicated to reduce ATP binding and catalytic activity of the kinase domain (Hanks and Hunter, 1995), yielded no significant change in the reporter gene suppression (Figure 2b). This suggests that ANKK1 has a biological function different from RIPK4, despite sharing similar protein domains.

Since our PBAT and FBAT analysis indicated that rs2734849 is significantly associated with ND, and rs2734849 is a non-synonymous polymorphism with G to A transition causing an alteration from arginine to histidine at amino-acid residue 490 (R490H) in ANKK1, we further investigated whether rs2734849 represents a functional polymorphism to change ANKK1 suppression on NF-κB activity. Using the NF-κB-luciferase reporter assay, we found that the 'A' allele of rs2734849 in *ANKK1* had greater suppression ($\sim$30%) on NF-κB-regulated luciferase activity than the 'G' allele of rs2734849 (Figure 2c). This indicates that rs2734849 is a functional polymorphism that may be responsible, at least partly, for the observed association of *ANKK1* with ND. In a protein sequence alignment and crystal structure comparison with a 12-ankyrin repeat domain (Michaely *et al*, 2002), we found that the residue at 490 position resides on the surface of protein (Figure 2d), a prerequisite for its involvement in mediating protein–protein interaction in the signal-transduction processes leading to inhibition of NF-κB activity.

DISCUSSION

In this study, we show that (1) *ANKK1* is significantly associated with ND in our family-based PBAT and FBAT analysis; (2) the strength of this association is greater than for *DRD2*; (3) SNP rs2734849 in *ANKK1* is significantly associated with ND in both AA and pooled samples, and a likely causative polymorphism for ND; and (4) SNP rs2734849 in *ANKK1* is functional in yielding differential suppression of NF-κB-regulated gene expression. Since transcription factor NF-κB is a necessary and sufficient signal to induce *DRD2* expression (Bontempi *et al*, 2007; Fiorentini *et al*, 2002), this suggests that variants of *ANKK1*, specifically rs2734849, may function to affect *DRD2* expression.

As a G-protein-coupled receptor in dopaminergic neurons, the dopamine D_2 receptor plays a prominent role in reward-mediating mesocorticolimbic pathways. As such, *DRD2* variants have been the focus of many genetic association studies of addictive behaviors. Of the loci at *DRD2* studied, the *Taq* IA polymorphism (rs1800497) has received the most attention, and has been implicated in smoking behavior and alcoholism (Li *et al*, 2004; Munafo *et al*, 2004, 2007), as well as in smoking cessation in pharmacogenetic studies (Berlin *et al*, 2005; David *et al*, 2007). However, its importance is controversial given inconsistent results from association studies. Recently, significant efforts have been expended to investigate additional genes in the *DRD2* region, including *TTC12*

Table 4 *Z*-Scores and Permutation *P*-Values for Some Major Haplotypes of rs10891545–rs7945132–rs4938013–rs7118900–rs1604671 in *ANKK1* with Three ND Measures under the Additive (Top Line) and Dominant (Bottom Line) Models in AA, EA, and Pooled Samples

			SQ			HSI			FTND		
Sample	**Haplotype**	**%**	**No. of family**	***Z* (*P*)-value**	**Global *P*-value**	**No. of family**	***Z* (*P*)-value**	**Global *P*-value**	**No. of family**	***Z* (*P*)-value**	**Global *P*-value**
AA	C-T-C-G-G	33	155	−0.61 (0.54)	0.68	157	−0.52 (0.60)	0.79	158	−0.46 (0.64)	0.87
			133	−1.47 (0.14)	0.45	136	−1.32 (0.19)	0.56	137	−1.22 (0.22)	0.67
	T-T-C-G-A	6	44	1.65 (0.098)	0.68	44	1.32 (0.19)	0.79	44	1.17 (0.24)	0.87
			43	1.98 (0.047)	0.45	43	1.70 (0.089)	0.56	43	1.55 (0.12)	0.67
	T-A-A-G-G	17	106	0.36 (0.72)	0.68	108	−0.24 (0.81)	0.79	107	−0.52 (0.61)	0.87
			100	−0.21 (0.83)	0.45	102	−0.76 (0.45)	0.56	101	−0.96 (0.34)	0.67
	C-T-C-A-G	23	152	−1.54 (0.12)	0.68	154	−1.16 (0.25)	0.79	153	−0.83 (0.40)	0.87
			134	−1.20 (0.23)	0.45	136	−0.83 (0.41)	0.56	135	−0.49 (0.62)	0.67
EA	C-T-C-G-G	13	48	−1.58 (0.12)	0.15	48	−1.81 (0.071)	0.13	48	−1.60 (0.11)	0.19
			45	−1.41 (0.16)	0.23	45	−1.56 (0.12)	0.14	46	−1.40 (0.16)	0.17
	T-T-C-G-A	47	87	2.13 (0.033)	0.15	87	2.38 (0.017)	0.13	87	2.09 (0.037)	0.19
			52	1.43 (0.15)	0.23	54	1.86 (0.063)	0.14	54	1.56 (0.12)	0.17
	T-A-A-G-G	18	50	−0.03 (0.98)	0.15	50	0.28 (0.78)	0.13	50	0.36 (0.72)	0.19
			46	−0.037 (0.97)	0.23	46	0.39 (0.70)	0.14	46	0.61 (0.54)	0.17
	C-T-C-A-G	—									
Pooled	C-T-C-G-G	26	199	−1.36 (0.17)	0.21	203	−1.31 (0.19)	0.26	210	−1.12 (0.26)	0.48
			178	−2.06 (0.039)	0.27	180	−1.95 (0.051)	0.15	188	−1.73 (0.083)	0.31
	T-T-C-G-A	20	129	**2.70 (0.0071)**	0.21	127	**2.72 (0.0066)**	0.26	132	2.39 (0.017)	0.48
			95	2.38 (0.017)	0.27	97	2.50 (0.012)	0.15	101	2.19 (0.028)	0.31
	T-A-A-G-G	17	155	0.46 (0.65)	0.21	156	0.08 (0.94)	0.26	159	−0.11 (0.91)	0.48
			147	−0.01 (0.99)	0.27	148	−0.33 (0.74)	0.15	153	−0.39 (0.69)	0.31
	C-T-C-A-G	15	148	−1.36 (0.17)	0.21	149	−1.11 (0.27)	0.26	156	−0.74 (0.46)	0.48
			131	−0.97 (0.33)	0.27	131	−0.76 (0.45)	0.15	138	−0.37 (0.71)	0.31

The significant *Z*-scores and permuted *P*-values are shown in bold.
The adjusted permutation *P*-values at the 0.05 significance level after Bonferroni correction for four (in EAs), five (in AAs), and six (in the pooled) major haplotypes are 0.013, 0.010, and 0.0083, respectively.
Age and sex were included as covariates for ethnic-specific analyses. Ethnicity was controlled for in the pooled sample.
% = haplotype frequency; — = haplotype frequency <5%; no. of family = number of informative families; global *P*-value = permutation-based global haplotypic *P*-value reported by FBAT.

(tetratricopeptide repeat domain 12), *ANKK1*, and *NCAM1* (neural cell adhesion molecular 1) (Gelernter *et al*, 2006; Yang *et al*, 2007). In a recent family-based association analysis using two distinct American populations, relatively weak evidence emerged for association of ND with markers at *DRD2* and *NCAM1*, whereas strong evidence for multiple SNPs at *TTC12* and *ANKK1* was noted (Gelernter *et al*, 2006). In conjunction with our results, the *Taq* IA polymorphism likely contributes to LD with *ANKK1*, rather than adjacent *DRD2*. This may explain previous inconsistent findings on ND and other psychiatric disorders regarding this polymorphism.

Our current family-based association study provides an independent replication of Gelernter *et al* (2006) in that *ANKK1* has a stronger association with ND than *DRD2*. However, some discrepancies exist at the individual SNP level. Potential reasons for these discrepancies include differences in sample characteristics across studies, as well as the assessment of ND. Gelernter *et al*'s sample was originally recruited for opioid and/or cocaine dependence, whereas the MSTF participants were recruited based on ND, specifically excluding other substance dependence except for alcohol. Another factor may be the statistical power. Our AA sample ($N = 1366$ in 402 families) was larger than that of Gelernter *et al* ($N = 854$ subjects in 319 families), whereas our EA sample was slightly smaller ($N = 671$ in 200 families in this study; $N = 761$ subjects in 313 families in Gelernter *et al*'s study). In addition, we employed SQ, HSI, and the FTND to assess ND, measures emphasizing the amount, frequency and pattern of tobacco consumption, while Gelernter *et al* used FTND and DSM-IV criteria, the latter of which addresses withdrawal, impairment, difficulty quitting, and other factors in a diagnostic framework.

In contrast with the weak association of *DRD2* with ND in the EA sample, based on both SNP and haplotype association analyses (Tables 2 and 3), we detected strong associations of *ANKK1* with ND in the AA and pooled samples, specifically for rs2734849. Evidence suggests

Table 5 *Z*-Scores and Permutation *P*-Values for Some Major Haplotypes of rs4938013–rs7118900–rs11604671–rs2734849–rs1800497 in *ANKK1* with Three ND Measures under the Additive (Top Line) and Dominant (Bottom Line) Models in AA, EA, and Pooled Samples

			SQ			HSI			FTND		
Sample	**Haplotype**	**%**	**No. of family**	***Z* (*P*)-value**	**Global *P*-value**	**No. of family**	***Z* (*P*)-value**	**Global *P*-value**	**No. of family**	***Z* (*P*)-value**	**Global *P*-value**
AA	C-G-G-A-G	34	159	−0.55 (0.59)	0.26	162	0.50 (0.62)	0.28	161	0.34 (0.73)	0.40
			139	−0.48 (0.63)	0.076	142	−0.40 (0.69)	0.056	142	−0.39 (0.70)	0.15
	C-G-A-G-G	5	43	2.60 (0.0092)	0.26	43	**2.64 (0.0083)**	0.28	43	2.37 (0.018)	0.40
			43	**2.97 (0.0029)**	0.076	43	**3.08 (0.0021)**	0.056	43	**2.83 (0.0047)**	0.15
	A-G-G-A-G	17	113	−0.17 (0.87)	0.26	114	−0.90 (0.30)	0.28	114	−0.86 (0.39)	0.40
			106	−0.86 (0.39)	0.076	107	−1.57 (0.12)	0.056	107	−1.36 (0.18)	0.15
	C-A-G-A-A	15	109	−1.94 (0.052)	0.26	110	−1.63 (0.10)	0.28	112	−1.59 (0.11)	0.40
			104	−1.72 (0.086)	0.076	105	−1.45 (0.15)	0.056	107	−1.28 (0.20)	0.15
EA	C-G-G-A-G	13	37	−1.17 (0.24)	0.67	37	−1.39 (0.17)	0.21	37	−1.26 (0.21)	0.32
			33	−0.97 (0.33)	0.79	34	−1.09 (0.27)	0.58	34	−1.02 (0.31)	0.69
	C-G-A-G-G	47	79	1.61 (0.11)	0.67	79	1.91 (0.056)	0.21	79	1.75 (0.081)	0.32
			44	1.20 (0.23)	0.79	44	1.27 (0.20)	0.58	44	1.11 (0.27)	0.69
	A-G-G-A-G	18	48	−0.18 (0.86)	0.67	48	0.06 (0.96)	0.21	48	0.06 (0.95)	0.32
			45	−0.20 (0.85)	0.79	45	0.15 (0.88)	0.58	45	0.28 (0.78)	0.69
	C-A-G-A-A	5	22	−0.87 (0.38)	0.67	22	−0.87 (0.39)	0.21	22	−0.69 (0.49)	0.32
			21	−0.66 (0.51)	0.79	21	−0.65 (0.52)	0.58	21	−0.45 (0.65)	0.69
Pooled	C-G-G-A-G	27	188	−0.01 (0.99)	0.12	192	−0.11 (0.91)	0.11	198	−0.17 (0.86)	0.23
			166	−0.89 (0.38)	0.052	170	−0.85 (0.39)	0.038	176	−0.79 (0.43)	0.14
	C-G-A-G-G	19	125	**2.78 (0.0055)**	0.12	121	**3.10 (0.0019)**	0.11	126	**2.82 (0.0048)**	0.23
			90	**2.93 (0.0034)**	0.052	88	**3.06 (0.0022)**	0.038	92	**2.75 (0.0059)**	0.14
	A-G-G-A-G	18	162	−0.23 (0.82)	0.12	162	0.75 (0.45)	0.11	163	−0.71 (0.48)	0.23
			152	−0.85 (0.40)	0.052	152	−1.32 (0.19)	0.038	153	−1.07 (0.29)	0.14
	C-A-G-A-A	12	128	−2.22 (0.026)	0.12	129	−1.95 (0.051)	0.11	133	−1.85 (0.069)	0.23
			122	−1.92 (0.055)	0.052	123	−1.69 (0.091)	0.038	127	−1.45 (0.15)	0.14

The significant *Z*-scores and permuted *P*-values are shown in bold.
The adjusted permutation *P*-values at the 0.05 significance level after Bonferroni correction for five (in EAs), six (in AAs), and seven (in the pooled) major haplotypes are 0.010, 0.0083, and 0.0071, respectively.
Age and sex were included as covariates for ethnic-specific analyses. Ethnicity was controlled for in the pooled sample.
% = haplotype frequency; no. of family = number of informative families; global *P*-value = permutation-based global haplotypic *P*-value reported by FBAT.

polymorphism rs11604671 may also contribute to this association (Tables 2, 4 and 5). In addition, the haplotypes with either 'A' allele of rs11604671 or 'A-G' by rs11604671 and rs2734849 yielded positive *Z*-scores in the haplotype analyses (Tables 4 and 5), implicating a protective function in the development of ND. Given both rs11604671 and rs2734849 are non-synonymous polymorphisms, possessing 'G' to 'A' transitions that cause amino-acid changes from arginine to either glycine or histidine in the C-terminal ankyrin repeat domain of ANKK1, we suspect these SNPs represent two causative polymorphisms in the observed association of *ANKK1* with ND in the AA and pooled samples.

ANKK1 is classified as one of the RIPKs, which have emerged as essential sensors of cellular stress, integrating both extracellular stress signals transmitted by various cell surface receptors, and signals emanating from intracellular stress (Meylan and Tschopp, 2005). Although RIPKs share high homolog kinase domains (Supplementary Figure 1) and have been shown to affect the NF-κB pathway, the kinase domain of RIPK4 is the only one required to activate NF-κB (Meylan *et al*, 2002). Despite the similarity between ANKK1 and RIPK4, ANKK1 appears to be a negative regulator of NF-κB (Figure 2b and c), suggesting that its biological function differs from that of RIPK4. Given the K51R mutant at the kinase domain showed no effect on suppression (Figure 2b), we concluded that the C-terminal ankyrin repeats acts as an inhibitory domain, just like its counterpart in RIPK4 (Meylan *et al*, 2002). As activation of transcription factor NF-κB is dependent on the formation of a multi-protein complex, the C-terminal ankyrin repeat domain in ANKK1 (or RIPK4) may function to block some protein interactions, and thus trigger a partial inhibitory effect. Variant R490H, caused by the rs2734849 polymorphism, may induce differential protein-binding affinity of ANKK1 in this NF-κB-inhibition process, leading to enhanced inhibition (Figure 2c). Since the promoter region of *DRD2* contains NF-κB-binding sites (Bontempi *et al*,

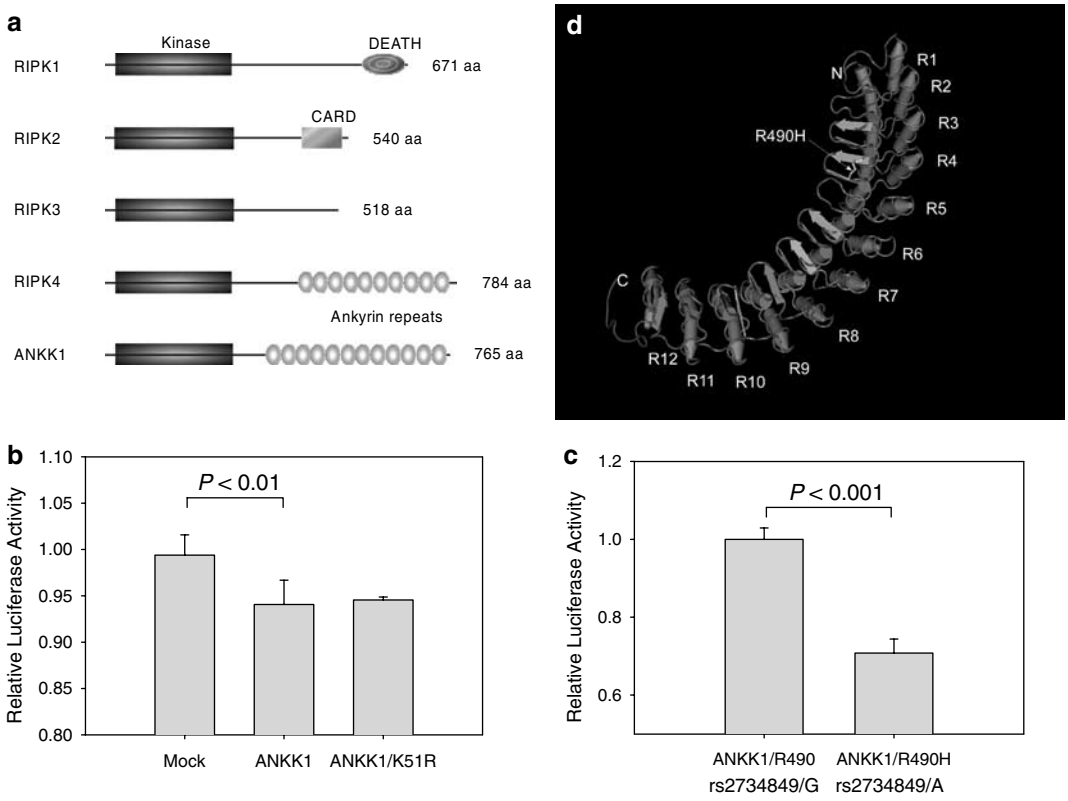

Figure 2 rs2734849 affects suppression of ANKK1 on NF-κB-regulated gene expression. (a) A subfamily of serine/threonine kinases, which share high similarity in their N-terminal kinase domains, but possess distinct C termini. RIPK1 has a so-called death domain (DD or DEATH); RIPK2 has a related caspase recruitment domain (CARD); RIPK4 has 10 ankyrin repeats; and ANKK1 has 12 ankyrin repeats. (b, c) NF-κB-reporter assay. Vector pcDNA3.1 was used as a mock control, and mutant K51R was used as a mutation control (b). ANKK1/R490 is encoded by *ANKK1* with rs2734849/G allele (b, c). Mutant R490H is encoded by *ANKK1* with rs2734849/A allele (c). Each plasmid construct was co-transfected with pNF-κB-Luc reporter vector, and their luciferase activities in human neuroblastoma SH-SY5Y cells were measured after 48 h of cell transfection. The result is representative of three independent experiments. Data are shown as mean ± SD ($N = 4$). (d) R490H mutation site resides on protein surface in the three-dimensional (3D) structure of ankyrin repeat domain. The 3D structure of 12 ankyrin repeats is modified from 1N11 in Protein Data Bank (PDB), using Cn3D 4.1 software (NCBI). R490H mutation site is determined by a protein sequence alignment.

2007; Fiorentini *et al*, 2002), our findings provide a putative connection between *ANKK1* variants and *DRD2* expression. In some *in vitro* tests, nicotine has been shown to inhibit transcriptional activity of NF-κB (Yoshikawa *et al*, 2006; Zhang *et al*, 2001). However, it is not clear whether nicotine inhibits NF-κB signaling via ANKK1.

Expression of *DRD2* determines density and availability of the dopamine D_2 receptor in dopaminergic neurons. Recent positron emission tomography (PET) studies have indicated that decreased dopamine D_2 receptor availability in the striatum of brain may be a predisposing neurobiological trait for substance dependence (Dalley *et al*, 2007; Morgan *et al*, 2002; Nader *et al*, 2006), as opposed to solely a consequence of chronic exposure to abused drugs (Heinz *et al*, 2004; Martinez *et al*, 2004; Volkow *et al*, 1997, 2004). The finding suggests that individual differences in *DRD2* expression (or dopamine D_2 receptor availability) relate to a specific behavioral process that confers addiction vulnerability to abused drugs. Specifically, low *DRD2* expression in the striatum predicts increased consumption of abused drugs (Dalley *et al*, 2007; Nader *et al*, 2006).

Although functional PET imaging studies have indicated that the *Taq* IA polymorphism is associated with reduced dopamine D_2 receptor density in the striatum as well (Jonsson *et al*, 1999; Pohjalainen *et al*, 1998), this finding is not universally accepted (Laruelle *et al*, 1998), similar to its genetic association with smoking behavior or alcohol dependence (Li *et al*, 2004; Munafo *et al*, 2004, 2007). In this report, we report a significant association of *ANKK1* with ND, but also identify a possible causative genetic variant, rs2734849, which functions to regulate NF-κB signaling, including *DRD2* expression. We suspect that polymorphism rs2734849 represents a more centromeric variant in *ANKK1* than the *Taq* IA polymorphism, which may account for conflicting reports on the association of the *Taq* IA polymorphism with ND. We expect future functional PET scans will confirm the association of rs2734849 in *ANKK1* with dopamine D_2 receptor availability, as well as the association of *ANKK1* with ND.

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DISCLOSURE

We have nothing to disclose.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)