Common and Unique Biological Pathways Associated with Smoking Initiation/Progression, Nicotine Dependence, and Smoking Cessation

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Twin and family studies reveal a significant genetic contribution to the risk of smoking initiation and progression (SI/P), nicotine dependence (ND), and smoking cessation (SC). Further, numerous genes have been implicated in these smoking-related behaviors, especially for ND. However, no study has presented a comprehensive and systematic view of the genetic factors associated with these important smoking-related phenotypes. By reviewing the literature on these behaviors, we identified 16, 99, and 75 genes that have been associated with SI/P, ND, and SC, respectively. We then determined whether these genes were enriched in pathways important in the neuronal and brain functions underlying addiction. We identified 9, 21, and 13 pathways enriched in the genes associated with SI/P, ND, and SC, respectively. Among these pathways, four were common to all of the three phenotypes, that is, calcium signaling, cAMP-mediated signaling, dopamine receptor signaling, and G-protein-coupled receptor signaling. Further, we found that serotonin receptor signaling and tryptophan metabolism pathways were shared by SI/P and ND, tight junction signaling pathway was shared by SI/P and SC, and gap junction, neurotrophin/TRK signaling, synaptic long-term potentiation, and tyrosine metabolism were shared between ND and SC. Together, these findings show significant genetic overlap among these three related phenotypes. Although identification of susceptibility genes for smoking-related behaviors is still in an early stage, the approach used in this study has the potential to overcome the hurdles caused by factors such as genetic heterogeneity and small sample size, and thus should yield greater insights into the genetic mechanisms underlying these complex phenotypes.
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INTRODUCTION

Cigarette smoking is the most common form of tobacco use (Smith and Fiore, 1999), and is one of the most significant sources of morbidity and death worldwide (Murray, 2006). In the United States, more than 20% of adults are current smokers (CDC, 2007), and cigarette smoking is responsible for ∼438 000 premature deaths and an estimated economic cost of $167 billion annually (CDC, 2005b). In addition, about 20% of high school students (CDC, 2006) and 8% of middle school students (CDC, 2005a) in the United States smoke. Moreover, every day, about 4000 teenagers in the United States initiate cigarette smoking, and more than 1000 of them may become daily cigarette smokers (SAMHSA, 2006). Although a large fraction of smokers try to quit (CDC, 2007), available treatments are effective for only a fraction of them (Hughes *et al*, 2007; Lerman *et al*, 2007). Thus, developing therapeutic approaches that can help smokers achieve and sustain abstinence from smoking, as well as methods that can prevent people, especially youths, from starting to smoke, remain a huge challenge in public health.

Cigarette smoking is a complex behavior that includes a number of stages such as initiation, experimentation, regular use, dependence, cessation, and relapse (Ho and Tyndale, 2007; Malaiyandi *et al*, 2005; Mayhew *et al*, 2000). Although the initiation of tobacco use, the progression from initial use to smoking dependence, and the ability to quit smoking are undoubtedly affected by various environmental factors, twin, family, and adoption studies have provided strong evidence that genetics has a substantial role in the etiology of these phenotypes (Goode *et al*, 2003; Lerman and Berrettini, 2003; Lerman *et al*, 2007; Osler *et al*, 2001). Earlier studies revealed a considerable genetic contribution to the risk of smoking initiation (SI) (Hardie *et al*, 2006; Kendler *et al*, 1999; Li *et al*, 2003; Mayhew *et al*, 2000; Morley *et al*, 2007; Vink *et al*, 2004), nicotine dependence (ND) (Kendler *et al*, 1999; Lessov *et al*, 2004a; Maes *et al*,

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2004; Malaiyandi *et al*, 2005; Sullivan and Kendler, 1999; True *et al*, 1999), as well as smoking cessation (SC) (Hamilton *et al*, 2006; Heath *et al*, 1999; Morley *et al*, 2007; Xian *et al*, 2003).

Nicotine is the main psychoactive ingredient in cigarettes and evokes its physiological effects by stimulating the mesolimbic brain reward system by binding with nicotinic acetylcholine receptors (nAChRs). So far, the majority of candidate gene-based association studies have focused on those genes that may predispose to addictive behavior by virtue of their effects on key neurotransmitter pathways (for example, dopamine and serotonin) and genes that may affect response to nicotine (for example, nAChRs and nicotine metabolism) (Ho and Tyndale, 2007). However, genetic studies have indicated that, for complex behaviors such as cigarette smoking, the individual differences can be attributed to hundreds of genes and their variants. Genes involved in different biological functions may act in concert to account for the risk of vulnerability to smoking behavior, with each gene having a moderate effect (Hall *et al*, 2002; Lessov *et al*, 2004b; Tyndale, 2003). Polymorphisms in related genes may cooperate in an additive or synergistic manner and modify the risk of smoking rather than act as sole determinants. Consistent with this belief, more and more genes have been found to be associated with smoking behavior over past decades, especially during the past few years. Whereas some plausible candidate genes (for example, nAChRs and dopamine signaling) have been reported and the findings have been partially replicated, numerous genes involved in other biological processes and pathways also have been associated with different smoking behaviors. This is especially true as the genome-wide association (GWA) study is being commonly used in genetic studies of complex traits such as smoking, and the underlying genetic factors can now be investigated in a high throughput and more comprehensive approach. In this situation, a systematic approach that is able to reveal the biochemical processes underlying the genes associated with smoking behaviors will not only help us understand the relations of these genes but also provide further evidence of the validity of the individual gene-based association studies.

In this study, we searched the literature to identify genes purportedly associated with SI/progression (P), ND, and SC. We then examined whether these genes are enriched in biochemical pathways important in neuronal and brain function.

MATERIALS AND METHODS

Identification of Smoking-Related Genes

Contemporary genetic association studies of smoking behaviors are focused primarily on SI, progression to smoking dependence, ND (assessed by various measures or scales such as DSM-IV, Fagerstrom Test for Nicotine Dependence, Fagerstrom Tolerance Questionnaire, and/or smoking quantity, etc.), or SC. Only limited studies have been conducted on SI and progression to ND, and considering the potential overlap of these two highly related behaviors, we combined them into the single category of SI/P.

The list of candidate genes for the three smoking-related phenotypes was created by searching all human genetics association studies deposited in PUBMED (http://www.ncbi.nlm.nih.gov/pubmed/). Similar to Sullivan *et al* (2004), we queried the item '(Smoking [MeSH] OR Tobacco Use Disorder [MeSH]) AND (Polymorphism [MeSH] OR Genotype [MeSH] OR Alleles [MeSH]) NOT (Neoplasms [MeSH])', and a total of 1790 hits was retrieved by September 2008. The abstracts of these articles were reviewed and the association studies of any of the three smoking-related behaviors were selected. From the selected publications, we narrowed our selection by focusing on those reporting a significant association of one or more genes with any of the three phenotypes. To reduce the number of false-positive findings, the studies reporting negative or insignificant associations were not included, although it is likely that some of the genes analyzed in these studies might be associated with the phenotypes that we were interested in. The full reports of the selected publications were reviewed to ensure that the conclusions were supported by the content. From these studies, genes reported to be associated with each phenotype were selected for the current study.

The results from several GWA studies were included. In the work of Bierut *et al* (2007), 35 of 31 960 SNPs were identified with *p*-values <0.0001, and several genes were suggested to be associated with ND, including neurexin 1 (*NRXN1*), vacuolar sorting protein (*VPS13A*), transient receptor potential channel (*TRPC7*), as well as a classic candidate gene related to smoking, neuronal nicotinic cholinergic receptor β3 (*CHRNB3*). All the genes nominated in this study were included in our list for ND. In another large-scale candidate gene-based association study, Saccone *et al* (2007) analyzed 3713 SNPs corresponding to more than 300 candidate genes. The top five SNPs with the smallest false discovery rate (FDR) values (ranging from 0.056 to 0.166) corresponded with neuronal nicotinic cholinergic receptor α3 (*CHRNA3*), α5 (*CHRNA5*), and *CHRNB3*. In the work of Uhl *et al* (2007), allele frequencies in nicotine-dependent and control individuals were compared for 520 000 SNPs, and 32 genes were suggested to be potentially associated with ND; all of them were included in the ND-related gene list. In a recent study on SC, Uhl *et al* (2008) performed GWA studies on three independent samples to identify genes facilitating SC success with bupropion hydrochloride *vs* nicotine replacement therapy. Various genes involved in cell adhesion, transcription regulation, transportation, and signaling transduction were suggested to be candidates contributing to successful SC. From this study, we included those genes that showed significant association with SC in all the three samples (eight genes) or in two samples with at least two nominally significant SNPs in each sample (55 genes).

Identification of Enriched Biochemical Pathways

By literature search, we collected a list of genes associated with each smoking-related phenotype. To get a better understanding of the underlying biological mechanisms, multiple bioinformatics tools were used to identify the significantly enriched pathways involved in the smoking phenotypes. The available pathway analysis tools can be

classified into three categories (Tarca *et al*, 2009): (1) Over-representation analysis (ORA), which compares the genes of interest with genes in predefined pathways and identifies the pathways that include a statistically higher number of genes in the list of interest as overrepresented; (2) functional class scoring (FCS), which compares the genes in chosen pathways with the entire list of genes sorted by certain criteria and identifies the pathways showing statistically significant correlation with the phenotypes under study; and (3) impact analysis, which is similar to ORA, but also considers the connections of genes in the pathways. The FCS approach, for example, GSEA (Subramanian *et al*, 2005), is not feasible for the current analysis as it requires gene expression measurements. The following is a brief description of the four pathway analysis tools used in the current study.

Ingenuity pathway analysis. The core of Ingenuity pathway analysis (IPA) (http://www.ingenuity.com/) is the Ingenuity Pathways Knowledge Base (IPKB), a manually curated knowledge database consisting of function, interaction, and other information of genes/proteins. On the basis of such information, IPA is able to perform analysis on global canonical pathways, dynamically generated biological networks, and global functions from a list of genes. Currently, the IPKB includes 81 canonical metabolic pathways involved in various metabolism processes such as energy metabolism, metabolism of amino acids, and complex carbohydrates. It also includes 202 signaling pathways, such as those related to neurotransmitter signaling, intracellular and secondary signaling, and nuclear receptor signaling.

In our analysis, the gene symbol and the corresponding GenBank accession numbers of genes associated with each smoking phenotype were uploaded into the IPA and compared against the genes in each canonical pathway included in the IPKB. All the pathways with one or more genes overlapping the candidate genes were extracted. A significance value was assigned by the program to measure the chance that the genes of interest participate in a given extracted pathway. Briefly, the *p*-value for a given pathway was calculated by considering: (1) the number of input genes that could be mapped to this pathway in the IPKB, denoted by *m*; (2) the number of genes involved in this pathway, denoted by *M*; (3) the total number of input genes that could be mapped to the IPKB, denoted by *n*; and (4) the total number of known genes included in the IPKB, denoted by *N*. Then the *p*-value was calculated using the right-tailed Fisher's exact test (which is identical to the hypergeometric distribution in this case):

$$p = 1 - \sum_{k=0}^{m-1} \frac{C(M, k)C(N - M, n - k)}{C(N, n)}$$

where $C(M, k)$, $C(N-M, n-k)$, and $C(N, n)$ are binomial coefficients. In general, a *p*-value < 0.05 indicates a statistically significant, non-random association.

As many pathways were examined, multiple comparison correction for the individually calculated *p*-values was necessary to permit reliable statistical inferences. The output *p*-values for the pathways associated with each smoking phenotype were analyzed separately using the MATLAB Bioinformatics Toolbox (The Mathworks, Natick, MA), which calculated the FDR by the method of Benjamini and Hochberg (1995).

The database for annotation, visualization, and integrated discovery. The database for annotation, visualization, and integrated discovery (DAVID) (http://david.abcc.ncifcrf.gov) (Hosack *et al*, 2003; Huang da *et al*, 2009) is a bioinformatics resource consisting of an integrated biological knowledge database and analytic tools aimed at extracting biological themes from gene/protein lists systematically. Compared with other tools, DAVID can provide an integrated and expanded back-end annotation database, multiple modular enrichment algorithms, and exploratory ability in an integrated data-mining environment. In our analysis, the input genes were analyzed using different text- and pathway-mining tools including gene functional classification, functional annotation chart or clustering, and functional annotation table. Pathway analysis was performed using its Functional Annotation Tool based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg) and Biocarta (www.biocarta.com) pathway databases. The enrichment of given pathways in the gene list was measured by EASE score, a modified Fisher exact test *p*-value. The program also performed *p*-value correction based on the method of Benjamini and Hochberg (1995).

GeneTrail. GeneTrail (Keller *et al*, 2008) (http://genetrail.bioinf.uni-sb.de) is web-based bioinformatics tool providing the statistical evaluation of gene/protein lists with respect to enrichment of functional categories. It can perform a wide variety of biological categories and pathway analysis based on multiple databases such as KEGG and Gene Ontology (GO; http://www.geneontology.org/). The GeneTrail analysis tool used in this work was the 'Over-representation Analysis' module, which compared the gene list with a reference set of genes and identified the overrepresented pathways.

Onto Pathway-Express. Onto Pathway-Express (http://vortex.cs.wayne.edu/ontoexpress/) is a pathway analysis tool based on the KEGG pathway database. Different from other tools such as IPA and DAVID, this bioinformatics tool integrates the pathway topology and the position information of each gene in the pathway into its enrichment analysis. By implementing an impact factor analysis, Onto Pathway-Express incorporates both the probabilistic component and the gene interactions into pathway identification (Draghici *et al*, 2007). Briefly, on the basis of the input gene list, Onto Pathway-Express calculates a perturbation factor for each gene by taking into account its expression level and the perturbation of genes downstream from it in each selected pathway. The impact factor of the entire pathway includes a probabilistic term that takes into consideration the proportion of differentially regulated genes in the pathway and the perturbation factors of all genes in the pathway. The output pathways are assigned significance levels according to their impact factors, and the FDR values are computed by the method of Benjamini and Hochberg (1995).

Of the four tools, IPA, DAVID, and GeneTrail use the ORA approach. However, the pathway databases underlying these tools are different, for example, a proprietary database is included in IPA, whereas public databases are adopted by DAVID (KEGG and Biocarta) and GeneTrail (KEGG). Although Onto Pathway-Express performs its analysis on the basis of the KEGG database, the analysis algorithm is different from other methods. With these methods, we expect to obtain a relatively comprehensive evaluation of the pathways associated with the genes important to each smoking-related phenotype.

RESULTS

Identification of Genes Reported to be Associated with Each Smoking Behavior

By searching PUBMED, we extracted publications on the genetic association studies related to tobacco smoking. In the current work, we focused only on the studies related to one of the three phenotypes: SI/P, ND, and SC. For each phenotype, only the publications reporting a significant association of a gene(s) with this phenotype were collected; those by the original authors reporting a negative result or insignificant association were not included. A detailed list of all genes reported to be associated with each of the three phenotypes is provided in Table 1.

For SI/P, 16 genes were identified in 15 studies, all of which were performed at individual gene level. Among them are five nAChR subunit genes, that is, *CHRNA3*, *CHRNA5*, *CHRNA6*, *CHRNB3*, and *CHRNB4*; dopamine receptor D2 (*DRD2*) and D4 (*DRD4*); and one serotonin receptor (*HTR6*). The genes encoding transporters of dopamine (*DAT1* or *SLC6A3*) and serotonin (*5-HTT* or *SLC6A4*) were also included. The other genes were those involving the functions related to nicotine or neurotransmitter metabolism/synthesis such as *COMT*, *CYP2A6*, and *TPH1*; signal transduction (for example, *PTEN* and *RHOA*); or immune response (for example, *IL8*).

Regarding ND, there were 76 publications, including 73 studies focused on either single or a few genes. In these papers, 63 genes were reported to be significantly associated with ND by the original authors. The other three studies were either on a genome-wide scale (Bierut *et al*, 2007; Uhl *et al*, 2007) or on hundreds of candidate genes (Saccone *et al*, 2007), and they nominated a total of 41 genes. Collectively, 99 unique genes are included in the final list. The most prominent genes were those encoding acetylcholine receptors (*CHRM1*, *CHRM5*, *CHRNA4*, *CHRNA5*, and *CHRNB2*), dopamine receptors (*DRD1*, *DRD2*, *DRD3*, and *DRD4*), GABA receptors (*GABRA2*, *GABRB2*, *GABARAP*, and *GABRA4*), serotonin receptors (*HTR1F* and *HTR2A*), as well as proteins involved in nicotine or neurotransmitter metabolism/synthesis (for example, *CYP2A6*, *DBH*, *MAOA*, and *TPH1*).

For SC, 63 genes were nominated by a GWA study (Uhl *et al*, 2008) and 12 by 23 candidate gene-based association studies. These genes were involved in various biological functions, such as dopamine receptor signaling (*DRD2*, *DRD4*, and *SLC6A3*), glutamate receptor signaling (*GRIK1*, *GRIK2*, *GRIN2A*, and *SLC1A2*), and calcium signaling (for example, *CACNA2D3*, *CACNB2*, *CDH13*, and *ITPR2*).

Among the genes associated with the three smoking phenotypes, five were included in all the three lists, that is, *COMT*, *CYP2A6*, *DRD2*, *DRD4*, and *SLC6A3*. Another six genes, that is, *CHRNA3*, *CHRNA5*, *CHRNB3*, *PTEN*, *SLC6A4*, and *TPH1*, were associated with SI/P and ND. Ten genes, that is, *A2BP1*, *ARRB2*, *CDH13*, *CHRNB2*, *CSMD1*, *CYP2B6*, *DBH*, *OPRM1*, *PRKG1*, and *PTPRD*, were associated with ND and SC.

Enriched Biological Pathways Associated with Each Smoking-Related Phenotype

On the basis of the genes related to each smoking phenotype, enriched biochemical pathways were identified by IPA and other bioinformatics tools. For SI/P, the 16 genes (Table 1) were overrepresented in 9 pathways defined in the IPA database ($p < 0.05$; Table 2). For five of these pathways (calcium signaling, dopamine receptor signaling, serotonin receptor signaling, cAMP-mediated signaling, and G-protein-coupled receptor signaling), the corresponding FDR values were < 0.05. For the other pathways (tryptophan metabolism, tight junction signaling, IL-8 signaling, and integrin signaling), they had slightly higher FDR values (0.085–0.116).

The IPA assigned 51 of the 99 genes associated with ND to 21 overrepresented pathways ($p < 0.05$; Table 3). Fourteen of these pathways (for example, dopamine receptor signaling, cAMP-mediated signaling, G-protein-coupled receptor signaling, and serotonin receptor signaling) had an FDR < 0.05, and the other pathways (for example, fatty acid metabolism and synaptic long-term potentiation (LTP)) had an FDR < 0.14.

For SC, 13 pathways were found to be enriched in 18 of the 75 genes associated with this phenotype ($p < 0.05$; Table 4). Four of the pathways (dopamine receptor signaling, glutamate receptor signaling, cAMP-mediated signaling, and calcium signaling) had an FDR < 0.05, and the remaining pathways (for example, synaptic LTP, G-protein-coupled receptor signaling, and synaptic long-term depression (LTD)) had an FDR ranging from 0.082 to 0.18.

Of the pathways enriched in the genes associated with each smoking phenotype, four, that is, calcium signaling, cAMP-mediated signaling, dopamine receptor signaling, and G-protein-coupled receptor signaling, were associated with all three smoking behaviors (Table 5). Two other enriched pathways (that is, serotonin receptor signaling and tryptophan metabolism) were shared by SI/P and ND, and three enriched pathways (neurotrophin/TRK signaling, synaptic LTP, and tyrosine metabolism) were shared by ND and SC.

The enrichment of these pathways in multiple smoking phenotypes was consistent with the fact that synaptic transmission-related biological processes, such as nicotine-nAChR and dopamine signaling, were the key biochemical components underlying different smoking-related behaviors. This also implies that the genes involved in these three smoking phenotypes indeed overlap highly. On the basis of these biochemical relationships, we present in Figure 1 a schematic representation of the major pathways associated with the three phenotypes.

Table 1 Genes Associated with Smoking-Related Behaviors

Gene symbol	Gene name	Reference	Category
A2BP1	Ataxin 2-binding protein 1	Uhl et al (2008)	Cessation
AKAP13	A kinase anchor protein 13	Uhl et al (2008)	Cessation
ARRB2	Arrestin, beta, 2	Ray et al (2007a)	Cessation
ATP9A	Adenosine triphosphatase class II, type 9A	Uhl et al (2008)	Cessation
BNC2	Basonuclin 2	Uhl et al (2008)	Cessation
CACNA2D3	Voltage-dependent calcium channel 32/3 subunit 3	Uhl et al (2008)	Cessation
CACNB2	Voltage-dependent calcium channel 32	Uhl et al (2008)	Cessation
CCDC73	Coiled-coil domain containing 73	Uhl et al (2008)	Cessation
CDH13	Cadherin 13	Uhl et al (2008)	Cessation
CHN2	Chimerin 2	Uhl et al (2008)	Cessation
CHRNB2	Cholinergic receptor, neuronal nicotinic, beta polypeptide 2	Conti et al (2008)	Cessation
CLSTN2	Calsyntenin 2	Uhl et al (2008)	Cessation
COMT	Catechol-O-methyltransferase	Berrettini et al (2007); Colilla et al (2005); Han et al (2008); Johnstone et al (2007)	Cessation
CREB5	cAMP responsive element-binding protein 5	Uhl et al (2008)	Cessation
CSMD1	Cub and Sushi multiple domains 1	Uhl et al (2008)	Cessation
CTNNA2	Catenin, alpha-2	Uhl et al (2008)	Cessation
CYP2A6	Cytochrome p450, subfamily IIA, polypeptide 6	Kubota et al (2006); Ozaki et al (2006)	Cessation
CYP2B6	Cytochrome p450, subfamily IIB, polypeptide 6	David et al (2007a); Lee et al (2007)	Cessation
DAB1	Disabled homolog 1	Uhl et al (2008)	Cessation
DAPK1	Death-associated protein kinase 1	Uhl et al (2008)	Cessation
DBH	Dopamine beta hydroxylase	Johnstone et al (2004)	Cessation
DRD2	Dopamine D2 receptor	David et al (2007a); Johnstone et al (2004); Lerman et al (2006); Morton et al (2006); Park et al (2005); Robinson et al (2007); Swan et al (2005); Yudkin et al (2004)	Cessation
DRD4	Dopamine D4 receptor	David et al (2008b)	Cessation
DSCAM	Down syndrome cell adhesion molecule	Uhl et al (2008)	Cessation
DSCAML1	Down syndrome cell adhesion molecule like 1	Uhl et al (2008)	Cessation
ERC2	ELKS/RAB6-interacting/CAST family member 2	Uhl et al (2008)	Cessation
ERG	V-ets erythroblastosis virus E26 oncogene-like	Uhl et al (2008)	Cessation
FGF12	Fibroblast growth factor 12	Uhl et al (2008)	Cessation
FLJ42220	FLJ42220 protein	Uhl et al (2008)	Cessation
FREQ	Frequenin, drosophila, homolog of	Dahl et al (2006)	Cessation
GALNT17	Polypeptide N-acetylgalactosaminyltransferase 17	Uhl et al (2008)	Cessation
GLIS3	GLI-similar family zinc finger 3	Uhl et al (2008)	Cessation
GRIK1	Inotropic glutamate receptor kainate 1	Uhl et al (2008)	Cessation
GRIK2	Inotropic glutamate receptor kainate 2	Uhl et al (2008)	Cessation
GRIN2A	Inotropic glutamate receptor N-methyl D-aspartate 2A	Uhl et al (2008)	Cessation
HINT1	Histidine triad nucleotide-binding protein 1	Ray et al (2007a)	Cessation
ITPR2	Inositol 1,4,5-triphosphate receptor 2	Uhl et al (2008)	Cessation
KCNIP4	Kv channel-interacting protein 4	Uhl et al (2008)	Cessation
KCNK2	K-type potassium channel 2	Uhl et al (2008)	Cessation
KIAA1026	Kazrin	Uhl et al (2008)	Cessation
LAMA1	Laminin 31	Uhl et al (2008)	Cessation
LARGE	Like-glycosyltransferase	Uhl et al (2008)	Cessation
LEPREL1	Leprecan-like 1	Uhl et al (2008)	Cessation
LYZL1	Lysozyme-like 1	Uhl et al (2008)	Cessation
MAGI1	Membrane-associated guanylate kinase, WW and PDZ domain containing 1	Uhl et al (2008)	Cessation
MTUS1	Mitochondrial tumor suppressor 1	Uhl et al (2008)	Cessation
MYO18B	Myosin XVIIIB	Uhl et al (2008)	Cessation
NEK11	NIMA (never in mitosis gene a)-related kinase 11	Uhl et al (2008)	Cessation

Table 1 Continued

Gene symbol	Gene name	Reference	Category
NELL1	NEL-like 1	Uhl et al (2008)	Cessation
NRXN3	Neurexin 3	Uhl et al (2008)	Cessation
OPRM1	Opioid receptor, mu-1	Lerman et al (2004); Munafo et al (2007); Ray et al (2007a)	Cessation
PARD3	Partitioning defective 3 homolog	Uhl et al (2008)	Cessation
PARK2	Parkin	Uhl et al (2008)	Cessation
PCDH15	Protocadherin 15	Uhl et al (2008)	Cessation
PEBP4	Phosphatidylethanolamine-binding protein 4	Uhl et al (2008)	Cessation
PPP2R2B	Protein phosphatase 2 regulatory subunit B, beta	Uhl et al (2008)	Cessation
PRKG1	cGMP-dependent protein kinase 1	Uhl et al (2008)	Cessation
PTPRD	Receptor protein tyrosine phosphatase D	Uhl et al (2008)	Cessation
PTPRN2	Receptor protein tyrosine phosphatase N2	Uhl et al (2008)	Cessation
PTPRT	Receptor protein tyrosine phosphatase T	Uhl et al (2008)	Cessation
RBM19	RNA-binding motif protein 19	Uhl et al (2008)	Cessation
SGCZ	Sarcoglycan zeta	Uhl et al (2008)	Cessation
SLC1A2	Solute carrier family 1 high-affinity glutamate transporter 2	Uhl et al (2008)	Cessation
SLC6A3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	Han et al (2008); O'Gara et al (2007); Stapleton et al (2007)	Cessation
SORCS1	Sortilin-related VPS10 domain containing receptor 1	Uhl et al (2008)	Cessation
SOX5	SRY box 5	Uhl et al (2008)	Cessation
ST6GALNAC3	ST6 (3-N-acetyl-neuraminyl-2,3-3-galactosyl-1,3)-N-acetylgalactosaminide 3-2,6-sialyltransferase 3	Uhl et al (2008)	Cessation
SUPT3H	Suppressor of Ty 3 homolog	Uhl et al (2008)	Cessation
TEK	TEK receptor tyrosine kinase	Uhl et al (2008)	Cessation
THSD4	Thrombospondin type 1 domain containing 4	Uhl et al (2008)	Cessation
TNIK	TRAF2 and NCK-interacting kinase	Uhl et al (2008)	Cessation
TRIO	Triple functional domain/PTPRF-interacting protein	Uhl et al (2008)	Cessation
UNC13C	Unc13 homolog C	Uhl et al (2008)	Cessation
USH2A	Usher syndrome 2A	Uhl et al (2008)	Cessation
ZNF423	Zinc finger protein 423	Uhl et al (2008)	Cessation
A2BP1	Ataxin 2-binding protein 1	Uhl et al (2007)	ND
ABCC4	Atp-binding cassette, subfamily c, member 4	Uhl et al (2007)	ND
ACTN2	Actinin, alpha-2	Uhl et al (2007)	ND
ADRA2A	Alpha-2a-adrenergic receptor	Prestes et al (2007)	ND
ANKK1	Ankyrin repeat and kinase domain containing 1	Gelernter et al (2006); Huang et al (2009); O'Gara et al (2008); Radwan et al (2007)	ND
ARRB1	Arrestin, beta, 1	Sun et al (2008)	ND
ARRB2	Arrestin, beta, 2	Sun et al (2008)	ND
BDNF	Brain-derived neurotrophic factor	Beuten et al (2005b); Lang et al (2007)	ND
CCK	Cholecystokinin	Comings et al (2001); Takimoto et al (2005)	ND
CD14	Monocyte differentiation antigen CD14	Hubacek et al (2002)	ND
CDH13	Cadherin 13	Uhl et al (2007)	ND
CHRM1	Cholinergic receptor, muscarinic, 1	Lou et al (2006)	ND
CHRM5	Cholinergic receptor, muscarinic, 5	Anney et al (2007)	ND
CHRNA3	Cholinergic receptor, neuronal nicotinic, alpha polypeptide 3	Berrettini et al (2008); Saccone et al (2007)	ND
CHRNA4	Cholinergic receptor, neuronal nicotinic, alpha polypeptide 4	Feng et al (2004); Li et al (2005)	ND
CHRNA5	Cholinergic receptor, neuronal nicotinic, alpha polypeptide 5	Berrettini et al (2008); Bierut et al (2007); Saccone et al (2007)	ND
CHRNA7	Cholinergic receptor, neuronal nicotinic, alpha polypeptide 7	De Luca et al (2004)	ND
CHRNB1	Cholinergic receptor, neuronal nicotinic, beta polypeptide 1	Lou et al (2006)	ND
CHRNB2	Cholinergic receptor, neuronal nicotinic, beta polypeptide 2	Ehringer et al (2007)	ND
CHRNB3	Cholinergic receptor, neuronal nicotinic, beta polypeptide 3	Bierut et al (2007); Saccone et al (2007)	ND
CLCA1	Chloride channel, calcium-activated, 1	Bierut et al (2007)	ND

Table 1 Continued

Gene symbol	Gene name	Reference	Category
CNR1	Cannabinoid receptor 1	Chen et al (2008)	ND
CNTN6	Contactin 6	Uhl et al (2007)	ND
COMT	Catechol-O-methyltransferase	Beuten et al (2006); Tochigi et al (2007)	ND
CREB1	cAMP response element-binding protein 1	Ray et al (2007b)	ND
CSMD1	CUB and SUSHI multiple domains 1	Uhl et al (2007)	ND
CTNNA3	Catenin, alpha-3	Bierut et al (2007)	ND
CYP17A1	Cytochrome p450, family 17, subfamily a, polypeptide 1	Liu et al (2005)	ND
CYP2A6	Cytochrome p450, subfamily IIA, polypeptide 6	Gambier et al (2005); Kubota et al (2006); Minematsu et al (2006); Tyndale et al (1999)	ND
CYP2B6	Cytochrome p450, subfamily IIB, polypeptide 6	Lee et al (2007)	ND
CYP2D6	Cytochrome p450, subfamily IID, polypeptide 6	Caporaso et al (2001)	ND
CYP2E1	Cytochrome p450, subfamily lie	Howard et al (2003); Tyndale (2003)	ND
DBH	Dopamine beta-hydroxylase, plasma	McKinney et al (2000)	ND
DDC	Dopa decarboxylase	Ma et al (2005); Yu et al (2006b); Zhang et al (2006a)	ND
DEFB1	Defensin, beta, 1	Uhl et al (2007)	ND
DLG4	Discs large, Drosophila, homolog of, 4	Lou et al (2007)	ND
DNM1	Dynamin 1	Xu et al (2009)	ND
DRD1	Dopamine receptor D1	Huang et al (2008a)	ND
DRD2	Dopamine receptor D2	Comings et al (1996); Costa-Mallen et al (2005); Noble et al (1994); Spitz et al (1998)	ND
DRD3	Dopamine receptor D3	Huang et al (2008b); Vandenbergh et al (2007)	ND
DRD4	Dopamine receptor D4	Lerman et al (1998); Shields et al (1998)	ND
ELMO1	Engulfment and cell motility gene 1	Uhl et al (2007)	ND
EPAC	Rap guanine nucleotide exchange factor 3	Chen et al (2004)	ND
EPHX1	Epoxide hydrolase 1, microsomal	Liu et al (2005)	ND
ESR1	Estrogen receptor 1	Liu et al (2005)	ND
FBXL17	F-box and leucine-rich repeat protein 17	Bierut et al (2007)	ND
FGF14	Fibroblast growth factor 14	Uhl et al (2007)	ND
FTO	Fat mass- and obesity-associated gene	Bierut et al (2007)	ND
GABRA2	Gamma-aminobutyric acid A receptor 2	Agrawal et al (2008)	ND
GABAB2	Gamma-aminobutyric acid B receptor 2	Beuten et al (2005a)	ND
GABARAP	GABA-a receptor-associated protein	Lou et al (2007)	ND
GABRA4	Gamma-aminobutyric acid receptor, alpha-4	Agrawal et al (2008); Bierut et al (2007)	ND
GCCR	Glucocorticoid receptor	Rogausch et al (2007)	ND
GIRK2	Potassium channel, inwardly rectifying, subfamily j, member 6	Bierut et al (2007)	ND
GPR154	G-protein-coupled receptor 154	Uhl et al (2007)	ND
GRM7	Glutamate receptor, metabotropic, 7	Uhl et al (2007)	ND
HHLA1	Human endogenous retrovirus-h long terminal repeat-associating 1	Uhl et al (2007)	ND
HRH4	Histamine receptor h4	Uhl et al (2007)	ND
HTR1F	Serotonin receptor 1F	Pomerleau et al (2007)	ND
HTR2A	5-αhydroxytryptamine receptor 2a	do Prado-Lima et al (2004)	ND
KCNQ3	Potassium channel, voltage-gated, kqt-like subfamily, member 3	Uhl et al (2007)	ND
LRRN6C	Leucine-rich repeat protein, neuronal, 6c	Uhl et al (2007)	ND
LRRN1	Leucine-rich repeat neuronal protein 1; lern1	Uhl et al (2007)	ND
MAOA	Monoamine oxidase a	Lewis et al (2007); McKinney et al (2000); Tochigi et al (2007)	ND
MAOB	Monoamine oxidase b	Costa-Mallen et al (2005); Lewis et al (2007); Tochigi et al (2007)	ND
MICALCL	MICAL C-terminal like	Uhl et al (2007)	ND
MLH1	MutL, E. coli, homolog of, 1	Yu et al (2006a)	ND
NFIB	Nuclear factor i/b	Uhl et al (2007)	ND
NRXN1	Neurexin 1	Bierut et al (2007); Nussbaum et al (2008)	ND

Table 1 Continued

Gene symbol	Gene name	Reference	Category
NTRK2	Neurotrophic tyrosine kinase receptor, type 2	Beuten et al (2007b)	ND
OC90	Otoconin 90	Uhl et al (2007)	ND
OGG1	8-αOxoguanine DNA glycosylase	Liu et al (2005)	ND
OPRM1	Opioid receptor, mu-1	Ray et al (2006); Zhang et al (2006c)	ND
OSBPL1A	Oxysterol-binding protein-like protein 1a	Uhl et al (2007)	ND
PAM	Peptidylglycine alpha-amidating monooxygenase	Gelernter et al (2007)	ND
PDE4D	Phosphodiesterase 4d, cAMP-specific	Uhl et al (2007)	ND
PDE1C	Phosphodiesterase 1C, calmodulin-dependent, 70 kda	Uhl et al (2007)	ND
PPP1R1B	Phosphatase 1 regulatory subunit 1B	Beuten et al (2007a)	ND
PRKG1	Protein kinase, cGMP-dependent, regulatory, type 1	Uhl et al (2007)	ND
PTC	Phenylthiocarbamide gene	Cannon et al (2005)	ND
PTEN	Phosphatase and tensin homolog	Zhang et al (2006b)	ND
PTHB1	Parathyroid hormone-responsive b1 gene	Uhl et al (2007)	ND
PTPRD	Protein-tyrosine phosphatase, receptor-type, delta	Uhl et al (2007)	ND
SEMA3C	Semaphorin 3c	Uhl et al (2007)	ND
SERT	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4; slc6a4	Gerra et al (2005); Kremer et al (2005)	ND
SHC3	SHC-transforming protein 3	Li et al (2007)	ND
SIPA1L2	Signal-induced proliferation-associated 1 like 2	Uhl et al (2007)	ND
SLC18A2	Solute carrier family 18 (vesicular monoamine), member 2	Schwab et al (2005)	ND
SLC6A3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	Erblich et al (2005); Lerman et al (1999); Ling et al (2004); Timberlake et al (2006)	ND
SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	Liu et al (2005); O'Gara et al (2008)	ND
SLC9A9	Solute carrier family 9 (sodium/hydrogen exchanger), isoform a9	Uhl et al (2007)	ND
TAS2R38	Taste receptor, type 2, member 38	Mangold et al (2008)	ND
TH	Tyrosine hydroxylase	Olsson et al (2004)	ND
TPH1	Tryptophan hydroxylase 1	Reuter and Hennig (2005); Reuter et al (2007)	ND
TPH2	Tryptophan hydroxylase 2	Reuter et al (2007)	ND
TRPC7	Transient receptor potential cation channel, subfamily m, member 2	Bierut et al (2007)	ND
TTC12	Tetratricopeptide repeat domain 12	Gelernter et al (2006)	ND
VPS13A	Vacuolar protein sorting 13, yeast, homolog of a	Bierut et al (2007)	ND
XKR5	XK, Kell blood group complex subunit-related family, member 5	Uhl et al (2007)	ND
CHRNA3	Cholinergic receptor, neuronal nicotinic, alpha polypeptide 3	Schlaepfer et al (2008)	SI/P
CHRNA5	Cholinergic receptor, neuronal nicotinic, alpha polypeptide 5	Schlaepfer et al (2008)	SI/P
CHRNA6	Cholinergic receptor, neuronal nicotinic, alpha polypeptide 6	Zeiger et al (2008)	SI/P
CHRNB3	Cholinergic receptor, neuronal nicotinic, beta polypeptide 3	Zeiger et al (2008)	SI/P
CHRNB4	Cholinergic receptor, neuronal nicotinic, beta polypeptide 4	Schlaepfer et al (2008)	SI/P
COMT	Catechol-O-methyltransferase	Guo et al (2007)	SI/P
CYP2A6	Cytochrome p450, subfamily IIa, polypeptide 6	Audrain-McGovern et al (2007)	SI/P
DRD2	Dopamine D2 receptor	Audrain-McGovern et al (2004); Laucht et al (2008)	SI/P
DRD4	Dopamine D4 receptor	Laucht et al (2008); Skowronek et al (2006)	SI/P
HTR6	5-αHydroxytryptamine receptor 6	Lerer et al (2006)	SI/P
IL8	Interleukin 8	Ito et al (2005)	SI/P
PTEN	Phosphatase and tensin homolog	Zhang et al (2006b)	SI/P
RHOA	RAS homolog gene family, member A	Chen et al (2007)	SI/P
SLC6A3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	Ling et al (2004); Segman et al (2007)	SI/P
SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	Skowronek et al (2006)	SI/P
TPH1	Tryptophan hydroxylase 1	Lerman et al (2001); Sullivan et al (2001)	SI/P

Abbreviations: Cessation, smoking cessation; ND, nicotine dependence; SI/P, smoking initiation/progression.

Table 2 Pathways Overrepresented by Genes Associated with Smoking Initiation/Progression[a]

Pathway	P-value	FDR	Genes included
Calcium signaling	2.24×10^{-6}	8.51×10^{-5}	CHRNA3, CHRNA5, CHRNA6, CHRNB3, CHRNB4
Dopamine receptor signaling	2.57×10^{-6}	4.88×10^{-5}	COMT, DRD2, DRD4, SLC6A3
Serotonin receptor signaling	1.12×10^{-5}	1.42×10^{-4}	HTR6, SLC6A4, TPH1
cAMP-mediated signaling	0.001	0.010	DRD2, DRD4, HTR6
G-protein-coupled receptor signaling	0.002	0.015	DRD2, DRD4, HTR6
Tryptophan metabolism	0.013	0.085	CYP2A6, TPH1
Tight junction signaling	0.018	0.099	PTEN, RHOA
IL-8 signaling	0.021	0.102	IL8, RHOA
Integrin signaling	0.028	0.116	PTEN, RHOA

[a]Pathways identified by IPA unless specified.

Table 3 Pathways Overrepresented by Genes Associated with Nicotine Dependence[a]

Pathway	P-value	FDR	Genes included
Dopamine receptor signaling	1.58×10^{-13}	1.03×10^{-11}	COMT, DDC, DRD1, DRD2, DRD3, DRD4, MAOA, MAOB, PPP1R1B, SLC18A2, SLC6A3, TH
cAMP-mediated signaling	3.16×10^{-12}	1.03×10^{-10}	ADRA2A, CHRM1, CHRM5, CREB1, DRD1, DRD2, DRD3, DRD4, GRM7, HTR1F, OPRM1, PDE1C, PDE4D, RAPGEF3
G-protein-coupled receptor signaling	5.01×10^{-12}	1.03×10^{-10}	ADRA2A, CHRM1, CHRM5, CREB1, DRD1, DRD2, DRD3, DRD4, GRM7, HTR1F, HTR2A, OPRM1, PDE1C, PDE4D, RAPGEF3
Serotonin receptor signaling	6.31×10^{-11}	1.03×10^{-9}	DDC, HTR2A, MAOA, MAOB, SLC18A2, SLC6A4, TPH1, TPH2
Tryptophan metabolism	3.80×10^{-7}	4.94×10^{-6}	CYP2A6, CYP2B6, CYP2D6, CYP2E1, DDC, MAOA, MAOB, TPH1, TPH2
Calcium signaling	3.55×10^{-6}	3.53×10^{-5}	CHRNA3, CHRNA4, CHRNA5, CHRNA7, CHRNB1, CHRNB2, CHRNB3, CREB1, TRPC7
Tyrosine metabolism	3.80×10^{-6}	3.53×10^{-5}	COMT, DBH, DDC, MAOA, MAOB, TH
GABA receptor signaling	2.04×10^{-5}	1.66×10^{-4}	DNM1, GABARAP, GABBR2, GABRA2, GABRA4
Linoleic acid metabolism	4.37×10^{-4}	3.16×10^{-3}	CYP2A6, CYP2B6, CYP2D6, CYP2E1, OC90
Phenylalanine metabolism	1.66×10^{-3}	0.011	DDC, MAOA, MAOB
Arachidonic acid metabolism	2.09×10^{-3}	0.012	CYP2A6, CYP2B6, CYP2D6, CYP2E1, OC90
Metabolism of xenobiotics by cytochrome P450	2.57×10^{-3}	0.014	CYP2A6, CYP2B6, CYP2D6, CYP2E1, EPHX1
Histidine metabolism	3.55×10^{-3}	0.018	DDC, MAOA, MAOB
Neurotrophin/TRK Signaling	0.011	0.049	BDNF, CREB1, NTRK2
LPS/IL-1-mediated inhibition of RXR function	0.012	0.051	ABCC4, CD14, CYP2A6, MAOA, MAOB
Fatty acid metabolism	0.013	0.051	CYP2A6, CYP2B6, CYP2D6, CYP2E1
PXR/RXR activation	0.013	0.051	CYP2A6, CYP2B6, NR3C1
Synaptic long-term potentiation	0.039	0.140	CREB1, GRM7, RAPGEF3
Gap junction[b]	0.005	0.078	DRD1, DRD2, HTR2A, PRKG1
MAPK signaling pathway[b]	0.006	0.078	ARRB1, ARRB2, BDNF, CD14, FGF14, NTRK2
Regulation of actin cytoskeleton[b]	0.012	0.096	ACTN2, CD14, CHRM1, CHRM5, FGF14

[a]Pathways identified by IPA unless specified.
[b]Pathway identified by Onto Pathway-Express.

DISCUSSION

Over recent decades, much has been learnt from animal or cell models about the molecular mechanisms underlying nicotine treatment. Numerous genes and pathways have been found to have a role, either directly or indirectly, in these important smoking-related phenotypes. However, it is less clear whether the same sets of genes and pathways are involved in tobacco dependence of humans. Epidemiologi-cal studies have shown that genetic factors are responsible for a significant portion of the risk for SI, ND, and SC (Hamilton et al, 2006; Lerman and Berrettini, 2003; Li et al, 2003; Mayhew et al, 2000; Sullivan and Kendler, 1999). Moreover, significant genetic overlaps have been identified among these three phenotypes (Ho and Tyndale, 2007; Kendler et al, 1999; Maes et al, 2004). Identifying vulnerability genes for the three phenotypes, especially the biochemical pathways associated with them, will not only

Table 4 Pathways Overrepresented by Genes Associated with Smoking Cessation[a]

Pathway	P-value	FDR	Genes included
Dopamine receptor signaling	2.29×10^{-6}	1.03×10^{-4}	COMT, DRD2, DRD4, FREQ, PPP2R2B, SLC6A3
Glutamate receptor signaling	1.82×10^{-4}	4.10×10^{-3}	GRIK1, GRIK2, GRIN2A, SLC1A2
cAMP-mediated signaling	1.15×10^{-3}	0.017	AKAP13, CREB5, DRD4, DRD2, OPRM1
Calcium signaling	1.91×10^{-3}	0.022	CHRNB2, CREB5, GRIK1, GRIN2A, ITPR2
Circadian rhythm signaling	9.12×10^{-3}	0.082	CREB5, GRIN2A
Amyotrophic lateral sclerosis signaling	0.012	0.086	GRIK2, GRIN2A, SLC1A2
Synaptic long-term potentiation	0.017	0.096	CREB5, GRIN2A, ITPR2
G-protein-coupled receptor signaling	0.017	0.096	CREB5, DRD2, DRD4, OPRM1
Synaptic long-term depression	0.034	0.170	ITPR2, PPP2R2B, PRKG1
Tyrosine metabolism	0.037	0.170	COMT, DBH
Neurotrophin/TRK signaling	0.043	0.180	CREB5, SORCS1
Tight junction[b]	0.007	0.103	CTNNA2, MAGI1, PARD3, PPP2R2B
Gap junction[b]	0.022	0.171	DRD2, ITPR2, PRKG1

[a]Pathways identified by IPA unless specified.
[b]Pathway identified by Onto Pathway-Express.

Table 5 Identified Common and Specific Pathways for Each Smoking Behavior Category

Pathways	Smoking initiation and progression	Nicotine dependence	Smoking cessation
Calcium signaling	+	+	+
cAMP-mediated signaling	+	+	+
Dopamine receptor signaling	+	+	+
G-protein-coupled receptor signaling	+	+	+
Serotonin receptor signaling	+	+	−
Tryptophan metabolism	+	+	−
Gap junction	−	+	+
Neurotrophin/TRK signaling	−	+	+
Synaptic long-term potentiation	−	+	+
Tyrosine metabolism	−	+	+
Integrin signaling	+	−	−
Tight junction signaling	+	−	+
Arachidonic acid metabolism	−	+	−
Fatty acid metabolism	−	+	−
GABA receptor signaling	−	+	−
Histidine metabolism	−	+	−
Linoleic acid metabolism	−	+	−
LPS/IL-1-mediated inhibition of RXR function	−	+	−
MAPK signaling pathway	−	+	−
Metabolism of xenobiotics by cytochrome P450	−	+	−
Phenylalanine metabolism	−	+	−
PXR/RXR activation	−	+	−
Regulation of actin cytoskeleton	−	+	−
Amyotrophic lateral sclerosis signaling	−	−	+
Circadian rhythm signaling	−	−	+
Glutamate receptor signaling	−	−	+
Synaptic long-term depression	−	−	+

provide a systematic overview of the genetic factors underlying different smoking behaviors but is also helpful in guiding selection of potentially important genes for further analysis. With a thorough review of the genes contributing to the genetic risk of smoking behaviors, and a systematic search for gene networks using various pathway
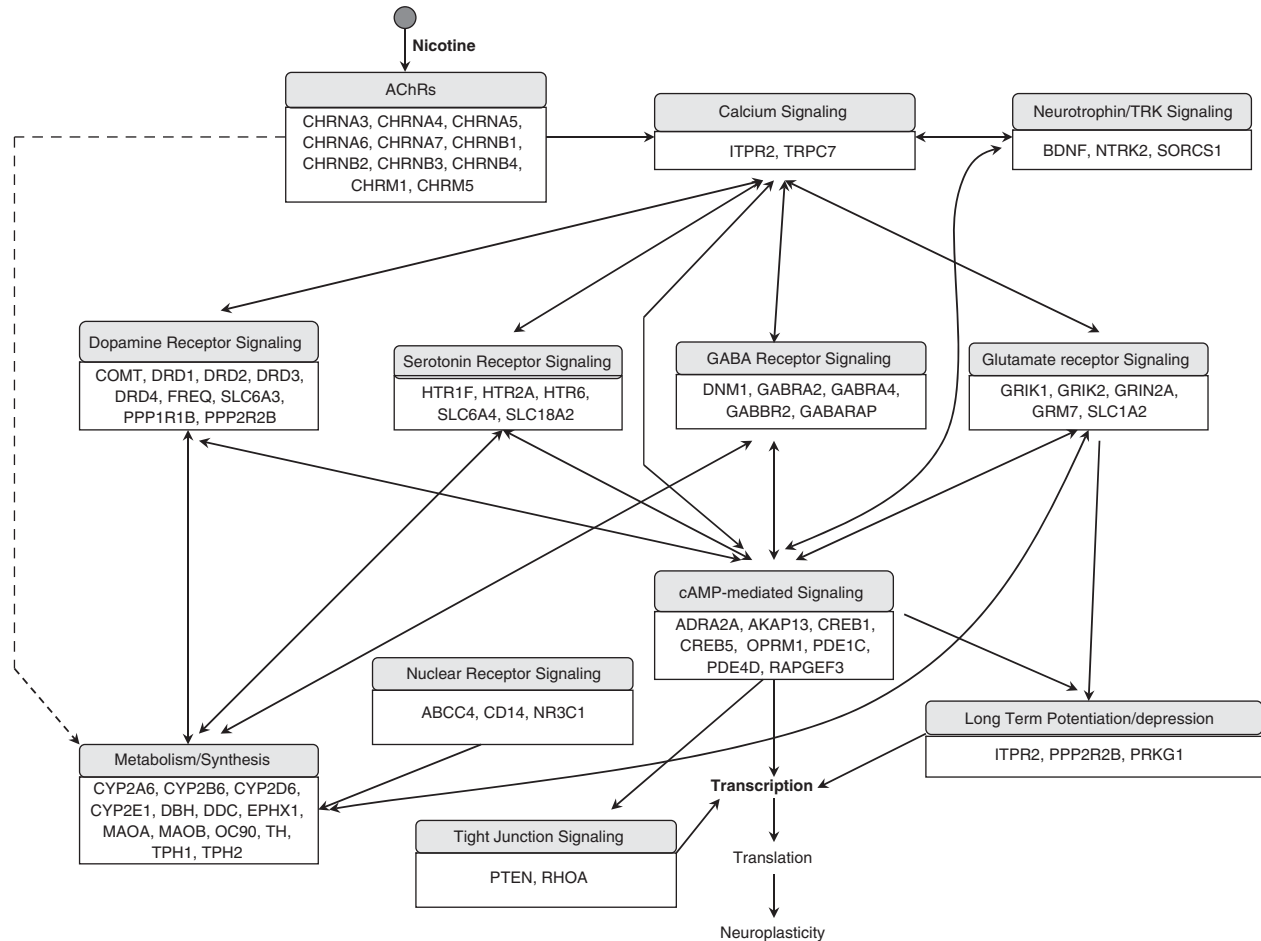
Figure 1 Schematic representation of the genes and major pathways involved in smoking initiation/progression (SI/P), smoking dependence, or smoking cessation (SC). Genetic studies have indicated that tobacco smoking is a complex disorder. On the basis of the genes associated with SI/P, ND, and SC, we identified various enriched pathways corresponding to each phenotype of interest. These pathways were then connected on the basis of their biological relations. Owing to the overlap of many pathways among these three phenotypes, for the sake of simplicity, all identified pathways are shown together.

analysis tools, herein, we provide a comprehensive view of the biochemical pathways involved in the three major smoking phenotypes (see Figure 1 for details).

Although candidate gene-based association studies have provided much of our knowledge about factors contributing to smoking behaviors, a systematic approach, as reported in this study, has significant advantages. For complex disorders such as tobacco smoking, the presence of genetic heterogeneity and multiple interacting genes, each with a small to moderate effect, are considered to be the major hurdle in genetic association studies (Ho and Tyndale, 2007; Lessov-Schlaggar et al, 2008). Numerous genetic factors have been implicated, but in many cases, these findings cannot be replicated in independent studies. At the same time, because of resource limitations, a significant proportion of reported genetic studies might not have sufficient sample size or enough replication samples to reduce the rate of false-positive associations evoked by multiple testing. This is especially true for GWA studies, in which tens of thousands of SNPs can be analyzed simultaneously. A pathway approach, which takes account of the biochemical relevance of genes identified from association studies, not only can be more robust to potential false positives caused by factors such as low density

of markers, small sample sizes, different ethnicities, and heterogeneity within and between samples but also may yield a more comprehensive view of the genetic mechanism underlying smoking behaviors. Moreover, although in candidate gene-based association studies, the selection of targets may be focused on some specific biological processes or pathways, the results from GWA studies seem to be more diverse. In such cases, pathway analysis becomes more necessary to detect the main biological themes from the genes involved in different functions. For example, in a recently reported GWA study, Vink et al (2009) identified 302 genes associated with SI and current smoking, but no gene involved in classic targets, such as dopamine receptor signaling or nAChRs, was detected. Instead, they identified genes related to glutamate receptor signaling, tyrosine kinase signaling, and cell-adhesion proteins. In our analysis based on genes other than those reported by Vink et al, glutamate receptor signaling was enriched among the genes associated with SC, and TRK signaling was enriched in both ND and SC (see Tables 3 and 4, and Figure 1). With the increased interest in conducting GWA studies for smoking behavior and other complex traits, a pathway approach will become more useful.

However, there are several limitations of this study. First, our pathway analysis results depend entirely on genes reported to be associated with each smoking phenotype of interest. Given that identification of susceptibility genes for each smoking phenotype is an ongoing process, the pathways identified in this report should be treated in the same way. Therefore, the pathways identified here are only some of the pathways that may be involved in the regulation of the three phenotypes. This is especially true for SI/P and SC, as significantly more genetic studies have been conducted on ND compared with the other smoking phenotypes. Second, we adopted the conclusions drawn by the original authors of each study in our pathway analysis. This means that some of our conclusions may be biased by some of those original reports because of their small sample size, the presence of heterogeneity, or absence of correction for multiple testing. Initially, we tried to apply a general standard to all those reported studies but had to give it up because different research groups conducted those studies over different time periods. It was challenging to redraw a conclusion from those studies reported by other researchers. However, we do not think this will affect our results greatly, as we have included as many reports as we could get from the literature. Third, for the sake of simplicity and increasing the number of genes included in each smoking phenotype, we classified more than 100 reports on smoking-related behaviors from different ethnic populations into three broad categories, that is, SI/P, ND, and SC. This is certain to bring a heterogeneity issue to the three phenotypes of interest, especially for SI/P and ND. Fourth, the direction of association is an important issue. For example, some variations may be associated with a protective effect against SI or ND, whereas others may increase the risk of such tendencies. Considering the fact that the direction of association depends on genetic variants under investigation for a given phenotype, we did not consider it in our current analyses. Because at this stage we are more interested in the genes and pathways potentially associated with smoking behaviors, focusing on the genes without considering the association directions will not create a serious problem. In addition, to simplify the analysis and reduce the number of false-positive genes, we did not include publications reporting negative or insignificant results. However, we realize that some genes from these studies may be among the factors associated with the smoking behaviors of interest. The fact that they were not found to be associated is likely attributable to other factors such as the small sample size or the presence of heterogeneity in their samples.

Although there are some limitations to this study, some interesting findings emerged, which probably never would have been identified in any single genetic study, including GWA, in one or a few samples. For example, we found that calcium signaling, dopamine receptor signaling, and cAMP-mediated signaling are the main pathways enriched in all three smoking phenotypes. The most prominent calcium signaling-related genes associated with each phenotype were nACh receptors. By mediating intracellular Ca^{2+} concentration, these ligand-gated cation channels have an important role in regulating various neuronal activities, including neurotransmitter release (Marshall *et al*, 1997; Wonnacott, 1997). Transcription factors, such as CREBs

(cAMP responsive element-binding proteins), are crucial for conversion of events at cell membranes into alterations in gene expression. Regulation of the activity of CREB by drugs of abuse or stress has a profound effect on an animal's responsiveness to emotional stimuli (Carlezon *et al*, 2005; Conti and Blendy, 2004). The CREB function in the neurons is normally regulated by glutamatergic and dopaminergic inputs (Dudman *et al*, 2003).

The mesolimbic dopamine pathway is believed to be one of the central pathways underlying addiction to various drugs of abuse (Nestler, 2005). Genes included in this pathway are among the major targets of association study for ND. Although this pathway is enriched in all the three smoking-related phenotypes, the genes associated with each smoking phenotype are different. For SI/P, the genes reported in literature, such as *COMT*, *DRD2*, *DRD4*, and *SLC6A3*, were shared by ND and SC. For SC, two genes, *FREQ* and *PPP2R2B*, were uniquely detected. The *FREQ* protein (also known as neuronal calcium sensor 1, *NCS1*), a member of the neuronal calcium sensor family, has been implicated in the regulation of a wide range of neuronal functions such as membrane traffic, cell survival, ion channels, and receptor signaling (Burgoyne, 2007). In mammalian cells, *FREQ* may couple the dopamine and calcium signaling pathways by direct interaction with *DRD2*, implying an important role in the regulation of dopaminergic signaling in normal and diseased brain (Kabbani *et al*, 2002). The interaction between variants of *DRD2* and *FREQ* significantly impacts the efficacy of nicotine replacement therapy (Dahl *et al*, 2006). *PPP2R2B* encodes a brain-specific regulatory subunit of protein phosphatase 2A (*PP2A*) and gives rise to multiple splice variants in neurons (Dagda *et al*, 2003; Schmidt *et al*, 2002). The product of this gene is suggested to be localized in the outer mitochondrial membrane and involved in neuronal survival regulation through the mitochondrial fission/fusion balance (Dagda *et al*, 2008). A CAG-repeat expansion in a non-coding region of this gene is responsible for the neurodegenerative disorder, spinocerebellar ataxia type 12 (*SCA12*) (Holmes *et al*, 1999). Although the dopamine receptor pathway has an important role in all three smoking phenotypes, it is possible that different parts of this pathway are involved in each smoking behavior, with SI/P and ND having greater similarity than SC. Given the importance of this pathway in the development of drug addiction, more genes need to be verified to obtain a more specific picture of its role underlying each phenotype.

Serotonin modulates dopamine release and has been implicated in nicotine reinforcement. Earlier study has shown that serotonin concentrations are increased by nicotine administration and decreased during withdrawal. Serotonin receptor signaling was enriched in the genes associated with SI/P and ND, but not in those associated with SC, in our analysis. In several recent studies designed to investigate the association between genes from the serotonin receptor signaling pathway and SC, no positive result was obtained (Brody *et al*, 2005; David *et al*, 2007b, 2008a; Munafo *et al*, 2006; O'Gara *et al*, 2008). Similar to the serotonin receptor signaling pathway, tryptophan metabolism, the pathway involved in the biological synthesis of serotonin, is enriched in the genes associated with SI/P, but not in those associated with SC. Consistent with this result,

to date, the clinical effects of serotonergic-based drugs in SC are largely negative (Fletcher *et al*, 2008). Although more studies are needed, these results suggest that the genetic variants in serotonin receptor signaling and tryptophan metabolism pathways may be less important in SC.

Glutamate receptor signaling was found to be enriched in the genes associated with SC, but not in those associated with the other two phenotypes. In a recent GWA study (Vink *et al*, 2009), multiple genes from the glutamate receptor signaling pathway were suggested to be associated with SI and current smoking. Similarly, the glutamate receptor signaling-related genes associated with SC were also identified by a GWA study (Uhl *et al*, 2008). The genes in this pathway that are associated with SC include *GRIK1*, *GRIK2*, *GRIN2A*, and *SLC1A2*, whereas *GRIN2A*, *GRIN2B*, *GRIK2*, and *GRM8* were associated with SI and current smoking (Vink *et al*, 2009). Another gene, *GRM7*, was suggested to be associated with ND in an earlier GWA study (Uhl *et al*, 2007). Taken together, these results suggest that glutamate receptor signaling is involved all three phenotypes of interest. In addition, till now, most of the genes from this pathway were identified by the GWA studies, showing the great potential of the GWA study in identifying genetic variants related to smoking behavior.

Our analysis indicates that the LTP pathway was enriched in genes associated with ND and SC, and the LTD pathway was enriched in genes associated with SC. Repeated exposure of neurons to nicotine eventually leads to the modulation of the functioning of the neural circuits in which the neurons operate. LTP and LTD are thought to be critical mechanisms that contribute to such modifications in neuronal plasticity (Kauer, 2004; Saal *et al*, 2003; Thomas and Malenka, 2003). In the development of ND, the LTP and LTD pathways may be essential for the neurons to form new synapses and eliminate some unnecessary ones to adapt to a new environment. In the process of SC, these pathways may be invoked to interrupt some neuron connections formed in the development of nicotine addiction in order to help the reward circuit return to normal. Until now, only a few genes related to LTP and LTD have been identified in the association studies. Considering the importance of these pathways in ND development and SC, other genes associated with these processes represent potential targets for future studies of these phenotypes.

In a recent study, five pathways were suggested to be associated with addiction to cocaine, alcohol, opioids, and nicotine in humans (Li *et al*, 2008). These pathways are gap junctions, GnRH signaling, LTP, MAPK signaling, and neuroactive ligand–receptor interaction. As shown in Table 5, three of the pathways (gap junction, LTP, and MAPK signaling) were enriched in genes associated with ND or SC. Although another pathway, neuroactive ligand-receptor interaction, was identified for all three smoking phenotypes by either Onto Pathway-Express or DAVID analysis in our work, it was not included in the current report because several more specific pathways, such as calcium signaling, dopamine receptor signaling, and serotonin receptor signaling, were also identified and reported herein. Our results provide further evidence that nicotine may share some biological mechanisms with other substances in addiction conditions. However, we also identified multiple specific pathways related to smoking

behavior, suggesting that the mechanisms underlying nicotine addiction are complex and may be different in certain ways from those associated with addiction to other drugs.

The significantly overrepresented pathways suggest a view of neuronal responses in different conditions of nicotine–neuron interaction (Figure 1). On binding by nicotine, the nAChRs open and cause the influx of Ca^{2+} and Na^+ into the presynaptic neuron, which evokes depolarization of the neuron, as well as activation of the Ca^{2+} signaling cascade. The Ca^{2+} signaling cascade is directly related to the presynaptic release of neurotransmitters, including dopamine, serotonin, GABA, and glutamate, in different neurons. The neurotransmitters interact with their specific receptors, provoking a series of signaling pathways, such as cAMP-mediated signaling and PKC signaling. With the regulation of these pathways, various physiological processes such as neuronal excitability and energy metabolism may be mediated. Variations in some of these genes may change the efficiency or function of the pathways and, eventually, the psychopathological phenotype. Although a significant number of genes associated with these pathways have been identified, our understanding of the genetic determinants of smoking is still in its early stages (Munafo and Johnstone, 2008). It can be expected that as more genetic factors are determined, more detailed pathways and more comprehensive understanding of the mechanisms of human smoking behavior will be obtained.

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DISCLOSURE

The authors declare no conflict of interest.

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