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Converging Findings from Linkage and Association Analyses on Susceptibility Genes for Smoking and Other Addictions

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Abstract

Experimental approaches to genetic studies of complex traits evolve with technological advances. How do discoveries using different approaches advance our knowledge of the genetic architecture underlying complex diseases/traits? Do most of the findings of newer techniques, such as genome-wide association study (GWAS), provide more information than older ones, e.g., genome-wide linkage study? In this review, we address these issues by developing a nicotine dependence (ND) genetic susceptibility map based on the results obtained by the approaches commonly used in recent years, namely, genome-wide linkage, candidate gene association, GWAS, and targeted sequencing. Converging and diverging results from these empirical approaches have elucidated a preliminary genetic architecture of this intractable psychiatric disorder and yielded new hypotheses on ND aetiology. The insights we obtained by putting together results from diverse approaches can be applied to other complex diseases/traits. In sum, developing a genetic susceptibility map and keeping it updated are effective ways to keep track of what we know about a disease/trait and what the next steps might be with new approaches.

Keywords

Addiction; GWAS; Linkage; Smoking; Susceptibility genes

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CONFLICT OF INTEREST

The authors declare no conflicts of interest relating to this report.

INTRODUCTION

Along with technological advances, experimental approaches for the genetic study of complex diseases/traits have evolved from genome-wide linkage study to candidate gene association study and from genome-wide association study (GWAS) to targeted sequencing. With improvements in accuracy, coverage, and cost, whole-exome and whole-genome sequencing studies seem to be the next mainstream approaches. Are the discoveries from all of these approaches consistent? Should we focus on results obtained with newer approaches; e.g., GWAS, and abandon findings from older ones, such as genome-wide linkage study, in the literature sea? How can we make the findings guide our understanding of the genetic architecture of the disease/trait in question? In this review, we use nicotine dependence (ND) as an example to investigate these issues.

Tobacco smoking poses significant threats to public health and kills more than 6 million people annually worldwide, making it one of the three leading components of the global disease burden in 2010.[1] Despite 50 years of prevention efforts, smoking remains the greatest cause of preventable diseases and deaths; each year, nearly 500,000 Americans die prematurely from smoking, and more than 16 million Americans suffer from a disease caused by smoking. Even though today's users smoke fewer cigarettes than those 50 years ago, they are at higher risk of developing adenocarcinoma, possibly because of ventilated filters and greater amounts of tobacco-specific nitrosamines in cigarettes.[2]

Since the 1980s, a broad scientific consensus has been established that nicotine dependence (ND) is the primary factor maintaining smoking behaviour.[3] We and others have shown strong evidence for the involvement of genetics in ND, with an average heritability of 0.56.[4, 5] In the past dozen years, considerable efforts have been exerted to identify the genetic factors underlying ND. However, only three widely accepted “successes;” i.e., the neuronal nicotinic acetylcholine receptor gene clusters on chromosomes 15 (*CHRNA5*/*A3*/*B4*)[6-20] and 8 (*CHRNB3*/*A6*)[13, 15, 18, 21-25] and the genes encoding nicotine-metabolizing enzymes on chromosome 19 (*CYP2A6*/*A7*),[16, 18, 26-28] meet community standards for significance and replication.[29] These few triumphs stand in contrast to the limited heritability they explain; e.g., the most significant synonymous single-nucleotide polymorphism (SNP) rs1051730 ($p = 2.75 \times 10^{-73}$) in *CHRNA3* accounted for only 0.5% of the variance in cigarettes smoked per day (CPD) in a meta-analysis of 73,853 subjects.[16] Researchers have suggested that “missing heritability” is merely hidden and that additional loci can be discovered in GWAS with larger samples,[30, 31] not to mention that the largest ND GWAS to date included 143,023 subjects,[16] and many relevant genetic loci have been revealed with other experimental approaches, such as genome-wide linkage, hypothesis-driven candidate gene association, and targeted sequencing. Despite the fact that many non-GWAS findings have an uncertain yield or failed to be replicated, sorting out genetic loci with evidence from multiple approaches is not only essential but also more cost effective than pursuing a formidable sample size for GWAS.

In this communication, we first review the literature on genetics studies for all smoking-related phenotypes using different approaches by highlighting the converging results from different approaches and then offer new hypotheses that have emerged across the allelic

spectrum, including common and rare variants. These findings provide insights into the preliminary genetic architecture of ND, data that are essential for guiding future research. Crucially, we show that developing a genetic susceptibility map with data from various approaches is an effective means of knowledge integration, research progress evaluation, and research direction forecast.

GENOME-WIDE LINKAGE STUDIES

For many years, linkage analysis was the primary approach for the genetic mapping of both Mendelian and complex traits with familial aggregation.[32, 33] This method was largely supplanted by the wide adoption of GWAS in the middle 2000s. In 2008, we published a comprehensive review of more than 20 published genome-wide linkage studies of smoking behaviour and identified 13 regions, located on chromosomes 3–7, 9–11, 17, 20, and 22, suggestively or significantly linked with various ND measurements in at least two independent samples.[34] Since then, only one genome-wide linkage study has been reported, by Hardin *et al.*,[35] finding a linked spot in the same region as in their previous analysis (6q26) using the same sample but a different phenotype.[36] In addition, Han *et al.*[37] conducted a meta-analysis of 15 genome-wide linkage scans of smoking behaviour and identified two suggestive (5q33.1–5q35.2 and 17q24.3–q25.3) and one significant (20q13.12–q13.32) linkage regions. In fact, the regions on chromosomes 5 and 20 expand two of the regions reported in our 2008 review. The region on chromosome 17 reported by Han *et al.*[37] verified one of the regions detected in only one sample before 2008, which makes it a newly nominated linkage peak (**Table 1**).[34] Please refer to Li[34] and **Table 1** for detailed information on the 14 nominated linkage regions. Figure 1 also shows updated linkage results after incorporating the findings reported after 2008 by Han *et al.*[37]

HYPOTHESIS-DRIVEN CANDIDATE GENE ASSOCIATION STUDIES

Candidate gene association studies usually have moderate sample sizes and are much cheaper than GWAS, where the genes examined are selected according to the linkage/GWAS study results or biological hypotheses. However, because of population heterogeneity and liberal statistical thresholds (compared with GWAS) that often are applied, hypothesis-driven candidate gene association studies generally are considered to have an uncertain yield.[38] On the other hand, the abundant results obtained using this approach provide greater depth of exploration of potential targets and offer valuable replication for other unbiased approaches; e.g., genome-wide linkage study and GWAS.

To eliminate concerns about potential false-positive results, especially for studies reported in earlier years, we focused primarily on the genes showing significance in at least two independent studies with a sample size of ≥ 1,000 or within (or close to) nominated linkage regions or overlapping with GWAS results but with a sample size of ≥ 500 based on the statistical thresholds set by each study. Because the reported sex-average recombination rate is 1.30 ± 0.80 cM/Mbp,[39] in this report, we defined candidate genes within 2 megabase pairs (Mbp) of any linkage region as “within” and 2–5 Mbp as “close to.” The sample size requirement was determined with the following parameters: two-tailed $\alpha = 0.05$, population risk = 0.30, minor allele frequencies = 0.20, and genotypic relative risk = 1.3 with an

approximate odds ratio (OR) of 1.5 or 0.7, which is similar to the statistics usually found in candidate gene association studies. For a statistical power of 0.80 (β = 0.20) using the allelic test, the minimum sample size for a case-control study is 1,062, with equal numbers of cases and controls. Of the reported 201 candidate gene association studies, only 88 have had a sample size of 1,000 or more. Considering the detected power of 0.54 for a sample size of 500 under the dominant genetic model, we also included genes implicated in studies with 500–1,000 subjects if the genes were located in a nominated linkage peak[34] or overlapped with GWAS signals. In total, 34 genetic loci with 43 genes met the criteria (**Table 2** and **Figure 1**), which were assigned to the following four groups. For details on those studies that failed to pass the thresholds but show positive associations, please see **Supplementary Table 1**.

Neurotransmitter system genes

Dopaminergic system—The dopaminergic system has long been acknowledged to play a critical role in nicotine addiction.[40] The most studied gene in this system is *DRD2*, located on chromosome 11q23.2 within a modest linkage peak.[41] The intriguing polymorphism *Taq*1A is located in *ANKK1* near *DRD2*, leading to an amino acid change in *ANKK1*.[42] Several other variants and haplotypes in regions adjacent to *DRD2*, within *TTC12* and *ANKK1*, or downstream of *DRD2* have been associated with smoking-related phenotypes.[13, 43-47] Besides *DRD2*, a modest number of studies have shown significant associations between ND traits and other dopamine receptor genes, such as *DRD1*[48] and *DRD4*,[49-51] and genes involved in dopamine metabolism, including dopamine β-hydroxylase (*DBH*),[13, 52, 53] DOPA decarboxylase (*DDC*),[54, 55] and catechol-O-methyl transferase (*COMT*).[56-61] All of these genes are within or close to the nominated linkage peaks[34] except for *DBH* and *DDC*, which have received support from GWAS results[16] and as ND-associated genes from two independent studies with sample sizes ≥ 1,000.[13,50-53]

Huang *et al*.[62] implicated *DRD3* as a susceptibility gene for ND, but this result has not yet been replicated. Meanwhile, Stapleton *et al*.[63] showed a significant association of a dopamine transporter gene (*SLC6A3*) with smoking cessation in a meta-analysis of 2,155 subjects (80% of European ancestry), although this finding received only weak support from another study on age at smoking initiation in 668 Asians.[64] This gene group includes two others, protein phosphatase 1 regulatory subunit 1B (*PPP1R1B*) and μ-opioid receptor (*OPRM1*), on the basis of their functional connections with dopamine in studies of other addictive substances. *PPP1R1B*, also known as dopamine- and cAMP-regulated neuronal phosphatase (*DARPP-32*), encodes a key phosphoprotein involved in the regulation of several signaling cascades for dopaminoceptive neurons in several areas of the brain, which also is required for the biochemical effects of cocaine.[65] Activation of OPRM1 in the ventral tegmental area suppresses the activity of inhibitory GABAergic interneurons, resulting in disinhibition of dopamine neurons and dopamine release from terminals in the ventral striatum.[66] *OPRM1* A118G variation is a genetic determinant of the striatal dopamine response to alcohol in men,[66] with a preliminary study of tobacco smoking confirming this result.[67] Although we believe in the importance of the above-mentioned genes in ND based on rigorous scientific evidence, the inconsistent results are worth further examination.[68-72]

GABAergic and serotonergic systems—For the GABAergic system, variants in the $GABA_B$ receptor subunit 2 (*GABBR2*),[73] $GABA_A$ receptor-associated protein (*GABARAP*),[74] and $GABA_A$ receptor subunits alpha-2 (*GABRA2*) and −4 (*GABRA4*)[13, 75, 76] were significantly associated with different ND phenotypes. Cui *et al*.[77] reviewed the significance of the GABAergic system in ND and alcohol dependence. In addition, the serotonergic system is implicated in susceptibility to ND because nicotine increases serotonin release in the brain, and symptoms of nicotine withdrawal are associated with diminished serotonergic neurotransmission.[78] Genes encoding serotonin receptor 3A, ionotropic (*HTR3A*),[79] 5A, G protein-coupled (*HTR5A*),[13] and serotonin transporter (*SLC6A4*)[80-82] showed significant association with smoking-related behaviors. All of these seven genes of the GABAergic and serotonergic systems are within or close to the nominated linkage peaks,[34] which strengthens the validity of the identified associations, although two studies reported negative results for association between serotonin transporter gene (*SLC6A4*) and smoking behaviour.[83, 84] Another gene worth mentioning for this group is serotonin receptor 2A, G protein-coupled (*HTR2A*), which is within a modest linkage peak (13q14) suggested by Li *et al*.[85] and was significantly associated with smoking status in a Brazilian sample of 625 subjects.[86] Replication in larger samples is needed to confirm association of this gene with ND.

Glutamatergic system and related genes—Two glutamate receptors, ionotropic, NMDA 3A (*GRIN3A*), within the nominated linkage peak on 9q21.33-q33,[34] and NMDA 2B (*GRIN2B*), suggested by one GWAS[87] and close to a modest linkage peak on 12p13.31-13.32,[88] were significantly associated with scores on the Fagerström Test for Nicotine Dependence (FTND).[89, 90] More genes in the glutamatergic system, such as *GRIN2A*, *GRIK2*, *GRM8*, and *SLC1A2*, showed suggestive association with smoking behaviour in the GWAS reported by Vink *et al*.[87] but without significant replication in candidate gene association studies. Accumulating evidence suggests that blockade of glutamatergic transmission attenuates the positive reinforcing and incentive motivational aspects of nicotine, inhibits the reward-enhancing and conditioned rewarding effects of nicotine, and blocks nicotine-seeking behaviour.[91] More attention may be paid to this neurotransmitter system in the future.

In the catch-all part, after showing suggestive association in the first ND GWAS,[92] neurexin 1 (*NRXN1*) association has been replicated in two independent studies with more than 2,000 subjects of three ancestries: African, Asian, and European.[93, 94] Although neurexin 3 (*NRXN3*) also showed a significant association with the risk of being a smoker,[95] this finding has not been verified in any other ND samples, and *NRXN3* is not within any detected linkage peak.[34] Neurexins are cell-adhesion molecules that play a key role in synapse formation and maintenance and have been implicated in polysubstance addiction.[96]

Nicotinic receptor (nAChR) subunit and other cholinergic system genes

As nAChR subunit gene clusters on chromosomes 15 (*CHRNA5*/*A3*/*B4*) and 8 (*CHRNB3*/*A6*) are major discoveries from ND GWAS, their candidate association results will be discussed together with the GWAS results. Significant association of variants in two other subunit genes (*CHRNA4* and *CHRNB1*) did not approach genome-wide significance

($p < 5 \times 10^{-8}$), but they are both close to nominated linkage peaks.[34] Association of *CHRNA4* with ND, close to the nominated linkage peak on 20q13.12–13.32,[34] has been demonstrated in five independent studies (**Table 2**).[90, 97-100] Variants within *CHRNB1*, located close to the nominated linkage peak on 17p13.1-q22,[34] are significantly associated with FTND and CPD scores.[90, 101] Two other genes encoding nAChR subunits, *CHRNB2* and *CHRNA2*, although associated with ND-related phenotypes in two studies,[102, 103] are not within any detected linkage peaks and have no replication studies reported that are of the required sample size. Thus, these two genes are considered to have only weak evidence of involvement in ND and therefore are not included in **Figure 1** and **Table 2**. Besides nAChR subunit genes, two cholinergic receptors, muscarinic 1 (*CHRM1*) and 2 (*CHRM2*), were found to be significantly associated with CPD and FTND, respectively.[90, 101] They are within nominated linkage peaks as well.[34] However, because of the inadequacy of knowledge of their biological functions, they have been less investigated.

Nicotine metabolism genes

Of the nicotine metabolism genes, those encoding nicotine-metabolizing enzymes (*CYP2A6* and *CYP2B6*) have been most investigated.[104] Six studies have provided consistent evidence that variants leading to reduced or absent CYP2A6 activity are associated with various smoking-related phenotypes, including the nicotine metabolite ratio,[105] time to smoking relapse,[27] exhaled carbon monoxide (CO),[28] initial subjective response to nicotine,[82] FTND,[13] and CPD.[106] All six samples consisted of subjects of European descent (**Table 1**). The negative result of *CYP2A6* in the 2004 meta-analytic review contrasts with the findings from more recent studies, which we believe offer stronger statistical evidence.[107] Such significant association of variants in the *EGLN2-CYP2A6-CYP2B6* region with ND is corroborated by GWAS results, as discussed in the next section.[18, 26]

MAPK signalling pathway and other genes

Although space limitations do not permit an exhaustive review, we want to acknowledge studies implicating other genes in ND, including brain-derived neurotrophic factor (*BDNF*),[108, 109] neurotrophic tyrosine kinase, receptor type 2 (*NTRK2*),[110] arrestin, beta 1 (*ARRB1*),[111] *MAP3K4*,[90] *SHC3*,[112] dynamin 1 (*DNM1*),[113] taste receptor type 2, member 38 (*TAS2R38*),[114] amyloid beta precursor protein-binding, family B, member 1 (*APBB1*),[115] *PTEN*,[116] and neuregulin 3 (*NRG3*).[117] It is worth noting that the first five of these genes belong to the MAPK signalling pathway, which was identified as significantly enriched in involvement with four drugs subject to abuse, namely, cocaine, alcohol, opioids, and nicotine.[118]

GENOME-WIDE ASSOCIATION STUDIES

Although the concept of GWAS was initially proposed in 1996, [119] no GWAS was conducted until 2005. [120] Since then, this technique became the preferred mapping tool for complex diseases/traits.[32] As of October 2015, nine published GWASs and meta-GWASs have yielded 11 genetic loci carrying variants of genome-wide significance (GWS; $p < 5\times10^{-8}$) associated with relevant ND phenotypes in subjects of European, African, and East Asian ancestries (**Table 3** and **Figure 1**). However, only three loci were replicated in more

than two independent GWASs or meta-GWASs, among which the *CHRNA5/A3/B4* gene cluster has the most evidence of significance.

Before the GWAS reports, Saccone *et al.*[13] reported significant association of a 3′-UTR variant (rs578776) in *CHRNA3* with dichotomized FTND in smokers in a candidate gene association study examining 348 genes. Then, in the GWAS era, five variants in this region reached genome-wide significance in five GWAS and meta-GWAS,[12, 16-19] among which four (rs1051730, rs16969968, rs64952308, and rs55853698) were found to be significant in Europeans, and one (rs2036527) was significantly associated with CPD in AAs. The SNPs rs1051730, rs16969968, and rs55853698 are close-tagging proxies (all pairwise $r^2 >$ 0.96), [12] and rs2036527 is correlated with rs1051730.[19] All the r^2s reported in the main text were extracted from the original studies. Thus, these variants were predicted to either tag or potentially cause the principal risk for high smoking quantity attributable to the 15q25 locus, with approximately one CPD step increase for each risk allele.[12, 16, 19] Although the synonymous SNP rs1051730 (Y188Y) in *CHRNA3* showed the strongest association, the nonsynonymous SNP rs16969968 (D398N) in *CHRNA5* and rs55853698 in the 5′-UTR of *CHRNA5* hold more promise of functional importance. In the European samples, conditional on rs16969968 or rs55853698, residual association was detected at rs588765, tagging high expression of *CHRNA5* and rs6495308 within *CHRNA3* as showing significant association with CPD unconditionally. Liu *et al.*[12] discovered better model fitting when conditioning on rs55853698 and rs6495308 compared with rs16969968 and rs588765 using the Bayesian information criteria (BIC). Both rs588765 and rs6495308 were reported to be in low linkage disequilibrium (LD) with each other ($r^2 = 0.21$) and both to be in only modest LD with the principal SNPs (maximum $r^2 = 0.47$) in subjects of European ancestry.[12] However, in the AA samples, no second association signal was detected in this region after conditioning on rs2036527, suggesting that rs20356527 and correlated SNPs in populations of African ancestry define a single common haplotype.[19] At the same time, the finding of importance of this gene cluster has been replicated by candidate gene association studies in persons of Asian ancestry[8, 11] and different ND phenotype-cotinine concentrations,[9] neural responses,[121] smoking cessation successes,[122-124] ages of initiation,[125] and CPD during pregnancy.[126] The two most replicated variants in candidate gene association studies, rs16969968 and rs1051730, are consistent with the GWAS results. Please refer to **Table 2** for details.

The three GWS SNPs on chromosome 8p11 in samples of African and European ancestries —rs13280604, rs6474412, and rs1451240—are in perfect LD with each other[18, 25] and also with a variant (rs13277254) suggestively associated with the ND status of smokers in the first ND GWAS.[92] As noted by Rice *et al.*,[25] although the dichotomized FTND appeared to have an equivalent relation with rs1451240 across ethnicities, the relation between this SNP and CPD was much weaker in AAs than in EAs. The other two SNPs were both significantly associated with CPD in Europeans.[18] These associated SNPs are either intergenic or intronic, which may tag causal variation(s) within the LD block that contains *CHRNB3* and *CHRNA6* or regulate the expression of the two genes directly. Significant association of variants in *CHRNB3* and *CHRNA6* with ND was confirmed in eight candidate gene association studies with diverse population ancestries and smoking traits (**Table**

2).[21-24, 106, 127-129] Cui *et al*.[21] obtained a close to GWS *meta-p* value for an upstream variant of *CHRNB3* (rs4736835) in a candidate gene association study of 22,654 subjects with African, European, and East Asian ancestries.

The last region detected by more than one GWAS or meta-GWAS is on chromosome 19q13.2 and includes genes such as *CYP2A6*/*A7*/*B6*, *EGLN2*, *RAB4B*, and *NUMBL*. Thorgeirsson *et al*.[18] identified rs4105144 and rs7937 as significantly associated with CPD in European samples. These two SNPs were reported to be in LD with each other (r^2 = 0.32 and D' = 0.82 in the HapMap CEU samples). Rs4105144 was also in LD with *CYP2A6*2* (rs1801272; r^2 = 0.13 and D' = 1.0 in the HapMap CEU samples), which reduces CYP2A6's enzymatic activity.[18] The SNP identified by the Tobacco and Genetics Consortium[16] (rs3733829) lies between these sites and was reported to show moderate LD with rs4105144 and rs7937. Besides association signals in samples with European ancestry, Kumasaka *et al*.[26] found a copy-number variant (CNV; rs8102683) with a strong effect on CPD (β = −4.00) in a Japanese population and another significantly associated SNP (rs11878604; β = −2.69) located 30 kb downstream of the *CYP2A6* gene after adjustment of the CNV. Rs8102683 shared a deletion region with other CNVs ranging from the 3′ end of the *CYP2A6* gene to the 3′ end of the *CYP2A7* gene; however, this common deletion was not significant in a European population.[26] Very recently, Loukola *et al*.[130] conducted the first GWAS on nicotine metabolite ratio (NMR) and identified 719 GWS SNPs within this region. Strikingly, the significant *CYP2A6* variants explain a large fraction of variance (up to 31%) in NMR in their sample.

All the other signals reported by only one GWAS or meta-GWAS can be found in **Table 3** and **Figure 1**, among which a missense variant rs6265 in *BDNF* was significantly associated with smoking initiation and an intergenic variant rs3025343 close to *DBH* was implicated in smoking cessation.[16] It is worth noting that GWASs without GWS variant identification still render valuable information in determining susceptibility loci for ND. The first ND GWAS, performed by Bierut *et al*.,[92] nominated *NRXN1* in the development of ND, which was validated by a subsequent candidate gene association study.[93] By using a network-based genome-wide association approach, Vink *et al*.[87] discovered susceptibility genes encoding groups of proteins, such as glutamate receptors, proteins involved in tyrosine kinase receptor signaling, transporters, and cell-adhesion molecules, many of which were confirmed in later candidate gene association studies.[89, 110] Please refer to **Supplementary Table 1** for a list of GWASs without GWS results.

TARGETED SEQUENCING STUDIES

As the “missing heritability” issue emerged in each field, researchers suspected that much of the missing heritability is attributable to genetic variants that are too rare to be detected by GWAS but may have relatively large effects on risk and thus are important to study using next-generation sequencing technologies.[131] Both population genetic theories and empirical studies of several complex traits suggest that rare alleles are enriched for functional and deleterious effects and thus are disproportionately represented among disease alleles.[132]

For the field of ND genetics, rare variant investigation started with the nAChR subunit genes, which not only are biologically important but also have yielded the most replicable results in both GWASs and candidate gene association studies, as presented above. Wessel *et al.*[133] first examined the contribution of common and rare variants in 11 nAChR genes to FTND in 448 EA smokers, which revealed significant effects of common and rare variants combined in *CHRNA5* and *CHRNB2*, as well as of rare variants only in *CHRNA4*. Xie *et al.*[134] followed up on the *CHRNA4* finding by sequencing exon 5, where most of the nonsynonymous rare variants were detected, in 1,000 ND cases and 1,000 non-ND controls with equal numbers of EAs and AAs. They discovered that functional rare variants within *CHRNA4* may reduce ND risk. Also, Haller *et al.*[135] detected protective effects of missense rare variants at conserved residues in *CHRNB4*. They examined *in vitro* the functional effects of the three major association signal contributors (i.e., T375I and T91I in *CHRNB4* and R37H in *CHRNA3*), finding that the minor alleles of the studied SNPs increased the cellular response to nicotine. The two rare variants in *CHRNB4* were confirmed to augment nicotine-mediated α3β4 nAChR currents in hippocampal neurons, as did a third variant, D447X, in the report of Slimak *et al.*[136] The fourth SNP they analyzed, R348C, reduced nicotine currents. They also observed that habenular expression of the β4 gain-of-function allele T374I resulted in strong aversion to nicotine in mice, whereas transduction of the β4 loss-of-function allele R348C failed to induce nicotine aversion. Later, Doyle *et al.*[137] reported an interesting rare variant in *CHRNA5* that could result in nonsense-mediated decay of aberrant transcripts in 250 AA heavy smokers. And recently, Yang *et al.*[138] performed a targeted sequencing study with the goal of determining both the individual and the cumulative effects of rare and common variants in 30 candidate genes implicated in ND. Rare variants in *NRXN1*, *CHRNA9*, *CHRNA2*, *NTRK2*, *GABBR2*, *GRIN3A*, *DNM1*, *NRXN2*, *NRXN3*, and *ARRB2* were found to be significantly associated with smoking status in 3,088 AA samples, and a significant excess of rare variants exclusive to EA smokers was observed in *NRXN1*, *CHRNA9*, *TAS2R38*, *GRIN3A*, *DBH*, *ANKK1*/*DRD2*, *NRXN3*, and *CDH13*. The 18 genetic loci implicated in targeted sequencing studies are marked in **Figure 1**.

IMPLICATIONS

According to our list, 242 candidate gene association, 22 genome-wide linkages, 18 GWAS, and 5 targeted sequencing, making a total of 287 studies, have been conducted in the ND genetics field. The numbers for genome-wide linkage and candidate gene association studies before 2004 are based on Li[34] and Munafò *et al.*,[139] respectively. As a summary and refining of the 286 ND genetic studies, we developed an ND genetic susceptibility map with 14 linkage regions and 47 unique loci of 60 susceptibility genes (**Figure 1**).

Both genome-wide linkage and GWAS are considered “unbiased” exploratory approaches. By comparing their results, we found that only two GWS signals are within the nominated linkage peaks, which are *LOC100188947* and *BDNF*.[34, 140] The other nine loci, including the three most replicable ones, are all outside of the linkage peaks, and the rest of the 12 linkage regions do not contain any GWS signal (**Tables 1** and **2**). This discrepancy might reflect not only the different natures of the two genome-wide approaches but also different ND measures used among those studies. Genome-wide linkage studies usually investigate

sparse microsatellites segregated with the trait of interest in different families, whereas GWAS takes advantage of dense common variants and thousands of unrelated individuals. Because of the distinct characteristics of family and case control samples and known locus heterogeneity for ND, we might not expect same sets of susceptibility alleles to be detected by both approaches. The relatively large nominated linkage regions tagged by microsatellites may implicate common or rare variants or both within the region of interest, on the other hand, it is generally believed that only common variants can be detected by GWAS. Moreover, even if a linkage region is driven by common variants, we may still not be able to locate them in GWAS because of the stringent *p* values applied for defining significance in GWAS. The presence of GWAS signals outside linkage peaks might also result from the lack of power for linkage studies to detect weak genetic effects exhibited by the loci involved in complex diseases compared with association studies.[119] As one can see, these unbiased approaches are powerful in marking areas in the genome; nevertheless, the areas they indicate are often large and may not be complete. In this case, hypothesis-driven studies are useful and necessary tools not only to scrutinize marked areas, but also to explore promising false-negative results and biologically plausible targets.

Both candidate gene association and targeted sequencing studies serve this purpose. Candidate gene association studies replicated and extended 5 of the 11 GWAS results; i.e., *CHRNB3*/*A6*, *DBH*, *BDNF*, *CHRNA5*/*A3*/*B4*, and *EGLN2*/*CYP2A6*/*B6*. For the other 29 non-GWS candidate genetic loci, 20 and 7 were selected from within and close to linkage peaks, respectively, the exceptions being *NRXN1* and *DDC* (**Table 2**), which reminds us of the importance of examining suggestive results in GWAS,[92] the other two examples being *GRIN2B* and *NTRK2*,[87] and biologically plausible genes separately. Although we have localized candidate genes within most of the nominated linkage regions, four linkage peaks, on chromosomes 3q26-q27, 5q11.2-q14, 9p21-p24.1, and 17q24.3-q25.3, are still empty, suggesting there are novel susceptibility genes to be discovered in the future. Overlaps and distinctions from the two unbiased approaches and the significant number of loci reproduced or proposed in candidate gene studies suggest that we have many more study targets with good statistical evidence besides the three most replicable GWAS loci. The fourth "immature" approach is also hypothesis driven and has verified the importance of rare variants in ND genetics.[133-135, 138] Besides the demonstrated aggregate effects of rare variants in 12 genetic loci implicated in previous studies, biological candidates showing equivocal or no association beforehand were found to be significantly associated with ND-related phenotypes, such as *CHRNB2*, *CHRNA9*, *CHRNA2*, *NRXN2*, *NRXN3*, and *CDH13*, among which *CHRNA9* and *NRXN2* are within linkage regions.[34, 141] Thus, we believe whole-exome and whole-genome sequencing studies focusing on rare variants, as the third unbiased experimental approach, will reveal new susceptibility genes/variants and further dissect the existing targets.

It is worth noting that to establish a replication of a genotype–phenotype association, every effort should be made to analyze phenotypes comparable to those reported in the original study.[29] However, the ND genetics studies mentioned above involved a plethora of smoking-related phenotypes. Generally speaking, they can be classified into the following groups: 1) categorical variables along smoking trajectories; e.g., smoking initiation, status, and

cessation; 2) ND assessed using DSM-IV or FTND; 3) smoking quantity such as CPD; and 4) endophenotypes such as NMR, cotinine and CO concentrations, or functional imaging results. At least two of the four phenotype groups have been used in genome-wide linkage studies (**Table 1**),[34] candidate gene association studies (**Table 2**), and GWASs (**Table 3**). Because of the sample source and size requirement differences, DSM- or FTND-ascertained ND definitions were commonly used in linkage studies, whereas CPD was more often applied in GWAS. For candidate gene association studies, more comprehensive smoking profiles were usually tested for association with positive results from unbiased studies as replication, or more importantly, extension by using different phenotypes (**Table 2**), because there is considerable evidence that the various smoking measures are not highly related to one another.[142] Even for measures with relatively high correlation, such as FTND and CPD, the slight change of phenotype from FTND-based ND to CPD would change the results.[25] Therefore, although several loci, such as *TTC12-ANKK1-DRD2*, *CHRNA5/A3/B4*, and *CYP2A6/B6* showed associations with different phenotypes (**Tables 2** and **3**), we should not expect positive associations with one phenotype to be replicated in samples with other phenotypes. It is important to keep in mind that a small change in phenotype may expose previously undiscovered variants, which underlie different biological processes and may have specific roles in distinguishing phenotypes.[25]

Additionally, gene–gene and gene–environment interactions are two pieces of information missing from the current map because of the small number of reported studies. We expect more results in these two areas will be published with the development of efficient algorithms and become important parts of the susceptibility map. It also is worth noting that half of the 48 ND loci were significantly associated with alcohol-related phenotypes, and ~30% were involved in illicit drug dependence (**Supplementary Table 2**), suggesting that the 60 genes on the ND map are good candidates for addiction studies of other drugs as well.

FUTURE DIRECTIONS

Technological advances enable the development of different experimental approaches. A genetic susceptibility map, as put together in this review, contains scientific evidence from diverse approaches and can serve as a draft of the “parts list” to be updated periodically until complete.[38] We hope such an enumeration will catalyze an array of specific targeted and nuanced scientific studies, as suggested by Sullivan *et al.*;[38] e.g., calculating the heritability explained by the 47 genetic loci, replicating association signals currently inadequately supported, identifying causal variant(s) within each locus through expression data integration and functional characterization, selecting appropriate phenotypic measures of ND, elucidating biological mechanisms between the genotype and ND, exploring gene–gene and gene–environment interactions, understanding the part played by epigenetic modifications, developing and evaluating treatment prediction models, and so forth.

Although the sample size of candidate gene association studies has increased over the years (**Supplementary Figure 1A**), genetic power calculation and corresponding sample size ascertainment should always be a top priority before conducting genetic studies. Additionally, only 18% and 10% of the 287 studies investigated subjects with African and Asian ancestries, respectively, compared with 69% for European ancestry (**Supplementary**

Figure 1B). Studying different populations is necessary to understand the genetic causes of ND in various ethnic groups. Concurrently, given the importance of rare variants suggested by targeted sequencing study results, thorough and well-powered genomic evaluations at the lower end of the allelic spectrum are needed. Whole-exome and whole-genome sequencing studies with enough statistical rigor would enable a substantial update of the ND genetic susceptibility map in the near future.

However, it is important to acknowledge that the genetic liability accounted for by each of the 47 loci is probably less than 1% of the phenotypic variance, considering their respective effect sizes, which may also explain why they can be identified through one type of unbiased study, but not the other. Anticipating future studies on the predictive power of these loci cumulatively, we are inclined to project that the amount of heritability explained will still be limited, which renders the susceptibility map as only a beginning. Furthermore, functional studies have been conducted for limited genetic variants with certain or uncertain smoking associations (**Table 4**). Nevertheless, the *TTC12/ANKK1-DRD2* cluster shows consistent association with smoking-related behaviors (**Table 2**), and the function of the most prominent variation in this region, *Taq1A*, still is largely unknown. [47] On the other hand, we have understood the molecular and neurobehavioral functional consequences of *BDNF* Met66Val polymorphism (rs6265) for more than a decade,[143] although its association with ND phenotypes is still relatively weak (**Table 2**). Combining the susceptibility map results with relevant functional annotations will facilitate determination of variations bearing higher translational values.[144] All in all, this map empowers us to sift through existing accomplishments and ponder future research strategies, an approach that may serve as a useful tool for other complex diseases/traits also.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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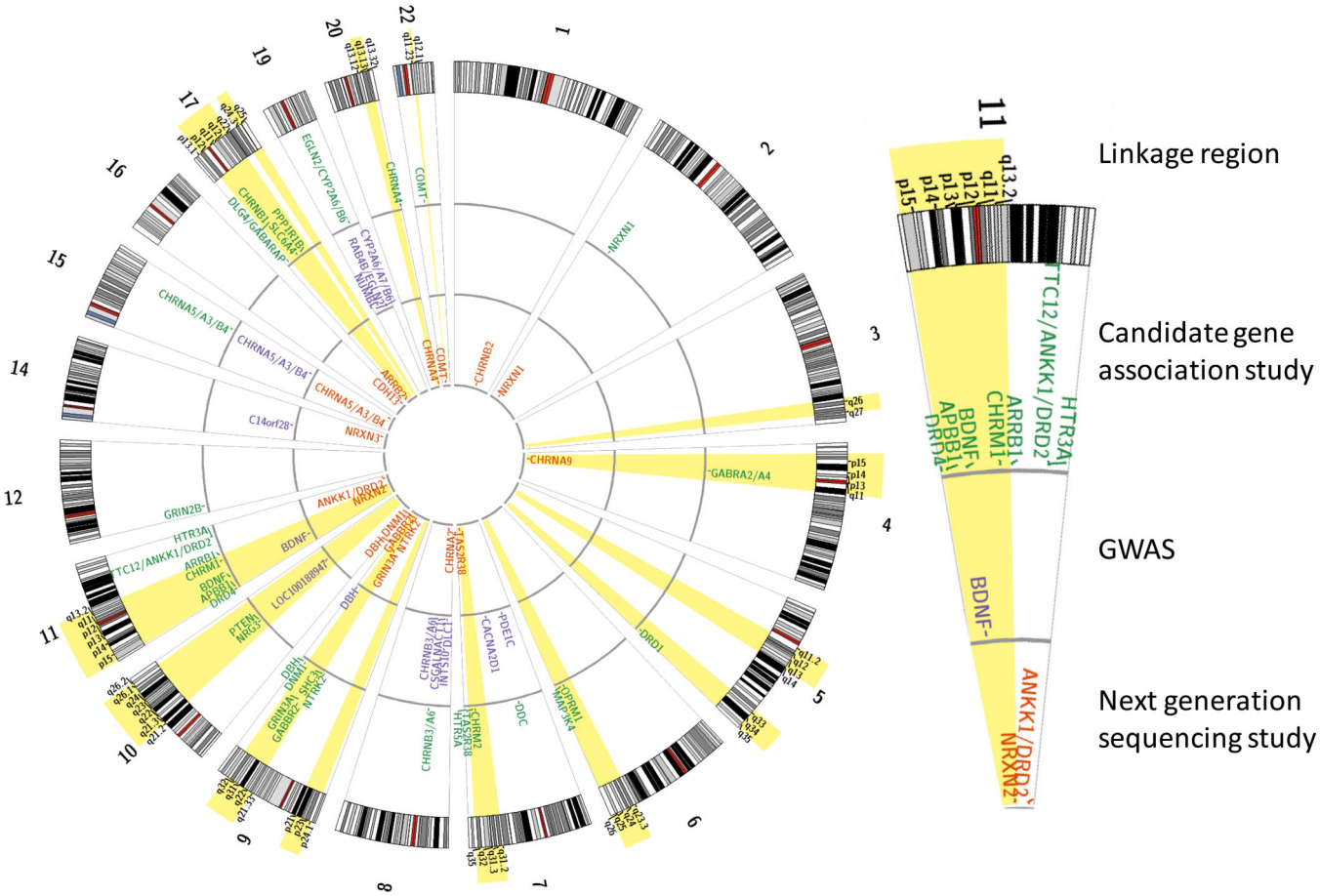

Figure 1.

The ND genetic susceptibility map with nominated linkage peaks and candidate genes, as suggested by genome-wide linkage, hypothesis-driven candidate gene association (CAS), genome-wide association (GWAS), and targeted sequencing (next-generation sequencing; NGS) studies. Linkage peaks are marked in light gray; CAS, GWAS, and NGS results are presented as gene names at the outer, middle, and inner rings, respectively.

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Table 1

Information on the Nominated Linkage Regions Updated According to Li [34]

Chromosome	Marker or marker region	Position	Chr. bands	Phenotype
3	D3S1763–D3S1262	167,239,681–186,223,727	3q26–q27	DSM-IVND, SQ
4	D4S403–D4S2632, D4S244	13,750,828–65,491,728	4p15–q13.1	FTND, CPD
5 (region 1)	D5S1969, D5S647, D5S428	53,242,832–85,410,963	5q11.2–q14	SQ, smoking status, FTND
5 (region 2)*	D5S400, D5S1354	149,800,001–179,631,902	5q33.1–q35*	FTND, CPD
6	D6S1009, D6S1581–D6S281, D6S446	137,302,085–170,552,657	6q23.3–q27	Smoking status, FTND, withdrawal severity
7	D7S486, D7S636	115,894,675–150,699,599	7q31.2–q36.1	FTND, DSM-IV
9 (region 1)	D9S2169–D9S925, D9S925–D9S319	5,200,390–29,560,115	9p21–p24.1	FTND, HSI, SQ
9 (region 2)	D9S257–D9S910, D9S283, D9S64, D9S1825	90,290,735–127,888,281	9q21.33–q33	SQ, FTND, smoking status
10	D10S1432, D10S2469/CYP17, D10S597, D10S1652-D10S1693, D10S129-D10S217	64,407,495–129,540,525	10q21.2–q26.2	SQ, FTND, smoking status
11	D11S4046, D11S4181, D11S2362-D11S1981, D11S1999-D11S1981, D11S2368–D11S2371, D11S1392–D11S1344, D11S1985–D11S2371	1,963,635–73,505,374	11p15–q13.4	FTND, SQ
17 (region 1)	GATA 193, D17S974–D17S2196, D17S799–D17S2196, D17S799–D17S1290	10,518,666–56,331,730	17p13.1–q22	CPD, SQ, HSI
17 (region 2)*	D17S968	67,100,001–81,195,210	17q24.3–q25.3*	Smoking status
20*	D20S119–D20S178, D20S481–D20S480	43,648,850–58,400,000	20q13.12–q13.32*	CPD, SQ
22	D22S345–D22S315, D22S315–D22S1144	24,488,587–27,683,302	22q11.23–12.1	CPD, age at first cigarette

Notes: This table was modified from Table 3 of Li.[34]

Abbreviations: Chr., chromosome; CPD, cigarettes smoked per day; DSM = Diagnostic and Statistical Manual (American Psychiatric Association), FTND, Fagerström Test for Nicotine Dependence; HSI, heaviness of smoking index; SQ, smoking quantity.

*denotes linkage regions expanded or newly ascertained after evaluating results published after our 2008 review. Genomic positions for microsatellite markers and corresponding chromosome bands were obtained through the UCSC Genome Browser (http://genome.ucsc.edu/), which are in the GRCh37/hg19 assembly.

Table 2

Significant Candidate Gene Association Results for ND-Related Phenotypes

Gene	Chr.	Linkage (distance)/GWAS	Sample size	Variant	Position	Variant Type	*P* value	Effect size	Phenotype	Ref
Neurotransmitter system genes										
Dpaminergic system										
TTC12-ANKK1-DRD2	11q23.2	Within the modest linkage peak on 11q23 (0 bp)[41]	638 (270 AAs+368 EAs)	rs2303380-rs4938015-rs11604671 (*TTC12*)-(*ANKK1*)-(*ANKK1*)			0.01	OR = 1.6	Regular smoking initiation	43
			752 (European)	rs1800497 (*Taq*1A)	113270828	Missense (*ANKK1*)	4.0×10^{-3} (interaction with sex and treatment)		Smoking cessation	145
			755 (European)	rs1800497 (*Taq*1A)	113270828	Missense (*ANKK1*)	0.04 (interaction with treatment)		Smoking cessation	146
			782 (European)	rs1799732 (−141C Ins/Del)	113346251: 113346252	Near Gene-5 (*DRD2*)	0.01 (interaction with treatment)		Smoking cessation	147
			1,026 (European)	rs1800497 (*Taq*1A)	113270828	Missense (*ANKK1*)	$<1.0 \times 10^{-8}$		Smoking status	148
			1,615 (854 AAs+761 EAs)	rs4938012	113259654	5′-UTR (*ANKK1*)	8.0×10^{-6} (pooled)		DSM-IV ND	44
			1,900 (European and other)	rs1800497 (*Taq*1A)	113270828	Missense (*ANKK1*)	0.01 (interaction with ADHD symptoms)		Initial subjective response to nicotine	82
			1,929 (European	rs4245150	113364647	Intergenic	0.01		FTND ≥ 4 vs. FTND = 0 in smokers	13
				rs17602038	113364691	Intergenic	0.01			
			2,037 (1,366 AAs+671 EAs)	rs2734849	113270160	Missense (*ANKK1*)	5.3×10^{-4} (AA)		HSI	45
			4,762 (European)	rs10502172	113199146	Intronic (*TTC12*)	9.1×10^{-6}	OR = 1.3	Smoking status	46
DRD1	5q35.2	Within the nominated linkage peak on 5q34–q35 (0 bp)	2,037 (1,366 AAs+671 EAs)	rs686	174868700	3′-UTR	4.8×10^{-3} (AA)		FTND	48

Gene	Chr.	Linkage (distance)/GWAS	Sample size	Variant	Position	Variant Type	*P* value	Effect size	Phenotype	Ref
DRD4	11p15.5	Within the nominated linkage peak on 11p15–q13.4 (1.3 Mbp)	720 (European)	VNTR		Exon 3	0.03		Smoking cessation	49
			839 (59% European)	VNTR		Exon 3	2.0×10^{-3} (interaction with neuroticism)	OR = 3.5	Progression to ND	50
			2,274 (European)	VNTR		Exon 3	6.0×10^{-3}	B = 0.1	CPD	51
DBH	9q34.2	GWAS[16]	793 (European)	rs1541333	136511385	Intronic	4.0×10^{-4} (interaction with ND)		Smoking cessation	52
			1,608 (European)	rs3025382	136502321	Intronic	3.3×10^{-4}		FTND ≥4 vs. 0 in smokers	90
			1,929 (European)	rs4531	136509370	Missense	5.1×10^{-3}		FTND ≥4 vs. 0 in smokers	13
			2,521 [149]	rs5320	136507473	Missense	7.0×10^{-3} (male)		CPD	53
DDC	7p12.1		1,590 (854 AAs+736 EAs)	rs12718541	50550144	Intronic	2.0×10^{-4} (pooled)		FTND	54
			2,037 (1,366 AAs+671 EAs)	rs921451	50623285	Intronic	0.01 (EA)		CPD	55
COMT	22q11.21	Close to the nominated linkage peak on 22q11.23–q12.1 (4.5 Mbp)	511 (81 AAs+430 EAs)	rs737865-rs165599			4.3×10^{-3} (EA)		Smoking cessation	56
			614 (91% European)	rs4680	19951271	Missense	<0.05	OR = 2.1	Increased smoking	57
			657 (European)	rs4680	19951271	Missense	0.02 (male)		Smoking status	58
			2,037 (1,366 AAs+671 EAs)	rs4680	19951271	Missense	9.0×10^{-3} (EA)		CPD	59
			6,310 (European)	rs4680	19951271	Missense	3.0×10^{-3}	OR = 0.7	Smoking cessation	60
			13,312 (European)	rs4680	19951271	Missense	7.0×10^{-3} (meta)	OR = 1.1	Smoking status before pregnancy	61
PPP1R1B	17q12	Within the nominated linkage peak on 17p13.1–q22 (0 bp)	2,037 (1,366 AAs+671 EAs)	rs2271309-rs907094-rs3764352-rs3817160			0.01 (EA)		CPD	150
OPRM1	6q25.2	Within the nominated linkage peak on 6q23.3–	710 (European)	rs1799971	154360797	Missense	5.0×10^{-3} (interaction with sex)		Smoking cessation	151

Gene	Chr.	Linkage (distance)/GWAS	Sample size	Variant	Position	Variant Type	*P* value	Effect size	Phenotype	Ref
		q27 (0 bp)	1,929 (European)	rs510769	154362019	Intronic	9.8×10^{-3}		FTND ≥ 4 vs. 0 in smokers	13
GABAergic system										
GABBR2	9q22.33	Within the nominated linkage peak on 9q21.33–q33 (0 bp)	1,276 (793 AAs+483 EAs)	rs1435252	101103591	Intronic	3.0×10^{-3} (EA)		CPD	73
				rs3750344	101340316	Synonymous	3.0×10^{-3} (EA)			
DLG4-GABARAP	17p13.1	Within the nominated linkage peak on 17p13.1–q22 (0 bp)	2,037 (1,366 AAs+671 EAs)	rs222843	7145981	nearGene-5 (*GABARAP*)	9.0×10^{-3} (EA)		FTND	74
GABRA2-GABRA4	4p12	Within the nominated linkage peak on 4p15–q13.1 (0 bp)	1,929 (European)	rs3762611	46997288	nearGene-5 (*GABRA4*)	9.0×10^{-4}	OR = 0.5	FTND ≥ 4 vs. 0 in smokers	13, 75, 76
Serotonergic system										
HTR3A	11q23.2	Within the modest linkage peak on 11q23 (0 bp)[41]	2,037 (1,366 AAs+671 EAs)	rs1150226-rs1062613-rs33940208-rs1985242-rs2276302-rs10160548			2.0×10^{-3} (AA)		HSI	79
HTR5A	7q36.2	Close to the nominated linkage peak on 7q31.2–q36.1 (4.2 Mbp)	1,929 (European)	rs6320	154862621	Synonymous	6.5×10^{-3}		FTND ≥ 4 vs. 0 in smokers	13
SLC6A4	17q11.2	Within the nominated linkage peak on 17p13.1–q22 (0 bp)	782 (European)	5-HTTLPR+intronic VNTR			1.0×10^{-4}	OR = 1.4	Smoking status	80
			1,098 (41% European)	5-HTTLPR			$<1.0 \times 10^{-3}$ (interaction with peer smoking)	HR = 5.7	Regular smoking initiation	81
			1,900 (European and other)	5-HTTLPR			0.02 (interaction with ADHD symptoms)		Initial subjective response to nicotine	82
Glutamatergic system and other										
GRIN3A	9q31.1	Within the nominated linkage peak on 9q21.33–q33 (0 bp)	2,037 (1,366 AAs+671 EAs)	rs17189632	104368002	Intronic	2.0×10^{-4} (pooled)		FTND	89
GRIN2B	12p13.1	GWAS,[87] close to the modest linkage peak on 12p13.31–	1,608 (European)	rs17760877	13819473	Intronic	1.5×10^{-5} (interaction with age of		FTND ≥ 4 vs. 0 in smokers	90

Gene	Chr.	Linkage (distance)/GWAS	Sample size	Variant	Position	Variant Type	*P* value	Effect size	Phenotype	Ref
			2,047 (European)	rs16969968	78882925	Missense (*CHRNA5*)	5.2×10^{-8}	B = 0.2	FTND	106
			2,206 (European)	rs16969968	78882925	Missense (*CHRNA5*)	4.4×10^{-3} (interaction with childhood adversity in male)	OR=1.8	DSM-IV ND	157
			2,284 (European)	rs17487223	78923987	Intronic (*CHRNB4*)	1.0×10^{-3}		Habitual smoking	7
			2,474 (European)	rs1051730	78894339	Synonymous (*CHRNA3*)	3×10^{-4}	OR=1.3	CPD during pregnancy	126
			2,633 (European)	rs1051730	78894339	Synonymous (*CHRNA3*)	<0.01 (NRT at 6 months)	OR=2.5	Smoking cessation	124
			2,772 (710 AAs+2,602 EAs)	rs16969968	78882925	Missense (*CHRNA5*)	4.5×10^{-8}	OR=1.4	FTND ≥4 vs. 0 in smokers	158
			2,827 (European)	rs680244-rs569207-rs16969968-rs578776-rs1051730 (*CHRNA5*)-(*CHRNA5*)-(*CHRNA5*)-(*CHRNA3*)-(*CHRNA3*)			2.0×10^{-5} (age of daily smoking ≤16)	OR=1.8	FTND≤4 vs. FTND≥6	14
			2,847 (European)	rs3743078	78894759	Intronic (*CHRNA3*)	5.0×10^{-9}	OR=0.7	Heavy vs. light smokers	159
				rs11637630	78899719	Intronic (*CHRNA3*)	5.0×10^{-9}	OR=0.7		
			4,150 (European)	rs1051730	78894339	Synonymous (*CHRNA3*)	5.7×10^{-3}	OR=1.3	CPD≤10 vs. CPD>10	160
			4,153 (European)	rs16969968	78882925	Missense (*CHRNA5*)	5.0×10^{-3}		FTND	161
			4,762 (European)	rs1051730	78894339	Synonymous (*CHRNA3*)	1.1×10^{-5}	OR=1.3	Smoking status	46
			8,842 [149]	rs951266	78878541	Intronic (*CHRNA5*)	1.0×10^{-3} (male)	OR=1.7	Indexed CPD	11
				rs11072768	78929478	Intronic (*CHRNB4*)	1.0×10^{-3} (male)	OR=1.2	Smoking status	
			32,587 (10,912 AAs + 6,889 Asians + 14,786 European)	rs16969968	78882925	Missense (*CHRNA5*)	1.1×10^{-17} (meta)	OR=1.3	CPD≤10 vs. CPD≥20	8
			32,823 (European)	rs1051730	78894339	Synonymous (*CHRNA3*)	$<1.0 \times 10^{-3}$		Pack-years	162

Gene	Chr.	Linkage (distance)/GWAS	Sample size	Variant	Position	Variant Type	*P* value	Effect size	Phenotype	Ref
			33,348 (European)	rs16969968	78882925	Missense (*CHRNA5*)	0.01	OR=1.5 (early-onset) OR=1.3 (late-onset)	CPD ≤10 vs. >20	163
			38,617 (European)	rs16969968	78882925	Missense (*CHRNA5*)	6.0×10^{-31}	OR=1.3	CPD ≤10 vs. >20	164
			965 (European)	rs4950	42552633	5′-UTR (*CHRNB3*)	$<1.0 \times 10^{-4}$ (patients with Parkinson's disease)	OR=1.5	Smoking status	129
			1,051 (132 AAs + 860 EAs + 28 Hispanics + 31 others)	rs7004381	42551161	nearGene-5 (*CHRNB3*)	2.4×10^{-3} (pooled)		Quit attempt	23
CHRNB3-CHRNA6	8p11.21	GWAS[18, 25, 92]	1,076 (189 AAs + 631 EAs + 154 Hispanics + 102 others)	rs892413	42614378	Intronic (*CHRNA6*)	$<1.0 \times 10^{-3}$ (interaction with ADHD symptoms)	β=−0.3	CPD	128
			1,929 (European)	rs13277254	42549982	nearGene-5 (*CHRNB3*)	4.0×10^{-5}	OR=1.4	FTND ≥4 vs. 0 in smokers	127
			2,047 (European)	rs6474412	42550498	nearGene-5 (*CHRNB3*)	1.3×10^{-4}	β=−0.2	WISDM tolerance	106
			2,580 (74% European)	rs4950	42552633	5′-UTR (*CHRNB3*)	$<1.0 \times 10^{-3}$		Initial subjective response to nicotine	24
				rs13280604	42559586	Intronic (*CHRNB3*)	$<1.0 \times 10^{-3}$			
			5,092 (1,661 AAs + 3,431 EAs)	rs13273442	42544017	NearGene-5 (*CHRNB3*)	8.6×10^{-5} (meta)	OR=0.8	FTND ≥4 vs. 0 or 1 in smokers	22
			22,654 (4,297 AAs + 9,515 EAs + 8,842 Asians)	rs4736835	42547033	nearGene-5 (*CHRNB3*)	5.1×10^{-8} (meta)	β=0.16	FTND, indexed CPD	21
CHRNA4	20q13.33	Close to the nominated linkage peak on 20q13.12–13.32 (3.6 Mbp)	621 (Asian male)	rs1044397	61981104	Synonymous	$<1.0 \times 10^{-3}$		FTND	97
			1,608 (European)	rs2236196	61977556	3′-UTR	9.3×10^{-4}		FTND ≥4 vs. 0 in smokers	90
			2,037 (1,366 AAs + 671 EAs)	rs2236196	61977556	3′-UTR	9.0×10^{-4} (AA female)		FTND	98

Gene	Chr.	Linkage (distance)/GWAS	Sample size	Variant	Position	Variant Type	*P* value	Effect size	Phenotype	Ref
			3,695 (2,394 EAs + 1,301 Hispanics)	rs1044396	61981134	Missense	0.02 (pooled)		DSM-IV ND symptom count	100
			5,561 (European)	rs2236196	61977556	3′-UTR	2.3×10^{-3}	β=0.1	FTND	99
CHRNB1	17p13.1	Close to the nominated linkage peak on 17p13.1–q22 (3.2 Mbp)	1,608 (European)	rs17732878	7362359	nearGene-3	1.7×10^{-3}		FTND ≥4 vs. 0 in smokers	90
			2,037 (1,366 AAs + 671 EAs)	rs2302763	7359277	Intronic	0.01 (EA)		CPD	101
CHRM1	11q12.3	Within the nominated linkage peak on 11p15–q13.4 (0 bp)	2,037 (1,366 AAs + 671 EAs)	rs2507821-rs4963323-rs544978-rs542269-rs2075748-rs1938677			8.0×10^{-3} (AA)		CPD	101
CHRM2	7q33	Within the nominated linkage peak on 7q31.2–q36.1 (0 bp)	1,608 (European)	rs1378650	136705151	nearGene-3	2.1×10^{-3}		FTND ≥4 vs. 0 in smokers	90
Nicotine metabolism genes										
EGLN2-CYP2A6-CYP2B6	19q13.2	GWAS[16, 18, 26, 130]	545 (European)	rs1801272	41354533	Missense (*CYP2A6*)	$<1.0 \times 10^{-4}$		Nicotine metabolite ratio	105
				rs28399433	41356379	nearGene-5 (*CYP2A6*)	$<1.0 \times 10^{-4}$			
				*CYP2A6*12*		crossover with *CYP2A7*	$<1.0 \times 10^{-4}$			
				*CYP2A6*1B*		conversion	$<1.0 \times 10^{-4}$			
			709 (European)	genotype-based metabolism (*CYP2A6*)			2.0×10^{-8} (interaction with treatment)	HR=0.4	Time to relapse	27
			1,355 (European)	rs3733829	41310571	Intronic (*EGLN2*)	3.8×10^{-5}	β=2.0	Carbon monoxide (CO)	28
			1,900 (European and other)	rs1801272	41354533	Missense (*CYP2A6*)	0.02 (interaction with ADHD symptoms)		Initial subjective response to nicotine	82
			1,929 (European)	rs4802100	41496025	nearGene-5 (*CYP2B6*)	6.8×10^{-3}		FTND ≥4 vs. 0 in smokers	13
			2,047 (European)	rs3733829	41310571	Intronic (*EGLN2*)	1.5×10^{-3}	β=0.1	CPD	106
MAPK signaling pathway & other genes										

Gene	Chr.	Linkage (distance)/GWAS	Sample size	Variant	Position	Variant Type	*P* value	Effect size	Phenotype	Ref
BDNF	11p14.1	GWAS,[16] within the nominated linkage peak on 11p15–q13.4 (0 bp)	628 [149]	rs6265	27679916	Missense	<0.05 (male)		Age of initiation	108
			2,037 (1,366 AAs + 671 EAs)	rs6484320-rs988748-rs2030324-rs7934165			9.0×10^{-4} (EA)		CPD	109
NTRK2	9q21.33	GWAS,[87] close to the nominated linkage peak on 9q21.33–33 (2.7 Mbp)	2,037 (1,366 AAs + 671 EAs)	rs1187272	87404086	Intronic	1.0×10^{-3} (EA)		HSI	110
ARRB1	11q13.4	Within the nominated linkage peak on 11p15–q13.4 (1.5 Mbp)	2,037 (1,366 AAs + 671 EAs)	rs528833-rs1320709-rs480174-rs5786130-rs611908-rs472112			8.0×10^{-4} (EA)		FTND	111
MAP3K4	6q26	Within the nominated linkage peak on 6q23.3–q27 (0 bp)	1,608 (European)	rs1488	161538250	3′-UTR	2.7×10^{-4}	OR=1.4	FTND ≥4 vs. 0 in smokers	90
SHC3	9q22.1	Within the nominated linkage peak on 9q21.33–33 (0 bp)	2,037 (1,366 AAs + 671 EAs)	rs1547696	91694120	Intronic	9.0×10^{-3} (pooled)		CPD	112
DNM1	9q34.11	Close to the nominated linkage peak on 9q21.33–33 (3.1 Mbp)	2,037 (1,366 AAs + 671 EAs)	rs3003609	130984755	Synonymous	3.1×10^{-3} (EA)		CPD	113
TAS2R38	7q34	Within the nominated linkage peak on 7q31.2–q36.1 (0 bp)	567 (European)	Haplotype conferring intermediate taste sensitivity (AAV)			1.0×10^{-3}		Smoking status	165
			2,037 (1,366 AAs + 671 EAs)	Taster (PAV) and non-taster (AVI) haplotypes			3.0×10^{-3} (AA female)		CPD	114
APBB1	11p15.4	Within the nominated linkage peak on 11p15-q13.4 (0 bp)	2,037 (1,366 AAs + 671 EAs)	rs4758416	6434149	Intronic	3.0×10^{-3} (pooled)		CPD	115
PTEN	10q23.1	Within the nominated linkage peak on 10q21.2–q26.2 (0 bp)	688 (European)	rs1234213	89689321	Intronic	2.0×10^{-4}		Smoking status	116
NRG3	10q23.1	Within the nominated linkage peak on 10q21.2–q26.2 (0 bp)	614 (European)	rs1896506	83874383	Intronic	4.0×10^{-4}		Smoking cessation	117

Notes: Genes significantly associated with ND-related phenotypes in at least two hypothesis-driven candidate gene association studies with a sample size of more than 1,000 or in studies with a sample size of 500 or more but overlapped with linkage or GWAS findings. The "Linkage (distance)/GWAS" column indicates whether a gene is within (< 2 Mbp) or close to (2–5 Mbp) any reported linkage region (**Table 1**) or found significant in GWAS. All the linkage peaks are based on the review by Li in 2008[34] unless otherwise noted. Distances

between candidate genes and nearby linkage regions are in parentheses. For genes with more than one significant variant in a particular study, only the variant(s) with the smallest *p* value(s) is presented, and only the most significant *p* value is shown for each variant if multiple phenotypes were tested in different ethnic/gender groups. Corresponding ethnic/gender group or special analysis methods, such as meta-analysis and interaction, for each *p* value are noted in parentheses right after. Variants composing the most significant haplotype are given if none of the single variants tested was statistically significant. Corresponding effect sizes are provided whenever available. The general term “smoking cessation” was used in the “Phenotype” column for ease of summarization, which represents abstinence at different time points for different studies. Variant positions are based on NCBI Build 37/hg19. For loci with multiple genes, symbols of the gene variants are indicated in parentheses following the variant type.

Abbreviations: Smoking status, smokers vs. non-smokers; HSI, heaviness of smoking index (0-6 scale); FTND, Fagerström Test for Nicotine Dependence (0-10 scale); CPD, cigarettes smoked per day; indexed CPD,[11, 21] CPD categorized as non-smoking, <10, 11-20, 21-30, and >31 CPD; habitual smoking,[7] ever smoking 20 CPD for 6 months or more; heavy vs. light smokers,[159] heavy smokers, defined as smoking at least 30 CPD for at least 5 years, and light smokers, defined as smoking <5 CPD for at least 1 year; WISDM, Wisconsin Inventory of Smoking Dependence motives; NRT, nicotine replacement therapy; AA, African American; EA, European American; VNTR, variable number tandem repeat; 5-HTTLPR, serotonin-transporter-linked polymorphic region; OR, odds ratio; HR, hazard ratio; ADHD, attention-deficit/hyperactivity disorder; bp, base pair; Mbp, megabase pair.

Table 3

Significant Genome-Wide Association Study (GWAS) Findings for ND-Related Phenotypes

Population	Phenotype	Nearest gene	Chr.	SNP [Effect Allele]	Physical Position	Variant Type	Sample size	*P* value	Effect size	Refs
European	CPD	*CHRNA5/A3/B4*	15q25.1	rs1051730[A]	78894339	Synonymous	73,853	2.8×10^{-73}	β = 1.02	12, 16-18
				rs16969968[G]	78882925	Missense	73,853	5.6×10^{-72}	β = 1.00	12, 16
				rs6495308[T]	78907656	Intronic	136,090	5.8×10^{-44}	β = 0.73	12
				rs55853698	78857939	5′-UTR	136,090	1.3×10^{-16}		12
		CYP2A6, EGLN2, RAB4B	19q13.2	rs4105144[C]	41358624	Intergenic	83,317	2.2×10^{-12}	β = 0.39	18
				rs7937[T]	41302706	3′-UTR	86,319	2.4×10^{-9}	β = 0.24	18
				rs3733829[G]	41310571	Intronic	73,853	1.0×10^{-8}	β = 0.33	16
		LOC100188947	10q23.32	rs1329650[G]	93348120	Intronic	73,853	5.7×10^{-10}	β = 0.37	16
				rs1028936[A]	93349797	Intronic	73,853	1.3×10^{-9}	β = 0.45	16
		PDE1C	7p14.3	rs215605[G]	32336965	Intronic	77,012	5.4×10^{-9}	β = 0.26	18
		CHRNB3/A6	8p11.21	rs13280604[A]	42559586	Intronic	76,670	1.3×10^{-8}	β = 0.31	18
				rs6474412[T]	42550498	Intergenic	84,956	1.4×10^{-8}	β = 0.29	18
	FTND	*CACNA2D1*	7q21.11	rs13225753	82158523	Intergenic	4,117	3.5×10^{-8}	NA	166
	Smoking initiation	*BDNF*	11p14.1	rs6265[C]	27679916	Missense	143,023	1.8×10^{-8}	OR = 1.06	16
	Smoking cessation	*DBH*	9q34.2	rs3025343[G]	136478355	Intergenic	64,924	3.6×10^{-8}	OR = 1.12	16
	NMR	*CYP2A6, CYP2B6, CYP2A7, EGLN2, NUMBL*	19q13.2	rs56113850[C]	41353107	Intronic	1,518	5.8×10^{-86}	β = −0.65	130
African American	CPD	*CHRNA5/A3/B4*	15q25.1	rs2036527[A]	78851615	Intergenic	15,554	1.8×10^{-8}	β < 1.00	19
	FTND	*C14orf28*	14q21.2	rs117018253	45337321	Intergenic	3,529	4.7×10^{-10}	NA	166
		CSGALNACT1, INTS10	8p21.3	rs6996964	19623911	Intergenic	3,529	1.1×10^{-9}	NA	166
		DLC1	8p22	rs289519	13237048	Intronic	3,529	4.5×10^{-8}	NA	166
European & African American	Dichotomized FTND	*CHRNB3*	8p11.21	rs1451240[A]	42546711	Intergenic	4,200	6.7×10^{-16}	OR = 0.65	25
Japanese	CPD	*CYP2A6, CYP2A7*	19q13.2	rs8102683[0 copy]	41363765	CNV	17,158	3.8×10^{-42}	β = −4.00	26
				rs11878604[C]	41333284	Intergenic	17,158	9.7×10^{-30}	β = −2.69	26

This table focuses on results achieving genome-wide significance (GWS). We used the significance threshold of 5×10^{-8}. The most significant GWAS finding from different studies for any specific variant is given. If numerous tightly mapped markers showed GWS in one study, only the most significant one is provided. Variant positions are based on NCBI Build 37/hg19. For many studies, it was not possible to extract the exact sample size used for each locus, so the sizes above are approximate. "Effect sizes" refers to beta coefficients for CPD and NMR and odds ratios for smoking initiation and cessation.

Abbreviations: CNV, copy number variation; CPD, cigarettes smoked per day; dichotomized Fagerström Test for Nicotine Dependence (FTND): scores ≥ 4 vs. < 4; NA, not available; NMR, nicotine metabolite ratio; OR, odds ratio; Smoking cessation, whether regular smokers had quit at the time of interview; Smoking initiation, ever versus never began smoking.

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Table 4

Functional studies of variations associated with smoking in the 47 ND susceptibility loci

Chr.	Gene	Experiment	Variation [Effect Allele]	Effect	Ref.
1	*CHRNB2*	*In vitro* gene expression assay	rs2072658 [A]	Reduced expression	167
6	*OPRM1*	PET brain imaging	rs1799971 [G]	Binding potential & receptor availability change	67, 168, 169
8	*CHRNA2*	Electrophysiology assay	rs141072985 rs56344740 rs2472553	nAChR function change	170, 171
	CHRNB3	*In vitro* gene expression assay	rs6474413 [C]	Reduced expression	172
		ChIP and *in vitro* gene expression assay	rs4950 [G]	Eliminated TF binding and reduced promoter activity	129
9	*DNM1*	*In vitro* gene expression assay	rs3003609 [T]	Reduced expression	113
11	*BDNF*	fMRI, ^{1}H-MRSI, and immunoenzyme assays	rs6265	Different brain activation, BDNF secretion, and subcellular distribution	143
	DRD4	fMRI	exon 3 VNTR	Different brain activation	173
15	*CHRNA5/A3/B4*	Imaging Series of *in vitro* assays Electrophysiology and FLEX station	rs16969968 [A]	Brain circuit strength prediction Altered response to nicotine agonist Lower Ca permeability and increased short-term desensitization	7, 174, 175
17	*SLC6A4*	*In vitro* gene expression assay In situ hybridization SPECT imaging	5-HTTLPR	Transcriptional efficiency and expression change	176-178
19	*CYP2A6/B6*	Please refer to Tricker[179] for a comprehensive summary			
20	*CHRNA4*	Electrophysiology assay	exon 5 haplotype	Different receptor sensitivity	180
22	*COMT*	Enzyme activity assay	rs4680 [A]	Less enzyme activity	181

Abbreviations: ChIP, chromatin immunoprecipitation; fMRI, functional magnetic resonance imaging; ^{1}H-MRSI, ^{1}H magnetic resonance spectroscopic imaging; nAChR, nicotinic acetylcholine receptor; PET, positron emission tomography; SPECT, single-photon emission computed tomography.