

New Approaches to Identifying Rare Genetic Variants Associated with Nicotine Dependence

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The publication of candidate gene studies related to nicotine dependence (ND) increased rapidly in the late 1990s and 2000s with a number of studies supporting a role for variation in nicotinic acetylcholine receptor (nAChR) genes. For example, several studies reported that variation in the *CHRNA4* gene was related to the stimulatory effects of nicotine and nicotine dependence as well as to treatment outcomes (1,2). The *CHRNA4* gene codes for the $\alpha 4$ subunit of the $\alpha 4/\beta 2$ receptor. The $\alpha 4/\beta 2$ is the most common nAChR in the brain and arguably the most studied nAChR with respect to ND. Other notable candidate gene findings include studies that reported an association between smoking and variations in other nAChR subunit genes, such as *CHRNA5* and *CHRNA3* (3). Thus, candidate gene studies have suggested that variation in the genes coding for subunits of nAChRs may play an important role in ND etiology and treatment response.

Within the last 5 years, there was a shift from an emphasis on candidate gene studies to an emphasis on genome-wide association studies (GWAS). One of the largest genome-wide analyses involved an analysis of 41,150 smokers (4). These analyses identified three loci that were significantly associated with smoking. Two of the three loci were previously identified in candidate gene studies, as noted above (3). Although confirmatory evidence regarding the role of nAChR genes is valuable and although genome-wide approaches have led to the identification of new genetic associations, others have noted some important limitations of the genome-wide approach. For example, it has been questioned why genome-wide studies with large sample sizes have identified genes that at best explain only 1-2% of the genetic variance in a phenotype. Although there has been some debate about the reasons, explanations include the ideas that GWAS do not adequately capture the effect of rare mutations, that they do not include neurobiological phenotypes, and that they rely heavily on statistical approaches that de-emphasize biological plausibility (5).

In response to these limitations, the field is now turning to the use of next-generation sequencing, made possible by recent technological developments that have greatly reduced the costs of sequencing, to identify and evaluate the role of rare mutations. In addition, scientists are including neurobiological phenotypes that more directly capture the putative underlying molecular effects of a given genetic variant and phenotypes that are mechanistically related to the disorder of interest. In this issue of *Biological Psychiatry*, Xie *et al.* (6) report findings from a study of rare variants in the *CHRNA4* gene and ND that utilizes both sequencing and neurobiological phenotypes, which allows for the identification of rare variants and addresses the biological function of these variants.

The authors sequenced exon 5 of the *CHRNA4* gene in 1000 nicotine-dependent cases and 1000 controls and identified 169 rare

variants, 63 of which were nonsynonymous. Analyses indicated that rare nonsynonymous variants were found more frequently in controls as opposed to nicotine-dependent individuals. In addition, this effect was specific to the intracellular cytoplasmic loop, which is an area that has important implications for the function of the receptor. In vitro functional assays suggested that some of these variants had a detrimental effect. Taking it one step further, the authors also examined the effect of rare variants on in vivo nAChR binding using single-photon emission-computed tomography (SPECT) in a subsample of 139 subjects. One of the rare variants was associated with substantially greater nAChR availability in the brain, compared with four age-matched individuals, suggesting that the variant may alter nAChR availability.

The present article is also one of the first to combine a sequencing approach to identify new variants with an in vitro assessment of the functional effect of these variants. In addition, the analyses included a neuroimaging approach to examine the functional significance of some of these variants on a phenotype (nAChR availability) that is proximate to the molecular function of the gene and central to ND. As expected for a cutting-edge article, the results lead to a number of important questions regarding the role of rare mutations in ND, the role of neurobiological phenotypes in these studies, and statistical approaches for testing the effect of rare variants in complex phenotypes.

Regarding the role of rare variants in nicotine dependence, at least with respect to the $\alpha 4/\beta 2$ receptor, the results suggest that the location of the rare mutations in specific areas within coding regions may be particularly important. To be more specific, differences between cases and controls were observed only for variants within the large cytoplasmic loop in exon 5, whereas there was little difference in the extracellular and transmembrane regions. In this case, the rare variants appear to have a protective effect. The caveat is that this is only one gene, of many genes that may influence ND. In addition, only one region (exon 5) within that one gene was sequenced. Clearly, sequencing this area has revealed important new information about the genetic bases of differences in nAChR function and ND, but much work remains to be done. Rare mutations in other coding and regulatory areas of *CHRNA4* may also influence these phenotypes through different mechanisms. Furthermore, analyses of rare variants outside coding regions are important because studies have suggested that the majority of disease-associated loci in GWAS are in intronic or intergenic regions of unknown function (7). Nonetheless, this study provides an important demonstration of an approach that combines sequencing with information on biological plausibility to identify rare variants in the *CHRNA4* gene that are protective against ND. In so doing, the study represents a reasonable attempt to resolve some of the missing heritability in ND.

On a related note, the present study highlights an important role for neuroimaging and other biological phenotypes because these phenotypes are more tightly coupled with specific genes, which in theory addresses the limitation noted above. In this paper, nAChR function is directly assessed in vivo using SPECT. The SPECT results add a very compelling element to the analysis by suggesting that at least one of these variants may have a substantial effect on in vivo nAChR binding. Thus, SPECT provides some confirmatory evidence

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for the functional significance of at least one variant. In this respect, the study is a great example of the usefulness of integrating cutting-edge approaches that include an *in vivo* measure of brain function. Given that one variation was associated with a 50% increase in binding (albeit in one individual), one cannot help but wonder what the analyses would look like if there were additional SPECT data, enough to perhaps run a traditional statistical test. Of course, there are important limitations related to the low frequency of these variants. Table 2 suggests that even if SPECT data were available for all 1000 nicotine-dependent individuals and controls, there would still be inadequate numbers to perform traditional statistical tests.

In terms of the bigger picture, an important question relates to the relative contribution of rare mutations on the risk for ND as well as its clinical implications. For example, how much of the variance in receptor function or ND is accounted for by rare mutations? This is a difficult question to answer, given the fact that the variants are rare and not adequately represented in normal samples sizes and therefore are not amenable to traditional statistical analyses (5,8). In the present article, the analysis approach compensates for this limitation by comparing the number of rare variants in controls versus nicotine-dependent individuals and comparing nAChR binding between one or two individuals with a rare variant and four age-matched individuals without the variant. Although others have hypothesized that rare mutations may be critical for understanding the genetic risk for a given disease, a notion largely based on the lack of findings from GWAS, which rely largely on the common disease common variant assumption, it is important that we verify the extent to which these variants actually impact risk. For example, in the present study, it is unclear whether all of the variants in the intracellular cytoplasmic loop are important, or only a select one or two. Clearly the SPECT data suggest that one or two of those may make a big difference in terms of receptor availability. Going forward, it is critical to identify new statistical and methodologic approaches for testing the effect of rare variants (5,8).

In conclusion, the article by Xie *et al.* (6) offers important new insights into the role of rare variants and the importance of the

location of those variants within *CHRNA4*. The article also offers a cutting-edge integration of sequencing, *in vitro* assays, and *in vivo* assays of nAChR function to identify new genetic associations. In so doing, a number of important questions are highlighted by this research. Ultimately the public health relevance of this research and sequencing of rare mutations hinges on the answers to these more fundamental questions.

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1. Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P, Labarca C, Whiteaker P, Marks MJ, Collins AC, Lester HA (2004): Nicotine activation of $\alpha 4^*$ receptors: sufficient for reward, tolerance, and sensitization. *Science* 306:1029–1032.
2. Hutchison KE, Allen D, Jepson C, Lerman C, Benowitz N, Stitzel J, *et al.* (2007): *CHRNA4* and tobacco dependence: from gene regulation to treatment outcome. *Arch Gen Psychiatry* 64:1078–1086.
3. Saccone SF, Hinrichs AL, Saccone NL, Chase GA, Konvicka K, Madden PA, *et al.* Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. *Hum Mol Genet* 2007;16:36–49.
4. Liu JZ, Tozzi F, Waterworth DM, Pillai SG, Muglia P, Middleton L, *et al.* (2010): Meta-analysis and imputation refines the association of 15q25 with smoking quantity. *Nat Genet* 42:436–440.
5. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorf LA, Hunter DJ, *et al.* (2009): Finding the missing heritability of complex diseases. *Nature* 461:747–753.
6. Manolio TA, Xie P, Kranzler HR, Krauthammer M, Cosgrove KP, Oslin D, Anton RF, *et al.* (2011): Rare nonsynonymous variants in Alpha-4 nicotinic acetylcholine receptor gene protect against nicotine dependence. *Biol Psychiatry* 70:528–536.
7. Hindorf LA, Sethupathy P, Junkins HA, Ramos EM, Mehta JP, Collins FS, Manolio TA (2009): Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proc Natl Acad Sci USA* 106:9362–9367.
8. Pritchard JK (2001): Are rare variants responsible for susceptibility to complex diseases? *Am J Hum Genet* 69:124–137.