Genome-Wide Association for Smoking Cessation Success: Participants in a Trial with Adjunctive Denicotinized Cigarettes

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The ability to quit smoking successfully displays substantial heritability in classical and molecular genetic studies. Twin studies suggest that some of the genetics for the ability to quit overlap with genetic components of nicotine dependence, but many do not. Genome-wide association (GWA) studies have demonstrated haplotypes that distinguish successful quitters from individuals who were not able to quit smoking in: i) clinical trials that employed nicotine replacement; ii) clinical trials that employed bupropion; and iii) community quitter samples. We now report novel GWA results from participants in a clinical trial that document the efficacy of adjunctive use of denicotinized cigarettes. These results buttress data from our prior GWA studies of smoking cessation. They suggest that ability to change smoking behavior using denicotinized cigarettes shares substantial underlying genetics with the ability to change this behavior in community settings or in response to treatments with nicotine replacement or bupropion.
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INTRODUCTION

Twin studies document that the ability to successfully abstain from smoking displays substantial heritability (1,2). Vulnerability to becoming dependent on addictive substances also displays substantial heritability that is largely shared between dependences on different addictive substances (3–7). Classical genetics suggest that some, but not most, of the influences on ability to quit smoking are shared with the genetics of developing dependence on an addictive substance (1,2).

We have reported recently genome-wide association (GWA) data for successfully quitting smoking in three independent samples of carefully monitored individuals who tried to quit smoking in the context of clinical trials that used placebo, nicotine replacement, or bupropion treatments (8), and in a fourth sample of research volunteers who reported achieving sustained abstinence in community settings (9). The molecular genetic results from these independent samples display substantial convergence with each other; the nominally positive results cluster in small chromosomal regions to extents much more than we would expect by chance. These results also display more modest convergence with molecular genetic data for vulnerability to dependence on nicotine and other addictive substances (8,10–14).

Reduced nicotine cigarettes are designed to progressively wean smokers from the smoking habit. Research demonstrating that smoking cessation requires withdrawal from nicotine, weaning from the habitual sensory, and behavioral reinforcement of smoking supports the idea that denicotinized cigarettes may help extinguish the reinforcing value of smoking-related behavioral and sensory cues. Recently, Becker *et al*. reported significantly increased success in smoking cessation when they added denicotinized cigarettes (Quest 1, Quest 2, and Quest 3; Vector Tobacco Inc., New York, NY, USA) to standard nicotine patch therapy (15). We now report GWA data that compares individuals from this trial who were successful in achieving biochemically monitored 4-week abstinence with those who were unsuccessful. Single nucleotide polymorphism (SNP) data from Affymetrix 6.0 (Affymetrix, Santa Clara, CA, USA) array studies of these samples identifies many of the same chromosomal regions previously identified by data for smoking ces-

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sation and for vulnerability to dependence on addictive substances. Copy number variant (CNV) data from this work provides an initial basis for future comparisons. We discuss the limitations of these modest-sized data sets, as well as the ways in which they buttress previous GWA results for the molecular genetics of smoking cessation from clinical trial and community-based samples of participants who attempted to quit using a variety of other behavioral and pharmacological strategies.

MATERIALS AND METHODS

Samples

DNA was available from most of the 262 participants in a study of denicotinized cigarettes who were followed for at least 4 wks after the end of treatment. Participants smoked ≥ 15 but < 60 cigarettes/d for at least 1 year prior to the study, displayed carbon monoxide measurements > 0.15 ppm, expressed motivation to quit, were 21 to 65 years of age, were recruited at 1 of 5 United States sites, and were generally healthy, as described (15). Participants were randomized to treatment in 1 of 3 conditions that utilized Quest (Quest 1, Quest 2, and Quest 3) denicotinized versus active control cigarettes as well as 24–h patches that contained 21-, 14-, or 7-mg nicotine doses, and standard behavioral support. Subjects were examined and evaluated every 2 wks after randomization until the end of wk 18 (3 months following the targeted quit date), and then 3 months later at the end of week 31 (6 months after the quit date). DNA extracted from the blood of self-reported European-American subjects (the largest self-reported ethnicity in this sample) was pooled into: i) three pools (n = 16 in each) containing DNA from all individuals who self reported abstinence, with biochemical confirmation, at the 4-wk follow-up time point ("successful quitters"); and ii) six pools of individuals who never displayed self-reported abstinence at any follow-up time ("unsuccessful quitters") who were selected based on matches to features noted for the successful quitters. Successful and unsuccessful quitters were similar for gender (% female 39 versus 42), baseline scores on the Fagerstrom Test for Nicotine Dependence (FTND) (5.8 versus 6.1), and the fraction who reported prior unsuccessful attempts to quit (92 versus 94%). Of these individuals who were abstinent for 4 wks, 73% and 65% both attended subsequent 3- and 6-month follow-up visits and reported continuous biochemically verified abstinence at these time points, respectively.

DNA Preparation and Assessment of Allelic Frequencies

DNA was prepared from blood (16–18) and carefully quantitated. DNA from groups of 16 individuals of the same phenotype was combined. Pooled genotyping reduced costs and allowed us to assess high densities of genotypes in these subjects while providing no threat of loss of genetic confidentiality to these individual research volunteers. Hybridization probes were prepared with precautions to avoid contamination, as described (Affymetrix assay 6.0, [19]). For each pool, 250 ng of pooled DNA was processed, labeled, and hybridized to Affymetrix 6.0 arrays according to the instructions of the manufacturer (Affymetrix) and previous studies (16,19,20). Quality controls for assays were performed as recommended (Affymetrix, Supplementary Information).

Identification of Nominally Positive SNPs

Allele frequencies for each SNP in each DNA pool were assessed based on hybridization to the 3–4 "perfect match" cells on each of three arrays, as described (19,20). We have validated this approach and identified approximately 0.98 correlations between SNP genotypes identified in individual versus pooled samples using this approach (19,20). We deleted data from SNPs on sex chromosomes, allowing us to combine data from male and female subjects and increase overall power. We also deleted data for SNPs whose chromosomal positions could not be determined adequately.

Identification of Genomic Regions and Genes that Contained Clustered Positive SNP Data from These Samples

We performed preplanned primary analyses as described previously. We identified SNPs that display t values with $P < 0.01$ "nominally positive" significance in comparison of data from quit success versus failure pools, and cluster in small chromosomal regions, so that at least four of these nominally positive SNPs lie within 25 kb of other reproducibly positive SNPs. A number of these clustered, nominally positive SNPs identify genes; many of them also lie between currently annotated genes. We identify different subsets of these clusters when we impose criteria for < 100 kb, < 50 kb, and < 10 kb distances between reproducibly positive SNPs.

To seek additional support for the chromosomal regions identified by these clusters of nominally positive SNPs, we sought additional association signals in these same regions from clustered, nominally positive SNPs identified in relevant independent GWA studies:

i) 500,000 to 600,000 SNP GWA studies of smokers who were successful versus unsuccessful in quitting in clinical trial settings (three independent samples from Uhl *et al*. [8]);
ii) 500,000 SNP GWA study of smokers who quit versus those who continued to smoke in community settings (Drgon *et al*. [9]);
iii) 38,000 SNP genome-wide association studies of nondependent (FTND) versus dependent (FTND) smokers (Bierut *et al*. [12]); and
iv) data from European-American subjects in studies of dependence on illegal addictive substances (21) (T Drgon, Q-R Liu, P-W Zhang, C Johnson, D Walther, C-Y Li, J Hess, and GR Uhl. Genome wide association for addiction: replicated results and comparisons of two analytic approaches [submitted]) (Table 1).

To provide insights into some of the genes likely to harbor variants that con-

Table 1. Genomic regions with variants likely to contribute to individual differences in smoking cessation success based on data from the current study. The data highlight the same small chromosomal regions identified by clustered nominally positive SNPs from at least one other GWA study of ability to quit smoking or of vulnerability to dependence on addictive substances.

chr[a]	bp:start	bp:end[b]	# SNPs[c]	Confirm sample[d]	# Confirm SNPs[e]	Genes[f]
1	29,920,187	29,948,179	4	C	2[g]	
1	***167,937,772***	***167,975,898***	***5***	***E***	***4***	***SELL, SELE*** [h]
2	**9,192,143**	**9,207,808**	**7**	**E**	**6**	
2	*100,649,980*	*100,682,660*	*9*	*C*	*3*	
2	139,403,085	139,450,494	4	B	2	
2	**221,891,738**	**221,913,606**	**9**	**E**	**4**	
2	***224,903,889***	***224,953,132***	***7***	***F***	***2***	***FLJ22746***
2	***231,987,255***	***232,009,914***	***5***	***F***	***1***	
3	10,972,362	10,995,033	9	C	2	
3	***35,749,993***	***35,762,465***	***5***	***E***	***4***	***ARPP-21[j], MIRN128B***
3	38,734,469	38,778,513	4	B	3	SCN10A
3	141,216,152	141,287,770	7	B	3	CLSTN2[j]
3	141,800,311	141,835,923	4	A	9	
3	**141,800,311**	**141,835,923**	**4**	**F**	**1**	
3	**144,085,356**	**144,113,673**	**4**	**E**	**16**	**PCOLCE2**
3	146,729,297	146,774,561	7	A	3	
4	***41,672,864***	***41,833,889***	***4***	***E***	***16***	***ATP1BL1, CCD4, SLC30A9***[k]
4	126,093,238	126,113,552	6	D, ***E***	4,***4***	
4	126,193,075	126,232,652	4	D	4	
4	168,191,419	168,193,048	4	C	3	SPOCK3[k]
5	168,177,383	168,207,535	5	C	3	SLIT3
6	**86,706,424**	**86,761,974**	**6**	**E**	**5**	
6	**98,227,102**	**98,268,675**	**5**	**E**	**4**	
6	105,161,737	105,193,915	4	B	2	
6	144,960,512	144,982,008	4	C	2	UTRN
7	**42,471,602**	**42,487,544**	**4**	**E**	**8**	
8	3,909,332	3,944,372	4	C,D,***E***	3,6,***9***	CSMD1
9	89,373,187	89,380,485	4	B	3	DAPK1
9	***102,617,616***	***102,659,836***	***6***	***E***	***12***	
9	***102,809,194***	***102,826,144***	***6***	***F***	***1***	***PRG-3***
10	2,734,009	2,811,707	8	B	2	[k]
10	49,758,250	49,810,114	6	A,B	4,8	LRRC18[k]
10	***49,758,250***	***49,810,114***	***6***	***F***	***1***	
10	***66,303,948***	***66,409,111***	***13***	***E***	***7***	
10	69,720,623	69,731,358	4	D	4	MAWBP
10	**88,086,066**	**88,108,932**	**5**	**F**	**3**	**GRID1**
10	108,879,748	108,964,249	9	B,C	3,2	SORCS1
10	118,957,450	118,976,703	4	B	4	KCNK18[k]
11	81,985,443	82,024,265	4	B,E	3,***4***	
11	***91,649,044***	***91,675,441***	***4***	***E***	***4***	
11	***108,260,231***	***108,310,082***	***7***	***E***	***4***	***DDX10***
11	117,090,924	117,119,023	4	C	2	DSCAML1[k]
12	221,728	248,990	5	B	2	SLC6A13[k]
12	**3,432,200**	**3,452,279**	**4**	**F**	**1**	
12	32,976,465	33,009,217	7	C,***F***	2,***1***	
12	33,039,131	33,072,493	6	C	2	
12	104,124,882	104,149,349	4	D	3	APPL2

Continued

tribute to individual differences in ability to quit, we identified candidate genes that are identified by overlapping clusters of positive SNPs from the current and at least one other quit success or addiction vulnerability sample in Table 1.

We compared observed results to those expected by chance using 10,000 Monte Carlo simulation trials, as described (21). For each trial, a randomly selected set of SNPs from the current data set was assessed to see if it provided results ≥ than the results that we actually observed. The number of trials for which the randomly selected SNPs displayed (at least) the same features as the observed results was then tallied to generate an empirical *P* value. These simulations thus correct for the number of repeated comparisons made in these analyses, and are an important consideration in evaluating these GWA data sets.

To assess the power of our current approach, we used current sample sizes and standard deviations, the program PS v2.1.31 (22,23) (see http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize) and $\alpha = 0.05$. To provide controls for the possibilities that abuser-control differences observed herein were due to either occult ethnic/racial allele frequency differences, or noisy assays, we assessed the overlap between the results obtained here and the SNPs that displayed the largest allele frequency differences between African-American versus European-American control individuals and the largest assay "noise."

Identification of Nominally Positive CNV Probes

Allele frequencies for each CNV probe in each DNA pool were assessed based on hybridization to the single "perfect match" cell on each of three arrays, as described (T Drgon, Q-R Liu, C Johnson, P-W Zhang, D Walther, and GR Uhl, Copy number variant probe data and vulnerability to substance dependence [in preparation]). In studies that have sought to validate this

Table 1. *Continued.*

chr[a]	bp:start	bp:end[b]	# SNPs[c]	Confirm sample[d]	# Confirm SNPs[e]	Genes[f]
12	**115,255,355**	**115,276,560**	**4**	**F**	**1**	
13	33,740,610	33,765,548	4	C	2	
13	44,202,415	44,209,971	4	C	2	
13	***60,112,584***	***60,160,245***	***15***	***E***	***6***	[k]
13	***61,658,287***	***61,687,708***	***4***	***F***	***1***	
14	***102,690,084***	***102,714,789***	***5***	***E***	***5***	***RPL21P12***
15	24,857,276	24,876,109	4	C	2	
16	4,508,770	4,534,672	4	B	2	C16 or f5, HMOX2[k]
16	80,978,225	81,035,927	6	A	3	
17	47,051,861	47,087,703	4	B	2	CA10[j]
18	4,636,261	4,670,052	4	A	6	
18	10,027,213	10,030,445	5	C	4	[k]
18	***54,104,584***	***54,136,429***	***4***	***E***	***5***	***NEDD4L***
20	17,715,035	17,728,921	6	B	4	
20	41,966,838	41,982,230	4	A	4	C20 or f100
20	53,283,389	53,307,708	5	B,D	2,5	[k]
21	32,721,019	32,799,427	9	A	3	C21 or f63
22	**48,374,819**	**48,392,082**	**4**	**B**	**2**	**C22 or f34**

[a]Chromosome for the genomic region identified by clustered nominally positive SNPs from the current study and supporting data from at least one other study.
[b]Basepair coordinates for the beginning and end of the genomic region.
[c]Number of nominally positive SNPs that lay in clusters within the region in the current sample
[d]Code for the confirmatory sample(s): A, Lerman; B, Brown; C, Rose from (8); D, Hamer (9); E, data from European-American samples from comparisons of substance dependent to control samples (Drgon submitted); and F, data from comparison of 38,000 SNPs from (12).
[e]Number of SNPs in the confirmatory sample that fall into the chromosomal region defined by the current sample.
[f]Annotated gene(s) that fall within the chromosomal region, if any.
[g]Boldfaced segments are those identified by data from at least two smoking cessation studies.
[h]Italicized segments are those identified by data from the current smoking cessation study and at least one addiction vulnerability GWA sample.
[j]Genes for which "NRT-selective" effects have been previously suggested in eTable 4 in (8).
[k]Genes (or chromosomal regions) for which some (but not all) of the nominally positive SNPs fall into the group of SNPs with the 2.5% largest differences between control European American versus African American research volunteers in prior studies (C Johnson, T Drgon, and GR Uhl, unpublished observations, 2008).

method, we have identified many CNV probes that display good (> 0.9) correlations between CNV genotypes in individual versus pooled samples using this approach, but also identified subsets of CNV probes with poor correlations (< 0.02) (Drgon *et al*. [same as above in this paragraph], in preparation). For primary analyses, we sought CNV probes that display *t* values with $P <$ 0.05 significance in quit success versus failure comparisons and cluster, so that 4 to 10 reproducibly positive CNV probes lie within 25 kb of other reproducibly positive CNV probes.

All supplementary materials are available online at www.molmed.org.

RESULTS

We assessed allele frequencies in multiple pools of DNA from smokers who remained abstinent for at least 4 wks after the end of treatment versus those who were not abstinent at any time point. For SNPs, there was modest variability among replicate arrays (n = 3) that assessed the same pool, the standard error of the mean (SEM) value was 0.015. There also was modest variability among the different pools that assessed the same phenotype; SEM was 0.024. For the quitter versus non-quitter comparison, these samples and these estimates of variance thus provided 0.17, 0.56, 0.89, and 0.98 nominal power to detect allele frequency differences of 2.5%, 5%, 7.5%, and 10%, respectively. The allele frequency differences used in these power calculations are comparable to the actual mean allele frequency differences for the nominally significant SNPs for unsuccessful versus successful quitters in these studies.

Unsuccessful Versus Successful Quitters

When we compared data from unsuccessful versus successful quitters, there was significant clustering of nominally positive SNPs in small chromosomal regions. There were 7,931 "nominally positive" SNPs that displayed allele frequency differences with nominal $P <$ 0.01. Of these nominally positive SNPs, 1,361 fell into 249 clusters of at least four nominally positive SNPs separated from each other by ≤ 25 kb. Monte Carlo P values for this degree of clustering were $P <$ 0.0001. By chance, we would have expected only 29 SNPs to cluster in this fashion. Applying differing sets of criteria based on distances of 100, 50, and 10 kb between nominally positive SNPs led to identification of 2,756, 2,041, and 661 clustered, nominally positive SNPs, in 464, 361, and 138 clusters, respectively (all $P <$ 0.0001, data not shown).

Overlap with Data from Four Previously Reported Quit Success Samples

These data for clustered, nominally positive SNPs from the current data set provide significant overlap with genes identified by other relevant data sets. They thus identify the same small chromosomal regions within genes that are identified by nominally positive results in other studies to extents much greater than

we would expect by chance. The clustered nominally positive SNPs from the current data set provide highly significant overlap with data from each of the three quit success samples reported by Uhl *et al*. (8) (for example, of ≥ four nominally positive SNPs within 25 kb of each other identified by comparisons between successful versus unsuccessful quitters). Overlaps with the samples from Lerman *et al*., Niaura *et al*., and Rose *et al*. identified 34, 80, and 98 clusters of nominally positive SNPs, respectively ($P < 0.0001$ for each comparison). These four SNP/25 kb clusters also displayed significant overlap with clustered, nominally positive SNPs reported by Drgon *et al*. (9). Overlaps with these data identified 27 clusters of nominally positive SNPs ($P < 0.0001$, 1 would have been expected by chance). These overlaps between the clustered, nominally positive SNPs from the current sample and the clustered, nominally positive SNPs from at least one other sample of successful versus unsuccessful quitters identify 40 chromosomal regions that contain 20 annotated genes (see Table 1).

Overlap with Data from Substance Dependence Phenotypes

The clusters of four nominally positive SNPs from the data that compare individuals who are successful versus those who are unsuccessful in quitting smoking in the current trial also overlap at greater than chance levels with data from studies that compare nondependent to dependent smokers and studies that compare substance dependent to nondependent individuals who have little lifetime use of any addictive substance. The overlaps between the clustered, nominally positive SNPs from the current sample and the clustered, nominally positive SNPs from at least one other sample comparing nondependent to dependent individuals identify 29 chromosomal regions that contain 13 annotated genes ($P < 0.001$, see Table 1).

Possible Alternative Explanations for Observed Results

We would anticipate the observed, highly significant clustering of SNPs that display nominally positive results if many of these reproducibly positive SNPs lay near, and were in linkage disequilibrium with functional allelic variants that distinguished subjects who were more able to quit smoking from those who were less able. We would not anticipate this degree of clustering if the results were due to chance. The Monte Carlo P values noted here are thus likely to receive contributions from both the extent of linkage disequilibrium among the clustered, nominally positive SNPs and the extent of linkage disequilibrium between these SNPs and the functional haplotype(s) that lead to the association with quit success.

Control for occult stratification was based on examining the overlap between the 1,361 clustered positive SNPs from the present quit success analyses with the 2.5% of the SNPs for which the racial/ethnic differences in control individuals from prior data sets were largest. There was a mild excess of such SNPs; we identified 64 when 35 would have been expected by chance. Principal components analyses did not provide evidence for overall stratification, however (data not shown). In Table 1, we thus indicate the SNP clusters that contain SNPs which do display sizable racial/ethnic differences, to allow additional cautions in interpreting these data. It is important to note that none of these genes were identified only by SNPs whose allele frequencies fell into those with the 2.5% largest differences between African-American versus European-American control individuals.

Controls for noisy SNPs identified many fewer than expected by chance. None of the clustered nominally positive SNPs overlapped with the set of SNPs that provided the largest variance, while 34 would be expected by chance.

Identification of Nominally Positive CNV Probes

Nominally significant differences between successful and unsuccessful quitters were displayed by 75,915 CNV probes. There was no significant difference from values expected by chance when we examined clusters of ≥ 4 to ≥ 8 nominally positive CNV probes. However, when we examined clusters of ≥ 9 nominally positive CNV probes, we identified 3,771 CNV probes that fell into 344 such clusters in ways that were found in only 79 of 10,000 Monte Carlo trials ($P = 0.0079$). The genes identified by these clusters of ≥ 9 nominally significant CNV probes are listed in Supplemental Table S1.

DISCUSSION

The current results provide independent support for GWA results from prior studies of smoking cessation success in clinical trial and community settings. The substantial overlaps between these data and those obtained previously support the idea that ability to quit in a circumstance in which the most participants used denicotinized cigarettes shares strong overall genetic influences with ability to quit when other pharmacological strategies were used. None of the current data exclude potential influences of allelic variants that are selective to success in using Quest denicotinized cigarettes. Confirmation of such allelic variants will require additional studies using independent samples, however. The current results also support a significant, though more modest, overlap between allelic variants that alter vulnerability to develop dependence on an addictive substance versus those that alter ability to quit smoking, as we have noted in prior analyses (24).

These observations can be discussed in light of the strengths and limitations of the current data sets. The data display several strengths: i) the similar clinical characteristics and the small pool-to-pool variation provide reassurance that these smoking cessation subjects may not differ markedly from the European-American smoking cessation subjects who were studied previously; ii) the subjects were followed carefully with biochemical monitoring of self-reported abstinence; iii) we used pooled genotyping methods that have been validated exten-

sively in prior work and that demonstrate small variance in the current studies; and iv) many more of the positive results from this work than we would expect by chance identify the same chromosomal regions that were identified by other studies of smoking cessation and/or vulnerability to develop substance dependence in European-American samples. In prior work, we have compared results from trials of nicotine replacement to results from trials of bupropion to tentatively nominate genes with "NRT-selective" and "bupropion-selective" effects. It is interesting that current results, from a study of reduced-nicotine cigarettes, identified three of the "NRT-selective" but none of the "bupropion-selective" genes (eTable 4 in [8]).

There are major and minor limitations of the present work. The major limitation is based on the relatively modest number of successful quitters in this trial for whom we have DNA available. Though we have worked to match these individuals to twice their number of nonquitters, limitations from this modest sample size reduce our confidence in the genes that are identified in this work but not in prior studies, and negative data concerning any gene that has been reproducibly identified in prior studies but not in the current work. In addition, the power of this sample is insufficient and does not allow us to split successful quitters based on the treatment to which they were assigned. While we can thus make general statements about the likelihood that the allelic variants that we identify here might impact individual differences in response to Quest or other denicotinized cigarettes based on the use of these cigarettes by most successful quitters, our results do include data from a small group who were able to quit without this aid. Finally, though we have provided extensive validation of the pooled genotyping methods for the SNP probes used here, the modest variance introduced by the pooling approach combined with the modest sample size does provide a modest additional limit to interpretation of data from SNP probes. The large number of CNV probes for which pooling data provides only a modest reflection of data that would be obtained from individual genotyping provides an even greater caution in interpreting the negative data from the CNV probe data sets. Conceivably, these differences could contribute to observations that significant results for the chromosomal clustering of these probes only arise when we require nominally significant differences in at least nine nearby CNV probes.

Despite these limitations, the current data add appreciably to the increasingly robust sets of studies that document clear molecular genetic contributions to the ability to quit smoking in research and community settings. It is conceivable that such findings also may identify some of the genetic determinants of generalized abilities to change health-related behaviors. Thus, for both dependent individuals and individuals with other health problems that can be modified through behavior change, this data adds to an increasingly rich basis for improved understanding and for development of personalized treatment strategies.

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DISCLOSURE

T Drgon, C Johnson, and D Walther report no conflict of interest for this study. JE Rose and GR Uhl are listed as inventors on a patent application filed by Duke University based on genomic markers that distinguish successful quitters from unsuccessful quitters in data from other clinical trials. AP Albino is an employee of Vector Tobacco Inc., which has a financial interest in the product upon which the work was based.

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