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Variants in nicotinic acetylcholine receptors α5 and α3 increase risks to nicotine dependence†

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Abstract

Nicotinic acetylcholine receptors bind to nicotine and initiate the physiological and pharmacological responses to tobacco smoking. In this report, we studied the association of α5 and α3 subunits with nicotine dependence and with the symptoms of alcohol and cannabis abuse and dependence in two independent epidemiological samples (n = 815 and 1,121, respectively). In this study, seven single nucleotide polymorphisms were genotyped in the CHRNA5 and CHRNA3 genes. In both samples, we found that the same alleles of rs16969968 (P = 0.0068 and 0.0028) and rs1051730 (P = 0.0237 and 0.0039) were significantly associated with the scores of Fagerström test for nicotine dependence (FTND). In the analyses of the symptoms of abuse/dependence of alcohol and cannabis, we found that rs16969968 and rs1051730 were significantly associated with the symptoms of alcohol abuse or dependence (P = 0.0072 and 0.0057) in the combined sample, but the associated alleles were the opposite of that of FTND. No association with cannabis abuse/dependence was found. These results suggested that the α5 and α3 subunits play a significant role in both nicotine dependence and alcohol abuse/dependence. However, the opposite effects with nicotine dependence and alcohol abuse/dependence were puzzling and future studies are necessary to resolve this issue.

Keywords

smoking; alcoholism; cannabis; comorbidity; genetic association

Introduction

Tobacco smoking is one of the most costly health issues facing the world today. According to the World Health Organization, billions of dollars were spent in smoking related health care and in the US alone, 440,000 deaths were attributed to smoking related disorders in 2002 [MMWR, [2004]]. While smoking is a complex behavior in which many environmental factors are involved, genetic-epidemiology studies in the last decade have provided compelling evidence that genetic factors play a significant etiologic role [True et al., [1997]; Lerman et al., [1999]; Sullivan and Kendler, [1999]; Li et al., [2003]; Munafo et

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al., [2004]]. Genome wide linkage scans [Bergen et al., [1999]; Duggirala et al., [1999]; Straub et al., [1999]; Bierut et al., [2004]; Vink et al., [2004]; Li et al., [2006]; Swan et al., [2006]] and association studies [Ishikawa et al., [1999]; Bierut et al., [2000]; Caporaso et al., [2001]; Sullivan et al., [2001]; Feng et al., [2004]; Ling et al., [2004]; Li et al., [2007]; Nussbaum et al., [2008]] have identified linkage regions and candidate genes for tobacco smoking and nicotine dependence.

Nicotinic acetylcholine receptors (nAChRs) are highly expressed in the central nerve system and their binding to nicotine, the most active pharmacological compound in cigarettes, triggers the physiological and pharmacological responses to tobacco smoking. Animal model studies have shown that these nAChRs are critical to tolerance, reward and the modulation of mesolimbic dopamine function that are essential to the development of nicotine dependence [Mineur and Picciotto, [2008]]. Many genes in the human genome encode for the nicotinic receptors and several genes have been studied in smoking and nicotine dependence in human subjects [Silverman et al., [2000]; Feng et al., [2004]; Li et al., [2005]; Saccone et al., [2007]; Winterer et al., [2007]]. In a recent study, Saccone et al. reported associations in multiple single nucleotide polymorphisms (SNPs) in CHRNA5, CHRNA3, and CHRNB4 gene cluster on 15q25. However, due to the large number of markers tested, the significance of these results did not survive correction for multiple testing. More recently, several other studies provided independent evidence that the CHRNA5, CHRNA3, and CHRNB4 locus is associated with heavy smoking and nicotine dependence [Berrettini et al., [2008]; Bierut et al., [2008]; Thorgeirsson et al., [2008]]. Interestingly, the associations of these genes with other substance abuse/dependence [Grucza et al., [2008]; Wang et al., [2008]] and with lung cancer [Amos et al., [2008]; Hung et al., [2008]; Thorgeirsson et al., [2008]] were also reported.

The comorbidity between nicotine dependence and alcohol abuse/dependence is well documented in the literature [True et al., [1999]; Daeppen et al., [2000]; John et al., [2003]; Falk et al., [2006]]. Nicotine dependent individuals are at increased risk for misusing a variety of other psychoactive substances [Dani and Harris, [2005]; Degenhardt et al., [2007]]. A recent large-scale twin study indicated that approximately two-thirds of the genetic risk factors for nicotine dependence were substance specific in their effect while one-third were non-specific and increased risk for abuse of a range of other psychoactive substances [Kendler et al., [2007]]. We were therefore interested in testing whether these variants in the α5 and α3 nicotinic receptors were associated solely with smoking and nicotine dependence or also with misuse of either another common licit (alcohol) or an illicit psychoactive substance (cannabis).

Materials and Methods

Sample Description

In this study, we selected two independent samples taken from the Virginia Adult Twin Study of Psychiatric and Substance Use Disorder [Kendler et al., [1999]; Prescott and Kendler, [1999]; Kendler and Prescott, [2007]]. These twin studies ascertained a variety of phenotypes, including substance use/abuse/dependence, neuroticism, and other common psychiatric disorders. Tobacco smoking and nicotine dependence were assessed by the Fagerström Tolerance Questionnaire (FTQ) and/or Fagerström Test of Nicotine Dependence (FTND) during the time of heaviest lifetime nicotine use [Fagerström and Schneider, [1989]; Heatherton et al., [1991]]. The VAANX-ND sample was a combined sample of two previously used panels, the Virginia study of nicotine dependence (VAND) panel and the Virginia study of anxiety and neuroticism (VAANX) panel [Chen et al., [2004], [2008]; Hettema et al., [2006]]. The main reason to combine these two panels together was to increase sample size and statistical power. Because the VAND panel was originally

designed to study both smoking initiation and nicotine dependence [Sullivan et al., [2001]; Chen et al., [2004], [2007]], never smokers were included. There were two groups of smokers in the VAND panel, one group was with low nicotine dependence (defined as those with an FTQ scores between 0 and 2) and the other group was with high nicotine dependence (defined as those with an FTQ score between 7 and 11). The VAANX panel was initially selected for the study of anxiety and neuroticism [Hettema et al., [2006]]. The inclusion criterion was the top and bottom 25 percentile of a genetic factor score - a composite index representing several internalizing anxiety disorders and the personality trait of neuroticism [Hettema et al., [2006]]. We denoted this composite index as internalizing anxiety and neuroticism genetic factor (IANGF) score. In this study, only regular smokers (defined as those who used at some point in their lives an average of at least seven cigarettes per week for a minimum of 4 weeks) were included (Table I and Supplementary Figure S1).

The VAFTND subjects were selected from the same sources as the VAANX-ND sample. This sample included almost all regular smokers who had FTQ/FTND assessments but were not used in the VAANX-ND sample. We excluded those subjects who themselves or their cotwins were used in previous sample panels to maintain the independence of these samples so they can be used to verify results obtained from previous panels. As in the previous panels, for each twin pair, only one twin was selected. The distributions of the IANGF and FTND scores in the two samples are compared in Supplementary Figure S1. The subjects in both samples were adults, with ages between 18 and 65 at the time of FTND ascertainment and all of them self-reported European ancestry.

In addition to FTQ/FTND measures, the subjects were assessed for abuse and dependence of alcohol and illicit substances using DSM-IV criteria. To evaluate whether the nAChR genes were associated with the abuse/dependence symptoms of these other substances, we queried our twin databases and selected categories with reasonable frequencies (>25%) in our combined VAANX-ND and VAFTND samples. We found that only the symptom counts of alcohol and cannabis abuse/dependence had sufficient frequencies to permit useful analysis. Therefore, we used the symptom counts of alcohol and cannabis abuse/dependence (SymAlcAD and SymCanAD, respectively) as phenotypes instead of a dichotomized affected/unaffected category. In parallel to FTND scores where only regular smokers were assessed, we used a “regular drinker” definition (those who used five or more drinks in a month) as the inclusion criterion for SymAlcAD. Similarly, a “regular cannabis user” definition (those who used cannabis five or more times in a month) was used as inclusion criterion for SymCanAD. The rationale for using these definitions was that we wanted to use only those subjects with sufficient exposures to drinking and cannabis use. In the combined sample, 1,801 and 614 subjects met inclusion criteria for SymAlcAD and SymCanAD, respectively. The frequency distributions of SymAlcAD and SymCanAD are shown in Supplementary Figure S2.

Marker Selection and Genotyping

We selected markers based on the Caucasian dataset of the HapMap project and positive association reports. For the interval covering the CHRNA5, CHRNA3, and CHRNB4 genes, we downloaded the Caucasian dataset from the HapMap website (http://www.hapmap.org) and used the HaploView program (version 4.0) to tag markers with frequency of 5% or greater. Markers with positive reports of association (rs16969968 and rs1051730) were included. Accordingly, we selected 7 SNPs covering the 55 kb genomic distance that encodes CHRNA5 and CHRNA3. No markers located in the CHRNB4 gene were selected, since markers in these three genes were in high LD as shown in HapMap database and previous reports, markers selected in CHRNA5 and CHRNA3 tagged those variants in CHRNB4 effectively.

Genotyping was performed with the TaqMan genotyping method [Livak, [1999]]. Briefly, the PCRs were conducted with 384-well microplates. To ensure the quality of genotyping, negative control samples were included in each plate. The PCRs were performed with 5 ng of genomic DNA, 0.25 μl of TaqMan assay mix (Applied Biosystems, Inc., Foster City, CA) and 2.5 μl of TaqMan universal PCR master mix in a total reaction volume of 5 μl. After activating the polymerase and denaturizing DNA by heating at 95°C for 10 min, 40 cycles of 92°C for 15 sec and 60°C for 1 min were performed. After the reaction, the fluorescence intensities of reporter 1 and 2 (reporter 1: VIC, excitation = 520 ± 10 nm, emission = 550 ± 10 nm; reporter 2: FAM, excitation = 490 ± 10 nm, emission = 510 ± 10 nm) were measured by the Analyst Fluorescence Plate Reader (LJL Biosytems, Sunnyvale, CA). Based on the ratio of fluorescence intensities, genotypes were scored by a Euclidean clustering algorithm developed in our laboratory [van den Oord et al., [2003]]. After genotyping, genotypes were cleaned and checked with the HaploView program [Barrett et al., [2005]] for deviation from Hardy-Weinberg Equilibrium. Details of marker characteristics were shown in Table II. Of the seven markers typed, rs6495038 and rs8192475 had mild deviations in the VAANX-ND and VAFTND samples, respectively (Table II).

Data Analyses

In this study, association analyses with FTND score were conducted with the UNPHASED program (version 3.12) [Dudbridge, [2008]], which uses a log likelihood retrospective regression model. Since the VAANX-ND sample contained individuals selected for high IANGF scores, we thought it prudent to control for its effect by including the IANGF score as a covariate in the regression model. In all association analyses, including those performed for SymAlcAD and SymCanAD, a standard Z-score transformation of raw scores was performed for the phenotypes. For association tests, the SNP spectral decomposition (SNPSpD) method [Nyholt, [2004]] was used to evaluate the effective number of independent tests and *P*-values reported were uncorrected. Multi-marker haplotype analyses were conducted similarly with the UNPHASED program, and 1% was used as the cutoff for minor haplotypes. Pairwise LD was estimated for all subjects by the HaploView 4.0 software [Barrett et al., [2005]]. Hardy-Weinberg equilibrium tests were also conducted with the HaploView program. To exclude the impact of FTND on the association of SymAlcAD and SymCanAD, FTND scores were included as a covariate when association with SymAlcAD and SymCanAD was analyzed.

Results

LD Structure

In this study, we genotyped seven SNPs covering the CHRNA5 and CHRNA3 genes in two independent samples selected from our previous twin studies. The first sample, denoted VAANX-ND (n = 815), was a combined sample of two previously used panels, the VAANX panel and the VAND panel [Chen et al., [2004], [2008]; Hettema et al., [2006]]. The second sample, denoted VAFTND (n = 1,121), included subjects who had FTQ/FTND assessments but were not used in the VAANX-ND sample. The characteristics of the markers typed were summarized in Table II. Two markers (rs6495308 and rs8192475) showed mild deviation from Hardy-Weinberg equilibrium. Their location and relative position to the genes were shown in Figure 1A. When linkage disequilibrium (LD) was examined, the two samples showed very similar pairwise LD (D′) among the typed SNPs (Fig. 1B,C). In both samples, rs16969968, rs578776, rs1051730, rs2869546, and rs6495308 were partitioned in a single LD block using default parameters. When haplotype structure was examined, both samples had the same major haplotypes and their frequencies were similar (data not shown). Our data were consistent with those from previous reports and the HapMap database.

Association With FTND

In this study, we used a quantitative design for both VAANX-ND and VAFTND samples. The distribution of FTND scores in the two samples are plotted in Supplementary Figure S1 in the Supplementary Material. The mean FTND score was slightly lower in the VAFTND sample (mean, s.d., 4.27, 2.62) than in the VAANX-ND sample (5.18, 2.57). In the VAANX-ND sample, rs16969968 and rs1051730 were nominally significant (Table III), but only rs16969968 survived correction for multiple testing given the effective number of independent tests estimated at 5 by the SNPSpD method. In VAFTND, four of the seven markers showed nominal significance (Table III), rs16969968 and rs1051730 remained significant after Bonferroni correction. In both samples, the same alleles were over-represented in those subjects with higher FTND scores. For rs16969968, the minor allele A (Asn) was more frequent in those with higher FTND scores; for rs1051730, it was also the minor allele (the A allele) that was over-represented in subjects with higher FTND scores. For both markers, the risk alleles were the same as reported in previous studies [Saccone et al., [2007]; Berrettini et al., [2008]; Thorgeirsson et al., [2008]].

We conducted haplotype analyses for markers rs16969968, rs1051730, and rs2869546 since these markers showed significant or marginal associations in both VAANX-ND and VAFTND samples. In these analyses, we found that the major haplotypes were the same in the two samples, and that their frequencies were similar. In association analyses, haplotype A-A-T or 2-2-1 was significantly associated FTND scores in both samples (Table IV). These results were consistent with that observed in the single marker analyses.

Association With SymAlcAD and SymCanAD

To address whether a variant increasing the risk for tobacco smoking and nicotine dependence also increases risks for alcohol and cannabis abuse or dependence, we tested the association with the symptoms of alcohol and cannabis abuse/dependence phenotypes in the combined sample. Table V summarizes the results of these analyses. Two markers (rs16969968 and rs1051730) were significantly associated with SymAlcAD when the effects of FTND scores were taken into consideration by using them as a covariate (P = 0.0072 and 0.0057, respectively) (Table V). Both markers remained significant after correction for multiple testing. However, the risk alleles for high SymAlcAD were the opposite alleles to what we found associated with FTND. Interestingly, the study of Wang et al. [2008] found the same pattern of results. No association was found with SymCanAD in the combined sample.

Discussion

During preparation of this article, two other reports of association between variants at the CHRNA5, CHRNA3, and CHRNB4 loci at 15q25 and heavy smoking/nicotine dependence were published [Bierut et al., [2008]; Thorgeirsson et al., [2008]]. These and our study replicated the association of CHRNA5 and CHRNA3 with smoking and nicotine dependence. Two previous studies [Berrettini et al., [2008]; Thorgeirsson et al., [2008]] used the number of cigarettes smoked as phenotype. By contrast, ours and other studies [Saccone et al., [2007]; Bierut et al., [2008]] used FTND as phenotype. To compare directly how much difference the phenotypes made, we examined our data using “the number of maximal cigarettes smoked daily” as a phenotype. In our combined sample, the P-value was 0.0010 (effect = 0.111, 95% CI, 0.045-0.178) for rs16969968, which was comparable with the results obtained with FTND scores (see Table III). But for rs2869546, using the number of cigarettes smoked produced a slightly stronger association (P = 0.0060, effect = 0.092, 95% CI, 0.026-0.159). As pointed out in the previous studies [Saccone et al., [2007]; Berrettini et al., [2008]; Thorgeirsson et al., [2008]], rs16969968 and rs1051730 were in high LD (r^2 =

0.96 in our combined sample), our results were consistent with this observation. From our study, it seems that a large sample is required to detect the small effect at this locus. For rs16969968, carrying one copy of the minor allele, the A allele (Asn), only increased the mean by 0.13-0.17 standard deviation. Of note, none of the markers typed in this study is located in the CHRNB4 gene. Since the markers used in our study were selected to tag the variants in the CHRNA5/CHRNA3/CHRNB4 genes, where extensive high LD was evident in both our samples and others reported [Saccone et al., [2007]; Berrettini et al., [2008]; Bierut et al., [2008]; Grucza et al., [2008]; Schlaepfer et al., [2008]; Wang et al., [2008]; Weiss et al., [2008]], we believe that the association signal observed at rs16969968 and rs1051730 could extend to CHRNB4 gene. In other words, while we did not observe association directly from CHRNB4 gene, due to its high LD with CHRNA5 and CHRNA3 genes, we cannot exclude it from the association observed at CHRNA5 and CHRNA3 genes.

We also found significant association with symptoms of alcohol abuse and dependence in our combined sample, but the associated alleles were the opposite of those associated with the FTND score. While this finding is similar to those reported recently by others [Grucza et al., [2008]; Wang et al., [2008]], it is not consistent with epidemiological studies where nicotine dependence is consistently found to be positively correlated with alcohol abuse and dependence. In analyses without using FTND as covariate, both rs16969968 and rs1051730 was nominally significant (P = 0.018 and 0.029 respectively, data not shown). However, the association became stronger when accounting for the effect of FTND scores. These analyses further support the hypothesis that the effects of FTND and SymAlcAD at these variants are truly in the opposite direction. It is possible that these findings reflect the different actions of the two substances: nicotine is a stimulant whereas alcohol is a depressant. So, for example, if those with Asn alleles are more predisposed to enjoy the action of stimulants, they may be less likely to enjoy the effects of depressants. Nevertheless, in the light of the strong comorbidity between nicotine and alcohol dependence in the general population, these findings are puzzling and future studies will be necessary to resolve this issue.

To summarize, there were two main findings of this study. First, in two large samples, we verified the association of rs16969968 and rs1051730 with nicotine dependence when FTND scores were used as phenotype. Of these two SNPs, rs16969968 is a non-synonymous polymorphism, changing Asp to Asn at the 398th amino acid position of the CHRNA5 gene. This provides an opportunity to directly test this polymorphism in animal models. Combining these results with those obtained from prior studies [Saccone et al., [2007]; Berrettini et al., [2008]; Bierut et al., [2008]; Thorgeirsson et al., [2008]], we believe that the α5 and α3 genes are established as one of the best replicated genes imposing risks to tobacco smoking and nicotine dependence. Functional studies of these genes in animal and cellular models should follow. Second, we also found significant association with SymAlcAD for the same markers associated with FTND scores. However, the effects were in the opposite direction. While this finding was in agreement with others' reports [Grucza et al., [2008]; Wang et al., [2008]], it is inconsistent with the findings of epidemiological studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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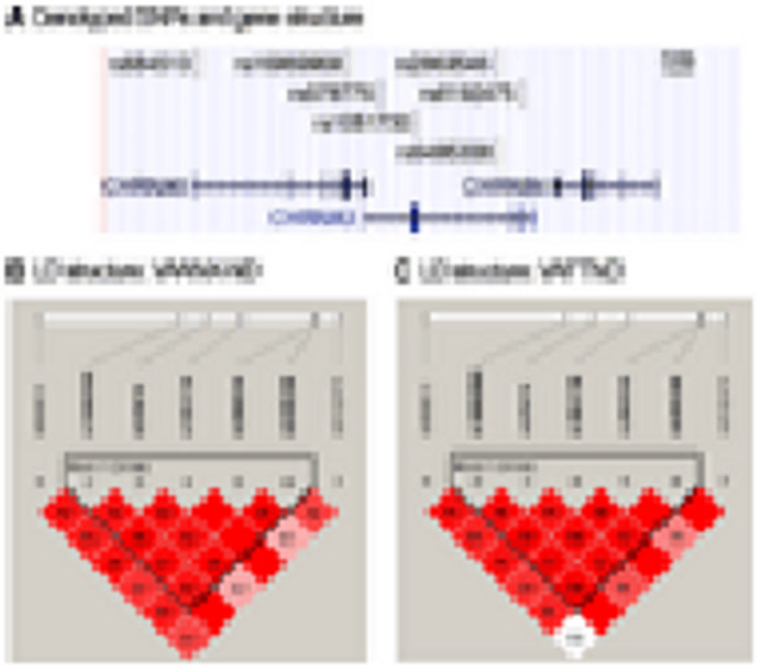

Figure 1. Genotyped SNPs and gene structure in the CHRNA5, CHRNA3, and CHRNB4 region (A), and pairwise LD (D′) in the VAANX-ND (B) and VAFTND (C) samples

Table I

Sample Description

Sample	FTND	SymAlcAD	SymCanAD
VAANX-ND	A combined sample from two panels: (1) VAANX, regular smokers with high and low IANGF scores; and (2) VAND, regular smokers with high and low FTQ scores. The combined sample (n = 815, 532 males and 283 females) had FTND scores (mean, s.d.) of 5.18, 2.57 and IANGF scores of 0.25, 0.87. See Supplementary Figure S1 for their distribution	DSM IV symptom counts for alcohol abuse and dependence. Only those subjects (n = 1,801) who used five or more drinks in a month were included in the analyses. See Supplementary Figure 2S for symptom count distribution	DSM IV symptom counts for cannabis abuse and dependence. Only those subjects (n = 614) who used cannabis five times or more in a month were included in the analyses. See Supplementary Figure 2S for symptom count distribution
VAFTND	Regular smokers (n = 1,128, 727 males, 399 females, and 2 with unknown sex) with FTND scores of 4.60, 2.62 and IANGF scores of 0.08, 0.46. See Supplementary Figure S1 for their distribution		

Table II

Marker Characteristics

Marker	Chromosomal position	Polymorphism	Minor allele	Gene/function	Minor allele frequency		HWE *P*-value[a]	
					VAANX-ND	VAFTND	VAANX-ND	VAFTND
rs684513	76645705	C/G	G	CHRNA5/intron	0.213	0.201	0.9051	0.3543
rs16969968	76670230	A/G	A	CHRNA5/Asp>Asn	0.314	0.356	0.5221	0.8627
rs578776	76675455	A/G	A	CHRNA3/intron	0.292	0.272	0.1220	1.0000
rs1051730	76681394	A/G	A	CHRNA3/intron	0.307	0.354	0.2008	0.9369
rs2869546	76694400	C/T	C	CHRNA3/intron	0.399	0.369	0.7343	0.5269
rs6495308	76694711	C/T	C	CHRNA3/intron	0.239	0.217	**0.0087**	0.3019
rs8192475	76698535	C/T	C	CHRNA3/intron	0.059	0.052	0.4116	**0.0266**

HWE, Hardy-Weinberg equilibrium.

[a] *P*-values ≤0.05 were in bold.

Table III

Single Marker Association With FTND Scores[a]

		VAANX-ND (n = 815)						VAFTND (n = 1,121)					
Marker	**Allele**	***P*-value**	**Cnt**	**Freq**	**Effect**	**95% CI low bound**	**95% CI high bound**	***P*-value**	**Cnt**	**Freq**	**Effect**	**95% CI low bound**	**95% CI high bound**
rs684513	C	0.9391	1,193	0.787	-	-	-	**0.0222**	1,772	0.799	0.122	0.017	0.226
rs16969968	A	**0.0068**	470	0.314	0.166	0.045	0.287	**0.0028**	785	0.356	0.133	0.046	0.221
rs578776	G	0.6122	1,060	0.708	-	-	-	0.3280	1,613	0.729	-	-	-
rs1051730	A	**0.0237**	464	0.307	0.139	0.018	0.260	**0.0039**	786	0.354	0.129	0.041	0.216
rs2869546	T	0.0589	891	0.601	0.043	-0.002	0.088	0.0705	1,399	0.604	0.080	-0.007	0.166
rs6495308	T	0.3486	1,147	0.761	-	-	-	**0.0384**	1,740	0.783	0.107	0.006	0.208
rs8192475	C	0.1741	89	0.059	-	-	-	0.5830	116	0.052	-	-	-

Cnt, count; Freq, frequency.

[a]The effect, the low bound, and high bound of 95% confident intervals were shown for only those markers with a *P*-value less than 0.1. *P*-values ≤0.05 were in bold.

Table IV

Three-Marker (rs169699698-rs1051730-rs2869546) Haplotype Analysis

	Global *P*-value	Haplotype	Count	Frequency	Effect	95% CI low bound	95% CI high bound	*P*-value
VAANX-ND	0.08149	G-G-T	421	0.290	0.000	0.000	0.000	0.5642
		G-G-C	576	0.396	-0.032	-0.172	0.109	0.0917
		A-A-T	438	0.302	0.120	-0.028	0.269	**0.0347**
VAFTND	**0.00685**	G-G-T	604	0.276	0.000	0.000	0.000	0.3450
		G-G-C	802	0.366	-0.021	-0.127	0.085	0.0545
		A-A-T	766	0.350	0.130	0.022	0.237	**0.0012**

P-values ≤0.05 were in bold.

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Table V

Association With SymAlcAD and SymCanAD in the Combined Sample[a]

	Marker	Allele	Count	Frequency	Effect	95% CI low bound	95% CI high bound	Chi square	*P*-value
SymAlcAD (1,801)	rs684513	G	737	0.207	-	-	-	2.68	0.1019
	rs16969968	G	2,350	0.662	0.224	0.060	0.387	7.23	**0.0072**
	rs578776	A	967	0.273	-	-	-	2.56	0.1093
	rs1051730	G	2,359	0.665	0.231	0.067	0.395	7.65	**0.0057**
	rs2869546	C	1,356	0.387	-	-	-	1.92	0.1655
	rs6495308	C	785	0.221	-	-	-	1.98	0.1594
	rs8192475	C	196	0.055	-	-	-	0.13	0.7197
SymCanAD (614)	rs684513	G	268	0.220	-	-	-	0.02	0.8870
	rs16969968	G	823	0.678	-	-	-	0.51	0.4769
	rs578776	A	338	0.280	-	-	-	0.67	0.4122
	rs1051730	G	832	0.684	-	-	-	0.51	0.4763
	rs2869546	C	472	0.393	-	-	-	0.01	0.9412
	rs6495308	C	286	0.235	-	-	-	0.18	0.6732
	rs8192475	C	61	0.051	-	-	-	0.00	0.9784

[a]The effect sizes were shown for only those markers with a *P*-value less than 0.1. *P*-values ≤0.05 were in bold.