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Genome-Wide Interaction Study Identifies *RCBTB1* as a Modifier for Smoking Effect on Carotid Intima-Media Thickness

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Abstract

Objective—Carotid intima-media thickness (cIMT), a marker for atherosclerosis, is affected by smoking and has substantial interindividual variation. We sought to identify the genetic moderators influencing the effect of smoking on cIMT

Approach and Results—With a multistage design using 722 379 single nucleotide polymorphisms (SNP), a genome-wide interaction study was performed in a discovery sample of 669 Hispanics, followed by replication in 589 subjects (264 Hispanics, 172 non-Hispanic blacks, 153 non-Hispanic whites). Assuming an additive genetic model, regression analysis was performed to test for smoking–SNP interaction on cIMT while controlling for age, sex, and the top 3 principal components of ancestry. The strongest interaction in Hispanics was found with a synonymous splicing SNP (rs3751383) in exon 9 of *RCBTB1* (P=2.5e^{-6} in discovery sample; P=0.01 in the Hispanic replication sample; P<8.8e^{-9} in the combined Hispanic sample). Stratification analysis in the combined Hispanic sample showed that smoking had no effect on cIMT among rs3751383 G homozygote (P=0.15), a moderate effect among rs3751383 heterozygote (P=0.01), and a strong effect among rs3751383 A homozygote (P=2.1e^{-7}). A consistent trend was observed in the non-Hispanic white and black data sets, leading to an interaction effect of P<2.9e^{-9} in the meta-analysis of all 1258 subjects.

Conclusions—Our study represents the first genome-wide smoking–SNP interaction study of cIMT and identifies *RCBTB1* as a modifier of the smoking effect on cIMT. Testing for gene–environment interactions can help uncover genetic factors that contribute to the interindividual variation in response to the same environmental exposure.

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Keywords

carotid arteries; carotid intima-media thickness; Hispanic Americans

Carotid intima-media thickness (cIMT) has been widely used as a quantitative measure of the artery wall thickening and shown to be predictive of vascular diseases.[1–5] Family studies have documented a moderate heritability of cIMT, ranging from 0.30 to 0.60, supporting its genetic component.[2,6–8] Genome-wide linkage studies of cIMT have nominated several regions of interest on chromosomes 2, 7, 12, and 14.[9] On chromosome 14, suggestive evidence for linkage has been reported near D14S606 by 2 independent studies.[10,11] Our fine mapping study on the chromosome 14 linkage region has identified *PRIMA1* (proline rich membrane anchor 1) as a novel candidate gene for cIMT.[12] Other attempts to elucidate the genetic basis for cIMT include candidate gene and genome-wide association studies (GWAS).[13] Although there is some consensus on the associations with apolipoprotein E, angiotensin-converting enzyme, and inter-leukin-6 genes, findings from candidate gene studies have been in general conflicting.[14] Recently, a large-scale candidate gene study using Illumina 200K CardioMetabochip to interrogate hundreds of candidate genes simultaneously found rs4888378 in the BCAR1-CFDP1-TMEM170A locus was associated with cIMT as well as coronary artery disease risk in multiple European cohorts.[15] Early GWAS on cIMT found no SNP meeting criteria for genome-wide significance.[16] A meta-analysis of GWAS, including >40 000 whites, reported 3 single nucleotide polymorphisms (SNPs) near *ZHX2* (zinc fingers and homeoboxes 2), *APOC1* (apolipoprotein C-I), and *PINX1* (PIN2/TERF1 interacting, telomerase inhibitor 1) to reach genome-wide significance for cIMT.[9] These loci, however, explained only a small proportion (≤1.1%) of the variance in cIMT.

Despite the success of GWAS in many complex traits, the missing heritability in GWAS is a well-acknowledged concern.[17] Given that GWAS is designed to detect common variants with a significant main effect, this approach misses the genetic variants that have no or little main genetic effects but interact with environmental factors to contribute to the phenotypic variance. Indeed, numerous epidemiological studies have shown that cIMT is strongly influenced by cigarette smoking.[18–21] Therefore, we performed a genome-wide interaction study (GWIS) with smoking to reveal genetic loci that interact with smoking to affect cIMT but may not have detectable main genetic effect in GWAS. We focused our interaction analysis on the total cIMT, which is a composite measure of cIMT at different carotid sites. In the discovery stage, we performed GWIS in 669 Hispanic subjects. The promising findings were then examined in the replication stage in 264 Hispanics, 172 Non-Hispanic blacks, and 153 Non-Hispanic whites.

Materials and Methods

Materials and Methods are available in the Table I online-only Supplement.

Results

Sample characteristics for the discovery sample and replication data sets are reported in Table 1. Compared with the Hispanic discovery sample, non-Hispanic blacks and whites were relatively older, had higher prevalence of smoking, and had larger total cIMT, whereas the Hispanic replication discovery sample was similar in age, sex, and smoking distributions but had higher total cIMT.

GWIS in the Discovery Hispanic Data Set

After QC, a total of 722 379 SNPs were available for GWIS in the discovery stage. Figure 1 is the Manhattan plot displaying the *P* values for the interaction effect between each SNP and cigarette smoking on total cIMT. Although no interaction reached the genome-wide significance ($P<7.0e^{-8}$; Figure 1), there was no suggestion of an inflated type I error with a genomic inflation factor of $\lambda=1.00$ (Figure I in the online-only Data Supplement). In total, there were 21 SNPs that had an interaction *P* value $<1.0e^{-5}$ (Table I in the online-only Data Supplement). Among them, 6 SNPs were found on chromosome 13 in or near the regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 1 (*RCBTB1*) gene, 2 SNPs on chromosome 2 in dynein assembly factor with WDR repeat domains 1 (*WDR69*) gene, and 2 SNPs on chromosome 8 in tumor protein D52 (*TPD52*) gene.

Replication in the Second Hispanic Data Set and Combined Analysis in All Hispanics

The replication Hispanic data set included 264 subjects with cIMT measurements (Table 1). Among the 21 SNPs derived from the discovery stage, 6 SNPs had a nominal interaction ($P<0.05$) in the replication Hispanic data (Table I in the online-only Data Supplement; Table 2). One SNP is located within solute carrier family 8 (sodium/calcium exchanger), member 1 gene (*SLC8A1*) on chromosome 2, 1 SNP is located within FERM domain containing 3 (*FRMD*3) gene on chromosome 9, and 4 SNPs are located within the *RCBTB1* gene.

The combined analysis of both Hispanic data sets showed that all 4 *RCBTB1* SNPs but none of other 2 SNPs reached genome-wide significance, with the most significant interaction observed with a synonymous splicing SNP (rs3751583) in exon 9 of *RCBTB1* ($\beta\pm SE$: 0.03 ± 0.01; $P=8.8e^{-9}$). Genotype-stratified analysis demonstrated that cigarette smoking had no effects on cIMT in subjects who are homozygous for rs3751583 G allele ($P=0.15$) and a moderate effect in those who are heterozygotes ($P=0.01$) but significantly increases cIMT in individuals who are homozygous for the A allele ($P=2.1e^{-7}$), suggesting a dose effect of the A allele (Figure 2).

Regional Analysis Surrounding *RCBTB1* in All Hispanics

To investigate the *RCBTB1* region thoroughly, we expanded our analysis in all Hispanic subjects to include SNPs within the 1 Megabase (Mb) flanking region of rs3751383. Figure 3 displays the smoking–SNP interaction *P* values, recombination rates, pairwise r^2 with rs3751583, and functional annotation of SNPs in the region. The 4 *RCBTB1* SNPs that reached genome-wide significance are in high linkage disequilibrium (LD) with each other ($r^2>0.8$), suggesting that they represent the same signal (Figure 3).

Replication in Other Data Sets and Meta-Analysis

To explore whether the promising findings in Hispanics can be generalized to other populations, we first examined interactions between smoking and the 6 SNPs, which showed a replication in Hispanic samples, in non-Hispanic white, and non-Hispanic black data sets. Among the 6 SNPs, only *RCBTB1* SNPs displayed the same direction of effect in non-Hispanic whites (β±SE: 0.02±0.02) and blacks (β±SE: 0.02±0.01), and the *P* value resulting from the meta-analysis of the 3 populations was more significant than in the combined Hispanic sample (Table 2).

Given that rs3751383 was the most significant marker but had a considerable difference in allele frequency across the 3 populations (minor allele frequency=0.16 in non-Hispanic whites, 0.44 in non-Hispanic blacks, and 0.26 in Hispanics), we extended the meta-analysis to include the SNPs within the 1 Mb flanking region of rs3751383 for the 3 population samples. Although the most significant interaction remained for rs3751383 (P=2.9e^{-9}), genome-wide significance was also detected for 2 other SNPs in low LD with rs3751383 (r^2=0.200 for rs7318481 and 0.254 for rs9568239 in Hispanics): rs7318481 (P=4.2e^{-8}) is within SET domain, bifurcated 2 (*SETDB2*) gene, and rs9568239 (P=8.8e^{-9}) is located between *RCBTB1* and PHD finger protein 11 (*PHF11*) gene (Table II in the online-only Data Supplement).

To determine whether the 2 SNPs represent independent signals, we performed conditional analysis on the top SNP rs3751383. Conditioning on rs3751383 removed substantial portion of the interaction effect at the other 2 SNPs (P=4e^{-8} versus 0.004 for rs7318481 and P=9e^{-9} versus 0.01 for rs9568239, before and after conditioning, respectively). This analysis suggests that these signals are not totally independent of each other, probably because of the limited LD between them. Given that rs3751383 did not completely account for the interaction signal at rs9568239 and rs7318481 (as evidenced by the remaining significance), we performed an allelic score analysis using the 3 SNPs together. The allelic score displays strong evidence for interaction with smoking in combined Hispanic sample (P=4.7e^{-11}; β=0.02±0.003), suggesting that the true variant(s) might reside in the haplotype delimited by all 3 SNPs or multiple variants collectively contribute to the signal in the region.

Discussion

This study represents the first genome-wide smoking–SNP interaction study of cIMT. Our previous studies have shown that non-Hispanic blacks had the greater IMT values and a greater incidence of stroke than non-Hispanic whites. Hispanics also had an increased risk for stroke in comparison with non-Hispanic whites.[22,23] To reduce the heterogeneity across population, we started with our screen in a Hispanic data set that was dominant in our first wave of GWAS sample, then examined the replication of the top findings in a Hispanic replication data set in our second wave of GWAS, and finally, investigated the generalizability of the replicated interactions in Hispanics to non-Hispanic whites and blacks. With a multistage design, we identified *RCBTB1* as a modifier gene for effect of smoking on interindividual variance in cIMT, which was replicated in an independent Hispanic data set and reached genome-wide significance in the meta-analysis of data sets from the 3 race-ethnic groups. This suggests that testing for gene–environment interactions

can help uncover genetic factors that contribute to the interindividual variation in response to the same environmental exposure.

A well-known problem in GWAS is that identified genetic factors only account for a small portion of the phenotype variation, suggesting missing or hidden heritability that requires other strategies to reveal. Like other multifactorial complex traits, cIMT is under the influence of both environmental and genetic factors. Factoring in gene–environment interactions in the association studies of cIMT might uncover novel associations compared with studies examining only genotype-pheno-type correlations.[24] Cigarette smoking by itself is a significant risk factor for atherosclerosis as measured by increased cIMT (P=0.003 in our data sets).[23] Understanding genetic modification in the link between smoking and atherosclerosis will help to elucidate causal mechanisms and provide opportunities for improvement of life quality and disease prevention.

Our study highlights the importance of incorporating environmental exposure to map the genetic architecture, which accounts for interindividual phenotypic variance. *RCBTB1* had no main effect on cIMT in our data sets (P values ranging 0.51–0.63 for the top 4 SNPs in the combined Hispanic sample) and was not reported in previous studies. Specifically, smoking (pack-years) only explained 0.7% variation of cIMT (P=0.027) and the top SNP rs3751383 explained <0.05% variation of cIMT (P values >0.50) in the main effect model, whereas their interaction explained 3.2% variation of cIMT in interaction model. Stratification analysis also demonstrated that cigarette smoking had no effect on cIMT in rs3751383 G homozygotes, a moderate effect among rs3751383 heterozygotes, and a strong effect among rs3751383 A homozygotes. Therefore, the effect of this gene may be missed in the traditional GWAS.

RCBTB1 encodes a protein with an N-terminal regulator of chromosome condensation (RCC1) domain and a C-terminal BTB (broad complex, tramtrack, and bric-a-brac) domain. A link between *RCBTB1* and atherosclerosis has not yet been described. However, links between SNPs in *RCBTB1* and both inflammatory and allergic pulmonary diseases have been previously reported.[25,26] These findings could be linked, at least in part, to the impact of cigarette smoking on diseases related to inflammatory response such as atherosclerosis, in subjects with *RCBTB1* genetic variants. *RCBTB1* is also characterized as a tumor suppressor gene. It is often deleted in Chronic Lymphocytic Leukemia and in many colon and cervical cancer cell lines.[27,28] This is not the first study reporting a potential link between tumor suppressor genes and atherosclerosis.

For example, polymorphisms of the tumor suppressor gene *LSAMP* (limbic system–associated membrane protein) have been associated with atherosclerosis in a nonbiased genetic study, showing that downregulation of *LSAMP* promotes smooth muscle cell proliferation in human aortic cells.[29] Similarly, *RCBTB1* has been highly expressed in rodent vascular smooth muscle cells and normal human epithelial cells. Overexpression of *RCBTB1* in rat vascular smooth muscle cells induce cellular hypertrophy, most likely mediated through its interaction with the angiotensin II receptor-1A at the C terminus.[30] Furthermore, reduced *RCBTB1* expression protects human epithelial cells against apoptosis triggered by DNA damage.[31]

The SNP displaying the strongest interaction with smoking (rs3751383) is a synonymous SNP in exon 9. The variations at rs3751383 do not change protein sequence, but this SNP is a potential exonic splicing enhancer (http://snpinfo.niehs.nih.gov), which is located in a region that is subject to a methylation transcription factor binding and Dnase hypersensitive site according to The Encyclopedia of DNA Elements (ENCODE) data annotation. Thus, it is possible that the sequence variation at rs3751383 modifies epigenetic changes induced by smoking and alters *RCBTB1* expression level, which in turn potentiates atherogenic smooth muscle cell hyperplasia or aberrant cell survival leading to increased cIMT. In a transcriptome coupled with GWAS in human peripheral monocytes, rs3751383 is indeed associated with expression of the *RCBTB1* gene and another nearby gene *EBPL* (P=6.6e^{-14} and 1.4e^{-20}, respectively; Table III in the online-only Data Supplement).[32] Given that circulating monocytes play a role in atherogenesis, the expression quantitative trait locus (eQTL) data suggest a functional impact of the polymorphism at rs3751383 and therefore provide additional support for the potential interaction of rs3751383 with smoking in determining cIMT. Future studies are warranted to investigate how polymorphisms within or nearby *RCBTB1* identified in our study interact with smoking and affect gene expression.

The existence of gene–environment interactions also emphasizes the importance of the heterogeneity of genetic effects introduced with modification by environment risk factors. Indeed, it is increasingly recognized that the heterogeneity arising from different environmental exposures is a potential source of variability between genetic findings in different cohorts. This is particularly true for strong environmental risk factors such as smoking.[33] In a validation study on genetic association with coronary artery disease, the initial effort to replicate the findings observed in the CATHGEN study with the Intermountain Heart Collaborative Study (IHCS) failed.[34] The CATHGEN cohort, however, had a much higher prevalence of smokers than the IHCS cohort. Therefore, the gene–smoking interactions analyses were performed and showed that the genetic variants with a significant interaction with smoking replicated in the association with coronary artery disease in the smokers but not in the overall samples. Although not observed in this study, it is also possible that some genetic associations can only be detected in nonsmokers and therefore can only be replicated in individuals without smoking exposure.

We acknowledge several limitations in the current study. First, despite that the eQTL data supported a functional role of rs3751383 (or any SNPs in high LD with it), additional follow-up studies are often required to identify and fully characterize the functional variants responsible for the detected interaction. Second, the white and black replication data sets were small although the results using these samples demonstrated the same trends. Additional validations are needed in larger data sets to further establish the contribution of the gene–smoking interaction identified here in cIMT variation.

Conclusions

Using a nonbiased genome-wide approach, we have identified *RCBTB1* as a modifier gene for effect of smoking on interindividual variance in cIMT. Further studies are required to validate and characterize the molecular mechanisms underlying the intriguing interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

cIMT	carotid intima-media thickness
GWAS	genome-wide association study
GWIS	genome-wide interaction study
SNP	single nucleotide polymorphism

Significance

Atherosclerosis is the pathology underlying most ischemic strokes and myocardial infarction. Smoking can initiate and accelerate atherosclerosis by multiple mechanisms. However, the degree of the cigarette smoking–induced damage also varies from individual to individual. This intersubject variability in response to the effects of smoking may be largely because of the between-individual difference in genetic susceptibility, in addition to the variability in the presence of other environmental risk factors. Thus, understanding genetic modification in the link between smoking and atherosclerosis will help to elucidate causal mechanisms and provide opportunities for improvement of life quality and disease prevention. The present study represents the first genome-wide smoking–SNP interaction study of carotid intima-media thickness and identifies *RCBTB1* as a modifier of the smoking effect on cIMT. Our findings suggest that testing for gene–environment interactions can help uncover genetic factors that contribute to the interindividual variation in response to the same environmental exposure.

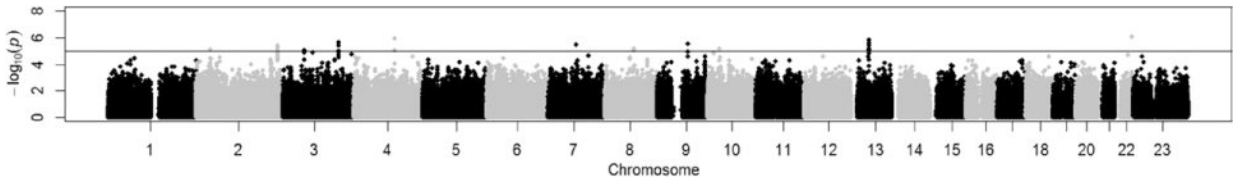

Figure 1.

Manhattan plot showing genome-wide interaction *P* values (−log10) of single nucleotide polymorphisms (SNPs) with smoking on carotid intima-media thickness (cIMT) in Hispanic discovery sample. Horizontal line indicates the threshold for suggestive significance (P=1.0e^{-5}) for replication.

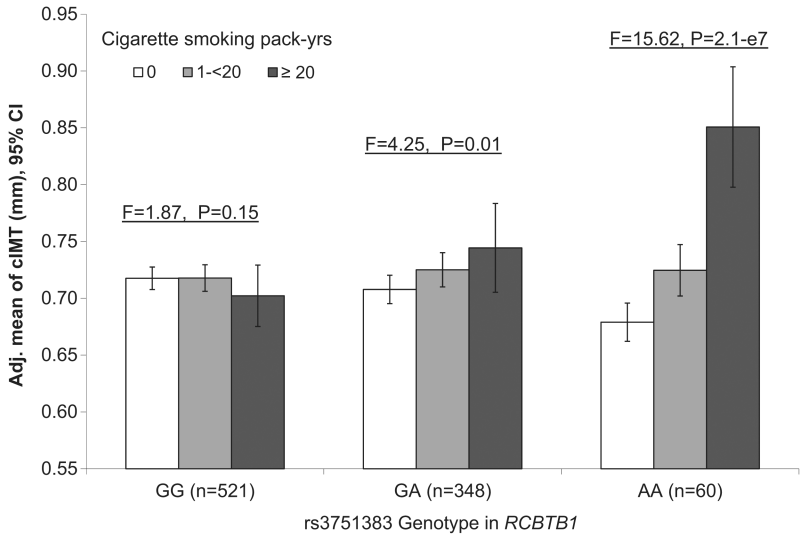

Figure 2.

Bar graph presenting the means of carotid intima-media thickness (cIMT) by rs3751383 genotype in RCBTB1 gene and cigarette smoking status in Hispanic pooled sample, adjusted for age, sex, the top 3 principal components of ancestry.

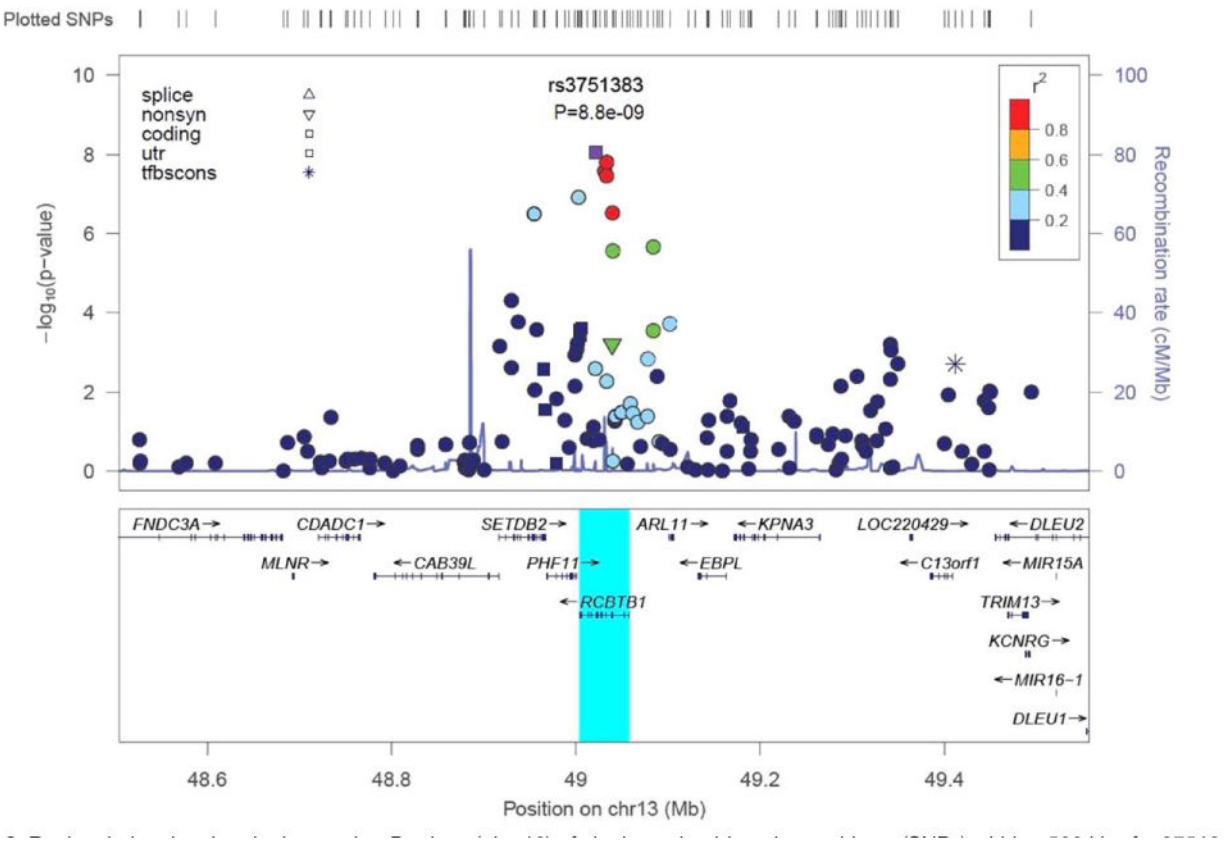

Figure 3.

Regional plot showing the interaction *P* values (–log10) of single nucleotide polymorphisms (SNPs) within ±500 kb of rs3751383 with smoking on carotid intima-media thickness (cIMT) in Hispanic pooled sample. Linkage disequilibrium (LD) is shown with top SNP rs3751383 and estimated based on the Hispanic sample.

Table 1

Sample Characteristics

Characteristics	Hispanic, Discovery (n=669)	Hispanic, Replication (n=264)	Non-Hispanic Black (n=172)	Non-Hispanic White (n=153)
Age, y, mean±SD	68±8	69±8	73±9**	73±9**
Male, n (%)	250 (37)	110 (42)	65 (38)	77 (50)**
Smoking pack-years, n (%)				
0	343 (51)	144 (54)	69 (40)	59 (39)
1–<20	220 (33)	68 (26)	54 (31)	42 (27)
≥20	106 (16)	52 (20)	49 (29)**	52 (34)**
Carotid IMT, mm, mean±SD	0.72±0.09	0.70±0.07**	0.75±0.10**	0.76±0.10**

* *P* value <0.05 and

** *P* value <0.01 compared with Hispanic discovery sample.

Table 2

Smoking–SNP Interactions with $P<1\times10^{-5}$ in Hispanic Discovery Sample and $P<0.05$ in Hispanic Replication Sample

SNP Information/Sample	MAF/Interaction Effect	Reference SNPs					
		rs10183585	**rs4111744**	**rs3751383**	**rs17069328**	**rs9596148**	**rs9568258**
Chromosome		2p22.1	9q21.32	13q14.2	13q14.2	13q14.2	13q14.2
Location, BP		40297172	85300194	49021650	49033205	49033309	49033464
Minor/major allele		C/T	G/A	A/G	C/T	C/T	A/C
Nearest gene		SLC8A1	FRMD3	RCBTB1	RCBTB1	RCBTB1	RCBTB1
Type		Intron	Intron	cds-synon	Intron	Intron	Intron
Hispanic, discovery	MAF	0.09	0.44	0.25	0.25	0.25	0.25
	β±SE	0.047±0.011	−0.027±0.006	0.034±0.007	0.033±0.007	0.033±0.007	0.032±0.007
	P	7.7E–06	2.9E–06	2.5E–06	4.2E–06	4.2E–06	9.0E–06
Hispanic, replication	MAF	0.08	0.45	0.27	0.27	0.27	0.27
	β±SE	0.039±0.013	−0.016±0.007	0.024±0.009	0.023±0.009	0.023±0.009	0.023±0.009
	P	0.003	0.03	0.01	0.01	0.01	0.01
Hispanic, combined	MAF	0.09	0.44	0.25	0.25	0.25	0.25
	β±SE	0.042±0.008	−0.023±0.005	0.033±0.006	0.032±0.006	0.032±0.006	0.032±0.006
	P	6.8E–07	7.0E–07	8.8E–09	1.6E–08	1.6E–08	3.6E–08
Non-Hispanic white	MAF	0.11	0.47	0.16	0.16	0.16	0.17
	β±SE	0.010±0.023	0.021±0.013	0.018±0.017	0.017±0.017	0.017±0.017	0.008±0.018
	P	0.65	0.12	0.30	0.34	0.34	0.67
Non-Hispanic black	MAF	0.10	0.42	0.44	0.46	0.46	0.46
	β±SE	−0.015±0.021	0.010±0.013	0.017±0.011	0.014±0.011	0.014±0.011	0.017±0.012
	P	0.47	0.43	0.13	0.21	0.21	0.18
Meta-Analysis*	β±SE	0.032±0.007	0.015±0.005	0.029±0.005	0.027±0.005	0.027±0.005	0.028±0.005
	P	1.5E–05	2.1E–04	2.9E–09	1.5E–08	1.5E–08	3.6E–08
	Direction	++−	−++	+++	+++	+++	+++

β indicates parameter estimate for smoking–SNP interaction effect; BP, base pair on the base of National Center for Biotechnology Information reference assembly build 18; MAF, minor allele frequency; SE, standard error; and SNP, single nucleotide polymorphism.

*Based on combined Hispanic, non-Hispanic white, and non-Hispanic black samples.