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## Regulation of *CARD8* Expression by *ANRIL* and Association of *CARD8* SNP rs2043211 (p.C10X) with Ischemic Stroke

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**Abstract**

**Background and Purpose**—*ANRIL* has long been considered as the strongest candidate gene at the 9p21 locus, robustly associated with stroke and coronary artery disease (CAD). However, the underlying molecular mechanism remains unknown. The present study works to elucidate such a mechanism.

**Methods**—Utilizing eQTL analysis we identified potential genes whose expression may be influenced by genetic variation in *ANRIL*. To verify the identified gene(s), knockdown and over-expression of *ANRIL* was evaluated in HUVECs and HepG2 cells. Ischemic stroke and CAD risk was then evaluated in the gene(s) demonstrated to be mediated by *ANRIL* in 3 populations of Chinese Han ancestry; two ischemic stroke populations including the Central China cohort (903 cases and 873 controls) and the Northern China cohort (816 cases and 879 controls), and one CAD cohort consisting of 772 patients and 873 controls.

**Results**—eQTL analysis identified *CARD8* among others, with knockdown of *ANRIL* expression decreasing *CARD8* expression and over-expression of *ANRIL* increasing *CARD8* expression. The minor T allele of a previously identified *CARD8* variant (rs2043211) was found to be significantly associated with a protective effect of ischemic stroke under the recessive model in two independent stroke cohorts. No significant association was found between rs2043211 and CAD.

**Conclusion**—*CARD8* is a downstream target gene regulated by *ANRIL*. SNP rs2043211 in *CARD8* is significantly associated with ischemic stroke. *ANRIL* may increase the risk of ischemic stroke through regulation of the *CARD8* pathway.

### Keywords

ischemic stroke; coronary artery disease; case-control study; single nucleotide polymorphisms; *CARD8*

### Introduction

Atherosclerosis is one of the most common causes of both ischemic stroke and coronary artery diseases (CAD).<sup>1</sup> Stroke is a leading cause of morbidity and mortality in China and other parts of the world.<sup>2</sup> Ischemic stroke accounts for about 87% of all strokes,<sup>3</sup> and is caused by both genetic and environmental factors. To date, large-scale genome-wide association studies (GWAS) have identified several risk loci for ischemic stroke, including *HDAC9*, *PITX2*, *ZFHX3*, 9p21, *PRKCH*, and *NINJ2*.<sup>4</sup> However, most loci have small effects and may explain a small proportion of the heritability of ischemic stroke. Nearly fifty risk loci for CAD have been identified by GWAS, but explain approximately 10% of the heritability of CAD only.<sup>5</sup> Therefore, more genetic factors for ischemic stroke and CAD remain to be discovered.

SNPs (single nucleotide polymorphisms) at the chromosome 9p21 locus were found to be associated with both ischemic stroke and CAD by GWAS.<sup>4, 5</sup> The 9p21 locus contains an annotated non-coding RNA, termed *ANRIL* (antisense non coding RNA in the *INK4* locus). *ANRIL* is considered as a prime candidate gene for atherosclerosis at the 9p21 locus.<sup>6</sup> First, SNPs associated with ischemic stroke and CAD (rs10116277, rs7865618, rs564398, rs496892, rs7044859) within the 9p21 region are located within the *ANRIL* gene.<sup>7</sup> Second, *ANRIL* is expressed in cell types and tissues that are involved in atherosclerosis. Third, several studies investigated *ANRIL*'s association with 9p21 SNP genotypes and showed a correlation of *ANRIL* expression with atherosclerosis severity, even though the direction of the effects is still in dispute.<sup>6, 7</sup> Moreover, the risk alleles of rs10811656 and rs10757278 disrupted a binding site for transcriptional factor STAT1 and STAT1 in turn regulated *ANRIL* expression.<sup>8</sup> The STAT1 signaling pathway mediates responses to inflammation upon stimulation of the pro-inflammatory cytokine interferon gamma.<sup>9</sup> These results supported the notion that *ANRIL* might play a role in the inflammatory response and atherosclerosis. The molecular mechanism by which *ANRIL* mediates atherosclerosis is unknown. However, as a long noncoding RNA, *ANRIL* may play its role in atherosclerotic processes by influencing the expression of other genes.

In this study, we identified *CARD8* as a downstream gene of *ANRIL*, and assessed the association between *CARD8* SNP rs2043211 and ischemic stroke or CAD in Chinese Han populations.

## Materials and Methods

### Analysis of Expression Quantitative Loci (eQTLs) for *ANRIL* SNPs

In order to identify potential downstream genes regulated by *ANRIL*, we analyzed ischemic stroke- and CAD-associated SNPs rs10116277, rs7865618, rs564398, rs496892, and rs7044859 in *ANRIL*.<sup>7</sup> These SNPs were shown to influence the mRNA level of *ANRIL*.<sup>7</sup> We performed eQTL analysis for these SNPs by searching the database at University of Michigan Center for Statistical Genetics (<http://www.sph.umich.edu/csg/liang/imputation/>). These studies identified several genes whose expression may be associated with SNPs evaluated in our study. We chose to evaluate *CARD8* over the other identified genes because of its increased expression in atherosclerotic lesions.<sup>10</sup>

### Cell Transfection and Quantitative Real-Time PCR (qRT-PCR) Analysis

Details of cell transfection and qRT-PCR were described in online SUPPLEMENTAL MATERIAL. The sequence of *ANRIL* siRNA was as follows: 5'-GGAATGAGGAGCACAGTGA-3'. Plasmid pcDNA3.1-*ANRIL* (NR\_003529.3) was synthesized by GENEWIZ (Beijing, China). The sequences of primers used for qRT-PCR are listed in Supplement Table I.

### Study Subjects

All study participants were selected from the GeneID database.<sup>11</sup> Diagnostic criteria for ischemic stroke, CAD, and related factors were described in detail in online SUPPLEMENTAL MATERIAL. This study followed the principals outlined in the Declaration of Helsinki and has been approved by local institutional review boards on human subject research. Written informed consent was obtained from all participants.

### Genotyping and Statistical Analysis

Details of isolation of genomic DNA, SNP genotyping, and statistical analysis were described in online SUPPLEMENTAL MATERIAL.

## Results

### *ANRIL* Regulates Expression of *CARD8*

Five 9p21 SNPs rs10116277, rs7865618, rs564398, rs496892, and rs7044859 are located within *ANRIL* and affect the expression level of *ANRIL* mRNA.<sup>7</sup> By searching a public eQTL database (<http://www.sph.umich.edu/csg/liang/imputation/>), we identified 87 genes whose expression may be associated with one of the five 9p21 SNPs (online-only Supplement Table I). One of the 87 genes, *CARD8*, became a strong candidate gene downstream of *ANRIL* because it also showed differential expression in a preliminary microarray analysis comparing HepG2 cells treated with *ANRIL* siRNA to those transfected with control siRNA (data not shown).

To verify that *CARD8* is a downstream gene regulated by *ANRIL*, HepG2 cells were transfected with *ANRIL* specific siRNA to knock *ANRIL* expression down (NC siRNA as negative control) and used for qRT-PCR analysis. Compared to NC siRNA, *ANRIL* siRNA successfully reduced its own expression by about 83% ( $P < 2.0 \times 10^{-5}$ ) and the expression of *CARD8* by about 55% ( $P < 2.4 \times 10^{-4}$ ) (Figure 1A). Similarly, HUVECs transfected with

*ANRIL* specific siRNA showed significant reduction of *ANRIL* by 70% ( $P < 1.5 \times 10^{-5}$ ) and *CARD8* by 48% ( $P < 6.7 \times 10^{-4}$ ) compared to cells with NC siRNA (Figure 1B). These data suggest that *ANRIL* regulates the expression of *CARD8*. Consistent with the siRNA studies, HepG2 cells transfected with pcDNA3.1-*ANRIL* for 48 hrs showed a 57-fold increase in *ANRIL* mRNA expression ( $P < 5.84 \times 10^{-5}$ ) and 1.6-fold increase in *CARD8* mRNA expression ( $P < 3.1 \times 10^{-3}$ ) (Figure 1A). Due to a difficulty in transfection of the specific line of HUVECs under this study with plasmid DNA, we did not obtain any data on the effect of *ANRIL* overexpression on *CARD8* in HUVECs.

### Characteristics of Study Subjects

Two independent cohorts were used to assess whether *CARD8* SNP rs2043211 is associated with ischemic stroke. The discovery cohort for the ischemic stroke study consisted of 903 cases and 873 controls enrolled from Hubei Province in Central China. The replication cohort for the ischemic stroke study consisted of 816 cases and 879 controls enrolled from hospitals in Northern China (Table 1). The case-control cohort for the CAD study consisted of 772 patients with CAD and 873 controls from Hubei Province in Central China (Table 1). Patients with ischemic stroke or CAD had a higher prevalence of conventional risk factors, including smoking, history of hypertension, diabetes and a lower level of HDL-C (Table 1).

Statistical power analysis was performed for all three cohorts prior to each study. Each cohort had >90% of power to detect an association between rs2043211 and ischemic stroke or CAD with an OR of 1.20 at the nominal type I error rate of >0.05 and a minor allelic frequency of >0.43 for rs2043211 in the Chinese population (HapMap HCB data).<sup>10</sup>

### Significant Genotypic Association between SNP rs2043211 and Ischemic Stroke in Two Independent Chinese Populations

The genotyping data for rs2043211 did not deviate from the Hardy-Weinberg equilibrium in the control group ( $P > 0.05$ ). In the discovery population for the ischemic stroke study, no significant allelic association was detected between rs2043211 and ischemic stroke ( $P_{\text{obs}} = 0.077$ ,  $P_{\text{adj}} = 0.092$ , online-only Supplemental Table III). Similarly, no significant association between rs2043211 and ischemic stroke was detected in the replication cohort or the combined discovery/replication population ( $P > 0.05$ , online-only Supplemental Table III).

Genotypic association analysis was then conducted because this type of study can provide genetic insights into the association under different inheritance models (additive, dominant or recessive). Interestingly, the minor allele T of SNP rs2043211 showed significant association with a protective effect of ischemic stroke under either a recessive model ( $P_{\text{obs}} = 3.0 \times 10^{-4}$ ) or an additive mode ( $P_{\text{obs}} = 2.88 \times 10^{-4}$ ) (Table 2). After multivariate logistic regression analysis by adjusting for covariates of the age, sex, BMI, smoking history, hypertension, diabetes mellitus and lipid concentrations, the genotypic association between rs2043211 and ischemic stroke remained significant only under the recessive model ( $P_{\text{adj}} = 0.028$ , OR = 0.68, Table 2).

To confirm the initial finding of genotypic association between rs2043211 and ischemic stroke in the discovery population, we validated the finding in an independent replication cohort. The results showed that rs2043211 was also significantly associated with a protective effect of ischemic stroke under either a recessive model ( $P_{\text{obs}} = 9.00 \times 10^{-3}$ ) or an additive mode ( $P_{\text{obs}} = 4.26 \times 10^{-5}$ ) (Table 2). The genotypic association between rs2043211 and ischemic stroke remained significant only under the recessive model in the replication population after multivariate logistic regression analysis ( $P_{\text{adj}} = 0.017$ , OR = 0.70, Table 2).

For the combined population of the discovery and replication cohorts, the *P* value for the genotypic association between rs2043211 and ischemic stroke under the recessive model became much more significant ( $P\text{-obs}=9.78\times 10^{-6}$ ,  $P\text{-adj}=5.83\times 10^{-4}$ , OR=0.70, Table 2 and online-only Supplemental Figure I). These data suggest that SNP rs2043211 confers a protective effect of ischemic stroke under a recessive model of inheritance.

The genotypic association between rs2043211 and ischemic stroke under the recessive model was more significant in the female group ( $P\text{-adj}=0.007$ , OR=0.65) than in the male group ( $P\text{-adj}=0.024$ , OR=0.73, Table 2 and online-only Supplemental Figure I). The genotypic association was significant in the early-onset (<60 years) ischemic stroke group under the recessive model ( $P\text{-adj}=0.001$ , OR=0.61, Table 2 and online-only Supplemental Figure I). The genotypic association was stronger under the recessive model for the female early-onset ischemic stroke group ( $P\text{-adj}=0.004$ , OR=0.51, Table 2 and online-only Supplemental Figure I).

### Lack of Significant Association between SNP rs2043211 and CAD

We also analysed SNP rs2043211 for its association with CAD. In a case-control study with 772 CAD cases and 873 controls, SNP rs2043211 did not show any significant association with CAD in the standard allelic association analysis ( $P\text{-obs}=0.235$ ,  $P\text{-adj}=0.300$ ) or in the genotypic association analysis under three different genetic models (all  $P>0.05$ ). The association remained non-significant in either male or female CAD groups (online-only Supplemental Table IV).

### Discussion

In the present study, we identified *CARD8* as a downstream gene regulated by *ANRIL*, and demonstrated an association between the *CARD8* SNP rs2043211 and ischemic stroke in two independent Chinese Han populations. *CARD8* encodes a member of the caspase recruitment domain (CARD)-containing family, and is also known as TUCAN/CARDINAL. Previous population-based studies found that the functional SNP rs2043211 (p.C10X) located in exon 5 of *CARD8* may be a genetic risk factor for chronic inflammatory diseases, such as inflammatory bowel disease (IBD) and rheumatoid arthritis (RA). Our finding that rs2043211 is associated with ischemic stroke could indicate a shared inflammatory response or pathway similar to IBD and/or RA.

SNP rs2043211 has been previously associated with other diseases. Roberts et al observed a significant genotypic association in New Zealand between rs2043211 and abdominal aortic aneurysm ( $P=0.047$ , OR=0.83),<sup>12</sup> which is a disease that shared some similar pathological characteristics and risk factors with atherosclerosis, such as inflammation and angiogenesis. However, Paramel et al found that there was no significant association between rs2043211 and MI (FIA Cohort:  $P=0.10$ , OR=1.1; SCARF:  $P=0.66$ , OR=1.0).<sup>10</sup> Garcia-Bermudez et al found that there was no evidence for the role of rs2043211 in the development of CV events in Spanish RA patients ( $P=0.67$ , OR=0.96).<sup>13</sup> Meanwhile, there were still some conflicting results in association studies between rs2043211 and some chronic inflammatory diseases. Roberts et al found that the minor allele T of rs2043211 conferred a potential protective effect against early disease onset of Crohn's disease in New Zealand Caucasians.<sup>14</sup> But other studies reported that the minor allele T of rs2043211 was associated with increased severity of IBD ( $P=0.001$ , OR=1.50)<sup>15</sup> and RA<sup>16</sup> as well as increased risk of Alzheimer's disease in women ( $P=0.01$ , OR=2.39)<sup>17</sup>. Intriguingly, SNP rs2043211 was associated with ischemic stroke, but not with CAD in this study. These results are consistent with the findings for MI and CV events by Paramel et al<sup>10</sup> and Garcia-Bermudez et al.<sup>13</sup> Although the underlying molecular mechanism for the positive association with ischemic stroke and negative

association with CAD by rs2043211 is not known, it is possible that the role of *ANRIL*-regulated *CARD8* pathway may be limited to cerebral infarction but not to CAD.

*CARD8* acts as an adaptor molecule that negatively regulates NF- $\kappa$ B activation, CASP1-dependent IL1 $\beta$  secretion, and apoptosis, and reduces the inflammatory response.<sup>18</sup> SNP rs2043211 results in an A to T transversion that changes codon 10 into a stop codon in *CARD8* mRNA (Cys10Stop). Previous studies showed that homozygotes for the stop codon allele T can reduce the expression of *CARD8* and impair the NF- $\kappa$ B-inhibiting property of *CARD8* *in vitro*.<sup>19</sup> Moreover, the expression of *CARD8* mRNA was significantly higher in atherosclerotic lesions from carotid artery plaque tissue in ischemic cerebrovascular patients compared to iliac arteries from brain dead transplant donors.<sup>10</sup> But, more studies will be needed to investigate how *CARD8* SNP rs2043211 influences the process of ischemic stroke.

These data suggest that *ANRIL* acts as an important modulator for expression of its downstream gene *CARD8*. This is consistent with the suggestion that *ANRIL* might modulate atherogenic diseases by cis- or trans-acting effects.<sup>6</sup> Recently, Holdt et al suggested a novel role for Alu elements in epigenetic gene regulation by long ncRNAs.<sup>20</sup> Importantly, trans-regulation was dependent on Alu motifs, which marked the promoters of *ANRIL* target genes and were mirrored in *ANRIL* RNA transcripts.<sup>20</sup> As with other downstream target genes, the detailed mechanism by which *ANRIL* regulates *CARD8* expression remains to be identified. We performed bioinformatic analysis of the promoter sequence of *CARD8* and surprisingly found that the same core Alu motif was also present in the DNA promoter sequence of *CARD8*. Thus, the mechanism by which *ANRIL* regulates *CARD8* expression may be that *ANRIL* binds to chromatin through interaction via the Alu motif.

There are some limitations in the present study. First, although the mRNA level of *CARD8* is regulated by *ANRIL*, the precise underlying molecular mechanism is not clear. Second, the sample sizes are fixed and frequencies of covariates including age, gender, smoking history, hypertension, diabetes as well as lipid concentrations were significantly lower in controls than in cases. Nevertheless, the observed association remained significant after adjustment of covariates using multiple logistic regression analysis. Third, although rs2043211 is a functional SNP, it may still serve as a genetic marker, and further studies are needed to establish the cause-effect relationship. Fourth, in addition to *CARD8*, our preliminary microarray analysis identified another gene, *DDX58*, which also showed >2-fold differential expression in HepG2 cells treated with *ANRIL* siRNA compared to those transfected with control siRNA. *DDX58*, also referred to as *RIG-I*, is involved in host antiviral immunity by sensing viral RNAs and triggering innate antiviral responses.<sup>21</sup> Because an antiviral response does not appear to be closely linked to atherosclerosis and stroke, *DDX58* was not assessed for its association with stroke in this study. However, because *DDX58* is regulated by *ANRIL*, it may still be interesting to investigate whether variants in *DDX58* are associated with atherosclerosis in the future. Finally, previous studies showed that the 9p21 locus (the *ANRIL* locus) is associated with large-vessel atherosclerotic stroke, thus, it may be interesting to investigate whether *CARD8* variant is associated with a specific stroke subtype in the future. Several schemes were reported to classify stroke subtypes, including the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification system (TOAST), the Causative Classification System (CCS), or the phenotypic System A-S-C-O (A for atherosclerosis, S for small vessel disease, C for cardiac source, O for other cause).<sup>22</sup> In the present study, many patients with specific subtypes were excluded, thus we were unable to perform association studies with specific stroke subtypes. In the future, we can collect more clinical information, including the data from the carotid study, classify the cases into different subtypes and assess the association between *CARD8* SNP rs2043211 and individual stroke subtype.

## Summary

In conclusion, here we show that *ANRIL* can regulate the expression level of *CARD8* mRNA and a functional SNP in *CARD8*, rs2043211 located in exon 5 of *CARD8* was significantly associated with ischemic stroke, but not with CAD in the Chinese Han population. Although the detailed mechanism of *CARD8* in the pathogenesis of ischemic stroke remains unclear, this study indeed links the *ANRIL* regulated *CARD8* pathway to the development of ischemic stroke.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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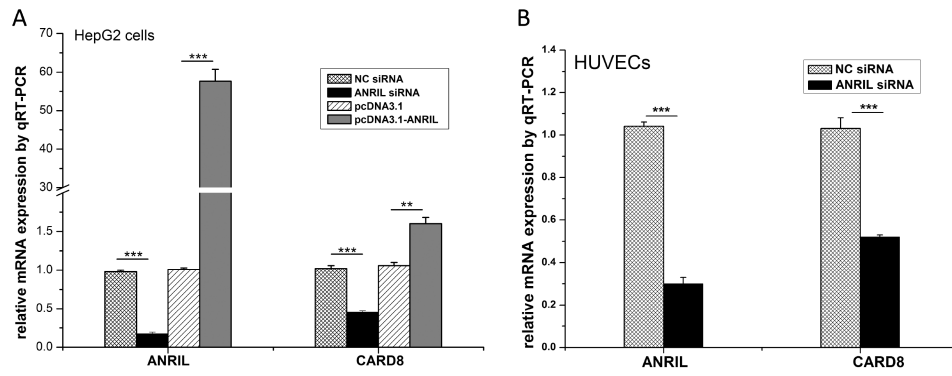
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**Figure 1.**

Effect of knockdown or over-expression of *ANRIL* on regulation of *CARD8* by quantitative RT-PCR (qRT-PCR) analysis. The mRNA samples were prepared from transfected HUVECs or HepG2 cells. *ACTIN* was used as a control for normalization. (A) *CARD8* gene was significantly down-regulated about two fold by *ANRIL* knockdown in HepG2 cells. Expression of *ANRIL* was decreased by 5.7-fold and *CARD8* expression was reduced by 2-fold. Transfection with pcDNA3.1-*ANRIL* increased the expression of *ANRIL* by 57-fold and *CARD8* expression by 1.6-fold. Each experiment was performed three times in triplicate. (B) *CARD8* gene was significantly down-regulated about two fold by *ANRIL* knockdown in HUVECs. \*\*\*:  $P < 0.001$ ; \*\*:  $P < 0.01$ ; \*:  $P < 0.05$ .

**Table 1**

Clinical and demographical characteristics of study populations.

Characteristics	GenelD-Central			GenelD-North			GenelD-Central		
	Ischemic Stroke	Control	<i>p</i> value	Ischemic Stroke	Control	<i>p</i> value	CAD	Control	<i>p</i> value
Subject numbers	903	873	N.A	816	879	N.A	772	873	N.A
Age (years) *	68.3±14.0	45.2±15.1	<1.00E-50	60.2±12.5	52.1±17.0	<1.00E-50	66.1±12.5	45.2±15.1	<1.00E-50
Sex (male%)	61.1	55.7	4.30E-03	57.7	55.9	4.71E-06	69.3	55.7	3.98E-15
Smoking (%)	18.6	6.6	5.98E-26	22.4	17.9	<1.00E-50	26.8	6.6	<1.00E-50
BMI (kg/m <sup>2</sup> )	24.3±0.3	23.4±2.0	<1.00E-50	24.4±2.7	24.6±1.3	3.89E-04	24.1±1.5	23.4±2.0	1.08E-28
Hypertension (%)	80.1	13.9	<1.00E-50	58.5	26.6	<1.00E-50	69.9	13.9	<1.00E-50
DM	24.4	3.6	<1.00E-50	10.3	13.3	<1.00E-50	25.3	3.6	<1.00E-50
Tch (mmol/l)	4.58±1.01	4.36±0.46	3.53E-17	4.57±0.51	4.36±1.13	1.64E-12	4.40±0.92	4.36±0.46	0.15
TG (mmol/l)	1.49±0.97	1.50±0.50	0.79	1.75±0.78	1.54±1.12	9.80E-11	1.59±1.00	1.50±0.50	0.67
HDL-c (mmol/l)	1.10±0.27	1.19±0.14	1.09E-35	1.20±0.19	1.36±1.24	7.23E-08	1.12±0.27	1.19±0.14	9.51E-19
LDL-c (mmol/l)	2.77±0.80	2.60±0.39	3.14E-15	2.61±0.41	2.40±0.83	1.57E-19	2.62±0.76	2.60±0.39	0.36

The data are presented as the means ± standard deviation or percent;

BMI, body mass index; DM, diabetes; Tch, total cholesterol; TG, triglyceride

HDL-C, high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol;

\* Age for the case group is the age at diagnosis; age for the control group is the age at which the study subject was enrolled;

**Table 2**

Genotypic association analysis between rs2043211 and ischemic stroke.

Cohorts	Model	Case(n)	Control(n)	<i>P-obs</i>	<i>P-adj</i>	OR(95%CI)
GeneID-Central IS (903/873)						
	Additive	173/521/209	230/430/213	2.88E-04	0.082	0.83(0.67-1.02)
	Dominant	694/209	660/213	0.535	0.520	1.12(0.80-1.56)
	Recessive	173/730	230/643	3.00E-04	0.028	0.68(0.48-0.96)
GeneID-North IS (816/879)						
	Additive	174/464/178	235/404/240	4.26E-05	0.692	0.97(0.81-1.15)
	Dominant	638/178	639/240	0.009	0.085	1.28(0.97-1.69)
	Recessive	174/642	235/644	0.009	0.017	0.70(0.53-0.94)
Combined IS (1719/1752)						
entire cohort	Additive	347/985/387	465/834/453	3.13E-08	0.324	0.94(0.83-1.06)
entire cohort	Dominant	1332/387	1299/453	0.021	0.075	1.19(0.98-1.45)
entire cohort	Recessive	347/1372	465/1287	9.78E-06	5.83E-04	0.70(0.57-0.86)
Female	Recessive	127/561	203/563	2.57E-04	0.007	0.65(0.48-0.89)
Male	Recessive	220/811	262/724	0.006	0.024	0.73(0.56-0.96)
Age<60	Recessive	104/442	360/965	2.17E-04	0.001	0.61(0.45-0.83)
Age 60	Recessive	243/930	105/322	0.100	0.107	0.79(0.59-1.05)
Female and Age<60	Recessive	43/206	155/421	0.003	0.004	0.51(0.32-0.80)
Male and Age<60	Recessive	61/236	205/544	0.022	0.104	0.71(0.48-1.08)

Model: Additive(TT/AT/AA), Dominant(TT+AT/AA), Recessive(TT/AT+AA); OR, odds ratio;

*P-obs*, *P* value using 2 × 2 contingency table  $\chi^2$  tests before adjustment for covariates;*P-adj*, *P* value adjusted by multivariate logistic regression analysis for traditional risk factors, including age, sex, BMI, smoking history, hypertension, DM, TC, TG, HDL- and LDL-C)