RESEARCH ARTICLE

HDAC9 Variant Rs2107595 Modifies Susceptibility to Coronary Artery Disease and the Severity of Coronary Atherosclerosis in a Chinese Han Population

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Abstract

A previous genome-wide association study showed that a single nucleotide polymorphism (SNP) rs2107595 in histone deacetylase 9 (HDAC9) gene was associated with large artery stroke (LAS) in Caucasians. Based on the similar atherosclerotic pathogenesis between LAS and coronary artery disease (CAD), we aimed to evaluate the associations of SNP rs2107595 with CAD risk and the severity of coronary atherosclerosis in a Chinese Han population, and explore the potential gene-environment interactions among SNP rs2107595 and conventional CAD risk factors. In a two-stage case-control study with a total of 2317 CAD patients and 2404 controls, the AG + AA genotypes of SNP rs2107595 were significantly associated with increased CAD risk (Adjusted odds ratio (OR) = 1.23, Padj = 0.001) and higher modified Gensini scores (Adjusted OR = 1.38, Padj < 0.001). These associations remained significant in subtype analyses for unstable angina pectoris (UAP), non-ST-segment elevation myocardial infarction (NSTEMI) and ST-segment elevation myocardial infarction (STEMI). Subgroup and multifactor dimensionality reduction analyses (MDR) further found the gene-environment interactions among SNP rs2107595 and conventional CAD risk factors. In a two-stage case-control study with a total of 2317 CAD patients and 2404 controls, the AG + AA genotypes of SNP rs2107595 were significantly associated with increased CAD risk (Adjusted odds ratio (OR) = 1.23, Padj = 0.001) and higher modified Gensini scores (Adjusted OR = 1.38, Padj < 0.001). These associations remained significant in subtype analyses for unstable angina pectoris (UAP), non-ST-segment elevation myocardial infarction (NSTEMI) and ST-segment elevation myocardial infarction (STEMI). Subgroup and multifactor dimensionality reduction analyses (MDR) further found the gene-environment interactions among SNP rs2107595, body mass index, type 2 diabetes and hyperlipidemia in CAD risk and the severity of coronary atherosclerosis. Moreover, patients with CAD had higher levels of HDAC9 mRNA expression and plasma HDAC9 than controls. Subsequent genotype-phenotype analyses observed the significant correlations of SNP rs2107595 with HDAC9 mRNA expression and plasma HDAC9 levels in controls and patients with NSTEMI and STEMI. Taken together, our data suggest that SNP rs2107595 may contribute to coronary atherosclerosis and CAD risk through a possible mechanism of regulating HDAC9 expression and gene-environment interactions.
Introduction

Coronary artery disease (CAD), the leading cause of death and disability worldwide[1], is mainly caused by multiple interactions between genetic and environmental risk factors of atherosclerosis[2]. Atherosclerosis in the coronary arteries is a continuous process of deposition of lipoproteins that results in the formation of atherosclerotic plaques, the progression of plaque instability, and eventually myocardial infarction (MI)[3, 4]. Therefore, the genetic and environmental determinants involved in CAD risk may vary across different stages of atherosclerosis, leading to the underlying heterogeneity between stable and unstable CAD[4, 5]. Moreover, this atherosclerotic pathogenesis also applies to the cerebral vessels and contributes to the development of ischemic stroke (IS), especially to the development of large artery stroke (LAS)[6]. Numerous studies have shown that CAD and LAS shared several conventional risk factors and susceptible loci[7, 8], such as smoking, hypertension and single nucleotide polymorphisms (SNPs) on chromosome 9p21, suggesting shared heritability for both diseases[6].

Recently, a genome-wide association study (GWAS) first reported that SNP rs2107595 in 3’-region of histone deacetylase 9 (HDAC9) gene was associated with LAS risk[9]. HDAC9 gene is located on chromosome 7p21.1, and encodes a member of histone decetylases, which is mainly responsible for the deacetylation of histones and subsequent gene transcription[10]. Accumulated evidence highlights the crucial roles of SNP rs2107595 and HDAC9 gene in the development of atherosclerosis. First, in a population-based cohort study, the minor allele of SNP rs2107595 was correlated with higher carotid intima-media thickness (CIMT)[11], which has been reported as a predictive marker for atherosclerosis[12]. Second, HDAC9 plays central roles in heart development[13], and is highly expressed in atherosclerotic plaques at human systemic arteries[11]. Finally, HDAC9 deletion could lead to down-regulation of inflammatory genes[14], up-regulation of lipid-associated genes[14], and dramatic reduction of atherosclerotic lesion size in mice[15]. Taking all these findings together, we hypothesize that SNP rs2107595 may also contribute to the development of coronary atherosclerosis and CAD risk by modifying HDAC9 expression.

Thus, in this two-stage case-control study, we assessed the associations of SNP rs2107595 with CAD risk and the severity of coronary atherosclerosis, and further performed a genotype-phenotype analysis by detecting HDAC9 mRNA expression and plasma HDAC9 levels, followed by subgroup and multifactor dimensionality reduction (MDR) analyses to explore the potential gene-environment interactions. This is the first study to assess the associations of SNP rs2107595 with CAD risk and the severity of coronary atherosclerosis in the Chinese population.

Materials and Methods

Study population

This two-stage case-control study included a total of 2317 CAD patients and 2404 age- and sex-matched controls. For reducing the impact of population stratification, we only recruited unrelated ethnic Han Chinese in Hubei Province, central China. In the discovery set (Study 1), 1172 patients and 1086 controls were enrolled from Wuhan Asia Heart Hospital between March 2011 and June 2016. The replication set (Study 2) with 1145 cases and 1318 controls was recruited from Zhongnan Hospital of Wuhan University between July 2013 and June 2016. In these two sets, CAD was angiographically defined as stenosis of more than 50% in at least one major coronary artery or their main branches. Then, according to the American College of Cardiology/American Heart Association Task Force on Practice Guidelines[16–18], CAD patients were further categorized based on the following clinical presentation: (1) stable
angina pectoris (SAP); (2) unstable angina pectoris (UAP); (3) non-ST-segment elevation MI (NSTEMI); (4) ST-segment elevation MI (STEMI) (S1 Appendix). Finally, for each case, modified Gensini scores[19] were calculated by two independent angiographers to assess the severity of coronary atherosclerosis. The control groups included participants without lumen stenosis validated by coronary angiography and healthy subjects without cardiovascular diseases identified by physical examinations. The following data for each participant were collected: (1) clinical information such as blood pressure, body mass index (BMI), fasting plasma glucose (FPG) and lipid levels; (2) conventional CAD risk factors including smoking status, alcohol drinking status, and histories of type 2 diabetes mellitus (T2DM), hyperlipidemia, and hypertension (S1 Appendix). The following subjects were excluded: (1) individuals with systemic diseases such as cerebrovascular diseases, cancers, autoimmune diseases and renal or hepatic diseases; (2) subjects with cardiac diseases including myocardial bridge, coronary artery spasm and congenital or valvular heart diseases. This study followed the Declaration of Helsinki, and was approved by the Ethics Committees of Wuhan Asia Heart Hospital and Zhongnan Hospital of Wuhan University. Written informed consent was obtained from all participants.

Criterion of modified Gensini scores
In the modified Gensini scoring system[19], angiographic stenosis of each coronary segment was first scored according to the degree of luminal narrowing: 1 for 0–25% stenosis, 2 for 26–50%, 4 for 51–75%, 8 for 76–90%, 16 for 91–99% and 32 for 100%. Then a multiplier was assigned to each segment depending on the physical importance of the area supplied by that segment: 5 for main left coronary artery (MLCA), 2.5 for proximal left anterior descending coronary artery (LAD) and proximal left circumflex branch, 1.5 for mid-segment of LAD, 0.5 for second diagonal branch and posterolateral branch, and 1 for other branches. Finally, the weighted scores for each segment were added to give modified Gensini scores. As acute coronary occlusion mainly occurs in a previous angiographically non-critical lesion, acute total occlusion was scored as a non-critical lesion (0–5 score) in the modified Gensini scoring system [19].

SNP rs2107595 genotyping
Genomic DNA of peripheral blood leukocytes was isolated by a phenol/chloroform method. SNP rs2107595 was genotyped by high resolution melting (HRM) analyses using a LightScanner 96 system (Idaho Technology, Salt Lake City, UT, USA)[20, 21], as described in S1 Table. Repeated assays and DNA sequence analysis were used to validate the accuracy of HRM genotyping (S1 Fig).

Reverse-transcription quantitative PCR analysis of HDAC9 mRNA
Total RNA was extracted from peripheral blood leukocytes using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). After eliminating DNA contamination using the RNase-Free gDNA eraser, 1μg of total RNA was prepared for reverse transcription using a reverse transcriptase kit (Takara Bio Inc, Kusatsu, Shiga, Japan).Then, to determine HDAC9 mRNA expression, the cDNA products were used for reverse-transcription quantitative PCR (RT-qPCR) analysis with the SYBR-Green kit on a CFX96 Touch system (Bio-rad, Hercules, CA, USA). RT-qPCR analysis for each sample was performed in triplicate and followed the MIQE guidelines [22]. The relative expression of HDAC9 was normalized to the expression of reference gene (GAPDH) using the 2-△△Cq method[23], Primer sequences and RT-qPCR conditions for HDAC9 and GAPDH were also summarized in S1 Table.
Assessment of plasma HDAC9 activity

Plasma samples were separated by centrifugation (at 2000 g for 10 minutes at 4°C) and stored at -80°C until detection. According to the manufacturer’s instructions, plasma HDAC9 levels were measured by an enzyme-linked immunosorbent assay (ELISA) (HDAC9 ELISA kit, Xin-fan Biosystems, Shanghai, China), followed by quantification using a standard curve with the detection limit of 0.1 ng/mL. CV values for intra- and inter-assays were 4.7% and 6.5%, respectively.

Statistical analyses

For clinical characteristics, the differences in categorical and continuous variables between cases and controls were analyzed by the Pearson $^2$ test and the Student’s t-test, respectively. For SNP rs2107595, Hardy-Weinberg equilibrium (HWE) was assessed by the Pearson $^2$ test. Allelic and genotypic association analyses were performed by logistic regression analyses with and without adjustment for age, sex, BMI, smoking status, alcohol drinking status and histories of T2DM, hyperlipidemia and hypertension. The homogeneity between the odds ratios (ORs) of two sets was evaluated by the Breslow-Day test. The association of SNP rs2107595 with modified Gensini scores was analyzed by the Mann-Whitney U test and logistic regression analyses. When samples were stratified, the multiplicative likelihood ratio test was carried out to test the possible gene-environment interactions in CAD risk and the severity of coronary atherosclerosis. The differences in HDAC9 expression and plasma HDAC9 levels between cases and controls, and the associations of SNP rs2107595 with HDAC9 expression and plasma HDAC9 levels were assessed by analyses of covariates (ANCOVA) after adjusting for covariates. The correlations of HDAC9 expression and plasma HDAC9 levels with modified Gensini scores were examined by the Spearman correlation test. All these statistical analyses were performed by SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and P values of less than 0.05 (two-sided) were considered as statistically significant.

In association analyses for SNP rs2107595, we performed the Bonferroni correction and Monte-Carlo permutation tests to control for multiple comparisons. The Monte-Carlo permutation test can formally calculated an empirical P value by repeating permutations 100,000 times to randomly redistribute genotype counts of cases and controls [24]. An empirical P value of less than 0.05 was considered as a stable result for multiple comparisons. A statistical power was calculated by PS 3.0 program (Vanderbilt University, Nashville, TN, USA). To further assess the high-order gene-environment interactions in CAD risk and the severity of coronary atherosclerosis, multifactor dimensionality reduction (MDR) analyses were carried out using MDR 2.0 beta 8.4 program (UPenn, Philadelphia, PA, USA) [25]. In brief, this program first constructed all possible combinations of included variables. Then, by using 100-time cross-validation and 1000-time permutation tests, the best n-factor models for predicting CAD risk and the severity of coronary atherosclerosis were found with the maximal cross-validation consistency (CVC) and the optimal testing accuracy. Finally, for these best n-factor models, the interaction entropy graphs were applied to visually depict the univariate effect of each variable and the pairwise interactions. The univariate and pairwise effects were expressed as the percentage of entropy.

Results

Characteristics of study population

As summarized in S2 Table, in two cohorts of our study, there were significant differences in blood pressure, BMI, FPG and lipid levels as well as the frequencies of smoking, alcohol...
drinking, T2DM, hyperlipidemia, and hypertension between cases and controls. The genotype distributions of SNP rs2107595 were in accordance with HWE in two control groups (in discovery and replication sets, $P_{HWE} = 0.734$ and 0.904, respectively).

### Allelic and genotypic association analyses between SNP rs2107595 and CAD risk

In the discovery set, allelic association analyses showed that the minor allele A of SNP rs2107595 was significantly associated with increased CAD risk ($OR = 1.19$, $P = 0.008$). This significant association was further identified in the replication set with an allelic OR of 1.20 and a $P$ value of 0.002. As the Breslow-Day test confirmed the homogeneity of ORs between the two sets ($P = 0.864$), a meta-analysis of these two cohorts was carried out. And the results indicated that the minor allele of SNP rs2107595 had a 1.19-fold ($P = 6.08 \times 10^{-5}$) increased risk of CAD in the merged set. All these associations remained significant after the permutation test for multiple testing and adjustment for covariates (Table 1). Given a minor allele frequency (MAF) of 0.3055 in controls, a MAF of 0.3442 in cases and the type I error of 0.05, the merged sample size could provide a statistical power of 81.0% to detect the association.

To further explore the inheritance pattern of SNP rs2107595, genotypic association analyses were performed based on additive, dominant and recessive models (Table 1). In both discovery and replication sets, significant associations were identified between SNP rs2107595 and increased CAD risk under both additive and dominant models (Table 1). When we combined

Table 1. Allelic and genotypic associations of SNP rs2107595 with CAD risk.

| Model               | Alleles (G/A)/Genotypes § | Without adjustment | With adjustment † |
|---------------------|---------------------------|--------------------|-------------------|
|                     | Cases, N | Controls, N | OR (95%CI) | $P$ | $P_{emp}$* | OR (95%CI) | $P_{adj}$ |
| Allelic association analyses ‡ |
| Discovery set       | 1548/796 | 1515/657   | 1.19 (1.05–1.34) | 0.008 | 0.009 | 1.18 (1.03–1.35) | 0.016 |
| Replication set     | 1491/799 | 1824/812   | 1.20 (1.07–1.36) | 0.002 | 0.002 | 1.16 (1.02–1.31) | 0.020 |
| Merged set          | 3039/1595 | 3339/1469 | 1.19 (1.09–1.30) | $6.08 \times 10^{-5}$ | $4.11 \times 10^{-5}$ | 1.16 (1.06–1.28) | 0.001 |

Genotypic association analyses §

| Model               | Alleles (G/A)/Genotypes § | Without adjustment | With adjustment † |
|---------------------|---------------------------|--------------------|-------------------|
|                     | Cases, N | Controls, N | OR (95%CI) | $P$ | $P_{emp}$* | OR (95%CI) | $P_{adj}$ |
| Discovery set       | 495/558/119 | 526/463/97 | 1.19 (1.05–1.36) | 0.007 | 0.014 | 1.19 (1.03–1.36) | 0.014 |
| Dominant            | 495/677 | 526/560 | 1.29 (1.09–1.52) | 0.003 | 0.003 | 1.26 (1.05–1.50) | 0.011 |
| Recessive           | 1053/119 | 989/97 | 1.15 (0.87–1.53) | 0.324 | 0.533 | 1.19 (0.88–1.60) | 0.257 |
| Replication set     | 481/529/135 | 632/560/126 | 1.21 (1.07–1.36) | 0.002 | 0.009 | 1.16 (1.02–1.32) | 0.020 |
| Dominant            | 481/664 | 632/686 | 1.27 (1.08–1.49) | 0.003 | 0.006 | 1.21 (1.02–1.43) | 0.026 |
| Recessive           | 1010/135 | 1192/126 | 1.26 (0.98–1.64) | 0.073 | 0.169 | 1.21 (0.92–1.58) | 0.169 |
| Merged set          | 976/1087/254 | 1158/1023/223 | 1.20 (1.10–1.31) | $5.22 \times 10^{-5}$ | $1.60 \times 10^{-4}$ | 1.17 (1.07–1.28) | 0.001 |
| Dominant            | 976/1341 | 1158/1246 | 1.28 (1.14–1.43) | $3.04 \times 10^{-5}$ | $3.35 \times 10^{-5}$ | 1.23 (1.09–1.39) | 0.001 |
| Recessive           | 2063/254 | 2181/223 | 1.20 (0.99–1.46) | 0.055 | 0.093 | 1.19 (0.98–1.45) | 0.088 |

* Empirical $P$ values were obtained from 100,000-time Monte-Carlo permutation test.
† Adjusted OR (95%CI) and $P_{adj}$ values were obtained from logistic regression analyses after adjusting for age, sex, BMI, smoking status, alcohol drinking status and histories of T2DM, hyperlipidemia and hypertension.
‡ In allelic association analyses, the major allele G was considered as the reference.
§ In genotypic association analyses, additive model = GG/AG/AA; dominant model = GG (Reference)/AA + AG; recessive model = AG + GG (Reference)/AA.

Bold values indicate statistically significant after the Bonferroni correction ($P < 0.05/24 \approx 0.002$)

doi:10.1371/journal.pone.0160449.t001
these two cohorts, the dominant model was considered as the most significant model with the largest OR of 1.28 and the smallest P value of $3.04 \times 10^{-5}$. After adjusting for covariates, all these associations remained significant (Table 1). In both allelic and genotypic association analyses, all significant results in the merged set were robust enough to withstand the Bonferroni correction (Table 1).

### Subtype and subgroup analyses for associations between SNP rs2107595 and CAD risk

To further evaluate the effects of SNP rs2107595 on different CAD subtypes and the potential gene-environment interactions, subtype and subgroup analyses were performed under a dominant model (Table 2). In subtype analyses, compared with the GG carriers, subjects with the AG + AA genotypes had a 1.32-fold ($P = 7.75 \times 10^{-4}$), a 1.53-fold ($P = 1.20 \times 10^{-5}$) and a 1.62-fold ($P = 9.61 \times 10^{-5}$) increased risk of UAP, NSTEMI and STEMI, respectively. In subgroup analyses, the association between the AG + AA genotypes and increased CAD risk was consistently significant in almost all subgroups, except for individuals with BMI $\leq 25$ and participants without T2DM and hyperlipidemia. Furthermore, we observed the multiplicative interactions of SNP rs2107595 with BMI ($P_{inter} = 0.009$), T2DM ($P_{inter} = 0.004$) and hyperlipidemia ($P_{inter} = 0.001$) status in CAD risk. All the above associations were still significant after adjusting for covariates (Table 2). Based on the Bonferroni correction, we also found significant associations in subtype analyses for UAP, NSTEMI and STEMI as well as in subjects with BMI $> 25$, T2DM and hyperlipidemia (Table 2).

### Association of SNP rs2107595 with the severity of coronary atherosclerosis

By using a modified Gensini scoring system, we assessed the severity of coronary atherosclerosis in CAD patients and found that patients with the AG + AA genotypes had higher modified Gensini scores than the GG carriers in discovery ($P = 0.028$), replication ($P = 0.001$) and merged sets ($P = 6.47 \times 10^{-5}$) (Table 3). When we classified CAD patients into two groups according to the median (30) of modified Gensini scores, we also observed that the variant genotypes (AG + AA) of SNP rs2107595 were associated with a 1.38-fold ($P_{adj} = 1.68 \times 10^{-4}$) increased risk of higher modified Gensini scores ($> 30$) in the merged set after adjusting for covariates. These associations remained significant in almost all subtypes and subgroups, except for SAP subtypes as well as subgroups of BMI $\leq 25$, non-T2DM and non-hyperlipidemia (Table 3). Moreover, the AG + AA genotypes multiplicatively interacted with higher BMI ($> 25$) ($P_{inter} = 0.043$), and histories of T2DM ($P_{inter} = 0.013$) and hyperlipidemia ($P_{inter} = 1.51 \times 10^{-4}$) to increase the severity of coronary atherosclerosis. After the Bonferroni correction, the associations in the merged set as well as in patients with BMI $> 25$, T2DM and hyperlipidemia remained significant (Table 3).

### MDR analyses for assessing the high-order gene-environment interactions in CAD risk and the severity of coronary atherosclerosis

Based on the results of multiplicative interaction analyses, data on SNP rs2107595, BMI, T2DM and hyperlipidemia status were included in the MDR analyses to further explore the high-order gene-environment interactions. As presented in Table 4, the four-factor model including all variables was selected as the best predictor for both CAD risk and higher modified Gensini scores, because it had the optimal testing accuracy and the maximal CVC values. For this four-factor model, when evaluating the univariate effects of different variables, history of
T2DM (0.58%) exerted the greatest independent effect on CAD risk (Fig 1A), while history of Hyperlipidemia (1.56%) was the strongest risk factor for higher modified Gensini scores (Fig 1B). When assessing the strength of pairwise effects, the strongest pairwise interactions were found between SNP rs2107595 and T2DM (3.66%) in MDR analyses for predicting CAD risk (Fig 1A), and between SNP rs2107595 and hyperlipidemia (2.89%) in MDR analyses for predicting the severity of coronary atherosclerosis (Fig 1B).
Table 3. Association of SNP rs2107595 with the severity of coronary atherosclerosis in 2317 CAD patients under a dominant model.

| Variables | Modified Gensini scores * | Modified Gensini scores † (≤ 30/> 30, N) | With adjustment ‡ |
|-----------|---------------------------|------------------------------------------|------------------|
|           | N  | GG  | N  | AG + AA | P † | GG  | AG + AA | OR (95%CI) | P adj | P inter § |
| Study set |    |     |    |         |     |     |         |           |       |           |
| Discovery | 549| 29.5 (18.0–63.0) | 757| 34.0 (20.0–71.0) | 0.028 | 276/219 | 317/360 | 1.32 (1.04–1.67) | 0.025 |
| Replication | 427| 30.0 (18.0–71.0) | 584| 35.0 (19.6–82.9) | 0.001 | 268/213 | 299/365 | 1.45 (1.14–1.85) | 0.002 |
| Merged set | 976| 29.5 (18.0–66.0) | 1341| 35.0 (20.0–75.0) | 6.47 × 10⁻⁶ | 544/432 | 616/725 | 1.38 (1.17–1.64) | 1.68 × 10⁻⁴ |
| CAD subtypes | | | | | | | | |
| SAP       | 335| 20.0 (14.0–29.5) | 345| 19.5 (14.0–28.8) | 0.355 | 266/58 | 289/67 | 0.76 (0.51–1.13) | 0.172 |
| UAP       | 320| 20.0 (17.0–30.0) | 456| 23.0 (18.0–36.0) | 0.016 | 246/75 | 304/151 | 1.49 (1.07–2.08) | 0.018 |
| NSTEMI    | 205| 71.0 (45.0–90.5) | 338| 76.8 (55.4–97.5) | 0.041 | 18/197 | 12/316 | 2.22 (1.01–4.87) | 0.047 |
| STEMI     | 116| 73.8 (53.3–93.5) | 202| 81.5 (54.0–110.6) | 0.023 | 14/102 | 11/191 | 2.51 (1.07–5.89) | 0.035 |
| Age, years | | | | | | | | |
| < 60      | 493| 28.5 (18.0–62.5) | 667| 32.0 (20.0–74.0) | 0.005 | 280/213 | 318/349 | 1.38 (1.09–1.76) | 0.008 |
| > 60      | 483| 30.0 (18.0–73.5) | 674| 38.0 (20.0–78.0) | 0.004 | 264/219 | 298/376 | 1.40 (1.10–1.77) | 0.007 |
| Sex       | | | | | | | | |
| Male      | 535| 30.0 (18.0–71.0) | 743| 33.0 (20.0–72.0) | 0.018 | 246/195 | 273/325 | 1.36 (1.09–1.71) | 0.008 |
| Female    | 441| 29.0 (18.0–63.5) | 598| 36.0 (19.9–79.6) | 0.001 | 298/237 | 343/400 | 1.41 (1.10–1.81) | 0.007 |
| BMI, kg/m² | | | | | | | | |
| ≤ 25      | 532| 30.0 (19.5–69.0) | 668| 31.0 (19.5–70.9) | 0.227 | 293/239 | 330/338 | 1.16 (0.91–1.46) | 0.229 |
| > 25      | 444| 28.5 (18.0–63.0) | 673| 41.0 (20.0–79.5) | 8.76 × 10⁻⁶ | 251/193 | 286/387 | 1.68 (1.32–2.15) | 3.26 × 10⁻⁵ |
| Smoking status | | | | | | | | |
| Yes       | 315| 29.0 (18.0–59.0) | 490| 34.3 (20.0–77.0) | 0.001 | 183/132 | 221/269 | 1.59 (1.19–2.13) | 0.002 |
| No        | 661| 30.0 (18.0–69.0) | 851| 35.0 (19.5–74.0) | 0.017 | 361/300 | 395/456 | 1.29 (1.05–1.59) | 0.015 |
| Drinking status | | | | | | | | |
| Yes       | 307| 29.0 (18.0–71.0) | 450| 33.5 (19.5–76.5) | 0.001 | 172/135 | 209/241 | 1.40 (1.14–1.71) | 0.001 |
| No        | 669| 30.0 (18.0–63.5) | 891| 35.0 (20.0–74.0) | 0.024 | 372/297 | 407/484 | 1.36 (1.01–1.83) | 0.045 |
| T2DM      | | | | | | | | |
| Yes       | 263| 30.0 (18.0–71.0) | 487| 48.0 (21.0–88.0) | 1.15 × 10⁻⁴ | 138/125 | 174/313 | 1.89 (1.39–2.58) | 5.48 × 10⁻⁵ |
| No        | 713| 29.0 (18.0–65.0) | 854| 30.0 (19.5–66.0) | 0.122 | 406/307 | 442/412 | 1.21 (0.99–1.48) | 0.068 |
| Hyperlipidemia | | | | | | | | |
| Yes       | 238| 29.0 (18.0–66.0) | 443| 46.5 (22.5–87.0) | 1.01 × 10⁻⁷ | 137/101 | 157/286 | 2.31 (1.67–3.21) | 5.40 × 10⁻⁷ |
| No        | 738| 30.0 (18.0–66.0) | 898| 30.0 (19.5–65.6) | 0.330 | 407/331 | 459/439 | 1.14 (0.94–1.39) | 0.197 |
| Hypertension | | | | | | | | |
| Yes       | 582| 29.0 (18.0–63.5) | 792| 33.0 (19.5–75.0) | 0.006 | 324/258 | 374/418 | 1.32 (1.06–1.64) | 0.013 |
| No        | 394| 30.0 (18.0–70.6) | 549| 36.0 (20.0–75.5) | 0.003 | 220/174 | 242/307 | 1.49 (1.14–1.94) | 0.004 |

Abbreviation: N, number; OR (95%CI), odds ratio (95% confidence interval); SAP, stable angina pectoris; UAP, unstable angina pectoris; NSTEMI: non-ST-segment elevation myocardial infarction; STEMI: ST-segment elevation myocardial infarction; BMI, body mass index; T2DM, type 2 diabetes mellitus.

* Modified Gensini scores are expressed as median (interquartile range) because of the skewed distributions.

† P values were obtained from the Mann-Whitney U test.

‡ CAD patients were classified into two groups based on the median (30) of modified Gensini scores, then logistic regression analyses were used to assess the association between SNP rs2107595 and modified Gensini scores after adjusting for age, sex, BMI, smoking status, alcohol drinking status and histories of T2DM, hyperlipidemia and hypertension.

§ P_inter values were obtained from the multiplicative likelihood ratio test to assess the multiplicative interactions between SNP rs2107595 and selected variables in the severity of coronary atherosclerosis.

Bold values indicate statistically significant after the Bonferroni correction (P < 0.05/54 ≈ 9.26 × 10⁻⁴).
The differences in HDAC9 mRNA expression and plasma HDAC9 levels between CAD patients and controls

We measured HDAC9 mRNA expression and plasma HDAC9 levels in 250 representative subjects (125 cases vs 125 controls, S3 Table) randomly selected from the replication set. In ANCOVA models adjusted for covariates, HDAC9 mRNA expression (1.20 ± 0.41 vs 1.06 ± 0.42) and plasma HDAC9 levels were significantly higher in CAD patients compared to controls (10.5 ± 4.5 vs 9.2 ± 3.9 ng/ml).

Table 4. The gene-environment interactions between SNP rs2107595 and conventional CAD risk factors in CAD risk and the severity of coronary atherosclerosis by MDR analyses.

| No. of risk factors | Best interaction models | CVC * | Testing accuracy (%) † | P for permutation test ‡ |
|--------------------|-------------------------|-------|------------------------|-------------------------|
| Gene-environment interactions in CAD risk, 2317 CAD patients/2404 controls | | | | |
| 1 | History of T2DM | 94/100 | 0.5440 | 0.0013 |
| 2 | History of T2DM, SNP rs2107595 (GG/AG + AA) | 100/100 | 0.6211 | < 0.0001 |
| 3 | History of T2DM, SNP rs2107595 (GG/AG + AA), BMI status | 100/100 | 0.6547 | < 0.0001 |
| 4 | History of T2DM, SNP rs2107595 (GG/AG + AA), BMI status, history of hyperlipidemia | 100/100 | 0.6869 | < 0.0001 |
| Gene-environment interactions in the severity of coronary atherosclerosis, 1160/1157 CAD patients with lower (< 30)/higher (> 30) modified Gensini scores | | | | |
| 1 | History of hyperlipidemia | 96/100 | 0.5691 | 0.0001 |
| 2 | History of hyperlipidemia, SNP rs2107595 (GG/AG + AA) | 100/100 | 0.6219 | < 0.0001 |
| 3 | History of hyperlipidemia, SNP rs2107595 (GG/AG + AA), BMI status | 100/100 | 0.6300 | < 0.0001 |
| 4 | History of hyperlipidemia, SNP rs2107595 (GG/AG + AA), BMI status, history of T2DM | 100/100 | 0.6698 | < 0.0001 |

Abbreviation: T2DM, type 2 diabetes mellitus; BMI, body mass index; CVC, cross-validation consistency.

* CVC means the number of times that a given combination of factors is identified in each testing set (a total of 100 times).
† Testing accuracy (%) is the percentage of participants for whom a correct prediction is made.
‡ The permutation test was carried out to repeat the MDR analyses 1000 times and calculate the CVC and testing accuracy of each n-factor model.

Bold values indicate the models that have the maximal CVC and the optimal testing accuracy.

The differences in HDAC9 mRNA expression and plasma HDAC9 levels between CAD patients and controls

We measured HDAC9 mRNA expression and plasma HDAC9 levels in 250 representative subjects (125 cases vs 125 controls, S3 Table) randomly selected from the replication set. In ANCOVA models adjusted for covariates, HDAC9 mRNA expression (1.20 ± 0.41 vs 1.06 ± 0.42) and plasma HDAC9 levels were significantly higher in CAD patients compared to controls (10.5 ± 4.5 vs 9.2 ± 3.9 ng/ml).

Fig 1. Interaction entropy graphs for the gene-environment interactions between SNP rs2107595, BMI status, and histories of T2DM and hyperlipidemia in CAD risk and the severity of coronary atherosclerosis. (A) Interaction entropy graphs for the gene-environment interactions between SNP rs2107595, BMI status, and histories of T2DM and hyperlipidemia in CAD risk; (B) Interaction entropy graphs for the gene-environment interactions between SNP rs2107595, BMI status, and histories of T2DM and hyperlipidemia in the severity of coronary atherosclerosis. In these graphs, the univariate effect of each variable and the pairwise interactions are expressed as the percentage of entropy. The univariate effect of each variable is presented in a box with the percentage of entropy. The pairwise interactions are shown as connected lines accompanied by the percentage of entropy. Different colors on lines indicate the strength of the interactions, i.e., red, orange and yellow lines mean strong, moderate and weak interactions, respectively.

doi:10.1371/journal.pone.0160449.g001
0.39, \( P = 0.031 \)) and plasma HDAC9 levels (54.2± 9.9 vs 50.6± 8.7, \( P = 0.015 \)) in CAD patients were significantly higher than those in controls. In subtype analyses, patients with NSTEMI and STEMI had higher levels of HDAC9 mRNA expression and plasma HDAC9 than controls (Table 5). Moreover, we also observed a positive correlation between plasma HDAC9 levels and modified Gensini scores (\( r = 0.184, P = 0.040 \)).

### Table 5. Associations of SNP rs2107595 with HDAC9 mRNA expression and plasma HDAC9 levels.

|                    | Control | CAD | SAP | UAP | NSTEMI | STEMI |
|--------------------|---------|-----|-----|-----|--------|-------|
| HDAC9 mRNA expression | Total   | N   | Mean ± SD | N   | Mean ± SD | N   | Mean ± SD | N   | Mean ± SD | N   | Mean ± SD |
| Total              | 125     | 1.06 ± 0.39* | 125 | 1.20 ± 0.41* | 45   | 1.15 ± 0.38* | 25   | 1.26 ± 0.44* | 21   | 1.34 ± 0.39* |
| SNP rs2107595      |         |     |           |     |           |     |           |     |           |     |           |
| GG                 | 65      | 0.97 ± 0.36   | 50  | 1.09 ± 0.40  | 17   | 1.16 ± 0.38  | 10   | 0.95 ± 0.42  | 6    | 0.98 ± 0.29  |
| AG                 | 46      | 1.13 ± 0.40†  | 54  | 1.25 ± 0.36† | 22   | 1.16 ± 0.40  | 11   | 1.16 ± 0.35  | 9    | 1.41 ± 0.34†  |
| AA                 | 14      | 1.23 ± 0.42†  | 21  | 1.34 ± 0.47† | 6    | 1.08 ± 0.35  | 6    | 1.13 ± 0.50  | 6    | 1.55 ± 0.31†  |
| AG + AA            | 60      | 1.16 ± 0.40†  | 75  | 1.28 ± 0.39† | 28   | 1.14 ± 0.39  | 17   | 1.15 ± 0.39  | 15   | 1.47 ± 0.32†  |
| Plasma HDAC9 levels (ng/mL) | Total | 125 | 50.6 ± 8.7 | 125 | 54.2 ± 9.9* | 45   | 52.3 ± 10.3 | 34   | 53.1 ± 10.4 | 25   | 56.6 ± 8.3* | 21   | 57.0 ± 9.1* |
| SNP rs2107595      |         |     |           |     |           |     |           |     |           |     |           |
| GG                 | 65      | 48.6 ± 8.2 | 50  | 52.3 ± 9.5 | 17   | 53.6 ± 8.7 | 17   | 52.9 ± 12.0 | 10   | 50.7 ± 6.0 | 6    | 49.7 ± 9.3 |
| AG                 | 46      | 51.6 ± 8.8 | 54  | 55.4 ± 10.2 | 22   | 51.9 ± 11.1 | 11   | 54.3 ± 9.8 | 9    | 59.8 ± 9.2† | 12   | 59.6 ± 7.8† |
| AA                 | 14      | 57.2 ± 7.5† | 21  | 55.3 ± 9.8 | 6    | 50.3 ± 13.1 | 6    | 51.2 ± 7.8 | 6    | 61.6 ± 3.5† | 3    | 60.9 ± 7.1† |
| AG + AA            | 60      | 52.9 ± 8.8† | 75  | 55.4 ± 10.0 | 28   | 51.6 ± 11.3 | 17   | 53.2 ± 9.0 | 15   | 60.5 ± 7.3† | 15   | 59.9 ± 7.5† |

Abbreviation: N, number; SD, standard deviation; CAD, coronary artery disease; SAP, stable angina pectoris; UAP, unstable angina pectoris; NSTEMI: non-ST-segment elevation myocardial infarction; STEMI: ST-segment elevation myocardial infarction.

* \( P < 0.05 \), in the comparisons between CAD patients and controls.
† \( P < 0.05 \), in the comparisons between different genotypes of SNP rs2107595.

doi:10.1371/journal.pone.0160449.t005

Associations of SNP rs2107595 with HDAC9 mRNA expression and plasma HDAC9 levels

Assuming a dominant model, significant correlations were found between the AG + AA genotypes and increased HDAC9 mRNA expression in both cases (\( P = 0.011 \)) and controls (\( P = 0.008 \)), and between the AG + AA genotypes and higher plasma HDAC9 levels only in controls (\( P = 0.009 \)) (Table 5 and Fig 2). When patients were stratified by CAD subtypes, we further observed the significant associations of the AG + AA genotypes with increased levels of HDAC9 mRNA expression and plasma HDAC9 in patients with NSTEMI and STEMI (Fig 2).

### Discussion

In 2012, the METASTROKE collaboration reported a significant association between SNP rs2107595 of HDAC 9 gene and LAS risk in Caucasians[9], suggesting an important role of this locus in atherosclerotic stroke. Subsequent GWAS datasets in Europeans identified that SNPs rs11984041 and rs2023938 in this gene were associated with the risk of LAS[26, 27] and CAD [6, 28], respectively. However, based on the data from a previous report[29] and 1000 Genome Database[30] (http://www.1000genomes.org/), these two SNPs in Caucasians are not polymorphic in the Chinese Han population, suggesting that the genetic architecture in HDAC9 region may differ across ethnicities. More importantly, no previous studies have assessed the effects of
SNP rs2107595 on coronary atherosclerosis and subsequent CAD risk. Therefore, in the current study, by using a two-stage case-control design with a total of 2317 CAD patients and 2404 controls, we genotyped SNP rs2107595 and found that the minor allele A of this locus was significantly associated with increased CAD risk and higher modified Gensini scores in a Chinese Han population. Then, by using subgroup and MDR analyses, the gene-environment interactions among the AG + AA genotypes, higher BMI and histories of T2DM and hyperlipidemia were identified to increase CAD risk and the severity of coronary atherosclerosis. Finally, the genotype-phenotype analyses further suggested that SNP rs2107595 might be functional by regulating HDAC9 mRNA expression and plasma proteins.

The present study, for the first time, reported the significant associations of SNP rs2107595 with CAD risk and the severity of coronary atherosclerosis. These findings are further supported by the current result that the minor allele A of SNP rs2107595 was correlated with higher HDAC9 mRNA expression and the previous reports that HDAC9 expression was involved in the development of atherosclerosis[11, 14]. By predicting the potential functions of SNP rs2107595 in the F-SNP database [31] (http://compbio.cs.queensu.ca/F-SNP/), we found that this variant is located in an evolutionarily conserved region, suggesting the importance of its physical location. Subsequent bioinformatics using the MatInspector [32] (http://www.genomatix.de/index.html) and SiteGA databases [33] (http://wwwmgs.bionet.nsc.ru/cgi-bin/mgs/sitega/index.pl) further showed that the minor allele of this locus disrupted the binding sites of several E2F transcription factors, including repressor E2F-4 and -6, which have been reported to repress gene transcription by forming a heterodimeric complex with RB protein [34]. Although the exact mechanism needs to be further elucidated, the above evidence reinforces the possibility that SNP rs2107595 may regulate HDAC9 expression and protein levels, then contribute to the development of coronary atherosclerosis and CAD risk.

In a GWAS for Caucasians, SNP rs2107595 was first associated with LAS, which was considered as an atherosclerotic subtype of IS[35]. In the current study, we also found stronger associations of this variant with unstable CAD (UAP, NSTEMI and STEMI) than with SAP. In general, the continuum from SAP to unstable CAD mainly results from the progression of plaque vulnerability caused by the aggravated inflammation in plaques[36]. Notably, this inflammatory process can be further induced by the polarized switching of macrophages from an anti-inflammatory M2 phenotype to a proinflammatory M1 state[37–39]. Recently, Cao et al.
have reported that HDAC9 deficiency could decrease the expression of M1 inflammatory genes and promote M2 polarization. More importantly, in the present study, besides the significant association of SNP rs2107595 with HDAC9 expression, we also found that HDAC9 mRNA expression in patients with NSTEMI and STEMI was significantly higher than that of controls. Taking all the evidence together, it is reasonable to hypothesize that SNP rs2107595 and HDAC9 expression may involve the progression of atherosclerosis, and thus exert stronger effects on unstable CAD than on SAP.

The above evidence also pinpoints the importance of subtype analyses when assessing the associations of susceptible loci with chronic, progressive diseases like CAD and IS. Recently, a case-control study by Su et al.[40] failed to find a significant association of SNP rs2107595 with IS in a southern Chinese population. Besides the relatively small sample size of their study (816 cases and 816 controls, a power of 37% and 60% based on the allelic ORs of previous GWAS (OR = 1.13) and the present study (OR = 1.19), respectively) and the genetic heterogeneity between central (a MAF of 0.306 in our controls) and southern Chinese (a MAF of 0.345 in their controls), another important reason for their non-significant results may be that Su et al.'s study did not define IS subtypes for their cases, and therefore could not detect the association of SNP rs2107595 with specific IS subtypes.

In this study, by using subgroup and MDR analyses, we found the gene-environment interactions among SNP rs2107595, T2DM, hyperlipidemia and BMI in CAD risk and the severity of coronary atherosclerosis. Subsequent interaction entropy graphs further observed the greatest pairwise effect of SNP rs2107595 and T2DM on CAD risk, and the strongest pairwise interaction between SNP rs2107595 and hyperlipidemia in higher modified Gensini scores. For T2DM and SNP rs2107595, recent studies have shown that HDAC9 ablation mice exhibited improved glucose tolerance and insulin tolerance[41], and that HDAC9 up-regulation enhanced gluconeogenesis in vitro[42]. The important roles of HDAC9 gene in glucose metabolism, combined with the known impact of dysglycemia on CAD risk[43] as well as the significant correlation of SNP rs2107595 with HDAC9 expression, support that the interaction between SNP rs2107595 and T2DM may greatly increase CAD risk. In clinical studies, hyperlipidemia has long been associated with the severity of coronary atherosclerosis[44, 45]. For HDAC9 gene, functional studies in mice also suggested that increased HDAC9 expression could inhibit cholesterol efflux through reduction of histone acetylation at promoters of cholesterol efflux genes[14]. As the first stage of reverse cholesterol transport[46], cholesterol efflux plays crucial roles in lipid metabolism, and is inversely correlated with dyslipidemia[47] and the severity of coronary atherosclerosis[48]. Therefore, by increasing HDAC9 expression, the minor allele of SNP rs2107595 may inhibit cholesterol efflux, then interact with hyperlipidemia to aggravate coronary atherosclerosis.

In conclusion, by regulating HDAC9 expression and interacting with T2DM, hyperlipidemia and BMI status, the minor allele A of SNP rs2107595 increased CAD risk and the severity of coronary atherosclerosis. Functional studies are needed to explain the underlying mechanism.

Supporting Information

S1 Appendix. Supplementary materials and methods (Diagnostic criterion of different CAD subtypes and Definition of clinical characteristics).

S1 Dataset. Clinical and genetic data in our population.
S2 Dataset. Clinical and genetic data of participants that were randomly selected to measure HDAC9 mRNA expression and plasma HDAC9 levels.

S1 Fig. HRM and sequencing analyses for different genotypes of SNP rs2107595. (A) HRM plots for different genotypes of SNP rs2107595. The normalized melting peaks are given in the left column, and the normalized melting curves are given in the right column. Arrows indicate the genotypes. Heterozygous samples are identified by a change in melting curve shape, and different homozygous are distinguished by melting temperature (Tm) shifts; (B) Direct sequencing analyses for different genotypes of SNP rs2107595.

S1 Table. Primer details and PCR conditions for HRM, direct sequencing and RT-qPCR analyses in our study.

S2 Table. Clinical characteristics of participants in our study.

S3 Table. Comparative analyses for clinical and genetic characteristics between the randomly selected subjects and the whole samples.

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