Associations of polymorphisms in TXNIP and gene–environment interactions with the risk of coronary artery disease in a Chinese Han population

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Abstract

Single nucleotide polymorphisms (SNPs) in thioredoxin-interacting protein (TXNIP) gene may modulate TXNIP expression, then increase the risk of coronary artery disease (CAD). In a two-stage case–control study with a total of 1818 CAD patients and 1963 controls, we genotyped three SNPs in TXNIP and found that the variant genotypes of SNPs rs7212 [odds ratio (OR) = 1.26, P = 0.001] and rs7211 (OR = 1.23, P = 0.005) were significantly associated with increased CAD risk under a dominant model. In haplotype analyses, compared with the reference haplotype, haplotype ‘G-T’ had a 1.22-fold increased risk of CAD (P = 0.003). We also observed the cumulative effects of SNPs rs7212 and rs7211 on CAD risk and the severity of coronary atherosclerosis. Moreover, the gene–environment interactions among the variant genotypes of SNP rs7212, smoking habit, alcohol drinking habit and history of type 2 diabetes were associated with a 3.70-fold increased risk of CAD (P < 0.001). Subsequent genotype-phenotype correlation analyses further observed the significant effects of SNP rs7212 on TXNIP mRNA expression, plasma TXNIP and malondialdehyde levels. Taken together, our data suggest that TXNIP SNPs may individually and cumulatively affect CAD risk through a possible mechanism for regulating TXNIP expression and gene–environment interactions.

Keywords: TXNIP SNPs ● TXNIP expression ● CAD risk ● severity of coronary atherosclerosis ● gene–environment interactions

Introduction

Coronary artery disease (CAD), which is mediated by multiple interactions of genetic and environmental factors [1], is the leading cause of death and disability worldwide [2]. The main pathogenesis of CAD is atherosclerosis, a process of accumulated deposition of lipoproteins in the coronary artery and its branches that results in impaired or absent blood supply to the heart and eventually myocardial infarction [3]. Atherosclerosis is seen as a chronic inflammatory process [4] and is influenced by multiple events, such as oxidative stress caused by the excessive production of reactive oxygen species (ROS) [5]. Oxidative stress can induce a series of molecular changes [6], including oxidative damage of macromolecules, proliferation and migration of vascular smooth muscle cells (VSMC), and apoptosis in the endothelial cells, all of which involve the atheroma formation [6].

Thioredoxin-interacting protein (TXNIP), a binding protein of thioredoxin (TRX), mainly acts as an oxidative stress modulator by inhibiting TRX’s antioxidant activity [7] and interacting with antioxidant transcription factors such as Nrf2 [8]. Moreover, molecular studies also demonstrated that TXNIP linked oxidative stress to inflammatory response through activating NLPR3 inflammasome [9] and regulating chromatin modification [10]. Besides its crucial role in oxidative damage and inflammation, in epidemiological studies, TXNIP was further correlated with higher carotid intima-media thickness [11] and abnormal glucose metabolism [12], which have been considered as a surrogate marker [13] and a traditional risk factor [14] for CAD respectively. All these findings, combined with the significant effect of a TXNIP polymorphism on arterial stiffness [15], support the hypothesis that

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single nucleotide polymorphisms (SNPs) in TXNIP gene may modify TXNIP expression and protein levels, and thus contribute to CAD risk.

Hence, in this study, we detected TXNIP mRNA expression, plasma TXNIP and malondialdehyde (MDA) levels, and carried out a two-stage case-control study to evaluate the associations of three TXNIP SNPs with CAD risk and the severity of coronary atherosclerosis, followed by multifactor dimensionality reduction (MDR) and classification and regression tree (CART) analyses to investigate the interaction effects of TXNIP SNPs and traditional cardiovascular (CV) risk factors on CAD risk.

Materials and methods

Study population

A two-stage case-control design was used in our study. The discovery set (Study 1) with 812 CAD patients and 957 controls was recruited from Wuhan Asia Heart Hospital between January 2011 and December 2012. In the replication set (Study 2), 1006 cases and 1006 controls were enrolled from Zhongnan Hospital of Wuhan University between May 2013 and December 2015. CAD was angiographically confirmed as stenosis of more than 50% in at least one major coronary artery or their main branches. Then, for each case, the severity of coronary atherosclerosis was assessed by vessel scores [16] and modified Gensini scores [17] (Data S1). Patients with cardiac diseases such as congenital or valvular heart diseases, coronary artery spasm and myocardial bridge, or systemic diseases such as renal or hepatic diseases, autoimmune diseases and cancers were excluded. The control groups consisted of participants without stenosis confirmed by coronary angiography (1054 controls) and healthy individuals without CV disease identified by physical examination (909 controls), and also excluded participants with the aforementioned cardiac and systemic diseases. The case and control groups from two sets were well matched for age, sex and geographical area. Data on traditional CV risk factors [14, 18] such as smoking status, alcohol drinking status and histories of hypertension, hyperlipidemia and type 2 diabetes mellitus (T2DM) (Data S1), and clinical data such as body mass index (BMI), blood pressure, lipid and fasting plasma glucose (FPG) levels were also recorded. This study was approved by the Ethics Committees of Wuhan Asia Heart Hospital and Zhongnan Hospital of Wuhan University and also followed the Declaration of Helsinki. All participants were ethnic Han Chinese and signed written informed consent.

Selection of SNPs and genotyping

TXNIP gene is located on chromosome 1q21.1 and spans a relatively small genomic region of less than 4200 base pairs. According to the data from HapMap database (http://hapmap.ncbi.nlm.nih.gov/; phase1, 2&3, Hapmap-CHB) and 1000 Genome Project (http://www.1000genomes.org/) [19], only three SNPs in TXNIP gene, which were rs9245 in 5’-untranslated region (UTR) as well as rs7211 and rs7212 in 3’-UTR, had a minor allele frequency of ≥5% in the Chinese population and were selected in our study (Table S1).

Genomic DNA was prepared from peripheral blood leucocytes using a phenol/chloroform method. SNP genotyping was conducted with high resolution melting (HRM) analyses on a LightScanner 96 system (Iodan Technology, Salt Lake City, UT, USA), as described previously [20, 21]. The accuracy of genotyping was confirmed by repeated assays (Fig. S1) and direct sequencing (Fig. S2). Detailed information for HRM and sequencing analyses, such as primer sequences and PCR conditions, was summarized in Table S2.

Reverse-transcription quantitative PCR analysis of TXNIP mRNA

Total RNA of peripheral blood leucocytes was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), followed by elimination of DNA contamination using the RNase-Free gDNA eraser and reverse transcription (1 μg of total RNA) using a reverse transcriptase kit (Takara Bio Inc, Kusatsu, Shiga, Japan). According to the MIQE guidelines [22], reverse-transcription quantitative PCR (RT-qPCR) analysis was carried out to determine TXNIP mRNA expression on a CFX96 Touch system (Bio-Rad, Hercules, CA, USA) using the SYBR-Green method (Bio-Rad). The relative expression of TXNIP was normalized to the internal reference gene (GAPDH) and was calculated using the 2^−ΔΔCq method [23]. Primer sequences and RT-qPCR conditions for TXNIP and GAPDH were also presented in Table S2.

Determination of plasma TXNIP levels

After the whole blood of each participant was centrifuged (2000 × g for 10 min. at 4°C), plasma samples were collected and stored at −80°C until use. Based on the manufacturer’s instructions, the concentrations of TXNIP were detected by ELISA (TXNIP ELISA kit; Xinfan Systems, Shanghai, China), and were then quantified by a standard curve with the detection range of 20–480 pg/ml. Plasma MDA, a well-known biomarker for oxidative damage [24], was also determined using the thiobarbituric acid reactive substances assay, as described by Girotti et al. [25]. The coefficient of variance values for intra- and inter-assays were 5.4% and 7.8% for TXNIP, and 6.5% and 8.1% for MDA respectively.

Methylation levels at cg19693031 determined by bisulphite pyrosequencing

Bisulphite treatment of genomic DNA (1 μg) was first conducted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA), followed by PCR amplification using the bisulphite-treated DNA (~20 ng) and the PyroMark PCR kit (Qiagen, Hilden, Germany). Then, methylation levels at cg19693031 within 3’-UTR of TXNIP were quantified by pyrosequencing assays using the PyroMark Q96 MD instrument (Qiagen), as described in Table S2. Non-CpG cytosines, fully methylated and unmethylated DNA were used for quality controls. Methylation levels for each sample were calculated as the mean of two independent runs.

MDR and CART analyses

To investigate the interaction effects of TXNIP SNPs and traditional CV risk factors on CAD risk, MDR and CART analyses were performed by MDR 3. 0. 2 (UPenn, Philadelphia, PA, USA) and Clementine 12.0 (SPSS Inc., Chicago, IL, USA) programs respectively. In MDR analyses [26], all possible combinations of included variables were constructed, then 1000-fold cross-validation and 1000-time permutation tests were conducted to...
SNPs with each marker were evaluated by analyses of covariance expression, plasma (TXNIP and MDA) and methylation (cg19691031)

Whitney associations of equilibrium (HWE) was examined by the Pearson chi-squared test. The possible gene performed, the multiplicative likelihood ratio test was used to test the hypertension, hyperlipidaemia and T2DM. When subgroup analyses were

ysed by the linear-by-linear association chi-squared test and the Mann

SNPs on vessel scores and modified Gensini scores were anal-

with CAD risk was assessed by logistic regression analyses.

Statistical analyses

For clinical data, the differences in quantitative and qualitative variables between cases and controls were assessed by the Student’s t-test and the Pearson chi-squared test respectively. For each SNP, Hardy–Weinberg equilibrium (HWE) was examined by the Pearson chi-squared test. The associations of TXNIP SNPs with CAD risk were evaluated by multivari-

ale genotype and environmental factors to predict CAD risk by hierarchically building a binary clas-

ification tree [27]. In CART analyses, Gini index was used as the splitting criterion and the minimal terminal node (TN) size was 50 [28]. When the final pruned tree was constructed, the association of each TN with CAD risk was assessed by logistic regression analyses.

Results

Population characteristics

In two sets of our study, age and gender distributions were similar between cases and controls, while there were significant differences in BMI, blood pressure, lipid and FPG levels as well as the rates of smoking, alcohol drinking, hypertension, hyperlipidaemia and T2DM among the two groups (Table S3). The genotype frequencies of all three SNPs did not deviate from HWE in controls (P > 0.05, Table S1).

Single locus analyses

In the combined population with 1818 cases and 1963 controls (Table 1 and Table S4), allelic association analyses showed that the minor alleles of SNP rs7212 (OR = 1.18, P = 0.008) and rs7211 (OR = 1.19, P = 0.006) were significantly associated with increased CAD risk. In genotypic association analyses, we also found significant associations of SNP rs7212 and rs7211 with increased CAD risk under both additive (rs7212: OR = 1.19, P = 0.007; rs7211: OR = 1.19, P = 0.006) and dominant models (rs7212: OR = 1.26, P = 0.001; rs7211: OR = 1.23, P = 0.005). All these associations remained significant after the Bonferroni correction (Table 1). Power analyses showed that the merged sample size could provide sufficient power (α = 0.05, 90.5% for rs7212; 83.2% for rs7211) to detect the associations with the dominant ORs of 1.26 and 1.23 for SNPs rs7212 and rs7211 respectively. However, there was no significant association between SNP rs9245 and CAD risk in this study. The gene-based association analysis indicated that TXNIP was a suscepti-

ble gene for CAD (P = 0.004), and SNP rs7212 was the most susceptible locus in this gene (P = 0.002).

Haplotype and cumulative analyses of SNPs rs7212 and rs7211

Because SNPs rs7212 and rs7211 constructed a moderate LD block (in all participants: D' = 0.86, r² = 0.76, Fig. S3), haplotype analyses of these two SNPs were performed. As presented in Table 2 and Table S5, significant differences in haplotype distributions were iden-

ified between the case and control groups. Compared with the most common haplotype ‘C-C’ (alleles in order of SNPs rs7212 and rs7211), haplotypes ‘G-C’ (OR = 1.53, P = 0.003, P_BON = 0.012) and ‘G-T’ (OR = 1.22, P = 0.003, P_BON = 0.012) showed significant associations with increased CAD risk. In a cumulative analysis of SNPs rs7212 and rs7211 based on a dominant model (i.e. risk genotypes: CC + CG for SNP rs7212; CC + CT for SNP rs7211), compared with participants without risk genotypes, individuals with one and two risk genotypes were associated with a 1.43-fold (P = 0.009, P_BON = 0.036) and a 1.25-fold (P = 0.003, P_BON = 0.012) increased risk of CAD respectively. When we merged individuals with risk genotypes (one or two) into one group, the cumulative effect of SNPs rs7212 and rs7211 on CAD risk became more significant (OR = 1.28, P < 0.001, P_BON = 0.001).

Subgroup analyses

To further explore the potential gene-environment interactions in CAD risk, subgroup analyses were performed based on a dominant
For SNP rs7212, after the Bonferroni correction, the associations of the variant genotypes (CC + CG) with increased CAD risk remained significant in participants who were thinner (BMI \( \leq 25; \ P_{\text{Bon}} = 0.040\)) and smokers (\( P_{\text{Bon}} = 0.004\)) as well as participants with T2DM (\( P_{\text{Bon}} = 0.008\)). Moreover, the multiplicative likelihood ratio test suggested the significant interactions of SNP rs7212 with smoking status (\( P_{\text{inter}} = 0.022\)), alcohol drinking status (\( P_{\text{inter}} = 0.014\)) and history of T2DM (\( P_{\text{inter}} = 0.022; \) Table 3). For SNP rs7211, the associations between the variant genotypes (CC + CT) and increased CAD risk were more significant to withstand the Bonferroni correction in participants who were thinner (\( P_{\text{Bon}} = 0.048\)) and smokers (\( P_{\text{Bon}} = 0.040; \) Table 3). When we combined the genotypes of these two SNPs, the combined risk genotypes were consistently associated with increased CAD risk in almost all subgroups.

### Table 1: Associations of TXNIP SNPs with CAD risk in 1818 CAD patients and 1963 controls

| SNPs | Alleles/genotypes N(%) | CAD | Controls | \( \text{OR (95% CI)*} \) | \( \text{P*/P_{\text{Bon}} †} \) |
|------|------------------------|-----|----------|--------------------------|--------------------------|
| rs7212 |                          |     |          |                          |                          |
| C    | 2936 (80.7)            | 3294 (83.9) | 1 (Ref) | \( 1.18 \ (1.04–1.34) \) | \( 0.008/0.024 \) |
| G    | 700 (19.3)             | 632 (16.1)  |          |                          |                          |
| CC   | 1171 (64.4)            | 1387 (70.7) | 1 (Ref) | \( 1.29 \ (1.11–1.50) \) | \( 0.001/0.003 \) |
| CG   | 594 (32.7)             | 520 (26.4)  |          |                          |                          |
| GG   | 53 (2.9)               | 56 (2.9)    |          | \( 1.01 \ (0.68–1.51) \) | 0.961                   |
| CG + GG | 647 (356)         | 576 (29.3)  |          | \( 1.26 \ (1.10–1.46) \) | \( 0.001/0.003 \) |
| Additive |                    |          |          | \( 1.19 \ (1.05–1.35) \) | \( 0.007/0.021 \) |
| rs7211 |                          |     |          |                          |                          |
| C    | 2944 (81.0)            | 3289 (83.8) | 1 (Ref) | \( 1.19 \ (1.05–1.34) \) | \( 0.006/0.018 \) |
| T    | 692 (19.0)             | 637 (16.2)  |          |                          |                          |
| CC   | 1191 (65.5)            | 1386 (70.6) | 1 (Ref) | \( 1.23 \ (1.06–1.42) \) | \( 0.007/0.021 \) |
| CT   | 562 (30.9)             | 517 (26.3)  |          |                          |                          |
| TT   | 65 (3.6)               | 60 (3.1)    |          | \( 1.25 \ (0.86–1.82) \) | 0.239                   |
| CT + TT | 627 (34.5)        | 577 (29.4)  |          | \( 1.23 \ (1.07–1.42) \) | \( 0.005/0.015 \) |
| Additive |                    |          |          | \( 1.19 \ (1.05–1.34) \) | \( 0.006/0.018 \) |
| rs9245 |                          |     |          |                          |                          |
| C    | 2791 (76.8)            | 3025 (77.1) | 1 (Ref) | \( 1.00 \ (0.90–1.12) \) | 0.984                   |
| A    | 845 (23.2)             | 901 (22.9)  |          |                          |                          |
| CC   | 1061 (58.4)            | 1156 (58.9) | 1 (Ref) | \( 1.01 \ (0.88–1.16) \) | 0.874                   |
| CA   | 669 (36.8)             | 713 (36.3)  |          | \( 0.98 \ (0.71–1.34) \) | 0.877                   |
| AA   | 88 (4.8)               | 94 (4.8)    |          | \( 1.01 \ (0.88–1.15) \) | 0.918                   |
| CA + AA | 757 (41.6)       | 807 (41.1)  |          | \( 1.00 \ (0.89–1.12) \) | 0.985                   |

\*P-value from logistic regression after adjustment for age, sex, BMI, smoking status, alcohol drinking status and histories of hypertension, hyperlipidaemia and T2DM. †Multiple testing by the Bonferroni correction, \( P\)-value multiplied 3 (3 SNPs) to get a \( P_{\text{Bon}} \) value. Bold values are statistically significant with \( P < 0.05\). CAD, coronary artery disease; \( N\): number; OR (95% CI): odds ratio (95% confidence interval); Ref: reference.
all subgroups, except for non-smokers, non-drinkers, non-T2DM participants and those with BMI >25 (Table S6).

**MDR and CART analyses**

According to the results of the multiplicative likelihood ratio test, data on SNP rs7212, smoking status, alcohol drinking status and history of T2DM were included in MDR and CART analyses to further scrutinize the best interaction models. As summarized in Table 4, in MDR analyses, the four-factor model including all variables had the maximal CVC of 100/100 and the optimal testing accuracy of 0.6215 as well as the most significant *P*-value (*P* < 0.0001) for permutation test, and was therefore considered as the best predictor for CAD risk.

In CART analyses (Fig. 1), ‘smoking status’, ‘alcohol drinking status’, ‘history of T2DM’ and ‘SNP rs7212’ variables were selected as the primary, secondary, tertiary and terminal split nodes, respectively, indicating that smoking habit exerted the strongest effect on CAD risk, followed by drinking habit, history of T2DM and the variant genotypes (CC + CG) of SNP rs7212. Furthermore, compared with the reference group, participants with smoking and drinking habits, T2DM and the CC + CG variant genotypes had a 3.70-fold (*P* < 0.001) increased risk of CAD, suggesting the existence of gene–environment interactions.

When the combined genotypes of SNPs rs7212 and rs7211 were included in MDR and CART analyses, similar gene–environment interactions were also found between the combined genotypes and three traditional risk factors (Table 4 and Fig. S4).

**Associations of TXNIP SNPs with the severity of coronary atherosclerosis**

As summarized in Table 5, in single locus analyses, only the variant genotypes (CC + CG) of SNP rs7212 were associated with higher modified Gensini scores (*P* = 0.011). In a cumulative analysis of SNPs rs7212 and SNP rs7211, CAD patients with 1-2 risk genotypes had higher modified Gensini scores than those without risk genotypes (*P* = 0.008). Moreover, when the severity of coronary atherosclerosis was assessed by vessel scores, we further observed a dose–response effect of the increasing number of risk genotypes on higher vessel scores (*P* = 0.025).

**Correlations of TXNIP SNPs with TXNIP mRNA expression, plasma TXNIP and MDA levels**

We detected TXNIP mRNA expression, plasma TXNIP and MDA levels in 240 participants (120 CAD patients and 120 controls) randomly selected from two sets. These participants had similar genetic and clinical characteristics as compared to the overall population (Table S7). After adjusting for covariates, ANCOVA models showed that CAD patients had higher levels of TXNIP mRNA expression (1.20 ± 0.28 versus 1.06 ± 0.28, *P* = 0.001), plasma TXNIP (289.3 ± 23.0 pg/ml versus 274.1 ± 23.0 pg/ml, *P* < 0.001) and MDA (1.76 ± 0.35 μmol/l versus 1.61 ± 0.32 μmol/l, *P* = 0.011) than the control group (Table S7).
| Variables          | SNP rs7212 (cases/controls, N) | OR (95% CI)* | P* / PBon† | Pinter‡ | SNP rs7211 (cases/controls, N) | OR (95% CI)* | P* / PBon† | Pinter‡ |
|--------------------|---------------------------------|--------------|------------|---------|---------------------------------|--------------|------------|---------|
| Age, years         |                                 |              |            |         |                                 |              |            |         |
| ≤60                | 580/633                         | 1.30 (1.05–1.62) | 0.018/0.072 | 0.817   | 588/627                         | 1.26 (1.01–1.56) | 0.038/0.152 | 0.929   |
| >60                | 591/754                         | 1.26 (1.04–1.53) | 0.021/0.084 |         | 603/759                         | 1.24 (1.02–1.51) | 0.033/0.132 |         |
| Sex                |                                 |              |            |         |                                 |              |            |         |
| Male               | 625/752                         | 1.24 (1.02–1.50) | 0.024/0.096 | 0.686   | 638/749                         | 1.19 (0.98–1.44) | 0.079/0.546 |         |
| Female             | 546/635                         | 1.29 (1.04–1.60) | 0.020/0.080 |         | 553/637                         | 1.29 (1.04–1.60) | 0.021/0.084 |         |
| BMI, kg/m²         |                                 |              |            |         |                                 |              |            |         |
| ≤25                | 636/868                         | 1.28 (1.06–1.54) | 0.010/0.040 | 0.791   | 646/872                         | 1.27 (1.05–1.53) | 0.012/0.048 | 0.903   |
| >25                | 535/519                         | 1.38 (1.06–1.80) | 0.018/0.072 |         | 545/514                         | 1.36 (1.04–1.77) | 0.023/0.092 |         |
| Smoking status     |                                 |              |            |         |                                 |              |            |         |
| Yes                | 374/381                         | 1.55 (1.21–2.00) | 0.001/0.004 | 0.022   | 397/384                         | 1.40 (1.08–1.80) | 0.010/0.040 | 0.234   |
| No                 | 797/1006                        | 1.14 (0.96–1.36) | 0.144      |         | 794/1002                        | 1.16 (0.98–1.38) | 0.093    |         |
| Drinking status    |                                 |              |            |         |                                 |              |            |         |
| Yes                | 317/330                         | 1.66 (1.26–2.18) | <0.001/0.001 | 0.014   | 356/332                         | 1.28 (0.97–1.68) | 0.082/0.778 |         |
| No                 | 854/1057                        | 1.13 (0.95–1.34) | 0.159      |         | 835/1054                        | 1.21 (1.02–1.43) | 0.028/0.112 |         |
| Hypertension       |                                 |              |            |         |                                 |              |            |         |
| Yes                | 693/547                         | 1.28 (1.04–1.56) | 0.019/0.076 | 0.889   | 702/541                         | 1.22 (0.99–1.49) | 0.059/0.883 |         |
| No                 | 478/840                         | 1.24 (1.01–1.51) | 0.040/0.160 |         | 489/845                         | 1.24 (1.01–1.52) | 0.038/0.152 |         |
| T2DM               |                                 |              |            |         |                                 |              |            |         |
| Yes                | 335/356                         | 1.53 (1.18–1.99) | 0.002/0.008 | 0.022   | 371/366                         | 1.36 (1.04–1.77) | 0.024/0.321 |         |
| No                 | 836/1031                        | 1.15 (0.97–1.36) | 0.116      |         | 820/1020                        | 1.18 (0.99–1.40) | 0.061    |         |
Table 3. Continued

| Variables         | SNP rs7212 (cases/controls, N) OR (95% CI)* | P* | P Bonferroni | P* | P Bonferroni |
|-------------------|------------------------------------------|----|--------------|----|--------------|
| Hyperlipidaemia   | Yes                                      | 334/326 1.38 (1.04–1.83) | 0.026 | 339/322 1.32 (1.00–1.75) | 0.050 |
|                   | No                                       | 837/1061 1.22 (1.03–1.44) | 0.020 | 852/1064 1.19 (1.01–1.75) | 0.040 |

P*-value from logistic regression after adjustment for age, sex, BMI, smoking status, alcohol drinking status and histories of hypertension, hyperlipidaemia and T2DM. Multiple testing was assessed by the Bonferroni correction, P*-value multiplied 4 (3 SNPs + 1 combined risk genotypes of SNPs rs7212 and rs7211) to get a P Bonferroni value. TXNIP mRNA expression and plasma TXNIP were selected to determine methylation levels at cg19693031. As presented in Figure S6, methylation levels at cg19693031 were inversely correlated with HbA1c (%) and FPG. In CAD patients, participants with the CG genotypes of SNP rs7212 were significantly correlated with increased plasma TXNIP levels (r = 0.220, P = 0.016).

In healthy controls (Fig. 2 and Table S8), the variant genotypes (CC + CG) of SNP rs7212 were significantly correlated with increased levels of TXNIP mRNA expression (P = 0.013) and plasma TXNIP (P < 0.001). In CAD patients, participants with the CG + GG genotypes had higher levels of TXNIP mRNA expression compared with the CC carriers (P = 0.036). Similarly, in a cumulative analysis of SNPs rs7212 and rs7211, significant correlations were also found between the risk genotypes and higher levels of TXNIP mRNA expression in both the case (P = 0.006) and control (P = 0.009) groups, and between the risk genotypes and higher plasma TXNIP levels only in the case group (P = 0.024). Moreover, the Spearman correlation test also found that the plasma TXNIP levels were positively correlated with modified Gensini scores in CAD patients (r = 0.220, P = 0.016).

Correlations between TXNIP SNPs and cg19693031

Cg19693031, a CPG site within 3’-UTR of TXNIP, was recently reported by two epigenome-wide association studies to be associated with T2DM, HbA1c (%) and FPG. To test the possible interactions between cg19693031 and TXNIP SNPs, 48 participants (24 CAD patients versus 24 controls) with data on HbA1c (%), FPG, TXNIP mRNA expression and plasma TXNIP were selected to determine methylation levels at cg19693031. As presented in Figure S6, methylation levels at cg19693031 were inversely correlated with HbA1c (%) (r = −0.318, P = 0.027), FPG (r = −0.299, P = 0.039), TXNIP mRNA expression (r = −0.383, P = 0.007) and CAD risk (P = 0.032), but were not associated with SNP rs7212 and rs7211.

Discussion

This study, for the first time, reported the significant associations of TXNIP SNPs with increased CAD risk. Several lines of evidence reinforced this finding. Firstly, using a two-stage case-control design with a total of 1818 CAD patients and 1963 controls, we found that TXNIP SNPs were significantly associated with increased CAD risk and the severity of coronary atherosclerosis in both single locus and cumulative analyses. Secondly, by performing subgroup, MDR and CART analyses, we further validated the interaction effects of SNP rs7212 and three traditional CV risk factors on CAD risk. Finally, subsequent genotype-phenotype correlation analyses supported that SNP rs7212 might be functional by affecting TXNIP expression and protein levels.

It is widely accepted that oxidative inflammatory response plays a vital role in the development of CAD. TXNIP, an up-regulated gene of oxidative stress, encodes a TXNIP that inhibits the antioxidant activity of TRX protein and Nrf2 transcription factor, activates NLPR3.
inflammasome [9], and thus links oxidative stress to inflammation [9]. Recently, several studies further showed that TXNIP could induce the inflammatory response in endothelial cells and VSMC by increasing leucocyte adhesion [34] and regulating the expression of adhesion molecules [35] and anti-inflammatory transcription factors (such as Kruppel-like factor 2) [34], and therefore involve the

![Classification and regression tree](image)

**Fig. 1** Classification and regression tree for smoking status, alcohol drinking status, history of T2DM and SNP rs7212 in all participants of our study. Terminal nodes (TN) are thick bordered. ORs and 95% CIs were calculated by logistic regression after adjusting for age, sex, BMI, smoking status, alcohol drinking status and histories of hypertension, hyperlipidaemia and T2DM, *P < 0.05.

| No. of risk factors | Best interaction models                                                                 | CVC* | Testing accuracy (%)† | P for permutation test‡ |
|--------------------|----------------------------------------------------------------------------------------|------|------------------------|-------------------------|
| 1                  | Smoking status                                                                         | 84/100 | 0.5585                | 0.0356                  |
| 2                  | Smoking status, alcohol drinking status                                                | 94/100 | 0.5873                | 0.0001                  |
| 3                  | Smoking status, alcohol drinking status, history of T2DM                               | 100/100 | 0.6011               | <0.0001                 |
| 4                  | Smoking status, alcohol drinking status, history of T2DM, SNP rs7212 (CC/CG + GG)    | 100/100 | 0.6215               | <0.0001                 |
| 4                  | Smoking status, alcohol drinking status, history of T2DM, no. of risk genotypes (0/1 + 2) | 100/100 | 0.6283               | <0.0001                 |

* 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 to 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 as well as the most significant P-value for permutation test. T2DM: type 2 diabetes mellitus; CVC: cross-validation consistency.
Table 5: Associations of TXNIP SNPs (rs7212 and rs7211) with the severity of coronary atherosclerosis in 1818 CAD patients

| The severity of coronary atherosclerosis | SNP rs7212 | SNP rs7211 | No. of risk genotypes |
|-----------------------------------------|-----------|-----------|----------------------|
| Vessel score-1                           | CC, 426 (66.4) | CG + GG, 216 (33.6) | 0.112 | 441 (68.7) | 201 (31.3) | 0.107 | 417 (65.0) | 225 (35.0) | 0.025 |
| Vessel score-2                           | 395 (64.6) | 216 (35.4) | 386 (63.2) | 225 (36.8) | 361 (59.1) | 250 (40.9) |
| Vessel score-3                           | 350 (61.9) | 215 (38.1) | 364 (64.4) | 201 (35.6) | 332 (58.8) | 233 (41.2) |
| Modified Gensini score*                 | 29.0 (18.0–70.5) | 35.0 (19.5–79.5) | 0.011 | 30.0 (18.0–74.0) | 33.0 (19.5–74.0) | 0.155 | 29.0 (18.0–72.0) | 35.0 (20.0–75.0) | 0.008 |

*Modified Gensini scores were expressed as median (interquartile range) because of the skewed distributions. Bold values indicate statistically significant with P < 0.05.
In this study, by performing subgroup, MDR and CART analyses, we also found the interaction effects of SNP rs7212 and three traditional CV risk factors (i.e. smoking status, alcohol drinking status and history of T2DM) on CAD risk. From a biological perspective, it is well known that smoking and drinking habits can cause excessive ROS production, either directly by inducing peroxidation, or indirectly through depletion of endogenous antioxidants (such as vitamin C) [43, 44]. Accordingly, their indirect impact on ROS generation is similar to the role of TXNIP protein in oxidative damage and CAD risk. Moreover, for history of T2DM, besides the known impact of dysglycaemia on CAD risk [45], numerous studies have also reported the effect of TXNIP protein on abnormal glucose metabolism [12, 46] and the significant associations between TXNIP SNPs and DM risk [36]. All these findings, combined with the significant correlations of SNP rs7212 with TXNIP expression and protein levels, suggest that the gene–environment interactions among SNP rs7212, smoking status, alcohol drinking status and history of T2DM may greatly increase CAD risk. In our CART analyses, we observed that compared with the reference group, participants with smoking and drinking habits, T2DM and the CC + CG variant genotypes had a 3.70-fold increased risk of CAD. This result partially supports the above view and needs to be further explained by functional studies.

In this study, we calculated modified Gensini scores and vessels scores, and found the significant correlations of SNP rs7212 and plasma TXNIP levels with the severity of coronary atherosclerosis in a Chinese population. These results are supported by the report that TXNIP-ApoE double knockout mice exhibited a dramatic reduction in atherosclerotic lesion size at aorta [35] and the evidence that SNP rs7212 was significantly associated with arterial stiffness in a Brazilian population [15]. All these findings validate the crucial role of SNP rs7212 and TXNIP protein in the development of atherosclerosis, and the detailed mechanism needs to be elucidated in future studies.

Some limitations of our study also merit consideration. First, although we have matched for age, sex and geographical location between cases and controls, the selection bias might be inevitable because of the inherent drawback of retrospective study. Second, in our study, we only genotyped common SNPs in TXNIP. Fine-mapping...
studies are encouraged to find low-frequency variants in this gene. Finally, although we have collected data on several CV risk factors, other risk factors might also involve gene–environment interactions.

In conclusion, our study shows that TXNIP SNPs may individually and cumulatively contribute to CAD risk by affecting TXNIP expression and protein levels as well as by interacting with smoking status, alcohol drinking status and history of T2DM. Future studies are needed to replicate these results and explore the underlying mechanism.

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Conflict of interest

The authors declare no competing financial interests.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 HRM plots for different genotypes of three SNPs.
Figure S2 Direct sequencing analyses for different genotypes of three SNPs.
Figure S3 Analysis of the LD structure.

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