Association Between a CCL17 Genetic Variant and Risk of Coronary Artery Disease in a Chinese Han Population

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Background: In the present study we investigated the effects of genetic variations in the C-C motif chemokine ligand 17 (CCL17) gene on serum CCL17 levels and risk of coronary artery disease (CAD).

Methods and Results: A case-control study was conducted to determine causal inferences among CCL17 single-nucleotide polymorphisms (SNPs), serum CCL17 levels, and risk of CAD. Luciferase assays, electrophoretic mobility shift assays (EMSA), and allele-specific quantitative chromatin immunoprecipitation (ChIP) assays were used to assess the function of the SNPs. In all, 947 participants (794 with CAD, 153 without CAD) were included in the study. The T allele in rs223828, located in intron of the CCL17 gene, was associated with increased serum CCL17 levels as well as increased CAD risk. A causal inference test using mediation analysis suggested that rs223828 had a significant indirect causal effect on the increased risk of CAD mediated via serum CCL17 levels. Luciferase assays confirmed that the rs223828T allele enhances CCL17 promoter activity. Protein-DNA binding studies using EMSA and allele-specific quantitative ChIP assays indicated preferential activator protein-1 (AP-1) complex formation and recruitment with the rs223828 T allele compared with the C allele.

Conclusions: We propose that the CCL17 SNP rs223828 is associated with increased risk of CAD, and that this site may be a potential AP-1 binding site.

Key Words: Atherosclerosis; CCL17; Chemokines; Coronary artery disease

It is well-established that atherosclerosis is an inflammatory process, and there is increasing evidence from experimental studies of the role of inflammation, and the underlying cellular and molecular mechanisms, in atherosclerosis.1 The immigration and infiltration of activated macrophages and T cells into atherosclerotic lesions guided by chemokines is one of the major processes in atherosclerotic disease.2 Chemokines are considered to be of great importance for maintaining leukocyte homeostasis in cell mobilization, differentiation, influx, proliferation, and apoptosis or survival to contribute to atherogenesis and neointima formation rather than solely effecting leukocyte recruitment.2

Chemokine CC-motif ligand 17 (CCL17), also known as the thymus and activation-regulated chemokine (TARC), is an important regulator of atherosclerosis that has been shown to drive atherosclerosis in an animal model by restraining regulatory T cell homeostasis.3 In previous studies, we found a link between serum CCL17 concentrations and coronary artery disease (CAD).4,5 In 971 consecutive patients who underwent coronary angiography, serum CCL17 levels remained associated with CAD even after adjusting for other traditional cardiovascular risk factors.5 Moreover, serum CCL17 concentrations were correlated with the subtype and severity of CAD.5 In order to determine the causal inference between CCL17 and CAD, we searched for and genotyped single-nucleotide polymorphisms (SNPs) in CCL17 among patients in our previous study.3 We then used a mediation model to explore the effects of these genetic variants on CAD risk and whether their effects are causally mediated through serum CCL17. Furthermore, we aimed to determine the effect and potential mechanisms underlying the effects of the SNPs on CCL17 expression in vitro.

Methods

Study Population
Patients visiting our hospital between January and December 2013 for coronary angiography were consecutively recruited...
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approximately 2.7% and 8.2%, respectively.  

Tag SNP Selection

Taking into consideration the fact that the study population was of Chinese ancestry, genotype data from the HCB panel (Han Chinese in Beijing, China) of the Phase I, II, and III HapMap Project were used to determine patterns of linkage disequilibrium (LD) and select tag SNPs in CCL17. Tag SNPs capturing common genetic variation in CCL17 were chosen using HAPLOVIEW (v.4.2) software. The criterion for tag SNPs selection was that every SNP in the HCB panel of the HapMap project Phases I, II, and III with 5% allele frequency was captured with a pairwise $r^2>0.8$ by at least 1 tag SNP. Five tag SNPs (rs223895, rs4784805, rs9302690, rs223899, and rs223828) were selected.

SNPs Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a Tiangen (Beijing, China) Genomic DNA Purification Kit, according to the manufacturer’s instructions. Genotyping was performed using TaqMan on an Applied Biosystems real-time Prism 7900HT Sequence Detection System (ABI, Foster City, CA, USA). Primers and the TaqMan fluorogenic probes bearing a suitable reporter dye on the 5'-end and a quencher dye on the 3'-end were designed using Primer Express software v2.0 (ABI) and procured from Applied Biosystems (Warrington, UK). One probe (for Allele 1) was labeled with the VIC dye and to the study (Table 1). The inclusion and exclusion criteria for this study population have been described previously. Briefly, the exclusion criteria included dermatitis, current infection, malignancy, autoimmune disease, vasculitis, asthma, cirrhosis, severe renal failure (estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 m$^2$) and shock.

Subjects with ≥50% stenosis in 1 or more of their main coronary arteries were defined as having CAD. Those with <50% stenosis in any main coronary artery were considered as non-CAD patients. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Hypertension and diabetes were diagnosed on the basis of current guidelines. Peripheral venous blood drawn was from the antecubital vein after a 12-h fast and was used to determine total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) concentrations.

Table 2. Characteristics of Selected Single Nucleotide Polymorphisms Found in the CAD Population

| SNP       | Position     | Minor allele | Major allele | Function | MAF1 | MAF2 |
|-----------|--------------|--------------|--------------|----------|------|------|
| rs223895  | Chr.16:57440896 | T            | C            | Intron   | 0.49 | 0.49 |
| rs4784805 | Chr.16:57441407 | A            | C            | Intron   | 0.07 | 0.10 |
| rs9302690 | Chr.16:57442968 | A            | G            | Intron   | 0.01 | 0.02 |
| rs223899  | Chr.16:57444576 | T            | G            | Intron   | 0.42 | 0.47 |
| rs223828  | Chr.16:57447414 | T            | C            | Intron   | 0.34 | 0.39 |

CAD, coronary artery disease; Chr., chromosome; MAF1, minor allele frequency in the Chinese Han population; MAF2, minor allele frequency in the present study population; SNP, single nucleotide polymorphism.

Table 1. Baseline Characteristics and Serum CCL17 Concentrations in Patients With or Without CAD

|                      | Non-CAD (n=153) | CAD (n=794) | P value |
|----------------------|-----------------|-------------|---------|
| Age (years)          | 59.6±10.4       | 62.2±10.3   | 0.005   |
| No. males (%)        | 74 (48.4)       | 574 (72.3)  | <0.001  |
| BMI (kg/m$^2$)       | 25.7±3.9        | 25.5±3.6    | 0.571   |
| Hypertension         | 84 (54.9)       | 575 (72.4)  | <0.001  |
| Diabetes             | 48 (31.4)       | 330 (41.6)  | 0.019   |
| Current smoker       | 55 (35.9)       | 449 (56.5)  | <0.001  |
| Family history of CAD| 33 (21.6)       | 165 (20.8)  | 0.828   |
| TC (mmol/L)          | 4.24±0.97       | 4.18±1.57   | 0.656   |
| TG (mmol/L)          | 1.31 [0.92–1.96]| 1.39 [1.02–2.01] | 0.069 |
| LDL-C (mmol/L)       | 2.39±0.91       | 2.31±0.88   | 0.267   |
| HDL-C (mmol/L)       | 1.27±0.58       | 1.15±0.57   | 0.017   |
| Serum CCL17 (pg/mL)  | 218.8 [144.5–293.9] | 254.9 [165.20–363.0] | <0.001 |

Data are presented as the mean±SD, n (%), or as the median [interquartile range]. BMI, body mass index; CAD, coronary artery disease; CCL17, C-C motif chemokine ligand 17; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

Determinaton of Serum CCL17 Concentrations

CCL17 concentrations were determined from blood samples collected from the radial or femoral artery after insertion of the sheath for coronary angiography. Serum CCL17 concentrations were measured using a commercially available ELISA for human CCL17/TARC (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Absorbance was assessed using a Labsystems Multiskan MS spectrophotometer (Thermo Labsystems, Helsinki, Finland) and calculations were performed using Ascent software v2.6 (Thermo Labsystems). The lower limit of detection of the assay was 7pg/mL CCL17. The intra- and interassay coefficients of variation (CV) were approximately 2.7% and 8.2%, respectively.
the other (for Allele 2), with a FAM (fluorescein) dye at the 5'-end, and serial dilutions were run to determine the optimal working concentrations. For each reaction, a 25-µL mixture was prepared by mixing 5 µL containing 50 ng DNA, 12.5 µL of 2× Universal mix (Eurogentec, Seraing, Belgium), 1.25 µL of 20× probe assay mix, and 6.25 µL DNase-free distilled water. Three no-template controls were included in each plate to normalize the emission signal. The thermal profile for amplification for the first cycle was as follows: 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 94°C for 15 s and 60°C for 30 s. The plates were then scanned for a fluorescence resonance energy transfer (FRET) signal using a 7900HT sequence detection system. Data were analyzed using SDS software version 2.0 (ABI).

Luciferase Assay
Genomic DNA samples from 2 patients with a C/C or T/T genotype at rs223828 variant were cloned into a pGM-T vector (Tiangen) and sequence fidelity was verified by DNA sequencing. Promoter inserts were then cloned into the pGL3-Basic vector (Promega, Mannheim, Germany) containing a luciferase reporter gene. To analyze the effects of SNPs on gene expression, a dual-luciferase reporter assay (Promega) was performed according to the manufacturer’s instructions. Briefly, cell lysates were obtained 48 h after Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) transfection of the pGL3 vector constructs into the THP-1 cell line, using a passive lysis buffer. Then, 100 µL luciferase assay reagent was added to 20 µL lysate and samples were incubated with Stop&Glo reagent, after which luciferase activity was determined using a luminometer (LB952; Berthold, Bad Wildbad, Germany). An empty pGL3 vector was used as a negative control. The activity of cotransfected Renilla luciferase was used to normalize values (firefly luciferase reporter gene). To analyze the effects of SNPs on gene expression, a dual-luciferase reporter assay (Promega) was performed according to the manufacturer’s instructions. Briefly, cell lysates were obtained 48 h after Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) transfection of the pGL3 vector constructs into the THP-1 cell line, using a passive lysis buffer. Then, 100 µL luciferase assay reagent was added to 20 µL lysate and samples were incubated with Stop&Glo reagent, after which luciferase activity was determined using a luminometer (LB952; Berthold, Bad Wildbad, Germany). An empty pGL3 vector was used as a negative control. The activity of cotransfected Renilla luciferase was used to normalize values (firefly luciferase values were divided by Renilla luciferase values).

Electrophoretic Mobility Shift Assay
Nuclear extracts were prepared from harvested THP-1 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents. THP-1 cells were harvested using ice-cold phosphate-buffered saline (PBS). After centrifugation, the pellet was resuspended in ice-cold sodium dodecyl sulfate (SDS) sample buffer supplemented with 100 mmol/L dithiothreitol. Cytoplasmic and nuclear extracts were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents containing 1× Halt Protease Inhibitor Cocktail according to the manufacturer’s instructions. Protein concentrations were determined using a BCA protein assay kit (Bio-Rad Laboratories, Berkeley, CA, USA). The DNA oligonucleotide probes used in the electrophoretic mobility shift assays (EMSA) were as follows: rs223828-C, 5'-CTGCTCCTGGTCACTCCCCGACACTTCTC-3'; rs223828-T, 5'-CTGCTCCTGGTCACTCCCCGACAC-3'. The underlined bases indicate the position of the SNP. For each oligonucleotide, a complementary oligonucleotide was used. The oligonucleotides were labeled with biotin using the Biotin Light Chemiluminescent EMSA kit (Exprogen Biotecnetology, Beijing, China). Then, 10 µg purified protein (in 3.2 µL) was incubated with the probe at 30°C for 20 min in a 15-µL binding reaction containing 1.5 µL of 10× binding buffer, 1.5 µg poly(deoxyinosinic-deoxycytidylic acid) (poly(dI-dC)), and 500 fmol biotin-labeled probe. To demonstrate the specificity of probe-protein binding, competitor experiments with an 100-fold excess of unlabeled probe as a specific competitor were performed. For super-shift assays, 4 µg specific antibodies against c-Jun or c-Fos, or non-immune IgG, was added to the binding reaction and the reaction mixture was incubated on ice for 10 min before labeled probe was added at 120 V in a 1% agarose gel in 0.5× Tris-borate-EDTA for 1.5 h. Then, the gel was then electrophoretically transferred to a nylon membrane at 380 mA for 60 min, and cross-linked DNA was transferred to the membrane using an ultraviolet light cross-linker (UVP, Upland, CA, USA). After cross-linking, biotin-labeled DNA was detected by chemiluminescence and exposed to X-ray film for 5–10 min.

Allele-Specific Quantitative Chromatin Immunoprecipitation (ChIP) PCR
Allele-specific quantitative chromatin ChIP PCR was performed as described previously. Briefly, human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient separation. PBMCs were washed with PBS and incubated for 10 min with 1% (v/v) formaldehyde. Cross-linking reactions were terminated by incubating the cells with 0.1 mol/L glycine for 5 min. Cells were then washed twice with PBS and lysed for 1 h at 4°C in a lysis buffer. Cell lysates were sonicated to obtain chromatin fragments with an average length of 500–800 bp, as assessed by agarose gel electrophoresis. The samples were precleared with protein A-agarose (Roche) for 1 h at 4°C on a rocking platform, after which 10 µg specific antibody against c-Jun (sc-1694; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and samples were incubated overnight at 4°C with shaking. The immunoprecipitates were captured with 10% (v/v) protein A-agarose for 4 h. Protein A-agarose was blocked overnight at 4°C with 1 µg/mL bovine serum albumin (BSA) and 1 µg/mL salmon sperm DNA that had been sheared to 500–1000 fragments. Quantitative (q) PCR was performed by the SYBR green method using allele-specific primers for rs4648889. The primers for the T and C alleles were 5'-CTGCTTGGTTTCTGCTCCTGGTT-3' and 5'-CTGCTTGGTTTCTGCTCCTGGTT-3', respectively, and the common reverse primer was 5'-ACGCACAGCTGGGTTCACATCC-3'.

Statistical Analysis
Baseline characteristics, including demographics and baseline measurements, are given as the mean±SD for normally distributed continuous variables and as the median with interquartile range (IQR) for continuous variables with a skewed distribution. Continuous variables between 2 groups were analyzed by Student’s t-test or the Mann-Whitney U-test depending on data distribution. Categorical variables are summarized as counts and percentages and were compared using the χ² test. Analysis of variance (ANOVA; for SNP with 3 genotypes) or Student’s t-test (for SNP with 2 genotypes) were used for comparisons of serum CCL17.
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Table 3. Association Between CCL17 Single Nucleotide Polymorphisms and Risk of CAD

| SNP / Genotype | Non-CAD group | CAD group | Unadjusted OR (95% CI) per effect allele | P value | Adjusted OR (95% CI) per effect allele | P value | Adjusted P valuea |
|----------------|---------------|-----------|----------------------------------------|---------|--------------------------------------|---------|-----------------|
| rs223895       |               |           |                                        |         |                                      |         |                 |
| CC             | 49 (32.0)     | 200 (25.2)| 1.10 (0.861–1.406)                     | 0.445   | 1.095 (0.841–1.424)                  | 0.501   | 1.000           |
| CT             | 65 (42.5)     | 408 (51.4)|                                        |         |                                      |         |                 |
| TT             | 39 (25.5)     | 186 (23.4)|                                        |         |                                      |         |                 |
| rs4784805      |               |           |                                        |         |                                      |         |                 |
| CC             | 127 (83.0)    | 636 (80.1)| 1.269 (0.821–1.959)                    | 0.284   | 1.318 (0.826–2.105)                  | 0.247   | 1.000           |
| AC             | 26 (17.0)     | 149 (18.8)|                                        |         |                                      |         |                 |
| AA             | 0 (0)         | 9 (1.1)   |                                        |         |                                      |         |                 |
| rs9302690      |               |           |                                        |         |                                      |         |                 |
| GG             | 149 (97.4)    | 755 (95.1)| 1.924 (0.677–5.465)                    | 0.219   | 1.789 (0.607–5.275)                  | 0.291   | 1.000           |
| AG             | 4 (2.6)       | 39 (4.9)  |                                        |         |                                      |         |                 |
| AA             | 0 (0)         | 0 (0)     |                                        |         |                                      |         |                 |
| rs223899       |               |           |                                        |         |                                      |         |                 |
| GG             | 62 (40.5)     | 201 (25.3)| 1.569 (1.215–2.025)                    | 0.001   | 1.591 (1.209–2.094)                  | 0.001   | 0.005           |
| GT             | 66 (43.1)     | 413 (52.0)|                                        |         |                                      |         |                 |
| TT             | 25 (16.3)     | 180 (22.7)|                                        |         |                                      |         |                 |
| rs223828       |               |           |                                        |         |                                      |         |                 |
| CC             | 73 (47.7)     | 271 (34.1)| 1.620 (1.239–2.117)                    | <0.001  | 1.644 (1.233–2.191)                  | 0.001   | 0.005           |
| CT             | 67 (43.8)     | 392 (49.3)|                                        |         |                                      |         |                 |
| TT             | 13 (8.5)      | 131 (16.5)|                                        |         |                                      |         |                 |

Unless indicated otherwise, data show n (%). aAdjusted by Bonferroni correction. CI, confidence interval; OR, odds ratio. Other abbreviations as in Tables 1,2.

correlations among different genotypes for 5 SNPs. All SNPs were encoded using an additive genetic model (0, wild-type; 1, heterozygosity; 2, homozygosity; minor allele as effect allele). The analysis in the present study included 3 steps: (1) association between SNPs and risk of CAD; (2) association between SNPs and serum CCL17 concentrations; and (3) causal inference analysis. The causal inference analysis was conducted only if the previous 2 analyses reached statistical significance. All models were adjusted for covariates of age, gender, BMI, hypertension, diabetes, lipid profile, smoking status, and family history of CAD. Linear regression analysis was used to investigate associations between the 5 SNPs and serum CCL17 concentrations. The results are reported as β coefficients and 95% confidence intervals (CI). The association between SNPs and risk of CAD was evaluated by logistic regression analysis and is described by odds ratios (OR). In causal inference analyses, Vanderweele’s mediation model was used to assess the indirect causal effect of an SNP that was mediated through serum CCL17, which has been used in previous studies.10,11 The results are described by indirect OR (ORIndirect) and 95% CI, calculated as follows:

\[
\text{ORIndirect} = e^{(\theta_{CCL17} \cdot \beta_{SNP} + \theta_{interaction} \cdot \beta_{SNP})}
\] (1)

where \(\theta_{CCL17}\) and \(\theta_{interaction}\) were estimated from Model 2 and \(\beta_{SNP}\) was estimated from Model 3. Bootstrap analysis with 1,000 replicates was used to estimate the 95% CI of ORIndirect and statistical significance as follows:

\[
\logit(p) = \theta_0 + \theta_{SNP} \cdot SNP + \theta_{CCL17} \cdot CCL17 + \theta_{interaction} \cdot SNP \cdot CCL17 + B \cdot Covariates
\] (2)

where \(\theta_{SNP}, \theta_{CCL17}, \text{and} \theta_{interaction}\) represent the coefficients of SNP, serum CCL17, and the SNP-CCL17 interaction, respectively, and B is a vector of coefficients of covariates. \(\beta_{SNP}\) was calculated using the following equation:

\[
\text{Serum CCL17} = \beta_0 + \beta_{SNP} \cdot SNP + B \cdot Covariates + e
\]

\[w = \begin{cases} \frac{\text{Prevalence/r}}{(1-\text{prevalence})(1-r)}, & \text{for cases} \\ \frac{\text{Prevalence/r}}{(1-\text{prevalence})(1-r)}, & \text{for controls} \end{cases}
\] (3)

where the prevalence represents the prevalence of CAD among the population that underwent coronary angiography and r represents the proportion of CAD cases in the analytical dataset.

Statistical significance was set at P<0.05. Bonferroni correction was used to adjust the P value for multiple comparisons. All analyses for the clinical studies were performed using SAS v.9.3.

For in vitro studies, 1-way ANOVA and 2-tailed Student’s t-test were used to determine statistical significance using GraphPad Prism v.5.03 (GraphPad Software, San Diego, CA, USA).

The sample size was calculated using Power and Sample Size Program 11.0 (NCSS, Kaysville, UT, USA.), assuming an α value of 0.05, a 2-sided test, and minor allele frequencies (MAF) for the Chinese population from the HapMap database. For the association between rs223828 and CAD, a sample size of 947 patients (794 in the CAD group, 153 in the non-CAD group) was calculated to achieve 90% power to detect an OR of 1.8 using an MAF of 0.34.

Ethics Approval
This study was approved by the Human Research Ethics Committee of Peking Union Medical College Hospital (Beijing, China), and was performed strictly in accordance with the approved guidelines. The study protocol con-
forms to the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from all subjects.

Results

In all, 971 patients who fulfilled the inclusion and exclusion criteria were selected for the present study. Of these patients, DNA samples were not available for 24 (2.5%). Thus, 947 patients (794 with CAD, 153 without CAD) were included in the study. The baseline characteristics of the CAD and non-CAD groups are given in Table 1. All study participants were from the Chinese Han population. Most of the patients in the CAD group were male and older than those in the non-CAD group. The proportion patients with hypertension, diabetes, and current smoker status was higher in the CAD group, whereas HDL-C concentrations were lower in the CAD group. In addition, serum CCL17 concentrations were significantly higher in the CAD than non-CAD group (Table 1).

Association of CCL17 SNPs With Risk of CAD

Five SNPs, namely rs223895, rs4784805, rs9302690, rs223899, and rs223828, were identified and included in the study. The minor alleles in each SNP were defined as the effect alleles, and the allele–frequency distribution in this study population was similar to that in general Han Chinese population (Table 2). Results from the Hardy-Weinberg equilibrium test revealed no significant differences between the present study population and the general Han Chinese population (P>0.05). Of the 5 SNPs, rs223899 and rs223828 were associated with a significantly increased risk of CAD, even after adjusting for other confounding factors (for rs223899, adjusted \( \text{OR}_{\text{aOR}} \) per effect allele=1.591, 95% CI 1.209–2.094, \( P=0.001 \), adjusted \( P=0.005 \); for rs223828, \( \text{aOR}_{\text{aOR}} \) per effect allele=1.644, 95% CI 1.233–2.191, \( P=0.001 \), adjusted \( P=0.005 \)). No association was found between CAD and the other 3 alleles (i.e., rs223895, rs4784805, and rs9302690; Table 3).

These results were confirmed by introducing another age- and sex-matched control group (2:1) from a community in Shunyi district, Beijing. The characteristics of the control population are presented in Table S1. After adjusting for other confounding factors, rs223899 and rs223828 were still associated with the risk of CAD (for rs223899, \( \text{aOR}_{\text{aOR}} \) per effect allele=1.317, 95% CI 1.076–1.612, \( P=0.007 \), adjusted \( P=0.035 \); for rs223828, \( \text{aOR}_{\text{aOR}} \) per effect allele=1.445, 95% CI 1.109–1.883, \( P=0.006 \), adjusted \( P=0.030 \); Table S2).

LD Analysis and Haplotype Association Study

Pairwise \( D' \) and \( r^2 \) values are shown in Figure S1. Haplotype blocks were constructed using the CI method described by Gabriel et al \(^{12} \) and Zhu et al \(^{13} \). No haplotype block was detected using the CI method of Gabriel et al \(^{12} \). Conversely, when the block was defined by a solid spine of LD \( D' > 0.8 \) \(^{13} \), a block consisting of 2 SNPs (rs223899 and rs223828) was detected. A haplotype association study was also performed using HAPLOVIEW software based on the method of Zhu et al \(^{13} \) (Table S3), which revealed that the G-C haplotype was linked to a reduced risk of CAD (OR 0.658, 95% CI 0.513–0.844, adjusted \( P=0.010 \)). In contrast, the T-T haplotype was associated with an increased risk of CAD (OR 1.661, 95% CI, 1.269–2.176, adjusted \( P=0.004 \)).
CCL17 Polymorphisms and CAD

Association of CCL17 SNPs With Serum CCL17 Concentrations
Serum CCL17 concentrations were compared among subjects with different genotypes. Across the entire study population, serum CCL17 concentrations were significantly different among the different genotypes for the rs223899, rs223895, and rs223828 SNPs (Figure 1). Using the multiple linear analysis model, only the effect allele T of rs223828 was associated with increased serum CCL17 concentrations (Table 4).

Serum CCL17, Increased CAD Risk, and CCL17 (rs223828) SNP
Because rs223828 was independently associated with both increased CAD risk and serum CCL17 concentrations, we hypothesized that serum CCL17 concentrations may be affected by the genetic effects of rs223828 and be an important causal mediator of CAD. To verify this hypothesis, a causal inference test using mediation analysis was conducted. Based on data in the present study, the prevalence of CAD among the at-risk population was found to be approximately 80%, which was used to calculate the weights for mediation analysis. The results revealed that the CCL17 SNP rs223828 had a significant indirect causal effect on the increased risk of CAD mediated via serum CCL17 concentrations (ORIndirect=1.077, 95% CI 1.015–1.161, P=0.047).

Effects of CCL17 SNP rs223828 on CCL17 Promoter Activity
Luciferase reporter assays were used to assess the effects of different rs223828 genotypes on promoter activity. Luciferase reporter assays were performed in human THP-1 cells transfected with pGL3-basic promoterless plasmid and the 1.4-kb PRE sequence with either the effect allele T or the major allele C. The effect allele T significantly increased luciferase activity compared with the major allele C (P<0.001; Figure 2A).

Potential Activator Protein-1 Binding Site on CCL17 SNP rs223828
Bioinformatics analysis using Transfac software and the JASPAR database indicated that rs223828 was located in the activator protein-1 (AP-1) DNA-binding motif. Data from the JASPAR database. (C) Prediction of the affinity of AP-1 binding to the T and C alleles of rs223828 according to JASPAR score. Abbreviations as in Figure 1.
100-fold competitor probe was added (Figure S2, Lanes 5–8), whereas excess unlabeled allele C probe did not compete with the labeled allele T probe (Figure S2, Lanes 9–12). The results of the quantitative competitive assay support our interpretation that THP-1 nuclear protein extracts bound more highly to the effect allele T probe than the allele C probe. By adding anti-AP-1 antibodies (c-jun and c-fos Ab) that competes with the endogenous complex (c-jun and c-fos) with the labeled allele T probe (Figure S2, Lanes 9–12), we also showed the presence of a higher mobility complex (super shift) associated with the rs223828 C allele (due to association with c-fos or c-jun antibodies) that competes with the endogenous complex (rs223828 C allele and THP-1 nuclear extract). Furthermore, ChIP with allele-specific qPCR was used to determine the DNA-binding capacity of c-fos and c-jun in peripheral mononuclear cells freshly isolated from 6 patients harboring heterozygous CCL17 (rs223828) SNP and CAD. The results indicated that both c-jun and c-fos were preferentially recruited to the effect T allele compared with the C allele (Figure 4).

Discussion

In the present study, we demonstrated that the T allele of the CCL17 SNP rs223828 is associated with increased risk of CAD as well as higher serum CCL17 concentrations. Causal inference analysis further showed serum CCL17 concentrations mediate the increased CAD risk, which is associated with the T allele of the CCL17 (rs223828) gene. Mechanistic studies highlighted that the T allele of the CCL17 (rs223828) gene: (1) had a relatively higher promoter activity than the C allele; and (2) preferentially binds to AP-1 and thus has a potential AP-1-binding site, which, together, may provide an explanation as to how the rs223828 SNP affects the risk of CAD.

Increased circulating CCL17 concentrations have been found in several allergy-related diseases, such as atopic dermatitis. Recently, Lee et al. found that CCL17 gene polymorphisms and their expression were associated with susceptibility and the formation of coronary artery aneurysms in Kawasaki disease. Several previous studies have reported that the CCL17 SNP rs223828 (previously known as −431C>T) is associated with increased CCL17 expression. For example, Sekiya et al. found that subjects carrying the −431T allele had significantly higher serum CCL17 concentrations than those without this allele, indicating that the −431T allele may have a major effect on CCL17 production. Similarly, another study showed that serum CCL17 concentrations in patients with asthma were significantly associated with the −431C>T allele. Moreover, −431C>T has been shown to enhance CCL17 promoter activity in vitro. Together, these findings indicate that rs223828 is a functional SNP, which is consistent with the findings of the present study.

Several animal studies have proven atherogenic effects of CCL17. For example, CCL17 deficiency has been found to significantly inhibit the formation of atherosclerotic lesions in ApoE−/− mice. Furthermore, CCL17 expression in dendritic cells was shown to limit the expansion of regulatory T cells by restricting their maintenance, and thus precipitated T cell-mediated atherosclerosis. In addition, platelets may provide another explanation for the effects of CCL17 on CAD. For example, platelets have been reported to be a major source of CCL17. During the clotting process, activated platelets release high concentrations of CCL17 in an autocrine manner, which could further enhance platelet aggregation. AP-1 (Fos/Jun) is a transcriptional regulator comprising members of the Fos and Jun families of DNA-binding proteins.
proteins, which play a vital role in the inflammatory process. It has been reported that AP-1 is increased in human carotid plaques and is associated with cholesterol esters, indicating that AP-1 could be a marker of plaque vulnerability. Upregulation of AP-1 was also associated with increased age-related vascular smooth muscle cell proliferation in a rabbit model. Conversely, Jun proteins need to be activated by c-Jun N-terminal kinases (JNKs), and its inhibition protects against endothelial dysfunction and oxidative stress, as well as against low shear stress-induced atherogenesis. The present study established a potential link between AP-1 and the CCL17 pathway, which may be a key component of the inflammatory network of atherosclerosis. The present study has certain limitations. Causal inference was verified by a case-control study; however, a prospective cohort study is needed to confirm the findings of this study. Although our in vitro studies evaluated the potential mechanism of the SNP, additional investigations are warranted to fully elucidate the mechanisms that underlie the association between the rs223828 SNP and CAD risk.

In summary, we conclude that the CCL17 SNP rs223828 is associated with both elevated serum CCL17 concentration and increased risk of CAD. Results from our in vitro studies indicate that rs223828 directly affects CCL17 promoter activity and may be a potential AP-1 transcription factor binding site.

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Conflict of Interest
The authors declare no conflicts of interest.

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Supplementary Files
Supplementary File 1
Figure S1. Positions of single nucleotide polymorphisms (SNPs) on the structure of the C-C motif chemokine ligand 17 (CCL17) gene and linkage disequilibrium (LD) among the SNPs in the tested population including both (A) D’ and (B) r² data.
Figure S2. Results of electrophoretic mobility gel shift assays.
Table S1. Baseline characteristics of the CAD group and additional community control population from Shunyi District, Beijing.
Table S2. Association between CCL17 SNPs and risk of CAD
Table S3. Haplootype association study of CCL17
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