Clinical significance of costimulatory molecules CD40/CD40L and CD134/CD134L in coronary heart disease

A case-control study

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Abstract
The aim of the study was to evaluate the potential role of CD40/CD40 ligand (CD40L) and CD134/CD134 ligand (CD134L) in the development of coronary heart disease (CHD) via the performance of a case-control study.

The research objects were 234 cases of CHD patients and 120 cases of well-matched normal controls. Following the separation of peripheral blood mononuclear cells (PBMCs), real-time quantitative PCR (qRT-PCR), Western blot, immunohistochemistry, and flow cytometry were applied for the detection of mRNA levels and expression levels of CD40/CD40L and CD134/CD134L; meanwhile, intercellular adhesion molecule-1 (ICAM-1) and Fas protein mRNA levels were detected using qRT-PCR. There was no statistical difference in the comparison of baseline characteristics between groups, indicating comparability between groups. qRT-PCR and Western blot analysis indicated that CD40/CD40L and CD134/CD134L mRNA and protein expression levels were all increased in the CHD group than those in the control group. Flow cytometry further confirmed the similar tendency. Meanwhile, ICAM-1 and Fas protein mRNA levels were elevated in the CHD group and positively correlated with the above parameters. Furthermore, CD40/CD40L expression rates were negatively correlated with gender and different types of CHD. Meanwhile, CD134/CD134L expressions were also higher in male patients, in patients with family history, previous history of hypertension, diabetes, and cerebrovascular diseases. CD40/CD40L and CD134/CD134L are increased and may have potential correlation with clinical pathological features of patients with CHD. Further in-depth exploration of costimulatory molecules for CHD guidance as well as intrinsic mechanisms are needed combined with in vivo and in vitro experiments.

Abbreviations: AMI = acute myocardial infarction, APCs = antigen-presenting cells, CD134L = CD134 ligand, CD40L = CD40 ligand, CHD = coronary heart disease, ECs = epithelial cells, FBS = fetal bovine serum, ICAM-1 = intercellular adhesion molecule-1, IL = interleukin, NK = natural killer, PBMCs = peripheral blood mononuclear cells, PBS = phosphate-buffered saline, qRT-PCR = real-time quantitative PCR, SA = stable angina pectoris, SDS-PAGE = sodium dodecyl sulfate poly acrylamide gel electrophoresis, SMCs = smooth muscle cells, Th1 = T-helper cells 1, Th2 = T-helper cells 2, TNF = tumor necrosis factor, UA = unstable angina pectoris.

Keywords: CD134/CD134L, CD40/CD40L, coronary heart disease

1. Introduction
Cardiovascular disease is still one of the dominant causes of disability and mortality worldwide, accounting for 30% to 40% of all human deaths among the delayed degenerative diseases. Coronary heart disease (CHD) is the most common type of cardiovascular diseases and the principal cause of heart attacks, identical to atherosclerotic heart disease, coronary artery disease, or ischemic heart disease.[12–14] The formation of damaged or diseased atherosclerotic plaque along the inner walls of the coronary arteries is mainly responsible for the pathogenesis of CHD, which may induce thrombotic occlusion or spasm, and thereby resulting in narrowed blood flow of arteries and reduced function of the heart.[6] With an estimated approximately 7 million deaths in 2010 in the world, CHD is predominantly prevalent in developed countries.[15] Although exact mechanisms of CHD are still not well understood, CHD is considered to be a complex and multifactorial disease which may be resulted from the interaction of genetic and environmental factors.[16–19] To date, previous evidence has documented that important extrinsic risk factors responsible for the progress of CHD include age, sex, obesity, physical inactivity, hypertension, family history, smoking, alcohol intake, work stress, and so on.[12–17] Besides, inflammatory response and related markers of the innate and adaptive immune system are suggested to be involved in the development of CHD.

A costimulatory molecule is a cell surface molecule existing mostly in the T/B cells, antigen-presenting cells (APCs), and target
cells. The role of the costimulatory system in the response of expressed on dendritic cells, B cells, and activated endothelial on the other hand, CD134 (OX40) is a member of the tumor for the conduction of antigen stimulation information, the activation process of immune cells, as well as antigen recognition and immune response. Furthermore, originally discovered on B-cells and other APCs, CD40 is a costimulatory molecule activated by CD40 ligand and is synthesized in inflammation by natural killer (NK) cells, monocytes, and lymphocytes B. On the other hand, CD134L (OX40L) is a member of the tumor necrosis factor (TNF) receptor family that is expressed on activated T lymphocytes; its ligand, CD134 ligand (CD134L), is expressed on dendritic cells, B cells, and activated endothelial cells. The role of the costimulatory system in the response of the immune system to pathogens is now widely accepted given by all the involved subjects. The research object of the study was 234 cases of CHD patients (120 males and 114 females, mean age of 52.32 ± 10.11 years ranging from 29 to 67 years old) who visited our Hospital from January 2013 to December 2014. CHD was defined as over 50% of diameter stenosis of left main coronary artery, left anterior descending artery, left circumflex artery, and right coronary artery or their branch arteries during coronary angiography. CHD patients with stroke, autoimmune disease, thyroid disease, infectious disease, chronic infection, and heart failure were excluded from the study. There were 120 cases of acute myocardial infarction (AMI), 80 cases of unstable angina pectoris (UA), and 34 cases of stable angina pectoris (SA), which were classified according to the clinical manifestation of incorporated patients associated with the diagnostic method of electrocardiogram in accordance with Cardiovascular Diseases Treatment Guidelines and Recommendations made by the editorial board of the Chinese Journal of Cardiology and the Chinese Medical Association in 2007. In addition, a sum up of 120 cases of well-matched normal controls were included as the control group, including 70 males and 50 females, with a mean age of 51.32 ± 10.11 years old, ranging from 27 to 69 years old. Inclusion criteria: (1) subjects aged from 25 years old to 72 years old; (2) study objects were involved in the study under the condition of their being fully acknowledged, and all the cases were in the observation voluntarily; (3) no gender limitation. Exclusion criteria: (1) patients who had combined systemic diseases related to infection at the admission; (2) patients who had their serum creatinine levels over 267 μmol/L or with renal failure; (3) patients who had previous history of suffering from malignant tumor, severe liver and kidney diseases, hereditary hyperlipidemia, or had diseases of the immune system in the past month; (4) patients who had previous administration history of immunosuppressive drugs; (5) pregnancy or lactating women; (6) patients with incomplete clinical data or without coronary angiography information.

2.2. Biochemical index detection

The whole blood sample (~20 mL) was collected from each subject in the early morning from each group after an overnight fasting, and blood samples were collected for blood lipid examination of cholesterol, triglyceride, high-density lipoprotein, and low-density lipoprotein, and were then analyzed using the full automatic biochemical analyzer provided by the Guangzhou Panyu Central Hospital. All the whole blood samples were under the condition of sterilization and heparin anticoagulation. Blood samples were collected using the EDTA anticoagulation tube and stored at low temperature of −4°C to prepare for extraction and separation of peripheral blood mononuclear cells (PBMCs).

2.3. Separation of PBMCs

In accordance with the above process of blood collection, peripheral venous blood samples (10 mL) were drawn by venipuncture from each subject. Heparin was the anticoagulant used in this study. PBMC isolation was performed using Ficoll density gradient centrifugation. Collected samples were inoculated in the culture dish and placed at 37°C and 50 mL/L CO2 culture box for 2 hours. Subsequently, the supernatant was discarded and the adherent cells (monocytes) were collected, sparing for further usage. The mononuclear cell purity was greater than or equal to 80% through density gradient centrifugation and adherence method.

2.4. Cell culture

Isolated and purified cells with various radio sensitivity (Gibico BRL Inc.) were cultured in the RPMI 1640 complete medium (Invitrogen Corporation) containing 10% of fetal bovine serum (FBS, Gibico BRL Inc.), 100 μL/mL of penicillin, and 100 μL/mL of streptomycin; serial subcultivation was performed afterward in an incubator under the environment of 5% CO2 and at 37°C.

2.5. Real-time quantitative PCR (qRT-PCR)

Collected cells were cultured in the RPMI 1640 medium to extract cell suspension with each culture flask containing about 2.5 × 10^6 cells and 5 mL of RPMI 1640 medium, followed by overnight culturing in the environment with 5% CO2 and 37°C of culturing temperature. The TRIZOL method (Invitrogen Inc., Carlsbad, CA) was implemented to extract RNA after the collection of the cells, the integrity of RNA samples was estimated by denaturing gel electrophoresis with 1% formaldehyde, and purity and density of RNA samples were assessed through the UV spectrophotometer (WP-93-1ZF, Shanghai Analytical
Instrument Factory). Subsequently, the RNA solution should be sub-packaged and preserved under the temperature of −70°C for further usage.

Quantitative PCR was performed by using the iCycler real-time quantity PCR instrument (Bio-Rad Hercules, CA). The qRT-PCR reaction system (100 μL) consisted of 10 μL of PCR buffer, 2.5 μL 10 mM dNTP, 0.8 μL Taq enzyme, and appropriate proportion of each primer. The conditions for the reaction system were 5 minutes of initial denaturation at 95°C, 1 minute of denaturation at 94°C, 1 minute of annealing at 55°C, 1 minute of extension with 30 circulation at 72°C, 10 minutes of extension at 72°C; and then preserved at 4°C, followed by agarose gel electrophoresis. CD28 was used as an internal reference because of its similar expression in CHD and normal controls samples. CD40/CD40L and CD134/CD134L as well as their up- and down-stream targets of ICAM-1 and Fas protein were detected; detailed information regarding primers of those proteins and their internal control were presented in Table 1. The gel image analysis system was applied to analyze the electrophoresis photograph and to measure its absorbance value. The quantitative comparisons were finished with the ratio of the target gene, and the net density of β-actin was regarded as an internal reference.

2.6. Western blot

Protein extraction was achieved by adding protein lysate (Beyotime Biotechnology Institute), and then, obtained samples were preserved at 4°C. After violent up-and-down shaking for 6 minutes, the samples were centrifuged at a centrifugation speed of 12,000 rpm for 10 minutes, and the supernatant was extracted, which was preserved in a centrifuge tube and stored at −70°C. The protein content was determined by the Bradford method. Lastly, electrophoresis was performed and finished until the bromphenol blue reached the bottom of the gel. After that, the protein was transferred to the PVDF membrane (purchased from Amersham Pharmacia, Biotech, Sweden); the transfer lasted for about 90 minutes at a voltage of 100V. After the end of the electorotation, the sample was closed with 5% skim milk and incubated at room temperature for 1 to 2 hours. Subsequently, the sample was added and mixed with mouse anti-human primary antibody (20 μL, provided by Abcam company, CA), which was incubated at 4°C overnight, and washed with TBST buffer for 3 times, 10 minutes per time. Soon afterward, horseradish peroxidase-conjugated goat anti-mouse secondary antibody was added and incubated at room temperature for 1 hour, followed by another washing with TBST buffer. β-actin was used as the internal control. The enhanced chemiluminencescence (ECL) method was then used for protein detection, band detection was performed following an exposure of the x-ray film for about 60 to 90 minutes, and the x-ray film was placed into the developer solution for 15 to 30 seconds, and in the fixer solution for about 1 minute, and then rinsed with tape water. Finally, the photo was analyzed by the IPP 6.0 image analysis software.

2.7. Immunohistochemistry

Cells were fixed with acetone and then washed with running water, followed by 3% H2O2 incubation at room temperature for 10 minutes and washing with distilled water, and then, samples were immerse in phosphate-buffered saline (PBS) for 5 minutes, digested with 0.05% trypsin at 37°C for 30 minutes; another immersion with PBS was conducted for 5 minutes. Subsequently, samples were blocked with 10% normal goat serum for 10 minutes at room temperature. Then, the serum was poured; mouse antihuman primary antibody was added and incubated overnight at 4°C. Following another 3 times of PBS washing for 5 minutes, biotin labeled secondary antibody (ready-to-use) was added and incubated at 37°C for 40 minutes. Then, samples were washed with PBS (3 × 5 min), a chromogenic agent was the DAB developer, with another washing with running water thoroughly, and redyeing mounting and microscopic examination were conducted afterwards. Determination of positive results: the positive target was brown after DAB coloration.

2.8. Flow cytometry

Following another washing, the collected sample was analyzed on an EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, FL). Immediately before flow cytometry, samples were filtered through a 40 nm nylon mesh to make into single cell suspension, with pre-cooling PBS washing for 2 times. Then, the 2 mL working solution of Permeabilization buffer were added and incubated for cells washing. The supernatant was discarded and the cell sedimentation was collected. Following another washing, a fluorescent-labeled secondary antibody was added directly and incubated in the dark at 4°C for at least 30 minutes. Subsequently, another washing with the supplementation of 2 mL working solution of Permeabilization buffer was performed to exclude supernatant without combination with the fluorescent labeled secondary antibody. Finally, the previous step of washing was repeated, and the detection of CD40/CD40L and CD134/CD134L was conducted at the flow cytometer (Becton Dickinson) to calculate fluorescent density values of cells.

2.9. Statistical analysis

SPSS 17.0 software (IBM Inc., Chicago, IL) was used for statistical analysis; measurement data were expressed as mean ± standard deviation (mean ± SD), and detected through normality test. The t test was applied for comparison between groups and the χ2 test was used to count the data. Correlation analysis was carried out to show the relationship of CD40/CD40L and CD134/CD134L with ICAM-1 and Fas protein. Single factor analyses and multiple factors logistic regression analyses were performed to identify the correlation of CD40/CD40L and

Table 1

| PCR primer sequences. | Primer sequences (5′–3′) |
|-----------------------|--------------------------|
| CD40                  | F: AGTGCTTTAAGGGACAAAC  |
|                       | R: CCACAGATTGTCATGTATG   |
| CD40L                 | F: CATACTGGTTCAGATTTCT   |
|                       | R: CACAGGTCATGTATGAG     |
| CD134                 | F: GAACTGTCTGTTGACGGTGC  |
|                       | R: AGGTCATAAGGACCTAAGAG  |
| CD134L                | F: GAGGGCTCAACTCAAAGG    |
|                       | R: GTCGCCCTCCACGTAAGG    |
| CD28                  | F: TGTTGTGCGTACATCCTT    |
|                       | R: TGAATGGAGGATGAGTACAG  |
| ICAM-1                | F: AGCAACAAAGAGAAGAGGAA  |
|                       | R: GAGAAGCCGCAACCCGTTAG  |
| Fas                   | F: CTCCAAGGATATTGAAATTGA |
|                       | R: TTGTGTTGCTAGGATGTG    |

CD134L = CD134 ligand, CD40L = CD40 ligand, ICAM-1 = intercellular adhesion molecule-1, PCR = polymerase chain reaction, CD28 was regarded as the internal control.
CD134/CD134L with different pathological features. $P < .05$ meant that the difference was statistically significant.

### 3. Results

#### 3.1. General data comparison between the CHD group and the control group

As shown in Table 2, there were no apparent differences with respect to age, gender, smoking, previous history of hypertension, diabetes, and cerebrovascular disease between the CHD group and the control group, suggesting that both groups were comparable (all $P > .05$). In addition, TC and HDL-C showed none significant statistical differences between groups (all $P > .05$). However, LDL-C, uric acid, BNP, and Gensini scores were obviously higher in the CHD group when compared to the control group, with statistical differences (both $P < .05$).

#### 3.2. CD40/CD40L and CD134/CD134L mRNA levels and protein expression by qRT-PCR and Western blot

Compared with levels in the control group, CD40/CD40L and CD134/CD134L mRNA and protein expression levels were all increased in the CHD group by applying qRT-PCR and Western blot analysis (all $P < .05$). Figures 1 and 2 illustrated changes of CD40/CD40L and CD134/CD134L mRNA and protein expression levels in the CHD group and the control group, as well as statistical alternation tendencies. Furthermore, as shown in Fig. 3, ICAM-1 and Fas mRNA expression levels were all increased in the CHD group when compared to those in the control group (all $P < .05$). Besides, there were positive correlations that ICAM-1 and Fas mRNA levels were increased along with the elevated levels of CD40/CD40L and CD134/CD134L mRNA levels (ICAM-1 and CD40: $r = 0.327$, $P < .001$; ICAM-1 and CD40L: $r = 0.683$, $P < .001$; Fas and CD40: $r = 0.277$, $P = .003$; Fas and CD40L: $r = 0.388$, $P < .001$).

#### 3.3. CD40/CD40L and CD134/CD134L expression by immunohistochemistry

Positive cells of CD40/CD40L and CD134/CD134L were detected in the CHD group, which were localized in the cell membrane mostly; a small amount was also stained in the cytoplasm. However, they were negative or weakly expressed in the control group, showing significant differences when compared between groups. Detailed information of their expression intensities were shown in Table 3.

#### 3.4. CD40/CD40L and CD134/CD134L expression levels by flow cytometry detection

Table 4 illustrated the expression levels of CD40/CD40L and CD134/CD134L on the surface of PBMCs by flow cytometry. Compared with the control group, expressions of CD40 and CD40L were significantly increased, showing statistical differences (both $P < .05$). In addition, expression levels of CD134 and CD134L were also significantly elevated in the CHD group than those in the control group (both $P < .05$).

Correlation analysis showed that the expression levels of CD40 and CD40L were positively correlated with Gensini scores; with the increase of Gensini scores, expression levels of CD40 and CD40L were also increased correspondingly ($r = 0.915$, $r = 0.830$; both $P < .05$). Furthermore, there was also positive correlation between CD134 and Gensini scores ($r = 0.340$, $P < .05$). However, there was no obvious correlation between CD134L and Gensini scores ($P > .05$).

#### 3.5. The relevance of CD40/CD40L and CD134/CD134L expression with clinical pathological index in CHD patients

Single factor analyses revealed that higher expression levels of CD40/CD40L were found to be much more in CHD male patients than female patients, and corresponding expression levels were significantly higher in UA patients and AMI patients.

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**Table 2**

| Variables                        | CHD group (n=234) | Control group (n=120) | $P$   |
|----------------------------------|-------------------|-----------------------|------|
| Age                              | 48.70±13.70       | 47.60±9.94            | .391 |
| Gender, male/female              | 150/84            | 85/35                 | .204 |
| Smoking, yes/no                  | 121/113           | 61/59                 | .876 |
| Body mass index                  | 24.75±7.92        | 26.67±5.95            | .011 |
| Medical history                  |                   |                       |      |
| Hypertension, yes/no             | 167/67            | 74/46                 | .064 |
| Diabetes, yes/no                 | 156/78            | 80/40                 | 1.000|
| Cerebrovascular disease, yes/no  | 121/113           | 68/52                 | .376 |
| Triglyceride, mmol/L             | 2.46±0.78         | 2.45±0.99             | .913 |
| Low-density                      | 3.16±1.21         | 3.33±0.98             | .167 |
| Lipoprotein-cholesterol, mmol/L | 1.39±0.68         | 1.10±0.57             | <.001|
| High-density                     |                   |                       |      |
| Lipoprotein-cholesterol, mmol/L | 190.63±61.72      | 172.86±52.73          | .005 |
| Uric acid, μmol/L                | 151.21±57.16      | 100.08±30.00          | <.001|
| Type B natriuretic peptide, pg/mL| 19.56±5.51        | 6.00±2.48             | <.001|

CHD = coronary heart disease.
than in SA patients; besides, levels in AMI patients were much higher than that in the UA patients (all \( P < .05 \)) (Table 5). Furthermore and obviously, increased expressions of CD134/CD134L were found to be much more in male patients, in patients with family history and hypertension, patients with past history of diabetes and cerebrovascular diseases, all revealing obvious statistical differences (all \( P < .05 \)). Detected indicators then entered the multiple factor logistic regression analysis model, and corresponding results indicated that CD40/CD40L expression was correlated with gender and clinical types, with statistical difference (\( P < .05 \)); meanwhile, there was statistical difference with respect to gender, family history, hypertension, and cerebrovascular diseases history in exploring the correlation with CD134/CD134L expression (\( P < .05 \)) (Table 6).

4. Discussion
Through the exploration of CD40/CD40L and CD134/CD134L in CHD, our study confirmed that CD40/CD40L and CD134/CD134L were increased in CHD patients and correlated with some clinical pathological features of CHD, suggesting important roles of costimulatory molecules in the development of CHD.

Human lymphocytes are divided into T lymphocytes, B lymphocytes, and natural killer cells.\(^{[27]}\) Theoretically, the involvement of T lymphocytes is of great significance in the course of coronary atherosclerotic plaque development.\(^{[28]}\) The aggregation of T lymphocytes to the local region of immune response resulting in signal enhancement, further stimulating macrophage phagocytosis, secretion of cytokines and growth factors, while promoting the vascular media proliferation of smooth muscle cells (SMCs), migration, and phagocytosis.\(^{[29,30]}\) CD40 was initially found in B lymphocytes, which has been suggested to be participated in the activation of B cells and has other key roles.\(^{[31]}\) Apoptosis induction can be inhibited by the interaction between CD40 and their ligands in B lymphocytes;\(^{[32]}\) but the expression level of CD40 is low under normal physiological conditions, which may be significantly up-regulated under pathological conditions.\(^{[33]}\) Importantly, as the key hub of the inflammatory and immune responses in vivo, the CD40/CD40L system may be responsible for antigen presentation and
Table 3
Expression of CD40/CD40L and CD134/CD134L detected by immunohistochemistry.

| Cases   | Expression intensity | P   |
|---------|----------------------|-----|
|         | -  | +  | ++ | +++ |
| CD40    |    |    |    |     |
| CHD group | 234 | 60 | 44 | 56  | 74  | <.001 |
| Control group | 120 | 85 | 20 | 11  | 4   |     |
| CD40L   |    |    |    |     |
| CHD group | 234 | 67 | 40 | 55  | 72  | <.001 |
| Control group | 120 | 75 | 23 | 16  | 6   |     |
| CD134   |    |    |    |     |
| CHD group | 234 | 62 | 59 | 53  | 60  | <.001 |
| Control group | 120 | 60 | 31 | 23  | 6   |     |
| CD134L  |    |    |    |     |
| CHD group | 234 | 63 | 67 | 49  | 55  | <.001 |
| Control group | 120 | 60 | 28 | 25  | 7   |     |

CD134L = CD134 ligand, CD40L = CD40 ligand, CHD = coronary heart disease.
Scoring was counted according to the cell staining intensity score: colorless, 0 points; light yellow, 1 points; brown yellow, 2 points; and deep yellow, 3 points. The product of the above two scores for 0 point was regarded as negative (−); 0–1 points, weakly positive (+); 2–3 points, positive (++); and ≥4 points, strongly positive (+++).

Table 4
Expression levels of CD40/CD40L and CD134/CD134L on the surface of PBMCs detected by flow cytometry detection.

| Variables | CHD group | Control group | P   |
|-----------|-----------|---------------|-----|
|           | CD40      | 1.76 ±0.28    | 0.65 ±0.11 | <.001 |
|           | CD40L     | 1.43 ±0.21    | 0.61 ±0.24 | <.001 |
|           | CD134     | 1.78 ±0.24    | 0.73 ±0.17 | <.001 |
|           | CD134L    | 0.65 ±0.09    | 0.56 ±0.11 | <.001 |

CD134L = CD134 ligand, CD40L = CD40 ligand, CHD = coronary heart disease, PBMCs = peripheral blood mononuclear cells.

Table 5
The relevance of CD40/CD40L and CD134/CD134L expressions in CHD patients and its clinical pathological indexes by single factor analyses.

| CD40/CD40L expression | CD134/CD134L expression |
|-----------------------|-------------------------|
| High (n = 171)        | Low (n = 63)             | P   | High (n = 167) | Low (n = 67) | P   |
| Gender                |                         |     |               |              |     |
| Male                  | 120                     | 30  | <.001         | 122           | 28  | <.001 |
| Female                | 47                      | 37  |               | 49            | 35  |     |
| Smoking               |                         |     |               |               |     |     |
| Yes                   | 85                      | 36  | .106          | 88            | 33  | .769 |
| No                    | 82                      | 31  |               | 83            | 30  |     |
| Family history        |                         |     |               |               |     |     |
| Yes                   | 80                      | 33  | .152          | 90            | 23  | .001 |
| No                    | 87                      | 34  |               | 81            | 40  |     |
| Hypertension          |                         |     |               |               |     |     |
| Yes                   | 104                     | 40  | .423          | 114           | 30  | .001 |
| No                    | 63                      | 27  |               | 58            | 33  |     |
| Diabetes history      |                         |     |               |               |     |     |
| Yes                   | 111                     | 45  | .812          | 130           | 26  | .015 |
| No                    | 56                      | 22  |               | 41            | 37  |     |
| Cerebrovascular diseases history |     |     |               |               |     |     |
| Yes                   | 87                      | 34  | .213          | 97            | 24  | <.001 |
| No                    | 80                      | 33  |               | 74            | 39  |     |
| Clinical types        |                         |     |               |               |     |     |
| AMI                   | 99                      | 21  | <.001         | 90            | 30  | .567 |
| UA                    | 58                      | 22  |               | 58            | 22  |     |
| SA                    | 10                      | 24  |               | 23            | 11  |     |

AMI = acute myocardial infarction, CD134L = CD134 ligand, CD40L = CD40 ligand, CHD = coronary heart disease, SA = stable angina pectoris, UA = unstable angina pectoris.
immune response, and associate significantly with the activation of T lymphocytes and macrophages, thereby exerting critical biological roles in different cell types by the modulation of different signaling pathways.\cite{35,36} With respect to the above, it was postulated in the present study that the combination of CD40 and CD40L might be partially involved in the development of CHD in view of their important effects implicated in activating immune cells and initiating the immune process. In this study, CD40/CD40L mRNA and expression levels were all increased in the CHD group when compared with the control group. Possible mechanisms in which the CD40 and CD40L system affected atherosclerosis might be correlated with the following contents: first, the CD40 and CD40L system can induce the activation of T cells and related cells; simultaneously, their binding can activate T lymphocytes, activating the immune response of T-helper cells 1 (Th1) and T-helper cells 2 (Th2) in the process of atherosclerotic plaques formation, and can also stimulate the activation of APT and T cells.\cite{37,38} Second, during the early stage of atherosclerosis, the interaction between CD40 and CD40L can contribute to the strengthened expression of a variety of adhesion molecules, helpful evidently for the induction of circulating T lymphocytes and monocytes adhering to the endothelium and entering the vascular wall.\cite{39} Third, another important effect of CD40 signal is to promote vascular cells and macrophages to express large amounts of cytokines, which can promote the release of interleukin (IL)-6 and IL-8 stimulated by epithelial cells (ECs) and SMCs; at the same time, through the activation of IL-1 converting enzyme, IL-1 stimulation of precursor processing, IL-1 activity and expression can be increased, in turn elevating blood vessel wall inflammation.\cite{40} Previous studies also emphasized the important role of CD40 and its ligand in the development of stable and unstable cardiovascular diseases\cite{41,42}; however, our investigation also indicated the clinical pathological significance in CHD, which was different from the above studies. It was found in our study that CD40/CD40L expression levels were significantly higher in male patients and UA/AMI patients than other subgroups, which might be attributed to the mediation of immune inflammatory reaction and suggested an important role of CD40/CD40L in the pathophysiological process of CHD.

In addition, CD134 is mainly expressed in the activated T lymphocytes\cite{43,44}, and CD134L in APC such as B lymphocytes and macrophage; their interaction can regulate the differentiation of T lymphocytes and thereby contribute to the immune responses of T lymphocytes, promoting the proliferation and differentiation of B lymphocytes to secrete immunoglobulin, which is critical for the pathogenesis of various inflammatory and autoimmune diseases.\cite{45,46} Few studies focused on the exploration of CD134 and CD134L in humans, not to mention the development of CHD. In this study, elevated CD134/CD134L mRNA and expression levels were detected in patients with CHD when compared to those in normal controls. As we all know, CD34 and CD134L have synergistic effects to promote the migration and differentiation of activated CD+T cells, contributing to the infiltration of activated T lymphocytes into the inflammatory sites, and to stimulate B cells to produce and differentiate to phlogocytes, thus influencing the cellular immune function.\cite{47} In addition, there were elevated trends of ICAM-1 and Fas mRNA expression levels in patients with CHD; meanwhile, ICAM-1 and Fas mRNA levels were positively correlated with the elevated levels of CD40/CD40L and CD134/CD134L mRNA levels. Those results, in turn, highlighted a possible mechanism that CD40/CD40L and CD134/CD134L involved in the development of CHD. ICAM-1 is a immunoglobulin superfamily member widely distributed in a variety of cells, which can provide stimulus signal for T cells and thus promote the activation of T cells; meanwhile, Fas can be expressed on the surface of many immune cells and solid tumor cells. Highly expressed ICAM-1 and Fas have been suggested to be correlated with cells escaping from immune surveillance and of course involved in the development of cardiovascular diseases.\cite{48,49} Furthermore, expression levels of CD40/CD40L and CD134/CD134L were relatively higher in CHD male patients, and in UA patients and AMI patients, suggesting that changes in these indicators might also be responsible for clinical pathological characteristics alternations in CHD. Significantly, the strength of the present study was that under the background of few research on this field, some significant results were found in the present study. Expression of CD134/CD134L were increased in CHD patients, especially of CD134; their expressions were obviously higher in male patients, in patients with hypertension, and in patients who had previous history of diabetes, cerebrovascular diseases, and those who had family history of CHD. Such findings would be valuable for the early screening and later prevention/treatment of CHD. Afterward, we intend to investigate relevant mechanisms of CD134/CD134L and corresponding possible therapeutic agents in the development of CHD in our future research.

Collectively, CD40/CD40L and CD134/CD134L are increased and may be involved in the pathophysiological process of CHD. However, we also keep our modest attitude that there were some limitations of this study. We have explored the expression of

### Table 6

Multiple factor logistic regression analysis for exploring the relevance of CD40/CD40L and CD134/CD134L expressions in CHD patients and its clinical pathological indexes.

| Variables B | SE | χ² | P  | OR (95%CI) |
|------------|----|----|----|-----------|
| CD40/CD40L Gender 0.732 | 0.280 | 10.947 | <.001 | 2.539 (2.005–9.350) |
| Clinical types CD134/CD134L Gender 0.865 | 0.305 | 10.826 | .001 | 1.975 (1.365–2.923) |
| Family history 1.156 | 0.541 | 8.507 | .025 | 3.785 (1.138–11.854) |
| Hypertension 1.511 | 0.229 | 6.841 | .041 | 3.260 (1.775–6.661) |
| Diabetes history 0.711 | 0.563 | 1.593 | .207 | 2.036 (0.675–6.174) |
| Cerebrovascular diseases history 0.680 | 0.227 | 8.793 | .003 | 2.539 (1.260–5.254) |

95% CI = 95% confidence interval, CD134L = CD134 ligand, CD40L = CD40 ligand, OR = odd ratio, SE = standard error.
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