Expression of inflammatory factors and oxidative stress markers in serum of patients with coronary heart disease and correlation with coronary artery calcium score

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Abstract. Expression characteristics of inflammatory factors interleukin-23 and interleukin-35; oxidative stress markers of malondialdehyde, which is a final product of lipid peroxidation; superoxide dismutase; microRNA-126 and microRNA-146a in serum of patients with coronary heart disease were investigated. Correlation between these biomarkers and CACS (calcification score), as well as the underlying clinical significance were evaluated. A total of 192 patients diagnosed with coronary heart disease were recruited as the observation group, and 69 healthy adults who provided their blood samples were selected as the control group. Enzyme linked immunosorbent assay was carried out to measure the levels of inflammatory factors interleukin-23 and interleukin-35, and the levels of oxidative stress markers of malondialdehyde and superoxide dismutase in serum of the patients and healthy subjects. Real-time fluorescence-based quantitative PCR was performed to measure the expression levels of microRNA-126 and microRNA-146a in serum. The differences in expression of these biomarkers were analyzed, and correlation between these biomarkers and coronary artery calcium score were assessed. The differences in expression levels of interleukin-23, interleukin-35, malondialdehyde, superoxide dismutase, microRNA-126 and microRNA-146a were statistically significant in both groups. The expression levels of interleukin-23, interleukin-35, malondialdehyde, superoxide dismutase, microRNA-126 and microRNA-146a in the observation group were closely associated with severity of the disease. There were positive correlations between coronary artery calcium score and interleukin-23, interleukin-35, malondialdehyde, microRNA-126 and microRNA-146a, respectively; while a negative correlation existed between coronary artery calcium score and superoxide dismutase in the observation group. In conclusion, biomarkers interleukin-23, interleukin-35, malondialdehyde, superoxide dismutase, microRNA-126 and microRNA-146a were abnormally expressed in serum of patients with coronary heart disease, implicating their association with onset and progression of the disease. The biomarkers were found to be correlated with coronary artery calcium score. Detection of changes of related biomarkers in serum may have certain value in diagnosis of disease formation, as well as assessment of disease severity.

Introduction

Coronary artery calcium score (CACS), as an indicator of severity of coronary artery calcification (1), is an important preoperative parameter for coronary atherosclerosis (2) and an important basis for diagnosis of coronary heart disease. The score can be used to assess the overall condition of patients with coronary heart disease. Studies found that related inflammatory factors and oxidative stress markers are associated with onset and progression of coronary heart disease. Abnormal expression of these indicators plays an important role in aggravating myocardial injury (3). Inflammatory factors such as interleukin-23 (IL-23) has pro-inflammatory effects. It is a related mediator of oxidative stress response and may be involved in inflammatory response, macrophage response, and immune disease progression. IL-35 is thought to be an inducing factor that activates the anti-inflammatory M2-like macrophage phenotype. Recent studies showed that both cytokines are abnormal in plaque formation (4). It was reported that both IL-23 can promote inflammatory response and regulate malondialdehyde (MDA) and superoxide dismutase (SOD) to promote tissue damage (5). Expression of IL-35 and SOD in peripheral blood of patients with coronary heart disease often decreased, while expression of MDA increased. IL-35 may have a synergistic effect with oxidative stress indicators during inflammatory activation, and MDA and SOD are inversely correlated (6). In MDA-mediated oxidative stress and inflammation, it was noted that the expression of microRNA (miRNA) in serum may also be

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abnormal (7). According to the literature reports on coronary heart disease, two factors were selected from the gene bank in association with inflammatory injury and oxidative stress, i.e. microRNA-126 (miR-126) and microRNA-146a (miR-146a) (8,9). In this study, expression levels of related factors in serum of patients with coronary heart disease were measured, and their correlation with CACS was analyzed for potential clinical application.

Patients and methods

Clinical information. A total of 192 patients diagnosed with coronary heart disease who were admitted to The Third Affiliated Hospital of Qiqihar Medical University (Qiqihar, China) from January 2018 to December 2018 were recruited into observation group. Patients who met following criteria were eligible for the study: Patients who experienced precordial pain during physical exertion and rest, and were diagnosed with coronary heart disease by coronary CT angiography; and new patients who were not treated before. Patients who met following criteria were excluded from this study: Patients who had other heart conditions such as myocarditis and valvular heart disease; patients who had rheumatic autoimmune diseases; patients who had malignant tumors; and patients who dropped out of the study halfway. In the observation group, there were 100 males and 92 females aged 46-88 years with an average age of 60.1±7.2 years. A total of 69 volunteers were selected as the control group. These volunteers underwent physical examination in the same period, they were adults with no obvious organic disease after cardiovascular system testing, and provided their blood samples. In the control group, there were 39 males and 30 females aged 40-62 years with an average age of 56.2±6.9 years. This study met the relevant requirements and was approved by the hospital ethics committee. Patients and their families signed an informed consent form.

Measurement of levels of IL-23, IL-35, MDA and SOD. Approximately 3 ml of fasting venous blood were taken from the subjects in the observation group in the morning after diagnosis, whereas fasting venous blood samples were taken from the control group in the morning, and were centrifuged at 300 x g at room temperature for 20 min. The supernatant was collected and stored for analysis. ELISA was performed to measure serum levels of IL-23 (ELISA kit #RLN2207 from Suzhou Ruiying Biotechnology Co., Ltd.), SOD (ELISA kit #RLT4364 from Suzhou Ruiying Biotechnology Co., Ltd.), IL-35 (ELISA kit #70-EK135-24 from Hangzhou Lianke Biotechnology Co., Ltd.) and MDA (ELISA kit #70-ab30841-050 from Hangzhou Lianke Biotechnology Co., Ltd.), respectively. The user manuals of these ELISA kits were strictly followed for reliable measurements.

Measurement of levels of miR-126 and miR-146a. Total RNA was extracted, and cDNA was synthesized via reverse transcription. Real-time fluorescence-based quantitative PCR was performed using cDNA as a template to measure levels of miR-126 and miR-146a. Mature sequences of miR-126 and miR-146a were obtained from the miRNA database (www.mirbase.org), and primers were designed accordingly. U6 was used as a reference gene. The primer sequences were as follows: for miR-126 primer (60 bp long) the forward sequence was 5'-GGG TGA GAA CTG AAT TCCA-3', and the reverse sequence was 5'-CAG GTG GCG TCG TGG ATG-3'; for miR-146a primer (79 bp long) the forward sequence was 5'-GCA TAA CAC TAG AGG GTC CA-3', and the reverse sequence was 5'-CAG GTG AAT TTC CCA GGT CGG-3'; and for U6 primer (75 bp long), the forward sequence was 5'-GCT TCG GCA CAT ATA CTA AAAA-3', and the reverse sequence was 5'-CGC TTC ACG AAT TTG CGT GTCA-3'. The primers were synthesized by Suzhou Ruiying Biotechnology Co., Ltd. The PCR kit was from Invitrogen; Thermo Fisher Scientific, Inc. and Suzhou Ruiying Biotechnology Co., Ltd. IQ5 PCR Instrument (Bio-Rad) was used. The PCR amplification reaction was performed as follows: Pre-denaturation at 95˚C for 10 min, 10 cycles of 95˚C for 15 sec and 60˚C for 20 sec, followed by another 35 cycles of 95˚C for 25 sec and 60˚C for 35 sec. The Ct value was calculated for each sample, and the relative expression level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

CACS assessment method. Coronary CT angiography (CTA) was performed in all patients using a Siemens SOMATOM Definition AS+ 64 Rows/128 Slices CT scanner. The phase of cardiac cycle with the best vessel visibility was chosen for image reconstruction. The image had a slice thickness of 0.625 mm. The coronary arteries comprise the left main trunk, the anterior descending branch, the circumflex branch, and the right coronary artery. The scan covered the area from the base to the apex of the heart. After data processing, images were reconstructed at 55% of the R-R interval using B35f convolution kernel, and the reconstructed slice thickness was 3 mm with an increment of 3 mm. Detection and quantitative analysis of coronary calcification plaques were performed using CaScoring software. There are three methods: Agatston integral method (AS), volume integral method (VS), and mass integration method (MS). The areas of calcified plaques were marked in each branch of the coronary arteries. AS, VS, MS of the left main trunk, the anterior descending branch, the circumflex branch, and the right coronary artery, as well as the total score, were obtained automatically. The calcification of the diagonal branch belongs to the left anterior descending branch, and the calcification of the obtuse round branch belongs to the left circumflex. The sum of the above four coronary artery calcification scores was calculated.

Statistical analysis. Statistical analysis was performed using the SPSS 17.0 software. The Chi-square test was used for comparison of rates, and the odds ratio (OR) for the evaluation. OR=1 showed that the factor had no effect, OR >1 indicated a risk factor, and OR <1 indicated a protective factor. Mean ± standard deviation was used for quantitative data (including ratio), Kolmogorov-Smirnov was used to detect the normal distribution of data, and t-test for comparison of normal distribution between two groups, analysis of variance was used among multiple groups (SNK method for pairwise comparison), and Mann-Whitney U test was to compare the non-normal distribution between groups. Spearman correlation test were conducted. A difference was statistically significant at $P<0.05$.

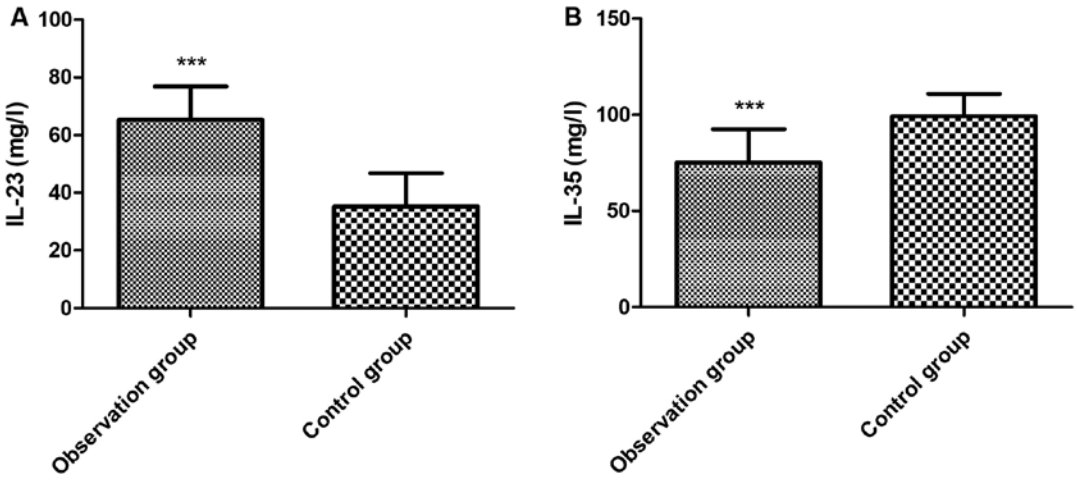

Figure 1. Comparison of IL-23 and IL-35 expression levels. (A) Comparison of IL-23 expression levels between two groups. (B) Comparison of IL-35 expression level between two groups. IL, interleukin. ***P<0.05.

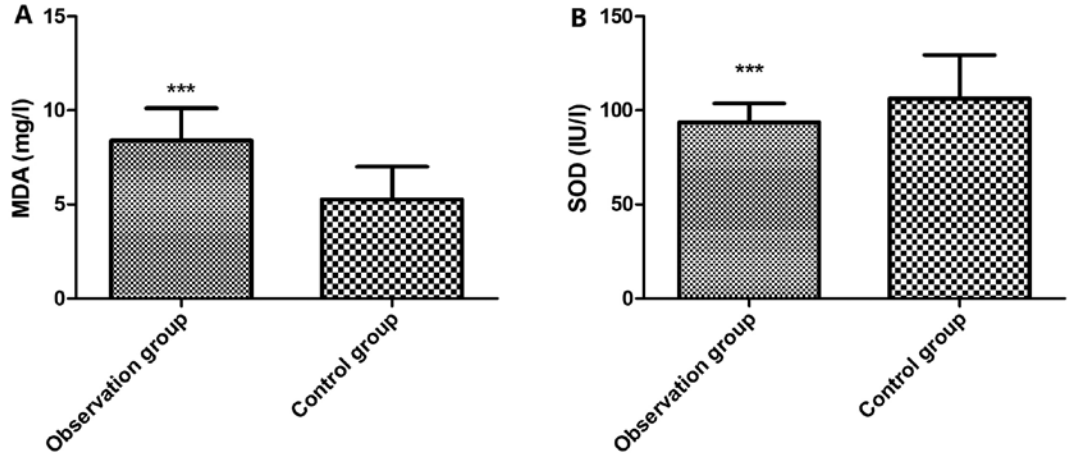

Figure 2. Comparison of MDA and SOD expression levels. (A) Comparison of MDA expression levels between two groups. (B) Comparison of SOD expression level between two groups. MDA, malondialdehyde; SOD, superoxide dismutase. ***P<0.05.

Results

Comparison of IL-23 and IL-35 expression levels. As shown in Fig. 1, IL-23 data in the two groups were normally distributed. The differences in IL-23 (65.32±6.39 vs. 35.26±6.28 mg/l, t=8.31, P=0.001) were statistically significant. The IL-35 data in the two groups were not normally distributed, and the Mann-Whitney U test was applied. The differences in IL-35 (75.26±6.84 vs. 99.36±9.21 mg/l, Z=12.298, P<0.001) were statistically significant. Expression levels of both IL-23 and IL-35 in the observation group were higher than those in the control group.

Comparison of MDA and SOD expression levels. As shown in Fig. 2, the data of MDA and SOD in the two groups were not normally distributed, and Mann-Whitney U test was applied. The differences in MDA (8.39±2.34 vs. 5.27±0.88 mg/l, Z=9.40, P<0.001) and SOD (95.24±13.94 vs. 106.33±14.29 IU/l, Z=7.689, P<0.001) were statistically significant. In the observation group, the MDA level was higher while the SOD level was lower than the corresponding level in the control group.

Comparison of expression levels of miR-126 and miR-146a. As shown in Table I, Figs. 3 and 4, the expression levels of miR-126 and miR-146a in the observation group were significantly higher than those in the control group. The difference in expression level of miR-126 and miR-146a between the two groups were statistically significant.

Comparison of levels of IL-23, IL-35, MDA, SOD, miR-126 and miR-146a at different stages of disease severity in the observation group. As shown in Table II, the difference in levels of IL-23, IL-35, MDA, SOD, miR-126 and miR-146a in serum of patients with coronary heart disease at different stages of severity was statistically significant.

Correlation analysis between CACS and level of IL-23, IL-35, MDA, SOD, miR-126 and miR-146a in the observation group. As shown in Table III, a Spearman correlation test was conducted and showed positive correlation between CACS and IL-23, MDA, miR-126 and miR-146a, respectively (P<0.05); while a negative correlation existed between CACS and IL-35, SOD (P<0.05) in the observation group.

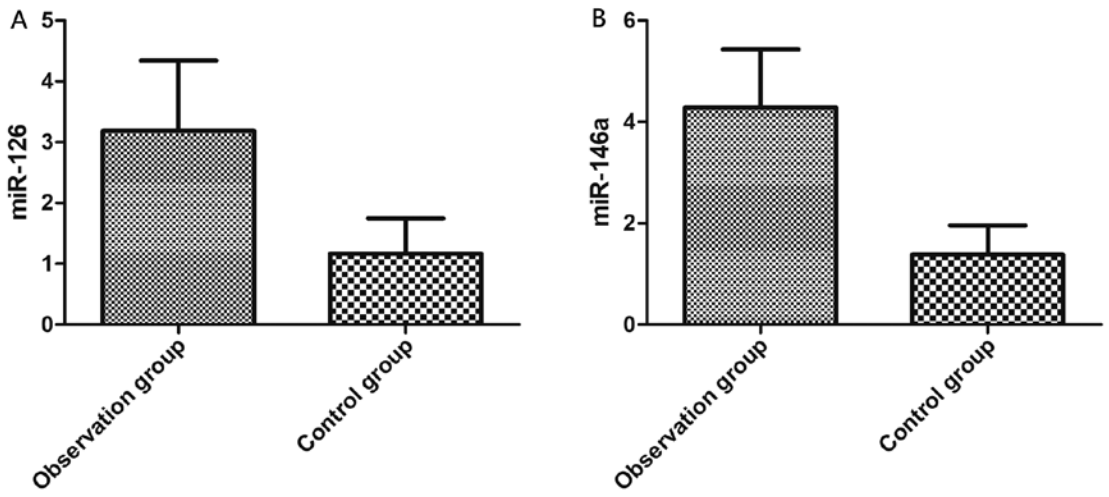

Figure 3. Comparison of expression levels of miR-126 and miR-146a. (A) Comparison of miR-126 expression levels between two groups. (B) Comparison of miR-146a expression levels between two groups.

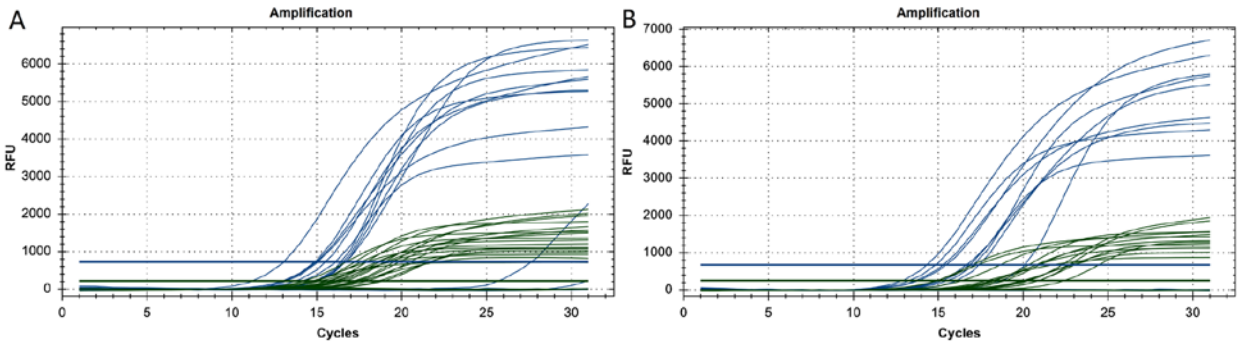

Figure 4. Expression of miR-126 and miR-146a in the two groups. (A) Expression of miR-126. (B) Expression of miR-146a.

Table I. Comparison of expression levels of miR-126 and miR-146a.

Items	Observation group	Control group
Patient no.	192	69
miR-126	3.19±0.25	1.17±0.20
t value	4.11	
P-value	0.024	
OR value	3.87	
95% CI	1.002-5.654	
miR-146a	4.28±0.54	1.38±0.49
t value	5.15	
P-value	0.011	
OR value	2.65	
95% CI	1.452-3.062	

OR, odds ratio.

Discussion

Coronary atherosclerosis with calcification is the result of disease progression of atherosclerotic plaques. In clinic, the severity of atherosclerotic plaques is often used as an important indicator of disease progression. Symptoms of coronary heart disease are often not evident in its early development. When the disease progresses to a more serious stage, it often leads to myocardial infarction and arrhythmia in clinic. Therefore, an early screening of the disease using objective indicators is important. Detection and quantitative analysis of coronary calcification are of great value in early diagnosis of the lesion and early preventive intervention. Interleukins are a group of cytokines that are involved in the formation of macrophages, and have obvious effects on formation of cholesterol crystals. In addition, some members of the interleukin family are involved in the body's oxidative stress response, such as IL-17, IL-23 and IL-35, of which IL-17 and IL-35 have the strongest effects. Jing *et al* (10) reported that IL-23 was upregulated in a mouse model of kidney injury and was associated with the body's inflammatory response and oxidative stress response. MDA and SOD are classic oxidative stress indicators. When the body is in an oxidative stress state, there is an increase in oxidation products such as MDA (11), which aggravate the damage to cells and tissues. Moreover, oxidative stress also render the deformability of neutrophils and weaken macrophages, induce activation of nuclear factor and activated protein-1, and modulate the release of inflammatory mediators. As a result, macrophages and damaged

Table II. Comparison of levels of IL-23, IL-35, MDA, SOD, miR-126 and miR-146a at different stages of disease severity in the observation group.

Item	Disease severity			F-value	P-value
	Single-vessel disease	Two-vessel disease	Three-vessel disease and above		
Case no.	50	11230			
IL-23	60.59±6.31	66.23±9.26	72.35±9.84	5.39	0.005
IL-35	85.02±10.31	75.98±12.30	65.31±11.97	6.23	<0.001
MDA	7.45±2.37	8.30±1.30	9.31±1.29	4.30	0.012
SOD	104.31±25.37	95.01±20.17	80.13±16.39	5.31	0.006
miR-126	2.54±0.37	3.27±0.64	4.12±0.54	2.95	0.025
miR-146a	3.51±0.64	4.20±0.71	5.34±0.81	3.02	0.021

IL, interleukin; MDA, malondialdehyde; SOD, superoxide dismutase.

Table III. Correlation between CACS and related biomarkers in serum of patients with coronary heart disease.

Biomarker	Correlation coefficient (r)	P-value
IL-23	0.46	0.025
IL-35	-0.49	0.020
MDA	0.51	0.016
SOD	-0.48	0.022
miR-126	0.53	0.013
miR-146a	0.54	0.012

CACS, Coronary artery calcium score; IL, interleukin; MDA, malondialdehyde; SOD, superoxide dismutase.

cells are induced to release a large number of oxygen free radicals and a variety of mediators, aggravating the imbalance of local protease/anti-protease and oxidation/anti-oxidation, which causes a vicious circle. In recent years, association of miRNA with coronary heart disease and coronary atherosclerosis has drawn considerable attentions from researchers. miRNAs are non-coding small RNA molecules that regulate lipoprotein metabolism and are associated with plaque formation (12). Both miR-126 and miR-146a are not only involved in inflammatory response and oxidative stress response, but also modulate macrophage functions (13).

The present study is CACS-related experimental research, in which correlations between CACS and inflammatory factors and related oxidative stress indicators were explored. The results showed that expression levels of IL-23, MDA, miR-126 and miR-146a were significantly higher, while the IL-35, SOD levels were significantly lower in serum of patients with coronary heart disease than those in the control group. Above findings suggested that abnormal expression of IL-23, IL-35, MDA, SOD, miR-126 and miR-146a is an important factor in promoting lesion formation. IL-23 is an important pro-inflammatory cytokine involved in immune response. IL-35 inhibits inflammatory response. Both are associated with the modulating effects of IL-1 (14,15). Studies have shown that overexpression of IL-1 can induce the release of higher levels of IL-23 by hepatocytes and macrophages, and cause disorders of lipid metabolism, triggering formation of an inflammatory microenvironment (16,17). It was also reported that IL-23 is overexpressed in local tissues of atherosclerotic plaques, while IL-35 expression is low, suggesting that IL-23 and IL-35 are associated with the formation of atherosclerotic plaques (18,19). In this study, the results showed that high expression of MDA and low expression of SOD were not only associated with the formation of atherosclerotic plaques, but also associated with the severity of the lesions, suggesting that abnormal expression of both markers may have some auxiliary significance for judging the extent of lesions, and can promote onset and progression of coronary heart disease. MDA and SOD are important indicators of oxidative stress in the body. Abnormal expression of both indicators suggested that the formation of atherosclerotic plaques may be associated with oxidative stress. Abnormal expression of MDA and SOD can lead to increased production of oxygen free radicals, aggravating local damage of vascular endothelial cells. Under this condition, platelets, granulation tissue and macrophages start to aggregate, causing local abnormal lipid metabolism and accelerated formation of plaques (20,21). Patients with coronary heart disease may experience high oxidative stress locally or systemically, leading to aggravated damage to local tissue of the arterial wall and increased release of mediators after activation of nuclear factors, including inflammatory mediators and oxygen free radicals. This condition may render imbalanced local redox reaction and promote disease progression. Treatments of coronary heart disease targeting oxidative stress and inflammation may have some effects. Although there are many factors that can modulate the release of cytokines, miRNAs have drawn attention that it may be associated with coronary heart disease. In this study, it was found that miR-126 and miR-146a were associated with the formation and severity of coronary heart disease. Our results were consistent with the findings in literature, in which Wang *et al* (22) found abnormal expression of miR-126 in patient serum. In this study, a clear positive correlation was found between CACS and miR-126 and miR-146a, respectively, suggesting that miR-126 and miR-146a played important roles in disease progression. Both

miR-126 and miR-146a may be promoting factors in disease formation (23,24), exhibiting a modulating effect on local inflammatory microenvironment and oxidative stress (25,26). In particular, IL-35, as a pro-inflammatory factor, forms a 'waterfall effect' to initiate the modulation of related cytokines (27-29). Detection of expression of IL-23, IL-35, MDA, SOD, miR-126 and miR-146a may offer a theoretical insight to diagnosis, prevention and severity assessment of coronary heart disease.

In conclusion, biomarkers IL-23, IL-35, MDA, SOD, miR-126 and miR-146a were abnormally expressed in serum of patients with coronary heart disease, and were found to be correlated with CACS. Detection of changes of related biomarkers in serum may have certain values in diagnosis of disease formation, as well as assessment of disease severity.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DC, ML and YL conceived and designed the study. DC, CJ, YS and DX were responsible for the collection and analysis of the experimental data. DC and ML interpreted the data and drafted the manuscript. CJ and YL revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Third Affiliated Hospital of Qiqihar Medical University (Qiqihar, China). Signed informed consents were obtained from the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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