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Original Article

Profiling of plasma circulating miRNA in coronary heart disease patients detected by next-generation small RNA sequencing

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Abstract: MiRNA levels in plasma have emerged as potential novel noninvasive biomarkers for some diseases. We aimed to identify a miRNA expression profile in plasma of patients with coronary heart disease. The levels of plasma miRNA from a cohort of 49 patients with coronary heart disease and 13 healthy controls were detected by small RNA deep sequencing, the differentially expressed miRNAs were validated using the reverse transcription real-time quantitative polymerase chain reaction from an additional cohort of 135 hospitalized patients and 140 controls. Five miRNAs were differentially expressed, miR-30c-2-3p and miR-5091 were upregulated and miR-125b-5p, miR-501-3p, and miR-31-5p downregulated in plasma of patients with coronary heart disease than in controls. This study identified 5 plasma miRNAs that might play an important role in the pathogenesis of coronary heart disease. The level of miR-30c-2-3p was 132 folds upregulation (P=0.000), it suggests that miR-30c-2-3p may be a potential noninvasive biomarker for the diagnosis of coronary heart disease, however, with further research.

Keywords: miRNA, plasma, profiling, coronary heart disease

Introduction

Coronary heart disease (CHD), involving atherosclerosis and myocardial infarction, is a leading cause of morbidity and mortality in the world. Although, pathogenesis of CHD is still not fully understood, it is considered that vascular endothelial cell injury, platelet reactivity, vascular smooth muscle cell proliferation, lipid infiltration, and increased synthesis of connective tissue are the main pathological processes of CHD [1]. Forty-six CHD susceptibility loci identified through GWAS do not appear to be mediating risk through effects on traditional risk factors, such as blood pressure and lipid levels, in subjects of European descent [2]. MicroRNA (miRNA) is reported as a group of short, single-stranded, noncoding RNA that can function as endogenous RNA interference to regulate expression of the targeted genes. MiRNAs play an essential role in fundamental biological processes such as proliferation, apoptosis, differentiation and homeostasis by decreasing mRNA stability or inhibiting translation [3]. To date, more than 2000 human miRNAs have been reported in the RNA database [4]. MiRNAs are found in plasma, serum and other body fluids, and exist in a stable extracellular form of the Ago2-miRNA complex and/or encapsulation in exosomes [5]. Effective predictive methods are important for reducing the burden of CHD on public health and its costs. Coronary angiography and coronary artery computed tomography are currently efficient but expensive approaches for diagnosis of CHD. Therefore, a cheap and convenient method for predicting CHD is urgently needed. Some studies have been undertaken searching for serum miRNA profiles being evaluated for cancer classification and prognosis [6]. A few studies have been undertaken exploring for miRNA expression in blood from patients. The aim of this study was to character-

Table 1. Baseline clinical characteristics of CHD patients recruited in the small RNA sequencing cohort

Characteristics	Patients (n=49)	Controls (n=13)	*P*-value
Gender (male/female)	37/12	10/3	0.916[a]
Age	57.6±13.5	56.5±13.8	0.283[b]
Systolic blood pressure (mmHg)	134.1±25.9	120.4±16.9	0.040[b]
Diastolic blood pressure (mmHg)	77.3±16.6	73.9±13.4	0.026[b]
Body mass index	25.2±3.2	23.8±0.8	0.019[b]
Fasting plasma glucose (mmol/L)	7.8±2.9	4.9±1.0	0.008[b]
Total cholesterol (mmol/L)	4.9±1.5	4.6±0.9	0.049[b]
Triglycerides (mmol/L)	1.7±1.5	1.3±0.6	0.017[b]
Smoking (past or current)	40.8%	38.5%	0.879[a]
Drinking	8.1%	7.7%	0.954[a]
Hypertension (past history)	49.0%	23.1%	0.094[a]
Diabetes mellitus (past history)	26.5%	7.7%	0.148[a]
Dyslipidemia (past history)	61.2%	15.4%	0.003[a]

[a]: Chi square test; [b]: *t* test.

ize miRNA profiles in plasma from CHD patients. Plasma samples from CHD patients and healthy individuals in a Chinese Han population were assessed for miRNA expression. We characterize a plasma miRNA profile in CHD that may serve as a non-invasive approach in early diagnosis for CHD in the future.

Materials and methods

Patients and controls

A cohort of 49 hospitalized CHD patients (baseline clinical characteristics summarized in **Table 1**) and 13 healthy volunteers were recruited in next-generation small RNA sequencing study, an additional cohort of 135 hospitalized CHD patients (baseline data summarized in **Table 2**) and 140 controls were recruited in quantitative reverse transcription-polymerase chain reaction analysis during June 2013 to July 2014 from Shenzhen Baoan Hospital, Southern Medical University. All CHD cases were diagnosed according to the following requirements: coronary angiography showed that stenosis of more than 50% was present in at least 1 of 3 main blood arteries (left circumflex artery, right coronary artery, and left anterior descending coronary artery) and stenosis can be revealed in 2 left main coronary arteries; patients suffered from symptoms of unstable angina pectoris: angina was present when at rest and lasted over 20 minutes, or newly occured (<2 months) severe angina, or aggravation angina with increased intensity, duration, and frequency; clinical symptoms of myocardial infarction appeared, such as consistent and intense chest pain over 30 minutes, characteristic change of electrocardiograph (e.g., ST segment of 2-3 adjacent leads depressed or elevated for ≥1 mm, and left bundle branch block emerged) and abnormal rise of myocardial markers [7]. Cases with coronary artery bypass surgery were also considered as CHD patients. Patients with cardiomyopathy, congenital heart disease, renal disease, liver disease, connective tissue disease, and tumors were excluded. Healthy volunteers had no history or any symptom of CHD. As control, we used healthy control subjects without other documented disorders. Physical examination, laboratory tests, ECG and other preliminary tests were performed for these control subjects. The study was approved by the ethics committee of Shenzhen Baoan Hospital, Southern Medical University. Written consent was obtained from all patients and control individuals.

RNA isolation

Five-ml peripheral blood was collected from each participant using a drying tube containing EDTA as anticoagulant. The whole blood was separated into plasma and cellular fractions by centrifugation at 3,000 rpm and 4°C for 10 min, followed by 12000 rpm for 15 min to completely remove cell debris. Plasma was again centrifuged at 25,000 rpm and 4°C for 10 min to remove additional cellular nucleic acids attached to cell debris, then kept plasma frozen at -80°C. Total RNA isolation from plasma samples was performed with the miRNeasy serum/plasma kit (Qiagen) according to the manufacturer's protocol. After the initial denaturizing step, we spiked in synthetic C.elegans miR-39 for all plasma in order to control variations in RNA extraction and purification procedures because of the absence of homologous sequences in humans. To assess the quality of

Table 2. Baseline clinical characteristics of CHD patients enrolled in the validated cohort

Characteristics	Patients (n=135)	Controls (n=140)	*P*-value
Gender (male/female)	100/35	102/38	0.819[a]
Age	57.9±12.9	57.5±13.1	0.929[b]
Systolic blood pressure (mmHg)	130.2±23.6	118.6±14.8	0.008[b]
Diastolic blood pressure (mmHg)	75.9±16.3	73.6±13.1	0.032[b]
Body mass index	25.1±3.1	23.1±0.6	0.006[b]
Fasting plasma glucose (mmol/L)	7.5±2.7	4.8±0.9	0.000[b]
Total cholesterol (mmol/L)	4.9±1.2	4.7±0.9	0.035[b]
Triglycerides (mmol/L)	1.6±1.2	1.2±0.6	0.002[b]
Smoking (past or current)	43.7%	35.7%	0.176[a]
Drinking	8.1%	7.1%	0.754[a]
Hypertension (past history)	48.1%	12.1%	0.000[a]
Diabetes mellitus (past history)	25.9%	2.9%	0.000[a]
Dyslipidemia (past history)	64.4%	14.3%	0.000[a]

[a]: Chi square test; [b]: *t* test.

RNA, the purified RNA was measured with using Agilent 2100 Bioanalyzer and ABI Step-One-Plus real-time PCR.

Small RNA sequencing

Sixty-two smRNA libraries were constructed from RNAs of 49 CHD patients and 13 healthy volunteers by using a smRNA sample prep kit (Illumina, USA) following the standard protocols. Briefly, the appropriate fractions ranged from 18nt to 30nt were separated, purified via 15% (w/v) denaturing polyacrylamide gel electrophoresis and then linked to RNA adaptors (5'-GTTCAGAGTTCTACAGTCCGACGATC and 3'-TCGTATGCCGTCTTCTGCTTG) using T4 RNA ligase and were subsequently reverse transcribed into cDNA by Superscript II Reverse Transcriptase. The cDNA fragments were amplified, and the RT-PCR products were sequenced by the Illumina Hiseq 2000 (Illumina, San Diego, CA, USA) according to Illumina's protocol at Beijing Genomics Institute-Shenzhen.

Data analysis

The smRNA sequencing data were analyzed referring to the methods described in the previous publications [8]. Briefly, after deleting 5' adaptor and trimming 3' acceptor sequences, filtering low quality reads (Q<10, the quality value was calculated by Q=ASCII character code-64) and cleaning up contaminated reads, the occurrence of each unique read was counted as a tag, the qualified unique tags were used to analyze length distribution and were mapped to the human genome GRCh37.p5 by introducing SOAP version2.0 ultrafast tool (http://soap.genomics.org.cn). Only tags with no more than one mismatch were picked out for further analysis. The conserved known miRNAs were identified by aligning the mapped tags to miRBase tool in Homo sapiens, and the tags not matched in miRBase were further aligned against the sequences of other non-coding RNAs (rRNAs, tRNAs, snRNAs, snoRNAs, scRNAs) presented on Rfam (http://rfam.sanger.ac.uk/) database, repeats database as well as piRNA database. The known miRNAs were annotated by aligning to miRBase 18.0 (http://www.mirbase.org/index.shtml). The remaining reads that cannot be annotated were used to predict novel miRNAs by Mireap (http://sourceforge.net/projects/mireap/). In consideration of a few smRNA tags might map to more than one category, this study followed the priority rules described in the previous publication to guarantee that every unique smRNA was mapped to unique annotation as follows: miRNA, Rfam, repeats, piRNA and mRNA [9]. All miRNA profiles were normalized using the geometric mean for each sample over all miRNAs. Profiles of the most differentially expressed miRNAs were subjected to hierarchical clustering to create a heat map. The predicted target genes of differentially expressed and miRNAs were subjected to analysis of gene ontology terms for gene ontology (GO) analysis. The predicted miRNA targets were mapped to the signaling pathway annotation databases downloaded from KEGG (http://www.genome.jp/kegg/) for pathway analysis.

Quantitative RT-PCR assay

To confirm the expression of miRNAs identified by the deep sequencing approach, reverse

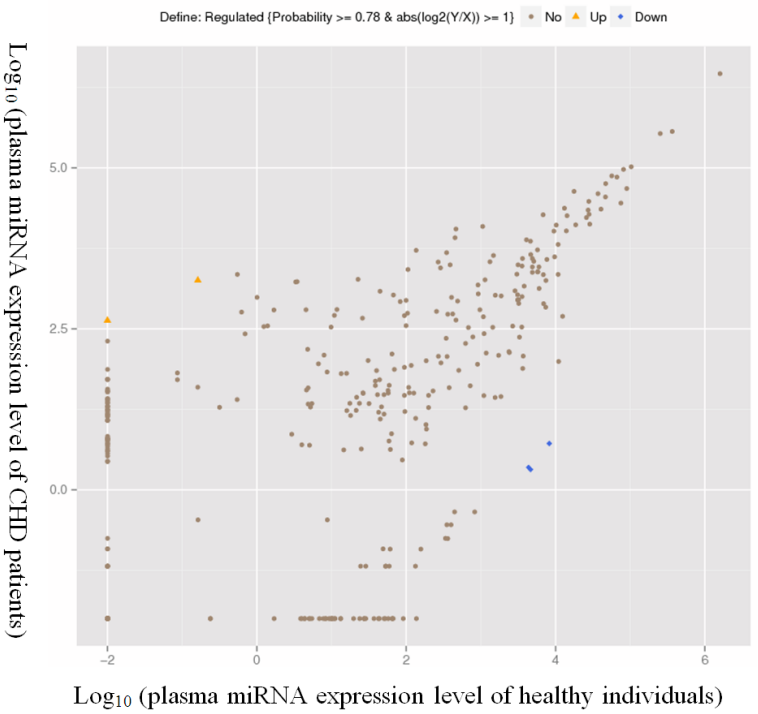

Figure 1. The miRNA expression profiles in CHD patients and healthy individuals are presented by next-generation RNA sequencing technology.

Table 3. Summary of differentially expressed plasma miRNAs by next-generation RNA sequencing technology[a]

miR ID	log_2Ratio (CHD/healthy)	Probability	Status[b]
miR-30c-2-3p	16.37457256	0.80200747	Upregulated
miR-5091	15.67550423	0.797619048	Upregulated
miR-125b-5p	-10.62370057	0.795238095	Downregulated
miR-501-3p	-10.93135424	0.791923436	Downregulated
miR-31-5p	-9.953194374	0.783753501	Downregulated

[a]: RNA sequencing cohort including 49 CHD patients and 13 healthy controls;
[b]status: probability ≥0.78 & abs (log_2 (Y/X)) ≥1.0 as regulated.

Table 4. Summary of differentially expressed plasma miRNAs by real-time PCR

miR ID	Fold-change (CHD/healthy)	*P*-value	Status
miR-30c-2-3p	132	0.000	Upregulated
miR-5091	3.78	0.013	Upregulated
miR-125b-5p	-6.23	0.000	Downregulated
miR-501-3p	-16.0	0.000	Downregulated
miR-31-5p	-14.4	0.000	Downregulated

transcription real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed using the total RNA samples from another 135 hospitalized CHD patients and 140 controls. The expressions of miRNAs were quantified by All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, USA) following reverse transcription according to the manufacturer's instructions. Reactions were loaded onto a 96-well plate and run in triplicate on a LightCycler 480 Real-Time PCR System (Roche). The reactions were firstly incubated at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 20 seconds, then 10 seconds of extension at 72°C. A melting curve analysis was performed immediately after qRT-PCR cycling to monitor the qRT-PCR reaction. The primers used for qRT-PCR in this study are as following: miR-30c-2-3p: CTGGGAGAAGGCTGTTTACTCT, miR-5091: CTGGGAGAAGGCTGTTTACTCT, miR-125b-5p: TCCCTGAGACCCTAACTTGTGA, miR-501-3p: AATGCACCCGGGCAAGGATTCT, miR-31-5p: AGGCAAGATGCTGGCATAGCT, RNU6-2: CAAAGTCAGTGCAGGTAGGCTTA and SNORD44: CCTGGATGATGATAAGCAAATGCTG. The snRNA levels of RNU6-2 and SNORD44 were used as internal controls. MiRNA expression was normalized to snRNAs of RNU6-2 and SNORD44 and to get the relative abundance. Averages of five independent experiments each performed in triplicate with standard errors were presented. The delta Ct method was used to determine relative number of copies of miRNA. To search for differentially expressed miRNAs between CHD group and healthy group, delta Ct values from two groups were evaluated using the t-Student test. Quantitative data from qRT-PCR were compared using unpaired t-tests. $P<0.05$ was considered statistically significant.

Table 5. GO analysis for predicted targets of differentially expressed plasma miRNA between CHD patients and healthy individuals

miR ID	Targeted genes
miR-30c-2-3p	CCL22, SSR2, GAGE12B, MS4A14, SHISA6, MOB3A, ZNF880
miR-5091	AL590235.1, FN3K, C6orf223, MIF4GD, C1orf147, ARPC5L, C10orf85, TNFRSF4, TMEM161A, BCL2
miR-125b-5p	FAM169B, MTFP1, ARMC7, EPO, RP4539M6.19, CXCL13, DRAM2, GCNT1, RFXANK, RTP1
miR-501-3p	TMEM213, RPRD1B, TMSB15A, KCTD9, TMEM170B, ZNF488, MYNN, SUM01, CLEC4D, SLC26A6
miR-31-5p	RNF144B, RSBN1, KCNJ6, SH2D1A, AK4, AC022498.1, KRTAP13-4, DKFZP779L 1853, PAX9, HAAO

Results

Overview of small RNA sequencing data

To determine the small RNA profile in plasma of CHD patients, we sequenced 62 (49 CHD patients and 13 healthy individuals) small RNA libraries using Solexa next-generation sequencing technology. The majority of these clean reads was 22nt in length, with sizes varying between 18nt and 30nt after removing the adaptor sequences and low quality reads. These clean reads were mapped to several filter databases, such as the Human Genome, rRNA, tRNA and Rfam sequence databases, and were subsequently mapped to miRbase (V14.1). After detecting other types of small RNAs, including rRNAs, snRNAs, snoRNAs, and tRNAs, 358 unique tags corresponding to 167419 reads in healthy individuals and 374 unique tags corresponding to 198554 reads in CHD patients were identified as known miRNAs.

Plasma miRNA differentially expressed in CHD patients and healthy individuals

To investigate possible differentially expressed plasma miRNAs in CHD patients and healthy individuals, the counts of each kind of miRNA were normalized based on the total number of all the clean reads mapped on the genome, and then compared between the CHD patients and healthy individuals. Five known miRNAs were differentially expressed between plasma from CHD patients and healthy individuals (**Figure 1**). Of these 5 miRNAs, miR-30c-2-3p and miR-5091 were upregulated, miR-125b-5p, miR-501-3p, and miR-31-5p were downregulated (**Table 3**). To validate the expression of these differentially expressed known miRNAs detected by next-generation sequencing technology, five differentially expressed miRNAs were chosen for quantification by real-time PCR. Real-time PCR showed that expression of plasma miR-30c-2-3p and miR-5091 were upregulated, miR-125b-5p, miR-501-3p, and miR-31-5p were downregulated in the CHD patient (**Table 4**). The levels of differentially expressed plasma miRNAs measured by quantitative real-time PCR were consistent with the results obtained from next-generation sequencing technology.

Prediction of the miRNA targeted genes and pathways

After the investigation of these five known miRNAs that were differentially expressed between CHD patients and healthy individuals, we then predicted the targeted genes that could potentially be targeted by these miRNAs using Targetscan. After prediction of the miRNA targeted genes, we found that five predicted targeted genes of differentially expressed miRNAs appeared to be involved in a broad range of biological processes (**Table 5**). Most of the targets related to metabolic process (e.g., macromolecule metabolic process, primary metabolic process and cellular metabolic process), cellular component organization or biogenesis (e.g., cell junction assembly, cell morphogenesis, multicellular organismal process), cellular process (e.g., cellular protein modification process, cell differentiation, cell maturation), developmental process (e.g., cardiac muscle tissue development, muscle tissue morphogenesis, mesoderm formation), cell proliferation, and reproduction (**Figure 2**).

Discussion

Both genetic and environmental factors cumulatively contribute to CHD risk in human populations. Recent studies have leveraged some genetic variations to identify multiple sites of CHD susceptibility; however, the causal mechanisms largely remain elusive [10]. MiRNA, first described in 1993, regulate gene translation by binding at complementary mRNA sequences,

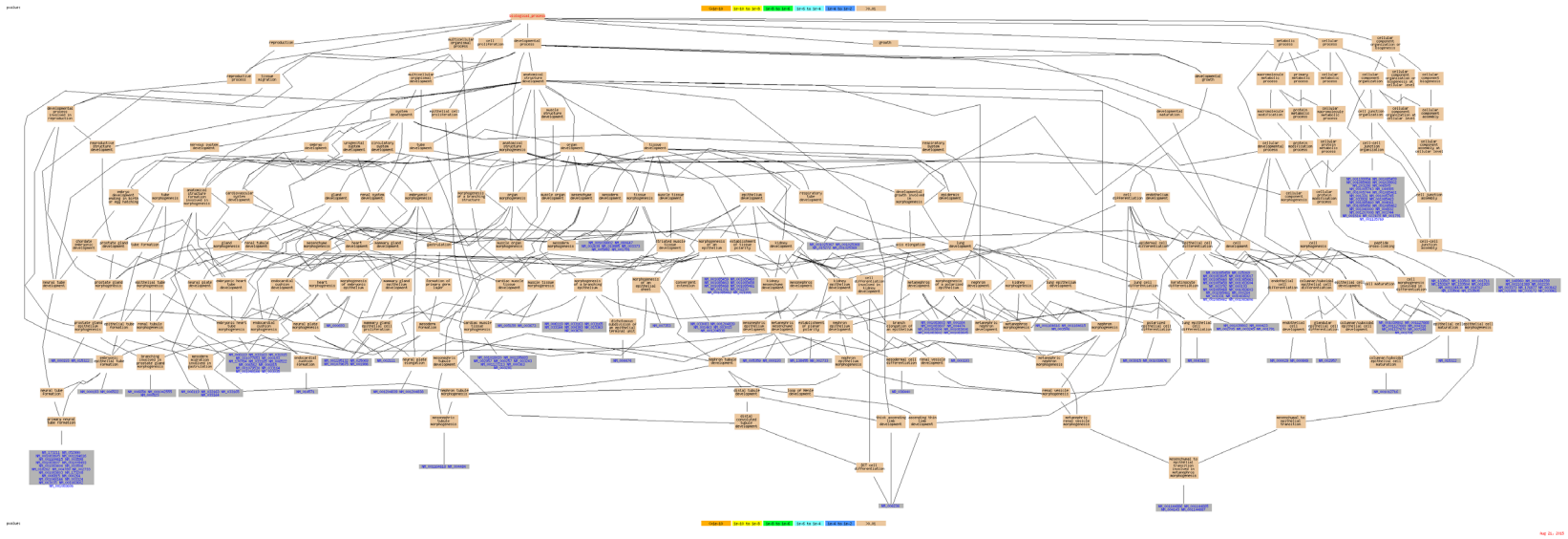

Figure 2. KEGG pathways involving miR-30c-2-3p, miR-5091, miR-125b-5p, miR-501-3p, and miR-31-5p.

then initiating mRNA cleavage or obstructing mRNA incorporation [11]. Studies have found that miRNA modulates a wide range of pathophysiological processes such as tumorigenesis, cardiovascular diseases, pain, viral infections, cellular proliferation, cellular differentiation, and apoptosis during the last decade [12].

Our study from the plasma miRNA expression profile of CHD patients found that miRNAs were differentially expressed between plasma from CHD and healthy group. Among differentially expressed miRNAs, miR-30c-2-3p and miR-5091 were upregulated. MiR-30 family members have been implicated in cancer, myocardial matrix remodeling, cellular senescence, osteoblast differentiation, adipogenesis, and epithelial-to-mesenchymal transition [13]. In vitro, overexpression miR-30 promotes angiogenic sprouting in epithelial cells. MiR-30 is among the most highly expressed miRNAs in cardiac myocytes [14]. A previous study showed that miR-30 downregulate connective tissue growth factor, a key profibrotic protein, thereby establish an important role for it in the control of structural changes in the extracellular matrix of the myocardium [15]. In our study, the level of plasma miR-30c-2-3p was 132 folds upregulation, it suggests that dysregulation of miR-30c-2-3p in the heart may contribute to myocardial matrix remodeling after myocardial infarction for CHD patients. Molecular markers have the potential to help in the disease diagnosis and treatment selection. Previous studies have identified a few serum markers, such as cardiac troponins, myoglobin, heart fatty acid binding protein, and glycogen phosphorylase BB are used to help in establishing the diagnosis of myocardial infarction [16]. New markers of early detection of myocardial damage are is still on. Our study identified that plasma miR-30c-2-3p was high upregulated between CHD patients and healthy controls, thereby it points to the possibility of miR-30c-2-3p as a circulating molecular marker in the diagnosis of CHD. Therefore, more studies from larger CHD patient groups will be required, which will allow to determine the cutoff values to classify the plasma miR-30c-2-3p expression level as “high” or “low”.

This study showed that plasma miR-5091 was 3.78-fold upregulated in CHD patients. The miR-5091 targeted genes predicted by Targetscan contain FN3K, MIF4GD, TNFRSF4, and BCL2, and so on. The FN3K gene encodes an enzyme which catalyzes the fructosamines phosphorylation which may result in deglycation [17]. The MIF4GD protein interacts with the N-terminus of the stem-loop binding protein and the 3’ end of histone mRNA, thereby, this interaction facilitates the activation of histone mRNA translation [18]. The TNFRSF4 protein is a member of the TNF-receptor superfamily shown to activate NF-kappaB signaling pathway [19]. The BCL2 gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of lymphocytes [20]. How the miR-5091 regulates the FN3K, MIF4GD, TNFRSF4, and BCL2 genes, thereby leading to CHD keeps unknown.

MiRNA can regulate cardiac and skeletal muscle function in both development and disease. It is noteworthy that miR-125b-5p have been shown to be functionally involved in cardiovascular diseases such as acute myocardial infarction, heart failure, and hypertrophy [21]. MiR-125b-5p is an abundant miRNA present in cardiac valve, and has been shown to protect cardiac muscle from ischemia/reperfusion injury by reducing infarct size by 60%. In addition, miR-125b-5p has been associated with atherosclerosis and CHD development [22]. The plasma level of miR-5091 was significantly lower in patients with CHD when compared with healthy controls in this study. Our study is consistent with previous studies [23]. It suggests that miR-5091 may be involved in the pathogenesis of CHD, but not be released from the myocardium.

MiR-31-5p had been widely explored in some cancer diseases. Downregulation of miR-31-5p has been found in T-cell non-Hodgkin lymphomas, it was shown to be associated with activation of the nuclear factor κB pathway in these lymphomas [24]. The role of miR-31-5p involving in tumor cellular proliferation, apoptosis, migration and invasion is generally recognized [25]. MiR-31-5p was also decreased in patients with celiac disease, little is known about the significance of miR-31-5p in celiac disease [26]. A previous study suggested that miR-501-3p could regulate GluA1 expression locally in dendrites in rat brains [27]. MiR-31-5p and miR-501-3p are down-regulated in plasma of CHD patients in our study. This suggests that

both of miR-31-5p and miR-501-3p may be closely involved in the pathogenesis of CHD.

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Disclosure of conflict of interest

None.

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