Original Article
Serum long non-coding RNA uc022bqs.1 is a potential biomarker for early diagnosis of human coronary heart disease

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Abstract: Aim: To determine the lncRNA expression profile in serum from patients with coronary heart disease (CHD) and investigate potential clinical significance of specific lncRNA. Methods: The difference of lncRNA expression profile between CHD patients and normal individuals was analyzed by lncRNA microarray. Quantitive real-time PCR (qRT-PCR) was adopted to confirm the expression of representative lncRNA in 30 cases of normal control (NC), 30 cases of stable angina pectoris (SAP), 42 cases of unstable angina pectoris (UAP) and 30 cases of acute myocardial infarction (AMI). The relationship between lncRNA expression levels and clinicopathological factors of patients with CHD was explored in our study. Receiver operating characteristic (ROC) curve was adopted to evaluate the diagnostic efficiency of the specific lncRNAs. Results: LncRNA expression profile showed 224 lncRNAs with expression alteration eight fold or above. The up-regulated and down-regulated candidate lncRNAs were 59 and 165 ($P<0.005$), respectively. The expression of uc022bqs.1 verified by qRT-PCR was consistent with that by microarray analysis. With ROC curves analysis, uc022bqs.1 was found to well distinguish the SAP, UAP and AMI from NC. And the areas under curve (AUC) was 0.796 (95% CI: 0.681~0.911), 0.821 (95% CI: 0.718~0.924), 0.846 (95% CI: 0.795~0.932), respectively. Particularly, the AUC for CHD and NC individuals was 0.895 (95% CI: 0.824~0.965), and the sensitivity and specificity were 79.5% and 83.3%, respectively. Conclusions: Uc022bqs.1 is highly expressed in serum of patients with CHD, and our data suggest that serum uc022bqs.1 can serve as a potential biomarker for early diagnosis of coronary heart diseases.

Keywords: Coronary heart disease, long noncoding RNA, serum, diagnosis

Introduction

Coronary heart disease (CHD) continues to be a major cause of death worldwide. The diagnosis of CHD mainly involves invasive and non-invasive methods. Non-invasive methods include the electrocardiogram, Holter monitor and myocardial nuclide imaging, etc. However, low sensitivity and low specificity were found with these methods in clinical. Currently, coronary angiography is a 'gold standard' of CHD diagnosis. Additionally, CT angiography has been widely used over the last decade [1]. Although the prognosis of CHD has been improved through both diagnostic and therapeutic methods (percutaneous coronary intervention, coronary artery bypass surgery and drug treatment, etc.), the high mortality of CHD still exists. Most

patients were diagnosed as advanced CHD when they first visited, which made them lose the opportunities to receive surgery. Therefore, early detection especially before left ventricular dysfunction, early diagnosis and early treatment of CHD are the key points to improve the cure rate of CHD and to reduce mortality due to CHD [2, 3].

Long non-coding RNAs (LncRNAs) are RNA molecules which are longer than 200 nt and do not encode proteins. It regulates gene expression at both transcriptional and post-transcriptional levels [4, 5]. The functions of lncRNAs are still poorly understood, yet there are a lot of evidence showing that lncRNAs are closely linked with human diseases [6]. LncRNAs are stable in body fluids (blood, plasma, urine, etc.) and

therefore serve as biomarkers in some diseases. For example, a prostate-specific lncRNA PCA3 in urine has been identified as the most specific biomarker for the diagnosis of prostate cancer, showing a higher specificity than the widely used prostate-specific antigen (PSA) test [7]. Another example is that LncRNA AA174084 in gastric juice has been found as indicator of gastric cancer [8]. Innumerable examples are lncRNA biomarkers in plasma include H19 for gastric cancer [9], lncRNA HULC for hepatocellular carcinoma [10], lncRNA HOTAIR for breast cancer and so on [11]. Despite of that, more lncRNAs remain to be disclosed, and specific circulating lncRNAs may be found as CHD biomarkers.

In this study, the serum lncRNAs expression profile in patients with CHD was investigated by microarray analysis, we found one lncRNA, uc022bqs.1, was stable in serum. The expression of uc022bqs.1 was confirmed by qRT-PCR in patients with CHD and normal individuals, then we tested the potential correlations between the serum uc022bqs.1 levels and clinicopathological factors in patients with CHD. Our data demonstrated that serum uc022bqs.1 could be a potential biomarker for early CHD screening.

Materials and methods

Medical records

Serum from 102 CHD patients admitted to the First Affiliated Hospital of Jinan University, China, between August 2014 and June 2015 were obtained. CHD was confirmed by coronary angiography and clinical manifestations. The 102 CHD patients included 30 cases stable angina pectoris (SAP), in which male patients were 27 cases, female patients were 3 cases, mean age (70.52±11.53); 42 cases unstable angina pectoris (UAP), including 22 males and 20 females, mean age (68.67±11.25); 30 cases acute myocardial infarction (AMI), in which 29 males and 1 female, mean age (64.53±13.34). In the meantime, 30 selected healthy people without a history of coronary heart disease were used as normal control (NC) group, including 18 males and 12 females, mean age (59.64±13.72). This study was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University. Written informed consents in accordance with Decla-

ration of Helsinkin were obtained from all subjects.

Serum preparation

Peripheral blood samples were collected into 3 ml drying tubes in the absence of anti-coagulation treatment. Fresh blood samples coagulated for 30 minutes at room temperature were centrifuged at 3500 g for 10 minutes to separate serum from any residual cells and remaining cellular debris. Supernatant were collected into fresh EP tubes and stored at -80°C until further processing.

RNA isolation

Serum samples from CHD patients or NC group were dissolved on ice before total RNA isolation. 200 μl of serum sample was prepared in a 1.5 ml RNase-free EP tube, and then 800 μl of Trizol reagent (Invitrogen, CA, USA) were added to a total volume of 1 ml. After blending, the mixture was incubated for 2 minutes at room temperature. Then 0.2 ml of chloroform was added to the tube and the mixture was homogenized for 15 seconds with votex. The sample was incubated for 15 minutes at 4°C and then centrifuged at 12,000 g for 15 minutes at 4°C to obtain obvious stratification. Next, 500 μl of the top aqueous layer was transferred into a new tube preparing with 0.5 ml of isopropanol refraining from interphase layer. To precipitate the RNA, the mixture was centrifuged at 12,000 g for 20 minutes at 4°C before mixing and keeping at room temperature for 10 minutes. The supernatant were removed and the pellet was washed with 1 ml 75% cold ethanol (diluted with DEPC water). After centrifugation, the supernatant were removed and the pellet was allowed to dry at 50°C for 10 minutes. The dried RNA sample was dissolved with 20 μl of DEPC water and then stored at -80°C until further processing.

Microarray analysis

Isolated RNA was pre-amplified and then analyzed by microarray (Arraystar, Human LncRNA array, version 3.0). LncRNAs were screened by microarray analysis of the RNA samples from CHD patients (n=3) and NC group (n=3). The screening was operated according to the manufacturer's protocol, and this procedure allowed the concurrent detection of 30586 lncRNAs. To
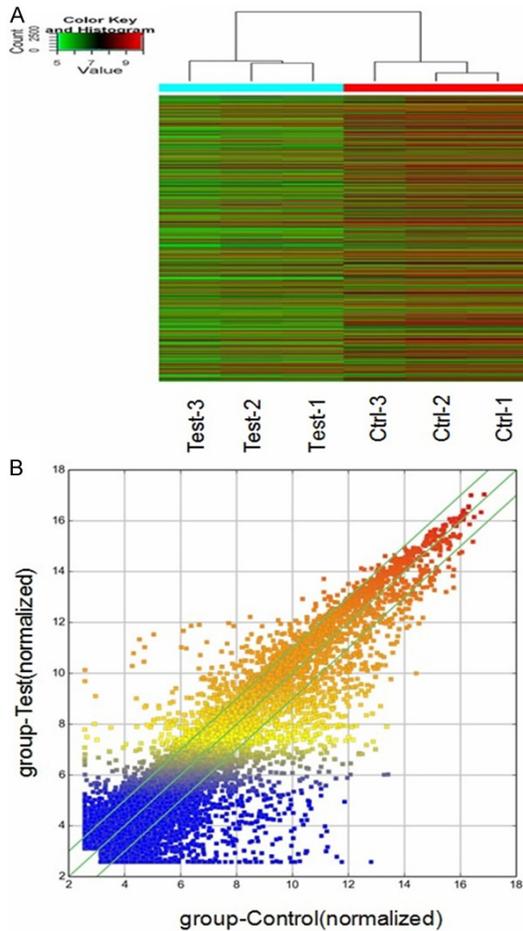
Figure 1. Differential expression of lncRNAs in CHD patients and NC individuals. A. Hierarchical clustering analysis of all lncRNAs that were differentially expressed in the two groups of participants. Expression values are represented in red and green, indicating expression value above and below the median in CHD patients (Test-1, Test-2, Test-3) or NC individuals (Ctrl-1, Ctrl-2, Ctrl-3), respectively. B. Volcano plot of fold change and corresponding *P* value for each lncRNA.

find specific lncRNAs as possible CHD biomarker candidates, the following criteria for up-regulated lncRNAs was adopted in our study: signal intensity >8, fold change >8 and *P*<0.005.

Quantitive real-time PCR (qRT-PCR)

The quantity and quality of total RNA from serum samples were determined by NanoDrop (ND-1000). Approximately 20-50 ng of RNA was obtained from 1 µl RNA dilution and samples were used in following experiment only if the ratio of A260/A280 was between 1.8 and 2.1. No difference in the amount of extracted RNA in a unit of serum samples was found between NC and CHD individuals. Then, 3 µl of purified RNA was used for cDNA synthesis.

According to the manufacturer's recommendations, 20 µl of final reaction mixture containing 10 µl of SYBR Green PCR Master Mix (Vyzame, China), 0.5 µl of sense primer, 0.5 µl of antisense primer, 0.5 µl ROX Reference Dye (50×), 6.5 µl of DEPC water and 2.0 µl of the synthesized cDNA. Primers were designed by Primer Premier 5.0 and synthesized by Sangon Biotech (Sangon, Shanghai, China). Their sequences were as follows: 5'-AACCAGGCGACCCAGACAA-3' (sense) and 5'-TTAGGGACGGATCGGA GAA-3' (antisense) for uc022bqs.1; 5'-CCTGGATACC-GCAGCTAGGA-3' (sense) and 5'-GCGGCGC-AATACGA ATGCCCC-3' (antisense) for human 18 s-rRNA. C_T value was detected by ABI Prism 7000 sequence Detector (Applied Biosystem, CA, USA). And the relative expression level of lncRNA was normalized to internal control 18s-rRNA and calculated by the comparative C_T ($\Delta\Delta C_T$) method.

Statistical analysis

All statistical data were analyzed by Statistical Program for Social Sciences (SPSS) 13.0 software (SPSS, Chicago, IL). The results were reported as mean ± standard deviation (SD) if normal distribution was met. In all statistical analyses, lncRNA levels were log-transformed by taking the base 10 logarithm to account for the skewness of their distributions. The comparison of measurement data between two groups were performed using one-way ANOVA and Least-Significant Difference-t test. Pearson's correlation analyses were employed to verify the relationship between clinical characteristics and serum uc022bqs.1 expression. A receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value for differentiating between CHD and NC. A two-tailed *P* value of 0.05 or less was considered statistically significant.

Results

LncRNA expression profile in the serum of CHD patients

The level of lncRNAs in serum differed significantly between the two groups (CHD *vs.* NC), as illustrated in the hierarchical clustering analy-

Table 1. The information of five selected lncRNAs

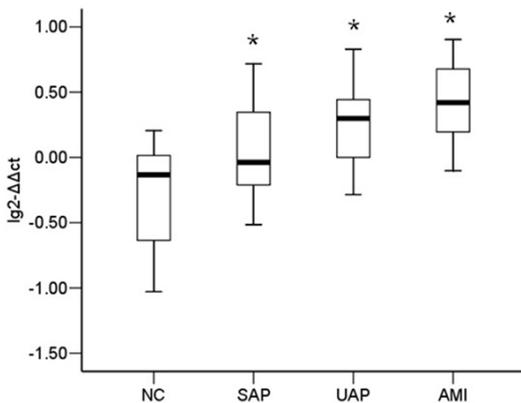| Sequence name	P-value	Fold Change	Regulation	Source	RNA length	Chromosome
ENST00000546135	4.92E-05	38.70	up	GENCODE	254	chr12
ENST00000428009	6.78E-06	112.26	up	GENCODE	4390	Chr20
uc022bqs.1	5.42E-05	90.15	up	UCSC	781	chrM
NR_027275	2.29E-04	79.60	up	knowngene	845	chr16
ENST00000430859	1.28E-05	47.81	up	GENCODE	740	Chr7

Figure 2. The expression of serum uc022bps.1 in NC individuals and CHD patients. SAP patients (0.03±0.33, n=27). UAP patients (0.26±0.29, n=42). AMI patients (0.42±0.29, n=30). NC (-0.29±0.39, n=30), *P<0.05 vs. NC.

Table 2. The correlation between uc022bqs.1 level and clinical parameters of CHD

Clinical parameters	Data (Mean ± SD)	uc022bqs.1	
		r	P-value
Age (year)	65.26±13.18	-0.113	0.360
Chol (mmol/L)	4.25±0.98	-0.088	0.487
TG (mmol/L)	1.59±0.77	0.012	0.407
LDL (mmol/L)	2.42±0.13	-0.067	0.547
Lp-a (mmol/L)	321.98±344.41	-0.074	0.566
BNP (ng/L)	661.21±970.89	0.211	0.185

00430859 (**Table 1**). Then we verified the expression levels of above five lncRNAs through qRT-PCR in CHD patients (n=8) and normal individuals (n=8). It was found that the serum expression levels of uc022bqs.1 was up-regulated in CHD patients, and was in accordance with the microarray results. While the expression levels of the other four lncRNAs were too low to analyze. These results indicated that uc022bqs.1 may be a good candidate biomarker to predict CHD.

Levels of serum uc022bqs.1 is associated with the severity of CHD

When performed normality and homogeneity of variance tests, all the data were skewed distributed, and the variances were not homogeneous. Data were log-transformed by taking the base 10 logarithm to account for the skewness. After the above processing, the treated data were normal distributed and had constant variances. When analyzed by one-way ANOVA and Least-Significant Difference-t test, the differences of serum uc022bqs.1's relative level between each group was statistically significant (F=22.50, P<0.05). At the same time, the serum uc022bqs.1 level in AMI (0.42±0.29, n=30) had a higher level than those in UAP (0.26±0.29, n=42), SAP (0.03±0.33, n=27) and NC (-0.29±0.39, n=30), respectively (**Figure 2**). This proved that the expression level of uc022bqs.1 in serum increased with pathological changes level of CHD with statistical significance (P<0.05), which suggested uc022bqs.1 may serve as a therapeutic target for CHD.

The relationship between uc022bqs.1 level and clinical parameters of CHD

To verify whether the diagnostic ability of uc022bqs.1 was independent of risk factors of CHD, we tested the correlation of uc022bqs.1 expression in 99 samples with age and blood lipid levels of CHD patients by using Pearson's

sis (**Figure 1**). Of the 30586 lncRNAs detected on the microarray, 224 were found to be differentially expressed in CHD patients with the criteria: a fold change >8 and P<0.005. Among them, 59 lncRNAs were up-regulated whereas 165 were down-regulated. To make potential lncRNA biomarkers easy to be measured in clinical, we selected up-regulated lncRNAs as our main study objects. The five lncRNAs of the most significantly up-regulated expression were ENST00000546135, ENST00000428-009, uc022bqs.1, NR_027275 and ENST000-
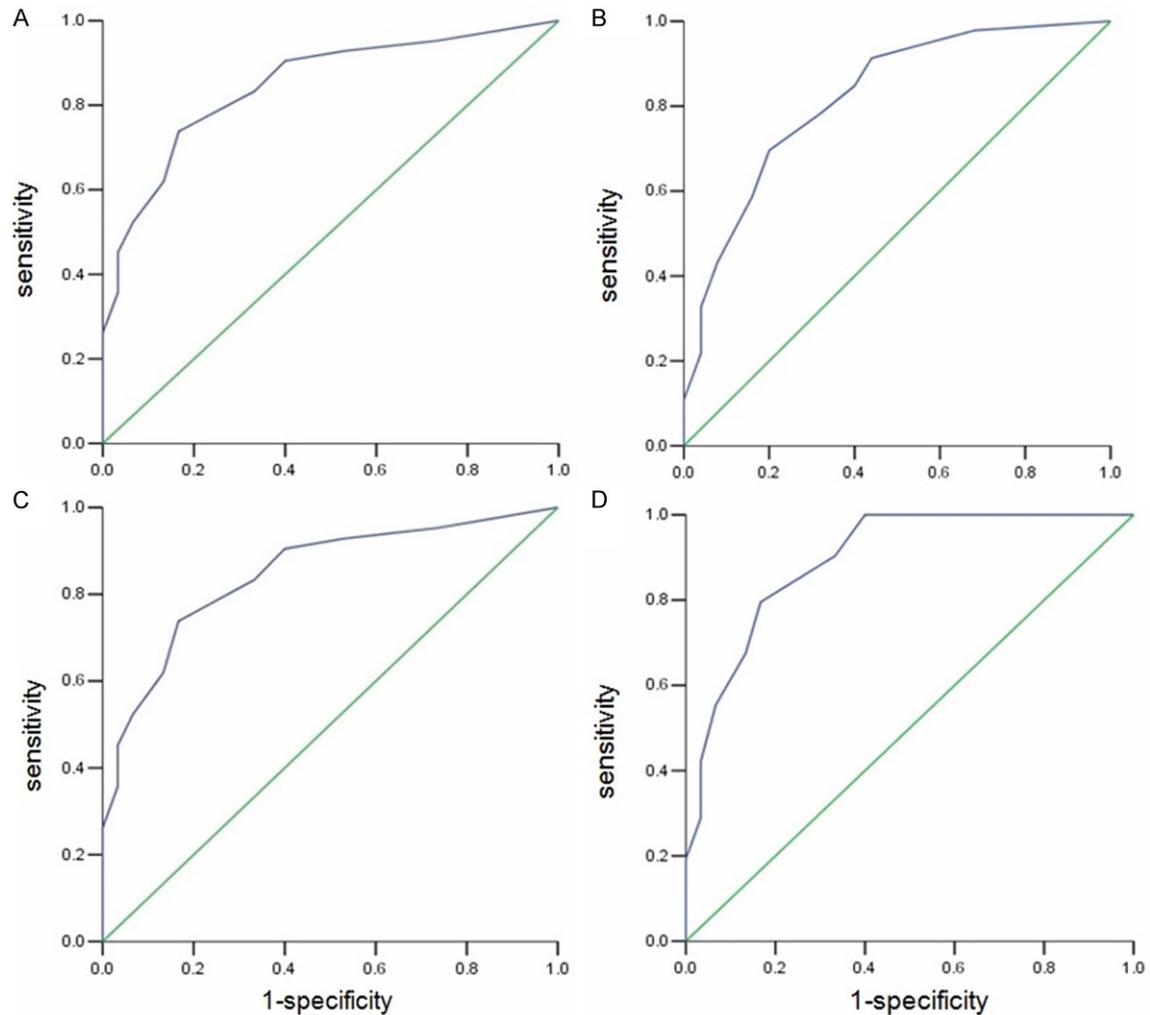
Figure 3. The ROC curves of serum uc022bqs.1 in different groups for the diagnosis of CHD. A. SAP patients (n=27). B. UAP patients (n=42). C. AMI patients (n=30). D. Total CHD patients (n=99).

correlation analysis. Statistical analysis indicated that high uc022bqs.1 expression was not associated with age ($P=0.360$), Chol ($P=0.487$), TG ($P=0.407$), LDL ($P=0.547$), Lp-a ($P=0.566$) or BNP ($P=0.185$), as shown in **Table 2**.

Serum uc022bqs.1 level is sensitive for CHD

ROC curve was used to determine the best positive serum uc022bqs.1 reference values to improve its diagnostic value of CHD. The area under the ROC curve (AUC) of serum uc022bqs.1 for diagnosing SAP, UAP, AMI were 0.730 (95% CI: 0.603~0.857), 0.782 (95% CI: 0.665~0.898), 0.821 (95% CI: 0.723~0.919), respectively. The AUC of serum uc022bqs.1 for diagnosing CHD was 0.895 (95% CI: 0.824~0.965), with the sensitivity and specificity were

89.0% and 76.7%, respectively (**Figure 3**). These suggest that serum uc022bqs.1 may be a potential biomarker for the diagnosis of CHD.

Serum uc022bqs.1 is stable

To be a reliable biomarker, its stability should be taken into consideration. We investigated the stability of uc022bqs.1 in serum through exposing it for various periods at room temperature and freezing and thawing it with different cycles ($P>0.05$) (**Figure 4**). Exposure of the serum to room temperature for 8 h had no effect on uc022bqs.1 expression. Even after 48 h, the uc022bqs.1 expression was still about 80%. Freeze-thawing cycle is another major factor affecting RNA levels in serum. We found that two-three freeze-thaw cycles had
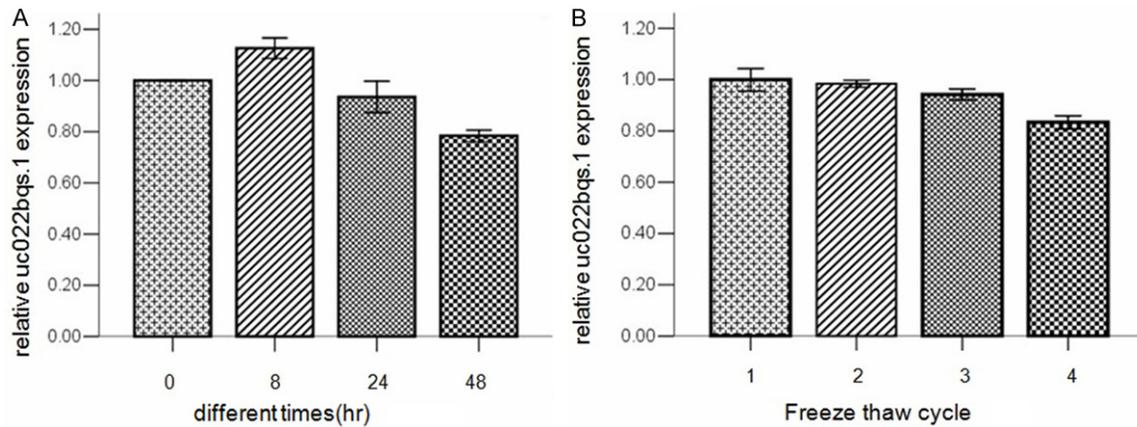
Figure 4. The stability of serum uc022bqs.1. Detection of uc022bps.1 in Serum from five NC individuals were exposed for different times at room temperature (A) or with different freeze-thawing cycles (B). P>0.05 vs. control, n=5.

almost no influence on uc022bqs.1 (P>0.05). Our data found uc022bqs.1 was stable in serum and could be can be a reliable biomarker for diagnosing CHD.

Discussion

CHD continues to be a leading cause of death worldwide, which is a common complex disorder that can be caused by single gene or multifactorial conditions. Prompt diagnosis means of CHD can improve its prognosis with percutaneous coronary intervention, coronary artery bypass surgery and drug treatments. Therefore, there is a clinical demand for specific and reliable non-invasive biomarkers for the early diagnosis of CHD. High-throughput methodologies have disclosed an unexpectedly large number of lncRNAs. These lncRNAs were found to be associated with human disease, most notably cancer. LncRNAs, such as MALAT1, CCAT-1, HOTAIR and H19, participate in the process of pathogenesis, progression, and metastasis of tumors [12, 15]. Several lncRNAs have been characterized as potential biomarkers in human body fluids [16, 17]. The most prominent biomarkers is PCA3, which highly expresses in prostate cancer [18]. Additionally, PCA3 in urine has been demonstrated to be a more specific marker for prostate cancer diagnose than the commonly used prostate-specific antigen (PSA) [19]. We chose serum as sample type, not only for its availability, but also for the stability of lncRNAs in it. These characters were clearly demonstrated by our data in **Figure 4**, which

enables it to be a screening biomarker for early CHD.

There are also studies finding that lncRNAs play an important regulatory role in adipogenesis, and participate in the occurrence of atherosclerosis and development [20]. The finding of a long-term prospective cohort study suggested that lncRNA ANRIL-related transcripts EU74-1058 and NR_003529 were associated with the severity of atherosclerosis (p=0.02 and 0.001, respectively) [21]. Another large scale case-control studies demonstrated a strong association of the ANRIL SNP (rs1333049) with Myocardial Infarction (MI) as well as familial hypercholesterolemia patients in a northern Pakistani population and could be used as a useful genetic marker for the screening of MI in the general Pakistani population. Therefore, lncRNAs are closely associated with atherosclerosis, and have potential to be a great breakthrough of the treatment of cardiovascular diseases.

This study screened lncRNAs expression profile of CHD patients' serum samples using microarray analysis. We then verified that serum expression level of uc022bqs.1 was in accordance with microarray results through qRT-PCR. We showed for the first time that lncRNA uc022bqs.1 was frequently up-regulated in CHD patients' serum than in normal individuals. A ROC curve was established for distinguishing CHD patients' serum from normal individuals' serum samples, and the results demonstrated that the AUC was 0.895. We believed

that this correlation would strengthen the clinical value of uc022bqs.1. The expression of serum uc022bqs.1 in CHD patients was up-regulated, and was significantly associated with the severity of CHD. In early 2014, researchers had reported uc022bqs.1 was a novel biomarker of cardiac remodeling and predicts future death in patients with heart failure, and then named it long intergenic noncoding RNA predicting cardiac remodeling (LIPCAR) [22], which strengthen our finding. LncRNA uc022bqs.1 derives from mitochondrial DNA, which suggests that it may regulate mitochondrial pathways, such as oxidative phosphorylation. While the underlying mechanism involving CHD and mitochondrial pathways deserve intensive study.

Taken together, our results revealed the level of serum uc022bqs.1 in CHD patients was higher than that in normal individuals. To make serum uc022bqs.1 as a successful biomarker used in clinic, multi-centers and large number experiments needs to be proceeded, and the mechanism of uc022bqs.1 participate in occurrence and progress of CHD also needs to be further explored.

Disclosure of conflict of interest

None.

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