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ORIGINAL ARTICLE

Plasma MicroRNA-133a as a Potential Predictor for Coronary Artery Stenosis Severity

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ABSTRACT

Background: Several scoring systems have tried to determine the severity of coronary artery stenosis to investigate its correlation with laboratory parameters.**Aim of the study:** To investigate the correlation between the expression levels of circulating miRNA-133a and the severity of coronary artery stenosis. **Methods:** The study was conducted in Medical Biochemistry & Molecular Biology Department and Cardiology Department - Faculty of Medicine, Zagazig University& Medical Scientific Research Center. It included 72 subjects classified into 2 groups; Group (1): included 18 healthy subjects served as a control. Group (2): included 54 acute coronary syndrome (ACS) patients. It was subdivided into 3 subgroups each 18 patient: unstable angina (UA) , non-ST-segment elevation myocardial infarction (NSTEMI) and ST-segment elevation myocardial infarction (STEMI). Plasma miRNA-133a expression level was analyzed by real time PCR. Cardiac Cathetrization was done for showing the severity of stenosis. **Results:** The present study showed high significant positive correlation between miRNA-133a level and coronary stenosis percentage in unstable angina group ($p<0.001$). The study showed high significant positive correlation between miRNA-133a level and coronary stenosis percentage in NSTEMI and STEMI groups ($p<0.001$). Interestingly, we suggested that miRNA -133a level may be considered a predictor marker of significant coronary stenosis percentage with cut off value = 4.56, specificity 92 % and sensitivity 86 % .**Conclusions:** The present study suggested that plasma miRNA-133a expression level can reflect the severity of coronary atherosclerosis in coronary heart disease (CHD) patients to predict the need for percutaneous coronary intervention (PCI) in clinical cardiology intervention.

Key words: ACS , STEMI , NSTEMI , real time PCR, miRNA 133a.

INTRODUCTION

Coronary artery disease (CAD) is a narrowing of the small blood vessels that supply blood and carry oxygen to the heart. Atherosclerosis is a complex process that involves mainly inflammation of the arterial wall. Coronary atherosclerosis can be manifested as endothelial dysfunction at the very early stage of the disease. The release of sensitive markers of myocardial necrosis (e.g. troponins) is regarded as indicative of myocardial cell necrosis and fulfills the definition of myocardial infarction. If no rise in markers is detected, the term unstable angina (UA) is used and noncardiac differential diagnoses must be considered [1].

For scientists and clinicians who carry out research about the pathogenesis of atherosclerosis, it has always been compelling to somehow quantify the grade of severity of coronary artery calcification and stenosis. The severity of coronary stenosis was suspected to be a prognostic factor for patients with coronary artery disease (CAD) and this hypothesis was proven in several clinical studies with long follow-up periods [2]. However, the quantification of CAD poses a problem due to the lack of consistent and

universally valid scoring systems. There is no doubt that visual anatomic evaluation with coronary artery stenosis severity, guiding decisions regarding revascularization with angioplasty or coronary artery bypass graft surgery, and evaluating the outcome of revascularization interventions [2].

Acute coronary syndrome (ACS) describes the spectrum of clinical manifestations which follow disruption of a coronary arterial plaque, complicated by thrombosis, embolization and varying degrees of obstruction to myocardial perfusion. It includes three diseases involving the coronary arteries: ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI) and unstable angina (UA) [3].

MicroRNAs (miRNAs) are endogenous small non-coding RNAs with 21-25 nucleotides in length. By pairing with the 3' UTR of target mRNAs, miRNAs can regulate protein-coding genes at the posttranscriptional level via degradation of mRNAs or repression of protein translation [4]. MicroRNAs are easily detected and quantified in body fluids by microarray assays, northern blot, and real-time polymerase chain reaction (qRT-PCR) [4].

MiRNA-133a is one of the most abundant miRNAs in the heart. The combination of experimental models of human pathologies with tools that modulate miRNA-133 activity has provided important insights on the role played by this miRNA and their targets in very prevalent cardiovascular pathologies [5].

Previous studies demonstrated that miRNA-133a had a low level presence in the plasma of healthy people, and it was expressed differentially in different cardiovascular diseases [6]. Furthermore, it has been reported that the elevated miRNA-133a is released into peripheral circulation from the injured myocardium after Ca2+ stimulation [7].

The aim of the work was to investigate the correlation between the levels of circulating miRNA-133a and the severity of coronary artery stenosis.

qualitative and quantitative angiography remained the cornerstone of the assessment of

METHODS

The study was conducted in Medical Biochemistry & Molecular Biology Department and Cardiology Department - Faculty of Medicine, Zagazig University& Medical Scientific Research Center in the period from July 2017 to September 2018. It included 72 subjects classified into 2 groups: Group (1): included 18 healthy subjects served as a control. Group (2): included 54 ACS patients. Patient (ACS) Group (2) was subdivided into 3 subgroups: Group (I): included 18 UA patients, group (II): included 18 NSTEMI patients and group (III): included 18 STEMI patients. All patients were subjected to the following: - Full history: including history of diabetes, hypertension, smoking & family history. - Complete physical & clinical examination. - Electrocardiography: to find out ischemic changes. - Laboratory investigations: Fasting blood glucose, lipid profile and high sensitive troponin T (Hs-cTnT) were taken from patient sheet. - Plasma miRNA-133a expression level by real time PCR (RT-qPCR). - Coronary angiography: for diagnosis and visual assessment of degree of coronary stenosis severity.

Ethical consideration: Written consent was obtained from every patient after explanation of the procedure. Medical research and ethics committee approved the study. The work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Real time PCR analysis for miRNA-133a expression

miRNA Extraction from plasma was done using miRNeasy kits from Qiagen, Germany, catalogue No RY43.Then miRNA was reverse transcribed using miScript IIRT kit Qiagen, Germany catalogue no: 218161. The amplification was performed in a 20 µL mixture containing 5µL of the cDNA, 100 pmol/mL of each primer miRNA-133 a Cat. no MS00045857) or RNU6 as internal control (Cat. no. MS00033740), 10 µL 2x

QuantiTect SYBR Green PCR Master Mix (Qiagen) and 4 µL DdH2O. The amplification was carried out using Real time PCR (Stratagene Mx3005P) qPCR System according to the following protocol; **95**ºC for 15 min initial activation step then 40 cycle of 95ºC for 15 sec, 55ºC for 30 sec then 70ºC for 30 sec. The amplitude of change of the miRNA expression observed in patients in relation to control group was analyzed by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All data were statistically analyzed using SPSS 20.0 for windows (SPSS Inc., Chicago, IL). Receiver operating characteristic (ROC) curve analysis was used to identify optimal cut-off values of miRNA-133a with sensitivity and specificity for diagnosis of ACS.

RESULTS

Regarding to demographic data, the present study showed that there were no significant differences between the ACS patients and healthy controls regarding age, BMI, gender, smoking, DM, dyslipidemia and family history (p>0.05) while there was statistically significant difference as regard the hypertension status (p<0.05) (Table 1).

The present study showed a significant up-regulation of miRNA-133a expression in ACS patients when compared to the controls (p<0.001)(table 2). The same results have been found for ACS subgroups (UA, NSTEMI and STEMI patients) when compared to the healthy control group (p<0.001 for each) (Table 2). In the present study, there were higher miRNA-133a expression levels in NSTEMI than UA patients (p<0.001). Also, there was a significant up-regulation of this microRNA expression in STEMI group compared to the NSTEMI group (p<0.001) (Table 2).

Furthermore, there was a significant positive correlation between miRNA-133a expression and cardiac troponin (Hs-cTnT) levels (fig 1).

When we studied the relation between cardiac troponin level (Hs-cTnT) level and the severity of coronary artery stenosis, we found insignificant correlation regarding UA group and a significant positive correlation (P < 0.05) in NSTEMI & STEMI .

When we studied the relation between miRNA-133a expression and the severity of coronary artery stenosis, we found a significant positive correlation (P < 0.001) between miRNA-133a expression level and coronary stenosis percentage in all patients groups (Table 3, fig 2).

Roc analysis revealed that miRNA -133a level may be considered as a predictor marker of significant coronary stenosis percentage with cut off value = 4.56, specificity 92 % and sensitivity 86 % (fig 3).

Table 1. Descriptive statistics of the demographic parameters in the studied groups:

Variable	Control group 1 (n=18)		ACS group2 (n=54)		T test	P-Value
	X ±SD		X ±SD			
Age	45.11 ± 6.5		48.54 ± 7.63		-1.71	0.091
BMI (Kg/m^2)	29.6 ± 2.81		30.98 ± 4.09		-1.32	0.188
Onset of chest pain	5.32±4.21		6.11±4.32		-0.68	0.501
Gender					0.46	0.49
Male	8	44.4	31	57.4		
Female	10	55.6	23	42.6		
Smoker					0.19	0.661
Yes	5	33.3	18	33.3		
No	13	66.7	36	66.7		
Hypertension					6.01	**<0.05**
Yes	5	33.3	33	61.1		**(S)**

No	13	66.7	21	38.9		
Dyslipidemia					0.46	0.49
Yes	8	44.4	29	53.7		
No	10	55.6	25	46.3		
DM					3.45	0.063
Yes	3	16.7	22	40.7		
No	15	83.3	32	59.3		
Family History					3.46	0.062
Yes	4	22.2	25	46.3		
No	14	77.8	29	53.7		

Table 2. showing comparison between UA group I , NESTMI group II , STEMI group III & the control group regarding miRNA-133a expression level.

Variable	UA group I (n=18)	NESTMI group II (n=18)	STEMI group III (n=18)	Control group (n=18)	F	P-Value	LSD
	X ±SD	X ±SD	X ±SD	X ±SD			
miRNA -133a expression level	3.6 ± 1.3	4.63 ± 0.8	5.89± 1.12	1.06 ± 0.06	84.2	< 0.001 (HS)	$< 0.001^1$ $< 0.001^2$ $< 0.001^3$ $< 0.001^4$ $<0.001^5$ $< 0.001^6$

F is for ANOVA test
LSD is for least significant difference
P1: Group IV compared to group I. `P2: Group IV compared to group II. P3: Group IV compared to group III. P4: Group II compared to group I. `P5: Group III compared to group I. P6: Group III compared to group II.

Table 3. Correlation analysis between miRNA-133a level and coronary stenosis percentage in the three subgroups of ACS patients (unstable angina group I, NESTMI group II & STEMI group III).

	Variables	miRNA -133a level (r)	P value
Coronary Stenosis %	**UA**	**0.338**	**< 0.001**
	NESTMI	**0.745**	**< 0.001**
	STEMI	**0.793**	**< 0.001**

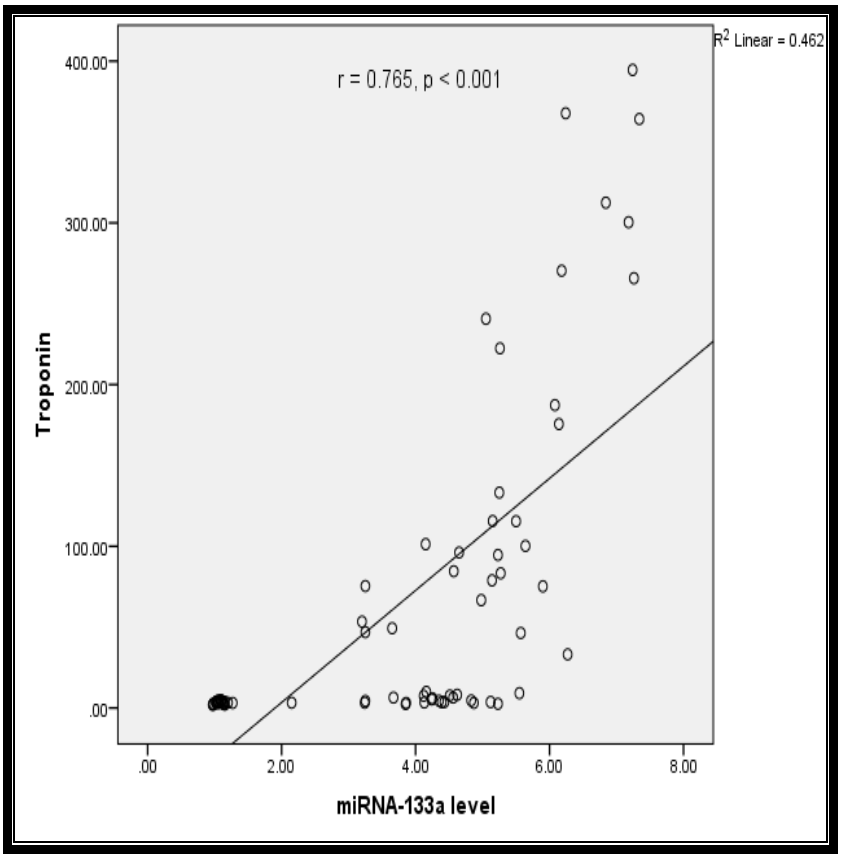
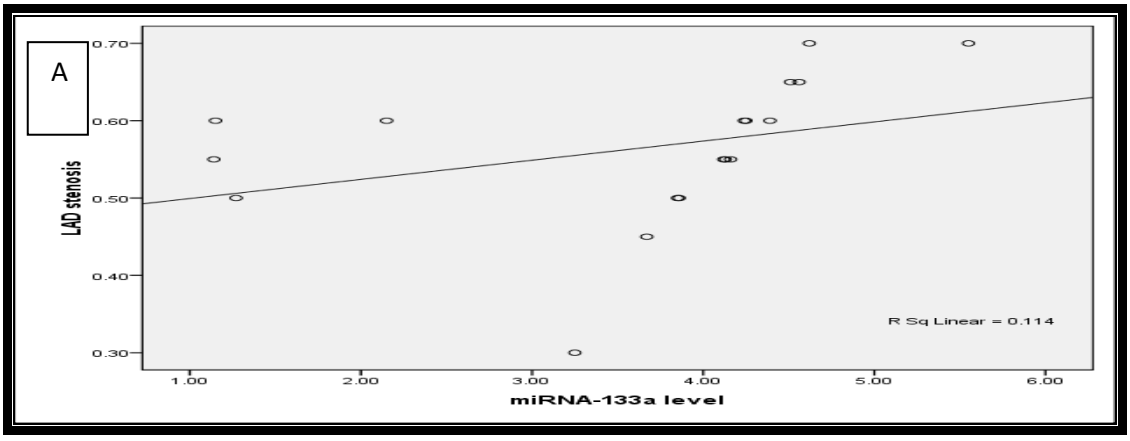


Figure 1. Correlation between cardiac troponin (Hs-cTnT) level and miRNA-133a expression level



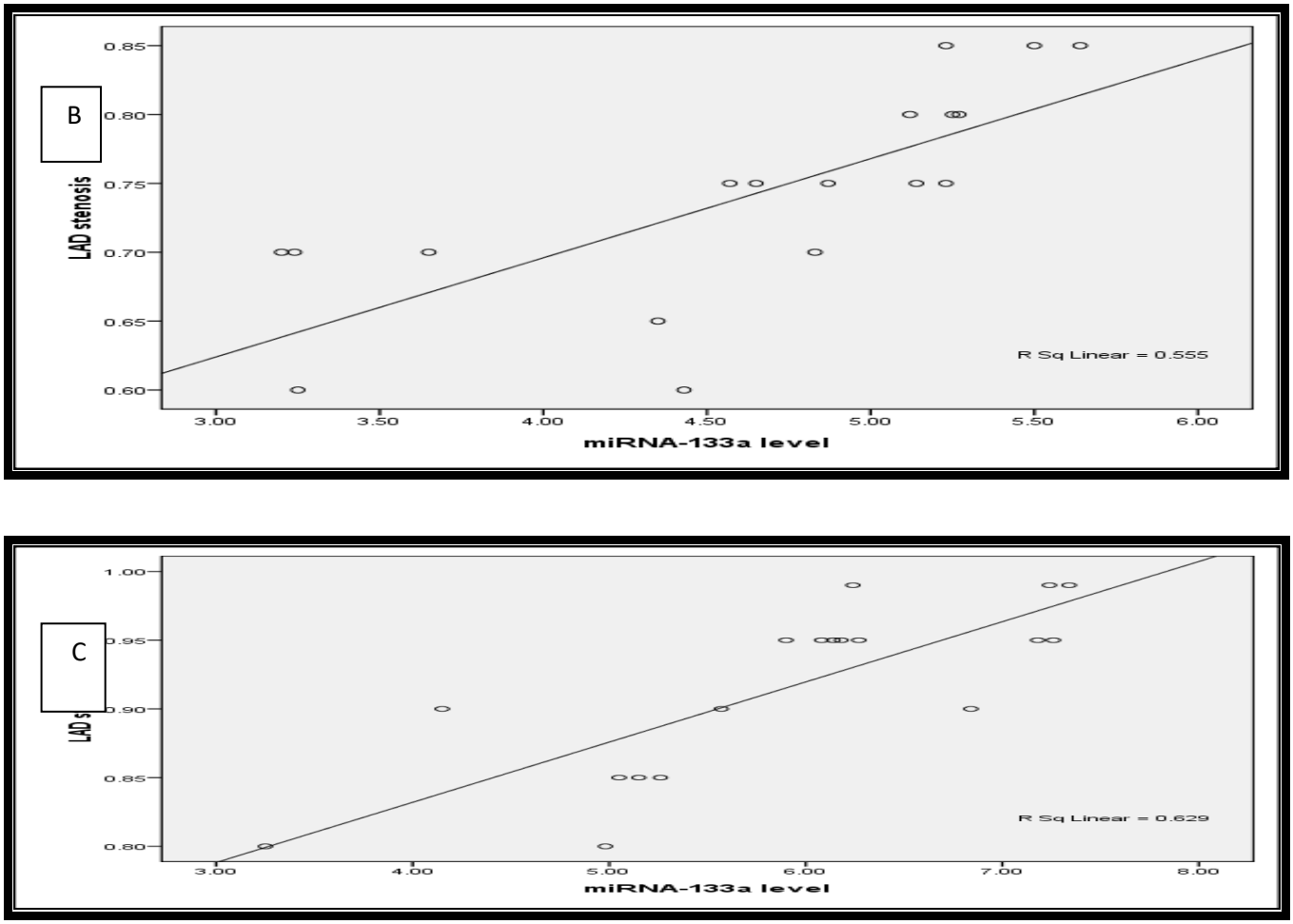


Figure 2. High significant positive correlation between miRNA-133a level and coronary stenosis percentage regarding: (A) unstable angina group (B) NSTEMI group (C) STEMI group

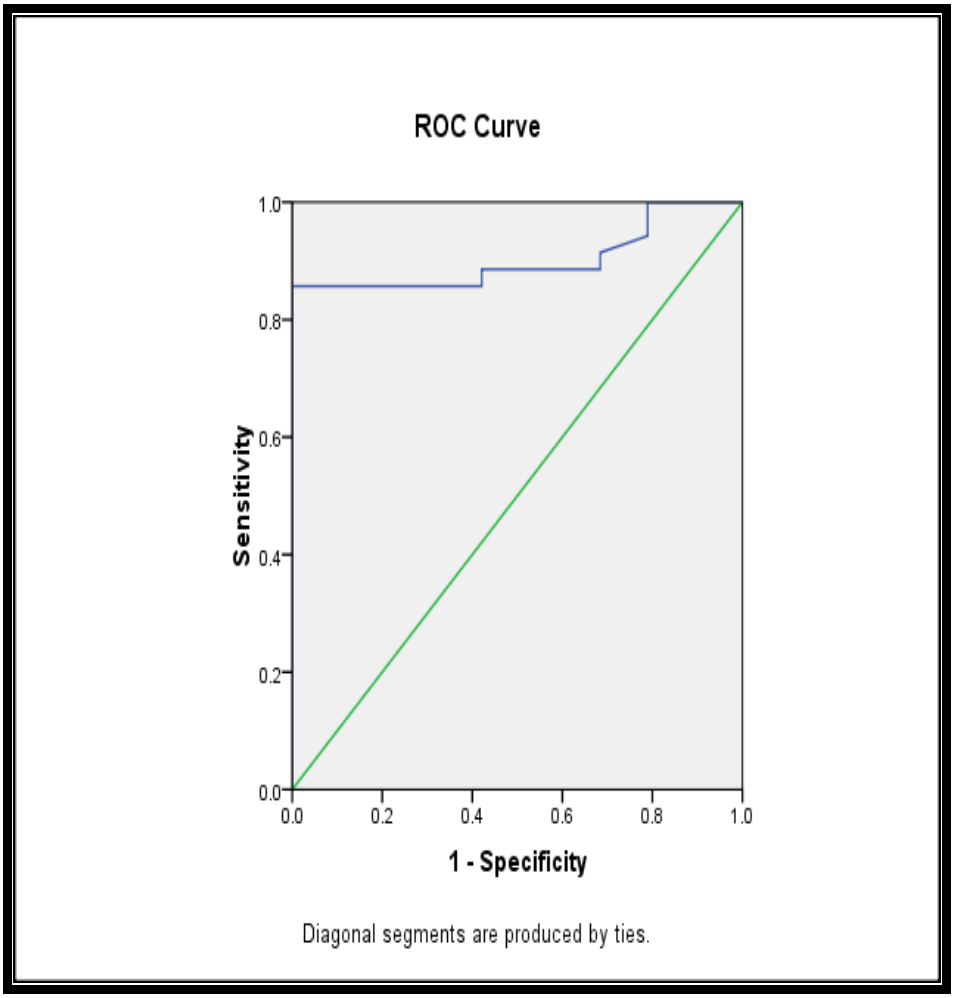


Figure 3. ROC Curve detecting sensitivity and specificity of miRNA-133a expression level as regards coronary stenosis percentage**.**

DISCUSSION

It is very critical to diagnose myocardial infarction in chest pain patients as soon as possible. Up to date, the most commonly used biomarkers for MI are cardiac troponins; cardiac troponin I and T (cTnI and cTnT). Unfortunately, these biomarkers are not consistently elevated within the first hours after onset of symptoms, demanding subsequent measurements and delaying early diagnosis [8]. So it is still a clinical need for novel biomarkers, which can reliably rule in or rule out ACS immediately on admission.

Circulating miRNAs fulfill a number of criteria that allow them to be considered as ideal clinical biomarkers that can be quantified by real time-PCR or microarrays [9].

It has been reported that the human genome includes thousands of miRNAs, 300 of which are roughly expressed in the heart [10]. Cardiomyocyte-enriched miRNAs, including miRNA-1, miRNA 208a, miRNA-208b, miRNA-133a, miRNA-133b, and miRNA-499 have been suggested as potential diagnostic markers in patients with AMI [11, 12].

We selected miRNA-133a because of its known high expression in cardiac muscle [13]. miRNA-133a shows low expression levels in the plasma of healthy individuals, while it is released into peripheral circulation from the injured myocardium after calcium stimulation [14].

The present study found a significant up-regulation of miRNA-133a expression in ACS patients when compared to the controls indicating that it can be used as a diagnostic

biomarker for ACS. Also, our study showed a statistically significant difference between control group and ACS patients regarding cardiac tropnonin (Hs-cTnT) values.

Although both cTnT and miRNA-133a showed significant elevation with ACS; the priority factors for using miRNA-133a as a biomarker for ACS might include: (i) microRNAs are detected in circulating blood in a remarkably stable form, which can withstand enzymatic degradation, repetitive freezing, and thawing cycles [15]; and (ii) miRNA-133 may be superior to cardiac troponin for detecting myocardial injury in individuals with renal dysfunction, since troponin would increase in end-stage renal disease, even in the absence of an ACS [16].

In the present study, we found that there was no significant difference between UA group and control group concerning Hs-cTnT while there was a significant up-regulation of miRNA-133a expression levels in UA patients when compared to controls.

Detectable quantities of cTnT are released only in the setting of irreversible myocardial injury, for example, myocardial necrosis, thereby leaving the patients with UA, which by definition indicates myocardial ischemia without necrosis, undiagnosed with cTnT [17]. In the present work, we observed the elevation of miRNA-133a in UA patients indicating that this miRNA might be helpful for accelerating the diagnosis of UA, so the better management could be allowed. The release mechanism of miRNAs is unclear, but they may be freed to the bloodstream as a consequence of passive release of the cell contents as apoptotic bodies, exosomes and microparticles [18]. Additionally, miRNA microarray analysis and in situ hybridization indicated that miRNA-133a was released from infarcted and peri-infarcted myocardium [19]. Therefore, we hypothesized that the elevated levels of miRNA-133a in the state of UA could result from its passive release from ischemic myocardial tissue.

Only one work studied the miRNA-133a expression levels in UA patients [19]. Although the sample sizes were small, the serum level of miRNA-133a increased significantly in patients with UA (n=8, $P<0.05$) compared with other patients without ACS [19].

In the present study, there were higher miRNA-133a expression levels in NSTEMI than UA patients. Also, there was a significant up-regulation of this microRNA expression in STEMI group compared to the NSTEMI group. These results indicated that miRNA-133a expression levels could be discriminate between UA, NSTEMI and STEMI patients. Our results are consistent with a large cohort comprised of 444 patients with ACS, [20]. They found that patients with myocardial infarction (NSTEMI or STEMI) presented with higher levels of miRNA-1, miRNA-133a, and miRNA-208b compared with patients with UA [20].

In our study, there was a significant up-regulation of miRNA-133a in both NSTEMI and STEMI patients compared to control group. Our results are consistent with many studies. It has been reported that miRNA-1, miRNA-133, miRNA-499 as well as miRNA-208a levels in plasma from AMI patients were significantly higher than those in healthy subjects, CHD patients without AMI, or patients with other cardiovascular diseases [13]. Recently, based on the meta-analysis of ten case-controlled studies including 1074 patients, it was found that the level of miRNA-133a in blood serum or plasma maybe used as a diagnostic biomarker of AMI [21].

However, other studies disagreed with our findings and showed that miRNA-133 is not a good biomarker for AMI diagnosis [22, 23]. Among these is a study done by Wang et al [13] which showed that miRNA-133a was detected with higher levels in plasma from the AMI group, but there were no statistically significant differences in it level among the healthy, non-CHD ,and CHD groups or miRNA-133 levels not significantly increased in patients with AMI (n=32) compared with healthy individuals (n=36) [13]. We suggest that these differences may be due to the variation in the time from the onset of symptoms to sampling the circulating blood

or the relative small patient numbers of the studies.

In the present study, Roc analysis revealed that the AUC of miRNA -133a level in plasma of ACS patients was 0.92, with specificity of 93 % and sensitivity of 88.2 %, indicating that miRNA-133a may be considered as a good predictor marker of ACS.

Roc analysis revealed also that the AUC of miRNA-133a in plasma of AMI patients was 0.93 with specificity of 91.6 % and sensitivity of 87.5%., indicating that miRNA-133 may be a clinically practicable biomarker for AMI diagnosis. ROC curve of Hs-cTnT was plotted and showed specificity of 61.1 % and sensitivity of 76.9 % in ACS patients and specificity of 88.9 % and sensitivity of 61.1% in AMI patients. These results revealed that circulating miRNA-133a is more informative than cTnT for ACS and AMI diagnosis.

In consistent with ours, miRNA-133 showed an AUC of 0.912, with a sensitivity of 81.1% and a specificity of 91.2% in AMI compared with non-AMI [24]. Wang et al. found that the ROC curves of CHD subcategories revealed that circulating miRNA-133a is more sensitive for AMI diagnosis than cTnI in CHD patients [25]. Recently, the meta-analysis of ten case-controlled studies including approximately 1 thousand AMI patients suggested that miRNA-133a could be used as a biomarker for AMI diagnosis, particularly considering that the pooled AUC of the ROC curve is 0.88 (95% CI 0.85–0.90) with the sensitivity of 0.83 % and the specificity of 0.78% [21].

In the present work, we found a significant correlation between miRNA-133a and coronary stenosis % in all patients groups. This is an additional advantage for miRNA-133a whose level in plasma can reflect the severity of coronary atherosclerosis in CHD patients and predict the need for PCI in clinical cardiology interventional practice.

We can consider miRNA-133a level as a predictor marker of significant coronary stenosis with cut off value = 4.56, AUC = 0.90, specificity 92 % and sensitivity 86 % in ACS patients. Cardiac troponin (Hs-cTnT) level also can indicate the coronary stenosis severity with lower specificity 88.9 % and sensitivity 72.2 % than microRNA-133a values.

Wang et al. showed that the levels of plasma miRNA-133a positively correlated with the severity of coronary artery stenosis in CHD patients with single left anterior descending coronary atherosclerosis, but there was no association between plasma cTnI and the degree of coronary stenosis [25]. Concerning ours and Wang's results, the circulating miRNA-133a is superior to troponin in detecting the severity of coronary artery lesions.

CONCLUSION

To conclude, our study analyzed the changes in expression levels of circulating miRNA-133a in ACS patients, and provided insights into the relation between plasma miRNA-133a and the severity of coronary atherosclerotic stenosis. Plasma miRNA-133a expression levels were up-regulated in all ACS groups when compared to controls indicating that circulating miRNA-133a can be a novel and potent biomarker for ACS, especially for UA. Our results suggested that circulating miRNA-133a may be a sensitive predictor for diagnosing ACS.

Also, plasma miRNA-133a can reflect the severity of coronary atherosclerosis in CHD patients and predict the need for PCI in clinical cardiology interventional practice. These results may provide theoretical foundation in improving the clinical diagnosis of ACS. Further studies should be conducted to reveal the exact time course of miRNA-133a in the plasma of ACS patients. Moreover, the underlying mechanisms of increased circulating miRNAs and whether they have pathophysiological functions in ACS require further investigation.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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