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Plasma MicroRNA-133a as a Potential Biomarker for Acute Coronary Syndrome

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Abstract

Background: In clinical practice, there is still a need for novel biomarkers, which can reliably rule in or rule out acute coronary syndrome (ACS) immediately on admission.

Aim of the study: To evaluate the role of plasma miRNA-133a (miR-133a) as a novel biomarker for early diagnosis of ACS.

Patients and Methods: A total of 72 subjects with chest pain suggestive of ACS were admitted as early as possible from the onset of chest pain to the ICU of Cardiology Department, Faculty of Medicine, Zagazig University and were enrolled in this cross sectional study. They included 18 controls (group 1) and 54 ACS patients (group 2). Group 2 was subdivided into 3 subgroups: unstable angina (UA), non ST-segment elevation myocardial infarction (NSTEMI) and ST-segment elevation myocardial infarction (STEMI). Immediately at enrollment, blood samples were taken for estimation of plasma miR-133a expression levels by real time PCR.

Results: The present study showed a significant up-regulation of miR-133a expression in ACS patients when compared to the controls ($p<0.001$). The same results have been found for ACS subgroups when compared to the healthy control group ($p<0.001$ for each). Also, there were higher miR-133a expression levels in NSTEMI than UA patients ($p<0.001$) and a significant up-regulation of this miRNA expression in STEMI group compared to the NSTEMI group ($p<0.001$). Furthermore, there was a significant positive correlation between miR-133a expression and cardiac troponin (Hs-cTnT) levels.

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

Conclusions: Plasma miR-133a expression level may represent a sensitive predictor for diagnosing ACS. This is of particular interest in patients with UA and NSTEMI in whom diagnostic uncertainty is high.

Keywords: ACS, STEMI, NSTEMI, Real time PCR, miR-133a

1. Introduction

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) (Condorelli *et al.,* 2014).

In clinical practice, there is still a need for novel biomarkers, which can reliably rule in or rule out ACS immediately on admission. This is of particular interest in patients with UA and NSTEMI in whom diagnostic uncertainty is high (Falk *et al.,* 2013).

The golden standard for diagnosing acute myocardial infarction is still cardiac troponins (cTn) but blood cTn concentrations may be falsely elevated in certain cardiac as well as non-cardiac diseases such as severe heart failure, atrial fibrillation, chronic kidney disease, severe sepsis and septic shock (Giannitsis and Katus, 2013). Therefore, it is necessary to investigate novel biomarkers with high sensitivity and specificity for early identification of ACS.

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 (Ha and Kim, 2014). MicroRNAs are easily detected and quantified in body fluids by microarray assays, northern blot, and real-time polymerase chain reaction (qRT-PCR) (Ha and Kim, 2014).

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

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cardiovascular pathologies (Van Rooij and Kauppinen, 2014).

Previous studies demonstrated that miR-133a had a low level presence in the plasma of healthy people, and it was expressed differentially in different cardiovascular diseases (D'Alessandra *et al.*, 2010). Furthermore, it has been reported that the elevated miR-133a is released into peripheral circulation from the injured myocardium after Ca2+ stimulation (Ahlin *et al.*,2016).

The aim of our work was to identify the role of plasma miR-133a as a novel biomarker for acute coronary syndrome.

2. Subjects and Methods

The study was conducted in Medical Biochemistry & Molecular Biology Department and Cardiology Department - Faculty of Medicine, Zagazig University & Medical Scientific Research Center, Zagazig University in the period from July 2017 to September 2018. A total of 72 subjects presenting with chest pain suggestive of ACS were admitted as early as possible from the onset of chest pain to the ICU of Cardiology Department and enrolled in this cross sectional study. Subjects were classified into 2 groups: Group (1): included (18) healthy subjects served as controls who were admitted to hospital for their complaints of pericardial discomfort. It was further confirmed by cardiac catheterization that those people turned out to have no coronary artery lesions. Therefore, they were selected as 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. We excluded patients with chronic kidney disease as the elevated levels of cTn due to impaired clearance may result in false positive data. Also, patients with heart faliure , severe aortic stenosis , stress cardiomyopathy, hypertrophic obstructive cardiomyopathy (HOCM) were excluded, as in these patients false positive results regarding plasma miR-133a level may interfere with the study. All participants 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. - Immediately at enrollment, blood samples were taken for analysis of plasma miR-133a expression levels by real time PCR. - Coronary angiography: for diagnosis of ischemia.

Written consent was obtained from every participant 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.

3. Real time PCR analysis for miR-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 from 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 Dd H2O. The amplification was carried out using real time PCR (Stratagene Mx3005P) qPCR System according to the following protocol; 95ºC for 15 min initial activation step then 40 cycles of (95ºC for 15 sec, 55ºC for 30 sec then 70ºC for 30 sec). A melting curve was analyzed to validate the results after the PCR amplification. 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.

4. 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 miR-133a with sensitivity and specificity for diagnosis of ACS.

5. Results

Regarding demographic data, the present study showed that there were no significant differences between the ACS patients and healthy controls regarding age, body mass index, gender, smoking, diabetes mellitus, 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 miR-133a expression in ACS patients when compared to the controls ($p<0.001$) (fig 1). 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 miR-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).

Regarding miR-133a expression levels, there was statistically highly significant difference between control group & case group ($p<0.001$) (Table 3). As for cardiac troponin (Hs-cTnT), there was statistically highly significant difference between control group & case group ($p<0.001$) (Table 3).

Furthermore, there was a significant positive correlation between miR-133a expression and cardiac troponin (Hs-cTnT) levels (fig 2).

ROC analysis showed that cardiac troponin level (Hs-cTnT) has specificity of 61.1 % and sensitivity of 76.9 % with cut off value = 3.65 when discriminating between ACS patients and controls (fig 3). ROC analysis revealed that the AUC of miR-133a level in plasma of ACS patients was 0.92, with specificity of 93 % and sensitivity of 88.2 % with cut off value = 1.24 when discriminating between ACS patients and controls indicating that miR-133a may be considered as a good predictor marker of ACS (fig 4). Additionally, ROC curve revealed that the AUC of miR-133a expression was 0.93 with specificity of 91.6 % and sensitivity of 87.5 % with cut off value = 4.3 when discriminating between myocardial infarction (MI) patients and controls in myocardial infarction (MI) patients

indicating that miR-133a level may be considered as a predictor marker of myocardial infarction (fig 5).

Table (1): Descriptive statistics of the demographic parameters in the studied groups: group 1(control group) and group 2 (ACS group). The data presented in table (1) showed that there were no significant differences between the ACS patients and healthy controls when comparing age, body mass index, onset of chest pain , gender, smoking, diabetes mellitus, dyslipidemia and family history (p>0.05) while there was statistically significant difference when comparing hypertensive status (p<0.05).

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
Body mass index (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

Continued Table (1):

Variable	**Control group 1 (n=18)**		ACS group2 (n=54)		χ^2	**P value**
	N	**%**	**N**	**%**		
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		
Diabetes mellitus					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): Comparison of miR-133a expression level between UA group I , NSTEMI group II , STEMI group III & the control group. The data presented in (table 2) showed that miR-133a expression level, was (3.6 ± 1.3) in UA group, (4.63 ± 0.8) in NSTEMI patients, (5.89 ± 1.12) in STEMI patients and (1.06 ± 0.06) in control group. The data indicated that there was high statistically significant difference when comparing miR-133a expression level between control group and different ACS subgroups (UA , NESTMI & STEMI) with (P < 0.001).

Variable	UA group I (n=18)	NSTEMI group II (n=18)	STEMI group III (n=18)	Control group (n=18)		P-Value	
	X ±SD	X ±SD	X ±SD	X ±SD	F		LSD
miR -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): Comparison of cardiac tropnonin (Hs-cTnT) values & miR-133a expression level between control group and case group. The data presented in table (3) showed that there was statistically highly significant difference between control group & case group (p<0.001).

Variable	**Control group 1 (n=18)**	**ACS group2 (n=54)**	Mann whitney test/ t-test	P-Value
Cardiac Troponin (pg/ml)			164.5	**< 0.001 (HS)**
X ±SD	3.51 ± 0.82	86.52 ± 111.16		
Median	3.6	46.64		
Range	(1.94-4.85)	(2.15-394.6)		
miR -133a expression level			-10.67	**< 0.001 (HS)**
X ±SD	1.06 ± 0.06	4.71± 1.43		
Median	1.055	4.74		
Range	(0.97-1.2)	(1.14-7.34)		

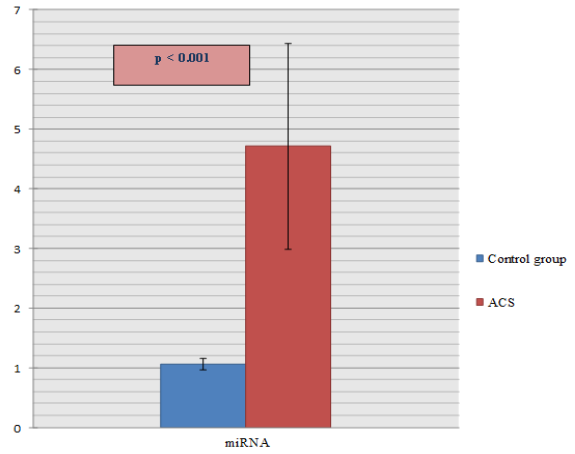

Figure 1. Showing comparison of miR-133a expression level between control group { (n=18) with mean ± SD : 1.06 ± 0.06 } and case group { (n= 54) with mean ± SD : 4.71 ± 1.43 } . The data in fig (1) showed a significant up-regulation of miR-133a expression in ACS patients when compared to the controls ($p<0.001$) .

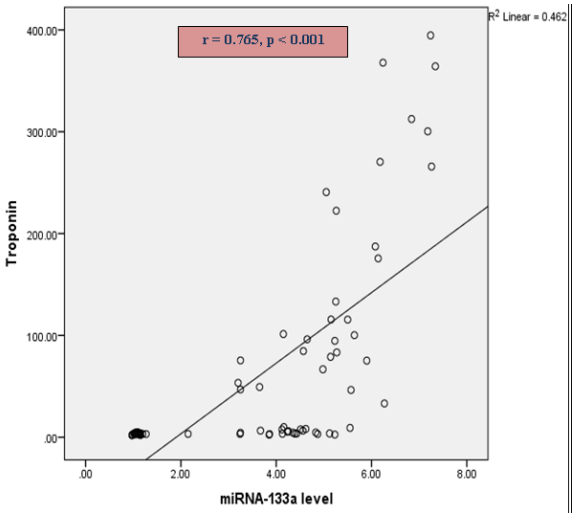

Figure 2. Correlation between cardiac troponin (Hs-cTnT) value and miR-133a expression level. The data in fig (2) showed a significant positive correlation between miR-133a expression level and cardiac troponin (Hs-cTnT) levels , r = 0.765 with $p < 0.001$.

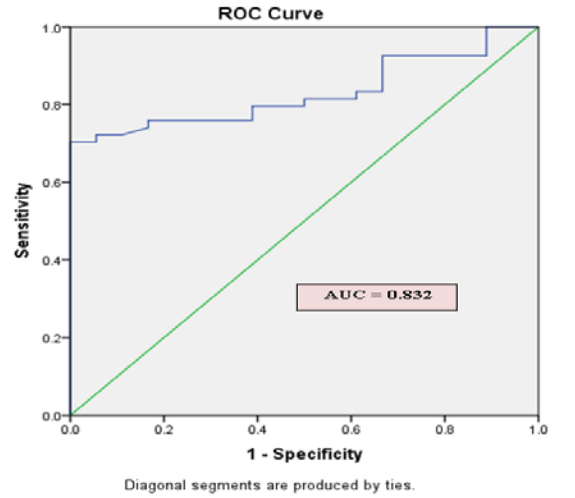

Figure 3. ROC analysis showed that cardiac troponin level (Hs-cTnT) has specificity of 61.1 % and sensitivity of 76.9 % with cut off value = 3.65 when discriminating between ACS patients and controls.

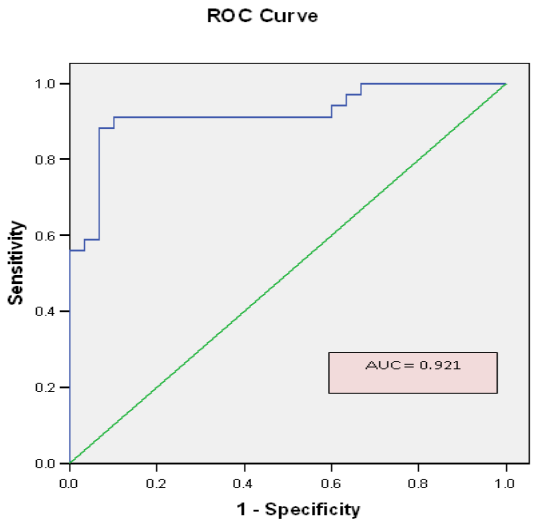

Figure 4. ROC curve detecting sensitivity and specificity of miR-133a level in ACS patients .The data in fig (4) revealed that the miR-133a level with specificity of 93 % and sensitivity of 88.2 % with cut off value = 1.24 when discriminating between ACS patients and controls.

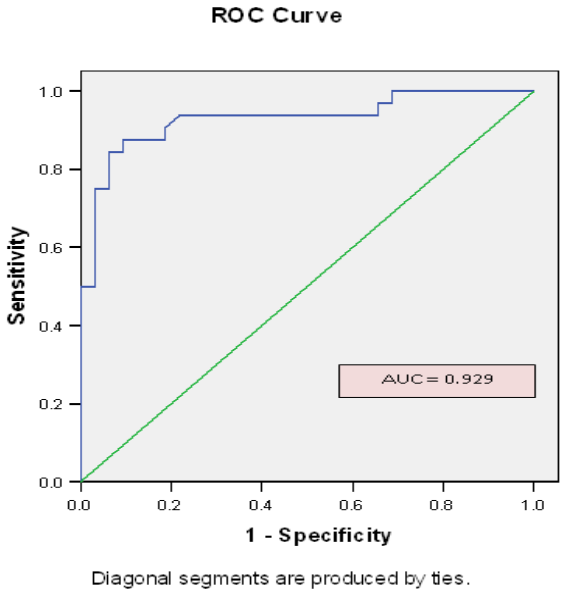

Figure 5. ROC curve detecting sensitivity and specificity of miR-133a level in myocardial infarction (MI) patients .The data in fig (5) revealed that the miR-133a level with specificity of 91.6 % and sensitivity of 87.5% with cut off value = 4.3 when discriminating between myocardial infarction (MI) patients and controls.

6. 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 the onset of symptoms, demanding subsequent measurements and delaying early diagnosis (Anderson *et al.*, 2011). Therefore, it is still a clinical need for novel biomarkers, which can reliably rule in or rule out ACS immediately on admission.

It has been reported that the human genome includes thousands of miRs, 300 of which are roughly expressed in

the heart (Hu G1 *et al.,* 2012). Cardiomyocyte-enriched miRNAs, including miR-1, miR 208a, miR-208b, miR-133a, miR-133b, and miR-499 have been suggested as potential diagnostic markers in patients with AMI (Shalaby *et al.,* 2016; Parizadeh *et al.,* 2018).

We selected miR-133a because of its known high expression in cardiac muscle (Wang GK *et al.,* 2010). MiR-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 (Liu *et al.,* 2008).

The present study found a significant up-regulation of miR-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 miR-133a showed significant elevation with ACS, the priority factors for using miR-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 (Dimmeler and Zeiher 2010); and (ii) miR-133a 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 (Abbas *et al,.* 2005).

In the present study, we found that there was no significant difference between UA group and control group concerning Hs-cTnT values while there was a significant up-regulation of miR-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 (Wu *et al.,* 2006). In the present work, we observed the elevation of miR-133a in UA patients indicating that this miRNA might be helpful for accelerating the diagnosis of UA, so 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 (Martinez *et al .,* 2011). Microparticles containing miRs may be formed during myocardial ischemia as well as during necrosis (Martinez *et al.,* 2011). Additionally, miRNA microarray analysis and in situ hybridization indicated that miR-133a was released from infarcted and peri-infarcted myocardium (Kuwabara *et al.,* 2011). Therefore, we hypothesized that the elevated levels of miR-133a in the state of UA could result from its passive release from ischemic myocardial tissue.

Kuwabara *et al.,* 2011 studied the miR-133a expression levels in UA patients (Kuwabara *et al.,* 2011). Although the sample sizes were small, the serum level of miR-133a increased significantly in patients with UA (n=8, $P<0.05$) compared with other patients without ACS (Kuwabara *et al.,* 2011).

In the present study, there were higher miR-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 miR-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 (Widera *et al.,* 2011).

In our study, there was a significant up-regulation of miR-133a in both NSTEMI and STEMI patients compared to control group. Our results are consistent with many studies. It has been reported that miR-1, miR-133, miR-499 as well as miR-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 (Wang *et al.,* 2010). However, the amplitudes of the increase were different among the 4 types of miRNAs; the highest increase was the miR-133 (Wang *et al.,* 2010). Recently, based on the meta-analysis of ten case-controlled studies including 1074 patients, it was found that the level of miR-133a in blood serum or plasma may be used as a diagnostic biomarker of AMI (Zhu *et al*., 2018).

However, other studies disagreed with our findings and showed that miR-133 is not a good biomarker for AMI diagnosis (D'Alessandra *et al.,* 2010 *;* Roberta De Rosa *et al.,* 2017) Among these is a study done by Wang et al which showed that miR-133a was detected with higher levels in plasma from the AMI group, but there were no statistically significant differences in its level among the healthy, non-CHD ,and CHD groups or miR-133 levels not significantly increased in patients with AMI (n=32) compared with healthy individuals (n=36) (Wang *et al.,* 2010). 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 sample size of the studies.

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

ROC analysis revealed also that the AUC of miR-133a in plasma of AMI patients was 0.93 with specificity of 91.6 % and sensitivity of 87.5%.; indicating that miR-133a 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 miR-133a is more informative than cTnT for ACS and AMI diagnosis.

Consistently, miR-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 (Liu P *et al*., 2014). Wang et al. found that the ROC curves of CHD subcategories revealed that circulating miR-133a is more sensitive for AMI diagnosis than cTnI in CHD patients (Wang *et al.,* 2013). Recently, the meta-analysis of ten case-controlled studies including approximately 1 thousand AMI patients suggested that miR-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% (Zhu *et al*., 2018).

In contrary to our results, Devaux *et al.* (2015) prospectively investigated the use of six different miRs in n=1155 acute chest pain patients. Finally, n=179 patients

were diagnosed as NSTEMI and n=45 patients as suffering from STEMI. MiR-133a, miR-208b and miR-499 were identified as univariate predictors of myocardial infarction as these three miRs control cardiomyocyte identity (Xin *et al*., 2013). However, their predictive value did not remain significant after correction for troponin levels. Furthermore, the AUC were low (AUC: 0.53–0.76) compared to high sensitive troponin (AUC: 0.94) (Devaux *et al.*, 2015).

The main concern of our study was that we analyzed miR-133a in suspected ACS patients at the time of their enrollment at the emergency department. Other studies investigated the miR-133a levels at later time points or in established diagnosed cases. Early diagnosis of ACS can definitely help decrease the morbidity and the mortality outcomes.

7. Conclusion

Plasma miR-133a expression levels were up-regulated in all ACS groups when compared to controls indicating that circulating miR-133a may be a novel and potent biomarker for ACS, especially for UA. Our results suggested that circulating miR-133a may be a potentially promising predictor for accelerating the diagnosis of ACS patients in the clinical practice.

Conflict of interest

None.

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