Původní sdělení | Original research article

Role of serum microRNA-499 as a diagnostic marker in acute myocardial infarction

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SOUHRN

Akutní infarkt myokardu je jednou z hlavních příčin morbidity a mortality v rozvojových zemích, jako např. v Indii, a dokonce po celém světě. Jeho etiologie je složitá vzhledem k několika zkreslujícím faktorům v jeho patogenezi. V poslední době se prokázalo, že významnou úlohu v predikci diagnózy a prognózy infarktu myokardu hrají malé molekuly RNA, označované jako mikroRNA (miR), z nichž miR-499 hraje zcela zásadní roli v zotavování kardiomyocytů po poranění. Od té doby několik studií identifikovalo několik typů miR spojených s kardiovaskulárními onemocněními; z nich se miR-499 exprimuje hlavně v myokardu. Hlavním cílem naší studie bylo zkoumat úlohu miR-499 v diagnostice AIM. Do studie bylo zařazeno 60 pacientů s AIM ve věku 30 až 60 let a stejný počet kontrolních osob odpovídajícího věku a pohlaví. Zařazení všech účastníků proběhlo na pracovišti terciární péče (Mahatma Gandhi Medical College and Research Institute). Analýza hodnot miR-499 se prováděla metodou RT-PCR, zatímco lipidový profil a hodnoty CK (NAC), CK-MB a srdečních troponinů (pomocí vysoce senzitivní metody) se stanovovaly metodami schválenými IFCC. Pro srovnání průměrných hodnot případů a kontrol byl použit nepárový Studentův test. Vztah mezi hodnotami miR a klasickými markery se zkoumal pomocí Pearsonova korelačního koeficientu. Senzitivita a specificita miR-499 u AIM se hodnotila pomocí vynesené křivky ROC (Receiver Operating Characteristic). Naše studie prokázala statisticky významně vysoké hodnoty miR-499 u pacientů s AMI ve srovnání se zdravými kontrolními osobami (p = 0,012). Hodnoty miR-499 pozitivně korelovaly s hodnotami hs-cnTnT (r = 0,582; p < 0,01) a CK-MB (r = 0,443; p < 0,01). Plocha pod křivkou ROC pro miR-499 měla hodnotu 0,974 se senzitivitou 93,33 % a specificitou 86,67 %; tedy poměrně vyššími než v případě v současnosti používaných markerů CK-MB (86,67 %, resp. 71,67 %) a hs-cnTnT (90,00 %, resp. 81,56 %). Analýza kombinované senzitivity uvedených biomarkerů prokázala, že jejich senzitivita se zvyšuje, zatímco specificita se snižuje. Z výsledků vyplývá, že miR-499 významně koreluje s ostatními klasickými markery při vyšší senzitivitě a specificitě, a mohl by tedy sloužit jako přesnější marker v časné diagnostice AIM.

ABSTRACT

Acute myocardial infarction is one of the leading causes of morbidity and mortality in developing countries such as India and even worldwide. Its etiology is complicated because of several confounding factors involved in its pathogenesis. Recently, MicroRNA has been recognized to play an important role in predicting the diagnosis and prognosis of myocardial infarction. MiR-499 plays a pivotal role in the recovery of cardiac cell, following injury. Since several studies have profiled several miRs in cardiovascular diseases and of which miR-499 is mainly expressed in the myocardium. Our study is primarily designed to explore the diagnostic role of miR-499 in AMI. The study included 60 AMI patients aged 30–60 years and an equal number of age and gender matched controls. All cases were taken from Mahatma Gandhi Medical College and Research Institute a tertiary healthcare set up and analyzed for miR-499 by RT-PCR, Lipid Profile, CK (NAC), CK-MB and high sensitivity cardiac Troponin T were analyzed by IFCC approved methods. Unpaired Student's test was performed to compare the mean of the cases with the controls. Pearson correlation was used to study the association of miR with conventional markers. Receiver Operating Characteristic curve was plotted to find

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out the sensitivity and specificity of miR-499 in AMI. Our study showed significantly high levels of miR-499 in AMI patients in comparison to the healthy controls ($p = 0.012$). MiR-499 levels positively correlated with hs-cnTnT ($r = 0.582$, $p < 0.01$) and CK-MB ($r = 0.443$, $p < 0.01$). ROC for miR-499 showed an AUC 0.974 with 93.33% sensitivity and 86.67% specificity which was comparatively higher than the current markers CK-MB (86.67% and 71.67%) and hs-cTnT (90.00% and 81.56%). When these biomarkers analyzed for combined sensitivity increases but the specificity decreases. The results showed that miR-499 has a significant correlation and also had higher sensitivity and specificity when compared to other conventional markers and thus it could serve as a better early diagnostic marker of AMI.

Introduction

An early diagnostic marker is critical and imperative for appropriate and timely management of acute myocardial infarction (AMI), which is one of the leading causes of mortality and morbidity worldwide.[1] Creatine kinase (CK NAC), CK-MB and high sensitivity cardiac troponins (hs--cnTnT) currently serve as a biomarker in diagnosing AMI[2] and their sensitivity and specificity in early hours of MI is still not convincing. Recently, several novel markers have been evaluated for their use in early diagnosis of MI and the role of microRNA is promising.

MicroRNAs (miR) are non-coding RNAs, approximately with 18–27 nucleotides which play an important role in the regulation of cellular processes such as proliferation, differentiation, development, and apoptosis.[3,4] MiR's are promising markers and play a cardinal role in the development and functions of the myocardium.[5–7] MiRs target most human protein coding genes; especially miR-499 encoded by myosin gene family shows a high expression in circulation in patients with AMI.[8] It is involved in the structural and functional differentiation of cardiac stem cell into cardiomyocytes[9] and also plays a pivotal role in the recovery of cardiac cell, following injury.[10,11] Few studies in the recent past have shown that miR in body fluids are highly stable and become specific molecular signatures for diseases thus enabling them as markers of diagnosis as well as prognosis.[5,12] Only a few studies have been reported thus far in the literature signifying the use of miR in the diagnosis of the AMI.[13–15] In this study, our primary aim was to assess the diagnostic utility of miR-499 in the acute myocardial infarction in comparison with other conventional markers.

Methodology

The study was undertaken at Mahatma Gandhi Medical College and Research Institute a tertiary healthcare center, Puducherry in collaboration with General Medicine unit. 60 patients in the age group between 30 to 60 were included in the study after obtaining Institutional Human Ethics Committee approval and informed consent from the subjects.

Inclusion and exclusion criteria

Patients were admitted to the hospital with a diagnosis of acute myocardial infarction. The final diagnosis of ACS was based on two out of three criteria as given below

1. Ischemic chest pain for at least 30 minutes.
2. Electrocardiogram evidence of ACS.
3. Rise and fall of the cardiac biomarker (CK-MB/troponin).

Patients were excluded if they are known or diagnosed to be having ischemic heart disease, chronic renal failure, and liver disorders. In the control group, healthy ambulatory volunteer was included in the study who visited the hospital as well as from Master health check-up after obtaining inform consent.

Laboratory data

Venous blood samples were obtained in BD vacutainer with clot activator at the time of addmission and immediately centrifuged. Serum lipid profile, CK (NAC) and CK-MB were done by autoanalyzer. Hs-troponin T was estimated using fully auto chemiluminescenceanalyzer (Roche Diagnostics – COBAS e411). The remaining serum sample was aliquoted in 1.8 ml cyro tube and storedat –20 °C until the time of microRNA analysis. All the estimations were subjected to stringent quality control.

MicroRNA analysis

Total RNA was isolated from serum using a mini spin column kit (Helini biomolecules, India) according to the manufacturer's instruction. From that cDNA was synthesized using cDNA synthesis kit (Helini biomolecules, India), as per documented specification. Quantification by RT-PCR was carried out in 25 µl using Helini *Pin the Tail Probe System* method, according to the protocol of the manufacturer (Helini, Biomolecules P. Ltd, India).

Statistical analysis

Statistical analyses were performed using Epi info. Data were entered in the excel sheet and in order to find whether it follows abnormal distribution or not Shapiro-Wilk test was performed. The data were expressed in mean and standard deviation. Student's *t*-test was performed to document the difference between case and control. To evaluate the diagnostic performance of miR and other biomarkers receiver operator characteristic curve (ROC), likelihood ratio (LR), positive predictive value (PPV), negative predictive value (NPV) and Lin's concordance were calculated.

Results

Based on inclusion and exclusion criteria, 60 AMI patients and an equal number of healthy volunteers were included in the study. The biochemical profiles are expressed in mean and standard deviation (Table 1). It showed a significant difference between case and control except high density lipoprotein and low density lipoprotein.

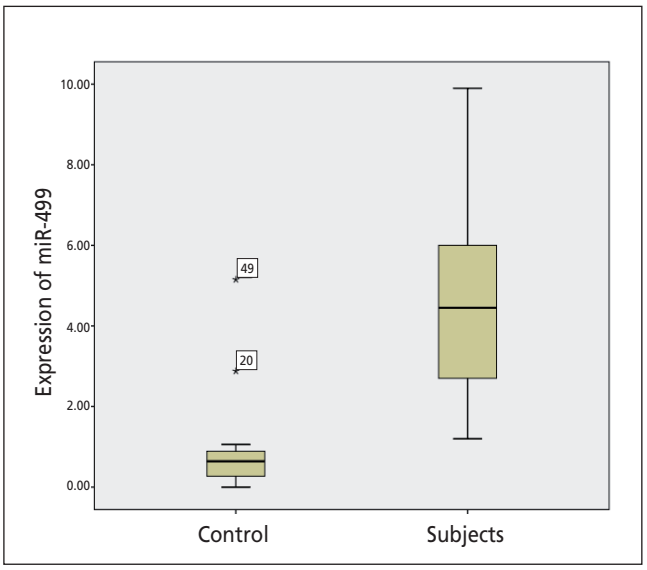

Fig. 1 – Expression of miR-499 in control and AMI patients

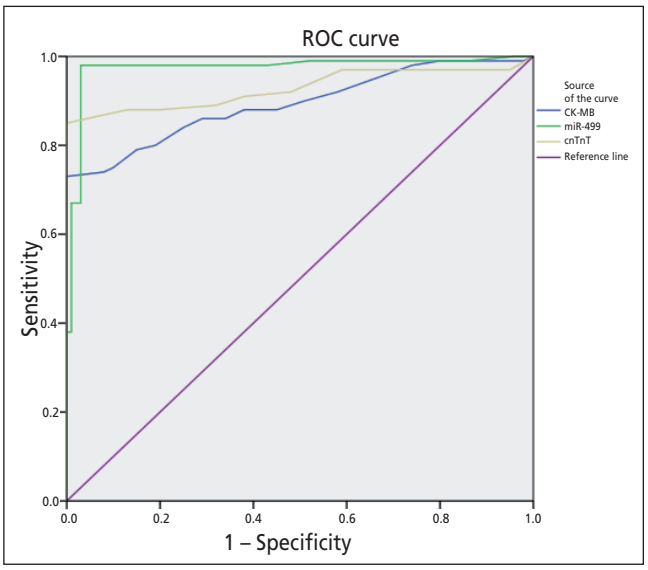

Fig. 2 – ROC of serum MiR-499, cnTnT, and CK-MB in diagnosis of acute myocardial infarction.

MiR-499 showed 6–8 folds increase in AMI patients when compared with healthy individuals (Fig. 1). To comprehend the diagnostic utility of miRs and other conventional markers, receiver operating characteristic curves (ROC) were plotted. MiR-499 with an area under curve (AUC) of 0.974 (95% CI 0.94–0.99); whereas hs-cnTnT AUC of 0.924 (95% CI 0.88–0.97) and CK-MB AUC of 0.893 (95% CI 0.84–0.94) as depicted in Table 2 and Fig. 2.

The sensitivity and specificity of miR-499 and other biomarkers were calculated. MiR-499 showed 93.33% sensitivity and 86.67% specificity; hs-cnTnT has 90% and 81.56% and CK-MB has 86.67% and 71.67% sensitivity and specificity respectively. MiR-499 possesses high sensitivity and specificity when compared with other biomarkers. These biomarkers are analyzed for combined sensitivity, they showed an increase in sensitivity, but their specificity was decreased. We computed likelihood ratio (LR), positive predictive value and negative predictive value for both individual and combined biomarkers results are tabulated in Table 3. As the result suggest as miR-499

Table 1 – Mean and SD of biochemical parameters in AMI cases and controls

S.No.	Parameter	Controls (N = 60)		AMI subjects (N = 60)		*p*-value
		Mean	SD	Mean	SD	
1.	Glucose (mg/dL)	100.61	14.16	219.79	118.22	0.001
2.	Total cholesterol (mg/dL)	155.48	24.68	182.47	55.33	0.002
3.	Triacylglycerol (mg/dL)	100.48	27.04	144.32	58.86	0.001
4.	HDL-C (mg/dL)	41.91	6.92	41.00	13.56	0.651
5.	LDL-C (mg/dL)	104.72	24.72	112.94	52.60	0.089
6.	VLDL (mg/dL)	20.10	5.41	28.85	11.80	0.001
7.	CK-NAC	266.34	61.73	912.64	950.79	0.001
8.	CK-MB	19.95	4.59	77.09	35.90	0.001
9.	Cardiac troponin T (ng/l)	3.25	1.5	47.58	20.83	0.001

** p-value < 0.05 is significant*

Table 2 – AUC of serum miR-499, CnTn T, and CK-MB

Test Result Variable(s)	Area	Sig	95% confidence interval	
			Lower bound	Upper bound
MiRNA-499	0.974	0.000	0.949	0.999
hs-cnTnT	0.924	0.000	0.877	0.970
CK-MB	0.893	0.000	0.847	0.939

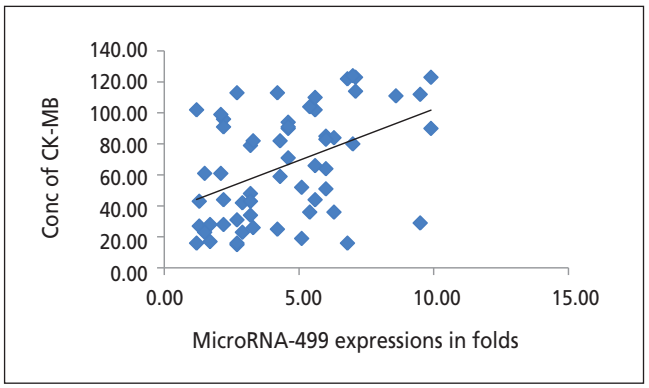

Fig. 3 – (A) Correlation of miR-499 expression with CK-MB.

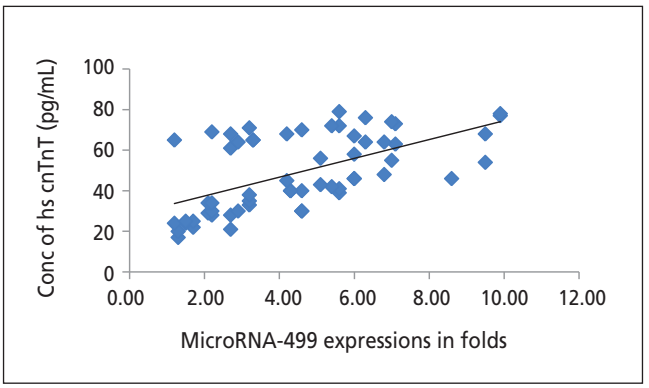

Fig. 3 – (B) Correlation of miR-499 expression with cnTnT.

individually has an LR of 7.00 but when it combined with other biomarkers, there was a decrease in LR.

In addition to above finding, we tried to correlate and find the concordance miR-499 with current cardiac markers. It showed a positive correlation with a marker (Fig. 3A and 3B). The results suggest that miR-499 can be used as a one of the diagnostic marker possessing a high diagnostic value.

Discussion

The main objective of the study was to find the diagnostic utility of circulating miR-499 in comparison with existing biomarkers such as CK-MB and hs-troponin T. We have quantified of miR-499 using RT-PCR and thereby looking into the results we found nearly 6–8 fold increase in the concentration of miR in AMI patients in comparison with healthy controls. In one of the earlier studies undertaken by Olivieri et al., it was demonstrated that there is an 80 fold increase of miR-499-5p a family member of miR-499 in elderly patients with NSTEMI.[16] Our finding correlates with a study by Li et al. which reports that miR-499 can be detected in the plasma with one hour of onset of chest pain in AMI patients.[17] MiR-499 also showed the positive predictive value of 93% in AMI patients within three hours of onset of chest pain.[18] Several other studies which were published recently have documented statistically significant difference of miR-499 in AMI patients relative compared with controls.[19–21]

To assess the diagnostic performance of miR-499, the area under curve (AUC) was plotted that correlated the diagnostic value with other conventional marker namely CK-MB and cnTnT in AMI. Our results showed AUC of 0.928 for miR 499, which was higher when compared with currently used biomarkers of CK-MB and cnTnT showed AUC of 0.893 and 0.794, respectively. Zhang et al. in their study showed AUC of 0.89 with sensitivity and specificity of 80.28%.[21] Shalaby et al. demonstrated the differential diagnostic value of miR-499 by recoding AUC of 0.98 and 0.97 in unstable angina and NSTEMI respectively.[22] Cheng et al. in a meta-analysis report also corroborate our result by reporting the sensitivity of 88% and specificity of 87%.[23] According to our study, miR-499 had a higher sensitivity of 94% and specificity of 89% when compared with previous studies.

Our result showed a positive correlation with traditional biomarkers (CK-MB and cnTnT). Similarly in the past miR-499 was shown to possess positive correlation with traditional marker and has been substantiated by cardiac expression and release from cardiac injury.[24] We also tried to correlate with most common risk factors of AMI, namely lipid profile, but we couldn't find any significant correlation between them. In previous studies implicate the role of miR expression in the regulation of lipid transport and metabolism.[25] Additionally, Vicker et al. demonstrated high density lipoprotein isolated from patients with family hypercholesterolemia to be enriched in other miR thereby suggesting lipid and protein as carrier molecular for miRNAs.[26]

Several studies have also cited the release of miR-499 into the circulation during pathophysiological conditions and nearly specific expression in myocytes. However, miR-499 is more stable, sensitive and specific for cardiac

Table 3 – Sensitivity and specificity of microRNA-499, cnTnT and CK-MB

Test Result Variable(s)	Sensitivity (%)	Specificity (%)	LR	PPV (%)	NPV (%)
MicroRNA-499	93.33	86.67	7.00	87.50	92.86
hs-cnTnT	90.00	81.56	4.91	83.08	89.09
CK-MB	86.67	71.67	3.06	75.36	84.31
MicroRNA-499 + Hs-cnT T	95.00	81.67	5.18	83.58	92.45
hs-cnTnT + CK-MB	90.00	71.67	3.17	76.05	87.75
CK-MB + microRNA-499	93.33	71.67	3.29	76.71	91.48
CK-MB + microRNA-499 + hs-cnTnT	95.00	71.67	3.35	76.71	91.48

injury, thus enhancing the diagnostic utility of miR-499 in AMI in comparison to the use of existing conventional novel biomarkers. This is a major finding that has emerged from our study and that has been attributed to both enhanced sensitivity and specificity.

Conclusion

Biological variation data of AMI patients showed expression of miR-499 was significantly higher than for a healthy individual. Moreover, it also exhibits positive correction with existing cardiac markers. The study concluded early detection of miR-499 in circulation will improve sensitivity and specificity for the appropriate clinical diagnosis of AMI patients along with conventional markers. The study concluded early detection of miR-499 in circulation will improve sensitivity and specificity for the appropriate clinical diagnosis of AMI patients along with conventional markers.

Limitation of the study

- Single miR was selected.
- Serial analysis of miR and biomarker were not included in the study.
- Unstable angina group of patients was not included in the study.

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