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Expression Profiling of Cardiac Specific miRNAs in Coronary Artery Disease

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Abstract

Acute Myocardial Infarction (AMI) is a major cause of death, worldwide. Advanced approaches for the identification of myocardial infractions are required to overcome the mortality caused by AMI. MicroRNAs (miRNAs) are small (~22 nt) regulatory RNAs that are often unregulated and serve as potential blood-based markers in the diagnosis and prognostication of various pathological conditions. This study shows that these circulating miRNAs are present in a significantly stable and cell free form in human plasma that is shielded from endogenic RNase activity. Real-time PCR analysis of some cardiac and muscle-specific miRNAs viz., miR-208a, miR-208b, miR-1 and miR-133a revealed significantly elevated levels of these miRNAs in the plasma of AMI patients (n=52) compared with healthy individuals (n=15, P < 0.001). Notably, miR-208a remained undetectable in healthy individuals but was easily detected in 90% AMI patients within 4 hours of the onset of symptoms. Markedly increased level of cardiac-specific miRNAs in plasma could serve as a novel biomarker for the early detection of myocardial injury in humans.

Keywords: Acute Myocardial Infarction (AMI), biomarker, MicroRNAs

Introduction

Coronary artery disease (CAD) is the most common type of heart disease. It is the leading cause of death in the world with a higher rate in men than women. CAD occurs when the arteries that supply blood to heart muscle become hardened and narrowed. This is due to the build-up of cholesterol and other material, called atherosclerotic plaque. As it grows, less blood can flow through the arteries. As a result, the heart muscle can't get the blood or oxygen it needs. This can lead to chest pain (angina) or a heart attack. Over time, CAD can also weaken the heart muscle and contribute to [1-2]. In the scenario of cardiovascular diseases, acute myocardial infarction (AMI) is a major cause of physical disability. In India and Pakistan it contributes significantly to cardiovascular diseases accounting for 75% of deaths in the world [3].

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Biomarkers are used for timely diagnosis, increasing number of novel markers have been identified to predict the outcome, following an acute myocardial infarction. This may facilitate to give required therapy to high-risk patients [1]. Presently, the only biochemical markers used for diagnosis of AMI are markers of myocardial necrosis. Cardiac troponin is one of them [4] but its limitation is the time lag of up to 12 h from the start of the infarction to the appearance of increased concentrations of cardiac troponins in the blood that can be detected by use of current troponin assays. Therefore, other biochemical markers must be sought that provide important information and might give an earlier sign of an on-going AMI [5].

Recently, circulating miRNAs (21–25 nucleotides that can pair with sites in 3'untranslated regions in mRNAs of protein coding genes to down regulate their expression) have been reported as promising biomarkers for various pathological conditions. In case of AMI these miRNAs leak into circulating blood from infracted myocardium and may be useful for expression profiling of coranory artery diseases. It has been reported that cardiac-specific miRNAs including miR-1, miR-133a, and miR-208a, play important roles in maintaining development and function [6]. Heart-specific miRNAs leak into the circulation during AMI and can be used to detect and monitor myocardial injury. These miRNAs are found to be consistently elevated in plasma of AMI patients within hours after the onset of infarction [7]. Most importantly, the technology used to detect miRNAs requires optimization. Although several RNA isolation protocols with appropriate recovery rates are available, normalization remains the major challenge. So in that case spiking of the plasma/serum samples with recombinant nonhuman miRNAs (eg, *Caenorhabditiselegans* miRNAs) might help to normalize for efficiency of RNA isolation and its subsequent quantification by real time PCR [8].

So in the light of this introduction we hypothesized that the cardiac specific miRNAs might release into the circulation during AMI and could be used to detect and monitor the myocardial expression profile. In this study, we detected the heart-specific and muscle-specific miRNAs in plasma from AMI patients and individuals who show no sign of this, to identify specific miRNAs that are elevated in plasma from AMI patients; these could be potential biomarkers for the diagnosis of AMI.

Methodology

Chemicals, Reagents and Kits

All chemicals and reagents used in this study were of the highest purity grade. For microRNA extraction, TRIzol LS Reagent (Cat# 10296-028) was used. Poly (A)

polymerase, (Cat# AM2030) was purchased from Ambion-Life Technologies. Reverse transcription was performed by using Improm-II™ Reverse transcriptase system (Cat# A3800 from Promega (USA). The study was performed at Institute of Biochemistry and Biotechnology and School of Biological Sciences, University of the Punjab, Lahore.

Sample Collection, Processing and Storage

Blood samples (3cc) were collected in EDTA tubes from patients admitted in ICU emergency for acute myocardial infarction from Punjab Institute of Cardiology, Lahore. Samples were collected within 2 to 4 hours from the onset of the symptoms. These samples were centrifuged at 3000g for 4 minutes at 4°C. Plasma was collected in RNase free sterile tubes. Aliquots were prepared (250µl each) for miRNA extraction and proteome analysis and were stored at -80°C.

Proteome Analysis

Protein Quantification

Total protein contents in plasma were estimated by Bradford assay [9], (Working reagent: 100mg Coomassie blue G250 in 50ml Ethanol added to 100ml 85% H_3PO_4, diluted to 1000ml with distilled H_2O) using Bovine Serum Albumin (BSA) as standard [9].

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For total plasma protein analysis SDS-PAGE was done. All reagents were prepared using a modification of the procedure described by [10].

Micro RNA (Mirna) Isolation and Quantification

miRNA Extraction using TRIzol

miRNA from plasma was isolated using Ambion TRIzol LS Reagent (Cat# 10296-028) following the protocol provided by the manufacturer. Briefly, 750ul of TRIzol LS Reagent was mixed with 250ul of plasma volume in RNase free tube and homogenized by pipetting the suspension up and down several times. The homogenized sample was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Then 200ul of chloroform per 750ul of TRIzol was added and again the mixture was homogenized, the tube was capped securely. The tube was then shaken vigorously by hand for 15 seconds tube was incubated for 15 minutes at room temperature. Then the sample was centrifuged at 12000g for 15 minutes at 4 °C. The aqueous phase of the sample was removed by angling the tube at 45° and pipetting the solution out. The aqueous phase was placed in a new RNase free tube and 500ul

of 100% isopropanol was added to it. The mixture was then incubated for 10 minutes at room temperature and centrifuged at 12000g for 10 minutes at 4°C. After centrifugation the supernatant was removed from the tube, leaving only the pellet. Then the RNA pellet was washed with 1ml 75% ethanol by vortexing the mixture. The sample was then centrifuged at 7500g for 5 minutes at 4°C, and the supernatant was discarded. The RNA pellet was air dried for 10 minutes and resuspended in RNase-free water. The miRNA was stored at -80° C for further downstream processing. The quantity of isolated miRNA was measured on Scandrop200/250. Concentration of RNA at 260nm was measured against RNase free water as blank.

Primer Designing

miRNA sequences were obtained from miRNA database miRBase (www.mirbase.org). With the help of these stem-loop sequences of mir-1, mir-133a, mir-208a and mir-208b "DNA primers" were synthesized.

Poly A Polymerase Reaction

Poly A tail (-A_{15}) was added to the isolated miRNA using poly (A) polymerase from Ambion, catalog# AM2030. For a 20µl reaction, in aRNase treated tubes, 100ng RNA was added with 1x Poly A polymerase buffer, 2.5mM $MnCl_2$, 1mM ATP and 1U Poly A polymerase. The reaction mixture was incubated at 37°C for 60 minutes. Enzyme activation was done at 65°C for 20 minutes.

Reverse Transcription Reaction

cDNA of poly A tailed miRNA was synthesised using Promega's Improm-II™ Reverse transcriptase system catalogue No. A3800. For 10µl reaction, 4µl of poly A tailed miRNA and 1µM of Universal Reverse transcription primer (Universal RT primer) was mixed. This primer template mixed was thermally denatured at 70°C for 5 minutes and then chilled on ice. Reverse transcriptase mixture was prepared by mixing 2.5mM $MgCl_2$, 1x RT buffer, 0.1mM dNTPs, and 100U of Improm-II™ Reverse Transcriptase. Annealing was done at 25°C for 5 minutes. And then strand synthesis was carried out at 42°C for 60 minutes. The product was then preceded to Real-time PCR analysis. The sequence of Universal RT primer was 5'CAGGTCCAGTTTTTTTTTTTTTTGT 3'.

Real-Time Polymerase Chain Reaction (qPCR)

For quantitative analysis of miRNA present in AMI sample and in normal samples quantitative real-time PCR was performed using CFX96™ Real-Time System with C1000™ Thermal Cycler from Bio-Rad. For 10µl reaction, 4µl of cDNA was mixed with 250nM of each primer and 5µl of 2X VeriQuest Fast SYBR Green qPCR master mix with Fluorescein (Cat # 75675) (Affymetrrix)

Cyclic conditions were 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds and at annealing temperature for primers set used for 20 seconds. Melting curve analysis from 65°C to 95°C was performed after the thermal profile. Quantification was based on determination of the quantification cycle (Ct values).

Characterization of Patients' Samples

The biochemical and clinical history reports of patients were collected from Pathology laboratory of Punjab Institute of Cardiology, Lahore. The study cohort includes age, gender, smoking, family history, hypertension, diabetes, CPK (U/L), Ck-MB (U/L), ALT (U/L) and ALP (U/L). Table 1 shows the clinical characteristics of AMI patients and normal samples.

SDS-PAGE Analysis

7 µg of protein of each sample was loaded on 12% SDS-gel (Fig.1). The gels were stained with colloidal coomassie and destained in water. These gels were analysed in gel documentation software. By comparing serum protein profile of patient samples with that of normal sample, we found that there was no significant over-expression or down-expression of any protein. One possibility of such results might be that these samples were collected in early hours from the onset of symptoms of AMI. Protein variations usually occur in late hours. These results can be improved by collecting samples twice of each patient: firstly, at early hours and secondly, at late hours.

miRNA Isolation

miRNA single-step isolation was done by using AmbionTRIzol LS Reagent (Cat# 10296-028). The method utilized here was (phenol/guanidiniumisothiocynate) organic phase extraction based isolation. After isolation, the isolated miRNA were stored at -80°C. Absorbance at 260nm of every sample was measured by Scandrop200/250 (Scandrop). Concentration in ng/ µl is given in Table 5.

Normalisation for qRT–PCR microRNA Expression Assays

cel-miR-39 (exogenous control) was used as a reference. Finally, the expression levels of miRNAs in (AMI) subjects were calculated with reference to Ct values of exogenous control [11].

Quantification of miRNA by Real-Time PCR

For real-time PCR analysis, the isolated miRNA were subjected to poly A polymerization. The size of miRNA is small, and the amount is relatively low than mRNA. So a poly A tail increases its length which aids in primer designing but also ensures specific detection with the use of universal reverse transcription primer.

Statistical Analysis

The Mann–Whitney U-test was used to detect differences with regard to Ct values. Relative miRNA expression levels between groups were calculated by using the Ct method (Livak and Schmittgen, 2001). Statistical analysis was performed using the IBM SPSS Statistics v.24 software package (IBM Corporation, Armonk, NY, USA).

Results and Discussion

Table 1. Clinical Characteristics of Patients with AMI and Normals

Characteristics	Total patients (n=52)	Total Normal (n=15)
AGE	52.98±9.77	43.53±16.5
SEX (F/M)	7/45	11/4
Smoking Habit (Y/N)	36/16	0/15
Diabetes mellitus (Y/N)	39/13	0/15
Streptokinase (Y/N)	46/6	0/15
Hypertension (Y/N)	42/10	3/12
Family History (Y/N)	15/37	4/11
Prothrombin Time (sec)	18.6±7.8	Ref range : (10-14 secs)
Creatine Phosphatase Kinase (U/L).	1232.13±972.5	Ref range : (24-195 U/L)
Creatine Kinase Isoform (U/L).	109±113.5	Ref range : (less than 24 U/L)
Alanine Transaminase U/L.	63.88±41.76	Ref range : (5-33U/L)
Alkaline Phosphatase (U/L).	84.2±29.1	Ref range : (35-104 U/L)

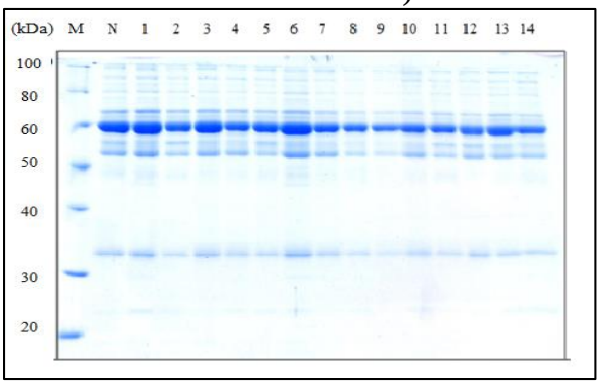
Table 2. Mean Value of Total Protein Concentration of AMI Samples and Normals

Number of samples	Sample type	Total protein conc. ug/µl (Mean Value)
52	AMI	104.36±85.6
15	Normal	80.83±62.7

Table 2 shows that the total amount of protein in patient plasma is higher than in a normal sample. Normal serum protein concentration ranges from 6 g/dl to

8g/dl (60-80 µg/ml). The values show increase in total serum protein in AMI patients. This could be due to an increase in serum globulins, as reported that serum globulins are elevated in AMI patients.

Figure 1. Protein Marker (Page Ruler™ Unstained Protein Ladder #SM0661fermentas)



12% SDS-Gel with 10µg of protein loaded N= Normal with M= Molecular marker, LANE 3-14 show representative patient samples. The gel images show that there is no significant variation in protein profile of normal and AMI sample.

No significant change was observed in terms of total protein concentration through SDS PAGE analysis of all the AMI patients against normal Figure 1.

Table 5. Mean Value of Total RNA Conc. of AMI and Normal Samples

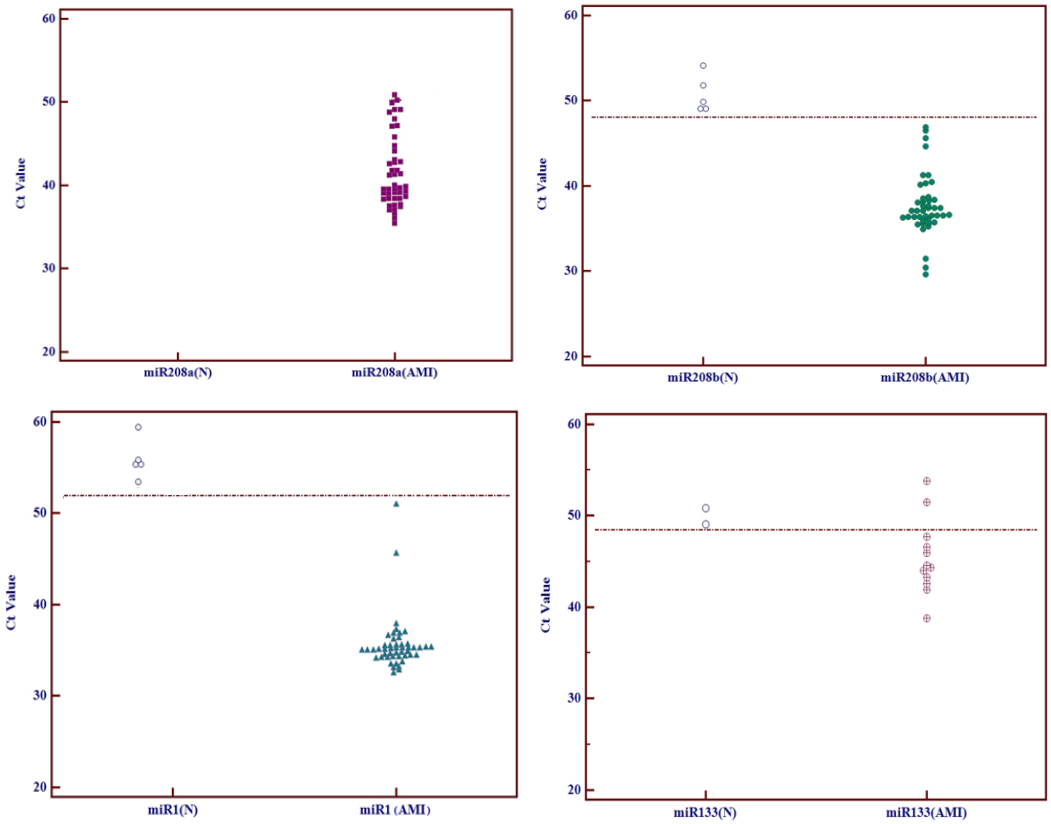
Number of samples	Sample type	Total RNA conc. ng/µl (Mean Value)
52	AMI	89.1±52.3
15	Normal	74.133±43.7

There is an overall increase in miRNA content in AMI patients as compared to healthy subjects.

In this study, the distribution of miRNA expression in plasma was analyzed using the strategy of isolating cell-free RNA from plasma and further poly A tailing and cDNA synthesis was done using Universal RT-primer. Furthermore real-time PCR analysis of U-cDNA was done using specific and efficient "DNA primers" and the results of qPCR showed significant variations. In significant numbers, miRNAs have been detected in cell-free human plasma preparations. They have been found to be stable and protected from endogenous RNase activity. In addition, levels of a specific miRNA (miR-208a) can distinguish

patients with AMI from healthy control. In our analysis, we found moderate to high levels of expression of miRNAs in plasma of patients with coronary disease.

Figure 2. The Statistical Distribution of the Ct Values Showed that Mir-208a Mir-1, Mir-133a and Mir-133b in Case of (AMI) were Significantly Upregulated in Comparison with Mir-208a, Mir-1, Mir-133a and Mir-133b of Normal Group



Several runs of qRT-PCR were performed to establish the miRNA profile associated with AMI. miR-1, is the most abundant heart and muscle-specific miRNA. Under physiological conditions, only a small amount of miR-1 is released into the blood. After damage, the released amount of miR-1 was associated with the extent and size of cardiac cell injury. The levels of circulating cell-free miR-1 were significantly increased in patients with AMI. These outcomes proposed serum miR-1 as a novel sensitive diagnostic biomarker for AMI [12].

miR-133 is expressed in skeletal muscle, and the increased level of these miRNAs in plasma might be due to skeletal muscle damage. This miR133 presented high levels in plasma from the AMI patients.

Our results from clinical samples in patients demonstrated that the levels of miR-1, miR-133a, miR-208b, and miR-208a in plasma from patients with AMI are elevated compared with those from healthy individuals. Real-time PCR analysis demonstrated that the miR-208a remained undetectable in the plasma from all healthy individuals. Furthermore statistical significance was determined by using the two-tailed Mann–Whitney non-parametric test that indicated ($P<0.001$) significance showing that these four miRNAs could be good biomarkers for AMI diagnosis.

Conclusion

This study provides clinical evidence that miRNAs are small RNAs that can be detected in cell-free plasma in a stable form and are related to select diseases. By examining few measurable miRNAs, and comparing plasma miRNA expression in patients with coronary disease, we begin to define specific miRNAs that are altered and provide potential targets that influence atherosclerosis. Our study demonstrated that circulating miR-208a as a marker of cardiac damage. However, research limitations exist in our study, including that the sample size is small. Therefore, additional investigations with larger cohorts of healthy people and patients are needed to extensively evaluate the miRNAs as practical biomarkers in comparison with other cardiac markers, as well as the false-positive rate. It may warrant further development of approaches for simple and rapid detection of miRNAs in blood. Furthermore the use of "qRT-PCR and DNA primers" is by far the most sensitive and specific means for the evaluation of miRNA profiles, elucidating clinical diagnosis and prognosis. It is possible that the detection of circulating miRNA biomarkers can be included in future routine clinical examinations for the diagnosis of early stages of cardiovascular disease [12].

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