ORIGINAL ARTICLE

MicroRNAs Expression Profile in Young Patients with Acute Myocardial Infarction

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ABSTRACT

INTRODUCTION: Acute myocardial infarction (AMI) is a severe coronary heart disease. Targeted miRNAs studies implicated two main pathways in the regulation of AMI namely pro-apoptosis (miR-29b and miR-194-5p on PTEN) and pro-necroptosis (miR-325 & miR-105 on RIPK3) pathways. This study aims to profile the miRNAs in Healthy Controls, Young AMI, and Mature AMI patients with matching criteria. **MATERIALS AND METHODS:** Total RNA was extracted from plasma and the miRNA expression profiling using small RNA was done on the BGISEQ500 SE5 sequencing platform with BGI sequencing libraries. The sequence data were analysed using Gene Ontology (GO) to determine the function of the differently expressed genes, while Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analyses were applied to identify the biological pathways in Young AMI against Mature AMI. **RESULTS:** Of 1497 differentially expressed miRNAs, 1090 miRNAs were upregulated, and 407 miRNAs were downregulated in Young AMI against Mature AMI. The top 10 upregulated miRNAs were miR-552, miR-4446-3p, miR-432-5p, miR-548j-5p, miR-219, miR-982, miR-181a-2-3p, miR-654-5p, miR-58 and miR-548k; while the top 10 downregulated were miR-16-5p, miR-1064, miR-431-5p, miR-790 miR-1177, miR-201, miR-105, miR-518, miR-419 and miR-1103. There were 9 novel miRNAs discovered in this study; miR-58, miR-982, miR -548k, miR-1064, miR-790, miR-1177, miR-201, miR-419, and miR-1103. The target genes of differentially expressed miRNAs that were mapped to the signal transduction pathway in KEGG indicated that 346 classes were enriched. **CONCLUSION:** Our miRNA profiling revealed differentially expressed miRNAs including 9 novel miRNAs in Young and Mature AMI that require further evaluations for their roles in AMI.

Keywords
microRNAs (miRNA), acute myocardial infarction (AMI), AMI pathogenesis

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INTRODUCTION

Acute myocardial infarction (AMI) is an early necrosis of heart muscle tissue that occurs due to acute occlusion or spasm of coronary arteries, which are characterised by ST and T waves changes in the reflecting leads and elevated levels of cardiac enzymes.[37] It is the most lethal presentation of coronary heart disease which is associated with high mortality and disability. The incidence of AMI is increasing in recent years and it is associated with younger ages where the prevalence in young populations less than 40 to 45 years old ranged between 2 and 10 percent around the world.[1,28] Current data from the Department of Statistics Malaysia, showed that the prevalence of AMI in adults aged 41-59 years old is 20% and in adults aged more than 60 years old is 18%.[5]

Currently, the pathogenesis of AMI has not been fully clarified as their complex regulatory mechanisms have not been completely understood. Inflammation, abnormal regulation of myocardial cell death including apoptosis, necrosis, and autophagy as well as cardiomyocytes regeneration were associated with myocardial cell injury and development of AMI[32, 39] where these processes are

regulated by miRNAs.[10, 39, 43, 46, 47]

miRNAs are short, endogenous, single-stranded, and highly conserved RNAs with around 20-25 nucleotide (nt).[35] miRNAs play vital roles in gene expression regulation, post-transcriptionally where they are estimated to account for 1-5% of the human genome and regulate about 60% of protein-coding genes.[20,30] miRNAs involve in numerous biological and cellular processes by modulating the signaling pathways of the target genes expressions.[34] They are available in all organs and tissues as well as the circulation where an alteration in their expression levels are associated with many diseases including AMI.[3,27,44] Recently, many related studies have demonstrated that miRNAs regulate various function of cardiomyocytes and abnormal miRNAs expressions could lead to the occurrence of cardiovascular diseases.[7, 8, 13, 19, 47]

AMI usually encompasses different cell death processes including apoptosis, necrosis, and autophagy.[32] Both pro- and anti-apoptotic miRNAs have been discovered to regulate apoptosis such as miR-29b and miR-194-5p were all anti-apoptotic in cardiomyocytes by targeting phosphatase and tensin homolog (PTEN) in vitro and in vivo.[15,45] In contrast, miR-19b-3p and miR-221 downregulate PTEN and thus promotes apoptosis in cardiomyocytes.[10, 13]

Additionally pro- and anti-necrotic or necroptotic miRNAs are also involved in regulating myocardial cell death and AMI. miR-325-3p and miR-105 were anti-necroptotic in cardiomyocytes by inhibiting receptor-interacting protein kinase 3 (RIPK3) and B-cell lymphoma 2 (BCL2) and adenovirus E1B 19 kDa-interacting protein 3 (BNIP3).[29, 43] Pro- and anti-autophagic miRNAs were also found to be involved in AMI cell death process such as miR-490-3p and miR-590-3p that were pro-autophagic in cardiomyocytes by targeting autophagy-related 4B cysteine (AT4B) and hypoxia-inducible factor 1α (HIF-1α).[7,40] The miRNAs from injured or apoptotic cardiomyocytes are released into the circulation by encapsulated in the apoptotic bodies, microvesicles, or exosomes, or associated with Argonaute protein or nucleophosmin-1 protein, and high-density lipoprotein (HDL) or low-density lipoprotein (LDL) as protection from digestion.[6] These circulating miRNAs are stable in the peripheral blood and therefore their use as biomarkers are reliable to detect early changes in AMI pathogenesis.[44] The expression profiles of circulating miRNAs in healthy individuals and patients with various diseases are significantly different.[4] The current study conducted a hospital-based case-control study where small-RNA sequencing (sRNA-seq) was used to detect the miRNAs in all participants. The differential expression of miRNAs in healthy subjects, Young AMI, and Mature AMI patients and their connections in the incidence and trend of AMI may provide new information to the early detection of myocardial injury, providing prognosis and predicting the development of complications.

MATERIALS AND METHODS

Subject

Subjects for normal controls were recruited from Klinik Kesihatan Bandar Kuantan and among International Islamic University Malaysia (IIUM) staff, Kuantan, Pahang. The AMI patients were recruited from the Emergency Department (ED) of Hospital Tengku Ampuan Afzan, and the ED of Sultan Ahmad Shah Medical Centre @ IIUM (SASMEC@IIUM), Kuantan, Pahang. The study was conducted following the Declaration of Helsinki[38] and guidelines from the Ethical Committee of Kulliyyah of Medicine, IIUM (IIUM/305/20/4/1/7) and Medical Research and Ethical Committee (MREC), Kementerian Kesihatan Malaysia (NMRR-16-2572-32869 (IIR)).

The participants were divided into 3 groups; healthy control, Young AMI, and Mature AMI groups. The criteria for healthy controls were Malaysian aged 18 to 45 years who were healthy with no known chronic illnesses, alcohol consumption, or on any medication. The inclusion criteria for AMI groups were Malaysian with a first episode of clinically confirmed ST-elevation myocardial infarction (STEMI), age ≤ 45 years for the Young AMI group, and age ≥ 46 for the Mature AMI group. Exclusion criteria for AMI groups were any prior thrombolytic therapy or percutaneous intervention and other known chronic diseases, alcohol consumption as well as those on any

medication. STEMI is defined by local guidelines as elevation of ST segment ≥ 1 mm in two contiguous electrocardiographic (ECG) leads or the presence of a new left bundle branch block (LBBB) with positive cardiac enzymes.[23] All subjects who fulfilled the study criteria and consented were enrolled.

For the sRNA-seq, 3 healthy controls, 3 Young AMI patients, and 3 Mature AMI patients were recruited. The present study only included Malay male participants to preserve the homogeneity of the data.

Sample Collection and Human Plasma Isolation

Three (3) millilitres of peripheral venous blood were collected into EDTA tubes from AMI patients upon presentation at the ED after confirmation of the clinical diagnosis of STEMI and from healthy controls after an overnight fast. The plasma was isolated within 1 hour by centrifugation at 2500 rpm for 10 minutes and aliquoted into several RNAse-free tubes and stored at -80°C until further use.

miRNA Extraction

Total RNA was extracted from plasma using miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) with final elution of 20 µL with RNase-free water according to the manufacturer's protocol. The quality and quantity of RNA were evaluated with SimpliNano spectrophotometer (GE Healthcare Life Sciences, Buckinghamshire, UK) and QIAxel RNA QC Kit v2.0 using QIAxel Advanced System (Qiagen, Hilden, Germany).

Small-RNA Sequencing (sRNA-seq)

The library construction and sRNA-seq were performed using BGISEQ500 SE50 (BGI, Shenzen, Guangdong, China). Small RNA libraries were constructed using the BGI protocol. Small RNAs were enriched and purified and the 3' end adapter was ligated. Then Unique molecular identifier (UMI) labelled Primer was added followed by the digestion of the unligated adaptors and 5' end adaptor ligation. Next, the cDNA was synthesized with UMI labelled primer followed by fragment selection. The ligation product was then amplified and subjected to the single-strand circularization process, deriving a single-strand circular DNA library. Following the library quality control (QC), the single-strand circular DNA library was amplified using PCR as per the manufacturer's protocol to produce DNA NanoBalls (DNBs). Next, the DNBs were loaded onto the sequencing chip, and finally, sequencing was done using the BGISEQ500 SE50 platform at BGI (Shenzen, Guangdong, China).

Sequencing Data Analysis

After filtering the raw data, the remaining clean data was stored in FASTQ format. *Bowtie2* was used to map the clean data to the reference genome and other sRNA databases including miRbase, primabank, snoRNA, Rfam, and also miRDeep2. RNAhybrid, TargetScan, and miRanda were used to find the target gene of miRNAs. DEGseq method was used to analyse the differentially expressed SRNAs (DESs).

Identification of Differently Expressed Genes (DEGs)

DEGseq method was used to analyse the differentially expressed miRNAs between the 3 groups. *P*-value was adjusted with the *q*-value where any *q*-value < 0.05 and [log2(fold change)] > 1 was put as the threshold for the significantly differential expression by default.

Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) Enrichment Analysis

For the gene ontology (GO) enrichment analysis, all genes were mapped to the GO-terms in the database (http://www.geneontology.org/) according to the principle of GO classification.[42] All the information were annotated and classified according to the biological processes, molecular functions, and cellular components. Kyoto Encyclopaedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), a major public pathway-related database, was used to perform the pathway enrichment analysis where it identified significantly enriched metabolic

pathways or signal transduction pathways in the target genes when compared to the whole genome background.[9] These pathways were classified into metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases, and drug development where each category was further divided into sub-classes.

Statistical Analysis

SPSS statistical software version 28.0 was used for the data analysis. The normally distributed data were reported as mean (SD) and the non-normally distributed data were reported as median (IQR). Chi-square and ANOVA tests were used to analyse the differences between the three groups while an unpaired T-test was used to analyse the differences between the two groups. *P*-value < 0.05 was considered as statistically significant.

RESULTS

Baseline Clinical Characteristics of the Study Subjects

The clinical characteristics of these three groups were matched as close as possible except for age, systolic blood pressure (SBP), and diastolic blood pressure (DBP). Mature AMI patients were older with a mean (SD) age of [53.00 (2.00) years] compared to Young AMI patients [40.00 (1.00) years] and healthy controls [39.33 (3.06) years]. Young AMI patients were also having higher SBP with mean (SD) of [140.2 (15.59) mmHG] and DBP [92.8 (9.41) mmHg] compared to Mature AMI patients [122.1 (2.42) and 74.9 (4.16) mmHg]] and healthy controls [115.6 (2.01) and 74.7 (3.28) mmHg].

Differentially Expressed miRNAs Profile

miRNAs were analysed with strict data quality control. These miRNAs were considered significantly upregulated if the fold change (FC) of the relative expressions are ≥ 1 and p ≤ 0.05 and considered significantly downregulated if the FC ≤ 1 and p ≤ 0.05.

The volcano plot in Figure 1 showed a total of 1599 miRNAs that were differentially expressed in AMI (Young and Mature AMI) patients compared to healthy controls, where 1288 miRNAs were upregulated and 311 were downregulated. When miRNAs expression of Young AMI patients were compared to Mature AMI patients, a total of 1497 miRNAs were noted to be differently expressed, where 1090 miRNAs were upregulated and 407 were downregulated.

The top 10 significantly upregulated miRNAs between Young AMI and Mature AMI patients were miR-552, miR-4446-3p, miR-432-5p, miR-548j-5p, miR-219, miR-982, miR-181a-2-3p, miR-654-5p, miR-58 and miR-548k, and the top 10 significantly downregulated miRNAs were miR-16, miR-1064, miR-431, miR-790 and miR-1177, miR-201, miR-105, miR-518, miR-419 and miR-1103 as displayed in Table 1.

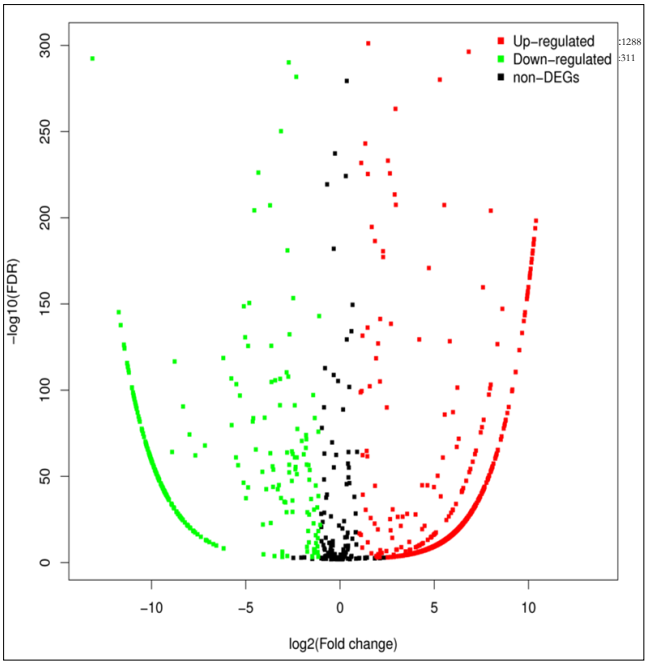

Figure 1: Volcano plot of differential miRNA expression between Controls and AMI (Young AMI and Mature AMI) patients. X-axis: log2 fold change; Y-axis: -$\log_{10}$ (corrected *q*-value) for each probe.

Target Prediction and Functional Analysis of Differentially Expressed miRNAs

The genes were classified according to Cellular Component, Molecular Function, and Biological Process by GO analysis. For 1497 differentially expressed miRNAs in Young AMI patients compared to Mature AMI patients, 34,195 target genes were predicted by GO analysis. The functional analysis revealed that 11,199 GO terms were involved in biological processes, 10,984 in molecular

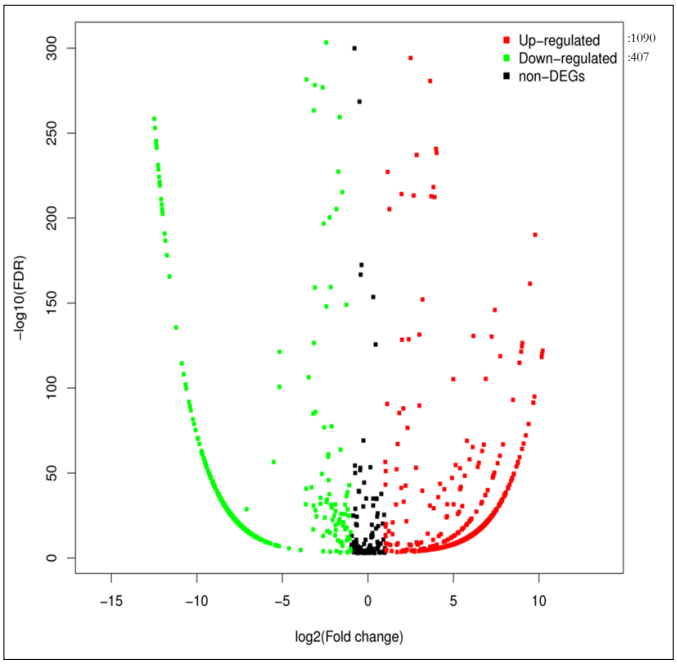

Figure 2: Volcano plot of differential miRNA expression between Young AMI and Mature AMI patients. X-axis: log2 fold change; Y-axis: -$\log_{10}$ (corrected *q*-value) for each probe.

Table 1: Top 20 differentially expressed miRNAs in Young AMI versus Mature AMI in sRNA-seq

miRNAs	Young AMI vs Mature AMI		
	Regulation	Fold change	p-value
miR-552	Up	13.74	< 0.0001
miR-4446-3p	Up	11.50	< 0.0001
miR-432-5p	Up	10.57	< 0.0001
miR-548j-5p	Up	10.21	7.65E-121
miR-219	Up	10.18	1.39E-118
miR-982	Up	10.16	3.86E-117
miR-181a-2-3p	Up	10.09	< 0.0001
miR-654-5p	Up	9.78	4.62E-189
miR-58	Up	9.74	9.55E-94
miR-548k	Up	9.67	2.90E-90
miR-16	Down	-15.91	< 0.0001
miR-1064	Down	-12.49	1.69E-257
miR-431-5p	Down	-12.45	4.13E-252
miR-790	Down	-12.39	2.42E-244
miR-1177	Down	-12.38	2.52E-243
miR-201	Down	-12.38	3.1E-243
miR-105	Down	-12.36	3.48E-241
miR-518	Down	-12.35	1.81E-240
miR-419	Down	-12.27	2.95E-230
miR-1103	Down	-12.25	1.20E-227

Note. Paired T-test; Significant difference at 95 % confidence interval, with fold change ≥ 1 or fold change ≤ 1.

Target Prediction and Functional Analysis of Differentially Expressed miRNAs

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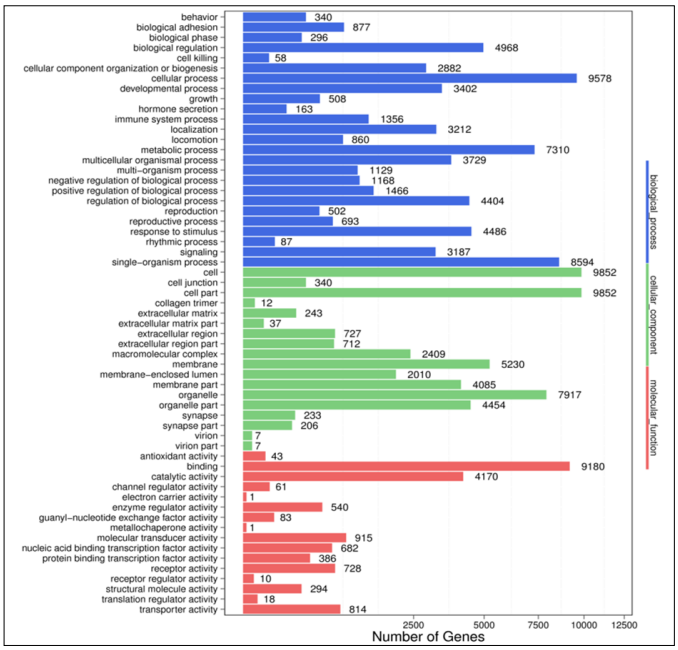

Figure 3: GO analysis of differentially expressed miRNAs that covers three domains: Biological Process, Cellular Component, and Molecular Function. X-axis: Number of genes (miRNAs). Y-axis on the left: GO terms of Biological process, cellular component, and molecular function. The blue row indicates the biological process, the green row indicates cellular component, and the red row indicates molecular function.

KEGG Pathway Analysis of Targets of Differentially Expressed miRNAs

To identify the biological pathway in Young AMI patients compared to Mature AMI patients, target genes of differently expressed miRNAs were mapped in the signal transduction pathway, KEGG, which revealed that 346 categories were enriched. The top 20 pathways according to p-value were displayed in Figure 4.

DISCUSSION

sRNA-seq is the next-generation sequencing that allows genome-wide profiling and analysis of the known, novel, and also miRNA variants as its high sensitivity enable for profiling of low input samples.[2] miRNA profile may give useful diagnostic and prognostic information, since the changes in the miRNA expression may reflect the genetic

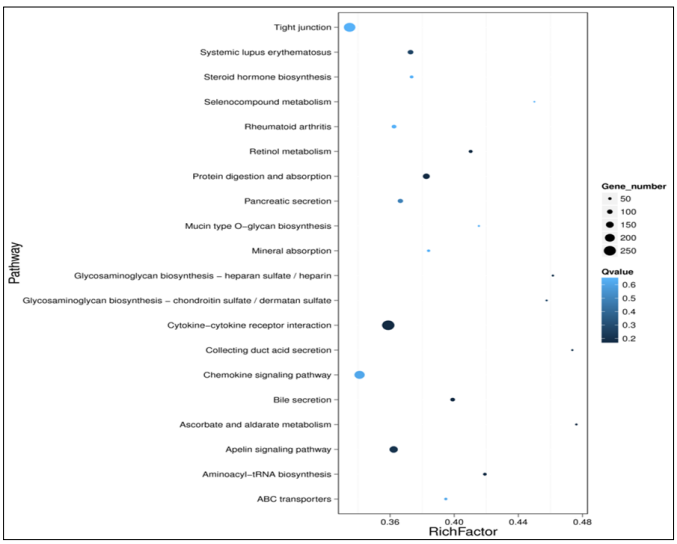

Figure 4: Scatter plot of enriched KEGG Pathway analysis of differentially expressed miRNAs between Young AMI and Mature AMI patients. Pathway analysis is a functional analysis mapping genes to the KEGG pathway and other pathway databases. The Rich factor is the ratio of differentially expressed gene numbers annotated in this pathway term to the total gene numbers. The greater the Rich factor, the greater the degree of pathway enrichment. A Q-value is the corrected p-value ranging from 0 – 1. The lower the p-value, the more significant the pathway. The colour and size of the dots represent the range of the Q-value and the number of DEGs mapped to the indicated pathways, respectively. The top 20 enriched pathways are shown in the figure.

and protein changes associated with the pathogenesis of many diseases.[26] The present study was done to identify the differently expressed miRNAs profiles in Young AMI patients compared to Mature AMI patients where it is hoped that it could lead to the identification of miRNAs that could be used to identify early myocardial injury and development of AMI as well as it's a complication in this young patients so that effective treatment could be administered in time.

The involvement of miRNAs in the pathogenesis of AMI in the young population has not been extensively studied. A previous study with a different study design on young STEMI and NSTEMI found that miR-183-5p was upregulated in NSTEMI while miR-134-5p, miR-15a-5p and let 7i-5p were downregulated in STEMI patients, compared to controls.[34]

Among the top 10 upregulated and downregulated miRNAs in our study, miR-552, miR-432-5p, miR-548j-5p, miR-219, miR-181a-2-3p, miR-654-5p, miR-16, miR-431-5p, miR-105, and miR-518 were associated with AMI pathogenesis in recent studies. Atherosclerosis is the main underlying cause of AMI and it is closely associated with cholesterol biosynthesis.[23] miR-552 is one of the top 10 upregulated miRNAs in Young AMI compared to Mature AMI patients in our study. In a previous study, it was identified as a new proprotein convertase subtilisin kexin 9 (PCSK9) inhibitor that reduces low-density lipoprotein-cholesterol (LDL-C) by promoting LDL-C reuptake and lower serum LDL-C in a high-fat diet fed-mice which subsequently reduces the risk for developing atherosclerosis and AMI.[18] Meanwhile, several miRNAs were implicated in the inflammatory responses that were involved in the progression of atherosclerosis and AMI. For instance, miR-654-5p that was upregulated in Young AMI patients in our study was reported to be involved in the inflammatory response in atherosclerosis through lncRNA ZFAS1 mediation, by targeting A Disintegrin and Metalloproteinase 10 (ADAM10) and Ras-related protein Rab-22A (RAB22A).[33] In contrast, miR-181a-5p, and miR-181a-3p, were shown to prevent the progression of atherosclerosis through inhibition of nuclear factor kappa B (NF-$\varkappa$B) activation and vascular inflammation by targeting transforming growth factor β (TGF-β) activated kinase 1 (TAK1) binding protein 2 (TAB2), and Nuclear factor-kappa B essential modulator (NEMO) and thus reduce the risk of developing AMI.[31]

Moreover, miR-548j-5p promotes angiogenesis in ischaemic tissue by targeting the nitric oxide synthase (NOS) and stromal cell-derived factor (SDF)-1 signaling pathways[11] while decreased expression of miR-548 upregulated folate receptor 3 (FOLR3) and interleukin-29 (IL-29), thus reducing blood pressure and thrombus burden.[36] In contrast, miR-432-5p was involved in cardiac hypertrophy by targeting toll-like receptor 4 (TLR4) through binding with lncRNA CASC15 that was activated by transcription factor vitamin D receptor (VDR).[12] The upregulation of miR-548j-5p and miR-432-5p in Young AMI against Mature AMI patients in our study may suggest higher cardiac remodeling activity through angiogenesis and cardiac hypertrophy and hence indicate better prognosis and recovery in the young population.

Myocardial cell death has a vital role in the pathogenesis of AMI where it is regulated by miRNAs.[32] Overexpression of miR-219a and suppression of miR-16 reduce cardiomyocyte apoptosis through blockade of TLR4 pathway by targeting Absent in melanoma 2 (AIM2)[16] and

by reversing beta2-adrenergic receptor downregulation respectively.[17] The dysregulated miR-219 and miR-16 in Young AMI against Mature AMI patients in our study might confirm the good prognosis of AMI in young patients compared to more mature patients[25] and they may be used to predict AMI prognosis. Other miRNAs that suppress apoptosis in cardiomyocytes are miR-431, 518a-5p, and miR-105, by targeting autophagy-relate 3 (ATG3), Granzyme B (GZMB), RIP3, and BNIP3 respectively.[29, 41, 47] The downregulation of miR-431, miR-518, and miR-105 in Young AMI against Mature AMI patients in our study, suggest that age factor may lead to differential expression of these miRNAs.

There are no or limited studies on miR-4446-3p, miR-58, miR-982, miR-548k, miR-1064, miR-790, miR-1177, miR-201, miR-419, and miR-1103 concerning the pathophysiology of AMI or atherosclerosis. However, a study on ischaemic stroke found that miR-548k has a potential diagnostic value for the disease with the area under the curve (AUC) value of 1.0 ($p = 0.047$; 95% CI, 1.00 to 1.00).[48] Another study on miR-4446-3p in mesenchymal stem cells (MSCs) revealed that inflammatory cytokines may cause changes in exosomal miRNAs that affect the cellular components, molecular functions, and biological processes in ischaemic diseases.[8] Therefore understanding the roles of these miRNAs in the pathogenesis of AMI are crucial.

According to the database from the KEGG pathway, the signal transduction pathways associated with AMI in our study include ascorbate and aldarate metabolism as well as glycosaminoglycans biosynthesis – heparan sulfate/heparin (Figure 4). Ascorbate and aldarate metabolism are part of the carbohydrate and glucose metabolism. However, there is no information on how the ascorbate and aldarate metabolism is involved in AMI, particularly in the young population. Park et al. (2018) demonstrated that glucose metabolism is associated with the sustainable proliferation of cancer cells.[22] However, the effect may be different in ischaemic and infarcted myocardial cells.

Glycosaminoglycans (GAG) are part of the non-structural components of the cardiac extracellular matrix (ECM) which is involved in cardiac development and remodeling.[24] During pathological cardiac remodelling, GAGs accumulate and mediate myocardial inflammation and fibrosis via direct binding of tumour necrosis factor-α to the GAG chains.[46] In the present study, the KEGG pathway revealed that the GAG biosynthesis is associated with AMI, particularly between Young AMI and Mature AMI patients. This suggests that the differentially expressed miRNAs may regulate the functions of the target genes in these signaling pathways during the formation and development of AMI in the Young population. However, further clarifications are needed as the number of samples in this study was relatively small.

In summary, our findings indicated that it has significant changes in the expression of various miRNAs in Young AMI compared to Mature AMI patients. We also discovered new unknown miRNAs, miR-58, miR-982, miR-548k, miR-1064, miR-790, miR-1177, miR-201, miR-419, and miR-1103; and suggested that these miRNAs regulatory mechanisms on gene expression may be more closely involved in Young AMI. In the course of formation and development of AMI in young patients in our study, the differential expression of miRNAs may be related to the ascorbate and aldarate metabolism signaling pathway as well as glycosaminoglycans synthesis - heparan sulfate/heparin signaling pathway. This study requires further elaboration with functional studies in a larger population.

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