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Unique MicroRNA signatures associated with early coronary atherosclerotic plaques

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ABSTRACT

The pathophysiology of coronary atherosclerotic plaques is a complex process. Early detection of coronary atherosclerotic plaques is critical in the prevention, prognostic and therapeutic intervention of cardiovascular disease. MicroRNAs (miRNAs), endogenous short non-coding RNAs, have been reported to play an important role in cardiovascular diseases and are also used as disease markers. However, the miRNA expression profile in early coronary atherosclerotic plaques has yet been reported. We hypothesize that miRNAs can be used as effective disease markers for detection of early coronary atherosclerotic plaques. In this analysis, coronary artery samples from three patients with early coronary atherosclerosis were harvested and miRNA expression profile determined using microarray analysis. Compared with healthy controls, a total of 44 miRNAs were upregulated and 57 miRNAs were downregulated. Among the dysregulated miRNAs, eight were significantly upregulated while five miRNAs were significantly downregulated, as determined by *t*-test ($P < 0.05$). Four of the significantly dysregulated miRNAs, including miR-221, miR-155, miR-100 and hsa-miR-1273, were selected and verified by real-time PCR. The real-time PCR results were consistent with the microarray data that miR-221, miR-155 and miR-100 were significantly downregulated in plaques, whereas miR-1273 was significantly upregulated. These results indicate that miRNAs expression level can be used as potential markers for early coronary atherosclerotic plaque formation.

1. Introduction

During the early stages of atherosclerosis, risk factors such as hypertension, hypercholesterolemia, advanced glycation end products (AGEPs), chemical stimulation (tobacco smoking), obesity, genetic abnormalities may contribute to endothelial cell damage. Macrophages are then recruited to the damage sites, followed by the uptake of oxidized LDLs and transformation into foam cells, resulting in the formation of early atherosclerotic plaques. Plaque volume seem to be positively correlated with the endothelial cell damage level, Plaques become unstable under shear stress and inflammatory factors, leading to vessel embolism and necrosis of important organs like heart and brain, ultimately resulting in organ death. Cardiovascular disease, such as CAP, is the second leading cause of death due to its complex etiology, prevalence, and high mortality and morbidity. Therefore, early detection of coronary atherosclerotic plaques is beneficial for the prognostics, prevention, and therapeutic intervention and control of atherosclerotic diseases progression.

MicroRNAs (miRNAs) are short (20–24 nt) non-coding RNAs that are endogenously expressed and play an important role in post-transcriptional regulation of gene expression in multicellular organisms by altering both the stability and translation. Several studies suggest the involvement of miRNAs in cardiovascular development and disease, and specifically miRNAs have been shown to influence heart formation [1], vascular development [2–4], smooth muscle differentiation and phenotypic modulation [5]. Furthermore, miRNAs have been demonstrated to be involved in cardiovascular diseases like myocardial infarction [6], cardiac hypertrophy [7–9], and atherosclerosis [10]. Endothelial cell-specific miR-126 modulates the expression of *Vcam1* to regulate

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tumor necrosis factor-α–stimulated leukocyte adherence to endothelial cells and vessel inflammation. In addition, it has been shown that in endothelial cell–derived apoptotic bodies miR-126 mediates intracellular communication to facilitate to recruit *Sca1*$^{+}$ endothelial progenitor cells, that attenuate plaque formation [11]. Vessel damage decreases the expression levels of miR-143 and miR-145, which in turn modulate smooth muscle cell differentiation and affect lesion formation [12].

miRNAs have been widely shown to have therapeutic and diagnostic application in cardiovascular diseases [13,14]. However, little is known about the miRNA expression profile in early coronary atherosclerotic plaques, and whether they can be used as markers for diagnosing early coronary atherosclerotic plaques. In this study, using microarray techniques, we analyzed the expression of miRNAs in early plaques to help in the management of early coronary atherosclerotic plaques. Although several miRNA profiling reports exist, we have first used microarray techniques on human coronary artery samples. Our results provide new candidates that contribute to the prevention, prognostics and therapy of atherosclerotic diseases.

2. Materials and methods

2.1. Materials

Human heart samples were obtained from three patients of 18–25 year old males. The subjects were donated to our hospital. Organs were harvested for transplantation. Valves were removed for cardiac transplantation, and the remaining heart tissues were used in this study. The research protocol was approved by the ethical committee of Qilu hospital, Shandong University.

2.2. Specimens

Coronary arteries were isolated and divided into right and left branches, each of which was further sub-divided into left anterior descending (LAD), circumflex (CB) and diagonal branches (DB). Coronary arteries were cut into 3–4 mm pieces, and vessels including atherosclerotic plaques and normal control vessels were separated and stored in liquid nitrogen. Normal artery wall consists of 3 layers: intima, media and adventitia, with a smooth and elastic intima, however, vessels with atherosclerotic plaques showed white or yellow lipid-fibrous, oval hard caps, and lack elasticity.

2.3. MicroArray analysis

MicroRNA microarray analysis was carried out using miRCURY™ LNA Array (v.14.0) (Exiqon), provided by Kangchen LTD, Shanghai.

2.4. Total RNA extraction

The samples were homogenized in 1 mL Trizol (Invitrogen) by Mini-Bead-Beater-16. After incubation for 5 min at room temperature, 0.2 mL chloroform was added to the homogenate. The mixture was then vortexed vigorously for 15 s and incubated at room temperature for 3 min and centrifuged at 12,000 rpm, at 4 °C for 15 min. After centrifugation, the aqueous phase was transferred into a new tube and mixed with 1.5 volumes of ethanol and vortexed. Then, 700 μl mixture, 700 μl RWT and 500 μl RPE were sequentially added into RNeasy Mini spin column (Qiagen) and spun at 8000 rpm for 15 s each. The columns were washed again with 500 μl RPE, centrifuged for 2 min at 8000 rpm for drying. The columns were then transferred into a new 1.5 mL tube, and lastly RNA was eluted with 20 μl RNAse free water at 8000 rpm for 1 min.

RNA concentration were determined by Nanodrop ND-1000, and quality were measured by bioanalyzer 2100. The quality of RNA was also assessed by agarose gel electrophoresis. A mixture comprised of 2 μl 10xMOPS buffer, 3.5 μl formaldehyde, 10 μl deionized formamide and 4.5 μl RNA sample in a DEPC treated 0.5 mL tube, was incubated in 60 °C water for 10 min and frozen on ice for 2 min. Next, 3 μl loading buffer was added into the mixture and the samples were subjected to electrophoresis using 1xMOPS buffer at 7.5 V/ml.

2.5. Fluorescent labeling, microarray hybridization, image capture and data analysis

RNA samples were labeled with fluorescent probes using miRCURY™ Array Power labeling kit (Cat #208032-A, Exiqon), according to the manufacturer's manual. The labeled RNAs were spun briefly and stored at 4 °C. A mixture of 12.5 μl labeled RNA, 90 μl 2xHybridization Buffer, 77.5 μl RNAse-free water were loaded into miRNA microarray, incubated at 95 °C in the dark for 2 min under standard conditions (PhalanxTM Hybridization bag at 56°C/2 rpm overnight) and chilled on ice for 2 min. After hybridization, the array was removed from the hybridization bag and washed with RCURY™ ArrayWash buffer kit. The array was then dried at 200 rpm for 5 min, immediately followed by imaging as below.

(1) Images were captured with GenePix 4000B, and the original fluorescence intensity was calculated and analyzed with GenePix proV6.0 (*t*-test). We used Lowess (Locally Weighted Scatter plot Smoothing) Normalization (MIDAS, TIGR Microarray Data Analysis System) for within-slide normalization. In the absence of a common reference standard, scale normalization between slides was performed) to adjust for scale differences. (2) After normalization, the statistical significance of differentially expressed miRNA was analyzed by *t*-test. (3) Unsupervised hierarchical clustering was performed using miRNA data.

2.6. Real-time RT-PCR

Several miRNAs related to plaque instability were selected for further validation with real-time RT-PCR: hsa-miR-1273, hsa-miR-100, hsa-miR-221, and hsa-miR-155. The miRNA specific primers sequences are listed below:

a) miR-100F: 5′-ACACTCCAGCTGGGAACCCGTAGATCCGA-3′
b) miR-1273F: 5′-ACACTCCAGCTGGGGGGCGACAAAGCAAGAC-3′
c) miR-221F: 5′-ACACTCCAGCTGGGAGCTACATTGTCTGCTG-3′
d) miR-155F: 5′-ACACTCCAGCTGGGTTAATGCTAATCGTGA-3′

2.7. HE staining

Isolated tissues were fixed, paraffin-embedded, and sectioned (4 μm). Sections were deparaffinized in xylene; and washed with ethanol and water: xylene I 5 min, xylene II 5 min, 100% ethanol 2 min, 95% ethanol 1 min, 80% ethanol 1 min, 75% ethanol 1 min and water 2 min. The sections were then stained with hematoxylin for 5 min, and then washed with tap water. The stains were removed using acid and alcohol for 30s, rinsed well in tap water for 15 min or in water at 50 °C for 5 min, and counterstained with Eosin for 2 min. The samples were then dehydrated, cleared and mounted: 95% ethanol (I) 1 min, 95% ethanol (II) 1 min, 100% ethanol (I) 1min, 100% ethanol (II) 1 min, Xylene phenolic (3:1) 1 min, Xylene (I) 1 min, Xylene (II) 1 min, and mounted in neutral resins.

3. Results

3.1. Early morphological change in atherosclerosis

For each of the three cases, normal coronary artery and arteries with plaques were analyzed in pairs. Fig. 1A shows intimal thickness of more than 0.9 mm. Intimal thickening indicates elevated endothelial permeability to lipids and is the earliest morphological change in atherosclerosis. A few rounded foam cells with plenty of vacuoles in the cytoplasm can be observed beneath the endothelial cells. Additionally, inflammatory cell infiltration is also observed. Fibrous plaques in early to mid-atherosclerotic lesions with plenty of collagen fibrils, and smooth muscle cells displaying hyaline degeneration, and large number of foam cells, inflammatory cells and cholesterol crystals were observed beneath fibrous cap (Fig. 1B).

3.2. Differential expression of miRNAs in coronal artery with atherosclerotic plaques

In the coronary miRNA microarray expression profiling, we find that in comparison with the miRNA expression level in healthy vessels and coronary artery with atherosclerotic plaques showed that 44 miRNAs were upregulated and 51 miRNAs were downregulated in atherosclerosis.

MicroRNAs with two-fold change in expression are shown here (Fig. 2A). Among these dysregulated miRNAs, 8 miRNAs were significantly upregulated: hsa-miR-221, hsa-miR-363, hsa-miR-29c, hsa-miR-199b-5p, hsa-miR-508-5p, hsa-miR-497,hsa-miR-100,hsa-miR-155 (Fig. 2B and Table 1), and 5 miRNAs were downregulated (Fig. 2C and Table 2), including hsa-miR-490-3p, sa-miR-1284, hsa-miRPlus-F1021, hsa-miR-1273, hsa-miRPlusF1195 by *t*-test ($P < 0.05$). These genetic changes reflect differences between the early plaque tissue and normal tissue expression, and the data suggest that these alteration can distinguish the molecular phenotype of early plaques from normal tissue and can be taken as a potential biomarker.

3.3. Real-time PCR validation of the microarray results

To verify the microarray results, the expression of hsa-miR-1273, hsa-miR-100, hsa-miR-221, hsa-miR-155 were analyzed with real-time PCR. The levels of miRNA microarray signal intensities were compared with the levels of PCR results and demonstrated that the qRT-PCR analysis confirmed the results obtained by microarray analysis. Relative expressions were shown as Fig. 3. The hsa-miR-100, hsa-miR-221, hsa-miR-155 were upregulated in plaque group, and hsa-miR-1273 was downregulated in plaque group. The results are consistent with microarray data.

4. Discussion

Atherosclerosis is attributed to endothelial cell injury and a result of deranged apoptosis, intimal invasion and lipid deposits, monocyte adherence, and macrophage transformation. MicroRNAs have been shown to participate in smooth muscle cell proliferation, and migration to intima and matrix synthesis. Elucidation of the mechanisms by which these miRNAs undergo is a top research priority.

To our knowledge, this is the first report in characterizing early plaques in atherosclerosis, directly from human coronary artery samples of 18–25 year old Chinese males. We compared the miRNA expression level of coronary artery tissues with early atherosclerotic plaques and normal coronary artery tissues using microarray analysis. Of the 101 differentially expressed miRNAs, 44 miRNAs were upregulated in plaque tissues, and 57 were downregulated. The miR-221, miR-155, miR-100, hsa-miR-378, hsa-miR-335*, and hsa-miR-2115* were significantly upregulated, while hsa-miR-1273 was significantly downregulated in plaque tissues. Furthermore, the upregulation of miR-100, miR-155, miR-221 and downregulation of miR-1273 in early atherosclerotic plaques were confirmed with real-time PCR. The specific regulation pattern of miRNAs in early atherosclerotic plaques may be useful in determining the formation and stability of plaques. Our work systematically compared the miRNA profile in atherosclerotic coronary arteries with normal coronary arteries. The results reported here provide new insights for diagnosis of early coronary atherosclerosis. Some of the identified dysregulated miRNAs including miR-221, miR-155 and miR-100, have been reported to correlate with vessel damage. The miR-221 was reportedly dysregulated in smooth muscle cells of balloon-injured artery wall, and knockdown of miR-221 decreased cell proliferation and inhibited neointima formation. Furthermore, high glucose induces miR-221 expression,

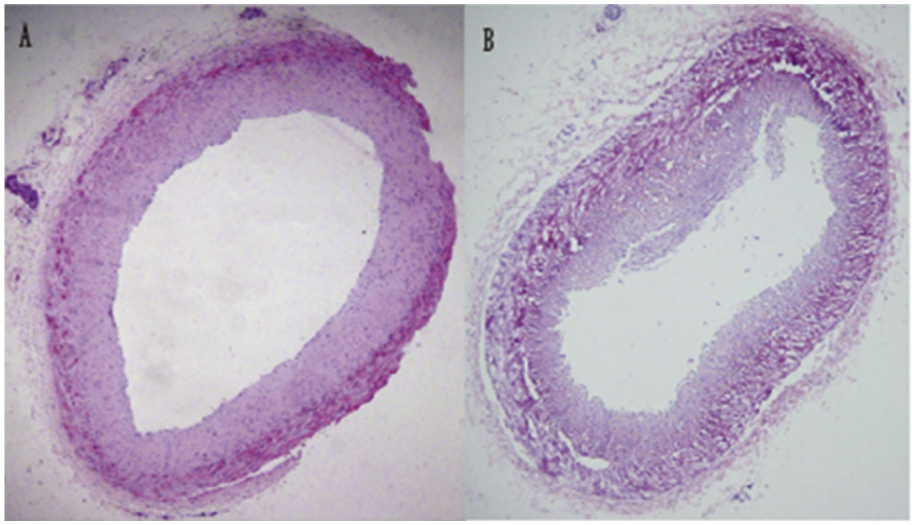

Fig. 1. HE staining of the atherosclerotic plaques Fig. 1A shows intimal thickness more than 0.9 mm. Intimal thickening indicates elevated endothelial permeability to lipids, and is the earliest morphological change in atherosclerosis. A few rounded foam cells with plenty of vacuoles in the cytoplasm can be observed beneath endothelial cells. Additionally, inflammatory cell infiltration is also seen. Fig. 1B shows fibrous plaques in early to mid-atherosclerotic lesions with plenty of collagen fibrils, and smooth muscle cells displaying hyaline degeneration, and large number of foam cells, inflammatory cells and cholesterol crystals beneath fibrous cap.

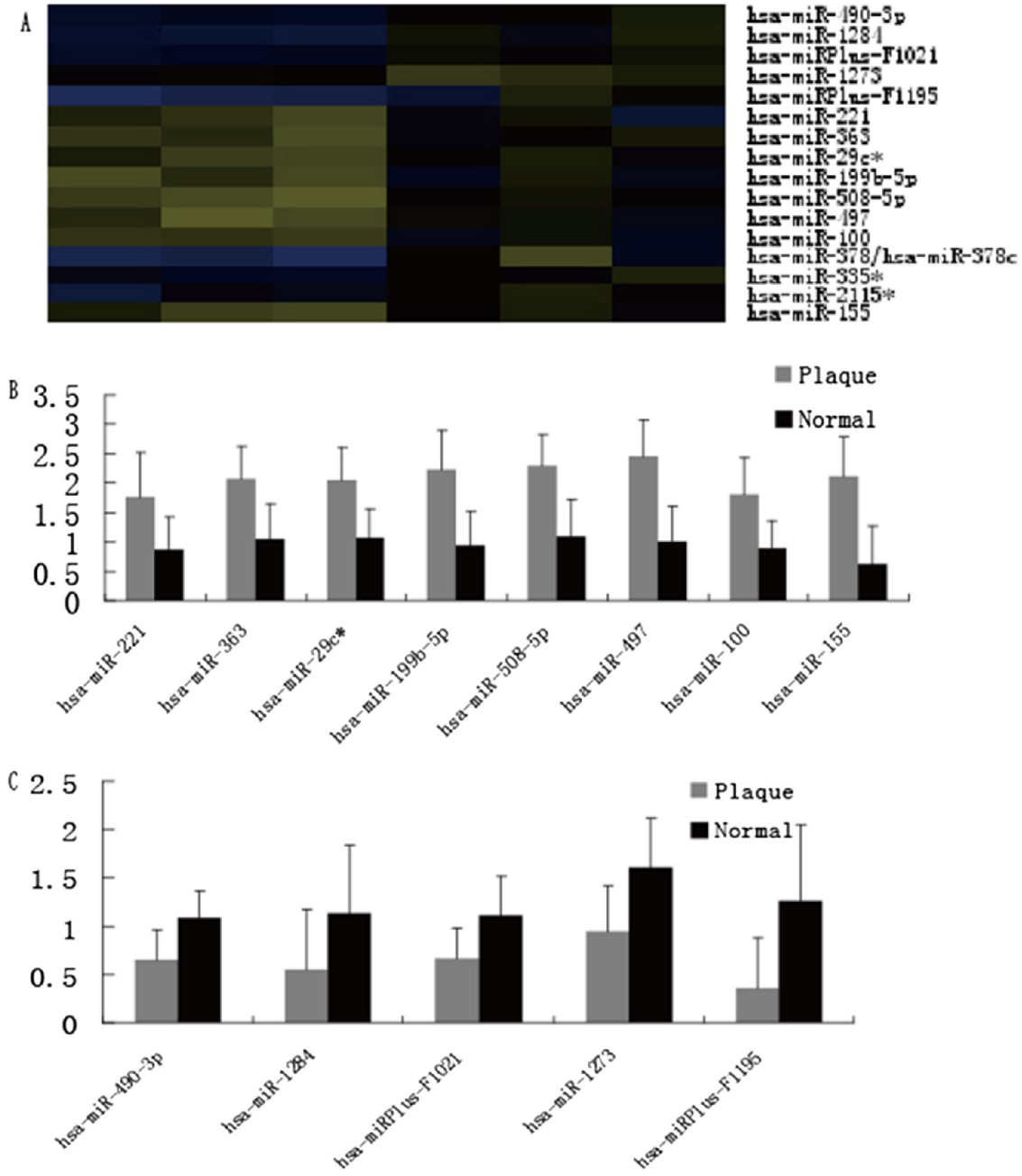

Fig. 2. MiRNAs expression in the coronary arteries. Fig. 2A:The heat map diagram shows the result of the two-way hierarchical clustering of miRNAs and samples. Each row represents a miRNA and each column represents a sample. The first three columns of the plaque group, the latter three as a normal group, The color scale shown the relative expression level of a miRNA in the certain slide: yellow color represents a higher expression level than control sample; blue color represents a lower expression level than the control sample. Fig. 2B, C Respectively, to calculate the mean of the three specimens of the plaque group and normal group, draw the standard curve after standardization, compare rates and lowered the MicroRNA gene expression differences between the two groups, $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

which repress c-kit to promote vessel permeability and angiogenesis [15,16].

Inflammation-related miR-155 decreased AT(1)R protein level by binding to 3′UTR, and the SNP in the target site (1166 A/C) was associated with cardiovascular diseases [17]. The miR-100 was downregulated in hind-limb ischemia in mice, and it inhibited anti-angiogenesis by attenuating endothelial cell proliferation and smooth muscle migration [18]. Our results show that these miRNAs were dysregulated in early atherosclerotic plaques, which was consistent with previous reports, confirming their important roles in atherosclerosis initiation via inflammation, endothelial cell injury and smooth muscle cell proliferation. The target genes of these miRNAs may relate to atherosclerosis initiation, and the expression dynamics of these miRNAs may reveal the process of atherosclerosis. Our results identified new miRNAs that were dysregulated, including hsa-miR-1273, hsa-miR-378, hsa-miR-335*, and hsa-miR-2115*. The miR-1273 is increasingly expressed in CD34 + hematopoietic progenitor cells, and may be involved in erythropoiesis [19]. The miR-378 is involved in cell survival and tumorigenesis [20]. However, it remains unclear whether these

Table 1
Up-regulated miRNAs in atherosclerosis.

Name	Average (ratio scale)		p-value	Standard deviation	
	Sick	Normal	Sick/normal	Sick	Normal
hsa-miR-221	1.772	0.866	0.04	0.56	0.742
hsa-miR-363	2.075	1.049	0.031	0.605	0.566
hsa-miR-29c*	2.045	1.061	0.017	0.499	0.565
hsa-miR-199b-5p	2.23	0.926	0.014	0.58	0.669
hsa-miR-508-5p	2.286	1.087	0.035	0.625	0.543
hsa-miR-497	2.459	0.996	0.049	0.619	0.61
hsa-miR-100	1.805	0.888	0.006	0.482	0.633
hsa-miR-155	2.113	0.613	0.003	0.657	0.682

Table 2
Down-regulated miRNAs in atherosclerosis.

Name	Average (ratio scale)		p-value	Standard deviation	
	Sick	Normal	Sick/normal	Sick	Normal
hsa-miR-490-3p	0.656	1.079	0.032	0.291	0.303
hsa-miR-1284	0.546	1.135	0.035	0.705	0.622
hsa-miRPlus-F1021	0.661	1.111	0.012	0.409	0.321
hsa-miR-1273	0.94	1.613	0.032	0.503	0.481
hsa-miRPlus-F1195	0.362	1.257	0.007	0.791	0.512

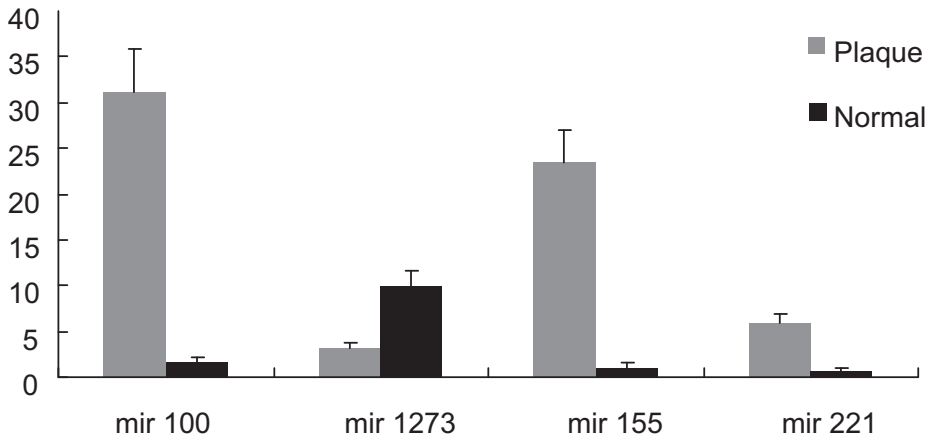

Fig. 3. Relative expression levels of selected miRNAs. The hsa-miR-100, hsa-miR-221, hsa-miR-155 were upregulated in plaque group, and hsa-miR-1273 was downregulated in plaque group. Blue columns indicate plaque group and purple columns indicate control. *P < 0.05.

miRNAs can regulate atherosclerotic plaque formation, although it has been reported that miR-378 targets VEGF [21], which plays a critical role in angiogenesis, and it may target LRP2, which plays an important role in lipid metabolism. Our future work will focus on the roles of these miRNAs and their targets in different cell types during plaque formation.

Results from our study investigated samples from young patients, with early atherosclerotic plaques, and therefore our finding shed light on prediction of early atherosclerosis. Due to sample scarcity, our results may be more appropriate for the miRNA expression profile of early atherosclerotic plaques. Our results are also important in identifying serum miRNA markers of early atherosclerotic plaques. Further work is required to confirm our findings with emphasis on identifying miRNA targets, e.g., by profiling the mRNAs in early atherosclerotic plaques using current target predication software. Identification of different cell types associated with miRNA dysregulation is also essential.

In conclusion, our work reveals the miRNA expression profile in atherosclerotic vessels, and identifies a series of upregulated and downregulated miRNAs by microarray analysis. Our real-time PCR results validated of the microarray results and the possibility of several miRNAs serving as potential indicators in the detection of early atherosclerosis and thereby, presenting as novel diagnostic markers for coronary atherosclerotic heart diseases. Our future research will focus on determination of the molecular targets for diagnosis and prevention of atherosclerosis by identifying serum miRNAs and their dysregulation in different cell types in early stages of the disease.

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Transparency document

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