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Profiling of Differentially Expressed Circulating Exosomal Micrornas among Patients with Coronary Artery Disease

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Abstract

Coronary Artery Disease (CAD) is the leading cause of death in developed countries. Studies have indicated that ischemic tissues may release exosome-carried microRNAs (miRNAs) into circulation. miRNAs are a class of small, endogenous, noncoding RNAs that regulate the expression of multiple target genes at the posttranscriptional level on the basis of sequences that are complementary to target mRNA molecules. However, the prognostic role of exosomal miRNAs in CAD remains unclear. In this study, we compared the profiles of circulating exosomal miRNA expression in patients with angiographically assessed absent and well-developed collateral vessels. A total of 109 patients who underwent coronary angiography were recruited, including 44 patients with CAD and well-developed collateral circulation, 31 patients with no collateral flow (as indicated by their Rentrop scores), and 34 patients with patent coronary arteries (control group). miRNAs were then extracted from exosomes collected from peripheral blood and amplified, and a miRNA microarray system was used to profile the expression of these miRNAs in the exosomes. Subsequently, the effects of specific miRNAs on angiogenesis were identified *in vitro*. On the basis of the array data, miRNAs with high differential expression ratios along with Grade 0-2 collateral flow were selected. Among patients with no collateral flow, the top five overexpressed candidate miRNA markers were miR-29a, miR-592, miR-518e, miR-32 and miR-766. Similarly, among patients with adequate collateral perfusion, the top five overexpressed miRNAs were miR-300, miR-576-3p, miR-642, miR-620 and miR-1255a. As a next step, the angiogenic ability and proangiogenic signaling pathway of two specific angiogenesis-related miRNAs (i.e., miR-300 and miR-29a) with high differential expression ratios were determined. According to the microarray data and study results, miRNAs with high expression levels can be used as biomarkers to distinguish between types of collateral circulation in patients with CAD and can be used in diagnostic or prognostic applications for high-risk patients with CAD. These findings may serve as a reference for the development of noninvasive and cost-effective approaches aimed at identifying the high risk of CAD and for the development of novel therapeutic targets for vascular diseases, including CAD with impaired collateral angiogenesis.

Keywords: Coronary artery disease; Collateral circulation; microRNA; Microarray analysis

Introduction

Coronary Artery Disease (CAD) is the leading cause of morbidity and mortality among people living in highly industrialized countries [1]. Percutaneous coronary intervention and coronary bypass surgery are the primary treatment strategies for CAD [2]. In addition to the functioning of stenotic lesions as a natural bypass, coronary collateral function enhancement through angiogenesis is an interesting approach for preserving the ischemic myocardium. Although percutaneous coronary interventions are a commonly used early management strategy for CAD [3], only a minority of patients with CAD present with coronary collaterals, which link proximal and distal parts of the arterial tree, bypassing areas of stenosis [4]. As a natural bypass, collateral arteries effectively restore blood flow to compromised tissues. Collateral circulation also plays a critical role in protecting tissues against ischemic damage. Recurrent and severe myocardial ischemia may stimulate the development of coronary collateral circulation [5]. However, collateral development does not occur in all patients with coronary occlusion, the reason for which has not yet been investigated.

MicroRNAs (miRNAs) are a class of small, endogenous, noncoding RNAs that coordinately regulate the expression of multiple target genes at the posttranscriptional level through the degradation or translational inhibition of their target mRNAs [6,7]. Studies have indicated that miRNAs, negative regulators of gene expression, are highly expressed in the vascular system and may be involved in neovascularization [8]. Other studies have revealed that miRNAs may be released into

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circulation from injured cells or tissues as a result of ischemia [9]. Evidence has indicated that exosomes (endosome-derived organelles) carry mRNAs and miRNAs, which are actively secreted by cells during exocytosis [10,11]. Although the secretory mechanisms and biological functions of CAD-associated exosomes remain unclear, circulating exosomal miRNAs derived from the ischemic myocardium of patients with CAD are regarded as potential noninvasive biomarkers of CAD.

Only a few studies have investigated the prognostic role of exosomal miRNAs in CAD. In this study, we investigated the differences in the miRNA profiles of plasma exosomes between patients with CAD and healthy controls. We also explored the prognostic role of exosomal miRNAs in CAD. Using a genomic-scale miRNA profiling approach, we investigated the differences in the expression of circulating exosomal miRNAs between patients with CAD and well-developed collateral and no collateral flow and healthy controls. We identified spectra of angiogenesis-related miRNAs. In addition, we used a computational approach to examine the angiogenic ability, proangiogenic signaling pathway, and target genes responsible for angiogenesis in two angiogenesis-related miRNAs, namely miR-300 and miR-29a, with high differential expression ratios. Overall, our findings may serve as a reference for the development of noninvasive and cost-effective approaches aimed at identifying patients with a high risk of CAD and for the development of novel therapeutic targets for vascular diseases, including CAD with impaired collateral angiogenesis.

Materials and Methods

Participants

This study aimed to evaluate the roles of miRNA in coronary collateral development by analyzing human samples and *in vitro* studies. In this study, 109 patients who underwent coronary angiography from Jan 4, 2012 to Jan 3, 2013 were recruited. Basic demographic data, procedural characteristics and clinical follow-up data were then collected and analyzed (Table 1). All patients provided written informed consent. Ethics approval for human sample collection was obtained from National Taiwan University Hospital Research Ethics Committee B (201109010RB). Before coronary angiography, 2 ml of peripheral venous blood was collected immediately at admission. Samples were collected in regular blood collection tubes containing ethylenediaminetetraacetic acid. These samples were processed within 30 min of collection.

Age	CTO (n=44)	MVD (n=31)	Patent (n=34)
	63 ± 11 (years)	62 ± 12 (years)	57 ± 11 (years)
Male gender (%)	36(82)	25(81)	21(62)
Hypertension (%)	32(81)	23(81)	20(81)
Diabetes (%)	15(34)	10(32)	6(18)
Dyslipidemia (%)	29(66)	17(55)	12(35)
Smoking (%)	19(44)	7(23)	8(24)
Antiplatelet (%)	43(98)	31(100)	31(91)
ACEI/ARB (%)	17(39)	10(32)	13(38)
Beta-blockers (%)	24(54)	14(45)	13(38)
Ca channel blockers (%)	22(49)	17(55)	12(35)
Diuretics (%)	7(17)	3(10)	8(24)
Nitrates (%)	25(56)	10(32)	9(26)
Lipid-lowering agents (%)	28(63)	14(45)	9(26)

Table 1: Clinical characteristics of patients used in this study.

CAD and chronic total occlusion

CAD is characterized by more than 50% stenosis in major coronary vessels [3]. Chronic Total Occlusion (CTO) is characterized by 100% occlusion with a thrombolysis in myocardial infarction score of 0 for at least 3 months. Collateral circulation is classified in accordance with the rentrop scoring system in which Grade 0 indicates no visible filling of any collateral channel, Grade 1 indicates filling of the side branches of the occluded epicardial segment (which is not reachable by the dye), Grade 2 indicates partial filling of the epicardial vessels, and Grade 3 indicates complete filling of the epicardial vessels through collaterals [12]. Of the 109 participants included in this study, 44 with CAD had adequate collateral flow (Grade 2, CTO), 31 had no visible flow (Grade 0, Multi Vessel Disease [MVD]), and the remaining 34 had patent coronary arteries (control group).

Exosome isolation

The blood samples were processed to separate the plasma and extract the exosomes. The plasma was centrifuged at 2000 × g for 10 min to remove dead cells and cellular fragments, and the supernatant was recovered. The supernatant was then centrifuged at 2000 × g for 20 min, recovered, and passed through a polyvinylidene fluoride filter with a pore size of 0.45 μm (Millipore, Billerica, MA, USA). Subsequently, the solution containing the exosomes was centrifuged at 12000 × g for 2 h, and an exosome pellet was obtained. Finally, the exosome pellet was resuspended in 20 μL of Phosphate-Buffered Saline (PBS) and stored at -80ºC for subsequent analysis.

Exosomal miRNA isolation and microarray analysis

To purify the miRNAs extracted from the exosomes and simplify the process, the exosomes were pooled as MVD, CTO and control groups. Total RNA was extracted from the exosome pool using TRIzol (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. miRNAs were then separated from the total RNA by using mirVana miRNA purification columns (Ambion; Thermo Fisher Scientific) and used for microarray analysis in accordance with the manufacturer's protocol. Each total RNA sample (500 ng) was labeled and hybridized using a FlashTag Biotin HSR RNA Labeling Kit (Thermo Fisher Scientific). Each biotin-labeled RNA sample was then hybridized for 16 h-18 h at 45ºC on an Affymetrix GeneChip miRNA 2.0 Array. Subsequently, the miRNA arrays were washed and stained using an Affymetrix Fluidics Station 450 and scanned using an Affymetrix GeneChip Scanner 3000 7G. Finally, the data were analyzed using the Affymetrix Expression Console software version 1.2.0.20 (Affymetrix; Thermo Fisher Scientific).

miRNA target prediction and gene ontology analysis

The miRNA sequences were downloaded from the miRBase website (http://www.mirbase.org), and the miRNA target sites were predicted using computer-aided algorithms on TargetScan version 7.1 (http://www.targetscan.org). On the basis of the Gene Ontology (GO)

database (http://www.geneontology.org), significant GO terms of the upregulated miRNA targeted genes were analyzed using the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/home.jsp) [13].

Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were procured from PromoCell (Heidelberg, Germany) [14]. All endothelial cells used in this study were isolated from the third to fourth passages.

Cell transfection and reverse transcription quantitative polymerase chain reaction

miR-29a and miR-300 mimics were procured from Thermo Fisher Scientific. The cells were first grown overnight and then transfected with 200 µL of miRNA (100 nM) and RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific) for 48 h in accordance with the manufacturer's protocol. The transfection efficacy was determined using Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific) in accordance with the manufacturer's instructions. To detect the expression of specific miRNAs, complementary DNA was generated using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific) under the following conditions: 16ºC for 30 min, 42ºC for 30 min and 85ºC for 5 min. PCR amplification was then performed using a TaqMan Small RNA Assay (Applied Biosystems; Thermo Fisher Scientific). U6 small nuclear RNA was used as an internal control to normalize the relative expression levels of miR-29a and miR-300. The primer sequences were as follows: miR-29a forward, 5′-CTGATTTCTTTTGGTGTTC-3′, reverse, 5′-GAACATGTCTGCGTATCTC-3′; miR-300 forward, 5′-TACAAGGG CAGACTCTC-3′; reverse, 5′-GAACATGTCTGCGTATCTC-3′; U6 forward, 5′-CTCGCTTCGGCAGCACAT-3′, reverse, 5′-TTTGCGTGT CATCCTTGCG-3′. The above primers (MIR29a Human qPCR Primer Pair Catalog number: HP300281, MIR300 Human qPCR Primer Pair Catalog number: HP300285, U6 Human qPCR Primer Pair Catalog number: MP300001) were all purchased from and designed by OriGene Technologies, Inc.. All reactions were performed in triplicate for each sample, and all experiments were repeated three times. The fold change for each miRNA was analyzed using the $2^{-\Delta\Delta Ct}$ method [15]. The miRNA mimics (miR-29a mimics: mirVana® miRNA mimic, Catalog number: MC12499 and miR-300 mimics: mirVana® miRNA mimic, Catalog number: MC13049) and negative control (mirVana™ miRNA Mimic, Negative Control #1; Catalog number: 4464061) were purchased from Thermo Fisher Scientific.

Cell proliferation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

HUVECs were first transfected for 48 h. The transfected cells were then plated on 96-well culture plates at a density of 6×10^3 cells/well in an Endothelial Cell Medium (ECM) for 24 h. Subsequently, Bromodeoxyuridine (BrdU) was added, and the culture was incubated for 4 h. BrdU incorporation was assessed using a Biotrak Cell Proliferation ELISA System (GE Healthcare, Amersham, UK) in accordance with the manufacturer's protocol. Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Subsequently, 48 h after transfection, MTT solution (0.5 mg/mL) was added to each well, and the cells were incubated for 4 h at 37ºC. The MTT solution was then extracted and 100 µL of dimethyl sulfoxide was added to lyse the mixture for 10 min. Finally, absorbance was photometrically measured at 490 nm.

Wound healing migration assay

HUVECs transfected with specific miRNAs were first trypsinized 48 h after transfection, and 1×10^4 cells were then plated on a six-well culture insert (ibidi plates). Subsequently, 24 h after cell attachment, the culture insert was gently removed using sterile tweezers. The cells were then washed with PBS and placed in a culture medium for 12 h. Finally, the migration region was analyzed using MetaMorph software (Molecular Devices, San Jose, CA, USA).

Transwell cell migration and tube formation assays

HUVECs transfected with specific miRNAs were trypsinized 48 h after transfection. The cells were then harvested through centrifugation, resuspended in 500 µL of ECM, counted, and placed on the upper side of a modified Boyden chamber (2×10^4 cells/chamber, pore size: 8 µm; BD Biosciences, San Jose, CA, USA) coated with 0.1% gelatin solution. The chamber was then placed in a 24-well culture dish containing ECM and 20 ng/mL vascular endothelial growth factor. After incubation for 5 h at 37ºC, the migrated cells on the upper side of the chamber were mechanically removed, and the remaining cells on the lower side were fixed with 4% paraformaldehyde. The migrated cells were then stained with Giemsa's staining solution and manually counted in five random microscopic fields. For the tube formation assay, the formation of capillary-like structures was assessed in a 24-well plate by using a matrigel growth factor reduced matrix (BD Biosciences). For this procedure, HUVECs (2×10^4 cells/well) were plated on matrigel (280 µL/well) and transfected with specific miRNAs. After 24 h, the cells were observed under a bright-field microscope. The total tube area was evaluated as the mean pixel density through image analysis of the five random microscopic fields in Image J software (http://rsb.info.nih.gov/nih-image).

Western blotting

To perform Western blotting [13], the total protein was first extracted using radioimmuno precipitation assay buffer. Equal protein amounts were then loaded and separated using gel electrophoresis. The protein was then transferred to nitrocellulose membranes and probed overnight using appropriate primary antibodies: Akt (Ak strain transforming protein), phosphorylated Akt, Extracellular Signal-Regulated Kinase (ERK), phosphorylated ERK, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, Danvers, MA, USA). After incubation with appropriate secondary antibodies, immune reactive bands were observed using chemiluminescence. The protein bands on the Western blots were then quantified using Image J densitometric analysis software.

Statistical analysis

All experiments were conducted at least three times. All statistical analyses were performed using one-way ANOVA and Bonferroni and Dunnett's T3 test. The data are presented as mean ± Standard Error of the Mean (SEM), and statistical significance was $p<0.05$.

Results

Differential expression of circulating exosomal miRNAs in CAD

The total RNA extracted from exosomes isolated from the participants was enriched and analyzed using miRNA microarrays. On the basis of the array data (Figures 1A and 1B), miRNAs with high differential expression ratios along with Grade 0-2 collateral flow were selected. Among patients with no collateral flow, the top five

overexpressed miRNAs were miR-29a, miR-592, miR-518e, miR-32 and miR-766. As predicted by Target Scan Human software and reported in the literature, the target genes of miR-29a, including genes *COL3AI, ELN* and *COL5A1*, were involved in the development of blood vessels (Table 2). Among patients with adequate collateral perfusion, the top five overexpressed candidate miRNA markers were miR-300, miR-576-3p, miR-642, miR-620, and miR-1255a. On the basis of the expression profiles of the adequate collateral perfusion group and the control group with patent coronary arteries, among patients with adequate collateral perfusion, the top five differentially overexpressed miRNAs were miR-300, miR-642, miR-30c, miR-485-3p and miR-1253. Similarly, in the control group, the top five candidate miRNAs were miR-5481, miR-664, miR-34a, miR-1255-5p and miR-658. Among the candidate miRNAs, the target genes of miR-300 were related to angiogenesis (Table 3). Among the overexpressed miRNAs in the plasma exosomes of patients with CAD (Figure 1), a strong correlation was observed between the top overexpressed miRNAs and the angiogenesis-related genes. Therefore, both miR-29a and miR-300 miRNAs were selected for further analysis.

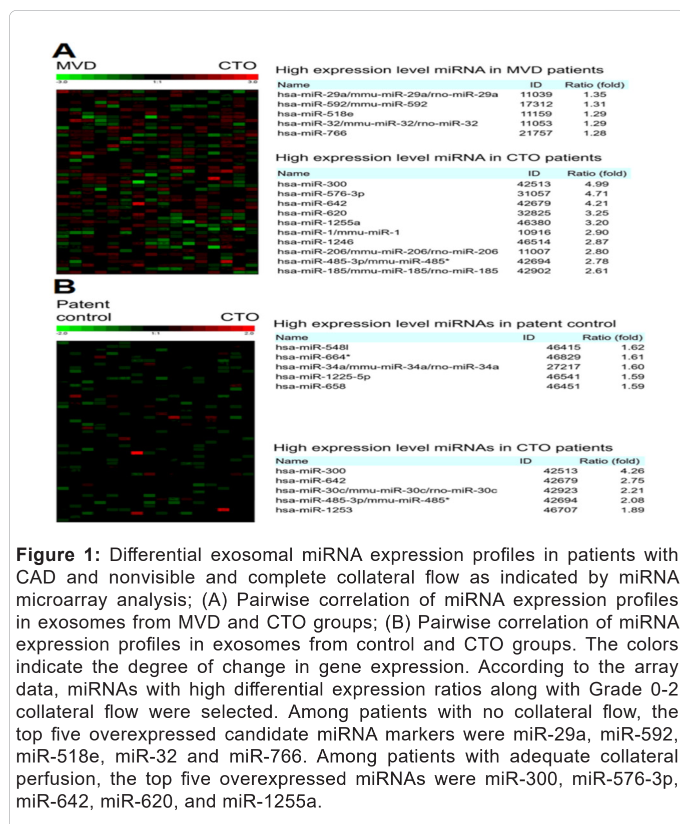

Figure 1: Differential exosomal miRNA expression profiles in patients with CAD and nonvisible and complete collateral flow as indicated by miRNA microarray analysis; (A) Pairwise correlation of miRNA expression profiles in exosomes from MVD and CTO groups; (B) Pairwise correlation of miRNA expression profiles in exosomes from control and CTO groups. The colors indicate the degree of change in gene expression. According to the array data, miRNAs with high differential expression ratios along with Grade 0-2 collateral flow were selected. Among patients with no collateral flow, the top five overexpressed candidate miRNA markers were miR-29a, miR-592, miR-518e, miR-32 and miR-766. Among patients with adequate collateral perfusion, the top five overexpressed miRNAs were miR-300, miR-576-3p, miR-642, miR-620, and miR-1255a.

miRNA	Targets	Pathway/GO ontology
Hsa-miR-29a	*COL3A1*	Blood vessel development
	COL5A1	Blood vessel development
	ELN	Blood vessel remodeling
	HBP1	Wnt receptor signaling pathway
Hsa-miR-592	*FAM1238*	Wnt receptor signaling pathway
Hsa-miR-518e	*PTPRU*	Wnt receptor signaling pathway through beta-catenin
	RAP1B	Cell proliferation
	PRICKLE2	Wnt signaling pathway
Hsa-miR-32	*FBXW7*	Cell adhesion
	PCDH11X	-
	PCDH11Y	-
Hsa-miR-766	*NR3C2*	Regulation of cell proliferation
	Mar-09	Protein ubiquitination
	CYLC2	Cell differentiation
	NAV1	-

Table 2: The top five up-expressed exosomal miRNAs and their putative target genes in MVD patients with poor collateral flow predicted with computational method including PicTar, miRanda, and TargetScan database.

miRNA	Targets	Pathway/Go ontology
Hsa-miR-300	*EIF4A1*	Cell growth
	SMAD4	Wnt signaling pathway
	PIAS1	Protein inhibitor of activated STAT 1
	BCL2L11	PI3K-AKT signaling pathway
	RICTOR	mTOR signaling pathway

Table 3: The top Up-regulated miRNA300 and their putative targets in CTO patients with good collateral flow predicted with computational method including PicTar, miRanda, and Target scan database.

Effects of two specific angiogenesis-related miRNAs (miR-29a and miR-300) on angiogenesis and the proangiogenic signaling pathway

To further understand the biological functions of miR-29a and miR-300, we investigated their effects on the activity of endothelial cells. RT-qPCR revealed successful overexpression of miR-29a and miR-300 in HUVECs (Figure 2). Random sequence pre-miR molecules, which have been extensively used to analyze human cell lines, were selected as Mock Controls (MCs). Cy3 dye–labeled pre-miR negative controls were used to monitor the efficiency of transfection in transfection experiments with pre-miR miRNA precursors. A fluorescent label was used to directly observe the cellular uptake, distribution, and localization of the control. Subsequently, 48 h after transfection, the MC precursors were expressed in the HUVECs, and the transfection efficiency was approximately 65%-75% (data not presented). RT-qPCR also revealed the successful overexpression of endogenous miR-29a and miR-300 in the HUVECs by the miR-29a and miR-300 mimics (Figures 2A and 2B). To examine the *in vitro* effects of miR-29a and miR-300 on endothelial

cell growth, the miR-29a and miR-300 transcripts were overexpressed in the HUVECs. The results indicate that the overexpression of miR-29a or miR-300 considerably increased the growth of the HUVECs *in vitro* (Figure 3). The proliferation and migration of miR-29a- or miR-300-overexpressed HUVECs considerably increased compared with those of the MCs (Figures 4 and 5). We then tested the effects of miR-29a and miR-300 on the formation of microtubules. The results indicate that the cumulative length of the tube was considerably greater in HUVECs with miR-29a or miR-300 overexpression than in those treated with the MC (Figure 6). Generally, phosphoinositide 3-kinase/Akt and Ras/Raf/mitogen-activated protein kinase kinase/ERK signaling pathways play a critical role in angiogenesis [16]. For this reason, we analyzed the effects of miR-29a and miR-300 on Akt and ERK phosphorylation. The results indicate that the phosphorylation of Akt and ERK was considerably higher in HUVECs with miR-29a or miR-300 overexpression than in those treated with the MC (Figure 7). These results demonstrate that miR-29a and miR-300 promoted the growth, proliferation, migration, microtubule formation ability, and Akt/ERK phosphorylation of endothelial cells and thus enhanced angiogenesis.

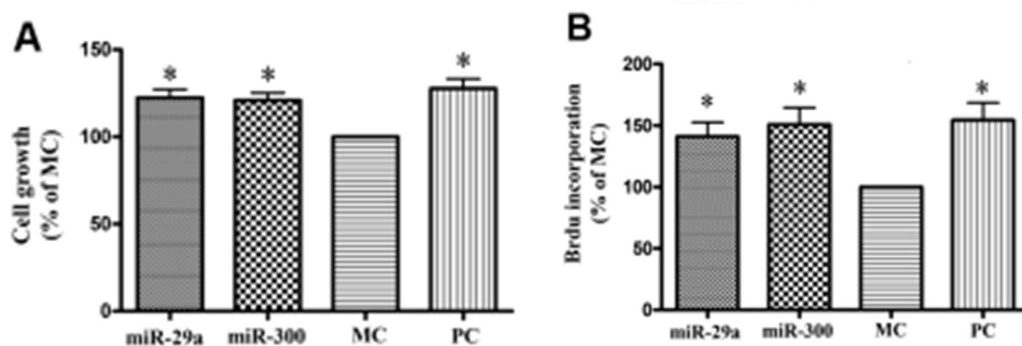

Figure 2: Efficiency of miRNA expression in pre-miR-transfected endothelial cells. The cells were collected and quantitated 48 h after transfection for the miRNA expression of miR-29a (A) and miR-300 (B) The cells were transfected with a random sequence pre-miR as MCs (*p<0.05 compared with the MC; data are presented as mean ± SEM, n=3).

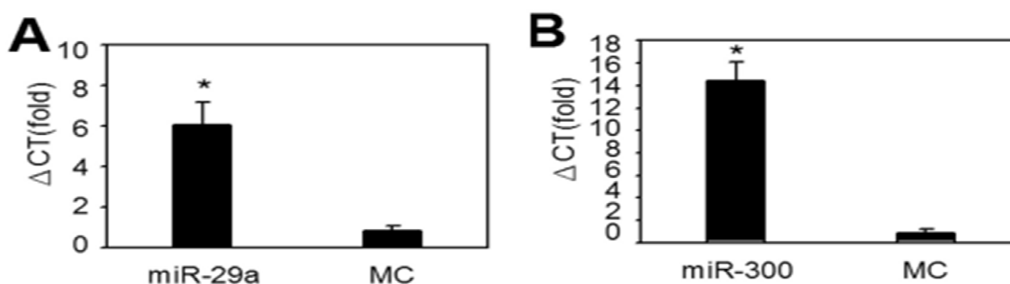

Figure 3: Proliferative effects of miR-29a and miR-300 on cultured HUVECs. (A) The MTT assay revealed that the growth of HUVECs considerably increased 24 h after the transfection of miR-29a and miR-300 (*p<0.05 compared with the MC; data are presented as mean ± SEM, n=5). The cells were transfected with a random sequence pre-miR as MC. Cells treated with 10% fetal bovine serum in a culture medium were used as Positive Controls (PC); (B) The cells were collected and stained with BrdU 24 h after the transfection of miR-29a and miR-300. BrdU incorporation was assessed using a Biotrak Cell Proliferation ELISA System. DNA synthesis signiicantly increased in both miR-29a and miR-300 transfected cells. **Note:** *p<0.05 compared with the MC; data are presented as mean ± SEM, n=6).

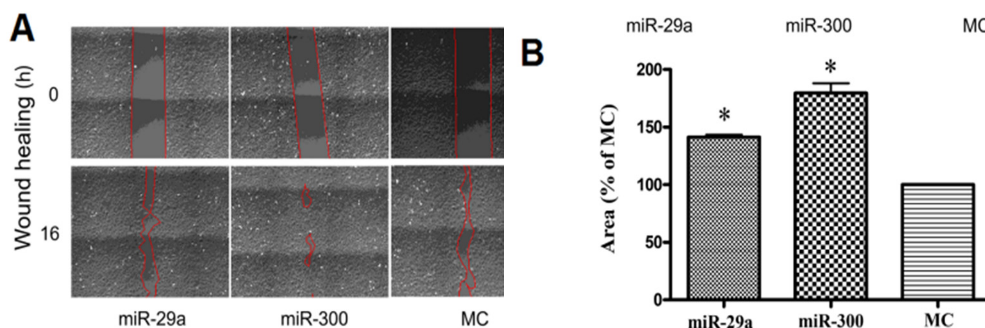

Figure 4: Migration of transfected HUVECs in the wound healing assay. (A) Representative images (magnification 40×) and (B) quantitative data of the wound healing assay of HUVECs transfected with miR-29a, miR-300, and MCs. **Note:** The edges of the scratch wound are indicated by red lines. The migrated cells were quantified as displayed in the bar graphs (mean ± SEM, n=12). The cells were transfected with a random sequence pre-miR as MCs (*p< 0.05 compared with the MC).

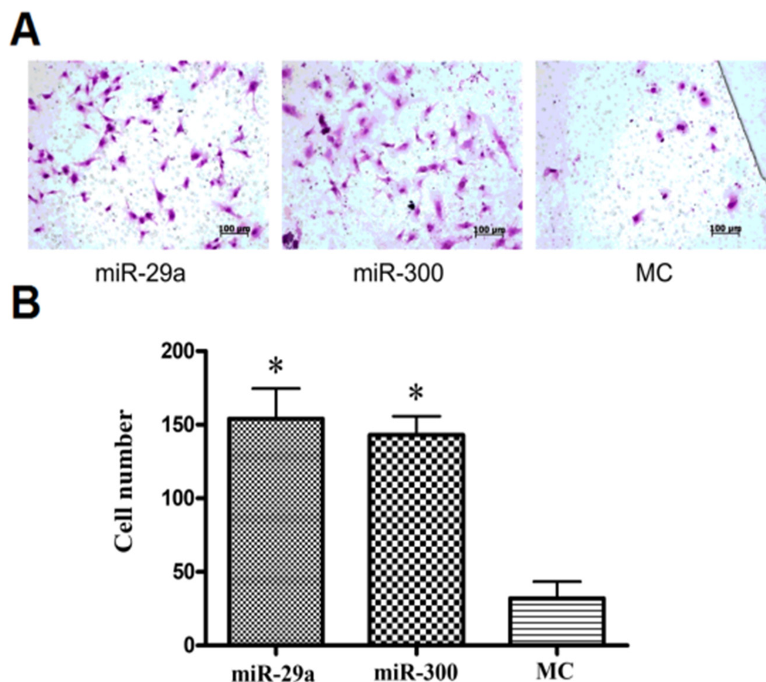

Figure 5: Effects of pre-miR on the migration of human endothelial cells. (A) Representative images (magnification 200 ×) and (B) quantitative data of the cell migration of HUVECs transfected with miR-29a, miR-300, and MCs. The migration capacity of the transfected HUVECs was measured by seeding the cells on the upper side of a Boyden chamber coated with 0.01% gelatin. After 8 h, the nonmigrated cells on the upper side of the chamber were mechanically removed, and the remaining cells on the lower side were stained with Giemsa's staining solution. The migrated cells were quantified as displayed in the bar graphs (mean ± SEM, n=4). The cells were transfected with a random sequence pre-miR as MCs. **Note:** *$p< 0.05$ compared with the MC).

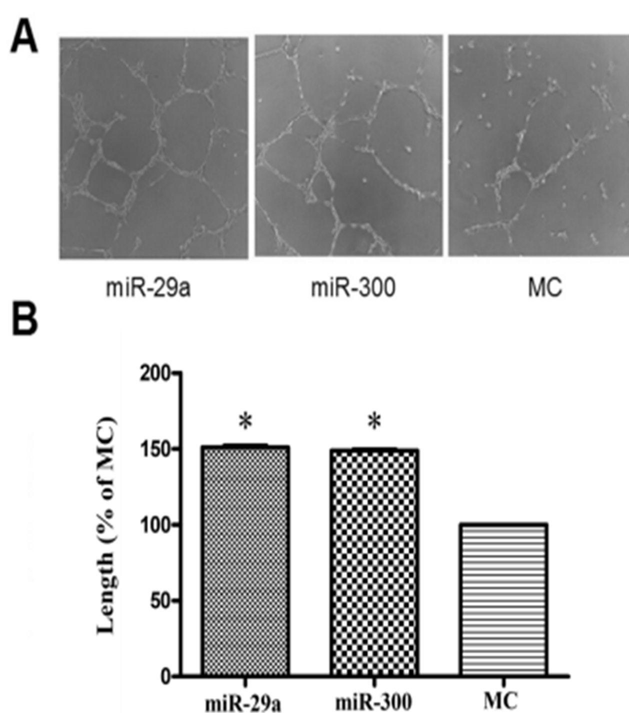

Figure 6: Effects of specific miRNA overexpression on capillary tube formation in HUVECs. (A) Representative images of tube formation (magnification 100 ×) and (B) quantitative data of cumulative tube length of HUVECs transfected with miR-29a, miR-300 and MCs. After transfection with specific pre-miR, the HUVECs were seeded on a growth-factor-enriched Matrigel Basement Membrane Matrix. The total length of the tube structure per field was measured using light microscopy 16 h after transfection. The cells were transfected with a random sequence pre-miR as MCs. **Note:** *$p<0.05$ compared with the MC; data are presented as mean ± SEM, n=4).

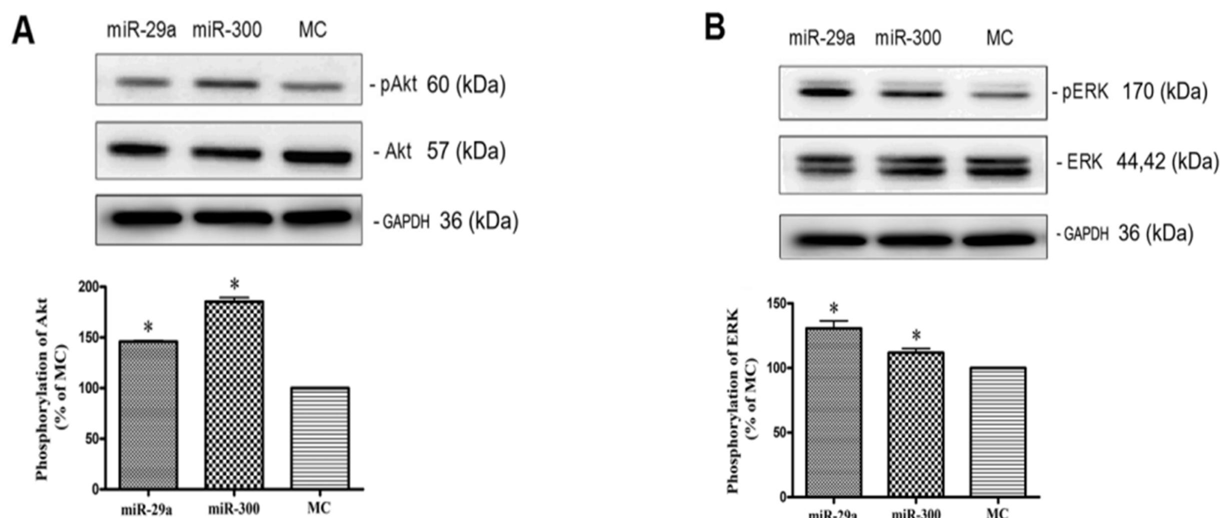

Figure 7: Effects of pre-miR on the migration of human endothelial cells. (A) Representative images (magnification 200 ×) and (B) quantitative data of the cell migration of HUVECs transfected with miR-29a, miR-300, and MCs. The migration capacity of the transfected HUVECs was measured by seeding the cells on the upper side of a Boyden chamber coated with 0.01% gelatin. After 8 h, the nonmigrated cells on the upper side of the chamber were mechanically removed, and the remaining cells on the lower side were stained with Giemsa's staining solution. The migrated cells were quantified as displayed in the bar graphs (mean ± SEM, n=4). The cells were transfected with a random sequence pre-miR as MCs. **Note:** *p< 0.05 compared with the MC).

Discussion

In this study, we used a genomic-scale miRNA profiling approach to elucidate the differences in the expression patterns of circulating exosomal miRNAs between patients with CAD and well-developed coronary collaterals, patients with CAD with no collateral flow, and patients with patent coronary arteries (control group). We then identified the spectra of angiogenesis-related miRNAs. Subsequently, we examined the angiogenic effects of two miRNAs, predicted their respective target genes responsible for angiogenesis, and determined the proangiogenic signaling pathways involved in collateral formation. Finally, we investigated the angiogenic ability, proangiogenic signaling pathways, and target genes responsible for angiogenesis of two specific angiogenesis-related miRNAs, namely miR-300 and miR-29a, among a set of miRNAs with high differential expression ratios.

CAD is a global health concern. The collateral circulation undergoes dynamic changes, thereby inducing cardioprotective effects [17]. Although the coronary collateral circulation has been recognized as an alternative source of blood supply to the ischemic myocardium, the reason underlying the selective collateral development in some patients with coronary occlusion but not in others has not yet been elucidated. Modern biomedical research has uncovered the molecular mechanisms responsible for the regulation of gene expression. As expression markers, miRNAs are used to decipher signaling pathways, and they may also serve as biomarkers for detecting the pathophysiological state of organs [18]. miRNAs are small, endogenous, noncoding RNA molecules that modulate the expression of their target genes on the basis of complementary sequencing with their target mRNA molecules in the 3′-UTR region [18]. Because each miRNA can have multiple mRNA targets, more than one-third of the human genes are estimated to be regulated by miRNAs. Because of their functional importance and modes of action, miRNAs have been increasingly recognized as key regulatory molecules that control a wide variety of fundamental cellular processes, such as proliferation, differentiation, motility, and angiogenesis. Therefore, understanding the applicability of miRNAs in the pathogenesis of human diseases may help promote the development of novel therapeutic strategies and the identification of diagnostic and prognostic biomarkers.

Surgical resection of damaged tissues is not a routine practice in cardiovascular disease management. This is why tissue specimens are not readily available for the investigation of cardiovascular ischemia. However circulating exosomes are secreted by different cell types, thereby affecting their biological functions and contributing to intercellular signaling transmission [19]. Emerging evidence has indicated that exosomal miRNAs are potent biomarkers of and therapeutic targets for cardiovascular diseases [19]. Therefore, the expression of exosomal miRNAs is regarded as a promising alternative for the detection of collateral vascularization and prognosis of patients with ischemic heart disease. Exosomes are small membrane vesicles (measuring approximately 30 nm-100 nm) that are actively secreted by cells during exocytosis. A variety of cells, such as dendritic cells, lymphocytes, mast cells, endothelial cells, epithelial cells, and tumor cells, release exosomes into the plasma. Although the functions of exosomes, which carry mRNAs and miRNAs, have not yet been thoroughly elucidated, they have been found to contribute to cardiovascular protection and repair [19]. In this study, we hypothesized that exosomes with miRNAs would be released from ischemic myocardial tissues into peripheral blood as signaling molecules that modulate intercellular communication and subsequently affect endothelial function and angiogenesis. To the best of our knowledge, this is the first study to profile differentially expressed circulating exosomal miRNAs among patients with CAD and correlate them to the clinical severity of CAD. We also discovered that miR-29a and miR-300 were upregulated in the serum exosomes of patients with CAD. Therefore, these two exosomal miRNAs may be potential biomarkers of CAD.

This study has some limitations. First, the sample size was not sufficiently large. Studies should include a larger sample to conduct validated and prospective clinical trials. Second, although the extracellular vesicles extracted from the plasma of the participants

were mainly composed of exosomes, other extracellular vesicles may have contaminated the samples during the isolation process. Third, although miRNAs can be delivered from donor cells to recipient cells by exosomes, whether candidate miRNAs can be functionally delivered to target cells could not be determined. Fourth, although this *in vitro* study partially explained the role of miR-29a and miR-300 in angiogenesis, their role in the pathogenesis of CAD warrants further investigation.

Conclusion

In summary, we successfully established a method for profiling the expression of miRNAs in circulating exosomes. This method helps determine the pathophysiological state of cardiovascular diseases. We obtained the peripheral exosomal miRNA expression profiles of patients with adequate and no coronary collaterals. However, the majority of the miRNA expression profiles remain to be further validated. Overall, our findings may serve as a reference for the development of noninvasive and cost-effective approaches aimed at identifying the high risk of CAD and for the development of novel therapeutic targets for vascular diseases, including CAD with impaired collateral angiogenesis.

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Author contributions statement

WRH and HJH conducted the experiments. YCW collected the human tissue samples from National Taiwan University Hospital. WRH and HJH analyzed the data. CYC performed ImageJ analysis. THC, JCL, YCW, and JJC conceived and designed the experiments. THC, JCL and YCW wrote the paper. All the authors contributed to data analysis, drafting, and paper revision, and they read and approved the final manuscript and agree to be accountable for all aspects of the work.

Additional Information

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Data availability statement

The data generated in the present study are not publicly available because some datasets were lost due to force majeure.

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the National Taiwan University Hospital Research Ethics Committee B (201109010RB), Taipei, Taiwan. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests statement

The authors declare that they have no competing interests.

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