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Circulating microRNAs are elevated in plasma from severe preeclamptic pregnancies

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Abstract

Until recently, the molecular pathogenesis of preeclampsia (PE) remained largely unknown. Reports have shown that circulating microRNAs (miRNAs) are promising novel biomarkers for cancer, pregnancy, tissue injury, and other conditions. The objective of this study was to identify differentially expressed miRNAs in plasma from severe preeclamptic pregnancies compared with plasma from normal pregnancies. By mature miRNA microarray analysis, 15 miRNAs, including 13 up- and two downregulated miRNAs, were screened to be differentially expressed in plasma from women with severe PE (sPE). Seven miRNAs, namely miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, and miR-574-5p, were validated to be elevated in plasma from severe preeclamptic pregnancies by real-time quantitative stem-loop RT-PCR analysis. Gene ontology and pathway enrichment analyses revealed that these miRNAs were involved in specific biological process categories (including regulation of metabolic processes, regulation of transcription, and cell cycle) and signaling pathways (including the MAP kinase signaling pathway, the transforming growth factor-β signaling pathway, and pathways in cancer metastasis). This study presents, for the first time, the differential expression profile of circulating miRNAs in sPE patients. The seven elevated circulating miRNAs may play critical roles in the pathogenesis of sPE, and one or more of them may become potential markers for diagnosing sPE.

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Introduction

Preeclampsia (PE), a pregnancy-related disease characterized by hypertension and proteinuria, is a major cause of maternal mortality, morbidities, perinatal deaths, preterm birth, and intrauterine growth restriction (Sibai *et al*. 2005). Although circulating soluble fms-like tyrosine kinase 1, soluble endoglin, and placental growth factor were recently suggested to contribute to the pathogenesis of PE (Levine *et al*. 2006), the mechanisms involved in this pathological condition remain poorly understood.

MicroRNAs (miRNAs) are a conserved group of ~22-nucleotide regulatory RNAs that play important roles in regulating gene expression by binding to 3′-UTR of mRNAs for either degradation or translation repression (Bartel 2004). miRNAs have been shown by oligonucleotide microarrays to be highly enriched in the placenta (Barad *et al*. 2004). However, miRNAs are differentially expressed in the human placentas of patients with PE, which indicates that miRNAs may have important roles in the pathogenesis of PE. In one report, among the 157 miRNAs manipulated by real-time quantitative RT-PCR (qRT-PCR) analysis, the expression of two miRNAs (miR-182 and miR-210) was significantly increased (2.1- and 3.0-fold respectively) in placentas of PE patients compared with that in women with normal pregnancy (Pineles *et al*. 2007). In addition, gene ontology (GO) analysis of the potential target genes of miR-182 and miR-210 indicated that specific biological process categories (anti-apoptosis for miR-182 and regulation of transcription for miR-210) were enriched (Pineles *et al*. 2007). A microarray analysis of 836 known human mature miRNAs in placental tissues of PE patients identified 91 dysregulated miRNAs, including 38 down- and 53 upregulated miRNAs (Roman *et al*. 2008). Two other reports (Hu *et al*. 2009, Zhu *et al*. 2009) further proved the importance of miRNAs in the pathophysiology of PE. Zhu *et al*. (2009) demonstrated that 11 miRNAs (including miR-210 and miR-181a) were overexpressed in the placentas of PE patients, whereas the levels of 23 miRNAs were

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decreased compared with women with normal pregnancies. The elevation of miR-181a in preeclamptic placentas was also identified by another group (Hu *et al*. 2009). In other studies, miRNAs specifically expressed in human placentas were detected in sera from pregnant women and found to be significantly elevated compared with those from nonpregnant women; their levels increased with gestational age and decreased after delivery, providing a new group of molecular markers for pregnancy monitoring (Chim *et al*. 2008, Gilad *et al*. 2008). In this study, a microarray analysis of the miRNA expression profile in plasma from severe PE (sPE) and normal pregnancies, as well as a real-time qRT-PCR validation, was performed to explore the association between maternal circulating miRNAs and the molecular pathogenesis of sPE.

Results

miRNA microarray analysis

To investigate whether maternal circulating miRNAs are associated with the pathogenesis of sPE, plasma samples were collected from women with normal pregnancies and pregnancies complicated by sPE. A comprehensive miRNA microarray analysis was performed on nine plasma samples, including five sPE plasma samples and four plasma samples from normal pregnancies. Among the 821 human miRNAs detected by microarray, 15 differentially expressed miRNAs were identified, of which 13 miRNAs, namely miR-574-5p, miR-26a, miR-151-3p, miR-130a, miR-181a, miR-130b, miR-30d, miR-145, miR-103, miR-425, miR-221, miR-342-3p, and miR-24, were upregulated in sPE plasma samples and two miRNAs, namely miR-144 and miR-16, were downregulated in sPE plasma samples, compared with those from normal pregnancies ($P<0.05$, twofold changes or more). As shown in Fig. 1, among all 15 dysregulated miRNAs, the fold changes of miR-574-5p and miR-26a appeared to be more pronounced.

miRNA expression validation by real-time quantitative stem-loop RT-PCR analysis

Real-time stem-loop qRT-PCR was performed to validate the 15 differentially expressed miRNAs identified in the miRNA microarray analysis. Nineteen plasma samples, consisting of ten sPE plasma samples and nine normal plasma samples, were used for RNA isolation with the mirVana PARIS kit. As shown in Fig. 2, seven miRNAs, namely miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, and miR-574-5p, were validated to be elevated in sPE plasma samples. Consistent with miRNA microarray analysis, the changes of all seven elevated miRNAs were either two- or threefold.

GO and pathway enrichment analyses

GO analysis of the predicted targets of the seven elevated miRNAs indicated that a large group of genes was connected to chromatin/nucleic acid/protein/ion binding, regulation of metabolic processes, regulation of transcription, embryonic development, and cell cycle (Table 1). Pathway enrichment analysis suggested that several pathways, including long-term potentiation, endocytosis, the transforming growth factor-beta (TGF-β) signaling pathway, cytokine–cytokine receptor interaction, the MAP kinase (MAPK) signaling pathway, and pathways in cancer metastasis, were mostly related to the seven significantly elevated miRNAs (Table 2).

Discussion

Circulating miRNAs have emerged as potential novel diagnostic biomarkers for cancer (Mitchell *et al*. 2008), pregnancy (Chim *et al*. 2008, Gilad *et al*. 2008), tissue injury (Wang *et al*. 2009), and other conditions. PE is a critical pregnancy-specific disease complicated by hypertension and proteinuria, and is a major cause of maternal mortality, morbidities, perinatal deaths, preterm birth, and intrauterine growth restriction (Sibai *et al*. 2005), affecting 3–5% of pregnancies worldwide

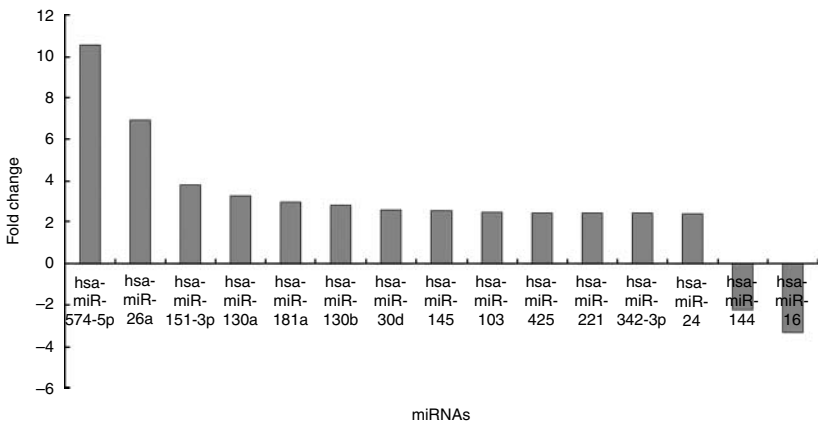

Figure 1 Differential expression profile of miRNAs in human plasma by miRNA microarray. Nine samples, including five sPE plasma samples and four normal pregnancy plasma samples, were analyzed by Agilent miRNA microarray chips. The expressions of 15 miRNAs were screened to be significantly ($P<0.05$) differential (twofold changes or more), of which 13 were upregulated and two were downregulated. The baseline denotes the mean expression level of miRNAs in four plasma samples from normal pregnancy.

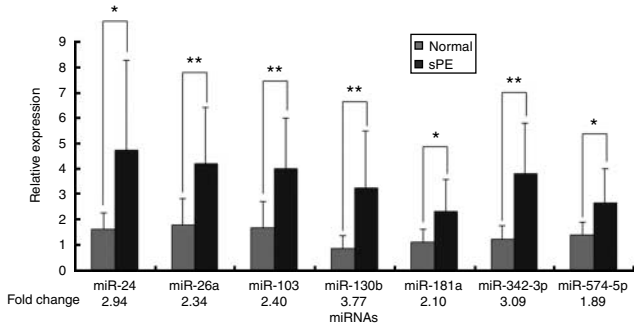

Figure 2 Expressions of miRNAs were validated by real-time quantitative stem-loop RT-PCR analysis. Synthetic *C. elegans* miRNAs, including cel-miR-39, cel-miR-54, and cel-miR-238, were added to normalize variation in RNA isolation from different samples. The experimental real-time qRT-PCR values were normalized by these three spiked-in *C. elegans* control miRNAs. Bar graphs show real-time qRT-PCR analysis of miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, and miR-574-5p in human plasma samples from sPE ($n=10$) and normal pregnancies ($n=9$). The data are presented as relative expression following normalization. The columns denote the mean; the bars denote the S.D. $^{*}P<0.05$ and $^{**}P<0.01$.

(Hogberg 2005). The mechanisms involved in PE remained poorly understood, despite advances in our understanding of this pathological condition (Levine *et al.* 2006). Exploration of the roles of differentially expressed circulating miRNAs in PE patients will enrich our understanding of the pathogenesis of this disease and contribute to its diagnosis and management. This study, for the first time, has profiled the differential expression of miRNAs in plasma samples from pregnant women with sPE compared with those from women with normal pregnancies. Seven miRNAs, namely miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, and miR-574-5p, were found to be elevated significantly in sPE plasma samples.

Abnormal placentation is one of the major pathological causes of PE (Myatt 2002), and delivery of the placenta remains the only definitive treatment for PE (Maynard *et al.* 2008). Several reports (Pineles *et al.* 2007, Roman *et al.* 2008, Hu *et al.* 2009, Zhu *et al.* 2009, Enquobahrie *et al.* 2011, Mayor-Lynn *et al.* 2011, Noack *et al.* 2011) have illustrated the differential expression of placental miRNAs in PE patients. However, except for miR-210 (elevated in preeclamptic placenta) (Pineles *et al.* 2007, Zhu *et al.* 2009, Enquobahrie *et al.* 2011), miR-181a (elevated in preeclamptic placenta) (Hu *et al.* 2009, Zhu *et al.* 2009), and miR-1 (decreased in preeclamptic placenta) (Roman *et al.* 2008, Zhu *et al.* 2009, Enquobahrie *et al.* 2011), there was little overlap among these data; this could have resulted from differences in the sample collections (including the gestational ages of the placentas and the processing of the placentas), profiling methods and patients' ethnicities (Hu *et al.* 2009). Interestingly, in this study, miR-181a was also validated to be elevated in plasma samples from sPE patients. miR-181a is one member of the hsa-miR-181 family (Ji *et al.* 2009) that also includes miR-181b, miR-181c, and miR-181d. miR-181a has been shown to be an intrinsic modulator of T cell sensitivity and selection; the inhibition of miR-181a expression in immature T cells decreased their sensitivity to antigen and weakened both positive and negative selection (Li *et al.* 2007), indicating its critical role in establishing proper development of immunity and tolerance, which are largely involved in placentation (Sibai *et al.* 2005, Bonney 2007). Since posttranscriptional silencing of 30% of protein-coding genes in

Table 1 Gene ontology analysis of circulating miRNAs elevated in sPE.

	Genes targeted by miRNAs						
Biological process category (*n*)	miR-24	miR-26a	miR-103	miR-130b	miR-181a	miR-342-3p	miR-574-5p
Anatomical structure morphogenesis	23		20		25	7	
Binding		168	121		164		
Biosynthetic process				53	67	17	
Cell communication	54						9
Cell cycle		19	14	15			
Cellular component organization		42	35				
Cellular developmental process	26		22				
Chromatin binding			4		5		2
Embryonic development	13		13		13	6	
Ion binding			51		64		
Nucleic acid binding			39	45	56		
Nucleoside-triphosphatase regulator activity		11	10	13			
Positive regulation of biological process	29			32		11	
Positive regulation of cellular process	27			29		10	
Protein binding		113	92	93	119	26	
Regulation of cellular process	91	99	69		101		
Regulation of metabolic process	46		40	52	63		
Transcription factor activity			15	16	21		
Transcription regulator activity			24	25	27		
Transport		48	39				

Values represent the number of genes targeted by miRNAs.

Table 2 Pathway enrichment analysis of circulating miRNAs elevated in sPE.

	Genes targeted by miRNAs						
Pathway (*n*)	miR-24	miR-26a	miR-103	miR-130b	miR-181a	miR-342-3p	miR-574-5p
Long-term potentiation	2	2		2	2	3	
Endocytosis	3		4	5	4		
TGF-β signaling pathway			2	3	3	3	
Adipocytokine signaling pathway		2		2	3		
Cytokine–cytokine receptor interaction	4			4	4		
Glycerophospholipid metabolism	4			2	4		
MAPK signaling pathway	8	7				5	
Pathways in cancer metastasis	6		4	6			
Regulation of actin cytoskeleton		4	3	3			
Vascular smooth muscle contraction	3	4			3		
SODD/TNFR1 signaling pathway		1	1		2		
Adherens junction			2		3		
Calcium signaling pathway	4				4		
Gap junction	2			3			
MTOR signaling pathway		2		3			
PPAR signaling pathway				2	2		
Wnt signaling pathway		3		3			
ErbB signaling pathway	2			2			
CDK regulation of DNA replication	1	1					
Mechanism of protein import into the nucleus	1	1					
Role of PI3K subunit p85 in regulation of actin organization and cell migration	1					2	
p53 signaling pathway		2			2		
Dicer pathway			1	1			

Values represent the number of genes targeted by miRNAs.

mammals were shown to be mediated by miRNAs (Lewis *et al*. 2005), the increased expression of miRNAs in sPE patients could have a profound impact on diverse biological functions (Table 1).

In addition to miR-181a, six other miRNAs, namely miR-24, miR-26a, miR-103, miR-130b, miR-342-3p, and miR-574-5p, were also found to be elevated in sPE plasma samples. These miRNAs, with the exception of miR-574-5p, which has been poorly investigated, had all been identified to be ubiquitously expressed in 40 normal human tissues, including brain, heart, kidney, liver, lymph node, and placenta (Liang *et al*. 2007). Since PE is a multisystem disorder, and several factors including renal disease, obesity and insulin resistance, and maternal susceptibility genes, have been identified with increased risk of PE (Sibai *et al*. 2005), further exploration of the sources of these significantly elevated circulating miRNAs in human tissues, especially in placenta due to its possible importance in the pathogenesis of PE, is needed.

The enrichment for specific biological process categories, including regulation of metabolic processes, regulation of transcription, and cell cycle, were revealed by the GO analysis of the predicted target genes of the seven elevated miRNAs (Table 1). Consistently, significant metabolism abnormalities in severe preeclamptic placenta have been found since the late 1980s, including metabolisms of glycogen, amino acids, and lipids (Bloxam *et al*. 1987, Walsh & Wang 1993). The placenta is also relatively hypoxic in PE, since the differentiation of cytotrophoblast is abnormal and the invasion (including interstitial invasion and endovascular invasion) is shallow (Genbacev *et al*. 1996). Hypoxia inducible factor 1 (HIF1), a transcriptional activator consisting of a constitutively expressed HIF1β subunit and an O_2-regulated HIF1α subunit, is an important global regulator of oxygen homeostasis (Semenza & Wang 1992, Wang *et al*. 1995). Under hypoxic conditions, HIF1α binds to the constitutively expressed HIF1β, and the complex subsequently translocates to the nucleus and binds to the HIF-responsive elements, initiating and enhancing the transcription of a series of genes counteracting hypoxia, which include increase in glucose uptake, activation of glycolysis, the kidney synthesis of erythropoietin, and angiogenesis stimulation (Tranquilli & Landi 2010). The expression of HIF1α has been reported to be upregulated in preeclamptic placentas obtained by cesarean section (Rajakumar *et al*. 2004). And a specific group of miRNAs, including miR-24, miR-26a, miR-103, and miR-181a, which were all found to be elevated in sPE plasma samples in this study, were revealed to be also elevated via a key involvement of HIF in human cancer cell lines, in response to low oxygen (Kulshreshtha *et al*. 2007). Besides, a very recent study reported that the miR-130 family members, including miR-130a and miR-130b, the latter of which was also found to be upregulated in sPE plasma samples in this study, regulated HIF1α signaling via targeting P-body protein DDX6, which promoted the translation of HIF1α under hypoxia (Saito *et al*. 2011). In

addition, it has long been believed that cytotrophoblast proliferation is upregulated in low oxygen concentrations (Fox 1964), which is further supported by the phenomenon that there are increased numbers of cytotrophoblast cells in the placenta at high altitude (Ali 1997), indicating the cell cycle is altered in preeclamptic placenta. However, more investigation is required to determine the mechanisms whereby the seven circulating miRNAs were elevated in sPE.

The results of pathway enrichment analysis suggested that these seven elevated miRNAs were involved in several pathways, including the MAPK signaling pathway, the TGF-β signaling pathway, and pathways in cancer metastasis. Consistent with the prediction shown in Table 2, miR-24 was reported to be able to stimulate MAPK signaling by directly targeting MAPK phosphatase 7 (Zaidi *et al*. 2009). miR-24 was also involved in TGF-β signaling, since miR-24 could repress erythropoiesis by directly targeting activin type I receptor *ALK4* and subsequently interfering with activin-induced SMAD2 phosphorylation (Wang *et al*. 2008).

All the seven elevated miRNAs presented in this study have been identified to be involved in the pathways in cancer metastasis. The miR-103/107 miRNA family was recently identified as a negative regulator of miRNA biosynthesis by targeting Dicer, which is a critical member of the miRNA-processing machinery; this resulted in decreased miR-200 expression, which induced epithelial-to-mesenchymal transition (Martello *et al*. 2010). miR-130b was shown to be involved in cell growth and self-renewal by directly targeting tumor protein 53-induced nuclear protein 1 (Yeung *et al*. 2008, Ma *et al*. 2010), and cancer metastasis (Su *et al*. 2010). Despite the fact that miR-342-3p, miR-574-5p, miR-26a, and miR-181a were not included in the pathways in cancer metastasis by pathway enrichment analysis (Table 2), these four miRNAs also had critical roles in cancer metastasis. miR-342-3p has been suggested as a potential marker for prion disease (Saba *et al*. 2008), multiple myeloma (Ronchetti *et al*. 2008), and breast cancer (Van der Auwera *et al*. 2010). miR-574-5p was recently reported to be significantly associated with chemoresistance in patients with small cell lung cancer (Ranade *et al*. 2010). miR-26a was recently found to greatly decrease the expression of *EZH2*, which resulted in the inhibition of cell growth and tumorigenesis of nasopharyngeal carcinoma (Lu *et al*. 2011). Conversely, *EZH2* expression could be elevated through negative modulation of its repressor miR-26a by *MYC* (Sander *et al*. 2008), which had been demonstrated to be directly targeted by miR-24 via binding to seedless miRNA recognition elements within its 3′-UTR (Lal *et al*. 2009). miR-181a has recently been identified as an oncogenic miRNA in MCF-7 cells (Oliveras-Ferraros *et al*. 2011).

In summary, through miRNA microarray assay and real-time stem-loop qRT-PCR analysis, this study demonstrated a maternally differential circulating miRNA expression profile in plasma samples from severe preeclamptic pregnancies compared with those from normal pregnancies. The relationship between sPE and dysregulated miRNA expression suggests critically functional roles of miRNAs in the pathology of this pregnancy-related disease. These differentially expressed miRNAs might be novel targets for the further investigation of the molecular pathogenesis and management of sPE. However, due to the high biological variability of human plasma samples, a study with a larger number of samples, which also profiles gestation from an early stage, is needed to prove miRNA analysis as an ideal and easily accessible diagnostic method for PE.

Materials and Methods

Sample collection

Plasma samples were obtained with informed consent from patients with late-onset sPE (sPE group; $n=10$) and term-matched normal pregnancies (control group; $n=9$); all pregnancies were between 37 and 40 weeks of gestation. All women were patients at the Department of Obstetrics and Gynecology, General Hospital of the People's Liberation Army in Beijing, China. A woman was determined to have sPE when either severe hypertension (either a systolic blood pressure of 160 mmHg or higher or a diastolic blood pressure of 110 mmHg or higher on two occasions at least 6 h apart while the patient was on bed rest) or severe proteinuria (either urinary excretion of 5 g protein or higher in a 24 h urine specimen or 3+ protein or greater in two random urine samples collected at least 4 h apart), or both, were present after 20 weeks of gestation (Practice ACoO 2002). All women with

Table 3 Demographic and clinical characteristics of normal and severe preeclamptic pregnancies.

Characteristics	**Control** ($n=9$)	**sPE** ($n=10$)	***P* value**
Maternal age (years)	30.4±1.3	29.9±3.1	NS
Current smoker (*n*)	0 (0%)	0 (0%)	
Preeclampsia onset (weeks)	None	34.4±1.8	
Complicated by SGA (*n*)	None	3 (30%)	
Gestational age at delivery (weeks)	38.8±0.4	37.7±1.0	NS
Primiparae (*n*)	9 (100%)	9 (90%)	
Birth weight (g)	3510.0± 482.7	2964.3± 567.7	NS
Female fetus (*n*)	5 (55.6%)	5 (50%)	
Prepregnancy weight (kg)	55.2±6.1	62.2±8.0	NS
Prepregnancy body mass index (kg/m^2)	20.5±2.6	23.3±3.4	NS
Han ethnicity (*n*)	9 (100%)	10 (100%)	
Proteinuria (g/24 h)	Normal	3.3±3.2	<0.01
Systolic blood pressure (mmHg)	112.0±4.5	161.1±15.4	<0.01
Diastolic blood pressure (mmHg)	70.0±0	105.0±13.2	<0.01

Some values are presented as mean±S.D., and statistical analyses were performed by one-way ANOVA. $P<0.05$ was considered to be statistically significant. sPE, severe preeclampsia; SGA, small for gestational age; NS, not significant.

sPE had no other maternal complications. The demographic and clinical characteristics of the study groups are summarized in Table 3. The study protocol was approved by the ethics committee of the Institute of Zoology, Chinese Academy of Sciences.

Peripheral blood was collected into $EDTAK_2$ tubes (San Li, Liuyang, China), and then immediately subjected to centrifugation at 820 ***g*** for 10 min. Supernatant plasma was transferred to RNase-free tubes and centrifuged at 16 000 ***g*** for 10 min to pellet any remaining cellular debris. Aliquots of the supernatant were transferred to fresh tubes and immediately stored at −80 °C.

Total RNA isolation from human plasma samples

Total RNA was isolated from 400 μl human plasma sample with the mirVana PARIS kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions, with the modification that samples were extracted twice with an equal volume of acid-phenol:chloroform (Mitchell *et al*. 2008). Synthetic *Caenorhabditis elegans* miRNAs, including cel-miR-39, cel-miR-54, and cel-miR-238 (GenePharma, Shanghai, China), were added to each denatured sample (after the addition of an equal volume of 2× denaturing solution to plasma to inhibit RNases) to normalize variation in RNA isolation from different samples (Mitchell *et al*. 2008). RNA was eluted with 110 μl elution solution.

Mature miRNA microarray analysis

Nine samples, including five sPE plasma samples and four normal pregnancy plasma samples, were analyzed by Agilent miRNA microarray chips (ShanghaiBio Corporation, Shanghai, China). Raw data were normalized with GeneSpring 11.2 software (Agilent Technologies, Santa Clara, CA, USA). miRNAs with significantly ($P<0.05$) differential expression of twofold changes or more were screened by Student's *t*-test for unpaired heteroscedastic samples without adjustment of *P* values.

Real-time quantitative stem-loop RT-PCR validation of mature miRNA microarray

miRNAs with significantly ($P<0.05$) differential expression of twofold changes or more were further validated in 19 plasma samples by real-time stem-loop qRT-PCR as described previously (Chen *et al*. 2005, Varkonyi-Gasic *et al*. 2007) with some modifications. In brief, a 'no RNA' RT master mix was first prepared by scaling the volume of each reaction that contained 0.5 μl 10 mM dNTP mix, 10.15 μl nuclease-free water, and 1 μl stem-loop RT primer (1 μM). The mixture was heated at 65 °C for 5 min and incubated on ice for 2 min. After a brief centrifugation, 4 μl 5× first-strand buffer, 2 μl 0.1 M DTT, 0.1 μl RNase inhibitor (40 units/μl, TaKaRa Biotechnology, Dalian, China), and 0.25 μl SuperScript II RT (200 units/μl, Invitrogen) were added into the mixture for each reaction. The RT master mix was then aliquoted to each reaction (18 μl), into which 2 μl RNA isolated from human plasma sample with spiked-in *C. elegans* control miRNAs was added. Stem-loop RT reactions were performed at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min and then held at 4 °C.

Real-time PCR was performed by a standard SYBR Premix Ex Taq II (Perfect Real Time) (TaKaRa Biotechnology) kit protocol. The 20 μl PCR consists of 10 μl SYBR Premix Ex Taq II (2×), 1 μl PCR forward primer (10 μM), 1 μl PCR reverse primer (10 μM), 2 μl stem-loop RT product, and 6 μl dH_2O. The reactions were incubated at 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 25 s, and then ended by a melting step with slow heating from 65 to 95 °C. All reactions were done in duplicate. The threshold

Table 4 Primers used in real-time quantitative stem-loop RT-PCR analysis.

miRNAs	**Primers**	**Sequence** (5′–3′)
hsa-miR-24	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGTTC
	PCR	GCGTGGCTCAGTTCAGCAG
hsa-miR-26a	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCCTA
	PCR	GGCAGGTTCAAGTAATCCAGGA
hsa-miR-103	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATAG
	PCR	GGCAGCAGCATTGTACAGGG
hsa-miR-130b	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCCC
	PCR	GCCGCCAGTGCAATGATGAAA
hsa-miR-181a	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCAC
	PCR	GCCGAACATTCAACGCTGTCG
hsa-miR-342-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACGGGT
	PCR	GGCTCTCACACAGAAATCGC
hsa-miR-574-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACACAC
	PCR	CCGCTGAGTGTGTGTGTGTGA
cel-miR-39	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGCT
	PCR	GCGCTCACCGGGTGTAAATC
cel-miR-54	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCGGA
	PCR	GGCCGTACCCGTAATCTTCATAA
cel-miR-238	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTGAA
	PCR	GGCGTTTGTACTCCGATGCCA
Universal reverse	PCR	GTGCAGGGTCCGAGGT

hsa, *Homo sapiens*; cel, *Caenorhabditis elegans*.

cycle (C_t) refers to the fractional cycle number at which the fluorescence passes the fixed threshold. In this study, the C_t was determined with the automatic threshold settings. The 'Delta–delta' method (Livak & Schmittgen 2001) was used to analyze real-time qRT-PCR data. Normalization of experimental real-time qRT-PCR data using spiked-in *C. elegans* control miRNAs was carried out as described previously (Mitchell *et al.* 2008). All primers synthesized by Invitrogen, Beijing, China are listed in Table 4.

Statistical analysis

Validation results from real-time qRT-PCR are displayed as the mean ± S.D. Statistical analysis was performed by one-way ANOVA. $P<0.05$ was considered to be statistically significant.

GO and pathway enrichment analyses

Differentially expressed miRNAs were further analyzed for predicted targets from TargetScan (www.targetscan.org) via GeneSpring 11.2 software, while the parameters were set as 'context score percentile: 90.0' and 'database: conserved'. GO and pathway enrichment analyses of predicted targets of the differentially expressed miRNAs were undertaken by the ShanghaiBio Corporation (SBC) analysis system (http://sas.ebioservice.com), which functions on the enrichment calculation and function annotation of differentially expressed genes by combining R-software (the R Project for Statistical Computing, http://www.r-project.org) with seven public databases that include NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/gene), GO (http://www.geneontology.org), KEGG (http://www.genome.jp/kegg), and Biocarta (http://www.biocarta.com). The enrichment *P* values of both GO and pathway enrichment analyses were calculated by Fisher's exact test (Fisher 1922), which were corrected by enrichment *q*-values (the false discovery rate) that were calculated by John Storey's method (Storey *et al.* 2004).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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