Clin Chem Lab Med 2009;47(8):923–929 © 2009 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2009.228

Differential expression of microRNAs in the placentae of Chinese patients with severe pre-eclampsia

Yali Hu[1,2], Pengfei Li[3], Sha Hao[3], Liu Liu[3], Junli Zhao[3] and Yayi Hou[2,3,*]

[1] The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing 210008, PR China
[2] Jiangsu Key Laboratory of Molecular Medicine, Nanjing University, Nanjing 210093, PR China
[3] Immunology and Reproductive Biology Lab, Medical School and State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, PR China

Abstract

Background: The pathogenesis of pre-eclampsia (PE) is incompletely understood. The placenta is considered to play a key role in this disease. Recent research showed that many microRNAs (miRNAs) are expressed in human placenta. Our aim in this study was to determine differential expression of miRNAs in placenta with severe PE, and normal placenta.
Methods: Differential expression of miRNAs in placenta (four severe PE and a control group of four normal pregnant women) was first screened using microarray analysis. Following this, some differential miRNAs were selected and validated using real-time quantitative reverse transcription-polymerase chain reaction in placenta from women with severe PE (n=24), and a healthy control group (n=26).
Results: We found the following miRNAs were significantly increased in placenta from women with severe PE: miR-16, miR-29b, miR-195, miR-26b, miR-181a, miR-335 and miR-222. Gene ontology analysis of the target genes revealed enrichment for specific biological process categories, i.e., regulation of cellular physiological process including miR-16, miR-29b, miR-195, miR-26b and miR-335, and signal transduction including miR-181a and miR-222.
Conclusions: These different miRNAs may play an important role in pathogenesis of PE and may become diagnostic markers for PE.
Clin Chem Lab Med 2009;47:923–9.

Keywords: gene ontology; microarray; microRNA; placenta; pre-eclampsia.

*Corresponding author: Yayi Hou, PhD, Immunology and Reproduction Biology Lab, Medical School, Nanjing University, Nanjing 210093, PR China
Phone: +86-25-83686441, Fax: +86-25-83686441,
E-mail: yayihou@nju.edu.cn
Received March 4, 2009; accepted May 28, 2009

Introduction

Pre-eclampsia (PE) is a disease of pregnancy characterized by hypertension and proteinuria, developing after 20 weeks of gestation. It has been estimated that 5%–7% of pregnancies world wide are complicated by this disorder, resulting in a very large disease burden (1). Although its pathogenesis is incompletely understood, the placenta is considered to play a key role in the disease (2).

Recent studies on microRNA (miRNA) offer the possibility for developing a new class of molecular markers for diagnosis of PE. MiRNAs are short (19–25 nucleotides), single-stranded, and non-protein-coding RNAs (3) that regulate gene expression by binding to the 3′ untranslated region of the target mRNAs (4). They function in diverse biological processes, including development, differentiation, apoptosis, and oncogenesis (5). Moreover, recent research shows that many miRNAs are expressed abundantly in the human placenta (6).

Published data on miRNAs in human PE is surprisingly sparse. Pineles et al. (7) first reported the miRNA expression patterns in placentae with distinct pathologies including PE and PE+small-for-gestational age (SGA). However, they only identified the expression of 157 miRNAs using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) for miRNA arrays. Roman et al. (8) reported the differential expression of miRNA in placenta of Hispanics with severe PE. Recently, there was another report published on miRNA in Chinese women with PE (9). This lack of data prompted us to determine and compare the expression profile of miRNA in placenta of Chinese patients with PE compared with normal placenta tissue. We wished to evaluate the potential clinical usefulness of miRNA as diagnostic markers.

Materials and methods

Patients and tissue samples

Placental tissue was obtained from women who were hospitalized in the Department of Gynecology and Obstetrics of The Affiliated Drum Tower Hospital of Nanjing University Medical School. Written consent was received from women prior to surgery. The hospital Ethics Committee approved the consent forms and the protocols for evaluation of the tissue. All placental tissues were obtained at the time of cesarean section. We selected 24 pregnancies complicated by severe late-onset PE with delivery occurring after 34 weeks. Also, 26 pregnant women with normal term pregnancy were recruited as the control group. The relevant clinical details for the patients are shown in Table 1. For the control group, women with chronic hypertension, cardiovascular disease, renal disease, hepatitis, diabetes, any evidence

Table 1 Clinical characteristics of the study population.

	PE (n=24)	Control (n=26)	p-Value
Age, years	28.1±1.3	28.7±1.1	NS
Gestational age at delivery, weeks	37.0±0.2	38.8±0.4	NS
% of primiparae	10 (41.6%)	15 (57.6%)	NS
Body mass index, kg/m^2	29.0±1.0	27.2±1.2	NS
Systolic blood pressure, mm Hg	161.5±4.1	119.6±3.9	<0.05
Diastolic blood pressure, mm Hg	113.4±2.8	81.7±3.3	<0.05
Proteinuria, mg/24 h	2209.4±23.6	0	<0.05
Alanine aminotransferase, U/L	33.9±8.6	30.5±6.5	NS
Blood urea nitrogen, mmol/L	4.1±0.3	3.8±0.2	NS
Platelet, $\times 10^9$/L	157.9±22.1	192.0±31.4	NS
Birth weight, g	2835.6±173.0	3415.3±158.3	NS
Placenta weight, g	485.8±25.6	526.3±28.4	NS

PE, pre-eclampsia; NS, non-significant.

of intrapartum infection or other complications of pregnancy complications, such as fetal anomalies or chromosomal abnormalities were excluded from this study.

PE was defined as gestational hypertension (systolic pressure >140 mm Hg or diastolic blood pressure >90 mm Hg on ≥2 occasions after gestational week 20) with proteinuria (>0.3 g/day). Severe PE was defined by the presence of ≥1 of the following: 1) severe gestational hypertension (systolic pressure >160 mm Hg or diastolic blood pressure >110 mm Hg on ≥2 occasions after gestational week 20); or 2) severe proteinuria (≥5 g protein in a 24-h urine specimen) (ACOG practice bulletin) (10). We collected chorionic tissue blocks (~1 cm^3) from the central part of the placenta only. Contamination with mother decidua and amniotic membranes was excluded by morphological observation. Tissues were thoroughly washed with normal saline and then frozen in liquid nitrogen and stored at –80°C until used.

RNA extraction

Total RNA included miRNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA integrity was determined using formaldehyde denaturalization agarose gel electrophoresis. RNA concentrations were measured with the smartspec™ plus spectrophotometer (BIO-RAD, Hercules, CA, USA).

MicroRNA microarray analysis

Eight samples, four normal placentae (4 normal placentae were pooled to form a control group) and four placentae from women with severe PE (matched for gestational age at delivery and mother age) were assayed using a miRNA microarray chip (CapitalBio Corp, Beijing, China). We undertook miRNA expression analysis according to their instructions. We reported only those genes with significant ($p<0.05$) differential expression of ≥2.0-fold changes. MiRNAs were further analyzed according to predicted targets found at miRBase (http://microrna.sanger.ac.uk/targets version 5).

Mature microRNA quantitative real-time reverse transcription PCR (qRT-PCR)

The method to quantify mature miRNA was performed as described previously (11). We used TaqMan™ MicroRNA Assays (Applied Biosystems, Foster City, CA, USA). Total RNA was purified using mirVana miRNA isolation kit (Ambion, Austin, TX, USA) to enrich the small RNA fraction. Then, 10 ng of total RNA was combined in the 15 μL RT reaction: 1.5 μL 10×RT-PCR buffer, 1 μL of 50 U/μL MultiScribe RT enzyme, 0.15 μL 100×dNTP mix, 0.19 μL 20 U/μL RNase-inhibitor, and 3 μL 5×specific RT-primer and nuclease-free water to a total volume of 15 μL. The transcription reaction was incubated at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. All reverse transcription reactions, including no-template controls and RT minus controls, were run in duplicate. Once the RT reactions are complete, the PCR reactions can be assembled. For a 20-μL reaction, 10 μL 2×Universal Master Mix, 1.32 μL of the RT product, 1 μL 20×TaqMan™ Assay, and 7.68 μL nuclease-free water were combined. Subsequently, qRT-PCR was performed using an Applied BioSystems 7300 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All experiments were done in triplicate. The threshold cycle (Ct) was determined using the default threshold settings. The Ct value was defined as the fractional cycle number at which fluorescence passes a fixed threshold. The relative amount of each miRNA to internal control was calculated using the equation $2^{-\Delta C_t}$, where $\Delta C_t = C_{tmiRNA} - C_{tU6}$. All primers used are listed in Table 2.

Statistical analysis

Results were expressed as mean±SEM. Statistical analysis was performed using unpaired Student's t-test, using GraphPad Prism 5 Demo software (GraphPad software, San Diego, CA, USA). A $p<0.05$ was considered to be statistically significant.

Results

Characteristics of RNA samples

The mean ratio of absorbance at 260 and 280 nm (A260/A280) of all 50 RNA samples amounted to 1.93±0.05 (arithmetic mean±SD). RNA integrity was determined using formaldehyde denaturalization agarose gel electrophoresis (data not shown). The criterion for RNA quality is an A260/A280 ratio >1.8 and a clearly visible 28S and 18S rRNA band with a ratio of 1.0~2.0 (28S:18S).

MicroRNA microarray expression data

We used the microarray platform CapitalBio Mammalian miRNA Array V3.0 consisting of 924 miRNA

Table 2 MicroRNA primer information.

Sanger_name	Forward primer (5′–3′)	Reverse primer (5′–3′)
hsa-miR-181a	ACACTCCAGCTGGGAACATTCAACGCTGTCG	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACTCACCG
hsa-miR-222	ACACTCCAGCTGGGAGCTACATCTGGCTA	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACCCAGTA
hsa-miR-26b	ACACTCCAGCTGGGTTCAAGTAATTCAGG	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACCTATCC
hsa-miR-29b	ACACTCCAGCTGGGTAGCACCATTTGAAA	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACACTGA
hsa-miR-16	ACACTCCAGCTGGGTAGCAGCACGTAAAT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCGCCAATA
hsa-miR-195	ACACTCCAGCTGGGTAGCAGCACAGAAAT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCCAATAT
hsa-miR-335	ACACTCCAGCTGGGTCAAGAGCAATAACG	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACATTTTT
U6snRNA	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
URP	TGGTGTCGTGGAGTCG	

probes (677 human miRNAs) corresponding to the Sanger Center database miRBase version 10.0. First, we assessed the expression profile in normal placenta and placenta from women with severe PE. Several previous in depth comparative studies between microarray platforms and analysis procedures have indicated the very high reproducibility, sensitivity, and specificity of similar expression microarrays when using the recommended procedures (12, 13). Microarray data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE15789. These miRNAs were retained for further statistical analysis including SAM (14) and hierarchical cluster analysis (15) (Figure 1). After the raw data were normalized, we found 20 miRNAs to be up-regulated and seven miRNAs down-regulated in patients with severe PE compared with nomal placenta. The changes in miRNA identified in placentas from women with severe PE are shown in Table 3.

Validation of microarray data by qRT-PCR analysis

Further validation of aberrant miRNAs was determined using qRT-PCR in placentas from 24 women with severe PE and 26 placentas from women with normal pregnancy. We identified the seven most up-regulated miRNAs (miR-181a, miR-195, miR-222, miR-16, miR-29b, miR-26b and miR-335). As shown in Figure 2, the expressions of miR-16, miR-29b, miR-195, miR-26b, miR-181a, miR-335 and miR-222 were significantly different in severe PE compared with normal placenta. These miRNAs were found to have the same expression trend as microarray analysis, but the discordance observed was not identical. This result was not surprising because qRT-PCR is a more sensitive technique compared with miRNA microarray.

Target prediction and GO analysis

Predicted targets of miRNAs differentially expressed in this study were determined using miRBase Targets (16) (Table 4). In addition, we used CapitalBio® Molecule Annotation System V4.0 to perform gene ontology (GO) analysis on the gene target lists of miR-16, miR-29b, miR-195, miR-26b, miR-181a, miR-335 and miR-222 and found that specific biological process categories were enriched (Table 5).

Discussion

In the present study, we profiled the expression of a number of miRNAs in placenta obtained from women with severe PE and from women with normal pregnancy. Of the 677 human miRNAs on the array of the expression values, 27 miRNAs were differentially expressed between placenta from women with severe PE and those with normal pregnancy (Table 3). There were 20 miRNAs that were up-regulated in PE compared with normal placenta, and seven miRNAs were

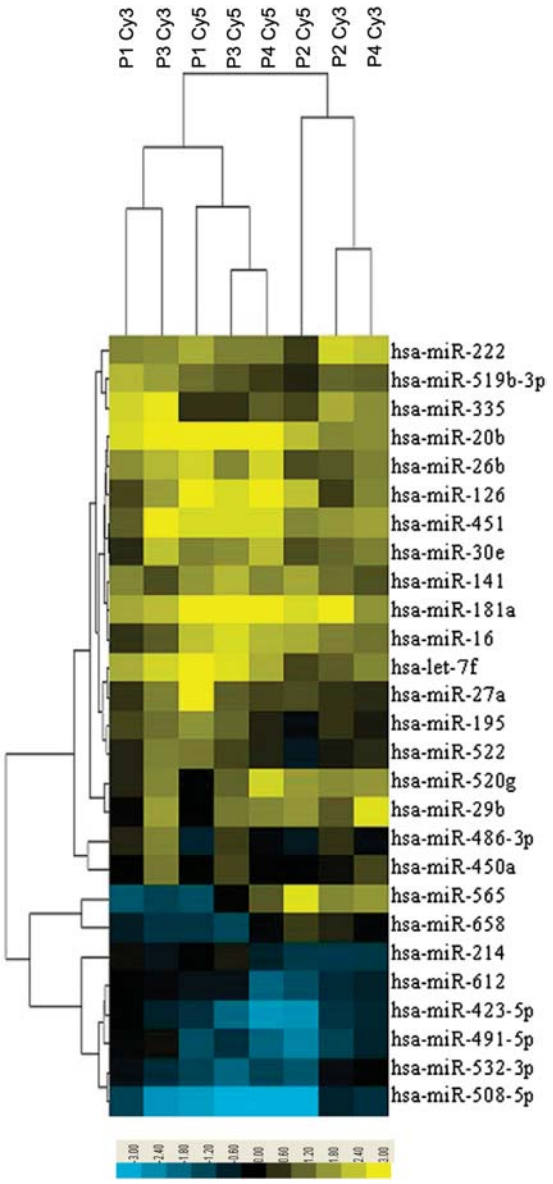

Figure 1 Hierarchical cluster analysis of differentially expressed miRNAs in placenta from women with severe pre-eclampsia (P1, P2, P3, P4) and normal placenta (composed of tissue from four normal placentas).
Each row represents a microRNA and each column represents a sample pair of placenta from women with severe pre-eclampsia and normal pregnancy. The color indicates high expression (yellow) or low expression (blue) according to the color legend shown below. The microRNA data were clustered according to their similarities in expression pattern in microRNAs and tissues. The dendrograms display similarity of expression among these cohorts.

down-regulated. Several studies have reported the differential expression of miRNA in normal placenta and PE placenta. Pineles et al. (7) found that expression of two miRNAs (miR-210 and miR-182) was significantly higher in PE than in the control group. Roman et al. (8) found that there were 91 dysregulated miRNAs (38 miRNAs were down-regulated and 53 were up-regulated). Zhu et al. (9) found that 11 miRNAs were overexpressed and 23 miRNAs were underexpressed in PE. When compared with their results, there was no overlap with our data. This might be attributed to the different array platforms and ethic groups and gestation age of the placenta.

It is estimated that ~30% of genes are potential targets of miRNA function (17, 18). Since each miRNA is

Table 3 The list of miRNAs that were differentially expressed between pre-eclampsia compared with normal placenta.

Name	Fold change
hsa-miR-181a	6.58
hsa-miR-195	4.23
hsa-miR-222	4.08
hsa-miR-16	3.65
hsa-miR-29b	3.57
hsa-miR-26b	3.24
hsa-miR-335	2.91
hsa-miR-126	2.76
hsa-miR-7f	2.68
hsa-miR-565	2.61
hsa-miR-20b	2.57
hsa-miR-27a	2.46
hsa-miR-141	2.42
hsa-miR-519b-3p	2.37
hsa-miR-451	2.34
hsa-miR-450a	2.25
hsa-miR-520g	2.20
hsa-miR-30e	2.16
hsa-miR-522	2.12
hsa-miR-486-3p	2.05
hsa-miR-214	–2.04
hsa-miR-658	–2.13
hsa-miR-532-3p	–2.38
hsa-miR-423-5p	–3.13
hsa-miR-491-5p	–3.70
hsa-miR-612	–4.35
hsa-miR-508-5p	–7.69

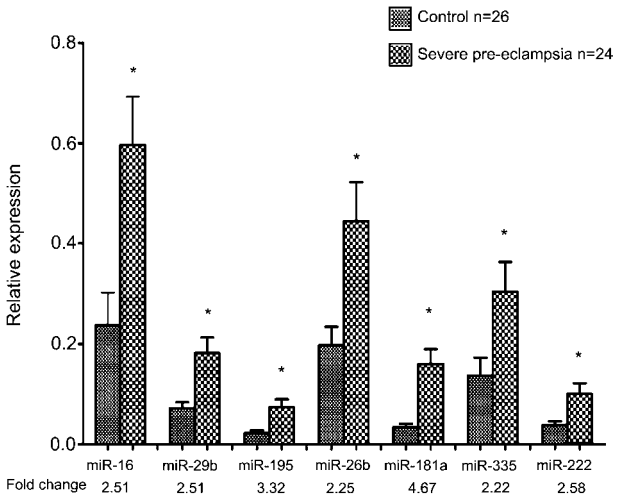

Figure 2 Real time PCR expression of miR-16, miR-29b, miR-195, miR-26b, miR-181a, miR-335 and miR-222 in normal placenta and placenta from women with severe pre-eclampsia.
Bar graphs show real time PCR expression of miR-16, miR-29b, miR-195, miR-26b, miR-181a, miR-335 and miR-222 in placenta from women with normal pregnancy and placenta from women with severe pre-eclampsia. The data are presented as relative expression following normalization. Data represent mean ± SE. *Significantly different forms. $p < 0.05$ was considered significant.

Table 4 MiRNAs and gene targets.

miRNA	Chromosomal location	Predicted target genes
hsa-miR335	7q21.1	PLGF,PGR,ACVR1C,IGR2BP2,HIF1AN,COL4A2,IL-17RD,KIAA 0256,MAP3K2,EBF4,TP53NP2,ECFN2,HOXB3,VAV2,MEF2D
hsa-miR181a	9q33.3/1q31.3	TGFBRAP1,IL18R1,IL1R1,FGFR2,KIR2DL1,IL12RB1,KIAA1632, TNFAIP6,HLA-C,HLA-B,HLA-DRA,TLR1, IL2,SAP30,TGFBI, CD164L2,CD1E,KLRD1,IFNG,CCL7,CCL11,CCL8, MMP7
hsa-miR222	xp11.3	IL18RAP,ADAM17,ADAM8,KIAA1279,AGTPBP1,KIR3DL1, ICAM4,HLA-F,PI16,ADAMTS16, CD180,IL17RB,ADAM22, IFRD1,CD99,CYR61,CD68,MMP1,IL18
has-miR16	3q26.1/13q14.3	C2orf34,hcG_1790474,IL18RAP,CD40,FGFR2,FGF8,KIAA1462, FLT3,ADAMTS18,GFER,TLE4,ADAMTSL1,CD274,CD37,CD97, PIGB,EGFL8,TKR1,NKKB1,CXCL3,PPAP2A,CD99,TGFBR3, CD48,ADAMTS4,IL20,VWF,CD163,CCL13,CCL4,CCL4L, FGFR1,IL10RB,CD151,MMP3
hsa-miR26b	2q35	IL18R1,MMP21,PPA1,KIAA1279,ANGPTL2,ADAMTSL1,PEG3, KIR3DL1,KIR2DL4,KIR2DL1,FGF21,CDC34,MAP3K2,STAT4, PRL,EGF,IRF2,CCL26,CSFIRA,IL13RA1,KIAA2013,ETV3,CD1C, CD1E,IL20,IFNG,CD68,CCL13,SERPINA10,MMP12
hsa-miR29b	1q32.2/7q32.3	ILIF9,PIGF,CD40,ADAMTS18,IL32,HIF3A,ADAMTS10,ANGPTL4, TNFRSF9,KIAA0101,ADAMTS7,HLA-DQA2,VEGFA,IL20RA, ADAMTS6,SRP19,CDC7,IL4,CD55,IFNG,IL-22,TGFB3,LPL, CD248,MMP8
hsa-miR195	17p13.1	CD40,ADAM12,FGF8,KIAA1462,ADAMTS1,CD37,MICA,LTB, EGFL8,VEGFA,TLR1,IL15,CXCL3,PPAP2A,ILF2,CD48,IL20, CD163,CCL4,CCL4L1,IL10RB,MMP3HLA-DRB1

Note: see citations in the text and http://microrna.sanger.ac.uk/sequences/regarding most updated predicted target genes.

Table 5 The results of gene ontology analysis of targets of differentially expressed miRNAs.

Biological process category, n	Targets of miR-16	Targets of miR-26b	Targets of miR-29b	Targets of miR-195	Targets of miR-335	Targets of miR-181a	Targets of miR-222
Cell death	16	8	9	12	10	7	7
Immune system process	12	9	9	20	9	17	12
Immune response	7	5	5	8	7	5	7
Regulation of cellular physiological process	44	38	33	44	45	29	29
Signal transduction	41	28	29	34	36	35	30
Organ development	10	12	14	14	16	13	12
System development	7	8	5	9	9	6	5
Regulation of signal transduction	6	6	5	6	4	7	5
Tissue development	5	1	3	6	5	3	3
Cell proliferation	4	3	3	5	6	4	4
Cell development	2	6	4	4	5	2	3

Values expressed as number of genes targeted by miRNA.

predicted to have a broad range of targets, even an alteration in the expression of a single miRNA could have a significant impact on the outcome of diverse biological functions associated with these genes. As such, the absence or altered expression of these and other miRNAs could result in expression re-programming of many of their target genes in PE. The increased miRNA expression in PE suggests the down-regulation of potential target genes which may contribute to the pathology of PE. Among the genes predicted as the target of miR-16, miR-26b, miR-29b, miR-335, miR-222, miR-181a and miR-195 (Table 4), we found the target genes of these miRNAs were related to angiogenic factors, such as vascular endothelial growth factor A (VEGF-A) and placental growth factor (PLGF) (19) which are important for the development of PE (see below). Furthermore, we performed GO analysis on the target genes, and found that a considerable number of genes have been identified related to immune response, signal transduction and angiogenesis. These are all thought to be involved in the maintenance of pregnancy and the development of PE (Table 5).

Perturbation of angiogenesis has been proposed as one of the key features of PE. It is speculated that there is an imbalance in the production of angiogenic growth factors at the maternal-fetal interface. Recent lines of evidence suggest that failed trophoblast invasion is linked to the maternal vascular pathology through abnormal placental production of vasculogenic and angiogenic factors, such as VEGF (20, 21). Some angiogenic growth factors, such as angiopoietin 2 (Ang2), PLGF, and VEGF-C, fibroblast growth factor (FGF), interferon-γ (IFN-γ), and transforming growth factor-β (TGF-β), are involved in pregnancy (22). These molecules may be useful markers for pre-

dicting PE (23, 24). Zhou et al. (25) demonstrated that the expressions of VEGF-A and VEGF receptor-1 are down-regulated in cytotrophoblasts of pre-eclamptic placenta. Several investigations have demonstrated that other growth factors and their receptors, such as PLGF and insulin-like growth factor I (IGF-I) are also dysregulated in serum or placental tissue of women with PE (26, 27). Mo et al. (28) found that cysteine-rich 61 (CYR61) is essential for placental development and vascular integrity. Gellhaus et al. (29) found that CYR61 is significantly decreased in pre-eclamptic placenta. Interestingly, our research revealed that these angiogenic growth factors were potential targets of the altered miRNA, such as CYR61, PLGF, VEGF-A which were targets of miR-222, miR-335 and miR-195, respectively. Moreover, it is reported that miRNA is strongly implicated in angiogenesis (30).

The association between PE and altered miRNA expression suggests the possibility of a functional role for miRNA in this disease. Our finding may provide novel targets for further investigation of the pathogenesis of PE and these differential miRNAs may be potential markers for the diagnosis of PE. Moreover, Chim et al. found that placental miRNAs represent a novel class of fetal nucleic acid markers in maternal plasma (31). Therefore, it will be interesting to determine whether the differential expression profiles of miRNAs found in placenta correlates with those from maternal plasma, especially since these may serve as diagnostic markers of PE.

Conflict of interest disclosures

None declared.

Acknowledgements

This work was supported by the Project Foundation of Jiangsu Province Department of Health, China (Grant No. H200754) and Special Research Grant (XK200709 to YH) for the Key Laboratory from the Department of Health, Jiangsu Province.

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