Variations of MicroRNAs in Human Placentas and Plasma From Preeclamptic Pregnancy

Peng Xu,* Yangyu Zhao,* Ming Liu, Yongqing Wang, Hao Wang, Yu-xia Li, Xiaoming Zhu, Yuanqing Yao, Haibin Wang, Jie Qiao, Lei Ji,* Yan-ling Wang*

Abstract—Preeclampsia is a major cause of maternal and fetal mortality and morbidity worldwide. The differential expression of several microRNAs (miRNAs) has been found in preeclamptic placentas. However, great conflict exists regarding this aspect, and detailed examinations have largely been lacking of miRNA profiles in different parts of the placenta and in maternal plasma of women with this disorder. In this study, a total of 9 downregulated miRNAs (miR-195, miR-223, miR-218, miR-17, miR-18a, miR-19b1, miR-92a1, miR-379, and miR-411) and 7 upregulated miRNAs (miR-210, miR-30a-3p, miR-518b, miR-524, miR-17-3p, miR-151, and miR-193b) were identified in severe preeclampsia (sPE) placentas when compared with normal pregnant controls. In addition, sampling position in the chorionic or basal plate of placenta led to evident variations in differential miRNAs of sPE placentas. In a prospective pregnant cohort, we found that the circulating levels of 3 members of miR-17-92 cluster (ie, miR-18a, miR-19b1, and miR-92a1) were significantly lower, whereas that of miR-210 was higher in sPE patients than those in normal controls at gestational weeks 15 to 18 and at term. The results of in situ hybridization revealed the localization of miR-18a, miR-92a1, and miR-210 in various subtypes of placental trophoblasts and endothelial cells. In human trophoblast cell line, HTR8/SVneo cells, miR-18a could promote trophoblast cell invasion via targeting and suppressing Smad2 expression. This study provides fundamental evidences for exploring the roles of miRNAs in the pathogenesis of preeclampsia. (Hypertension. 2014;63:1276-1284.) • Online Data Supplement

Key Words: microRNAs ▪ placenta ▪ plasma ▪ preeclampsia ▪ miR-17-92

Preeclampsia affects ≈2% to 7% pregnancies worldwide and is a major disorder that leads to maternal, fetal, and neonatal mortality and morbidity.1 Placental defects, especially the dysregulation of trophoblast cell behaviors, have been assumed to substantially mediate the clinical disease.2,3 Identifying the genes that are specifically dysregulated in preeclamptic placentas may be a useful manner for exploring the biomarkers and the cause of preeclampsia.

MicroRNAs (miRNAs) are a class of 20- to 22-nt, endogenous, noncoding RNAs.4 These small molecules play roles in a diverse range of cell events.5,6 Interestingly, the human placenta is an organ rich in miRNA expression, and a great number of differentially expressed miRNAs have been found in preeclamptic placentas.7-14 Some of these differential miRNAs are demonstrated to participate in the regulation of trophoblast cell invasion,15 placent al immune activation, and platelet aggregation.16,17 However, the relevant investigations have been far less sufficient.

A notable fact is that there exists a great conflict in describing miRNA profiles in preeclamptic placenta. Such inconsistencies might be because of the differences in sample sizes or in experimental settings and platforms. However, the location in placenta from where the samples were taken may have more influence. The human placenta is not a homogenous tissue, and different parts of the placenta are composed of various cell types. The expression profiles and functions of miRNAs in these cell types may vary significantly. Accordingly, detailed investigations on these issues are of great value in elucidating the roles of small RNAs during physiological and pathological pregnancies.

In this study, we attempted to identify differential miRNAs in severe preeclampsia (sPE) placentas derived from Chinese Han pregnant women. The expression patterns of differential miRNAs in the chorionic and basal plates of placent al tissues were separately measured. Members of miR-17-92 cluster were subjected to further investigation, including their localization in placentas, their differences in maternal plasma between sPE and normal pregnancy, as well as the modulation of trophoblast cell invasion by miR-18a. This study gives a detailed demonstration on miRNA expression patterns in preeclamptic placenta and demonstrates the invasion...
modulating effect of miR-18a. The data provide important evidences for exploring the pathogenesis of the disease from a noncoding RNA viewpoint.

Materials and Methods
All the details of the materials and methods are provided in the online-only Data Supplement.

Study Subjects
In this study, the collection of human placenta tissues and plasma specimens was performed with the permission of the local ethical committee in the Institute of Zoology, Chinese Academy of Sciences, and informed consent was obtained from all patients enrolled in this study.

Placentas and maternal blood samples from normal pregnant and preeclamptic women were obtained from pregnant women who underwent perinatal care in Peking University Third Hospital from August 2010 to October 2012. Totally 20 severe preeclamptic patients who delivered at 35th to 39th weeks and 33 normal pregnant women who delivered at 37th to 39th weeks were enrolled in this study. Their placentas at deliveries and plasma samples at gestational weeks 15 to 18 and weeks 35 to 38 were used. The clinical characteristics of these women are summarized in Table 1.

miRNA Microarray Analysis
Large-scale profiling of miRNA expression was achieved by mammalian miRNA chip array (V2.0, Capitalbio, Beijing, China), which stems from the miRbase release 8.2 (Wellcome Trust Genome Campus, Cambridge, United Kingdom; ftp://mirbase.org/pub/mirbase/8.2). The data were analyzed using Significance Analysis of Microarrays software (http://www-stat.stanford.edu/~tibs/SAM).

RT-qPCR
SYBR Premix Ex Taq assays (Takara, Dalian, China), MiRcute miRNA qPCR assays (Tiangen, Beijing, China), and TaqMan Gene Expression Assays (Life Technologies, CA) were separately performed for the quantitative analysis of mRNA and miRNA expression in human placenta and plasma.

In Situ Hybridization for miRNAs
Frozen sections were subjected to in situ hybridization using anti-sense 3′-digoxigenin-labeled miRCURY LNA miRNA Detection probes (Exiqon, Copenhagen, Denmark).

Cell Culture and Transient Transfection
HTR8/SVneo cells were cultured in RPMI 1640 culture medium (Life technologies) supplemented with 10% fetal bovine serum. Transient transfection of siRNA or miRNA duplexes or plasmids was performed using Lipofectamine 2000 reagent (Life technologies).

Transwell Invasion Assay
Transwell insert invasion assay was conducted in 24-well fitted inserts (8 μm pore size; Millipore Corp, MA). Cell invasion index was presented as a percentage of invaded cell number compared with the corresponding control.

Western Blotting
Protein levels of Smad2 in cells or placental tissues were measured by Western blotting with specific antibodies (Cell Signaling Technology, MA).

Luciferase Assays
Luciferase assays were performed using a reporter plasmid carrying the wild type (WT) or mutated 3′-UTR of human Smad2 gene.

Statistical Analysis
All statistical analyses were performed with Statistical Package for the Social Sciences software (version 17.0; SPSS Inc, Chicago, IL). Multiple comparisons were performed using R software (version 3.0.2; http://www.r-project.org/), and the differences are considered significant when $q<0.05$. Comparisons between 2 groups were estimated using independent samples t test, and $P<0.05$ was considered statistically significant.

Table 1. Clinical Characteristics of Pregnant Women Enrolled in This Study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal Pregnancy (n=33)</th>
<th>Preeclampsia (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, y</td>
<td>31±4</td>
<td>30±4</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22±2</td>
<td>22±3</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>116±6</td>
<td>161±17*</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>77±5</td>
<td>104±8*</td>
</tr>
<tr>
<td>24-h urine protein, g</td>
<td>ND</td>
<td>5±5*</td>
</tr>
<tr>
<td>50 g GCT, mmol/L</td>
<td>7±1</td>
<td>7±1</td>
</tr>
<tr>
<td>Gestational age at delivery, d</td>
<td>268±7</td>
<td>243±16*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD, and compared by independent samples t-test. BMI indicates body mass index; DBP, diastolic blood pressure; GCT, glucose challenge test; ND, not determined; and SBP, systolic blood pressure.

$^*P<0.05$ vs normal pregnancy.

Table 2. List of Differential MicroRNAs (miRNAs) in Severe Preeclamptic Placentas as Detected by miRNA Microarray

<table>
<thead>
<tr>
<th>miRNA Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-214</td>
<td>0.64</td>
</tr>
<tr>
<td>miR-218</td>
<td>0.56</td>
</tr>
<tr>
<td>miR-590</td>
<td>0.66</td>
</tr>
<tr>
<td>miR-18a</td>
<td>0.50</td>
</tr>
<tr>
<td>miR-19a</td>
<td>0.60</td>
</tr>
<tr>
<td>miR-379</td>
<td>0.62</td>
</tr>
<tr>
<td>miR-411</td>
<td>0.48</td>
</tr>
<tr>
<td>miR-195</td>
<td>0.47</td>
</tr>
<tr>
<td>miR-223</td>
<td>0.57</td>
</tr>
<tr>
<td>miR-363</td>
<td>0.60</td>
</tr>
<tr>
<td>miR-542-3p</td>
<td>0.59</td>
</tr>
<tr>
<td>miR-181a</td>
<td>1.98</td>
</tr>
<tr>
<td>miR-584</td>
<td>2.04</td>
</tr>
<tr>
<td>miR-30a-3p</td>
<td>1.82</td>
</tr>
<tr>
<td>miR-151</td>
<td>1.54</td>
</tr>
<tr>
<td>miR-31</td>
<td>1.52</td>
</tr>
<tr>
<td>miR-210</td>
<td>2.95</td>
</tr>
<tr>
<td>miR-17-3p</td>
<td>1.51</td>
</tr>
<tr>
<td>miR-193b</td>
<td>1.76</td>
</tr>
<tr>
<td>miR-638</td>
<td>1.80</td>
</tr>
<tr>
<td>miR-525*</td>
<td>1.90</td>
</tr>
<tr>
<td>miR-515-3p</td>
<td>1.55</td>
</tr>
<tr>
<td>miR-519e*</td>
<td>1.64</td>
</tr>
<tr>
<td>miR-518b</td>
<td>2.03</td>
</tr>
<tr>
<td>miR-524</td>
<td>1.79</td>
</tr>
<tr>
<td>miR-296</td>
<td>2.31</td>
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<tr>
<td>miR-362</td>
<td>1.70</td>
</tr>
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</table>
Results
Screening of Differential miRNAs in sPE Placenta by miRNA Microarray
A comprehensive miRNA microarray analysis was performed using RNAs extracted from random sites of 3 sPE placentas and 3 normal placentas. Of the 184 human miRNAs detected by the microarray, 11 and 16 miRNAs were, respectively, found to be down- and upregulated by at least 1.5-fold in sPE placentas, compared with normal placentas ($P<0.05$). These 27 miRNAs were chosen as candidates of differential miRNAs in sPE placentas (Table 2).

Expression Patterns of Differential miRNAs in Preeclampsia Placenta
RT-qPCR was performed to validate the differential expression of the chosen 27 miRNAs in sPE placentas. Placenta specimens were derived from 14 sPE patients and 33 normal pregnant women, and the chorionic and basal plates of each specimen were separately analyzed. According to expression patterns, the miRNAs were classified into 3 groups, as described below.

The first group included 13 miRNAs, the differential expression of which in sPE placentas, as revealed by genechip, was fully validated by RT-qPCR. As shown in Figure 1A to

![Figure 1. Differential expression patterns of microRNAs (miRNAs) in severe preeclamptic placentas (n=14) and gestational week–matched normal pregnant placentas (n=33), as revealed by qPCR. The expression of miRNAs was separately measured in the chorionic plate (A, C, E, G) and in the basal plate (B, D, F, H). The relative expression of each unique miRNA was normalized by the value of the U6 gene. Data are presented as mean±SEM, according to the results of 3 independently repeated experiments. A statistical comparison between the severe preeclampsia (sPE) group and the corresponding control group was performed using multiple comparison. *$q<0.05$. Double vertical axis (A to F) was separately corresponded to the columns at the left and right of the dotted line.](http://hyper.ahajournals.org/content/full/63/6/1278)
1D, 6 miRNAs (miR-195, miR-223, miR-218, miR-18a, miR-379, and miR-411) were significantly downregulated, and 7 miRNAs (miR-210, miR-30a-3p, miR-518b, miR-524, miR-17-3p, miR-151, and miR-193b) were significantly upregulated in sPE placentas, compared with normal pregnant controls. These data were in agreement with genechip results. One interesting observation was that these miRNAs exhibited different patterns between the chorionic plate and basal plate of the placenta. Specifically, miR-223, miR-218, and miR-411 were downregulated in both chorionic and basal plates of sPE placentas, whereas downregulation of miR-195, miR-18a, and miR-379 only occurred in chorionic plates, but not in basal plates, of sPE placentas. Upregulated expression was found in basal plates of sPE placentas for miR-210, miR-30a-3p, miR-518b, miR-524, miR-17-3p, miR-151, and miR-193b.

The second group included 12 miRNAs (miR-181a, miR-638, miR-515-3p, miR-525-3p, miR-19a, miR-542-3p, miR-584, miR-362, miR-31, miR-363, miR-590, and miR-296). Inconsistent with genechip results, their expression in sPE placentas did not evidently differ from those in normal controls, as revealed by qPCR. In addition, their patterns were not different between the chorionic and basal plates of placentas (Figure 1E and 1F).

For the other 2 miRNAs (miR-519e-5p and miR-214), qPCR results were in opposite to those of the genechip (Figure 1G and 1H).

Expression Patterns of miR-17-92 Cluster Members in sPE Placentas

The miR-17-92 cluster encodes 6 unique mature miRNA sequences: miR-17, miR-18a, miR-19a, miR-19b1, miR-20a, and miR-92a1. According to the results of miRNA microarray, 5 of 6 members in the miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-19b, and miR-20a) were downregulated in sPE placentas. We, therefore, expanded the validation study to include all of the 6 members in the placentas. As shown in Figure 2A and 2B, miR-17, miR-18a, miR-19b1, and miR-92a1 were significantly downregulated in chorionic plates, but not in basal plates, of sPE placentas, compared with the corresponding controls. The expression of miR-19a and miR-20a exhibited no significant differences between sPE and control, in either chorionic or basal plate.
Differences in miR-17-92 Cluster Members in the Plasma Between sPE Patients and Normal Controls

We further measured the levels of miR-17, miR-18a, miR-19b1, miR-92a1, and miR-210 in plasma derived from sPE patients and normal pregnant women at weeks 15 to 18 and weeks 35 to 39. In agreement with our expectations, TaqMan qPCR data demonstrated that the circulating levels of miR-18a, miR-19b1, and miR-92a1 were significantly lower, and that of miR-210 was clearly higher in sPE plasma at both gestational stages, when compared with the corresponding normal controls (Figure 2C and 2D). Plasma miR-17 did not vary between sPE and control groups at the 2 gestational stages.

Localization of miR-18a, miR-92a1, and miR-210 in Human Normal Placentas

The localization of miR-18a, miR-19b1, miR-92a1, and miR-210 in human placentas was examined using specific in situ hybridization. The probe for miR-19b1 did not work, and the results for the other 3 miRNAs are shown in Figure 3. The localization patterns for miR-18a, miR-92a1, and miR-210 were similar in normal placental tissues derived from the first trimester (weeks 7-8) and from full term. These small RNAs were predominately produced by various subtypes of trophoblasts, including villous cytotrophoblast cells, syncytiotrophoblasts, column cytotrophoblast cells (Figure 3A, 3D, and 3G), and interstitial trophoblast cells invading the decidual stroma (Figure 3C, 3F, and 3I) that were identified by CK-7 immunostaining in adjacent sections (Figure 3L). In term placentas, staining for these miRNAs was also found in villous core capillary endothelial cells (Figure 3B, 3E, and 3H). No positive signals were found in decidual stromal cells (Figure 3C, 3F, and 3I).

Validation of Smad2 as the Direct Target of miR-18a in Human Trophoblast Cells

Target gene prediction using TargetScan software (http://www.targetscan.org/) indicated Smad2 as one of the targets of miR-18a. We performed target validation study in human trophoblastic cell line HTR8/SVneo. As shown in Figure 4A to 4C, the transfection of miR-18a mimics in cells resulted in the downregulation of Smad2 expression at both mRNA and protein levels to 73% and 60% of scramble controls, respectively. According to bioinformatics analysis, the seed sequence of miR-18a was complementary to the 136 to 142 nt of 3' UTR in Smad2 mRNA. The luciferase reporter construct (WT-UTR) carrying a 300-bp DNA fragment of 3' UTR in human Smad2 mRNA or the point-mutated report construct (MUT-UTR) was transfected into HTR8/SVneo cells together with miR-18a mimics and renilla luciferase vector (pRL-TK). Forty-eight hours after transfection, miR-18a mimics could evidently reduce the relative luciferase activity of WT-UTR construct by ≈56% compared with scramble control, but could not affect the relative luciferase activity of MUT-UTR construct (Figures 4D–4F). The data strongly proved Smad2 as the target gene of miR-18a in human trophoblast cells.

miR-18a Could Regulate Trophoblast Cell Invasion via Targeting Smad2

To demonstrate whether miR-18a can influence trophoblast cell behaviors via targeting Smad2, we performed transwell
insert invasion assay in HTR8/SVneo cells. As shown in Figure 5 and Figure S1A and S1B in the online-only Data Supplement, the overexpression of miR-18a in HTR8/SVneo cells significantly promoted cell invasiveness to ≈1.8-fold of control cells. The overexpression of Smad2-expressing construct (pcDNA4-Smad2) could reduce basal cell invasiveness by 30% and completely block the invasion-promoting effect caused by miR-18a in HTR8/SVneo cells (Figure 5). These data demonstrated that Smad2 was directly involved in invasion regulation by miR-18a in human trophoblast cells.
Inverse Correlation Between the Expression Levels of miR-18a and Smad2 Protein in Severe Preeclamptic Placentas

We then examined the expression pattern of Smad2 in sPE placentas. Data of qPCR revealed that the mRNA expression of Smad2 had no obvious difference in sPE and normal placentas, in either chorionic or basal plates (Figure 6A and 6B). However, Western blotting results demonstrated that Smad2 protein levels in the chorionic plate of sPE placentas were ≈1.6-fold of the corresponding normal control, whereas that in the basal plates did not differ (Figure 6C and 6D). The differential pattern of Smad2 in sPE placenta exhibited inverse correlation to that of miR-18a expression as shown in Figure 2A and 2B.

Discussion

It has been well recognized that any single molecule or signaling pathway can hardly be responsible for the occurrence of the complex syndrome, preeclampsia. miRNAs have been indicated to be fine-tuned factors in maintaining homeostasis in many organs, including the placenta, by targeting a large number of genes and participating in various cellular events. The identification of dysregulated miRNAs in preeclamptic placentas and the clarification of gene networks that are regulated by those miRNAs are, therefore, likely to be novel steps in understanding the pathogenesis of such compromised pregnancies.

In the present study, we started from examining the differences in miRNA expression between preeclampsia and control placentas. Thirteen differential miRNAs were identified in sPE placenta. Some of our data are consistent with other reports, such as the downregulation of miR-18a, miR-195, miR-223, and miR-411,8,11 and the upregulation of miR-30a-3p, miR-193b, miR-210, and miR-518b in preeclamptic placentas.7–12,21,22 Meanwhile, for the first time to our knowledge, several miRNAs (miR-17, miR-17-3p, miR-19b1, miR-92a1, miR-379, miR-524, miR-151, miR-218) were found to be aberrantly expressed in sPE placentas.

To date, several studies have shown aberrant miRNA profiles in preeclamptic placentas, using various platforms and techniques.7–14,21,23 Among these studies, there have been some conflicting results. We proposed that the position from which the samples were taken from the placenta is a crucial issue to consider. This notion has been well accepted in terms of mRNA expression, but has not been demonstrated in miRNA respect. In this study, several miRNAs exhibited distinct expression in either chorionic or basal plates of sPE placentas. These results indicate that different sampling methods could result in varying, or even opposite, results in miRNA expression analyses. This has been further supported by different expression patterns of several known differential miRNAs7–11,13,14,23 in different parts of sPE placenta, which may partly explain the controversial reports regarding their differences between preeclamptic placentas and

![Figure 6](http://hyper.ahajournals.org/)

Figure 6. Differential expression patterns of Smad2 in placentas derived from severe preeclampsia (sPE) patients. Quantitative RT-PCR (A and B) and Western blotting (C and D) were performed to measure the expression of Smad2 in chorionic (A and C) and basal plates (B and D) of placentas derived from sPE patients (n=14) and from normal pregnant women (n=33). Data are presented as mean±SEM; ^*P<0.05.
normal controls (Figure S2). Whereas, the spatially specific expression profiles of miRNAs in human placenta may help to speculate about the pathophysiological roles of these small RNAs in the occurrence of preeclampsia.

One important question to be addressed is whether these differential placental miRNAs contribute to the pathological change of preeclampsia or they are just the consequence of the disorder at late gestation. For the pregnancy-associated disease of preeclampsia, there are challenges in addressing the discrepancy between the key pathophysiological changes that are initiated well before 20 weeks gestation and the clinical symptoms that are not manifest until after 20 weeks gestation.24 Circulating miRNAs in pregnant women are suggested to be mainly coming from the placenta.25,26 The prospective plasma samples at early gestation are, therefore, valuable species for exploring potentially pathological factors of the disorder. Our observations on the circulating levels of several miRNAs in sPE patients at 15th to 19th weeks of gestation strongly indicated their possibly aberrant expression in sPE placenta at the first half gestation and their participation in the early pathological events of sPE.

The next step is to clarify the contribution of differential miRNAs to the defects in early placental development. It has been proposed that members in the same miRNA cluster might have similar functions.27 In the present study, we chose miR-17-92 cluster to perform the functional study in human trophoblast cells. The miR-17-92 cluster has been reported to be frequently amplified in malignancies and solid tumors,28 and its oncogenic activity has been well demonstrated.29 Considering the many similarities in proliferative and invasive properties between placental trophoblast cells and cancer cells, and our finding that several members in this cluster are largely located in various subtypes of trophoblast cells, we propose that these small RNAs may participate in the regulation of some principal trophoblast cell events. The downregulation of 4 members in this cluster, that is, miR-17, miR-18a, miR-19b1 and miR-92a1, in severe preeclamptic placenta further suggests their involvement in modulating trophoblast cell activities. Our results revealed that miR-18a could modulate trophoblast cell invasion via repressing Smad2 expression. Bioinformatic analysis also reveals that miR-17 and miR-19b1 can potentially modulate TGF-β signaling, with Smad6/Smad7 and Smad4/Smad5 as the predicted targets, respectively. These targets are central mediators of TGF-β signaling, which plays an essential role in regulating many trophoblast cell activities.30–32 It is most likely that the miR-17-92 cluster may regulate placenta development via, at least in part, finetuning TGF-β signaling. A pathway enrichment analysis for the predictive targets of miR-17-92 cluster using the DAVID Gene Functional Classification Tool (version 6.7; http://david.abcc.ncifcrf.gov/home.jsp)33,34 also reveals the potential involvement of this cluster in modulating several signaling pathways being essential to placental development, such as gonadotropin-releasing hormone signaling, p53 pathway, Wnt signaling, extracellular matrix–receptor interaction, etc (Figure S3). Further in-depth functional study is necessary to eventually discover the working mechanisms of these small molecules that account for the occurrence of preeclampsia.

Another interesting finding in this study is the differences in plasma miRNAs between preeclampsia patients and normal controls. Plasma miRNAs have been reported to be not affected by incubation temperature or pH or even by RNase A treatment.15,36 The stable properties, as well as the early differential patterns of plasma miRNAs in preeclampsia patients, as shown in our study and other reports,23,37 strongly suggest the significant potential of using these molecules in the noninvasive prenatal diagnosis of preeclampsia. However, their low abundance in circulation26 and the specificity and sensitivity in measuring circulating miRNAs hamper the identification of plasma miRNA biomarkers for diseases of pregnancy.

Perspectives
Further in vitro and in vivo studies using specific miRNA-deficient cells/animals are warranted to comprehensively understand the roles of the aberrantly expressed miRNAs in the onset or progression of preeclampsia. Meanwhile, it would be of great clinical interest to further test these small RNAs as potential biomarkers for sPE in a larger and prospective cohort.

Acknowledgments
We thank Dr C.H. Graham at Queen’s University, Canada, for the kind gift of HTR8/SVneo cells, and Dr Qinghua Shi at University of Science and Technology of China for his technical support in in situ hybridization experiment.

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Disclosures
None.

References


**Novelty and Significance**

**What Is New?**

- The unique expression patterns of 16 microRNAs (miRNAs) in the choriocarcinoma and basal plates of severe preeclamptic placentas are demonstrated, strongly supporting the notion that the sampling position in the placenta should be considered when identifying differential miRNAs with regard to this disorder.

- The differences of miR-18a, miR-19b1, miR-92a1, and miR-210 in severe preeclamptic placenta at early to mid-gestation indicated their participation in the early pathological events of severe preeclampsia (pEPE).

- miR-18a could promote trophoblast cell invasion via targeting and suppressing Smad2 expression.

**What Is Relevant?**

- Preeclampsia is a serious hypertensive disorder in pregnancy, which is a major cause of maternal and fetal mortality and morbidity worldwide.

Identifying the miRNAs that are specifically dysregulated in preeclamptic placentas and maternal plasma has been demonstrated to be a useful manner for exploring the biomarkers and cause of the disease. Moreover, identifying the targets of miRNAs and demonstrating the relevance of miRNAs and their functional targets in preeclamptic placenta would be of great value in elucidating the molecular mechanisms that are likely involved in the pathogenesis of preeclampsia.

**Summary**

The findings illustrate the detailed differential miRNA profile in preeclamptic placenta and provide fundamental evidence for better understanding the roles of noncoding miRNAs in the processes leading to impaired placenta activities in preeclampsia.
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Variations of MicroRNAs in Human Placentas and Plasma from Preeclamptic Pregnancy

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Expanded Materials and Methods

Study subjects

In this study, all the collection of human placenta tissues and plasma specimens were performed with the permission of the Local Ethical Committee in the Institute of Zoology, Chinese Academy of Sciences, and the informed consents were obtained from all patients enrolled in this study.

Tissues of human chorionic villi at gestational weeks 7–8 were obtained at Beijing Haidian Hospital (Beijing, China) from patients who underwent therapeutic termination of pregnancy. All the patients accepted no special medical treatment before termination of pregnancy. The gestational week of specimens was determined according to morphological observation of the villi and pathological examination, with the record of menstrual cycles as a reference.

Placentas and maternal blood samples from normal pregnancies and severe preeclamptic women were obtained from a pregnant cohort that included 2500 pregnant women who underwent perinatal care in Peking University Third Hospital from August 2010 to October 2012. For each of the pregnant women in this cohort, 5 ml peripheral blood samples were collected in heparin tubes at the 15-19th and 35-39th gestational weeks. Within 1h after collection, the blood samples were centrifuged at 1,500 x g for 15 min at 4°C, and the supernatants were further centrifuged at 12,000 x g for 15 min at 4°C. The cell-free plasma samples were aliquoted in DNase/RNase-free tubes and stored at -80°C. The placentas of the pregnant women were collected within 1h of caesarean birth, and specimens at the chorionic plate, basal plate and random site were separately taken from the placenta disc near the position of umbilical cord insertion. The specimens were snap-frozen in liquid nitrogen and subjected to RNA extraction. Meanwhile, a full thickness specimen encompassing the chorionic and basal plates was subjected to embedding in tissue-Tek O.C.T compound (Sakura Finetek, Torrance, CA) and frozen sectioning.

Totally 20 severe preeclamptic patients delivered at the 35-39th weeks and 33 normal pregnant women delivered at the 37-39th weeks were randomly selected from the cohort and enrolled in this study. Among these women, the placentas from 14 sPE patients and 33 normal pregnant ones were used for the examination of miRNA expression in the placentas, and the plasma samples derived from 20 sPE patients and 20 normal pregnant ones at gestational weeks 15-18 and weeks 35-38 were used for the measurement of plasma miRNAs. The clinical characteristics of the patients were summarized in Table 1.

Severe PE was diagnosed according to the definition in Williams Obstetrics (23rd edition)\(^1\). In brief, the patients had no history of preexisting or chronic hypertension, but they showed systolic blood pressure of >160 mm Hg or diastolic blood pressure of >110 mm Hg on at least 2 occasions, accompanied by significant proteinuria (>2 g per 24 h or ≥3+ by dipstick in 2 random samples collected at >4 h intervals) after 20 weeks of gestation. Normal or uncomplicated pregnancy was defined as gestation in
a previously normotensive woman who did not suffer from any complications during pregnancy and who delivered a healthy neonate with a weight adequate for a gestational age of more than 37 weeks of pregnancy\(^2\). Women who developed renal disease, transient hypertension during pregnancy, multiple pregnancy, gestational diabetes, spontaneous abortion, intrauterine fetal death, fetal chromosomal or congenital abnormalities or pregnancies conceived by fertility treatment were all excluded from this study.

**MicroRNA microarray analysis**

Random parts of placental tissues derived from 3 late onset sPE and 3 normal pregnancies were subjected to miRNA microarray analysis. In brief, total RNA was extracted using Trizol reagent (Life Technologies, CA, USA), according to the manufacturer's instructions, and miRNAs were isolated from the total RNA using the PEG method\(^3\). The RNA integrity was evaluated using the absorbance at 260nm to 280nm with Nanodrop 2000 (Thermo Fisher Scientific, MA, USA), and further confirmed by the agarose gel electrophoresis assay. High quality RNA samples with A260/A280 ratio>2.0 and clear 18S and 28S tRNA bands in agarose gel electrophoresis were used for microarray assay.

The miRNAs from sPE and control placentas were separately labeled with Cy3 and Cy5, and were subjected to hybridization using a mammalian microRNA chip array (version 2.0, Capitalbio, Beijing, China), which was stemming from the miRbase release 8.2 (ftp://mirbase.org/pub/mirbase/8.2/)\(^4\) (Wellcome Trust Genome Campus, Cambridge, UK). The chip was scanned with a LuxScan 10K/A laser scanner (Capitalbio, Beijing, China), and raw data were extracted using LuxScan image analysis software (Capitalbio, Beijing, China), version 3.0. After the low-intensity spots were removed according to the cut-off value of the median background plus two times its standard deviation, the data were analyzed by using Significance Analysis of Microarrays software ([http://www-stat.stanford.edu/_tibs.SAM](http://www-stat.stanford.edu/_tibs.SAM))\(^5\), version 2.1, and the miRNAs with 1.5-fold changes between the PE and control groups (\(p<0.05\)) were identified as candidate differential genes.

**Reverse transcription-real-time quantitative polymerase chain reaction (RT-qPCR)**

Two micrograms of total RNA were reverse-transcribed into cDNA using ologo(dT) primer (TIANGEN, Beijing, China) and Moloney murine leukemia virus reverse transcriptase (Promega, WI, USA). Reverse transcription of miRNA was performed with the use of the miRcute miRNA first-strand cDNA synthesis kit (TIANGEN, Beijing, China).

Quantitative real-time PCR was performed using Applied Biosystems 7500 (Life Technologies, CA, USA). For the detection of cDNA, the experiment was carried out following the instructions of the SYBR® Premix Ex Taq™ kit (Takara, Dalian, China), the reaction for each sample was carried out in duplicate at 95°C for 30s, followed by 40 cycles of 95°C for 5s, 60°C for 31s. While for the quantitation of miRNA cDNA, the reaction was performed according to the instructions of the miRcute miRNA qPCR
detection kit (TIANGEN, Beijing, China). The reaction for each sample was carried out in duplicate at 94°C for 2 min, followed by 40 cycles of 94°C for 20 s, 60°C for 30 s and 72°C for 30 s. Human GAPDH and U6 RNA was amplified in parallel as an internal control. The fold changes in mRNA and miRNA expression between the PE and normal pregnant groups were calculated using the 2^ΔΔCq method, where ΔCq indicated the subtraction of the Cq of GAPDH or U6 from the mRNA or miRNA of interest, and ΔΔCq was calculated by subtracting the ΔCq of the normal pregnant group from that of the PE group.

The sequences of primers of GAPDH and Smad2 were listed in Table S1. The primers of miR-1, miR-16, miR-17-3p, miR-34c-5p, miR-155, miR-223 and miR-377 were purchased from Qiagen (Qiagen, Hilden, Germany). The sequences of other miRNAs’ primers are listed in Table S2.

Because the SYBR Green-based technique used to measure placenta miRNA levels might generate false positive results due to primer dimer formation or some nonspecific PCR products for the specificity of primer design and/or the quality of RNA or miRNA samples, several experiments was performed to prove that these cases were unlikely to appear in our experiments. The results are shown in Figure S4B-H.

Plasma total RNA was extracted with Trizol LS reagent (Life Technologies, CA, USA). Total RNA (10 ng) was reverse transcribed into microRNA cDNA for quantitative real-time PCR for miR-210, miR-17, miR-18a, miR-19b, miR-92a1 and U6, using TaqMan® Gene Expression Assays (Life Technologies, CA, USA) with the 7500 Fast Real-Time PCR system (Life Technologies, CA, USA). The miRNA level was normalized to the U6 transcript level in the same sample.

In situ hybridization for miRNAs

Frozen sections of 7 µm in thickness were treated with protease K and were fixed in 4% paraformaldehyde (PFA) for 15 min, followed by hybridization with the miRCURY LNA microRNA Detection probes (Exiqon, Copenhagen, Denmark) specifically against miR-18a, miR-92a1 and miR-210, at 55°C overnight. The sections were serially washed in 5x, 2x, and 0.2x SSC buffer and then were incubated with AP-conjugated anti-DIG antibody (Roche, IN, USA) at 37°C for 2 h. The stained signals were visualized using BCIP/NBT (Promega, WI, USA) as a substrate. The scramble miRNA (NC) probe was used as a negative control. The probe sequences were as follows (5’ to 3’): miR-18a, CTATCTGCACTAGATGCACCTTA; miR-92a1, ACAGGCCGGGACAAATGCAATA; miR-210, TCAGCCGCTGTCACACGCACAG; and NC, GTGTAACACGTCTATACGCCCA.

Immunohistochemistry

Frozen sections of 7 µm in thickness were subjected to routine rehydration and antigen retrieval before being incubated with antibodies against human CK-7 (Cell Signaling Technologies, MA, USA). The sections were further incubated with secondary antibody conjugated with horseradish peroxidase (Zhongshan Goldenbridge, Beijing, China) and were visualized with diaminobenzidine
(Zhongshan Goldenbridge, Beijing, China) as a substrate.

**Cell culture and transient transfection**

Htr8/SVneo cell line was kindly provided by Dr. CH Graham at Queen’s University, Canada. The cells were maintained in RPMI 1640 culture medium (Life technologies, CA, USA) supplemented with 10% fetal bovine serum and were passaged at a ratio of 1:5 every 5 days.

After cells were grown to 60-70% confluence, transient transfection of siRNA or miRNA duplexes was carried out using Lipofectamine 2000 (Life technologies, CA, USA) following the manufacturer’s protocol. The sequences of siRNA and miRNA are shown in Table S3.

**Western blotting**

The proteins from the cells transfected for 48h or the homogenized tissues were extracted with RIPA buffer containing 150mM NaCl, 10mM Tris (pH7.6), 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 1mM NaF, Na 3VO 4 supplemented with protease inhibitor cocktail (Sigma Aldrich, MO, USA). The supernatants were harvested after centrifuging, and the protein concentration were measured by a BCA protein assay (Boster Biological Technology, Wuhan, China). Forty microgram protein was subjected to 10%SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare, CT, USA). The membrane was blocked for 1h with 5% nonfat milk in PBST (PBS and 0.1%Tween 20) and incubated at 4°C overnight with Smad2 (Cell Signaling technology, MA, USA) or GAPDH (Ambion, TX, USA) antibody. The membrane was washed with PBST for 3 times, incubated further with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, PA, USA) at room temperature for 2h, and washed again 3 times with PBST. Final visualization was achieved by ECL kit (Thermo Scientific, MA, USA), and the signals were exposed to X-ray films (Kodak, NY, USA) and analyzed by the Quantity One® 1-D Analysis Software, Version 4.4 (Bio-Rad, CA, USA). The relative densities of Smad2 were determined by normalization with the density value of GAPDH in the same blot.

**Luciferase assays**

According to the bioinformatics analysis, the seed sequence of miR-18a was complementary to the 136-142 nt of 3’UTR in Smad2 mRNA. We generated a luciferase reporter construct (WT-UTR) by cloning a 300bp DNA fragment of the 3’UTR in human Smad2 mRNA (50-349nt) at downstream of the luciferase reporter gene. A point-mutation was incorporated into the 139nt of the 3’UTR in the WT-UTR plasmid to generate MUT-UTR construct. The sequences of primers for vectors construction are listed in Table S1.

For the luciferase assay, Htr8 cells (40000 per well) were seeded into 24-well plates 12h before transfection. The cells were transfected with 80ng of WT-UTR or MUT-UTR, 8ng of Renilla luciferase reporter vectors (Promega, WI, USA) and 40nM chemically synthesized mature hsa-miR-18a or scramble miRNAs with Lipofectamine
Luciferase activity was assayed 48h after transfection with a Dual-luciferase reporter assay system (Promega, WI, USA) and was measured with a luminometer (Promega, WI, USA). All transfections were carried out with triplicate in at least three independent experiments.

**Expressing plasmids construction**

For the construction of the Smad2 expressing plasmid (pcDNA4-Smad2), the coding sequence of Smad2 was amplified and inserted into pcDNA4.0 vector at the EcoRI and XhoI restriction sites. The sequences of primers for Smad2 expressing plasmid construction are also listed in Table S1.

**Transwell invasion assay**

Transwell insert invasion assay was conducted in 24-well fitted inserts (8µm pore size, Millipore Corp, Massachusetts, USA). Briefly, 48 hours after transfection, cells were treated with 10mg/ml mitomycin C for another 2 hours. Then they were trypsinized and seeded in the transwell inserts pre-coated with 20µg matrigel (BD Biosciences, NJ, USA) with 200µl serum-free culture medium. Lower chambers were loaded with the same medium supplemented with 10% fetal bovine medium. After incubating for 28 h, cells on the upper surface of membranes were completely removed, and the cells migrated to the lower surface of membranes were fixed with 0.25% glutaraldehyde and stained with hematoxylin. The number of invaded cells was counted in the whole field of the membranes under light microscope. Cell invasion index was presented as the percentage of invaded cell number compared with the corresponding control.

**Statistical analysis**

All statistical analyses were performed with Statistical Package for the Social Sciences software (Version 17.0; SPSS Inc., Chicago, IL, USA). Multiple comparisons were carried out using R software (Version 3.0.2; [http://www.r-project.org/](http://www.r-project.org/)) as previously reported, and q-values were calculated after the simple t-test. The differences are considered significant when q<0.05. Statistical comparisons between two groups were estimated using the independent samples t-test, and p<0.05 was considered statistically significant.
Supplementary References


Supplementary Tables

Table S1. Sequence of the primers used for plasmid construction or real-time PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad2 real-time PCR</td>
<td>F: CATGCATCACAGCCCTCACT</td>
</tr>
<tr>
<td></td>
<td>R: CATTITCTACCGTGGAATTTTCT</td>
</tr>
<tr>
<td>GAPDH real-time PCR</td>
<td>F: AAGGTCATCCCTGAGCTGAAC</td>
</tr>
<tr>
<td></td>
<td>R: ACGCCTGCTTCACCACCTT</td>
</tr>
<tr>
<td>WT-UTR</td>
<td>F: GGACTAGTTCTAGCATGGTGTTGGTGCTCC</td>
</tr>
<tr>
<td></td>
<td>R: CCCAAGCTTTAAACAGACAATACACTTGTA</td>
</tr>
<tr>
<td>MUT-UTR</td>
<td>F: GGTCTCATCAATTAAAGCAGCTTGAATGTTCTCT</td>
</tr>
<tr>
<td></td>
<td>R: AGGAAACAGATTCCACAAGCTGCTTTAATTGATGAGACC</td>
</tr>
<tr>
<td>Smad2 expressing plasmid</td>
<td>F: GGAATTCAGAGGCTTTTTCATAGCCT</td>
</tr>
<tr>
<td></td>
<td>R: CCGCTCGAGCATTAAAGTCTTTTTCATGGGAC</td>
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</table>

Abbreviations: F, forward; R, Reverse.
Table S2 Primers for real-time PCR of miRNAs

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR primers (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6</td>
<td>F: CGCAAGGATGACACGCAAATTC</td>
</tr>
<tr>
<td>miR-18a</td>
<td>F: TAAGGTCATCTAGTGCAAGATAG</td>
</tr>
<tr>
<td>miR-19a</td>
<td>F: TGTGCAAAATCTATGCAAAACTGA</td>
</tr>
<tr>
<td>miR-30a-3p</td>
<td>F: CGCCTTCCAGTGATGGTTTTG</td>
</tr>
<tr>
<td>miR-31</td>
<td>F: AAGCAAGATGCTGGCATAGCT</td>
</tr>
<tr>
<td>miR-151</td>
<td>F: CAGTCGAGTCAGATCTACAGTCTAGTA</td>
</tr>
<tr>
<td>miR-181a</td>
<td>F: AACCTTTAAGCGGCGTTAGT</td>
</tr>
<tr>
<td>miR-193b</td>
<td>F: GGGGATCGCGTGCTAGGAGTT</td>
</tr>
<tr>
<td>miR-195</td>
<td>F: TAGCAGCAGAATATATGAGC</td>
</tr>
<tr>
<td>miR-210</td>
<td>F: CCGTGGAGAGGCTGAGTT</td>
</tr>
<tr>
<td>miR-214</td>
<td>F: TGGCTGTCTACAGTCTAGA</td>
</tr>
<tr>
<td>miR-218</td>
<td>F: TTGCTGCTGGATCTAACCAGT</td>
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<tr>
<td>miR-296</td>
<td>F: GGGTCCACCTCAATCTCGTAA</td>
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<tr>
<td>miR-362</td>
<td>F: TCTTTGGAACCTAGGGTGAGT</td>
</tr>
<tr>
<td>miR-363</td>
<td>F: CGGGTGGGATCATGAGTT</td>
</tr>
<tr>
<td>miR-379</td>
<td>F: GGGCGACTGAGTCAGGAGT</td>
</tr>
<tr>
<td>miR-411</td>
<td>F: GGTAGAGCGAAGTCAGGAGT</td>
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<tr>
<td>miR-515-3p</td>
<td>F: GAGTGCCTTTTCTGAGG</td>
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<tr>
<td>miR-518b</td>
<td>F: AAAGCGCTCCCTTTTGGAGT</td>
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<tr>
<td>miR-519e-5p</td>
<td>F: TGGTCTAGAAGGGAGCAGT</td>
</tr>
<tr>
<td>miR-524</td>
<td>F: CTACAAGGAAACGAGTCTTTCAG</td>
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<tr>
<td>miR-525-3p</td>
<td>F: CGTCAGGCCTTCTCTTTAGA</td>
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<tr>
<td>miR-542-3p</td>
<td>F: CGTGCTGTGAAGATGAAATCTGAAA</td>
</tr>
<tr>
<td>miR-584</td>
<td>F: TATGGTGTGTGCTGGAGT</td>
</tr>
<tr>
<td>miR-590</td>
<td>F: ACCAGGCTTTACATAAAAGTGAGA</td>
</tr>
<tr>
<td>miR-638</td>
<td>F: AGGGATCGCCGGCCGTO</td>
</tr>
</tbody>
</table>

Abbreviations: F, forward.
Table S3 The sequences of siRNA and miRNA

<table>
<thead>
<tr>
<th>Mimics</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scramble control</td>
<td>UUCUCCGAACGUGUCACGUTT</td>
<td>ACGUGACACGUUCGGAGAATT</td>
</tr>
<tr>
<td>si-Smad2</td>
<td>GGUUUACUCUCCAAUGUUATT</td>
<td>UAACAUUGGAGAGUAAACCTA</td>
</tr>
<tr>
<td>hsa-miR-18a</td>
<td>UAAGGUGCAUCUAGUCAGAUAG</td>
<td>AUCUGCACUAUGCACCUUAUU</td>
</tr>
</tbody>
</table>
To validate the accuracy of pcDNA4-Smad2 plasmid, the expression of Smad2 in HTR8/SVneo cells transfected with pcDNA4-Smad2 or pcDNA4 plasmid were measured by real-time PCR and western blotting experiments.
Figure S2. Measurement of other reported differential miRNAs in sPE placenta, as revealed by qPCR.

The expressions of 5 miRNAs (miR-16, miR-155, miR-377, miR-1 and miR-34c-5p), which had been controversially reported regarding their differences between PE and normal placentas and (or) their contribution to the onset of preeclampsia, were measured in severe preeclamptic placentas (n=14) and gestational-week-matched normal pregnant placentas (n=33). The expressions were separately measured in the chorionic plate (A) and in the basal plate (B). The relative expression of each unique miRNA was normalized according to the value of the U6 gene. The data are presented as the Mean ± SEMs, according to the results of 3 independently repeated experiments. A statistical comparison between the sPE group and the corresponding control group was performed using multiple comparison, and * indicates q<0.05. Note in Supplementary Figures S2A-B, double vertical axis was separately corresponded to the columns at the left and right of the dotted line.

As shown, miR-1 and miR-34c-5p were evidently downregulated in the chorionic plate, but not in the basal plate. The differences of miR-155 and miR-377 between sPE and normal placentas in the chorionic plate were opposite to that in the basal plate. The expression of miR-16 exhibited no difference in the sPE and normal placentas.
Figure S3
Pathway enrichment analysis showing the associations of miR-17, miR-18a, miR-19b1 and miR-92a1 with various signaling pathways and physiological processes.
Figure S4

Technical considerations for the measurement of microRNAs by quantitative PCR. A. Typical results of RNA electrophoresis showing clear bands for RNA samples extracted from human placenta. B. Agarose gel electrophoresis showing specificity of PCR for certain microRNAs (i.e, U6, miR-1, miR-16, miR-34c-5p, miR-155 and miR-377). Negative control was set using RNA templates without reverse transcription. MW: 20 base pair molecular weight markers (TakaRa, Dalian, China). C-H, Melting curves with single peaks showing amplification specificity for specific microRNA. Negative control was set using RNA templates without reverse transcription.