BASIC SCIENCE: OBSTETRICS

Distinct subsets of microRNAs are expressed differentially in the human placentas of patients with preeclampsia

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OBJECTIVE: Preeclampsia and small-for-gestational age (SGA) neonates have partially overlapping clinicopathologic features. MicroRNAs (miRNAs) are critical posttranscriptional regulators of gene expression. This study was performed to determine whether preeclampsia and SGA are associated with alterations in placental miRNA expression.

STUDY DESIGN: Placentas were obtained from patients with (1) preeclampsia (n = 9); (2) SGA (n = 9); (3) preeclampsia + SGA (n = 9); and (4) a control group with spontaneous preterm labor and delivery (PTL; n = 9). The expression of 157 miRNAs was assessed by real-time quantitative reverse transcription-polymerase chain reaction.

RESULTS: Differential expression between preeclampsia and the control group (miR-210, miR-182) and between preeclampsia + SGA and the control group (miR-210, miR-182*, and others) was found. Gene Ontology analysis of the target genes revealed enrichment for specific biological process categories (antiapoptosis: miR-182; regulation of transcription: miR-210).

CONCLUSION: This study reports, for the first time, increased expression of specific placental miRNAs in preeclampsia with and without SGA. The findings also provide novel targets for further investigation of the pathophysiology of preeclampsia.

Key words: microRNA, placenta, preeclampsia, small-for-gestational age

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MicroRNAs (miRNAs), members of a class of small, noncoding RNA molecules, have a critical role in posttranscriptional regulation of protein-coding genes. Originally identified in *Caenorhabditis elegans*, miRNAs have been shown to operate in a wide range of species, including humans.[1] Computational estimates predict that up to 30% of human genes are potential targets of miRNA and that miRNAs compose 1%-5% of animal genomes.[2] A primary miRNA, transcribed from DNA, is processed to a looped, imperfectly base-paired approximately 70-mer oligonucleotide termed the miRNA precursor. Either arm of the miRNA precursor can be processed enzymatically to the mature, approximately 22-mer miRNA. Most precursors lead to exclusive maturation of 1 arm of the double-stranded segment of the precursor, but some end in the maturation of both arms. If 1 arm clearly produces the predominant miRNA, an asterisk (*) is added to the end of the name of 1 mature miRNA to denote the less abundant product of the opposite arm.[1] The mechanism of translational inhibition by miRNA is dependent on complementarity between a 6-7 nucleotide "seed" region in the miRNA and targets in the 3′ untranslated region of messenger RNA. High complementarity between these regions results in degradation of messenger RNA target sequences by cleavage, whereas mismatches preferentially lead to translational repression.[1] Studies of miRNA expression across several organs have revealed that miRNA expression is tissue-specific and that sequences may possess functions unique to organs or cell types.[3,4] Targeted miRNA depletion in *Drosophila melanogaster* embryos demonstrated that miRNA is essen-

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tial for proper cellularization and cell survival during embryonic development.[5]

Pathologic alterations are shared in placentas of mothers with preeclampsia or small-for-gestational age (SGA) neonates, which suggests that these 2 conditions are related and may have similar pathogenetic mechanisms.[6] Preeclampsia is complicated by SGA in 1 of 5 cases.[7] Morphologic changes associated with placental underperfusion are encountered frequently in preeclampsia and SGA, and the expression of several placental genes is affected also. Failure of physiologic transformation of spiral arteries in the placental bed[8,9] and altered expression of both membrane-bound[10,11] and soluble[12,13] angiogenic and antiangiogenic factors are also well-known examples. However, the fundamental mechanisms that underlie placental pathophysiology and their contribution to clinical manifestations remain to be elucidated.

Many miRNAs are expressed abundantly in the human placenta,[14] but their expression patterns in placentas with distinct pathologies have not been reported. In the present study, we explored the miRNA expression profiles of placentas obtained from patients with preeclampsia and patients with SGA to determine whether these conditions are associated with specific alterations compared with gestational age-matched placentas from control patients with appropriate-for-gestational age neonates after spontaneous preterm labor and delivery (PTL).

MATERIALS AND METHODS

Study design

A cross-sectional, case-control study was conducted to examine the patterns of miRNA expression in placentas obtained from patients in the following groups: (1) preeclampsia (n = 9); (2) SGA (n = 9); (3) preeclampsia + SGA (n = 9); and (4) a control group (PTL; n = 9). All preeclampsia, SGA, and preeclampsia + SGA cases were matched for gestational age at delivery (within 2 weeks) to control cases. PTL was defined by the presence of regular uterine contractions at a frequency of at least 2 contractions every 10 minutes that were associated with cervical changes and resulted in delivery at <37 completed weeks of gestation. Preeclampsia was defined as hypertension (systolic blood pressure, ≥140 mm Hg, or diastolic blood pressure, ≥90 mm Hg, on at least 2 occasions, 4 hours to 1 week apart) and proteinuria (≥300 mg in a 24-hour urine collection or 1 dipstick measurement of ≥2+). Severe preeclampsia was defined as either severe hypertension (highest systolic blood pressure, ≥170 mm Hg, or highest diastolic blood pressure, ≥110 mm Hg) plus mild proteinuria or mild hypertension plus severe proteinuria (a 24-hour urine sample that contained 3.5 g protein or urine specimen ≥3+ protein by dipstick measurement). SGA was defined as a birthweight below the tenth percentile for gestational age.[15] Control patients delivered normal infants with birthweights appropriate for gestational age (10th–90th percentile).[15] Patients with chronic hypertension and superimposed preeclampsia, premature rupture of membranes, multiple gestation, histologic chorioamnionitis, stillbirth, or fetal anomalies were excluded. The use of tissue samples for research purposes was approved by the Institutional Review Boards of both Wayne State University and the National Institute of Child Health and Human Development. All patients who participated in this study provided written informed consent.

RNA isolation

Snap-frozen placenta samples kept at −80°C were used. Small RNAs (<200 nucleotides) were obtained with the mirVana RNA Isolation kit (Ambion, Austin, TX) according to the manufacturer's recommendations. Briefly, homogenized samples were lysed, followed by an acid-phenol:chloroform extraction. Total RNA samples were collected, and small RNAs were purified by precipitation with ethanol. Small RNA species were immobilized on glass-fiber filters, followed by several washes and elution of small RNA with nuclease-free water. In accordance with the mirVana protocol, small RNA integrity was determined by polyacrylamide gel electrophoresis. Small RNA concentration was determined with the RNA 6000 Nano Assay on the 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for miRNA assay

miRNA expression was determined with the TaqMan MicroRNA Assays Human Panel–Early Access Kit (Applied Biosystems, Foster City, CA). The kit uses gene-specific stem-loop reverse transcription primers and TaqMan primer/probe sets to detect mature miRNA transcripts in a 2-step real-time qRT-PCR assay.[16] The 157 miRNAs were placed in 2 96-well plates, each plate containing one-half of the total number of miRNAs and 3 replicates of the endogenous normalization control, 5S ribosomal RNA. Assays for 3 miRNAs known not to be expressed in humans (cel-lin-4, cel-miR-2, and ath-miR-159a) were used as negative controls. The qRT-PCR assay was run in triplicate for each case to allow assessment of technical variability. Equal amounts (3 ng) of small RNA were reverse transcribed with the gene-specific primers from the TaqMan MicroRNA Assay Human Panel Kit with the protocol and reagents from the TaqMan MicroRNA Reverse Transcription Kit (3 μL gene-specific miRNA primer; 50 units MultiScribe Reverse Transcriptase; 1.0 mmol/L deoxyribonucleotide triphosphate; 0.25 units/μL RNase inhibitor; 1X reverse transcription buffer, and nuclease-free water to 15 μL [Applied Biosystems]). Reverse transcription was performed with the following thermal cycling parameters: 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C (Applied Biosystems). Complementary DNA to assay for the endogenous normalization control was prepared as described earlier; 3 μL random hexamers were substituted for gene-specific primers (High Capacity cDNA Archive Kit; Applied Biosystems). Reactions for qRT-PCR consisted of 1 μL RT reaction product, 1 μL TaqMan MicroRNA Assay, 5 μL Universal PCR Master Mix (Applied Biosystems), and

nuclease-free water to a total volume of 10 μL. Reactions were run in triplicate with the following thermal cycling parameters: 95°C for 10 minutes for the activation of AmpliTaq Gold DNA polymerase (Applied Biosystems), 40 cycles of 95°C for 15 seconds (denaturation), and 60°C for 60 seconds (annealing/extension). A custom-designed TaqMan assay for endogenous 5S ribosomal RNA (forward primer: 5′-CGCCCGATCTCGTCTGAT-3′; reverse primer 5′ GGTCTCCCATCCAAGTACTAACCA-3′) was used to normalize miRNA expression (Applied Biosystems).

Statistical analysis

The miRNA expression of patients in the control group was compared separately with that of patients in each of the preeclampsia, SGA, and preeclampsia + SGA groups. Real-time qRT-PCR was used to assess expression of 157 miRNAs. The most common method to analyze qRT-PCR data is the "Delta-delta" method.[17] Traditionally, a change in expression of ≥2-fold is considered sufficient to assert that the change is significant.[18] However, there are major drawbacks to the use of only fold change in inferring differential expression.[19] Control of the false discovery rate was necessary because of the multiple comparisons made in this study.

The method proposed by Fu et al[20] as an alternative to the "Delta-delta" approach allows deriving *P* values by modeling qRT-PCR data with the generalized estimating equations (GEE). This method uses weighted combinations of observations to extract information from correlated measurements. GEE has specific benefits over other approaches, such as repeated measures analysis of variance. The GEE provides a more robust estimate of the regression parameters (in this case the fold change estimate) and yields standard errors,[21] which are reliable and, thus, allow computation of *P* values for inferential purposes.

For each miRNA, a GEE model was fit to estimate the effect of the covariate (obstetric condition) on the observed expression level with the use of the "*gee*" package in the R statistical software.[22] We chose an *exchangeable* correlation structure (every pair of technical replicates has the same correlation). A *P* value was calculated to assess the significance of the association between obstetric condition and differential expression of each miRNA.

In high-dimensional biology, it is acceptable for investigators to tolerate a small fraction of false-positive results (for example, up to 10%).[23] The false discovery rate, defined as the proportion of false positive results among all positive results, was controlled at 10% with the method proposed by Benjamini and Yekutieli.[24] This means that 10% of the miRNAs deemed to be significant may actually be false positive results. For example, if 10 miRNAs have corrected *P* values of <.1, we expect 0.1 × 10 = 1 miRNA to be incorrectly declared significant. Correction for both multiple assays and multiple group comparisons is still an unsettled issue in high-dimensional biology.[19] Because only 3 group comparisons were made, the impact of the latter was minimal, and the *P* values within each comparison were corrected for the 157 different miRNAs.

Gene Ontology analysis of potential miRNA targets

miRBase Targets[25] was used to determine putative messenger RNA targets of differentially expressed miRNAs. The Gene Ontology (GO) project has developed structured vocabularies to categorize genes by cellular component, molecular function, and biological process.[26] To better understand the function of the targets of miRNAs, we used Onto-Express[27-29] to categorize targets by GO biological processes. GO biological process "refers to a biological objective to which the gene or gene product contributes."[26] The number of targets in each GO category was tallied and compared with the number of targets expected to be found in the GO category. The union of target genes predicted by miRBase for all assayed miRNAs was used as a reference. Significant differences from the expected number of genes were calculated with the assumption of a hypergeometric distribution; *P* values were adjusted with

TABLE 1
Demographic characteristics of the study population

Demographic	Preeclampsia (n = 9)	SGA (n = 9)	Preeclampsia + SGA (n = 9)	Control (PTL) (n = 9)
Age (y)*	28 (19-39)	30 (19-44)	23 (19-36)	24 (18-37)
Nulliparity (n)	5 (55.6%)	3 (33.3%)	4 (44.4%)	3 (33.3%)
Smoking (n)	0 (0%)	2 (22.2%)	0 (0%)	1 (11.1%)
Weight (kg)*†	77 (50-125)	79 (43-91)	64 (52-114)	82 (63-93)
Body mass index (kg/m^2)*†	29.6 (19.5-44.6)	29.9 (20.4-32.0)	32.2 (25.3-35.4)	26.4 (22.9-49.9)
Black ethnicity (n)	9 (100%)	9 (100%)	7 (77.8%)	7 (77.8%)
Gestational age at delivery (wk)*	33.1 (25.6-36.0)	33.1 (26.6-36.6)	32.8 (27.4-35.9)	31.7 (26.7-35.9)
Birth weight (g)*	1785 (775-2715)	1530 (460-1975)	1480 (471-1775)	1625 (1010-2810)
Female fetus (n)	2 (22.2%)	5 (55.6%)	5 (55.6%)	4 (44.4%)

* Values expressed as median (range).

† Anthropometric data available for 86% of patients.

TABLE 2
Clinical characteristics of the study population

Characteristic	Preeclampsia (n = 9)	SGA (n = 9)	Preeclampsia + SGA (n = 9)	Control (PTL) (n = 9)
Systolic blood pressure (mm Hg)*†	167 (148-190)	140 (108-176)	176 (149-197)	121 (105-155)
Diastolic blood pressure (mm Hg)*†	105 (84-111)	84 (56-107)	113 (103-132)	68 (60-94)
Severe preeclampsia (n)	5 (55.6%)	—	8 (88.9%)	—
Chronic hypertension (n)	0	4 (44.4%)	0	0

* Values are presented as median (range).

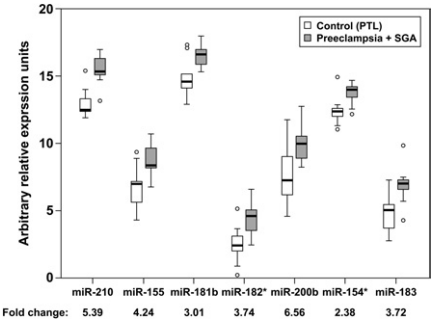
† Highest measured blood pressures.

the false discovery rate correction[24] based on the number of GO categories that were tested.

RESULTS

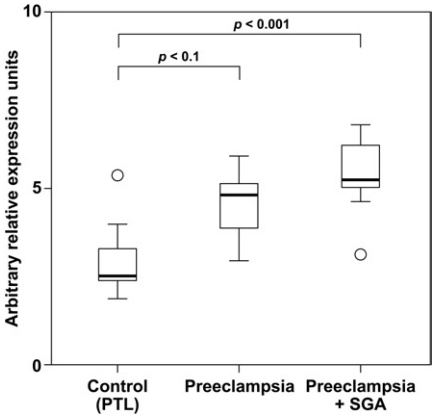
Demographic and clinical characteristics of the study population are summarized in Tables 1 and 2, respectively. Among the 157 miRNAs screened by qRT-PCR, the expression of 153 miRNAs (97%) was detected readily in the placenta. Seven miRNAs (miR-210, miR-155, miR-181b, miR-182*, miR-200b, miR-154*, and miR-183) were differentially expressed between preeclampsia + SGA and the control group (PTL) ($P < .1$ and >2-fold change; Figure 1). The expression of 2 miRNAs (miR-182 and miR-210) was significantly higher in preeclampsia than in the control group ($P < .1$). Increases in the expression of miR-182 and miR-210 in preeclampsia were 2.1-fold and 3.0-fold, respectively.

FIGURE 1
miRNAs differentially expressed between preeclampsia + SGA and the control group (PTL)



Schematic representation of the miRNAs found to be expressed differentially between preeclampsia + SGA and the control group after multiple comparison adjustment for the testing of 157 miRNAs (all $P < .1$ and >2-fold change). The *y-axis* represents units of delta Ct ($Ct_{5S} - Ct_{miRNA}$), with an arbitrary zero point, so that each unit measures a 2-fold change. The fold change between preeclampsia + SGA and the control group is displayed below each miRNA name.

FIGURE 2
miR-210 expression in the control group (PTL), preeclampsia, and preeclampsia + SGA



Schematic representation of miR-210 expression in the control group, preeclampsia, and preeclampsia with SGA. The *y-axis* represents units of delta Ct ($Ct_{5S} - Ct_{miRNA}$), with an arbitrary zero point, so that each unit measures a 2-fold change. The fold changes and *P* values (after multiple comparison adjustment for the testing of 157 miRNAs) are shown for the comparisons between the control group and preeclampsia and between the control group and preeclampsia + SGA. This figure is different from the one published in the Journal describing this work in abstract form. This was due to a directional graphing error.[30]

All differentially expressed miRNAs were more highly expressed in preeclampsia or preeclampsia + SGA than in the control group. miR-210 expression was consistently higher in both comparisons (preeclampsia vs control group and preeclampsia + SGA vs control group; Figure 2). Please note that an abstract describing this work contains a graphing error.[30] No differences in miRNA expression were found between SGA and the control group.

GO analysis performed on the gene target lists of miR-182 and miR-210 found that specific biological process categories were enriched (Table 3). Processes with significantly higher than expected representation in the gene target lists included antiapoptosis for miR-182 ($P < .01$; n = 13 genes) and regulation of transcription for miR-210 ($P < .01$; n = 11 genes). Processes involved in the immune response were enriched in the target lists for both miR-182 and miR-210 (Table 3).

COMMENT

Preeclampsia is characterized by the upregulation of specific miRNAs in the placenta; higher expression of miR-182, miR-182*, and miR-210 segregate preeclampsia from SGA and the control group; and GO analysis revealed that antiapoptosis, immune response, and lipid metabolism were enriched among the target genes of miR-182, miR-182*, and miR-210, respectively.

The increased miRNA expression in preeclampsia suggests the downregulation of potential target genes, which may contribute to the pathology of preeclampsia. The association between pre-

TABLE 3
The results of Gene Ontology analysis of targets of differentially expressed miRNAs

Biologic process category (n/N)	Targets of miR-182	Targets of miR-182*	Targets of miR-210	Corrected *P* value
Immune response	23/239	—	—	<.05
Antiapoptosis	13/87	—	—	<.01
Cation transport	—	10/123	—	<.05
Cellular defense response	—	7/59	—	<.05
Response to pest, pathogen, or parasite	—	4/20	—	<.05
Regulation of lipid metabolism	—	—	2/3	<.01
Regulation of transcription	—	—	11/72	<.01
Innate immune response	—	—	8/53	<.05
Response to stimulus	—	—	6/350	<.05
Complement activation, classic pathway	—	—	5/25	<.05

n/N: Values expressed as number of genes targeted by miRNA of interest/number of genes targeted by all analyzed miRNAs.

eclampsia and altered miRNA expression suggests the possibility of a functional role for miRNA in this disease and, at the same time, contrasts with studies that have shown biochemical similarities between preeclampsia and SGA.[6] Marked increases in the expression of miR-182, miR-182*, and miR-210 seen in isolated preeclampsia and preeclampsia associated with SGA indicate that these miRNAs may be related to the presence of preeclampsia, regardless of the presence of SGA. Of note, the group of patients with SGA without preeclampsia included individuals with chronic hypertension but did not demonstrate any alterations in miRNA expression.

Identification of potential target genes is essential for the further elucidation of the biologic role of individual miRNAs in vivo. Perturbation of angiogenesis has been proposed as 1 of the key features of preeclampsia, and miRNA is strongly implicated in angiogenesis. Interestingly, our search revealed angiogenin and vascular endothelial growth factor-B as potential targets of miR-182 and miR-182*, respectively. Defective angiogenesis that led to embryonic lethality was observed in mice deficient of all miRNAs.[31] The yolk sacs of murine embryos lacking the miRNA processing enzyme "Dicer" had fewer, smaller, and less-organized vessels. The Dicer-null mutants also demonstrated marked increases in pro- and antiangiogenic factors. Among the affected molecules were 3 members of the vascular endothelial growth factor family, which are also regulated aberrantly in human pregnancies complicated by preeclampsia.[10,32] In human umbilical vein endothelial cells, 2 miRNAs of the same family, miR-221 and miR-222, target the receptor c-kit, which diminishes angiogenic activity normally induced by its ligand, stem cell factor.[33] It is noteworthy that miR-182 and miR-182* originate from a common miRNA precursor transcript, thus the expression of both is controlled by a common promoter and transcription factors.

GO analysis revealed certain categories of biological processes that differentially regulated miRNAs can affect potentially in a systematic way. Individual miRNAs are involved in a variety of biologic responses, and certain categories such as antiapoptosis and immune response contain genes that would be relevant targets for the study of preeclampsia. Because miR-182 may downregulate antiapoptosis genes, high expression of miR-182 in preeclampsia may contribute to the increased apoptosis in the placentas of patients with preeclampsia. The targets of both miR-182 and miR-210 are enriched in immune processes, which supports the association between abnormal immune responses and preeclampsia.[34]

A major strength of this study is its robust analysis of the expression of 157 miRNAs in 36 placentas with the use of real-time qRT-PCR, which is the most sensitive and specific RNA quantification method available. However, a limitation is that a comprehensive list of the 474 human miRNAs in the miRBase database[25] could not be screened because of the method (qRT-PCR) that we used for analysis. Other experimental tools such as miRNA microarrays may reveal more alterations in miRNA expression of patients with preeclampsia and SGA.

This study provides the first evidence that variations in miRNA expression occur in obstetric diseases. We report distinct placental miRNA profiles of preeclampsia that do not overlap with those of SGA. Future studies on the biologic effects of these miRNAs may provide novel targets for further investigation of the fundamental molecular pathophysiology of preeclampsia. Identification of target messenger RNAs is a critical step in determining the biologic pathways affected by the miRNAs that are overexpressed in preeclampsia. Several web-based programs are helpful in target prediction,[35] but the confirmation of miRNA target interactions is critical because of the complexity of the bioinformatic target prediction and the limitations of the available algorithms. A reporter vector containing the 3′-untranslated region of a putative target

mRNA can be cotransfected with the miRNA into relevant cells to determine their specific binding. Transfection of target cells with miRNA precursors and inhibitors and subsequent analyses of the cellular and molecular changes is also a valuable tool in the assessment of the biologic significance of a miRNA. A few in vivo studies have treated mice with specific miRNA-expressing vectors[36] or specific miRNA inhibitors[37] All of these in vitro and in vivo approaches could be useful for future investigation of our findings. ■

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