Reference values for circulating pregnancy-associated microRNAs in maternal plasma and their clinical usefulness in uncomplicated pregnancy and hypertensive disorder of pregnancy

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Running title: Normal ranges of plasma placental miRNAs

Abstract

Aim: To establish the reference values for circulating pregnancy-associated placental microRNAs in maternal plasma and clarify their clinical significance in patients with hypertensive disorder of pregnancy (HDP).

Methods: Blood samples were collected from 145 women with uncomplicated pregnancies (24, 26, 31, and 32 women at 12, 23, 30, and 36 weeks of gestation, respectively, and 32 women 1 day after delivery). Plasma concentrations of pregnancy-associated placental microRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b) were measured by quantitative real-time reverse-transcription polymerase chain reaction. Reference values for each microRNA were determined by the line of best fit and 95% prediction interval and are expressed as logarithmic transformation. To clarify the clinical significance of these reference values, we measured the plasma concentrations of pregnancy-associated microRNAs in a different population comprising 33 pregnant women with HDP and 44 women with uncomplicated pregnancies.

Results: Reference values for circulating pregnancy-associated placental microRNAs on chromosome 19 miRNA cluster showed an increasing tendency as pregnancy progressed and decreased significantly 1 day after delivery ($P<0.05$). The sensitivity and specificity of each reference value were 57.6% and 93.2% for miR-515-3p, 63.6% and 75.0% for miR-517a, 75.8% and 79.5% for miR-517c, and 63.6% and 75.0% for miR-518b, respectively. The positive and negative predictive values of each reference value were 86.4% and 74.5% for miR-515-3p, 65.6% and 73.3% for miR-517a, 73.5% and 81.4% for miR-517c, and 65.6% and 73.3% for miR-518b, respectively.

Conclusion: Establishing the reference values for circulating pregnancy-associated

placental microRNAs in maternal plasma could be useful for the evaluation of HDP.

Key words: Pregnancy-associated placental microRNAs, Maternal plasma, Biological marker, Reference values, Obstetric management

Introduction

The pathophysiology of hypertensive disorders of pregnancy (HDP) is not yet fully understood. However, circulating placental factors have been hypothesized to contribute to the pathogenesis of preeclampsia (PE), which is a dangerous type of HDP.[1] Therefore, the development of novel tests using circulating placental molecules for evaluation of HDP are useful in obstetric management.

MicroRNAs (miRNAs), which are non-protein-coding small RNAs (21–25 nucleotides), function as regulators of gene expression by antisense complementarity to specific messenger RNAs.[2-4] Several pregnancy-associated miRNAs in the maternal circulation have recently been identified, and their circulating levels are measurable during pregnancy and decrease significantly after delivery.[5-8] Additionally, because miRNAs are stable in plasma samples, pregnancy-associated miRNAs in maternal plasma may serve as biomarkers to monitor the pregnancy status.[9-12] In our and other previous studies, miR-515-3p, miR-517a, miR-517c, and miR-518b were reported as pregnancy-associated placental miRNAs circulating in maternal plasma.[6,7,13] These miRNAs are included within chromosome 19 miRNA cluster (C19MC), which contains 46 highly related miRNAs within an approximately 100-kb region.[14] Circulating levels of pregnancy-associated placental miRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b) are measurable and were significantly associated with placental weight.[6,7,13] Moreover, aberrant circulating levels of pregnancy-associated placental miRNAs on C19MC in plasma from women with preeclampsia (PE) have been reported.[15-17] In addition, aberrant circulating levels of pregnancy-associated placental miRNAs on C19MC in maternal plasma were associated with placenta previa, placenta abruption, or abnormal pregnancies (molar pregnancy, ectopic pregnancy, and spontaneous

abortion).[18-22] Therefore, circulating pregnancy-associated placental miRNAs on C19MC in maternal plasma may be potential biomarkers for pregnancy complications linked to a placental pathogenesis.

However, no universally accepted internal control suitable for quantification of pregnancy-associated miRNAs in plasma has been established.[12] Therefore, it is difficult to compare the differences in circulating miRNA levels among plasma samples. U6 snRNA is used as an internal control in quantitative studies of miRNAs in blood samples.[23-25] To use U6 snRNA as an internal control for comparison of the differences in circulating pregnancy-associated placental miRNA levels in plasma as pregnancy progresses, the stability of both RNA extraction and U6 snRNA levels in maternal plasma during pregnancy should be confirmed. Moreover, circulating levels of pregnancy-associated placental miRNAs in maternal plasma show individual variations,[12] because their levels depend on the efficacy of RNA extraction from the plasma samples, pregnancy condition (e.g., placental weight, and uterine contraction), and gestational age during a normally progressing pregnancy.[13,26] Therefore, information regarding normal ranges of pregnancy-associated placental miRNAs is desired for effective clinical use of these molecules.

In this study, to develop a quantitative analysis of circulating pregnancy-associated placental miRNAs as a clinical test, we investigated the reference values [value for line of best fit and 95% prediction interval (PI)] for the plasma concentrations of C19MC pregnancy-associated placental miRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b) during pregnancy in a population of women with uncomplicated pregnancies. Next, to clarify the clinical significance of these reference values, we measured the plasma concentrations of the same pregnancy-associated

placental miRNAs in another population of pregnant women with HDP and uncomplicated pregnancies and compared them with the above reference values.

Methods

Sample collection

This study was conducted from July 2013 to April 2016 at the Nagasaki University Hospital. The study protocol was approved by the Institutional Review Board for Ethical, Legal, and Social Issues of Nagasaki University (approval numbers: 121026236 and 13052715), and all samples were obtained after receiving written informed consent from each pregnant woman.

First, to investigate the normal ranges of plasma pregnancy-associated placental miRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b on C19MC) for a given gestational age, plasma samples were collected from women with uncomplicated pregnancies from September 2014 to November 2015; i.e., those without complications (e.g., HDP, multiple gestations, infection, fetal anomalies, fetal chromosomal abnormalities, fetal growth restriction [FGR], placenta previa, or invasive placentation) and subsequent full-term delivery of singleton healthy infants weighing >2500 g after 37 weeks of gestation. The ultrasound dating of pregnancy based on crown–rump length was performed at 9 to 11 weeks. Maternal blood samples were obtained from 145 women with uncomplicated pregnancies, including 24 women at 12 weeks of gestation, 26 at 23 weeks of gestation, 31 at 30 weeks of gestation, 32 at 36 weeks of gestation, and 32 at 1 day after delivery. The clinical characteristics of the pregnant women at each gestational age are listed in Table 1.

Next, we evaluated another population of women to confirm the clinical significance of the normal ranges of circulating C19MC miRNAs in maternal plasma. The maternal blood samples from 33 women with HDP (18 with PE, 4 with gestational hypertension, and 11 with pregnancy complicated by chronic hypertension) and 44 women with uncomplicated pregnancies were collected to investigate the sensitivity, specificity, and positive and negative predictive values of the reference values of each circulating miRNA level. The samples from women with HDP were obtained from July 2013 to April 2016, and the samples from women with uncomplicated pregnancies were obtained from December 2015 to April 2016.

In accordance with the definition of the Japan Society of Obstetrics and Gynecology (JSOG), HDP (PE, chronic hypertension, and chronic hypertension with superimposed PE and gestational hypertension) was diagnosed as previously described.[16] We subsequently compared the data in patients with PE based on the different criteria for PE among the JSOG, American Congress of Obstetricians and Gynecologists (ACOG), and International Society for the Study of Hypertension in Pregnancy (ISSHP).[27,28]

Using a double centrifugation method as described previously,[29-31] cell-free plasma samples were prepared from maternal blood in tubes containing ethylenediaminetetraacetic acid. After the first centrifugation at 3,000×*g* for 10 min, the supernatant was centrifuged at 16,000×*g* for 10 min to remove blood cells. Using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA), total RNA containing small RNA molecules was extracted from 1.2 mL of maternal plasma according to the manufacturer's instructions. To correct for variations in RNA extraction efficiency, plasma samples were spiked with 5 μL of 200 nM synthetic *Caenorhabditis elegans*,

(cel)-miR-39 (Sigma-Aldrich, St. Louis, MO, USA) after RNases were inactivated.[32]

Real-time quantitative polymerase chain reaction analysis of miRNAs

Plasma concentrations of pregnancy-associated placental miRNAs (hsa-miR-515-3p, hsa-miR-517a, hsa-miR-517c, and hsa-miR-518b on C19MC region), cel-miR-39 (exogenous control), and U6 snRNA (endogenous control) were measured by real-time quantitative reverse-transcription polymerase chain reaction (PCR) using a LightCycler 480 Real-Time PCR System (Roche, Pleasanton, CA, USA).[16,19,32] All specific primers and TaqMan in the TaqMan MicroRNA Assays were purchased from Life Technologies (Carlsbad, CA, USA). For each miRNA assay, by 10-fold serial dilution of single-stranded cDNA oligonucleotides corresponding to each miRNA sequence, a calibration curve was prepared from 1.0×10^2 to 1.0×10^8 copies/mL. Each sample and calibration dilution were analyzed in triplicate, and three water blanks were included as negative controls for each of the reverse transcription and PCR steps. The minimum detectable concentration of each assay was 300 RNA copies/mL.[16,19] We performed absolute quantitation of the miRNA concentration in the plasma samples because this was recommended in a previous study.[33] The plasma concentrations of target miRNAs (copies/μL plasma) were adjusted by the plasma concentrations of U6 snRNA, which was used as an internal control during quantitative PCR in each sample. Finally, the target miRNA concentrations were normalized to the concentration of cel-miR-39.

Statistical analysis

The patients' backgrounds were compared among the gestational weeks using the

Kruskal–Wallis test or chi-square test. The differences in circulating C19MC miRNA levels among the gestational ages were evaluated using the Steel–Dwass test. Each circulating miRNA level in maternal plasma was expressed as logarithmic transformation. Linear regression analysis was used to derive the line of best fit for the plot for each circulating C19MC miRNA level against gestational age. The 97.5th and 2.5th percentiles for each circulating C19MC miRNA level at each gestational age were defined by the upper and lower borders, respectively, of the 95% PI around each regression analysis. Therefore, reference values are expressed as predictive values (value for line of best fit for the plot for each circulating C19MC miRNA level against gestational age) and 95% PI. Statistical analyses were performed with JMP v11 Pro (SAS Institute Inc., Cary, NC, USA). Significant differences were accepted at $P<0.05$.

Results

Concentration of synthetic spike-in miRNA and circulating level of U6 snRNA in maternal plasma during pregnancy and after delivery

The concentrations of cel-miR-39 (synthetic spike-in miRNA) in RNA samples extracted from maternal plasma were measured to confirm the stability of RNA extraction from the plasma samples. The concentrations of cel-miR-39 showed no differences during pregnancy and day 1 postpartum (Steel–Dwass test, $P>0.05$) (Figure 1a); the median (minimum–maximum) copies/μL of cel-miR-39 were 458.55 (365.44–538.5) at 12 weeks of gestation, 452.205 (401.02–598.2) at 23 weeks, 458.235 (391.7–551.7) at 30 weeks, 470.685 (403.12–579.19) at 36 weeks, and 463.395 (401.52–574.92) on day 1 postpartum, respectively. Therefore, the stability of RNA extraction from the plasma samples was confirmed. The coefficient of variation of cel-miR-39

intra-assay variation was 9.09%.

Next, the circulating levels of U6 snRNA in maternal plasma were measured to investigate the utility of U6 snRNA as the endogenous control. Their concentrations did not change significantly during pregnancy and day 1 postpartum (Steel–Dwass test, $P>0.05$) (Figure 1b); the median (minimum–maximum) copies/μL of plasma U6 snRNA were 268.58 (14.99–5447.95) at 12 weeks of gestation, 271.31 (1.59–2157.55) at 23 weeks, 260.51 (23.59–6406.81) at 30 weeks, 261.87 (7.76–3577.22) at 36 weeks, and 437.37 (6.78–4822.57) on day 1 postpartum, respectively. The coefficient of variation of U6 snRNA intra-assay variation was 8.16%. Therefore, U6 snRNA was used as the endogenous control during pregnancy and on day 1 postpartum.

Reference values for log_{10} pregnancy-associated miRNA levels in maternal plasma

The predictive value and 95% PI of log_{10} pregnancy-associated miRNA levels in maternal plasma were determined as reference values. The relation between the plasma concentration of miRNA and gestational age for each pregnancy-associated placental miRNA is shown in Figure 2a–d, together with the lines of best fit and the 95% PI of log_{10} pregnancy-associated placental miRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b). The circulating levels of each log_{10} pregnancy-associated placental miRNA increased significantly in a linear fashion as pregnancy progressed ($Y=0.015\times X-2.53$ and $P=0.0466$ for miR-515-3p, $Y=0.016\times X-1.13$ and $P=0.0051$ for miR-517a, $Y=0.016\times X-1.59$ and $P=0.0049$ for miR-517c, $Y=0.015\times X-1.13$ and $P=0.0002$ for miR-518b; linear regression analysis) (Table 2, Figure 2a–d) and decreased significantly on day 1 postpartum ($P<0.0001$ for each miRNA; *t*-test) (Figure 2a–d).

Clinical significance of reference values for log_{10} pregnancy-associated miRNA levels in maternal plasma

We measured the circulating levels of log_{10} pregnancy-associated placental miRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b) in 33 women with HDP (18 with PE, 4 with gestational hypertension, and 11 with pregnancy complicated by chronic hypertension) and 44 women with uncomplicated pregnancies to compare them with the reference values (Figure 3a–d). The sensitivity and specificity of each reference value were 57.6% (19/33 women) and 93.2% (41/44) for miR-515-3p, 63.6% (21/33) and 75.0% (33/44) for miR-517a, 75.8% (25/33) and 79.5% (35/44) for miR-517c, and 63.6% (21/33) and 75.0% (33/44) for miR-518b, respectively (Table 3). The positive and negative predictive values of each normal range were 86.4% (19/22 women) and 74.5% (41/55) for miR-515-3p, 65.6% (21/32) and 73.3% (33/45) for miR-517a, 73.5% (25/34) and 81.4% (35/43) for miR-517c, and 65.6% (21/32) and 73.3% (33/45) for miR-518b, respectively (Table 3). Three women with chronic hypertension in the first trimester showed increased plasma concentrations of all four pregnancy-associated placental miRNAs and later developed chronic hypertension with superimposed PE (Figure 3a–d).

Of 33 women with HDP, 25 samples were obtained within 12 months, while 8 samples were stored for more than 12 months from sampling to analysis. In the 25 samples from women with HDP obtained within 12 months from sampling to analysis, the sensitivity of each reference value was 76.0% (19/25 women) for miR-515-3p, 84.0% (21/25) for miR-517a, 96.0% (24/25) for miR-517c, and 88.0% (22/25) for miR-518b (Supplementary Figure 1a-d). Conversely, in the eight samples from women with HDP stored for more than 12 months from sampling to analysis, the sensitivity of each

reference value was 0.0% (0/8) for miR-515-3p, 0.0% (0/8) for miR-517a, 12.5% (1/8) for miR-517c, and 0.0% (0/8) for miR-518b (Supplementary Figure 1a-d).

Among the 18 women with PE, 13 samples were obtained within 12 months and 5 samples were stored for more than 12 months. In consideration of sample quality, the plasma concentrations of pregnancy-associated placental miRNAs in the 13 samples obtained within 12 months were used for statistical analysis.

The circulating levels of pregnancy-associated placental miRNAs (miR-515-3p, -517a, -517c, and -518b) were significantly higher in patients with PE with FGR (n=8) than in those with uncomplicated pregnancies (n=44) (Mann–Whitney U-test, $P<0.0001$ for miR-515-3p, -517c, and -518b and $P=0.0001$ for miR-517a) (Supplementary Table 1 and Supplementary Figure 2a-d). The circulating levels of pregnancy-associated placental miRNAs (miR-515-3p, -517a, -517c, and -518b) were significantly higher in patients with PE without FGR (n=5) than in those with uncomplicated pregnancies (n=44) ($P=0.0003$ for each miRNA) (Supplementary Table 1 and Supplementary Figure 2a-d). The circulating levels of pregnancy-associated placental miRNAs (miR-517a, -517c, and 518b) in patients with PE with FGR (n=8) were significantly lower than those in patients with PE without FGR (n=5) ($P=0.0157$ for miR-517a and $P=0.0338$ for miR-517c and -518b) (Supplementary Table 1 and Supplementary Figure 2b-d). The plasma concentration of miR-515-3p in patients with PE with FGR showed a tendency toward lower circulating levels than in patients with PE without FGR (Supplementary Table 1 and Supplementary Figure 2a).

Comparison of plasma pregnancy-associated placental miRNA levels in patients with PE based on JSOG, ACOG, and ISSHP criteria for PE

Because the JSOG diagnostic criteria for PE seem to be considerably different from the more widely accepted ACOG and ISSHP criteria, we compared the data in patients with PE based on each set of criteria for PE (JSOG, ACOG, and ISSHP). Consequently, we confirmed that there were no significant differences in the test's precision, including the sensitivity, specificity, and positive and negative predictive values (Supplementary Table 2).

Discussion

In this study, we determined the reference values for circulating pregnancy-associated miRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b) in maternal plasma as pregnancy progressed and clarified their clinical significance.

First, we confirmed that the cel-miR-39 concentration in each sample after RNA extraction showed no significant difference and that the circulating levels of U6 snRNA in maternal plasma also showed no significant difference during pregnancy (Figure 1), suggesting that the efficacy of the RNA extraction process from maternal plasma sample is stable and that U6 snRNA is a suitable internal control for the quantification of plasma miRNAs in pregnant women. In accordance with our findings, another study also used U6 snRNA as an endogenous control as pregnancy progressed.[25] However, with respect to quantification of plasma miRNAs in patients with carcinoma, the stability of U6 snRNA as an endogenous control remains controversial; some studies used U6 snRNA as an endogenous control,[34,35] while some showed that U6 snRNA was an unsuitable endogenous control.[36] Because the circulating level of each miRNA in a plasma sample is affected by the patient's background,[13,25] discrepancies in the stability of the circulating U6 snRNA levels in plasma samples may be caused by biological

differences between carcinoma and pregnancy. Therefore, in this study, cel-miR-39 and U6 snRNA were used as external and internal controls to normalize the circulating levels of pregnancy-associated placental miRNAs in maternal plasma.

To establish the reference values for circulating C19MC pregnancy-associated placental miRNAs (miR515-3p, miR-517a, miR-517c, and miR-518b) in maternal plasma, we included a larger number of plasma samples at several gestational time points (145 women with uncomplicated pregnancies; 24, 26, 31, and 32 women at 12, 23, 30, and 36 weeks of gestation, respectively, and 32 women 1 day after delivery) in comparison with previous studies.[5-7] In accordance with our and other previous studies,[5-7] the reference values of pregnancy-associated placental miRNAs on C19MC (miR515-3p, miR-517a, miR-517c, and miR-518b) increased as pregnancy progressed and rapidly decreased 1 day after delivery. Additionally, one study showed that the expression of C19MC miRNAs in pNK cells were significantly upregulated in the third trimester compared with the first-trimester, and the rapid clearance of C19MC miRNAs from the pNK cells occurred after delivery.[37] This suggests that C19MC miRNA regulates migratory and invasive behaviors of extravillous trophoblasts and plays a role in the establishment of the maternal–fetal interface.[38,39] In several studies, aberrant levels of pregnancy-associated placental miRNAs on C19MC in maternal plasma were detected in complicated pregnancies (e.g., PE, gestational diabetes, placenta previa, and placental abruption) compared with uncomplicated pregnancies.[10-12,15-22] Therefore, taking the above-mentioned reports into consideration, our reference values (predictive value and 95% PI) of circulating pregnancy-associated placental miRNAs (miR515-3p, miR-517a, miR-517c, and miR-518b) in maternal plasma as pregnancy progresses make it possible to evaluate and/or predict pregnancy complications as novel test.

To clarify the clinical significance of the above reference values for circulating C19MC pregnancy-associated placental miRNAs (miR515-3p, miR-517a, miR-517c, and miR-518b) in maternal plasma as pregnancy progresses, a second population different from the first population was evaluated. We measured the circulating levels of each C19MC pregnancy-associated placental miRNA in plasma samples from 33 pregnant women with HDP (18 with PE, 4 with gestational hypertension, and 11 with pregnancy complicated by chronic hypertension) and 44 women with uncomplicated pregnancies and compared their circulating levels with the above reference values. Most circulating levels of C19MC miRNAs in plasma samples from women with uncomplicated pregnancies were within the reference ranges, and those in plasma samples from pregnant women with HDP were outside of the reference ranges (Figure 3). In particular, the sensitivity and specificity of the reference value for circulating $\log_{10}$ miR-517c in maternal plasma were 75.8% and 79.5%, and the positive and negative predictive values were 73.5% and 81.4%, respectively (Table 3). Our data are in accordance with previous studies showing increased levels of circulating C19MC miRNAs in maternal plasma in women with PE than in women with uncomplicated pregnancies.[15,16] Our reference values for circulating C19MC pregnancy-associated placental miRNAs (especially miR-517c) in maternal plasma seem to distinguish HDP from uncomplicated pregnancy. Additionally, in patients with PE, the measurement of pregnancy-associated miRNA concentrations in maternal plasma obtained within 12 months from sampling to analysis showed a higher sensitivity than measurement of these concentrations in maternal plasma stored for more than 12 months (Supplemental Figure 1a-d). Therefore, the time from sampling to analysis can affect the precision of plasma miRNA quantification, and fresh samples (within 12 months from sampling to

analysis) could be suitable for clinical application.

Additionally, all women with chronic hypertension in the first trimester showed aberrant levels of four pregnancy-associated placental miRNAs in their plasma samples and subsequently developed chronic hypertension with superimposed PE, suggesting that pregnancy-associated placental miRNAs in patients with chronic hypertension may serve as potential predictive markers of superimposed PE. Moreover, the levels of pregnancy-associated miRNAs in patients with PE were significantly lower in those with than without FGR. This result is consistent with previous data showing that the expression levels of placental miRNAs in FGR-affected placenta tissues are significantly lower than those in placental tissues from cases of uncomplicated pregnancy.[17,40,41] Therefore, the lower levels of pregnancy-associated placental miRNAs in patients with PE with FGR may reflect placental insufficiency in cases of FGR. As a next step, we should use plasma samples collected before the onset of disease to determine whether our reference values can predict subsequent HDP.

This study has several limitations. Although we confirmed the stable levels of circulating U6 snRNA in maternal plasma during pregnancy and used U6 snRNA as the internal control for normalization of four pregnancy-associated placental miRNA levels in maternal plasma, other internal controls (e.g., U1, U43, U44, U48, miR-16, miR-19b, miR-24, miR-30e, miR-142-3p, miR-192, miR-638, let-7a, 5S, and 18S) were not investigated. We confirmed the possibility of the clinical application of our reference values for circulating pregnancy-associated placental miRNAs in HDP. However, in plasma samples from patients with carcinoma, several miRNAs (miR-16, miRNA-106a, and miRNA-21) and a combination of Let-7d, Let-7g, and Let-7i were reported as more suitable internal controls than U6 snRNA for the quantification of circulating miRNAs

in plasma samples,[34,42,43] although this conclusion remains controversial. To establish our approach as a more accurate clinical test, we should select a suitable internal control that would most effectively normalize circulating pregnancy-associated placental miRNAs levels in plasma. In addition, because we calculated the reference values for circulating levels of pregnancy-associated placental miRNAs from only 145 women with uncomplicated pregnancies, the sample volumes were limited. Therefore, the present findings need to be confirmed in larger studies.

In conclusion, we determined the reference values for circulating pregnancy-associated placental miRNAs on C19MC in maternal plasma throughout pregnancy and confirmed their clinical significance for evaluation of HDP. In addition, the same precision in the measurement of plasma pregnancy-associated placental miRNA levels was confirmed in patients with PE based on three different criteria (JSOG, ACOG, and ISSHP), suggesting that our reference values in this study can be used not only in Japan but also in other countries. The reference values for circulating pregnancy-associated placental miRNAs in maternal plasma showed a tendency to distinguish HDP from uncomplicated pregnancy, suggesting that our approach in this study may help to diagnose HDP. Circulating placental molecules (e.g., placental growth factor and placental exosome) were reported to be potential predictive markers of PE or gestational diabetes.[25,44-46] As novel parameters for prenatal monitoring and diagnosis, the reference values for circulating pregnancy-associated placental miRNAs in maternal plasma may contribute to the prediction of HDP (especially PE) before the onset of disease.

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Disclosure

No author has any potential conflict of interest.

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Figure Legends

Figure 1. Plasma concentrations of cel-miR-39 and U6 snRNA throughout the progression of pregnancy

(a) cel-miR-39 and (b) U6 snRNA. The upper and lower limits of the boxes and the horizontal line within the boxes indicate the 75th and 25th percentiles and the median, respectively. The whisker caps indicate the maximum value and minimum value, excluding outliers. The plot shows the outliers. The plasma concentrations of cel-miR-39 and U6 snRNA did not significantly change during pregnancy or on day 1 postpartum, respectively (Steel–Dwass test, $P>0.05$).

Figure 2. Correlation plot between circulating pregnancy-associated placental miRNAs levels and gestational age

(a) miR-515-3p, (b) miR-517a, (c) miR-517c, and (d) miR-518b. Linear regression analysis was used to derive the line of best fit for the plot for each circulating pregnancy-associated placental miRNA level against gestational age. The circulating level of each $\log_{10}$ pregnancy-associated placental miRNA is on the vertical axis, and gestational age is on the horizontal axis. Reference values are expressed as the predictive value (value for the line of best fit for the plot for each circulating pregnancy-associated placental miRNA level against gestational age) and 95% prediction interval. The dotted lines are the borders of the 95% prediction interval, and the straight line shows the linear regression line fit. The circulating levels of each $\log_{10}$ pregnancy-associated placental miRNA increased significantly in a linear fashion as pregnancy progressed ($P=0.0466$ for miR-515-3p, $P=0.0051$ for miR-517a, $P=0.0049$ for miR-517c, and $P=0.0002$ for miR-518b; linear regression analysis). Asterisks (*)

indicate that each circulating pregnancy-associated placental miRNA decreased significantly on day 1 postpartum ($P<0.0001$ for each miRNA; *t*-test).

Figure 3. Plots of circulating $\log_{10}$ pregnancy-associated miRNA levels vs. gestational age together with line of best fit and 95% prediction interval.

(a) miR-515-3p, (b) miR-517a, (c) miR-517c, and (d) miR-518b. The lightly shaded area represents the reference values (line of best fit and 95% prediction interval). The white square plots (□) indicate 11 women with pregnancy complicated by chronic hypertension, the triangular plots (△) indicate 18 women with preeclampsia, the diamond plots (◇) indicate 4 women with gestational hypertension, and the black square plots (■) indicate 44 women with uncomplicated pregnancy.

Supporting information legend

Supplementary Table 1 lists the plasma log_{10} pregnancy-associated placental miRNAs levels in uncomplicated pregnancy, preeclampsia with fetal growth restriction, and preeclampsia without fetal growth restriction.

Supplementary Table 2 lists the data regarding comparison of the test's precision in patients with preeclampsia based on the different criteria for preeclampsia among the JSOG, ACOG, and ISSHP.

Supplementary Figure 1 indicates the plots of circulating log_{10} pregnancy-associated placental miRNA levels between the preeclamptic samples obtained within 12 months and those stored for more than 12 months.

(a)miR-515-3p, (b) miR-517a, (c) miR-517c, and (d) miR-518b.

The reference values (line of best fit and 95% prediction interval) are represented between the broken lines. The black circle plots (●) indicate the hypertensive disorders of pregnancy (HDP) samples obtained within 12 months from sampling to analysis (n=25), while the white circle plots (○) indicate the HDP samples stored for more than 12 months from sampling to analysis (n=8).

Supplementary Figure 2 compares the circulating log_{10} pregnancy-associated placental miRNAs levels in maternal plasma from cases of uncomplicated pregnancy, preeclampsia with fetal growth restriction, and preeclampsia without fetal growth restriction.

(a) miR-515-3p, (b) miR-517a, (c) miR-517c, and (d) miR-518b. *Significant

difference. The circulating levels of pregnancy-associated placental miRNAs (miR-515-3p, 517a, -517c, and 518b) were significantly higher in cases of preeclampsia (PE) with fetal growth restriction (FGR) (n=8) than in cases of uncomplicated pregnancy (n=44) (P<0.0001 for miR-515-3p, -517c, and -518b and P=0.0001 for miR-517a; Mann–Whitney U-test). The circulating levels of pregnancy-associated placental miRNAs (miR-515-3p, -517a, -517c, and -518b) were significantly higher in cases of PE without FGR (n=5) than in cases of uncomplicated pregnancy (normal control [NC], n=44) (P=0.0003 for each miRNA, Mann–Whitney U-test). The circulating levels of pregnancy-associated placental miRNAs (miR-517a, -517c, and -518b) in cases of PE with FGR (n=8) were significantly lower than those in cases of PE without FGR (n=5) (P=0.0157 for miR-517a and P=0.0338 for miR-517c and -518b).

Table 1. Clinical characteristics of the pregnant women

Clinical characteristics	Gestational age				1 day after delivery (n=32)	p value
	12 weeks (n=24)	23 weeks (n=26)	30 weeks (n=31)	36 weeks (n=32)		
Maternal age (years)	30.7 (4.2)†	29.8 (5.3)†	29.6 (4.7)†	30.7 (4.2)†	29.1 (5.6)†	0.60
Parity (cases)						0.13
Primiparous	7	12	20	16	14	
Multiparous	17	14	11	16	18	
BMI (kg/m2)	20.9 (2.1)†	20.8 (2.4)†	20.5 (3.2)†	20.5 (3.5)†	20.9 (2.6)†	0.51
Gestational age at delivery (weeks)	39.8 (1.1)†	39.9 (0.9)†	39.8 (1.2)†	39.5 (1.1)†	40.0 (1.1)†	0.50
Placental weight (g)	642.3 (120.7)†	577.7 (106.2)†	607.7 (103.9)†	602.3 (102.5)†	598.9 (79.9)†	0.25
Fetal birth weight (g)	3181.5 (400.4)†	3101.7 (303.1)†	3090.8 (295.8)†	3026.9 (323.5)†	3140.1 (294.3)†	0.58
Newborn Sex (cases)						0.49
Male	13	11	12	19	16	
Female	11	15	19	13	16	

BMI: body mass index, NS: not significant. † Values are expressed as mean (standard deviation). Significant differences between groups were analyzed by the Kruskal–Wallis test or chi-square test.

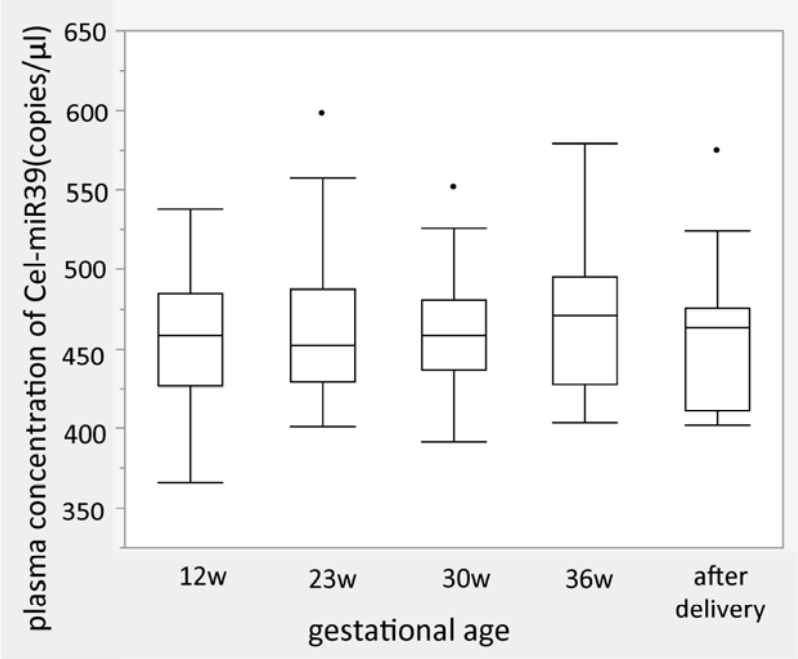
Table 2. Reference values for $\log_{10}$ pregnancy-associated placental miRNA levels in maternal plasma

Pregnancy-associated placental microRNAs	95% prediction interval	Gestational age			
		12 weeks	23 weeks	30 weeks	36 weeks
miR-515-3p	2.5th percentile	-3.782	-3.600	-3.494	-3.409
	Predictive value†	-2.345	-2.178	-2.072	-1.981
	97.5th percentile	-0.907	-0.756	-0.650	-0.552
miR-517a	2.5th percentile	-2.020	-1.830	-1.716	-1.623
	Predictive value	-0.935	-0.756	-0.642	-0.545
	97.5th percentile	0.150	0.318	0.431	0.533
miR-517c	2.5th percentile	-2.452	-2.266	-2.154	-2.063
	Predictive value	-1.394	-1.219	-1.108	-1.012
	97.5th percentile	-0.337	-0.173	-0.061	0.039
miR-518b	2.5th percentile	-1.685	-1.516	-1.414	-1.329
	Predictive value	-0.953	-0.792	-0.689	-0.601
	97.5th percentile	-0.221	-0.067	0.035	0.127

†Value for line of best fit.

Table 3. Clinical significance of reference values for $\log_{10}$ pregnancy-associated placental miRNA levels in maternal plasma

Pregnancy-associated placental microRNAs		Positive cases	Negative cases	Total
miR-515-3p	Hypertensive disorders of pregnancy	19	14	33
	Uncomplicated pregnancy	3	41	44
	Total	22	55	
miR-517a	Hypertensive disorders of pregnancy	21	12	33
	Uncomplicated pregnancy	11	33	44
	Total	32	45	
miR-517c	Hypertensive disorders of pregnancy	25	8	33
	Uncomplicated pregnancy	9	35	44
	Total	34	43	
miR-518b	Hypertensive disorders of pregnancy	21	12	33
	Uncomplicated pregnancy	11	33	44
	Total	32	45	


plasma concentration of Cel-miR39(copies/μl)
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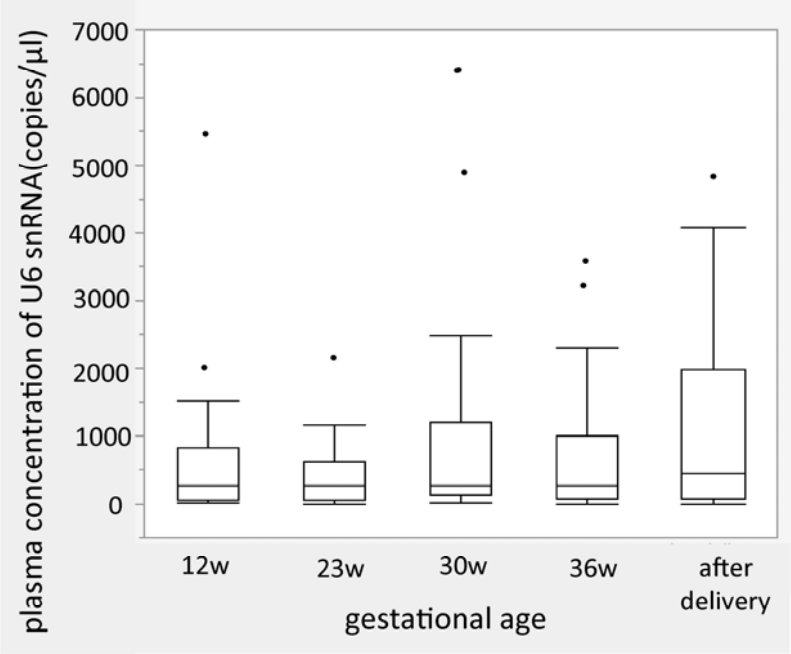
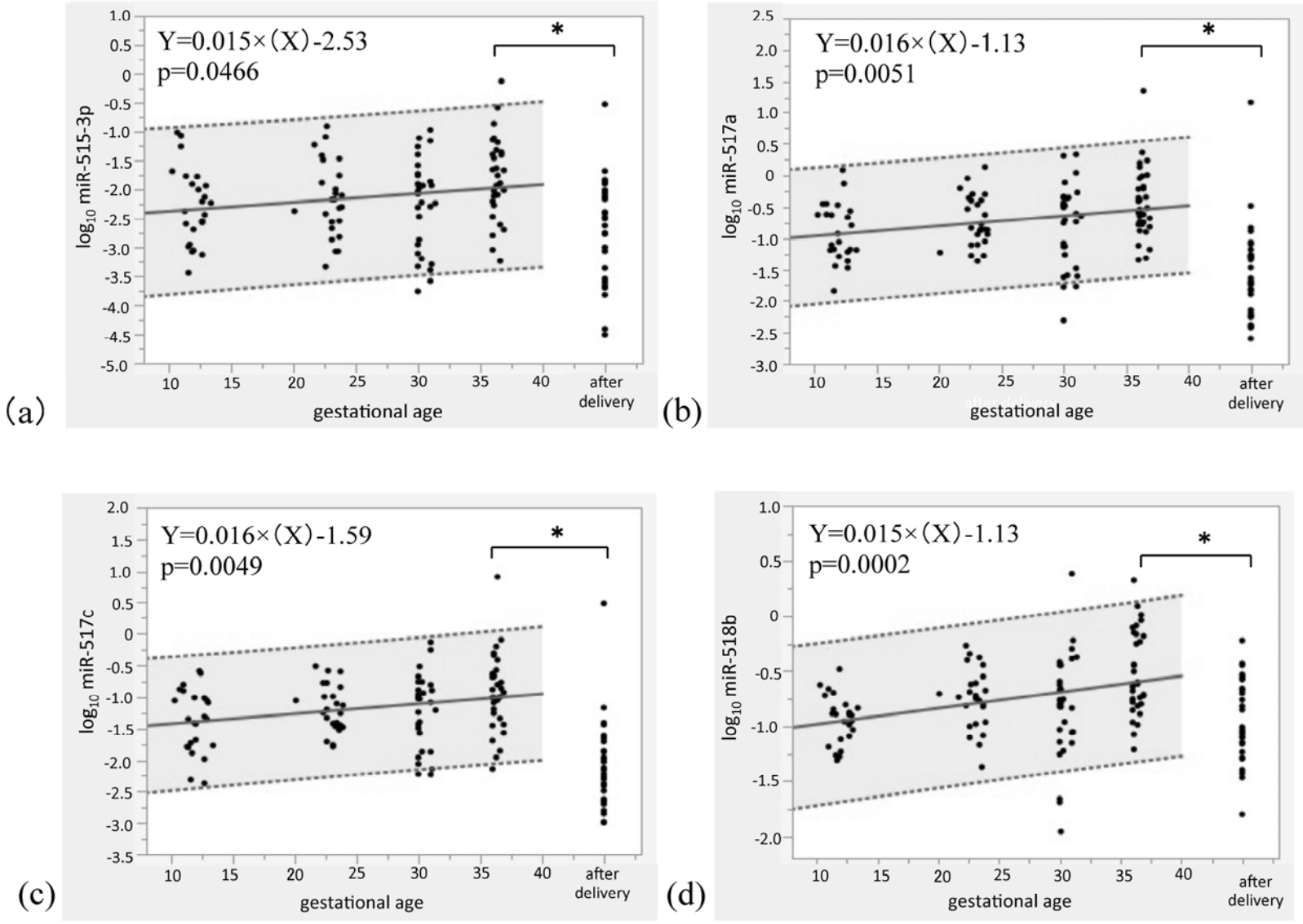
600
550
500
450
400
350
12w
23w
30w
36w
after delivery
gestational age

(a)


plasma concentration of U6 snRNA(copies/μl)
7000
6000
5000
4000
3000
2000
1000
0
12w
23w
30w
36w
after delivery
gestational age

(b)

Figure1


(a)
Y=0.015×(X)-2.53
p=0.0466
log10 miR-515-3p
gestational age
after delivery
(b)
Y=0.016×(X)-1.13
p=0.0051
log10 miR-517a
gestational age
after delivery
(c)
Y=0.016×(X)-1.59
p=0.0049
log10 miR-517c
gestational age
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(d)
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gestational age
after delivery

Figure 2

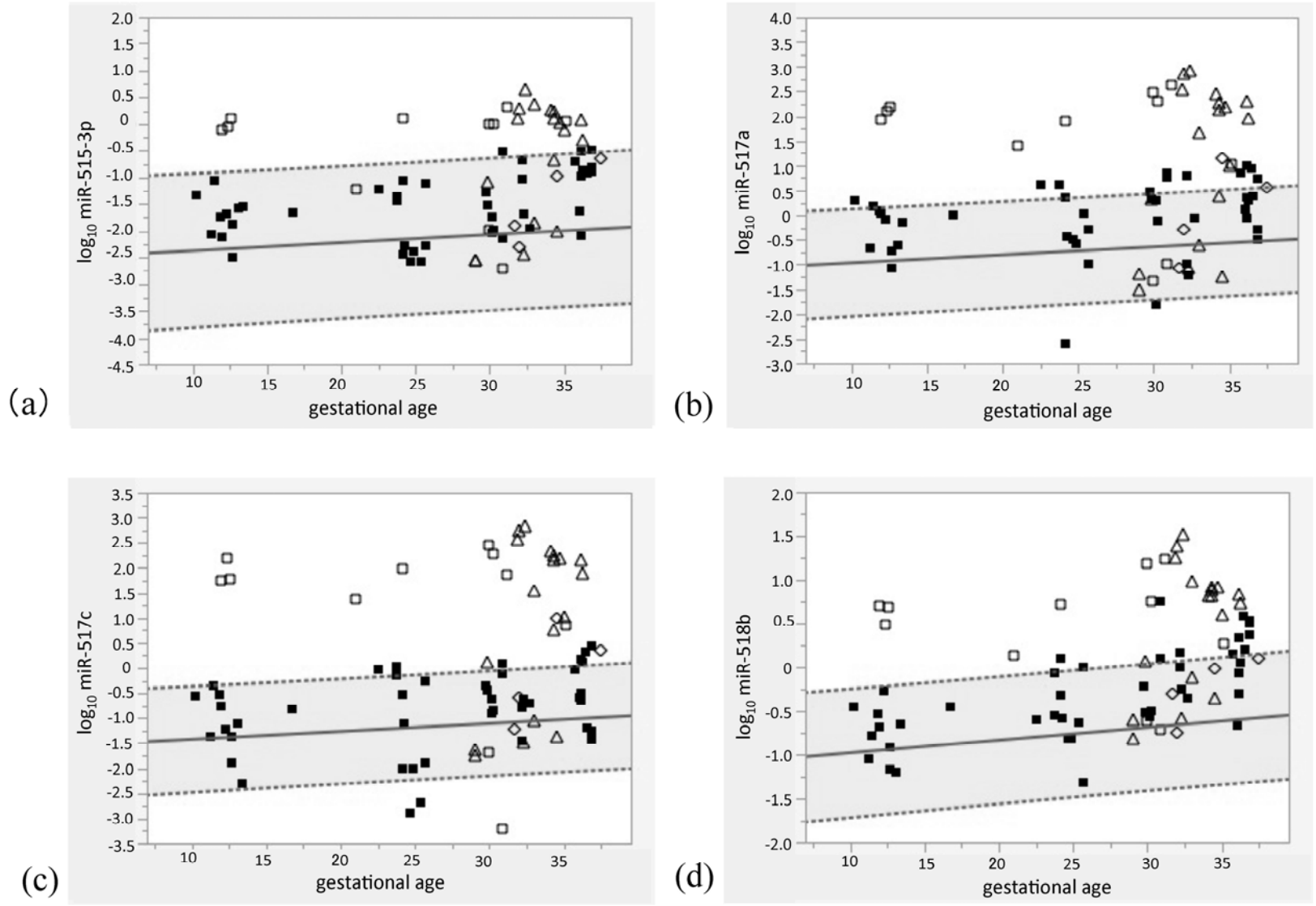
(a)
log10 miR-515-3p
gestational age
(b)
log10 miR-517a
gestational age
(c)
log10 miR-517c
gestational age
(d)
log10 miR-518b
gestational age

Figure3.

Reference values for circulating pregnancy-associated microRNAs in maternal plasma and their clinical usefulness in uncomplicated pregnancy and hypertensive disorders of pregnancy

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Running title: Normal ranges of plasma placental miRNAs

Reference values for circulating pregnancy-associated microRNAs in maternal plasma and their clinical usefulness in uncomplicated pregnancy and hypertensive disorders of pregnancy

Supplementary Table 1 lists the plasma $\log_{10}$ pregnancy-associated placental miRNAs levels in uncomplicated pregnancy, preeclampsia with fetal growth restriction, and preeclampsia without fetal growth restriction.

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Supplementary Table 1. Plasma log_{10} pregnancy-associated placental miRNAs levels in uncomplicated pregnancy, preeclampsia with fetal growth restriction, and preeclampsia without fetal growth restriction

Pregnancy-associated placental miRNAs	Uncomplicated pregnancy	PE with FGR	PE without FGR
miR-515-3p	−1.568 (−2.575 to −0.502)	3.09×10^{-5} (−1.084–0.362)	0.107 (0.034–0.654)
miR-517a	0.023 (−2.606–0.983)	1.800 (0.334–2.455)	2.534 (2.200–2.914)
miR-517c	−0.667 (−2.893–0.433)	1.718 (0.114–2.331)	2.561 (2.157–2.828)
miR-518b	−0.397 (−1.309–0.748)	0.824 (0.073–0.980)	1.248 (0.840–1.515)

PE: preeclampsia, FGR: fetal growth restriction

Data are presented as median (minimum–maximum)

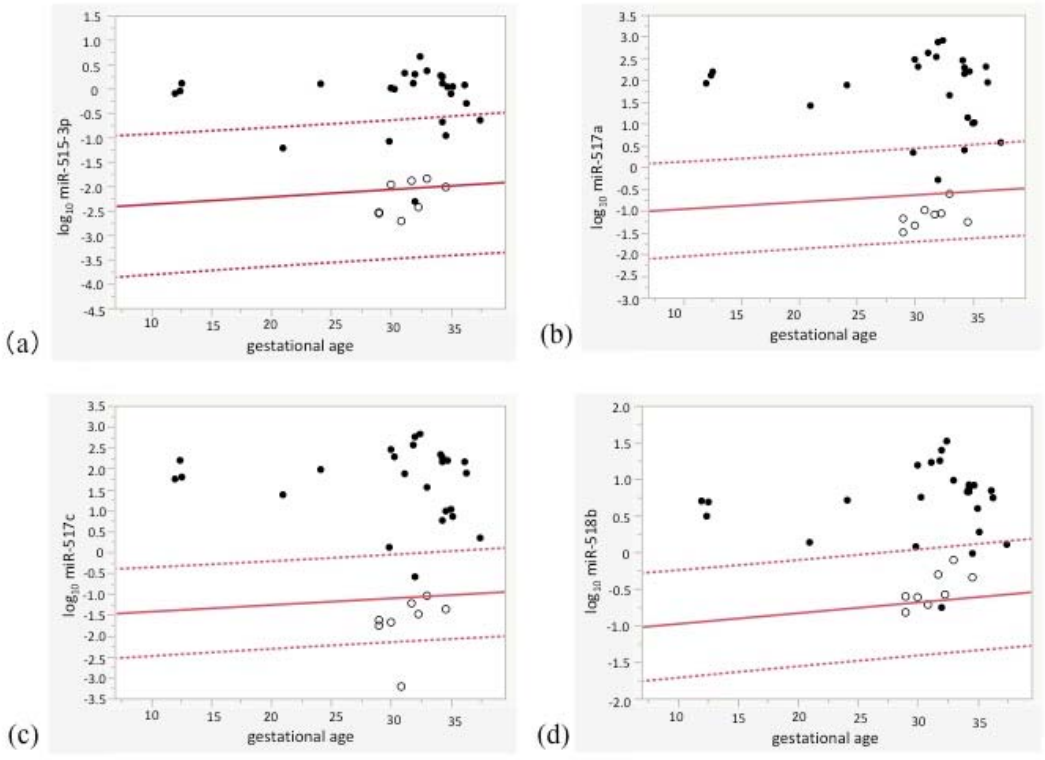
Supplementary Table 2. Comparison of the test's precision in patients with preeclampsia based on the different criteria for preeclampsia among the JSOG, ACOG, and ISSHP

Pregnancy-associated placental miRNAs		JSOG (n=13)	ACOG (n=13)	ISSHP (n=14)
miR-515-3p	Sensitivity (%)	84.6	84.6	78.6
	Specificity (%)	93.2	93.2	93.2
	PPV (%)	78.6	78.6	78.6
	NPV (%)	95.3	95.3	93.2
miR-517a	Sensitivity (%)	84.6	84.6	85.7
	Specificity (%)	75.0	75.0	75.0
	PPV (%)	50.0	50.0	52.2
	NPV (%)	94.3	94.3	94.3
miR-517c	Sensitivity (%)	100.0	100.0	100.0
	Specificity (%)	79.5	79.5	79.5
	PPV (%)	59.1	59.1	60.9

	NPV (%)	100.0	100.0	100.0
miR-518b	Sensitivity (%)	100.0	100.0	92.9
	Specificity (%)	75.0	75.0	75.0
	PPV (%)	54.2	54.2	54.2
	NPV (%)	100.0	100.0	97.1

1 PPV: positive predictive value, NPV: negative predictive value

2



Supplementary Figure 1

Supplementary Figure 1. Plots of circulating log_{10} pregnancy-associated placental miRNA levels between hypertensive disorders of pregnancy samples obtained within 12 months and those stored for more than 12 months

(a) miR-515-3p, (b) miR-517a, (c) miR-517c, and (d) miR-518b. The reference values (line of best fit and 95% prediction interval) are represented between the broken lines. The black circle plots (●) indicate the hypertensive disorders of pregnancy (HDP) samples obtained within 12 months from sampling to analysis (n=25), while the white circle plots (○) indicate the HDP samples stored for more than 12 months from

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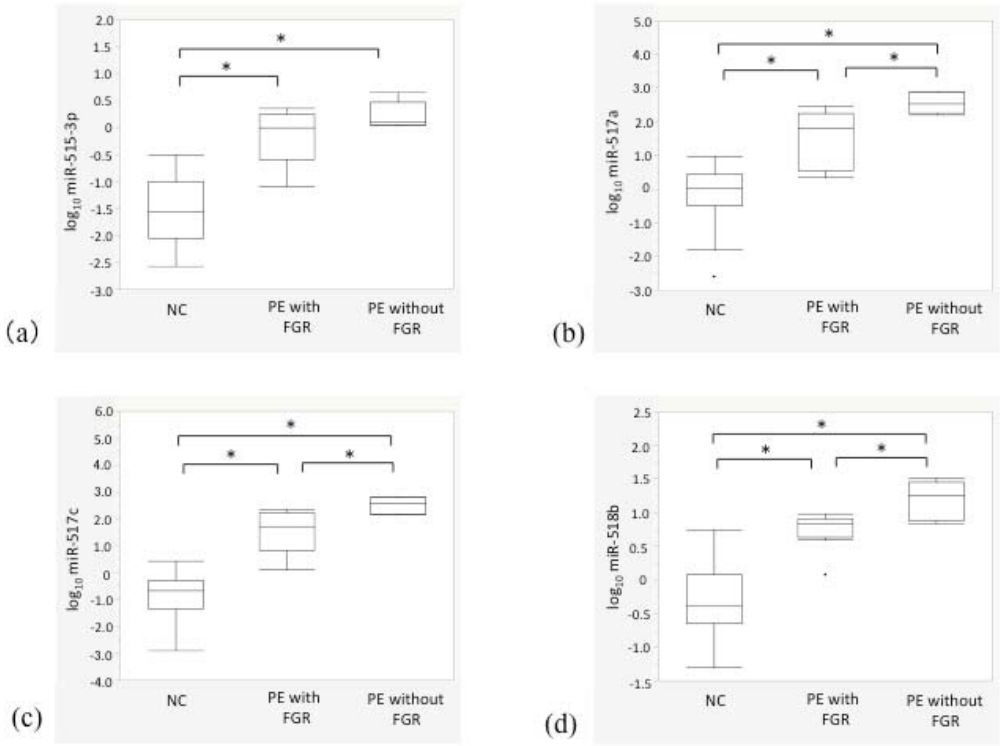

Supplementary Figure 2

Supplementary Figure 2. Comparison of circulating pregnancy-associated placental miRNAs levels in maternal plasma from cases of uncomplicated pregnancy, preeclampsia with fetal growth restriction, and preeclampsia without fetal growth restriction

(a) miR-515-3p, (b) miR-517a, (c) miR-517c, and (d) miR-518b. *Significant difference. The circulating levels of pregnancy-associated placental miRNAs (miR-515-3p, 517a, -517c, and 518b) were significantly higher in cases of preeclampsia (PE) with fetal growth restriction (FGR) (n=8) than in cases of uncomplicated

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