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Decreased plasma concentrations of pregnancy-associated placenta-specific microRNAs in pregnancies with a diagnosis of fetal trisomy 18

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Summary

Objectives: This study aimed to clarify the association between circulating pregnancy-associated, placenta-specific microRNAs in the maternal plasma of women with fetal trisomy 18 pregnancies. *Materials and Methods:* All samples were obtained after receiving written informed consent and the study protocol was approved by the Institutional Review Board. Maternal blood samples (7 ml) were obtained at 16–19 weeks of gestation from 13 pregnant women carrying fetuses with trisomy 18 (trisomy 18 pregnancy group) and from 24 pregnant women carrying fetuses with normal karyotypes (normal karyotype pregnancy group). The plasma concentrations of pregnancy-associated, placenta-specific microRNAs (miR-515-3p, -517a, -517c, and -518b) were measured by quantitative RT-PCR. *Results:* No significant differences in clinical characteristics were observed between the two groups. miR-515-3p, miR-517a, miR-517c, and miR-518b were shown to be present in significantly lower plasma concentrations in the trisomy 18 pregnancy group than in the normal karyotype pregnancy group. Plasma concentrations of cell-free miR-517c could distinguish pregnancies with fetal trisomy 18 from those with normal fetal karyotypes, yielding an area under the curve of 0.733 (95% confidence interval: 0.559–0.881). *Conclusion:* Cell-free pregnancy-associated, placenta-specific microRNAs may therefore be potential molecular markers to estimate the risk of pregnancies with fetal trisomy 18.

Key words: Placenta-specific microRNA; Pregnancy; Fetal trisomy 18; Maternal plasma; Molecular marker.

Introduction

Trisomy 18, known as Edwards syndrome, is the second most common chromosomal aneuploidy, with an average incidence of one in 6,000 pregnancies. It is caused by the presence of an extra copy of all or part of chromosome 18 [1], and its prevalence increases in a maternal age-dependent manner. Clinical features of fetuses carrying trisomy 18 include fetal growth restriction, polyhydramnios, overlapping fingers, and other congenital abnormalities [2, 3].

Amniocentesis and chorionic villus sampling are diagnostic procedures for fetal chromosomal aneuploidies, but both carry a risk of procedure-associated fetal loss [4], hence pregnant women and their families should be made aware of their invasive nature. As an alternative, attempts are being made to develop non-invasive prenatal tests based on markers to estimate the risk of pregnancies carrying fetuses with trisomy 18 [5]. To date, cell-free DNA in the maternal plasma and maternal serum biochemical markers in the first and second trimesters have been used as non-invasive prenatal tests [6, 7]. Because these are both of placental origin [7, 8], it is conceivable that other placenta-specific molecules in the maternal circulation could be used as candidate markers to estimate the risk of pregnancies with fetal trisomy 18.

MicroRNAs (miRNAs) act as regulators of gene expression, and recent studies have reported that pregnancy-associated miRNAs circulate in the maternal plasma [9, 10]. The authors also identified cell-free pregnancy-associated, placenta-specific miRNAs (miR-515-3p, -517a, -517c, and -518b) in the plasma of pregnant women [11]. These miRNAs are located at 19q13.42, which is commonly referred to as the chromosome 19 miRNA cluster (C19MC) [10-13]. The plasma concentration of miRNAs from the C19MC region can be measured by quantitative RT-PCR, and their aberrant levels have been reported in various pregnancy-associated diseases such as preeclampsia, placenta previa, and placental abruption [14-18]. This suggests that they could be used as biomedical markers of placental abnormalities [19-22], and as a means of estimating the risk of pregnancy with fetal chromosomal aneuploidy. However, the association between C19MC miRNA plasma concentrations and pregnancies carrying fetuses with chromosomal aneuploidies remains unknown.

In this study, we collected 49 plasma samples of high-age (≥ 35-years-old) pregnancies (13 cases carrying fetuses with trisomy 18, and 36 cases carrying fetuses of normal karyotype) during the second trimester (16–19 weeks of

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Table 1. — *Clinical characteristics of the pregnant women.*

Characteristic	Trisomy 18 pregnancy group (n=13)	Normal karyotype pregnancy group (n=36)	*p*-value
Maternal age, years; mean (SD)	37.7 (3.0)	39.8 (2.7)	NS [a]
Gestational age at sampling, weeks (range)	16 (16–18)	16 (16–19)	NS [a]
Gender			NS [b]
Male	5	22	
Female	8	14	

NS: not significant; [a] t-test; [b]chi-square test

gestation). The authors measured the circulating levels of miR-515-3p, miR-517a, miR-517c, and miR-518b in the maternal plasma samples to determine their association with pregnancies carrying fetuses with trisomy 18.

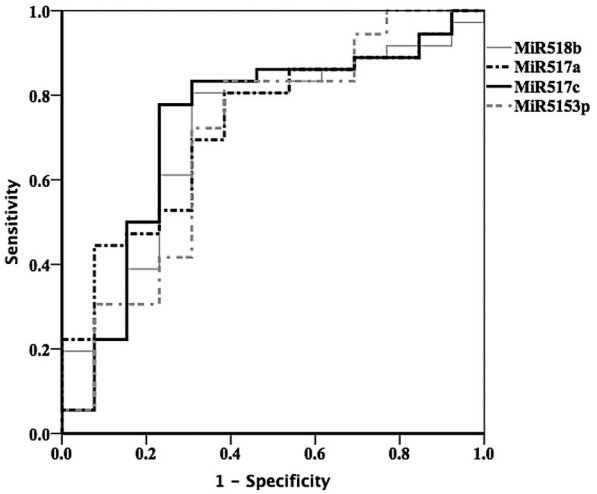

Figure 1. — ROC curve analysis using plasma pregnancy-associated placenta-specific miRNAs for discriminating trisomy 18 pregnancies from normal karyotype pregnancies. ROC analysis reveals high AUC values for each plasma C19MC miRNA (trisomy 18 pregnancy, $n = 13$; normal karyotype pregnancy, $n = 36$): miR-515-3p, miR-517a, miR-517c, and miR-518b yielded AUCs of 0.611 (95% CI: 0.422–0.800), 0.724 (95% CI: 0.570–0.879), 0.733 (95% CI: 0.559–0.881), and 0.709 (95% CI: 0.542–0.877), respectively.

Materials and Methods

All samples were obtained after receiving written informed consent and the study protocol was approved by the Institutional Review Board for Ethical, Legal and Social Issues of Nagasaki University.

Because of the high-age pregnancy (≥ 35-years-old), all pregnant women included in this study attended the Department of Obstetrics and Gynecology at Nagasaki University Hospital to undertake a prenatal diagnostic test for fetal chromosomal abnormalities. Genetic counseling was made available to the women and their partners during the first trimester, then amniocentesis was performed during the second trimester (16–19 weeks of gestation), and the fetal karyotype was determined by G-banding. Blood sampling was performed before the amniocentesis. Maternal blood samples (7 ml) were obtained from 13 pregnant women carrying fetuses with trisomy 18 (trisomy 18 pregnancy group) and from 36 pregnant women carrying fetuses with normal karyotypes (normal karyotype pregnancy group). All cases of fetal trisomy 18 in this study were full trisomy 18, in which an extra copy of the entire chromosome 18 was present. Table 1 summarizes the clinical characteristics of the women included in this study. There was no significant difference in clinical characteristics between the trisomy 18 pregnancy and the normal karyotype pregnancy groups.

Blood samples were collected in tubes containing ethylenediaminetetraacetic acid. Cell-free RNA was stable in plasma samples [23, 24]; therefore, cell-free plasma samples were prepared from maternal blood using a double centrifugation method as described previously [16-18, 25]. Briefly, after the first centrifugation at 3,000 × *g* for ten minutes, the supernatant was immediately stored at −80°C. Within six months, plasma samples were centrifuged at 16,000 × *g* for 10 minutes, and 1.2 mL of the supernatant (double-centrifuged plasma sample) was used as a plasma sample to extract cell-free RNA according to the manufacturer's instructions.

Pregnancy-associated, placenta-specific miRNAs (miR-515-3p, -517a, -517c, and -518b), and U6 snRNA (mature miRNA internal control) were selected for quantitative analysis [11]. The plasma concentration of each miRNA was measured by quantitative RT-PCR, as described previously [16-18, 25]. A total of 2.5 ng RNA sample was used for reverse transcription. Next, a calibration curve was prepared using ten-fold serial dilutions of single-stranded cDNA oligonucleotides corresponding to each miRNA sequence, representing 1.0×10^2–1.0×10^8 copies/mL. Each sample and each calibration dilution was analyzed in triplicate. Each assay had a detection limit of 100 RNA copies/mL [16-18, 25]. Every batch of amplifications included three water blanks as negative controls for each of the reverse transcription and PCR steps. All data were collected and analyzed. It was recommended that quantitative measurements of plasma mRNA be expressed as an absolute concentration [26]; therefore, we performed absolute quantitative RT-PCR analysis. Concentrations of U6 snRNA in each sample were determined as an internal control.

In each sample, the plasma concentrations of target miRNAs were adjusted relative to that of U6 snRNA. By using a compact benchtop instrument that enables rapid, high-precision PCR setup, the authors analyzed 49 samples in triplicate with standard curves and negative controls on a single 384-well plate. All experiments were run on the same 384-well plate. The intra-assay coefficients of variation, which were the ratios of the standard deviation to the mean for the probes in absolute quantitative RT-PCR, were 7.3% for miR-515-3p, 7.5% for miR-517a, 6.7% for 517c, 8.3% for miR-518b, and 7.8% for U6 snRNA.

Patient clinical characteristics were compared between the trisomy 18 pregnancy and the normal karyotype pregnancy groups using the Student's *t*-test for continuous variables, and the chi-square test for discrete variables. Differences between the two groups were evaluated using the Mann–Whitney U-test. Statistical analyses were performed using SPSS software version 22. *P* values < 0.05 were considered statistically significant. To evaluate the ability of miRNAs to discriminate trisomy 18 pregnancies from pregnancies with normal fetal karyotypes, receiver operating characteristic (ROC) curves were plotted using SPSS software version 22.

Table 2. — *Circulating levels of plasma cell-free pregnancy-associated miRNAs in the trisomy 18 pregnancy group and normal karyotype pregnancy group.*

Pregnancy-associated miRNA	Trisomy 18 pregnancy group (n=13)	Normal karyotype pregnancy group (n=36)	*p*-value
miR-515-3p	553.5 (113.55–4,531.7)	1,375.07 (208.7–12,666.57)	0.035
miR-517a	94,940.3 (1351.9–394,898.3)	231,438.9 (1799.5–1,791,652.8)	0.017
miR-517c	1,935.4 (157.8–24,277.9)	6,698.1 (122.0–33,983.9)	0.014
miR-518b	6,265.6 (247.4–45,698.5)	20,097.6 (207.4–120,248.1)	0.035

Circulating levels are indicated as the median with (minimum–maximum) copies/mL. Significant differences between the two groups were analyzed by the Mann–Whitney U-test.

Results

Circulating plasma levels of miR-515-3p, miR-517a, miR-517c, and miR-518b were significantly lower in the trisomy 18 pregnancy group than in the normal karyotype pregnancy group (Table 2; Mann–Whitney U-test, p = 0.035 for miR-515-3p, p = 0.017 for miR-517a, p = 0.014 for miR-517c, and p = 0.035 for miR-518b).

The diagnostic value of miR-515-3p, miR-517a, miR-517c, and miR-518b concentrations in maternal plasma samples was evaluated. ROC curves to discriminate trisomy 18 pregnancies from pregnancies with normal fetal karyotypes were constructed based on miR-515-3p, miR-517a, miR-517c, and miR-518b plasma concentrations. ROC analysis revealed high area under the curve (AUC) values for each plasma miRNA (Figure 1): miR-515-3p, miR-517a, miR-517c, and miR-518b yielded AUCs of 0.611 (95% confidence interval (CI): 0.422–0.800), 0.724 (95% CI: 0.570–0.879), 0.733 (95% CI: 0.559–0.881), and 0.709 (95% CI: 0.542–0.877), respectively. In addition, the authors attempted to combine the four miRNAs together. Although the miRNA signature (miR-517c and miR-517a) yielded AUC of 0.7415 (95% CI: 0.595–0.888), this was not significantly superior to AUC yielded by the miR-517c.

When 3861.3 copies/mL of miR-517c (representing the highest AUC) was selected as a cut-off negative/positive value (best for both sensitivity and specificity), sensitivity and specificity were 76.9% (10/13) and 77.8% (28/36), respectively. Positive predictive and negative predictive values for miR-517c were 55.6% (10/18) and 90.3% (28/31), respectively.

Discussion

In the present study, we clarified the association between plasma concentrations of cell-free pregnancy-associated, placenta-specific miRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b) from the C19MC region with pregnancies carrying fetuses with trisomy 18.

miR-515-3p, miR-517a, miR-517c, and miR-518b plasma concentrations were shown to be significantly lower in pregnant women carrying fetuses with trisomy 18 than in those carrying fetuses with normal karyotypes. Regarding the diagnostic value of these plasma miRNA levels, ROC curve analysis revealed that plasma miR-515-3p miR-517a, miR-517c, and miR-518b could all discriminate trisomy 18 pregnancies from normal karyotype pregnancies with high AUCs (Figure 1). This suggests that cell-free pregnancy-associated placenta-specific microRNAs in maternal plasma could be potential markers for the identification of pregnancies carrying fetuses with trisomy 18.

The villous trophoblast releases exosomes containing miRNAs into the maternal circulation, which is a source of these miRNAs in the plasma of pregnant women [10, 27]. The miRNAs analyzed in this study were located in the C19MC region, which is imprinted in the placenta with expression from the paternally inherited chromosome [28]. Because miRNAs function as regulators of gene expression by antisense complementarity to specific messenger RNAs [28-30], placenta-specific miRNAs on C19MC regulate placental development and function. A recent study reported that circulating levels of C19MC miRNAs in maternal plasma are positively associated with placental weight [31], while the placental weight of the early human fetus with trisomy 18 was shown to be typically decreased compared with that of the normal karyotype fetus [32]. This indicates that decreased plasma concentrations of C19MC miRNAs in pregnancies with fetal trisomy 18 may be associated with their placental weight.

Other studies found that aberrant levels of cell-free C19MC miRNAs in the maternal plasma were associated with pregnancy-associated diseases caused by abnormal placentation, such as preeclampsia, placenta previa, placental abruption [14-22]. Although the functional association between C19MC miRNAs and placentas with chromosomal aneuploidies remains unknown, circulating levels of miRNAs from the C19MC region in the maternal plasma may reflect the difference in placental status between fetal trisomy 18 and fetuses of normal karyotype. Further investigation of the biological function of C19MC miRNAs in placentas with chromosomal aneuploidy may lead to the discovery of treatments for placental dysfunction caused by chromosomal aneuploidy.

In conclusion, we clarified for the first time that circulating levels of cell-free pregnancy-associated, placenta-specific miRNAs (miR-517a, miR-517c, miR-518b, and miR-515-3p) are decreased in pregnancies carrying fetuses with trisomy 18 compared with those with normal karyotypes. Although the present data are still preliminary because of the small sample size, the measurement of cell-free

pregnancy-associated placenta-specific miRNAs in plasma may be used as non-invasive prenatal tests to estimate the risk of pregnancies carrying fetuses with trisomy 18.

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