CEOG Clinical and Experimental Obstetrics & Gynecology

Decreased cell-free but not exosomal miR-518b in maternal plasma is caused by amniocentesis

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Summary

Aim: To obtain knowledge of the relationship between cell-free and exosomal pregnancy-associated microRNAs in maternal plasma, the authors investigated their circulating levels before and after amniocentesis. *Materials and Methods:* In 16 maternal plasma samples collected before and after amniocentesis, circulating levels of cell-free and exosomal pregnancy-associated microRNAs (miR-515-3p, -517a, -517c, -518b, and 323-3p) were measured by real-time quantitative RT-PCR. Changes in the plasma concentration and the association between circulating levels of cell-free and exosomal microRNAs were analyzed. Statistical significance was defined as $p < 0.05$. *Results:* The plasma concentration of cell-free miR-518b was significantly decreased after amniocentesis (Wilcoxon signed rank test, $p = 0.001$), but no significant difference was observed in any other cell-free or exosomal microRNA ($p > 0.05$). There was no association between the circulating levels of cell-free and exosomal microRNAs (miR-515-3p, -517a, -517c, -518b) in chromosome 19 microRNA cluster region. However, a significant association was detected between plasma cell-free and exosomal miR-323-3p levels in chromosome 14 microRNA cluster region (r and P values before amniocentesis; 0.617 and 0.011, those after amniocentesis; 0.899 and < 0.001). *Conclusions:* Amniocentesis caused a decreased cell-free but not exosomal miR-518b levels in maternal plasma. Correlations between plasma concentrations of cell-free and exosomal pregnancy-associated microRNAs were different for each microRNA.

Key words: MicroRNA; Amniocentesis; Cell-free; Exosome; Maternal plasma.

Introduction

MicroRNAs (miRNAs) are non-protein coding small RNAs (21-25 nucleotides) that function as regulators of gene expression by antisense complimentarily to specific messenger RNAs [1-3]. Recently, pregnancy-associated placental miRNAs in the plasma of pregnant women have been identified, and are potential molecular markers to monitor the status of pregnancy [4, 5]. The present authors previously showed that plasma concentrations of cell-free pregnancy-associated placental miRNAs (miR-515-3p, -517a, -517c, -518b, and 323-3p) were associated with diseases during pregnancy (preeclampsia, placenta previa, and placenta abruption) [6-8], or with abnormal pregnancy (molar pregnancy, ectopic pregnancy, and abortion) [9, 10]. However, the association between plasma concentrations of pregnancy-associated placental miRNAs and invasive prenatal diagnostic procedures (for example; amniocentesis, chorionic villus sampling) remains unknown.

In plasma samples from pregnant women, both cell-free and exosomal pregnancy-associated placental miRNAs (miR-515-3p, -517a, -517c, -518b, and 323-3p) are circulating and both types (cell-free or exosomal) miRNAs are measurable by quantitative RT-PCR [4, 5, 11, 12]. Recent study showed that cell-free plasma and exosomes prepared from maternal plasma had different profiles and concentrations of miRNAs [12]. However, the correlations between cell-free and exosomal pregnancy-associated placental miRNA levels in maternal plasma samples also remains unknown.

In this study, to elucidate the aforementioned questions, the authors performed a quantitative analysis of cell-free and exosomal pregnancy-associated placental miRNAs (miR-515-3p, -517a, -517c, -518b, and 323-3p) in maternal plasma samples that were collected before and after amniocentesis. Then they evaluated the correlations between the quantified levels circulating cell-free and exosomal miRNAs in the same maternal plasma samples.

Materials and Methods

Sixteen women with singleton pregnancies at 16 weeks of gestation, who attended the Department of Obstetrics and Gynecology at Nagasaki University hospital to undergo amniocentesis because of their advanced ages (ranging from 35- to 42-years-old), participated in the study. For all 16 cases, amniocentesis was performed using a 22G needle, and there was no case of transplacental puncture. Written and informed consent was obtained from all participants and the study was approved by the Research Ethics Committee of Nagasaki University. Blood samples (7 ml) from each woman were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) before and 10 minutes after amniocentesis. Analysis of amniocentesis samples showed that the

Revised manuscript accepted for publication September 4, 2017

karyotypes of all fetuses were normal. The women included in this study had no complications during their pregnancies.

Cell-free RNA is stable in plasma samples [13, 14]; therefore, cell-free plasma samples were prepared from maternal blood using a double centrifugation method as described previously [4, 5, 15]. Briefly, after a first centrifugation at 3,000 ×*g* for 10 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 supernatant (double-centrifuged plasma sample) was used for the extraction of cell-free RNA. Cell-free RNA containing small RNA molecules (ranging from 40 to 60 ng/μL) was extracted, according to the manufacturer's instructions.

After a first centrifugation of plasma samples at 2,000 ×*g* for 20 minutes, the supernatants were centrifuged at 10,000 ×*g* for 20 minutes. Exosomes were then isolated from 1.2 mL of supernatant (from plasma) according to the manufacturer's instructions. Briefly, 1.0 mL of plasma was transferred to a new tube and 0.5 mL of PBS was added and mixed, and then 50 μL of Proteinase K were added and incubated at 37°C for 10 minutes. Subsequently, 300 μL of the exosome precipitation reagent (from plasma) were added to the sample. After incubation, the sample was centrifuged at 10,000 × *g* for five minutes at room temperature. Exosomes were contained in the pellet at the bottom of the tube. The pellet was resuspended in 100 μL of PBS. Resuspended exosomes were immediately stored at -80°C. Within six months, total exosomal RNA was extracted, according to the manufacturer's instructions.

The concentration of extracted RNA was determined using a and is expressed as nanograms per milliliter of plasma. Pregnancy-associated placenta-specific miRNAs (miR-515-3p, 517a, miR-517c, and miR-518b) on chromosome 19 miRNA cluster region (C19MC), pregnancy-associated but not placenta-specific miRNA (miR-323-3p) on chromosome 14 miRNA cluster region (C14MC), and U6 snRNA (mature miRNA internal control) were analyzed in this study [10]. Real-time qRT-PCR of miRNAs in plasma samples was performed as described previously [4, 5, 9, 10, 16]. Initially, 2.5 ng of RNA was used for reverse transcription. Subsequently, for the qRT-PCR of miRNAs, the authors prepared a calibration curve by ten-fold serial dilutions of single-stranded cDNA oligonucleotides corresponding to each miRNA sequence at 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 300 RNA copies/mL [5, 9, 10, 16]. 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 is recommended that quantitative mRNA measurements in plasma samples are expressed as an absolute concentration [17]; therefore, the authors applied the same recommendation to the quantitative measurement of miRNAs in plasma. Hence, absolute qRT-PCR analysis was performed, and the concentration of U6 snRNA in each sample was used as an internal control. In each sample, the plasma concentrations of target miRNAs were adjusted relative to the plasma concentration of U6 snRNA. Using a compact benchtop instrument that enables a rapid, high-precision PCR setup, the authors analyzed 16 samples in triplicate on a plate together with the standard curves and negative controls. All experiments were run on the same 384-well plate.

For cell-free and exosomal miRNAs, changes in the plasma concentration of miRNAs before and after amniocentesis were compared using the Wilcoxon signed-rank test. Pearson product-moment correlation coefficients between circulating levels of cell-free and exosomal miRNAs in maternal plasma were analyzed. Statistical analyses were performed using SPSS version 22. Statistical significance was defined as $p < 0.05$.

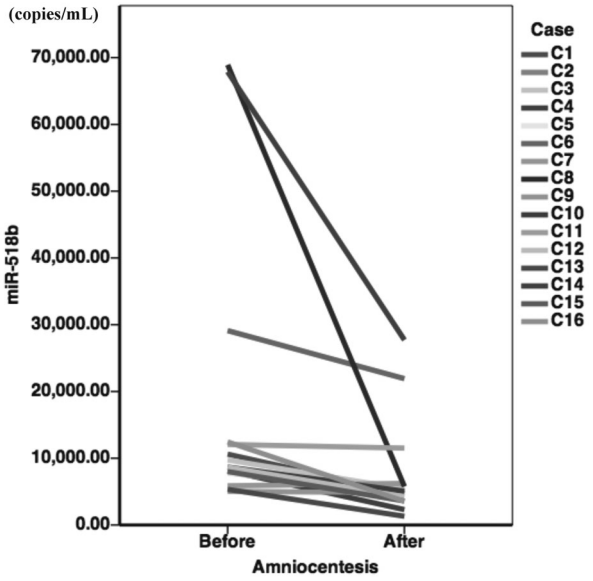

Figure 1. — Changes in plasma concentrations of cell-free miR-518b before and after amniocentesis. Circulating levels of cell-free miR-518b in maternal plasma are expressed as copies/mL. Plasma level of miR-518b is significantly decreased after amniocentesis (Wilcoxon signed-rank test, $p = 0.001$).

Results

Quantitative data for cell-free and exosomal miRNAs (miR-515-3p, -517a, -517c, -518b, and -323-3p) before and after amniocentesis are shown in Table 1. The plasma concentration of cell-free miR-518b was significantly decreased after amniocentesis (Figure 1; Wilcoxon singed rank test, $p = 0.001$), although there was no significant difference in the plasma concentration of exosomal miR-518b before and after the procedure ($p > 0.05$). With regards to the other miRNAs (miR-515-3p, -517a, -517c, and -323-3p), there were no significant differences in plasma concentrations of cell-free or exosomal miRNAs before and after the procedure ($p > 0.05$).

Table 2 summarizes the association between levels of cell-free and exosomal pregnancy-associated miRNAs in maternal plasma. Both before and after amniocentesis, no association was seen between the circulating levels of cell-free and exosomal pregnancy-associated placenta-specific miRNAs (miR-515-3p, -517a, -517c, and -518b) on C19MC. On the other hand, with regards to the pregnancy-associated but not placenta-specific miRNA (miR-323-3p) on C14MC, a significant association was detected between cell-free and exosomal miRNA levels in maternal plasma (*r* and *p* values before amniocentesis; 0.617 and 0.011, those after amniocentesis; 0.899 and < 0.001).

Table 1. — *Circulating levels of cell-free and exosomal pregnancy-associated miRNAs in maternal plasma before and after amniocentesis.*

miRNA	Type	Circulating levels of miRNA in maternal plasma*		*p*-value**
		Before amniocentesis	After amniocentesis	
miR-518b	Cell-free	8674.34 (5036.41-68915.82)	5007.07 (1308.63-27721.59)	0.001
	exosome	10451.11 (2623.08-63018.29)	9028.93 (3667.26-45516.03)	0.796
miR-517a	Cell-free	48160.927 (17951.20-998515.34)	50542.67 (15363.20-154819.19)	0.234
	exosome	142200.93 (70429.70-631105.48)	181286.220 (82747.17-658797.12)	0.063
miR-515-3p	Cell-free	5210.30 (2458.48-55089.01)	6128.78 (825.49-29660.28)	0.679
	exosome	41559.73 (19972.48-227457.99)	53517.37 (20175.17-247564.84)	0.215
miR-517c	Cell-free	39580.05 (22995.44-416539.17)	43344.29 (15503.16-113688.11)	0.379
	exosome	108548.57 (62265.39-349309.45)	136671.36 (73964.88-397383.48)	0.056
miR-323-3p	Cell-free	3822.6 (1599.1-10348..8)	3872.7 (2056.3-28758.6)	0.605
	exosome	34125.6 (6810.2-426415.7)	43054.1 (12741.2-734792.9)	0.501

**median (minimum-maximum) copies/mL, **Wilcoxon signed-rank tests (significance is defined as p < 0.05).*

Table 2. — *Summary of correlation coefficient analysis between plasma concentrations of cell-free and exosomal pregnancy-associated miRNAs.*

Pregnancy- associated miRNA	Amniocentesis	Chromosomal location	Expression pattern	Statistical analysis	*r*-value	*p*-value
miR518b	Before	C19MC	Placenta- specific expression	Pearson product-moment correlation coefficient	0.359	0.172
	After				0.388	0.137
miR517a	Before				-0.058	0.831
	After				-0.089	0.744
miR515-3p	Before				-0.104	0.702
	After				-0.252	0.346
miR517c	Before				0.022	0.936
	After				-0.155	0.566
miR323-3p	Before	C14MC	Embryonic and placental expression	Pearson product-moment correlation coefficient	0.617	0.011
	After				0.899	<0.001

Significances are defined as p < 0.05. C19MC; chromosome 19 miRNA cluster region, C14MC; chromosome 14 miRNA cluster region.

Discussion

Here, for the first time, the authors measured the circulating levels of cell-free and exosomal pregnancy-associated placental miRNAs in maternal plasma before and after amniocentesis. Subsequently, the authors investigated the correlations between circulating levels of cell-free and exosomal pregnancy-associated placental miRNAs in the same maternal plasma samples.

First, among pregnancy-associated miRNAs (miR-515-3p, -517a, -517c, -518b, and -323-3p), the level of cell-free miR-518b in maternal plasma was significantly decreased after amniocentesis, while no significant change was observed for the other cell-free and exosomal miRNAs after the invasive procedure. In addition, it is noteworthy that plasma concentrations of C19MC miRNAs (miR-515-3p, -517a, and -517c, but excluding miR-518b) showed a non-significant tendency to increase after amniocentesis (Table 1). In previous studies by the present authors and others [18, 19], plasma concentrations of cell-free fetal DNA increased after amniocentesis, suggesting that increased levels of cell-free fetal DNA in maternal plasma might be explained by direct connection between the placenta and maternal circulation. However, the plasma concentration of cell-free miR-518b was significantly decreased after amniocentesis, but that of exosomal miR-518b did not change significantly before or after amniocentesis. Although the relationship between cell-free and exosomal miRNA in maternal plasma remains unknown, this discrepancy between cell-free and exosomal miR-518b in maternal plasma may reflect different profiles of miRNAs in cell-free plasma and exosomes [12]. This phenomenon was also seen in cases of placenta previa [7]. Compared with plasma samples from uncomplicated pregnancies, levels of cell-free fetal DNA in maternal plasma were significantly higher in the cases of placenta previa. On the other hand, increased levels of cell-free miR-517a and decreased levels of cell-free miR-518b were seen in maternal plasma samples from placenta previa pregnancies. Furthermore, increased expression of miR-517a and decreased expression of miR-518b were also seen in placentas with fetal growth restriction (FGR), compared with placentas that were large or of adequate size for the gestational age [20]. How the plasma concentration of cell-free miR-518b decreased after amniocentesis remains

unknown; however, the above results suggest that a decrease in cell-free but not exosomal miR-518 is a potential miRNA signature that can reflect the placental condition of invasive procedure. Pregnancy-associated placental miRNAs in the maternal circulation may participate in fetoplacental-maternal communication by influencing local and distant target tissues [21]. In this study, the decrease in cell-free miR-518b levels in maternal plasma after amniocentesis may be caused by changes in the utero-placental condition (e.g. uterine contraction and/or maternal fetal hemorrhage due to amniocentesis), though further examination in cases of other invasive procedures will be necessary.

Subsequently, to clarify the status of cell-free and exosomal pregnancy-associated miRNAs in maternal plasma, the authors analyzed the association between plasma concentrations of cell-free and exosomal pregnancy-associated miRNAs. There was no association between the levels of cell-free and exosomal C19MC miRNAs (miR-515-3p, -517a, -517c, and -518b) in maternal plasma, suggesting that cell-free C19MC miRNAs are markers that are independent of exosomal C19MC miRNAs in maternal plasma. On the other hand, with regards to the C14MC miRNA (miR-323-3p), a significant association was seen between cell-free and exosomal miRNA levels in maternal plasma. Both C19MC miRNAs (miR-515-3p, -517a, -517c, and -518b) on 19q13.41 and the C14MC miRNA (miR-323-3p) on 14q32 are pregnancy-associated placental miRNAs. However, expression patterns of both clusters are known to have various differences. C19MC contains placenta-specific miRNAs [11, 22, 23], while C14MC miRNAs are predominantly expressed in the placenta but are not placenta-specific [23]. From the first to the third trimester, expression of C19MC miRNAs increases, but that of C14MC miRNAs decreases [24, 25]. From the first to the third trimester, levels of C19MC miRNAs in maternal plasma increase, while those of C14MC miRNAs do not change [5]. In addition, both C19MC and C14MC miRNAs are known to be located within imprinted loci [23, 26, 27]. C19MC miRNAs are expressed from the paternally inherited chromosome [26], while C14MC miRNAs are expressed from the maternal chromosome [27]. In the maternal circulation, the expression patterns of each pregnancy-associated placental miRNA may affect the association between the levels of cell-free and exosomal miRNAs in maternal plasma.

A limitation of this study is that the sample size was too small to detect differences before and after amniocentesis and to evaluate differences in the circulating plasma levels between cell-free and exosomal miRNAs. Further large-scale studies are necessary to confirm the clinical significance of the results in this pilot-study. Another limitation of this study was that only five pregnancy-associated placental miRNAs were analyzed. To date, numerous placental miRNAs have been reported as pregnancy-associated molecules [4, 5, 23, 28]. Also, the present authors collected blood samples at only two time points to investigate changes in plasma concentration of miRNAs in maternal plasma before and after amniocentesis. In future studies, both measurement of other pregnancy-associated miRNAs and frequent sampling should be performed to determine the time-dependent changes in circulating miRNAs profiles after amniocentesis.

In conclusion, the authors showed that a decrease in cell-free but not exosomal miR-518b in maternal plasma was seen after amniocentesis. Also, the circulating levels of cell-free and exosomal C19MC miRNAs were not associated with each other in maternal plasma, while significant correlation between cell-free and exosomal C14MC miRNA was detected, suggesting that correlations between plasma concentrations of cell-free and exosomal miRNAs are different among miRNAs and may depend on their expression patterns. Further investigation of how cell-free and exosomal pregnancy-associated miRNAs circulate in maternal plasma could provide a new approach to monitor pregnancy.

Acknowledgement

The authors would like to thank Yasuko Noguchi for her technical assistance in sample preparation. K.M., A.H., S.M., A.Y., and H.M. were supported by JSPS KAKENHI, grant numbers 17K11241, 17K16302, 15K10677, 15K10677, and 17K11283, respectively.

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