Increased levels of cell-free miR-517a and decreased levels of cell-free miR-518b in maternal plasma samples from placenta previa pregnancies at 32 weeks gestation

Yuri Hasegawa M.D. [1], Kiyonori Miura M.D., Ph.D. [1,*], Ai Higashijima M.D., Ph.D. [1], Shuhei Abe M.D., Ph.D. [1], Shoko Miura M.D., Ph.D. [1], Koh-ichiro Yoshiura M.D., Ph.D. [2], Hideaki Masuzaki M.D., Ph.D. [1]

[1]Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[2]Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

***Address correspondence to:** Kiyonori Miura
Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
Tel: +81-95-819-7363; fax: +81-95-819-7365; e-mail: kiyonori@nagasaki-u.ac.jp

Running head: Placenta-specific microRNAs and placenta previa

19 **Abbreviated Short Title:** Plasma concentrations of placenta-specific
20 microRNAs in placenta previa pregnancies

21

22 Manuscript word, table/figure counts; Words: 3,441, Tables: 3, Figures: 2

23

24 **Grant/funding Support:** K.M., S.M., and H.M. were supported by JSPS
25 KAKENHI (grant numbers 26462495, 24791712, and 25462563,
26 respectively).

27

28 **Conflict of interest:** None of the authors have any conflict of interest.

29

Abstract

Objective: The aim of this study was to clarify the association between placenta previa and circulating levels of cell-free pregnancy-associated placenta-specific miRNAs in maternal plasma.

Method: Twenty singleton pregnancies with placenta previa (placenta previa group) and 26 uncomplicated pregnancies (control group) were recruited. Blood sampling was performed at 32 weeks gestation and cesarean delivery in all cases of placenta previa was performed at a mean gestational age of 37 weeks. The maternal plasma concentrations of cell-free pregnancy-associated placenta-specific miRNAs (miR-517a and miR-518b) were measured by absolute quantitative real-time RT-PCR.

Results: Plasma concentrations of cell-free miR-517a in the placenta previa group were significantly higher than that in the control group (P=0.011), while the plasma concentration of cell-free miR-518b in the placenta previa group was significantly lower than that in the control group (P=0.004). Plasma concentrations of cell-free miR-517a in placenta previa were significantly higher in placenta previa with alert bleeding later group than those in placenta previa without alert bleeding group or control group (P =

0.030 or 0.047, respectively), and correlated with the volume of hemorrhage at delivery (R and P-value: 0.512 and 0.025).

Conclusion: Plasma concentrations of cell-free miR-517a and miR-518b at 32 weeks gestation were altered in pregnant women with placenta previa, and the circulating level of cell-free miR-517a in placenta previa may be a predictive marker for the risks of alert bleeding later and massive hemorrhage at delivery.

Keywords: pregnancy-associated microRNA, maternal plasma, placenta previa, alert bleeding, hemorrhage

Introduction

Placenta previa shows the abnormal location of the placenta, and women with placenta previa should be delivered by cesarean section. Although the prevalence of placenta previa has been approximately 0.5% of all pregnancies, placenta previa has the risks of emergency cesarean section following alert bleeding and massive hemorrhage at cesarean section[1]. Therefore, placenta previa is one of major causes of maternal morbidity and mortality. However, alert bleeding during pregnancy and massive hemorrhage at cesarean section are not observed in all women with placenta previa. The ability to predict alert bleeding during pregnancy and massive hemorrhage at cesarean delivery is critical in the management of placenta previa prenatally, because prenatal diagnosis allows for a planned approach under a more controlled condition with a possible treatment.

Risk factors for placenta previa include prior cesarean delivery, pregnancy termination, intrauterine surgery, smoking, multifetal gestation, increasing parity, and maternal age[1]. Placenta previa also has a high risk of placenta accreta, which is defined as an abnormal adherence of the placenta to the uterine wall[1]. Although placenta accreta

71 has the risk of massive hemorrhage, this condition is usually found at the time of
72 delivery and its final diagnosis was confirmed by pathological examination after the
73 surgery. Therefore, placenta previa is screened by transvaginal ultrasonography
74 prenatally, and anterior placentation is a risk factor for massive hemorrhage during
75 cesarean section in patients with placenta previa[2]. Although the prediction of the risks
76 of alert bleeding and massive hemorrhage prenatally seems to be made by
77 ultrasonography and magnetic resonance imaging (MRI), which are readily available in
78 most centers[3-5], the diagnostic accuracies of ultrasonography and MRI are still
79 unsatisfactory. Information regarding prediction of hemorrhage in placenta previa at
80 delivery is limited. Therefore, advent of a noninvasive prenatal diagnostic procedure to
81 estimate the risks of alert bleeding during pregnancy and massive hemorrhage during
82 cesarean section in patients with placenta previa is undoubtedly a great advance for
83 clinical management of placenta previa.

84 Recently, pregnancy-associated placenta-specific miRNAs circulating in
85 maternal plasma have been reported[6,7], and we identified pregnancy-associated
86 placenta-specific miRNAs (miR-515-3p, miR-517a, miR-517c, miR-518b and

87 miR-526b) in the plasma of pregnant women[8]. A major source of cell-free placental
88 miRNAs in maternal plasma is the villous trophoblast, which is able to release
89 exosomes containing miRNAs into the maternal circulation[9,10]. To date, several
90 placental miRNAs on chromosome 19 miRNA cluster (C19MC) region are involved in,
91 or associated with, preeclampsia that is caused by abnormalities in the development of
92 placental vessels in early pregnancy[11]. Therefore, miRNAs predominantly expressed in
93 the placenta are probably involved in placental differentiation and in the maintenance of
94 pregnancy[12]. Because the placenta is a source of supply for cell-free
95 pregnancy-associated placenta-specific miRNAs[6-8], the measurement of cell-free
96 pregnancy-associated placenta-specific miRNAs in maternal plasma may have the
97 potential to become novel biomarkers to predict the condition of placenta previa.
98 However, circulating levels of cell-free pregnancy-associated placenta-specific miRNAs
99 in plasma samples with placenta previa remains unknown.

100 Here, to get the knowledge of association between placenta previa and
101 cell-free pregnancy-associated placenta-specific miRNAs, we measured the circulating
102 levels of cell-free pregnancy-associated placenta-specific miRNAs (miR-517a and

103 miR-518b) and cell-free pregnancy-associated but not placenta-specific miRNA
104 (miR-323-3p) in maternal plasma with placenta previa at 32 weeks gestation[8].
105
106 **Materials and Methods**
107
108 ***Sample Collection***
109 All pregnant women recruited for this study attended Nagasaki University
110 Hospital. All samples were obtained after receiving written informed consent, and the
111 Institutional Review Board for Ethical, Legal and Social Issues of Nagasaki University
112 approved the study protocol.
113 Women with multiple gestations, preterm labor, infection, fetal anomalies or
114 aneuploidy, fetal growth restriction, or preeclampsia, or who were current smokers were
115 excluded. From April, 2013 to January, 2014, we obtained blood samples (7 mL) from
116 20 singleton pregnancies with placenta previa (placenta previa group) and 26
117 uncomplicated pregnancies (control group) at 32 weeks gestation. Gestational age was
118 calculated from the date of their last menstrual period, and then confirmed by

119 ultrasonographic examination, which the crown rump length (CRL) of fetus at 8-10
120 week gestation was measured. Cesarean delivery was performed in all cases of placenta
121 previa at a mean gestational age of 37 weeks. The volume of hemorrhage of each case
122 was defined as the volume of bleeding at cesarean delivery. However, Case 11
123 underwent cesarean hysterectomy and was excluded from the analysis (Table 1). In this
124 case, the placenta was located anterior to the uterine wall with a history of previous
125 cesarean section and was diagnosed as placenta previa–increta by pathological
126 examination of the placenta and uterus. There were no significant differences in clinical
127 variables, including maternal age (years), gestational age (weeks), number of prior
128 cesarean deliveries, artificial abortion, parity, placental weight (g), and birth-weight of
129 the newborn (g) between the two groups (Table 2). At the time of blood sampling, the
130 women showed no signs of labor and no history of vaginal bleeding (alert bleeding).
131 Blood samples were collected in tubes containing EDTA. Cell-free plasma samples
132 were prepared from maternal blood by a double centrifugation method as described
133 previously[6,8,14]. After the first centrifugation at 3,000 ×g for 10 min, the plasma samples
134 were frozen at -80 °C for a median 3 months (ranging from 1 month to 9 months).

When the quantification analysis was performed, 1.6 mL of each plasma sample was centrifuged at 16,000 ×g for 10 min to remove blood cells. Total RNA containing small RNA molecules was extracted from 1.2 mL of maternal plasma using a *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The concentration of extracted RNA was determined measuring the absorbance at 260 nm on a spectrophotometer (NanoDrop ND-2000; NanoDrop Technologies, Wilmington, DE, USA) and expressed as nanograms per milliliter of plasma.

Real-time qRT-PCR analysis of miRNAs

As pregnancy-associated placenta-specific miRNAs, miR-517a and miR-518b, which locates on chromosome 19 miRNA cluster region (C19MC), were used for this study, because these two miRNAs were also reported as placental miRNA in maternal plasma in three other studies[8,10,15-18]. Furthermore, by public database search of the expression patterns of miR-517a and miR-518b, these two miRNAs show a placenta-specific expression pattern (www.microRNA.org). Our previous study also identified the miR-323-3p as pregnancy-associated miRNA in maternal plasma[7].

151 However, the expression pattern of miR-323-3p, which is located on chromosome 14
152 miRNA cluster region (C14MC), is expressed in embryonic and placental tissues, and in
153 adults, it is restricted to the brain[17]. Therefore, we measured the plasma concentration of
154 cell-free miR-323-3p as the pregnancy-associated but not placenta-specific miRNA[17].
155 All specific primers and TaqMan probes were purchased from TaqMan MicroRNA
156 Assays (Applied Biosystems, Foster City, CA, USA). Real-time quantitative RT-PCR
157 (qRT-PCR) of miRNAs in plasma samples was performed as described previously[6,8,19,20].
158 For each miRNA assay, 2.5 ng of total RNA samples were analyzed for qRT-PCR. We
159 prepared a calibration curve by 10-fold serial dilution of single-stranded cDNA
160 oligonucleotides corresponding to each miRNA sequence from 1.0×10^2 to 1.0×10^8
161 copies/mL. The concentrations of each single-stranded cDNA oligonucleotides were
162 decided by QX100 droplet digital PCR (BioRad, Hercules, CA, USA). Each sample and
163 each calibration dilution was analyzed in triplicate. Each assay could detect down to
164 356 RNA copies/mL[8,19,20]. Every batch of amplifications included three water blanks as
165 negative controls for each of the reverse transcription and PCR steps. All data were
166 collected and analyzed using a LightCycler® 480 Real-Time PCR System (Roche,

167 Pleasanton, CA, USA). The intra-assay coefficients of variation (CV), which were the
168 ratios of the SD to the mean for the probes (miR-517a, miR-518b and miR-323-3p) in
169 the absolute qRT-PCR, were 7.2, 8.1 and 6.7%, respectively. There are no universally
170 accepted internal controls in either placental tissue or maternal circulation for miRNA
171 analysis, because snoRNAs and snRNAs, including RNU48 and RNU6B, have been
172 suggested as reference RNAs, but exhibit high variability[21,22]. Additionally, it was
173 recommended that the quantitative mRNA measurements in plasma were to be
174 expressed as an absolute concentration[23]. Therefore, we considered that the quantitative
175 miRNA measurements might be the same as quantitative mRNA measurements in
176 plasma. Here, absolute real-time qRT-PCR analysis was performed.
177
178 ***Ultrasonographic examination of placenta previa***
179 The prenatal prediction of placenta accreta in the placenta previa group was estimated
180 by ultrasonographic findings, which were suggestive of placenta accreta, including: (1)
181 irregularly shaped placental lacunae within the placenta; (2) thinning of the
182 myometrium overlying the placenta; (3) loss of retroplacental clear space; and (4)

183 increased vascularity of the uterine serosa-bladder interface[24]. When the cases of
184 placenta previa fulfilled two or more of four ultrasonografic features, these cases were
185 managed as suspected placenta previa-accreta and MRI test was performed to attempt to
186 further define the diagnosis.
187
188 ***Statistical analysis***
189 Patients' backgrounds were compared by Student's t-test and chi-square test
190 for continuous and discrete variables, respectively, in the placenta previa and control
191 groups. Circulating levels of cell-free plasma concentrations of pregnancy-associated
192 placenta-specific miRNAs in both groups were converted into multiples of the median
193 (MOM) in the control group and adjusted for gestational age. Differences between the
194 two groups were evaluated with the Mann–Whitney U test. Differences between the
195 three groups were evaluated using the Kruskal-Wallis test, and then differences between
196 the two groups were evaluated using Bonferroni correction for post hoc analysis of
197 multiple comparisons. Pearson product-moment correlation coefficients between
198 circulating levels of pregnancy-associated placenta-specific miRNAs in maternal

199 plasma and the volume of hemorrhage at delivery were analyzed. Statistical analyses
200 were performed with SPSS version 19 (IBM Corporation, Armonk, NY, USA).
201 Significances were defined as $P<0.05$.
202
203 **Results**
204 ***Circulating levels of plasma cell-free pregnancy-associated placenta-specific miRNAs***
205 ***in the placenta previa and control groups***
206 Table 1 summarizes cell-free miR-517a levels, cell-free miR-518b levels,
207 volumes of hemorrhage at cesarean delivery, and ultrasonographic features in cases of
208 placenta previa pregnancies. Cases 3, 9, 10, 11, 15 and 17 were managed as suspicion of
209 placenta previa-accreta and high risk of massive hemorrhage at delivery, because these
210 cases fulfilled two or more of four ultrasonografic features, which were suggestive of
211 placenta previa-accreta. In these six cases of suspected placenta previa-accreta, MRI test
212 was performed to further define the diagnosis. However, five cases except for Case 10
213 were diagnosed as suspicion of placenta previa-accreta by MRI test. Case 11 was
214 excluded from this study, because placenta removal was impossible at delivery and

215 cesarean hysterectomy was performed in this case. Case 11 was diagnosed as placenta
216 previa-increta by histological examination of placenta–uterus. Cases 9, 10, and 15
217 showed 2,000ml or more hemorrhage at delivery. However, 2,000ml or more
218 hemorrhage was also seen in Cases 4, 5, 12, 19, and 20, which were managed as low
219 risk of massive hemorrhage because these cases fulfilled one or none of four
220 ultrasonografic features, which were suggestive of placenta accreta.

221 Median (minimum–maximum) MoM values of plasma cell-free
222 pregnancy-associated placenta-specific miRNAs in the control (n=26) and placenta
223 previa (n=19) groups were 1.00 (0.11–37.58) and 5.15 (0.47–24.09) for miR-517a, 1.00
224 (0.46–6.89) and 0.12 (0.01–9.86) for miR-518b, respectively (Figure 1). Circulating
225 levels of plasma cell-free miR-517a levels in the placenta previa group were
226 significantly higher than that in the control group (Mann–Whitney U test, P=0.011),
227 while circulating levels of plasma cell-free miR-518b levels in the placenta previa group
228 were significantly lower than that in the control group (Mann–Whitney U test, P=0.004)
229 (Figure 1).

230 Tentatively, although sample numbers were small (Cases 3, 9, 10, 11, 15 and 17), to

231 evaluate the potential value of plasma pregnancy-associated placenta specific miRNAs
232 in cases of suspected placenta previa-accreta, median (minimum–maximum) MoM
233 values of plasma cell-free pregnancy-associated placenta-specific miRNAs in the
234 control (n=26) and placenta previa (n=19) groups were 1.00 (0.11–37.58) and 14.09
235 (0.85–265.12) for miR-517a, 1.00 (0.46–6.89) and 31.3 (0.01–17.45) for miR-518b,
236 respectively (Figure 1). Circulating levels of plasma cell-free miR-517a levels were
237 significantly higher in six cases of suspected placenta previa-accreta than in the control
238 group (Mann–Whitney U test, P=0.022), while there was no significant difference in
239 circulating levels of plasma cell-free miR-518b in both the cases of suspected placenta
240 previa-accreta and the control group (P>0.05).
241
242 ***Circulating levels of plasma cell-free pregnancy-associated placenta-specific miRNAs***
243 ***in placenta previa with alert bleeding group, placenta previa without alert bleeding***
244 ***group, and control group***
245 In 19 cases of placenta previa, seven cases had alert bleeding later, and
246 remaining 12 cases had no alert bleeding during pregnancy. Median (minimum–

247 maximum) MoM values of plasma cell-free pregnancy-associated placenta-specific
248 miRNAs in placenta previa with alert bleeding group (n=7), placenta previa without
249 alert bleeding group (n=12), and uncomplicated pregnancies as control group (n=26)
250 were 10.66 (5.15–24.09), 2.20 (0.47–7.72) and 1.00 (0.11–37.58) for miR-517a, 1.48
251 (0.01–9.86), 0.064 (0.01–7.35) and 1.00 (0.46–6.89) for miR-518b, respectively (Table
252 3). Plasma concentrations of cell-free pregnancy-associated miRNAs (miR-517a and
253 miR-518b) were confirmed to have significantly different plasma concentrations in
254 women with the placenta previa with alert bleeding, placenta previa without alert
255 bleeding or control groups (Kruskal-Wallis test; Table 3). Plasma concentrations of
256 cell-free miR-517a were significantly higher in the placenta previa with alert bleeding
257 group than in placenta previa without alert bleeding group or control group ($P = 0.030$
258 or 0.047, respectively; Table 3). However, there was no significantly difference of
259 plasma cell-free miR-517a levels between the placenta previa without alert bleeding and
260 control groups (Table 3). There was no significantly difference of plasma cell-free
261 miR-518b levels between the placenta previa with and without alert bleeding groups,
262 between the placenta previa with alert bleeding and control groups, or between the

263 placenta previa without alert bleeding and control groups. (Table 3).

264

265 ***Circulating levels of plasma cell-free pregnancy-associated but not placenta-specific***

266 ***miRNA in the placenta previa and control groups***

267 As pregnancy-associated but not placenta-specific miRNA, median (minimum–

268 maximum) MoM values of plasma cell-free miR-323-3p in the control and placenta

269 previa groups were 1.000 (0.24–13.76) and 0.747 (0.04–3.52), respectively. There was

270 no significant difference in circulating levels of plasma cell-free miR-323-3p in both the

271 placenta previa and the control groups ($P>0.05$).

272

273

274 ***Association between the volume of hemorrhage in placenta previa at cesarean***

275 ***delivery and plasma concentrations of cell-free pregnancy-associated***

276 ***placenta-specific miRNAs***

277 We investigated the association between the volume of hemorrhage in

278 placenta previa at cesarean delivery and plasma concentrations of cell-free

279 pregnancy-associated placenta-specific miRNAs (miR-517a or miR-518b). Median
280 (minimum–maximum) circulating levels of plasma cell-free pregnancy-associated
281 placenta-specific miRNAs in the placenta previa group were 19,600 (1,830–93,600)
282 copies/mL for miR-517a, and 6,210 (626–452,000) copies/mL for miR-518b,
283 respectively (Figure 2a, b). Median (minimum–maximum) volume of hemorrhage in the
284 placenta previa group at cesarean delivery was 1,650 (778–5,400) mL (Figure 2a, b).
285 The circulating levels of cell-free miR-517a and miR-518b in maternal plasma were
286 significantly associated with the volume of hemorrhage in placenta previa at cesarean
287 delivery (R and P-values: 0.512 and 0.025 for 517a, and 0.472 and 0.041 for 518b,
288 respectively) (Figure 2a, b). Median (minimum–maximum) circulating levels of of
289 plasma cell-free pregnancy-associated placenta-specific miRNAs in the placenta previa
290 group were 19,600 (1,830–93,600) copies/mL for miR-517a, and 6,210 (626–452,000)
291 copies/mL for miR-518b, respectively (Table 1, Figure 2a, b). Median (minimum–
292 maximum) volume of hemorrhage in the placenta previa group at cesarean delivery was
293 1,650 (778–5,400) mL (Table 1, Figure 2). The circulating levels of cell-free miR-517a
294 in maternal plasma were significantly associated with the volume of hemorrhage in

295 placenta previa at cesarean delivery (R and P-values: 0.512 and 0.025) (Figure 2a).
296 While, the correlation between the plasma concentrationss of cell-free miR-518b and
297 the volume of hemorrhage in placenta previa at cesarean delivery was weak (R and
298 P-values: 0.472 and 0.041) (Figure 2b).
299
300 **Discussion**
301 This is the first study to investigate maternal plasma concentrations of
302 cell-free pregnancy-associated placenta-specific miRNAs in placenta previa.
303 Increased levels of cell-free miR-517a and decreased levels of cell-free
304 miR-518b in maternal plasma at 32 weeks gestation were detected in the placenta previa
305 group. On the other hand, the plasma concentration of cell-free pregnancy-associated
306 but not placenta- specific miRNA, miR-323-3p on C14MC, which is expressed in the
307 placenta, embryo and mother, showed no significant difference between the control and
308 the placenta previa groups. Increased levels of placental miRNAs on C19MC have been
309 reported in cases of preeclampsia, because apoptotic changes in the placenta lead to
310 release of syncytiotrophoblast microparticles (STBM) and exosomes including

311 fetal/placental DNAs/RNAs into maternal circulation[25]. Our previous study showed that
312 the increased level of cell-free placental mRNA (human placental lactogen and human
313 chorionic gonadotropin) in placenta accreta might be explained by possible
314 direct-connection between the placenta and maternal circulation[24]. Here, although a case
315 of placenta previa-increta was excluded, cases of potential placenta accreta may be
316 included, because placenta previa is the risk factor of placenta accreta. Therefore, as
317 with the association of circulating levels of placental mRNAs and placenta previa, we
318 expected that circulating levels of miR-517a and miR-518b would be increased in the
319 placenta previa group compared with the control group. However, our result was
320 contrary to our expectation. In another study, the discrepancy of expression pattern of
321 placenta-specific miRNAs (increased expression of miR-519a and decreased expression
322 of miR-518b) was also seen in placentas with fetal growth restriction (FGR), compared
323 with placentas that were large or adequate for gestational age[26]. At present, although we
324 have no explanation regarding this discrepancy of cell-free miRNAs levels on the
325 C19MC region, the increased level of miR-517a and decreased level of miR-518b in
326 placenta previa may account for the discrepant concentration of these molecules in

maternal plasma. Therefore, the altered circulating levels of cell-free miR-517a and miR-518b on C19MC seem to be associated with the pathogenesis of placenta previa pregnancy. And, the measurement of cell-free miRNAs on the C19MC region in maternal plasma may reflect the functional status of placenta previa.

Plasma concentrations of cell-free miR-517a in the placenta previa with alert bleeding later group were significantly higher than those in placenta previa without alert bleeding group or uncomplicated pregnancy group. While, there was no significantly difference of plasma cell-free miR-518b levels between the placenta previa with and without alert bleeding later groups, between the placenta previa with alert bleeding later and uncomplicated pregnancy groups, or between the placenta previa without alert bleeding later and uncomplicated pregnancy groups. Therefore, the measurement of plasma cell-free miR-517a level may predict the risk of alert bleeding later in cases of placenta previa.

Also, cell-free miR-517a level in maternal plasma collected at around 32 weeks correlated with the volume of hemorrhage in placenta previa pregnancies at delivery. While, the correlation between the plasma concentrations of cell-free miR-518b and the

343 volume of hemorrhage in placenta previa at cesarean delivery was weak. A recent study
344 could not confirm the diagnostic benefit of cell-free fetal DNA to predict invasive
345 placentation in placenta previa[27], while other studies continue to suggest that the
346 placental mRNAs may be a marker for prediction of invasive placenta among women
347 with placenta previa[24,28]. Our results of placenta-specific miRNAs would be clinically
348 more useful if the profile/levels of placenta-specific miRNAs could be correlated to
349 invasive placenta disorders (e.g., previa–increta or previa–percreta). The prenatal
350 prediction of placenta previa-accreta is estimated by ultrasonographic findings.
351 However, ultrasonographic assessment for the risk of massive hemorrhage in cases of
352 placenta previa at delivery was difficult (Table 1). Furthermore, ultrasonographic
353 assessment for the placenta locating the posterior wall of uterus is more difficult,
354 compared with that for the placenta locating the anterior wall of uterus[1]. In this study,
355 six cases (Cases 3, 9, 10, 11, 15 and 17) were managed as suspected placenta
356 previa-accreta, because these cases had two or more of four ultrasonographic features,
357 which were suggestive of placenta previa-accreta. In six cases of suspected placenta
358 accreta, MRI tests were performed to further define the diagnosis. However, five of six

359 cases were diagnosed as suspicion of placenta accrete, suggesting that MRI test seems
360 to give no additional information for the predictions of placenta previa-accreta or
361 massive hemorrhage, compared with ultrasonografic examination[1]. It might have been
362 more useful to evaluate the potential value of plasma pregnancy-associated placenta
363 specific miRNAs in cases of suspected placenta accreta, but numbers are small in this
364 study and further examination should be necessary. Therefore, plasma concentrations of
365 cell-free placental RNAs (placental mRNA and miR-517a) combined with an
366 ultrasonographic examination may be used as a noninvasive obstetrical examination for
367 prenatal prediction of the risk of massive hemorrhage in pregnant women with placenta
368 previa at delivery. In turn, this may allow a reduction in perinatal maternal mortality
369 from not only placenta previa but also other placental abnormalities.

370 In conclusion, our data showed increased levels of cell-free miR-517a and
371 decreased levels of cell-free miR-518b in maternal plasma samples from placenta previa
372 pregnancies, compared with plasma samples from uncomplicated pregnancies.
373 Therefore, measurement of cell-free miR-517a and miR-518b concentrations in
374 maternal plasma at 32 weeks gestation has the potential to reflect the placental status of

placenta previa pregnancy. Subsequently, plasma concentration of cell-free miR-517a at 32 weeks gestation was significantly higher in placenta previa with alert bleeding later group than in placenta previa without alert bleeding or uncomplicated pregnancy groups, and correlated with the volume of hemorrhage in placenta previa at delivery, suggesting that plasma concentration of cell-free miR-517a in placenta previa may predict the risks of alert bleeding later and massive hemorrhage at cesarean delivery. As this pilot study was limited by its small sample size, further large-scaled study is necessary to our hypothesis.

Acknowledgements

We would like to thank Ayako Ueyama, Shizuka Yoshii, Hiroko Sakakida, and Yasuko Noguchi for their technical assistance. K.M., A.H., S.A., S.M., and H.M. were supported by JSPS KAKENHI (grant numbers 26462495, 26000000, 25861498, 24791712, and 25462563, respectively).

389 **References**

390 1. Oyelese Y, Smulian JC. Placenta previa, placenta accreta, and vasa previa. Obstet
391 Gynecol 2006; 107:927–41.
392 2. Baba Y, Matsubara S, Ohkuchi A, *et al.* Anterior placentation as a risk factor for
393 massive hemorrhage during cesarean section in patients with placenta previa. J
394 Obstet Gynaecol Res 2014; 40:1243–8.
395 3. Hasegawa J, Nakamura M, Hamada S, *et al.* Prediction of hemorrhage in placenta
396 previa. Taiwan J Obstet Gynecol 2012; 51:3–6.
397 4. Ghourab S. Third-trimester transvaginal ultrasonography in placenta previa: does
398 the shape of the lower placental edge predict clinical outcome? Ultrasound Obstet
399 Gynecol 2001; 18:103–8.
400 5. Zaitoun MM, El Behery MM, Abd El, et al. Does cervical length and the lower
401 placental edge thickness measurement correlates with clinical outcome in cases of
402 complete placenta previa? Arch Gynecol Obstet 2011; 284:867–73.
403 6. Chim SS, Shing TK, Hung EC, *et al*. Detection and characterization of placental
404 microRNAs in maternal plasma. Clin Chem. 2008; 54:482–90.

405 7. Chiu RW, Lo YM. Pregnancy-associated microRNAs in maternal plasma: a channel
406 for fetal-maternal communication? Clin Chem 2010; 56:1656–7.
407 8. Miura K, Miura S, Yamasaki K, *et al.* Identification of pregnancy-associated
408 microRNAs in maternal plasma. Clin Chem 2010; 56:1767–71.
409 9. Donker RB, Mouillet JF, Chu T, *et al.* The expression profile of C19MC
410 microRNAs in primary human trophoblast cells and exosomes. Mol Hum Reprod
411 2012; 18:417–24.
412 10. Luo SS, Ishibashi O, Ishikawa G, *et al.* Human villous trophoblasts express and
413 secrete placenta-specific microRNAs into maternal circulation via exosomes. Biol
414 Reprod 2009; 81:717–29.
415 11. Hromadnikova I, Kotlabova K, Ondrackova M, *et al.* Circulating C19MC
416 microRNAs in preeclampsia, gestational hypertension, and fetal growth restriction.
417 Mediators Inflamm 2013; 2013:186041.
418 12. Mouillet JF, Chu T, Sadovsky Y. Expression patterns of placental microRNAs. Birth
419 Defects Res A Clin Mol Teratol. 2011; 91:737–743.
420 13. Chiu RW, Poon LL, Lau TK et al. Effects of blood-processing protocols on fetal

421 and total DNA quantification in maternal plasma. Clin Chem. 2001;47:1607–13.

422 14. Bentwich I, Avniel A, Karov Y, *et al.* Identification of hundreds of conserved and

423 nonconserved human microRNAs. Nat Genet 2005; 37:766–70.

424 15. Liang Y, Ridzon D, Wong L, Chen C. Characterization of microRNA expression

425 profiles in normal human tissues. BMC Genomics 2007; 8:166.

426 16. Bortolin-Cavaillé ML, Dance M, Weber M, Cavaillé J. C19MC microRNAs are

427 processed from introns of large Pol-II, non-protein-coding transcripts. Nucleic Acids

428 Res 2009; 37:3464–73.

429 17. Morales-Prieto DM, Ospina-Prieto S, Chaiwangyen W *et al.*

430 Pregnancy-associated miRNA-clusters. J. Reprod. Immunol. 2013; **97**:

431 51–61.

432 18. Morales Prieto DM, Markert UR. MicroRNAs in pregnancy. J Reprod Immunol

433 2011; 88:106–11.

434 19. Higashijima A, Miura K, Mishima H, *et al.* Characterization of placenta-specific

435 microRNAs in fetal growth restriction pregnancy. Prenat Diagn 2013; 33:214–22.

436 20. Hasegawa Y, Miura K, Furuya K, *et al.* Identification of complete hydatidiform

437 mole pregnancy-associated microRNAs in plasma. Clin Chem 2013; 59:1410–2.

438 21. Mouillet JF, Chu T, Sadovsky Y. Expression patterns of placental microRNAs. Birth

439 Defects Res A Clin Mol Teratol 2011; 91:737–43.

440 22. Zhao Z, Moley KH, Gronowski AM. Diagnostic potential for miRNAs as

441 biomarkers for pregnancy-specific diseases. Clin Biochem 2013; 46:953–60.

442 23. Ng EK, Tsui NB, Lam NY, *et al.* Presence of filterable and nonfilterable mRNA in

443 the plasma of cancer patients and healthy individuals. Clin Chem 2002; 48:1212–7.

444 24. Miura K, Miura S, Yamasaki K, *et al*. Increased level of cell-free placental mRNA

445 in a subgroup of placenta previa that needs hysterectomy. Prenat Diagn.

446 2008;28:805–9.

447 25. Knight M, Redman CW, Linton EA, *et al*. Shedding of syncytiotrophoblast

448 microvilli into the maternal circulation in pre-eclamptic pregnancies. Br J Obstet

449 Gynaecol 1998; 105:632–40.

450 26. Wang D, Na Q, Song WW, *et al*. Altered expression of miR-518b and miR-519a in

451 the placenta is associated with low fetal birth weight. Am J Perinatol. 2014;31:729–

452 34.

453 27. Samuel A, Bonanno C, Oliphant A, *et al.* Fraction of cell-free fetal DNA in the
454 maternal serum as a predictor of abnormal placental invasion: a pilot study. Prenat
455 Diagn 2013; 33:1050–3.
456 28. Kawashima A, Sekizawa A, Ventura W, *et al.* Increased levels of cell-free human
457 placental lactogen mRNA at 28-32 gestational weeks in plasma of pregnant women
458 with placenta previa and invasive placenta. Reprod Sci 2014; 21:215–20.
459

460 **Figure Legends**

461 **Figure 1. Circulating levels of plasma cell-free miR-517a and miR-518b at 32**
462 **weeks gestation in placenta previa and control groups.**

463 **a.** miR-517a, and **b.** miR-518b.

464 Circulating levels were expressed as multiple of median (MoM) values. Gray bars
465 indicate the data from the placenta previa group, and white bars indicate the data from
466 the control group. Circulating levels of miRNAs in maternal plasma are expressed as
467 copies/mL. * indicated P=0.011, ** indicated P=0.004 (Mann–Whitney U test).

468

469 **Figure 2. Graph showing relationship between plasma concentration of cell-free**
470 **pregnancy-associated placenta-specific miRNAs at 32 weeks gestation and the**
471 **volume of hemorrhage in placenta previa at cesarean delivery.**

472 The correlation coefficient between the volume of hemorrhage at cesarean delivery and
473 cell-free pregnancy-associated placenta-specific miRNAs levels for **a.** miR-517a is
474 0.512 (P=0.025), and for **b.** miR-518b is 0.472 (P=0.041).

Table 1. Cell-free miRNA levels, volumes of hemorrhage at delivery and ultrasonographic features in cases of placenta previa pregnancies.

"O" indicated a case with the feature, while "X" a case without the feature. * Histological examination of placenta–uterus confirmed a case of placenta previa–increta.

Cases	Cell-free placenta-specific miRNA levels (copies/mL)		Ultrasonographic features				MRI	Volumes of hemorrhage (mL)
	517a	518b	Irregularly shaped placental lacunae	Thinning of the myometrium overlying the placenta	Loss of the retroplacental clear space	Increased vascularity of the uterine serosa-bladder interface	Suspected Placenta accrete	
1	9890	3590	X	X	X	X	-	1650
2	9640	1720	X	X	X	X	-	1200
3	22400	67300	O	O	O	X	Suspected	1700
4	57300	2260	O	X	X	X	-	2550
5	5050	2280	O	X	X	X	-	3500
6	7470	6780	X	X	X	X	-	1500
7	12600	13100	X	X	X	X	-	820
8	1830	626	X	X	X	X	-	1050
9	93600	452000	O	O	O	O	Suspected	5400
10	20000	649	O	X	O	X	Undefined	2100
11*	1030000	800000	O	O	O	O	Suspected	8250
12	6870	747	X	X	X	X	-	3100
13	23400	5640	X	X	X	X	-	1158
14	30000	337000	O	X	X	X	-	1200
15	87100	114000	O	O	O	X	Suspected	2250
16	27000	78200	X	X	X	X	-	778
17	3300	950	O	O	X	X	Suspected	980
18	41400	37500	X	X	X	X	-	1600
19	21000	13300	X	X	X	X	-	2250

20	6230	1630	X	X	X	X	-	2500

Table 2. Clinical characteristics of the pregnant women included in this study.

All parameters of the control group and placenta previa group are indicated as the mean and (standard deviation [SD]). Significant differences between groups were analyzed by Student's t-test or chi-square test. A P-value of <0.05 was considered significant. NS, not significant; a, mean (SD)

Characteristics	Control group (n=26)	Placenta previa group (n=19)	P-value
Maternal age (years)	33.11 (5.38)	33.42 (8.79)	NS [a]
Gestational age at sampling (weeks)	32.69 (0.47)	32.32 (0.94)	NS [a]
Gestational age at delivery (weeks)	38.31 (1.67)	36.89 (1.33)	0.004 [a]
Alert bleeding during pregnancy	0	7	<0.001 [b]
Parity			NS [b]
Primiparous	14	13	
Multiparous	12	6	
Placental weight (g)	565.50 (95.87)	577.53 (106.85)	NS [a]
Fetal birth weight (g)	3031.31 (531.65)	2755.16 (351.39)	NS [a]
Previous history of cesarean section	7	5	NS [b]
Previous history of artificial abortion	9	2	NS [b]

[a] t-test

[b] chi-square test

Table 3. Circulating levels of plasma cell-free pregnancy-associated placenta-specific miRNAs in placenta previa with alert bleeding group, placenta previa without alert bleeding group, and control group

Plasma cell-free microRNA levels are indicated as median (minimum-maximum) MoM. Significant differences between groups were analyzed by Kruskal-Wallis test or Bonferroni correction for post hoc analysis of multiple comparisons. A *P*-value < 0.05 was considered significant. NS: not significant.

pregnancy-associated placenta-specific miRNAs	A group Uncomplicated pregnancy (n = 26)	B group placenta previa with alert bleeding (n = 7)	C group placenta previa without alert bleeding (n = 12)	P-values Kruskal-Wallis test	A vs B	A vs C	B vs C
miR-517a (MoM)	1.00 (0.11–37.58)	10.66 (5.15–24.09)	2.20 (0.47–7.72)	0.002	0.047[a]	NS[a]	0.030[a]
miR-518b (MoM)	1.00 (0.46–6.89)	1.48 (0.01–9.86)	0.064 (0.01–7.35)	0.001	NS[a]	NS[a]	NS[a]

[a]Bonferroni correction

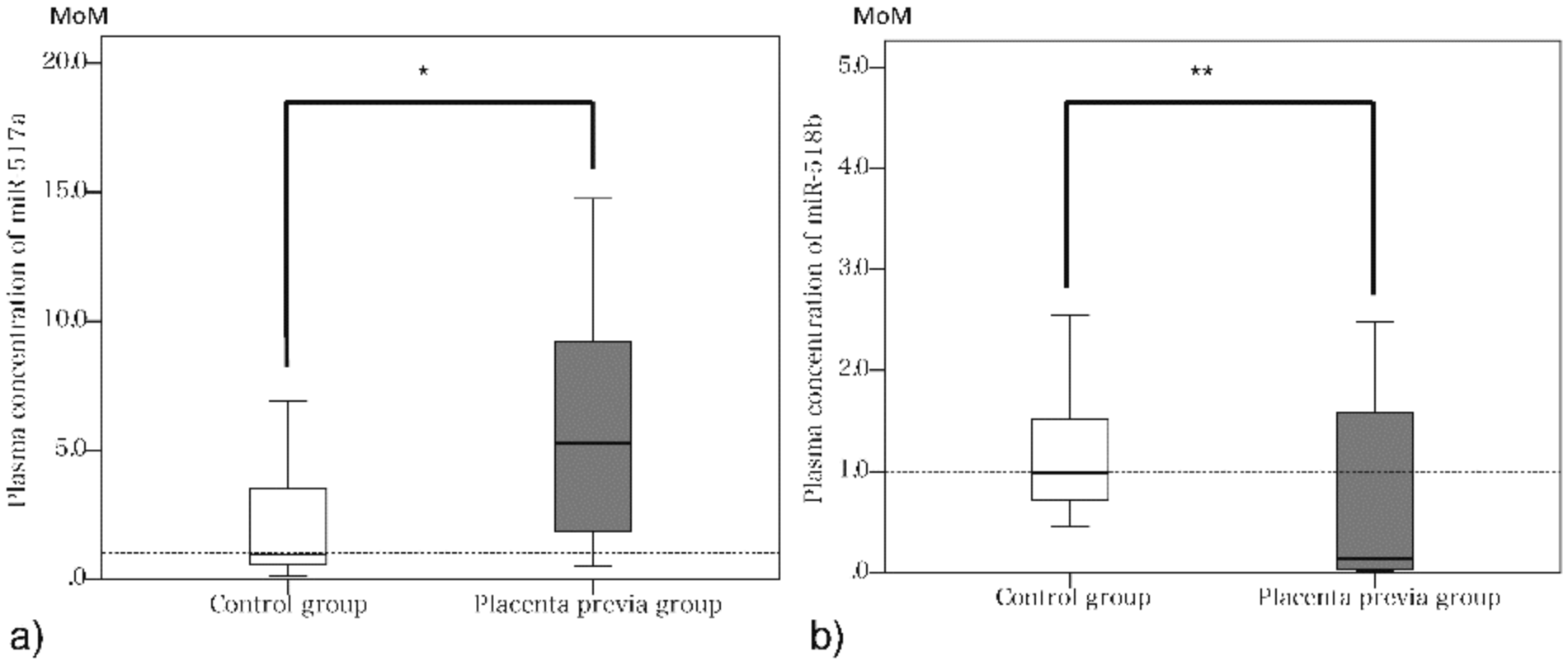
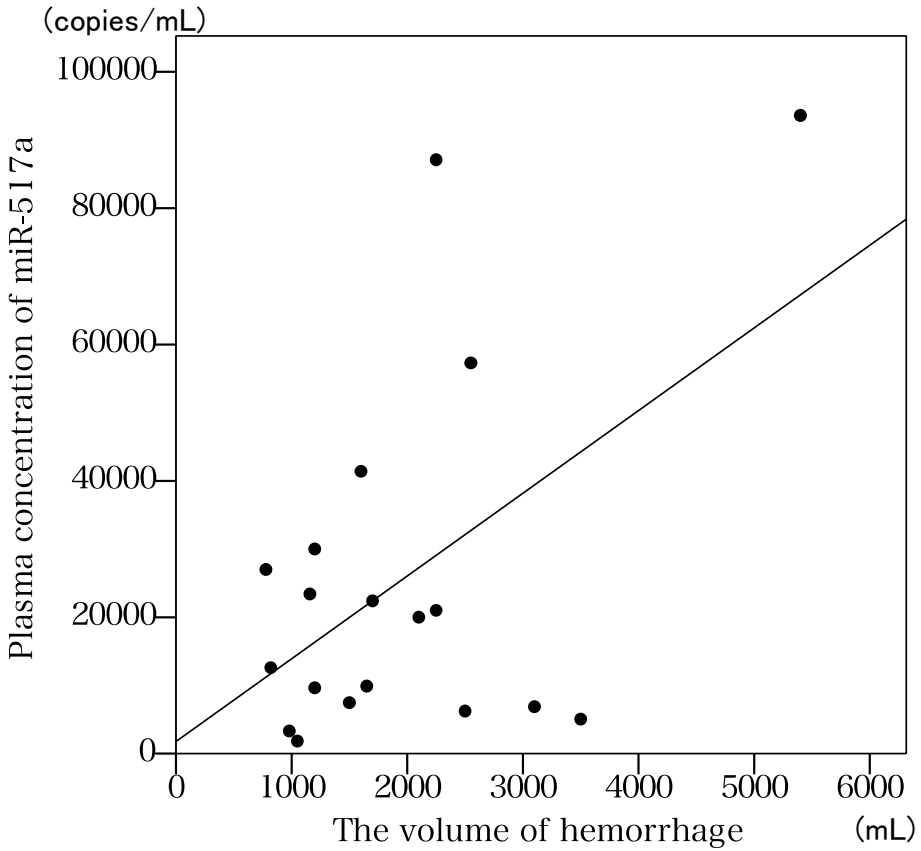
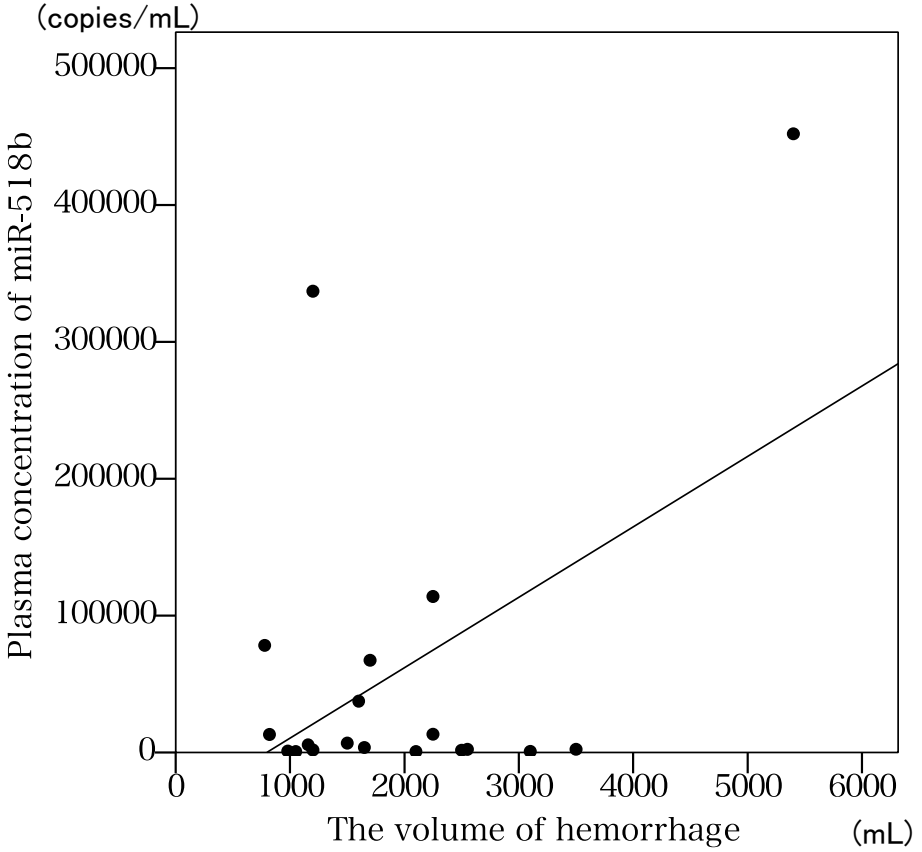

MoM
Plasma concentration of miR 517a
20.0
15.0
10.0
5.0
.0
*
Control group
Placenta previa group
a)
MoM
Plasma concentration of miR-518b
5.0
4.0
3.0
2.0
1.0
.0
**
Control group
Placenta previa group
b)

Figure 1


(copies/mL)
100000
80000
60000
40000
20000
0
Plasma concentration of miR-517a
0
1000
2000
3000
4000
5000
6000
The volume of hemorrhage
(mL)

a


(copies/mL)
500000
400000
300000
200000
100000
0
Plasma concentration of miR-518b
0
1000
2000
3000
4000
5000
6000
The volume of hemorrhage
(mL)

b

Figure 2