ORIGINAL ARTICLE

Characterization of placenta-specific microRNAs in fetal growth restriction pregnancy

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ABSTRACT

Objective The aim of this study was to characterize placenta-specific microRNAs in fetal growth restriction (FGR) pregnancy.

Method Placenta-specific miRNAs were identified by next-generation sequencing analysis. Subsequently, quantitative real-time reverse-transcription polymerase chain reaction was used to identify FGR placenta-specific miRNAs whose level of expression was significantly decreased in FGR placenta ($n=45$) compared with uncomplicated placenta ($n=50$). FGR pregnancy-associated, placenta-specific microRNAs were identified in maternal plasma after delivery at significantly decreased concentrations, and their circulating levels in maternal plasma was compared between FGR pregnancies ($n=10$) and uncomplicated pregnancies ($n=10$).

Results Out of the ten placenta-specific microRNAs that we identified, seven placenta-specific microRNAs (hsa-miR-518b, hsa-miR-1323, hsa-miR-516b, hsa-miR-515-5p, hsa-miR-520h, hsa-miR-519d, and hsa-miR-526b) from the chromosome 19 microRNA cluster were identified as FGR placenta-specific microRNAs. Four FGR placenta-specific microRNAs (hsa-miR-518b, hsa-miR-1323, hsa-miR-520h, and hsa-miR-519d) were confirmed as FGR pregnancy-associated, placenta-specific miRNAs, but their circulating levels in maternal plasma showed no significant differences between FGR pregnancy and uncomplicated pregnancy.

Conclusion Our data suggest that reduced expression in placenta of certain FGR placenta-specific miRNAs is associated with FGR and that the discrepancy between expression in FGR placenta and their circulating levels in maternal plasma will be crucial to understanding how placenta-specific microRNAs are released into the maternal circulation. © 2013 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

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INTRODUCTION

Fetal growth restriction (FGR) is usually defined as a birth weight below the tenth percentile for gestational age and gender and greatly increases the risk of perinatal complications, including fetal compromise in labor, fetal death *in utero*, neonatal morbidity, and neonatal death.[1,2] Although maternal complications (such as preeclampsia, diabetes mellitus, and connective tissue disorders) and fetal complications (such as aneuploidy, infections, genetic syndromes, and abnormality of cord insertion) are among the known causes of FGR, idiopathic FGR accounts for 70% of all cases and may be associated with uteroplacental insufficiency at the interface between fetal and maternal circulation.[3,4] However, information regarding the molecular mechanisms of FGR etiology is still limited.[5,6]

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.[7–9] miRNAs are expressed in tissue-specific patterns,[7,8] and miRNAs predominantly expressed in the placenta are probably involved in placental differentiation and in the maintenance of pregnancy.[10] Recently, by searching a panel of microarray assays, we have identified pregnancy-associated, placenta-specific miRNAs in the plasma of pregnant women. It is possible that such miRNAs serve to communicate between the fetus and mother.[11,12] The data regarding miRNAs in placenta and plasma of FGR pregnancies are limited; therefore, investigation of FGR pregnancy-associated, placenta-specific miRNAs is likely to shed light on the molecular mechanisms of human FGR etiology.

Microarray technology is high-throughput but can only detect a limited number of miRNAs because of the nature of probe hybridization.[13] Next-generation sequencing (NGS) technology using Illumina technology generates short reads (35 bp) but more than 1G bp of sequence data per run and can be used to measure the abundance of small RNA sequences in a sample. Because miRNAs are only 21–25 bp in length, this technology can enable in-depth characterization of the global repertoire of miRNAs.[14]

In this study, we tried to characterize placenta-specific miRNAs in FGR pregnancy. First, by comparative analysis of NGS-generated miRNA expression profiles of placenta tissue, cord blood cells, and maternal blood cells from the same pregnancy, we isolated placenta-specific miRNAs that were expressed only in the placenta. Then, by comparative analysis of FGR and uncomplicated pregnancy placenta tissues using real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR), we identified FGR placenta-specific miRNAs that were expressed at significantly lower levels in FGR placentas. Subsequently, when FGR placenta-specific miRNAs showed significantly decreased concentrations in maternal plasma after delivery of the placenta, these miRNAs were identified as FGR pregnancy-associated, placenta-specific miRNAs. Finally, we investigated the circulating levels of FGR pregnancy-associated, placenta-specific miRNAs in maternal plasma in women with FGR or uncomplicated pregnancies.

METHODS

Sample collection

All pregnant women attended Nagasaki University Hospital or its associated hospitals. 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.

For NGS analysis, we obtained placental tissue and corresponding maternal blood and cord blood from two women with uncomplicated pregnancies of a singleton at 38 weeks to exclude the possibility of preterm labor. Gestational age was assessed using ultrasonography. Maternal blood samples (7 mL) were collected prepartum and placed in tubes containing ethylenediaminetetraacetic acid. Placental tissue samples and cord blood samples were obtained immediately postpartum. Placental tissue samples were placed in RNAlater™ (Ambion, Austin, TX, USA), and cord blood samples were placed in tubes containing ethylenediaminetetraacetic acid. Using a *mir*Vana miRNA Isolation Kit (Ambion), total RNA containing small RNA molecules was extracted from each sample immediately after sampling. Quality assessment and concentration measurements of total RNA, including small RNAs, were performed using a Bioanalyzer (Agilent Technologies, South Queensferry, UK) and a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively.

For subsequent expression analysis by real-time qRT-PCR analysis, placental tissues were obtained from 50 cases of uncomplicated pregnancies (control group) and 45 cases of idiopathic FGR pregnancy (FGR group). Clinical characteristics of both groups are listed in Table 1. Placental tissue samples were collected in RNAlater™ (Ambion). Each sample was stored at −80 °C until use. Idiopathic FGR pregnancy was defined as a birth weight below the tenth percentile for the gestational age, but the cause of FGR was unknown (pregnancy-associated disease including preeclampsia, diabetes mellitus, connective tissue disorders, *in utero* infections, fetal genetic abnormalities, or non-genetic diseases were excluded). All uncomplicated pregnancy cases delivered infants whose birth weight was appropriate for gestational age (tenth–90th percentile). Total RNA containing small RNA molecules was extracted from placenta using a *mir*Vana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions.

Maternal blood samples (7 mL) before and after delivery were obtained from ten uncomplicated pregnancies cases, and maternal blood samples (7 mL) before delivery were obtained from ten idiopathic FGR pregnancy cases. Clinical characteristics of both pregnancy groups are listed in Table 2. Cell-free plasma samples were prepared from maternal blood by a double centrifugation method as described previously.[12] After the first centrifugation at 3000 g for 10 min, the supernatant 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) according to the manufacturer's instructions.

Table 1 **Clinical characteristics of the pregnant women included in the expression analysis of placenta-specific microRNAs in placenta tissue**

Characteristics	Uncomplicated pregnancy ($n = 50$)	FGR pregnancy ($n = 45$)	P value
Maternal age (years)	33.8 (6.5)[a]	29.9 (6.2)[a]	NS
Gestational age at sampling (weeks)	37.8 (1.6)[a]	37.2 (1.7)[a]	NS
Placental weight (g)	490.4 (111.2)[a]	311.2 (114.5)[a]	0.002
Fetal birth weight (g)	2749.2 (505.3)[a]	1706.1 (419.8)[a]	<0.001
Newborn gender			NS
Male	24	22	
Female	26	23	

All parameters of the uncomplicated pregnancy group and FGR pregnancy group are indicated as 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.

FGR, fetal growth restriction; NS, not significant;

[a]mean (SD)

Table 2 Clinical characteristics of the pregnant women included in the study of maternal cell-free plasma concentration of FGR pregnancy-associated, placenta-specific microRNAs

Characteristics	Uncomplicated pregnancy ($n=10$)	FGR pregnancy ($n=10$)	P value
Maternal age (years)	36.6 (3.0)	33.7 (5.5)	NS
Gestational age at sampling (weeks)	35.9 (2.3)	35.7 (2.2)	NS
Parity			NS
Primiparous	7	7	
Multiparous	3	3	
Placental weight (g)	556.4 (88.4)	351.8 (68.8)	<0.001
Fetal birth weight (g)	3007.0 (330.0)	1926.8 (330.0)	<0.001
BMI (kg/m^2)	23.2 (4.4)	19.5 (2.4)	NS
Newborn gender			NS
Male	4	5	
Female	6	5	

All parameters of the uncomplicated pregnancy group and FGR pregnancy group are expressed as the mean and (SD). Significant differences between groups were analyzed by the Student's *t*test or chi-square test. A *P* value <0.05 was considered significant.
BMI, body mass index; FGR, fetal growth restriction; NS, not significant.

In miRNA expression analysis of placental tissue, there is no consensus on universal endogenous normalization controls, because SnoRNAs and snRNAs, including RNU48 and RNU6B, have been suggested as reference RNAs but exhibit high variability.[10] In addition, the quantitative mRNA measurements in plasma have been recommended to be expressed as an absolute concentration.[15] Therefore, we considered that the quantitative miRNA measurements may be the same as quantitative mRNA measurements in plasma. Therefore, in this study, absolute real-time qRT-PCR analysis was performed, although up- and down-regulation of miRNA expression are more adequately presented as CT and 2- CCT values.

Small RNA library construction and next-generation sequencing

Next-generation sequencing was applied to two sets (sample set A and B) of placental tissue, maternal blood, and cord blood from uncomplicated pregnancies to screen for placenta-specific miRNAs. Small RNAs were extracted from each sample using a mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. RNA quality and the presence of small RNAs were verified on a Bioanalyser (Agilent Technologies). The concentration of RNA was measured on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). After RNA quality was stringently assured, 5 μg of total RNA was used for small RNA library construction using a Small RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. A pair of Illumina proprietary adapters was ligated to the 5′ and 3′ ends of RNAs, followed by reverse transcription. The small cDNA libraries were amplified by PCR with primers complementary to the adaptor sequences. Subsequently, the libraries were deep sequenced using an Illumina Genome Analyzer II (Illumina), and sequencing data were processed with the Illumina pipeline v1.4 (Illumina), according to the manufacturer's instructions.

miRNA mapping and differential expression analysis of miRNAs by NGS

Millions of short reads from miRNA NGS were processed by several steps. An initial filtering step was performed to exclude reads of low quality. Then, data were filtered to eliminate adaptor contaminants to generate usable reads of 18 bp or more. All reads were then aligned to Rfam and Genbank to discard rRNA, tRNA, snoRNA, and snRNA contamination. For mapping miRNAs of 18 to 32 bp, two software programs were used: a short oligonucleotide alignment program (SOAP), which analyzes numerous short sequences and aligns millions of short sequencing reads to genomic sequences,[16] and 'miRExpress', which was designed to construct miRNA expression profiles through the direct alignment of millions of short sequences with the sequences of known miRNAs.[17] Human genome data were from human genome Feb. 2009 (hg19) (http://genome.ucsc.edu/cgi-bin/hgGateway) and that of known miRNAs was from miRbase18.0 (http://mirbase.org/). For both programs, each set of sequencing data was mapped with miRBase 18.0 to search for complete matches of known miRNAs. Raw expression values (read counts) were obtained after these steps. Because low copy numbers are less reliable, miRNAs with fewer than ten counts were defined as 'negative'. To compare miRNA levels across data sets, the sequencing read count for each miRNA was normalized to the total read count of 1 000 000 in each sample and expressed as reads per million (RPM). For SOAP mapping data, when the normalized miRNA read count in placental tissue was >100 RPM and that in other samples was 'negative' in both sample sets A and B, these miRNAs were selected as placenta-specific miRNAs. For SOAP and miRExpress mapping data, when the normalized miRNA read count was 'negative' in maternal blood and cord blood samples, and was >100 RPM by SOAP and >1 000 RPM by miRExpress in placenta of both sample sets A and B, these miRNAs were selected as placenta-specific miRNAs. These miRNAs were then analyzed by RT-PCR in placental tissue from FGR and uncomplicated pregnancies.

Real-time quantitative reverse-transcription polymerase chain reaction analysis of miRNAs

For real-time qRT-PCR of placenta samples, each RNA preparation was diluted to a concentration of 100 ng/μL (measured using a NanoDrop ND-1000 spectrophotometer, Thermo Fisher Scientific). Five microliters total RNA was reverse-transcribed using a TaqMan MicroRNA Reverse-Transcription Kit according to the manufacturer's instructions (Applied Biosystems). For quantitative real-time RT-PCR of plasma samples, 2.5 μL total RNA was reverse-transcribed using a TaqMan MicroRNA Reverse-Transcription Kit according to the manufacturer's instructions (Applied Biosystems). Quantitative PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems), as described previously.[12] For each miRNA assay, we prepared a calibration curve by tenfold serial dilution of single-stranded cDNA oligonucleotides corresponding to each miRNA sequence from 1.0×10^2 to 1.0×10^8 copies/mL. Each sample and each calibration dilution were analyzed in triplicate. Each assay could detect down to 100 RNA copies per mL. 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 using an ABI Prism 7900 Sequence Detector (Applied Biosystems).

Statistical analysis

Patient backgrounds were compared by Student's *t*-test and chi-square test for continuous and discrete variables, respectively, of FGR pregnancy cases and uncomplicated pregnancy cases. Absolute quantification data were analyzed with SDS 2.3 software (Applied Biosystems). The expression levels of placenta-specific miRNAs in placenta tissues and the cell-free plasma concentrations of FGR pregnancy-associated, placenta-specific miRNAs in cases of FGR pregnancy and uncomplicated pregnancy were converted into multiples of the median of uncomplicated pregnant women adjusted for gestational age. Differences between the two groups were evaluated with Mann–Whitney's *U*-test. Changes in the cell-free plasma concentration of FGR placenta-specific miRNAs in uncomplicated pregnancy before and after delivery were compared by the Wilcoxon signed-rank test. Statistical analyses were performed with SPSS software version 19 (IBM Japan, Tokyo, Japan). Significant differences were defined as *P* values of less than 0.05.

RESULTS

Screening placenta-specific miRNAs by next-generation sequencing

Next-generation sequencing analysis of sample sets A and B yielded 11 584 810 and 10 601 784 reads from placenta tissues, 12 027 880 and 12 071 436 reads from maternal blood cells, and 12 442 620 and 11 033 531 reads from cord blood cells, respectively. High-throughput sequencing assays can be susceptible to noise and variability; therefore, measurement of miRNA expression was normalized using the library size (1 000 000 reads). Twenty placenta-specific miRNAs were identified by SOAP, and 23 placenta-specific miRNAs were identified by miRExpress (Supplemental data, Tables 1 and 2). All placenta-specific miRNAs identified were located on 19q13.42. Ten miRNAs (hsa-miR-518b, hsa-miR-1323, hsa-miR-516b, hsa-miR-516a-5p, hsa-miR-525-5p, hsa-miR-515-5p, hsa-miR-520h, hsa-miR-520a-5p, hsa-miR-519d, and hsa-miR-526b) were detected as placenta-specific miRNAs by both SOAP and miRExpress and were, therefore, subsequently analyzed by RT-PCR (Table 3). Placenta-specific miRNAs located on other chromosomes were excluded on the basis of our screening criteria. The placenta-specific miRNAs on the chromosome 19 miRNA cluster (C19MC), for example, miR-517 and miR-526a, were detected but excluded because they were outside of the top ten expressed placenta-specific miRNAs as determined by our screening criteria (data not shown).

Expression levels of placenta-specific miRNAs in placentas of FGR and uncomplicated pregnancies

Expression levels of the ten placenta-specific miRNAs in placentas from FGR and uncomplicated pregnancies were measured by qRT-PCR. The expression levels of seven placenta-specific miRNAs (hsa-miR-518b, hsa-miR-1323, hsa-miR-516b, hsa-miR-515-5p, hsa-miR-520h, hsa-miR-519d, and hsa-miR-526b) were significantly lower in placentas from FGR pregnancies than in placentas from uncomplicated pregnancies (Mann–Whitney *U*-test, *P* values were <0.001, <0.001, 0.049, 0.036, <0.001, <0.001, and 0.003, respectively). Meanwhile, there was no significant difference in the levels of three placenta-specific miRNAs (hsa-miR-516a-5p, hsa-miR-525-5p, and hsa-miR-520a-5p) between placentas from FGR and uncomplicated pregnancies (Mann–Whitney *U*-test, *P* values were 0.248, 0.964, and 0.128, respectively; Table 4 and Figure 1)

Identification of FGR pregnancy-associated, placenta-specific miRNAs that showed significantly decreased concentrations in maternal plasma after delivery

Of the seven FGR placenta-specific miRNAs, the four that showed the most significantly decreased expression level in placentas of FGR pregnancies compared with placentas in uncomplicated pregnancies (*P* values < 0.001; Table 4, hsa-miR-518b, hsa-miR-1323, hsa-miR-520h, and hsa-miR-519d) were selected for analysis of cell-free maternal plasma before and after pregnancy because sample volumes were not sufficient to perform absolute real-time qRT-PCR analysis of all ten placenta-specific miRNAs. The maternal plasma concentrations of all four miRNAs were significantly decreased after delivery of the placenta and were identified as FGR pregnancy-associated, placenta-specific miRNAs (Wilcoxon signed-rank tests, *P* values = 0.005, Table 5 and Figure 2).

Circulating levels of FGR pregnancy-associated, placenta-specific miRNAs in maternal plasma in FGR and uncomplicated pregnancies cases

We also investigated the circulating levels of FGR pregnancy-associated, placenta-specific miRNAs in maternal plasma in cases of FGR or uncomplicated pregnancies. However, no significant differences in FGR pregnancy-associated, placenta-specific miRNAs levels in cell-free maternal plasma between FGR pregnancy and uncomplicated pregnancy were detected (Mann–Whitney's *U*-test, *P* > 0.05; Table 6).

Table 3 **List of the ten placenta-specific microRNAs detected by both short oligonucleotide alignment and miRExpress programs, which are software programs for mapping of microRNA sequencing data. Normalized read counts are derived from the short oligonucleotide alignment program**

		Sample set A			Sample set B		
		Cord miRNA (total number of reads: 12 442 620)	Maternal miRNA (total number of reads: 12 027 880)	Placental miRNA (total number of reads: 11 584 810)	Cord miRNA (total number of reads: 11 033 531)	Maternal miRNA (total number of reads: 12 071 436)	Placental miRNA (total number of reads: 10 601 784)
miRNA	Chromosomal location	Normalized read counts (reads per million)	Normalized read counts (reads per million)	Normalized read counts (reads per million)	Normalized read counts (reads per million)	Normalized read counts (reads per million)	Normalized read counts (reads per million)
hsa-miR-518b	19q13.42	Negative	Negative	3210.151914	Negative	Negative	3073.633645
hsa-miR-1323	19q13.42	Negative	Negative	1062.770991	Negative	Negative	1198.288892
hsa-miR-516b	19q13.42	Negative	Negative	749.4296411	Negative	Negative	911.5446985
hsa-miR-516a-5p	19q13.42	Negative	Negative	469.235145	Negative	Negative	632.9123476
hsa-miR-525-5p	19q13.42	Negative	Negative	403.3730376	Negative	Negative	687.6201213
hsa-miR-515-5p	19q13.42	Negative	Negative	261.463071	Negative	Negative	354.4686441
hsa-miR-520h	19q13.42	Negative	Negative	212.0880705	Negative	Negative	361.9202202
hsa-miR-520a-5p	19q13.42	Negative	Negative	203.0244777	Negative	Negative	318.248325
hsa-miR-519d	19q13.42	Negative	Negative	174.4525806	Negative	Negative	250.4295503
hsa-miR-526b	19q13.42	Negative	Negative	124.6459804	Negative	Negative	186.9496681

The expression levels of miRNAs with fewer than ten read counts were defined as 'Negative'.

Table 4 Expression levels of placenta-specific microRNAs in placenta tissues from cases of FGR pregnancy and from uncomplicated pregnancy

Placenta-specific microRNA	Expression level in placenta tissue		P value
	FGR pregnancy ($n=45$)	Uncomplicated pregnancy ($n=50$)	
hsa-miR-518b	0.42 (0.00–2.44)	1.00 (0.00–5.57)	<0.001
hsa-miR-1323	0.40 (0.00–1.68)	1.00 (0.38–2.37)	<0.001
hsa-miR-516b	0.79 (0.00–1.59)	1.00 (0.12–2.64)	0.049
hsa-miR-516a-5p	0.84 (0.00–2.35)	1.00 (0.20–3.53)	0.248
hsa-miR-525-5p	0.94 (0.03–2.28)	1.00 (0.25–2.60)	0.964
hsa-miR-515-5p	0.64 (0.00–2.16)	1.00 (0.26–2.48)	0.036
hsa-miR-520h	0.41 (0.00–1.00)	1.00 (0.00–1.76)	<0.001
hsa-miR-520a-5p	0.71 (0.01–2.42)	1.00 (0.14–3.58)	0.128
hsa-miR-519d	0.61 (0.00–1.55)	1.00 (0.57–2.39)	<0.001
hsa-miR-526b	0.69 (0.00–2.39)	1.00 (0.33–2.33)	0.003

Expression levels are described as multiple of median (MoM) values [median (minimum-maximum)]. Significant differences between groups were analyzed by the Mann–Whitney U-test. A P value <0.05 was considered as significant.
FGR, fetal growth restriction.

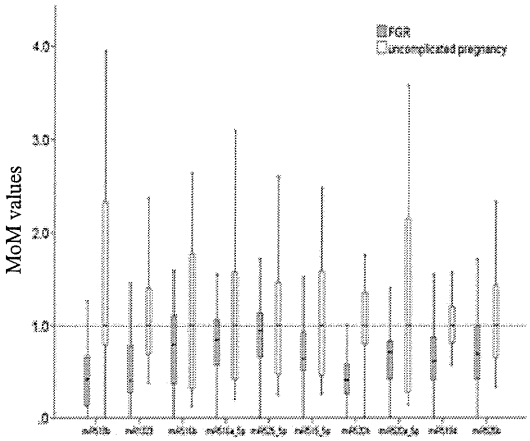

Figure 1 Expression levels of placenta-specific miRNAs in fetal growthrestriction (FGR) placentas and placentas from uncomplicatedpregnancies. Expression levels were expressed as multiple of median (MoM) values. Gray bars indicate the data from FGR cases, and white bars indicate the data from cases of uncomplicated pregnancy

DISCUSSION

In this study, we characterized placenta-specific miRNAs in FGR pregnancy. First, by NGS analysis, ten placenta-specific miRNAs (hsa-miR-518b, hsa-miR-1323, hsa-miR-516b, hsa-miR-516a-5p, hsa-miR-525-5p, hsa-miR-515-5p, hsa-miR-520h, hsa-miR-520a-5p, hsa-miR-519d, and hsa-miR-526b) were identified. These miRNAs are all located at 19q13.42, which is commonly referred to as C19MC. Two of ten placenta-specific miRNAs (miR-1323 and -516a-3p) were newly identified compared with our previous microarray data. Although NGS can enable a more in-depth characterization of the global repertoire of miRNAs compared with oligonucleotide microarray analysis, both technologies only detected placenta-specific miRNAs on C19MC, confirming that placenta-specific miRNAs mainly reside at C19MC. C19MC contains 46 highly related miRNAs within a ~100 kb region.[18] Although the regulation of expression of C19MC miRNAs is poorly understood, C19MC is imprinted in the placenta, with expression from the paternally inherited chromosome.[19] As miRNAs exhibit tissue-specific expression and function,[18] alterations to placental miRNA expression have been associated with *in utero* exposures[20,21] and pregnancy-associated disease.[22,23] In particular, because imprinted genes are known to be crucial for placental and fetal development in mammals,[24] the decreased expression level of FGR placenta-specific miRNAs, which are paternally expressed, in the placenta may cause lower placental weight and FGR. Our

Table 5 Circulating levels of fetal growth restriction (FGR) placenta-specific microRNAs in maternal plasma from ten uncomplicated pregnant women before and after delivery

Placenta-specific microRNA	Circulating level of FGR-related, placenta-specific microRNA in maternal plasma		P value
	Before delivery	After delivery	
hsa-miR-518b	37 340 (14 391–200 695)	6479 (1097–65 303)	0.005
hsa-miR-1323	13 193 (10 128–38 291)	5216 (2923–8666)	0.005
hsa-miR-520h	4684 (1151–18 881)	465 (236–1880)	0.005
hsa-miR-519d	1752 (633–6496)	217 (189–289)	0.005

Circulating levels are expressed as median (minimum–maximum) copies/mL.

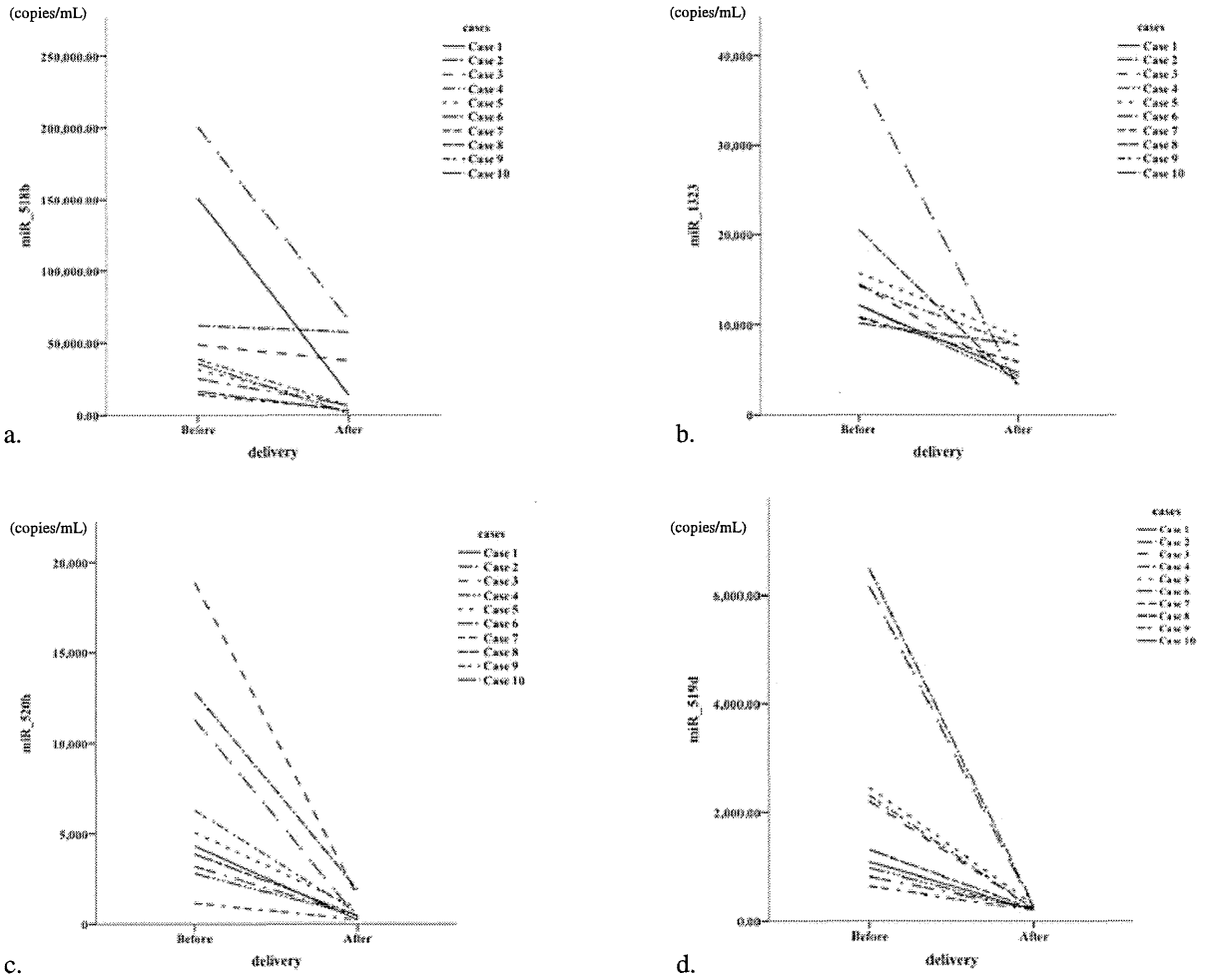

Figure 2 Circulating levels of fetal growth restriction placenta-specific miRNAs in maternal plasma before and after delivery. (a) miR-518b, (b) miR-1323, (c) miR-520h, and (d) miR-519d. Circulating levels of miRNAs in maternal plasma are expressed as copies/mL. The plasma levels of all four miRNAs were significantly decreased after delivery of the placenta (Wilcoxon signed-rank tests, *P* values = 0.005)

Table 6 Circulating levels of FGR pregnancy-related, placenta-specific microRNA in maternal plasma from cases of FGR pregnancy and uncomplicated pregnancy

	Circulating level of FGR pregnancy-related, placenta-specific microRNA in maternal plasma		
Placenta-specific microRNA	FGR pregnancy (n = 10)	Uncomplicated pregnancy (n = 10)	*P* value
hsa-miR-518b	0.68 (0.15–6.65)	1.00 (0.38–12.10)	0.481
hsa-miR-1323	0.85 (0.00–4.25)	1.00 (0.38–4.09)	0.529
hsa-miR-520h	0.55 (0.26–10.66)	1.00 (0.26–3.80)	0.353
hsa-miR-519d	1.15 (0.37–14.58)	1.00 (0.33–57.63)	0.912

Circulating levels are expressed as multiple of median (MoM) values [median (minimum–maximum)].
FGR, fetal growth restriction.

study supports the consensus that C19MC harbors a significant portion of placenta-specific miRNAs and that the regulation of placental C19MC play a role in the biology of placental trophoblasts.[25] Furthermore, the aberrant expression of placental C19MC may cause placental dysfunction resulting in FGR.

Accordingly, we investigated placenta-specific miRNA expression in FGR and identified seven FGR placenta-specific miRNAs (hsa-miR-518b, hsa-miR-1323, hsa-miR-516b, hsa-miR-515-5p, hsa-miR-520h, hsa-miR-519d, and hsa-miR-526b) with reduced placental expression. Maccani *et al.* [26] also showed that low levels of miR-16 and miR-21 expression in the placenta are associated with poor fetal growth. Our data support the evidence that FGR may be associated with the down-regulation of placenta-specific miRNAs on C19MC.

The placenta is of critical importance to ensure the proper growth and development of the fetus. Alterations of placental miRNA expression (epigenetic alterations) may serve as a record of *in utero* exposures and of intrauterine and extrauterine environments during pregnancy.[27] Fetal development represents a critical period during which perturbations to the intrauterine environment through various factors in the extrauterine environment can have major ramifications on not only the proper growth and development of the fetus but also on the risk for disease later in life.[28] Low birth weight is linked with morbidity and mortality in early infancy as well as with an increased risk for certain diseases later in life, particularly coronary heart disease, diabetes mellitus type 2, and hypercholesterolemia.[29] Our data are an important step in discovering miRNA expression profiles associated with FGR, which may be powerful predictions of risk for disease later in life.[26] The decreased expression of FGR placenta-specific miRNAs in FGR placenta suggests that target genes may be upregulated, which may contribute to the pathology of FGR. Given the importance of miRNAs in gene regulation, detailed investigation is required to elucidate the biological significance of these differentially expressed miRNAs by validating their specific target genes and determining the relevance of these target genes to placental development and functional activities. Knowledge of the molecular mechanisms underlying placental gene regulation may be enhanced by identifying miRNA biomarkers for FGR risk. In addition, these aberrant patterns of miRNA expression may lead to the identification of previously unknown pathways that are perturbed and that are targets for novel drug treatment or prevention strategies. Advances in characterizing placental mRNA expression associated with FGR placenta-specific miRNAs could provide a better understanding of the epigenetic regulatory mechanisms in the placenta. Future work characterizing associations between dysregulated FGR placenta-specific miRNA expression and assessments of fetal growth will be the key in understanding how dysregulated FGR placenta-specific miRNA expression may lead to FGR. Moreover, understanding how dysregulation of these miRNAs leads to dysregulation of their target gene protein levels will also be essential in further understanding the effects of environmental exposures on placental function and potentially downstream fetal programming.

Four FGR placenta-specific miRNAs (hsa-miR-518b, hsa-miR-1323, hsa-miR-520h, and hsa-miR-519d) were identified as FGR pregnancy-associated, placenta-specific miRNAs that were at significantly decreased concentrations in maternal plasma after delivery. The association between FGR placenta and altered expression of FGR placenta-specific microRNAs suggests the possibility of a functional role for miRNAs in FGR pregnancy. Placental miRNAs are released into the maternal circulation where they are found in a surprisingly stable form,[12,30,31] owing to their presence in circulating microparticles.[25,32] Our previous study [12] and those of Kotlabova *et al.* [31] revealed increased levels of placenta-specific miRNAs in maternal circulation during progression of normal pregnancies, which may be linked to the accruing mass, the aging, and/or the development of the placenta. In addition, placenta weight in FGR pregnancies was significantly lower compared with cases of uncomplicated pregnancies (Table 1). Therefore, even though systemic targets of circulating placental miRNAs remain to be defined, it will be interesting to determine whether possible changes in the circulating levels of FGR pregnancy-associated, placenta-specific miRNA in maternal plasma can serve as diagnostic markers of FGR. However, although FGR pregnancy-associated, placenta-specific miRNAs are expected to be a molecular marker for FGR pregnancy, their circulating levels in cell-free maternal plasma showed no significant differences between FGR pregnancy and uncomplicated pregnancy. Our finding is supported by other studies. Mouillet *et al.*[23] have recently observed no significant difference in circulating placenta-specific miRNA levels (mir-517a and miR-518b) in maternal plasma samples from normally progressing pregnancies and FGR pregnancies, although they found that the expression of individual miRNAs is altered by trophoblast hypoxia.[33] The studies of Hromadnikova *et al.*[34] also observed no significant difference in the maternal plasma levels of placenta-specific miRNA levels (miR-520a*, -520h, -525, -526a, -516-5p, -517*, and -518b) between normally progressing pregnancies and those with clinically established FGR. The negative correlation between no significant change of maternal plasma miRNA levels in FGR versus reduced expression levels of placenta miRNAs in FGR is intriguing. This may reflect related process where placental dysfunction in FGR attenuates miRNA biogenesis and alters exosome-dependent or independent release of miRNAs into the plasma.[23]

A possible concern in our study protocol is the discrepancy of miRNA levels between the placenta and plasma. Because the sample volumes were limited, we selected only four of the seven FGR placenta-specific miRNAs to assess the changes of miRNA levels in maternal plasma before and after delivery. Furthermore, to select the pregnancy-associated molecules, the decreased plasma concentrations of FGR placenta-specific miRNAs levels after delivery of the placenta were confirmed in women with uncomplicated pregnancies but not in FGR pregnant women. In addition, the most significantly decreased expression level in the placentas of FGR pregnancies compared with those in placentas from uncomplicated pregnancies does not necessarily indicate a major change of miRNA levels in the maternal plasma of FGR women before and after delivery. Therefore, to obtain more valuable information in future analyses, the FGR placenta-specific miRNAs in maternal plasma should be tested in FGR pregnancies before and after delivery.

In summary, we characterized placenta-specific miRNAs in FGR pregnancy. Our data suggest that reduced expression of FGR placenta-specific miRNAs may be important in the FGR condition. It will be important to determine the specific pathways in which these miRNAs are involved and how their altered expression contributes to FGR. Furthermore, our study indicates the potential to predict future health outcomes on the basis of the altered expression of miRNAs in the placentas of FGR infants.[26] In

addition, there was a discrepancy between reduced expression levels of FGR pregnancy-associated, placenta-specific miRNAs in placental tissue and the normal levels in maternal plasma. Further investigation of this discrepancy will be important to better understand how placenta-specific miRNAs are released into maternal circulation.

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WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Previous microarray analysis identified placenta-specific microRNAs on C19MC.
- Some placenta-specific microRNAs were pregnancy-associated in maternal plasma.

WHAT DOES THIS STUDY ADD?

- This study characterized placenta-specific microRNAs in fetal growth restriction (FGR) pregnancy by next-generation sequencing.
- We present evidence that down-regulation of placenta-specific miRNAs located on C19MC is associated with FGR.
- No significant change of circulating levels of placenta-specific miRNAs was detected in maternal plasma of FGR pregnancy cases.

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ORIGINAL ARTICLE

Copy number variation of the antimicrobial-gene, *defensin beta 4*, is associated with susceptibility to cervical cancer

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The aim of this study was to investigate association between copy number variation of the *defensin beta 4 gene* (*DEFB4*) and susceptibility to cervical cancer in a population at high risk of persistent oncogenic human papillomavirus (HPV) infection. The study subjects comprised 204 women with cervical cancer, a population having a high risk of persistent oncogenic HPV infection (cervical cancer group), and 200 healthy women from the general population (control group). Copy number variation of *DEFB4* in each test sample was determined by relative quantitation using the comparative CT ($^{\Delta\Delta}$CT) method. Differences between the two groups were evaluated. The median *DEFB4* copy number in the cervical cancer group was four and in the control group was five ($P=2.77e{-}4$, *t*-test). The odds ratio of cervical cancer in individuals with four *DEFB4* copies or less was higher (odds ratio 2.02; 95% confidence interval odds ratio 1.36–3.02), compared with that in individuals with five or more copies (odds ratio 0.49; 95% confidence interval odds ratio 0.33–0.74). We found copy number variation of *DEFB4* was a host genetic factor conferring susceptibility to cervical cancer. A lower *DEFB4* copy number was associated with susceptibility to cervical cancer.
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Keywords: cervical cancer; copy number variation; defensin beta 4; human papillomavirus; susceptibility

INTRODUCTION

In humans, β-defensins are a family of small cationic antimicrobial peptides that exert an important first-line antimicrobial activity against a broad spectrum of bacteria, fungi and viruses.[1] The β-defensins are expressed in a variety of epithelia.[2] In particular, the *defensin beta 4 gene* (*DEFB4*; MIM 602215) gene is expressed in the non-pregnant female reproductive tract, including in the uterus, cervix and vagina.[3] A cluster of antimicrobial β-defensin genes on 8p23.1, including *DEFB4*, *SPAG11*, *DEFB103*, *DEFB104*, *DEFB105*, *DEFB106* and *DEFB107*, exhibits copy number variation (CNV) in the population.[4] Individuals have 2–12 copies of this cluster per diploid genome. Levels of *DEFB4* mRNA are significantly correlated with *DEFB4* copy number.[5] Higher copy numbers of *DEFB4*, result in significantly upregulated transcription after induction by tumor necrosis factor-α, which results in better antimicrobial activity *in vitro*.[6] Conversely, a lower *DEFB4* copy number may lead to decreased antimicrobial activity. The median copy number of *DEFB4* per diploid genome was significantly lower in human immunodeficiency virus-positive children (3.54) compared with that in human immunodeficiency virus-exposed uninfected children (4.49).[7] CNV of *DEFB4* is associated with susceptibility to human immunodeficiency virus infection.[8] Therefore, CNV of *DEFB4* on 8p23.1 may be associated with susceptibility to infectious disease.

Cervical cancer is the third most common cancer in women worldwide. Persistent infection with oncogenic human papillomavirus (HPV), including types HPV-16, HPV-18, HPV-31, HPV-33, HPV-45, HPV-52 and HPV-58, is recognized as a major risk factor for cervical cancer,[9] but is not sufficient on its own for cancer development. Only a small proportion of women with persistent infection of oncogenic HPV over many years develop carcinoma *in situ* and invasive cervical cancer. Genital infections with HPV are very common, and these infections are transmitted by sexual contact.[10] The HPV life cycle is intimately linked to the differentiation program of the epithelium targeted by HPV. HPVs are thought to infect the basal cells of the epithelium through microtraumas, and viral genomes are maintained as episomes in the basal layer, with viral gene expression being tightly controlled as the infected cells move toward the epithelial surface.[11] However, HPV infections in most cases disappear naturally in a

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relatively short period, and pose little risk of developing disease.[12–14] Additional cofactors; for example, host genetic factors and/or environmental factors, may be involved in cervical carcinogenesis. HPV cofactors have been identified, including oral contraceptive use and smoking.[15] Possible factors in persistent infection could be related to the host, such as the host immune reaction against an oncogenic HPV infection. For example, several reports suggest that genetic variations in human leukocyte antigen class II regions influence the risk of cervical cancer by altering the efficiency of the immune response to HPV infections.[16–20] However, we do not fully understand the pathological mechanism that results in HPV infection developing into invasive cervical cancer.

In this study, we hypothesized that CNV in the *DEFB4* gene may be associated with susceptibility to cervical cancer. To investigate our hypothesis, we compared *DEFB4* CNV in women with cervical cancer, a population with a high risk for persistent oncogenic HPV infections, with that in healthy women from the general population.

MATERIALS AND METHODS

Patient backgrounds

The study populations comprised 204 women with cervical cancer, a population having a high risk of persistent oncogenic HPV infection (cervical cancer group), and 200 healthy women from the general population (control group). All of these women attended the Department of Obstetrics and Gynecology at Nagasaki University Hospital or its satellite hospitals from August 2006 to December 2009. 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. All cases of cervical cancer were diagnosed as squamous cell carcinoma by histopathological examinations, whereas healthy women had no abnormal cytological findings in pap smear tests of the uterine cervix. There were no significant differences between the cervical cancer group and the control group for the following factors: age, parity, body mass index, smoking habit and use of oral contraceptives ($P > 0.05$, Table 1).

Genomic DNA samples

Genomic DNA was extracted from peripheral whole blood using a DNA Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA samples were prepared at 5 ng μl^{-1} using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Analysis of genomic DNA samples with known copy numbers of DEFB4 by TaqMan real-time PCR

The *albumin* gene (*ALB*) served as a two copies per diploid genome reference gene. The method of quantitative real-time PCR using LightCycler Relative Quantification Software 1.0 (Roche, Mannheim, Germany) requires an appropriate calibrator. Genomic DNA sample, C0913, with three copies of *DEFB4* per diploid genome and two copies of *ALB* per diploid genome, was purchased from the European Collection of Cell Cultures (Wiltshire, UK) as the calibrator.[21] Real-time PCR was performed using the LightCycler480 (Roche). The set of primers and the hybridization probe sequence for *ALB* (GenBank accession no. M12523) were: forward primer: 5′-TGGAAAATGATG AGATGCCTG-3′, reverse primer: 5′-CATAGTTTTTGCAAACATCC-3′, FAM reporter: 5′-CTTTCAACAAAATCAGCAGCTAATGAAGGC-3′, and those for *DEFB4* exon 2 (NM_04942) were: forward primer: 5′-TTTTGGTGGTATA GG-3′, reverse primer: 5′-TCTAGGGCAAAAGACTG-3′, FAM reporter: 5′-AT GGCTCCACTCTTAAGGCAGGTAACAG-3′. All PCR reaction mixtures were prepared automatically using QIAgility (Qiagen). Real-time PCR reactions (10 µl) were carried out in triplicate with 10 ng of unknown genomic DNA or calibrator DNA (three copies of *DEFB4* and two copies of *ALB* per diploid genome), 2 × Quanti-Tect-Multiplex-PCR NoROX buffer (Qiagen), 0.125 µM of each primer and 0.0625 µM of each TaqMan probe. Thermal cycling was initiated with a 2-min incubation at 50 °C, followed by a first denaturation step of 15 min at 95 °C, and then by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by one cycle at 5 °C for 6 min. The number of copies of *DEFB4* in each test sample was determined by relative quantitation using the comparative CT ($^{\Delta\Delta}$CT) method.[22] This method measured the cycle threshold (CT) difference ($^{\Delta}$CT) between *DEFB4* as a target and *ALB* as a reference and then compared the $^{\Delta}$CT values of test samples to the calibrator sample known to have three copies of the target sequence. All samples were analyzed in triplicate. Compared with the genomic DNA calibrator, with 3 *DEFB4* copies per diploid genome, genomic DNA samples, with 2, 4, 5, 6, 7 and 8 copies of *DEFB4* and 2 copies of *ALB* per diploid genome, were identified from blood samples of healthy women.

Determination of DEFB4 CNV in genomic DNA samples

To create standard curves for each known *DEFB4* copy number (2, 3, 4, 5, 6, 7 and 8 copies) and for 2 copies of *ALB* in the diploid genome, we selected individuals from the general population known to have each known copy number and compared these with C0913, which has three *DEFB4* copies per diploid genome. In all, 20, 10, 5 and 2.5 ng of genomic DNA samples for each known copy number individual were prepared as a two-step dilution series. Then, for each dilution, the *DEFB4* and the *ALB* genes were amplified in a single assay to acquire the relative quantification standard curves for both genes (Figure 1). The genomic DNA samples with unknown copies of *DEFB4* were amplified in each run in parallel with the samples with known copy numbers of *DEFB4*. In addition, each PCR run was referenced to the control. A no-template control (negative control) was also included in each assay.

Statistical analysis

Differences between the two groups were evaluated with Student's *t*-test. Odds ratios are estimated with 95% confidence intervals using logistic regression. Statistical analyses were performed with SPSS software version 19 (IBM, Armonk, NY, USA). Significant differences were defined as *P*-values of <0.05.

Table 1 Study population characteristics

Characteristics	*Cervical cancer group (n = 204)*	*Control group (n = 200)*	*P-value*
Age at sampling (years)[a]	32.2 (6.5)	31.6 (5.09)	NS[b]
Parity			NS[b]
Nulliparous	74	76	
Parous	130	124	
BMI (kg m^{-2})[a]	21.9 (3.1)	22.5 (3.6)	NS[b]
Smokers	3	3	NS[b]
Users of oral contraceptives	4	3	NS[b]

Abbreviation: BMI, body mass index.
[a]Data are shown as mean (s.d.).
[b]NS indicates no significant difference between two groups (*t*-test and χ^2 comparisons for continuous and discrete variables, respectively, of cervical cancer and control groups).

RESULTS

Characteristics of the two groups are described in Table 1. The cervical cancer group and the control group were similar with regard to age, parity, body mass index, smoking and oral contraceptive use.

DEFB4 CNV is described as the mean (s.d.) number of copies, and its distribution in cervical cancer and control groups is shown in Figure 2. CNV *of DEFB4* in the control group was 4.69 (1.45) copies, whereas that in the cervical cancer group was 4.25 (1.25) copies, showing that the *DEFB4* copy number in the cervical cancer group was significantly lower than in the control group ($P = 2.77e{-}4$, *t*-test).

The odds ratio of cervical cancer in individuals with four copies or less of *DEFB4* was relatively higher (odds ratio 2.02; 95% confidence interval odds ratio 1.36–3.02), compared with that in individuals with five copies or more (odds ratio 0.49; 95% confidence interval odds

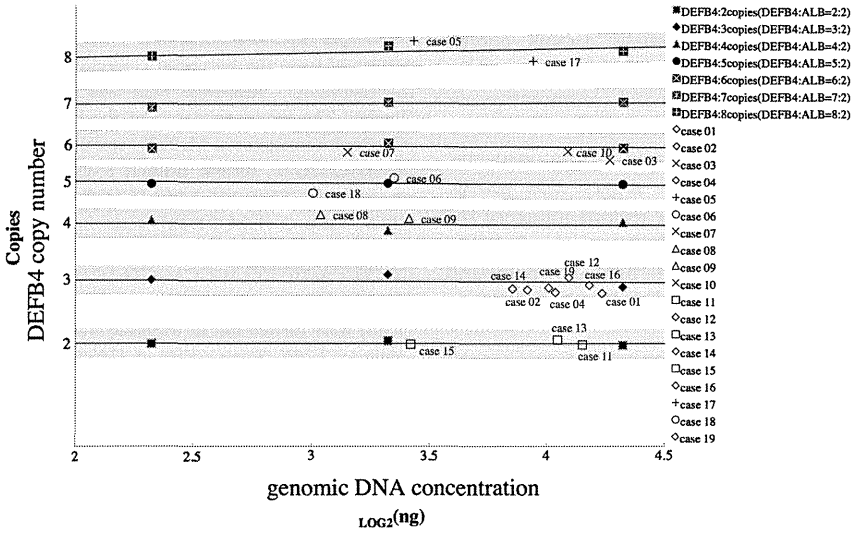

Figure 1 Standard curve for copy number quantification of *defensin beta 4 gene* (*DEFB4*). The standard curves of each known copy number of *DEFB4* (2, 3, 4, 5, 6, 7 and 8 copies) and 2 copies of *albumin* gene (*ALB*) in the diploid genome are shown as black lines and ±2 s.d. areas. The vertical axis indicates *DEFB4* copy number, whereas the horizontal axis shows log2 genomic DNA concentrations. Each plot reflects *DEFB4* assay copy numbers versus log2 (ng) genomic DNA concentration. The *DEFB4* copy number for genomic DNA samples with unknown copy number is classified into each copy number area.

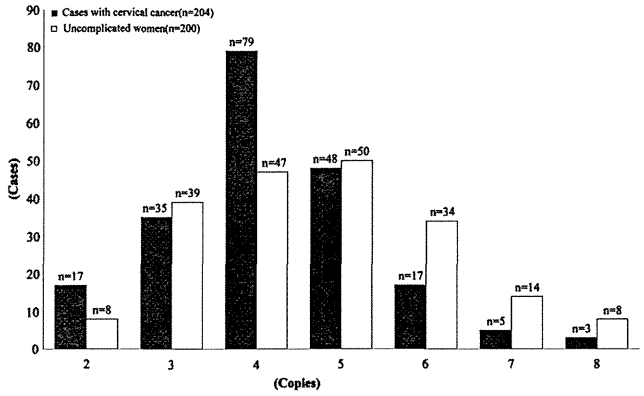

Figure 2 Distribution of *defensin beta 4 gene* (*DEFB4*) copy number variations (CNVs) in 204 women with cervical cancer and in 200 healthy women. Mean (s.d.) *DEFB4* CNV was 4.25 (1.25) copies in the cervical cancer group, and 4.69 (1.45) copies in healthy women. There was a significant difference in *DEFB4* CNV between the two groups ($P=2.77e{-}4$, *t*-test). The black bar indicates the distribution of *DEFB4* CNVs in the cervical cancer group, while the white bar indicates the distribution in healthy women.

ratio 0.33–0.74). Therefore, both values indicated a two-tailed significant difference from an odds ratio of 1.00 at the 5% level.

DISCUSSION

To our knowledge, this is the first study to investigate the association of *DEFB4* copy number with susceptibility to cervical cancer in a population at high risk of persistent oncogenic HPV infection. We found that a lower *DEFB4* gene copy number is associated with susceptibility to cervical cancer.

Cervical cancer develops via two critical transition steps: persistent HPV infection and progression to cervical intraepithelial neoplasia grade 3/invasive cancer (invasion through the basement membrane of the epithelium). Thus, HPV infection alone is not sufficient to cause cervical intraepithelial neoplasia grade 3/cervical cancer, and host genetic factors are very likely to contribute to cervical cancer pathogenesis.[23] A number of studies have evaluated the association of host genetic variations with cervical cancer and have implicated a role for human leukocyte antigens class II.[16–20] Many case–control studies have consistently reported a protective effect of human leukocyte antigen DRB1*13 against cervical cancer. Several class II alleles, such as DRB1*1501, DRB1*1302, DQB1*0602 and DQB1*03 are associated with an increased risk of cervical cancer, although the strength of the associations vary across racial and ethnic groups. In the population-based cohort studies of women in Guanacaste, Costa Rica, single-nucleotide polymorphisms in the Fanconi anemia complementation group A (*FANCA*) gene were associated with cervical cancer susceptibility. Single-nucleotide polymorphisms in the interferon regulatory factor 3 (*IRF3*) gene, which is an innate immune gene, and in the 2′,5′ oligoadenylate synthetase 3 (*OAS3*), sulfatase 1 (*SULF1*), deoxyuridine triphosphate (*DUT*) and general transcription factor IIH, polypeptide 3 (*GTF2H4*) genes were associated with HPV persistence. However, single-nucleotide polymorphisms in the interferon gamma (*IFNG*), epidermal dysplasia verruciformis (EV)-associated *EVER1/EVER2*, peroxiredoxin 3 (*PRDX3*) and ribosomal protein S19 (*RPS19*) genes were associated with progression to cervical intraepithelial neoplasia grade 3/cancer.[24–26] In this study, a lower *DEFB4* copy number was associated with susceptibility to cervical cancer, although the copy number at which *DEFB4* exerts its protective effect is not fully understood. The copy number of *DEFB4* affects its level of expression.[5] Thus, lower *DEFB4* CNV may increase susceptibility to cervical cancer because of persistent oncogenic HPV infection via a dosage effect. However, *DEFB4* only displayed mild anti-HPV activity *in vitro*.[27] Another study has shown substantial genetic heterogeneity in the human β-defensin gene cluster that may be a result of selection.[28] Hence, more widespread associations may be needed to determine whether such genetic heterogeneity accounts for some of the geographical or ethnic variations in different populations. And, as a cluster of β-defensin genes at 8p23.1 includes six genes (*DEFB4*, *DEFB103*, *DEFB104*, *DEFB105*, *DEFB106* and *DEFB107*)

within 100 kb region, there is a possibility that the copy number of *DEFB4* must closely link to that of other defensin genes. Therefore, to obtain more valuable information regarding the relation between CNVs of *DEFB4* and susceptibility to cervical cancer, the expression level of *DEFB4* should be analyzed in cervical tissues with low copy number and those with high copy number. Also, genome-wide association studies or exome sequencing analysis of cervical cancer should shed additional light on host genetic factors involved in cervical cancer. This new information may clarify the interplay between viral and host genetic factors in HPV persistence and the risk of progression to cervical intraepithelial neoplasia grade 3/cancer.

In conclusion, *DEFB4* CNV was observed in both cervical cancer and healthy control groups, and a lower *DEFB4* copy number may be associated with susceptibility to cervical cancer, which is a population at high risk of persistent oncogenic HPV infection. We found CNV of *DEFB4* to be an additional host genetic susceptibility factor of cervical cancer. Further studies are required to determine the reproducibility of our findings in larger patient groups and different study populations.

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ORIGINAL ARTICLE

Predominantly placenta-expressed mRNAs in maternal plasma as predictive markers for twin–twin transfusion syndrome

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ABSTRACT

Objective This study aimed to identify a set of predominantly placental (PP) mRNAs, which are associated with later-developing twin-to-twin transfusion syndrome (TTTS).

Method First, out of 50 PP mRNAs we previously reported, we select target mRNAs that are ordinarily detectable in maternal plasma. Plasma concentrations of these PP mRNAs were measured in monochorionic diamniotic twin (MCDA-T) pregnancies complicated by TTTS later ($n=11$) and in uncomplicated MCDA-T pregnancies ($n=17$). Finally, the diagnostic values of the PP mRNAs in plasma were evaluated.

Results From 50 PP mRNAs, nine [human placental lactogen (*hPL*); pregnancy-specific glycoproteins 2 (*PSG2*); human pregnancy-specific glycoproteins 3 (*PSG3*); *syncytin*; *syncytin 2*; retinoic acid-induced 14; A disintegrin and metalloproteinase domain-containing protein 12 (*ADAM12*); chorionic glycoprotein hormones, alpha polypeptide; and chorionic glycoprotein hormones, and beta polypeptide] were selected as target mRNAs. Changes in six PP mRNAs [increased *hPL*, *PSG2*, and *PSG3* and decreased *syncytin*, *syncytin2*, and *ADAM12*] in maternal plasma were detected in MCDA-T pregnant women who subsequently developed TTTS. Finally, mRNA signatures gave elevated AUCs (*hPL*/*PSG2*: 0.8717; *hPL*/*PSG3*: 0.8449; *hPL*/*ADAM12*: 0.8396) compared with single *hPL* mRNA.

Conclusion Quantitative aberration of plural cell-free PP mRNAs in maternal plasma precedes the appearance of clinically apparent TTTS. This suggests that pathophysiological changes in the placenta are associated with morbid conditions of TTTS. © 2013 John Wiley & Sons, Ltd.

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INTRODUCTION

Twin–twin transfusion syndrome (TTTS) is a serious complication in monochorionic diamniotic twins (MCDA-T) pregnancies that involves unequal blood flow via placental vascular anastomoses from the donor to the recipient twin. Although placental anastomoses are present in all cases of MCDA-T and both fetuses are genetically identical, TTTS occurs in only 15% of MCDA-T. Much of the pathophysiological basis of TTTS is unclear. Clinically, a staging system based on ultrasound features of TTTS is widely used for its management[1] but not to predict TTTS. New predictive markers are therefore desirable for the early detection and prevention of TTTS.

Recently, placental mRNA, such as human placental lactogen (*hPL*) and other hormones became detectable in maternal plasma; concentrations of each marker were measureable through quantitative real-time reverse transcription polymerase chain reaction (RT-PCR).[2–4] Thus, circulating cell-free mRNA in maternal plasma became an attractive target for non-invasive monitoring of pregnancy disorders.[4,5] Our previous studies indicated that increased levels of cell-free *hPL* mRNA in maternal circulation may be a novel predictive marker for TTTS,[6] although the statistical difference was small and the sample size was too small to give sufficient strength to the analysis. However, a combination of *plural* cell-free placental mRNA markers could effectively predict of TTTS, similar to the situation for tumor markers. Our previous other study to identify predominantly placental (PP) mRNAs, which are only expressed in the placenta, but not in blood cells, identified 50 PP mRNAs by comparing GeneChip signal intensities between placental tissues and corresponding whole blood samples.[7] These 50 PP mRNAs might both help predict and prevent TTTS and further elucidate the pathophysiological condition of TTTS.

In this study, we attempted to identify a set of PP mRNAs, which are associated with later-developing TTTS. We first selected (out of 50 PP mRNAs we had previously reported) PP mRNAs that are constantly detectable in maternal plasma.

Next, to identify PP mRNAs in maternal plasma that predict for TTTS, plasma concentrations of the PP mRNAs were measured in 28 cases of MCDA-T, and differences between MCDA-T pregnancies were complicated by TTTS later ($n=11$), and uncomplicated MCDA-T pregnancies ($n=17$) were evaluated. Finally, we analyzed diagnostic values of PP mRNAs that predicted TTTS.

MATERIALS AND METHODS

Sample collection

The study participants included 28 pregnant women who visited the Obstetrics Clinic of Nagasaki University Hospital at 12 to 21 weeks of gestation for management of their pregnancies with MCDA-T. All of the participants gave written informed consent, and the study was approved by the Research Ethics Committee of Nagasaki University. TTTS was defined as the presence of polyhydramnios (maximum vertical pocket of ≥8 cm) and oligohydramnios (maximum vertical pocket of ≤2 cm).[1] Although none of the 28 cases of MCDA-T were complicated by TTTS at the time of blood sampling, 11 cases subsequently developed TTTS (TTTS group), whereas the remaining 17 cases did not develop TTTS (non-TTTS group). Gestational ages at TTTS diagnosis were 12 to 24 weeks. For a control group, 50 singleton pregnant women without medical complications at similar gestational age were included. The two groups did not significantly differ in population characteristics, including maternal age, number of nulliparous women, and gestational age at the time of sampling (Table 1).

Blood (8 ml) was collected with ethylenediaminetetraacetic acid from the women, and cell-free mRNA was extracted from their plasma (1.6 ml) as described previously.[8]

Quantitative real-time RT-PCR

Because 50 PP mRNAs were identified in our previous study,[7] 50 primer sets for placenta-specific genes (TaqMan® Gene Expression Assays) were purchased from Applied Biosystems (Warrington, UK).[7] One-step quantitative real-time RT-PCR assay was performed using Applied Biosystems (ABI) 7900 T sequence detector (Applied Biosystems) as described previously.[8] RT-PCR amplifications of the placenta mRNA samples were carried out, and the PCR products were sequenced to confirm the accuracy of each gene-specific primer set. As the primer sets for *CSH1* and *HERV-FRD* failed to amplify their exact cDNA sequences, custom primers were made for these genes as described previously.[4,9] The PCR product was cloned into the TOPO II vector (Invitrogen), and the plasmid DNA was extracted to use for a calibration curve. For each mRNA assay, we prepared a calibration curve by tenfold serial dilution of plasmid DNA corresponding to each mRNA sequence from 1.0×10^2 to 1.0×10^8 copies/mL. Each sample and each calibration dilution were analyzed in triplicate. Each assay could detect down to 100 copies/mL. 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 using an ABI Prism 7900 sequence detector (Applied Biosystems).

Table 1 Clinical characteristics of pregnant women with monochorionic diamniotic twin included in the quantitative analysis of predominantly placental mRNAs in maternal plasma

Characteristics	TTTS group ($n=11$)	Non-TTTS group ($n=17$)	P
Maternal age (years)	29.2 (3.7)[a]	26.9 (3.8)[a]	NS
Gestational age at sampling (weeks)	17.0 (6.3)[a]	15.4 (2.2)[a]	NS
Parity			NS
Primiparous	6	13	
Multiparous	5	4	

TTTS, twin-to-twin transfusion syndrome; NS, no significant difference between two groups (Student t-test and χ^2 comparisons for continuous and discrete variables, respectively, of TTTS and non-TTTS groups).
$P<0.05$ is considered significant.
[a]Mean (standard deviation).

Selection criteria for PP mRNAs that are ordinarily found in maternal plasma

Because of the limitation of sample volumes, 50 cases of singleton pregnancy at 12 to 24 weeks of gestation were used to select PP mRNAs that are ordinarily found in maternal plasma. Fifty cases were grouped in five sets of ten cases. Each set was used for measurement of ten kinds of mRNAs. Each sample and each calibration dilution were analyzed in triplicate. Each assay could detect down to 100 copies/mL. When cell-free PP mRNA in plasma was consistently detectable (>100 copies/mL) in all of the ten cases, these mRNAs were selected as the target mRNAs in this study.

Statistical analysis

Patient backgrounds were compared by Student's t-test and Pearson's χ^2-test for continuous and discrete variables, respectively, of TTTS and non-TTTS groups. Absolute quantification data were analyzed with SDS 2.3 software (Applied Biosystems). Cell-free plasma concentrations of PP mRNAs in the TTTS and non-TTTS groups were converted into multiples of the median of the controls adjusted for gestational age, as described previously.[6,10] Differences between the two groups were evaluated with Mann–Whitney U-test, and differences between the three groups were examined with the Kruskal–Wallis test. Statistical analyses were performed with SPSS version 19 (IBM Japan, Tokyo, Japan). To determine the use of PP mRNAs to classify TTTS and non-TTTS later, receiver operating characteristic (ROC) curves were plotted with an R package, pROC.[10] To develop mRNA signatures that most accurately distinguished between patients who developed TTTS later and those who did not, we used a multivariate logistic regression model. The obtained regression models were evaluated with Wald tests. Statistical analyses used R (R Core Team, Vienna, Austria). $P<0.05$ was considered significant.

RESULTS

PP mRNAs that could ordinarily be measured in maternal plasma

Of 50 PP mRNAs, nine mRNA [*hPL*; human pregnancy-specific glycoproteins 2 (*PSG2*); human pregnancy-specific glycoproteins 3 (*PSG3*); *syncytin*; *syncytin 2*; retinoic acid-induced 14 (*RAI14*); A

disintegrin and metalloproteinase domain-containing protein 12 (*ADAM12*); chorionic glycoprotein hormones, alpha polypeptide (*CGA*); and chorionic glycoprotein hormones, and beta polypeptide (*CGB*)] were detectable in all ten singleton pregnancy plasma samples; these were selected as target mRNAs in this study.

Identification of cell-free PP mRNAs, which are associated with later-developing TTTS

Circulating levels of the nine PP mRNAs in plasma from the cases of MCDA-T and the control cases were measured by quantitative RT-PCR. The quantitative aberrations of six cell-free PP mRNAs (increased *hPL*, *PSG2*, and *PSG3* and decreased *syncytin*, *syncytin2*, and *ADAM12*) in maternal plasma were detected in MCDA-T pregnant women who subsequently developed of TTTS (Kruskal–Wallis test, *P* values were 0.005, 0.001, 0.002, 0.044, 0.027, and 0.012, respectively; Table 2), whereas there was no significant differences of three cell-free PP mRNAs (*RAI14*, *CGA*, and *CGB*) levels and cell-free *GAPDH* mRNA level in maternal plasma between TTTS, non-TTTS, and control groups (Table 2). The circulating levels of three PP mRNAs (*hPL*, *PSG2*, and *PSG3*) were significantly higher in plasmas from those who developed TTTS later than in plasmas from patients who did not (non-TTTS) (Mann–Whitney *U*-test, *P* values were 0.014, 0.007, and 0.003, respectively; Table 2, Figure 1). Circulating levels of three PP mRNAs (*syncytin*, *syncytin2*, and *ADAM12*) were significantly lower in plasmas from those who developed TTTS later than in plasmas from non-TTTS patients (Mann–Whitney *U*-test, *P* values were 0.041, 0.029, and 0.003, respectively; Table 2, Figure 1). Plasma levels of three PP mRNAs (*RAI14*, *CGA*, and *CGB*) and *GAPDH* mRNA did not significantly differ between the two groups (Mann–Whitney *U*-test, *P* values were 0.525, 0.655, 0.944, and 0.778, respectively; Table 2, Figure 1).

Diagnostic value of PP mRNAs in plasma

The ROC curves to discriminate the TTTS group from the non-TTTS group were constructed on the basis of PP mRNAs in plasma samples (TTTS, $n=11$; non-TTTS, $n=17$). Analysis of ROCs revealed high area under the curve (AUC) values for each

Table 2 Circulating levels of predominantly placenta-expressed miRNAs in plasma samples from patients with monochorionic diamniotic twin pregnancies who developed twin-to-twin transfusion syndrome (TTTS) later (TTTS group) and from such patients who did not develop TTTS (non-TTTS group) and singleton pregnant women without medical complications (control group)

mRNA	TTTS group ($n=11$)	non-TTTS group ($n=17$)	Control group ($n=10$)	*P*
hPL	1.78 (1.12–2.80)	1.15 (0.89–1.87)	1.0 (0.68–2.56)	0.005
PSG2	2.60 (1.28–4.04)	1.37 (0.58–3.20)	1.0 (0.52–2.74)	0.001
PSG3	3.13 (0.70–12.3)	0.99 (0.42–4.23)	1.0 (0.39–2.86)	0.002
Syncytin	0.46 (0.22–1.89)	1.12 (0.21–3.43)	1.0 (0.21–3.18)	0.044
Syncytin2	0.69 (0.48–0.99)	0.95 (0.46–3.53)	1.0 (0.42–3.37)	0.027
ADAM12	0.49 (0.31–1.39)	1.18 (0.31–2.31)	1.0 (0.25–1.46)	0.012
RAI14	1.13 (0.50–1.54)	1.21 (0.31–2.31)	1.0 (0.35–2.06)	NS
CGA	1.05 (0.36–1.49)	1.19 (0.29–1.68)	1.0 (0.33–1.61)	NS
CGB	0.95 (0.41–1.34)	0.92 (0.27–1.76)	1.0 (0.39–1.76)	NS
GAPDH	1.10 (0.51–2.17)	1.01 (0.49–2.62)	1.0 (0.47–2.17)	NS

NS, not significant.

Expression levels are shown as multiple of median, with median (minimum–maximum range). Significant differences between groups were analyzed by Kruskal–Wallis test. $P<0.05$ is considered significant.

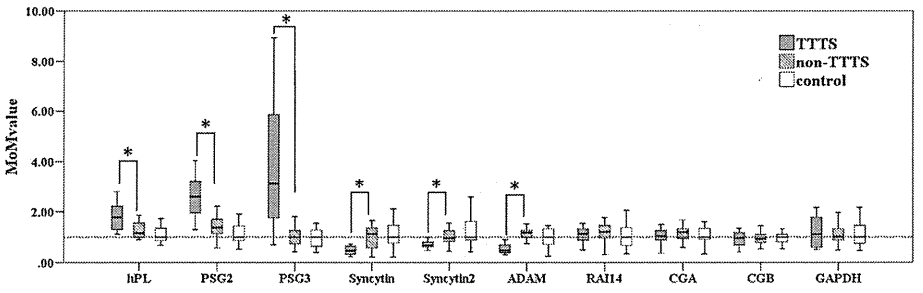

Figure 1 Circulating levels of predominantly placental mRNAs in maternal plasma samples from twin-to-twin transfusion syndrome (TTTS) group and non-TTTS group. Circulating levels of cell-free mRNA in maternal plasma were expressed as multiple of median (MoM) values. Gray bars indicate data from cases that later developed TTTS; shaded bars indicate the data from cases that did not develop TTTS (non-TTTS); white bars indicate the data from singleton pregnant women (control). *Significant difference ($P<0.05$)

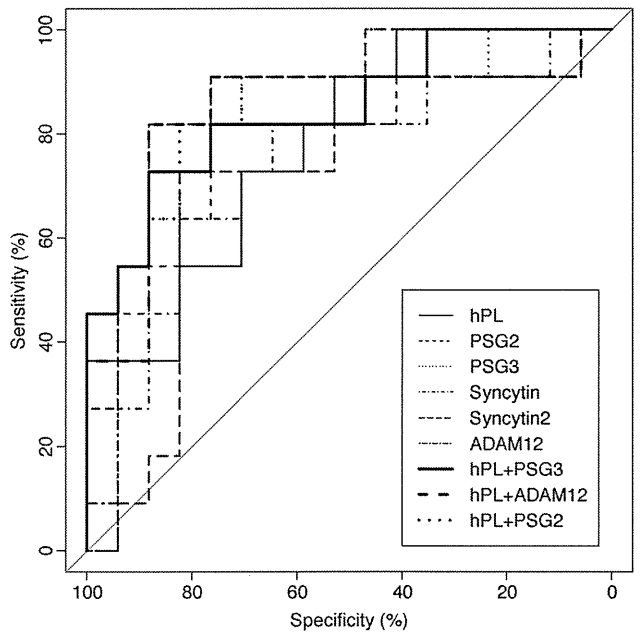

Figure 2 Receiver operating characteristic analysis of plasma mRNA profiles to discriminate cases of twin-to-twin transfusion syndrome (TTTS) later from cases of non-TTTS later. Analysis of receiver operating characteristics revealed high AUC values for each predominantly placental mRNA in plasma (TTTS: $n=11$; non-TTTS: $n=17$). The mRNA signatures consisting of two predominantly placental mRNAs yielded elevated AUCs compared with single *hPL* mRNAs

PP mRNA in plasma (Figure 2): *hPL*, 0.7807 (95% CI: 0.6083–0.9532), *PSG2*: 0.8075 (95% CI: 0.6383–0.9767), *PSG3*: 0.8396 (95% CI: 0.6785–1.0), *syncytin*: 0.0.7326 (95% CI: 0.5291–0.9362), *syncytin2*: 0.7487 (95% CI: 0.559–0.9383), and *ADAM12*: 0.8342 (95% CI: 0.648–1.0).[11]

The mRNA signatures consisting of two PP mRNAs yielded elevated AUCs in comparison to single *hPL* mRNAs (Figure 2): *hPL/PSG2*: 0.8717 (95% CI: 0.7400–1.0, $P=0.1124$, Wald test), *hPL/PSG3*: 0.8449 (95% CI: 0.6893–1.0, $P=0.0929$, Wald test), and *hPL/ADAM12*: 0.8396 (95% CI: 0.6579–1.0, $P=0.9571$, Wald test).

DISCUSSION

In this study, we found that changes in plasma concentrations of plural PP mRNAs were detectable before the appearance of clinically apparent TTTS. In addition, we evaluated the diagnostic significance of a set of PP mRNAs in maternal plasma.

As non-invasive potential predictive markers for patients who develop TTTS later, six PP mRNAs in maternal plasma were identified – three that increased (*hPL*, *PSG2*, and *PSG3*) and three that decreased (*syncytin*, *syncytin2*, and *ADAM12*). *PSG2*, *hPL*, and *PSG3* are synthesized in large amounts by the placental syncytiotrophoblast and are released into the maternal circulation during pregnancy.[12,13] *Syncytin1* and *syncytin2*, which are placenta-specific env genes of endogenous retroviruses, have fusogenic activity in promoting the formation of multinucleated syncytiotrophoblast cells in the placenta.[14,15] *ADAM12* is one of the most promising protein candidates for participation, as well as *syncytin*, in the process of trophoblast fusion in the placenta.[15,16] The current study found that changes in plasma concentrations of PP mRNAs, which are important molecules for placentation and maintenance of pregnancy, preceded the appearance of clinically apparent TTTS. This suggests that abnormal placentation and abnormalities in the placenta are associated with the pathophysiological basis of TTTS.

Although a staging system based on the ultrasound features of TTTS is widely used for its management,[1] this system is not generally used to predict TTTS.

These plasma-based biomarkers may lead to development of a non-invasive means of predicting later TTTS. Expression patterns for mRNA are known to be aberrant in pregnancy-related diseases (e.g. preeclampsia, placenta previa)[2,3,7,8]; placenta-derived mRNAs in circulation may be stored in microvesicles.[17] Additionally, cell-free mRNAs are remarkably stable in plasma.[2–4] Our observations suggest that PP mRNAs in plasma might serve as a non-invasive biomarker for early detection of TTTS later. Although the source of plasma PP mRNAs has not been determined, they might derive from exosomes shed from apoptotic or broken placental cells.[8,17,18] Therefore, although this is a matter of speculation, abnormal placentation caused by a decreased level of PP mRNAs (*syncytin*, *syncytin2*, and *ADAM12*) at the early stage of pregnancy may lead to increased levels of circulating microparticles, which include placental molecule (*hPL*, *PSG2*, and *PSG3*) synthesized by syncytiotrophoblast, into maternal plasma.

The specific conditions that regulate levels of the maternal plasma mRNA in the TTTS group remain unknown; possibly, unapparent pathophysiological changes had already occurred in the women who subsequently developed TTTS. ROC curve analysis revealed that six single-regulated PP mRNAs in plasma samples from MCDA-T pregnancies could distinguish between TTTS and non-TTTS pregnancies later, yielding high AUCs (Figure 2). The mRNA signatures consisting of two PP mRNAs that yield elevated AUCs compared with single *hPL* mRNAs, although the PP mRNA signatures and a single *hPL* mRNA did not significantly differ. The known predictive findings (e.g. growth discordancy, nuchal translucency) by ultrasonographic examination are only detectable in a small portion of TTTS cases,[19] although they can be measured simply and non-invasively. In contrast, expression profiles of PP mRNAs in maternal plasma differ between MCDA-T pregnancies that are, and are not, complicated by TTTS later, suggesting that PP mRNAs signatures in maternal plasma may be used as additional markers for TTTS prediction in MCDA-T pregnancies. As the pathogenesis of TTTS may be associated with multiple factors, TTTS could be regulated by a large variety of genes. Furthermore, another study demonstrated a significant difference in cell-free vascular endothelial growth factor A, endoglin, and angiopoietin-2 mRNA levels in maternal plasma between MCDA-T pregnancies complicated by TTTS and uncomplicated MCDA-T pregnancies.[20] The aforementioned observations suggest that placental factor and other factors are associated with TTTS pathogenesis of TTTS. Therefore, as with markers for

malignant tumors, a combination of plural mRNA markers (placental factor, PP mRNAs; other factors, vascular endothelial growth factor A, *endoglin*, and angiopoietin-2 mRNAs, etc.) and ultrasonographic findings (e.g. growth discordancy, nuchal translucency) may predict MCDA-T pregnancies later complicated by TTTS more efficiently, compared with a single PP mRNA marker.[20]

In conclusion, a cohort of MCDA-T pregnancies, in which some were complicated by TTTS later, showed that changes in plural cell-free PP mRNA levels in maternal plasma preceded the appearance of clinically apparent TTTS. This was a preliminary study with a small sample size. Therefore, a large prospective study measuring placental factors in MCDA-T pregnancies would help to confirm the diagnostic significance of a set of cell-free PP mRNAs as a predictive test for TTTS. Future studies regarding the biological pathway of PP mRNAs in maternal plasma as predictors of TTTS may help elucidate the molecular pathogenesis of TTTS.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Predominantly placental (PP) mRNAs, for example, human placental lactogen (*hPL*), in maternal plasma are measurable by quantitative reverse transcription polymerase chain reaction.
- Increased cell-free *hPL* mRNA in maternal plasma may predict twin-to-twin transfusion syndrome (TTTS).
- We found 50 PP mRNAs using microarray analysis.

WHAT DOES THIS STUDY ADD?

- In addition to *hPL* mRNA, quantitative aberration of plural PP mRNAs precedes the appearance of clinically apparent TTTS.
- We evaluated these PP mRNAs in maternal plasma as TTTS predictors.

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Initial Viral Load in Cases of Single Human Papillomavirus 16 or 52 Persistent Infection Is Associated With Progression of Later Cytopathological Findings in the Uterine Cervix

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The aim of this study was to investigate the relationship between viral load in single human papillomavirus (HPV) 16 or 52 persistent infection and the progression of later cytopathological findings in the uterine cervix. Cervical cytology and HPV genotyping tests were repeated within 3–6 months in 305 women with oncogenic HPV. Twenty-four cases of single HPV 52 persistent infection and 24 cases of single HPV 16 persistent infection were identified. Cases with later cytopathological findings showing progression were defined as the progression group, while those with no change or regression were the non-progression group. Relative HPV DNA loads were determined by quantitative real-time polymerase chain reaction and expressed relative to human albumin (*ALB*) DNA. Differences between the two groups were evaluated. The median relative HPV 52 DNA load was 2.211 in the progression group and 0.022 in the non-progression group (Mann–Whitney *U*-test, $P = 0.003$). The median relative HPV 16 DNA load was 4.206 in the progression group and 0.103 in the non-progression group ($P = 0.001$). HPV 52 and 16 DNA loads assessed by quantitative real-time methods may be useful short-term markers for identifying women at high risk for progression of cervical cytological pathology. ***J. Med. Virol. 85:2093–2100, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: cervical cytology; oncogenic human papillomavirus; persistent infection; progression; virus load

INTRODUCTION

Persistent infections with oncogenic human papillomaviruses (HPVs), including 16 HPV genotypes (16, 18, 31, 33, 35, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) are recognized as a major risk factor for cervical cancer [Muñoz et al., 2003]. Genital HPV infections are common and are transmitted by sexual contact [Shimada et al., 2007]. However, most HPV infections disappear naturally over a relatively short period and are associated with little risk of developing disease [Moscicki et al., 1993; Ho et al., 1998; Woodman et al., 2001]. The presence of HPV per se therefore has a low predictive value for the risk of developing cervical cancer.

Screening of high-grade squamous intraepithelial lesion and cervical cancer are currently based on cervicovaginal pap smears with detection of oncogenic HPV [Inoue et al., 2010]. However, clinical interest in viral-load quantification has been demonstrated in human immunodeficiency virus and hepatitis B virus infections, where it is used routinely as a tool for diagnosis, prognosis, and therapeutic management [Berger and Preiser, 2002]. By contrast, the association between HPV viral load and evolution towards malignant cervical lesions remains debatable

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[Dalstein et al., 2003; Carcopino et al., 2006; Xi et al., 2009].

The HPV DNA load seems to predict the risk of developing cervical carcinoma prior to the appearance of any cytological alterations, and certainly long before the appearance of tumors [Ylitalo et al., 2000]. In particular, high HPV 16 loads in normal cervical smears are a risk marker for later development of cervical intraepithelial neoplasia or carcinoma in situ of the cervix [van Duin et al., 2002; Moberg et al., 2003]. In addition, high HPV 16 load is a risk marker for subsequent invasive cervical cancer [Moberg et al., 2005]. Testing for HPV 16 DNA load during gynecological health checks could thus strikingly improve our ability to distinguish between high- and low-risk infections in terms of progression to cervical cancer [Josefsson et al., 2000]. However, one study showed an increased odds ratio of prevalent high-grade squamous intraepithelial lesion/cancer for HPV 16 load, but no similar trend for HPV 18 [Gravitt et al., 2003], while another study reported possible predictive values of viral loads in HPV 16- and 18-positive patients with low-grade squamous intraepithelial lesion cytology [Botezatu et al., 2009]. The clinical utility of viral-load testing for all oncogenic HPV genotypes therefore remains unclear.

HPV 16 and 18 account for approximately 70% of cancers and 50% of high-grade cervical intraepithelial neoplasia [Smith et al., 2007]. However, previous study showed that HPV 52 was a more common genotype in Nagasaki, Japan, compared with the distribution of high-risk HPV genotypes in other countries [Yamasaki et al., 2011a,b]. In addition, HPV 52 was the most common genotype among HPV-infected pregnant Japanese women. The second most common genotype was HPV 16, and these two genotypes collectively accounted for around 60% of HPV-positive pregnant women [Yamasaki et al., 2011b].

Another complication of using viral load to predict neoplasias of cervical intraepithelial neoplasia 2 or greater is the high prevalence of multiple oncogenic HPV infections detected in cervical samples. Recent studies have focused on longitudinal observations of viral load to predict viral clearance or lesion progression [Monnier-Benoit et al., 2006; Marks et al., 2011]. Initial data indicate that repeated measurements can improve prediction of persistence or clearance, but these data are currently limited to HPV 16. Because the screening of cervical disease is based on cervicovaginal Pap smears with detection of oncogenic HPV, it is both important and necessary to clarify the association between the viral load of individual oncogenic HPV types in each region and the progression of later cytopathological findings in the uterine cervix.

In this study, the relationship between viral loads in single HPV 16 or 52 persistent infections and the progression of later cytopathological findings in the uterine cervix was investigated to improve the understanding of the clinical utility of oncogenic HPV DNA loads in Japanese women.

MATERIALS AND METHODS

Study Patients

A total of 305 women with oncogenic HPV underwent repeat cervical cytology and HPV DNA tests within 3–6 months, between August 2007 and April 2011. These 305 women included patients negative for intraepithelial lesion or malignancy, with atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion or high-grade squamous intraepithelial lesion. Among these, 24 cases of single HPV 52 persistent infection and 24 cases of single HPV 16 persistent infection were included in this study, and the samples with multiple HPV types including HPV52 or HPV16 were excluded. The study protocol was approved by the Ethical Review Board of Nagasaki University and the other hospitals involved. All women were informed of the purpose of the study and gave their consent.

Sample Collection and Cytological Diagnoses

Specimens were collected using a Cervex Brush (Rovers Medical Devices, Oss, the Netherlands) and suspended in 10 ml of SurePath preservative fluid (Becton, Dickinson & Company, Franklin Lakes, NJ). Samples from the same vial for cytological testing with the Bethesda III system (2001) and for HPV genotype testing were used [Yamasaki et al., 2011a, b]. Cervical specimens for cytology and HPV genotyping were obtained at each visit from participants who received regular follow-up examinations. Cytologic diagnoses of the specimens were performed by the same experienced cytoscreener in a commercial laboratory (SRL, Tokyo, Japan), who was blinded to the results of the HPV genotyping test. Regarding cervical cytopathological findings after 3–6 months, cases showing progression were defined as the progression group, and cases showing no change or regression were defined as the non-progression group.

Histopathological Examinations

Colposcopies and biopsies were performed only when cervical cytopathological findings were detected. Histopathological diagnoses were made by two different pathologists, and cervical intraepithelial neoplasia is categorized according to World Health Organization Classification as follow: cervical intraepithelial neoplasia 1, 2, and 3 [Tavassoli and Devilee, 2003]. H&E photos of cervical intraepithelial neoplasia 1, 2, and 3 are shown in Figure 1.

HPV Genotyping Test

Genotyping of HPV DNA in SurePath preservative fluid was carried out after preparing glass slides, using the Linear Array HPV Genotyping