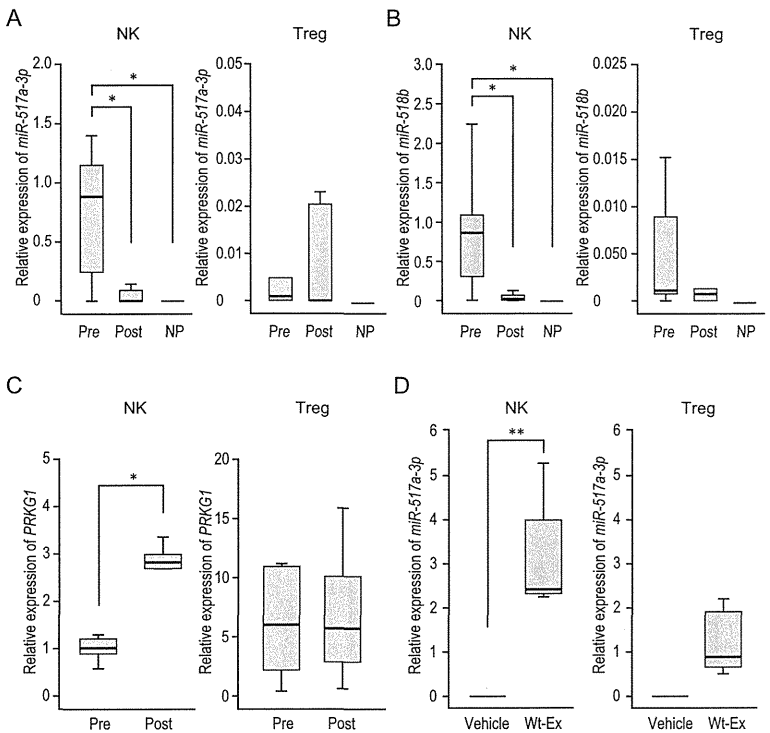

FIG. 5. Transferred placenta-associated miRNAs in the peripheral blood immune cells of pregnant women. **A** and **B**) Quantification of transferred placenta-associated miRNAs in maternal immune cells. Transferred *miR-517a-3p* (**A**) and *miR-518b* (**B**) were measured by real-time PCR in NK and Treg cells isolated from maternal peripheral blood samples at 1 day before delivery (Pre) and 4 days after delivery (Post). NK, n = 15; Treg, n = 9. The NK and Treg cells were also obtained from nonpregnant women with no history of pregnancy (NP; n = 5). Data were normalized to *SNORD44*. Expression in NK before delivery was defined as 1. Lines inside boxes denote medians; boxes represent the interquartile range; and whiskers extend to the most extreme values within 1.5 times the interquartile range. **C**) Differential expression of *PRKG1* mRNA in maternal peripheral blood immune cells before and after delivery. *PRKG1* expression in maternal NK cells (n = 5) and Treg cells (n = 6) obtained from pregnant women both 1 day before delivery (Pre) and 4 days after delivery (Post) was investigated by real-time PCR. Data were normalized to *GAPDH*. Expression in NK before delivery was defined as 1. **D**) In vitro transfer of BeWo *miR-517a-3p* into male NK and Treg cells. After incubation of each cell type in the presence (Wt-Ex) or absence (Vehicle) of BeWo wild-type exosomes (50 μg/ml) for 48 h at 37°C, levels of transferred *miR-517a-3p* were analyzed by real-time PCR (n = 5 each). Data were normalized to *SNORD44*. Expression in Treg cells incubated with exosomes was defined as 1. Data are means ± SD. Kruskal-Wallis test; $^{*}P < 0.05$, $^{**}P < 0.01$.

elucidated. Exosomes differ in their cellular origin [48]. Thus, cell type-specific molecules on the cell surface of exosomes may promote specific delivery to target cells. In addition to the exosome-mediated communication between BeWo and Jurkat cells, we investigated the possible involvement of *miR-517a-3p* in exosome-mediated communication between BeWo and human umbilical vein endothelial cells. Although BeWo exosomal *miR-517a-3p* was transferable to human umbilical vein endothelial cells as well as Jurkat cells, it suppressed the expression of different target genes within the nonimmune recipient cells (our unpublished data). The mechanism by which placental exosomes preferentially target and modulate specific cells has not yet been determined. In the present study, cell lines were primarily employed. Differential ultracentrifugation employed in this study could not separate relatively large vesicles (e.g., 150- to 200-nm vesicles). As reported by Bobrie et al. [49], improved techniques will be needed to accurately purify the different populations of extracellular vesicles. To further characterize trophoblast-derived exosomes (e.g., the cell surface properties and target cell-specific delivery) and reveal the functional roles of exosome-mediated placenta-associated miRNAs in maternal NK cells, future work should be performed using a combination of primary immune cells and exosomes isolated from primary human trophoblasts.

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Intestinal Microbiota is Different in Women with Preterm Birth: Results from Terminal Restriction Fragment Length Polymorphism Analysis

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Abstract

Preterm birth is a leading cause of perinatal morbidity and mortality. Studies using a cultivation method or molecular identification have shown that bacterial vaginosis is one of the risk factors for preterm birth. However, an association between preterm birth and intestinal microbiota has not been reported using molecular techniques, although the vaginal microbiota changes during pregnancy. Our aim here was to clarify the difference in intestinal and vaginal microbiota between women with preterm birth and women without preterm labor. 16S ribosomal ribonucleic acid genes were amplified from fecal and vaginal DNA by polymerase chain reaction. Using terminal restriction fragment length polymorphism (T-RFLP), we compared the levels of operational taxonomic units of both intestinal and vaginal flora among three groups: pregnant women who delivered term babies without preterm labor (non-PTL group) (n = 20), those who had preterm labor but delivered term babies (PTL group) (n = 11), and those who had preterm birth (PTB group) (n = 10). Significantly low levels of *Clostridium* subcluster XVIII, *Clostridium* cluster IV, *Clostridium* subcluster XIVa, and *Bacteroides*, and a significantly high level of *Lactobacillales* were observed in the intestinal microbiota in the PTB group compared with those in the non-PTL group. The levels of *Clostridium* subcluster XVIII and *Clostridium* subcluster XIVa in the PTB group were significantly lower than those in the PTL group, and these levels in the PTL group were significantly lower than those in non-PTL group. However, there were no significant differences in vaginal microbiota among the three groups. Intestinal microbiota in the PTB group was found to differ from that in the non-PTL group using the T-RFLP method.

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Introduction

We harbor more than 100 trillion microbes in and on our body and these microbes constitute our microbiota [1,2]. Although only a small part of this microbiota can be cultured in a medium, culture-independent analyses, like 16S ribosomal ribonucleic acid (rRNA)-based genomic analysis [3,4] and metagenomic analysis [5], have recently revolutionized our understanding of the microbiota in our body. The microbiota in our intestine plays a major role in health and disease [1] in our body. The gut microbiota interacts with the immune system, providing signals to promote the maturation of immune cells and the normal development of immune functions [6,7,8].

Preterm birth (PTB) is the leading cause of perinatal morbidity and mortality in developing and developed countries [9]. The frequency of preterm births is about 12 to 13% in the USA and 5 to 9% in many other developed countries including Japan. Spontaneous PTB is regarded as a syndrome resulting from multiple causes, including intrauterine infection or inflammation, stress, socioeconomic environment, and uterine over-distension. Risk factors for PTB in Western countries include a previous preterm birth, black ethnicity, periodontal disease, low maternal body mass index, short cervical length, and an elevated cervical-vaginal fetal fibronectin concentration, while multiple pregnancy, short cervical length, part-time worker, steroid use for asthma or collagen disease, low educational level, and male fetus were shown to be risk factors for PTB in Japan [10]. Although antibiotic treatment can eradicate bacterial vaginosis (BV) in pregnancy, the overall risk of PTB in pregnant women with BV was found not to be significantly reduced by antibiotic treatment [11].

An association between preterm birth and intestinal microbiota has not been reported using molecular techniques, although the vaginal microbiota changes during pregnancy. Aagaard et al. [12] showed that the vaginal microbiome signature in pregnancy was distinct from that in non-pregnant women with variation of taxa across vaginal subsites and depending on gestational age. Romero et al. reported that, in a longitudinal study, *Lactobacillus* spp. were the predominant members of the microbial community in normal pregnancy [13], and they did not detect a difference in human vaginal microbiota between women who subsequently had a spontaneous preterm delivery and those who delivered at term [14]. Koren et al. described dramatic remodeling of the gut microbiota over the course of pregnancy [15]. These changes of intestinal microbiota induced insulin resistance during pregnancy. Although abnormal vaginal microbiota such as BV was studied using 16S rDNA-based genomic analysis [16], there are no reports on a link between PTB and gut microbiota as determined by genomic analysis.

Terminal restriction fragment length polymorphism (T-RFLP) analysis has used non-targeted approaches to identify differences and similarities in microbial communities, but it does not provide direct sequence information. Because of its relative simplicity, T-RFLP analysis has been applied to the analysis of bacterial 16S rRNA genes and provides a facile means to assess changes in microbial communities [17]. In this prospective and cross-sectional study using T-RFLP analysis, we examined bacterium-derived 16S rRNA genes in feces and vaginal discharge to determine whether the microbiota differs among three groups as follows: pregnant women who delivered term babies without preterm labor (non-PTL group), those who had preterm labor but delivered term babies (PTL group), and those who had preterm birth (PTB group).

Materials and Methods

This study is a prospective and cross-sectional study. The study protocol and informed consent documents were reviewed and approved by the University of Toyama Institutional Review Board. Written informed consent was obtained from all subjects prior to participation in the study. From 2011 to 2013, the participants were categorized into three groups as follows: (1) pregnant women who delivered term babies without preterm labor during pregnancy (non-PTL group) (n = 20), (2) those who had regular uterine contraction and received tocolytic agents but delivered term babies (PTL group) (n = 11), and (3) those who had regular uterine contractions, received tocolytic agents, and finally delivered preterm babies (PTB group) (n = 10). Women who were at earlier than 22 weeks of gestation and those who took any antibiotics, tocolytics and steroids during pregnancy were excluded. Clinical characteristics of all participants are shown in Table 1. Vaginal samples were obtained from the posterior vaginal fornix using a swab. Swabs were immersed in vaginal discharge for 10 seconds, then immediately placed into 3 mL of a buffer medium (100 mM Tris-HCl, pH 9.0, 40 mM Tris-EDTA, pH 8.0, and 4 M guanidine thiocyanate), and stored in a freezer at −20°C. Fecal samples were collected at home or our hospital by the participants. Briefly, the toilet was covered with a sterile sheet of paper, which was temporarily waterproof but would dissolve in the water of the toilet within a few minutes and could be flushed away with the remaining stool. The defecated fecal samples were quickly collected using a sterile spoon or a swab, immediately placed in 3 mL of buffer medium, and stored in a freezer at −20°C.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

In order to investigate the microbiota of the fecal and vaginal samples obtained from all the subjects, terminal restriction fragment length polymorphism (T-RFLP) analysis was performed, as previously reported [18].

Fecal and vaginal samples were suspended in a solution containing 100 mM Tris-HCl, pH 9.0, 40 mM Tris-EDTA, pH 8.0, and 4 M guanidine thiocyanate, and kept at −20°C until deoxyribonucleic acid (DNA) extraction. An aliquot of 0.8 mL of the suspension was homogenized with zirconia beads in a 2.0 mL screw cap tube using a FastPrep FP120A Instrument (MP Biomedicals, Irvine, CA) and placed on ice. After centrifugation (at 5000×g, for 1 min), the supernatant was transferred to the automated nucleic acid isolation system 12GC (Precision System Science, Chiba, Japan). Thereafter, DNA was extracted from the bead-treated suspension using the Magtration-MagaZorb DNA Common Kit 200 N (Precision System Science, Chiba, Japan).

The 16S rDNA was amplified from human fecal DNA using fluorescent-labeled 516f primer (5′-(6-FAM)-TGCCAG-CAGCCGCGGTA-3′) and 1492r primer (5′-GGTTACCTTGT-TACGACTT-3′) and from human vaginal DNA using fluorescent-labeled 27f (5′-(6-FAM)-AGAGTTTGATCCTGGCTCAG-3′) and 1492r primer (5′-GGTTACCTTGTTACGACTT-3′), with Hot-starTaq DNA polymerase using Gene Amp PCR system 9600 (Applied Biosystems, CA, USA). The amplification program used was as follows: preheating at 95°C for 15 minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute; and finally terminal extension at 72°C for 10 minutes. The amplified DNA was purified using a MultiScreen^{R} PCRμ96 Filter Plate (Millipore, MA, USA) and verified by electrophoresis.

The restriction enzymes were selected according to Nagashima *et al.* [19]. In brief, the PCR product purified from fecal samples was digested with 10 U of *Bsl* I (New England BioLabs, Inc., Ipswich, USA) at 37°C for 3 hours or that from vaginal samples with 10 U of *Msp* I (TAKARA, Shiga, Japan) at 37°C for 3 hours. The resultant DNA fragments, namely, fluorescent-labeled terminal restriction fragments (T-RFs), were analyzed using an ABI PRISM 3130xl genetic analyzer, and their length and peak area were determined using the genotype software GeneMapper (Applied Biosystems).

According to the methods described by Nagashima *et al.* [19], the T-RFs were divided into 29 operational taxonomic units (OTUs) for fecal samples or 22 OTUs for vaginal samples. The OTUs were quantified as the percentage values of individual OTU per total OTU areas, which were expressed as the percent of the area under the curve (%AUC). The bacteria predicted for each classification unit OTU were identified with reference to Human Fecal Microbiota T-RFLP profiling (http://www.tecsrg.co.jp/t-rflp/). To minimize inter- and intra-observer coefficient of variations of the OTU, 1 author (TM) conducted all T-RFLP analyses.

For objective interpretation of the difference in T-RF patterns, cluster analyses were performed using the software SPSS (IBM Statistics, ver. 20.0, NY, USA). T-RF patterns produced by digestion with restriction enzymes (*Bsl* I or *Msp* I) were quantified as the proportion of the total peak area of all T-RFs. The levels of similarity among fecal and vaginal samples were calculated as correlation coefficients, and represented graphically by a scatter plot using principal component analysis.

Table 1. Clinical characteristics of all participants.

Characteristics	Non-PTL (N = 20)	PTL (N = 11)	PTB (N = 10)	Analysis of variance Bonferroni's multiple comparison
Maternal age (year), median (range)	34.0 (27–41)	30.5 (22–37)	33.4 (22–41)	$P = 0.2134$
Gestational weeks at sample collection, average (range)	28.6 (23–34)	28.5 (22–33)	28.0 (22–34)	$P = 0.9351$
Gestational weeks at birth, average (range)	39.2 (37–41)	37.9 (37–40)	33.4 (27–36)	$P = 0.0000$
Previous PTB, percent (n/N)	10.0 (2/20)	9.1 (1/11)	10.0 (1/10)	$P = 0.7897$
Smoking during pregnancy, n/N	0/20	0/11	0/10	-
Parity number, average (range)	0.9 (0–2)	1.1 (0–2)	0.4 (0–2)	$P = 0.1651$
Nulliparous, percent (n/N)	40.0 (8/20)	27.3 (3/11)	70.0 (7/10)	$P = 0.3169$
BMI at sample collection, average (range)	23.3 (17.6–29.1)	23.2 (18.9–29.4)	21.7 (17.0–27.2)	$P = 0.3263$

BMI: Body mass index (kg/m^2).
non-PTL: non-preterm labor.
PTL: preterm labor.
PTB: preterm birth.
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Statistical analysis

All values of OTU are expressed as the mean ± standard error (SE). Comparisons of quantitative data among the three groups were carried out by a multiple comparison technique (Bonferroni). False discovery rates were calculated using Benjamini-Hochberg method. Differences were considered significant at P values of less than 0.05. For multivariate analysis of the data, principal component analysis and cluster analysis were used. The principal component analysis was performed unsupervised.

Results

Of 21 women with preterm labor (the PTL group plus the PTB group), 10 women (47.6%) finally delivered their babies before 37 weeks of gestation.

Principal component analysis (PCA)

Fecal samples. PCA was performed based on the relative abundance of OTUs in fecal samples. The first two principal component scores, which accounted for 19.7% and 10.4% of the total variation, were calculated. Hierarchical clustering of fecal samples on the basis of their first two principal component scores separated the fecal samples into two primary clusters (Figure 1A). One cluster (labeled as 'cluster 1', thick dotted line, left in Figure 1A) included all 10 cases of the PTB group, as well as 2/20 of the non-PTL group and 7/11 of the PTL group. The other cluster (labeled as 'cluster 2', thick solid line, right in Figure 1A) was dominated by 90% of the non-PTL group (18/20) and 36.4% of the PTL group (4/11). No PTB cases were included in cluster 2 (0/10). The components of the PTL group occupied an intermediate position between the non-PTL group and the PTB group.

Vaginal samples. In vaginal samples, the first two principal component scores were calculated (the first and second principal components accounted for 18.5% and 15.8% of the total variation, respectively) (Figure 1B). The PCA showed that there was similarity among these three groups.

Terminal restriction fragment length polymorphism profile

The percentages of OTU (percentage values of individual OTU area per total OTU) of representative OTUs for fecal samples after *Bsl* I digestion are shown in Table 2 and those for vaginal samples after *Msp* I digestion in Table 3.

Fecal samples. When comparing respective OTUs of fecal samples between the non-PTL group and the PTB group, the peak areas of the OTU 650, OTU 657, OTU 749, OTU 853, and OTU 955 (digested with *Bsl* I) were significantly different (Table 2). The amounts of *Clostridium* cluster XVIII (OTU 650), *Clostridium* cluster IV (OTU 749), *Bacteroides* (OTU 853), and *Clostridium* subcluster XIVa (OTU 955) were significantly lower in the PTB group than those in the non-PTL group ($P = 0.0125$, $P = 0.0289$, $P = 0.0348$, and $P = 0.0005$, respectively). The amounts of *Clostridium* cluster XVIII and *Clostridium* subcluster XIVa in the PTL group were significantly higher than those in the non-PTL group (P = 0.0449 and P = 0.0125, respectively).

When comparing respective OTUs between the PTL group and the PTB group, the amount of *Clostridium* cluster XI and *Clostridium* subcluster XIVa (OTU 919) was significantly lower in the PTB group than that in the PTL group ($P = 0.0285$).

Vaginal samples. In contrast, the % OTU for vaginal samples after *Msp* 1 digestion from the non-PTL group was quite similar to that from the PTL or the PTB group (Table 3).

Discussion

In this study, we first showed that the fecal microbiota in the PTL group was substantially different from that in the non-PTL group using T-RFLP analysis. Average scores of OTU 650, OTU 749, OTU 853, and OTU 955 were significantly lower in the fecal microbiota from the PTB group than those from the non-PTL group. Nagashima *et al.* [19] reported that OTU 749 and OTU 955 most likely represent *Clostridium* cluster IV species and *Clostridium* subcluster XIVa species, respectively, as determined by the 16S rDNA clone library method. Taking these findings together, it was revealed that the levels of *Clostridium* cluster XVIII, *Clostridium* cluster IV, *Clostridium* subcluster XIVa, and *Bacteroides* were significantly reduced in the fecal microbiota from the PTB group. Our result first showed that there was a significant difference in the average OTU scores of *Clostridium* cluster XVIII, *Clostridium* cluster IV, and *Clostridium* subcluster XIVa between mothers with non-PTL and those with PTB, while mothers with PTL had intermediate scores between them. This finding does not mean that the intestinal microbiota accidentally changed in the case of the PTB group, but rather it suggests that some alterations in intestinal microbiota are associated with the clinical findings of mothers with PTB.

Our result is compatible with the report by Romero, who found no differences in human vaginal microbiota between women who subsequently had a spontaneous preterm delivery and those who

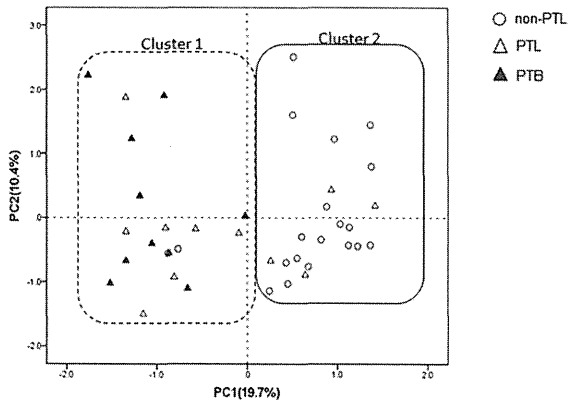

Figure 1B. Principal component analysis for vaginal microbiota

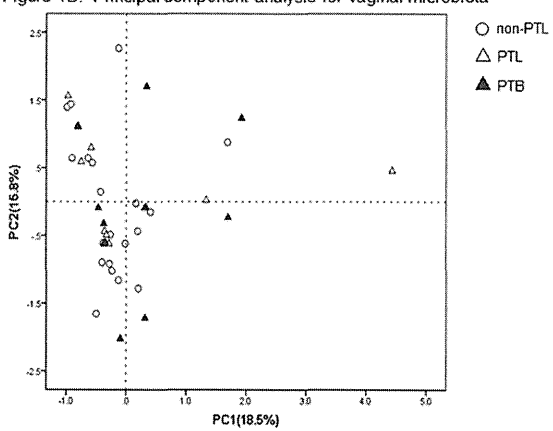

Figure 1. A. Principal component analysis of fecal microbiota. Principal component analysis scores are plotted based on the relative abundance of OTUs of vaginal microbiota. The percentage of variation explained by the principal coordinates is indicated on the axis. Open circles (○) represent the non-PTL group, open triangles (△) the PTL group, and closed triangles (▲) the PTB group. A dotted line, on the left in Figure 1A, shows 'cluster 1', which contains all 10 cases of the PTB group, as well as 2/20 of the non-PTL group and 7/11 of the PTL group. A solid circle, on the right in Figure 1A, shows 90% of the non-PTL group (18/20) and 36.4% of the PTL group (4/11). The PTL group occupied an intermediate position between the non-PTL group and the PTB group. B. Principal component analysis of vaginal microbiota. Principal component analysis scores are plotted based on the relative abundance of OTUs of vaginal microbiota. The percentage of variation explained by the principal coordinates is indicated on the axis. Open circles (○) represent the non-PTL group, open triangles (△) the PTL group, and closed triangles (▲) the PTB group.
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Table 2. Average OTU sores in fecal samples.

OTU	Bacteria predicted by T-RF length	Non-PTL (N = 20)	PTL (N = 11)	PTB (N = 10)	Bonferroni's adjustment *P* value	False Discovery Rate *P* value
106	*Clostridium* subcluster XIVa	1.85±0.75	0.33±0.27	0.19±0.13	NS	NS
110	*Clostridium* cluster IV	1.25±0.42	3.89±2.99	3.03±1.30	NS	NS
124	*Bifidobacterium*	11.19±1.98	14.10±4.08	5.91±2.61	NS	NS
168	*Clostridium* cluster IV	0.52±0.15	0.18±0.18	0.08±0.08	NS	NS
317	*Prevotella*	4.58±2.79	0.31±0.16	0.99±0.60	NS	NS
332	*Lactobacillares*	1.29±0.34	1.91±0.60	0.87±0.42	NS	NS
338	*Clostridium* cluster IV	0.57±0.16	0.37±0.19	0.00±0.00	NS	NS
366	*Bacteroides*	3.56±1.13	4.62±1.96	5.71±2.38	NS	NS
369	*Clostridium* cluster IV	0.27±0.18	0.89±0.86	2.76±1.32	NS	NS
469	*Bacteroides*	37.10±3.33	30.44±5.66	22.76±4.44	NS	NS
494	*Clostridium* subcluster XIVa	5.90±1.51	10.49±4.46	10.51±5.04	NS	NS
520	*Lactobacillares*	0.79±0.33	0.18±0.12	0.00±0.00	NS	NS
650	*Clostridium* cluster XVIII	2.12±0.34	0.70±0.35	0.26±0.21	Non-PTL vs. PTB, *P* = 0.0016; non-PTL vs. PTL, *P* = 0.0150	Non-PTL vs. PTB, *P* = 0.0125; non-PTL vs. PTL, *P* = 0.0449
657	*Lactobacillares*	5.18±2.54	17.63±8.29	24.24±7.17	Non-PTL vs. PTB, *P* = 0.0497	NS (*P* = 0.1089)
749	*Clostridium* cluster IV	6.41±1.15	2.90±1.22	0.80±0.80	Non-PTL vs. PTB, *P* = 0.0060	Non-PTL vs. PTB, *P* = 0.0289
754	*Clostridium* subcluster XIVa	1.70±0.28	1.63±1.03	0.00±0.00	NS	NS
853	*Bacteroides*	0.84±0.13	0.60±0.27	0.07±0.07	Non-PTL vs. PTB, *P* = 0.0087	Non-PTL vs. PTB, *P* = 0.0348
919	*Clostridium* cluster XI, *Clostridium* subcluster XIVa	1.30±0.23	0.36±0.16	2.06±0.55	PTL vs. PTB, *P* = 0.0047	PTL vs. PTB, *P* = 0.0285
940	*Clostridium* subcluster XIVa, *Enterobacteriales*	3.79±0.68	3.62±1.10	11.43±3.72	NS	NS
955	*Clostridium* subcluster XIVa	5.37±0.64	1.72±0.80	0.34±0.34	non-PTL vs. PTB, *P* = 0.0000; non-PTL vs. PTL, *P* = 0.0012	non-PTL vs. PTB, *P* = 0.0005; non-PTL vs. PTL, *P* = 0.0125
990	*Clostridium* subcluster XIVa	3.61±0.69	2.87±0.86	7.98±6.66	NS	NS
Others		0.83±0.42	0.26±0.20	0.00±0.00	NS	NS

OTU: operational taxonomic unit.
T-RF: terminal restriction fragment.
non-PTL: non-preterm labor.
PTL: preterm labor.
PTB: preterm birth.
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delivered at term. In addition, more recent studies have shown that bacteria from the oral cavity are most often found in the amniotic fluid of patients with preterm labor, demonstrating that periodontal pathogens/byproducts may reach the placenta and spread to the fetal circulation and amniotic fluid [20]. Taken together, these findings may suggest that oral and/or intestinal, not vaginal, microbiota could induce pathogens and the secretion of elevated levels of inflammatory mediators, which in turn may cause premature birth or suggest that dysbiosis caused by oral and/or intestinal, not vaginal, microbiota may render the uterus and/or the placenta susceptible to infection.

Recently, bacterial vaginosis-associated bacteria (BVAB) in the order *Clostridiales:* BVAB1, BVAB2, and BVAB3, were identified in association with bacterial vaginosis [21]. Subsequently, Foxman et al. reported that, in vaginal fluids, one of the Clostridia-like bacteria, BVAB-3, was consistently associated with a reduction in the risk of preterm birth for all ethnic groups (risk ratio, 0.55; 95% confidence interval, 0.39–0.78) [22]. Their result is compatible with our findings in that the level of *Clostridium* was significantly lower in preterm birth, although its level in our studies was lower in the samples not from vaginal fluid but from feces.

Among the microbiota indigenous to the murine and human colon, the genus *Clostridium* belonging to clusters XIVa and IV is reported to be an outstanding inducer of colonic $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (Treg) cells [7,8]. *Bacteroides fragilis* has also been shown to induce Treg cells in mouse [23]. It was suggested that polysaccharide A (PSA) produced by *Bacteroides fragilis potentiates the suppressor activity of Treg cells. The balance* between beneficial and potentially harmful species in the commensal microbial community, known as dysbiosis, has often been linked to the development of inflammatory bowel disease (IBD) in humans and analogous intestinal inflammation in mice [18]. Importantly, intestinal Treg cells play a key role in regulating inflammation by the production of IL-10 [8,23,24,25] and decreased intestinal Treg cells were observed in IBD patients. Preterm labor may be viewed as inflammation caused by

Table 3. Average OTU scores in vaginal samples.

OTU	Bacteria predicted by T-RF length	non-PTL (N = 20)	PTL (N = 11)	PTB (N = 10)	*P* value
57	Unknown	8.78±1.77	12.58±4.12	10.12±2.61	NS
74	*Enterococcus*	0.47±0.27	0.36±0.31	0.72±0.49	NS
93	*Chitinophagaceae*	1.20±0.53	4.25±2.74	4.16±2.41	NS
98	*Prevotella*	0.54±0.24	2.39±2.19	5.45±2.30	NS
134	*Bifidobacterium*	0.89±0.86	0.05±0.05	2.23±1.78	NS
147	*Firmicutes*	2.37±1.56	0.28±0.13	3.94±3.55	NS
163	*Actinobacteria*	3.17±1.67	0.01±0.01	5.78±4.93	NS
170	*Firmicutes*	0.33±0.17	0.74±0.35	0.53±0.28	NS
179	*Lactobacillus*	27.42±8.09	34.34±12.45	25.40±8.72	NS
189	*Lactobacillus*	43.41±8.38	39.68±13.11	29.01±10.90	NS
230	*Firmicutes*	0.41±0.15	0.59±0.38	0.37±0.25	NS
281	*Actinobacteria*	6.34±4.12	1.16±1.04	2.95±2.95	NS
300	*Clostridiales*	1.03±0.59	0.08±0.08	5.73±4.40	NS
563	*Streptococcus*	0.18±0.15	0.78±0.78	0.92±0.41	NS
573	*Lactobacillus*	3.24±1.29	2.39±1.35	2.62±0.91	NS
Others		0.20±0.15	0.33±0.33	0.05±0.05	NS

OTU: operational taxonomic unit.
T-RF: terminal restriction fragment.
non-PTL: non-preterm labor.
PTL: preterm labor.
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inappropriately expanded components of the normal bacterial community. In IL-10 knockout mice, a very small amount of lipopolysaccharide (LPS) induces preterm delivery [26]. These findings suggest that decreased intestinal *Clostridium* cannot induce an adequate volume of Treg cells, resulting in susceptibility to inflammation. Indeed, decreased Treg cell volume and function in the peripheral blood have been reported in preterm labor [27,28,29,30,31], although the level of intestinal Treg cells is unclear in these papers and this study. Further studies are needed to clarify the relationship between intestinal microbiota and peripheral Treg cells or intestinal Treg cells.

The peak area of the 919-bp OTU (*Clostridium* cluster XI, *Clostridium* subcluster XIVa) in the PTB group was significantly larger than that in the PTL group ($P = 0.0285$). This is a limitation of the T-RFLP method. We should thus reevaluate the bacterial species using metagenomic analysis.

Several limitations were identified in this study. Because of the small number of cases (fewer than 30) in each group, information that can only come from patient participation in well-designed clinical trials is needed to improve the management of PTB. Second, we did not check the level of Treg cells in peripheral blood and therefore could not compare the relationship between the fecal microbiota of the mother and the level of Treg cells. Further investigation is needed to address whether the difference in gut microbiota is related to the expression of immunoregulatory Treg cells or inflammation-induced Th17 cells that are induced by gut *Clostridium* strains and segmented filamentous bacteria, respectively. Third, it is possible that the unidentified confounders may have an effect on changes in the microbial communities. An alternative interpretation is that, as Aagaard et al. [12] reported, the vaginal microbial 16S rRNA gene catalogue may uniquely differ in pregnancy, with variation of taxa across vaginal subsites and depending on gestational age. Further study is required to determine the relationship between the difference in average OTU for some phylotypes and PTB. Fourth, owing to the cross-sectional nature of the study, our finding about the causal relationships between fecal microbiota and PTB should be interpreted with caution. Fifth, we didn't measure the concentrations of organic acids, indole, and ammonia. And we also didn't measure pH and moisture and didn't compare these values with those from normal pregnancy. Sixth, the selection of optimal T-RFLP probes is a subject of considerable ongoing discussion in the field of microbiome research. T-RFLP analysis only identify differences and similarities in microbial community and the resulting OTUs aren't assigned to a specific species. Unfortunately, there is no perfect set of T-RFLP probes for fecal and vaginal microbiome. Yet, the T-RFLP data can readily be analyzed using various statistical algorithms to quantitatively ascertain similarities and differences among communities and to infer plausible community of intestine and vagina. Seventh, we are unable to grasp the importance of the results that patients had preterm babies had a higher average OTU for Lactobacillares phylotypes than those who delivered term babies. Yet, the identification of significant differences in average OTUs provides evidence that the study of the intestinal microbiota during pregnancy can yield important insights into the relationship between the fluctuation of microbial communities and adverse pregnancy outcome like PTB. Further studies are required to confirm this finding and elucidate the role of intestinal microbiota in PTB. Eighth, the principal component analysis is not a prefect technique to analyze the T-RFLP data. Therefore, the performance of principal component analysis should be interpreted with caution since there were no validation samples to test.

Many studies have been performed to identify the differences in microbial diversity between healthy individuals and patients with rheumatoid arthritis (RA) [32], inflammatory bowel disease (IBD) [33], and type 1 diabetes [34]. However, the results about the fecal level of *Clostridium* in these patients are conflicting (decrease [32],

no change [33], and increase [34]). When abnormality in fecal microbiota in the PTL group is found, the question arises of whether microbiotic alteration, dysbiosis, is a cause or a result of preterm delivery. We speculate that the changes in the immune system through fecal dysbiosis may change the uterine activity. In addition, the underlying mechanisms resulting in alteration of the microbiota remain to be clarified. Recently, intake of probiotic food was found to be associated with a reduced risk of spontaneous preterm delivery and preeclampsia [35,36]. Prospective studies are needed to clarify whether intestinal dysbiosis before pregnancy might cause uterine inflammation and induce uterine contraction or cervical ripening during pregnancy.

Conclusion

Disturbance of the intestinal flora, dysbiosis, during pregnancy was first observed in the PTB group in this study. This may cause inflammatory reactions in the uterus leading to PTB. Further study is needed to clarify the relationship between PTB and dysbiosis of intestinal bacterial flora.

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Author Contributions

Conceived and designed the experiments: AS SY NY RY TM GS SS. Performed the experiments: AS SY NY RY TM GS SS. Analyzed the data: AS TM GS SS. Contributed reagents/materials/analysis tools: AS SY NY RY TM GS SS. Contributed to the writing of the manuscript: AS TM GS SS.

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Original Article

Intrauterine growth restriction modifies gene expression profiling in cord blood

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Abstract ***Background***: Small-for-gestational-age (SGA) newborns are at an increased risk for perinatal morbidity and mortality and development of metabolic syndromes such as cardiovascular disease and type 2 diabetes mellitus (T2DM) in adulthood. The mechanism underlying this increased risk remains unclear. In this study, genetic modifications of cord blood were investigated to characterize fetal change in SGA newborns.

Methods: Gene expression in cord blood cells was compared between 10 SGA newborns and 10 appropriate-for-gestational-age (AGA) newborns using microarray analysis. Pathway analysis was conducted using the Ingenuity Pathways Knowledge Base. To confirm the microarray analysis results, quantitative real-time polymerase chain reaction (RT-PCR) was performed for upregulated genes in SGA newborns.

Results: In total, 775 upregulated and 936 downregulated probes were identified in SGA newborns and compared with those in AGA newborns. Of these probes, 1149 were annotated. Most of these genes have been implicated in the development of cardiovascular disease and T2DM. There was good agreement between the RT-PCR and microarray analyses results.

Conclusions: Expression of certain genes was modified in SGA newborns in the fetal period. These genes have been associated with metabolic syndrome. To clarify the association between modified gene expression in cord blood and individual vulnerability to metabolic syndrome in adulthood, these SGA newborns will be have long-term follow up for examination of genetic and postnatal environmental factors. Gene expression of cord blood can be a useful and non-invasive method of investigation of genetic alterations in the fetal period.

Key words cord blood, gene expression, intrauterine growth restriction, metabolic syndrome, microarray analysis.

Intrauterine growth restriction (IUGR) is used to designate a fetus that has not reached its growth potential; it can be caused by fetal, placental, or maternal factors.[1] IUGR results in the birth of an infant who is small for gestational age (SGA).[1] SGA newborns are at increased risk for perinatal morbidity, mortality, and adverse developmental outcome.[2,3] After Barker *et al.* reported an association between restricted fetal growth and increased risk of mortality from cardiovascular disease in adulthood,[4] several studies noted the same or similar results in a variety of ethnicities.[5–7] Using several animal models of IUGR, other studies have confirmed the association between restricted fetal growth and increased risk of developing metabolic and cardiovascular disease in the future.[8–10] Despite the evidence of the relationship between fetal undernutrition and adult diseases,[5–10] the specific mechanism underlying this increased risk remains unclear. In the recent studies, they hypothesized that fetal and neonatal undernutrition may lead to epigenetic alterations, resulting in an increased susceptibility to development of metabolic syndrome and other chronic diseases in adulthood.[11–13] The high rate of DNA synthesis of the fetal and neonatal genome is particularly vulnerable to environmental factors, and the elaborate DNA methylation pattern required for normal tissue development is established during the early stages of fetal development.[14]

The microarray method provides a means to examine the expression profiles of several hundred genes. In combination with this method, pathway analysis technologies allow the mapping of gene expression data into the relevant pathway maps on the basis of their functional annotation and known molecular interactions.[15] Previous studies have shown that the total RNA derived from circulating blood can be used to distinguish control subjects and patients with certain diseases.[16,17] Cord blood is a non-invasive and readily available source of fetal cells. In this study, the global gene expression profiling of SGA newborns was characterized in cord blood cells using Affymetrix Gene Chip technology by comparing appropriate-for-gestational-age (AGA) with SGA newborns.

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Methods

Subjects

In this study, SGA was defined as birthweight and height below the 10th percentile of the local sex-specific distribution for gestational age, and AGA as these parameters between the 10th and 90th percentiles for gestational age. Singleton newborns were selected, and those with congenital malformations or exposure to intrauterine infection were excluded. After application of these criteria, 10 SGA newborns and 10 AGA newborns were included in the study. The AGA newborns were randomly selected from among infants whose birthweight and height matched these criteria. This study was approved by the Research Ethics Committee of the University of Toyama. Written informed consent was obtained from the families of all participants before the study.

RNA extraction from whole blood

Whole blood samples (0.5 mL) were obtained from umbilical cords, conserved in RNA later® (Ambion, Austin, TX, USA) following the manufacturer's instructions and stored at −20°C. Following complete thawing, RNA later® was eliminated by centrifugation. Total RNA was extracted from cord blood cells using a RiboPure-Blood Kit (Ambion) and treated with DNase I (Ambion) for 30 min at 37°C to remove residual genomic DNA. Whole blood messenger (m)RNA consists of a relatively large population of globin mRNA transcripts. Because globin mRNA interferes with the expression profiling of whole blood samples, α and β globin mRNA were depleted from the total RNA preparations using a GLOBIN clear Kit (Ambion).

Microarray hybridization and pathway analysis

Gene expression profiling of cord blood cells from AGA and SGA newborns was performed on an Affymetrix HG-U133A plus 2.0 array that was spotted with 54 674 probe sets (Affymetrix, Santa Clara, CA, USA). In this study, a total of 20 arrays were used: 10 for the AGA newborns and 10 for the SGA newborns. Samples were prepared for array hybridization following the manufacturer's instructions. In short, biotin-labeled complementary (c)RNA was prepared from 500 ng of globin-depleted total RNA using a 3′-IVT Express Kit (Affymetrix) and hybridized to the array. The arrays were scanned with a probe array scanner. The data files (CEL files) resulting from the analysis using the Affymetrix GCOS software were imported into GeneSpring GX 11.0 software (Agilent Technologies, Santa Clara, CA, USA) to extract the differentially expressed genes in the cord blood of SGA and AGA newborns.

Quantitative real-time polymerase chain reaction

To confirm the microarray analysis results, quantitative real-time polymerase chain reaction (RT-PCR) was performed to examine mRNA expression in four genes: fibronectin 1 (*FN1*), collagen type 1alpha2 (*COL1A2*), famesyl diphosphate synthase (*FDPS*) and mitochondrial tumor suppressor 1 (*MTUS1*). Complementary DNA (cDNA) was synthesized from total RNA (1 μg) using an oligo (dT) 16 primer and Omniscript reverse transcriptase (Qiagen, Hilden, Germany). RT-PCR was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Tokyo, Japan) and a Mx3000P QPCR system (Agilent Technologies). The following primer pairs were used: *FN1*: forward, 5′-gagagaagtgggaccgtcag-3′; reverse, 5′-tggcacc gagatattccttc-3′; *COL1A2*: forward, 5′-gcccaacctgaaaacatccc-3′; reverse, 5′-aggttgccagtctcctcatc-3′; *FDPS*: forward, 5′-aggacaaca aatgcagctgg-3′; reverse, 5′-ggtgctgcgtactgttcaat-3′; *MTUS1*: forward, 5′-gaaagctcggatggacaage-3′; reverse, 5′-gtcccattgtg cagtttcc-3′. RT-PCR was performed under the following conditions: 95°C for 10 s and 60°C for 30 s. All mRNA expression was normalized with respect to the mRNA of glyceraldehyde phosphate dehydrogenase (*GAPDH*).

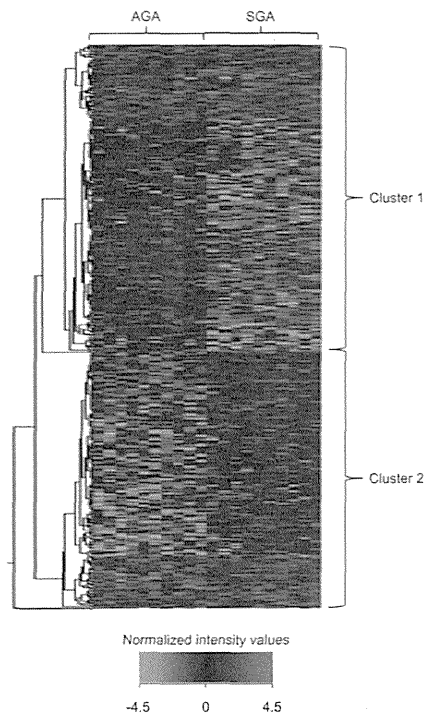

Fig. 1 Hierarchical clustergram. Each probe set is represented by a single row of colored bars. Red, upregulated; and green, downregulated; black, no change. Each line represents a cord blood sample from among the appropriate-for-gestational-age (AGA; $n = 10$) and small-for-gestational-age (SGA; $n = 10$) newborns.

Table 1 Neonatal and maternal characteristics

	GA	Sex	Delivery	Birthweight (g)	Birth height (cm)	Cord blood pH	Placental weight (g)	Cord insertion	Age (years)	Maternal Height (cm)	Maternal Weight (g)	BMI	Pregnancy weight gain (kg)
SGA 1	39 weeks 5 days	M	V	2400	46	7.31	440	Central	30	157	55	22.3	8.9
2	39 weeks 4 days	M	V	2430	46	7.25	430	Marginal	31	154	46.2	19.5	7.8
3	39 weeks 5 days	F	V	2066	43	7.30	330	Velamentous	28	159	48	19	8
4	40 weeks 0 days	F	V	2194	45	7.36	340	Marginal	26	155	48	20	12.5
5	37 weeks 2 days	F	C	1960	43.5	7.23	345	Eccentric	31	160	64	25	7.8
6	37 weeks 6 days	M	C	2214	43	7.40	270	Central	21	151	52	21.9	3.5
7	37 weeks 0 days	M	V	2070	40	7.35	440	Marginal	32	153	40	17.1	8
8	38 weeks 1 days	F	V	2398	43.5	7.33	510	Marginal	25	148	40	18.3	10
9	39 weeks 4 days	F	C	2054	44	7.33	430	Marginal	33	154	48	20.2	8
10	38 weeks 3 days	M	C	2355	44.2	7.22	375	Central	34	153	47	20.1	8
Mean	38 weeks 4 days			2214	43.8	7.31	391		29.1	154.4	48.8	20.3	8.3
AGA 1	40 weeks 3 days	F	V	3224	50.5	7.31	605	Central	35	155	58	24.1	10.8
2	38 weeks 2 days	F	V	2814	50	7.35	584	Central	26	157	52	21.1	9
3	38 weeks 0 days	M	V	2684	46.5	7.30	580	Marginal	31	149	44	19.8	5.9
4	38 weeks 1 days	F	C	2840	48.5	7.31	400	Central	39	168	61	21.6	6
5	39 weeks 2 days	F	V	2904	49.5	7.31	640	Marginal	37	149	42	18.9	6.5
6	37 weeks 2 days	M	C	3220	47	7.25	622	Central	35	167	54.5	19.5	9
7	39 weeks 2 days	M	V	3334	51	7.30	800	Central	25	168.5	56	19.7	11.3
8	40 weeks 3 days	M	V	3392	50.5	7.33	600	Central	25	162	65	24.8	14.8
9	39 weeks 5 days	M	V	3014	49.5	7.39	520	Marginal	37	154	50	21.1	9.3
10	37 weeks 0 days	F	C	2530	47.5	7.30	590	Central	39	149	44	19.9	9
Mean	38 weeks 5 days			2996	49.1	7.31	595		33	157.9	52.7	21.1	9.2

AGA, appropriate for gestational age; BMI, body mass index; GA, gestational age; SGA, small for gestational age.

Statistical analysis

Statistical analysis was done using unpaired *t*-test. Statistically significant differences were identified at $P < 0.05$. A fold change >2 (upregulated) or <2 (downregulated) was considered to be biologically important.[18] We used Ingenuity Pathways Analysis (IPA) tools (Ingenuity Systems, Mountain View, CA, USA) to investigate the following characteristics of upregulated and downregulated genes from cord blood of SGA newborns: biological processes, cellular components, molecular functions, genetic networks, and toxicity functions. IPA allowed the estimation of the likelihood of SGA newborns developing disease. Differences in demographic characteristics and clinical measures between SGA and AGA newborns were also examined using unpaired *t*-test.

Results

Cord blood samples were collected from 10 SGA and 10 AGA newborns. The characteristics of the newborns and their mothers are given in Table 1. Mothers were free of diseases such as gestational diabetes mellitus and hypertension. Significantly lower birthweight, height, and placental weight were recorded for SGA newborns than for AGA newborns (Table 1). Abnormal cord insertion was identified in 60% of placentas from SGA infants, suggesting that the placenta tended to be light or cord insertion tended to be abnormal in SGA newborns. There was no significant difference in maternal height, weight, body mass index before pregnancy, alcohol consumption or pregnancy weight gain between the two groups (Table 1). One mother had a smoking history in each group.

Gene expression was compared between cord blood samples from SGA and AGA newborns to characterize fetal changes in SGA newborns. Hierarchical clustering based on similarities in gene expression using differentially expressed genes (>twofold increase or decrease in RNA) demonstrated a clear distinction between SGA and AGA newborns (Fig. 1). In SGA newborns, 936 downregulated (Fig. 1, cluster 1) and 775 upregulated probes (Fig. 1, cluster 2) were identified for a total of 1711 changed probes. Of these 1711 probes, 1149 were annotated and labeled as SGA-specific genes.

To identify the biologically relevant networks and pathways of these genes, pathway analysis was conducted on the SGA-specific genes using the Ingenuity Pathways Knowledge Base. SGA-specific genes were classified into functional groups related to diseases and disorders, and the top 10 diseases are identified in Table 2 and the top five genes in Table 3. Most of these listed genes overlapped with biological processes and were related to metabolic syndromes, including cardiovascular disease and diabetes mellitus.

Four genes (*FN1*, *COL1A2*, *FDPS* and *MTUS1*) from Table 3 were selected for confirmation of the microarray analysis results. Overall, there was good agreement between the RT-PCR and microarray analyses results (Fig. 2).

Discussion

To characterize gene expression of the IUGR fetus, we compared cord blood between SGA and AGA newborns. IUGR was found to modify the expression patterns of certain genes during the fetal period. These genes are associated with development of metabolic syndromes including cardiovascular disease and type 2 diabetes mellitus (T2DM; Table 2). In the top five upregulated SGA-specific genes (Table 3), *FN1* is outstanding. Fibronectin regulates a wide variety of cellular interactions with the extracellular matrix (ECM) and plays crucial roles in cell adhesion, migration, growth, differentiation and embryogenesis.[19,20]

Table 2 Functional analysis of the genes specific for SGA newborns

Category	Function	*P*	Associated genes
Immunological disease	Autoimmune disease	1.55×10^{-9}	198
	Immunological disorder	1.25×10^{-8}	219
	Insulin-dependent diabetes mellitus	2.74×10^{-7}	101
Genetic disorder	Genetic disorder	1.64×10^{-9}	483
	Alzheimer's disease	5.96×10^{-7}	86
	Coronary artery disease	3.14×10^{-6}	104
Metabolic disease	Metabolic disorder	1.22×10^{-7}	220
	Diabetes mellitus	3.72×10^{-7}	186
Endocrine system disorder	Endocrine system disorder	3.35×10^{-7}	198
	Glucose intolerance	1.10×10^{-2}	7
Cardiovascular disease	Arteriosclerosis	3.51×10^{-7}	115
	Atherosclerosis	4.49×10^{-7}	114
	Cardiovascular disorder	1.69×10^{-6}	190
Neurological disease	Neurodegenerative disorder	5.41×10^{-7}	90
	Neurological disorder	7.45×10^{-6}	286
Inflammatory disease	Inflammatory disorder	1.47×10^{-6}	231
	Arthritis	4.09×10^{-6}	146
Cardiovascular system development	Development of blood vessel	1.67×10^{-6}	50
Organismal development	Angiogenesis	3.88×10^{-5}	39
Cancer	Tumorigenesis	2.82×10^{-6}	245

SGA, small for gestational age.

Table 3 Most strongly upregulated genes specific for SGA newborns

Category	AffyID	Gene symbol	Description	Log ratio
Immunological disease	211719_x_at	*FN1*	Fibronectin 1	2.235
	206683_at	*ZNF165*	Zinc finger protein 165	1.164
	221796_at	*NTRK2*	Neurotrophic tyrosine kinase, receptor, type 2	1.123
	211151_x_at	*Gh*	Growth hormone 1	0.991
	202404_s_at	*COL1A2*	Collagen, type I, alpha 2	0.956
Genetic disorder	211719_x_at	*FN1*	Fibronectin 1	2.235
	236517_at	*MEGF10*	Multiple EGF-like-domains 10	1.641
	217344_at	*FDPS*	Farnesyl diphosphate synthase	1.308
	1569701_at	*PER3*	Period homolog 3 (Drosophila)	1.267
	221796_at	*NTRK2*	Neurotrophic tyrosine kinase, receptor, type 2	1.123
Metabolic disease	211719_x_at	*FN1*	Fibronectin 1	2.235
	217344_at	*FDPS*	Farnesyl diphosphate synthase	1.308
	206683_at	*ZNF165*	Zinc finger protein 165	1.164
	211151_x_at	*Gh*	Growth hormone 1	0.991
	202404_s_at	*COL1A2*	Collagen, type I, alpha 2	0.956
Endocrine system disorder	211719_x_at	*FN1*	Fibronectin 1	2.235
	217344_at	*FDPS*	Farnesyl diphosphate synthase	1.308
	212093_s_at	*MTUS1*	Mitochondrial tumor suppressor 1	1.204
	206683_at	*ZNF165*	Zinc finger protein 165	1.164
	221796_at	*NTRK2*	Neurotrophic tyrosine kinase, receptor, type 2	1.123
Cardiovascular disorder	211719_x_at	*FN1*	Fibronectin 1	2.235
	1562301_at	*C8orf34*		1.079
	208190_s_at	*LSR*	Lipolysis-stimulated lipoprotein receptor	0.762
	211506_s_at	*IL8*	Interleukin 8	0.678
	202988_s_at	*RGS1*	Regulator of G-protein signaling 1	0.663
Neurological disorder	236517_at	*MEGF10*	Multiple EGF-like-domains 10	1.641
	243483_at	*TRPM8*	Transient receptor potential cation channel, subfamily M, member 8	1.417
	217344_at	*FDPS*	Farnesyl diphosphate synthase	1.308
	1569701_at	*PER3*	Period homolog 3 (Drosophila)	1.267
	206084_at	*PTPRR*	Protein tyrosine phosphatase, receptor type, R	1.207
Inflammatory disorder	211719_x_at	*FN1*	Fibronectin 1	2.235
	221796_at	*NTRK2*	Neurotrophic tyrosine kinase, receptor, type 2	1.123
	209278_s_at	*TFPI2*	Tissue factor pathway inhibitor 2	0.936
	220406_at	*TGFB2*	Transforming growth factor, beta 2	0.847
	226189_at	*ITGB8*	Integrin, beta 8	0.718
Cardiovascular system development and function	211719_x_at	*FN1*	Fibronectin 1	2.235
	208357_x_at	*CSH1*	Chorionic somatomammotropin hormone 1 (placental lactogen)	1.417
	221796_at	*NTRK2*	Neurotrophic tyrosine kinase, receptor, type 2	1.123
	211151_x_at	*GH1*	Growth hormone	0.991
	202404_s_at	*COL1A2*	Collagen, type I, alpha 2	0.956
Organismal development	211719_x_at	*FN1*	Fibronectin 1	2.235
	208357_x_at	*CSH1*	Chorionic somatomammotropin hormone 1 (placental lactogen)	1.417
	221796_at	*NTRK2*	Neurotrophic tyrosine kinase, receptor, type 2	1.123
	211151_x_at	*GH1*	Growth hormone	0.991
	202404_s_at	*COL1A2*	Collagen, type I, alpha 2	0.956
Cancer	211719_x_at	*FN1*	Fibronectin 1	2.235
	243483_at	*TRPM8*	Transient receptor potential cation channel, subfamily M, member 8	1.417
	217344_at	*FDPS*	Farnesyl diphosphate synthase	1.308
	1555471_a_at	*Fmn2*	Formin 2	1.160
	221796_at	*NTRK2*	Neurotrophic tyrosine kinase, receptor, type 2	1.123

SGA, small for gestational age.

Interestingly, ECM such as integrin and collagen were also upregulated in SGA cord blood (Table 3). Although these activated interactions could affect the fetal development of SGA newborns, we need further research to account for this pathological meaning.

Many studies have reported an association between low birthweight and the development of cardiovascular diseases in the future.[4–7] In the present study, 190 SGA-specific genes were associated with cardiovascular disease and 115 were related to arteriosclerosis (Table 2). Hence, we speculated that cardiovascular disease could develop during the fetal period. Several reports noted that the aortic wall in SGA newborns was thicker and stiffer than that in AGA newborns.[21–24] Fetal IUGR has also been associated with significant thickening of the aortic intima–media, suggesting that atherosclerosis may occur during the fetal period.[24] Interestingly, systolic blood pressure in these SGA infants significantly increased at a mean postnatal age of 18 months compared with AGA infants,[24] suggesting that fetal

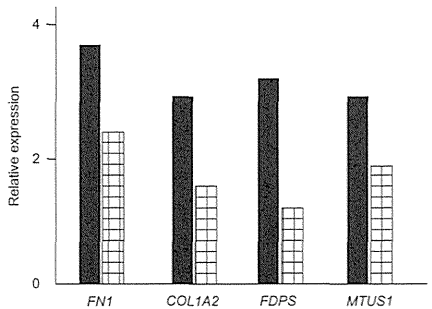

Fig. 2 (▤) Real-time polymerase chain reaction (RT-PCR) confirmation of (■) microarray analysis results. Complete agreement was found between RT-PCR and microarray results. GAPDH expression was used for normalization of RT-PCR. *COL1A2*, collagen type 1alpha2; *FDPS*, famesyl diphosphate synthase; *FN1*, fibronectin 1; *MTUS1*, mitochondrial tumor suppressor 1.

atherosclerosis may have an effect on blood pressure in the future. Thus, the risk for cardiovascular disease may originate from as early as the fetal period.

Many reports of the relationship between low birthweight and increased incidence of T2DM have been reviewed.[13,25] The precise mechanisms of this relationship, however, remain unknown. Genome-wide association studies have identified T2DM susceptibility variants in various ethnic groups.[26–29] In addition, several reports have proposed an association between variants of T2DM risk genes and reduced birthweight because insulin is a key fetal growth factor.[29,30] This fetal insulin hypothesis proposes that genetic variants reduce insulin secretion or action, which may predispose toward the development of T2DM and reduce birthweight. Among the SGA-specific genes identified here, 186 have been associated with T2DM (Table 2). Some of these may be consistent with the fetal insulin hypothesis.

Maternal undernutrition exposes the fetus to hormonal, metabolic, and vascular alterations.[31] Maternal stress or undernutrition during gestation also disturbs the hypothalamic-pituitary-adrenal (HPA) axis in SGA newborns.[32,33] This disturbance may provide a mechanism for susceptibility to cardiovascular and metabolic diseases.[32] In this study, 220 genes related to metabolic disorders and 198 genes related to endocrine system disorders were found among the SGA-specific genes (Table 2). These data suggest that fetal undernutrition induces dramatic changes in the fetal HPA axis, which may induce metabolic diseases in the future.

Although many animal models of fetal undernutrition have been reported, biopsy analysis of tissues of healthy infants for research is unethical. Therefore, gene expression in cord blood was investigated in this study. Genes in cord blood have had direct contact with many organs such as the pancreas, liver, and heart. Hayashi *et al.* showed that the transcriptional levels of genes in leukocytes could be a diagnostic marker for T2DM.[34] The present analysis has demonstrated that cord blood can be utilized as a good resource for non-invasive investigation of fetal genetic alterations.

This study has some limitations that must be considered in the interpretation of the results. First, family history of metabolic syndrome was not included in the analysis; hence, the effect of heredity on the results cannot be excluded. Second, SGA newborns may be a mixture of IUGR and constitutionally small newborn. Although maternal height, weight and parity are risk factors for SGA, we did not recognize any difference in risk factors between the AGA and SGA group.[35] Moreover, the SGA group had significantly lower placenta weight and a high percentage of abnormal cord insertion. This implies that most of the present SGA newborns were IUGR. Third, the association between modified gene expression in cord blood and development of metabolic disease in adulthood was not clarified. In addition, the relationship between low birthweight and future risk of disease may be mediated by epigenetic mechanisms.[14,36] Modified gene expression in SGA newborns has been associated with metabolic disease, but epigenetic factors were not investigated in this study. Previous studies have found an association between rapid weight gain during infancy and higher blood pressure and risk of coronary heart disease in the future.[37,38] Moreover, not all SGA newborns are at risk of insulin resistance and diabetes.[25] The results of these reports indicate that the association between IUGR and future development of diseases may be due to genetic and postnatal environmental interactions. In the future, long-term follow up of these SGA newborns will be conducted for the analysis of genetic and postnatal environmental factors to determine the precise pathological consequences of the genetic findings of this study.

Conclusion

A total of 1149 genes were differentially expressed in SGA cord blood in comparison with AGA cord blood. Interestingly, most of these genes have been implicated in the development of cardiovascular disease and T2DM. To clarify the association between modified gene expression in cord blood and individual vulnerability to metabolic syndrome in future, these SGA newborns will undergo long-term follow up for examination of genetic and postnatal environmental factors. Gene expression of cord blood can be a useful and non-invasive method of investigation of genetic alterations in the fetal period.

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Original Article

Incidence and prediction of outcome in hypoxic–ischemic encephalopathy in Japan

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Abstract ***Background***: Hypoxic–ischemic encephalopathy (HIE) is one of the most critical pathologic conditions in neonatal medicine due to the potential for neurological deficits in later life. We investigated the incidence of term infants with moderate or severe HIE in Japan and identified prognostic risk factors for poor outcome in HIE.

Methods: Data on 227 infants diagnosed with moderate or severe HIE and born between January and December 2008 were collected via nationwide surveys from 263 responding hospitals. Using logistic regression, we examined the relationship between maternal, antepartum, intrapartum, and neonatal risk factors and clinical outcome at 18 months following birth.

Results: In Japan, the incidence of moderate or severe HIE was 0.37 per 1000 term live births. Outborn births, low Apgar score at 5 min, use of epinephrine, and low cord blood pH were intrapartum factors significantly associated with neurodevelopmental delay and death at 18 months. Serum lactate, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase (all, $P < 0.001$) and creatine kinase ($P = 0.002$) were significantly higher in infants with poor outcome compared to those with favorable outcomes. Abnormal brain magnetic resonance imaging (MRI), an important prognostic factor, was significantly associated with poor outcome (odds ratio, 11.57; 95% confidence interval: 5.66–23.64; $P < 0.001$).

Conclusions: Risk factors predicting poor outcome in HIE include outborn birth, low Apgar score at 5 min, use of epinephrine, laboratory abnormalities, and abnormal MRI findings.

Key words hypoxic–ischemic encephalopathy, magnetic resonance imaging, neurodevelopmental outcome, risk factor.

Hypoxic–ischemic encephalopathy (HIE) is one of the most critical pathologic conditions in neonatal medicine. Infants with HIE suffer neurological sequelae in later life.[1–4] Some studies have reported predictive factors for neurodevelopmental outcome in infants with HIE.[5–7] Electroencephalography (EEG), magnetic resonance imaging (MRI), and laboratory data at birth are useful tools for predicting outcome based on neonatal risk factors. Whereas maternal and antenatal factors may foretell the development of HIE, these variables do not predict mortality or neurodevelopmental outcome.[8–10]

Neonatal encephalopathy (NE) refers to neurological abnormalities manifesting in the neonatal period and may be caused by multiple variables, among which, HIE is a key contributing factor. The incidence of NE has been reported in several studies.[8,11,12] The incidence of NE is 1–4 per 1000 live births,[8,11] but there are few reports of the incidence of HIE.[12–14] This may be due to the fact that establishing a diagnosis of HIE may be challenging because infants may present with non-specific symptoms and HIE is not always caused by a sentinel event.[4,15] Further, in some cases, an obvious hypoxic–ischemic event may have not been apparent during the intrapartum period or immediately after birth.[15] Because of the diagnostic difficulty, neonatologists and obstetricians are not always able to recognize brain insult in infants who suffer partial asphyxia at birth. Therefore, the incidence of HIE might be underestimated.

Accordingly, the aim of this study was to describe the incidence of HIE in term babies in Japan. Additionally, we investigated the risk factors for neurological sequelae and death.

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Methods

This retrospective survey study was approved by the ethics committees of the National Center for Child Health and Development (approval number, 575; date of approval, 5 June 2012). We conducted a nationwide cohort study to retrospectively collect data on term infants with HIE who were born between January and December 2008. The survey was designed to include term infants (≥37 weeks) who had moderate or severe HIE caused by obvious perinatal asphyxia. Term infants without obvious perinatal asphyxia were also included if they demonstrated any of the following during the first 72 h after birth: abnormal consciousness, difficulty maintaining respiration, abnormal tone and reflexes, or neonatal seizures. We excluded infants with acute encephalopathy resulting from causes other than hypoxic–ischemic events, that is, congenital abnormality, chromosomal abnormality, electrolyte abnormality, hypoglycemia, metabolic disease, neuromuscular disease, neurocutaneous syndrome, idiopathic stroke, intracranial hemorrhage, and central nervous infection.

Questionnaires were sent to 290 hospitals associated with the authorized educational facilities of the Japanese Society of Maternal Perinatal Medicine. Of the 290 hospitals, 263 responded, resulting in a response rate of 90.7%. Two hundred and ninety-four infants fulfilled the inclusion criteria. Due to the nature of the survey, patient data were not collected in entirety, and 67 cases had missing outcomes data for the 18 month period following birth. Incidence was estimated based on the total number of eligible subjects ($n = 294$), whereas risk factors were analyzed using data on 227 infants.

The questionnaire consisted of items concerning maternal, ante/intrapartum and neonatal factors. Maternal factors included age (≥35 or <35 years), gravidity, parity, fertility treatment, underlying disease, and medication (with the exception of tocolysis). Ante/intra-partum factors consisted of plurality, hospital of delivery, mode of delivery, induced delivery, instrumental delivery (forceps and/or vacuum delivery), meconium staining, umbilical abnormalities, and placental abnormalities. Fetal heart rate abnormalities included non-reassuring fetal status, bradycardia, deceleration, and loss of or decrease in variability. Fetal heart rate monitoring was evaluated according to the modified definition established by the Japan Society of Obstetrics and Gynecology.

Neonatal factors included gender, gestational age, birthweight, fetal growth, Apgar score (at 1 min and 5 min), and resuscitation. Blood gas analysis of cord blood and the patient's blood as well as the results of blood gas tests performed during admission to the neonatal intensive care unit (NICU) were evaluated.

Brain MRI performed during hospitalization was also reviewed. Decisions regarding whether to perform MRI, technical specifications (such as T1/T2 weighting and image sections), and the timing of imaging were determined by individual clinicians and were based on institutional policy. Abnormal findings included bilateral basal ganglia thalamic lesions, parasagittal injury, subcortical leukomalacia, multicystic encephalomalacia, periventricular leukomalacia, and intracranial hemorrhage.

Neurodevelopmental outcomes were evaluated at age 18 months by the attending physician using neurodevelopmental assessment tools and/or via medical interviews and physical examination. The primary endpoint of this study was outcome at 18 months. Poor outcome was defined as neurodevelopmental delay or death occurring within the first 18 months following birth.

Statistical analysis

In Japan, 1 027 890 term infants were born in 2008; at the time of the survey in 2012, there were a total of 2765 NICU beds. The incidence of HIE among term neonates was calculated based on these data. Statistical analysis was performed using SPSS version 19.0 (SPSS, Chicago, IL, USA) and included the chi-squared test, Fisher's exact test for categorical variables, and logistic regression. The main outcome measures were expressed as odds ratios (OR) and the respective 95% confidence intervals (CI). Continuous variables, such as maternal age, gestational age, birthweight and laboratory data, are reported as median and interquartile range (IQR). $P < 0.05$ was considered to be statistically significant.

Results

The median maternal age was 31 years (IQR, 28–35 years), gestational age was 36.6 weeks (IQR, 38.4–40.6 weeks), and birthweight was 2957 g (IQR, 2640–3253 g). Boys comprised 59.5% (135/227) of the study sample; 72 (24.5%) infants were inborn.

Incidence

In 2012, the number of NICU beds in Japan totaled 2765. Among the 263 hospitals responding to the questionnaire, the total number of NICU beds was 2138, which represented 77.3% (2138/2765) of all NICU beds in Japan. Based on the 294 infants meeting the inclusion criteria, the number of infants with moderate or severe HIE in 2008 was projected to be 380 (294/0.773). In 2008, 1 027 890 term infants were born in Japan. Therefore, the birth incidence of moderate or severe HIE was approximately 0.37 per 1000 term live births.

Risk factors for poor outcome

Table 1 lists the potential maternal risk factors for poor outcome. Of these, maternal age (≥35 years), gravidity, parity, fertility

Table 1 Maternal factors

	Good outcome *n* (%)	Poor outcome *n* (%)	OR (95%CI)	*P*
Maternal age (years)				
<35	61 (68.5)	94 (74.0)	1	
≥35	28 (31.5)	33 (26.0)	0.76 (0.342–1.39)	0.379
Gravida				
0	55 (61.1)	64 (51.2)	1	
≥1	35 (38.9)	61 (48.8)	1.50 (0.86–2.60)	0.149
Parity				
0	64 (72.7)	82 (65.1)	1	
≥1	24 (27.3)	44 (34.9)	1.43 (0.79–2.59)	0.237
Fertility treatment				
No	75 (90.4)	110 (92.4)	1	
Yes	8 (9.4)	9 (7.6)	0.77 (0.28–2.08)	0.601
Underlying diseases				
No	70 (78.7)	111 (86.0)	1	
Yes	19 (21.3)	18 (14.0)	0.60 (0.29–1.22)	0.153
Maternal medications				
No	82 (90.1)	118 (90.8)	1	
Yes	9 (9.9)	12 (9.2)	0.93 (0.37–2.30)	0.869

CI, confidence interval; OR, odds ratio.

treatment, maternal underlying disease, and maternal medication were not associated with poor outcome. Of the potential antepartum risk factors (Table 2), multiple conceptions did not portend an unfavorable outcome, but outborn birth was associated with a twofold increase in the odds of a poor outcome (OR, 2.07; 95%CI: 1.17–3.36). Mode of delivery, induced labor, and instrumental delivery were not associated with poor outcome, nor were umbilical and placental abnormalities. Fetal heart rate patterns were not associated with neurodevelopmental outcome in infants with HIE (Table 3).

Table 2 Intrapartum factors

	Good outcome *n* (%)	Poor outcome *n* (%)	OR (95%CI)	*P*
Plurality				
Singleton	92 (100)	133 (99.3)		
Twins	0 (0)	1 (0.7)	NA	0.406
Hospital of delivery				
Inborn	38 (41.3)	34 (25.4)	1	
Outborn	54 (58.7)	100 (74.6)	2.07 (1.17–3.66)	0.012
Mode of delivery				
Transvaginal	47 (52.2)	70 (56.0)	1	
Caesarean section	43 (47.8)	55 (44.0)	0.85 (0.50–1.48)	0.583
Labor				
Spontaneous	42 (60.9)	63 (63.6)	1	
Induced	27 (39.1)	36 (36.4)	0.89 (0.47–1.67)	0.716
Instrumental delivery				
No	60 (72.3)	82 (73.9)	1	
Yes	23 (27.7)	29 (26.1)	0.92 (0.47–1.75)	0.805
Meconium stain				
No	48 (53.9)	69 (60.5)	1	
Yes	41 (46.1)	45 (39.5)	0.76 (0.44–1.34)	0.346
Umbilical abnormalities				
No	72 (87.8)	88 (80.0)	1	
Yes	10 (12.2)	22 (20.0)	1.80 (0.80–4.05)	0.151
Placental abnormalities				
No	52 (67.5)	72 (69.9)	1	
Yes	25 (32.5)	31 (30.1)	0.90(0.47–1.69)	0.734
Abruptio placentae				
No	54 (70.1)	78 (75.7)	1	
Yes	23 (29.9)	25 (24.3)	0.75 (0.39–1.46)	0.401

CI, confidence interval; NA, not available; OR, odds ratio.

Table 3 Fetal heart rate monitoring

	Good outcome *n* (%)	Poor outcome *n* (%)	OR (95%CI)	*P*
Non-reassuring fetal status				
No	13 (16.3)	11 (10.3)	1	
Yes	67 (83.8)	96 (89.7)	1.69 (0.72–4.01)	0.227
Bradycardia				
No	52 (65.0)	59 (55.1)	1	
Yes	28 (35.0)	48 (44.9)	1.51 (0.83–2.74)	0.714
Deceleration				
No	13 (28.9)	11 (19.6)	1	
Yes	32 (71.1)	45 (80.4)	1.66 (0.66–4.18)	0.278
Loss/decrease in variability				
No	74 (92.5)	99 (92.5)	1	
Yes	6 (7.5)	8 (7.5)	0.99 (0.33–3.00)	0.995

CI, confidence interval; OR, odds ratio.

Female infants had a significantly higher odds for poor outcome compared to male infants (OR, 1.76; 95%CI: 1.01–3.05; $P = 0.004$). Gestational age and birthweight had no association with poor outcome, whereas low Apgar score (<7) at 5 min more than doubled the odds of poor outcome (OR, 2.31; 95%CI: 1.42–5.23; $P = 0.003$). Similarly, use of epinephrine during resuscitation significantly increased the odds of a poor outcome by nearly sevenfold (OR, 6.90; 95%CI: 1.42–33.30; $P = 0.017$; Table 4).

With respect to laboratory indices, pH and base excess (BE) as determined by blood gas analysis at admission were significantly

Table 4 Neonatal factors

	Good outcome *n* (%)	Poor outcome *n* (%)	OR (95%CI)	*P*
Gender				
Male	62 (67.4)	73 (54.1)	1	
Female	30 (32.6)	62 (45.9)	1.76 (1.01–3.05)	0.044
Gestational age (weeks)				
37	16 (17.4)	18 (13.3)	0.87 (0.38–2.02)	0.754
38	15 (16.3)	30 (22.2)	1.56 (0.70–3.44)	0.275
39	20 (21.7)	33 (24.4)	1.28 (0.61–2.70)	0.511
40	28 (30.4)	36 (26.7)	1	
41	10 (10.9)	16 (11.9)	1.24 (0.49–3.15)	0.645
42	3 (3.3)	2 (1.55)	0.52 (0.08–3.32)	0.488
Birthweight (g)				
<2499	12 (13.0)	19 (14.3)	0.97 (0.42–2.24)	0.947
2500–2999	35 (38.0)	57 (42.9)	1	
3000–3499	30 (32.6)	41 (30.8)	0.84 (0.47–1.58)	0.586
3500–3999	13 (14.1)	12 (9.0)	0.57 (0.23–1.38)	0.211
≥4000	2 (2.2)	4 (3.0)	1.23 (0.21–7.04)	0.818
Centile birthweight				
<10th	10 (11.4)	23 (17.6)	1.60 (0.72–3.60)	0.249
10th–90th	67 (76.1)	96 (73.3)	1	
>90th	11 (12.5)	12 (9.2)	0.76 (0.32–1.83)	0.542
Apgar score at 1 min				
<7	80 (39.4)	123 (60.6)	2.31 (0.90–5.89)	0.074
≥7	12 (60.0)	8 (40.0)	1	
Apgar score at 5 min				
<7	63 (36.2)	111 (63.8)	2.80 (1.416–5.529)	0.003
≥7	27 (30.0)	17 (13.35)	1	
Resuscitation				
None	5 (5.4)	4 (3.0)	1	
Oxygen	5 (5.4)	8 (6.0)	2.00 (0.36–11.24)	0.431
Bagging/intubation	72 (78.3)	75 (56.0)	1.30 (0.34–5.05)	0.702
Chest compression	4 (4.4)	14 (10.4)	4.37 (0.78–24.39)	0.093
Epinephrine	6 (6.5)	33 (24.6)	6.90 (1.42–33.30)	0.017

CI, confidence interval; OR, odds ratio.

Table 5 Laboratory data

	Good outcome Median (IQR)	Poor outcome Median (IQR)	*P*
Cord blood			
pH	6.97 (6.87–7.14)	6.88 (6.69–7.17)	0.044
BE (mmol/L)	−16.4 (−20.3 to −10.6)	18.4 (−25.8 to −12.1)	0.057
On admission			
pH	7.24 (7.14–7.33)	7.18 (6.92–7.30)	0.003
BE (mmol/L)	−9.9 (−15.2 to −3.65)	18.4 (−21.6 to −6.95)	<0.001
Lactate (mmol/L)	9.4 (4.7–15.0)	11.9 (5.8–17.6)	0.086
WBC (/mm^3)	20520 (14393–26525)	21500 (16300–29900)	0.030
CRP (mg/dL)	0.01 (0.00–0.15)	0.02 (0.00–0.20)	0.901
LDH (IU/L)	673 (507–1204)	987 (662–1866)	<0.001
AST (IU/L)	68 (45–150)	126 (67–20)	<0.001
ALT (IU/L)	17 (10–40)	34 (14–81)	<0.001
CK (IU/L)	642 (433–1328)	1022 (538–2603)	0.002

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BE, base excess; CK, creatine kinase; CRP, C-reactive protein; LDH, lactate dehydrogenase; WBC, white blood cells.

lower in infants with poor outcome compared to those with favorable outcomes. Conversely, serum lactate, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) were markedly higher in infants with poor outcome compared to infants with good outcome (Table 5).

Infants who had abnormal findings on brain MRI had a significantly higher risk for poor outcome compared to infants with normal MRI findings (Table 6).

Discussion

Incidence

In this study, the incidence of term infants with moderate/severe HIE in Japan was estimated to be approximately 0.37 per 1000 term live births. A few authors have reported the birth incidence of moderate or severe HIE with rates ranging from 0.46 to 1.26 per 1000 live births.[12,14,16] The variation among the reported data may be due primarily to the difficulty in diagnosing HIE. The diagnosis of neonatal HIE is challenging and typically inferred from non-specific signs.[17] Some infants with HIE have failed to exhibit obvious fetal distress, but nevertheless have suffered from neurological abnormalities immediately after birth.[15] In this study, the subjects consisted of infants with neurological abnormalities due to hypoxic–ischemic events, but not other causes, and included all types of HIE.

Neonatal encephalopathy is a heterogeneous syndrome characterized by signs of central nervous system dysfunction in newborn infants. NE occurs as a consequence of intracranial hemorrhage, hypoglycemia, severe hyperbilirubinemia, various metabolic disorders, and intracranial infection, among other disorders. The reported incidence of NE is 3.8 per 1000 term live births in Western Australia[8] and 1.64 per 1000 term live births in France.[12] Because NE may be caused by events other than hypoxic–ischemic events, the incidence of HIE may differ from that of NE.

Risk factors

Whereas several published studies have reported the ante-/intrapartum risk factors for developing NE and/or HIE,[8–10] none has evaluated the relationship between ante-/intrapartum risk factors and outcome in childhood. The present study found that outborn infants had a significantly higher risk of poor outcome. In Japan, approximately 50% of all neonates are delivered in private clinics. Therefore, it is important that medical staff working in facilities lacking organized perinatal centers receive education on neonatal resuscitation.

Fetal heart rate pattern was not associated with neonatal outcome. The reason for this finding may be the poor specificity of cardiotocography.[18] Similarly, fetal heart rate pattern and abnormalities of the placenta and umbilicus were not related with outcome. We speculated that the inability to estimate the severity of placental and umbilical abnormalities due to the retrospective design of the present study may have contributed to this finding.

Low Apgar score is caused by hypoxic–ischemic injury. Apgar scores at 1 min and 5 min reflect the neonate's general condition immediately after birth and are predictive

Table 6 Brain MRI in hospital

	Good outcome *n* (%)	Poor outcome *n* (%)	OR (95%CI)	*P*
Brain MRI				
Normal	54 (63.5)	14 (13.1)	1	
Abnormal	31 (36.5)	93 (86.9)	11.57 (5.66–23.64)	<0.001

CI, confidence interval; OR, odds ratio; MRI, magnetic resonance imaging.

of neurological outcome, respectively. Several authors have reported that low Apgar score at 5 min is a risk factor for serious morbidity and mortality.[19–21] In the present study, Apgar score at 1 min was not associated with poor outcome, but infants with low Apgar score at 5 min had greater risk of poor outcome compared to infants with higher Apgar score at 5 min. This finding was compatible with that reported in previous studies.

In this study, neonatal resuscitation level was predictive of death or neurological sequelae. The incidence of poor outcome in the infants who received epinephrine was significantly higher than in infants who were not given epinephrine. The need for a high level of resuscitation at delivery has been previously cited as a sensitive predictor of subsequent adverse outcome.[13,22] When the need for cardiopulmonary resuscitation coexisted with severe acidemia, an adverse outcome was likely in >90% of cases.[23]

Both cord arterial lactate and pH are measures of acidemia. Fetal arterial lactate measures anaerobic metabolism whereas fetal pH reflects both anaerobic metabolism and acidemia due to increasing fetal carbon dioxide level. LDH is an important biomarker of cellular damage and is commonly designated as an outcome variable in experimental studies of HIE.[24,25] AST, ALT, and CK as well as LDH may reflect cellular damage occurring in conjunction with extensive tissue damage in one or several organs.

Brain MRI is an essential method for establishing prognosis. One systematic review indicated that diffusion weighted and conventional MRI play an important role in prognostic evaluation.[5] MRI findings in HIE infants are heterogeneous.[2,3,15,26,27] In term neonates with brain injury, the specific regional distribution of injury was associated with different durations and severities of ischemia. Partial asphyxia caused cerebral white matter injury,[15,26] whereas acute and profound asphyxia produced basal ganglia and thalamus injury.[27] In this study, abnormal brain MRI findings were associated with poor outcome. We did not, however, evaluate the relationship between outcome and type of brain injury seen on MRI. Further investigation is necessary to confirm the relationship between outcome and type of MRI abnormality.

Limitations and strengths

This study has some limitations. First, the retrospective study design resulted in missing data; 67 cases (22.8%) did not provide outcome data. But whether or not the follow-up rate affected the true incidence of severe disabilities, is unclear.[28,29] A second limitation was the lack of uniformity among techniques for evaluating neurodevelopment. In Japan, methods for assessing neurodevelopment are subject to the individual clinician's practices and institutional policies. Therefore, it is important to establish a standardized protocol for following high-risk infants.

Nevertheless, this study has several strengths. Notably, the response rate was high at 90.7%. In Japan, approximately 50% of neonates are delivered in private clinics. Mothers and newborns suffering from complications are generally transferred to a regional perinatal center, and it is likely that all infants with moderate or severe HIE are treated in NICU. Therefore, the present results accurately describe the current status of infants with HIE in Japan. Additionally, the findings may contribute information that may be useful for prenatal counseling of parents and for cross-national research.

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A review of the mechanism for poor placentation in early-onset preeclampsia: the role of autophagy in trophoblast invasion and vascular remodeling

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ABSTRACT

Shallow trophoblast invasion and impaired vascular remodeling of spiral arteries have been recognized in early-onset preeclampsia. Placentation and vascular remodeling are multistep processes, and hypoxia, placental oxidative stress, excessive or atypical maternal immune response to trophoblasts, exaggerated inflammation, and increased production of anti-angiogenic factors such as the soluble form of the vascular endothelial growth factor (VEGF) receptor (sFlt-1) and soluble endoglin (sENG) may play a role in poor placentation in preeclampsia. Recent findings suggest that autophagy plays an important role in extravillous trophoblast (EVT) invasion and vascular remodeling under hypoxia, and sENG inhibits EVT invasion and vascular remodeling by the inhibition of autophagy under hypoxic conditions. In this review, we discuss the relationship between inadequate autophagy and poor placentation in preeclampsia.

1. Introduction

The pathogenesis of preeclampsia remains largely unknown. However, many researchers support the two-step theory (Steegers et al., 2010) or the three-step theory (Redman and Sargent, 2010). In normal pregnancy, extravillous trophoblasts (EVTs) deeply invade the uterine spiral arteries, disrupt the muscular coat and elastica, and replace the vascular endothelial cells (Pijnenborg et al., 1980). This remodeling dilates the spiral arteries and triggers increased uteroplacental blood flow. The placental oxygen curve estimated by Jauniaux et al. shows that this vascular remodeling starts from a gestational age of 10–12 weeks (Jauniaux et al., 2001). Therefore, failed remodeling at this stage leads to reduced uteroplacental blood flow and hypoxic stress to the fetus and placenta. In stage 1 of early-onset preeclampsia, impaired EVT invasion into maternal spiral arteries causes poor vascular remodeling and induces placental and endothelial damage (Khong et al., 1986).In stage 2, these damaged tissues release anti-angiogenic factors such as the soluble form of the vascular endothelial growth factor (VEGF) receptor (sFlt-1) and soluble endoglin (sENG), a co-receptor for transforming growth factor (TGF)-β_1 and -β_3, which induces maternal intravascular systemic inflammatory responses and endothelial dysfunction, resulting in hypertension and proteinuria after 20 weeks' gestation, especially in early-onset preeclampsia (Venkatesha et al., 2006; Levine et al., 2006).

During early pregnancy, the placental oxygen concentration is only 2%, while the decidual oxygen concentration is around 8% (Jauniaux et al., 2001). Furthermore, glucose concentration in the intervillous space at 5–12 weeks' gestation is only one quarter to one fifth of that in maternal serum, coelomic fluid, and amniotic fluid (Jauniaux et al., 2005). Nevertheless, EVTs invade the maternal decidua and myometrium, and induce vascular

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remodeling under harsh conditions. Indeed, the hypoxia-inducible factor (HIF) 1 system plays a critical role in EVT functions. Therefore, we should understand the mechanism by which EVTs can invade decidua or myometrium and induce vascular remodeling of spiral arteries under physiological conditions of low oxygen in normal pregnancy. In addition, we should understand why shallow EVT invasion and inadequate vascular remodeling occur in early-onset preeclampsia. Burton et al. have shown that total blood flow and hence oxygenation of the placenta was only slightly changed in patients with failure to transform the spiral arteries (Burton et al., 2009); thus, not only hypoxia, but also other unknown factors, may play important roles in the pathogenesis of early-onset preeclampsia.

Recent studies have demonstrated that autophagy is a process of self-degradation of cellular components in which double-membrane autophagosomes sequester organelles and fuse with lysosomes so that the contents can be digested by lysosomal enzymes (Mizushima et al., 2010, 2011; Ichimura and Komatsu, 2011). By using this system, cells can survive under starvation or stress conditions such as hypoxia or oxidative stress.

The expression of autophagy-related proteins in the placenta (Oh et al., 2008; Signorelli et al., 2011) and activated autophagy in the placenta of intrauterine growth restriction (IUGR) pregnancies (Hung et al., 2012) have been reported. Recently, we reported that autophagy is recognized in deeply invaded EVTs in the uterine myometrium and perivascular region. We have found that autophagy is essential for EVT functions, invasion, and vascular remodeling under physiological conditions of low oxygen (Nakashima et al., 2013; Saito and Nakashima, 2013). We also reported that anti-angiogenic factor sENG inhibits autophagy in EVTs under hypoxia, resulting in poor EVT invasion and vascular remodeling (Nakashima et al., 2013).

In this review, we discuss the mechanisms of poor placentation in preeclampsia from the perspective of autophagy.

1.1. Previous reports on the mechanisms of poor endovascular trophoblast invasion in preeclampsia

In normal pregnancy, invasive extravillous trophoblasts (EVTs) express integrin $\alpha_1\beta_1$, a receptor for collagen 1, collagen IV, and laminin. However, in preeclampsia, the expression of integrin $\alpha_1\beta_1$ is downregulated, and this failure to acquire the vascular repertoire of adhesion molecules may explain the impaired invasion of EVTs (Zhou et al., 1993) (Fig. 1). Early in the first trimester (<10 weeks), *placental oxygen tension is very low* (~2%; 25.6 *mmHG* O_2) (Jauniaux et al., 2001), and this low-oxygen environment maintains trophoblasts in an immature, proliferative state mediated by TGF-β_3 through HIF-1α (Caniggia et al., 1999, 2000). After the gestational age of 10 weeks, increased placental oxygen tension increases, and may reduce the pool of proliferating trophoblasts and increase the number of invasive trophoblasts. Caniggia et al. (1999) *speculated that* increased placental oxygen tension reduces the expression of HIF-1α and TGF-β_3 and the failure of TGF-β_3 production at around 9 weeks' gestation results in shallow trophoblast invasion. However, Lyall et al. (2001) reported that TGF-β_1 and -β_2, and to a much lesser extent, TGF-β_3, were present within the placental bed, and no change in the expression of either isoform of TGF-β was found in the placenta and placental bed in preeclampsia and fetal growth restriction (FGR) compared with those in normal pregnancy.

Smith et al. (2009) reported that NK cells and *macrophages were present in the vascular wall at the* stage of remodeling (gestational age of 9~10 weeks). These NK cells produce matrix metalloproteinase −7 and −9, and urokinase plasminogen activator (uPAR) (Fig. 1) (Smith et al., 2009; Naruse et al., 2009a,b). These enzymes can break down the extracellular matrix and induce the separation of vascular smooth vessel cells. Cell culture supernatant of uterine NK cells at a gestational age of 12–14 weeks stimulated EVT invasion (Lash et al., 2010; Lash and Bulmer, 2011), and this effect was partially abrogated in the presence of neutralizing antibodies to IL-8 and IP-10 (Fig. 1) (Hanna et al., 2006). Adequate NK cell stimulation might be necessary for EVT invasion and vascular remodeling of uterine spiral arteries. In this regard, Fraser et al. (2012) reported some very interesting findings. They studied the resistance indices using uterine artery Doppler ultrasound. Uterine NK cells isolated from pregnant women with higher resistance indices, i.e., impaired vascular remodeling, were less able to promote invasive behavior of trophoblasts. Furthermore, uterine NK cells isolated from high-resistance-index pregnancies failed to induce vascular apoptosis (Fraser et al., 2012). These findings suggest that dysregulation of uterine NK cells may contribute to the impaired vascular remodeling (Fig. 1). Indeed, Hiby et al. (2004) reported that the combination of maternal killer-cell immunoglobulin-like receptor (KIR) AA and fetal HLA-C2 was a risk factor for preeclampsia (Fig. 1). KIR AA lacks the activation receptor for HLA-C2; therefore, inadequate NK cell activation might induce poor EVT invasion and vascular remodeling, and adequate NK cell activation might be necessary for the placentation. Kaufmann et al. (2003) reported that activated macrophages induce trophoblast apoptosis by the secretion of TNFα and by the expression of indoleamine 2,3-dioxygenase (IDO), which depletes the local level of tryptophan. They speculated that activated macrophages reduce EVT invasion by the induction of apoptosis of EVTs in preeclampsia (Fig. 1). Indeed, the serum level of TNFα is elevated (Meekins et al., 1994) and peripheral blood mononuclear cells produce a lot of TNFα in such cases (Saito et al., 1999).

2. The mechanisms of autophagy

Extravillous trophoblast s invade the myometrium and maternal spiral arteries and replace the endothelial cells at low oxygen concentration and under stressful conditions. Autophagy may explain this mechanism, because autophagy is a cellular bulk degradation system to maintain cellular homeostasis under stress (Mizushima et al., 2010, 2011; Ichimura and Komatsu, 2011). By the degrada*tion of cellular components, autophagy supplies energy so* that cells can survive under starvation conditions (Fig. 2). In nutrient-sufficient conditions, amino acids, glucose, and insulin activate mTORC1, resulting in the regulation of autophagy (Fig. 3). However, under starvation

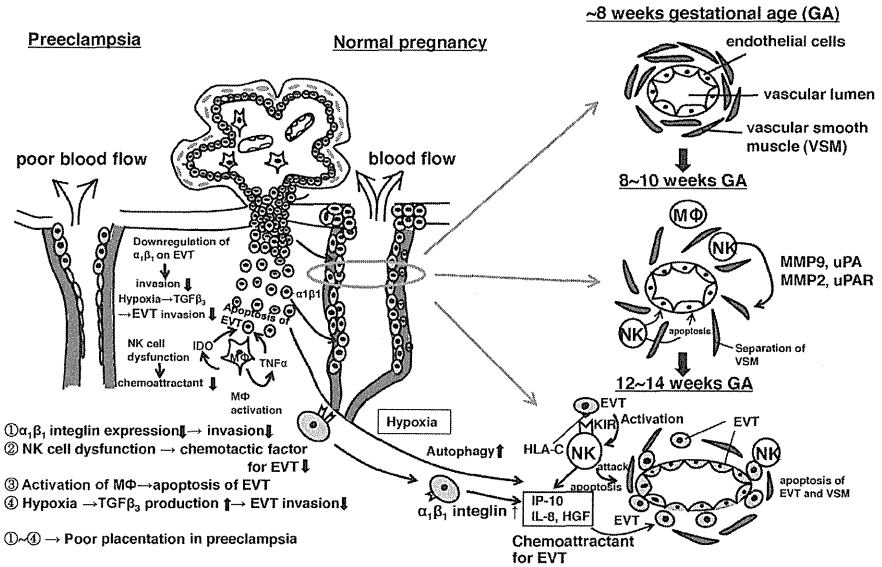

Fig. 1. The mechanisms of vascular remodeling in normal pregnancy and inadequate vascular remodeling in preeclampsia that have already been reported. At a gestational age of 8–10 weeks, uterine NK cells produce MMP-2, MMP-9, and uPA, and these enzymes induce the separation of vascular smooth muscle. NK cells also induce the apoptosis of endothelial epithelial cells and vascular smooth muscle, partly through Fas–Fas ligand signaling. These initial morphological changes have been called "trophoblast-independent remodeling." At a gestational age of 12–14 weeks, extravillous trophoblasts (EVTs) accumulate in spiral arteries owing to chemoattractant factors such as IL-10, IL-8, and HGF, and they invade the wall of the myometrial spiral arteries and replace endothelial cells. These changes have been called "trophoblast-dependent remodeling". In preeclampsia, poor EVT invasiveness and inadequate vascular remodeling have been considered to result from poor α1β1 integrin expression on EVTs, NK cell dysfunction, and the activation of macrophages, resulting in the induction of apoptosis of EVTs, and TGF-β production by hypoxic stress.

conditions, mTORC1 are suppressed, resulting in release of the mTORC1-ULK complex. Free ULK complex induces autophagy (Fig. 3). Hypoxia also induces autophagy, and HIF-1α plays an important role in its induction (Fig. 3). Hypoxia stabilizes HIF1α protein and activates BNIP3. BNIP3 releases the Baclin 1 and Bcl-2 complex. Then, free Bcl-2 inhibits PI3 kinase, resulting in the activation of autophagy (Fig. 3). Autophagy also plays an important role in the removal of old or misfolded proteins and damaged organelles before they cause damage (Fig. 2). This is a cellular homeostasis system, and basal autophagy acts as quality control machinery for cytoplasmic components (Mizushima et al., 2010, 2011). Autophagy also plays a role in the cell defense mechanism to clear intracellular bacteria or viruses. However, excessive autophagy can promote cell death, which is called autophagic cell death.

When cells recognize stress, an isolation double-membrane arises from the endoplasmic reticulum or the Golgi apparatus. Next, this isolation membrane elongates to engulf cytoplasmic components, including mitochondria and endoplasmic reticulum (Fig. 2). Association with the Atg5–Atg12 complex forms the isolation membrane. LC3-II derived from LC3-I localizes to the elongated isolation membrane during the latter step of autophagosome formation. Finally, the isolation membrane is enclosed to form an autophagosome. The diameter of the autophagosome is approximately 1 μm. Lastly, the lysozome fuses with the autophagosome to form an autolysosome, and lysosomal enzymes degrade the cell components in the autolysosome. They obtain energy by eating themselves (autophagy; Fig. 2).

2.1. Autophagy in trophoblasts

Hypoxia induces autophagy in choriocarcinoma cell lines JEG-3 and BeWo (Oh et al., 2008; Curtis et al., 2013), and in EVT cell lines HTR/SV40-neo and HchEpc1b (Nakashima et al., 2013). Autophagy was also observed in primary cultured trophoblasts under hypoxic conditions (Chen et al., 2012) or hypoxia–reoxygenation (Hung et al., 2010). Hypoxia was also shown to promote autophagy in placental chorionic plate-derived mesenchymal stem cells (Lee et al., 2013). Increased autophagy in the placenta was also found in fetal growth restriction (FGR) (Hung et al., 2012; Curtis et al., 2013; Chang et al., 2013) and preeclampsia (Oh et al., 2008).

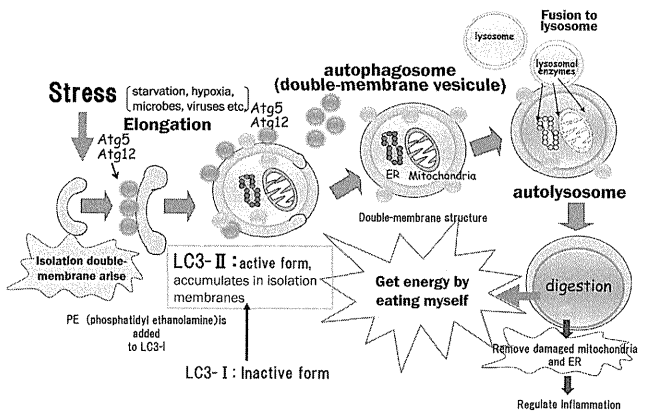

Fig. 2. Schematic diagram of autophagy. When cells experience stress, the isolation membrane arises and elongates, finally forming an autophagosome. Lastly, the autophagosome fuses with lysosomes, resulting in degradation of the cytoplasmic components by lysosomal hydrolases. Then, the cells obtain energy. At the same time, damaged mitochondria and ER are removed by autophagy, resulting in the regulation of inflammation.

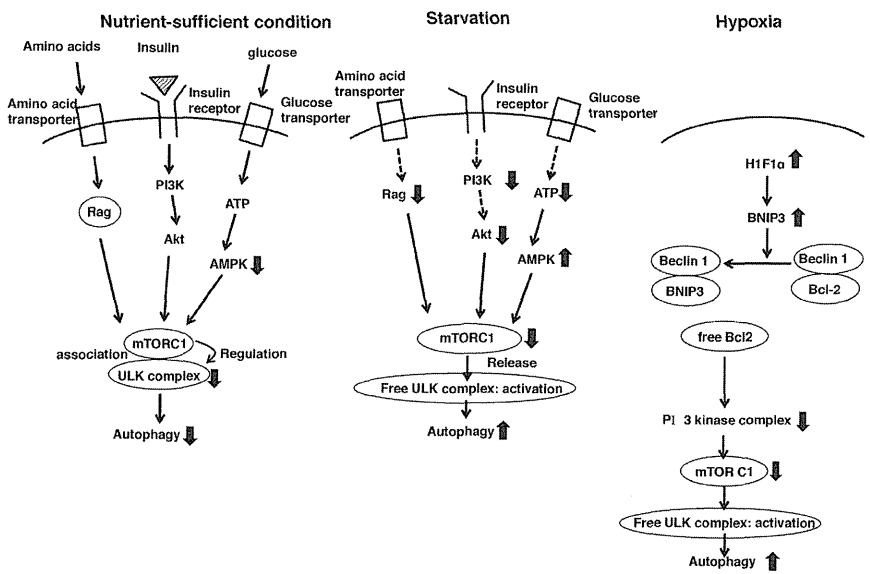

Fig. 3. The molecular mechanisms that induce autophagy. Under nutrient-sufficient conditions, amino acids, glucose, and insulin induce mTORC1 activation. mTORC1 associates with the UCL complex, resulting in the regulation of autophagy. Under starvation conditions, mTOR activity is reduced, resulting in autophagy. Hypoxia induces HIF-1α expression. HIF-1 induces BNIP3 expression, resulting in the release of Baclin 1 and Bcl2 complex. Free Bcl2 reduces mTORC1 activity, resulting in autophagy.

When primary cultured trophoblasts were cultured under hypoxic conditions, autophagy was mainly observed in syncytiotrophoblasts. Tuuli et al. (2011) proposed that hypoxia results in marked increases in p53 activity and yields apoptosis in cytotrophoblasts. In syncytiotrophoblasts, hypoxia results in a diminished level of p53 and promotes autophagy. Indeed, autophagy has been shown to localize predominantly in the syncytiotrophoblasts in FGR cases (Curtis et al., 2013).

Autophagic cell death is also observed in amniotic epithelial cells near the ruptured parts (Shen et al., 2008). Autophagic cell death may be involved in the development of membrane rupture of the human amnion in term gestation.

A higher level of autophagy in placental villi from cesarean section was observed compared with that in placentas from spontaneous vaginal delivery (Signorelli et al., 2011). Maternal fasting prior to the operation is commonly performed, so that the lower glucose level of the cord blood of infants may increase the autophagy in the placenta from cesarean section. Indeed, maternal starvation in a mouse model results in enhanced placental autophagy (Broad and Keverne, 2011). These findings suggest that autophagy is activated as a survival mechanism during nutrient deprivation (Bildirici et al., 2012).

2.2. The role of autophagy in EVT invasion and vascular remodeling by EVT

We have studied autophagy in EVTs obtained from induced abortion cases before 10 weeks' gestation (Nakashima et al., 2013). Interestingly, LC3B puncta, which exhibited autophagosomes, were present in cytokeratin 7-positive EVT cells in the decidua basalis at a deeper site than the fetal site (interstitial EVTs) and perivascular site (perivascular EVTs). We could not obtain the decidual samples after 10 weeks' gestation; thus further analysis is necessary whether EVTs experiencing higher oxygen during invasion (e.g., at 14–16 weeks' gestation) show a different autophagy pattern or not. These findings suggest that autophagy might play a role in EVT invasion and vascular remodeling by them under hypoxic conditions. To clarify the specific roles of autophagy in EVT functions, we constructed two autophagy-deficient EVT cells such as SV40 large T transformed-HTR8/SV neo and human papilloma virus (HPV) E6- and hTERT-transfected EchEpc 1b cells by stable transfection of $ATG4B^{C74A}$, an inactive mutant of ATG4B, which inhibits autophagic degradation and lipidation of LC3B paralogs.

Under 20% O_2, EVT invasion was similar in wild-type EVT cell lines and autophagy-deficient EVT cell lines. However, importantly, the depth of invasion of autophagy-deficient cell lines was significantly shallower than that of wild-type EVT cell lines in three-dimensional culture assays under hypoxic conditions (O_2: 2%), although levels of cell viability and proliferation were similar in wild-type EVT cells and autophagy-deficient EVT cells (Nakashima et al., 2013). The oxygen level in the decidua is 6–8%; thus, a 2% oxygen level in this study is considered a pathological hypoxic condition. Thus, it may be different from the chronically mild hypoxic condition. Kadyrov et al. reported that there was a much deeper invasion of more EVTs (Kadyrov et al., 2003). Under pathological hypoxic conditions, autophagy may play a role in EVT invasion. Supplementation of ATP under hypoxic conditions rescued the impairment of cell invasion in our recent study (unpublished data); thus, obtaining energy by autophagy plays a role in EVT invasiveness under pathological hypoxia.

We can evaluate vascular remodeling by tube formation assays with EVT cells and human umbilical vascular endothelial cells (HUVECs) (Kalkunte et al., 2010). Under a pathologically low oxygen level, 2% O_2, HUVEC did not undergo vascular formation on a Matrigel-coated plate; thus, we set the oxygen concentration at 8%. This concentration reflects the physiologically low oxygen level at a gestational age of 10–18 weeks in placenta and decidua, when vascular remodeling occurs. In the culture with wild-type EVT cells and HUVECs, tube areas were formed by EVT cells and HUVEC at 6 h, but the tubes were mostly occupied by EVT cells at 12 h or later. Meanwhile, tubes were still occupied by HUVEC when autophagy-deficient EVT cells were co-cultured with HUVECs, suggesting that replacement of endothelial cells by EVT under typical hypoxic conditions might require autophagy.

2.3. Impaired autophagy by soluble endoglin might be involved in poor placentation in preeclampsia

We have studied whether preeclampsia-related substances such as soluble endoglin (sENG), sFlt-1, TGF-β, and TNF-α affect autophagy in EVTs under hypoxia (2% O_2) (Fig. 4). Only sENG inhibited the number of autophagosomes in cytoplasm using confocal microscopy and reduced the LC3B-II conversion in western blotting under hypoxia. Importantly, sENG inhibited the cell invasion of EVT under 2% O_2 and reduced the vascular remodeling under 8% O_2, although sENG did not affect the EVT invasion and vascular remodeling under 20% O_2 (Nakashima et al., 2013). These findings were from an in vitro study; thus, we studied the evidence showing impaired autophagy in EVTs in preeclampsia using placental bed biopsy samples. It has been reported that p62/SQSTM1 is selectively digested by autolysosome; therefore, p62/SQSTM1 levels were suppressed under hypoxia in hepatic carcinoma cells (Pursiheimo et al., 2009) and EVT cells (Nakashima et al., 2013), but not in autophagy-deficient EVT cells. p62/SQSTM1 expression in interstitial EVTs and endovascular EVTs was markedly enhanced compared with that in normal pregnancy. The ratio of p62/SQSTM1 to CK7 expression was significantly higher in preeclampsia than in normal pregnancy in both interstitial EVTs and endovascular EVTs (Nakashima et al., 2013). These findings suggest that impaired autophagy in EVTs might be present in preeclampsia. It is unknown whether the sENG level is elevated at a gestational age of 10–18 weeks in that EVTs invade the myometrium and replace the endovascular cells of spiral arteries. Farina et al. (2008) examined the mRNA expression in chorionic villi at 11 weeks' gestation obtained by chorionic villous sampling. Five patients subsequently developed preeclampsia, while 25 did not. They showed that sENG mRNA expression was significantly higher in patients who subsequently developed preeclampsia than

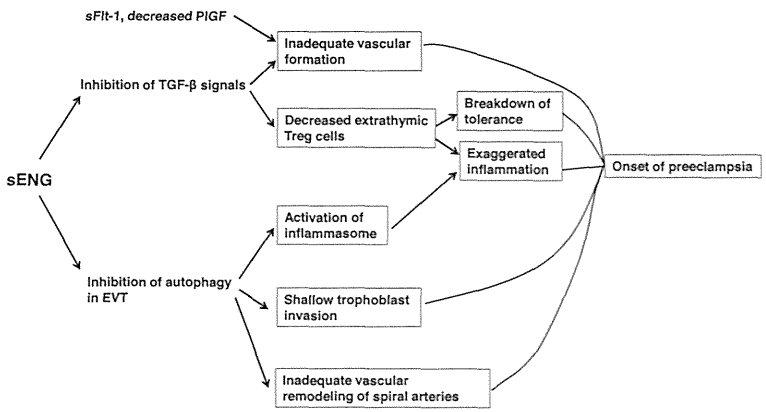

Fig. 4. The mechanisms for the pathophysiology of preeclampsia from the perspective of autophagy. The high level of sENG inhibits the autophagy in EVTs under hypoxic conditions, resulting in shallow trophoblast invasion and inadequate vascular remodeling of spiral arteries. Inhibition of autophagy reduces the recycling of damaged mitochondria and endoplasmic reticulum, resulting in the activation of inflammasomes. Inhibition of TGF-β signals, in association with increased sFlt-1 and decreased PlGF, induces inadequate vascular formation. Inhibition of TGF-β signals also decreases the induction of extrathymic Treg cells, resulting in the breakdown of tolerance and exaggerated inflammation.

in the controls. This report may show that sENG level at the feto-maternal interface is already upregulated before the onset of clinical symptoms of preeclampsia, although serum sENG during early pregnancy did not increase in a longitudinal study.

Poor early placentation is associated with early onset of preeclampsia and FGR patients without hypertension and proteinuria (Steegers et al., 2010; Redman and Sargent, 2010). Thus, the next question that arises is whether impaired autophagy is present in the EVTs of FGR patients. Our preliminary data showed that the ratio of p62/SQSTM1 to CK7 expression in FGR cases without preeclampsia was similar to that in normal pregnancy. The serum sENG level of FGR patients was reported to be lower than that of preeclampsia (Stepan et al., 2007; Jeyabalan et al., 2008); therefore, elevated sENG might play some role in the impairment of autophagy in EVTs, resulting in poor EVT invasion and inadequate vascular remodeling. This impaired autophagy reduces the 'trophoblast-dependent vascular remodeling' in preeclampsia (Fig. 1). Meanwhile, dysfunction of uterine NK cells may disturb the 'trophoblast-independent vascular remodeling' in preeclampsia (Fig. 1).

2.4. Correlation between impaired autophagy and systemic inflammation and inadequate tolerance in preeclampsia

Redman et al. (1999) proposed that an excessive maternal inflammatory response to pregnancy induces endothelial dysfunction, resulting in hypertension, proteinuria, edema, and clotting dysfunction. Oxidative stress (Stark, 1993) and system inflammation play an important role in the pathophysiology of preeclampsia. Recent studies have revealed that impaired autophagy in macrophages and adipocytes induce inflammation (Saitoh et al., 2008; Yoshizaki et al., 2012). Autophagy plays an important role in the elimination of damaged mitochondria or endoplasmic reticulum (ER) (Fig. 5). Signals from damaged mitochondria induced by uric acid or toll-like receptors induce oxidative stress and activate inflammasome components such as NALP-3 and caspase-1, resulting in overproduction of IL-1β and IL-18 (Saitoh et al., 2008; Yoshizaki et al., 2012) (Fig. 5). Uric acid also induces trophoblast IL-1β production via the inflammasomes (Mulla et al., 2011) (Fig. 5). Impaired autophagy additionally leads to ER stress, resulting in excessive inflammation. These findings suggest that autophagy impairment by sENG might play a part in the systemic inflammation of preeclampsia (Fig. 5).

Regulatory T cells (Treg) play essential roles in implantation and allogeneic pregnancy maintenance (Aluvihare et al., 2004; Sasaki et al., 2004). Treg cells are classified into thymic (naturally occurring) Treg cells that differentiate in the thymus and extrathymic (peripheral or inducible) Treg cells that differentiate in the periphery upon stimulation of naïve $CD4^+$T cells in the presence of TGF-β. Thymic Treg cells account for 70% of Treg cells, while extrathymic Treg cells account for the rest. Samstein et al. (2012) reported that extrathymic Treg cells play an essential role in the maintenance of allogeneic pregnancy. Interestingly, sENG inhibits TGF-β activity by competition with the binding of TGF-β receptor. Therefore, it can be readily speculated that increased sENG disturbs the differentiation of extrathymic Treg cells, resulting in the breakdown of maternal tolerance to the fetus (Fig. 4). Indeed, decreased Treg cells in preeclampsia have been reported (Sasaki et al., 2007; Saito et al., 2007). One of the important roles of Treg

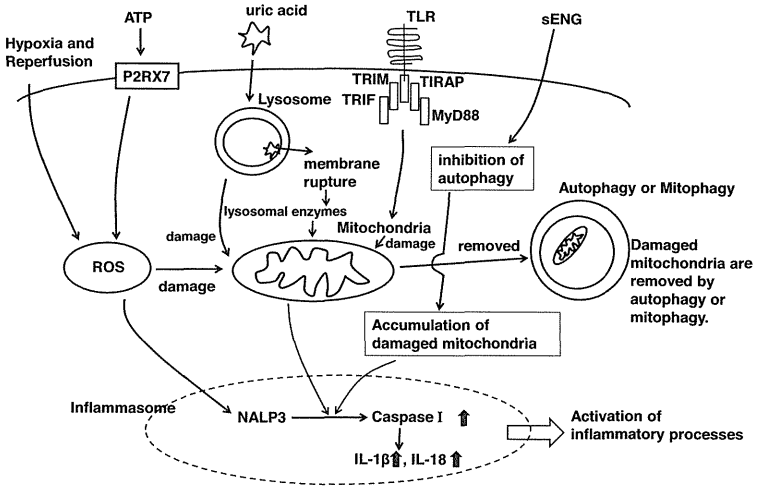

Fig. 5. The role of autophagy in NALP3 inflammasome activation. Quality control of mitochondria is essential for cellular homeostasis, and this process is achieved through autophagy. When autophagy is inhibited, damaged mitochondria activate inflammasomes, resulting in exaggerated inflammation.

cells consists of the regulation of inflammation; therefore, decreased Treg cells induce exaggerated inflammation (Fig. 4). This inflammation induces Th1-type immunity, which subsequently induces rejection (Saito et al., 2007), inhibits the function of Treg cells (Yang et al., 2004), and induces endothelial dysfunction (Redman et al., 1999). Redman and Sargent (2010) proposed that the inflammatory drive of preeclampsia results from the three-way interactions among oxidative stress, ER stress and inflammatory responses. Inadequate autophagy of EVTs may accelerate oxidative stress, ER stress and inflammation. Further studies are needed to clarify this hypothesis.

3. Conclusion

Autophagy plays an important role in trophoblast function under physiologically low oxygen conditions in normal pregnancy. Autophagy plays an important role in EVT invasion and vascular remodeling under a physiologically low oxygen concentration. Autophagy impaired by sENG may be involved in poor placentation due to impaired EVT invasion and poor vascular remodeling, systemic inflammation by activating inflammasome and induction of ER stress, and inadequate tolerance by reducing the pool of Treg cells in preeclampsia.

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Whole exome sequencing revealed biallelic *IFT122* mutations in a family with CED1 and recurrent pregnancy loss

To the Editor:

Cranioectodermal dysplasia-1 (CED1), also known as Sensenbrenner syndrome (MIM 218330), is characterized by skeletal, craniofacial, and ectodermal abnormalities (1). Here, we report a family with CED1 and recurrent abortions.

I-2, 39-year-old woman, was referred to our hospital for consultation regarding recurrent abortions. Although she had one healthy boy (II-2), she suffered from two artificial abortions due to fetal hydrops at 13 weeks of gestation (II-1) and skeletal anomalies at 21 weeks of gestation (II-8), one intrauterine fetal death with hydrops at 13 weeks of gestation (II-7), and four recurrent miscarriages (II-3 at 6 weeks, II-4 at 8 weeks, II-5 at 8 weeks, and II-6 at 7 weeks) (Fig. 1a). Postmortem physical findings of II-8 included a skull deformity and blisters beside the nasal bridge due to obstetric intervention, low set ears, nuchal edema, a narrow thorax, and acromelic shortening of the limbs, posterior bowing of the lower legs and bilateral 2–3 toe syndactyly (Fig. 1b). Postmortem radiography and 3-D computed tomography showed generalized skeletal alterations which were thought to fit to CED1, though the sharp angulation of the tibiae was very unusual (Fig. 1b).

Exome sequencing was performed in I-2, II-2, and II-8 as previously described (2). We identified compound heterozygous mutations in *IFT122* in the fetal skeletal anomalies (II-8): c.1108delG (p.E370Sfs*51) in exon 11 and c.1636G>A (p.G546R) in exon 14 was inherited from his mother (Fig. 1c,d). Of note, we confirmed the same compound heterozygous mutations (c.1108delG: 2 of 29 clones, c.1636G>A: 13 of 30 clones) by capillary sequence of polymerase chain reaction (PCR) product from cloned DNA from paraffin-embedded chorionic villi (II-6) (Fig. 1e). Accordingly, c.1108delG found in II-6 and II-8 was presumed to be inherited from the father, though it was not directly confirmed.

Walczak-Sztulpa et al. reported homozygous missense mutations of *IFT122* in three patients from two consanguineous pedigrees with CED1: p.V553G in family CED-01, p.S373F in family CED-02, and compound heterozygous mutations, c.502+5G>A and p.W7C in one sporadic case, family CED-03 (1) (Fig. 1c). The four patients with *IFT122* mutations showed skeletal anomalies. We found compound heterozygous mutations in a fetus with skeletal anomalies (II-8) and the villous tissues (II-6). Clinical skeletal features of the aborted fetus (II-8) are consistent with those of the reported CED patients carrying biallelic *IFT122* mutations. Because they were found in the villous tissues (II-6) that was sequenced, biallelic *IFT122* mutations may have caused some of the recurrent abortions in this family. All four *IFT122* missense mutations (including ours) are predicted to be damaging for IFT122 function (Table 1).

CED1 belongs to a group of short rib dysplasias, including Ellis van Creveld syndrome, Jeune asphyxiating thoracic dysplasia, short rib polydactyly syndrome (SRPS) I (Saldino-Noonan), SRPS II (Majewski), SRPS III (Verma-Naumoff) and SRPS IV (Beemer-Langer) (3). The skeletal manifestation of the present fetus shared some features with other short rib dysplasias. For example, humeral bowing resembles that commonly seen in Ellis van Creveld syndrome. Severe tibial angulation was somewhat reminiscent of tibial hypoplasia in SRPS II (Majewski type). Generally, CED1 is a non-lethal disorder. However, the present family showed recurrent abortions, which can be considered as the severest phenotypes caused by biallelic *IFT122* mutations in human. Interestingly, *Ift122*-null mice show multiple developmental defects with embryonic lethality consistent with recurrent pregnancy loss in our family (4).

On the basis of the assumption that recessive mutations may cause recurrent pregnancy loss, the literatures have been carefully reviewed, but only a homozygous *HERG* mutation in a family were found to show recurrent intrauterine fetal loss (5).

In conclusion, we were able to find causative *IFT122* mutations in a non-consanguineous family with recurrent abortions. This information is useful for future counseling including preimplantatory diagnosis in this family.

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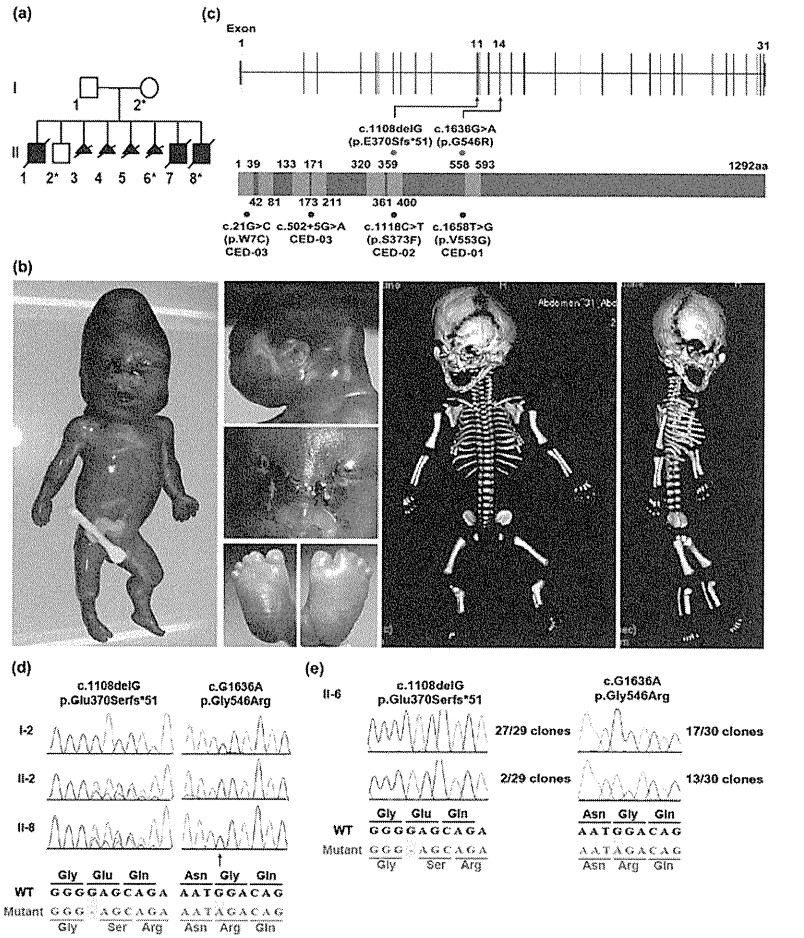

Fig. 1. Clinical finding of fetal skeletal anomalies (II-8) and genetic studies. (**a**) Familial pedigree. II-1 and II-8 were artificially terminated, II-3, II-4, II-5, II-6 and II-7 were spontaneously aborted. * DNA was available. (**b**) Clinical photographs and 3D computed tomography of II-8. (i–v) Postmortem physical findings included a skull deformity due to obstetric intervention, blisters beside the nasal bridge, low set ears, nuchal edema, a narrow thorax, and acromelic shortening of the limbs, anterior bowing of the lower legs and bilateral 2–3 toe syndactyly. (vi–vii) Postmortem 3-D computed tomography showed generalized skeletal alterations. The thorax was narrow with short ribs. The lower ribs showed a wavy appearance. The spine and ilia were normal. The long bones were not apparently short. However, the humeri were medially bowed, and the tibiae showed sharp bending at their proximal part. Ossification of the proximal and middle phalanges was defective. (**c**) The gene structure of *IFT122* (upper) and its protein structure (lower) containing seven WD40 domains (blue). Mutations found in this family and in the previous report are depicted above and below the protein, respectively. (**d**) Sequence electropherogram of family members. Compound heterozygous mutations are indicated in II-8, while healthy members (I-2 and II-2) only carry one of the two mutations. (**e**) Sequence electropherogram of the villous tissue at 7 weeks of gestation (II-6). Amplified PCR products were cloned into pCR4-TOPO vector and each clone was subjected to sequencing. Compound heterozygous mutations are indicated.