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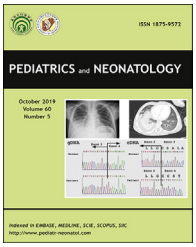

Original Article

DNA methylation of the *Rtl1* promoter in the placentas with fetal growth restriction

Kazumichi Fujioka [a,*], Kosuke Nishida [a], Mariko Ashina [a], Shinya Abe [a], Sachiyo Fukushima [a], Toshihiko Ikuta [a], Shohei Ohyama [a], Ichiro Morioka [b], Kazumoto Iijima [a]

[a] *Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan*
[b] *Department of Pediatrics and Child Health, Nihon University School of Medicine, Tokyo, Japan*

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Background: Small for gestational age (SGA) babies experience fetal growth restriction because of placental insufficiency, and aberrant fetal growth has been linked to DNA methylation in the placenta. An imprinted gene encoding retrotransposon-like protein 1 (*RTL1*) is regulated by DNA methylation in the promoter region and plays a key role in placental development. We therefore investigated the DNA methylation status of *RTL1* in the placenta of infants with severe SGA.
Methods: We extracted DNA from the placenta of appropriate for gestational age (AGA; gestational age 35 ± 6 weeks, birthweight 2292 ± 1006 g; n = 12), SGA (birthweight z-score ≤−2 SD, 33 ± 5 weeks, 1373 ± 580 g; n = 11), and severe SGA (birthweight z-score ≤−3 SD, 33 ± 4 weeks, 1145 g ± 423 g; n = 7) infants, and we determined the methylation rates of five CpG sites in the CG4 (82,275,427–82,275,737 in NT_026437 sequence, NCBI database) region of the *RTL1* promoter by pyrosequencing. We defined hypermethylation (>75.5%) and hypomethylation (<45.6%) based on the average methylation rate exceeding ± two standard deviations (SD) in the AGA group, respectively, and compared these among groups.
Results: There was no significant difference in the average methylation of CpG1-5 (control 59%, SGA 60%, severe SGA 63%), but abnormal methylation (hyper-/hypo-methylation) in CpG1 differed significantly among the groups (control 0%, SGA 36%, severe SGA 71%).
Conclusion: Infants with severe SGA have abnormal placental DNA methylation of CpG1 in the CG4 region of *RTL1*, suggesting the existence of disturbed epigenetic control *in utero*.

* Corresponding author. Department of Pediatrics, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Fax: +81 78 382 6099.
E-mail address: fujiokak@med.kobe-u.ac.jp (K. Fujioka).

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1. Introduction

Fetal growth restriction (FGR) occurs as a consequence of fetal environmental deterioration.[1] It results in small for gestational age (SGA) newborns who are smaller in size than expected, with birth weight (BW) more than two standard deviations (SDs) below the mean BW of newborns of the same gestational age.[2,3] The number of low BW infants has recently risen in Japan because of increases in FGR,[4] and children born SGA have an increased risk of developing adult non-communicable diseases such as developmental origins of health and disease (DOHaD).[1,5] Therefore, an understanding of the pathophysiology of FGR and the introduction of preventative strategies are urgently needed.

In animal studies, intrauterine undernutrition was shown to result in low BW and the development of postnatal obesity, which were associated with altered gene expression in adipose tissue.[6] Epigenetic regulation was recently proposed to be a major link between postconceptional fetal stress and gene expression changes.[7] Moreover, in humans, the epigenetic changes caused by FGR were also suggested to affect postnatal growth; however, the evidence for this is limited.[8]

Placental insufficiency is regarded as the main etiology for FGR.[9] Placental development is highly unique in eutherians and is regulated by numerous factors.[10] For example, the novel retrotransposon-derived gene retrotransposon-like 1 (*RTL1*) was recently shown to play a key role in placental development.[11–13] *RTL1* is a paternally expressed imprinted gene located on human chromosome 14, which is highly expressed during late pregnancy in both the fetus and placenta.[13] Intriguingly, *RTL1* expression levels are regulated by DNA methylation in its promoter regions.[14]

In this study, we hypothesized that the DNA methylation status of *RTL1* promoter regions would vary between healthy placentas and those of non-syndromic severe SGA fetuses.

2. Materials and methods

2.1. Study design and patient groups

This retrospective study was conducted under the approval of the ethical committee of Kobe University Graduate School of Medicine and parental consent. Thirty infants born at our center between 2008 and 2011 whose placentas were obtained at birth were enrolled in the study. Patients with congenital or chromosomal anomalies were excluded. Patients were classified into three groups based on their BW Z-score: appropriate for gestational age (AGA: up to −2SD of mean BW of Japanese newborns of the same gestational age,[3] n = 12), SGA (more than −2SD and less than −3SD of the mean BW, n = 11), and severe SGA (more than −3SD of the mean BW, n = 7). Clinical and DNA methylation status data were compared among the groups.

Clinical data were collected from patient records and included gestational age (GA), BW, BW Z-score, Apgar scores at 5 min, sex, maternal age, primiparity, multiple birth, pregnancy-induced hypertension (maternal systolic blood pressure >140 mmHg and/or diastolic pressure >90 mmHg during pregnancy), premature rupture of the membranes (rupture of the membranes more than 24 h before delivery), and birth by Cesarean section.

2.2. *Rtl1* methylation status

Genomic DNA was extracted from the placenta using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The methylation status of the CG4 regions (82,275,427–82,275,737 in NT_026437 sequence at NCBI Database (http://www.ncbi.nlm.nih.gov/)) in the intergenic differentially methylated region of the *Rtl1* promoter was then analyzed by pyrosequencing (EpigenDx Inc., Worcester, MA, USA). This CG4 region includes five CpG sites (**CGCG**GTT**CG**CCATTTGCC**CGCG**), and its methylation status has been reported to be associated with *RTL1* expression levels.[14] Abnormal methylation was defined as hypo-methylation (more than −2SD of the mean % DNA methylation of the AGA group) or hyper-methylation (more than 2SD of the mean % DNA methylation of the AGA group).

Data are expressed as the median (range) or mean ± SD. The Mann–Whitney nonparametric rank test and chi-square test were used to compare SGA or severe SGA data with AGA data (control group). Differences were deemed statistically significant when $p < 0.05$. Analyses were performed using GraphPad Prism 7 software (Graphpad Software, Inc., San Diego, CA, USA).

3. Results

The clinical characteristics are described in Table 1. In comparison with the AGA group, the BW and BW Z-score were significantly lower in SGA and severe SGA groups (BW $p = 0.037$ and 0.018; BW Z-scores both $p < 0.001$). Moreover, the incidence of pregnancy-induced hypertension was significantly higher in the SGA group than in the control group ($p = 0.043$). Additionally, significantly more male infants were found in the severe SGA group than in AGA and SGA groups ($p = 0.024$ and 0.017, respectively).

DNA methylation was analyzed at five CpG dinucleotides in *RTL1* promoter regions. Table 2 shows the percentage of DNA methylation at each CpG dinucleotide and the average percentile methylation of the combined CpG dinucleotides of *RTL1*. Mean CpG methylation levels at each CpG site ranged from 53.6% to 61.4% in the AGA group, 54.0%–62.4% in the SGA group, and 58.5%–68.4% in the severe SGA group. There was no significant difference in the methylation status of *RTL1* at each CpG site among the groups.

Regarding the effect of gestational age on the methylation condition, there was no significant correlation between the gestational age and the % methylation in CpG1 ($r^2 = 0.004$, $p = 0.731$), CpG2 ($r^2 = 0.005$, $p = 0.723$), CpG3 ($r^2 = 0.009$, $p = 0.614$), CpG4 ($r^2 = 0.052$, $p = 0.225$), and CpG5 ($r^2 = 0.066$, $p = 0.172$), respectively.

Based on the methylation status of the AGA placenta, abnormal methylation was determined as follows: GpG1 (hypomethylation, < 45.7%; hypermethylation, > 75.5%), CpG2 (<43.5% and >77.8%, respectively), CpG3 (<40.5% and >79.4%, respectively), CpG4 ((<35.8% and >87.1%,

Table 1 Clinical characteristics of AGA, SGA, and severe SGA infants.

	AGA	SGA	severe SGA
	n = 12	n = 11	n = 7
Gestational age, weeks (range)	38 (24–41)	35 (26–38)	33 (28–38)
Average birth weight, g (range)	2677 (590–3280)	**1650 (604–2034)**[a]	**1214 (590–1844)**[a]
Average birth weight Z-score (SD)	−0.1 (−0.5 to 1.1)	**−2.4 (−2.8 to −2.0)**[b]	**−3.7 (−4.0 to −3.1)**[b,d]
Average Apgar score at 5 min (range)	9 (3–10)	9 (5–10)	9 (6–10)
Pregnancy-induced hypertension (%)	8	**45**[a]	14
Premature rupture of the membranes (%)	17	9	29
Primiparity (%)	58	64	71
Cesarean section (%)	42	73	86
Male births (%)	50	45	**100**[a,c]
Maternal age, years (range)	34 (19–38)	28 (24–40)	35 (29–42)
Multiple pregnancies (%)	17	0	29

Bold value signifies $p < 0.05$. AGA, appropriate for gestational age; SGA; small for gestational age.
[a] vs. AGA, $p < 0.05$.
[b] vs. AGA, $p < 0.01$.
[c] vs. SGA, $p < 0.05$.
[d] vs. SGA, $p < 0.01$.

respectively), and CpG5 (<35.9% and >71.3%, respectively). There was no significant difference in the incidence of abnormal methylation among the groups for CpG2 to CpG5 sites, but significantly higher abnormal methylation was observed at the CpG1 site in SGA (4/11; 36.4%) and severe SGA (5/7; 71.4%) groups than in the AGA (0/12; 0%) group ($p = 0.022$ and $p < 0.001$, respectively) (Fig. 1).

Further analysis revealed that the incidence of abnormal methylation between the infants with and without pregnancy-induced hypertension was not statistically significantly different in CpG1 (3/7; 42.9%, vs. 6/23; 26.1%), CpG2 (2/7; 28.6%, vs. 2/23; 8.7%), CpG3 (1/7; 14.3%, vs. 0/23; 0%), CpG4 (0/7; 0%, vs. 0/23; 0%), and CpG5 (0/7; 0%, vs. 2/23; 8.7%), respectively ($p > 0.05$ for each). In addition, the incidence of abnormal methylation between female and male infants was not statistically significantly different in CpG1 (2/12; 16.7%, vs. 7/18; 38.9%), CpG2 (0/12; 0%, vs. 4/18; 22.2%), CpG3 (0/12; 0%, vs. 1/18; 5.6%), CpG4 (0/12; 0%, vs. 0/18; 0%), and CpG5 (0/12; 0%, vs. 2/18; 11.1%), respectively ($p > 0.05$ for each).

4. Discussion

Although the development of SGA caused by FGR is strongly associated with placental insufficiencies such as aberrant vasculature and malpositioning, the etiology and severity of SGA is heterogeneous and varied. Moreover, the definition of SGA is also variously defined as a BW below the 10th percentile and more than −1.5 SD of the AGA.[15] In this study, we postulated that placental insufficiency caused by aberrant *RTL1* expression might result in marked FGR. To

Table 2 DNA methylation status and the incidence of abnormal *Rtl1* methylation.

	AGA	SGA	severe SGA
	n = 12	n = 11	n = 7
DNA methylation status of *Rtl1* (%)			
CpG1	60.6 ± 7.5	62.4 ± 13.6	59.5 ± 22.6
CpG2	60.6 ± 8.6	61.6 ± 9.2	63.6 ± 17.1
CpG3	59.9 ± 9.7	62.3 ± 8.9	63.6 ± 8.0
CpG4	61.4 ± 12.8	59.3 ± 12.0	68.4 ± 7.7
CpG5	53.6 ± 8.9	54.0 ± 10.0	58.5 ± 9.0
Average CpG1-5	59.2 ± 6.7	60.0 ± 7.4	62.7 ± 10.9
Abnormal methylation of *Rtl1* (%)			
CpG1	**0/12 (0)**	**4/11 (36.4)**[a]	**5/7 (71.4)**[b]
CpG2	1/12 (8.3)	1/11 (9.1)	2/7 (28.6)
CpG3	0/12 (0)	1/11 (9.1)	0/7 (0)
CpG4	0/12 (0)	0/11 (0)	0/7 (0)
CpG5	0/12 (0)	1/11 (9.1)	1/7 (14.3)
Average CpG1-5	0/12 (0)	0/11 (0)	1/7 (14.3)

Bold value signifies $p < 0.05$. AGA, appropriate for gestational age; SGA; small for gestational age.
[a] vs. AGA, $p < 0.05$.
[b] vs. AGA, $p < 0.01$.

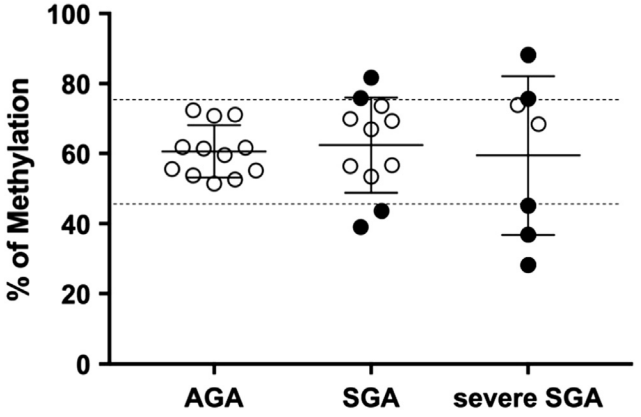

Figure 1 Dotted lines represent ± 2SD of mean % DNA methylation of the AGA group. White circles represent normal methylation, and black circles represent abnormal methylation.

exclude incidental or non-pathologic SGA, we selected strict inclusion criteria of more than −2SD of the mean BW, and we defined severe SGA as more than −3SD of the mean BW. Interestingly, the incidence of pregnancy-induced hypertension was higher in the SGA group than in the severe SGA group, suggesting that the etiology of the latter is associated with placental pathologic conditions rather than complications of maternal hypertensive disorders.

We found that infants with severe SGA had a higher incidence of abnormal CpG1 methylation in the placenta, though there was no significant difference in the average methylation of each CpG site among the three groups. It is noteworthy that not only hypomethylation but also hypermethylation were predominant in CpG1 of the severe SGA placenta. As a counterpart of paternally expressed *RTL1*, the maternally expressed *RTL1* antisense transcript overlaps and targets the *RTL1* transcript through an RNA interference mechanism.[16] *Rtl1* null pregnant mice were previously shown to suffer from placental hypoplasia, while pregnant mice overexpressing *Rtl1* demonstrated placental hyperplasia. Moreover, fetuses and newborns of both genotypes showed severe pre- and postnatal growth restriction, resulting in fatal outcomes.[11,17] Thus, we speculated that not only placental CpG1 hypomethylation but also hypermethylation at this site would result in fetal growth restriction via abnormal placental formation. It was recently reported that the placental DNA methylation pattern in *RTL1* promoter regions was negatively correlated with BW gain at 1 year of age in healthy term infants,[12] suggesting that abnormal *RTL1* methylation may affect future life and could be the trigger of DOHaD.

A limitation of this study was that we failed to measure *RTL1* expression levels in our placental samples. Because RNA degradation of clinical placenta samples occurs rapidly,[18] it is difficult to extract intact RNA from samples originally used for DNA extraction. Additionally, the quantitative analysis of *RTL1* that is exclusively of a paternal origin is technically challenging, requiring the 3′ rapid amplification of cDNA ends technique.[14] Therefore, further study to confirm the *RTL1* expression profile using intact RNA is warranted. Moreover, because of the technical limitations of pyro-sequencing, we could not survey entire CpG islands in the promoter regions of *RTL1*. A confirmation study using a high-throughput technique such as methylation-specific microarray or chromatin immunoprecipitation is therefore necessary.[19]

In conclusion, infants with severe SGA have abnormal placental DNA methylation of CpG1 in the CG4 region of *RTL1*, suggesting disturbed epigenetic control *in utero*. This might contribute to extrauterine growth restriction or the future development of DOHaD in these infants.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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