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Abstract: Notch signaling pathway has shown to be dysregulated in placentas with preeclampsia, but there has been a lack of studies on methylation of Notch family genes in this disorder. We therefore executed methylation specific PCR and immunostaining for Notch 1 receptor and the activating ligand, Delta-like (DLL)1 with placental tissues from cases of preeclampsia (early-onset, n=18; late-onset, n=19) and other placental disorders, including maternal complications like diabetes mellitus and collagen disease (n=10), fetal growth restriction (n=17), fetal anomaly (n=23), preterm birth (n=15), miscarriage (n=25), and hydatidiform moles (n=9), as well as term births (n=12). The frequency of DLL1 methylation was significantly higher with early-onset preeclampsia (61%) than the other groups (0-36%; p ≤ 0.016). None of the samples showed Notch 1 methylation among the groups studied. On gestational period-matched analysis, the rate of DLL1 methylation was significantly higher in the early-onset preeclampsia group (83.3%) than preterm birth group (13.3%; p < 0.001), with no significant differences in clinical backgrounds between the two. In this analysis, increase of syncytial knots and accelerated villous maturation were also most prominent in DLL1 methylated placentas with early-onset preeclampsia. Expression of Notch 1 and DLL1 in villous trophoblasts and endothelial cells was significantly lower in the early-onset preeclamptic placentas as compared to preterm birth controls. In conclusion, altered Notch signaling via methylation of DLL1 might play roles in the pathogenesis of early-onset preeclampsia. Assessment of DLL1 methylation might offer an alternative approach for presymptomatic diagnosis and a biomarker for disease severity with this disorder.

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Human Pathology

Editorial Office

Dear Editor

Please find enclosed our manuscript entitled "Altered Notch signaling via methylation of Delta-like ligand 1 in placental tissues with early-onset preeclampsia", which we would like to submit for consideration of publication in the *Human Pathology.*

The present paper documents evidence that altered Notch signaling via methylation of Delta-like (DLL) 1 gene promoter and decreased expression of DLL1 and Notch 1 proteins might have roles in the pathogenesis of early-onset preeclampsia.

The authors guarantee that the article is an original report in the public domain, and that it has not previously been published, submitted, or accepted for publication elsewhere. The authors also declare no conflict of interest.

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- *DLL1* methylation was frequent in early-onset preeclamptic placentas.

- None of the placental samples showed *Notch 1* methylation.

- Expression of Notch 1 and DLL1 was decreased in early-onset preeclamptic placentas.

- Altered Notch signaling play roles in the pathogenesis of early-onset preeclampsia.

Origiunal contribution

Altered Notch signaling via methylation of Delta-like ligand 1 in placental tissues with early-onset preeclampsia

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Abstract:

Notch signaling pathway has shown to be dysregulated in placentas with preeclampsia, but there has been a lack of studies on methylation of Notch family genes in this disorder. We therefore executed methylation specific PCR and immunostaining for Notch 1 receptor and the activating ligand, Delta-like (DLL)1 with placental tissues from cases of preeclampsia (early-onset, n=18; late-onset, n=19) and other placental disorders, including maternal complications like diabetes mellitus and collagen disease (n=10), fetal growth restriction (n=17), fetal anomaly (n=23), preterm birth (n=15), miscarriage (n=25), and hydatidiform moles (n=9), as well as term births (n=12). The frequency of *DLL1* methylation was significantly higher with early-onset preeclampsia (61%) than the other groups (0-36%; $p \leq$ 0.016). None of the samples showed Notch 1 methylation among the groups studied. On gestational period-matched analysis, the rate of *DLL1* methylation was significantly higher in the early-onset preeclampsia group (83.3%) than preterm birth group (13.3%; $p < 0.001$), with no significant differences in clinical backgrounds between the two. In this analysis, increase of syncytial knots and accelerated villous maturation were also most prominent in *DLL1* methylated placentas with early-onset preeclampsia. Expression of Notch 1 and DLL1 in villous trophoblasts and endothelial cells was significantly lower in the early-onset preeclamptic placentas as compared to preterm birth controls. In conclusion, altered Notch signaling via methylation of *DLL1* might play roles in the pathogenesis of early-onset preeclampsia. Assessment of *DLL1* methylation might offer an alternative approach for presymptomatic diagnosis and a biomarker for disease severity with this disorder.

1. Introduction

Preeclampsia (PE) is one of the important complications of human pregnancy and a leading cause of both maternal and neonatal morbidity and mortality; women with PE are at increased risk of cardiovascular complications [1] and intrauterine growth restriction as an important trigger of perinatal mortality [2] and development of coronary heart disease, hypertension and diabetes in adult life with low-birth-weight babies [3]. The two stage model of PE proposes that a poorly perfused placenta (Stage 1) produces factors leading to clinical manifestations (Stage 2); stage 1 is not sufficient to cause the maternal syndrome but interacts with maternal constitutional factors (genetic, behavioral or environmental) to result in Stage 2 [4, 5]. Although the pathophysiology of PE remains largely unknown, shallow trophoblast invasion and deficient remodeling of uterine spiral arteries are thought to contribute to the disease pathogenesis [6, 7]. In addition, morphological studies have confirmed an increase of syncytial knots in PE-complicated placental tissue [8, 9]. It is also known that PE is heterogeneous in etiology and can be further subclassified into early-onset and late-onset, with differences regarding clinical presentation and outcome [2, 10], as well as morphological characteristics of the placental tissues [11].

The Notch signaling family of receptor-ligands regulates cellular processes as diverse as proliferation, apoptosis, differentiation, invasion and adhesion, with particularly important roles in vascular patterning [12, 13]. In humans, there are four Notch receptors (1-4) and five membrane-bound Delta-like (DLL1, 3, 4) and Jagged (1, 2) ligands for canonical Notch signaling [14]. Notch ligand-receptor binding leads to receptor cleavage by proteases, and release of the intracellular domain that translocates to the nucleus and induces gene transcription of Notch target genes [15]. Several studies have identified dysregulated expression of Notch family members in preeclamptic placentas [16 - 24]. In

the Notch 3 case results have been varied, *i.e.*, up-regulated [18] , down-regulated [21, 23], or normoregulated [24], while Notch 1, Notch 2, and Notch 4 expression appears to be consistently down-regulated in placentas with this disorder [16, 21]. At the same time, DLL4 expression has been shown to be increased in PE placentas [24].

Although the exact cause remains unknown, epigenetic changes are implicated in the pathogenesis of PE. Alterations of non-imprinted genes have been suggested to be involved; for example the *TIMP3* or *maspin* promoter was found to be hypomethylated in PE-complicated placenta [25, 26]. This suggests that epigenetic variation may be associated with reduced trophoblastic invasion and that this might be used as a potential biomarker. Since there has been a relative lack of studies on methylation of Notch family genes in PE, we investigated the methylation status of *DLL1* and *Notch 1* and expression of those proteins in placental tissues with early-onset and late-onset PE as compared with other diseases. In addition, we assessed clinicopathological differences in early-onset PE with and without methylation of these Notch family genes.

2. Materials and methods

2.1. Patients and materials

The materials for our study were 148 samples of placental tissue (from 136 patients and 12 healthy individuals) obtained by vaginal delivery, cesarean section or intrauterine curettage at Juntendo University Hospital between 2011 and 2014. These comprised PE (early-onset, 25 - 35 weeks of gestation, *n*=18; late-onset, 34 - 40 weeks of gestation, *n*=19), maternal complications (34 - 39 weeks of gestation, *n*=10) including gestational diabetes mellitus (*n*=2), diabetes mellitus (*n*=3) and collagen disease (*n*=5), fetal growth restriction (25 - 40 weeks of gestation, *n*=17), fetal anomaly (31 - 39 weeks of gestation, *n*=23), preterm birth (24 - 32 weeks of gestation, *n*=15), miscarriage before 21 weeks' gestation (6 - 20 weeks of

gestation, *n*=25), and hydatidiform mole (8 - 11 weeks of gestation, *n*=9), as well as term births (37 - 40 weeks of gestation, *n*=12). A review of all the medical records of patients or individuals who had their placental tissues included in this study was conducted, aimed at collecting information on the gynecological background.

PE was defined as hypertension (systolic blood pressure $\geq$ 140 mmHg and diastolic blood pressure $\geq$ 90 mmHg after 20 weeks' gestation) and proteinuria ($\geq$ 300 mg in a 24 h urine collection or one dipstick measurement of $\geq$ 1+) according to the criteria of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy [27]. PE was further subclassified into early-onset (< 34 weeks) and late-onset ($\geq$ 34 weeks) according to previous reports [2, 10].

This study was approved by the ethical committee of our hospital (registration # 2012174).

2.2. *Methylation analysis of DLL1 and Notch1*

Genomic DNA was extracted from five 10-µm-thick formalin-fixed paraffin-embedded sections using a QIAamp DNA FFPE Tissue kit (Qiagen GmbH , Hilden, Germany), according to the manufacturer's instructions. Sections were stained lightly with hematoxylin and areas of normal mucosa were excluded by modified microdissection with observation of the tissue directly under a light microscope. The quality and integrity of the DNA were checked spectrophotometrically.

Sensitive methylation-specific PCR was used to detect promoter methylation. Bisulfite modification was conducted using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). The bisulfate-treated DNA was then amplified using specifically designed primers for methylated and unmethylated alleles. Sequences of the primers, annealing temperature, and product size are listed in Table 1. After amplification, products

were electrophoresed using 2% agarose gels, stained with ethidium bromide and visualized under UV illumination. Experiments in this study were run in triplicate.

2.3. Histological evaluation of preeclamptic and preterm birth placental tissues

Sections of placental tissues of PE and preterm birth cases stained with hematoxylin and eosin (H&E) were morphologically examined independently by two of the authors (YS and HM). The following variables were assessed among the groups studied: the number of syncytial knots (the mumbers of syncytial knots were counted in 90 villi and calculated per villus according to previous methodology with modification [8], infarction, accelerated villous maturation, *i.e.*, slender stem villi with reduced branching and very small terminal villi (positive, > 30% of total villous area), acute atherosis of spiral arteries, *i.e.*, mural fibrinoid necrosis with accumulation of foamy macrophages and neutrophils, and stromal inflammation. Interobserver variation was resolved by reevaluation and discussion to reach consensus.

2.4. Immunohistochemistry for preeclamptic and preterm birth placental tissues

Four μm-thick serial tissue sections prepared from formalin-fixed and paraffin-embedded placental tissues with preeclampsia and preterm birth were subjected to immunohistochemistry. Polyclonal antibodies used in this study were against DLL1 (LS-B4663, 1:200 dilution, LifeSpan BioSciences, Seattle, WA, USA) and Notch 1 (sc-6014, 1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antigen retrieval was executed by heating in an autoclave in Tris-EDTA buffer (pH 6.0). The sections were then incubated at 4℃ overnight with primary antibodies. Immunohistochemical staining was performed using an Envision Kit (Dako, Grostrup, Denmark) with substrate-chromogen solution.

Immunohistochemical staining intensity for DLL1 and Notch 1 was evaluated for villous trophoblasts and endothelial cells in the villi, and then scored as 0 (no expression), 1 (weak), or 2 (strong). Immunoreactive areas (distributions) for those proteins were also scored as 0 (no staining cell), 1 (1 - 24%), 2 (25 - 49%), 3 (50 - 74) or 4 ($\geq$ 75%). Placental villi from six different areas were randomly selected by one of the authors (YS) and immunohistochemical images of selected areas were captured using a digital microscope camera. Then, images were imported into a PowerPoint presentation and evaluated independently by two researchers (YS and HM), without prior knowledge of clinicopathological data or the methylation status. Finally, average immunoreactive scores were used for comparisons.

2.5. Statistical analysis

All statistical analyses were carried out using StatView for Windows Version 5.0 (SAS Institute Inc., Cary, NC, USA). Continuous data were compared with the Mann-Whitney *U*-test. Categorical analysis of variables was performed using either the Chi-squared test (with Yates' correction) or the Fisher's exact test, as appropriate. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Methylation of DLL1 *and* Notch 1 *in the various placental tissues studied*

Frequencies of *DLL1* and *Notch 1* methylation among the groups studied are summarized in Table 2 and representative results of methylation-specific PCR analysis for *DLL1* are illustrated in Fig. 1. *DLL1* methylation was detected significantly more frequently in the early-onset PE group (61%) than other groups (0 - 36%; $p \leq 0.016$). None of the samples showed *Notch 1* methylation among the groups studied (Table 2).

3.2. Methylation of DLL1 *and clinical background in PE and preterm birth*

We compared the frequency of *DLL1* methylation in placental tissues and clinical backgrounds between gestational period-matched (24 - 33 weeks) women with early-onset PE (*n*=12) and those with preterm birth as controls (*n*=15). In this analysis, *DLL1* methylation was detected significantly more commonly in the early-onset PE group (83.3%) than in the preterm birth group (13.3%; $p < 0.001$), but no significant differences were observed in clinical backgrounds between the two groups (Table 3). Furthermore, no significant differences in clinical features were found between early-onset PE cases with *DLL1* methylation and those without (*data not shown*).

3.3. Comparison of histological characteristics and immunohistochemstry for DLL1 and Notch 1 between placental tissues with DLL1 *methylated or unmethylated early-onset PE and with preterm birth*

We further comparatively analyzed differences in histological characteristics and immunoreactivity of DLL1 or Notch 1 between *DLL1* methylated or unmethylated early onset PE groups and preterm birth groups (*n*=10, all samples harboring no *DLL1* methylation).

The number of syncytial knots per one villus was higher in the *DLL1* methylated placental tissues compared with unmethylated samples ($p = 0.014$) or preterm birth samples ($p < 0.001$). Infarction and accelerated villous maturation were found in 7 of 11 (64%) and 9 of 11 (81%) *DLL1* methylated placentas but appeared absent in preterm birth placentas ($P \leq 0.004$); in addition, the latter feature was more frequently observed in placentas with *DLL1* methylation than those without ($p = 0.049$; Table 4).

In placental tissues with preterm birth, DLL1 and Notch 1 were largely expressed in the cytoplasm of cytotrophoblasts and syncytiotrophoblasts in the villi. Likewise, Notch 1

was localized in the venous endothelial cells of the villi. DLL1 and Notch 1 expression for villous trophoblasts and endothelial cells was lower in early-onset PE than preterm birth ($P \leq 0.048$). In the early-onset PE group, there were no significant differences in immunoreacivity of DLL1 or Notch 1 between *DLL1* methylated and unmethylated placentas (Table 4). Representative illustrations of expression of DLL1 and Notch 1 are given in Fig. 2.

4. Discussion

Here, we used methylation-specific PCR to detect a significantly elevated frequency of *DLL1* methylation in placentas with early-onset PE as compared with those with other diseases, while none of the samples showed *Notch 1* methylation. To our knowledge, this is the first study of methylation for DLL1 and Notch 1 in preeclamptic placentas. While several studies of the gene expression profile for members of Notch signal pathway in human placentas with PE have been reported [18, 21, 24], placental RNA can degrade during parturition and rapidly after delivery [28], making it difficult to obtain useful samples. The methylation-specific PCR we adopted is the most widely used approach as it is very sensitive, cost-effective, and does not require specialized equipment. Placental DNA from formalin-fixed paraffin-embedded tissue can be stored for decades and still be appropriate for retrospective studies for PE. DNA methylation is generally stable and provides an alternative marker for processes underlying PE, as stressed in previous reports [25, 26].

In placentas with PE, *Notch 1* gene expression may be down-regulated [21] while *DLL4* expression is up-regulated [24]. One earleir study searched for epigenetic alterations in members of the Notch signaling family in PE; the promoter of Notch 4 was confirmed to be hypomethylated in early-onset pleeclamptic placentas [25]. In the present study, Notch

ligand *DLL1* methylation was a frequent finding in placentas with early-onset PE. We thus hypothesized that Notch signal cannot be activated by *DLL1* methylation in preeclamptic placenta. In adult heterozygous *DLL1* mutants, ischemia-induced arteriogenesis is severely impaired [29], indicating that DLL1 regulates postnatal arteriogenesis. It is also required for maintaining arterial identity during mouse fetal development [30]. These findings support our hypothesis that DLL1 mediated Notch signaling disorders may contribute to PE.

Here, we also compared the frequency of *DLL1* methylation in placental tissues and clinical backgrounds between gestational period-matched women with early-onset PE and those with preterm birth as controls. In most previous studies on expression of Notch signaling members in placentas with PE, the gestational period of the samples was not matched between PE and control cases [16 - 23]. In our gestational age-matched analysis, *DLL1* methylation was detected significantly more frequently in the early-onset PE than in preterm birth control samples in spite of no significant differences in clinical backgrounds between the two.

Finally, we further comparatively analyzed histological characteristics and immunoreactivity of DLL1 or Notch 1 between *DLL1* methylated or unmethylated early-onset PE groups and preterm birth groups harboring no *DLL1* methylation. In previous reports, placental ischemia and hypoxia associated with PE might have accounted for increase in syncytial knots [8, 9] and accelerated villous maturation [31]. Our data also showed increased syncytial knots and accelerated villous maturation to be prominent in *DLL1* methylated placentas, further supporting roles for severe ischemia / hypoxia. Marked decrease in the immunoreactive level of Notch 1 has been described in placentas with PE [16]. Similarly, we found expression of Notch 1, together with DLL1, to be low in villous trophoblasts and endothelial cells in early-onset PE. In the current study, there was no inverse correlation between immunoreactivity of DLL1 and *DLL1* methylation in the group

of early-onset PE. This discrepancy might be due to other processes involved in transcriptional regulation of DLL1.

In conclusion, our present research demonstrated that *DLL1* is methylated, corresponding to decreased expression of DLL1 and Notch1, in placentas with early-onset PE compared to other pregnancy-associated disorders. Altered Notch signaling via methylation of *DLL1* might play roles in the pathogenesis of early-onset PE. Assessment of *DLL1* methylation may also expected to be an alternative approach for presymptomatic diagnosis and offer a biomarker for disease severity of PE. Further studies with larger sample sizes and experimental approaches should be designed and performed to clarify the epigenetic regulation of Notch family members in preeclamptic placentas.

Acknowledgements

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Figure legends

Fig. 1. Electrophoresis of methylation specific PCR products spanning *DLL1* promoters from bisulfate-treated DNA from cases of early-onset PE (A), late-onset PE (B), fetal growth restriction (C), preterm birth (D), and miscarriage (E). Each lane features separate PCR reactions specific for methylated (*M*) or unmethylated (*UM*) DNA templates. Commercially available CpG dinucleotides for completely methylated DNA (*C+*) and unmethylated DNA (*C-*) (methylated and unmethlyated EpiTect Control DNA, Qiagen) were used as positive controls. A 100-bp ladder was applied for molecular weight markers. Blanks without any DNA template were used as negative controls (*not shown*).

Fig. 2. Localization of DLL1 and Notch 1 proteins in placental tissues with early-onset PE. (A) Representative H&E staining of an early-onset preeclamptic placenta shows prominent syncytial knots. (B) At higher magnification, a syncytial knot displays crowding of nuclei and projection into the intervillous space. (C) Diffuse expression of DLL1 in the villous syncytio-cytotrophoblast layers in preeclamptic placenta. (D) At higher magnification, DLL1 staining in a granular pattern is evident at a moderate level in the villous trophoblasts. (E) Notch 1 is expressed in the villous trophoblasts of placenta complicated with PE. (F) An higher magnification, Notch 1 staining at a low level is evident in the villous trophoblasts together with the endothelial cells.

Figure 1
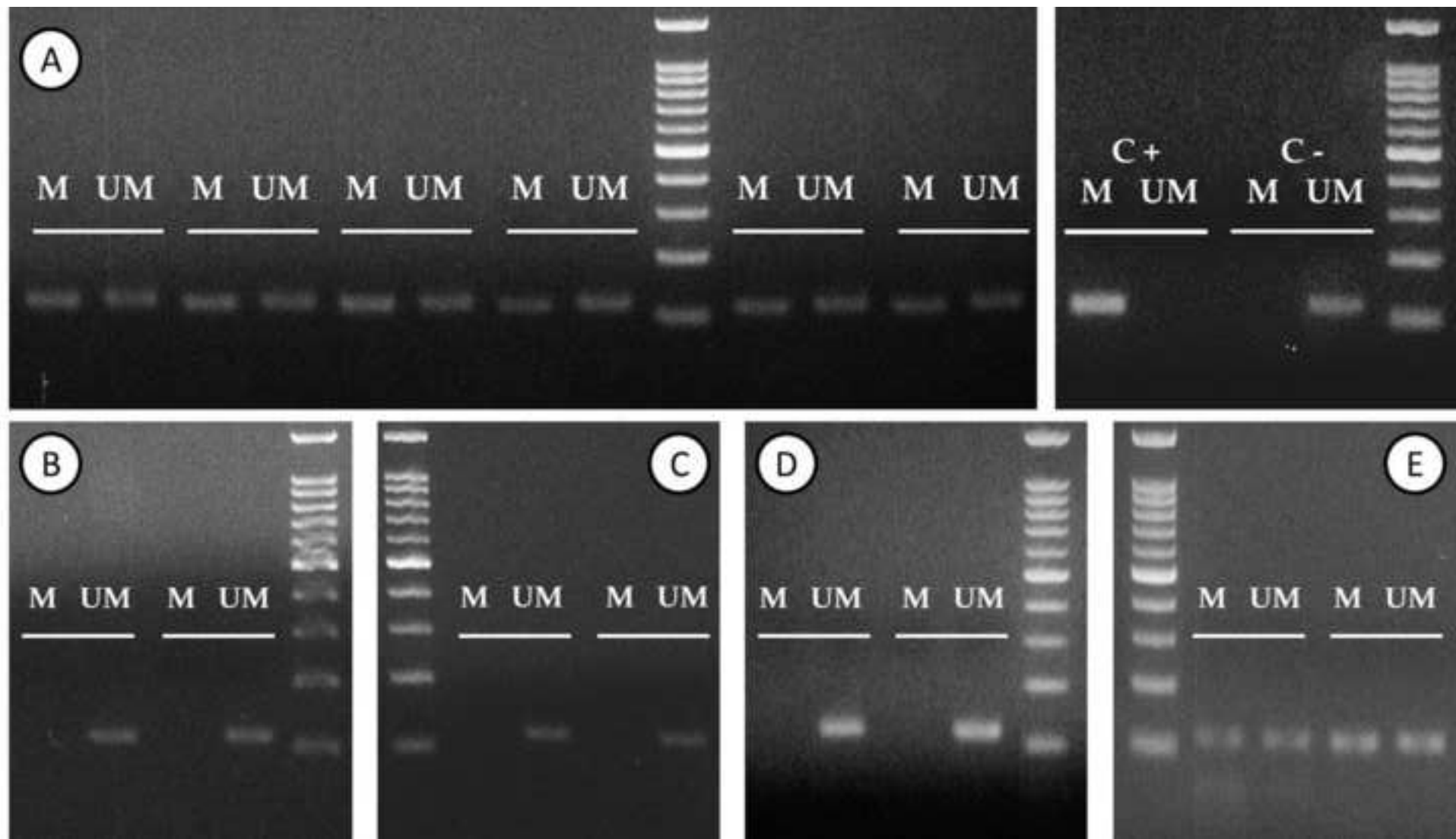**Click here to download high resolution image**

Figure 2
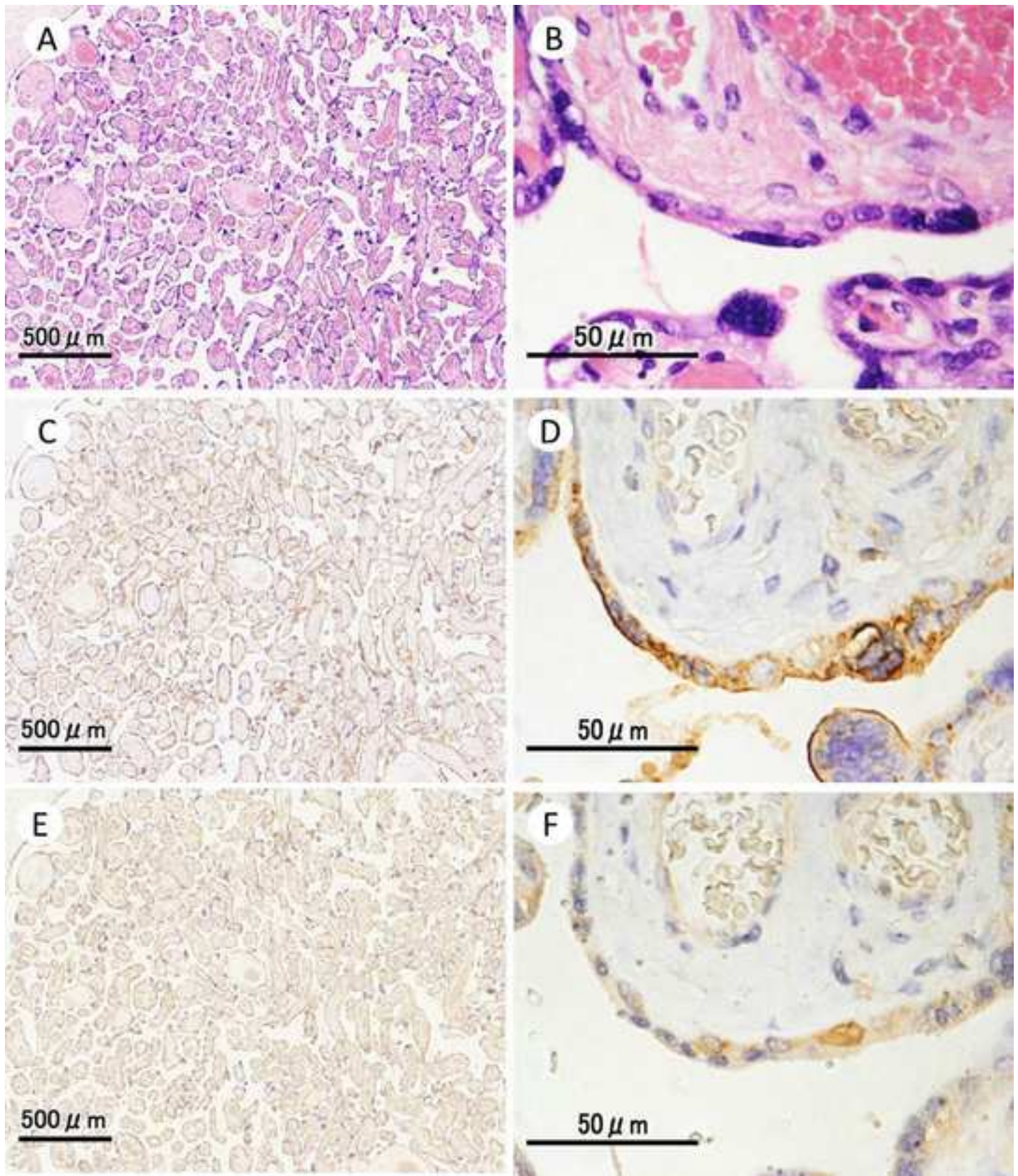**Click here to download high resolution image**

Table 1

Table 1 Primers used for the methylation-specific PCR analysis.

Gene	Forward primers (5' - 3')	Reverse primers (5' - 3')	Tm (°C)	Product size (bp)	PCR cycles
DLL1 Reg1	M: ATATTCGTCGTCGTCGATC	M: CCGAACCGATTAAAAAACC	56	100	40
	UM: GTATATTTGTTGTTGTTGATT	UM: TCCCAAACCAATTAAAAAACC	56	100	40
DLL1 Reg2	M: AAGGGCGTTTTTTTGTTTAC	M: ATACTACTTCGCTCCACGC	56	114	40
	UM: GGTAAGGGTGTTTTTTTGTTTAT	UM: ATACTACTTCACTCCACACACA	61	114	40
Notch 1	M: CGGTTTAGCGTACGGTGTATAC	M: GACTCTAACGATTACCGCGC	55	127	45
	UM: TTTGGTTTAGTGTATGGTGTATATG	UM: ATCCAACTCTAACAATTACCACACT	55	133	45

DLL1 Reg1, spanning between -532 and -432; DLL1 Reg2, spanning between -112 and ATG start codon; Positive for *DLL1* promoter methylation is defined as the presence of methylated DNA using both Reg 1 and Reg 2 primers; M, methylated DNA; UM, unmethylated DNA; Tm, annealing temperature

Table 2

Table 2 Frequency of *DLL1* and *Notch 1* methylations in the placental tissues studied.

	PE		Maternal complication	Fetal growth restriction	Fetal anomaly	Preterm birth	Term birth	Miscarriage	Hydatidiform mole
	Early-onset	Late-onset							
	(n = 18)	(n = 19)	(n = 10)	(n = 17)	(n = 23)	(n = 15)	(n = 12)	(n = 25)	(n = 9)
DLL1 [a]	11 (61 %)	1 (5 %)	0 (0 %)	3 (18 %)	3 (13 %)	2 (13 %)	0 (0 %)	9 (36 %)	2 (22 %)
Notch 1	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)

a: Statistically significant differences were as follows: early-onset PE *vs.* late-onset PE ($p < 0.001$), maternal complication ($p = 0.002$), fetal growth restriction ($p = 0.015$), fetal anomaly ($p = 0.002$), preterm birth ($p = 0.011$), term birth ($p < 0.001$), or miscarriage ($p = 0.016$); miscarriage *vs.* late-onset PE ($p < 0.027$), maternal complication ($p = 0.036$), or term birth ($p = 0.018$).

Table 3

Table 3 Comparison of *DLL1* methylation rate and clinical characteristics between early-onset PE and preterm birth.

Variable	Early-onset PE (*n* =12)	Preterm birth (*n* =15)	*p* value
Gestational period (weeks) *			
At onset	27.5 (21 - 33)	—	—
At delivery	30 (25 - 33)	30 (24 -32)	0.284
Neonatal birth weight (g) *	1148 (440 - 1940)	1364 (614 - 1973)	0.097
Maternal age (years)	32 (27 - 45)	35 (19 - 47)	0.419
Body mass index before pregnancy *	21 (16 - 30)	19.7 (17 - 25)	0.193
Body weight gain (kg) *	6.5 (- 6.4 - 15.0)	5.9 (0.0 - 15.0)	> 0.999
Methylation rate of *DLL1* **	83.3 (10)	13.3 (2)	< 0.001

* Data are expressed median (range); ** Data are expressed percentages (number of methylated cases).

Table 4

Table 4 Comparison of histological characteristics and immunohistochemstry for DLL1 and Notch 1 between placental tissues with *DLL1* methylated or unmethylated early onset PE cases and preterm births

	Early-onset PE (n = 18)			Preterm birth
	DLL1 methylation (+)	*DLL1* methylation (-)	Total	(n = 10)
Histological variables				
Number of syncytial knots per one villus	0.46 (0.17-0.61) [a, b]	0.26 (0.17-0.42) [a, c]	0.41 (0.17-0.61) [d]	0.14 (0.02 - 0.26) [b, c, d]
Infarction				
Positive (n)	7 [e]	1	8 [f]	0 [e, f]
Negative (n)	4	6	10	10
Accelerated villous maturation				
Positive (n)	9 [g, h]	2 [g]	11 [i]	0 [h, i]
Negative (n)	2	5	7	10
Acute atherosis of spiral artery				
Positive (n)	1	1	2	0
Negative (n)	10	6	16	10
Stromal inflammation				
Positive (n)	2	1	3	0
Negative (n)	9	6	15	10
Immunohistochemistry				
DLL1				
Villous trophoblasts				
Intensity	9.5 (6.0-12.0) [j]	8.5 (6.5-9.5) [k]	9.0 (6.0-12.0) [l]	11.5 (6.5-12.0) [j, k, l]
Distribution	13.5 (8.5-19.0)	13.5 (8.0-16.5)	13.5 (8.0-19.0)	12.8 (8.0-16.0)
Endothelial cells in the villi				
Intensity	0.5 (0.0-2.5)	0.5 (0.0-1.0) [m]	0.5 (0.0-2.5)	1.5 (0.5-4.0) [m]
Distribution	0.5 (0.0-3.0)	0.5 (0.0-1.0) [n]	0.5 (0.0-1.0) [o]	1.5 (0.5-8.5) [n, o]
Notch 1				
Villous trophoblasts				
Intensity	6.0 (6.0-8.5) [p]	6.0 (4.0-8.0) [q]	6.0 (4.0-8.5) [r]	9.3 (7.0-12.0) [p, q, r]
Distribution	12.5 (8.5-14.5)	8.5 (5.0-11.0) [s]	10.8 (5.0-14.5) [t]	12.8 (9.0-19.0) [s, t]
Endothelial cells in the villi				
Intensity	3.0 (1.5-5.0) [u]	3.0 (2.0-4.0) [v]	3.0 (1.5-5.0) [w]	4.5 (3.0-5.5) [u, v, w]
Distribution	5.0 (2.5-9.5)	3.0 (2.0-7.0) [x]	4.8 (2.0-9.5) [y]	7.0 (3.5-9.0) [x, y]

a, $p = 0.014$; b, $p < 0.001$; c, $p = 0.013$; d, $p < 0.001$; e, $p = 0.004$; f, $p = 0.025$; g, $p = 0.049$; h, $p < 0.001$; i, $p = 0.002$; j, $p = 0.035$; k, $p = 0.005$; l, $p = 0.005$; m, $p = 0.013$; n, $p = 0.013$; o, $p = 0.041$; p, $p = 0.001$; q, $p = 0.002$; r, $p < 0.001$; s, $p = 0.003$; t, $p = 0.007$; u, $p = 0.012$; v, $p = 0.012$; w, $p = 0.003$; x, $p = 0.021$; y, $p = 0.048$; Data are expressed medain (range)

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