

SYBR Green dye using the LightCycler 480 SW1.5 apparatus (Roche Molecular Biochemicals). HPV16 primers were as follows. (i) HPV16E6E7 forward primer: 5'-ATC ATC AAG AAC ACG TAG AG-3'; reverse primer: 5'-GAT CAG TTG TCT CTG GTT GCA AAT-3'. (ii) HPV16E7 forward primer: 5'-CAGCTCAGAGGAGGAGGATG-3'; reverse primer: 5'-ATTGTAATGGGCTCTGTCCG-3'. (iii) HPV16E6 forward primer: 5'-ACTGCAATGTTTCAGGACCC-3'; reverse primer: 5'-GCATAAATCCCGAAAAGCAA-3'. The qPCR was set up in a reaction volume of 20 µl. Each qPCR contained, in 1 µl of total plasma DNA, 2 µl of 5 µM each forward and reverse primers, 10 µl of 2 × Quantitect SYBR Green PCR Master Mix (Qiagen). For amplification of HPV16E6E7, the qPCR setting was initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing and extension at 60°C for 1 min. For amplification of HPV16E6 and HPV16E7, the qPCR setting was initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 1 min and extension at 72°C for 1 min.

DETERMINATION OF VIRAL LOAD

To prepare plasmids containing HPV16E6 and/or HPV16E7 sequences for standard curves, we used the HPV16-positive cervical cancer cell line (Caski cell). Total cellular DNA was isolated by using Genomic DNA purification kit (Promega, Madison, WI, USA) according to the instructions provided by the manufacturer. HPV16E6 and/or HPV16E7 were re-cloned in PCR2.1-TOPO for standard curves. From the known molecular weights of the recombinant plasmids, the amount of plasmid DNA equivalent to 5 × 10¹⁰ copies of HPV16E6 and/or HPV16E7 was first determined. Standard curves were generated automatically by plotting the threshold cycle values against the logarithm of the copy numbers of plasmid DNA standards (serial 10-fold dilution from 5 × 10¹⁰ to 0.5 copies of HPV16E6 and/or HPV16E7). The copy numbers of each sample were calculated from the standard curve. The concentration of plasma HPV DNA was expressed as copies of HPV genome per milliliter of plasma. The results are expressed as mean ± SD (n = 3).

STATISTICAL ANALYSIS

The Mann–Whitney test was used to compare the HPV DNA copy numbers among cancers of different stages. Probability values <0.05 were regarded as statistically significant.

RESULTS

Quantitative PCR was highly sensitive in detection of HPV16 DNA with as little as 50 copies/µl. Plasma HPV16E6E7 DNA was detected in 6 of 20 (30.0%) patients with HPV16-positive invasive cervical cancer, but in none

Table 2. Incidence of plasma HPV16E6E7 DNA according to the stage of cervical cancers and detection levels of plasma HPV16E6E7 DNA in cervical cancer patients and precancerous disease

	Cases	Positive	Plasma HPV16E6E7 DNA (copies/ml of plasma)		
			Frequency (%)	Maximum	Median
Normal	20	0	0	0	0
NILM	3	0	0	0	0
L-SIL	1	0	0	0	0
H-SIL	20	0	0	0	0
Cancer (FIGO stage)					
Ia1	4	1	25	507	300
Ia2	1	0	0	0	0
Ib1	2	0	0	0	0
Ib2	2	0	0	0	0
IIa	4	1	25	96 583	96 583
IIb	4	2	50	342 833	186 624
IIIb	1	0	0	0	0
IVa	1	1	100	229 792	135 381
IVb	1	1	100	17 768	11 935

HPV, human papillomavirus; normal, negative control; NILM, negative for intraepithelial lesion or malignancy; L-SIL, low-grade squamous intraepithelial lesion; H-SIL, high-grade squamous intraepithelial lesion.

of those with CIN3 and normal controls (Table 2). The copy numbers of plasma HPV16E6E7 DNA in patients with invasive cancer were higher than in micro-invasive cancer, CIN3 and normal individuals (Table 2).

Table 3 shows the plasma HPV16E6E7 DNA viral loads, disease stage and serum levels of SCCA. Only 4 out of 13 (30.8%) patients with high serum SCCA levels had detectable levels of plasma HPV16E6E7 DNA before operation. On the other hand, 2 out of 7 (28.6%) patients with normal serum SCCA levels had detectable levels of plasma HPV16E6E7 DNA before operation.

We also checked plasma HPV16 DNA using other primers, including HPV16E7 forward primer and HPV16E7 reverse primer to detect part of E7 (70 bp) as well HPV16E6 forward primer and HPV16E7 reverse primer to detect part of E6 (159 bp), to rule out differences in HPV DNA detection rate in the same patient based on differences in primers' position (Table 3). HPV16E6 and/or HPV16E7 DNA were detected in the plasma of two to three out of six patients with cervical cancer who were positive for HPV16 DNA by the HPV16E6E7 primer.

DISCUSSION

The major finding of the present study was the detection of HPV16 DNA in 60% of primary cervical cancers and in plasma samples of 30.0% of the patients with HPV16 DNA-positive primary tumors.

Table 3. Pre-treatment plasma HPV16 DNA levels and SCCA levels in HPV16-positive patients with cervical cancer

Patient	Diagnosis	SCCA	Copies/ml of plasma		
			HPV16E6E7	HPV16E7	HPV16E6
CC1	Ia1	2.1	0	0	0
CC2	Ia1	0.6	0	0	0
CC3	Ia1	0.9	0	0	0
CC4	Ia1	1.3	300	0	0
CC5	Ia2	1.7	0	0	0
CC6	Ib1	1.1	0	0	0
CC7	Ib1	1.3	0	0	0
CC8	Ib2	14.3	0	0	0
CC9	Ib2	9.9	0	0	0
CC10	Ila	3.0	96 583	334 000	0
CC11	Ila	2.6	0	0	0
CC12	Ila	2.7	0	0	0
CC13	Ila	1.7	0	0	0
CC14	Iib	12.9	0	0	0
CC15	Iib	9.3	342 833	129 000	1 190 000
CC16	Iib	18.2	0	0	0
CC17	Iib	1.5	30 417	406 000	556 667
CC18	IIIb	1.4	0	0	0
CC19	Iva	35.9	135 381	0	0
CC20	IVb	12.2	11 935	0	0

SCCA, squamous cell carcinoma antigen.

The detection rate of HPV DNA in plasma of patients with uterine cervical cancer remains controversial. Pornthanakasem et al. (11) detected HPV DNA in the plasma of 12% of their HPV-positive patients with cervical cancer, whereas the same rate was 70% in another study (12). Furthermore, Yang et al. (13) reported detection of HPV16 DNA in plasma samples of 50% of their patients with cervical cancer. In this context, Capone et al. (15) reported that they could not detect HPV16 DNA in the plasma of 65 patients with nasopharyngeal SCC using L1 primer but detected the same DNA in the plasma of 2 of their 65 patients by real-time PCR using E7 primer. These differences in the detection rate may be due to differences in sample numbers and method of analysis.

In the present study, plasma HPV16E6E7 DNA was detected in 6 of 20 (30.0%) HPV16-positive patients with invasive cervical cancer but in none of the normal controls. The copy number of plasma HPV16 DNA in patients with invasive cancer was higher than in CIN3 and micro-invasive cancer and in normal individuals. On the other hand, the copy number of plasma HPV16 E6E7 DNA in cervical cancer stage IVa was higher than that of cervical cancer stage IVb. More sample collection and further studies are required to determine the relationship between the detection

level of HPV DNA in plasma and clinical stage of uterine cervical cancer.

The source of plasma HPV DNA remains unclear. However, the DNA level is probably related to tumor size, stage and presence or absence of metastasis. In this regard, Pornthanakasem et al. (11) reported that the plasma level of HPV DNA in metastatic patients was three times higher than that of patients without metastasis. Their results suggested that the amount of plasma HPV DNA could be a useful tumor marker for prediction of disease progression and clinical outcome after treatment of patients with cervical cancer.

In our study, only 30.8% of the patients with high levels of serum SCCA were plasma HPV16 DNA-positive. And detection rate of plasma HPV16 DNA was different based on differences in primers' position (Table 3). In this context, the form of HPV DNA in the plasma of cervical cancer patients is not clear. It is possible that various HPV DNA fragments are present in the peripheral circulation since the HPV DNA detection rates in the plasma varied with the use of different primers in the same patients with cervical cancer.

What is the source of HPV DNA in the peripheral circulation? While the exact source is not clear at this stage, circulating HPV DNA levels could represent lysis of circulating cancer cells or micrometastasis shed from the tumor (16,17). Others have suggested that circulating tumor DNA in plasma might reflect tumor cell metastasis because of the high *in vitro* transforming activity (18,19).

The results of the present study indicated that HPV16E6E7 DNA in plasma might not be a sensitive marker of cervical cancer recurrence because plasma HPV16E6E7 DNA before operation was detected in only 4 out of 13 (30.8%) patients with high serum SCCA levels. However, HPV DNA could be potentially used as a marker of low-invasive cervical cancer tumors in patients with normal SCCA levels before treatment because two out of seven (28.6%) patients with normal serum SCCA levels had detectable levels of plasma HPV16E6E7 DNA before operation. We recommend the use of several HPV DNA primers to detect various HPV DNA fragments in the peripheral circulation in patients with cervical cancer.

Funding

Grant-in-Aid for Young Scientists (B) (No. 20791151) from the Ministry of Education, Sports, Culture, Science and Technology of Japan.

Conflict of interest statement

None declared.

References

1. Kato H, Torigoe T. Radioimmunoassay for tumor antigen of human cervical squamous cell carcinoma. *Cancer* 1977;40:1621–8.

2. Murakami A, Suminami Y, Sakaguchi Y, Nawata S, Numa F, Kishi F, et al. Specific detection and quantitation of SCC antigen 1 and SCC antigen 2 mRNAs by fluorescence-based asymmetric semi-nested reverse transcription PCR. *Tumour Biol* 2000;21:224–34.
3. Mitsuishi K, Nakamura T, Sakata Y, Yuyama N, Arima K, Sugita Y, et al. The squamous cell carcinoma antigens as relevant biomarkers of atopic dermatitis. *Clin Exp Allergy* 2005;35:1327–33.
4. Gutman LT, St Claire K, Herman-Giddens ME, Johnston WW, Phelps WC. Evaluation of sexually abused and non-abused young girls for intravaginal human papillomavirus infection. *Am J Dis Child*. 1992;146:694–9.
5. Stevens-Simon C, Nelligan D, Breese P, Jenny C, Douglas JM Jr. The prevalence of genital human papillomavirus infections in abused and nonabused preadolescent girls. *Pediatrics* 2000;106:645–9.
6. Kari S, Stima S. Infections in Human Pathology. Wiley, 117–26.
7. Franco EL, Villa LL, Sobrinho JP, Prado JM, Rousseau MC, Desy M, et al. Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer. *J Infect Dis* 1999;180:1415–23.
8. Kjaer SK, van den Brule AJ, Paull G, Svare EI, Sherman ME, Thomsen BL, et al. Type specific persistence of high risk human papillomavirus (HPV) as indicator of high grade cervical squamous intraepithelial lesions in young women: population based prospective follow up study. *Br Med J* 2002;325:572.
9. Schlecht NF, Kulaga S, Robitaille J, Ferreira S, Santos M, Miyamura RA, et al. Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia. *JAMA* 2001;286:3106–14.
10. Cason J, Kaye JN, Jewers RJ, Kambo PK, Bible JM, Kell B, et al. Perinatal infection and persistence of human papillomavirus types 16 and 18 in infants. *J Med Virol* 1995;47:209–18.
11. Pornthanakasem W, Shotelersuk K, Termrungleert W, Voravud N, Niruthisard S, Mutirangura A. Human papillomavirus DNA in plasma of patients with cervical cancer. *BMC Cancer* 2001;1:2.
12. Duenas A, Lizano M, Carrillo AL, Wojcik J, Trejo C. DNA of human papillomavirus detected by PCR in plasma of cervical cancer patients: a potential marker of residual disease. *Clin Chem* 2001;47:361–70.
13. Yang HJ, Liu VWS, Tsang PCK, Yip AMW, Tam KF, Wing LC, et al. Quantification of human papillomavirus DNA in the plasma of patients with cervical cancer. *Int J Gynecol Cancer* 2004;14:903–10.
14. Ho CM, Yang SS, Chien TY, Huang SH, Jeng CJ, Chang SF. Detection and quantitation of human papillomavirus type 16, 18 and 52 DNA in the peripheral blood of cervical cancer patients. *Gynecol Oncol* 2005;99:615–21.
15. Capone RB, Pai SI, Koch WM, Gillison ML, Danish HN, Westra WH, et al. Detection and quantitation of human papillomavirus (HPV) DNA in the sera of patients with HPV-associated head and neck squamous cell carcinoma. *Clin Cancer Res* 2000;6:4171–5.
16. Pao CC, Hor JJ, Yang FP, Lin CY, Tseng CJ. Detection of human papillomavirus mRNA and cervical cancer cells in peripheral blood of cervical cancer patients with metastasis. *J Clin Oncol* 1997;15:1008–12.
17. Czegledy J, Iosif C, Hansson BG, Evander M, Gergely L, Wadell G. Can a test for E6/E7 transcripts of human papillomavirus type 16 serve as a diagnostic tool for the detection of micrometastasis in cervical cancer? *Int J Cancer* 1995;64:211–5.
18. Anker P, Lyautey J, Lefort F, Lederrey C, Stroun M. Transformation of NIH/3T3 cells and SW480 cells displaying K-ras mutation. *C R Acad Sci III* 1994;317:869–74.
19. Garcia-Olmo D, Garcia-Olmo DC, Ontanon J, Martinez E, Vallejo M. Tumor DNA circulating in the plasma might play a role in metastasis: the hypothesis of the genomestasis. *Histopathology* 1999;4:1159–64.

A case of Kallmann syndrome carrying a missense mutation in alternatively spliced exon 8A encoding the immunoglobulin-like domain IIIb of fibroblast growth factor receptor 1

Kiyonori Miura^{1,*}, Shoko Miura¹, Koh-ichiro Yoshiura²,
Stephanie Seminara³, Daisuke Hamaguchi¹, Norio Niikawa⁴,
and Hideaki Masuzaki¹

¹Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, Japan
²Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan ³Reproductive Endocrine Unit, Massachusetts General Hospital, Boston, MA 02114, USA ⁴Research Institute of Personalized Health Sciences, Health Sciences University of Hokkaido, Hokkaido, Japan

*Correspondence address. Tel: +81-95-819-7363; Fax: +81-95-819-7365; E-mail: kiyonori@nagasaki-u.ac.jp

Submitted on August 24, 2009; resubmitted on December 17, 2009; accepted on January 4 2010

ABSTRACT: Fibroblast growth factor receptor 1 (*FGFR1*) is one of the causative genes for Kallmann syndrome (KS), which is characterized by isolated hypogonadotropic hypogonadism with anosmia/hyposmia. The third immunoglobulin-like domain (D3) of *FGFR1* has the isoforms *FGFR1-IIIb* and *FGFR1-IIIc*, which are generated by alternative splicing of exons 8A and 8B, respectively. To date, the only mutations to have been identified in D3 of *FGFR1* are in exon 8B. We performed mutation analysis of *FGFR1* in a 23-year-old female patient with KS and found a missense mutation (c.1072C>T) in exon 8A of *FGFR1*. The c.1072C>T mutation was not detected in her family members or in 220 normal Japanese and 100 Caucasian female controls. No mutation in other KS genes, *KS1*, *prokineticin-2*, *prokineticin receptor-2* and *FGF-8* was detected in the affected patient or in her family members. Therefore, this is the first case of KS carrying a *de novo* missense mutation in *FGFR1* exon 8A, suggesting that isoform *FGFR1-IIIb*, as well as isoform *FGFR1-IIIc*, plays a crucial role in the pathogenesis of KS.

Key words: Kallmann syndrome / *FGFR1b* mutation / fibroblast growth factor receptor 1 isoform expression

Introduction

Kallmann syndrome (KS), which is characterized by isolated hypogonadotropic hypogonadism (IHH) and anosmia/hyposmia, is a clinically and genetically heterogeneous disorder. To date, five causative genes for KS have been reported: *KS1* (*KALI*, GenBank accession M97 252), *prokineticin-2* (*PROK2*, GenBank accession NM 021935), *prokineticin receptor-2* (*PROKR2*, GenBank accession NM 144773), *fibroblast growth factor-8* (*FGF-8*, GenBank accession NM 033163) and *fibroblast growth factor receptor 1* (*FGFR1*, GenBank accession NM 023110.2).

Although sporadic cases of KS are more frequent, families with KS have been reported with X-linked recessive or autosomal dominant or recessive modes of inheritance. Mutations in *KALI* have been found in familial cases with X-linked recessive inheritance (Franco *et al.*, 1991; Legouis *et al.*, 1991). Mutations in *PROK2* were detected in the

heterozygous state, whereas *PROKR2* mutations were found in the heterozygous, homozygous or compound heterozygous state (Dodé *et al.*, 2006). *PROKR2/PROK2* mutations with true pathogenic potential were found only in the homozygous state (Abreu *et al.*, 2008), and any dominant-negative effect of *PROKR2* mutations was ruled out (Monnier *et al.*, 2009). Mutations in *FGFR1* or *FGF8* underlie an autosomal dominant form with incomplete penetrance. Therefore, KS families harbouring heterozygous *FGFR1* or *FGF8* mutations display variable olfactory phenotypes (Dodé *et al.*, 2003; Falardeau *et al.*, 2008), and a few cases with heterozygous *FGFR1* mutations show a normosmic IHH (Pitteloud *et al.*, 2006a). The *FGFR1* gene, which is located on chromosome 8p12, comprises 18 exons (Ruta *et al.*, 1989), and various mutations, including missense and protein truncation mutations, have been reported (Trarbach *et al.*, 2007). The third immunoglobulin-like domain (D3) of *FGFR1* has the isoforms *FGFR1-IIIb* and *FGFR1-IIIc*, which are generated by alternative splicing

of exons 8A and 8B, respectively (Johnson *et al.*, 1991). To date, mutations in D3 of *FGFR1* have only been identified in exon 8B, which encodes immunoglobulin domain IIIc, suggesting that isoform *FGFR1-IIIc* plays a crucial role in the pathogenesis of KS (Pitteloud *et al.*, 2006b; Trarbach *et al.*, 2006; Dodé *et al.*, 2007).

Here, we report for the first time a KS case carrying a *de novo* mis-sense mutation in the alternatively spliced exon 8A of *FGFR1-IIIb*.

Materials and Methods

Patient and family

Patient (Subject II-2) was a 23-year-old Japanese woman. When she was 18 years old, she was treated at Nagasaki University Hospital because of primary amenorrhea with anosmia. Her height was 159.2 cm and her weight was 72.0 kg. Her serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and estradiol (E2) were less than 0.5 and 1.5 mIU/ml and 10 pg/ml, respectively. Her LH frequent sampling study (sampling performed every 15 min) showed a low-amplitude pattern of LH pulsation (Fig. 1). Her brain magnetic resonance imaging (MRI) examination was negative for tumors and showed no anatomical abnormalities of the hypothalamic–pituitary region and olfactory bulbs. A scratch-and-sniff test (UPSIT, Sensonics, Haddon Hts, NJ, USA) (Doty *et al.*, 1985), which determines ability to smell, indicated anosmia. She was diagnosed as having KS and received hormone replacement therapy for 5 years. Her mother (Subject I-1) was normosmic and had normal puberty and regular menstrual cycles. Her father (Subject I-2), elder brother (Subject II-1) and younger brother (Subject II-3) were also normosmic and had normal puberty (Fig. 2).

Molecular analysis

DNA extraction

Whole blood samples were obtained from the KS patient and from her mother, father, elder and younger brothers. All samples were collected after obtaining written informed consent and the study protocol was approved by the Institutional Review Board of Nagasaki University. Genomic DNA from lymphocytes was extracted using a QIAamp DNA

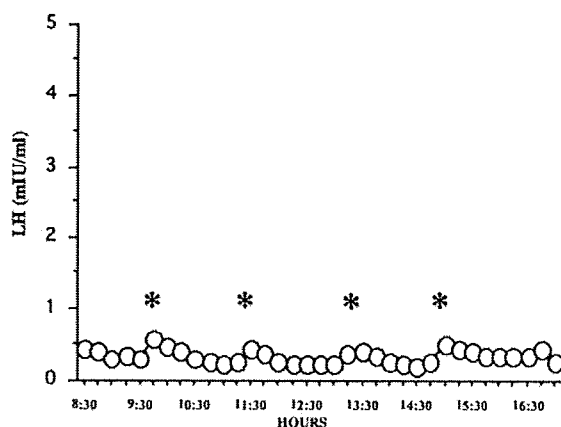


Figure 1 LH pulsation pattern in a case of KS, assayed using an LH frequent sampling study. LH frequent sampling was performed every 15 min. *Low-amplitude pattern of LH pulse.

blood mini kit (Qiagen, Düsseldorf, Germany), according to the manufacturer's instructions.

Sequence analysis

FGFR1 consists of 18 coding exons. Intragenic mutations were investigated by PCR amplification and sequence analysis using 14 pairs of primers, as previously described (Dodé *et al.*, 2003; Sato *et al.*, 2004). Genomic DNA was PCR amplified using conditions of 95°C for 12 min followed by 95°C for 30 s, 59°C for 30 s and 72°C for 60 s for 35 cycles and a final cycle of 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis, purified with ExoSAP-IT and subjected to sequencing reactions. Sequencing reactions were performed using the BigDye terminator v.3.1 kit and analyzed with an ABI PRISM 3100 Genetic Analyzer™ (Applied Biosystems). The KS patient carrying a mutation in *FGFR1* and her family members were also screened for mutations in the other genes known to be involved in KS [*KALI*, *PROK2*, *PROKR2* and *FGF8*]. Whether the mutation leads to a change in the protein structure and function was predicted bioinformatically using the ExPASy proteomics server (<http://au.exPASy.org/>) and PolyPhen (<http://genetics.bwh.harvard.edu/pph/>).

Confirmation of the alternatively spliced exon

Isolation of a full-length murine *Fgfr1-IIIb* showed that *Fgfr1-IIIb* was a transmembrane receptor (Beer *et al.*, 2000). Although the mRNA encoding exon IIIb has been found in human (Johnson *et al.*, 1991), the presence of sequences encoding the intracellular domain has not yet been demonstrated. Therefore, to determine the splice site of exon 8A and to detect *FGFR1-IIIb* mRNA encoding the intracellular domain, we performed RT-PCR using specific primers to amplify the splice isoform containing exon 8A. Kal23 is designed to span exons 7 and 8A for specific annealing to the *FGFR1-IIIb* isoform, which is spliced from exon 7 to exon 8A (Fig. 3A). Kal5 is designed within exon 8B for specific annealing to the *FGFR1-IIIc* isoform, which is spliced from exon 7 to exon 8B. Kal2 and Kal6 are designed within exons 7 and 9, respectively, for annealing to the D3 isoforms of *FGFR1*. Primer sequences were as follows: kal2: 5'-GACAGAAGGTCGGTTATGTC-3', Kal23: 5'-CAGATCTTGAAGC ATTCGGG-3', Kal5: 5'-GGTGGTATTAACCTCCAGCAG-3' and Kal6: 5'-GTACAGGGGCGAGGTCATCA-3'. The BD multiple tissue complementary DNA (cDNA, MTC) panels Human I and Human II (BD Biosciences Clontech, Mountain View, CA, USA) were used to detect the expression of each isoform of *FGFR1*. PCR amplification was performed on cDNAs as follows: 94°C, 30 s; 62°C, 30 s; 72°C, 1 min; 40 cycles. PCR products were analyzed by agarose gel electrophoresis and sequenced using then ABI PRISM 3100 Genetic Analyzer™.

Results

Sequence analysis of the entire coding region of *FGFR1*, including exon–intron boundary regions, showed that the KS patient had a mutation (c.1072C>T) in exon 8A of *FGFR1-IIIb*, while the other family members did not (Fig. 2). However, the full-length *FGFR1* mRNA that includes exon 8A is not deposited in the full-length cDNA database (GenBank accession no. NM 023110.2). RT-PCR analysis indicated that most transcripts containing exon 8A were spliced to exon 8B in all adult tissues except bone marrow (data not shown). We wished to demonstrate the existence of an alternative transcript, exon 8A which was spliced to exon 9 encoding the transmembrane helix; therefore, RT-PCR products from human fetal brain were cloned and sequenced. In 1 of 27 clones exon 8A was spliced to exon 9 (designated here '*FGFR1-IIIb*', GenBank accession FJ809917, see Fig. 3B), while in the other clones exon 8A was spliced to exon 8B (designated here

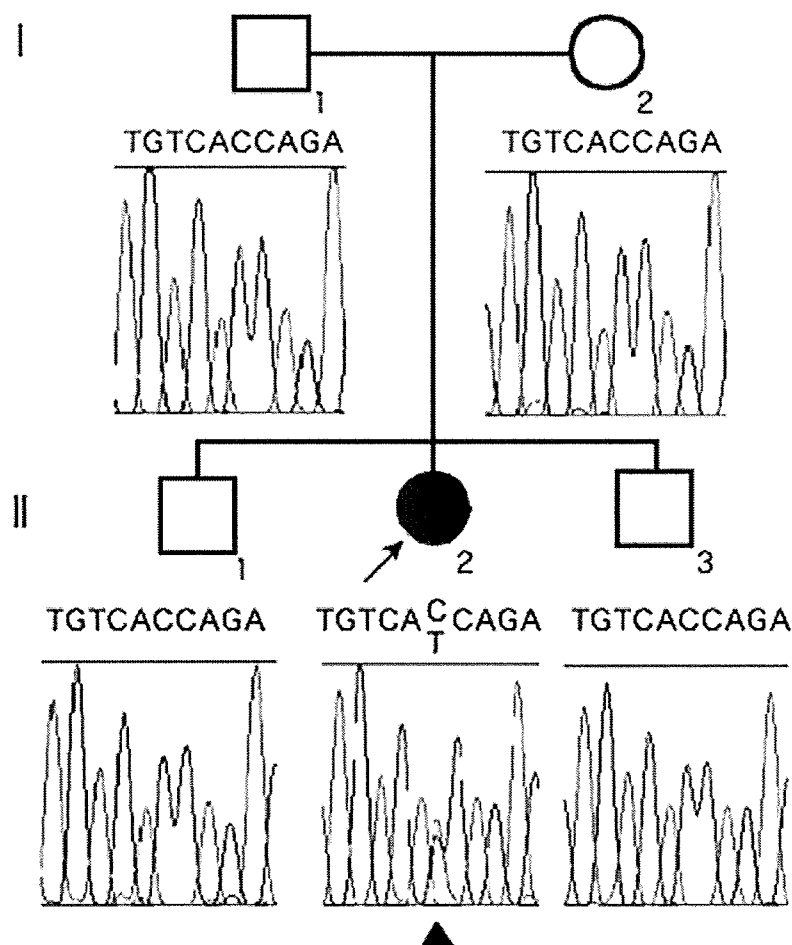


Figure 2 Pedigree of patient's family and the results of fibroblast growth factor receptor I (*FGFR1*) sequence analysis. II-2 is a 23-year-old woman with KS. The patient had a mutation in *FGFR1* (C>T) but the other family members did not. The mutation was *de novo* because parentage was assured. Arrowhead under the electropherogram indicates the mutation site.

'*FGFR1-sec*', GenBank accession FJ809916, see Fig. 3B). The exact acceptor and donor sites of exon 8A in '*FGFR1-IIIb*' mRNA, which produces a membrane-bound *FGFR1*-containing D3, were determined by sequence analysis of splice isoforms, '*FGFR1-IIIb*' and '*FGFR1-sec*', (Fig. 3). As most full-length *FGFR1* cDNAs in the database were transcripts containing exon 7–exon 8B–exon 9 (designated here '*FGFR1-IIIc*', GenBank accession NM 023110.2, see Fig. 2B) without exon 8A, '*FGFR1-IIIc*' is likely to be the most abundantly expressed human isoform. The EST, CA488712.1, was the only isoform in the EST database corresponding to '*FGFR1-IIIb*'. Although both '*FGFR1-IIIb*' and '*FGFR1-IIIc*' encode membrane-bound *FGFR1*, '*FGFR1-sec*' encodes a secreted form of *FGFR1* because of a sequence frameshift and a termination codon in exon 9.

The exons 8A and 8B of the human *FGFR1* isoforms shared the amino acid sequence at 354–357; WLTV. However, exon 8A ends with six extra amino acids at 358–363, TRPVAK, whereas exon 8B ends with only two, LE. These sequences are identical in the mouse *Fgfr1* isoforms (Beer et al., 2000). The mutation in exon 8A of

FGFR1-IIIb (GenBank accession no. FJ809917 bankit1193625) is c.1072C>T at the cDNA level and p.T358I at the amino acid level. Bioinformatic analysis shows the mutated amino acid residue to be conserved between human and mouse and to be located in D3 of *FGFR1-IIIb*, which is a critical region for FGF ligand binding. However, the mutation was not predicted to produce a change in the human protein structure. The c.1072C>T mutation was not detected in 220 normal Japanese women or in 100 normal Caucasian women. The patient had no mutation in any of the other four KS genes.

Discussion

Mouse *Fgfr1-IIIb* has a low level of expression in a wide variety of adult tissues, but a high level of expression in skin and brain, indicating the existence of specific splicing factors in skin and brain that recognize the relatively weak *Fgfr1-IIIb* splice site (Beer et al., 2000). Consistent with the expression pattern of mouse *Fgfr1-IIIb*, we could isolate human *FGFR1-IIIb* from a fetal brain cDNA library but not from adult

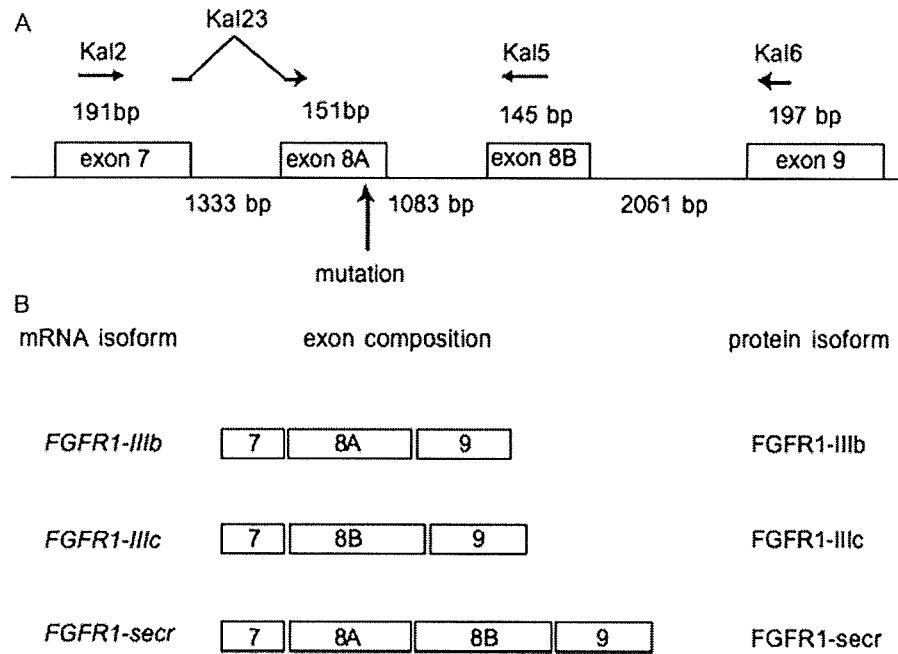


Figure 3 Genomic organization of *FGFR1* around exon 8A. (A) The numbers with bp indicate exon length (over the line) and intron length (under the line). The mutation is located near the end of exon 8A. Horizontal arrows indicate the locations of primers used to perform RT-PCR amplification of the isoform containing exons 7 and 8A. Primer Kal3 is designed to span exons 7 and 8A for specific annealing to the *FGFR1-IIIb* isoform, which is spliced from exon 7 to exon 8A (Fig. 3A). Kal5 is designed within exon 8B for specific annealing to the *FGFR1-IIIc* isoform, which is spliced from exon 7 to exon 8B. Kal2 and Kal6 are designed within exons 7 and 9, respectively, for annealing to the D3 isoforms of *FGFR1*. Vertical arrow indicates mutation site. (B) Composition of mRNA isoforms and of putative protein structures. *FGFR1-IIIb*: membrane-bound form of *FGFR1* with immunoglobulin-like domain IIIb encoded by exon 8A, *FGFR1-IIIc*: membrane-bound form of *FGFR1* with immunoglobulin-like domain IIIc encoded by exon 8B, *FGFR1-secr*: a secreted form of *FGFR1*.

tissues. Most full-length *FGFR1* cDNAs in the database represent *FGFR1-IIIc*. *FGFR1-IIIc* is expressed at high levels, but *FGFR1-IIIb* is expressed at very low levels (Johnson *et al.*, 1991). We can amplify only a tiny amount of *FGFR1-IIIb* that has exon 8A spliced to exon 9 by RT-PCR using the primers Kal23 and Kal6. Most of the sequenced RT-PCR products corresponded to *FGFR1-secr*, suggesting that the expression level of the three isoforms is '*FGFR1-IIIc*' >> '*FGFR1-IIIsecr*' >> '*FGFR1-IIIb*' (Fig. 3B).

Several studies suggested that mutations in exon 8B of isoform *FGFR1-IIIc* are implicated in the pathogenesis of KS (Pitteloud *et al.*, 2006b; Trarbach *et al.*, 2006; Dodé *et al.*, 2007). Mice homozygous for alleles with a stop codon in exon IIIc displayed phenotypes resembling those of embryos homozygous for null alleles, while mice carrying an in-frame stop codon in exon IIIb were viable and fertile (Partanen *et al.*, 1998). Therefore, *Fgfr1-IIIc* is the dominant isoform that carries out the majority of the biological functions of the *Fgfr1* gene, whereas *Fgfr1-IIIb* plays a minor and to some extent redundant role (Partanen *et al.*, 1998). A receptor-binding analysis revealed no difference in the binding specificity between the endogenous *Fgfr1-IIIb* and an artificially created *Fgfr1-IIIb*, which had two different amino acids in the 3'-end of the unique IIIb exon (Beer *et al.*, 2000), suggesting that the carboxyl terminus of D3 may not overtly influence binding specificity. However, being expressed at low levels does not imply that

'*FGFR1-IIIb*' has an unimportant role. The mutation we found, p.T358I, was located in exon 8A of *FGFR1-IIIb*. Therefore, p.T358I may affect ligand binding and cause the KS phenotype, although how this mutation affects the loss of function of *FGFR1-IIIb* is unknown. The spatio-temporal expression of any gene involved in development is key; therefore, the expression and functional involvement of *FGFR1-IIIb* may be important in the early embryonic brain, in particular during GnRH neuronal development. KS missense mutations in *FGFR1* are distributed in the first, second and third immunoglobulin-like domains (D1-D3), in the tyrosine kinase domain and also in the intracellular domain (Dodé *et al.*, 2003; Sato *et al.*, 2004; Albuisson *et al.*, 2005; Pitteloud *et al.*, 2006a; Trarbach *et al.*, 2006; Dodé *et al.*, 2007); therefore, the membrane-bound form of *FGFR1* is probably important for the KS phenotype. The membrane-bound form of *FGFR1-IIIb* could, therefore, be the critical isoform for the KS phenotype.

We present here, for the first time, a case of KS carrying a missense mutation in exon 8A of *FGFR1*, suggesting that the minor isoform '*FGFR1-IIIb*' as well as the major isoform '*FGFR1-IIIc*' has a crucial role in the pathogenesis of KS. Therefore, immunoglobulin-like domain IIIb may have an essential role in GnRH neuronal migration, which is initiated from the nasal placode and runs towards the forebrain following the olfactory sensory neuron axonal connection with the developing olfactory bulb. Further experiments are needed to show that the mutation in

exon 8A causes KS, such as expression of the mutated isoform in transfected cells to analyze receptor stability and signaling efficiency. Although *FGFR1* containing immunoglobulin-like domain IIIb has not been analyzed intensively, our mutation report should encourage researchers to analyze immunoglobulin-like domain IIIb function and the spatio-temporal expression of exon 8A in fetal brain development.

Acknowledgements

We thank Ms Yasuko Noguchi and Miho Ooga for their technical assistance and we thank Drs Nelly Pitteloud and William Crowley for their valuable contribution.

Funding

K.M. was supported, in part, by Seeds (No.15-B09) from the Japan Science and Technology Agency (JST), by grants from the Naito Foundation, by Grant-in-Aid for Young Scientists (B) (no. 21791567) from the Ministry of Education, Sports, Culture, Science and Technology of Japan, by a Grant for Child Health and Development (20C-1) from the Ministry of Health, Labor and Welfare, and by Grant-in-Aid for Scientific Research from Nagasaki University, Japan.

References

- Abreu AP, Trarbach EB, de Castro M, Frade Costa EM, Versiani B, Matias Baptista MT, Garmes HM, Mendonca BB, Latronico AC. Loss-of-function mutations in the genes encoding prokineticin-2 or prokineticin receptor-2 cause autosomal recessive Kallmann syndrome. *J Clin Endocrinol Metab* 2008;**93**:4113–4118.
- Albuisson J, Pêcheux C, Carel JC, Lacombe D, Leheup B, Lapuzina P, Bouchard P, Legius E, Matthijs G, Wasniewska M et al. Kallmann syndrome: 14 novel mutations in *KAL1* and *FGFR1* (*KAL2*). *Hum Mutat* 2005;**25**:98–99.
- Beer HD, Vindevoghel L, Gait MJ, Revest JM, Duan DR, Mason I, Dickson C, Werner S. Fibroblast growth factor (FGF) receptor I-IIIb is a naturally occurring functional receptor for FGFs that is preferentially expressed in the skin and the brain. *J Biol Chem* 2000;**275**:16091–16097.
- Dodé C, Levilliers J, Dupont JM, De Paepe A, Le Dû N, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F et al. Loss-of-function mutation in *FGFR1* cause autosomal dominant Kallmann syndrome. *Nature Genet* 2003;**33**:463–465.
- Dodé C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, Lespinasse J, Lienhardt-Roussie A, Mathieu M, Moerman A et al. Kallmann syndrome: mutations in the genes encoding prokineticin-2 prokineticin receptor-2. *PLoS Genet* 2006;**2**:e175.
- Dodé C, Fouveaut C, Mortier G, Janssens S, Bertherat J, Mahoudeau J, Kottler ML, Chabrolle C, Gancel A, François I et al. Novel *FGFR1* sequence variants in Kallmann syndrome, and genetic evidence that the *FGFR1c* isoform is required in olfactory bulb and palate morphogenesis. *Hum Mutat* 2007;**28**:97–98.
- Doty RL, Applebaum S, Zusho H, Settle RG. Sex differences in odor identification ability: a cross-cultural analysis. *Neuropsychologia* 1985;**23**:667–672.
- Falardeau J, Chung WC, Beenken A, Raivio T, Plummer L, Sidis Y, Jacobson-Dickman EE, Eliseenkova AV, Ma J, Dwyer A et al. Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. *J Clin Invest* 2008;**118**:2822–2831.
- Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, Carozzo R, Maestrini E, Pieretti M, Taillon-Miller P et al. A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. *Nature* 1991;**353**:529–536.
- Johnson DE, Lu J, Chen H, Werner A, Williams LT. The human fibroblast growth factor receptor genes: a common structure arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. *Mol Cell Biol* 1991;**11**:4627–4634.
- Legouis R, Hardelin JP, Levilliers J, Claverie JM, Compain S, Wunderle V, Millasseau P, Le Paslier D, Cohen D, Caterina D et al. The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* 1991;**67**:423–435.
- Monnier C, Dodé C, Fabre L, Teixeira L, Labesse G, Pin JP, Hardelin JP, Rondard P. PROKR2 missense mutations associated with Kallmann syndrome impair receptor signalling activity. *Hum Mol Genet* 2009;**18**:75–81.
- Partanen J, Schwartz L, Rossant J. Opposite phenotypes of hypomorphic and Y766 phosphorylation site mutations reveal a function for *Fgfr1* in anteroposterior patterning of mouse embryos. *Genes Dev* 1998;**12**:2332–2344.
- Pitteloud N, Acierno JS Jr, Meysing A, Eliseenkova AV, Ma J, Ibrahim OA, Metzger DL, Hayes FJ, Dwyer AA, Hughes VA et al. Mutations in fibroblast growth factor receptor I cause both Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci USA* 2006a;**103**:6281–6286.
- Pitteloud N, Meysing A, Quinton R, Acierno JS Jr, Dwyer AA, Plummer L, Fliers E, Boepple P, Hayes F, Seminara S et al. Mutations in fibroblast growth factor receptor I cause Kallmann syndrome with a wide spectrum of reproductive phenotypes. *Mol Cell Endocrinol* 2006b;**254–255**:60–69.
- Ruta M, Burgess W, Givol D, Epstein J, Neiger N, Kaplow J, Crumley G, Dionne C, Jaye M, Schlessinger J. Receptor for acidic fibroblast growth factor is related to the tyrosine kinase encoded by the *fms*-like gene (*FLG*). *Proc Natl Acad Sci USA* 1989;**86**:8722–8726.
- Sato N, Katsumata N, Kagami M, Hasegawa T, Hori N, Kawakita S, Minowada S, Shimotsuka A, Shishiba Y, Yokozawa M et al. Clinical assessment and mutation analysis of Kallmann syndrome I (*KAL1*) and fibroblast growth factor receptor I (*FGFR1*, or *KAL2*) in five families and 18 sporadic patients. *J Clin Endocrinol Metab* 2004;**89**:1079–1088.
- Trarbach EB, Costa EM, Versiani B, de Castro M, Baptista MT, Garmes HM, de Mendonca BB, Latronico AC. Novel fibroblast growth factor receptor I mutations in patients with congenital hypogonadotropic hypogonadism with and without anosmia. *J Clin Endocrinol Metab* 2006;**91**:4006–4012.
- Trarbach EB, Silveira LG, Latronico AC. Genetic insights into human isolated gonadotropin deficiency. *Pituitary* 2007;**10**:381–391.

胎児死亡例からみた常位胎盤早期剥離の検討

Vaginal delivery and cesarean section: comparison of
maternal outcome in placental abruption with fetal death.

谷川輝美
Terumi TANIGAWA

三浦清徳
Kiyonori MIURA

吉田 敦
Atushi YOSHIDA

中山大介
Daisuke NAKAYAMA

増崎英明
Hideaki MASUZAKI

日本産婦人科・新生児血液学会誌

Vol.18, No.2, 2009

胎児死亡例からみた常位胎盤早期剥離の検討

Vaginal delivery and cesarean section: comparison of maternal outcome in placental abruption with fetal death.

谷川輝美
Terumi TANIGAWA

三浦清徳
Kiyonori MIURA

吉田 敦
Atushi YOSHIDA

中山大介
Daisuke NAKAYAMA

増崎英明
Hideaki MASUZAKI

要 旨

目的：胎児死亡を伴う常位胎盤早期剥離（早剥）において、分娩様式の違いによって母体予後に差があるか否か検討した。

方法：当院で胎児死亡を伴う早剥と診断された19例を対象とした。ただちに帝王切開術を行った5例（帝切群）、最初に経膣分娩を試みたが後に帝王切開術に変更した4例（変更群）、および経膣分娩を完遂した10例（経膣群）、以上3群について母体予後を検討した。

結果：母体年齢および分娩週数、妊娠合併症、入院時の頸管開大度、血液検査所見、分娩時出血量は3群間に差がなかった。診断から分娩までの時間は経膣群で帝切群と比較して分娩までの時間が長かった。輸血を要した例は、帝切群+変更群で全例、経膣群で5/10例であった。

考察：今回の検討から、胎児死亡を伴う早剥例に対し、帝王切開術と経膣分娩いずれがよいかを判断することはできなかった。しかし、多くの例で安全に経膣分娩を完遂することが可能であった。

ABSTRACT

Objective: This study investigated whether the mode of delivery was associated with maternal prognosis in placental abruption with fetal death.

Method: Between January 1994 and March 2008, there were 19 cases of placental abruption with fetal death. We investigated the relationship between the mode of delivery and maternal prognosis. Five of 19 cases were delivered by cesarean section (group 1), 4 were delivered by cesarean section after attempting vaginal delivery (group 2), 10 had a vaginal delivery (group 3).

Result: There was no significant difference between the 3 groups for maternal age, gestational age at delivery, complications during pregnancy, cervical dilatation on admission and blood test results. Group 3 showed a longer time until delivery compared with that in group 1. There was no significant difference between the amounts of intrapartum hemorrhage, but the amount in group 3 was slightly less. All patients in group 1 and group 2, and half of the patients in group 3 received a blood transfusion. None of the patients in group 1 and one case each in group 2 and group 3 developed disseminated intravascular coagulation (DIC) before delivery. The period of hospitalization was 12 ± 8 days in group 1, 14 ± 7 days in group 2, and 7 ± 2 days in group 3.

Conclusion: We could not demonstrate whether vaginal delivery or cesarean section was better in placental abruption with fetal death in this study. However, this study suggested that patients with vaginal delivery had less intrapartum hemorrhage and lower frequency of blood transfusion rather than patients undergoing cesarean section.

Key words; placental abruption, fetal death, vaginal delivery, cesarean section

長崎大学医学部産婦人科 : Department of Obstetrics and Gynecology, Nagasaki University School of Medicine

著者連絡先 : 〒852-8501 長崎市坂本1丁目7番1号 長崎大学医学部産婦人科 谷川輝美

TEL: 095-819-7363 FAX: 095-819-7365 e-mail: t-terumi@nagasaki-u.ac.jp

緒言

常位胎盤早期剥離（早剥）は、正常位置に付着している胎盤が、妊娠中または分娩経過中の胎児娩出以前に子宮壁より剥離するものと定義されている。¹⁾

早剥は出血性ショックやDICを合併しやすく、胎児死亡や妊産婦死亡の原因となる。日本産科婦人科学会がまとめた周産期統計によると、2001年から2004年の妊産婦死亡28例のうち、前置胎盤ないし胎盤早期剥離が原因であったのは3例（10.7%）であり、2005年の周産期死亡930例のうち、早剥が原因であったのは87例（9.4%）であった。²⁾

胎児が生存している場合は急速遂娩が基本であるが、早剥により胎児が既に死亡している場合、ふたつの異なる管理方針がある。ひとつはDICの進行を防止するため死亡胎児や剥離した胎盤の早期娩出が重要であるとの観点から、すみやかに帝王切開術を行う方針である。もうひとつは分娩を急がず、まず十分な輸液、輸血あるいは凝固因子の補充を行い、全身状態を安定させた後に積極的に経腔分娩を試みる方針である。いずれの方針をとるか症例毎に検討されているが、明確な判断の基準があるわけではない。母体の全身状態を安定させた後の経腔分娩方針と急速遂娩方針を比較して母体予後に差がなく、分娩までの時間が長くなっても母体予後には影響がないという報告から、胎児死亡を伴う早剥の場合、出血が大量で外科的処置が必要と判断された例や凝固障害が悪化し続ける例以外は、人工破膜やオキシトシンを併用した積極的な経腔分娩が推奨されている^{3~5)}。産婦人科診療ガイドラインでは、施設のDIC対応能力や患者の状態により、経腔分娩促進、あるいは緊急帝王切開のいずれかの方針を採用するとされている。⁶⁾

今回、子宮内胎児死亡を伴う早剥例を、ただちに帝王切開術を行った例、経腔分娩を行った例、およびはじめ経腔分娩を試みたが後に帝王切開術に変更した例に分け、母体の転帰を後方視的に比較検討した。

方法

1994年1月から2008年3月（15年間）の当院受診例のうち、分娩前に早剥による子宮内胎児死亡と診断された19例を対象とした。うち16例は他院からの

救急搬送例であった。分娩前の早剥の診断は性器出血や下腹痛などの自覚症状および超音波検査によりなされ、分娩時の胎盤の肉眼所見や病理学的所見により確認した。当科では胎児死亡例は基本的に経腔分娩を選択する方針としているが、最終的な判断は母体の状態などにより症例毎に担当医が決定した。

ただちに帝王切開術を行った例（帝切群）、はじめ経腔分娩を試みたが後に帝王切開術に変更した例（変更群）、および経腔分娩を完遂した例（経腔群）、以上3群について母体年齢、分娩週数、分娩歴、妊娠合併症、入院時の内診所見、分娩所要時間、分娩時出血量と輸血、血液検査所見、血液凝固検査所見、および入院期間について検討した。推計学的検討はスチューデントのt検定あるいはクラスカル・ワリス検定によった。

結果

胎児死亡を伴う早剥19例のうち帝切群は5例（26%）、変更群は4例（21%）、経腔群は10例（53%）であった。

母体年齢（才）は帝切群 32 ± 2.7 （平均±標準偏差、以下同じ）、変更群 32 ± 6.5 、経腔群 33 ± 5.2 および分娩週数（週）帝切群 34 ± 4 、変更群 34 ± 1 、経腔群 34 ± 5 は3群間に差がなかった。経腔群には経産婦が多い傾向があった（帝切群3/5例（60%）、変更群1/4例（25%）、経腔群8/10例（80%））。早剥の危険因子である妊娠高血圧症候群あるいは子宮内胎児発育遅延あるいは喫煙の合併率（%）は、帝切群2/5例（40%）、変更群2/4例（50%）、経腔群5/10例（50%）であり3群間に差がなかった（Table 1）。

入院時の頸管開大度（cm）は帝切群 2 ± 1 、変更群 3 ± 1 、経腔群 2 ± 1 であり、3群間に差がなかった。経腔群の7/10例（70%）および変更群の2/4例（50%）では人工破膜、機械的頸管拡張あるいはオキシトシン点滴静注のいずれか、または併用による陣痛誘発ないし促進を行った。診断から分娩までの時間（分）は帝切群 116 ± 39 、変更群 253 ± 281 、経腔群 342 ± 149 で、経腔群は帝切群と比較して約3倍時間がかかっていた（Table 2）。

入院時のヘモグロビン値（g/dl）は3群間に差がなかった（帝切群 10.1 ± 0.8 、変更群 9.5 ± 2.7 、経腔

群 9.5 ± 1.4)。血小板 ≤ 10 万/ μ lの例は帝切群にはなかった(帝切群 0/5 例、変更群 2/4 例 (50%)、経膣群 3/10例 (30%))。フィブリノーゲン ≤ 150 mg/dlの例の割合(帝切群 2/5 例 (40%)、変更群 1/4 例 (25%)、経膣群 5/10例 (50%))、プロト

ロンビン時間 $\leq 50\%$ の例の割合(帝切群 0/5 例、変更群 0/4 例、経膣群 3/10例 (30%)) および FDP $\geq 10 \mu$ g/mlの例の割合(帝切群 3/5 例 (60%)、変更群 2/4 例 (50%)、経膣群 7/10例 (70%))は3群間で差がなかった (Table 3)。

Table 1. Maternal background

	Group 1 (n=5)	Group 2 (n=4)	Group 3 (n=10)
Maternal age (years)	32 \pm 2.7	32 \pm 6.5	33 \pm 5.2
Gestational age at delivery (weeks)	34 \pm 4	34 \pm 1	34 \pm 5
pluripara	3 (60%)	1 (25%)	8 (80%)
Risk factor	2 (40%)	2 (50%)	5 (50%)
PIH	2	1	1
IUGR	0	1	1
smoking	0	0	2

PIH: Pregnancy induced hypertension, IUGR: Intrauterine growth restriction (mean \pm SD)
Group 1: cesarean section, Group 2: cesarean section after attempting vaginal delivery, Group 3: vaginal delivery

Table 2. Cervical dilatation, induction of labor, time until delivery

	Group 1 (n=5)	Group 2 (n=4)	Group 3 (n=10)
cervical dilatation (cm)	2 \pm 1	3 \pm 1	2 \pm 1
induction of labor	0	2 (50%)	7 (70%)
time until delivery (min)	116 \pm 39	253 \pm 281	342 \pm 149

(mean \pm SD)

Table 3. Blood test results

	Group 1 (n=5)	Group 2 (n=4)	Group 3 (n=10)
Hb (g/dl)	10.1 \pm 0.8	9.5 \pm 2.7	9.5 \pm 1.4
Plt $\leq 100,000/\mu$ l	On admission	0	2 (50%)
	After delivery	3 (60%)	1 (25%)
Fib ≤ 150 mg/dl	2 (40%)	1 (25%)	5 (50%)
PT $\leq 50\%$	0	0	3 (30%)
FDP $\geq 10 \mu$ g/ml	3 (60%)	2 (50%)	7 (70%)

Hb: hemoglobin, Plt: platelet, Fib: fibrinogen, PT: prothrombin time, FDP: fibrin degradation products (mean \pm SD)

Table 4. The amounts of intrapartum hemorrhage, blood transfusion, disseminated intravascular coagulation (DIC) score

	Group 1 (n=5)	Group 2 (n=4)	Group 3 (n=10)
The amounts of intrapartum hemorrhage (ml)	2,089±797	1,581±327	1,682±1537
blood transfusion	5 (100%)	4 (100%)	5 (50%)
blood transfusion after delivery	5	2	1
DIC score≥8 points	0	1 (25%)	1 (10%)

(mean±SD)

分娩時出血量 (ml) は帝切群2,089±797、変更群1,581±327、経膣群1,682±1,537で、3群間で有意な差を認めなかったが、帝切群でやや多い傾向にあった。輸血を要した例は帝切群+変更群では全例 (9/9例)、経膣群では5/10例 (50%) であった。経膣群の4/5例および変更群の2/4例は分娩前、帝切群の5/5例は分娩後に輸血を開始した。分娩前に産科DICスコア (真木ら) が8点以上であったのは、帝切群0/5例、変更群1/4例 (25%)、経膣群1/10例 (10%) であった (Table 4)。

分娩から退院までの入院期間 (日) は帝切群12±8、変更群14±7、経膣群7±2であった。

帝王切開術を行った理由は、帝切群ではDICの進行 (1/5例) および横位 (1/5例)、本人の希望 (1/5例)、不明 (2/5例)、変更群では分娩停止 (3/4例) および急性腎不全 (1/4例) であった。帝切群、変更群、経膣群いずれにおいても、子宮出血のために子宮全摘術を要した例はなかった。

考 察

今回胎児死亡を伴う早剥19例を検討し、最終的に10例が経膣的に、9例が帝王切開術で分娩した。分娩様式は母体の状態により主治医が判断したが、分娩様式を選択する基準は明確ではない。来院時の頸管の開大度やヘモグロビン値、血液凝固系検査結果、DICスコアの値などは経膣群、帝切群あるいは変更群の間で違いはみられなかったが、血小板数が10万以下の例および経産婦では経膣分娩が多く選択されていた。血小板が減少していると、術中の出血コントロールが困難になると判断された可能性がある。また、経産婦では分娩が進行しやすいため、経膣分

娩が選択されたと考えられる。

Williams obstetrics 22版には、死亡した胎児をいつまでに分娩させればよいかというエビデンスはないが、分娩までにかかった時間より、適切な輸液・輸血が行われたかどうか母体予後に影響するという報告が記載されている⁷⁾。このことから、欧米では出血が大量で外科的処置が必要と判断された例や凝固障害が悪化し続ける例以外は、積極的な経膣分娩が推奨されている⁸⁾。

わが国での検討で、野田らは⁸⁾、経膣分娩した15例と帝王切開術を行った7例を比較し、経膣分娩で母体予後が良好であったと報告している。

当然ながら経膣分娩では帝王切開術と比較して分娩時間が長い。したがって子宮内に残存する死亡胎児や剥離した胎盤がDICを増悪させるおそれがある。今回の検討では分娩までにかかった時間は経膣群は帝切群の約3倍であった。しかし分娩時の平均の出血量をみると帝切群と経膣群で差はなく、むしろ分娩後に輸血を要した割合は帝切群の方が高かった。分娩までに要した時間よりも輸液・輸血などの適切な補充療法が行われたか否かが母体の転帰を左右するという報告を支持する結果であった。経膣から帝切に変更された例は4例あったが、うち3例は分娩停止、1例は急性腎不全が変更の理由であり、経膣分娩を完遂した10例と合わせ、経膣分娩の経過中に凝固障害の急激な悪化や大量出血を見た例はなかった。

今回の検討からは、胎児死亡を伴う早剥例に対し、帝王切開・経膣分娩どちらが望ましいかを判断する材料は得られなかった。しかし、分娩中にDICと判定された例を含め、多くの例で安全に経膣分娩を完

遂ることが可能であり、帝王切開術を行った場合と比較して分娩時の平均の出血量は少ない傾向にあり、輸血を要する割合も低いことが示唆された。

文 献

- 1) 産科婦人科用語集・用語解説集 第2版. 日本産科婦人科学会編. 金原出版, 2008.
- 2) 岡村州博. 周産期委員会. 日本産科婦人科学会雑誌 2007; 59: 1151-224.
- 3) Brame RG, Harbertet GM, Gaughey HS, Thornton WN. Maternal risk in abruption. *Obstet Gynecol* 1968; 31: 224-7.
- 4) Pritchard JA, Brekken AL. Clinical and laboratory studies on severe abruption placentae. *Am J Obstet Gynecol* 1967; 97: 681-700.
- 5) Oyelese Y, Ananth CV. Placental Abrup-tion. *Obstet Gynecol* 2006; 108: 1005-16.
- 6) 常位胎盤早期剥離（早剥）の診断・管理は？. 産婦人科診療ガイドライン 産科編2008. 日本産科婦人科学会/日本産婦人科医会 編集・監修. 日本産科婦人科学会, 2008; 91-4.
- 7) Antepartum Hemorrhage, Placental Abrup-tion. In: Williams Obstetrics 22th edition. Cunningham FG, Kenneth JL, Steven LB, John CH, Larry CG III, Katharine DW, eds. 2005; 810-9.
- 8) 野田清史. 胎児死亡例の分娩方針. 臨床婦人科産科 2005; 59: 194-7.

周産期学シンポジウム No.27

[別刷]

日本周産期・新生児医学会

第27回周産期学シンポジウム記録集

平成21年1月16, 17日 (於：郡山)

会長：大戸 齊

メジカルビュー社

Severe preterm IUGRにおける confined placental mosaicismの関与

—特に周産期から出生後12カ月について—

長崎大学産婦人科

三浦 清徳 山崎健太郎 三浦 生子 嶋田 貴子
 藤本 洋子 吉田 敦 中山 大介 増崎 英明

目的

原因不明のsevere preterm IUGR (以下, IUGR) には, しばしばconfined placental mosaicism (CPM) が認められる¹⁾。CPMとは, 胎児は正常核型であるにもかかわらず胎盤にのみ染色体異常を認める病態であり, 直接的には染色体異常に伴う胎盤機能不全, 間接的には胎児のuniparental disomy (UPD) あるいはインプリンティング異常のためIUGRをきたす可能性が指摘されている(図1)^{2,3)}。

私どもは, 原因不明のFGR (IUGR) における周産期事象や出生後の発育・発達とCPMとの関連について検討した。

対象・方法

本研究は倫理委員会の承認を得て, 説明と同意のもとに行われた。

1 対象

1996年から2007年に当科で分娩した3,257例のうち, 多胎妊娠, 染色体異常, 感染, 妊娠高血圧症候群, 糖尿病, 喫煙, アルコール, 臍帯因子を有する例はIUGRのリスク因子として除外した。妊娠週数は妊娠初期の超音波検査で確認し, 原因不明のFGR (IUGR) (<-2SD) 50例および患者背景をマッチさせた正常妊娠50例を対象とした。それぞれ臍帯血, 胎盤組織, および両親の末梢血を一組として採取した。

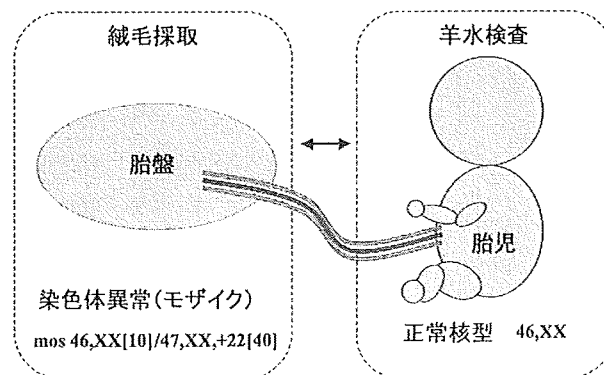
2 細胞遺伝学的検査

胎盤組織を胎盤の2カ所以上の部位から採取し, 50~100個の培養細胞についてGバンド法で染色体検査を行った。そして, 2つのフラスコにおけるモザイクの染色体核型が一致したものをモザイクと判定した。臍帯血についても同様に染色体検査を行った。胎盤組織の染色体核型がモザイクで, 臍帯血のそれが正常核型のものをCPMと判定した。

3 分子遺伝学的検査

CPMと判定された例について, それぞれ臍帯血, 胎盤組織, および両親の末梢血よりキットを用いてDNAを抽出した。DNA多型マーカー (ABI PRISM linkage-mapping set-MD10, Applied Biosystems) を用いて, 遺伝子型解

図1 Confined placental mosaicism (CPM)



析を行った。胎盤組織の遺伝子型と両親のそれを比較することにより、胎盤に認めるトリソミーアレルの由来が母親由来なのか父親由来なのか判定した。また、臍帯血DNAの遺伝子型と両親のそれを比較して、胎児の遺伝子型が両親由来 (biparental disomy ; BPD) なのか片親由来 (uniparental disomy ; UPD) なのか判定した。

4 臨床所見

CPMを伴うFGR (IUGR) (CPM群) およびCPMを認めないFGR (IUGR) (non-CPM群) に分類し、両群間における周産期事象 (母体年齢, 分娩週数, 出生時体重, アプガースコア, 臍帯動脈血pH, 性別, 子宮動脈血流波形における両側性ノッチ (以下, 両側ノッチ) の有無, 合併奇形の有無, 胎盤重量) や出生後12カ月における体重, 身長および発達について比較検討した。発達の程度は, 微細運動, 粗大運動, 言語発達, 社会適応について, デンバー式発達スケールを用いて評価した⁴⁾。

結果

1 Confind placental mosaicism

CPMは原因不明のIUGR 50例のうち8例 (16%) に認められたが, 正常妊娠には認められなかった (表1)。症例1では, 臍帯血の核型は46, XYであったが, 胎盤組織のそれはすべての細胞において22トリソミーを検出した。症例2~5では, 臍帯血の核型は正常であったが, 胎盤組織のそれは正常核型とそれぞれ2番染色体, 7番染色体, 13番染色体, および22番染色体のトリソミーとの

モザイクを認めた。モザイクの程度は3~80%までさまざまであった。症例6および症例7では, 臍帯血の核型は正常であったが, 胎盤組織のそれは正常核型と複数の染色体のトリソミーとのモザイクを認めた。症例8では, 臍帯血の核型は正常であったが, 胎盤組織のそれは正常核型と2番染色体の部分欠失とのモザイクを呈していた。

2 UPDの有無とトリソミーアレルの由来

胎盤組織において正常核型と7トリソミーおよび13トリソミーとのモザイクを認めた症例6について, 7番染色体上のDNA多型マーカー (D7S640) を用いて遺伝子型解析を行った (図2)。母親の遺伝子型は133bpアレルと141bpアレルのヘテロ接合で, 父親のそれは124bpアレルと145bpアレルのヘテロ接合であった。新生児の遺伝子型は141bpの母親由来アレルと145bpの父親由来アレルを有していた。一方, 胎盤組織の遺伝子型は母親由来の133bpおよび141bpのアレルと父親由来の145bpアレルを有していた。つまり, トリソミーアレルの由来は母親由来であった。同様に, 症例1~7の胎盤組織で検出されたトリソミーアレルについてそれぞれDNA多型マーカーを用いて遺伝子型解析を行った。すべての例において, 臍帯血DNAの遺伝子型は両親由来であり, UPDを伴うものは認められなかった (表1)。症例5のトリソミーアレルの由来のみ父親由来で, その他のそれはすべて母親由来であった。また, 症例1~5では組み換えが確認されたことからトリソミーは第一減数分裂の不分離に由来し, 症例6および症例7では組み換えを確認しえなかったことから第2減数分裂の不分離もしくは体細胞エラ

表1 CPMの核型と発生機転

	年齢	胎盤組織	臍帯血	トリソミーアレルの由来	UPD
1	29	47,XY,+22[50]	46,XY	Mat MI	—
2	28	46,XY[97]/47,XY,+2[3]	46,XY	Mat MI	—
3	25	46,XX[62]/47,XX,+7[8]	46,XX	Mat MI	—
4	26	46,XY[45]/47,XY,+13[5]	46,XY	Mat MI	—
5	31	46,XX[10]/47,XX,+22[40]	46,XX	Pat MI	—
6	29	46,XY[98]/48,XY,+7,+13[2]	46,XY	Mat MII mitotic	—
7	22	46,XY[48]/50,XY,+2,+7,+15,+20[2]	46,XY	Mat MII mitotic	—
8	29	46,XX[97]/46,XX,del(2)(p16)[3]	46,XX		

Mat:母親由来, Pat:父親由来, MI:第1減数分裂の不分離, MII:第2減数分裂の不分離

(Miura K et al. Am J Med Genet 2006改変)

一によるものと考えられた。

③ 周産期事象

CPM群の出生児体重を出生児体重曲線にプロットすると、いずれの例も極端なFGR (IUGR) を呈していた(図3)。CPMを伴うFGR (IUGR) の特徴として、妊娠中のプレグノグラムで妊娠早期からFGR (IUGR) を認めているにもかかわらず、胎内発育を認め、かつ羊水過少や娩出が必要なnon-reassuring fetal heart rate patternを呈することなく、妊娠末期(あるいはその付近)まで妊娠が継続されていることがあげられる(図4)。CPM群とnon-CPM群における周産期事象を比較したところ、子宮動脈血流波形における両側ノッチは、CPM群では8例のうち4例に、一方non-CPM群では42例のうち5例に認められた(図5)。一方、他の周産期事象については、両群間に有意差は認められなかった(表2)。

④ 出生後12カ月の発育・発達および生命予後

出生後の発育については、non-CPM群に比較してCPM群において低身長 (<-2SD) が高頻度に認められたが(それぞれ53.3%, 87.5%), 発達の程度については両群間に明らかな差は認められなかった(表3)。症例1の男児は心房中隔欠損と尿道下裂と診断され、外科治療が行われた(表4)。症例6の男児は逆三角形の顔貌、口唇裂および身体の左右非対称を認めRussell-Silver Syndromeと診断され、生後10カ月に原因不明で死亡した。死亡したものは本例のみであった。

考察

本研究では、原因不明のFGR (IUGR) 50例のうち8例(16%)にCPMが検出された。この結果は諸家の報告と一致していたが⁵⁾、私どもは培養細胞についてのみ染色体検査を行い、検出されたモザイクの程度も8例のうち4例は2~5%と低率であった。すなわち、non-CPM群に分類されたものの中に、培養細胞では検出し得ないcytotrophoblastにのみトリソミーを認めるタイプのCPMや、より低率のモザイクが含まれている可能性が

図2 DNA多型マーカーを用いた遺伝子型解析

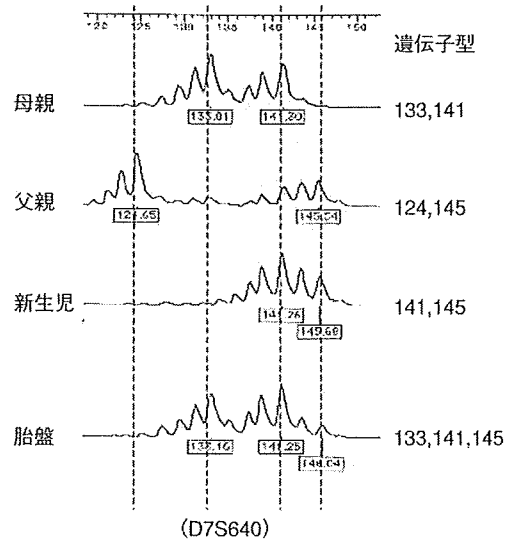
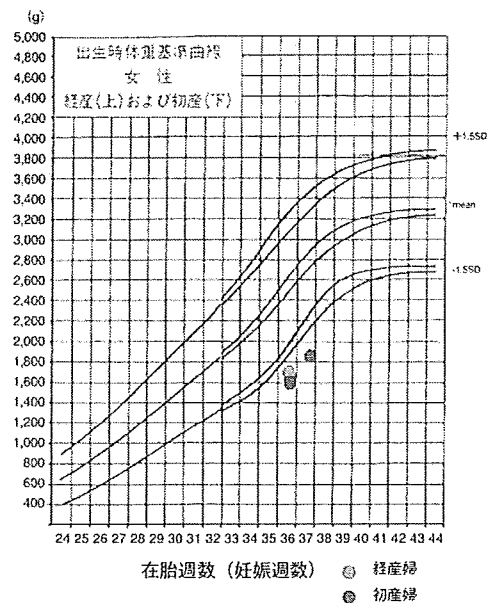
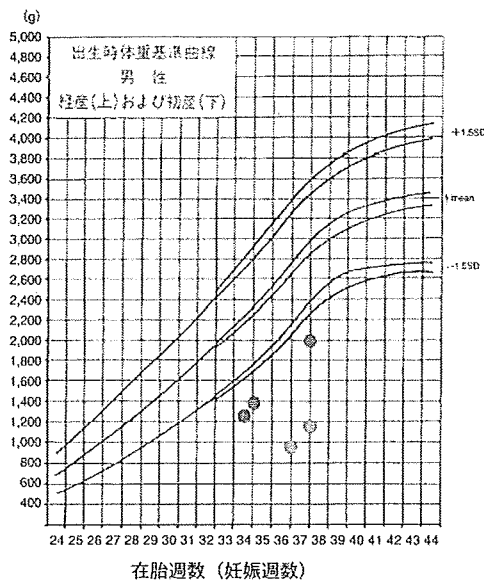


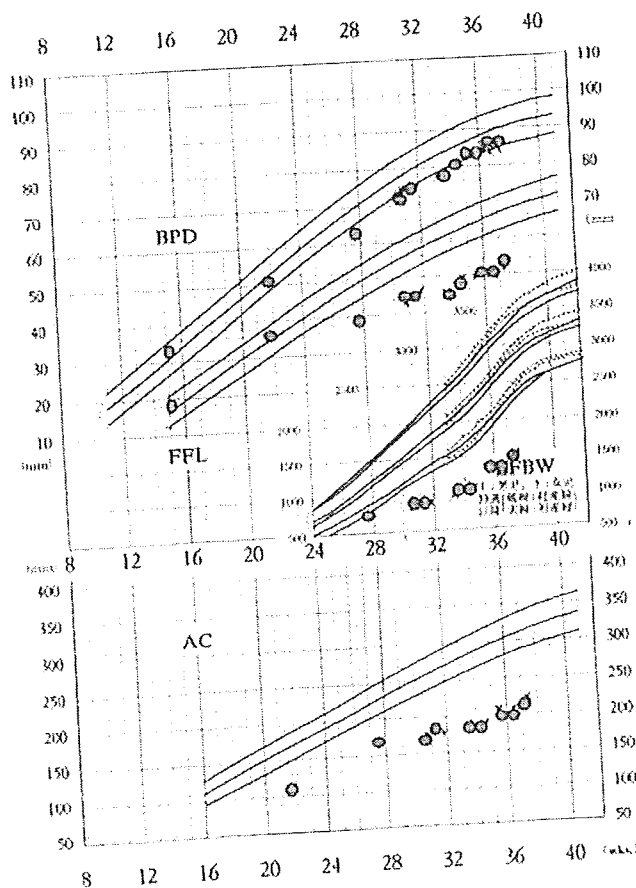
図3 CPM症例の出生時体重



考えられ、原因不明のFGR (IUGR) に関連するCPMの割合は16%より高率と考えられた。CPMによるFGR (IUGR) の発症機序として、胎盤組織におけるCPM自体の直接的な影響、あるいはUPDに伴うインプリンティング異常の関与が考えられるが、CPMを伴う8例にUPDを認めるものは存在しなかったことから、少なくとも後者の関与は低いものと考えられた。また、トリソミーアレルの大部分は減数分裂の不分離で発症し、その由来は母親由来のものがほとんどであったことから、減数分裂の不分離で発症したCPMはFGR (IUGR) の危険性が高いとする報告と一致した⁷⁾。

CPM群ではnon-CPM群と比較して子宮動脈血流波形の拡張期に両側ノッチを認める頻度が有意に高かった。子宮動脈血流波形の両側ノッチは子宮動脈の高い血管抵抗を反映しており、FGR (IUGR) を伴う妊娠高血圧症候群における子宮胎盤循環不全との関連が報告されている^{8,9)}。したがって、CPM群で認めた子宮動脈血流波形の両側ノッチは、正常核型の胎盤と比較してCPMを伴う胎盤で高率に認められた胎盤梗塞や脱落膜の血管形成不全などの所見を反映していると考えられた^{5,6)}。

図4 Pregnogram (症例6)



出生12カ月の発達については両群間に有意差は認められなかったが、原因不明のFGR (IUGR) 50例のうちRussell-Silver Syndrome 3例 (6%) が認められた。本症候群の発症頻度は1/3,000~100,000人ということを考慮すると¹⁰⁾、原因不明のFGR (IUGR) ではRussell-Silver Syndromeなどの疾患が関与していることを念頭において管理する必要があると考えられた。また、出生12カ月の発育について、CPM群8例のうち7例 (87.5%) の身長は-2SD未満であったのに対して、追跡可能であったnon-CPM群15例のうち8例 (53.3%) の身長は-2SD未満であった。両群間に有意差は認められないものの、CPMは出生後の発育に影響している可能性が示唆された。

まとめ

本研究により、原因不明のFGR (IUGR) のなかには

図5 母体の子宮動脈血流波形におけるノッチ
白矢印はノッチを示す。

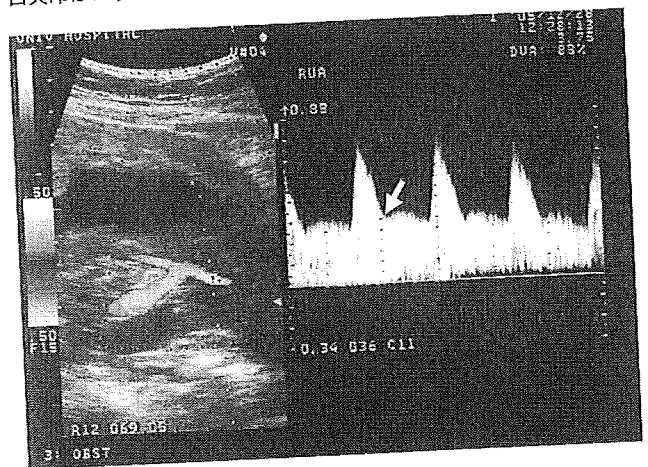


表2 CPMとnon-CPM群における周産期事象の比較

	CPM群	Non-CPM群
・症例数	8	42
・年齢	1-10	1-10
・男児/女児	5/3	27/15
・母体年齢	22-31	23-40
・出生時週数	34-38	34-39
・出生時体重 (SD)	-2.2~5.0	-2.0~4.7
・臍帯動脈 pH	7.14-7.34	7.11-7.39
・胎盤重量 (g)	369±130	346±81
・両側ノッチ (子宮動脈) *	5/8	5/42
・表現型の異常		
Russell-Silver Syndrome	1/7	2/42
その他の奇形	1/7	1/42 多趾症 尿道下裂

* : p<0.05

(Miyra K et al. Am J Med Genet 2006改竄)

表3 生後12カ月における発育および発達評価

	CPM群	non-CPM群
・症例数	7	15
・発育遅延 (<-2SD) (遅延した症例/全症例)	7/8 (87.5%)	8/15 (53.3%)
体重 (SD)	-1.7~3.5	-0.7~3.5
身長 (SD)	-0.4~3.3	-0.7~3.6
・発達遅延 (<75%) (遅延した症例/全症例)		
粗大運動	1/7	2/13
微細運動	1/7	2/13
言語発達	0/7	1/13
社会順応	2/7	2/13

(Miura K et al. Am J Med Genet 2006改変)

CPMを伴うものが存在した。CPMを伴うFGR (IUGR) には、子宮動脈血流波形の両側ノッチを有する例が有意に多く、子宮胎盤循環異常との関連が示唆された。また、CPMは出生後の発育に影響している可能性が考えられ

表4 CPM群におけるデンバー発達スケール・生命予後

	運動		言語発達	社会適応	生命予後
	微細	粗大			
1	N	N	N	N	心房中隔欠損・尿道下裂 生存
2	D	D	N	D	生存
3	N	N	N	N	生存
4	N	N	N	D	生存
5	N	N	N	N	生存
6	-	-	-	-	Russell-Silver Syndrome 生後10ヶ月で死亡
7	N	N	N	N	生存
8	N	N	N	N	生存

N: within normal range, D: delayed

(Miura K et al. Am J Med Genet 2006改変)

た。以上の成果は、FGR (IUGR) とCPMを伴う児に関する周産期管理と遺伝カウンセリングに有用な情報となりうるものと考えられた。

文 献

- Miura K, Yoshiura K, Miura S, Kondoh T, Harada N, Yamasaki K, Fujimoto Y, Yamasaki Y, Tanigawa T, Kitajima Y, Shimada T, Yoshida A, Nakayama D, Tagawa M, Yoshimura S, Wagstaff J, Jinno Y, Ishimaru T, Niikawa N, Masuzaki H: Clinical outcome of infants with confined placental mosaicism and intrauterine growth restriction of unknown cause. Am J Med Genet A 2006; 140A: 1827-33.
- Kalousek DK, Dill FJ: Chromosomal mosaicism confined to the placenta in human conceptions. Science 1983; 221: 665-7.
- Kalousek DK, Vekemans M: Confined placental mosaicism. J Med Genet 1996; 33: 529-33.
- Frankenburg WK, Dodds JB: The Denver developmental screening test. J Pediatr 1967; 71: 181-91.
- Wilkins-Haug L, Roberts DJ, Morton CC: Confined placental mosaicism and intrauterine growth retardation: a case-control analysis of placentas at delivery. Am J Obstet Gynecol 1995; 172: 44-50.
- Wilkins-Haug L, Quade B, Morton CC: Confined placental mosaicism as a risk factor among newborns with fetal growth restriction. Prenat Diagn 2006; 26: 428-32.
- Robinson WP, Barrett IJ, Bernard L, Telenius A, Bernasconi F, Wilson RD, Best RG, Howard-Peebles PN, Langlois S, Kalousek DK: Meiotic origin of trisomy in confined placental mosaicism is correlated with presence of fetal uniparental disomy, high levels of trisomy in trophoblast, and increased risk of fetal intrauterine growth restriction. Am J Hum Genet 1997; 60: 917-27.
- Sağol S, Ozkinay E, Oztekin K, Ozdemir N: The comparison of uterine artery Doppler velocimetry with the histopathology of the placental bed. Aust N Z J Obstet Gynaecol 1999; 39: 324-9.
- Coleman MA, McCowan LM, North RA: Mid-trimester uterine artery Doppler screening as a predictor of adverse pregnancy outcome in high-risk women. Ultrasound Obstet Gynecol. 2000; 15: 7-12.
- Abu-Amero S, Monk D, Frost J, Preece M, Stanier P, Moore GE: The genetic aetiology of Silver-Russell syndrome. J Med Genet 2008; 45: 193-9.