Results

We found five sequence variants, including two one-base deletions and three non-synonymous one-base substitutions, in six out of thirty-seven patients without imprinting defects or paternal UPD11 (Table 2; Fig. 1). Four mutations were novel and one had been previously reported (Li et al., 2001). The deletions observed in Patients 1, 2, and 3 caused frameshift mutations (p.G234fsX36 and p.L154fsX117). Patients 2 and 3 were siblings sharing the same variants. The substitution observed in Patient 4 resulted in a nonsense mutation (p.Q241X), while the substitutions observed in Patients 5 and 6 resulted in missense mutations (p.W61R and p.Y91H). The non-synonymous substitutions were not found in 100 normal individuals and databases, such as dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and 1000 genomes (http://www.1000genomes.org/). Two of the five variants occurred in the CKI domain, one in the PAPA repeat, and two in and near the QT domain. We predicted functional effects of these sequence variants with *in silico* prediction programs, such as MutationTaster (http://www.mutationtaster.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and SIFT (http://sift.bii.a-star.edu.sg/). The deletions in Patients 1, 2, and 3 were predicted as "DISEASE CAUSING" by MutationTaster and "DAMAGING" by SIFT-indels. The substitution in Patient 4 was also predicted as "DISEASE CAUSING" by MutationTaster. As for the substitutions in Patients 5 and 6, PolyPhen-2 and SIFT-genome predicted them as "PROBABLY DAMAGING" and "DAMAGING", respectively; however, MutationTaster did not predict this mutation as deleterious, but rather as just a polymorphism. We additionally used Align GVGD (http://agvgd.iarc.fr/index.php) and PANTHER (http://www.pantherdb.org/), which were prediction programs specific for missense mutations. Both programs predicted the mutations as deleterious (data not shown).

As for inheritance of these mutations, all mutations except for that of Patient 4 were maternally inherited (Fig. 1). The deletion observed in Patient 1 was inherited from the maternal grandfather and also inherited by the patient's mother and aunt. The mother and maternal aunt did not show any features of BWS in their childhood because of paternal transmission. The substitution in Patient 6 was inherited from the maternal grandmother. Furthermore, the patient's mother exhibited macroglossia, abdominal wall defects, and atrial septal defects, which are features strongly suggestive of BWS. On the other hand, the substitution in Patient 4 was a de novo mutation. We confirmed the expression of all mutant alleles except for Patients 2 and 3 in peripheral blood or placenta (data not shown). RNA from Patients 2 and 3 was unavailable.

Table 2 *CDKN1C* mutations observed in BWS patients

Patient no. (Laboratory ID)	Nucleotide change	Amino acid change	Protein domain	Inheritance	Prevalence in normal Individuals	mutationtaster	PolyPhen-2	SIFT-genome	SIFT-indels	Reference
Patient 1 (BWS059)	c.701delG	p.G234fsX36	QT	Maternal (grandfather)	n.a.	Disease causing	Invalid	Invalid	Damaging	Novel
Patient 2 (bwsh21-055A)	c.460delC	p.L154fsX117	PAPA	Maternal	n.a.	Disease causing	Invalid	Invalid	Damaging	Novel
Patient 3 (bwsh21-055B)	c.460delC	p.L154fsX117	PAPA	Maternal	n.a.	Disease causing	Invalid	Invalid	Damaging	Novel
Patient 4 (bwsh21-068)	c.721C>T	p.Q241X	QT	De novo	0/100	Disease causing	Invalid	Invalid	Invalid	Li et al. (2001)
Patient 5 (bwsh21-073)	c.181T>C	p.W61R	CKI	Maternal	0/100	Polymorphism	Probably damaging	Damaging	Invalid	Novel
Patient 6 (bwsh21-098)	c.271T>C	p.Y91H	CKI	Maternal (grandmother)	0/100	Polymorphism	Probably damaging	Damaging	Invalid	Novel

Patient 2 and 3 were siblings. Mutations are notated according to NCBI RefSeq accession NM_000076

n.a. not analyzed, *invalid* analysis of mutation unsupported by prediction program

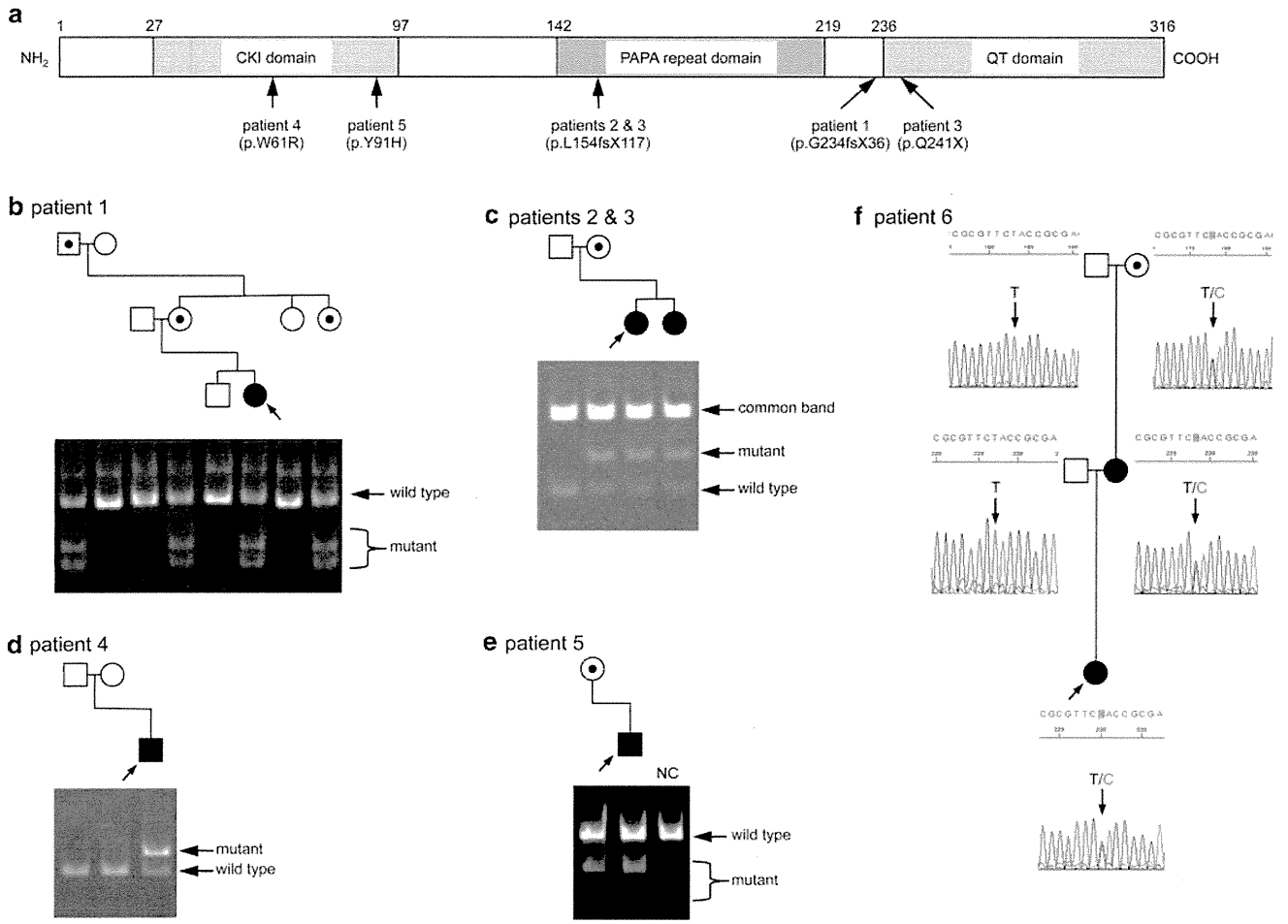

Fig. 1 *CDKN1C* mutations and their inheritance. **a** Domain structure of the CDKN1C protein and position of each mutation. Amino acid residues are indicated above. **b** Pedigree chart of Patient 1. *Bss*HII digestion of PCR region F was used to distinguish between the mutant and wild type alleles. The mutant allele was inherited from the maternal grandfather. **c** Pedigree chart of Patients 2 & 3. *Ava*II digestion of region C was used to distinguish between the mutant and wild type alleles. The mutant allele was inherited from the mother. **d** Pedigree chart of Patient 4. *Pvu*II digestion of region F was used to distinguish between the mutant and wild type alleles. The mutant allele was not found in the parents, indicating a de novo mutation. **e** Pedigree chart of Patient 5. *Nci*I digestion of region B was used to distinguish between the mutant and wild type alleles. The mutant allele was inherited from the mother. *NC* normal control. **f** Pedigree chart of Patient 6. Patient 6 and her mother were heterozygous (T/C) for the wild type and mutant alleles. The mutant allele was inherited from the maternal grandmother. The patient's mother was also affected

Regarding the clinical features of patients with *CDKN1C* mutations, the triad of macrosomia, abdominal wall defects, and macroglossia were seen with high frequency (Table 3). Four of the six patients showed all three traits, and two showed two traits. In addition, ear creases and/or ear pits were frequently seen in five of the six patients. In contrast, hemihyperplasia, abdominal organomegaly and/or malformation, and genital abnormality were not generally seen. Neonatal hypoglycemia was seen in three patients, nevus flammeus in two patients, and cleft palate in two patients. Patients 2 and 3 showed slight differences in the extent of hypoglycemia and abdominal organomegaly, suggesting variability in expressivity of the *CDKN1C* mutation. There was no tumor development in any patients except for Patient 6, whose cardiac rhabdomyoma was likely due to tuberous sclerosis. The cardiomegaly observed in Patient 5 was likely due to long QT syndrome.

Discussion

In this study, we found five mutations from six Japanese BWS patients. Four were novel mutations that were maternally inherited, and one was a de novo mutation that has been reported previously (Li et al. 2001). These variants consisted of two frameshift (p.G234fsX36 and p.L154fsX117), one nonsense (p.Q241X), and two missense mutations (p.W61R and p.Y91H). Since the positions of the frameshift mutations and the nonsense mutation occur after the PAPA repeat domain, these mutations

Table 3 Clinical information of BWS patients with *CDKN1C* mutations

Patient no. (Laboratory ID)	Age	Conception	Karyotype	Birth weight (gestational age)	Macrosomia	Abdominal wall defect	Macroglossia	Ear creases /Ear Pits	Neonatal hypoglycemia
Patient 1 (BWS059)	2 m	Natural	46,XX	3,804 g (37w1d)	+	+	+	+	–
Patient 2 (bwsh21-055A)	11y2 m	n.i	46,XX	4,424 g (38w0d)	+	+	+	+	+
Patient 3 (bwsh21-055B)	1 m	n.i	46,XX	4,025 g (38w0d)	+	+	+	+	–
Patient 4 (bwsh21-068)	1 m	Natural	46,XY	3,056 g (34w4d)	+	+	–	+	–
Patient 5 (bwsh21-073)	3y11 m	n.i	46,XY	3,000 g (34w0d)	+	+	+	+	+
Patient 6 (bwsh21-098)	3y9 m	n.i	46,XX	2,560 g (35w5d)	–	+	+	–	+

Patient no. (Laboratory ID)	Facial nevus flammeus	Cleft Palate	Hemihyperplasia	Abdominal organomegaly /Malformation	Genital abnormality	Tumor	Other features	Complication
Patient 1 (BWS059)	+	–	–	–	–	–	Advanced bone age	–
Patient 2 (bwsh21-055A)	–	–	–	–	–	–		–
Patient 3 (bwsh21-055B)	–	–	–	+ (hepato megaly)	–	–		–
Patient 4 (bwsh21-068)	+	+	–	–	–	–		–
Patient 5 (bwsh21-073)	–	+	–	–	–	–	Inguinal hernia, accessory ear, transient cardiomegaly	Long QT syndrome type 3 (*SCN5A* mutation)
Patient 6 (bwsh21-098)	–	–	–	–	–	+(cardiac rhabdomyoma)	Atrial septal defect	Tuberous sclerosis

Patient 2 and 3 were siblings

n.i no information

would abolish the QT domain. The QT domain contains a PCNA-binding domain, which can prevent DNA replication in vitro and S phase entry in vivo. Disruption of PCNA-binding partially reduces the suppressive activity of the CDKN1C protein (Watanabe et al. 1998). The QT domain also contains NLS; thus a *CDKN1C* mutant without an NLS would be expressed in the cytoplasm and excluded from the nucleus (Bhuiyan et al. 1999). Very recently, missense mutations in the PCNA-binding domain were reported in the undergrowth-associated condition of intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenita, and genital anomalies (IMAGe) syndrome (OMIM # 300290). These missense mutations resulted in excess inhibition of growth and differentiation, suggestive of gain of function mutations. The gain of function might be due to abolishment of PCNA-dependent CDKN1C monoubiquitination (Arboleda et al. 2012). On the other hand, we found that the two missense mutations occurred in the CKI domain, which contains a cyclin-binding region, a CDK-binding region, and a 3_{10} helix. This domain is both necessary and sufficient to bind and inhibit CDK activity (Lee et al. 1995; Matsuoka et al. 1995; Borriello et al. 2011). The p.W61R and p.Y91H mutations occurred within the CDK binding region and the 3_{10} helix, respectively, suggesting insufficient inhibition of CDK activity. Since we confirmed the expression of all mutant alleles, except for c.460delC (p.L154fsX117), and their maternal transmission, except for c.721C>T (p.Q241X), this suggests, in addition to the results of *in silico* prediction analyses and the absence of the mutations in the general population, that the mutations found in this study must be causative for BWS.

Among the patients analyzed in this study, the BWS triad was frequently seen, but hemihyperplasia, abdominal organomegaly and/or malformation, and genital abnormality were generally not observed. Neonatal hypoglycemia, nevus flammeus, and cleft palate were seen with moderate frequency. It has been reported that genital abnormalities, cleft palate, polydactyly, and supernumerary nipples were more frequently observed in BWS patients with *CDKN1C* mutations (Romanelli et al. 2010). In this study, no genital abnormalities were observed, and cleft palate was observed in two patients. Information regarding polydactyly and supernumerary nipples was not available. Because the number of patients in this study was small, we could not confirm aspects of Romanelli's data, indicating necessity for investigating a larger number of BWS patients with *CDKN1C* mutations. The overall tumor incidence in BWS is approximately 10 %; however, it has been reported to be 0–4 % in BWS with *CDKN1C* mutations (Weksberg et al. 2001; Rump et al. 2005). In this study, Patient 6 actually developed cardiac rhabdomyoma. However, since this patient also suffered from tuberous sclerosis, in which approximately 50 % of such cases develop cardiac rhabdomyoma, tumor development in this instance would likely be due to tuberous sclerosis. Therefore, tumor incidence is thought to be lower in BWS with *CDKN1C* mutations than in other alterations. Two of the six patients showed complicating diseases, such as long QT syndrome and tuberous sclerosis. These complications would affect clinical features and necessitate careful clinical examination. Furthermore, since only 16 % of BWS patients have *CDKN1C* mutations among the patients without imprinting defects or paternal UPD11, the existence of other causative genes for BWS is strongly indicated. Although a frameshift mutation in *NLRP2* was reported in a familial case of BWS (Meyer et al. 2009), there have been no other reports of new patients with *NLRP2* mutations to date. Exome sequencing analysis of patients without any causative alterations should be performed in order to identify novel causative genes.

In conclusion, we found four novel and one known *CDKN1C* mutations in Japanese patients with BWS. Since the total number of patients with *CDKN1C* mutations reported to date is still small, at less than thirty, a larger number of BWS patients should be analyzed to understand genotype-phenotype correlations more precisely.

Acknowledgments This study was supported in part by a Grant for Research on Intractable Diseases from the Ministry of Health, Labor, and Welfare, a Grant for Child Health and Development from the National Center for Child Health and Development, and a Grant-in-Aid for Challenging Exploratory Research and a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science.

Conflict of interests The authors have no conflicts of interest to declare.

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Endocrine Journal 2013, **60** (4), 403-408

ORIGINAL

Congenital hyperinsulinism in an infant with paternal uniparental disomy on chromosome 11p15: Few clinical features suggestive of Beckwith-Wiedemann syndrome

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Abstract. Beckwith-Wiedemann syndrome (BWS) is the most common congenital overgrowth syndrome involving tumor predisposition. BWS is caused by various epigenetic or genetic alterations that disrupt the imprinted genes on chromosome 11p15.5 and the clinical findings of BWS are highly variable. Hyperinsulinemic hypoglycemia is reported in about half of all babies with BWS. We identified an infant with diazoxide-unresponsive congenital hyperinsulinism (HI) without any apparent clinical features suggestive of BWS, but diagnosed BWS by molecular testing. The patient developed severe hyperinsulinemic hypoglycemia within a few hours after birth, with macrosomia and mild hydronephrosis. We excluded mutations in the K_{ATP} channel genes on chromosome 11p15.1, but found a rare homozygous single nucleotide polymorphism (SNP) of *ABCC8*. Parental SNP pattern suggested paternal uniparetal disomy in this region. By microsatellite marker analysis on chromosome 11p15, we could diagnose BWS due to the mosaic of paternal uniparental disomy. Our case suggests that some HI of unknown genetic etiology could involve undiagnosed BWS with no apparent clinical features, which might be diagnosed only by molecular testing.

Key words: Beckwith-Wiedemann syndrome, Congenital hyperinsulinism, ^{18}F-fluoro-L-DOPA positron emission tomography, Uniparental disomy 11p15

BECKWITH-WIEDEMANN SYNDROME (BWS) is the most common congenital overgrowth syndrome involving tumor predisposition and congenital malformations [1, 2]. BWS is caused by various epigenetic or genetic alterations that disrupt the imprinted genes in two imprinted domains on chromosome 11p15.5. In domain 1, insulin-like growth factor 2 (*IGF2*) and *H19* are monoallelically expressed, and in domain 2, *CDKN1C*, a growth repressor, and *KCNQ1OT1* are monoallelically expressed. In each domain, an imprinting center, *H19-DMR* or *KvDMR1*, regulates the expression of imprinted genes. In BWS, several mechanisms result in increased expression of *IGF2* and/or decreased expression of *CDKN1C*. *KvDMR1* loss of methylation occurs in 50% of BWS patients, and paternal uniparental disomy (UPD) on chromosome 11p15 is found in 20%.

The clinical findings of BWS are highly variable because of the heterogeneity of the underlying molecular etiology, and milder phenotypes may not be readily identified [1, 2]. Classically, BWS must be considered when exomphalos, macroglossia, or gigantism is noted; however, recent advances in molecular testing have expanded the diagnostic potential for BWS for patients with no or few clinical features [3].

Congenital hyperinsulinism (HI) comprises various genetic disorders due to inappropriate insulin secretion by pancreatic β-cells [4, 5]. Severe hypoglycemia is the major feature of HI and has a risk of seizures and brain damage if untreated. Mutations in ATP-sensitive potassium (K_{ATP}) channel genes, *ABCC8* and *KCNJ11*, on chromosome 11p15.1, are the most common causes of HI and account for 40-45% of all cases but, in nearly half of the cases, the genetic etiology remains unknown. HI is usually isolated, but in rare cases may be part of a

Submitted Jul. 5, 2012; Accepted Nov. 7, 2012 as EJ12-0242
Released online in J-STAGE as advance publication Nov. 30, 2012

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genetic syndrome, such as BWS and Sotos syndrome.

We report an infant with HI but without apparent clinical features suggestive of BWS, but diagnosed BWS by molecular testing due to the somatic mosaicism of paternal UPD on chromosome 11p15.

Clinical Report

This female patient was the first child of nonconsanguineous parents and had been conceived naturally. Fetal sonography suggested bilateral mild hydronephrosis at the prenatal age of 23 weeks, but the pregnancy was uncomplicated. The patient was delivered by cesarean section at 38 weeks gestation due to breech presentation. Her birth weight was 3,738 g (>90th percentile), height was 52 cm (>90th percentile), and she was physically evaluated as normal.

She developed severe hyperinsulinemic hypoglycemia 1.5 hours after birth and was diagnosed with hyperinsulinemic hypoglycemia (plasma glucose 17 mg/dL and serum insulin 37.3μU/mL with undetectable ketone bodies, normal lactate). The serum GH and cortisol were 9.18 ng/mL and 11 μg/dL, respectively. The glucose infusion rate required to maintain a blood glucose concentration >60 mg/dL was 20 mg/kg/min. She was apparently normal, without macroglossia, exopmphalos, hemihypertrophy or ear anomaly. Light brown irregular nevi on the shoulder, back and upper limb were apparent. Renal ultrasonography showed bilateral mild hydronephrosis, as observed on prenatal ultrasound. Her hypoglycemia failed to respond to maximum doses of diazoxide (20 mg/kg/d). Instead of diazoxide, continuous intravenous infusions of octreotide were started at the age of two weeks and the dose was slowly titrated up to 40 μg/kg/d. While continuing medical therapy, the surgical indication was also considered as a case of unresponsive HI. To determine the histopathological form, ^{18}F-fluoro-L-DOPA ([^{18}F]DOPA) positron emission tomography (PET) was performed, as described by Ribeiro *et al.* [6]. The patient demonstrated uptake in the head and body of the pancreas (Fig. 1a). The standardized uptake of the head, body and tail was 5.5, 4.4 and 3.7, respectively. As the result was a non-single focal form, *i.e.* multi-focal or diffuse form, it seemed that partial pancreatectomy was impossible.

At the age of one month, a few days after the maximum dose of octreotide, the glucose infusion rate could be decreased gradually. Normoglycemia without glucose infusion could be maintained one week later and the treatment was changed to continuous subcutaneous octreotide injection at the age of two months. The dose of octreotide was reduced in a stepwise manner and was discontinued at the age of 3 months. Subsequently, there were no episodes of hypoglycemia.

At the ages of 2 and 8 months, computed tomography (CT) with contrast demonstrated a mass adjacent to the upper segment of the left kidney (Fig. 1b). The mass measured 38 × 17 mm, with homogeneous density comparable to the spleen, and was not enhanced. Renal ultrasonography demonstrated no blood flow inside the mass. CT and MRI imaging also showed an enlarged mass occupying the anterior mediastinum, totally covering the heart to 20 mm thickness, indicating thymic hyperplasia (Fig. 1c). Tumor markers were

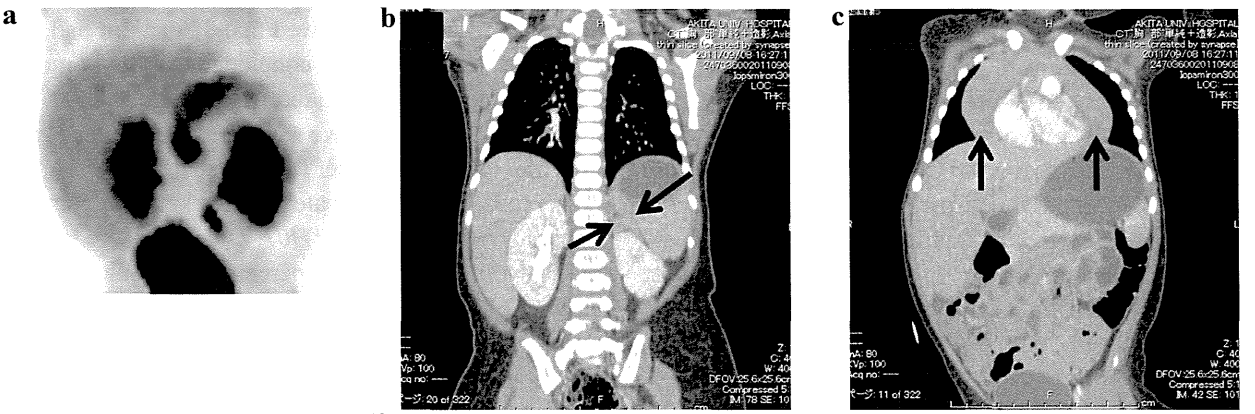


Fig. 1 (a) Representative patterns of [^{18}F]DOPA uptake. Maximum intensity projection obtained 30 min after injection. Multifocal or diffuse uptake in the head and body of the pancreas. (b) CT with contrast showed a mass adjacent to the upper segment of the left kidney (arrows). (c) CT with contrast showed an enlarged mass occupying the anterior mediastinum (arrows), indicating thymic hyperplasia.

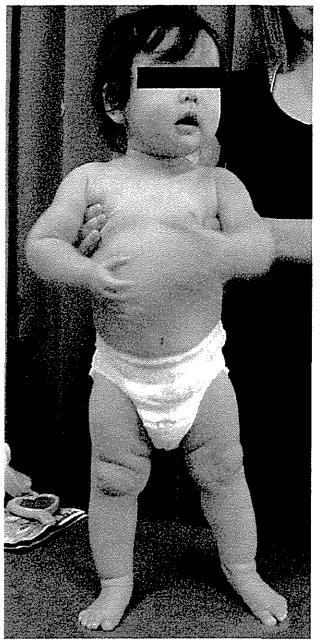


Fig. 2 Patient at the age of 8 months without apparent clinical features suggestive of BWS.

not elevated and these masses showed gradual regression, therefore, histological evaluation could not be performed. At the age of 8 months, she demonstrated normal growth and neurodevelopmental progress, with no apparent clinical features of BWS (Fig. 2).

Materials and Methods

K_{ATP} genes analysis

Genomic DNA was extracted from peripheral leukocytes. Mutation analysis of K_{ATP} genes, *ABCC8* and *KCNJ11*, was performed by sequencing coding exons and flanking intronic regions including 30-100bp. The PCR products were purified on 1.0% agarose gel and were sequenced directly with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) using an automated sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystems). Multiple ligation-dependent probe amplification (MLPA) of *ABCC8* was performed by using Salsa MLPA Kit (MRC-Holland, Amsterdam, Netherlands).

Molecular analysis of BWS

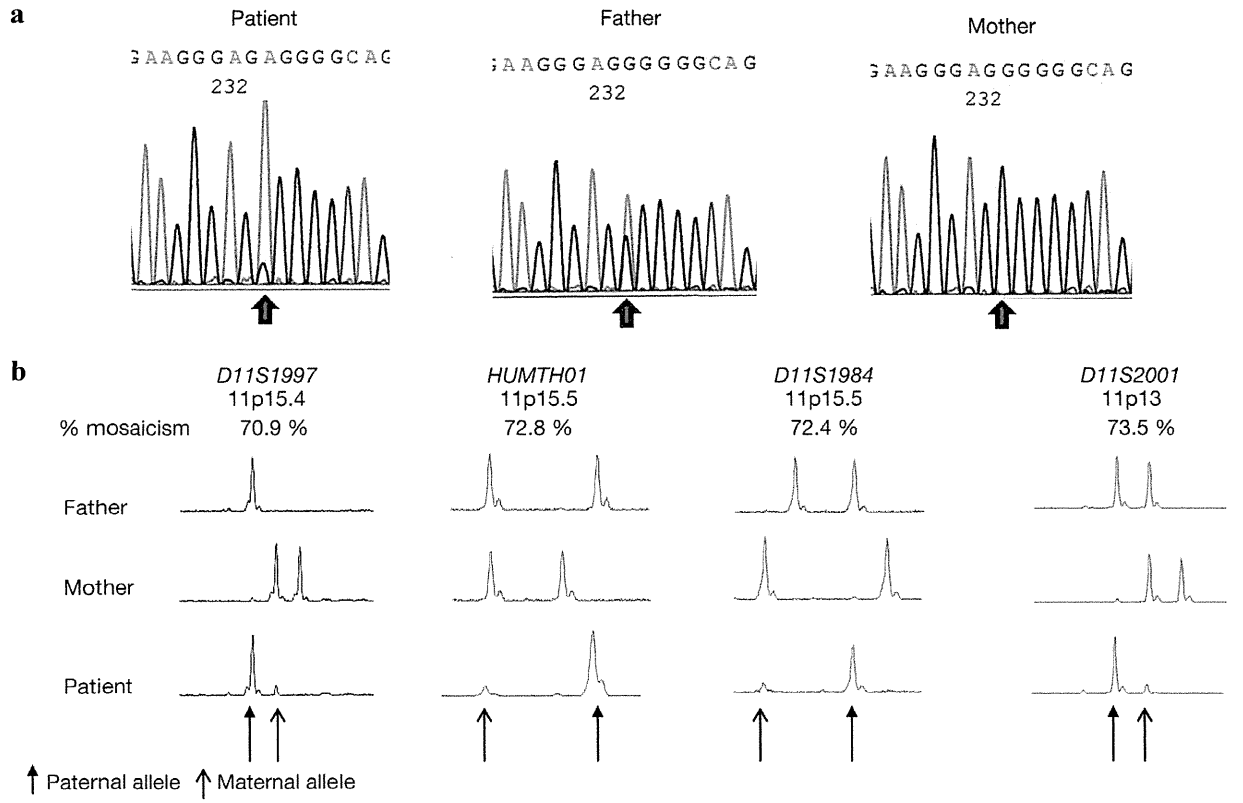
To analyze paternal UPD, genomic DNA was extracted from peripheral blood lymphocytes of the patient and her parents. For quantitative polymorphism analyses, tetranucleotide repeat markers (*D11S1997*, *HUMTH01*, and *D11S1984*) from 11p15.4-p15.5 were amplified and separated by electrophoresis on an Applied Biosystems 3130 genetic analyzer (Applied Biosystems,); data were quantitatively analyzed with GeneMapper software (Applied Biosystems). The peak height ratios of the paternal allele to maternal allele were calculated. The percentage mosaicism of paternal UPD was calculated as: % mosaicism = $(k - 1) / (k + 1) \times 100$, where k is the ratio of the intensity of the paternal to maternal alleles of the sample [7]. To confirm the range of UPD, we also used another marker *D11S2001* on 11p13 region. We also investigated methylation status in *KvDMR1* and *H19-DMR*, mutation analysis of *CDKN1C* by sequencing as described previously [8].

These studies were approved by ethical committee of Akita University Graduate School of Medicine and written informed consent was obtained from her parents.

Results

We first suspected mutations in the K_{ATP} channel genes. We obtained written informed consent for molecular testing from her parents, and genomic DNA was extracted from peripheral blood lymphocytes of the patient for direct sequencing of *ABCC8* and *KCNJ11*, but no mutations were found; however, a rare homozygous single nucleotide polymorphism (SNP) was found in intron 8 of *ABCC8* (rs1800850; A>G change, minor allele frequency was 6.7%). Then, the SNP in her parents was directly sequenced. The patient had A/A genotype, her father had G/A genotype, but her mother had G/G genotype, which suggested deletion of her maternal allele or paternal UPD on chromosome 11p15 (Fig. 3a). MLPA of *ABCC8* showed that the patient had two copies of all exons, and we concluded that the homozygous SNP might have resulted from paternal UPD. At the age of three months, we started chromosome 11p15 molecular analysis in order to define her diagnosis.

The results of microsatellite marker analysis for markers *D11S1997*, *HUMTH01*, *D11S1984*, *D11S2001* are shown in Fig. 3b. The percentage mosaicism was 70.9%, 72.8%, 72.4% and 73.5%, respectively. These results were consistent with a diagnosis of mosaic paternal UPD on chromosome 11p15. Methylation-sensitive Southern blots showed *H19-DMR* hypermethylation and *KvDMR1* hypomethylation, supporting her genetic diagnosis (data not shown). No *CDKN1C* mutation was detected.



	D11S1997						*HUMTH01*					
	size1	height1	size2	height2	*k*	% mos	size1	height1	size2	height2	*k*	% mos
Father	211	429					252	254	264	246		
Mother	215.02	620	219.46	572			252	224	260.15	229		
Patient	210.6	612	215.07	104	5.88	70.9%	252	166	264.27	1053	6.34	72.8%
	D11S1984						*D11S2001*					
	size1	height1	size2	height2	*k*	% mos	size1	height1	size2	height2	*k*	% mos
Father	192.15	215	200	205			129	810	133	732		
Mother	188.32	455	204	441			133	983	137	887		
Patient	188.27	72	200.18	449	6.24	72.4%	129	1548	133	236	6.56	73.5%

k = height (Father)/height (Mother)

Fig. 3 (a) SNP(rs1800850) of *ABCC8*. The patient had A/A genotype, her father had G/A genotype, but her mother had G/G genotype. (b) Microsatellite marker analysis for markers *D11S1997*, *HUMTH01*, *D11S1984* and *D11S2001*. The percentage mosaicism of paternal UPD was 70.9%, 72.8%, 72.4% and 73.5%, respectively.

Discussion

The neonatal hypoglycemia, macrosomia and hydronephrosis observed in our patient fulfill the generally accepted criteria of BWS (*i.e.* two major findings and one minor finding) [2]; however, we had difficulty in the diagnosis of BWS because macrosomia is commonly involved in HI and, above all, there were no apparent clinical features of BWS. She also showed an extrarenal mass and an enlarged thymus, but whether they are symptoms of BWS is uncertain at present. Balcom *et al.* reported hyperplasia of the thymus that caused pulmonary hypoplasia in an infant with BWS [9], but there are few reports about an association between the thymus and BWS.

There are no absolute criteria for the clinical diagnosis of BWS and there exist milder phenotypes of BWS which do not fulfill the criteria [1, 2]. Recently, with the development of molecular genetic analysis, epigenetic alterations of chromosome 11p15 have been detected in patients with no or few clinical features of BWS; for example, isolated hemihyperplasia [10], isolated Wilms tumor [11], and isolated cardiac tumor [3].

In BWS, it has been estimated that the incidences

of hypoglycemia, macrosomia, and renal abnormalities are 50%, 88%, and 59%, respectively [12]; however, to our knowledge, there have been no other reports of BWS phenotype only with hypoglycemia, macrosomia, and renal abnormalities. Goldman *et al.* reported that BWS with paternal UPD was associated with a higher incidence of renal abnormalities [13]. The most common findings are nephromegaly, simple cysts, hydronephrosis and medullary cysts [12-14]. The grade of hydronephrosis was reported to be mild to severe with vesiculoureteral reflux (VUR). Our case did not demonstrate VUR and diuretic renography with 99mTc-MAG3 showed a normal washout pattern. Although this information supported the diagnosis, it might be difficult to reach a diagnosis for less characteristic cases in the neonatal period. Given that the genetic etiology is still unknown in nearly half of HI, some HI might be involved in undiagnosed BWS with no apparent clinical features.

The underlying mechanism leading to HI in BWS remains unclear, and the severity, duration, and response to treatment with diazoxide and octreotide are variable [15, 16]. In the majority of BWS patients, hypoglycemia will be asymptomatic and resolve within the first few days of life. Less than 5% of patients will have hypoglycemia beyond the neonatal period and, in rare cases, there will be no response to medical theapy and partial pancreatectomy will be required. Hussain *et al.* reported histological and functional studies of BWS with paternal UPD using a pancreas obtained at partial pancreatectomy [16]. Histological findings showed marked proliferation of endocrine tissue forming irregular nodules and functional studies suggested a K_{ATP} trafficking defect. In their case, as in our case, the clinical features of BWS were not obvious at birth, but developed postnatally.

BWS caused by paternal uniparental disomy is basically a mosaic, that is, originates as a consequence of postzygotic error [17]. The clinical features, therefore, is inherently variable since the features depend on the timing of the error during the postzygotic process. If an error occurred in the earlier stage of development, the clinical features are more evident. Conversely, if the error occurred in the later stage of development and confined to certain somatic organs (e.g., pancreas), the BWS features are less evident. The mosaic ratio of peripheral blood is reasonably high to diagnose BWS, however this does not tell the mosaic ratio in other somatic tissues. Therefore, we consider that diagnosis of UPD11.5 mosaicism is important for differential diagnosis of unknown HI.

Precise genetic analysis of the K_{ATP} channel and [^{18}F]DOPA PET scan diagnosis are essential in the management of diazoxide-unresponsive patients [4, 5, 18]. The focal form is due to the combination of a paternally-inherited mutation and paternal isodisomy of the 11p15 region, which is specific to islet cells within the focal region. Recessive mutations are responsible for the diffuse form. However, some previous papers report that dominant mutations also have diffuse histology. Interestingly, [^{18}F]DOPA PET in our patient showed a non-single focal form, *i.e.* multi-focal or diffuse form. To our knowledge, there have been no reports of [^{18}F]DOPA PET in HI due to BWS. If no mutations are found in known genes and [^{18}F]DOPA PET does not show a typical form, there is a possibility that HI is caused by undiagnosed BWS with no apparent clinical features.

Early diagnosis of BWS is particularly important because patients with BWS have a predisposition to embryonal tumors, most commonly Wilms tumor and hepatoblastoma, and a variety of other malignant and benign tumors [19, 20]. The risk is approximately 7.5% and most of the tumors occur in the first 8−10 years of life; therefore, tumor surveillance is recommended for all children with confirmed or suspected BWS every 3 months to the age of 8 years by abdominal ultrasound and every 3 months to the age of 4 years by alpha fetoprotein assay [3]. In this regard, it is significant to recognize the existence of BWS patients with no or few clinical features, which might be diagnosed only by molecular testing.

In summary, we identified an infant with HI but without apparent clinical features suggestive of BWS, which was diagnosed by molecular testing as being due to somatic mosaicism of paternal UPD on chromosome 11p15. BWS could be very difficult to diagnose on clinical examination and should be taken into consideration also in children presenting with apparently isolated congenital anomalies of the spectrum of the syndrome, such as hyperinsulinism. Many cases without the typical and well-known facial phenotype are emerging, imposing a new clinical paradigm on the approach to this condition.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Beckwith-Wiedemann 症候群，Sotos 症候群

副島 英伸

Beckwith-Wiedemann 症候群（BWS）

1．概念

BWS は，過成長，巨舌，腹壁欠損（臍帯ヘルニア，臍ヘルニア）を特徴とする先天異常症候群で，ゲノムインプリンティングが関与する代表的な疾患である。症状は多様で，上記症状のほかに，耳垂の線状溝・耳輪後縁の小窩，新生児期の低血糖，腹腔内臓腫大，片側肥大，火焔状母斑，腎奇形などを呈する（**図 1** A）。また，約 10% の患児に Wilms 腫瘍，肝芽腫，横紋筋肉腫など胎児性腫瘍が発生する。

2．頻度

約 13,700 出生に 1 人の頻度と報告されている[1]。男女比は 1：1。85% は孤発例で，15% が家族例である。体外受精や顕微授精などの生殖補助医療（ART）で出産した児では，IC2 低メチル化による BWS 発症のリスクが高まることが報告されているが，議論の余地がある[2]。

3．遺伝子

原因として，IC1 高メチル化，IC2 低メチル化，11p の父性片親性ダイソミー（patUPD），*CDKN1C* の機能喪失変異，11p の染色体構造異常（重複，転座，逆位等）が知られており，いずれもゲノムインプリンティングが関与する。ゲノムインプリンティングは，両親から受け継いだ一対の対立遺伝子のうち，その親の性に従って一方の親由来の遺伝子のみが発現する現象である。責任遺伝子座 11p15.5 には，ドメイン 1 とドメイン 2 の二つのインプリンティングドメインが存在する（**図 2**）。ドメイン内のインプリント遺伝子の発現は，それぞれのインプリンティングセンターである IC1 と IC2 によって独立して制御されている[3,4]。IC は，両アレル間で親由来により DNA メチル化に違いがあるため，DNA メチル化可変領域（differentially

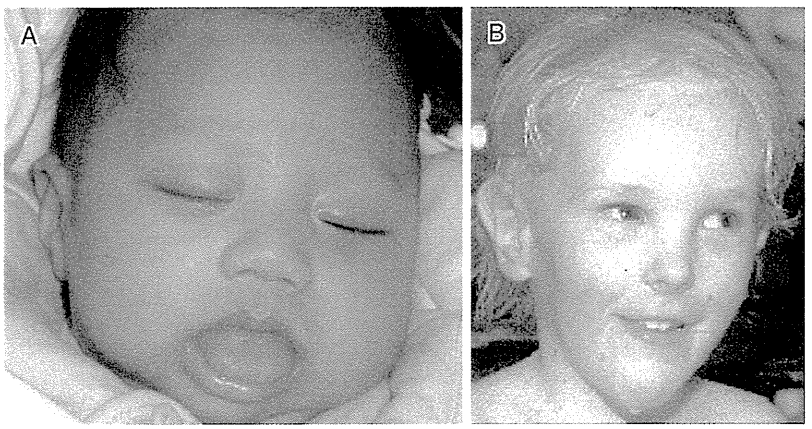

図 1　Beckwith-Wiedemann 症候群（A）と Sotos 症候群（B）（A：Kong ら，2007[7] より転載，B：Tatton-Brown ら，2005[8] より転載）
A：BWS 男児。巨舌を認める
B：5q35 微小欠失の Sotos 症候群

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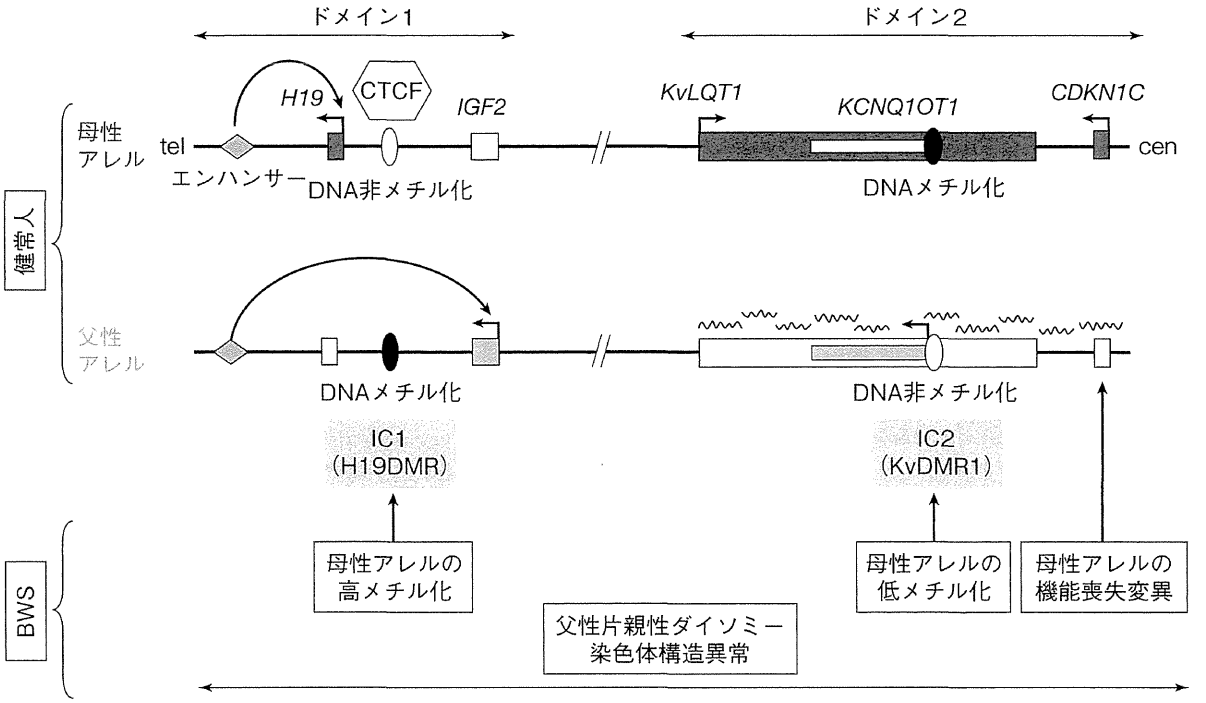

図2　11p15.5のインプリンティングドメインとBWSの遺伝子異常

正常組織のドメイン1では，母性アレルの非メチル化IC1にCTCFが結合してインスレーターとして働き，*H19*下流のエンハンサーをブロックして，*IGF2*への作用を阻害する。BWSでは，母性アレルのIC1が高メチル化されるために両アレルでCTCFの結合が阻害される結果，*IGF2*が両アレル発現する。正常組織のドメイン2では，父性アレルの非メチル化IC2からnon-coding RNAである*KCNQ1OT1*が発現し，シスに作用して*CDKN1C*の発現を抑制している。BWSでは，母性アレルIC2が低メチル化となり*KCNQ1OT1*が発現するようになるため*CDKN1C*の発現が抑制される。*CDKN1C*は母性発現するため，変異が母親から伝わった場合は発症するが，父親から伝わっても発症しない。patUPDの場合は，IC1とIC2の両者を含むため，IC1高メチル化とIC2低メチル化を同時に認める

濃茶：母性発現遺伝子，薄茶：父性発現遺伝子。波線：non-coding RNA，tel：テロメア側，cen：セントロメア側

methylated region：DMR）と呼ばれる。

ドメイン1では，*H19*の上流2-5 kbにIC1（H19-DMR）が存在する。非メチル化母性アレルにCTCFが結合してインスレーターとして働き，*H19*下流のエンハンサーをブロックして，*IGF2*への作用を阻害する。エンハンサーは*H19*に作用するため，母性発現を示す。一方，父性アレルはメチル化によりCTCF結合が阻害されるため，エンハンサーが*IGF2*に作用し，父性発現する。BWSでは，母性アレルのIC1が高メチル化されるために両アレルでCTCFの結合が阻害される結果，*IGF2*が両アレル発現（loss of imprinting：LOI）する。IGF2は細胞増殖因子であるため，その発現量増加がBWSの症状を引き起こす。

IC2（KvDMR1）は*KCNQ1OT1*のプロモーター領域にあり，母性メチル化を示す。父性アレルでは，非メチル化IC2からnon-coding RNAである*KCNQ1OT1*が発現し，シスに作用して*CDKN1C*の発現を抑制している。母性アレルでは，IC2がメチル化しているため*KCNQ1OT1*が発現せず，その結果*CDKN1C*が発現する。BWSでは母性アレルIC2が低メチル化となり，*KCNQ1OT1*が発現するようになるため*CDKN1C*の発現が抑制される。*CDKN1C*は，CDKインヒビターをコードしているので，発現が抑制されることでBWSの症状を引き起こす。

patUPDは11pの部分的な領域の父性ダイソミーであり，IC1とIC2の両者を含む。そのため，IC1高メチル化とIC2低メチル化を同時に認める。遺伝子発現としては，*IGF2*が増加し*CDKN1C*が低

下する。

CDKN1C は母性発現するため，機能喪失変異が母親から伝わった場合は発症するが，父親から伝わっても発症しない。染色体構造異常では，11p の部分トリソミーが比較的多く，父性 11p15 が重複している。

4. 出生前診断

通常 24 週以降，超音波検査における各種の成長パラメーターが在胎週数に比べて過大となる。羊水過多，過長な臍帯，腫大胎盤，腹壁欠損，臓器腫大，腎奇形，口蓋裂，心奇形，巨舌などが認められる。

5. 出生後診断

統一的な診断基準はないが，**表 1** に示した症状のうち主症状三つ以上，または主症状二つと副症状一つ以上を認めた場合に診断できる[3]。症例の約半数が出生 3 日以内に臨床的に診断されている。遺伝学的解析で，IC1 高メチル化，IC2 低メチル化，patUPD，*CDKN1C* 変異のいずれかを検出すると確定診断となる。しかし，異常を検出できるのは 75～80％の症例で，20～25％の症例では認めない。

染色体検査では，11p の重複，転座，逆位の検出が可能である。

6. 診療上の留意

孤発例 BWS の発症原因の頻度と臨床症状との関連を**表 2** に示した。IC1 高メチル化や patUPD の場合，Wilms 腫瘍，肝芽腫のリスクが高くなる。IC2 脱メチル化でも肝芽腫とほかの腫瘍のリスクが高くなるが，IC1 高メチル化や patUPD ほど高くはない。腫瘍発生は 6 歳以下が大半を占めるが，それ以上の年齢でも認めることがあるので，少なくとも小学校卒業まで定期的に検査することが望ましい。

染色体検査で検出できるレベルの 11p15.5 の重複，未熟児，コントロール不良な低血糖があると発達異常を呈することがある。また，成長とともに腹腔内臓器腫大や巨軀などの過成長症状は正常化する。

表 1　BWS 診断基準（Weksberg ら，2010 より引用一部改変）[3]

主症状	・臍帯ヘルニアまたは臍ヘルニア ・巨舌 ・97 パーセンタイルを超える過成長（身長と体重） ・耳垂の線状溝・耳輪後縁の小窩 ・腹腔内臓器腫大（例：肝，腎，脾，膵，副腎） ・小児期の胎児性腫瘍 ・片側肥大 ・胎児副腎皮質の細胞腫大（一般にびまん性，両側性） ・腎奇形（medullary dysplasia，medullary sponge kidney を含む） ・BWS の家族歴 ・口蓋裂
副症状	・羊水過多，腫大胎盤，臍帯肥厚，早期陣痛，早産 ・新生児期低血糖 ・顔面の火焔状母斑 ・心肥大，心奇形，心筋症 ・特徴的顔貌（眼球突出を伴う眼窩下部の溝，顔面中部後退，幅が広く突出した下顎） ・腹直筋解離 ・骨年齢亢進
診断	主症状三つ以上，または主症状二つと副症状一つ以上

鑑別診断として Simpson-Golabi-Behmel 症候群，Costello 症候群，Perlman 症候群，Sotos 症候群，ムコ多糖症 VI 型（Maroteaux-Lamy 症候群）などがある。

7. 治療

低血糖については，生直後から血糖値をモニタリングする。臍帯ヘルニアに対しては外科的根治術を行う。過度の巨舌は，幼児期の食物摂取や呼吸の障害となる。また，下顎骨の成長が過剰に促進され咬合不正を引き起こし，発音障害の原因にもなる。このため舌縮小術が適応となるが，形成外科，歯科口腔外科，言語聴覚の専門家などと手術時期，美容的問題，発語の問題等を総合的に検討する必要がある。片側肥大による下肢長の左右差が顕著な場合は，手術の適応に関して整形外科医にコンサルトする。

表2　BWSにおける遺伝子異常の頻度と症状との関連

遺伝子異常のタイプ	頻度(%)	BWS症状および腫瘍リスクとの関連性
IC2低メチル化	～50	臍帯ヘルニア，片側肥大，ART出生BWS 腫瘍リスク中(～10%) 肝芽腫，Wilms腫瘍以外の腫瘍
IC1高メチル化	2～7	片側肥大 腫瘍リスク高(20～30%) Wilms腫瘍，肝芽腫
patUPD	～20	低血糖・片側肥大 腫瘍リスク高(20～30%) Wilms腫瘍，肝芽腫
*CDKN1C*変異	～10	臍帯ヘルニア，口蓋裂 腫瘍リスク低(<5%)
11番染色体異常 (重複，転座，逆位等)	<2	発達遅滞(重複) 腫瘍リスク不明
上記の異常を認めない	～25	不明 腫瘍リスク不明

8. 予後

小児期以降の予後は良好である。

9. フォローアップ

12歳くらいまでは腹部超音波検査で3カ月ごとに腫瘍をスクリーニングする。また，4歳までは2～3カ月ごとにα-fetoprotein(AFP)も測定する。腎奇形に伴う石灰沈着や腎結石に関しては，年1回の頻度で思春期中期まで腹部超音波検査を行う。

10. 家族会など

BWS親の会(大森敏秀胃腸科クリニック内)

連絡先：〒362-0075　埼玉県上尾市柏座2-8-2

Tel：090-1435-2301

mail：air@m2.hinocatv.ne.jp

ホームページ：www.beckwith-wiedemann.com

なお，遺伝子解析は筆者の研究室で行っているので，詳細はお問い合わせいただきたい。

Sotos症候群

1. 概念

Sotos症候群は，特徴的な顔貌，過成長，学習障害を基本症状とする過成長症候群である(図1D～F)。主な症状として，行動障害，先天性心奇形，新生児黄疸，腎奇形，脊柱側彎，てんかん発作などがある[5]。脳性巨人症ともいわれる。

2. 頻度

約14,000出生に1人とされている。95%以上は孤発例であり，家族例の場合は常染色体優性遺伝形式をとる。

3. 遺伝子

NSD1(nuclear receptor binding SET domain protein 1)のハプロ不全で発症し，約80～90%の患者で*NSD1*遺伝子の異常を認める[6]。*NSD1*の遺伝子座は5p35で，ヒストンH3リシン36(H3K36)メチル化酵素をコードしている。H3K36メチル化は遺伝子の転写伸長にかかわるが，NSD1の標的遺伝子は明らかでない。日本人症例では，*NSD1*を含む5p35領域の微小欠失が約50%を占めるが，日本人以外の症例では遺伝子内変異が60～80%を占める。5p35欠失症例は遺伝子内変異症例に比べて学習障害が重度である。一方，遺伝子内変異症例のほうが高身長を示す。また，心奇形は5p35欠失症例に多い傾向がある[7]。

4. 出生前診断

巨頭症や高身長などは非特異的な所見であるため，超音波検査では正確な診断は難しい。家系内に*NSD1*異常症例が存在する時に限り，羊水およ

表 3　Sotos 症候群の臨床症状(Tatton-Brown ら，2005 より引用一部改変)[7]

基本症状 (90%以上の症例でみられる)	・特徴的顔貌 ・学習障害 ・過成長	
主症状 (15～89%の症例でみられる)	・行動障害 ・頭部 MRI/CT の異常 ・てんかん ・骨年齢促進 ・脊柱側彎 ・関節の過弛緩，扁平足	・新生児期の黄疸，筋緊張低下，哺乳不全 ・心奇形 ・腎奇形 ・母体の妊娠高血圧腎症
関連症状 (2～15%の症例でみられる)	・近視 ・遠視 ・乱視 ・斜視 ・白内障 ・眼振 ・コレステリン腫 ・伝音性難聴 ・頭蓋骨癒合 ・歯数不足 ・漏斗胸 ・脊椎異常 ・第 2・第 3 趾間合指症 ・内反尖足 ・拘縮 ・胃食道逆流	・便秘 ・甲状腺機能低下症 ・高カルシウム血症 ・新生児低血糖 ・臍帯ヘルニア ・鼠径ヘルニア ・停留睾丸 ・陰嚢水腫 ・尿道下裂 ・包茎 ・片側肥大 ・発育不全 ・皮膚の色素沈着過剰 ・皮膚の低色素沈着 ・腫瘍 ・血管腫

び絨毛組織を用いた出生前診断が可能である。

5. 出生後診断

臨床診断は基本症状三つ(特徴的顔貌，学習障害，過成長)があれば可能であり，*NSD1* 異常症例の 90%以上でこれらの基本症状を呈する(**表 3**)。3 症状を満たさない場合は，*NSD1* の遺伝子解析により確定診断できる。特徴的顔貌は，Sotos 症候群に最も特異的であり，特に 1～6 歳にかけて顕著である。頬部紅潮，前頭部の疎な毛髪，前額部の突出，眼瞼裂斜下，細長い顔，細く突き出た下顎を呈する。学習障害については，早期からの発達遅延が非常によくみられる。また，大きな体格，筋緊張低下，協調運動性の低さから運動技能が遅れ，言語発達遅滞もよくみられる。学習障害の程度は軽度から重度まで多様である。過成長については，身長あるいは頭囲が＋2 SD 以上を示す。

鑑別診断として Weaver 症候群，Beckwith-Wiedemann 症候群，Simpson-Golabi-Behmel 症候群，Bannayan-Riley-Ruvalcaba 症候群，脆弱 X 症候群などがある。

6. 診療上の留意

学習障害・心奇形・腎奇形・てんかん発作・脊柱側彎についての詳細な病歴を聴取する。小児の場合，診断がついたら心エコーと腎臓の超音波検査を行い，成人の場合は，腎臓超音波で慢性膀胱尿管逆流による腎障害を検査して，重篤な合併症を検出することが重要である。伝音声難聴の検査の検査も行う。

7. 治療

新生児黄疸に対して光線療法を行う。哺乳不全に対しては経鼻チューブ栄養を行うこともある。胃食道逆流に対しては，体位に注意する。各臨床症状については適切な専門家にコンサルトする。

8. 予後

予後はよい。身長は成長とともに目立たなくなるが，巨頭症は成人後も認められる。

9. フォローアップ

幼児期には，1，2年に一度のフォローを行う。小児期の腫瘍リスクは低いため，腫瘍スクリーニングは推奨されない。

10. 家族会など

Show's Home Page：http://www.askashow.com/

Sotos 症候群についての解説や，相談の場として開設。

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Human Reproduction, Vol.27, No.8 pp. 2541–2548, 2012
Advanced Access publication on June 6, 2012 doi:10.1093/humrep/des197

human reproduction ORIGINAL ARTICLE *Reproductive genetics*

Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies

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Submitted on February 9, 2012; resubmitted on March 29, 2012; accepted on May 1, 2012

BACKGROUND: There is an increased incidence of rare imprinting disorders associated with assisted reproduction technologies (ARTs). The identification of epigenetic changes at imprinted loci in ART infants has led to the suggestion that the techniques themselves may predispose embryos to acquire imprinting errors and diseases. However, it is still unknown at what point(s) these imprinting errors arise, or the risk factors.

METHODS: In 2009 we conducted a Japanese nationwide epidemiological study of four well-known imprinting diseases to determine any association with ART. Using bisulfite sequencing, we examine the DNA methylation status of 22 gametic differentially methylated regions (gDMRs) located within the known imprinted loci in patients with Beckwith-Wiedemann syndrome (BWS, $n = 1$) and also Silver-Russell syndrome (SRS, $n = 5$) born after ART, and compared these with patients conceived naturally.

RESULTS: We found a 10-fold increased frequency of BWS and SRS associated with ART. The majority of ART cases showed aberrant DNA methylation patterns at multiple imprinted loci both maternal and paternal gDMRs (5/6), with both hyper- and hypomethylation events (5/6) and also mosaic methylation errors (5/6). Although our study may have been limited by a small sample number, the fact that many of the changes were mosaic suggested that they occurred after fertilization. In contrast, few of the patients who were conceived naturally exhibited a similar pattern of mosaic alterations. The differences in methylation patterns between the patients who were conceived naturally or after ART did not manifest due to the differences in the disease phenotypes in these imprinting disorders.

CONCLUSION: A possible association between ART and BWS/SRS was found, and we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable.

Key words: assisted reproduction technologies / genomic imprinting / DNA methylation / gametic differentially methylated regions / genomic imprinting disorders

Introduction

Human assisted reproduction technologies (ARTs) are used in the treatment of infertility and involve the manipulation of eggs and/or sperm in the laboratory. Several recent studies have identified an increased incidence of some normally very rare imprinting disorders after ART, including Beckwith-Wiedemann syndrome (BWS: ONIM 130650), Angelman syndrome (AS: ONIM 105830) and Silver-Russell syndrome (SRS: OMIM 180860) but not Prader-Willi syndrome (PWS: OMIM 176270; DeBaun *et al.*, 2003; Gosden *et al.*, 2003; Svensson *et al.*, 2005). Additionally, there are several reports suggesting that epigenetic alterations (epimutations) at imprinted loci occur during the *in vitro* manipulation of the gametes, with both IVF and ICSI approaches implicated (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Moll *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Rossignol *et al.*, 2006; Bowdin *et al.*, 2007; Kagami *et al.*, 2007). However, some studies do not support a link between ART and imprinting disorders (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007).

Epigenetic marks laid down in the male or female germ lines, and which are inherited by the embryos, establish the imprinted expression of a set of developmentally important genes (Surani, 1998). Because imprinted genes are regulated by these gametic epigenetic marks, and by further epigenetic modifications in the somatic cell, they are particularly vulnerable to environmentally induced mutation. One of the best studied epigenetic marks is DNA methylation. DNA methylation is established in either the maternal or paternal germline at discrete genomic loci. This methylation is preserved in the fertilized embryo to generate differentially methylated regions (DMRs) which then signal to nearby genes to establish domains of imprinted chromatin by mechanisms that are not fully understood (John and Lefebvre, 2011). These germline or gametic DMRs (gDMRs) can orchestrate the monoallelic expression of genes over megabases of DNA (Tomizawa *et al.*, 2011) and are reset with every reproductive cycle (Lucifero *et al.*, 2002; Obata and Kono, 2002).

The increased frequency of epimutation(s) at imprinted loci in ART infants has led to the suggestion that ART procedures may induce imprinting error(s). However, these studies are confounded because ART populations are, by their very nature, different from populations who were conceived without the use of ART, with a low fertility rate, an increased frequency of reproductive loss and usually of advanced age, all of which are associated with increased occurrence of fetal and neonatal abnormalities. Furthermore, it is difficult to determine the causality of imprinting errors in any specific abnormality reported after ART. Both IVF and ICSI appear to be associated with an increased relative risk of imprinting disorders (Savage *et al.*, 2011). These procedures are often undertaken for unexpected infertility and require ovarian stimulation, oocyte collection and *in vitro* culture before the embryos are implanted. It has been suggested that infertility and any resulting ovarian stimulation may predispose to epigenetic errors (Sato *et al.*, 2007). Animal studies suggest that *in vitro* embryo culture may be associated with epigenetic alterations. In particular, the large offspring syndrome in cattle undergoing ART is associated with the loss of maternal allele methylation at insulin-like growth factor 2 receptor (*IGF2R*) gDMR (Young *et al.*, 2001) and has phenotypic similarity to BWS. It is still unknown when these imprinting errors arise and what factors predispose to epigenetic changes. Previously, Chang *et al.* (2005) reported no phenotypic differences between BWS patients who were conceived after ART and naturally. However, Lim *et al.* (2009) reported that patients who were conceived after ART had a significantly lower frequency of exomphalos and higher risk of non-Wilms tumor neoplasia. Phenotypic differences between patients who were conceived after ART and naturally are largely unreported, while any changes to phenotype may be altered by the frequency and the degree of epimutations. Studies revealed that some patients with BWS born after ART presented with epimutations that were not restricted to the 11p15 region (Rossignol *et al.*, 2006; Bliek *et al.*, 2009; Lim *et al.*, 2009). Further analysis of abnormal methylation patterns in imprinting disorders may provide clues as to the cause of disease and identify the ART-related risk factor(s).

To address these questions in this study, we engaged in a nationwide epidemiological study of the Japanese population to determine the frequency of four imprinting disorders after natural conception and after ART. We then analyzed the DNA methylation status of 22 gDMRs in BWS and SRS patients conceived by the two routes. Finally, we compared the abnormal methylation patterns and the phenotypes reported for both sets of patients. As a result we found that both BWS and SRS were more frequent after ART and that ART patients exhibited a higher frequency of aberrant DNA methylation patterns at multiple loci with, in some cases, mosaic methylation errors.

Materials and Methods

Nationwide investigation of imprinting disorders

The protocol was established by the Research Committee on the Epidemiology of Intractable Diseases. The protocol consisted of a two-stage postal survey. The first-stage survey was used to estimate the number of individuals with any of the four imprinting diseases: BWS, SRS, PWS and AS. The second-stage survey was used to identify the clinico-epidemiological features of these syndromes.

In the first-stage survey, the pediatric departments of all hospitals were identified based on a listing of hospitals, as at 2008, supplied by the R&D Co. Ltd (Nagoya, Japan). Hospitals were classified into seven categories according to the type of institution and the number of hospital beds. The survey was mailed to a total of 3158 departments in October 2009 with letters of request for participation in recording these diseases. A simple questionnaire was used to ask about the number of patients with any of the four imprinting disorders. Diagnosis was determined by karyotype analyses, genetic analyses and clinical phenotypes by their clinical doctors. In December 2009, a second request was sent to departments that had not responded to the earlier deadline (at the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

The second questionnaires were forwarded to the departments that had reported patients with the imprinting disorders on the first questionnaires. Detailed clinical information for the patients with these imprinting disorders was collected, including the age, gender, growth and development pattern, the methods of the diagnosis, the presence of infertility treatment and the methods of ART where applicable. Duplicate results were excluded using the information regarding the patient's age and gender where available. The study was approved by the Ethics Committee of Tohoku University School of Medicine.

Estimation of prevalence of imprinting disorders

The number of patients, who were diagnosed by genetic and cytogenetic testing and by clinical phenotypes, was obtained from data from the departments who responded to the first survey. The 95% confidence interval (CI) was calculated as previously described (Wakai *et al.*, 1997). The prevalence was determined, based on the population of Japan in 2009 (127 510 000) with data from the Statistics Bureau of the Ministry of Internal Affairs and Communications.

DNA preparation

Genomic DNA was obtained from blood or buccal mucosal cell samples from patients with one of the imprinting disorders using standard extraction methods (Kobayashi *et al.*, 2007). For control DNAs, DNA was prepared from the sperm and cord blood samples from unaffected individuals. The study was performed after obtaining patients or their parents' consent.

Bisulfite-treatment PCR including the SNPs

We first searched for single nucleotide polymorphisms (SNPs) within 22 previously reported human gDMRs (Kikyo *et al.*, 1997; Smith *et al.*, 2003; Kobayashi *et al.*, 2006, 2009; Wood *et al.*, 2007) using 20 control Japanese blood DNA samples. PCR primer sets were designed to span these SNPs (Supplementary data, Table SI) and human sperm DNA and blood DNA was used to confirm that these PCR assays detected the methylation status of the 22 DMRs. Paternal DMRs were shown to be fully methylated in sperm DNA, maternal DMRs were fully unmethylated and in blood DNA, both paternal and maternal DMRs showed ~50% methylation (Supplementary data, Fig. S1). The human gDMRs and the non-imprinted repetitive long interspersed nucleotide element (*LINE1*) and *Alu* repetitive sequences were examined by bisulfite sequencing using established protocols (Kobayashi *et al.*, 2007). Briefly, PCR products were purified and cloned into the pGEM-T vector (Promega, Madison, WI, USA). Individual clones were sequenced using M13 reverse primer and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). On average, 20 clones were sequenced for each sample.

Statistics

The frequency of the manifestation in patients who were conceived after ART was compared with that observed in patients conceived naturally using Fisher's exact test.

Results

Frequency of four imprinting disorders and their association with ART

We first investigated the nationwide frequency of four imprinting disorders (BWS, AS, PWS and SRS) in Japan in the year 2009. Of a total of 3158 departments contacted, 1602 responded to the first-stage survey questionnaire (50.7%). The total number of cases was calculated using a second-stage survey ensuring the exclusion of duplicates (Table I). Using this information, and taking into account the number of patients with suspect clinical signs but without a formal diagnosis, we identified 444 BWS patients (95% CI: 351–538), 949 AS patients (95% CI: 682–1217), 2070 PWS patients (95% CI: 1504–2636) and 326 SRS patients (95% CI: 235–416). From these figures (and using the 2009 population of Japan: 127 510 000) we estimated the prevalence of these syndromes to be 1 in 287 000, 1 in 134 000, 1 in 62 000 and 1 in 392 000, respectively, for BWS, AS, PWS and SRS. Further details are given in Supplementary data, Table SII and Supplementary data, Fig. S2.

Table I The 2009 frequency of four imprinting diseases in Japan in relation to use of assisted reproduction techniques (ART).

Imprinting disorders	Total estimated patient number (95% CI)	The total prevalence of the syndrome	The number of patients after ART/ total (%)
BWS	444 (351–538)	1 in 287 000	6/70 (8.6)
AS	949 (682–1217)	1 in 134 000	2/123 (1.6)
PWS	2070 (1504–2636)	1 in 62 000	4/261 (1.5)
SRS	326 (235–416)	1 in 392 000	4/42 (9.5)

Results of a nationwide epidemiological investigation of four imprinting disorders in Japan, under the governance of the Ministry of Health, Labor and Welfare of the Japanese government. Precise diagnosis was performed using fluorescence *in situ* hybridization and DNA methylation analyses. The type of ART, obtained from the questionnaires, was compared with the frequencies of these diseases and the epimutation rates. BWS, Beckwith-Wiedemann syndrome, AS, Angelman syndrome, PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome.

Between 1997 and 2008, the period during which the ART babies in this study were born, 0.64–0.98% of the total number of babies born in Japan were born as a result of IVF and ICSI. We ascertained the frequency of ART procedures in the cases of BWS, AS, PWS and SRS via the questionnaire sent to doctors (Table I, Supplementary data, Table SIII). The numbers of patients with PWS and AS we identified was low; however, the frequency of ART in these cases was not dissimilar to that expected, based on the population rate of ART use, with 2/123 (1.6%) cases of AS and 4/261 (1.5%) cases of PWS born after ART. In contrast, for BWS and SRS the frequency of ART was nearly 10-fold higher than anticipated with 6/70 (8.6%) BWS and 4/42 (9.5%) SRS patients born after ART.

After analyzing the second questionnaire, the blood or buccal mucosal cell samples were obtained from 15 individuals with BWS, 23 with SRS, 73 with AS and 29 with PWS. Using polymorphic bisulfite-PCR sequencing, we examined the methylation status of gDMRs within these samples at the imprinted regions implicated in these syndromes. For BWS we assayed *H19* and *KCNQ1OT1* (*LIT1*) gDMRs, for SRS we assayed the *H19* gDMR and for PWS and AS we assayed the *SNRPN* gDMR. For all patients (conceived naturally and with ART), the frequencies of DNA methylation errors (epimutations) corrected were 7/15 (46.7%) for BWS, 9/23 (39.1%) for SRS, 6/73 (8.2%) for AS and 2/29 (6.9%) for PWS. When looking at the ART cases exclusively, epimutation rates were 3/5 (BWS), 3/7 (SRS), 0/2 (AS) and 0/2 (PWS).

Abnormal methylation patterns in the ART and naturally conceived SRS patients with epimutations.

While hypomethylation of *H19* at chromosome 11 is known to be a frequent occurrence in SRS (Bliek *et al.*, 2006), various additional loci at chromosomes 7, 8, 15, 17 and 18 have been implicated as having a