Table 1 Original primers used in this study

	Analyzed region	*5′-Forward primer-3′*	*5′-Reverse primer-3′*
qPCR for copy number analysis	*DIS3L2* exon 9	GGCGTGGATTTCTCTGATTT	AAGCCTAGCCCCTAGGAAAG
RT-PCR	Between exons 8 and 11	TTTATGTGCCTCTCAAGGAC	AGCAATGTGAACTCCCACTT
Identification of deletion junction	Deletion junction	1a: ACTGATTGAAGCAGCCAACT	2: AGGACAAAAGGAAGCAAGTG
		1b: TGAAGCAGCCAACTCCAAAT	
		3: CCTCTTACCTCAGCCTACCA	4: GAAGTCAGTGTGGCGATTCC
		5: TATTCCCCTTCCTGTGTCCA	6: GGTGACATGATGAAACCTCACTT

All coding exons, from exon 2 to exon 21, of *DIS3L2* were amplified using primer pairs described previously.[2] Primer sequences for *GAPDH* and *TAT*, which were used as internal controls for qPCR, were the same as described in previous reports.[3,4]

L1-B in the parents were amplified and sequenced directly. L1-A sequences were full-length and identical between father and mother with 99.2% similarity to the L1Hs reference sequence obtained from Repbase on the Genetic Information Research Institute (GIRI) website.[5] L1-B sequences, which produced a 5′ truncated form with 98.6% similarity to the reference, were also identical between father and mother. The sequence similarity was 99.0% between L1-A and L1-B; however, nucleotide differences were found at 45 positions (Figures 1d and f). In addition, the mutant alleles in both father and mother were successfully amplified by PCR from the parents and the patient (Figure 1e). A sequence comparison among L1-A, L1-B and the mutant alleles revealed that the deletion junctions of each parental allele were different. The paternal deletion junction lay within an interval of 1578 nt corresponding to nucleotides 3377 to 4954 of the reference, whereas the maternal junction lay within an interval of 565 nt corresponding to nucleotides 4956 to 5520 of the reference (Figure 1f, Supplementary Figure S1). Furthermore, a nucleotide difference at position 4955 was heterozygous (T/C) in the patient, supporting the existence of both mutant alleles in the patient (Figure 1f). The results indicated that the deletion was caused by NAHR between the two L1 elements and strongly suggested that the two NAHR events occurred independently in the ancestors of each parent.

DISCUSSION

In this study, we found NAHR between the two L1 elements as the causative mechanism of *DIS3L2* exon 9 deletion. We also found that the deletion junctions of each parental allele were different, suggesting the occurrence of two independent NAHRs in the ancestors of each parent.

L1s account for 17% of the human genome.[6] A full-length L1 is ~6 kb and encodes two ORFs (ORF1 and ORF2), which are required for retrotransposition. Mobilization of L1s created several hundred species-specific insertions in humans and chimpanzees, and L1s are still actively expanding in humans, resulting in polymorphisms of L1 elements among individuals.[7,8] L1s are mutagenic agents capable of causing human disease as a result of insertion mutations or insertion-mediated deletions by retrotransposition and NAHR between L1 elements. Twenty-five L1 retrotransposition events have been reported to result in single-gene diseases to date.[6] Although *Alu*-mediated NAHR contributes to a large variety of genetic disorders, L1-mediated NAHR and human endogenous retrovirus-mediated NAHR are very rare causes of human diseases.[9–12] Only three human diseases – glycogen storage disease type IXb, Alport syndrome-diffuse leiomyomatosis, and Ellis-van Creveld syndrome – have been reported to be caused by L1-mediated NAHR.[13–15] To our knowledge, this is the fourth NAHR event to cause human disease, in this case Perlman syndrome. Several possible explanations for the rareness of L1-mediated NAHR have been posed: (1) L1s locate in gene-poor regions, such that recombination events are clinically silent; (2) frequent and extensive mutations over evolutionary time have limited the homology among elements; (3) L1s occur at longer intervals, rendering recombinations involving collinear elements unlikely.[13] The NAHR found in this study occurred in a gene, *DIS3L2*. The similarity between L1-A and L1-B was high (99.0%), and the interval was shorter than that of the human lineage-specific L1 recombination-associated deletion (~450 kb).[16] These conditions might enable the L1-mediated NAHR to cause disease, although the possibility of microhomology-mediated replication-dependent recombination models, such as fork stalling and template switching, microhomology-mediated break-induced replication and serial replication slippage, could not be ruled out.[17] The deletion size of exon 9 in the patients reported by Astuti *et al*[2], found in two Dutch pedigrees and one cell line established from a Caucasian patient, strongly suggests the same mechanism at work, although this was not mentioned. In our study, we suggest that two independent NAHRs in ancestors of a Japanese patient occurred. Taken together, this suggests that the region including exon 9 of *DIS3L2* might be a hot spot of L1-mediated NAHR. Other disease-causing L1-mediated NAHRs should be studied and analyzed to clarify the precise mechanism.

Perlman syndrome predisposes to Wilms tumor, the most common childhood malignancy, whereas the other three diseases caused by L1-mediated NAHR are not associated with malignancy. The difference in a predisposition to malignancy would depend on the function of the causative genes, not on the genomic instability because of NAHR, because unlike the other genes, *DIS3L2* shows tumor-suppressor activity.[2]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Novel mutations of CDKN1C *in Japanese patients with Beckwith-Wiedemann syndrome*

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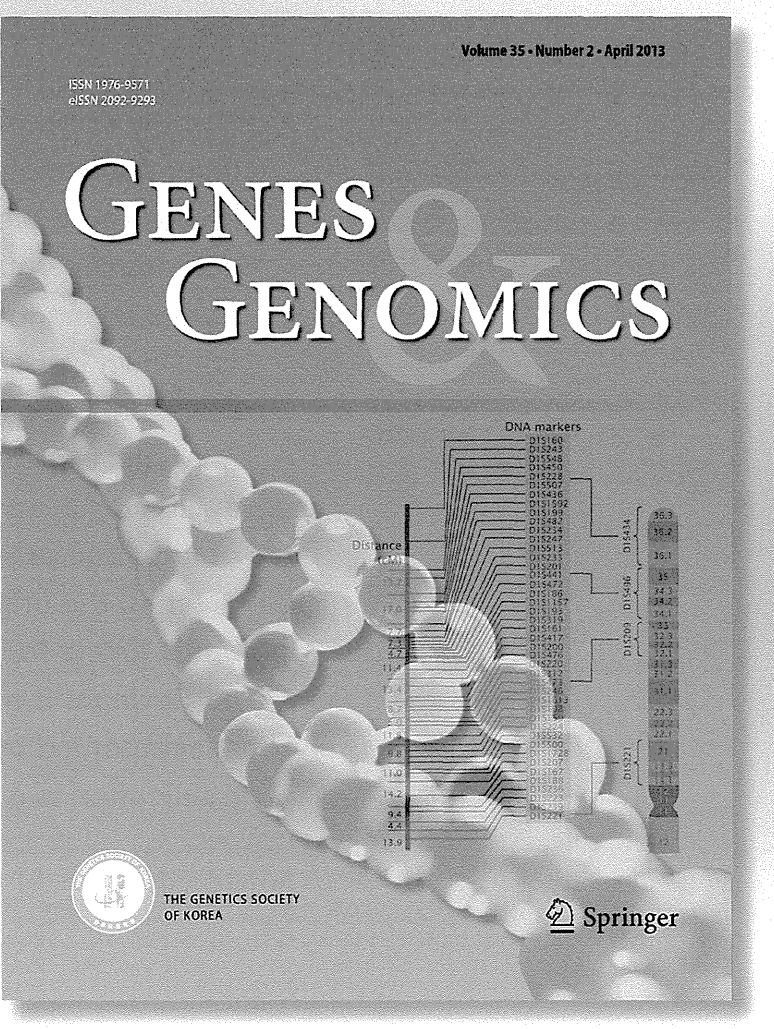

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RESEARCH ARTICLE

Novel mutations of *CDKN1C* in Japanese patients with Beckwith-Wiedemann syndrome

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Abstract Beckwith-Wiedemann syndrome (BWS) is an imprinting-related human disease that is characterized by macrosomia, macroglossia, abdominal wall defects, and variable minor features. BWS is caused by several genetic/epigenetic alterations, such as loss of methylation at KvDMR1, gain of methylation at H19-DMR, paternal uniparental disomy of chromosome 11, *CDKN1C* mutations, and structural abnormalities of chromosome 11. *CDKN1C* is an imprinted gene with maternal preferential expression, encoding for a cyclin-dependent kinase (CDK) inhibitor. Mutations in *CDKN1C* are found in 40 % of familial BWS cases with dominant maternal transmission and in ~5 % of sporadic cases. In this study, we searched for *CDKN1C* mutations in 37 BWS cases that had no evidence for other alterations. We found five mutations—four novel and one known—from a total of six patients. Four were maternally inherited and one was a de novo mutation. Two frame-shift mutations and one nonsense mutation abolished the QT domain, containing a PCNA-binding domain and a nuclear localization signal. Two missense mutations occurred in the CDK inhibitory domain, diminishing its inhibitory function. The above-mentioned mutations were predicted by *in silico* analysis to lead to loss of function; therefore, we strongly suspect that such anomalies are causative in the etiology of BWS.

Keywords Beckwith-Wiedemann syndrome · *CDKN1C* · Gene mutation · Genomic imprinting

Introduction

Beckwith-Wiedemann syndrome (BWS) (OMIM #130650) is an imprinting-related human disease that is characterized by the peculiar traits of prenatal and postnatal macrosomia, macroglossia, abdominal wall defects, and variable minor features. Genomic imprinting, an epigenetic phenomenon, is responsible for parent-of-origin-specific gene expression. The relevant imprinted chromosomal region in BWS, 11p15.5, consists of two independent imprinted domains, *IGF2/H19* and *CDKN1C/KCNQ1OT1*. Imprinted genes within each domain are regulated by two imprinting control regions (ICR): the differentially methylated region associated with H19 (H19-DMR) or KvDMR1 (Weksberg et al.

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2010; Choufani et al. 2010). Approximately 85 % of BWS cases are sporadic; the other 15 % are familial. Several causative alterations have been identified for sporadic cases of BWS: loss of methylation (LOM) at KvDMR1 (~50 %), gain of methylation (GOM) at H19-DMR (2–7 %), mosaic paternal uniparental disomy (UPD; ~20 %), *CDKN1C* mutations (~5 %), duplications of 11p15 (<1 %), and inversions or translocations involving 11p15 (<1 %) (Weksberg et al. 2010; Choufani et al. 2010; Sasaki et al. 2007). However, for approximately 15 % of all BWS cases, no alteration of 11p15.5 has been found.

CDKN1C is an imprinted gene with maternal preferential expression and contains three exons divided by two introns. The first two exons encode a 316 amino acid protein, a cyclin-dependent kinase (CDK) inhibitor, which is a strong inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation (Lee et al. 1995; Matsuoka et al. 1995). The CDKN1C protein consists of three distinct domains, including a CDK inhibitory (CKI) domain, a proline and alanine (PAPA) repeat domain, and a QT domain. The CKI domain contains a cyclin-binding region, a CDK-binding region, and a 3_{10} helix, which is both necessary and sufficient to bind and inhibit CDK activity (Lee et al. 1995; Matsuoka et al. 1995; Borriello et al. 2011). PAPA repeats interact with the LIM domain kinase 1 (LIMK-1) and regulates actin dynamics (Yokoo et al. 2003; Vlachos and Joseph 2009; Borriello et al. 2011). The QT domain contains a PCNA-binding domain, which can prevent DNA replication in vitro and S phase entry in vivo, and a nuclear localization signal (NLS) (Lee et al. 1995; Watanabe et al. 1998; Borriello et al. 2011). Dominant maternal transmission of germline *CDKN1C* mutations causes 40 % of familial BWS cases, and the mutation is found in ~5 % of sporadic cases as mentioned above (Weksberg et al., 2010; Choufani et al. 2010). Since it is located within the *CDKN1C/KCNQ1OT1* domain and is regulated by KvDMR1, LOM at KvDMR1 induces suppression of its transcription, leading to BWS phenotypes (Diaz-Meyer et al. 2003; Higashimoto et al. 2003; Soejima et al. 2004). Therefore, a loss of CDKN1C function due to either genetic or epigenetic alterations causes BWS, indicating its importance in the pathogenesis of this disease.

In this study, we searched for *CDKN1C* mutations in 37 BWS cases that did not show any alterations like LOM at KvDMR1, GOM at H19-DMR, paternal UPD, and chromosomal abnormalities. We found four novel mutations and one known mutation in six patients.

Materials and methods

Patients

Thirty-seven patients who were clinically diagnosed with BWS, but who did not display causative alterations like LOM at KvDMR1, GOM at H19-DMR, paternal UPD of chromosome 11, and structural chromosomal abnormalities (data not shown), were subjected to a *CDKN1C* mutation search. We used three criteria for clinical diagnosis (Elliott et al. 1994; DeBaun and Tucker 1998; Weksberg et al. 2001), and all patients met at least one of them. Patients 2 and 3 were siblings. Patient 5 was also diagnosed as a long QT syndrome type 3 case (OMIM #603830) with confirmed mutation of *SCN5A* (data not shown). Patient 6 was clinically diagnosed as a tuberous sclerosis case (OMIM #191100) based on medical criteria. This study was approved by the Ethics Committee for Human Genome and Gene Analyses of the Faculty of Medicine, Saga University, Japan.

Mutation search of *CDKN1C*

Genomic DNA was extracted from the peripheral blood of patients and their family members. Five regions covering coding sequences and all exon–intron borders were amplified by polymerase chain reaction (PCR) and directly sequenced with Applied Biosystems 3130 Genetic Analyzer (New York, USA) as previously described (Hatada et al. 1996; Hatada et al. 1997). The primers used in this study are shown in Table 1. The mutations in Patients 1, 2, 3, 4, and 5 were confirmed by digestion at restriction sites, which were affected by the mutations, with appropriate restriction enzymes. The mutation in Patient 6 was confirmed by sequencing of the plural clones into which PCR fragments were cloned. Genomic DNA from 100 volunteer individuals was collected with written informed consent and used to search the prevalence of non-synonymous substitutions.

Table 1 Primers used for mutation search of *CDKN1C*

Analyzed region	Forward primer	Reverse primer
A	5′-CGTTCCACAGGCCAAGTGCG-3′	5′-GCTGGTGCGCACTAGTACTG-3′
B	5′-CGTCCCTCCGCAGCACATCC-3′	5′-CCTGCACCGTCTCGCGGTAG-3′
C	5′-TGGACCGAAGTGGACAGCGA-3′	5′-AGTGCAGCTGGTCAGCGAGA-3′
F	5′-CCGGAGCAGCTGCCTAGTGTC-3′	5′-CTTTAATGCCACGGGAGGAGG-3′
H	5′-CGGCGACGTAAACAAAGCTG-3′	5′-GGTTGCTGCTACATGAACGG-3′

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

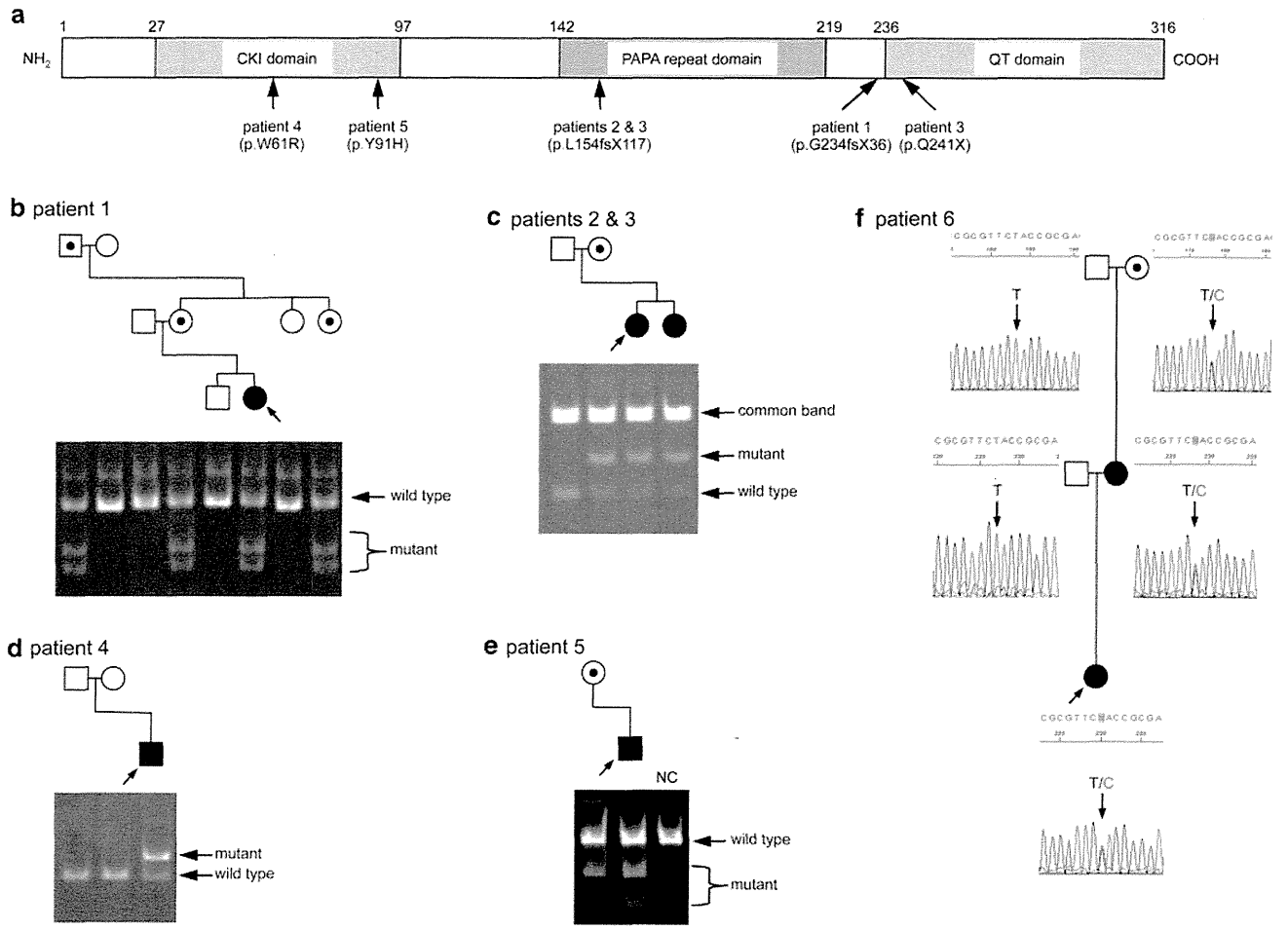

Fig. 1 *CDKN1C* mutations and their inheritance. **a** Domain structure of the CDKN1C protein and position of each mutation. Amino acid residues are indicated above. **b** Pedigree chart of Patient 1. *Bss*HII digestion of PCR region F was used to distinguish between the mutant and wild type alleles. 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.

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

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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

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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

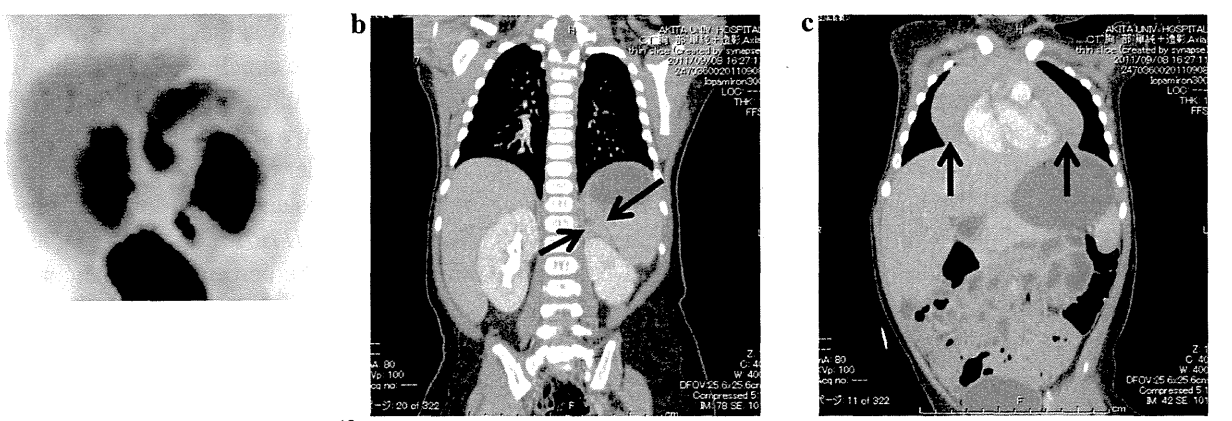


Fig. 1 (a) Representative patterns of [^{18}F]DOPA uptake. Maximum intensity projection obtained 30 min after injection. Multifocal or diffuse uptake in the head and body of the pancreas. (b) CT with contrast showed a mass adjacent to the upper segment of the left kidney (arrows). (c) CT with contrast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

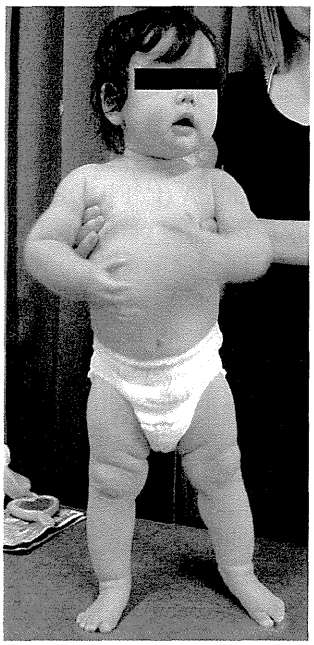


Fig. 2 Patient at the age of 8 months without apparent clinical features suggestive of BWS.

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

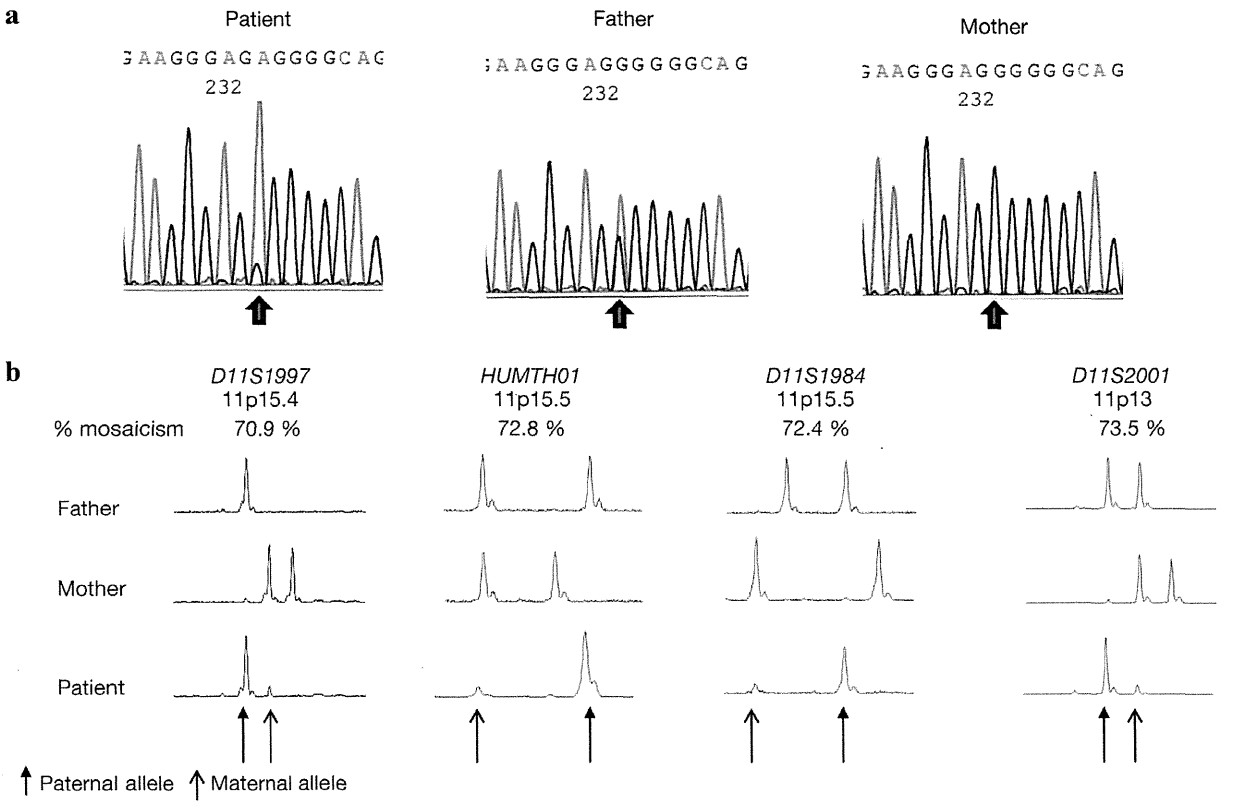
To analyze paternal UPD, genomic DNA was extracted from peripheral blood lymphocytes of the patient and her parents. For quantitative polymorphism analyses, tetranucleotide repeat markers (*D11S1997*, *HUMTH01*, and *D11S1984*) from 11p15.4-p15.5 were amplified and separated by electrophoresis on an 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

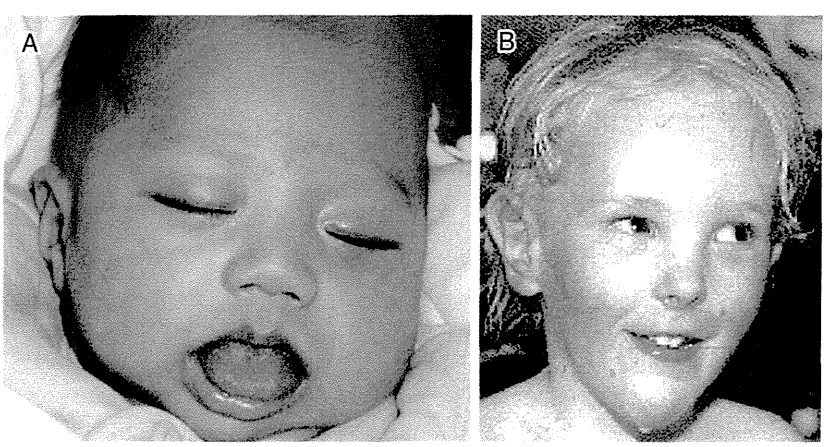

図 1　Beckwith-Wiedemann 症候群(A)と Sotos 症候群(B)(A：Kong ら，2007[7]より転載，B：Tatton-Brown ら，2005[8]より転載)
A：BWS 男児。巨舌を認める
B：5q35 微小欠失の Sotos 症候群

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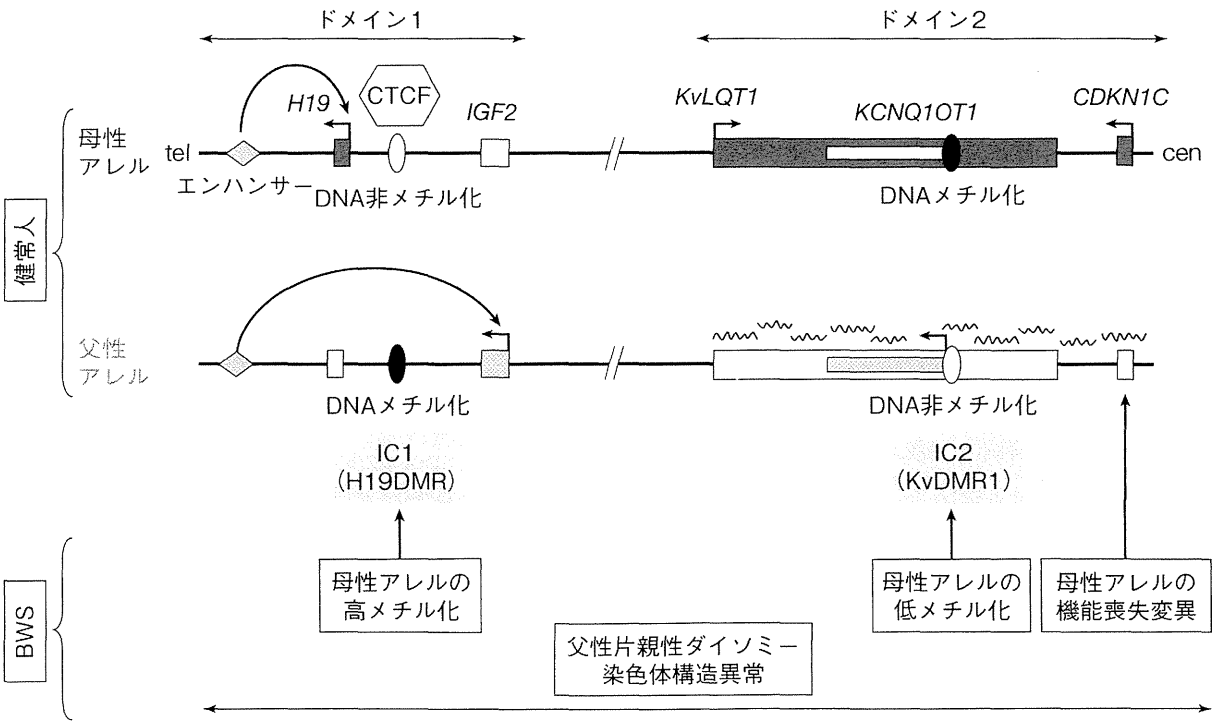

図2　11p15.5のインプリンティングドメインとBWSの遺伝子異常

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。