Choufani S, Shuman C, Weksberg R. Beckwith-Wiedemann syndrome. *Am J Med Genet Part C Semin Med Genet* 2010;**154C**:343–354.

Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL, Horsthemke B. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am J Hum Genet* 2002;**71**:162–164.

Cui H, Horon IL, Ohlsson R, Hamilton SR, Feinberg AP. Loss of imprinting in normal tissue of colorectal cancer patients with microsatellite instability. *Nat Med* 1998;**4**:1276–1280.

DeBaun MR, Niemitz EL, Feinberg AP. Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet* 2003;**72**:156–160.

Doornbos ME, Maas SM, McDonnell J, Vermeiden JP, Hennekam RC. Infertility, assisted reproduction technologies and imprinting disturbances: a Dutch study. *Hum Reprod* 2007;**22**:2476–2480.

Emiliani S, Van den Bergh M, Vannin AS, Biramane J, Englert Y. Comparison of ethylene glycol, 1,2-propanediol and glycerol for cryopreservation of slow-cooled mouse zygotes, 4-cell embryos and blastocysts. *Hum Reprod* 2000;**15**:905–910.

Galli-Tsinopoulou A, Emmanouilidou E, Karagianni P, Grigoriadou M, Kirkos J, Varlamis GS. A female infant with Silver Russell syndrome, mesocardia and enlargement of the clitoris. *Hormones (Athens)* 2008; **7**:77–81.

Gicquel C, Gaston V, Mandelbaum J, Siffroi JP, Flahault A, Le Bouc Y. In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. *Am J Hum Genet* 2003;**72**:1338–1341.

Gosden R, Trasler J, Lucifero D, Faddy M. Rare congenital disorders, imprinted genes, and assisted reproductive technology. *Lancet* 2003; **361**:1975–1977.

Honda S, Weigel A, Hjelmeland LM, Handa JT. Induction of telomere shortening and replicative senescence by cryopreservation. *Biochem Biophys Res Commun* 2001;**282**:493–498.

John RM, Lefebvre L. Developmental regulation of somatic imprints. *Differentiation* 2011;**81**:270–280.

Kagami M, Nagai T, Fukami M, Yamazawa K, Ogata T. Silver-Russell syndrome in a girl born after in vitro fertilization: partial hypermethylation at the differentially methylated region of PEG1/ MEST. *J Assist Reprod Genet* 2007;**24**:131–136.

Kikyo N, Williamson CM, John RM, Barton SC, Beechey CV, Ball ST, Cattanach BM, Surani MA, Peters J. Genetic and functional analysis of neuronatin in mice with maternal or paternal duplication of distal Chr 2. *Dev Biol* 1997;**190**:66–77.

Kobayashi H, Suda C, Abe T, Kohara Y, Ikemura T, Sasaki H. Bisulfite sequencing and dinucleotide content analysis of 15 imprinted mouse differentially methylated regions (DMRs): paternally methylated DMRs contain less CpGs than maternally methylated DMRs. *Cytogenet Genome Res* 2006;**113**:130–137.

Kobayashi H, Sato A, Otsu E, Hiura H, Tomatsu C, Utsunomiya T, Sasaki H, Yaegashi N, Arima T. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. *Hum Mol Genet* 2007;**16**:2542–2551.

Kobayashi H, Yamada K, Morita S, Hiura H, Fukuda A, Kagami M, Ogata T, Hata K, Sotomaru Y, Kono T. Identification of the mouse paternally expressed imprinted gene Zdbf2 on chromosome 1 and its imprinted human homolog ZDBF2 on chromosome 2. *Genomics* 2009; **93**:461–472.

Lidegaard O, Pinborg A, Andersen AN. Imprinting diseases and IVF: Danish National IVF cohort study. *Hum Reprod* 2005;**20**:950–954.

Lim D, Bowdin SC, Tee L, Kirby GA, Blair E, Fryer A, Lam W, Oley C, Cole T, Brueton LA *et al.* Clinical and molecular genetic features of Beckwith-Wiedemann syndrome associated with assisted reproductive technologies. *Hum Reprod* 2009;**24**:741–747.

Lim DH, Maher ER. Human imprinting syndromes. *Epigenomics* 2009; **1**:347–369.

Lucifero D, Mertineit C, Clarke HJ, Bestor TH, Trasler JM. Methylation dynamics of imprinted genes in mouse germ cells. *Genomics* 2002; **79**:530–538.

Ludwig M, Katalinic A, Gross S, Sutcliffe A, Varon R, Horsthemke B. Increased prevalence of imprinting defects in patients with Angelman syndrome born to subfertile couples. *J Med Genet* 2005;**42**:289–291.

Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, Macdonald F, Sampson JR, Barratt CL, Reik W *et al.* Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). *J Med Genet* 2003;**40**:62–64.

Marques CJ, Carvalho F, Sousa M, Barros A. Genomic imprinting in disruptive spermatogenesis. *Lancet* 2004;**363**:1700–1702.

Marques CJ, Costa P, Vaz B, Carvalho F, Fernandes S, Barros A, Sousa M. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. *Mol Hum Reprod* 2008;**14**:67–74.

Miura K, Niikawa N. Do monochorionic dizygotic twins increase after pregnancy by assisted reproductive technology? *J Hum Genet* 2005; **50**:1–6.

Moll AC, Imhof SM, Cruysberg JR, Schouten-van Meeteren AY, Boers M, van Leeuwen FE. Incidence of retinoblastoma in children born after in-vitro fertilisation. *Lancet* 2003;**361**:309–310.

Obata Y, Kono T. Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *J Biol Chem* 2002; **277**:5285–5289.

Okamoto K, Morison IM, Taniguchi T, Reeve AE. Epigenetic changes at the insulin-like growth factor II/H19 locus in developing kidney is an early event in Wilms tumorigenesis. *Proc Natl Acad Sci USA* 1997; **94**:5367–5371.

Orstavik KH, Eiklid K, van der Hagen CB, Spetalen S, Kierulf K, Skjeldal O, Buiting K. Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. *Am J Hum Genet* 2003;**72**:218–219.

Rossignol S, Steunou V, Chalas C, Kerjean A, Rigolet M, Viegas-Pequignot E, Jouannet P, Le Bouc Y, Gicquel C. The epigenetic imprinting defect of patients with Beckwith-Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region. *J Med Genet* 2006;**43**:902–907.

Sato A, Otsu E, Negishi H, Utsunomiya T, Arima T. Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Hum Reprod* 2007;**22**:26–35.

Savage T, Peek J, Hofman PL, Cutfield WS. Childhood outcomes of assisted reproductive technology. *Hum Reprod* 2011;**26**:2392–2400.

Shah PS, Weksberg R, Chitayat D. Overgrowth with severe developmental delay following IVF/ICSI: a newly recognized syndrome? *Am J Med Genet A* 2006;**140**:1312–1315.

Shimizu Y, Fukuda J, Sato W, Kumagai J, Hirano H, Tanaka T. First-trimester diagnosis of conjoined twins after in-vitro fertilization-embryo transfer (IVF-ET) at blastocyst stage. *Ultrasound Obstet Gynecol* 2004;**24**:208–209.

Smith RJ, Dean W, Konfortova G, Kelsey G. Identification of novel imprinted genes in a genome-wide screen for maternal methylation. *Genome Res* 2003;**13**:558–569.

Surani MA. Imprinting and the initiation of gene silencing in the germ line. *Cell* 1998;**93**:309–312.

Svensson J, Bjornstahl A, Ivarsson SA. Increased risk of Silver-Russell syndrome after in vitro fertilization? *Acta Paediatr* 2005;**94**:1163–1165.

Tomizawa S, Kobayashi H, Watanabe T, Andrews S, Hata K, Kelsey G, Sasaki H. Dynamic stage-specific changes in imprinted differentially methylated regions during early mammalian development and prevalence of non-CpG methylation in oocytes. *Development* 2011;**138**:811–820.

Wakai K, Tamakoshi A, Ikezaki K, Fukui M, Kawamura T, Aoki R, Kojima M, Lin Y, Ohno Y. Epidemiological features of moyamoya disease in Japan: findings from a nationwide survey. *Clin Neurol Neurosurg* 1997;**99**(Suppl. 2):S1–S5.

Wood AJ, Roberts RG, Monk D, Moore GE, Schulz R, Oakey RJ. A screen for retrotransposed imprinted genes reveals an association between X chromosome homology and maternal germ-line methylation. *PLoS Genet* 2007;**3**:e20.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* 2001;**27**:153–154.

ORIGINAL

Advance Publication
doi: 10.1507/endocrj.EJ12-0242

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

Hiroyuki Adachi[1], Ikuko Takahashi[1], Ken Higashimoto[2], Satoko Tsuchida[1], Atsuko Noguchi[1], Hiroaki Tamura[1], Hirokazu Arai[1], Tomoo Ito[1], Michiya Masue[3], Hironori Nishibori[4], Tsutomu Takahashi[1] and Hidenobu Soejima[2]

[1] *Department of Pediatrics, Akita University Graduate School of Medicine, Akita, Japan*
[2] *Division of Molecular Genetics and Epigenetics, Department of Biomolecular Sciences, Saga University, Saga, Japan*
[3] *Department of Pediatrics, Kizawa Memorial Hospital, Gifu, Japan*
[4] *Department of Radiology, Kizawa Memorial Hospital, Gifu, Japan*

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
Correspondence to: Ikuko Takahashi, M.D., Department of Pediatrics, Akita Graduate School of Medicine, Akita University, 1-1-1 Hondo, Akita-shi, Akita, 010-8543, Japan.
E-mail: takaiku@doc.med.akita-u.ac.jp

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 ultrasosography 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

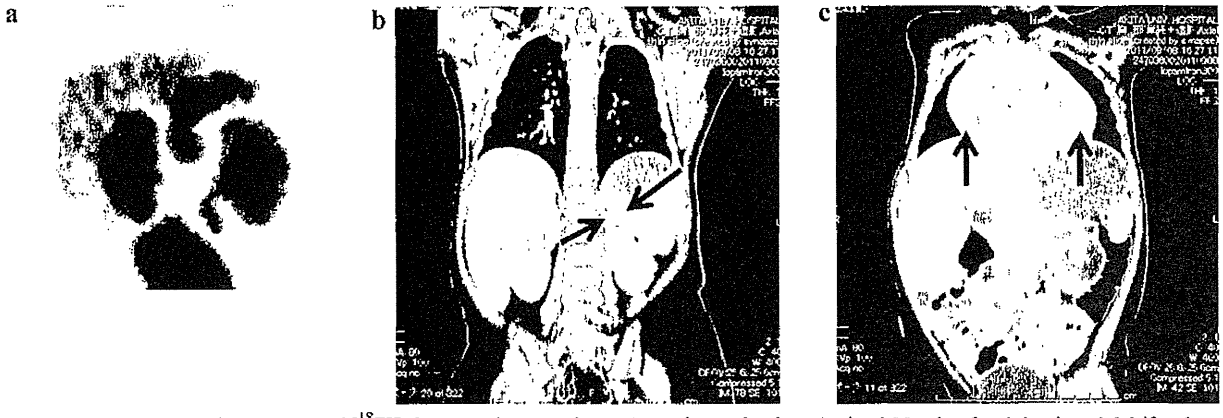


Fig. 1 (a) Representative patterns of [^{18}F]DOPA uptake. Maximum intensity projection obtained 30 min after injection. Multifocal or diffuse uptake in the head and body of the pancreas. (b) CT with contrast showed a mass adjacent to the upper segment of the left kidney. (c) CT with contrast showed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

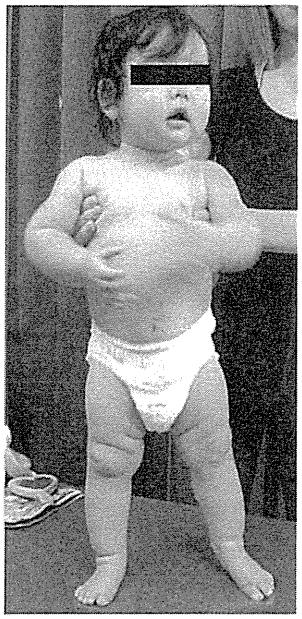


Fig. 2 Patient at the age of 8 months without apparent clinical features suggestive of BWS.

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

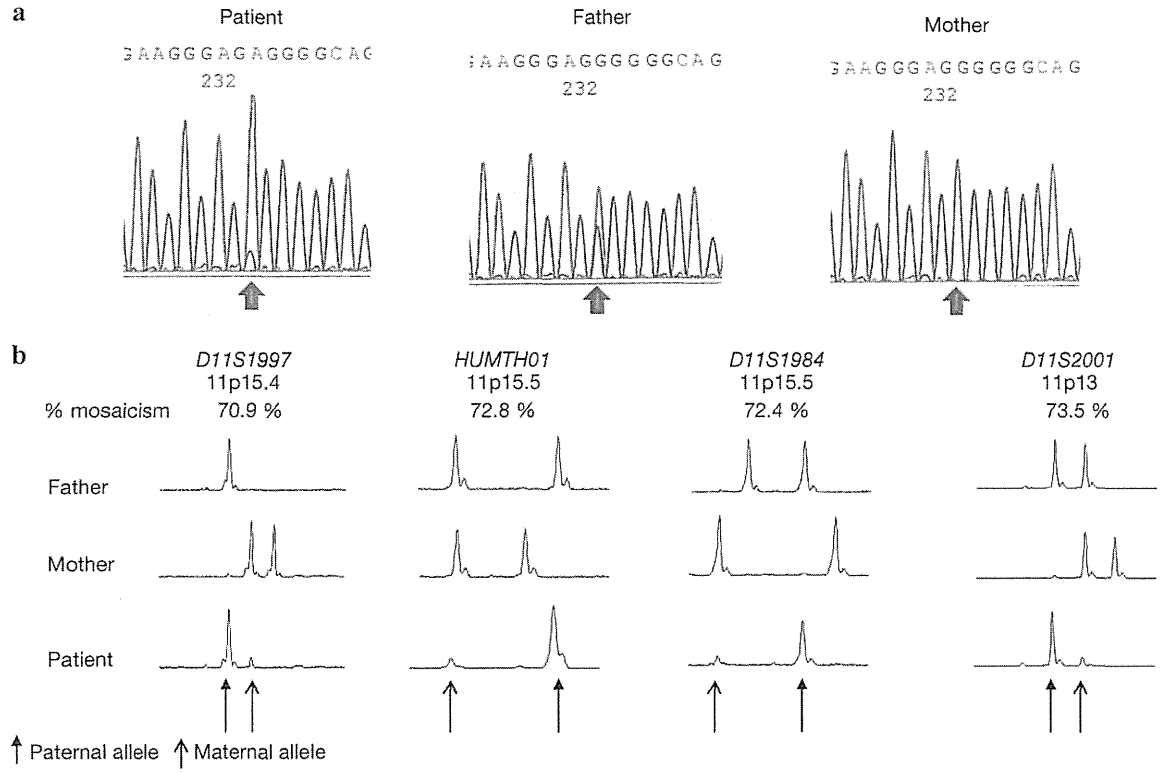
To analyze paternal UPD, genomic DNA was extracted from peripheral blood lymphocytes of the patient and her parents. For quantitative polymorphism analyses, tetranucleotide repeat markers (*D11S1997*, *HUMTH01*, and *D11S1984*) from 11p15.4-p15.5 were amplified and separated by electrophoresis on an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

References

1. Choufani S, Shuman C, Weksberg R (2010) Beckwith-Wiedemann syndrome. *Am J Med Genet C* 154C: 343-354.
2. Weksberg R, Shuman C, Beckwith JB (2010)Beckwith-Wiedemann syndrome. *Eur J Hum Genet* 18: 8-14.
3. Descartes M, Romp R, Franklin J, Biggio JR, Zehnbauer B (2008) Constitutional H19 hypermethylation in a patient with isolated cardiac tumor. *Am J Med Genet A* 146A: 2126-2129.
4. Arnoux JB, de Lonlay P, Ribeiro MJ, Hussain K, Blankenstein O, et al. (2010) Congenital hyperinsulinism. *Early Hum Dev* 86: 287-294.
5. Arnoux JB, Verkarre V, Saint-Martin C, Montravers F, Brassier A, et al. (2011) Congenital hyperinsulinism: current trends in diagnosis and therapy. *Orphanet J Rare Dis* 6: 63.
6. Ribeiro MJ, De Lonlay P, Delzescaux T, Boddaert N, Jaubert F, et al. (2005) Characterization of hyperinsulinism in infancy assessed with PET and 18F-fluoro-L-DOPA. *J Nucl Med* 46: 560-566.
7. Sasaki K, Soejima H, Higashimoto K, Yatsuki H, Ohashi H, et al. (2007) Japanese and North American/European patients with Beckwith-Wiedemann syndrome have different frequencies of some epigenetic and genetic alterations. *Eur J Hum Genet* 15: 1205-1210.
8. Hatada I, Ohashi H, Fukushima Y, Kaneko Y, Inoue M, et al. (1996) An imprinted gene $p57^{KIP2}$ is mutated in Beckwith–Wiedemann syndrome. *Nature Genet* 14:171-173.
9. Balcom RJ, Hakanson DO, Werner A, Gordon LP, et al. (1985) Massive thymic hyperplasia in an infant with Beckwith-Wiedemann syndrome. *Arch Pathol Lab Med* 109: 153-155.
10. Shuman C, Smith AC, Steele L, Ray PN, Clericuzio C, et al. (2006) Constitutional UPD for chromosome 11p15 in individuals with isolated hemihyperplasia is associated with high tumor risk and occurs following assisted reproductive technologies. *Am J Med Genet A* 140A: 1497-1503.
11. Scott RH, Douglas J, Baskcomb L, Huxter N, Barker K, et al. (2008) Constitutional 11p15 abnormalities, including heritable imprinting center mutations, cause nonsyndromic Wilms tumor. *Nat Genet* 40: 1329-1334.
12. Goldman M, Smith A, Shuman C, Caluseriu O, Wei C, et al. (2002) Renal abnormalities in beckwith-wiedemann syndrome are associated with 11p15.5 uniparental disomy. *J Am Soc Nephrol* 13: 2077-2084.
13. Wong CA, Cuda S, Kirsch A (2011) A review of the urologic manifestations of Beckwith-Wiedemann syndrome. *J Pediatr Urol* 7: 140-144.
14. Mussa A, Peruzzi L, Chiesa N, De Crescenzo A, Russo S, et al. (2012) Nephrological findings and genotype-phenotype correlation in Beckwith-Wiedemann syndrome. *Pediatr Nephrol* 27: 397-406.
15. Munns CF, Batch JA (2001) Hyperinsulinism and Beckwith-Wiedemann syndrome. *Arch Dis Child Fetal Neonatal Ed* 84: F67-69.
16. Hussain K, Cosgrove KE, Shepherd RM, Luharia A, Smith VV, et al. (2005) Hyperinsulinemic hypoglycemia in Beckwith-Wiedemann syndrome due to defects in the function of pancreatic beta-cell adenosine triphosphate-sensitive potassium channels. *J Clin Endocrinol Metab* 90: 4376-4382.
17. Kotzot D (2008) Complex and segmental uniparental disomy updated. *J Med Genet* 45:545-556.
18. Otonkoski T, Näntö-Salonen K, Seppänen M, Veijola R, Huopio H, et al. (2006) Noninvasive diagnosis of focal hyperinsulinism of infancy with [18F]-DOPA positron emission tomography. *Diabetes* 55: 13-18.
19. Rahman N (2005) Mechanisms predisposing to childhood overgrowth and cancer. *Curr Opin Genet Dev* 15: 227-233.
20. Rump P, Zeegers MP, van Essen AJ (2005) Tumor risk in Beckwith-Wiedemann syndrome: A review and meta-analysis. *Am J Med Genet A* 136: 95-104.

Genes Genom
DOI 10.1007/s13258-013-0079-3

RESEARCH ARTICLE

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

Hitomi Yatsuki · Ken Higashimoto · Kosuke Jozaki · Kayoko Koide · Junichiro Okada · Yoriko Watanabe · Nobuhiko Okamoto · Yoshinobu Tsuno · Yoko Yoshida · Kazutoshi Ueda · Kenji Shimizu · Hirofumi Ohashi · Tsunehiro Mukai · Hidenobu Soejima

Received: 12 July 2012 / Accepted: 13 August 2012

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.

H. Yatsuki · K. Higashimoto · K. Jozaki · K. Koide · H. Soejima (✉)
Division of Molecular Genetics & Epigenetics, Department of Biomolecular Sciences, Faculty of Medicine, Saga University, Saga 849-8501, Japan
e-mail: soejimah@med.saga-u.ac.jp

J. Okada · Y. Watanabe
Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume 830-0011, Japan

N. Okamoto
Department of Medical Genetics, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi 594-1101, Japan

Y. Tsuno
Perinatal Medical Center, Wakayama Medical University Hospital, Wakayama 641-8510, Japan

Y. Yoshida · K. Ueda
Department of Pediatrics, Kitano Hospital, The Tazuke Kofukai Medical Research Institute, Osaka 530-8480, Japan

K. Shimizu · H. Ohashi
Division of Medical Genetics, Saitama Children's Medical Center, Saitama 339-8551, Japan

T. Mukai
Nishikyushu University, Kanzaki 842-8585, Japan

Published online: 29 January 2013

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

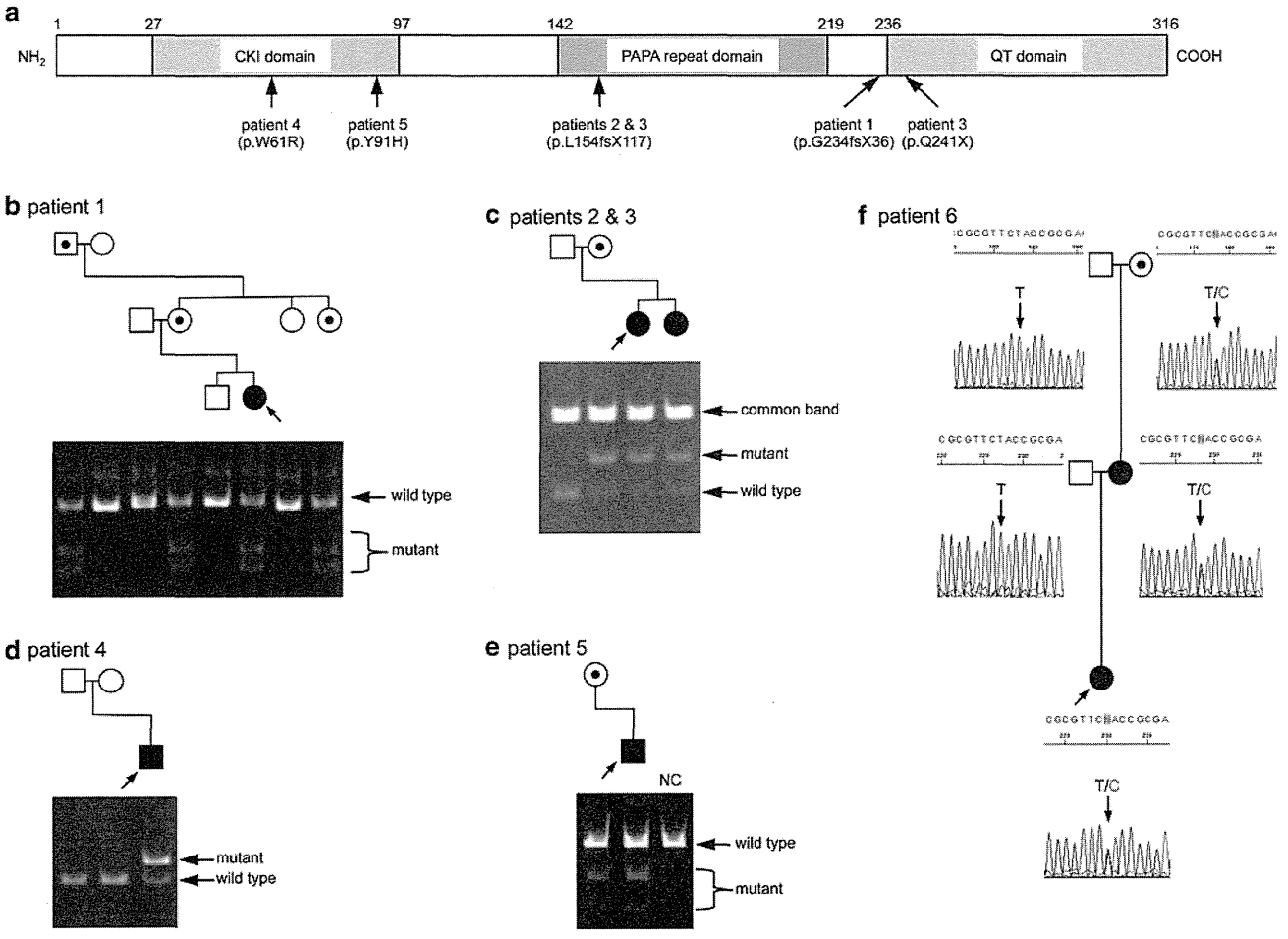


Fig. 1 *CDKN1C* mutations and their inheritance. **a** Domain structure of the CDKN1C protein and position of each mutation. Amino acid residues are indicated above. **b** Pedigree chart of Patient 1. *Bss*HII digestion of PCR region F was used to distinguish between the mutant and wild type alleles.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

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.

References

Arboleda VA, Lee H, Parnaik R, Fleming A, Banerjee A, Ferraz-de-Souza B, Délot EC, Rodriguez-Fernandez IA, Braslavsky D, Bergadá I et al (2012) Mutations in the PCNA-binding domain of CDKN1C cause IMAGe syndrome. Nat Genet 44:788–792

Bhuiyan ZA, Yatsuki H, Sasaguri T, Joh K, Soejima H, Zhu X, Hatada I, Morisaki H, Morisaki T, Mukai T (1999) Functional analysis of the p57KIP2 gene mutation in Beckwith-Wiedemann syndrome. Hum Genet 104:205–210

Borriello A, Caldarelli I, Bencivenga D, Criscuolo M, Cucciolla V, Tramontano A, Oliva A, Perrotta S, Della Ragione F (2011) p57(Kip2) and cancer: time for a critical appraisal. Mol Cancer Res 9:1269–1284

Choufani S, Shuman C, Weksberg R (2010) Beckwith-Wiedemann syndrome. Am J Med Genet C Semin Med Genet 154C:343–354

DeBaun MR, Tucker MA (1998) Risk of cancer during the first four years of life in children from The Beckwith-Wiedemann syndrome registry. J Pediatr 132:398–400

Diaz-Meyer N, Day CD, Khatod K, Maher ER, Cooper W, Reik W, Junien C, Graham G, Algar E, Der Kaloustian VM et al (2003)

Silencing of CDKN1C (p57KIP2) is associated with hypomethylation at KvDMR1 in Beckwith-Wiedemann syndrome. J Med Genet 40:797–801

Elliott M, Bayly R, Cole T, Temple IK, Maher ER (1994) Clinical features and natural history of Beckwith-Wiedemann syndrome: presentation of 74 new cases. Clin Genet 46:168–174

Hatada I, Ohashi H, Fukushima Y, Kaneko Y, Inoue M, Komoto Y, Okada A, Ohishi S, Nabetani A, Morisaki H et al (1996) An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome. Nat Genet 14:171–173

Hatada I, Nabetani A, Morisaki H, Xin Z, Ohishi S, Tonoki H, Niikawa N, Inoue M, Komoto Y, Okada A et al (1997) New p57KIP2 mutations in Beckwith-Wiedemann syndrome. Hum Genet 100:681–683

Higashimoto K, Urano T, Sugiura K, Yatsuki H, Joh K, Zhao W, Iwakawa M, Ohashi H, Oshimura M, Niikawa N et al (2003) Loss of CpG methylation is strongly correlated with loss of histone H3 lysine 9 methylation at DMR-LIT1 in patients with Beckwith-Wiedemann syndrome. Am J Hum Genet 73:948–956

Lee MH, Reynisdóttir I, Massagué J (1995) Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev 9:639–649

Li M, Squire J, Shuman C, Fei YL, Atkin J, Pauli R, Smith A, Nishikawa J, Chitayat D, Weksberg R (2001) Imprinting status of 11p15 genes in Beckwith-Wiedemann syndrome patients with CDKN1C mutations. Genomics 74:370–376

Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, Harper JW, Elledge SJ (1995) p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. Genes Dev 9:650–662

Meyer E, Lim D, Pasha S, Tee LJ, Rahman F, Yates JR, Woods CG, Reik W, Maher ER (2009) Germline mutation in NLRP2 (NALP2) in a familial imprinting disorder (Beckwith-Wiedemann Syndrome). PLoS Genet 5:e1000423

Romanelli V, Belinchón A, Benito-Sanz S, Martínez-Glez V, Gracia-Bouthelier R, Heath KE, Campos-Barros A, García-Miñaur S, Fernandez L, Meneses H et al (2010) CDKN1C (p57(Kip2)) analysis in Beckwith-Wiedemann syndrome (BWS) patients: genotype-phenotype correlations, novel mutations, and polymorphisms. Am J Med Genet A 152A:1390–1397

Rump P, Zeegers MP, van Essen AJ (2005) Tumor risk in Beckwith-Wiedemann syndrome: a review and meta-analysis. Am J Med Genet A 136:95–104

Sasaki K, Soejima H, Higashimoto K, Yatsuki H, Ohashi H, Yakabe S, Joh K, Niikawa N, Mukai T (2007) Japanese and North American/European patients with Beckwith-Wiedemann syndrome have different frequencies of some epigenetic and genetic alterations. Eur J Hum Genet 15:1205–1210

Soejima H, Nakagawachi T, Zhao W, Higashimoto K, Urano T, Matsukura S, Kitajima Y, Takeuchi M, Nakayama M, Oshimura M et al (2004) Silencing of imprinted CDKN1C gene expression is associated with loss of CpG and histone H3 lysine 9 methylation at DMR-LIT1 in esophageal cancer. Oncogene 23:4380–4388

Vlachos P, Joseph B (2009) The Cdk inhibitor p57(Kip2) controls LIM-kinase 1 activity and regulates actin cytoskeleton dynamics. Oncogene 28:4175–4188

Watanabe H, Pan ZQ, Schreiber-Agus N, DePinho RA, Hurwitz J, Xiong Y (1998) Suppression of cell transformation by the cyclin-dependent kinase inhibitor p57KIP2 requires binding to proliferating cell nuclear antigen. Proc Natl Acad Sci USA 95:1392–1397

Weksberg R, Nishikawa J, Caluseriu O, Fei YL, Shuman C, Wei C, Steele L, Cameron J, Smith A, Ambus I et al (2001) Tumor development in the Beckwith-Wiedemann syndrome is associated with a variety of constitutional molecular 11p15 alterations including imprinting defects of KCNQ1OT1. Hum Mol Genet 10:2989–3000

Weksberg R, Shuman C, Beckwith JB (2010) Beckwith-Wiedemann syndrome. Eur J Hum Genet 18:8–14

Yokoo T, Toyoshima H, Miura M, Wang Y, Iida KT, Suzuki H, Sone H, Shimano H, Gotoda T, Nishimori S et al (2003) p57Kip2 regulates actin dynamics by binding and translocating LIM-kinase 1 to the nucleus. J Biol Chem 278:52919–52923

European Journal of Human Genetics (2013), 1–4

www.nature.com/ejhg

SHORT REPORT

Homozygous deletion of *DIS3L2* exon 9 due to non-allelic homologous recombination between LINE-1s in a Japanese patient with Perlman syndrome

Ken Higashimoto[1,7], Toshiyuki Maeda[1,7], Junichiro Okada[2,7], Yasufumi Ohtsuka[1], Kensaku Sasaki[3], Akiko Hirose[2], Makoto Nomiyama[4], Toshimitsu Takayanagi[5], Ryuji Fukuzawa[6], Hitomi Yatsuki[1], Kayoko Koide[1], Kenichi Nishioka[1], Keiichiro Joh[1], Yoriko Watanabe[2], Koh-ichiro Yoshiura[3] and Hidenobu Soejima*[,1]

Perlman syndrome is a rare, autosomal recessive overgrowth disorder. Recently, the deletion of exon 9 and other mutations of the *DIS3L2* gene have been reported in patients; however, the mechanism behind this deletion is still unknown. We report the homozygous deletion of exon 9 of *DIS3L2* in a Japanese patient with Perlman syndrome. We identified the deletion junction, and implicate a non-allelic homologous recombination (NAHR) between two LINE-1 (L1) elements as the causative mechanism. Furthermore, the deletion junctions were different between the paternal and maternal mutant alleles, suggesting the occurrence of two independent NAHR events in the ancestors of each parent. The data suggest that the region around exon 9 might be a hot spot of L1-mediated NAHR.
European Journal of Human Genetics (2013) **0**, 000–000. doi:10.1038/ejhg.2013.45

Keywords: LINE-1; non-allelic homologous recombination; Perlman syndrome; exon deletion

INTRODUCTION

Perlman syndrome (OMIM #267000) is a rare, autosomal recessive overgrowth disorder characterized by polyhydramnios with neonatal macrosomia, nephromegaly, distinctive facies, renal dysplasia, nephroblastomatosis and a predisposition to Wilms tumor. The clinical features are reminiscent of Beckwith–Wiedemann syndrome; however, genetic and epigenetic alterations at 11p15.5 have been excluded from the etiology.[1] Recently, *DIS3L2* at 2q37.1 was reported as a causative gene, showing homozygous deletions of exon 6 or exon 9 (82.8 and ~22 kb, respectively) and compound heterozygous mutations in such patients.[2] However, the mechanisms behind these deletions are still unknown. In this report, we explore a parentally transmitted homozygous deletion of exon 9 in *DIS3L2* responsible for Perlman syndrome in a Japanese patient. We detected the sequence of the deletion junction and found that a rare, non-allelic homologous recombination (NAHR) between two collinear LINE-1 (L1) elements was the causative mechanism of the deletion. To our knowledge, this is the fourth NAHR event to be documented as causing a human disease. Furthermore, the deletion junctions were different between the paternal and maternal mutant alleles, suggesting the occurrence of two independent NAHR events in the ancestors of each parent. Our data suggest that the region around exon 9 of *DIS3L2* is a hot spot of L1-mediated NAHR.

MATERIALS AND METHODS

Patient

The male infant was the first child of non-consanguineous, healthy, Japanese parents. Prenatal ultrasound examination showed polyhydramnios and bilateral nephromegaly. He was delivered at 29 weeks and 4 days of gestation. He weighed 2267 g (+6.4 SD) and measured 45.5 cm (+4.3 SD) in length. Low-set ears, large fontanels, micrognathia, a depressed nasal bridge, an everted upper lip, prominent forehead, flexed digits, a micropenis and cryptorchidism were observed. He suffered from cholestasis with coagulation disorder and recurrent adrenal crisis, and died at 175 days of life due to a sepsis. Autopsy revealed visceromegaly and nephroblastomatosis, and he was diagnosed with Perlman syndrome. His karyotype was normal (46,XY). Causative alterations of Beckwith–Wiedemann syndrome, such as loss of methylation at KvDMR1, gain of methylation at H19DMR, paternal uniparental disomy of chromosome 11 and *CDKN1C* mutations, were ruled out (data not shown). This study was approved by the ethics committee for Human Genome and Gene Analyses of the Faculty of Medicine, Saga University, Japan.

Polymerase chain reaction and sequencing

Genomic DNA was extracted from cord blood, placenta and amniotic fluid of the patient and peripheral blood of his parents. All coding exons, from exon 2 to exon 21, of *DIS3L2* were amplified by PCR using primer pairs described previously.[2] The copy number of *DIS3L2* exon 9 was analyzed by quantitative real-time PCR (qPCR) based on SYBR-Green I. Normalization was performed against *GAPDH* and *TAT*.[3,4]

[1]Division of Molecular Genetics and Epigenetics, Department of Biomolecular Sciences, Faculty of Medicine, Saga University, Saga, Japan; [2]Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume, Japan; [3]Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [4]Department of Obstetrics and Gynecology, Perinatal Center, National Hospital Organization Saga National Hospital, Saga, Japan; [5]Department of Pediatrics, Perinatal Center, National Hospital Organization Saga National Hospital, Saga, Japan; [6]Department of Pathology and Laboratory Medicine, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan
[7]These authors contributed equally to this work.
*Correspondence: Professor H Soejima, Division of Molecular Genetics and Epigenetics, Department of Biomolecular Sciences, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501 Japan. Tel: +81 952 34 2260; Fax: +81 952 34 2067; E-mail: soejimah@med.saga-u.ac.jp
Received 2 November 2012; revised 6 February 2013; accepted 14 February 2013

L1-A and L1-B, which were located collinearly upstream and downstream of exon 9, were amplified with primer pairs 1a/2, and 3/4 and 5/6, respectively (Figure 1d). The paternal and maternal mutant alleles containing the deletion junctions were amplified with primer pair 1b/6. All PCR products were directly sequenced.

Total RNA was also extracted from placenta and amniotic fluid and cDNA was synthesized with random primers. RT-PCR was performed with a forward primer in exon 8 and a reverse primer in exon 11. The RT-PCR product was sequenced directly. The novel primers used in this study are shown in Table 1.

RESULTS

We first examined whether all coding exons of *DIS3L2* were amplified by PCR for the patient. In all patient samples, exon 9 could not be amplified whereas all exons were amplified in the patient's parents and normal control individuals (Figure 1a, data not shown). This result indicated a homozygous deletion of exon 9 in the patient, one that has been previously reported.[2] qPCR showed that the copy number of exon 9 in the parents was approximately half that of controls, indicating heterozygosity for the deletion (Figure 1b). No PCR amplification was observed in the patient's samples, supporting homozygosity for the deletion. Therefore, the parents were carriers of the deletion and one deleted allele was transmitted to the patient by each parent.

Next, the expression of the mutant allele was investigated by RT-PCR using primers on exons 8 and 11 (Figure 1c). In normal placentas, RT-PCR products matched the estimated normal size and contained the 174-bp exon 9 sequence. In contrast, the product size of the patient's placenta and amniotic fluid was smaller than normal. Sequencing revealed a missing exon 9 sequence and the existence of a junction at exons 8 and 10, indicating the expression of the mutant allele (Figure 1c). The expressed mutant allele would be translated to a mutant protein harboring an in-frame deletion of 58 amino acids, resulting in an abolished RNA-binding domain. Wild-type DIS3L2 has ribonuclease activity, but the mutant lacking exon 9 loses it. Accordingly, it has been speculated that alterations in mRNA turnover might be responsible for the phenotypes of Perlman syndrome.[2]

Finally, we tried to identify the deletion junction to clarify the deletion mechanism. Two L1 sequences, L1-A and L1-B, were located directly upstream and downstream of exon 9. The directions of the two L1 sequences were opposite to *DIS3L2* (Figure 1d). L1-A and

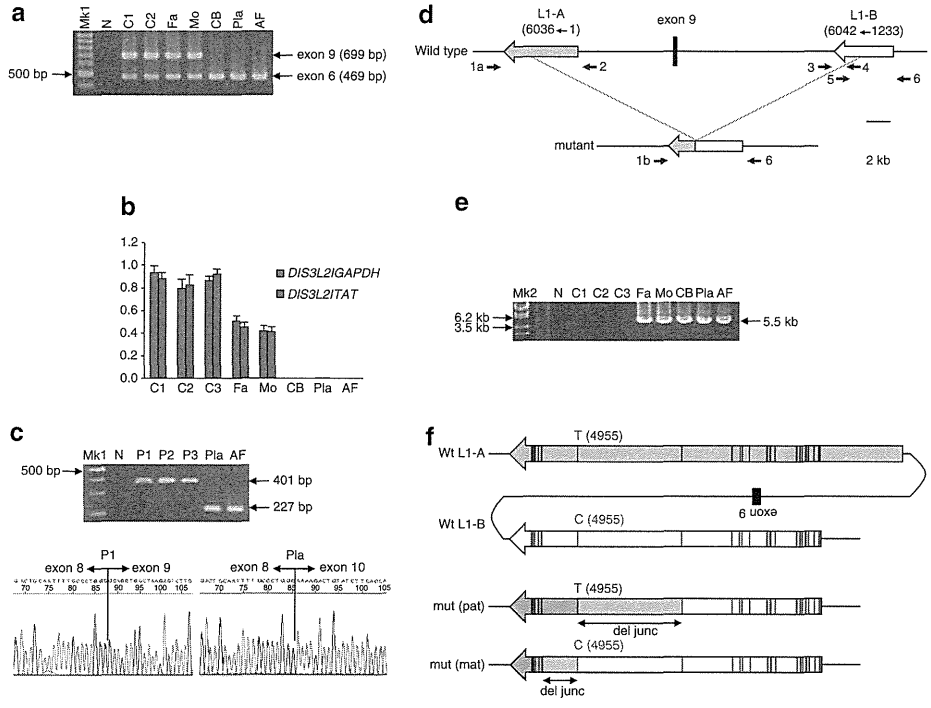


Figure 1 Homozygous deletion of *DIS3L2* exon 9 in a patient with Perlman syndrome. (**a**) Homozygous deletion of *DIS3L2* exon 9. Duplex PCR of exons 6 and 9 showed no amplification of exon 9 in any patient sample, whereas both exons were amplified in the parents and normal controls. Mk1, 100-bp ladders; N, negative control; C1, unrelated normal control #1; C2, unrelated normal control #2; Fa, father of the patient; Mo, mother of the patient; CB, cord blood of the patient; Pla, placenta of the patient; AF, amniotic fluid of the patient. (**b**) Copy number analysis of exon 9. qPCR of *DIS3L2* exon 9 normalized with *GAPDH* or *TAT* showed that the copy number of exon 9 in the parents was approximately half that of controls. No PCR product was amplified in any patient sample. The y axis displays arbitrary units. (**c**) RT-PCR of *DIS3L2*. RT-PCR was performed with a forward primer in exon 8 and a reverse primer in exon 11. The exon 9 deleted products were seen in the patient samples. Sequencing of the PCR products showed a lack of the exon 9 sequence, which was supposed to be 174 bp in length, in the patient's placenta, whereas the exon 9 sequence existed in normal placentas. P1, normal placenta #1; P2, normal placenta #2; P3, normal placenta #3. (**d**) Map of exon 9. Two L1 sequences, L1-A and L1-B, were located collinearly upstream and downstream of exon 9. The L1s were in the opposite direction to the *DIS3L2* gene. L1-A was full-length, corresponding to nucleotides 1 to 6036 of the reference sequence of L1Hs from Repbase on the GIRI website.[5] L1-B was a 5′ truncated form, corresponding to nucleotides 1233 to 6042 of the reference. Primers for PCR amplification are depicted as blue arrows. (**e**) PCR products containing the deletion junction. Approximately 5.5-kb products were amplified by PCR with primer pair 1b/6 in the patient and the parents, whereas no product was seen in normal controls. Mk2, lambda DNA digested with *Styl*. (**f**) The deletion junction. Comparing the sequences among L1-A, L1-B and the PCR products revealed that the deletion junctions of each parental allele were different. Vertical blue or red bars showed the positions of nucleotide differences between L1-A and L1-B. As the patient had two parental mutant alleles, a nucleotide at position 4955 was heterozygous (T/C) in the patient. The deletion junction is shown as a green box.

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.

ACKNOWLEDGEMENTS

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; 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.

1 Alessandri JL, Cuillier F, Ramful D *et al*: Perlman syndrome: report, prenatal findings and review. *Am J Med Genet A* 2008; **146A**: 2532–2537.
2 Astuti D, Morris MR, Cooper WN *et al*: Germline mutations in DIS3L2 cause the Perlman syndrome of overgrowth and Wilms tumor susceptibility. *Nat Genet* 2012; **44**: 277–284.
3 Ponchel F, Toomes C, Bransfield K *et al*: Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene

rearrangements, gene amplifications and micro gene deletions. *BMC Biotechnol* 2003; **3**: 18.
4 Pop R, Zaragoza MV, Gaudette M, Dohrmann U, Scherer G: A homozygous nonsense mutation in SOX9 in the dominant disorder campomelic dysplasia: a case of mitotic gene conversion. *Hum Genet* 2005; **117**: 43–53.
5 Kohany O, Gentles AJ, Hankus L, Jurka J: Annotation, submission and screening of repetitive elements in Repbase: RepbaseSubmitter and Censor. *BMC Bioinformatics* 2006; **7**: 474.
6 Hancks DC, Kazazian HH: Active human retrotransposons: variation and disease. *Curr Opin Genet Dev* 2012; **22**: 191–203.
7 Mills RE, Bennett EA, Iskow RC *et al*: Recently mobilized transposons in the human and chimpanzee genomes. *Am J Hum Genet* 2006; **78**: 671–679.
8 Wang J, Song L, Grover D, Azrak S, Batzer MA, Liang P: dbRIP: a highly integrated database of retrotransposon insertion polymorphisms in humans. *Hum Mutat* 2006; **27**: 323–329.
9 Belancio VP, Deininger PL, Roy-Engel AM: LINE dancing in the human genome: transposable elements and disease. *Genome Med* 2009; **1**: 97.
10 Sun C, Skaletsky H, Rozen S *et al*: Deletion of azoospermia factor a (AZFa) region of human Y chromosome caused by recombination between HERV15 proviruses. *Hum Mol Genet* 2000; **9**: 2291–2296.
11 Kamp C, Hirschmann P, Voss H, Huellen K, Vogt PH: Two long homologous retroviral sequence blocks in proximal Yq11 cause AZFa microdeletions as a result of intrachromosomal recombination events. *Hum Mol Genet* 2000; **9**: 2563–2572.
12 Blanco P, Shlumukova M, Sargent CA, Jobling MA, Affara N, Hurles ME: Divergent outcomes of intrachromosomal recombination on the human Y chromosome: male infertility and recurrent polymorphism. *J Med Genet* 2000; **37**: 752–758.
13 Burwinkel B, Kilimann MW: Unequal homologous recombination between LINE-1 elements as a mutational mechanism in human genetic disease. *J Mol Biol* 1998; **277**: 513–517.
14 Segal Y, Peissel B, Renieri A *et al*: LINE-1 elements at the sites of molecular rearrangements in Alport syndrome-diffuse leiomyomatosis. *Am J Hum Genet* 1999; **64**: 62–69.
15 Temtamy SA, Aglan MS, Valencia M *et al*: Long interspersed nuclear element-1 (LINE1)-mediated deletion of EVC, EVC2, C4orf6, and STK32B in Ellis-van Creveld syndrome with borderline intelligence. *Hum Mutat* 2008; **29**: 931–938.
16 Han K, Lee J, Meyer TJ, Remedios P, Goodwin L, Batzer MA: L1 recombination-associated deletions generate human genomic variation. *Proc Natl Acad Sci USA* 2008; **105**: 19366–19371.
17 Zhang F, Carvalho CM, Lupski JR: Complex human chromosomal and genomic rearrangements. *Trends Genet* 2009; **25**: 298–307.

Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)

REVIEW

Cardiovascular Therapeutics

Novel Effects of Macrolide Antibiotics on Cardiovascular Diseases

Jun-ichi Suzuki,[1] Masahito Ogawa,[1] Keiichi Hishikari,[2] Ryo Watanabe,[2] Kiyoshi Takayama,[3] Yasunobu Hirata,[1] Ryozo Nagai,[4] & Mitsuaki Isobe[2]

1 Department of Advanced Clinical Science and Therapeutics, University of Tokyo, Tokyo, Japan
2 Department of Cardiovascular Medicine, Tokyo Medical and Dental University, Tokyo, Japan
3 NB Health Laboratory, Saitama, Japan
4 Department of Cardiovascular Medicine, University of Tokyo, Tokyo, Japan

Keywords
Inflammation; Macrolide; Matrix metalloproteinases; Myocardial remodeling.

Correspondence
Jun-ichi Suzuki, Department of Advanced Clinical Science and Therapeutics, University of Tokyo, 7–3-1 Hongo, Bunkyo, Tokyo 113–8655, Japan.
Tel.: +81-3-5800-9116;
Fax: +81-3-5800-9182;
E-mail: *junichisuzuki-circ@umin.ac.jp*

doi: 10.1111/j.1755-5922.2011.00303.x

SUMMARY

Macrolide antibiotics are broadly used for the treatment of various microbial infections. However, they are also known to have multiple biologic effects, such as alteration of inflammatory factors and matrix metalloproteinases (MMPs). Because of controversial results in clinical trials, the effects of macrolides on cardiovascular diseases are still to be elucidated. It has been reported that MMP activity is upregulated in various cardiovascular diseases, such as myocarditis, cardiac transplant rejection and myocardial infarction. However, little is known about the effects of macrolides on cardiovascular diseases. We have reported that clarithromycin suppressed the development of myocarditis, cardiac rejection and myocardial ischemia using animal models. In this article, we reviewed the roles of MMPs in cardiovascular diseases and the effects of macrolides on the prevention of adverse tissue remodeling.

Introduction

Several studies have demonstrated that there is a significant association between chronic inflammation and atherosclerotic cardiovascular disease [1]. Recent studies have accumulated evidence that suggests bacterial infection, such as periodontitis [2] and pneumonia [3], contribute to cardiovascular disease. Thus, antibiotic therapies might be useful in the prevention of cardiovascular events [4]. Sinisalo et al. reported the effect of antibiotic therapy on the secondary prevention of acute coronary syndrome in the Clarithromycin in Acute Coronary Syndrome Patients in Finland (CLARIFY) trial. They randomly assigned the patients with acute non-Q-wave infarction or unstable angina to receive double-blind treatment with either clarithromycin or placebo for three months. The results showed that clarithromycin reduced the risk of ischemic cardiovascular events in patients with acute non-Q-wave infarction or unstable angina [5]. They also revealed that long-term clarithromycin therapy was beneficial in the prevention of recurrent cardiovascular events in nonperiodontitis patients [6] and in patients with C4 deficiency [7]. On the other hand, Jespersen et al. demonstrated that short-term clarithromycin therapy significantly increased cardiovascular mortality in patients with stable coronary heart disease [8]. They also demonstrated that concomitant statin treatment in the stable patients with coronary heart disease abrogated increased cardiovascular mortality [9]. Berg et al. tried to reveal the pathological mechanism of the controversial results of secondary prevention trials. However, clarithromycin did not change the levels of inflammatory markers (C-reactive protein, interleukin (IL)-2 receptor, IL-6, IL-8, and tumor necrosis factor [TNF]-alpha) in the patients with atherosclerosis [10]. Originally, the CLARIFY investigators tried to exploit the antimicrobial effects of clarithromycin, however, their results did not exclude nonantimicrobial effects [11]. Therefore, the effects of clarithromycin on cardiovascular diseases are still to be elucidated.

Clarithromycin suppresses transcription factors, such as activator protein (AP)-1 and nuclear factor-kappa B (NF-κB). The downregulation of the transcription factors inhibited pro-inflammatory cytokine production [12,13] and matrix metalloproteinase (MMP) activity. It has been reported that MMP activity is upregulated in various cardiovascular diseases, such as myocarditis [14–17], cardiac transplant rejection [18,19], myocardial infarction (MI) [20,21], and abdominal aortic aneurysm [22,23]. However, little is known about the effect of clarithromycin on cardiovascular diseases via MMPs. In this article, we reviewed the roles of MMP activity in cardiovascular diseases and the potent effect clarithromycin has on the prevention of tissue remodeling.