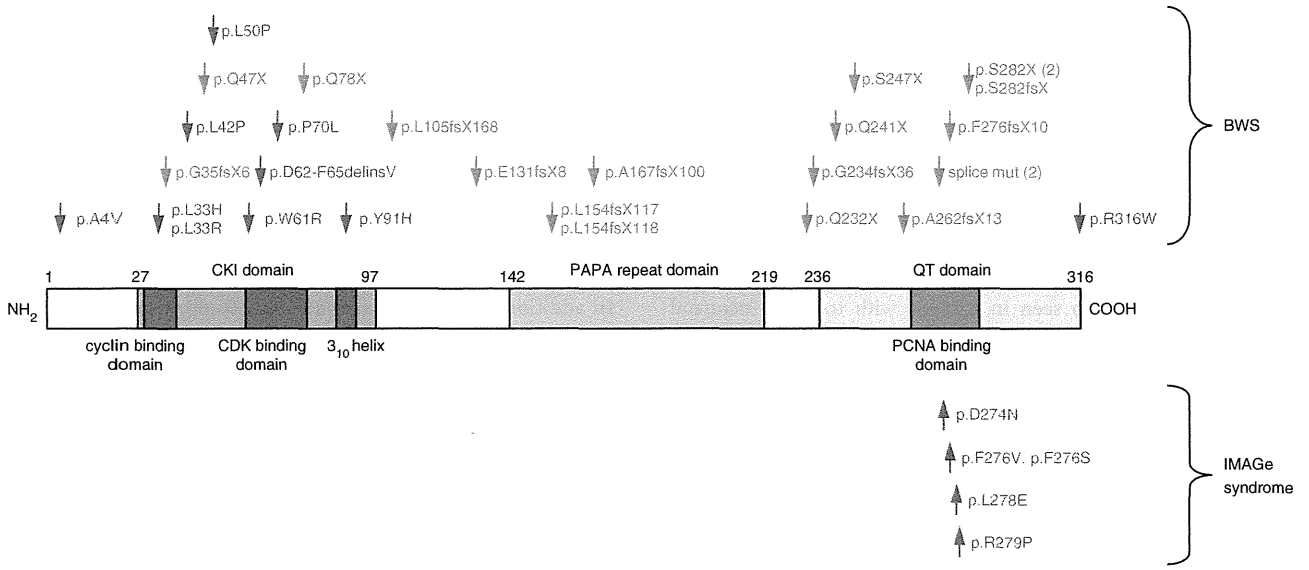

Figure 2 Mutations of *CDKN1C* in BWS and IMAGe syndrome.[67–69] The mutations in BWS are loss-of-function mutations, which are either amino-acid substitution mutations localized to the cyclin-dependent kinase inhibitory domain or truncating mutations. The mutations in IMAGe syndrome that lead to growth restriction are missense mutations specific to the PCNA-binding domain, considered a gain-of-function mutation. Blue: amino-acid substitution mutations; red: truncating mutations.

reported.[72] In this family, a SRS child was born from a mother with BWS phenotypes due to paternal duplication. Representative phenotypes of BWS due to duplication causes developmental delay (Table 2).[12]

So far at least 12 cases harboring translocations or inversions have been reported, with most break points of the translocations and inversions falling in the *KCNQ1* locus.[73–77] BWS develops when these are transmitted maternally. Three cases harboring inv(11)(p13;p15.5), inv(11)(p11.2;p15.5) and t(11;17)(p15.5;q21.3), respectively, have been seen to exhibit KvDMR1-LOM. However, a fibroblast with inv(11)(p15.5;q13) and a rhabdoid tumor line with t(11;22) have shown signs of reduced expression of *CDKN1C* with normal methylation at KvDMR1. These are consistent with the enhancer blocking insulator model mentioned before.[75–77] However, the remaining cases showed neither KvDMR1-LOM nor reduced expression of *CDKN1C*. Therefore, the developmental mechanism for BWS harboring translocations and inversions is largely unknown.

DIFFERENT RISKS FOR CHILDHOOD TUMORS IN EACH ALTERATION TYPE

Embryonal malignancies are the tumors most commonly associated with BWS—for example, Wilms' tumor, hepatoblastoma, adrenocortical carcinoma, rhabdomyosarcoma and neuroblastoma—but other malignant or benign tumors are occasionally observed.[6,7] Although overall tumor risk is ~7.5%, it is different for each causative alteration (Table 2). H19DMR-GOM and patUPD show the highest tumor risk, at >25%, especially for Wilms' tumor and hepatoblastoma. KvDMR1-LOM has a rate of developing hepatoblastoma, rhabdomyosarcoma and gonadoblastoma other than Wilms' tumors of ~5%.[10] The lowest risk is found in *CDKN1C* mutations with <5% of cases affected. Only neuroblastomas have been found in patients with *CDKN1C* mutations.[78,79] Wilms' tumors are frequently seen in patients with H19DMR-GOM or patUPD, but never seen in patients with KvDMR1-LOM or *CDKN1C* mutations, suggesting a critical role of *IGF2* overexpression in Wilms' tumor development. In fact, *IGF2* loss of imprinting is found in 60–70% of sporadic Wilms' tumors without 11p LOH.[80,81] Furthermore, *IGF2* loss of imprinting was also observed in ~21% of sporadic hepatoblastomas without 11p LOH, and aberrant methylations at H19DMR, H19 promoter, IGF2-DMR0 or IGF2-DMR2 were observed in ~55% of sporadic hepatoblastomas without 11p LOH, suggesting the importance of *IGF2* overexpression for hepatoblastoma development as well (Rumbajan JM *et al.*, submitted).[82] In addition, although many kinds of adult tumors display reduced *CDKN1C* expression, of which certain cases show KvDMR1-LOM, the risk of embryonal tumorigenesis is low in BWS patients with KvDMR1-LOM or *CDKN1C* mutations, suggesting different contributions of CDKN1C to tumor development between adulthood and childhood.

ART AND BWS

The worldwide usage of ART has increased. Several reports have raised concerns that the risk of imprinting disorders, such as BWS and Angelman syndrome, are increased in children conceived by ART, especially through *in vitro* fertilization and intracytoplasmic sperm injection, as the first reported associations in 2002 and 2003 between Angelman syndrome and BWS, respectively, with ART.[83–85] The risk of BWS is estimated to be six to nine times higher in children conceived by ART than in children conceived naturally.[86] The causative alteration for most of ART-related BWS is KvDMR1-LOM. The cause of Angelman syndrome is also LOM at *SNRPN*.

Animal studies have suggested that ovarian stimulation and culture medium for the embryo can affect DNA methylation and the expression of several imprinted genes.[87–90] In fact, 'large offspring syndrome' has been described as caused by LOM of the maternal *Igf2r* after sheep embryo culture.[91] However, in humans, although ovarian stimulation may predispose to aberrant methylation at imprinted loci,[92] it is still unclear whether the procedure of ART affects methylation at imprinted loci because ART populations are different from naturally conceived populations having low fertility rates, increased frequency of reproductive loss and advanced age.[93] Indeed, male infertility is strongly associated with aberrant methylation at both maternal and paternal alleles.[94,95] It has been

reported that there are no phenotypic differences between ART-related BWS and naturally conceived BWS.[96] However, Lim *et al.*[97] provided evidence that ART-related BWS had a significantly lower frequency of exomphalos and higher risk of tumor development than Wilms' tumor. Larger size studies are needed to better understand the correlation between ART and BWS.

MULTILOCUS HYPOMETHYLATION DISORDERS

Hypomethylations at several other imprinted loci have been reported to occur in BWS patients with KvDMR1-LOM.[47–49,97] As this phenomenon was also seen in patients with transient neonatal diabetes mellitus type 1 and SRS, a new entity of imprinting disorders such as MHD has been proposed.[49,98–101] The literature indicates an overall frequency of multilocus hypomethylation in BWS patients with KvDMR1-LOM of 20% (49/244).[49,98–101] *IGF2R-DMR2*, *GNAS*, *NESPAS*, *PEG1* and *PLAGL1* are frequently hypomethylated DMRs. In BWS patients, only maternally methylated DMRs displayed hypomethylation; however, several SRS patients with H19DMR-LOM showed hypomethylation at *DLK1/GTL2* IG-DMR, another paternally methylated DMR, indicating involvement of both maternally and paternally methylated DMRs. In addition, a certain SRS showed hypomethylation at both H19DMR and KvDMR1.[48,100] As these hypomethylations were mosaic, they were presumed to be due to a post-fertilization event.

Lim *et al.*[97] reported that ART-related BWS show multilocus hypomethylation more frequently than naturally conceived BWS; however, no such difference was observed by Rossignol *et al.*[47] One study reported that BWS with multilocus hypomethylation displayed characteristics not usually associated with BWS, such as speech retardation, peri/postnatal apnea, feeding difficulties and hearing problems; additionally, nevus flammeus and hemihypertrophy were significantly lower in patients with multilocus hypomethylation.[49] However, three other studies reported no difference in clinical features between MHDs and monolocus hypomethylation disorders.[47,48,97] As the studies so far have analyzed only limited numbers of DMRs, further investigation of all known DMRs are needed.

The involvement of trans-acting factors in these MHD has been suggested. In fact, in one study, homozygous and compound heterozygous mutations of *ZFP57*, which encodes a KRAB zinc-finger protein and is required for the post-fertilization maintenance of maternal and paternal methylation imprinting at multiple loci, were found in transient neonatal diabetes mellitus type 1 patients with multilocus hypomethylation.[102] However, no mutations were found in 27 BWS patients with KvDMR1-LOM probably without multilocus hypomethylation.[103] KAP1, a protein associated with ZFP57, interacts with DNMT1 and binds to many ICRs in embryonic stem cells to maintain DNA and histone methylation.[104,105] Mice with maternal deletions of *Trim28*, a homolog of human *KAP1*, show aberrant DNA demethylation at a few ICRs.[106] Mutation searches of *KAP1* in MHD patients have not been reported to date.

Other candidates for trans-acting factors are NLRP2 and NLRP7, which are members of the Nod-like receptor protein (NLRP) family. Some NLRPs are components of the inflammasome, an assembly that is implicated in the sensing of, and inflammatory reaction to, extracellular pathogens and intracellular noxious compounds.[107] Mutations of *NLRP2* were identified in a familial case of BWS with KvDMR1-LOM and PEG1-LOM, suggesting a role of NLRP2 in the establishment or maintenance of ICRs.[108] However, the mutation has not been corroborated by other studies yet. Mutations of *NLRP7* and *C6ORF221* account for familial biparental hydatidiform mole, which is a maternal effect recessive disorder resulting from failure of maternal imprints.[109,110] Mutation searches of *NLRP7* were performed on the mother of a patient showing both transient neonatal diabetes mellitus type 1 and BWS features with multilocus hypomethylation, but they were unsuccessful.[99] In addition, DNMT3L, which is required for establishing maternal imprints, was not mutated in two BWS patients with severe multilocus hypomethylation.[49] Mutation searching of all candidate trans-acting factors should be performed over a large number of MHD patients to explore this matter further.

In addition, one circular chromosome conformation capture (4C) study revealed that maternal H19DMR interacts with the autosomal region, and imprinting domains were strongly overrepresented in the 4C library, suggesting the involvement of higher order chromatin interaction in the regulation of imprinting.[111] The involvement of physical chromosome interactions in MHD should also be further elucidated.

CONCLUSIONS

Although H19DMR-GOM, KvDMR1-LOM, patUPD and *CDKN1C* mutations, and chromosomal rearrangements account for ~80% of BWS phenotypes, several questions about these alterations still remain to be clarified. In addition, at least 20% of patients do not have these associated alterations, suggesting the existence of other, unknown epigenetic/genetic defects. Furthermore, other issues, such as the effect of ART on imprinting disorders and the mechanism of multilocus imprinting establishment/maintenance, should be clarified. Further investigations of all of these issues must be elucidated in order to understand the molecular basis of BWS and related imprinting disorders.

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増刊 臨床医が知っておきたい先天異常
Ⅱ．各論

3．エピジェネティクスが関連する先天異常

2）Beckwith-Wiedemann 症候群とSilver-Russell 症候群

佐賀大学医学部　分子生命科学講座
分子遺伝学・エピジェネティクス分野
前田寿幸（まえだとしゆき），東元　健（ひがしもとけん），副島英伸（そえじまひでのぶ）

KEY WORDS　Beckwith-Wiedemann 症候群（BWS），Silver-Russell 症候群（SRS），ゲノム刷り込み遺伝子，成長障害

はじめに

常染色体にある一対の対立遺伝子（アレル）は，原則としてその親由来にかかわらず両方のアレルが等しく発現している。しかし，遺伝子の中にはその親由来に従って片アレルのみ発現する遺伝子が存在する。このような遺伝子を刷り込み遺伝子という。刷り込み遺伝子は，個体の発生，発育，成長に重要な役割を担っており，一般的に，父性発現する遺伝子は成長を促進し，母性発現する遺伝子は成長を抑制する[1]。また，多くの刷り込み遺伝子はクラスターをなし，刷り込みドメインを形成しており，遺伝子発現はドメインレベルで制御されている。このドメインレベルでの制御に中枢的役割をするDNA領域のことを刷り込み制御領域（imprinting control region：ICR）という。この領域は，配偶子形成過程で性特異的（卵特異的あるいは精子特異的）にDNAメチル化を受け，メチル化の差異は受精後も維持されるため，両アレル間でDNAメチル化状態が異なるメチル化可変領域（differentially methylated region：DMR）を形成する。刷り込みドメインを含む領域のエピゲノム異常やゲノム異常は，Beckwith-Wiedemann 症候群（BWS），Silver-Russell 症候群（SRS），Prader-Willi 症候群，Angelman 症候群などの刷り込み疾患を引き起こすことが知られている。本稿では，BWS と SRS に焦点をあて解説する。

Ⅰ．Beckwith-Wiedemann 症候群（BWS）

BWS は，新生児期の過成長，巨舌，腹壁欠損（臍帯ヘルニア，臍ヘルニア）を3主徴とする刷り込み疾患である。その他に，耳垂の線状溝・耳輪後縁の小窩，新生児低血糖，腹腔内臓腫大，片側肥大などの多彩な症状を呈し，約7.5％の患者に小児腫瘍（Wilms 腫瘍，肝芽腫，神経芽腫など）の合併がみられる[2)3)]。BWS の頻度は13,700人に1人で，性差はなく，孤発例が85％，家族例が15％を占める。統一された診断基準はないが，広く受け入れられている診断基準として，Weks-

〒849-8501　佐賀県佐賀市鍋島5-1-1

表1　BWSの診断基準（文献4より，改訂）

大基準	・腹壁欠損（臍ヘルニア，臍帯ヘルニア） ・巨舌 ・巨大児（身長と体重＞97th percentile） ・耳垂の線状溝・耳輪後縁の小窩（両側あるいは片側） ・腹腔内臓腫大（肝臓，腎臓，脾臓など） ・小児期の胎児性腫瘍 ・片側肥大 ・胎児の副腎皮質巨大細胞（通常，びまん性で両側） ・腎奇形（腎髄質異形成，腎石灰沈着症，腎結石症を含む） ・BWSの家族歴 ・口蓋裂
小基準	・羊水過多，巨大な胎盤，臍帯の肥厚，早産 ・新生児低血糖 ・火焔状母斑 ・心肥大，心奇形，心筋症 ・特徴的顔貌 ・腹直筋離解 ・骨年齢の亢進
診断	大基準3つ以上，もしくは大基準2つと小基準1つ

bergらが報告したものがある（**表1**）[4]。鑑別診断としては，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）が挙げられる。BWSの原因遺伝子座11p15には，セントロメア側の*CDKN1C/KCNQ1OT1*ドメインとテロメア側の*IGF2/H19*ドメインという2つの刷り込みドメインが存在する。各ドメイン内の刷り込み遺伝子の発現は，それぞれのドメインのICR（セントロメア側はICR2，テロメア側はICR1）によって制御されており，ICRのエピジェネティックな異常，あるいはジェネティックな異常により発症する。具体的な異常は，ICR2の低メチル化（～50%），ICR1の高メチル化（2～7%），父性片親性ダイソミー（patUPD）（～20%），*CDKN1C*の変異（～5%），11番染色体構造異常（～2%）である[4)~6)]。また，11p15の領域に異常を認めないBWSが約25%存在する。発症原因別に代表的な症状が異なる（**表2**）。

1．BWSの発症原因

1）*CDKN1C/KCNQ1OT1*ドメインの異常

健常人では，*CDKN1C*は母性優位に発現し，*KCNQ1OT1*は父性アレルより発現する。この刷り込み状態は，*KCNQ1*遺伝子のイントロン10に位置するICR2によって制御されている。ICR2は母性アレルがメチル化し，父性アレルが非メチル化されている。ICR2は*KNCQ1OT1*のプロモーター領域に重なるように存在し，非メチル化の父性アレルでは，*KCNQ1*の転写方向とは逆向きに*KNCQ1OT1*が発現する。また，*KCNQ1OT1*はlong non-cording RNAであり，シスにドメイン内の遺伝子発現を抑制する[7)]。その結果，周囲の遺伝子は母性発現を示す（**図1**）。BWSでは，母性アレルICR2の低メチ

表2　BWSの原因と臨床症状との関係

原因	頻度	代表的な臨床症状	腫瘍リスク	腫瘍の種類
H19DMR-GOM	2～7％	片側肥大	>25%	Wilms腫瘍 肝芽腫
KvDMR1-LOM	～50%	臍帯ヘルニア 片側肥大	～5％	肝芽腫 横紋筋肉腫 性腺芽腫 （Wilms腫瘍は認めない）
patUPD	～20%	片側肥大	>25%	Wilms腫瘍 肝芽腫
CDKN1C変異	～5％	臍帯ヘルニア 口蓋裂	＜5％	神経芽細胞腫
染色体異常	＜2％	発達遅滞 （重複例において）	不明	不明
上記の異常を認めない	～25%	不明	不明	不明

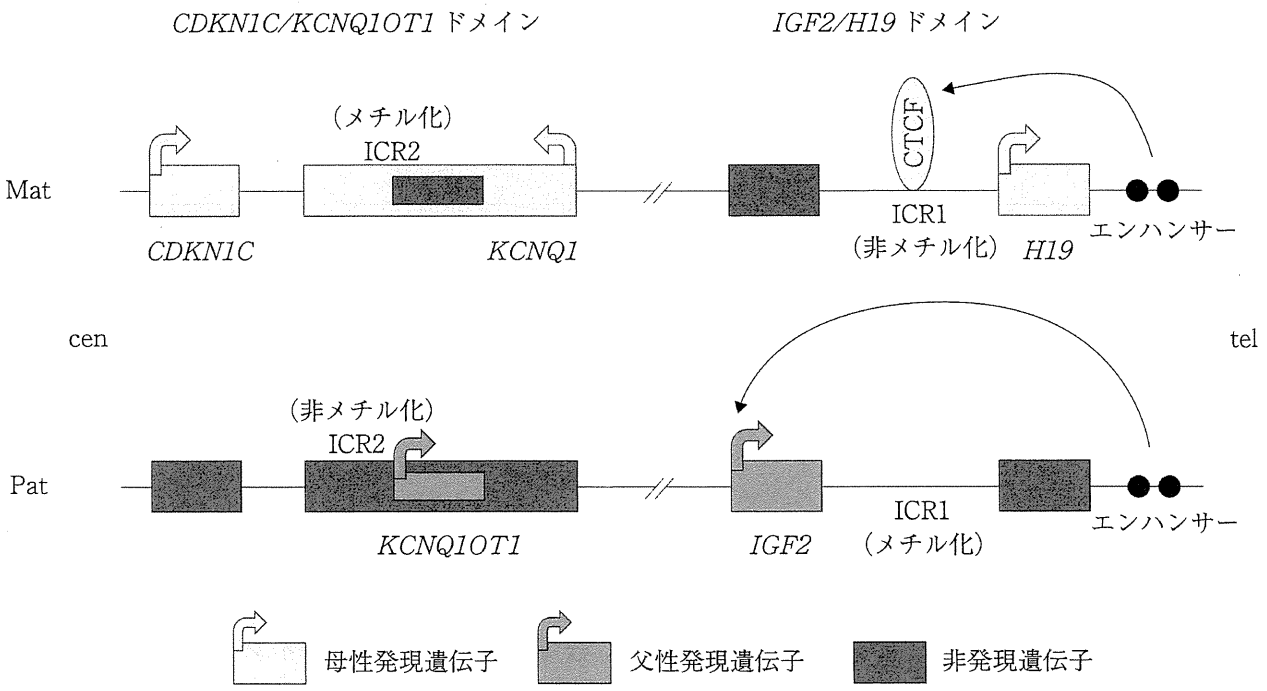

図1　11p15の刷り込みドメイン

11p15には，2つの刷り込みドメインが存在する．テロメア側の*IGF2/H19*ドメインはICR1，セントロメア側の*CDKN1C/KCNQ1OT1*ドメインはICR2により制御されている．ICR1は，父性アレルがメチル化されている．メチル化されていない母性アレルのICR1は，インシュレーターとして働き，CTCFタンパクが結合することで*H19*下流にあるエンハンサーを遮断する．ICR2では，母性アレルがメチル化されている．非メチル化父性ICR2よりnon-coding RNAである*KCNQ1OT1*が転写される．*KCNQ1OT1*はシスに働き，周辺の遺伝子発現を抑制する．Mat：母性アレル，Pat：父性アレル，tel：テロメア側，cen：セントロメア側．

ル化が生じるため，母性アレルからも*KCNQ1OT1*が発現し，*CDKN1C*を含む周辺遺伝子の母性発現を抑制する。*CDKN1C*は，細胞周期の進行を阻害するCDKインヒビター（CKI）をコードしており，その発現低下がBWSを引き起こす（**図2**）。

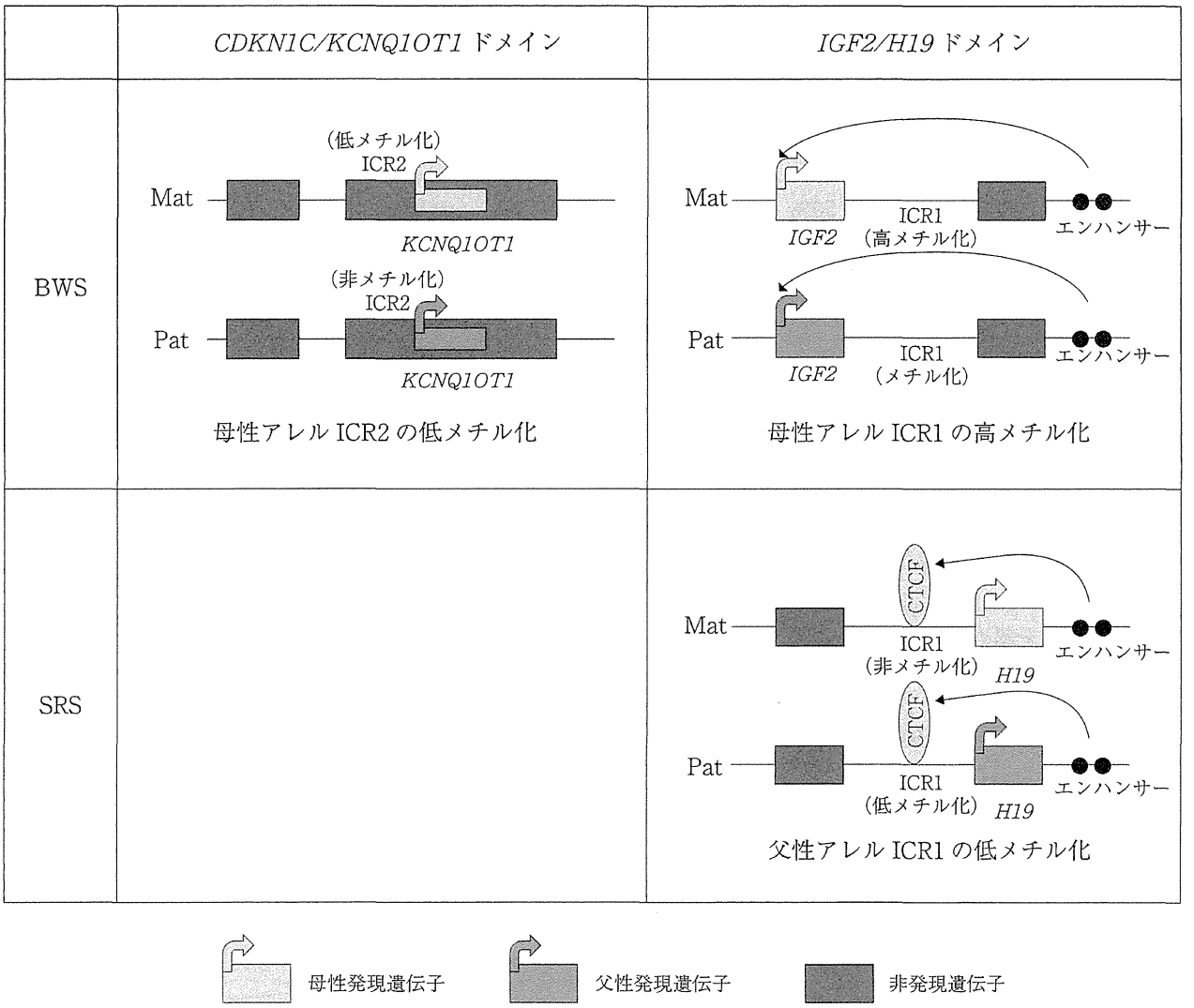

図2　BWS と SRS で見られるメチル化異常

BWS では，*CDKN1C* ／ *KCNQ1OT1* ドメイン，もしくは *IGF2/H19* ドメインのメチル化異常を認める．母性アレル ICR2の低メチル化により *KCNQ1OT1* が発現し，*CDKN1C* の発現低下を来す．また，母性アレル ICR1の高メチル化が *IGF2* 発現増加と *H19* 発現低下を来す．逆に，SRS では父性アレルの ICR1の低メチル化が *IGF2* 発現低下と *H19* 発現増加を来す．SRS では ICR2単独のメチル化異常は報告されていない．

2）*IGF2/H19* ドメインの異常

健常人では，*IGF2* は父性発現，*H19* は母性発現を示す。この刷り込み状態は，*H19* の上流にある ICR1によって制御されている。ICR1は母性アレルが非メチル化し，父性アレルがメチル化されている。母性アレルの非メチル化 ICR1はインシュレーターとして機能し，zinc finger タンパクである CTCF が結合することで *H19* 下流にあるエンハンサーが *IGF2* プロモーターに作用することを阻害する。その結果，母性アレルの *IGF2* は転写されず，エンハンサーは *H19* に作用するため，母性発現を示す。一方，父性アレルの ICR1はメチル化されているため CTCF が結合できない。そのためインシュレーターとして機能せず，*H19* 下流のエンハンサーは *IGF2* プロモーターに作用し，*IGF2* が転写される[8)9)]。ICR1のメチル化は *H19* プロモーターにまで及ぶため，*H19* の発現は抑制される。BWS では，母性アレルの ICR1が高メチル化の結果，*IGF2* の発現上昇と *H19* 発現低下を来す（**図2**）。*IGF2* は細胞増殖因子であり，その発現量増加が BWS を引き起こす。

表3　SRSの診断基準

必須基準	体重 and/or 身長が－2SD 以下
大基準	・出生時の相対的大頭 （頭囲の SD と体重 and/or 身長の SD の差が1.5を超える） ・出生後の発育遅延 （－2SD 以下，2歳時もしくは測定可能な年齢で） ・幼少期の前額突出 ・体の非対称性 ・乳幼児期の重度の哺乳・摂食障害 and/or 出生後の BMI －2SD 未満
診断	必須基準かつ大基準を3つ以上満たす

3）父性片親性ダイソミー（patUPD）

ほとんどが11p に限局した部分 UPD を示し，11p15の2つの刷り込みドメインがともに父親由来となる。そのため，ICR2は低メチル化，ICR1は高メチル化となり，*CDKN1C* の発現低下と *IGF2* の過剰発現が生じることにより BWS を引き起こす。また，patUPD は体細胞モザイクを示すことから，受精後の体細胞組み換えにより生ずると考えられている。

4）*CDKN1C* の変異

上述のように CDKN1C は CKI をコードしており，BWS でみられる変異はN末端の CKI ドメインのミスセンス変異かC末端の QT ドメインを失う機能喪失変異である。このため，細胞増殖を抑制できず BWS が発症する。変異は，孤発例では5％に認められるが，家族例の場合は40％に認める[4)6)]。*CDKN1C* は母性アレル優位の発現を示すため，その変異が母由来であるとき，BWS が発症する。最近，成長障害を示す IMAGe 症候群の原因が *CDKN1C* の機能獲得変異であることが報告された[10)]。

5）染色体構造異常

頻度としては低いが，母性アレルにおける *KCNQ1* 遺伝子内の転座・逆位や，11p15における父性アレルの重複を認める場合がある[11)]。

2．BWS 発症原因別の小児腫瘍の合併リスク

ICR1の高メチル化や patUPD では，腫瘍（Wilms 腫瘍や肝芽腫）の合併リスクが25％以上と高いが，*CDKN1C* の発現低下を起こす ICR2低メチル化や *CDKN1C* 変異の腫瘍合併リスクは低く5％以下である（**表2**）[12)]。しかしながら，健常者と比べると腫瘍合併リスクは高く，特に ICR2低メチル化では Wilms 腫瘍以外の肝芽腫や横紋筋肉腫などが発症しやすい。したがって，発症原因にかかわらず，定期的な腫瘍スクリーニングは重要である。

Ⅱ．Silver-Russell 症候群（SRS）

SRS は，子宮内発育遅延，キャッチアップしない低身長，逆三角形の顔貌（前額突出，とがった顎），相対的大頭，身体の左右非対称を特徴とする刷り込み疾患である。BWS と同様に臨床症状は多様であり，耳介低位，第5指彎指症，カフェオレ斑，性器異常，低血糖，過度の発汗，青色強膜，合指症，重度の摂食障害などが認められる[1)]。多様な臨床症状を示すことから臨床診断が困難であったため，臨床スコアリングシステムが提唱された（**表3**）[1)]。SRS の多くは孤発例だが，家族例の報告もある[13)]。SRS の発症原因としては，ICR1低メチル化（30～60％）

と7染色体母性片親性ダイソミー（matUPD7）（7～10%）がある[1]。ICR1低メチル化では左右非対称がよくみられ，matUPD7では言語の遅れがよくみられる[14)15)]。

1．SRSの発症原因

1）*IGF2/H19*ドメインの異常

健常人ではメチル化されている父性アレルのICR1が低メチル化となり，その結果*H19*の発現上昇，*IGF2*の発現低下によりSRSを引き起こす（**図2**）[1]。ICR1低メチル化はSRSの30～60%にみられるが，BWSでみられるICR1高メチル化と正反対の異常である。この相反する二種類のメチル化異常による*IGF2*の発現異常は，相反する表現型を示すBWSとSRSという二種類の疾患を引き起こすのである。BWSの原因としてはICR2の低メチル化もあるが，SRSではICR2単独の高メチル化を示した報告はない[16]。しかし，11p15の母性アレル重複を認めたSRSの2症例では重複領域に2つの刷り込みドメインを含んでいること，さらに，*CDKN1C/KCNQ1OT1*ドメインに限局した母性アレルの重複を認めたSRS患者が報告されたことから，母性アレル重複による*CDKN1C*の過剰発現が関与している可能性がある[17)18)]。

2）7染色体母性片親性ダイソミー（matUPD7）

SRSにおけるmatUPD7の多くは，7番染色体全体がUPDであることが多い。SRS発症機序として，増殖促進因子をコードする父性発現遺伝子の発現消失，あるいは増殖抑制因子をコードする母性発現遺伝子の発現増加が推測される。7p11.2-p13の母由来重複例と7q31-qterの部分的matUPD症例が報告され，両領域が責任座位として注目された[19)20)]。7p11.2-p13には，*GRB10*が存在する。マウス*Grb10*は母性発現し，過剰発現で成長障害とインスリン抵抗性を示す。一方，7q32には，父性発現する*MEST*（*PEG1*），*MESTIT1*，*COPG2*，*CIT1/COPG2IT1*，母性発現する*CPA4*が存在する（このうち*MESTIT1*と*CIT1/COPG2IT1*はnon-coding RNAである）。しかし，matUPD7を示さないSRS患者において，これらの刷り込み遺伝子の変異やDNAメチル化異常は認められず，SRSの原因遺伝子同定までは至っていない[21)22)]。

2．SRSに対する治療

対症療法が中心となる。SGA（small-for-gestational age）低身長症の基準を満たせば成長ホルモン治療を行うことができる。左右非対称に対する整形外科的な介入が必要となる場合がある。

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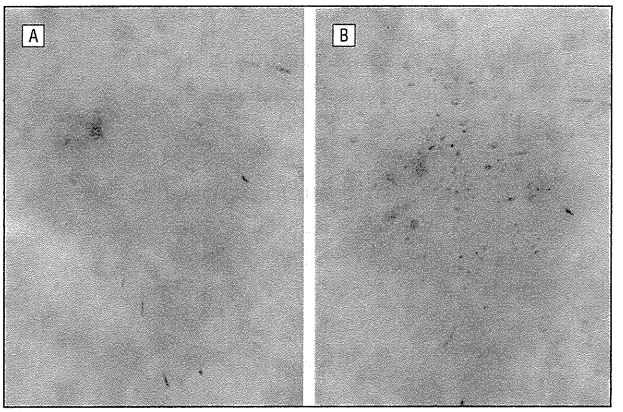

Figure 2. Dermoscopic images presented at a conference. A, At a recent dermoscopy conference with more than 200 participants, over 50% thought that the pictured lesion was a melanocytic tumor. B, After the ink test, over 90% of participants diagnosed this lesion as a seborrheic keratosis.

ings or crypts, gyri and sulci creating networklike structures, moth-eaten borders, hairpin blood vessels, and sharp demarcation. Of the SK features, comedolike openings and gyri and sulci are examples of 3-dimensional structures that may prove difficult to identify under 2-dimensional dermoscopy, especially in early macular lesions and in lesions that are less heavily pigmented. Dermoscopic networklike and globulelike structures present in SKs are quite common with gyri and sulci present in 52% to 61% of lesions and comedolike openings present in 71% to 80%.[2,3] These structures may be missed entirely or else confused for a pigment network or globules, which may lead to diagnostic uncertainty or misclassification as a melanocytic lesion.

However, with use of the ink test, these features can be highlighted and clarified. The procedure that we have used to successfully and reliably identify these 2 elements is as follows: First, mark the lesion thoroughly with a felt-tipped surgical marking pen. Next, remove the ink from the surface of the lesion with an alcohol wipe. Finally, view the lesion under dermoscopy; the ink will remain within the sulci and comedolike openings. The image may be viewed side-by-side with an image taken from before the ink test to confirm that the inked areas align with previously classified areas of globules and/or network (**Figure 2**).

The same process has been applied reliably to highlight the cornoid lamella in porokeratosis or in acral melanocytic lesions to distinguish the furrows of the skin from the ridges.[4,5] With the expanded application of the ink test for the visualization of features of SKs, we hope to limit the misdiagnosis of SKs as melanocytic lesions.

Sarah Yagerman, BS
Ashfaq A. Marghoob, MD

Author Affiliations: Weill Cornell Medical College of Cornell University, New York, New York (Ms Yagerman); Dermatology Service, Memorial Sloan-Kettering Cancer Center, New York, New York (Drs Marghoob and Ms Yagerman).
Correspondence: Dr Marghoob, Memorial Sloan-Kettering Skin Cancer Center Hauppauge, 800 Veterans Memorial Hwy, Second Floor, Hauppauge, NY 11788 (marghooa@mskcc.org).
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The Possibility That Multiple Mucocutaneous (Palisaded Encapsulated and Nonencapsulated) Neuromas May Be a Distinct Entity

Early childhood–onset mucosal neuromas of the lips, tongue, and eyelids are pathognomonic for multiple endocrine neoplasia (MEN) 2b syndrome, which is also associated with medullary thyroid carcinoma and pheochromocytoma. It has recently been suggested that multiple mucocutaneous neuromas may also represent an early manifestation of *PTEN* hamartoma-tumor syndrome (PHTS), including Cowden syndrome and others.[1,2] Mucocutaneous neuromas in PHTS appear in early childhood and characteristically involve acral sites.[1,2]

MEN 2b syndrome is caused by germline mutations in the *RET* proto-oncogene (usually in exons 15 and 16), while PHTS is associated with *PTEN* germline mutations. We herein report a case of adult-onset multiple mucocutaneous (palisaded encapsulated and nonencapsulated) neuromas without any features of either MEN 2b syndrome or PHTS.

Report of a Case. A 50-year-old man presented with a 15-year history of multiple, occasionally painful, papules and nodules that had increased in number and were distributed all over the body. A physical examination revealed approximately 100 skin-colored, translucent or brownish papules and nodules measuring 2 to 10 mm in diameter with a predilection for the upper and lower lips (**Figure 1**A) and bilateral acral sites (the back and palmar sides of the hands and fingers) (Figure 1B and C); they were also spread throughout the periorificial area, trunk (Figure 1D), and extremities. No other abnormal findings, including corneal nerve hypertrophy, were seen. Forty-eight lesions were surgically removed from various sites and histopathologically showed features of either palisaded encapsulated neuroma (**Figure 2**A and B), neuroma (resembling the features of mucosal neuroma in MEN

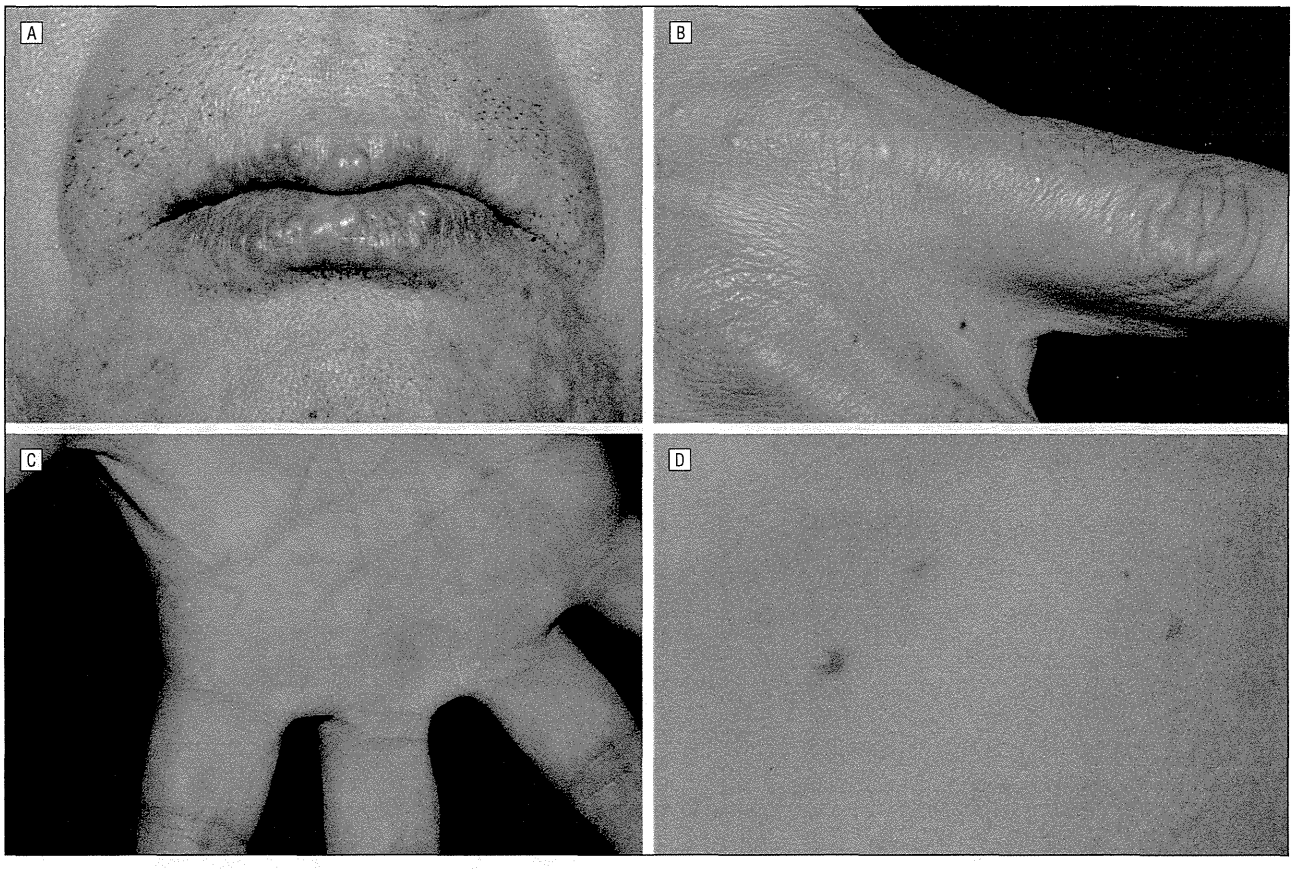

Figure 1. Skin-colored, translucent, or brownish papules and nodules. Lesion appear on the upper and lower lips (A), back side of the left hand and middle and index fingers (B), palmar side of the right hand and fingers (C), and lower back (D).

2b syndrome) (Figure 2C and D), or combined features of these 2 neoplasms.

The patient's medical and family histories were unremarkable. An intensive workup, including laboratory investigations and magnetic resonance imaging studies, revealed no abnormalities, and there were no findings indicative of either MEN 2b syndrome or PHTS. A DNA analysis of the *RET* proto-oncogene (exons 13, 15, and 16) and *PTEN* gene (exons 1-9) showed no mutations. His two daughters (ages 24 and 22 years) and son (age 20 years) had no similar papules or nodules.

Comment. The present case of multiple mucocutaneous (palisaded encapsulated and nonencapsulated) neuromas involved the following characteristics: (1) adult onset; (2) whole-body involvement with a predilection for the lips and acral sites; (3) isolated clinical features without any other abnormalities; and (4) the absence of germline mutations in either the *RET* proto-oncogene or *PTEN* gene.

A few cases of multiple mucocutaneous neuromas as an isolated clinical feature without germline mutations of the *RET* proto-oncogene have been reported.[3-5] However, no analyses of the *PTEN* gene were performed in these cases,[3-5] thus suggesting the possibility of PHTS.[1] Based on no mutations either in the *RET* proto-oncogene or the *PTEN* gene, the present case is the first to demonstrate that this rare clinical presentation may truly be a distinct entity.

The relationship between palisaded encapsulated neuroma and neuroma in MEN 2b syndrome has been noted.[1,5] The present case is the first demonstration that the 2 neoplasms lie within a spectrum of the same neoplastic entity, as they simultaneously occurred in the same patient.

Noriyuki Misago, MD
Keiichiro Joh, PhD
Hidenobu Soejima, MD, PhD
Yutaka Narisawa, MD

Author Affiliations: Department of Internal Medicine, Division of Dermatology (Drs Misago and Narisawa), and Department of Biomolecular Sciences, Division of Molecular Genetics & Epigenetics (Drs Joh and Soejima), Faculty of Medicine, Saga University, Saga, Japan.
Correspondence: Dr Misago, Department of Internal Medicine, Division of Dermatology, Faculty of Medicine, Saga University, Nabeshima 5-1-1, Saga 849-8501, Japan (misago@post.saga-med.ac.jp).
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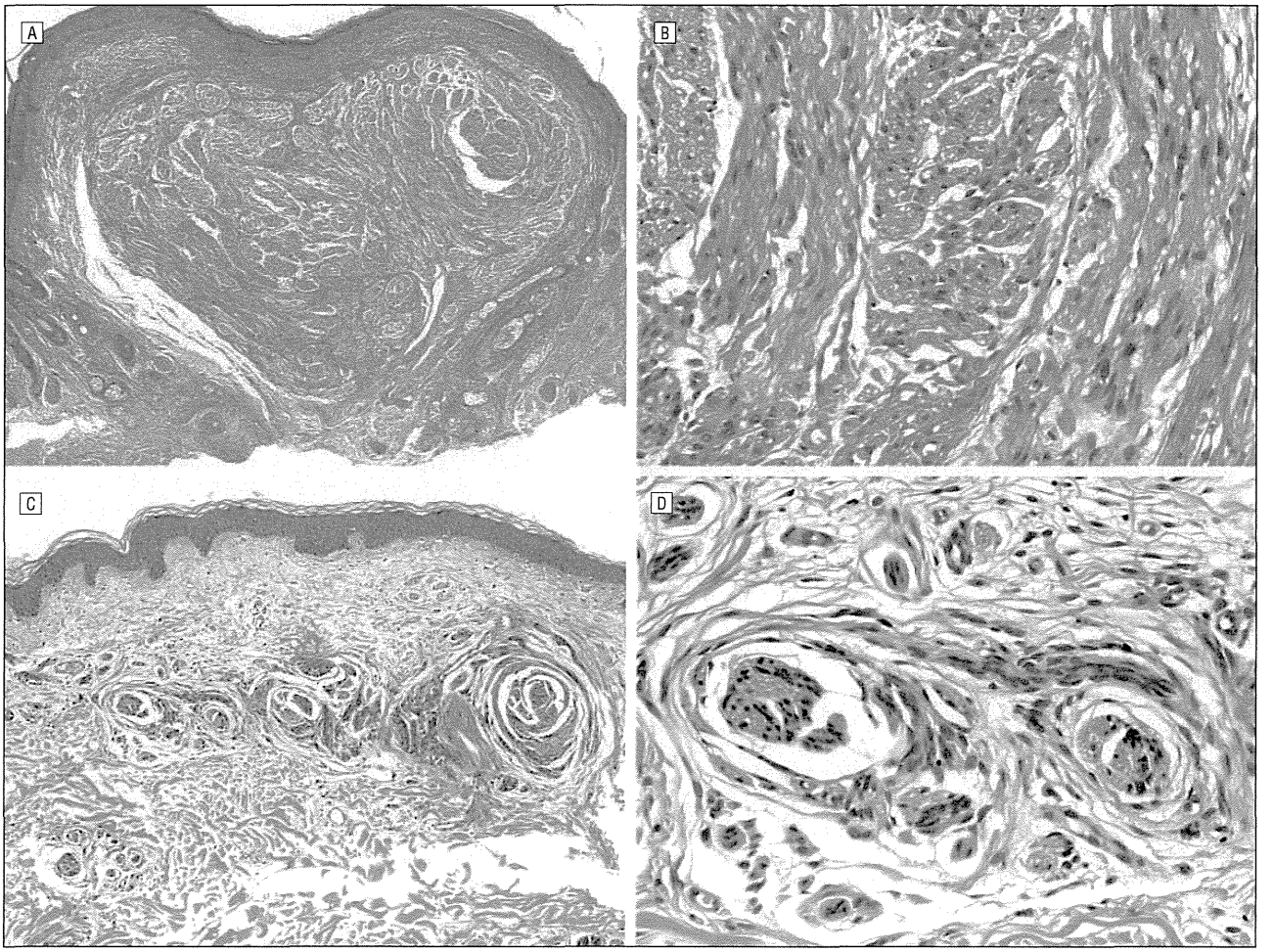

Figure 2. Hematoxylin-eosin–stained specimens of the palisaded encapsulated neuroma (A and B) and neuroma lesions (C and D). A, Large neoplastic aggregation with a smaller adjacent aggregation in the dermis, both of which are composed of intersecting fascicles separated by clefts (original magnification ×10). B, Close-up view of the intersecting fascicles with vertical and cross-sections (original magnification ×100). C, Several neighboring or scattered, thick and proliferated nerve bundles in the dermis (original magnification ×25). D, A close-up view of the proliferated nerve bundles with vertical and cross-sections associated with mucinous stroma (original magnification ×100).

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Neuropsychiatric Symptoms Associated With Topical Imiquimod Therapy: Report of 2 Cases

Imiquimod is an immune response modulator approved for topical treatment of actinic keratoses, basal cell carcinomas, and genital and perianal warts. Although the most common adverse effects are cutaneous reactions, systemic reactions—notably flulike symptoms—have been described.[1] Official prescribing information also lists "neuropsychiatric" symptoms among adverse reactions in postmarketing experience, but no details are provided.[1] We describe 2 patients who experienced acute neuropsychiatric symptoms—delirium in one case and mania in the other—during overuse of imiquimod. To our knowledge, these specific reactions have not been described previously.

Reports of Cases. *Case 1.* A dermatologist prescribed thrice-weekly application of imiquimod cream for a 91-year-old woman with a forehead basal cell carcinoma. She returned 2 weeks later with a vigorous inflammatory reaction covering much of her face. She admitted to using the cream more frequently—and over a much broader area—than prescribed; whether she washed her face after overnight use was unclear. During the ensuing weeks, she developed progressive insomnia, agitation, difficulty concentrating, and cognitive impairment. Results of laboratory testing and brain magnetic resonance imaging were unremarkable.

The patient was previously in excellent health. She took no medications, lived independently, drove a car, and socialized with friends. However, during the year following this episode, she required substantial help from family members and met criteria for mild dementia. Her physicians concluded that imiquimod triggered de-

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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.
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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
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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

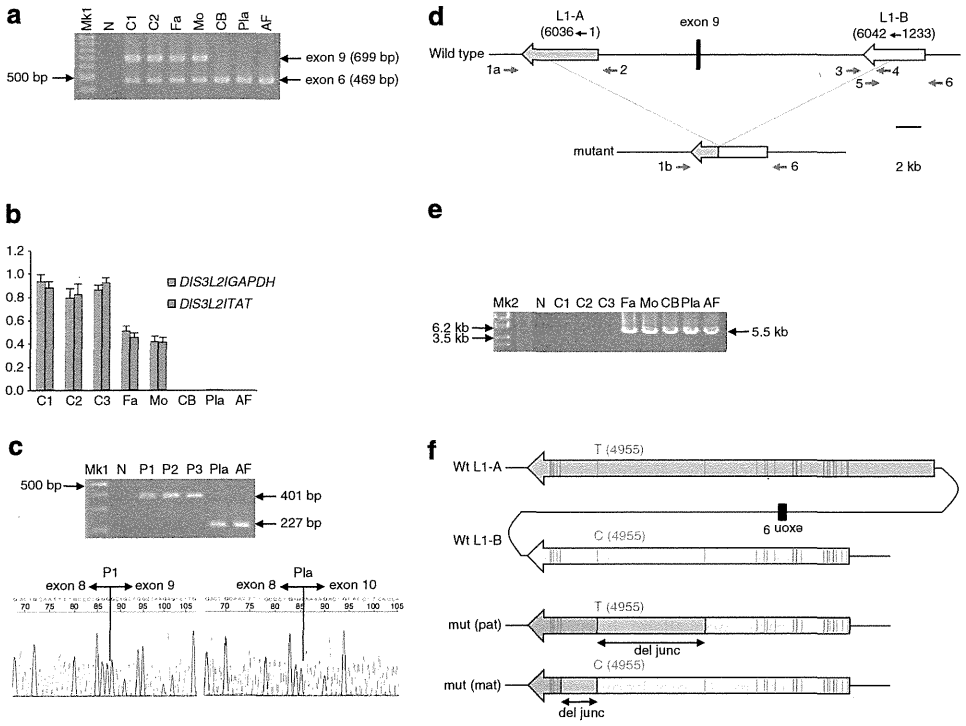

Figure 1 Homozygous deletion of *DIS3L2* exon 9 in a patient with Perlman syndrome. (**a**) Homozygous deletion of *DIS3L2* exon 9. Duplex PCR of exons 6 and 9 showed no amplification of exon 9 in any patient sample, whereas both exons were amplified in the parents and normal controls. Mk1, 100-bp ladders; 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 *Sty*I. (**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.

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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

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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.

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

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′