

CDKN1C (*p57^{Kip2}*) Analysis in Beckwith–Wiedemann Syndrome (BWS) Patients: Genotype–Phenotype Correlations, Novel Mutations, and Polymorphisms

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Beckwith–Wiedemann syndrome (BWS) is an overgrowth syndrome characterized by macroglossia, macrosomia, and abdominal wall defects. It is a multigenic disorder caused in most patients by alterations in growth regulatory genes. A small number of individuals with BWS (5–10%) have mutations in *CDKN1C*, a cyclin-dependent kinase inhibitor of G1 cyclin complexes that functions as a negative regulator of cellular growth and proliferation. Here, we report on eight patients with

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BWS and *CDKN1C* mutations and review previous reported cases. We analyzed 72 patients (50 BWS, 17 with isolated hemihyperplasia (IH), three with omphalocele, and two with macroglossia) for *CDKN1C* defects with the aim to search for new mutations and to define genotype–phenotype correlations. Our findings suggest that BWS patients with *CDKN1C* mutations have a different pattern of clinical malformations than those with other molecular defects. Polydactyly, genital abnormalities, extra nipple, and cleft palate are more frequently observed in BWS with mutations in *CDKN1C*. The clinical observation of these malformations may help to decide which genetic characterization should be undertaken (i.e., *CDKN1C* screening), thus optimizing the laboratory evaluation for BWS.

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Key words: overgrowth syndrome; mutations; cleft palate; omphalocele; polydactyly; extra nipple

INTRODUCTION

Beckwith–Wiedemann syndrome [BWS (OMIM 130650)] is a phenotypically variable and genotypically heterogeneous overgrowth syndrome characterized by somatic overgrowth, macroglossia and abdominal wall defects. Other findings include hemihyperplasia, embryonal tumors, adrenocortical cytomegaly, ear anomalies, visceromegaly, renal abnormalities, neonatal hypoglycemia, and occasionally cleft palate, polydactyly and a positive family history [Beckwith, 1963; Wiedemann, 1964; Pettenati et al., 1986; Elliott and Maher, 1994; Elliott et al., 1994; Weng et al., 1995; Engstrom et al., 1998]. BWS is a complex, multigenic disorder caused in up to 90% of patients by an alteration in growth regulatory genes located on chromosome 11p15 [Li et al., 1997, 1998]. Several molecular abnormalities are associated with BWS. Chromosomal rearrangements are relatively rare (~2–3% of cases) and comprise translocations or inversions (typically maternally inherited), and paternal duplications. The largest molecular subgroup (~60–70% of cases) is represented by patients carrying an epigenetic error in one or more genes on 11p15 [Maher and Reik, 2000; Cooper et al., 2005; Weksberg et al., 2005; Enklaar et al., 2006]. This region spans approximately 1 Mb and includes two differentially-methylated imprinted domains that control imprinting of genes in this cluster [Weksberg et al., 2003]. Patients (~15%) may also have paternal uniparental disomy, two paternally derived copies of 11p15 and no maternal contribution for that region [Henry et al., 1991]. Finally, a small number of individuals with BWS carry point mutations in *CDKN1C* (also known as *p57^{kip2}*; OMIM 600856). These mutations have been found in 5–10% of sporadic BWS cases [Lee et al., 1997; Li et al., 2001] and in approximately 40% of cases with a positive family history [O’Keefe et al., 1997].

CDKN1C maps centromeric to the region 11p15 and it is paternally imprinted in humans with preferential expression of the maternal allele [Hatada and Mukai, 1995]. It encodes a Cyclin-dependent Kinase (CdK) that functions as a potent tight-binding inhibitor of several G1 Cyclin/CdK complexes, thus acting as a

negative regulator of cellular proliferation [Lee et al., 1995]. Alterations of genes involved in cell cycle regulation are tightly linked to tumorigenesis, which led us to consider *CDKN1C* as a putative tumor suppressor gene. Despite this, and the fact that BWS patients have a 1,000-fold increased risk of embryonal tumors, including Wilms tumor, hepatoblastoma, and rhabdomyosarcoma [Wiedemann, 1983], there is no conclusive evidence that BWS patients with *CDKN1C* mutations have this increased risk of neoplasia.

CDKN1C (ENST00000414822) contains three exons (two coding) and two GC-rich introns of 535 and 83 bp (Fig. 1). Alternative splicing generates the heterogeneity in the translational initiations [Tokino et al., 1996]. The *CDKN1C* protein has 316 amino acids and is expressed in the heart, brain, lung, skeletal muscle, kidney, pancreas and testis. In addition, high levels are seen in placenta, and this fact may have importance in the pathophysiology of preeclampsia/HELLP syndrome [Romanelli et al., 2009]. The protein consists of three structurally distinct domains: (i) the N-terminal domain (aa 1–110) which is significantly similar to the CdK-inhibitors p21Cip1 and p27^{Kip1} and has been shown to be necessary for CdK inhibition; (ii) a central highly polymorphic hexanucleotide repeat encoding a proline-alanine series of repeats, PAPA-repeats (aa 156–213), and (iii) a highly conserved C-terminal region (QT domain) that presents homology with p27^{Kip1} (Fig. 1) [Lee et al., 1995; Matsuoka et al., 1995].

In this investigation we analyzed *CDKN1C* by direct bidirectional sequencing in a series of BWS patients who did not have chromosomal or epigenetic abnormalities at the 11p15 locus. The aim of this work was to look for new mutations, review reported *CDKN1C* aberrations and to evaluate genotype–phenotype correlations. We also performed *CDKN1C* mutational analysis in a series of patients with isolated hemihyperplasia, macroglossia or omphalocele, since these manifestations are frequent features of BWS and theoretically, mutations in *CDKN1C* might be present in patients with mild symptoms of the disorder.

We identified several novel mutations and polymorphisms of *CDKN1C*. We also carried out a genotype–phenotype correlation in our patients and found useful clinical findings that may aid in the laboratory workflow for diagnosis [Percepe et al., 2008]. Nucleotide c.845 appears to be a mutation hotspot as 4 patients had an alteration at this position.

PATIENTS AND METHODS

Patients

To date we have collected a total of 149 patients (127 BWS, 17 IH, 3 omphalocele, 2 macroglossia) which are included in the Spanish Overgrowth Syndrome Registry. We analyzed *CDKN1C* in 50 patients with BWS and 17 with IH who were negative for chromosomal or epigenetic alterations in 11p15. We also included 5 patients with isolated omphalocele (3 patients), and isolated macroglossia (2 patients). Data documented were: clinical and family history, biochemical analysis, X-rays and follow-up information. The institutional research board at Hospital Universitario La Paz approved this investigation and consent was obtained from all cases or their parents.

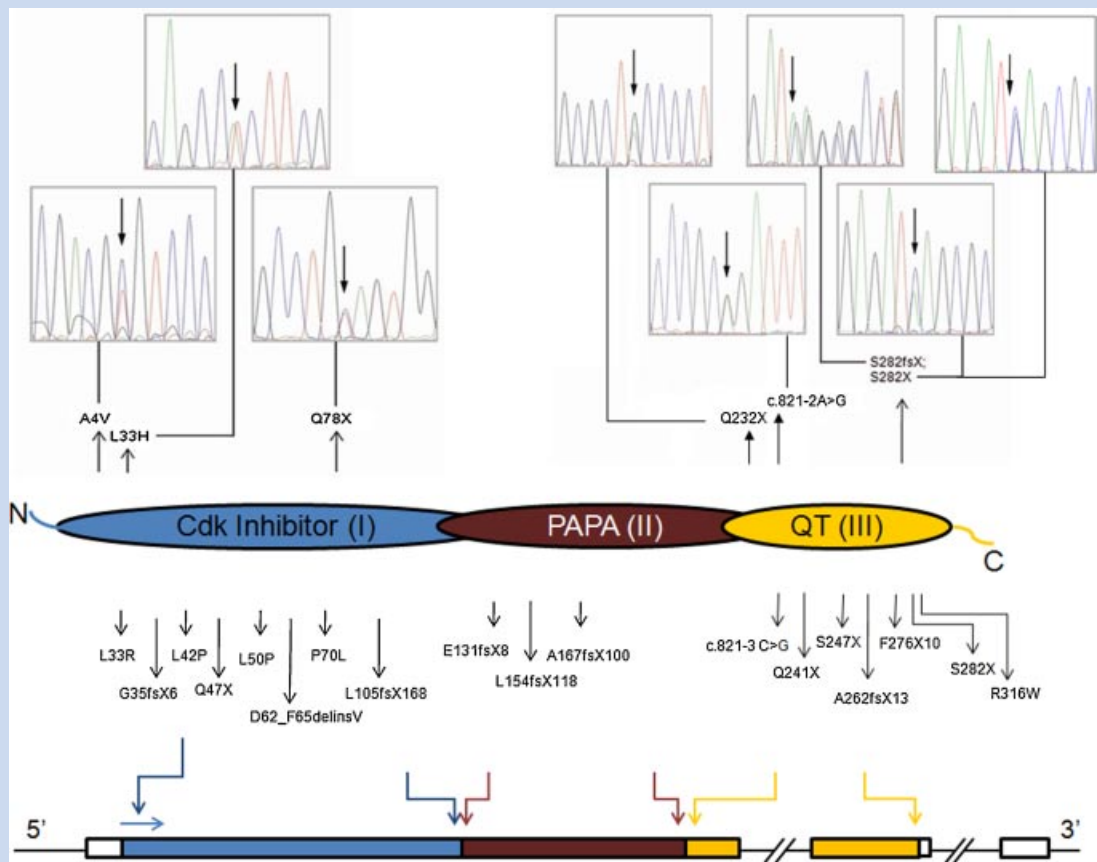


FIG. 1. Schematic representation of *CDKN1C* and mutations identified. Chromatograms of the eight novel mutations identified in this study are shown above the protein structure, while the previously reported mutations are shown below the protein. At the bottom, schematic representation of the gene; rectangles denote the three exons, and the broken lines represent the two introns. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PCR Amplification and Sequencing of *CDKN1C*

DNA samples were obtained from peripheral blood leukocytes using Puregene Blood Core Kit B (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

Primers for the amplification of *CDKN1C* were designed with the help of the OLIGO 6 software (Molecular Biology Insights, Inc., Cascade, CO). The following set of primers was used to obtain a single amplicon (1,675 bp) that comprises the entire coding region of *CDKN1C*: sense primer, 5’-cgccctctctctctctcttcccttc-3’ and antisense primer: 5’-tcgggctcttgggctctaaact-3’. The PCR reaction mixture consisted of: 10× PCR buffer HotStartTaq QIAGEN (containing 15 mM MgCl₂), 0.4 mM dNTPs, 0.4 μM of each primer, 10% DMSO, 100 ng DNA, 2 units HotStart Taq polymerase (Qiagen) in a final volume of 25 μL. A Tetrad2 thermocycler (Bio-Rad Laboratories, Inc., Hercules, California) was used for PCR; PCR conditions were a touchdown PCR consisting of an activation step at 95°C for 15 min; followed by 15 cycles of 95°C for 1 min 30 sec, 68°C for 1 min (with a touchdown of 0.5°C every cycle) and 72°C for 2 min and subsequently 23 cycles of 95°C for 1 min 30 sec, 60°C for 1 min and 72°C for 2 min and a final primer extension step

for 72°C for 10 min. PCR fragments were purified by ExoSAP-IT (USB) and sequenced by BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California). Due to the high polymorphic structure of the PAPA domain, sequencing of *CDKN1C* was performed with the help of eight different primers (Table I). Finally, sequences were precipitated by CleanSEQ (Agencourt) and sequenced on an ABI 3130 automatic sequencer.

TABLE I. Primers Used for Sequencing of *CDKN1C*

	Primer for sequencing	Exons
1F	5’-ctccttcccttctctctcg-3’	1
2F	5’-tggaccgaagtggacagcga-3’	1
3R	5’-cctgcaccgtctcgcgtag-3’	1
4F	5’-ccggagcagctgcctagtgc-3’	1
5R	5’-ggggccaggaccgcgacc-3’	1
6R	5’-gggaggaggcgggaaccctgcga-3’	1
7F	5’-cggcgacgtaaacaaagctgac-3’	2
8R	5’-tcgggctcttgggctctaaact-3’	2

3D Structure Modeling

Structural model of the CdK-inhibitor domain of human CDKN1C protein (UniProtKB/Swiss-Prot code P49918, aa 26–96) bound to cyclin A-CDK2 complex was constructed by standard comparative modeling methods and the software DeepView [Guex and Peitsch, 1997], using the structure of p27^{kip1}/cyclin a/Cdk2 complex deposited in the Protein Data Bank (PDB) [Berman et al., 2000] with code 1JSU [Russo et al., 1996] as template. Sequence identity between p27 and CDKN1C modeled domain was 47%, with a Blast e-value of 1.4×10^{-14} . The quality of the model was checked using the analysis programs (Anolea, Gromos and Verify3D) provided by the SWISS-MODEL server [Peitsch, 1996; Guex et al., 1999; Schwede et al., 2003].

RESULTS

Patients with either a complete phenotype of BWS ($n = 50$) or IH, omphalocele or macroglossia ($n = 22$) were studied. We found mutations in 8 BWS patients; all were novel, 6 of 7 were inherited from the mother, 1 was a de novo mutation and in 1 (patient 8) the inheritance is not known. Five of 8 mutations were nonsense mutations (Table II, Fig. 1). We also identified 6 synonymous non-described variants in our series (Table III). Novel mutations and clinical findings are listed in Tables II and IV.

DISCUSSION

To date, 25 different mutations have been reported in *CDKN1C* including the 7 novel mutations reported here. Among these, we found a novel splice mutation in intron 1 (c.821-2 A>G) which may lead to either inclusion of the intron 1 or exon-skipping of exon 2. Only two amino acids were affected in more than one patient; Leu33 (in two patients, L33H and L33R) and Ser282 (in four patients, S282X and S282fsX) (Table II). These residues comprise 23% of reported mutations and may well behave as mutation hot spots. Using homology-based modeling, we constructed a 3D structural model of the CdK-inhibitor domain of CDKN1C bound to cyclin A-Cdk2 (Fig. 2). The model suggested that the pair of CDKN1C residues (Leu33 and Phe34) conforms to a small hydrophobic patch in close contact with a hydrophobic groove composed by cyclin A residues Met210, Ile213, Leu214, Trp217, and Leu253. The introduction of a positively charged His at residue 33 (L33H) probably affects the stabilization of the complex (as suggested in the L33R mutant [Engel et al., 2000]) and may modify the local protein-protein interaction, which may result in a loss of binding affinity.

We searched for putative Exonic Splicing Enhancer (ESE) sequences in the CDS of *CDKN1C* using ESEfinder [Cartegni et al., 2003; Smith et al., 2006], RESCU-ESE [Fairbrother et al., 2002] and the Regulatory Sequence Database of the ASD Project [Stamm et al., 2006]. ESEs are short sequences found within coding exons that are

TABLE II. *CDKN1C* Mutations Identified to Date in BWS Patients*

Nucleotide change	Amino acid change	Protein domain	Inheritance	References
c.11 C>T	p. A4V	I	Maternal	Novel
c.98 T>A	p. L33H	I	Maternal	Novel
c.98 T>G	p. L33R	I		Engel et al. [2000]
c.105delG	p. G35fsX6	I		Lee et al. [1997]
c.125 T>C	p. L42P	I		Li et al. [2001]
c.139 C>T	p. Q47X	I		Hatada et al. [1996]
c.149 T>C	p. L50P	I		Li et al. [2001]
c.185_193delATTACGACT	p. D62_F65delinsV	I		O'Keefe et al. [1997]
c.209 C>T	p. P70L	I		Lam et al. [1999]
c.232 C>T	p. Q78X	I	De novo	Novel
c.310_311delCTinsG	p. L105fsX168	I		Hatada et al. [1997]
c.391_392insT	p. E131fsX8	I/II		Engel et al. [2000]
c.461delT	p. L154fsX118	I/II		Lee et al. [1997]
c.499_514delGCTCCGGTCGCGGCTC	p. A167fsX100	II		Lam et al. [1999]
c.694 C>T	p. Q232X	III	Maternal	Novel
c.721 C>T	p. Q241X	III		Li et al. [2001]
c.740 C>A	p. S247X	III		Hatada et al. [1996]
c.784_785delGC	p. A262fsX13	III		Li et al. [2001]
c.821-2 A>G	Splice mutation		Maternal	Novel
c.821-3 C>G	Splice mutation			Lam et al. [1999]
c.826delTinsAG	p. F276fsX10	III		Hatada et al. [1996]
c.845 C>G	p. S282X	III		Lam et al. [1999], current paper
c.845 C>A	p. S282X	III	Maternal	Novel
c.845delC	p. S282fsX	III	Maternal	Novel
c.946 C>T	p. R316W	III		Lam et al. [1999]

*Mutations affecting the same residues as previously reported mutation [Lam et al., 1999; Engel et al., 2000].

^aMutations are numbered according to ensembl ENST00000414822.

TABLE III. Reported and Novel *CDKN1C* Variants*

Number and percentage of patients (n = 72)	Nucleotide change	Amino acid residue	Protein domain	References
11 [15.3]	c.1–84 G>A			Lam et al. [1999]
1 [1.4]	c.1–83 G>A			Lam et al. [1999]
1 [1.4]	c.456 G>A	p. V152V	I/I I	Current paper
1 [1.4]	c.504 G>A	p. P168P	II	Current paper
32 [44.4]	c.511_522delGCTCCGGTCGCG	p. A171 A174del	II	Tokino et al. [1996]
3 [4.2]	c.528 G>C	p. A176A	II	Current paper
26 [36.1]	c.555 T>C	p. A185A	II	Tokino et al. [1996]
2 [4.2]	c.598_609delCCAGCCCCGGCC	p. P200 A203del	II	Tokino et al. [1996]
2 [2.8]	c.599 A>G	p. P200P	II	Current paper
3 [4.2]	c.612 G>A	p. P205P	II	Current paper
1 [1.4]	c.616_627delCCGGCCCCGGCC	p. P206 A209del	II	Tokino et al. [1996]
1 [1.4]	C.7Q8 G>A	p. E236E	III	rs3741341
45 [62.5]	c.951 + 29_951 + 30insG			rs34289096
1 [1.4]	c.951 + 29_951 + 30insGG			Current paper

*Mutations are numbered according to ensembl transcript ENST00000414822.

often predicted to be binding sites for splicing factors. ESEs are required for efficient splicing and may influence splice site recognition during both constitutive and alternative splicing [Blencowe, 2000]. We analyzed all known and novel missense mutations to find possible alterations in ESE sequences. ESEfinder identified a putative SRp40 element that is disrupted by the cytosine to thymine substitution at position 139 (c.139 C>T, Q47X; from score 3.04 to score 0). Both RESCU-ESE and the ASD-Regulatory Sequence Database identified a putative ESE sequence that is disrupted by the deletion at position 185 (c.185_193delATACGACT, D62_F65delinsV). RESCU-ESE identified a putative ESE sequence that is disrupted by the thymine insertion at position 391 (c.391_392insT, E131fsX8). These findings suggest that these mutations (Q47X, D62_F65delinsV and E131fsX8), apart from leading to the synthesis of a truncated protein, may also affect the splicing of the *CDKN1C* pre-mRNA. However, experimental evidence is necessary to confirm that these mutations disrupt ESE motifs. Finally, we also observed known and novel non-described variants not only SNPs but also the 12 bp ins/del polymorphism in the PAPA domain.

CDKN1C mutations were identified in 8 of 50 patients with BWS and in none of the patients with isolated omphalocele, hemi-hyperplasia or macroglossia. The absence of mutations in these three other malformations was expected due to the small number of cases evaluated and to the observation that no mutations in *CDKN1C* have previously been reported in these patients with isolated findings. The percentage of cases presenting with *CDKN1C* mutations in our series is in agreement with previously reports (8/127 = 6.2%) [Lee et al., 1997; Li et al., 2001]. In most BWS cases (6/7) the mutation was inherited from apparently asymptomatic mothers, who either inherited the change from their fathers or had de novo mutations in the paternal chromosome. Three of these mothers developed preeclampsia/HELLP syndrome during pregnancy [Romanelli et al., 2009]. In one adult case, the pattern of inheritance could not be evaluated due to lack of parental samples.

Most patients with BWS had omphalocele or umbilical herniae and three displayed cleft palate, which is considered as a major finding but is not frequently reported in BWS cases [Weksberg et al., 2010]. The *cdkn1c* $-/-$ mice have cleft palate, which suggests the possibility of an increased frequency of this malformation in BWS caused by *CDKN1C* mutations [Takahashi et al., 2000]. Among the 126 patients with BWS present in our cohort only three had cleft palate, all with *CDKN1C* mutations located in the QT domain of the protein. However, previously reported patients with mutations affecting domain III did not show cleft palate [Hatada et al., 1996; Lam et al., 1999; Li et al., 2001] indicating that our finding may be merely coincidental or caused by unknown factors. Interestingly, of the 26 mutations reported, 15 were mutations that altered or lacked the QT domain, which would leave the cyclin/Cdk binding and inhibitory region of the protein intact [Matsuoka et al., 1995]. Therefore, the QT domain seems to have a regulatory function for the p57^{kip2} protein.

Furthermore, two patients had polydactyly and two had extra nipples. Lam et al. [1999] reported a high frequency of omphalocele in patients with *CDKN1C* mutations, but found no cases with polydactyly, extra nipple, or cleft palate. One patient with polydactyly and an accessory nipple was previously reported, but his affected sister had neither polydactyly nor polythelia [Hatada et al., 1996]. Finally, one patient had hypospadias and another cryptorchidism. Genital anomalies have been recently noted as important clinical findings in adults and they seem to be more frequent than initially reported [Greer et al., 2008].

Altogether, these findings suggest that BWS with *CDKN1C* mutations may sometimes exhibit a different, heterogeneous pattern of clinical malformations than those with epigenetic/chromosomal abnormalities. These anomalies include polydactyly, extra nipple, genital anomalies and cleft palate. Identifying those characteristics may be useful to focus the molecular analysis undertaken.

BWS patients have a 5–7% risk of neoplasia [Lapunzina, 2005]. *CDKN1C* has been implicated in several types of human cancer such

TABLE IV. Clinical Findings of Eight BWS Patients With *CDKN1C* Mutations

	Patient							
	1	2	3	4	5	6	7	8
Sex	Male	Female	Male	Female	Male	Male	Male	Male
Age	1 y 6 m	3 y 6 m	11 y 3 m	2 y 10 m	6 y 7 m	1 y 7 m	7 y 7 m	32 y
Nucleotide change	c.11 C>T	c.98 T>A	c.232 C>T	c.694 C>T	IVS2- 2A>G	c.845delC	c.845 C>A	c.845 C>G
Amino acid change	p. A4V	p. L33H	p. Q78X	p. Q232X	Splicing mutation	p. S282fsX	p. S282X	p. S282X
Overtgrowth	Generalized	Generalized	Generalized	Generalized	Generalized	Generalized	Generalized	Generalized
Birth weight (g)	4,240	4,830	2,890 (33 ws)	4,700	3,650 (36 ws)	1,940 (29 ws)	2,440 (34 ws)	—
Craneofacial	Macroglosia; posterior ear pits	Macroglosia; ear pits; glabellar flat vascular malformations	Macroglosia; ear creases; nevus flammeus	Macroglosia; cleft palate; flat vascular malformation	Mild macroglosia; flat vascular malformation in glabella	Macroglossia; cleft palate; ear creases; nevus flammeus	Macroglossia; cleft palate; ear creases; nevus flammeus	Macroglossia; ear creases
Cardiovascular	—	—	—	—	—	—	VSD-PDA	—
Abdomen	Bilateral nephromegaly; hepatomegaly; splenomegaly	Large umbilical hernia	Umbilical hernia; inguinal hernia	Omphalocele	Omphalocele; inguinal hernia	Omphalocele; inguinal hernia; renal cystis	Omphalocele; hepatomegaly	Omphalocele
Neurologic (CNS)	—	—	—	—	—	Hypotonia	—	—
Limbs	Polydactyly	—	—	Polydactyly (postminimi)	—	—	—	—
Skin	—	Extra nipple	—	—	—	Capillary malformation	Extra nipple	Psoriasis
Genital	—	—	—	—	—	Hypospadias	—	Cryptorchidism
Other	Apneas	—	Hypoglycaemia	Hypoglycaemia	—	—	—	Hypoglycaemia; strabismus

y, year; m, month.

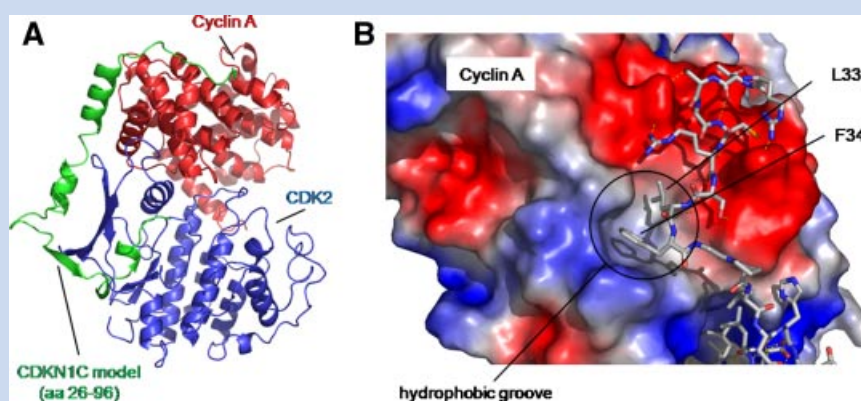


FIG. 2. 3D structural model of the CDK-inhibitor domain of CDKN1C. **A:** Ribbon-plot representation of 3D model for CDKN1C/cyclin A-CDK2 complex interaction. Model for CDKN1C includes only CDK-inhibitor domain (aa 26–96). **B:** Detail of the interaction of CDKN1C Leu33 residue with Cyclin A surface, colored according to electrostatic properties (Red: negative, Blue: positive). The pair of hydrophobic residues L33 and F34 are located in a hydrophobic groove of Cyclin A. Plots were generated using PyMOL [W.L. DeLano (2002) DeLano Scientific, San Carlos, CA]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

as colorectal, lymphohematologic and breast cancer, and it has been suggested as a putative breast cancer tumor suppressor [Larson et al., 2008]. To date, none of our eight patients with *CDKN1C* mutations developed neoplasia and as far as we know, only one patient has been previously described with neuroblastoma [Lee et al., 1997]. Follow-up of these patients was carried out until the age of 10 years as recommended [Lapunzina, 2005], though some patients have not yet reached this age. In summary, no patient had Wilms tumor, hepatoblastoma, or rhabdomyosarcoma, which are the commonest neoplasms observed in BWS patients with aberrant methylation and/or paternal UPD. While the total number of cases is small we suggested that familial and sporadic cases with *CDKN1C* mutations seem not to have an increased risk of Wilms tumor or other neoplasias [Cooper et al., 2005].

Simpson–Golabi–Behmel syndrome (OMIM 312870) has overlapping findings with BWS with *CDKN1C* mutations [Romanelli et al., 2007; Romanelli et al., 2009], mainly cleft palate, polydactyly, abnormal genitalia, and extra nipple. It is tempting to speculate that there may be a common pathway between CDKN1C protein and the *MYOD1/CIB2/p73*-dependent pathway. In contrast, no patient with isolated IH, macroglossia or omphalocele had mutations in *CDKN1C* suggesting that these malformations alone are not observed in association with *CDKN1C* mutations. However, further cases are needed to confirm this.

Finally, we suggest that this gene should be analyzed first, not only in BWS with cleft palate (as previously described) but also in BWS patients presenting with polydactyly, extra nipple and/or genital anomalies.

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