Facultad de Ciencias

Departamento de Biología Molecular

ANÁLISIS GENÉTICO
Y CORRELACIONES CLÍNICO-MOLECULARES
EN PACIENTES CON SÍNDROME DE SOBRECRECIMIENTO

Memoria para optar al grado de doctor europeo

de la licenciada

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Madrid, 2010

Hospital Universitario La Paz

SaludMadrid

Comunidad de Madrid

UAM UNIVERSIDAD AUTONOMA DE MADRID

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CERTIFICA:

Que Doña **Valeria Romanelli**, licenciada en Biotecnología, ha realizado bajo mi dirección, en el Instituto de Genética Médica y Molecular del Hospital La Paz de Madrid (INGEMM), Universidad Autónoma de Madrid el trabajo titulado:

ANÁLISIS GENÉTICO Y CORRELACIONES CLÍNICO-MOLECULARES EN PACIENTES CON SÍNDROME DE SOBRECRECIMIENTO

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Madrid, 4 de Octubre de 2010

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SUMMARY

Overgrowth Syndromes (OGS) are rare diseases and constitute a heterogeneous group of pathologies. The main clinical features of classic OGS are that weight, size and head circumference are over 2-3 standard deviations from the mean for age and sex. Beckwith-Wiedemann (BWS), Macrocephaly-Capillary Malformation (M-CM), Sotos (SS) and Simpson-Golabi-Behmel (SGBS) syndromes are the disorders thoroughly discussed in this doctoral thesis.

We were able to study different aspects of the BWS, the most frequent overgrowth syndrome, because it is a disorder with several etiologic causes. The cohort of patients of our hospital, that centralizes samples of many Spanish hospitals, has been analyzed according to an algorithm exploring the genetic causes of OGS. The obtained results enable us to communicate new point mutations in *CDKN1C* gene, altered in ~ 10% of sporadic cases and to detect a correlation between non-sense mutations in patients and HELLP/preeclampsia in their mothers during the pregnancy. We were able to set up a new and favorable method to assess the methylation level of the two imprinting centers on 11p15, the BWS locus. Moreover we re-studied cases due to paternal uniparental disomy (pUPD), thanks to the availability of molecular techniques allowing us to assess the degree of UPD mosaicism and its extension. With these new data we could carry out some phenotype/genotype correlations. Finally the reevaluation of BWS patients with pUPD through SNP-arrays, an innovative technique assessing the genetic dosage and the allele frequency of the whole genome, allowed us to identify a patient with pUPD of the whole genome in a high mosaicism degree.

Macrocephaly-Capillary Malformation is a syndrome with unknown etiology; we have studied in depth the clinical features of our patients and compared them with patients reported in the literature. We also performed a molecular analysis through SNP-arrays looking for altered regions to identify possible genetic causes.

We studied SBGS patients by means of direct sequencing and deletions/duplications analysis of *GPC3* and *GPC4* genes on Xq26, considered the critical region for this disease. Then, in those patients without alterations on Xq26 region and with a clear phenotype, we ruled-out mutations on the candidate gen *OFD1* on Xp22. Moreover, the study of these patients allowed us to find evidences that SGBS is another X-linked disease in which germinal mosaicism exists; information that should be taken into account in genetic counseling.

Finally we took part in a study about Sotos syndrome in adults to outline the disease's natural history and to suggest a suitable medical follow-up of these patients.

RESUMEN

Los Síndromes de Sobrecrecimiento (SSC) son enfermedades raras y constituyen un grupo heterogéneo de patologías. Los clasificados como SSC clásicos tienen como principales características clínicas peso, talla y perímetro cefálico por encima de 2-3 desviaciones estándar de la media para el sexo y la edad. Los síndromes de Beckwith-Wiedemann (BWS), Macrocefalia-Malformación Capilar (M-CM), Sotos (SS) y Simpson-Golabi-Behmel (SGBS) son los tratados más detenidamente en esta tesis doctoral.

Del BWS, síndrome de sobrecrecimiento con mayor frecuencia, hemos podido estudiar distintos aspectos siendo un trastorno generado por varias causas etiológicas. Así la cohorte de pacientes de nuestro hospital, que centraliza muestras de muchos hospitales de España, ha sido analizada según un algoritmo que prevé explorar sus causas genéticas desde las más frecuentes, como los defectos epigenéticos, hasta las menos representadas. Han surgido así resultados que nos han permitido comunicar nuevas mutaciones puntuales en el gen *CDKN1C*, alterado en un ~ 10% de casos esporádicos y detectar una correlación entre mutaciones sin sentido en los pacientes y desarrollo durante el embarazo de HELLP/preeclampsia en las madres de los mismos. Hemos podido poner a punto una nueva y ventajosa técnica para el estudio del nivel de metilación de los dos centros de imprinting en 11p15, locus BWS. Además hemos vuelto a estudiar los casos debidos a disomía uniparental paterna (UPDp), teniendo a disposición técnicas moleculares que nos han permitido estimar el nivel de mosaicismo de la UPDp y su extensión; también hemos podido realizar correlaciones con el fenotipo observado. Finalmente el reestudio de los pacientes BWS con UPDp por array de SNPs, técnica novedosa que permite estudiar dosis génica y frecuencia alélica de todo el genoma, nos ha permitido identificar un paciente con UPDp de todo el genoma en elevado grado de mosaicismo.

El síndrome de M-CM es un síndrome de etiología todavía desconocida, hemos así querido profundizar el análisis clínico de los casos de nuestro hospital y compararlos con los demás reportados hasta ahora en literatura. También hemos realizado un análisis molecular por array de SNP en búsqueda de regiones alteradas para identificar posibles causas de la patología.

Hemos estudiado los pacientes con SGBS realizando primero secuenciación directa y análisis de deleciones y duplicaciones de los genes *GPC3* y *GPC4* en Xq26, considerados responsables del síndrome. En aquellos pacientes con fenotipo evidente y sin alteraciones en la región Xq26, hemos querido descartar la presencia de mutaciones en el gen candidato *OFD1* de la región Xp22. Además, el estudio de estos pacientes y sus historias familiares nos ha permitido encontrar evidencias que el SGBS se tenga que incluir entre los síndromes ligados al cromosoma X con mosaicismo germinal, dado por tener en cuenta en el consejo genético de las familias.

Finalmente hemos participado en un estudio sobre los casos de síndrome de Sotos en adultos para trazar la evolución de la enfermedad y poder sugerir un adecuado seguimiento médico de los pacientes.

RIASSUNTO

Le sindromi di eccesso di crescita (OGS) sono malattie rare e costituiscono un gruppo eterogeneo di patologie. Quelle OGS definite classiche hanno come caratteristica clinica principale peso, taglia e perimetro cefalico di 2-3 deviazioni standard superiore alla media di etá e sesso.

Le sindromi di Beckwith-Wiedemann (BWS), Macrocefalia-Malformazione Capillare (M-CM), Sotos (SS) e Simpson-Golabi-Behmel (SGBS) sono quelle trattate piú dettagliatamente in questa tesi di dottorato.

Del BWS, sindrome di eccesso di crescita con maggiore frequenza, abbiamo potuto studiare differenti aspetti visto che si tratta di una malattia con varie cause eziologiche. Quindi la coorte di pazienti del nostro ospedale, che centralizza campioni di molti ospedali di Spagna, é stata analizzata seguendo un algoritmo che prevede esplorare le cause genetiche dalla piú frequente, come i difetti epigenetici, alle meno rappresentate.

Cosí sono sorti risultati che ci hanno permesso comunicare nuove mutazioni puntiformi del gene *CDKN1C*, alterato in un ~ 10% dei casi sporadici e identificare una correlazione tra le mutazioni senza senso nei pazienti e la manifestazione durante la gravidanza di HELLP/preeclampsia nelle loro madri. Abbiamo potuto mettere a punto una nuova e vantaggiosa tecnica per lo studio del livello di metilazione dei due centri di imprinting in 11p15, locus BWS. Inoltre abbiamo studiato di nuovo i casi dovuti a disomia uniparentale paterna (UPDp), avendo a disposizione tecniche molecolari che ci hanno permesso stimare il grado di mosaicismo della UPDp e la sua estensione; abbiamo anche potuto realizzare correlazioni con il fenotipo osservato. Infine la reanalisi dei pazienti BWS con UPDp con SNP-array, tecnica innovativa che permette studiare il dosaggio genico e la frequenza allelica di tutto il genoma, ci ha permesso identificare un paziente con UPDp dell'intero genoma con elevato grado di mosaicismo.

La sindrome di M-CM é una sindrome con eziologia sconosciuta, abbiamo quindi voluto profondizzare l'analisi clinica dei casi del nostro ospedale confrontandoli con gli altri descritti in letteratura. Inoltre abbiamo realizzato un'analisi molecolare con SNP-array alla ricerca di regioni alterate per identificare possibili cause della patologia.

Abbiamo studiato i pazienti SGBS realizzando prima di tutto sequenziamento diretto e analisi di delezioni e duplicazioni dei geni *GPC3* e *GPC4* in Xq26, considerati responsabili della sindrome. Nei pazienti con fenotipo evidente e senza alterazioni della regione Xq26, abbiamo voluto scartare la presenza di mutazioni del gene candidato *OFD1* nella regione Xp22. Inoltre, lo studio di questi pazienti e delle loro storie familiari ci ha permesso incontrare evidenze che la SGBS si debba includere tra le sindromi legate al cromosoma X con mosaicismo germinale, dato da tenere in considerazione nella consulenza genetica alle famiglie.

Infine abbiamo partecipato a uno studio sui casi della sindrome di Sotos negli adulti per tracciare l'evoluzione della malattia e poter suggerire un adeguato monitoraggio medico dei pazienti.

ÍNDICE

ABREVIATURAS

A	Alanina
ADN	Ácido desoxirribonucléico
AIP	Aryl hydrocarbon Receptor Interacting Protein
BWS	Beckwith-Wiedemann Syndrome
C	Citosina
CDKN1C	Cyclin-Dependent Kinase Inhibitor 1C
CNV	Copy Number Variant
CTCF	CCCTC-binding factor zinc finger protein
DE	Desviación Estándar
DMR	Differentially Methylated Region
et al.,	Y colaboradores
FBN1	Fibrillin 1
FGFR3	Fibroblast Growth Factor Receptor 3
G	Guanina
GH	Hormona de crecimiento, Growth Hormone
GNAS1	GNAS complex locus
GPC3	Glypican 3
GPC4	Glypican 4
H19	Imprinted maternally expressed transcript non-protein coding
HELLP	Hemolysis, Elevated Liver enzimes, Low Platelets count
HRAS	v-Ha-ras Harvey Rat Sarcoma viral oncogene homolog
IGF2	Insulin-like Growth Factor 2 somatomedin A

KCNQ1	Potassium voltage-gated Channel, KQT-like subfamily, member 1
KCNQ1OT1	KCNQ1 overlapping transcript 1 non-protein coding
LOH	Loss Of Heterozygosity
LOI	Loss Of Imprinting
Mb	Megabase de ADN
M-CM	Macrocefalia Malformación Capilar
MLPA	Multiplex Ligation Probe Amplification
MS-HRM	Methylation-Sensitive High Resolution Melting
MS-MLPA	Methylation Specific-Multiplex Ligation Probe Amplification
NSD1	Nuclear receptor binding SET Domain protein 1
OFD1	Oral-Facial-Digital Syndrome 1
PCR	Polimerase Chain Reaction
PTEN	Phosphatase and Tensin homolog
RMN	Resonancia Magnética Nuclear
SGBS	Simpson-Golabi-Behmel syndrome
SHOX	Short Stature HOmeobox-containing gene
SNC	Sistema Nervioso Central
SNP	Single Nucleotide Polymorphism
SRS	Silver-Russell Syndrome
SS	Sotos Syndrome
SSC	Síndrome de Sobrecrecimiento
STR	Short Tandem Repeat

T	Timina
TAC	Tomografía Axial Computarizada
TGFBR1	Transforming Growth Factor Beta Receptor 1
TGFBR2	Transforming Growth Factor Beta Receptor 2
U	Uracilo
UPD	Disomía Uniparental

INTRODUCCIÓN

1. Síndromes de sobrecrecimiento

Los Síndromes de Sobrecrecimiento (SSC) son enfermedades raras y relativamente nuevas cuya incidencia en menores de edad es cada vez mayor. Esta incidencia cada vez mayor tiene que ver, no con la extensión de la enfermedad, sino con que anteriormente sencillamente se desconocían o tenían una clasificación nosológica distinta.

Técnicamente hablando, los SSC constituyen un grupo heterogéneo de patologías cuya principal característica es que el peso, talla o perímetro cefálico, y frecuentemente todas estas mediciones conjuntamente, están por encima del percentil 97 o de 2-3 desviaciones estándar por encima de la media para el sexo y la edad [Weaver, 1994].

Además del aumento de los parámetros antropométricos, los SSC se caracterizan principalmente por presentar una frecuencia mayor de retraso mental que la población general y un alto índice de desarrollo de tumores [Cohen et al., 2002]. De hecho, la frecuencia de retraso mental en la población general es de aproximadamente del 1-3%, mientras en el grupo de SSC es cercana al 15% considerada en su conjunto. A la vez, la incidencia de cáncer en la edad pediátrica (menores de 15 años) en la población general es de 1:14.000, mientras es mucho mayor en el grupo de SSC, siendo entre el 3 y el 40% dependiendo de cuál patología con sobrecrecimiento se trate.

2. Clasificación de los síndromes de sobrecrecimiento

Los síndromes de sobrecrecimiento (SSC) comprenden distintos tipos de trastornos del crecimiento, pues son un grupo de patologías heterogéneas y de diversa etiología, existiendo causas nutricionales, hormonales y genéticas que pueden producirlas (Tabla I). Algunos SSC se manifiestan ya a nivel prenatal, con datos característicos como polihidramnios, un crecimiento fetal acelerado que se manifiesta principalmente a partir de la semana 25 y una placenta anormalmente grande; otros SSC no presentan síntomas en el periodo prenatal, manifestándose sólo a nivel postnatal. Al nacimiento, la definición de SSC no es fácil ya que existen diversos términos cuyo uso está muy extendido, como son “macrosomía”, “bebé macrosómico”, “gigante”, “grande para la edad gestacional”, etc [Lapunzina et al., 2002].

La mayoría de los SSC resultan de hiperplasia, hipertrofia, incremento en el intersticio, o alguna combinación de estos tres factores [Cohen, 1989]. De este modo, un SSC puede definirse como aquella condición en la que hay excesivo crecimiento o desarrollo, generalizado o localizado, para la edad y el sexo del individuo. Los SSC generalizados podrían incluir los síndromes clásicos (o verdaderos) en los que todos los parámetros de crecimiento y desarrollo físico están por encima de 2-3 desviaciones estándar de la media para la edad y sexo [Weaver, 1994]. Los sobrecrecimientos parciales, localizados o regionales, incluyen aquellas patologías en las que el crecimiento excesivo está confinado a una o a unas pocas regiones del cuerpo.

En esta sección se introducirán de forma resumida algunos de los síndromes de sobrecrecimiento clásicos, siguiendo la clasificación etiológica propuesta por Sotos JF [Sotos, 1996] y se hablará con más detenimiento de los SSC estudiados con mayor profundidad.

Tabla I: Clasificación etiológica del hipercrecimiento [Sotos, 1996]

Síndromes de sobrecrecimiento clásicos	
A. Alteraciones cromosómicas típicas	
Trisomía X (47, XXX femenino)	
Síndrome de Klinefelter XXY, XXXY	
Síndrome XYY	
B. Síndromes genéticos	
Síndrome de Marfan	Síndrome de Beckwith-Wiedemann
Síndrome de Bannayan-Riley-Ruvalcaba	Macrocefalia Malformación Capilar
Síndrome de Marshall-Smith	Síndrome de Simpson-Golabi-Behmel
Síndrome de Perlman	Síndrome de Sotos
Síndrome de Costello	
Síndrome de Weaver	
C. Alteración hormonal	
Acromegalia y gigantismo hipofisario	
D. Alteraciones no sindrómicas	
Trisomía 4p16	Monosomía 9q22
Trisomía 5p	Trisomía 15q25-ter
Trisomía 8p	Monosomía 22q13

3. Síndromes de sobrecrecimiento clásicos

Los síndromes clásicos (o verdaderos) incluyen los síndromes en que todos los parámetros de crecimiento y desarrollo físico están por encima de 2-3 desviaciones estándar de la media para la edad y el sexo, y que se deben principalmente a causas reconocidas.

3.1 Alteraciones cromosómicas típicas

3.1.1 Trisomía X (47, XXX)

Las pacientes afectadas por trisomía XXX suelen ser altas, con talla por encima del percentil 90. Las características clínicas más frecuentes son retraso del desarrollo y trastornos del comportamiento aunque el fenotipo sea muy variable, hasta asintomático en un porcentaje de casos. Entre las aneuploidías, la trisomía X es una de la más frecuente, con una incidencia de una de cada 1000 recién nacidas. El hipercrecimiento en estas pacientes se puede explicar por la presencia del cromosoma X extra, portador de regiones cromosómicas y genes que escapan a la inactivación. Entre los genes candidatos como causantes del hipercrecimiento, en ésta y otra aneuploidías sexuales, está el gen *SHOX* (Short Stature HOmeobox-containing gene), situado en la región pseudoautosómica (Xp22, Yp11.3), y que no resulta inactivado. La deleción de este gen provoca talla baja y retraso del crecimiento [Rao et al., 1997], también se han descrito algunos pacientes con estas características y mutaciones puntuales afectando a este gen [Clement-Jones et al., 2000]. Además se ha visto que la haploinsuficiencia de *SHOX* no sólo causa baja talla, si no también otras anomalías esqueléticas características del síndrome de Turner (cuartos metacarpos cortos, cubitus valgus y discondrosteosis de Leri-Weill) [Kosho et al., 1999].

3.1.2 Síndrome de Klinefelter (XXY, XXYY)

Los pacientes que presentan esta anomalía suelen ser altos y delgados, con piernas largas, y en algunos casos manifiestan hipogonadismo e infertilidad en la pubertad. El síndrome de Klinefelter tiene una incidencia de 1 de cada 1.000 recién nacidos. En los casos en que haya más de dos cromosomas X, variantes del síndrome de Klinefelter, se observa un fenotipo más

llamativo, con un desarrollo sexual más deficiente y retraso mental acusado. Tal como se comentó para la trisomía X, la talla alta se podría bien explicar por la presencia del gen *SHOX* no inactivado en el cromosoma X extra. La confirmación de esto la encontramos en pacientes afectos por el síndrome de Klinefelter que no presentan talla alta, teniendo un isocromosoma Xq (47; XiXqY), con ausencia de un brazo corto extra del segundo cromosoma X, donde se ubicaría el gen *SHOX*.

3.1.3 Síndrome XYY

Los varones afectos por el síndrome XYY presentan en un 50% de los casos talla alta; otros hallazgos clínicos son problemas del comportamiento y retraso en la adquisición del lenguaje. De nuevo podemos decir que la talla alta podría ser debida a una copia adicional del gen *SHOX*, presente también en el cromosoma Y.

3.2 Síndromes genéticos

3.2.1 Síndrome de Marfan

El síndrome de Marfan es una fibropatía que consiste en una alteración del tejido conectivo debida a la inadecuada síntesis de la fibrilina 1 por alteraciones del gen *FBN1* (Fibrillin 1, 15q21). La fibrilina es el componente más importante del tejido conectivo y muy probablemente un elemento funcional esencial en la organización normal de las microfibrillas; con esto se pueden explicar los signos clínicos y los cambios histológicos observados en los pacientes. El síndrome de Marfan tiene una incidencia de 1 de cada 15.000 recién nacidos, es un trastorno autosómico dominante caracterizado por alteraciones del tejido conectivo que afectan al esqueleto humano. Los signos clínicos característicos son elongación de los huesos tubulares, alteraciones en el sistema cardiovascular y ocular, moderada laxitud articular, dedos y extremidades desproporcionadamente largos, dientes apiñados, escoliosis y lordosis torácica. La esperanza de vida de los pacientes está disminuida por complicaciones cardiovasculares, ya que suelen presentar debilidad de la túnica media de los grandes vasos originando una dilatación de la aorta ascendente o arteria pulmonar y/o aneurisma disecante; con frecuencia pueden presentar

también prolapso de la válvula mitral. Se ha definido un síndrome de Marfan tipo II o Loeys Dietz por el hallazgo de mutaciones en los genes *TGFBR1* (Transforming Growth Factor Beta Receptor 1, 9q33) y *TGFBR2* (Transforming Growth Factor Beta Receptor 2, 3p22) en algunos pacientes en los que previamente no se habían encontrado mutaciones afectando al gen *FBN1*. Estos pacientes presentan un fenotipo bastante similar al producido por el síndrome de Marfan clásico, aunque no presentan anomalías oculares y su esperanza de vida parece ser menor. Los genes *TGFBR1* y *TGFBR2* codifican para el receptor TGF-beta, se sabe que este factor tiene generalmente un efecto negativo sobre el crecimiento celular, y la inactivación de su vía contribuye a la tumorigénesis [Disabella et al., 2006; Loeys et al., 2006; Matyas et al., 2006; Mizuguchi et al., 2004]

3.2.2 Síndrome de Bannayan-Riley-Ruvalcaba

Este síndrome se presenta como una enfermedad autosómica dominante; entre las causas conocidas están las mutaciones y las deleciones del gen supresor de tumores *PTEN* (Phosphatase and Tensin homolog, 10q23.31) [Marsh et al., 1997]. El gen *PTEN* puede bloquear el crecimiento celular y la tumorogenicidad bloqueando la progresión celular en la fase G1 [Li and Sun, 1998], por lo que su alteración probablemente desregule el crecimiento celular. Las características clínicas de los pacientes son macrocefalia, lipomas múltiples, pólipos intestinales, retraso del desarrollo, malformaciones vasculares y nevus pigmentarios localizados en los genitales en los pacientes varones. Además casi la mitad de los pacientes sufren hipotonía y retraso mental de moderado a severo. Los pacientes presentan también un riesgo aumentado de sufrir cáncer. En conjunto, el cuadro clínico del síndrome de Marfan es similar a la enfermedad de Cowden, un síndrome de predisposición al cáncer, especialmente de colon, tiroides y mama. Es interesante notar que en algunos pacientes con un fenotipo sugerente de enfermedad de Cowden, se han encontrado mutaciones en *PTEN*, lo que parece sugerir que en realidad ambos trastornos son alélicos presentando una variabilidad a diferentes edades.

3.2.3 Síndrome de Marshall-Smith

El síndrome de Marshall-Smith es un síndrome de sobrecrecimiento de etiología desconocida y ocurrencia esporádica. Se cree que está causado por mutaciones dominantes *de novo* y todavía

no se conoce su prevalencia ya que se han reportado poco más de 30 casos en la literatura. Los signos clínicos principales de este síndrome son edad ósea adelantada, retraso mental, anomalías faciales y dificultades de crecimiento postnatal. Las dismorfias presentadas son varias, entre ellas hay frente prominente, ojos protuberantes, escleróticas azules, micrognatia y narinas antevertidas. Los síntomas clínicos son graves, con dificultades para alimentarse, déficit de crecimiento y una gran tendencia a contraer infecciones respiratorias. Aunque los pacientes manifiesten con la edad una tendencia a tener peso bajo con crecimiento lento, el síndrome de Marshall-Smith se incluye entre los síndromes de sobrecrecimiento porque los pacientes recién nacidos presentan talla alta y edad ósea adelantada. El pronóstico es pobre y este síndrome suele causar mortalidad en los primeros años de vida [Adam et al., 2005].

3.2.4 Síndrome de Perlman

Este síndrome de sobrecrecimiento se presenta como una patología autosómica recesiva, aunque no se conozca la causa genética subyacente y no se haya encontrado ninguna alteración cromosómica ni genética en los pacientes estudiados. Los signos clínicos que lo caracterizan son gigantismo fetal, visceromegalia, facies inusual, hamartomas renales bilaterales con nefroblastomatosis y tumores de Wilms. No se ha estimado su prevalencia ya que hasta la fecha sólo se han descrito alrededor de 25 casos [Lapunzina et al., 2001].

3.2.5 Síndrome de Costello

El síndrome de Costello se presenta con una prevalencia desconocida ya que, hasta hora, se han descrito alrededor de 200 casos en la literatura. Se caracteriza por sobrecrecimiento prenatal, pues los pacientes nacen con alto peso y luego suelen desarrollar progresivamente dificultades en la alimentación y talla baja. Este trastorno conlleva alteraciones congénitas múltiples y retraso mental y los signos clínicos que lo caracterizan son facies tosca, piel redundante, cardiomiopatía y predisposición a tumores. El riesgo de tumores parece estar aumentado (17%), siendo los más frecuentes los rabdomiosarcomas. El síndrome de Costello tiene un diagnóstico diferencial que incluye el síndrome de Noonan y el síndrome cardio-facio-cutáneo. La causa genética ha sido recientemente identificada en mutaciones puntuales de pérdida de sentido en el protooncogén

HRAS (v-Ha-ras Harvey Rat Sarcoma Viral Oncogene homolog, 11p15.5) [Aoki et al., 2005], además existe una mutación recurrente que afecta al codón 12 de la proteína [Kerr et al., 2006]. El gen *HRAS* parece ser un regulador de la proliferación celular dependiente de factores de crecimiento, esa función puede explicar el mecanismo de hipertrofia.

3.2.6 Síndrome de Weaver

El síndrome de Weaver es un síndrome de sobrecrecimiento de prevalencia desconocida, con incidencia muy baja. Se han reportado alrededor de 50 casos en la literatura, la mayoría de ellos esporádicos, aunque se han identificado dos familias afectas con un patrón de herencia autosómico dominante. Este trastorno se caracteriza por hipercrecimiento pre- y postnatal, retraso en el desarrollo, hipertonía, edad ósea adelantada, camptodactilia y apariencia facial característica, consistente en una frente alta y ancha, hipertelorismo, línea media del labio superior prominente y larga y micrognatia. Además estos pacientes suelen presentar uñas de implantación profunda, llanto ronco y de tono bajo y yemas de los dedos prominentes [Cole et al., 1992; Opitz et al., 1998; Proud et al., 1998]. El fenotipo de los pacientes afectos tiene características parecidas a las presentadas por los pacientes con síndrome de Sotos y además en algunos de ellos se encontraron mutaciones en *NSD1*, gen responsable de SS [Douglas et al., 2003]. Estas evidencias y el hecho que se desconoce la causa molecular subyacente al síndrome de Weaver, han llevado a la hipótesis que este síndrome y el de Sotos sean la misma enfermedad en variantes alélicas. De todas formas se siguen considerando entidades distintas pues estudios realizados en series más largas de pacientes con síndrome de Weaver no han evidenciado alteraciones en el gen *NSD1* [Douglas et al., 2003; Tatton-Brown et al., 2005; Turkmen et al., 2003].

3.3 Alteraciones hormonales

3.3.1 Acromegalia y gigantismo hipofisario

Los pacientes afectos por acromegalia y gigantismo hipofisario presentan hipertrofia de las extremidades, talla alta y una facies tosca característica; estas dos enfermedades de sobrecrecimiento están causadas por una secreción excesiva de hormona de crecimiento o somatotropina (GH, Growth Hormone) que también puede llevar problemas cardiacos y diabetes. Los síntomas tienes un progreso lento, con lo cual el diagnostico suele ser tardío; se estima que la acromegalia tenga una prevalencia de 40-70 afectados por cada 1.000.000 de personas. La falta de regulación en la secreción de esta hormona es debida a adenoma de la glándula pituitaria o hipófisis (adenoma también conocido como somatotropinoma) o, en algunos casos, a hiperplasia del lóbulo anterior hipofisario o adenohipófisis. Hay situaciones excepcionales en que la acromegalia es debida a secreción hormonal ectópica, por cáncer de ovario, tiroides, páncreas o broncopulmonar. La acromegalia puede manifestarse aislada o asociada a otros trastornos; en un 40% de los casos los adenomas pituitarios que la generan presentan mutaciones somáticas heterocigotas en el gen *GNAS1* (GNAS complex locus, 20q13.2) [Hayward et al., 2001; Thakker et al., 1993]. La acromegalia suele presentarse de forma esporádica, aunque en algunos pacientes se han descrito formas familiares de predisposición que presentaban mutaciones germinales en el gen *AIP* (Aryl hydrocarbon Receptor Interacting Protein, 11q13) [Vierimaa et al., 2006].

3.4 Alteraciones no sindrómicas

El sobrecrecimiento, entre otros hallazgos clínicos, se manifiesta también en pacientes que presentan alteraciones genéticas no reconocidas como causantes del SSC. Estas alteraciones están descritas en la literatura y entre ellas cabe destacar:

Duplicación de la región p16 del cromosoma 4: esta duplicación también se ha asociado a sobrecrecimiento y la deleción recíproca causa a su vez hipocrecimiento. La región 4p16 comprende el gen *FGFR3* (Fibroblast Growth Factor Receptor 3), que codifica por un receptor del factor de crecimiento de los fibroblastos, por lo que se ha hipotetizado que la alteración del crecimiento sea debida a efectos de dosis de este gen [Partington et al., 1997].

Trisomía del brazo corto del cromosoma 5: esta alteración parece causar un trastorno en el crecimiento y se ha asociado con sobrecrecimiento [Avansino et al., 1999; Lorda-Sanchez et al., 1997]. Los pocos pacientes descritos presentan también macrocefalia, dilatación de los ventrículos cerebrales, inclinación antimongoloide de las fisuras palpebrales, micrognatia, cuello corto y defectos cardíacos.

Trisomía del brazo corto del cromosoma 8: esta anomalía ha sido largamente asociada al hipercrecimiento [Sotos, 1996].

Monosomía de la región 9q22.3: recientemente se ha propuesto esta alteración como una posible causa de sobrecrecimiento, acompañado por macrocefalia, trigonocefalia, retraso del desarrollo psicomotor e hiperactividad [Redon et al., 2006].

Trisomía 15q25-qter: esta alteración se ha encontrado en pacientes que tienen como característica clínica común el sobrecrecimiento y luego presentan otros numerosos hallazgos clínicos muy variables [Bonati et al., 2005; Faivre et al., 2004; Nagai et al., 2002; Zollino et al., 1999].

Deleción intersticial de la región 22q13: esta alteración se ha encontrado en pacientes que presentan crecimiento acelerado, problemas de lenguaje, retraso del desarrollo psicomotor e hipotonía [Fujita et al., 2000; Romain et al., 1990].

3.5 Síndromes estudiados en esta tesis

3.5.1 Síndrome de Beckwith-Wiedemann

El síndrome de Beckwith-Wiedemann (BWS, MIM #130650) ha sido originalmente descrito por Beckwith JB y Wiedemann HR [Beckwith , 1969; Wiedemann, 1969]. El BWS es el síndrome de sobrecrecimiento pediátrico con predisposición a tumores más frecuente, con una incidencia de aproximadamente 1 de 13.500-13.700 recién nacidos [Cohen, Jr., 1999]. Es incluso probable que esta cifra sea una subestimación visto que los fenotipos más suaves podrían no ser reconocidos [Weksberg et al., 2010]. La incidencia es igual en niños y niñas, con la importante excepción que, en los gemelos homocigotos, hay un fuerte exceso de casos femeninos. El síndrome de Beckwith-

Wiedemann suele presentarse de forma esporádica (85%), pero ocurre por transmisión familiar en ~ 15% de casos.

Descripción clínica

El síndrome de Beckwith-Wiedemann presenta características clínicas altamente variables, incluso algunos casos no presentan todos los signos distintivos originariamente descritos, como onfalocele, macroglosia y gigantismo. Existe un número importante de hallazgos en el BWS, los más frecuentes se enumeran en la Tabla II. Aunque no haya consenso absoluto sobre los criterios clínicos diagnósticos para el BWS, varios autores han sugerido en publicaciones diferentes criterios mayores y menores [Rump et al., 2005].

Los pacientes afectos por BWS crecen con velocidad aumentada respecto a la normal durante la segunda parte de la gestación y en los primeros años de vida. Los parámetros de crecimiento muestran un valor de altura y peso alrededor del percentil 97; en general los pacientes ya adultos muestran valores en los rangos normales [Pettenati et al., 1986; Weng et al., 1995]. Además, debido a que las características de BWS a menudo se normalizan a lo largo de la infancia, en la evaluación de los pacientes con sospecha de BWS es útil disponer de fotos de la primera infancia.

Tabla II: Características clínicas observadas en el síndrome de Beckwith-Wiedemann.

Muy frecuentes	**Poco frecuentes**
Macrosomía	Diástasis de rectos
Pliegues en lóbulos de la oreja	Facies características
Fosetas en helix posterior	Pie equinovaro
Macroglosia	Nistagmo y estrabismo
Onfalocele	Tumores embrionarios
Hernia umbilical	Déficit de atención y hiperactividad
Hipotonía	**Ocasionales**
Hipercrecimiento en la infancia	Malformación anatómica cerebral
Nefromegalia	Convulsiones
Hepatoesplenomegalia	Retraso mental leve/Fracaso escolar
Hemihiperplasia	Escoliosis
Hipoglucemia	Cardiopatía congénita o arritmias
Frecuentes	Diabetes o prediabetes
Citomegalia adrenocortical	
Polihidramnios	
Edad ósea avanzada	
Erupción prematura de los dientes	
Prematuridad	
Gemelaridad	
Malformación capilar (hemangioma plano)	

La cara de los pacientes BWS presenta características típicas como ojos prominentes con pliegues infraorbitarios, nevus flammeus facial, hipoplasia de mitad central de la cara, cara alargada con mandíbula prominente, pliegues en el lóbulo anterior, fosetas en hélix posterior y macroglosia [Pettenati et al., 1986]. Este último signo clínico puede cursar con dificultad respiratoria, infecciones incontrolables de la vía área superior, dificultad para alimentarse y hablar y mejora con el tratamiento quirúrgico de reducción [Gasparini et al., 2002; Giancotti et al., 2003; Kacker et al., 2000].

La hipoglucemia en un síntoma descrito en un 30-50% de niños con BWS, probablemente debida a hiperplasia de los islotes de Langerhans y a hiperinsulinemia. La mayoría de los niños no presentan retraso en la maduración neurológica, excepto un pequeño grupo de pacientes que han sufrido hipoglucemias insuficientemente tratadas [DeBaun et al., 2000; Engstrom et al., 1988; Hussain et al., 2005; Pettenati et al., 1986].

En los individuos con BWS existe una elevada frecuencia de malformaciones y complicaciones médicas, como asimetría (hemihipertrofia) de miembros, torso o cara; defectos de la pared abdominal como onfalocele y hernia umbilical; organomegalia de uno o más órganos entre hígado, bazo, páncreas, riñones y glándulas adrenales. Malformaciones cardíacas se encuentran en ~ 20% de casos, la mitad de los cuales son representados por cardiomegalia que se resuelve espontáneamente [Elliott and Maher, 1994; Pettenati et al., 1986].

El asunto de más importancia a tener en cuenta en los pacientes afectos por el síndrome de BW es el riesgo aumentado de desarrollar tumores. La mayoría de los tumores asociados con BWS se manifiestan en los primeros 8-10 años de vida [DeBaun et al., 1998; Tan and Amor, 2006] y entre ellos, los más frecuentes son: tumor de Wilms, hepatoblastoma, carcinoma adrenocortical, rabdomiosarcoma y neuroblastoma [DeBaun et al., 1998; Lapunzina, 2005b; Pettenati et al., 1986; Sotelo-Avila et al., 1980; Tan and Amor, 2006; Wiedemann, 1983]. No obstante, también se han descrito otros tumores malignos de forma ocasional y algunos tumores benignos en pacientes con BWS [Lapunzina, 2005b; Sotelo-Avila et al., 1980; Wiedemann, 1983]. El riesgo global de tumores en BWS ha sido calculado por varios autores y se estima en un 7.5% [DeBaun and Tucker, 1998; Wiedemann, 1983], además se describe una asociación entre la presencia de hemihiperplasia y nefromegalia y el incremento de riesgo de cáncer en niños con BWS. Aunque aún sean datos preliminares, estudios en distintos subgrupos moleculares de pacientes han evidenciado que el riesgo y el tipo de tumores es distinto según el defecto genético subyacente [Weksberg et al., 2001].

El síndrome de Beckwith-Wiedemann presenta varios diagnósticos diferenciales que incluyen los síndromes de Simpson-Golabi-Behmel, de Costello, de Perlman y de Sotos. En geneeral se pueden distinguir los casos de BWS de los demás síndromes por los hallazgos clínicos, test auxiliares como los análisis moleculares y bioquímicos y por seguimento del paciente.

Base genética

El síndrome de Beckwith-Wiedemann es una patología compleja y multigénica causada, en ~ 85% de casos, por alteraciones en la expresión o función de uno o de más genes reguladores del crecimiento de la región 11p15.5 [Maher and Reik, 2000]. En la región 11p15.5 está el Dominio de

imprinting 1 donde mapean los genes de expresión materna *H19* (Imprinted maternally expressed transcript non-protein coding) y paterna *IGF2* (Insulin-like Growth Factor 2 somatomedin A) y el centro de imprinting 1 telomérico, llamado H19DMR (Differentially Methylated Region) de imprinting paterno; en la zona más centromérica mapea el Dominio de imprinting 2, con seis genes de expresión materna, entre los cuales están *KCNQ1* (Potassium voltage-gated Channel, KQT-like subfamily, member 1) y *CDKN1C* (Cyclin-Dependent Kinase Inhibitor 1C), el trascrito paterno *KCNQ1OT1* (KCNQ1 overlapping transcript 1 non-protein coding) y el centro de imprinting 2 materno, llamado KvDMR (Figura 1).

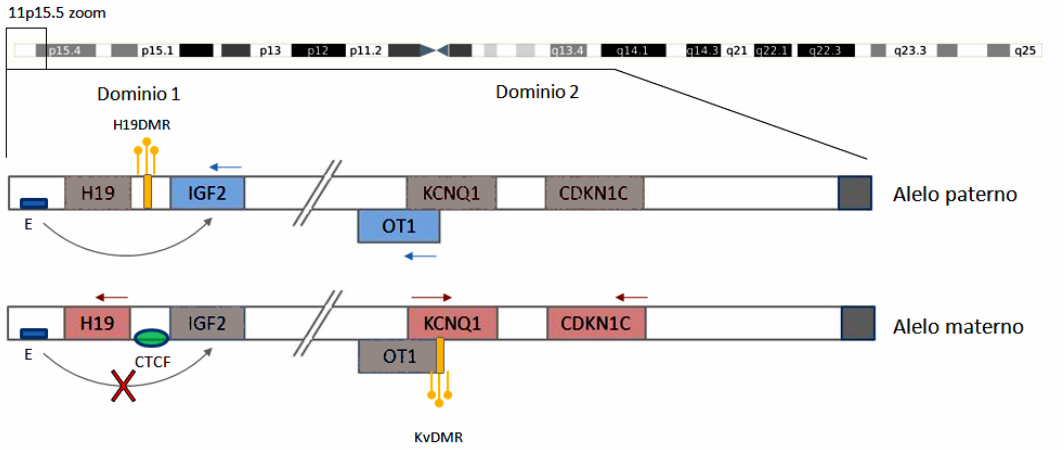

Figura 1: BWS locus y su regulación.
En los dos alelos se representan en azul y en rosa los genes de expresión paterna y materna respectivamente. Las flechas indican el sentido de lectura, las barritas amarillas la metilación en los centros de imprinting, la caja azul oscuro E representa el enhancer de *IGF2* y el ovalo verde CTCF, la proteína represora que se une al elemento insulator.

Regulación

Para poder entender mejor los mecanismos moleculares del síndrome de Beckwith-Wiedemann, es útil tener claro en qué consiste el mecanismo de *Imprinting Genómico*. El *Imprinting Genómico* es el proceso por el cual una copia de un gen es preferencialmente silenciada de acuerdo al origen parental del mismo. Aunque los genes que tienen imprinting se heredan de una forma mendeliana, la expresión de cada alelo es específica según el origen parental. Las bases moleculares del imprinting incluyen las modificaciones epigenéticas. Esto significa que dos alelos que son idénticos en su secuencia nucleotídica, pero de diferente origen parental, serán regulados en forma diferente en el mismo núcleo. La modificación epigenética del locus 11p15.5 es la

metilación diferencial de los centros de imprinting H19DMR y KvDMR; dicha modificación comprende también la estructura y función de la cromatina y define la actividad transcripcional de los alelos con imprinting. El imprinting genómico es reversible, el alelo silente (el imprintado) puede ser reactivado cuando pasa a través de la línea germinal al sexo opuesto [Koerner and Barlow, 2010; Pfeifer, 2000; Ubeda and Wilkins, 2008]. Los genes regulados por imprinting suelen ubicarse en amplios clusters largos más de 1 Mb. El imprinting de los genes en estos dominios es regulado en *cis* por uno o más centros de imprinting; estos centros de imprinting se consideran responsables de la generación del estatus "parent-of-origin-specific" de la cromatina que se propaga de forma bidireccional para regular el restablecimiento del imprinting en la línea germinal.

Dominio 1

H19: H19 es un gen de imprinting paterno, expresando el alelo materno, que codifica por un mRNA no traducido pero biológicamente activo que parece funcionar como supresor de tumores [Hao et al., 1993].

IGF2: *IGF2* codifica para un factor de crecimiento fetal (Insulin-like Growth Factor 2) y se expresa en el alelo paterno. Su patrón de expresión tejido-específica se muestra muy paralelo a los órganos que están implicados en el BWS [Ohlsson et al., 1993].

H19DMR: H19DMR es una región de metilación diferencial y constituye el centro de imprinting telomérico del locus BWS. H19DMR está ubicado 3' del gen *H19* y regula de forma recíproca la expresión de *H19* y *IGF2*; el imprinting silenciará la expresión de *H19* en el alelo paterno, permitiendo que *H19* se exprese en el alelo materno, con el DMR no metilado. Además regulará la expresión paterna de *IGF2* funcionando como elemento "insulator": H19DMR lleva de hecho una secuencia que constituye un sitio de unión para la proteína represora CTCF (CCCTC-binding factor zinc finger protein) que puede unirse al DNA no metilado del cromosoma materno e inhibir la interacción del alelo materno de *IGF2* con su enhancer ubicado corriente abajo de *H19*; con efecto de silenciar la expresión de *IGF2* en el alelo materno [Hark et al., 2000; Hark and Tilghman, 1998].

Dominio 2

KCNQ1: El gen *KCNQ1* codifica para una subunidad de un canal del potasio y está relacionado con diferentes tipos de síndromes de arritmia cardiaca. *KCNQ1* tiene imprinting paterno y se expresa en el alelo materno en muchos tejidos, con la importante excepción del corazón [Lee et al., 1997b].

KCNQ1OT1: Este gen se transcribe en el alelo paterno en anti sentido respecto a *KCNQ1*, es un mRNA no-codificante de función todavía desconocida y su promotor se encuentra en el intrón 10 de *KCNQ1*.

CDKN1C: El gen *CDKN1C* codifica para la proteína $p57^{Kip2}$, miembro de la familia de inhibidores del las quinasas dependientes de ciclinas (CdK). *CDKN1C* se expresa en el alelo materno y actúa como regulador negativo de la proliferación celular y supresor de tumores [Matsuoka et al., 1995].

KvDMR: KvDMR es el centro de imprinting centromérico del locus BWS. Se encuentra en la zona centromérica de la región y solapa con extremidad 5' del *KCNQ1OT1* [Lee et al., 1999; Smilinich et al., 1999]. El KvDMR, metilado en el alelo materno, inhibe la expresión del *KCNQ1OT1* de este alelo y permite la expresión de muchos genes de imprinting del dominio 2.

Subgrupos moleculares

El síndrome de Beckwith-Wiedemann presenta heterogeneidad molecular, pues se pueden reconocer subgrupos moleculares de esta patología (Figura 2). El grupo más frecuente es el de los pacientes que muestran pérdida de metilación del centro de imprinting materno centromérico KvDMR (~ 50% de casos esporádicos). La ganancia de metilación del centro de imprinting telomérico H19DMR se presenta en un subgrupo de ~ 2%-7% de pacientes. Entonces ~ 60% de casos son debido a un defecto epigenético de uno de los dos centros de imprinting del locus 11p15.5. El otro gran subgrupo es el representado por la disomía uniparental paterna (UPDp, ~ 20%). Las alteraciones cromosómicas son relativamente raras, incluyendo duplicaciones paternas (< 1%) y translocaciones/inversiones/deleciones maternas (< 1%). Finalmente, entre las causas genéticas, encontramos las mutaciones puntuales del gen *CDKN1C* (~ 10%) y las de los centros de

imprinting H19DMR y KvDMR (muy raras). En ~ 10%-15% de casos la etiología es desconocida [Enklaar et al., 2006; Weksberg et al., 2005].

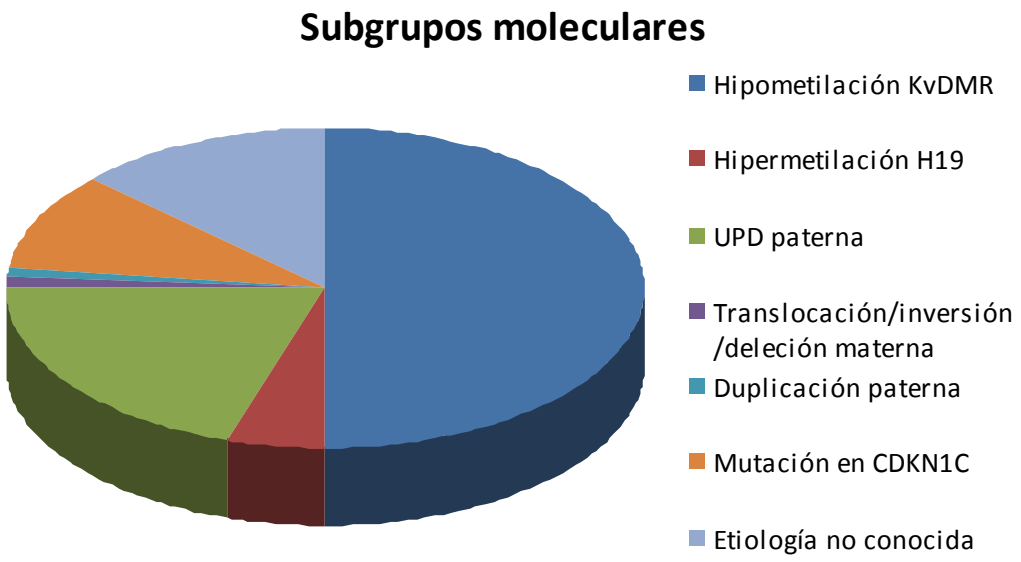

Figura 2: Representación gráfica del porcentaje de pacientes con BWS pertenecientes a cada subgrupo molecular.

Pérdida de metilación del KvDMR:

Este defecto se presenta en ~ 50% de casos esporádicos, se ha asociado con pérdida de imprinting del *KCNQ1OT1* y, en los tejidos humanos, se manifiesta por expresión bialélica de *KCNQ1OT1* y reducción de la expresión materna de *CDKN1C* [Gaston et al., 2001; Lee et al., 1999] (Figura 3). La disrupción del imprinting en el KvDMR puede ser debida, en rarísimos casos, a microdeleciones del mismo centro de imprinting [Niemitz et al., 2004; Zollino et al., 2009]. Los pacientes con este tipo de defecto tienen un bajo riesgo de desarrollar tumores siendo menos frecuente el tumor de Wilms en este subgrupo. Entre los signos clínicos mayormente representados hay hemihiperplasia y onfalocele. Por otra parte se han asociado las técnicas de fecundación asistida con un riesgo aumentado de BWS por pérdida de metilación del KvDMR [DeBaun et al., 2003; Gicquel et al., 2003; Halliday et al., 2004; Maher et al., 2003].

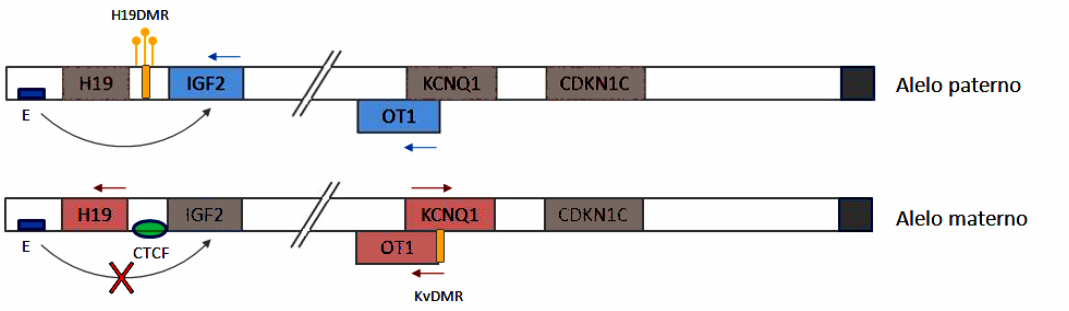

Figura 3: Hipometilación del KvDMR materno. La pérdida de metilación de KvDMR en el alelo materno lleva a la expresión bialélica de *KCNQ1OT1* y a la reducción de expresión del *CDKN1C*.

Ganancia de metilación del H19DMR:

La hipermetilación del centro de imprinting telomérico es un evento raro (~ 2%-7% de casos esporádicos) y conlleva pérdida de expresión del alelo materno de *H19* y expresión bialélica del *IGF2* [Joyce et al., 1997]. La pérdida de imprinting de *IGF2* puede también ser debida a microdeleciones raras del H19DMR [Prawitt et al., 2005; Sparago et al., 2004] (Figura 4). Los pacientes que muestran este defecto tienen un riesgo muy alto de desarrollar tumor de Wilms o hepatoblastoma [Bliek et al., 2001; Bliek et al., 2004; DeBaun et al., 2002; Rump et al., 2005].

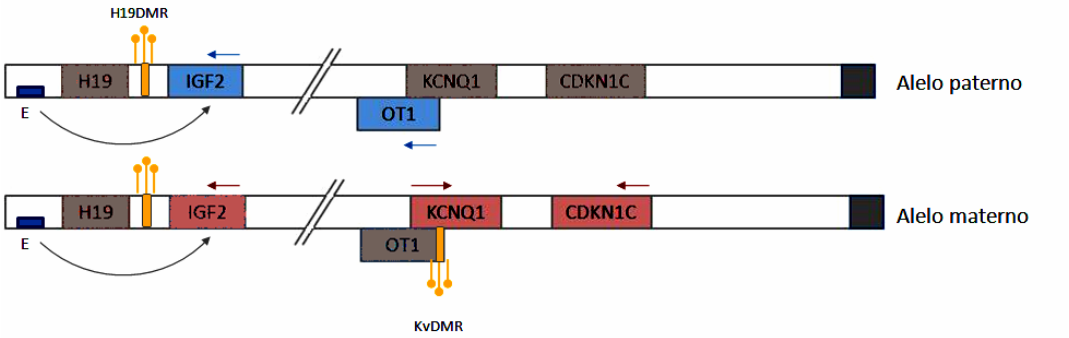

Figura 4: Hipermetilación del H19DMR paterno. La ganancia de metilación de H19DMR en el alelo materno lleva a la expresión bialélica de *IGF2* y a la pérdida de expresión de *H19*.

Disomía uniparental paterna (UPDp):

La disomía uniparental paterna consiste en la presencia de dos copias derivadas del padre del locus 11p15 con ausente o baja contribución materna para esta región [Henry et al., 1991; Slatter et al., 1994] (Figura 5). En estos pacientes, además de los efectos debidos a la

sobreexpresión de *IGF2*, es posible que una disminución del nivel de *CDKN1C* materno contribuya al fenotipo BWS. Ha sido demostrado que los pacientes que pertenecen a este subgrupo presentan el defecto genético en mosaico; esto sugiere que sea debido a un evento postcigótico [Hatada et al., 1996]; es posible también pensar que la pérdida total de uno o más genes de expresión materna no sea compatible con la vida [Cooper et al., 2007]. El fenotipo patológico de los pacientes está fuertemente asociado con el grado de mosaicismo y con la localización del defecto genético en los distintos tejidos [Itoh et al., 2000]. Clínicamente existe una asociación evidente entre la UPD paterna y la hemihiperplasia [Cooper et al., 2005]; además se ha visto que estos pacientes tienen un riesgo aumentado de desarrollar neoplasias y tumores de Wilms respecto a los otros subgrupos moleculares [Bliek et al., 2001; DeBaun et al., 2002].

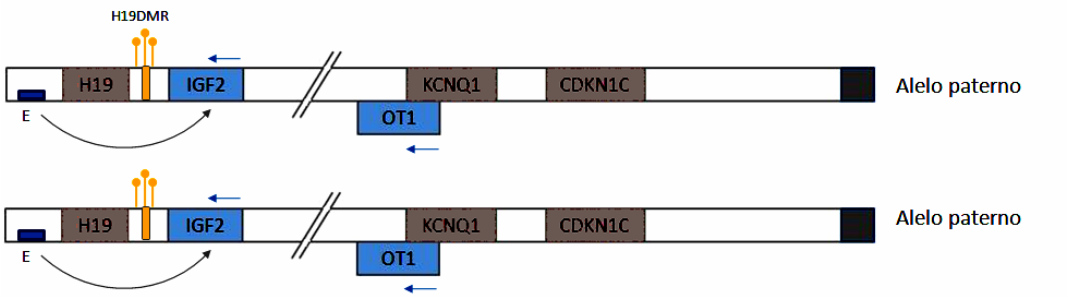

Figura 5: Disomía uniparental paterna (UPDp). La presencia de dos copias paternas lleva a la sobreexpresión de *IGF2* y a la disminución de la expresión de *CDKN1C*.

Alteraciones cromosómicas:

Las anomalías cromosómicas en la región 11p15 son eventos muy raros, acontecen sólo en ~ 2% de casos. Las translocaciones e inversiones muestran típicamente una herencia materna (Figura 6a), mientras las duplicaciones son de origen paterno (Figura 6b) [Algar et al., 2007]. El sitio de breakpoint de las translocaciones/inversiones maternas corresponde con el centro de imprinting centromérico KvDMR y estas alteraciones causan su disrupción mientras el centro de imprinting telomérico H19DMR queda intacto. El efecto de dicha desregulación del imprinting centromérico *in cis* lleva a una inactivación bialélica de la expresión de *CDKN1C*, lo cual causaría el síndrome de BWS [Horike et al., 2000]. Las duplicaciones paternas se caracterizan por una pérdida de imprinting de *IGF2* asociada a silenciamiento de *H19*; el efecto de esta alteración cromosómica se caracteriza por un riesgo aumentado de desarrollar tumor de Wilms [Algar et al., 2007] y además los pacientes suelen presentar retraso mental [Slavotinek et al., 1997].

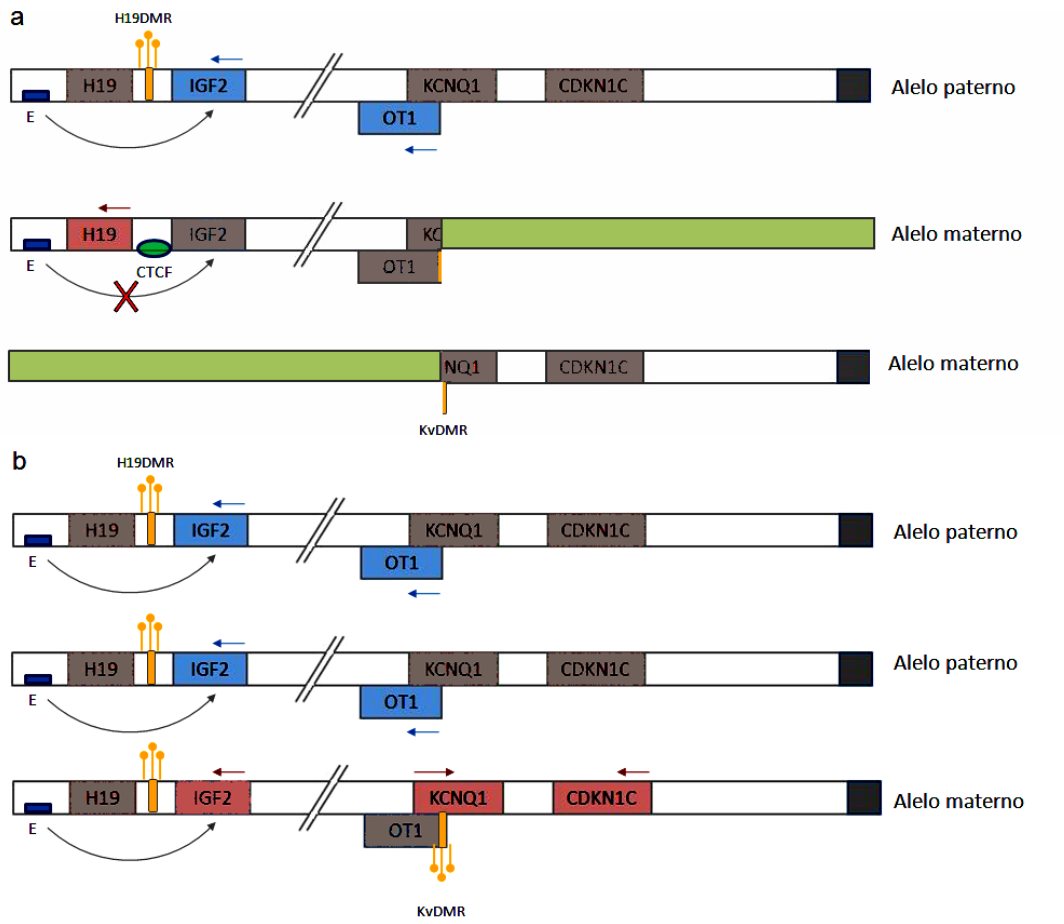

Figura 6: Alteraciones cromosómicas. a) Las translocaciones/inversiones maternas llevan a inactivación de la expresión de *CDKN1C*. b) Las duplicaciones paternas llevan a silenciamiento de *H19*.

Mutaciones puntuales de CDKN1C:

Este subgrupo molecular incluye ~ 10% de casos de BWS esporádicos y ~ 40% de casos con historia familiar positiva [Lee et al., 1997a; Li et al., 2001; O'Keefe et al., 1997]. Las mutaciones en este gen, a pesar de ser considerado un gen supresor tumoral putativo, no están claramente relacionadas con un riesgo aumentado de neoplasias. Los pacientes que presentan mutaciones en *CDKN1C* suelen tener un fenotipo caracterizado por la presencia de onfalocele, polidactilia, mamilas extras y anomalías genitales.

3.5.2 Síndrome de Macrocefalia Malformación Capilar

Descripción clínica

El síndrome de Macrocefalia Malformación Capilar (M-CM; MIM 602501) fue descrito por primera vez en 1997 como Macrocefalia Cutis Marmorata Telangiectatica Congenita [Clayton-Smith et al., 1997; Moore et al., 1997]. El síndrome M-CM es un síndrome genético raro y de etiología desconocida; los signos clínicos típicos son macrocefalia, retraso mental, telangiectasia congénita, cutis marmorata y sobrecrecimiento, además de otras anomalías como hemihipertrofia, hemangioma del labio y/o *philtrum*, sindactilia, anomalías del tejido conectivo y retraso del desarrollo [Lapunzina et al., 2004]. Se han descrito hasta ahora ciento treinta pacientes con este trastorno y el riesgo estimado de desarrollar tumores es de un 3-5%.

3.5.3 Síndrome de Simpson-Golabi-Behmel

El síndrome de Simpson-Golabi-Behmel (SGBS; MIM #312870) es un síndrome de sobrecrecimiento cuya prevalencia se desconoce. Se han reportado alrededor de 100 casos en la literatura después de los primeros pacientes descritos por Simpson JL, Golabi M and Behmel A [Behmel et al., 1988; Golabi and Rosen, 1984; Simpson et al., 1975].

Descripción clínica

El síndrome de Simpson-Golabi-Behmel presenta una amplia variabilidad clínica, desde niñas portadoras con un fenotipo leve hasta niños con afección severa y elevado riesgo de mortalidad neonatal. El sobrecrecimiento se observa en general tanto en el período prenatal como en el postnatal y continúa en general a lo largo de toda la vida. Los pacientes pueden llegar a medir 190-200 cm y suelen tener alto peso. El crecimiento esquelético no está en general acelerado y suele ser concordante con la edad cronológica, hecho que en ocasiones ayuda a diferenciarlo de otros síndromes de sobrecrecimiento.

Con mucha frecuencia se observan alteraciones de columna tales como escoliosis, xifosis o combinación de ambas. La hipotonía es habitual en el período neonatal y la infancia temprana. La maduración neurológica suele ser algo más lenta y puede existir retraso mental de grado variable que se manifiesta con retraso en el lenguaje y fracaso escolar. Algunos niños pueden tener dificultades en la deglución o en la respiración en los primeros meses de vida debido a la macroglosia, que puede estar presente hasta la infancia temprana. En los pacientes con SGBS debería realizarse una TAC (Tomografía Axial Computarizada) o RMN (Resonancia Magnética Nuclear) de cerebro para evaluar las posibles alteraciones del SNC (Sistema Nervioso Central).

La facies suele ser característica, consistente en macrocefalia, cráneo grande y rasgos faciales toscos. Es frecuente el hallazgo de alteraciones de la línea media tales como un pliegue profundo central en la lengua, paladar hendido con o sin labio leporino. Las orejas suelen ser muy grandes y carnosas, y a veces simplificadas, con un lóbulo redundante. Los hallazgos más frecuentes son manos grandes y toscas con pliegues palmares profundos y pulgares muy gruesos. Finalmente se han encontrado también varias alteraciones cardiovasculares en pacientes con SGBS [Neri et al., 1998].

Los pacientes presentan un riesgo aumentado de neoplasia, sobre todo tumor de Wilms, neuroblastoma y hepatoblastoma [Gracia and Lapunzina, 2005; Lapunzina, 2005b; Neri et al., 1988; Rodriguez-Criado et al., 2005], lo que confiere un riesgo incrementado de cáncer en el SGBS. Este incremento del riesgo de tumores es una característica común a todos los síndromes de sobrecrecimiento y en esta patología es de aproximadamente 7-10%.

Base genética

El síndrome de SGB es una patología recesiva ligada al cromosoma X. Las mujeres portadoras pueden presentar un fenotipo leve debido al fenómeno de lyonización. El SGBS fue mapeado en la región Xq26 por varios investigadores, pero fueron Pilia y colaboradores quienes identificaron el gen responsable de esta patología: un glipican al que denominaron glipican 3 (*GPC3,* Glypican 3) [Pilia et al., 1996; Veugelers et al., 1998]. El *GPC3* contiene 8 exones en una región de 500kb de DNA genómico. El RNA se expresa en casi todos los tejidos durante el desarrollo embrionario y en los adultos sólo se expresa en la placenta, los ovarios, los pulmones y el tejido mamario. El glipican

3 es un proteoglicano tipo heparan sulfato. Estos proteoglicanos se hallan en la superficie celular y posen la capacidad de ligar proteínas y ligandos y modular la actividad de la superficie celular. El producto del *GPC3* está involucrado probablemente en la regulación de la proliferación celular, la apoptosis y la modulación de la respuesta celular a los factores de crecimiento. Se han descrito deleciones y translocaciones en los dos extremos del gen en diferentes pacientes con SGBS. Las deleciones del gen *GPC3* se han observado en aproximadamente el 40% de los pacientes. Mutaciones puntuales del gen también han sido descritas por otros investigadores.

Es importante comentar que una forma aparentemente más grave de esta patología fue recientemente mapeada en la región Xp22, con mutaciones en el gen *OFD1* (Oral-Facial-Digital Syndrome 1, *CXORF5*) [Budny et al., 2006].

3.5.4 Síndrome de Sotos

El síndrome de Sotos (SS; MIM 117550) es un trastorno de sobrecrecimiento descrito en 1964 por Sotos JF y colaboradores [Sotos et al., 1964]. Es uno de los síndromes de sobrecrecimiento más frecuentes, tras el BWS, con una prevalencia de 1 de cada 14.000 nacidos y la gran mayoría de los casos se producen de novo sin historia familiar.

Descripción clínica

Los pacientes con SS presentan sobrecrecimiento prenatal, en peso, talla y perímetro craneal. La talla es el parámetro más importante e indicativo y se encuentra incrementada de 2 DE por encima de la media por edad y sexo en el 85% de los recién nacidos. El excesivo crecimiento es uno de los signos clínicos principales durante la infancia, de hecho el 90% de los individuos afectos presenta estatura o/y perímetro cefálico por encima del percentil 98. La altura y el peso tienden a normalizarse hacia la pubertad, aunque los adultos suelen superar la media. Una característica común en al menos el 76% de los pacientes es la edad ósea adelantada. Los pacientes con SS presentan una configuración facial característica, especialmente entre el primer y el sexto año de vida. Los rasgos craneofaciales más evidentes incluyen: frente alta y prominente, inclinación antimongoloide de las fisuras palpebrales y mentón ancho y prominente [Root and Diamond, 2006; Tatton-Brown et al., 2005; Tatton-Brown et al., 2009].

El 90% de los casos presentan retraso mental de grado variable y no progresivo que se manifiesta como retraso en las adquisiciones del desarrollo en todas sus aéreas. También hay problemas de pronunciación y balbuceo [Ball et al., 2005]. Las dificultades de aprendizaje están presentes en el 97% de los pacientes, aunque el retraso cognitivo oscila desde ligero a moderado y rara vez es severo [Cole and Hughes, 1994]. En múltiples pacientes se han descrito también problemas neurológicos como convulsiones y coordinación pobre, esta última manifestación parece mejorar con el tiempo.

El periodo neonatal se caracteriza por manifestación frecuente de hipotonía, ictericia prolongada y dificultades de alimentación en un 70% de casos [Tatton-Brown et al., 2005].

En un tercio de los pacientes se encuentran manos grandes y pies grandes y planos y curvaturas anómalas de la columna [Tatton-Brown et al., 2005]. Entre el 60% y el 80% de los pacientes presentan erupción prematura de los dientes. Las anomalías cardiacas congénitas se encuentran en un 21-25% y en alrededor del 15% se manifiestan problemas urinarios [Cole and Hughes, 1994; Tatton-Brown et al., 2005].

Los pacientes que presentan el síndrome de Sotos tienen un mayor riesgo de desarrollar tumores malignos; la predisposición ha sido estimada entre el 2% y el 7% [Hersh et al., 1992; Maldonado et al., 1984]. Los tumores más frecuentes son linfomas, leucemias, tumores de Wilms, neuroblastomas, carcinoma hepatocelular y teratomas sacrococcígeos. El rango de edad en el momento del diagnóstico va desde los 15 meses hasta la edad adulta, cuando el riesgo disminuye para la mayoría de los otros SSC. Además es importante destacar que el riesgo de tumor tiene un ratio hombre:mujer de 2:1 [Lapunzina, 2005a].

Base genética

El síndrome de Sotos se debe a la haploinsuficiencia de gen *NSD1* (Nuclear receptor binding SET Domain protein 1), bien por deleciones que incluyen todo el gen o por mutaciones puntuales causantes de pérdida de función. El gen *NSD1* codifica por un receptor nuclear y mapea en la región 5q35. Fue originariamente descrito en 2002 [Kurotaki et al., 2002] y luego se han comunicado varias series de pacientes europeos. La gran diferencia entre las series de pacientes japoneses y los de población europea estriba en el porcentaje de pacientes que presentan

microdeleción cromosómica. De hecho, aproximadamente el 45% de los pacientes japoneses presentan microdeleción de la región 5q35 [Kurotaki et al., 2003] mientras las microdeleciones son minoritarias en la población europea (0-18%) que tiende a presentar mutaciones puntuales del gen *NSD1* [Douglas et al., 2003].

El gen *NSD1* presenta 23 exones, de los cuales el primero no se traduce, y codifica una proteína de 2.696 aminoácidos con múltiples dominios funcionales (Figura 7).

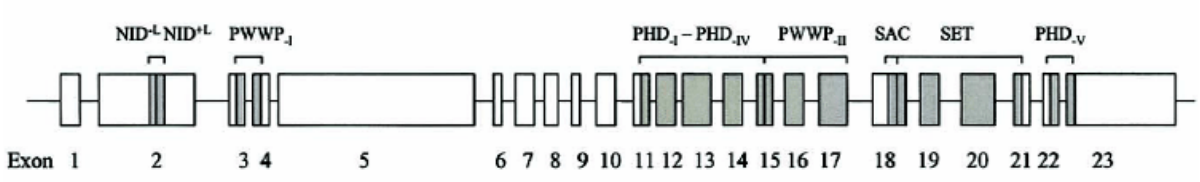

Figura 7: Representación esquemática del gen *NSD1*. Los rectángulos son los diferentes exones y en gris se evidencian los diferentes dominios funcionales de la proteína.

La proteína NSD1 es una metiltransferasa histónica que actuar como regulador de la cromatina, activando o inhibiendo la transcripción según el contexto celular [Berdasco et al., 2009]. A nivel nucleotídico el gen *NSD1* presenta una identidad del 86% con su homólogo en ratón *nsd1* y del 83% a nivel aminoacídico [Kurotaki et al., 2001]. Se han estudiado ratones *nsd1* knock-out y se ha visto que en heterocigosis no muestran ninguna característica propia del SS, mientras los ratones homocigotos tienen una muerte temprana durante la gestación. Este resultados sugiere que la proteína juega un papel esencial en el desarrollo inicial post-implantacional [Rayasam et al., 2003].

4 Técnicas moleculares

Son varias las técnicas moleculares que se han utilizado para el estudio de los defectos genéticos de los pacientes. Esto se debe a que hemos tenido que caracterizar alteraciones moleculares distintas, como mutaciones puntuales, deleciones/duplicaciones, defectos epigenéticos y reordenamientos cromosómicos como la disomía uniparental.

4.1 Secuenciación directa

La técnica que mejor nos permite observar los cambios puntuales y las pequeñas inserciones/deleciones de una región determinada es la secuenciación directa, precedida por amplificación por PCR de la región de interés. En los trabajos aquí reportados, dicha técnica ha sido utilizada para el estudio de los genes *GPC3* y *OFD1 (CXORF5)* en los pacientes con SBGS y del gen *CDKN1C* en los pacientes con BWS.

4.2 Multiplex Ligation-dependent Probe Amplification (MLPA) y Methylation-Specific MLPA (MS-MLPA)

La técnica de MLPA (Multiplex Ligation Probe Amplification) [Schouten et al., 2002] permite detectar y cuantificar el número de copias de varias regiones en una misma reacción multiplex. El principio básico de esta técnica consiste en la hibridación específica en las secuencias en estudio de dos oligonucleótidos adyacentes y su ligación. Después se realiza la PCR de las sondas ligadas con una única pareja de primers, eso es posible gracias a que todas las sondas tienen en sus extremos secuencias comunes homólogas a los primers de PCR. Los productos de la amplificación tienen tamaños distintos, gracias a las secuencias espaciadoras presentes en uno de los dos oligonucleótidos y los primers están marcados con el fluorocromo FAM. Los productos de PCR se analizan en un secuenciador y las áreas de los picos correspondientes a los distintos productos se normalizan con una hoja de cálculo Excel generada por nosotros (MetHULP v1.1). Esta normalización nos permite detectar deleciones y duplicaciones en varias regiones y sin que sea necesario analizar en paralelo las muestras de los progenitores como en el análisis de los microsatélites. La compañía MRC-Holland (Amsterdam, Holanda) ha desarrollado un kit específico

para el estudio de los genes *GPC3* y *GPC4* (Glypican 4) (SALSA P154) que ha sido utilizado para descartar deleciones y duplicaciones en nuestros pacientes con SGBS.

La técnica de MS-MLPA (Methylation Specific-MLPA) tiene el mismo principio de un MLPA de dosis génica, pero nos permite también cuantificar el nivel de metilación de los centros de imprinting de interés [Dikow et al., 2007; Scott et al., 2008]. Las diferencias de este método con el convencional se basan sobre el uso de sondas específicas de metilación y del enzima de restricción sensible a la metilación HhaI. Nuestra muestra se hibrida como en un MLPA normal pero luego se divide en dos alícuotas, una para determinar la dosis génica y la otra para determinar el nivel de metilación. La primera alícuota de hecho sólo se liga mientras la segunda se digiere con HhaI y se liga. La comparación entre la señal de la alícuota digerida con la no digerida, nos permite calcular el nivel de metilación del centro de imprinting que contiene sitios de restricción para HhaI. Para el estudio de la dosis génica y de la metilación del locus 11p15.5 en nuestros pacientes con BWS, utilizamos un kit desarrollado por MRC-Holland (SALSA ME030) y normalizamos los datos con una hoja de cálculo Excel realizada por nosotros (MetHULP v1.1).

4.3 Análisis de microsatélites

El análisis de los marcadores microsatélites es una de las técnicas más utilizadas para detectar deleciones y siempre requiere estudiar en paralelo las muestras de los progenitores para realizar un análisis de segregación alélica. En nuestro estudio se ha utilizado esta técnica para detectar los casos de disomía uniparental [Slatter et al., 1994] entre los pacientes con BWS, definir el origen parental de la disomía y cuantificar el nivel de mosaicismo en los casos positivos [Sasaki et al., 2007].

4.4 Methylation-Sensitive High Resolution Melting (MS-HRM)

La técnica de High-Resolution Melting es una nueva tecnología que nos permite detectar pequeñas alteraciones en con alto rendimiento y fiabilidad a bajo coste [Graham et al., 2005; Zhou et al., 2005]. Este método se basa en el análisis del perfil de fusión de cada muestra. La técnica consiste en realizar una PCR en presencia de un agente intercalante fluorescente, cuya incorporación en los productos nos permite seguir la caída de la fluorescencia a lo largo de una

rampa de temperatura y así trazar el perfil de fusión del fragmento. Cada segmento, según sus características, generará una curva patrón que se verá alterada en presencia de polimorfismos, mutaciones puntuales o micro-inserciones y deleciones, con resolución de 1bp.

La técnica de HRM se ha demostrado válida para la detección de diferencias en el nivel de metilación de las islas CpG de los centros de imprinting [Wojdacz et al., 2008b; Wojdacz et al., 2008a; Wojdacz and Dobrovic, 2007]. El DNA se somete primero a una reacción de bisulfitación que convierte las citosinas no metiladas de los dinucleótidos CpG en uracilos, convertidos luego en timinas por la reacción de PCR. Así los alelos metilado y no metilado tendrán una distinta temperatura de fusión según el propio nivel de metilación. Esto lleva a la generación de una curva con dos puntos de fusión distintos, el más bajo relativo al alelo no metilado y el más alto al alelo metilado. El plateau de la curva, con su valor de fluorescencia, representa la relación entre el alelo metilado y el no metilado [Alders et al., 2009], relación que nos permite definir el estado de metilación de las muestras.

4.5 Pirosecuenciación

La pirosecuenciación es un método de secuenciación “by-synthesis” que nos permite monitorear de forma cuantitativa la incorporación de los nucleótidos a través de la conversión enzimática del pirofosfato liberado en una señal luminosa proporcional. La pirosecuenciación ofrece también una cuantificación absoluta y directa de los patrones de metilación de cada dinucleótido CpG en muestras de DNA previamente tratadas con bisulfito [Tost and Gut, 2007]. Esta técnica ha sido utilizada para definir el nivel de metilación de los centros de imprinting H19DMR y KvDMR en pacientes con BWS.

HIPÓTESIS

Síndrome de Beckwith-Wiedemann

A) Un porcentaje pequeño de casos esporádicos (~10%) y un porcentaje importante de casos familiares (~40%) de pacientes con síndrome de Beckwith-Wiedemann se deben a mutaciones puntuales del gen de expresión materna *CDKN1C.* No se han identificado hasta el momento deleciones del gen entero ni micro deleciones de *CDKN1C* ni tampoco se han podido identificar puntos calientes de mutaciones.

B) Existen diferencias fenotípicas en los pacientes con síndromes de Beckwith-Wiedemann que pueden tener una base molecular y una correlación genotípica con los subgrupos moleculares de la patología.

C) Alteraciones del gen *CDKN1C* podrían ser responsables de preeclampsia en humanos debido a que mutaciones con pérdida de función del gen *cdkn1c* han sido observadas en modelos murinos de preeclampsia.

D) Las alteraciones epigenéticas son responsables de la gran mayoría de anomalías genéticas en los pacientes con síndrome de Beckwith-Wiedemann. Dichas alteraciones epigenéticas deben estudiarse mediante un análisis del estado de metilación del los dos centros de imprinting de la región 11p15.

E) El nivel de mosaicismo y la extensión de la zona de UPD paterna tienen relevancia en las características fenotípicas de los pacientes con síndrome de Beckwith-Wiedemann. Aunque se ha podido definir perfectamente un sitio de breakpoint para las inversiones/translocaciones maternas, los sitios de recombinación mitótica de UPD no están correctamente estudiados y pueden ser distintos de los de recombinación meiótica. El estudio de los pacientes con UPD paterna por SNP-array permitirá definir en detalle las características genómicas de esta enfermedad.

Síndrome de Macrocefalia-Malformación Capilar

F) Los arrays de SNPs permitirán una evaluación detallada de la arquitectura genómica de los pacientes con Macrocefalia-Malformación Capilar permitiendo tal vez confirmar o descartar los reordenamientos genómicos en esta patología.

Síndrome de Simpson-Golabi-Behmel

G) Existen pacientes con fenotipo de síndrome de Simpson-Golabi-Behmel y sin alteraciones del gen *GPC3.* Estos pacientes podrían tener una variante más severa de esté síndrome, cuyo gen afectado podría ser el *OFD1*.

H) La observación de más de un varón afecto de síndrome de Simpson-Golabi-Behmel en hermanos con resultados negativos en la madre sugiere que pueda existir en esta patología mecanismos de mosaicismo germinal que expliquen el hallazgo.

Síndrome de Sotos

I) El conocimiento de la evolución del síndrome de Sotos en la edad adulta permitirá definir un protocolo de seguimiento médico adecuado.

OBJETIVOS

Síndrome de Beckwith-Wiedemann

1. Identificar las alteraciones genéticas del gen *CDKN1C* en pacientes con fenotipo de Síndrome de Beckwith-Wiedemann sin diagnostico molecular, y valorar la frecuencia de deleciones completas del gen, mutaciones puntuales, microdeleciones y microduplicaciones y la presencia o ausencia de puntos calientes dentro del gen (Hipótesis A).
2. Realizar una correlación genotipo-fenotipo en los pacientes con Síndrome de Beckwith-Wiedemann (Hipótesis B).
3. Evaluar y secuenciar el gen *CDKN1C* en madres de pacientes con Síndrome de Beckwith-Wiedemann y preeclampsia o Síndrome HELLP con el objeto de identificar potenciales mutaciones (Hipótesis C).
4. Evaluar el estado de metilación mediante tres diferentes técnicas moleculares para la detección de defectos epigenéticos. Comparar las técnicas de MS-MLPA y MS-HRM en la cuantificación del nivel de metilación de los dos centros de imprinting de la región 11p15 (Hipótesis D).
5. Analizar mediante técnicas de SNP-arrays los pacientes con Síndrome de Beckwith-Wiedemann con UPD paterna, y comprar los resultados con otras técnicas con el objeto de evaluar el nivel de mosaicismo, el porcentaje de metilación y la extensión de la disomía uniparental. Correlacionar fenotipo-genotipo según la entidad del defecto. Analizar un caso de UPD paterna de todo el genoma con elevado nivel de mosaicismo; inicialmente diagnosticado como Beckwith-Wiedemann por UPD paterna de 11p15. Discutir el mecanismo molecular y la importancia del diagnostico por SNP-array en los casos de síndromes de imprinting (Hipótesis E).

Síndrome de Macrocefalia-Malformación Capilar

6. Analizar mediante array de SNPs la arquitectura genómica de los pacientes con Macrocefalia-Malformación Capilar y realizar una correlación fenotipo-genotipo y una correcta clasificación y valoración de los criterios clínicos de diagnóstico (Hipótesis F).

Síndrome de Simpson-Golabi-Behmel

7. Analizar las deleciones y mutaciones del gen *GPC3* y las deleciones de *GPC4* y secuenciar el gen *OFD1* en pacientes con síndrome de Simpson Golabi Behmel sin diagnostico molecular confirmado (Hipótesis G).
8. Analizar mediante técnicas moleculares el gen *GPC3* pacientes hermanos con fenotipo Síndrome de Simpson Golabi Behmel cuya madre no presenta alteraciones moleculares en este gen. Identificar un probable mosaicismo germinal en la madre, visto su estado de no portadora de la mutación presentada por sus dos hijos (Hipótesis H).

Síndrome de Sotos

9. Revisar los signos clínicos de pacientes mayores con síndrome de Sotos. Identificar características comunes y sugerir líneas guía para su seguimiento en la edad adulta (Hipótesis I).

ARTÍCULOS

ARTÍCULOS PUBLICADOS

ARTÍCULO I

CDKN1C (p57Kip2) Analysis in Beckwith-Wiedemann syndrome (BWS) Patients: Genotype-phenotype Correlations, Novel Mutations, and Polymorphisms. *Am J Med Genet A. 2010. 152A(6):1390-7.*

ARTÍCULO II

CDKN1C mutations in HELLP/preeclamptic mothers of Beckwith-Wiedemann syndrome (BWS) patients. *Placenta. 2009. 30(6):551-4.*

ARTÍCULO VII

The *OFD1 (CXORF5)* gene at Xp22 is not mutated in a subset of Simpson-Golabi-Behmel syndrome patients. *Current Topics in Genetics. 2008. 3:45-47.*

ARTÍCULO VIII

Germinal mosaicism in Simpson-Golabi-Behmel syndrome. *Clin Genet. 2007. 72: 384-386.*

ARTÍCULOS ACEPTADOS

ARTÍCULO IV

Beckwith Wiedemann syndrome and Uniparental Disomy 11p: Fine mapping of the recombination breakpoints and evaluation of several techniques. *Eur J Hum Genet. 2010.*

ARTÍCULO V

Constitutional Mosaic Genome-Wide Uniparental Disomy due to Diploidization: an Unusual Cancer-Predisposing Mechanism. *J Med Genet. 2010.*

ARTÍCULO VI

Macrocephaly-Capillary Malformation: Analysis of 13 Patients and Review of the Diagnostic Criteria. *Am J Med Genet A. 2010.*

ARTÍCULOS ENVIADOS

ARTÍCULO III

Quantification of Methylation Index of the 11p15-Differentially Methylated Regions in the BWS/SRS locus by Methylation-Sensitive High Resolution Melting (MS-HRM) analysis. *Hum Mutat.*

ARTÍCULO IX

Adults with Sotos syndrome: Case Report of the Oldest Person and Review of 20 New Patients. *Am J Med Genet.*

CDKN1C (p57Kip2) Analysis in Beckwith-Wiedemann syndrome (BWS) Patients: Genotype-phenotype Correlations, Novel Mutations, and Polymorphisms.

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, López-Siguero JP, Guillén-Navarro E, Gómez-Puertas P, Wesselink JJ, Mercado G, Esteban-Marfil V, Palomo R, Mena R, Sánchez A, del Campo M, Lapunzina P.

Am J Med Genet A. 2010. 152A(6):1390-7.

Análisis de *CDKN1C* (p57Kip2) en pacientes con síndrome de Beckwith-Wiedemann: correlaciones genotipo-fenotipo, nuevas mutaciones y polimorfismos.

El síndrome de Beckwith-Wiedemann (BWS) es un síndrome de sobrecrecimiento caracterizado por macroglosia, macrosomía y defectos de la pared abdominal. Se encuentran mutaciones de *CDKN1C* en el alelo materno en un 10% de casos esporádicos y en un 40% de las familias con BWS autosómico dominante. *CDKN1C* se encuentra en el locus BWS, región 11p15 y es un gen de expresión materna. Codifica para un miembro de la familia de inhibidores de quinasas dependientes de ciclina (CdK) que actúa como supresor del ciclo celular.

Se realizó un estudio del gen *CDKN1C* por secuenciación bidireccional en nuestros pacientes BWS sin diagnostico molecular. Fueron analizados 49 pacientes con fenotipo BWS y 22 con hemihiperplasia, onfalocele o macroglosia; se encontraron 8 pacientes con mutaciones no descritas de las cuales 6 eran heredadas de la madre, 1 era *de novo* y 1 de origen no conocida.

Hasta la fecha se han reportado 25 mutaciones distintas, incluyendo las nuestras. Entre las que presentamos hay una nueva mutación de splicing en el intrón 1 y además sugerimos que los residuos Leu33 y Ser282 sean puntos calientes de mutación, pues representan el 23% de los casos conocidos.

Finalmente hemos estudiado las características clínicas de nuestros pacientes, buscando correlación entre los signos clínicos presentados y la alteración molecular.

Analisi di *CDKN1C* (p57Kip2) in pazienti con sindrome de Beckwith-Wiedemann: correlazioni genotipo-fenotipo, nuove mutazioni e polimorfismi.

La sindrome di Beckwith-Wiedemann (BWS) é una sindrome di hipercrescimento caratterizzata da macroglossia, macrosomia e difetti della parete addominale. Si trovano mutazioni di *CDKN1C* sul allele materno in un 10% di casi sporadici e in un 40% delle famiglie con BWS autosomico dominante. *CDKN1C* si trova nel locuso BWS, regione 11p15 ed é un gene di espressione materna. Codifica per un membro della famiglia di inibitori delle chinasi ciclina-dipendenti (CdK) che agisce da soppressore del ciclo cellulare.

Si é realizzato uno studio del gene *CDKN1C* con sequenziamento bidirezionale nei nostri pazienti BWS senza diagnosi moleculare. Si analizzarono 49 pazienti con fenotipo BWS y 22 con emiiperplasia, onfalocele o macroglossia; si incontrarono 8 pazienti con mutazioni non descritte di cui 6 ereditate dalla madre, 1 *de novo* e 1 di origine sconosciuta.

Fino ad oggi sono state riportate 25 mutazioni diverse, includendo le nostre. Tra quelle che presentiamo c'é una nuova mutazione di splicing nell'introne 1 e inoltre suggeriamo che i residui Leu33 y Ser282 siano punti caldi di mutazione, visto que rappresentano il 23% dei casi noti.

In ultimo abbiamo studiato le caratteristiche cliniche dei nostri pazienti, cercando correlazione tra i segni clinici presentati e l'alterazione molecolare.

RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

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

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Received 27 November 2009; Accepted 5 March 2010

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

Grant sponsor: Fondo de Investigación Sanitaria (FIS); Grant number: PI08/1360.

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Published online 14 May 2010 in Wiley InterScience (www.interscience.wiley.com)
DOI 10.1002/ajmg.a.33453

How to Cite this Article:
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, López-Siguero JP, Guillén-Navarro E, Gómez-Puertas P, Wesselink J-J, Mercado G, Esteban-Marfil V, Palomo R, Mena R, Sánchez A, del Campo M, Lapunzina P. 2010. *CDKN1C* ($p57^{Kip2}$) analysis in Beckwith–Wiedemann syndrome (BWS) patients: Genotype–phenotype correlations, novel mutations, and polymorphisms.
Am J Med Genet Part A 152A:1390–1397.

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.

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 [Percesepe 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.

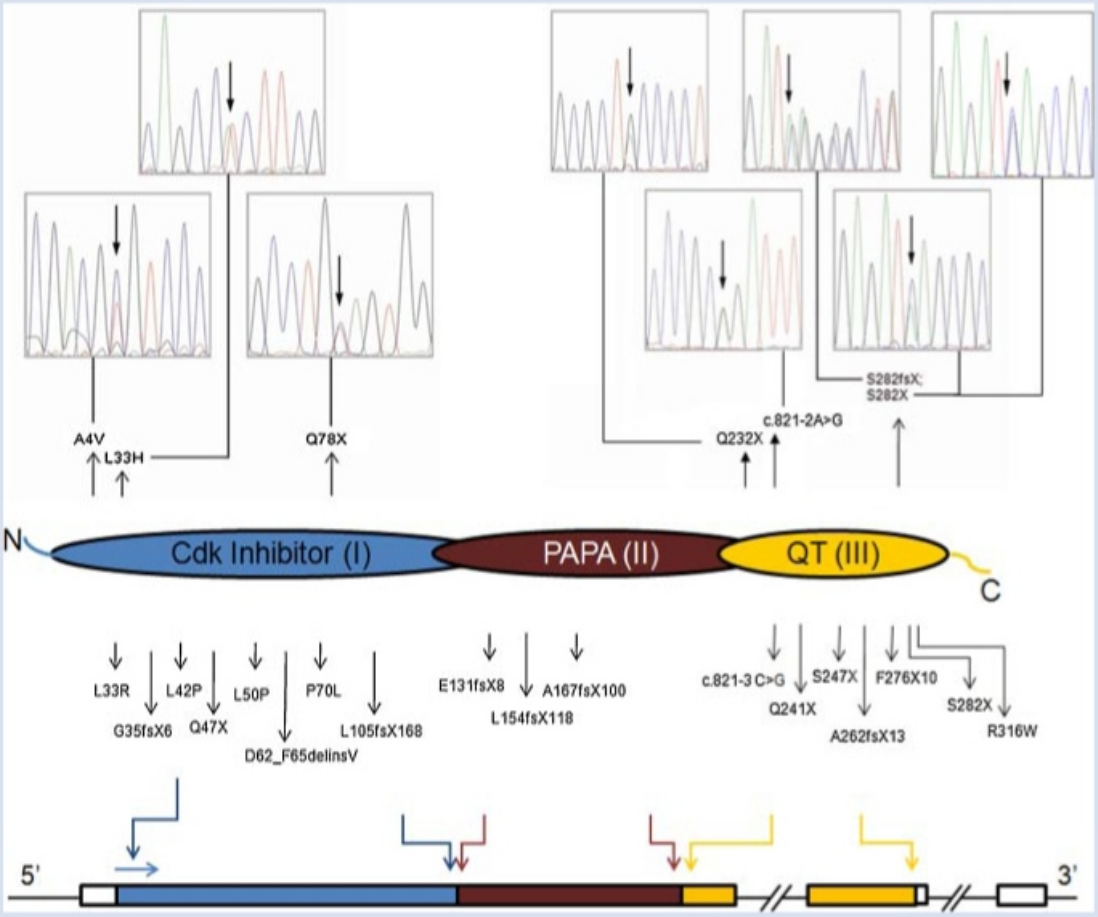

FIG. 1. Schematic representation of *CDKN1C* and mutations identified. Chromatograms of the eight novel mutations identified in this study are shown above the protein structure, while the previously reported mutations are shown below the protein. At the bottom, schematic representation of the gene; rectangles 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′-cgccctctcctcctctcctttccccttc-3′ and antisense primer: 5′-tcggggctctttgggctctaaact-3′. The PCR reaction mixture consisted of: 10× PCR buffer HotStartTaq QIAGEN (containing 15 mM $MgCl_2$), 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′-ctcctttcccctttctctcg-3′	1
2F	5′-tggaccgaagtggacagcga-3′	1
3R	5′-cctgcaccgtctcgcggtag-3′	1
4F	5′-ccggagcagctgcctagtgtc-3′	1
5R	5′-ggggccaggaccgcgacc-3′	1
6R	5′-gggaggaggcgggaaccctgcga-3′	1
7F	5′-cggcgacgtaaacaaagctgac-3′	2
8R	5′-tcggggctctttgggctctaaact-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].

[a]Mutations 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.708 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, hemihyperplasia 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
Overgrowth	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.

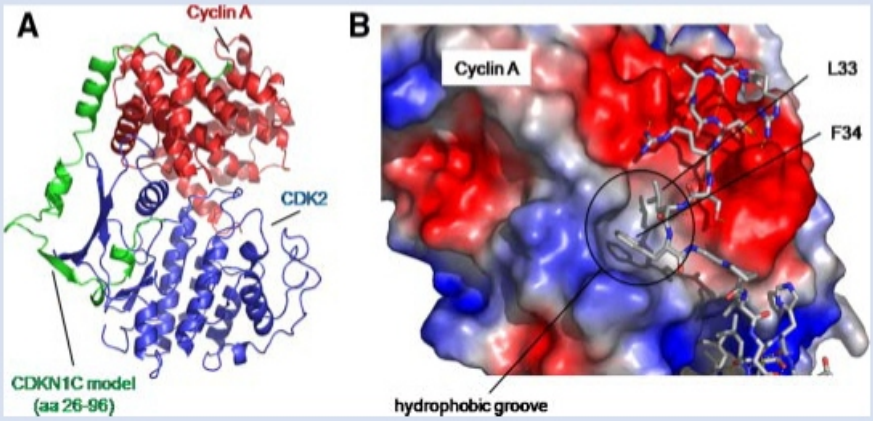

FIG. 2. 3D structural model of the CDK-inhibitor domain of CDKN1C. A: Ribbon-plot representation of 3D model for CDKN1C/cyclin A-CDK2 complex interaction. Model for CDKN1C includes only CDK-inhibitor domain (aa 26–96). B: Detail of the interaction of CDKN1C Leu33 residue with Cyclin A surface, colored 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, macroglosia 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.

ACKNOWLEDGMENTS

This work was supported by grants from the "Fondo de Investigación Sanitaria" (FIS; PI08/1360). Valeria Romanelli was a recipient of a PhD fellowship from FIS and currently is supported by Fundación Mutua Madrileña. Alberta Belinchón was supported by CIBERER Lanzadera PhD fellowship. Biomol-Informatics SL is partially supported by Torres-Quevedo funds from Ministerio de Ciencia e Innovación.

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CDKN1C mutations in HELLP/preeclamptic mothers of Beckwith-Wiedemann syndrome (BWS) patients.

Romanelli V, Belinchón A, Campos-Barros A, Heath KE, García-Miñaur S, Martínez-Glez V, Palomo R, Mercado G, Gracia R, Lapunzina P.

Placenta. 2009. 30(6):551-4.

Mutaciones de *CDKN1C* en madres con HELLP/preeclampsia de pacientes con síndrome de Beckwith-Wiedemann (BWS).

La preeclampsia es la manifestación de hipertensión con proteinuria después de 20 semanas de embarazo. El síndrome HELLP (hemolisis, enzimas hepáticas elevadas y recuento de plaquetas bajo) es una forma severa de preeclampsia, con elevada morbilidad para el recién nacido y para la madre.

Recientemente se ha visto que modelos de ratón *cdkn1c*$^{-/-}$ muestran el espectro completo de síntomas de preeclampsia.

En este trabajo se han estudiado los períodos de gestación de las madres de nuestros 96 pacientes con síndrome de Beckwith-Wiedemann con diagnostico genético definido. Los resultados obtenidos nos permiten comunicar que de las 7 madres de pacientes con BWS debido a mutaciones del gen *CDKN1C*, 3 han manifestado HELLP/preeclampsia; mientras que las 89 madres de pacientes BWS con otro diagnostico genético, sólo 3 han tenido algunos síntomas. Este hallazgo demuestra un aumento significativo en el riesgo de desarrollar HELLP/preeclampsia en los casos debido a mutación de *CDKN1C*.

Es interesante notar que en los 3 casos de HELLP/preeclampsia por mutaciones de *CDKN1C*, se trata de mutaciones sin sentido que generan una forma truncada de la proteína. Además es importante destacar que 2 de la 3 mutaciones han sido heredadas por la madre, mientras una de ellas es *de novo*. Este dato nos permite avanzar la hipótesis que el trastorno HELLP/preeclampsia pueda ser debido a la contribución de factores feto-placentarios y maternos.

Mutazioni di *CDKN1C* in madri con HELLP/preeclampsia di pazienti con sindrome de Beckwith-Wiedemann (BWS).

La preeclampsia é la manifestazione di ipertensione e proteinuria dopo la settimana 20 di gestazione. La sindrome HELLP (emolisi, alti valori di enzimi nel fegato e bassa conta piastrinica) é una forma grave di preeclampsia, con elevata morbositá per il neontato e per la madre.

Recentemente si é visto que modelli di topo cdkn1c$^{-/-}$ mostrano lo spettro completo dei sintomi di preeclampsia.

In questo lavoro sono stati studiati i periodi di gestazione delle madri dei nostri 96 pazienti con sindrome di Beckwith-Wiedemann con diagnosi molecolare definita. I risultati ottenuti ci permettono comunicare che delle 7 madri di pazienti con BWS dovuto a mutazioni del gene *CDKN1C*, 3 hanno manifestado HELLP/preeclampsia; invece delle 89 madri di pazienti BWS con un' altra diagnosi genetica, solamente 3 hanno avuto qualche sintomo. Questa scoperta dimostra un aumento significativo del rischio di sviluppare HELLP/preeclampsia nei casi dovuti a mutazioni di *CDKN1C*.

É interessante notare que nei 3 casi di HELLP/preeclampsia per mutazioni di *CDKN1C*, si tratta di mutazioni nonsense che generano una forma tronca della proteina. Inoltre é importante enfatizzare che 2 delle 3 mutazioni sono state ereditate dalla madre, mentre una di loro é de novo. Questo dato ci permette avanzare l'ipotesi che il disturbo HELLP/preeclampsia sia dovuto al contributo di fattori feto-placentari e materni.

Placenta 30 (2009) 551–554

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Short Communication

CDKN1C Mutations in HELLP/Preeclamptic Mothers of Beckwith–Wiedemann Syndrome (BWS) Patients

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ARTICLE INFO

Article history:
Accepted 22 March 2009

Keywords:
Preeclampsia
HELLP
CDKN1C
$p57^{KIP2}$
Beckwith Wiedemann syndrome
Overgrowth syndrome
Placenta
Mutations

ABSTRACT

Preeclampsia is the development of new-onset hypertension with proteinuria after 20 weeks of gestation. HELLP syndrome (haemolysis, elevated liver enzymes, and low platelet count) is a severe form of preeclampsia with high rates of neonatal and maternal morbidity. In recent years, loss of function of *cdkn1c* (a tight-binding inhibitor of G1 cyclin/cyclin-dependent kinase complexes and a negative regulator of cell proliferation) has been observed in several mouse models of preeclampsia. In this paper, we report on three women with HELLP/preeclampsia who had children with Beckwith Wiedemann syndrome, a complex genetic disorder characterised, among other findings, by overgrowth, omphalocele and macroglossia. All three children displayed mutations in *CDKN1C* predicted to generate truncated proteins. Two of the mutations were maternally inherited while the third was *de novo*. This finding suggests a fetal contribution to the maternal disease. To the best of our knowledge this is the first report of *CDKN1C* mutations in children born to women with preeclampsia/HELLP syndrome, thus suggesting the involvement of an imprinted gene in the pathophysiology of preeclampsia.

1. Introduction

Preeclampsia is a pregnancy-specific disorder defined by the sudden onset of hypertension and proteinuria in the second half of pregnancy (>20 weeks). It is a multi-system and potentially devastating syndrome associated with substantial risks for both mother and fetus [1]. Similarly, the related disorder HELLP (haemolysis, elevated liver enzymes and low platelets) is a severe disease characterised by thrombotic microangiopathy with microvascular endothelial activation, cell injury and thrombosis that affects a small proportion of pregnancies.

Beckwith Wiedemann syndrome (BWS, MIM 130650) is a genetic overgrowth disorder characterised by macrosomia, macroglossia, visceromegaly, hemihypertrophy, abdominal wall defects, ear creases/pits, neonatal hypoglycaemia, polyhydramnios, placentomegaly, placental mesenchymal dysplasia, cardiac defects, nevus flammeus, and an increased frequency of embryonic tumours [2]. This condition is caused by a variety of genetic or epigenetic alterations within two imprinting domains on human chromosome *11p15*. Several genes in this imprinted cluster encode proteins involved in growth regulation, the paternally expressed *IGF2* and the maternally expressed cell-cycle regulator cyclin dependent kinase inhibitor (*CDKN1C*; also known as $p57^{KIP2}$) among others [2].

In mammalian cells the encoded protein CDKN1C localizes to the nucleus. CDKN1C is a potent tight-binding inhibitor of several G1 cyclin/cyclin-dependent kinase complexes and a negative regulator of cell proliferation [3]. It is also a tumour suppressor gene and a negative regulator of fetal growth. Mutations in *CDKN1C* have been identified in approximately 5% of patients with BWS. Loss of methylation at the maternal differentially methylated region 2 is sometimes associated with decreased expression of *CDKN1C* [4].

In this paper, we report on three women with HELLP/preeclampsia who had children with BWS due to mutations in *CDKN1C*. Although downregulation and loss of function of the orthologous gene *cdkn1c* have been linked to several mouse models of preeclampsia in the past years [5,6], to our knowledge the present report constitutes the first confirmation of the involvement of *CDKN1C* mutations in mothers with HELLP/preeclampsia who gave birth to children with BWS.

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0143-4004/$ – see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.placenta.2009.03.013

2. Reports and results

All patients with a diagnosis of BWS included in the Spanish Overgrowth Syndrome Registry (RESSC) ($n = 96$) were evaluated according to a laboratory screening protocol that included karyotype determination, fluorescent in situ hybridization (FISH), methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) or Southern blot and microsatellite analysis of the chromosome 11p region as well as mutation screening of *CDKN1C*. The *CDKN1C* coding sequences and intron/exon boundaries were screened for mutations by PCR amplification of genomic DNA samples and subsequent DNA sequencing. Specific primers (available upon request) were designed with the help of Oligo 6 (V6.67; Molecular Biology Insights, Inc., Cascade, CO) software.

Clinical data of the participating BWS children including personal and family history, clinical, laboratory and X-rays, MRI, and CT scan findings, pedigree, and follow-up evolution, were registered in a database. Maternal data were also recorded in all cases including age, previous disease history, consanguinity, ethnic background, and pregnancy events.

From a total of 96 BWS patients analysed, we identified seven children with mutations in *CDKN1C*. All seven mothers were subsequently screened for mutations in *CDKN1C*. Six out of seven children inherited the mutation from their apparently mothers, and one mutation was *de novo*. Grandparents were evaluated in three families, one of the grandfathers did transmit the mutation and the other two were *de novo*. Consequently none of these women showed the clinical phenotype of BWS. Three out of the seven mothers developed preeclampsia/HELLP (Table 1); remarkably all three preeclamptic mothers gave birth to children with BWS due to *CDKN1C* mutations predicted to generate truncated proteins forms: c.232C>T (Q78X) located in the CDI protein domain and c.845C>A (S282X) and c.845delC (S282fsX) both located in the carboxy terminus (QT domain; Fig. 1). Clinical and molecular data from the three children and their mothers are listed in Table 1 and shown in Fig. 1. The remaining four mothers who did not develop preeclampsia, had children harbouring three different *CDKN1C* missense and one non-sense mutations, this last mutation was located in the QT domain. By contrast, only three out of 89 mothers of BWS patients without mutations in *CDKN1C* developed some symptoms of preeclampsia: two women had persistent hypertension during pregnancy and one also had proteinuria. In summary, there were seven women who gave birth to BWS children with *CDKN1C* mutations (three with preeclampsia and four without) whilst 89 women gave birth to BWS without *CDKN1C* mutations (three preeclampsia and 86 not) (p 0.004; Fisher exact test).

3. Discussion

Unlike most other human disorders, eclampsia, preeclampsia or HELLP syndrome have impact on two individuals, the mother and the child, both of whom can be severely affected. The frequency and severity of the disease are substantially higher in women with pre-existing maternal hypertension, diabetes, previous preeclampsia, pre-existing thrombophilia and a family history of preeclampsia. Genetic assessment of preeclampsia is further complicated by the potential contribution of both maternal and feto-placental factors to the disease pathogenesis, as well as by significant clinical and genetic heterogeneity [6]. Some susceptibility loci of preeclampsia have been mapped; the preeclampsia (PEE) 1 locus on chromosome 2p13, PEE2 in 2p25, PEE3 in 9p13 and PEE4 in the putative imprinted 10q22 region (*STOX1*) [7,8]. However, the contribution of *STOX1* is controversial in light of recent reports that failed to validate *STOX1* as a common preeclampsia susceptibility gene [9–11] and to demonstrate that the gene is imprinted [11]. Some epidemiological association/risk factors have also been described, the *MTHFR* homozygous 677T variant [12], the epoxide hydroxylase 1 gene [13], the glutathione *S*-transferase gene [14] and the coagulation factor V (R506V and R485K factor V mutation/polymorphism) [15–17]. Furthermore, the contribution of both environmental and genetic factors to preeclampsia has greatly hampered attempts to define the disease pathogenesis at the molecular level in humans [6].

On the other hand, mouse models of preeclampsia like the catechol-O-methyltransferase (*COMT*) knockout and *COMT* deficient transgenic mice [18], the bph/5 spontaneous hypertensive mice [1] and the *cdkn1c* ($p57^{KIP2}$) [5] mutants have greatly contributed to the understanding of this complex disorder. However, only the *cdkn1c*-mutant mouse recapitulates the full spectrum of preeclampsia symptoms. These mice display not only abnormal placental development but also premature labour, increased blood pressure, proteinuria and glomerular lesions [5,6,19]. Heterozygous female mice inheriting a mutant allele from their carrier dams express *cdkn1c* at wild-type levels. These carriers dams develop symptoms of preeclampsia when carrying a litter in which half of the embryos lack *cdkn1c*, demonstrating that preeclampsia is caused by a feto-placental defect rather than a maternal defect [6]. This is in agreement with our observation regarding one of the three preeclamptic women reported herein who does not carry the Q78X mutation detected in her newborn, emphasizing that the preeclamptic effects are probably caused by feto-placental abnormalities rather than by isolated maternal abnormalities.

All three BWS patients in this report had truncating mutations of *CDKN1C* (Q78X, S282X and S282fsX) predicted to generate a non-functional protein. Interestingly, two of the mutations affected the same nucleotide (cytosine 845; S282); a mutation previously reported in one patient with BWS [20]. Though we identified *CDKN1C* mutations in the genomic DNA sequence of three BWS children and two of their respective mothers, the functional consequences in placental tissue remain to be elucidated. Assessments of *CDKN1C* expression levels, either by RNA or protein analysis are difficult to interpret because most studies rely on tissue samples evaluated well after the onset of preeclampsia symptoms [6].

CDKN1C is a negative regulator of epithelial cell proliferation and a positive regulator of the transforming growth factor beta

Table 1
Clinical findings and molecular data of the three patients with *CDKN1C* mutations born to preeclampsia/HELLP syndrome mothers.

Patient	Pregnancy	Clinical findings in newborns	Mutation in *CDKN1C*	Inheritance
1	Preeclampsia Proteinuric hypertension	Macrosomia, hypoglycaemia, nevus flammeus, ear creases, omphalocele	c. 232 C > T (Q78X)	*de novo*
2	Preeclampsia Proteinuric hypertension	Macrosomia, omphalocele, macroglossia, ventricular septal defect	c. 845 C > A (S282X)	Maternal
3	HELLP syndrome	Macrosomia, hypoglycaemia, flammeus, ear creases, omphalocele, hypospadias, cleft palate	c. 845delC (S282fsX)	Maternal

Mutations are numbered according to ensembl transcript ENST00000313407.

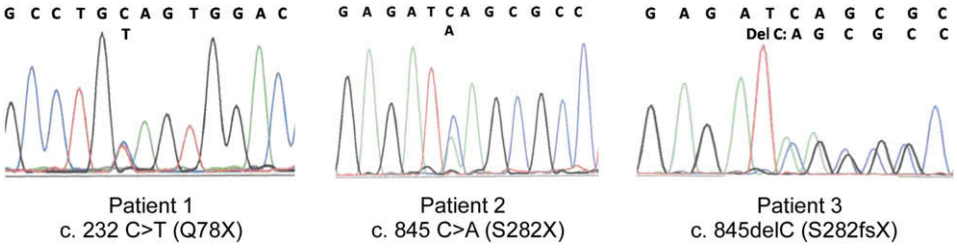

Fig. 1. *CDKN1C* sequence chromatograms showing the mutations in patients with BWS born to preeclamptic mothers.

receptor signalling pathway [21]. *CDKN1C* is imprinted in the paternal allele, thus only the maternal allele is expressed. *CDKN1C* is also imprinted in the placenta and it is strongly expressed throughout gestation in cytotrophoblast, villous mesenchyme and intervillous trophoblast islands whereas it is not expressed in the syncytiotrophoblast [22]. Thus, placentas of *CDKN1C*-mutant patients with preeclampsia may potentially show any degree of dysfunction of either the villous or the extravillous trophoblast. It has been demonstrated that lack of expression of *CDKN1C* may lead to increased mRNA levels of vascular endothelial growth factor, a potent stimulator of the migration and proliferation of endothelial cells and a known inducer of preeclampsia in mice [23]. In some BWS patients without mutations in *CDKN1C*, there is an extreme downregulation of *CDKN1C* with no apparent molecular cause [24–26]. Reduced levels of *CDKN1C* expression may be due to several mechanisms including *CDKN1C* mutations, hypomethylation of the centromeric imprinting domain, biallelic hypermethylation of the *CDKN1C* promoter (only in tumour cells) and aberrant chromatin structure of the *CDKN1C* promoter [24,25]. Enquobahrie et al. [27] studied the expression of 15,000 genes in placentas from 18 patients with preeclampsia, identifying 58 genes, including *CDKN1C*, that were differentially expressed in preeclampsia cases as compared with control subjects.

Isolated cases of women with "toxaemia" or proteinuric hypertension who had children with BWS were described by Irving [28] (1 case) and Lage [29] (1 case). In 1994, McCowan and Becroft reported on three BWS cases associated with maternal gestational proteinuric hypertension, placental anomalies and perinatal death [30]. Unfortunately, none of these authors carried out molecular analyses on their patients. Another paper reported a pregnant woman who developed HELLP syndrome and her baby presented with BWS due to hypermethylation of the telomeric imprinting centre [31]. Although all three women reported herein had children with truncating mutations in *CDKN1C* they showed a different clinical course. One of them developed HELLP syndrome, and the remainder two women showed different degrees of preeclampsia symptoms. Heterogeneity of clinical symptoms and disease course in women with preeclampsia may be due to different environmental factors and/or concomitant genetic background. Thus, it seems clear that alternative factor/s other than *CDKN1C* aberrations probably contribute to the preeclamptic phenotype. This is reinforced by the observation of other women with *CDKN1C* mutations who did not present proteinuric gestational hypertension [Ref. 4 and unpublished data]. A role for environmental factors in the disease pathogenesis has been extensively commented in previous work [reviewed in Ref. 6]. In mice, these factors could include diet, water, air filtration, temperature, seasonality, stress and housing conditions. In humans, obesity, age, smoking, altitude, and month of conception have also been shown to affect preeclampsia risk [32–34].

Nevertheless, in contrast to preeclampsia that affects ~3–4% of pregnancies, mutations in *CDKN1C* are rare accounting for approximately 1:300,000 births. This fact might be the reason for the lack of previous reports showing the association of *CDKN1C* mutations with preeclampsia/HELLP. Thus, though the epidemiological contribution of *CDKN1C* mutations in preeclampsia is probably low it should be taken into account particularly in preeclamptic women when ultrasonography reveals placental enlargement.

In summary, to our best knowledge this is the first report on the involvement of maternal and/or fetal *CDKN1C* mutations in women with preeclampsia/HELLP syndrome suggesting the implication of a human imprinted gene in the pathophysiology of preeclampsia. Similarly to the mutant mice, the role of other gene/s in the *CDKN1C* pathway in human placentae from both preeclamptic and *CDKN1C*-mutant women warrants further investigations.

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Quantification of Methylation Index of the 11p15-Differentially Methylated Regions in the BWS/SRS locus by Methylation-Sensitive High Resolution Melting (MS-HRM) analysis.

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Hum Mut. Submitted.

Cuantificación del índice de metilación de las regiones 11p15 diferencialmente metiladas del locus BWS/SRS con análisis de Methylation-Sensistive High Resolution Melting (MS-HRM).

El síndrome de Beckwith-Wiedemann está causado, en más del 90% de los casos, por alteraciones de genes que regulan el crecimiento ubicados en la región 11p15. El subgrupo molecular mayormente representado, ~ 75% de casos, lleva una alteración epigenética en los dos centros de imprinting presentes en este locus; el tipo de alteración puede ser hipometilación del centro de imprinting materno KvDMR, hipermetilación del centro de imprinting paterno H19DMR o de ambos centros en los casos de disomía uniparental paterna (UPDp).

El síndrome de Silver-Russell (SRS), caracterizado por retraso del crecimiento pre y postnatal, se genera por hipometilación del centro de imprinting paterno H19DMR (~ 30%), contrariamente a lo ya reportado para el BWS.

El estudio del nivel de metilación de los dos centros de imprinting del locus BWS/SRS se ha realizado durante años mediante Southern blot y análisis de microsatélites. Recientemente se han introducido técnicas como el MS-MLPA y la pirosecuenciación.

En este trabajo se estudian de nuevo los pacientes BWS, con diagnostico molecular conocido y nivel de metilación definido por pirosecuenciación, a través de MS-MLPA y se introduce la técnica de MS-HRM.

El MS-HRM es por definición una técnica no cuantitativa, pero la introducción de un logaritmo ideado por nosotros, nos permite utilizar los datos brutos para definir el nivel de metilación. Con el análisis estadístico de los datos obtenidos con las distintas técnicas, hemos evaluado la fiabilidad del nuevo método MS-HRM en comparación al MS-MLPA. Los resultados de este estudio nos llevan a afirmar que la técnica alternativa MS-HRM tiene la misma eficiencia del MS-MLPA y además tiene las ventajas de permitirnos analizar más muestras en un mismo ensayo, de no necesitar fases de hibridación y análisis de los padres, de poder realizarse con menor manipulación de las muestras.

Además cabe destacar que el diseño de los experimentos para el análisis de otras islas CpG es mucho más sencillo por la técnica MS-HRM que por MS-MLPA.

Quantificazione dello stato di metilazione delle regioni 11p15 con differente metilazione del locus BWS/SRS con analisi di Methylation-Sensistive High Resolution Melting (MS-HRM).

La sindrome di Beckwith-Wiedemann é causata, in piú del 90% di casi, da alterazioni dei geni che regolano il crescimento localizzati nella regione 11p15. Il sottogruppo molecolare piú rappresentato, ~ 75% di casi, porta un' alterazione epigenetica dei due centri di imprinting presenti in questo locus; il tipo di alterazione puó essere ipometilazione del centro di imprinting materno KvDMR, ipermetilazione del centro di imprinting paterno H19DMR o di entrambi i centri nei casi di disomia uniparentale paterna (UPDp).

La sindrome di Silver-Russell (SRS), caratterizzata da ritardo della crescita pre e postnatale, si genera per ipometilazione del centro di imprinting paterno H19DMR (~ 30%), al contrario di quanto riportato per il BWS.

Lo studio dello stato di metilazione dei due centri di imprinting del locus BWS/SRS é stato realizzato per anni con analisi Souther blot e dei microsatelliti. Recentemente sono state introdotte tecniche come il MS-MLPA e il pirosequenziamento.

In questo lavoro si studiano di nuovo i pazienti BWS, con diagnosi molecolare nota e stato di metilazione definito con pirosequenziamento, con MS-MLPA e si introduce la tecnica di MS-HRM.

Il MS-HRM é per definizione una tecnica non quantitativa, peró la introduzione di un algoritmo ideato da noi, ci permette utilizzare i dati crudi per definire lo stato di metilazione. Con l'analisi statistica dei risultati ottenuti con le diverse tecniche, abbiamo valutato l'affidabilitá del nuovo metodo MS-HRM in confronto al MS-MLPA. I risultati di questo studio ci hanno portatoad affermare che la tecnica alternativa MS-HRM ha la stessa efficenza del MS-MLPA e inoltre ha i vantaggi di permetterci di analizzare piú campioni nello stesso esperimento, di non aver bisgno di fasi di ibridazione e analisi dei genitori, di realizzarsi con minore maneggio dei campioni.

Inoltre é importante evidenziare che il disegno degli esperimenti per l'analisi de altre isole CpG é molto piú semplice per la tecnica MS-HRM che per MS-MLPA.

Quantification of Methylation Index of the 11p15-Differentially Methylated Regions in the BWS/SRS locus by Methylation-Sensitive High Resolution Melting (MS-HRM) analysis.

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ABSTRACT

Beckwith-Wiedemann syndrome (BWS) is a phenotypically and genotypically heterogeneous overgrowth syndrome characterised by somatic overgrowth, macroglossia and abdominal wall defects. 70% of BWS patients carry an epigenetic error in one or more members of a cluster of imprinted genes located on chromosome11p15. On the other hand, hypomethylation of the paternal imprinting centre (H19DMR) at 11p15 and maternal duplication of this region have recently been described as major epigenetic disturbances in Silver-Russell syndrome (SRS), a congenital disorder characterized by pre- and postnatal growth retardation, relative macrocephaly and a characteristic triangular face.

In this report we analyze and compare the methylation status of the two Differentially Methylated Regions (DMR) of 11p15 (H19DMR and KvDMR) in a series of patients with known molecular diagnosis of BWS or SRS, using Methylation-Sensitive High Resolution Melting analysis (MS-HRM) and Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA). With the scope to evaluate the specificity and sensitivity of these techniques for detecting methylation defects, we compared their performance in 40 BWS and 11 SRS patients who had been previously molecularly characterized by other methods such as Southern blot, SNP-arrays, pyrosequencing and/or microsatellite analysis.

Our results showed that MS-HRM is a suitable technique for the quantification of the 11p15 DMRs methylation index and that the relative methylation percentages obtained by this technique are comparable to those obtained by other quantitative techniques such as pyrosequencing or MS-MLPA. To the best of our knowledge, this is

the first report assessing the idoneity of MS-HRM for the analysis and quantification of methylation defects at both H19DMR and Kv11DMR.

INTRODUCTION

Beckwith-Wiedemann syndrome [BWS (MIM 130650)] is a phenotypically and genotypically heterogeneous overgrowth syndrome characterised by somatic overgrowth, macroglossia and abdominal wall defects [Beckwith 1963, Wiedemann, 1964]. In up to 90% of patients it is caused by alterations in growth regulatory genes located on chromosome 11p15 [Li et al., 1997; Li et al., 1998]. The largest molecular subgroup (70% of patients) is represented by individuals carrying an epigenetic defect in one or more members of a cluster of imprinted genes located at 11p15 region [Cooper et al., 2005; Enklaar et al., 2006; Maher and Reik, 2000; Weksberg et al., 2005]. This region spans approximately 1 Mb and includes two differentially methylated regions (DMR), which control the expression of genes in this cluster. H19DMR, the paternal imprinting centre, is altered in ~10% of cases, and KvDMR, the maternal imprinting centre in ~60% of cases [Weksberg et al., 2003]. The second largest BWS molecular subgroup (~10-20% of patients), is represented by patients with paternal uniparental disomy (patUPD), i.e. patients with two paternally derived copies of chromosome 11p15 and no maternal contribution for that region [Henry et al., 1991]. Finally, a small percentage of BWS cases may also present with *CDKN1C* point mutations (5-7%) [Romanelli et al., 2010; Romanelli et al., 2009], paternal duplications (2%) or maternal translocation/inversions (2%). Thus, overall ~ 85% of patients will have abnormal methylation in one or both DMRs at chromosome 11p15 [Lapunzina, 2005] (Figure 1).

Conversely, Silver-Russell syndrome [SRS (MIM 180860)] is a congenital disorder characterized by pre- and postnatal growth retardation, relative macrocephaly, and a triangular face [Price et al., 1999; Wollmann et al., 1995]. No single genetic

cause for SRS has been found. In addition to maternal uniparental disomy of chromosome 7, hypomethylation of the paternal imprinting centre (H19DMR) in 11p15 and maternal duplication of 11p15 have recently been described as major epigenetic defects in SRS [Bliek et al., 2006; Eggermann et al., 2006; Gicquel et al., 2005]. Therefore, it is expected that 30-40% of SRS patients will have a methylation disturbance in H19DMR.

The paternal H19DMR is located in the distal telomeric end of the cluster and regulates the imprinted genes *IGF2* (insulin-like growth factor 2) and *H19* [Bell et al., 2001; Bell and Felsenfeld, 2000; Hark et al., 2000]. *IGF2* encodes a paternally expressed foetal growth factor and *H19* encodes a maternally expressed non-coding RNA whose function is not entirely known. H19DMR is located upstream of the *H19* promoter and is unmethylated in the maternal allele while the paternal copy is methylated. The maternal imprinted domain (KvDMR) is centromeric with respect to H19DMR and contains the imprinted genes *KCNQ1*, *KCNQ1OT1* and *CDKN1C*. In contrast to H19DMR, KvDMR is methylated on the maternal allele and such differential methylation exists within a CpG island that overlaps with the *KCNQ1OT1* promoter [Mitsuya et al., 1999; Smilinich et al., 1999] (Figure 1).

Molecular diagnosis of BWS patients with hypomethylation of the KvDMR or patUPD11p has been performed for years by Southern Blot and microsatellite analysis, respectively [Gaston et al., 2001; Gicquel et al., 2005]. In recent years, other techniques such as combined bisulphite restriction analysis (COBRA), pyrosequencing, Methylation-Sensitive Multiplex Ligation-dependent Probe Amplification (MS-MLPA) and SNP-arrays became available and have been applied to the analysis of BWS and SRS individuals [Murrell et al., 2004; Reik et al., 1994; Scott et al., 2008].

MS-MLPA is a variant of the MLPA technique which combines copy number detection with the use of a methylation-sensitive restriction enzyme (H*haI*) allowing the interrogation of gene dosage and methylation in the same assay [Dikow et al., 2007; Scott et al., 2008]. This technique has been previously demonstrated to be useful in the diagnosis of both BWS and SRS in a large series of patients [Scott et al., 2008].

Methylation-sensitive High-Resolution Melting analysis (MS-HRM) represents a relatively novel technical approach for the evaluation of allelic changes based on differences in the melting profile of fragments differing in 1 bp or more. It has been shown to be able to detect differences in the methylation status of amplicons derived from bisulphite-modified DNA samples with highest sensitivity [Wojdacz et al., 2008b; Wojdacz et al., 2008a; Wojdacz and Dobrovic, 2007]. Due to the conversion of unmethylated cytosine to uracil by sodium bisulfite treatment and subsequent PCR-mediated conversion of uracil to thymine, methylated and unmethylated alleles are predicted to differ in their thermal stability because of their different GC contents.

In this report we analyze the methylation status of the two DMRs of region 11p15, using MS-HRM and MS-MLPA in a cohort of BWS and SRS patients with the aim to evaluate whether both methods are sensitive and specific enough to detect methylation defects in this region.

MATERIALS and METHODS

Subjects and controls

In order to determine the reliability of the MS-MLPA and MS-HRM assays, we reanalyzed a cohort of patients with a known molecular diagnosis of BWS or SRS,

obtained by Southern Blot, microsatellite analysis, SNP-arrays and/or CpG island mapping by pyrosequencing.

The experimental cohort consisted of a series of 40 patients with BWS and 11 patients with SRS: 22 BWS patients with hypomethylation of KvDMR (BWS h group); two BWS patients with hypermethylation of H19DMR (BWS H group); 10 BWS patients with patUPD (patUPD group); six SRS patients with hypomethylation of H19DMR (SRS h group); and six BWS and five SRS patients without epigenetic defects (BWS N and SRS N, respectively).

To define normal values in the MS-MLPA assay, a group of 50 DNA controls was also analyzed. Likewise, to determine the normal methylation variations of the two imprinting centres in the MS-HRM assay, we also analyzed 16 DNA controls (group C) in parallel. DNA controls and samples were obtained from the INGEMM sample repository. Samples were matched by age and gender.

In all cases, informed consents were obtained from the donors or legal representatives. The Institutional IRB approved this study as part of the research study at the Spanish Overgrowth Syndrome Registry [Gracia and Lapunzina, 2005] (# CEIC-HULP-PI446).

MS-MLPA assay

MS-MLPA reactions (SALSA MLPA Kit ME030-B1, MRC-Holland, Amsterdam, Holland) were performed with 100ng DNA according to the manufacturer's instructions. H*haI* was supplied by Promega (Madison, Wisconsin, USA). Briefly, after the initial hybridisation step, samples were divided in two aliquots: one to determine the gene dosage as in a conventional MLPA assay, and the second one was ligated and digested with the methylation-sensitive restriction enzyme H*haI* to evaluate the

methylation level. All samples were amplified by PCR and then separated by size by capillary electrophoresis in an ABI3130 Sequencer (Applied Biosystems, Foster City, California, USA). The analysis of results was based on the comparison between the signals of undigested samples and undigested controls, to calculate the gene dosage. Calculation of the methylation level of probes containing the H*haI* restriction site was performed comparing the signals of restriction digested samples with undigested samples. The analysis of raw data was carried out using an Excel-based in-house program (Meth-HULP v1.1; available upon request). We normalised the raw value of peak areas in both controls and patient samples for undigested and digested samples and quantified gene dosage and methylation level for each sample.

MS-HRM assay

Prior to MS-HRM, controls and patients genomic DNA samples were treated with sodium bisulphite using the EZ DNA methylation Kit (Zymo research, Orange, CA, USA) according to the manufacturer's instructions. To estimate the percentage of methylation, we included two commercial DNA samples with standard methylation of 0% and 100%, respectively, as controls (EpiTect PCR Control DNA Set, Qiagen). Specific primers to amplify bisulphite-converted DNA were designed by means of Methylation Primer Express Software v1.0 (Applied Biosystems). The primers amplify both the methylated and unmethylated templates, according to the principles set up to compensate for PCR bias [Wojdacz and Hansen, 2006]. The specificity of primers was confirmed by sequencing of the PCR products.

PCR amplification and MS-HRM were performed in a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Germany). PCR was carried out in a final volume of 10 µl containing 10 ng of bisulphite-treated DNA, 5 µl of LightCycler 480 High

Resolution Melting Master (2X), 0.2 µM of each primer and 1.2 mM Mg^{+2}. The amplification consisted of 10 min at 95ºC, followed by 45 cycles of 10 sec at 95ºC, 15 sec at the primer annealing temperature (56ºC for KvDMR and 58ºC for H19DMR) and 10 sec at 72ºC. High resolution melting was performed at the ramping temperatures starting from 75ºC and ending at 92ºC, rising by 0.02ºC/s and fluorescence acquisition setting was at a rate of 25 acquisitions/ºC as recommended by the manufacturer (25 acquisitions/ºC). Melting curves were normalised by calculation of the "line of best fit" between two normalisation regions before and after the major fluorescence decrease representing the melting of the PCR product. Normalisation was carried out using the software provided with the LightCycler 480 (Gene Scanning software v1.5). The methylation level was calculated by evaluating the area under the melting curve in the plateau phase as shown in Fig. 2. The fluorescence value of the plateau at the vertical scale represents the ratio between the methylated and unmethylated allele. In case of hypomethylation, there is an increased contribution of the unmethylated allele resulting in a relatively longer melting phase of the unmethylated allele, which results in a plateau phase at a lower fluorescence level [Alders et al., 2009]. The evaluation of the area under the curve in the plateau phase was done by means of an algorithm which allows relative quantification (patent pending) using three values of relative fluorescence signal corresponding to three equidistant temperature values (Figure 2).

Statistical Analysis

Quantitative data are described as mean methylation index (MI), standard deviation (SD), minimum and maximum. The mean methylation index (MI) of the controls was used to calculate the range of normal values, which was defined as: mean MI ± 2SD.

For each assay (MS-MLPA and MS-HRM) the comparison of the patients MI vs. the controls MI for each group and for both imprinting centres was carried out by the U-test of Mann-Whitney. Differences were considered significant if *p-values* < 0.05.

In order to estimate the reliability of the MS-MLPA and MS-HRM assays, we defined samples as correctly classified (C) or incorrectly classified (I) for each patient according to the correspondence with his/her previously known molecular diagnosis, independently for each assay, and considering H19DMR and KvDMR both individually and globally. We estimated the percentage of correct classification (95% confidence interval) and compared the correct classification of patients for each technique using a McNemar test [Berger and Sidik, 2003].

Finally, for each group the correlation between the two assays was evaluated by means of linear regression analysis. All statistic analyses were undertaken with the SAS 9.1 software (SAS Institute, Cary, USA).

RESULTS

Methylation index estimation

Mean H19DMR and KvDMR MI, SD, minimum and maximum were calculated for both MS-HRM and MS-MLPA assays for all groups of patients and controls (Table Ib). Mean MI data are presented as percentage of methylation.

Establishment of the range of normality and delineation of patient molecular subgroups

H19DMR and KvDMR MI normality ranges were calculated from the values obtained for the control groups by MS-MLPA or MS-HRM assays (Table Ia). We

subsequently assigned the patient groups according to the MI value obtained in each assay: those BWS patients with hypomethylation of KvDMR were assigned to the BWS h group, defined by a normal H19DMR MI (35.5<MI<43.78 by MS-HRM and 35.91<MI<64.59 in the MS-MLPA assay) and by a MI at KvDMR <52.2 in the MS-HRM assay and <45.45 in the MS-MLPA assay. Similarly, BWS patients with H19DMR hypermethylation were assigned to the BWS H group, defined by a normal MI at KvDMR (52.2<MI<67.12 in the MS-HRM assay and of 45.45<MI<54.69 in the MS-MLPA assay) and by a MI at H19 >43.78 (MS-HRM assay) and >64.59 (MS-MLPA assay).

The BWS patUPD group was defined by a MI at H19DMR >43.78 in the MS-HRM assay and >64.59 in the MS-MLPA assay, and by MI at KvDMR <52.2 in the MS-HRM assay and <45.45 in the MS-MLPA assay, respectively.

The SRS h group (patients with H19DMR hypomethylation) was defined by MI at H19DMR <35.5 in the MS-HRM assay and <35.91 in the MS-MLPA assay, and by normal MI at KvDMR (52.2<MI<67.12 in the MS-HRM assay and 45.45<MI<54.69 in the MS-MLPA assay).

BWS N and SRS N group (patients without epigenetic defects) were defined by normal MI at H19DMR (35.5<MI<43.78 in the MS-HRM assay and 35.91<MI<64.59 in the MS-MLPA) and by a normal MI at KvDMR too (52.2<MI<67.12 in the MS-HRM assay and 45.45<MI<54.69 in the MS-MLPA assay) (Table Ic).

Comparison of MI with U-test for Mann Whitney

For each group of patients by means of the U-test for Mann Whitney we compared the mean MI of H19DMR and KvDMR obtained by the two different assays with the respective control group. Differences with *p-value* <0.05 were considered

significant. As shown in Table Ib the results obtained by the U-test showed a statistically significant difference in the MI of the KvDMR for the BWS h group; in the MI of the H19DMR region for the BWS H and SRS h groups, in both KvDMR and H19DMR regions for the patUPD group and in none of the regions for the BWS N and SRS N group.

Estimation of "correct classification" percentage and McNemar test

We defined samples as C (correctly classified) and I (incorrectly classified) for each patient according to the correspondence with its previously known molecular diagnosis. According to this estimation the percentage of correct classification with a 95% confidence interval (95% CI) of the H19 methylation level was 96.1% (86.5%-99.5%) for MS-MLPA and 94.1% (83.7%-98.7%) for MS-HRM. The percentage of correct classification for the KvDMR methylation level was 78.4% (64.7%-88.7%) by MS-MLPA and 94.1% (83.7%-98.7%) by MS-HRM. The McNemar test calculated a *p-value* of 1 when comparing the percentages of correct classification of each technique for *H19* methylation level and a nearly significant *p-value* of 0.057 for the KvDMR.

Finally we compared the both techniques by means of computing globally the two imprinting centres, i.e. considering as correct score only the assignments that were correct for both imprinting centres. We obtained a cumulative percentage of 74.5% (60.3%-85.6%) for MS-MLPA and 88.2% (76.1%-95.5%) for MS-HRM. A *p-value* of 0.143 was obtained, when comparing the percentage of correct classification between the two techniques using the McNemar test (Table II).

Correlation analysis between MS-MLPA and MS-HRM

Evaluation of the MI values obtained by both MS-MLPA and MS-HRM assays for H19DMR and KvDMR showed an apparent high correlation between the results obtained in each assay. To confirm this observation, we performed a correlation analysis of the data by means of linear regression analysis (Figure 3). The regression coefficients between the MS-MLPA and the MS-HRM MI results for the two differentially methylated regions were 0.717 for the imprinting centre H19DMR and 0.917 for the imprinting centre KvDMR.

DISCUSSION

MS-MLPA has been demonstrated to be useful in diagnosing the majority of molecular abnormalities observed in patients with BWS and SRS [Scott et al., 2008]. In recent years quantitative PCR has been also applied to the analysis of BWS samples with similar results [Coffee et al., 2006]. Traditionally, diagnosis of BWS patients with hypomethylation of the KvDMR or patUPD11p has been performed by Southern Blot and microsatellite analysis, respectively [Gaston et al., 2001; Gicquel et al., 2005]. For the last three years we have been using MS-MLPA as a complementary test for the molecular diagnosis of these disorders and more recently we started evaluating MS-HRM as an alternative technique. However, the sensitivity and specificity of these assays had not been yet evaluated.

With the aim of evaluating and comparing these techniques we applied both MS-MLPA and MS-HRM to the analysis of BWS and SRS patients with different subtypes of molecular defects, namely isolated hypomethylation of KvDMR (BWS h), hypomethylation of KvDMR and hypermethylation of H19DMR (patUPD11p), isolated

hypermethylation of H19DMR (BWS H), BWS without epigenetic defects BWS N), hypomethylation of H19DMR (SRS h) and SRS patients without epigenetic defects (SRS N). All these patients had been previously diagnosed using Southern blot, microsatellite analysis, SNP-arrays and/or pyrosequencing. As far as we know, this is the first work comparing the reliability of MS-MLPA and MS-HRM for the diagnosis of patients with known methylation defects at both DMR of chromosome 11p15.

According to our data MS-HRM is a highly reliable technique to correctly estimate the methylation status of both DMRs at 11p15 not only qualitatively but also quantitatively. In our hands, the MS-HRM assay was able to detect a statistically significant difference between the KvDMR MI of the control group and the BWS group h; between the H19DMR MI of the control group and BWS group H and SRS group h, in both DMRs for the patUPD group and in none of the two DMRs in the BWS N and SRS N groups.

When comparing the accuracy and reliability of both assays we obtained a cumulative percentage of correct detection of the imprinting defect on both DMRs of 74.5% by MS-MLPA and of 88.2% by MS-HRM assay suggesting an apparently higher accuracy of the MS-HRM assay. However, the McNemar test *p-value* of 0.143 indicated that this difference in the efficiency of detection was not statistically significant, probably because of the limited number of samples evaluated. We also evaluated the correlation between both techniques by means of linear regression analysis. The regression coefficient of the two curves showed a high correlation between the results obtained for both DMRs. Altogether, these results demonstrate that MS-HRM can be used with confidence as a test for the detection of epigenetic defects in BWS and SRS patients.

Although HRM, by definition, is not a quantitative method, we have developed an algorithm which allows relative quantification of the 11p15 DMRs MI by MS-HRM and have shown that the methylation percentages obtained by this method are comparable to other quantification techniques such as pyrosequencing or MS-MLPA.

Potential benefits and advantages of the application of MS-HRM to methylation analysis of the 11p15 DMRs are its scalability to a high throughput (96 or 384 well plate) format, no need of hybridization or parental samples and less manipulation and hands-on requirements than MS-MLPA. In addition, the experimental design and set-up for the analysis of any new DMR is easier by MS-HRM than MS-MLPA. On the other hand it can be argued that the no need of performing DNA bisulphite conversion and the possibility of performing simultaneous copy number detection do represent specific advantages of the MS-MLPA assay, not accomplished by the MS-HRM assay. In conclusion, we have shown that ~~as similarly reported for MS-MLPA [Scott et al., 2008],~~ MS-HRM is a reliable technique for the quantitative determination of the MI in the DMRs of chromosome 11p15.

Acknowledgements

We thank the families with BWS and SRS who contributed with samples to this work. V. R. is supported by a FIBHULP grant kindly funded through Biotools S.A. H.M. is a Ph student from the UFRJ, Brazil. We also thank the cooperation of some colleagues from the CIBERER network. P.L. is recipient of a FIS, ISCIII grant PI08/1260.

Figure 1. Schematic representation of normal and altered imprinted gene cluster on chromosome 11p15. From left to right: normal status, epigenetic errors, genetic errors (*i.e*. *CDKN1C* mutations) and chromosomal abnormalities (paternal UPD, paternal duplication and maternal translocation/inversion). The first imprinting centre, H19DMR, is located in the distal telomeric end of the cluster and it is unmethylated in the maternal allele while the paternal copy is methylated. This allows the expression of maternal *H19* and paternal *IGF2*. In contrast, the second imprinting centre, KvDMR, is located centromerically and is methylated on the maternal allele while the paternal allele is unmethylated; this allows paternal transcription of the *KCNQ1OT1* transcript and maternal expression of *KCNQ1* and *CDKN1C*.

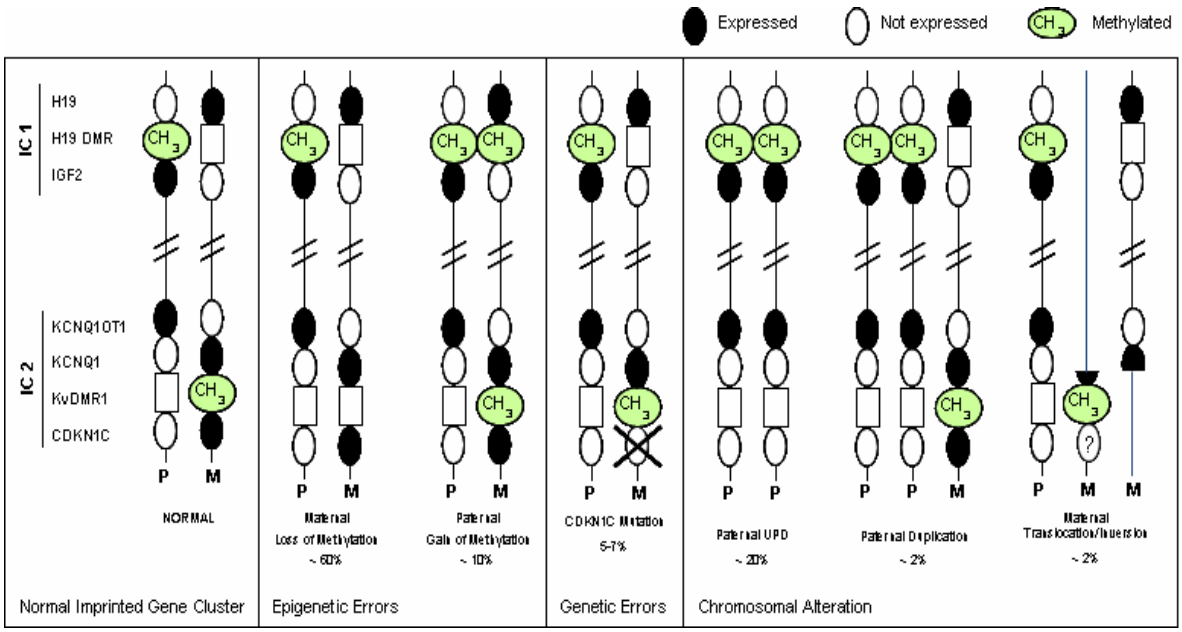

Figure 2. MS-HRM curve of the H19DMR. a) One case for each group of patients is here represented to appreciate the differences in melting profiles. 0% and 100% represent the melting curves of the commercially standard methylated & unmethylated DNAs. One case for each group of patients is here represented to appreciate the differences in melting profiles. Blue curves: normal controls, SRS and BWS patients without known molecular defects at H19DMR. Yellow curve: patient SRS h with hypomethylation at H19. Pink curve: BWS H patient with H19 hypermethylation. Green curve: BWS patient with patUPD. b). Zoom of the plateau phase of each melting curve is highlighted.

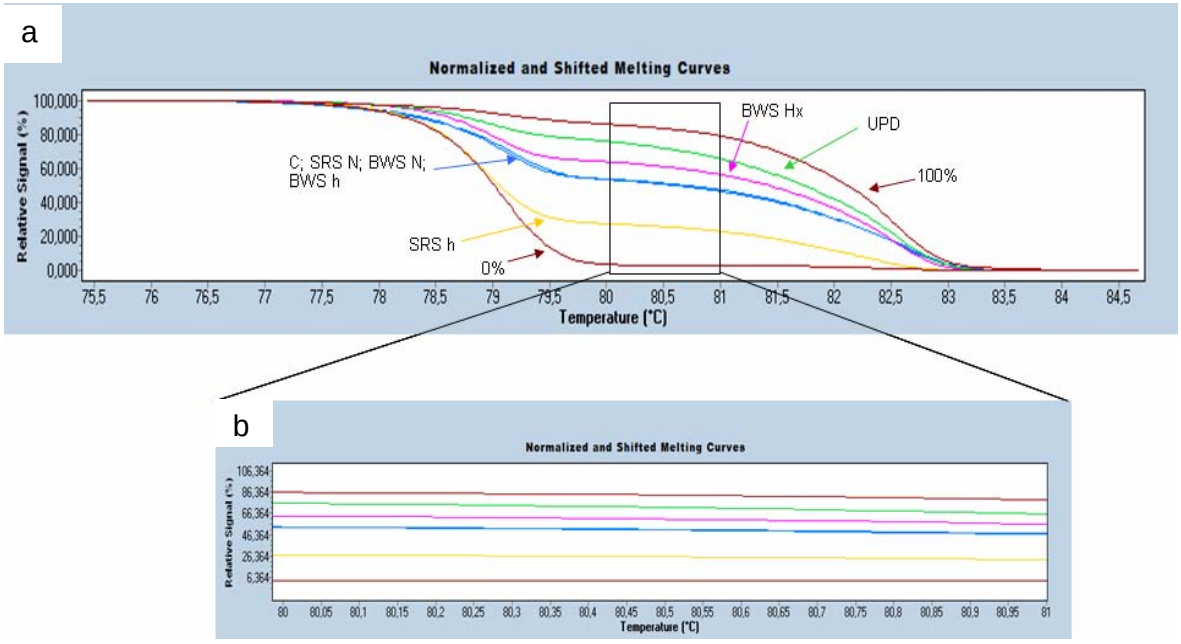

Figure 3. Linear regression analysis. Comparison of the relative Methylation Index values (MI), obtained using MS-MLPA vs MS-HRM, of each imprinting centre for each patient group. The slopes of the curves show a high correlation between the results. R^2 represent the calculated linear coefficient, which is higher for the KvDMR (0.91) than for the H19DMR (0.71). SRS N and BWS N: Silver-Russell and Beckwith-Wiedemann patients without known molecular defects; SRS h: Silver-Russell syndrome patients with hypomethylation at H19DMR; BWS H: Beckwith-Wiedemann patients with hypermethylation at H19DMR; patUPD: Beckwith-Wiedemann patients with paternal uniparental disomy; BWS h: Beckwith-Wiedemann patients with hypomethylation at KvDMR; C: controls.

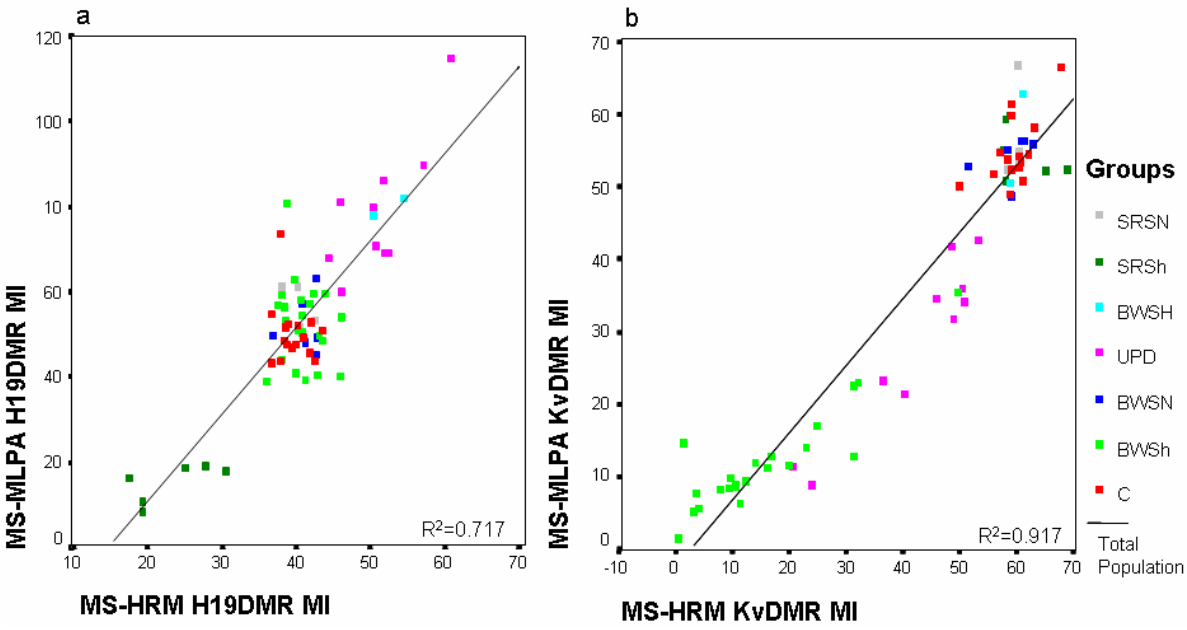

Table I. a) MI normality ranges calculated from the values obtained for the control groups (age and gender matched). **b)** Mean MI, Standard Deviation, Minimum and Maximum for each group of patients analyzed by MS-HRM and MS-MLPA. Groups carrying an epigenetic defect whose values are out of the normality and differences are considered statistically significant (*p-value* <0.05) by U-test of Mann-Whitney, are bolded and italiced. **c)** Group definitions through upper and lower bound values of MI normality ranges.

a

	MI Normality Range	
	Lower Bound	Upper Bound
MS-HRM (H19)	35.5	43.78
MS-HRM (KvDMR)	52.2	67.12
MS-MLPA (H19)	35.91	64.59
MS-MLPA (KvDMR)	45.45	54.69

b

	Group	N	Mean MI	*p-value*	SD	Min	Max
MS-HRM (H19)	C	16	39.64		2.07	36.66	43.54
	BWS h	22	40.87	0.191	2.69	36.01	46.27
	BWS H	2	***52.34***	***0.013***	***2.95***	***50.26***	***54.43***
	UPD	10	***51.23***	***<10^{-5}***	***5.10***	***44.37***	***60.98***
	SRS h	6	***23.26***	***<10^{-5}***	***5.28***	***17.49***	***30.54***
	BWS N	6	41.24	0.115	2.25	36.98	42.88
	SRS N	5	40.49	0.354	1.60	38.08	42.49
MS-HRM (KvDMR)	C	16	59.67		3.74	49.97	67.92
	BWS h	22	***15.65***	***<10^{-5}***	***12.32***	***0.47***	***49.85***
	BWS H	2	59.92	0.941	1.55	58.83	61.02
	UPD	10	***41.93***	***<10^{-5}***	***11.50***	***20.56***	***53.24***
	SRS h	6	61.20	0.858	4.69	57.88	68.95
	BWS N	6	59.10	0.802	3.98	51.69	63.05
	SRS N	5	59.42	0.660	1.48	57.28	60.63
MS-MLPA (H19)	C	16	50.26		7.18	43.14	73.67
	BWS h	22	52.55	0.234	9.78	38.98	80.49
	BWS H	2	***79.74***	***0.013***	***2.83***	***77.74***	***81.74***
	UPD	10	***78.87***	***<10^{-5}***	***15.65***	***59.81***	***114.77***
	SRS h	6	***15.13***	***<10^{-5}***	***4.52***	***8.34***	***19.22***
	BWS N	6	52.21	0.494	6.71	45.31	63.21
	SRS N	5	55.20	0.062	5.74	48.63	61.35
MS-MLPA (KvDMR)	C	16	54.69		4.63	48.86	66.55
	BWS h	22	***12.03***	***<10^{-5}***	***7.34***	***1.33***	***35.39***
	BWS H	2	***56.71***	***0.941***	***8.76***	***50.52***	***62.91***
	UPD	10	***28.53***	***<10^{-5}***	***11.86***	***8.89***	***42.76***
	SRS h	6	54.92	0.971	3.90	50.66	59.88
	BWS N	6	54.17	0.641	2.97	48.74	56.27
	SRS N	5	56.53	0.313	5.82	52.40	66.78

c

	H19DMR		KvDMR	
	MS-HMR	MS-MLPA	MS-HMR	MS-MLPA
BWS h	35.5<MI<43.78	35.91<MI<64.59	MI<52.2	MI<45.45
BWS H	MI>43.78	MI>64.59	52.2<MI<67.12	45.45<MI<54.69
UPD	MI>43.78	MI>64.59	MI<52.2	MI<45.45
SRS h	MI<35.5	MI<35.91	52.2<MI<67.12	45.45<MI<54.69
BWS N and SRS N	35.5<MI<43.78	35.91<MI<64.59	52.2<MI<67.12	45.45<MI<54.69

Table II. Percentages of classification and McNemar test. Number of cases and relative percentages of correctly (C) and incorrectly (I) classification for the two imprinting centres analyzed individually and combined, for MS-HRM and MS-MLPA assays. The comparison of both assays performance was statistically assessed using the McNemar test.

Technique		**DMR**		
MS-HRM	MS-MLPA	H19DMR	KvDMR	Both DMRs
C	C	46/51 (90.2%)	37/51 (72.5%)	33/51 (64.7%)
I	C	7/51 (5.6%)	3/51 (5.9%)	5/51 (9.8%)
C	I	2/51 (3.9%)	11/51 (21.6%)	12/51 (23.5%)
I	I	0/51	0/51	1/51 (2.0%)
	p-value	1	0.057	0.143

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Eur J Hum Genet. 2010. Accepted.

Síndrome de Beckwith-Wiedemann (BWS) y disomía uniparental 11p: mapeo fino de los puntos de rotura y evaluación de varias técnicas.

El síndrome de Beckwith-Wiedemann está causado, en ~ 20% de los casos, por disomía uniparental paterna (UPDp), pues la presencia de dos copias derivadas del padre del locus 11p15, sin o con baja contribución materna para esta región. El efecto de esta alteración es la sobreexpresión del gen *IGF2* (Insulin-like Growth Factor 2) y el reducido nivel del materno *CDKN1C*.

Se ha demostrado que los pacientes BWS con UPDp presentan la alteración en mosaico, esta evidencia sugiere que en todos los casos la UPDp se genere por un evento postcigótico y que la pérdida total del locus materno no sea viable con la vida.

En este trabajo hemos querido analizar detalladamente nuestros casos BWS con UPDp y hemos utilizado distintas técnicas moleculares que nos permiten explorar varias características del defecto.

Hemos evaluado el porcentaje de mosaicismo utilizando el análisis de STR y los SNP-arrays; la extensión de la región involucrada ha sido finamente mapeada con los SNP-arrays y además hemos estimado el nivel de metilación de los dos centros de imprinting en 11p15 por MS-MLPA, MS-HRM y pirosecuenciación.

Estos análisis nos han permitido evaluar las correlaciones entre la extensión de la UPDp, el porcentaje de mosaicismo, el nivel del defecto epigenético y las características clínicas de los pacientes y su riesgo de desarrollar tumores. Además hemos podido excluir una tendencia común entre nuestros casos, bien en el porcentaje de mosaicismo, bien en el punto de ruptura de la UPDp.

La sindrome di Beckwith-Wiedemann (BWS) e la disomia uniparentale 11p: mappatura fine dei punti di rottura e valutazione di varie tecniche.

La sindrome di Beckwith-Wiedemann é causata, nel ~ 20% dei casi, da disomia uniparentale paterna (UPDp), ossia la presenza di due copie derivate dal padre del locus 11p15, senza o con basso contributo materno per questa regione. L'effetto di questa alterazione é la iperespressione del gene *IGF2* (Insulin-like Growth Factor 2) e il ridotto livello del materno *CDKN1C*.

É stato dimostrato che i pazienti con UPDp presentano la alterazione in mosaico, questa evidenza suggerisce che in tutti i casi la UPDp si genera per un evento postzigotico e che la perdida totale del locus materno non sia compatibile con la vita.

In questo lavoro abbiamo voluto analizzare dettagliatamente i nostri casi BWS con UPDp e abbiamo utilizzato diverse tecniche molecolari che ci hanno permesso esplorare varie carattersitiche del difetto.

Abbiamo valutato la percentuale di mosaicismo utilizzando l'analisi de STR e gli SNP-arrays; l'estensione della regione coinvolta é stata mappata finemente con gli SNP-arrays e inoltre abbaimo stimato il livello di metilazione dei due centri di imprinting in 11p15 con MS-MLPA, MS-HRM e pirosequenziamento.

Queste analisi ci hanno permesso valutare le correlazioni tra la estensione della UPDp, la percentuale di mosaicismo, il ivello del difetto di metilazione e le carattersitiche cliniche dei pazienti e il loro rischio di sviluppare tumori. Inoltre abbiamo potuto escludere una tendenza comune tra i nostri casi, sia nella percentuale di mosaicismo, sia nel punto di rottura della UPDp.

Beckwith Wiedemann syndrome and Uniparental Disomy 11p: Fine mapping of the recombination breakpoints and evaluation of several techniques.

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KEYWORDS: Beckwith-Wiedemann syndrome, uniparental disomy, breakpoint, SNP-array.

RUNNING TITLE: BWS and paternal uniparental disomy.

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Conflict of Interest: The authors declare no conflict of interest.

ABSTRACT

Beckwith-Wiedemann syndrome (BWS) is a phenotypically and genotypically heterogeneous overgrowth syndrome characterized by somatic overgrowth, macroglossia and abdominal wall defects. Other usual findings are hemihyperplasia, embryonal tumors, adrenocortical cytomegaly, ear anomalies, visceromegaly, renal abnormalities, neonatal hypoglycaemia, cleft palate, polydactyly and a positive family history.

BWS is a complex, multigenic disorder associated, in up to 90% of patients, with alteration in the expression or function of one or more genes in the 11p15.5 imprinted gene cluster. There are several molecular anomalies associated with BWS and the large proportion of cases, about 85%, is sporadic and karyotypically normal. One of the mayor categories of BWS molecular alteration (10-20% of cases) is represented by mosaic paternal uniparental disomy (pUPD11), namely patients with two paternally derived copies of chromosome 11p15 and no maternal contribution for that. In these patients, in addition to the effects of *IGF2* overexpression, a decreased level of the maternally expressed gene *CDKN1C* may contribute to the BWS phenotype.

In this paper, we reviewed a series of 9 patients with BWS due to pUPD11 using several methods with the aim to evaluate the percentage of mosaicism, the methylation status at both loci, the extension of the pUPD11 at the short arm and the breakpoints of recombination. Fine mapping of mitotic recombination breakpoints by SNP-array in individuals with UPD and fine estimation of epigenetic defects will provide a basis for understanding the aetiology of BWS, allowing more accurate prognostic predictions and facilitating management and surveillance of individuals with this disorder.

INTRODUCTION

Beckwith-Wiedemann syndrome [BWS (MIM 130650)] is a phenotypically and genotypically heterogeneous overgrowth syndrome characterized by somatic overgrowth, macroglossia and abdominal wall defects. Other usual findings are hemihyperplasia, embryonal tumors, adrenocortical cytomegaly, ear anomalies, visceromegaly, renal abnormalities, neonatal hypoglycaemia, cleft palate, polydactyly and a positive family history [1-4] .

BWS is a complex, multigenic disorder associated, in up to 90% of patients, with alteration in the expression or function of one or more genes in the 11p15.5 imprinted gene cluster [5] (Figure 1a). There are several molecular anomalies associated with BWS and the large proportion of cases, about 85%, is sporadic and karyotypically normal.

Chromosomal rearrangements are relatively rare (~2-3% of cases) and comprise translocations or inversions (typically maternally inherited), and paternal duplications. Point mutations in *CDKN1C*, a cyclin-dependent kinase inhibitor acting as negative regulator of cell proliferation, have been found in 5-7% of sporadic BWS cases [6-9] and in approximately 40% of cases with a positive family history [10] .

The largest molecular subgroup, about 60% of cases, is represented by BWS patients that carry an epigenetic error on one or more members of 11p15.5 imprinted gene cluster. Two main epigenetic alterations in BWS patients have been described, one is represented by gain of methylation at the paternal H19DMR (observed in 5-10% of cases), and it is associated with loss of *H19* expression and *IGF2* biallelic expression [11,12] . The other alteration is loss of maternal methylation of KvDMR (observed in ~50% of BWS patients), usually accompanied by biallelic expression of the *KCNQ1OT1* transcript and down-regulation of *CDKN1C* [13-15] . Finally, another large category of BWS molecular alteration, 10-20% of cases, is represented by paternal uniparental disomy (pUPD), namely patients with two paternally derived copies of chromosome 11p15 and no maternal contribution for that region [16,17] . In these patients, in addition to the effects

of *IGF2* overexpression, a decreased level of the maternally expressed gene *CDKN1C* may contribute to the BWS phenotype [7,10,18] .

It has been demonstrated that BWS patients with paternal UPD always show mosaicisms, suggesting that all cases had arisen as a postzygotic event [17,19,20] ; a possible explanation is that lack of one or more chromosome 11 maternally expressed genes may lead to embryonic lethality [21] . It was also suggested that the degree of mosaicism and the location of the genetic abnormality in different tissue is strongly associated with the pathological phenotype [22] . The extent of pUPD at chromosome 11p has been studied by means of STR markers; the critical region for pUPD is telomeric to chromosome 11p13 and always includes the region where map some BWS genes (*IGF2, H19* and *CDKN1C)* [17,19,21] . Some cases with mosaic pUPD for the whole chromosome 11 have been also described and the clinical findings did not differ from patients with pUPD restricted to a small part of 11p [21,23] . While the extent of segmental disomy and proportion of cells with pUPD is variable, in all BWS cases the paternal UPD is isodisomic. This suggests that cells with paternal UPD of chromosome 11 may have a selective growth advantage and that maternal UPD cells die or there is a partition to different parts of the conceptus [24] .

Clinically, a clear association between mosaic UPD and hemihyperplasia exists [13,17,20,25] . In addition, it was noted that neoplasias and Wilms' tumors are more frequent in BWS patients with pUPD or H19DMR hypermethylation than in BWS patients with other molecular defect [25-27] . Moreover, it has been hypothesized that extremely high levels of UPD might drive severe phenotypic expression of BWS [28] ; but it is often difficult to determine if levels of UPD correlate with severity of the phenotype especially when the tissues/organs involved are not usually directly tested. Finally, in view of the evidence of imprinted transcripts at the Wilms' tumour suppressor gene (*WT1* at 11p13) [29-31] , it is interesting to evaluate whether disomy extended to WT1 influenced the risk of neoplasia (Figure 1a).

In this paper, we evaluated a series of 9 patients with BWS due to patUPD11p using several methods with the aim to evaluate the percentage of mosaicism, the methylation status at both loci, the extent of the pUPD at the short arm and their breakpoints. Fine mapping of mitotic recombination breakpoints by SNP-array in UPD and fine estimation of epigenetic defects will provide a basis for understanding the aetiology of BWS. In addition, it would allow more accurate prognostic predictions providing a better management and surveillance of BWS children.

PATIENTS AND METHODS

Patients

From a total of 132 patients with presumptive diagnosis of BWS included in the Spanish Overgrowth Syndrome Registry, 92 had confirmed molecular diagnosis of the disorder. Nine out of these 92 patients had laboratory results indicative of pUPD11. Clinical data of BWS individuals including personal and family history, clinical, laboratory and X-rays, pedigree, and follow-up evolution were included in a database. Parental data were also recorded. The Institutional IRB at Hospital Universitario La Paz approved this study as part of the research study at the Spanish Overgrowth Syndrome Registry (# HULP-PI446).

Cytogenetics and DNA extraction

Karyotypes were performed by standard methods in all patients. A minimum of 20 cells were counted with a resolution of at least 550 bands. FISH analysis using the D11S2071 probe was applied to all patients. DNA was extracted from blood using Qiagen kits Puregene Blood Core Kit (Maryland, USA) in all patients and available parents.

STRs segregation analyses

A panel of 6 microsatellites mapping to the short arm of chromosome 11 was evaluated in patients and their parents (when available) (Figure 1b). We used the following STRs: TH01, D11S1318, D11S4088, D11S1338, D11S1346 and D11S4046. Sequences of the primers were obtained from the NCBI public database (http://www.ncbi.nlm.nih.gov/sites/entrez). We calculated the percentage of mosaicism as recommended by Sasaki et al. [32] . The calculation was as follows: (K-1)/(K+1) x 100; where K is the ratio of the intensity of the parental alleles (paternal/maternal ratio) of the test sample.

Methylation-Specific MLPA (MS-MLPA)

MS-MLPA with 100 ng of genomic DNA, the SALSA ME030-B1 (11p15 region, BWS/SRS; MRC-Holland, Amsterdam, Holland) and the methylation-sensitive restriction enzyme H*haI* (Promega Corporation) was performed according to the manufacturer's recommendations. MS-MLPA PCR products were analyzed on an ABI 3130 automated sequencer (Applied Biosystems, Foster City, California, USA). Analysis of raw data was performed by means of an Excel-in-house based software (Meth-HULP v1.1) developed at our Institution (free available upon request). Normalization of the raw value of peak areas for both controls and patient samples and for undigested and digested samples allowed the visualization of gene dosage and methylation level for each loci and each sample.

Sodium bisulphite conversion and Methylation Specific-High Resolution Melting analysis (MS-HRMA)

Before MS-HRM assay, genomic DNA of controls and patients were treated with sodium bisulphite using the EZ DNA methylation Kit (Zymo research, Orange, CA, USA) according to the manufacturer's instructions. Bisulphite converts unmethylated cytosines to uracil, whereas methylated cytosines remain unreactive.

Specific primers (available upon request) to amplify H19 and KvDMR from bisulphite-converted DNA were designed by means of Methylation Primer Express Software v1.0 (Applied Byosistems). The primers amplify both methylated and unmethylated template, according to the principles set out to compensate for PCR bias [33] . Specificity of primers was confirmed by sequencing of the PCR products. PCR amplification and MS-HRM analysis were done in a LyghtCycler 480 Real-Time PCR System (Roche Diagnostics, Germany). Normalisation of the melting curves was carried out using the software provided with the LyghtCycler 480 (Gene Scanning software v1.5). To visually estimate the curves, we included two synthetic DNA with standard methylation of 0% and 100% as controls (EpiTect PCR Control DNA Set, Qiagen). We calculated the level of methylation by the MS-HRM assay, evaluating the area under the curve in the plateau phase. The position of the plateau at the vertical scale represents the ratio between the methylated and unmethylated allele.

Pyrosequencing of imprinted centres at 11p15

Quantitative analysis of methylation at the KvDMR and H19 imprinting centres was also performed by pyrosequencing. Specific primers (available upon request) to amplify and to pyrosequence the two amplicons were designed by means of Pyrosequencing Assay Design Sofware (Biotage, Charlottesville, VA). The forward primer for H19 and reverse primers for KvDMR amplification were 5'-biotinylated to facilitate single-strand DNA template isolation for the pyrosequencing reaction. Preparation of the single-strand DNA template for pyrosequencing was performed using the PSQ Vacuum Prep tool (Biotage, Charlottesville, VA) according to the manufacturer's instruction. The biotinylated PCR product was immobilized on streptavidin-coated Sepharose high-performance beads (Amersham Biosciences, Piscataway, NJ) and processed to obtain single strand DNA using the PSQ 96 Sample Preparation Kit (Biotage) according to the manufacturer's instructions. The sequencing-by-synthesis reaction of the complementary strand was automatically performed on a

PSW 96MA instrument (Biotage) at room temperature using PyroGold reagents (Biotage). As nucleotides were dispensed, a light signal was generated proportional to the amount of each incorporated nucleotide. These light signals were detected by a charge-coupled device camera and converted to peaks in a sequencing program that was automatically generated in real time for each sample. The Pyro Q-CpG software (Biotage) automatically determines individual methylation frequencies for all CpG sites in the amplicon; the degree of methylation is calculated from the ratio of the peak heights of C and T.

High Density Single Nucleotide Polymorphism-arrays (SNP-arrays)

DNAs were extracted by routine methods and were quantified using PicoGreen (Invitrogen Corporation, Carlsbad, CA). A genome-wide scan of 620,901 tag SNPs was conducted on the patients, using the Illumina Human610-Quad BeadChip according to the manufacturer's specifications (Illumina, San Diego, CA). GenCall scores < 0.15 at any locus were considered "no calls". Image data was analyzed using the Chromosome Viewer tool contained in Beadstudio 3.2 (Illumina, San Diego, CA). The metric used was the log R ratio which is the log (base 2) ratio of the observed normalized R value for a SNP divided by the expected normalized R value [34] . In addition, an allele frequency analysis was applied for all SNPs. All genomic positions were based upon NCBI Build 36 (dbSNP version 126).

RESULTS

Paternal UPD and establishment of mosaicism percentage

All 9 cases of UPD have been diagnosed by analysis of STRs markers mapping at the short arm of chromosome 11, and have shown paternal isodisomy (Figure 2a). We calculated the percentage of mosaicism by means of STRs and SNP-arrays (Figure 3a). The calculation of mosaicism using STRs has been done as recommended by

Sasaki et al. [32] (Table Ia). Taking into account that isodisomy is paternal we could estimate the percentage of mosaicism in the SNP-array as the higher allele B frequency (Table II).

Correlation between the percentage of the UPD cells and the metylation index

As expected, the degree of mosaicism, calculated by SNP-array, and the level of methylation defect, estimated by pyrosequencing (Table Id), showed very high and statistically significant correlation: r=+0.933 for H19DMR and r=-0.833 for KvDMR(Figure 5a; Table III).

Extent of the pUPD and correlation with methylation index and mosaicism

Fine mapping of mitotic recombination breakpoint in pUPD of chromosome 11 has been performed by SNP-array (Figure 3a, 3b). To estimate the extent of pUPD we mapped the latest SNP in pUPD and the first SNP in heterozygosis and we evaluated the minimum and the maximum size of pUPD region for each patient (Table II). We calculated the methylation index in both imprinting centres of 11p15.5 region, H19 and KvDMR, through MS-MLPA, MS-HRM and pyrosequencing analyses (Figure 2b, 4a, 4b; Table Ib, Ic, Id). We also performed a correlation analysis between the extent of the pUPD and the level of methylation (obtained by CpG Q-Pyro) and mosaicism (calculated by STR analysis). As we expected, there has been no correlation among these parameters (Figure 5b; Table III).

Fine mapping of the breakpoints

Fine mapping of mitotic recombination breakpoints in pUPD of chromosome 11 by SNP-arrays did not provide strong evidence for recombination hot-spots. The locations of mitotic breakpoints were different in all patients (Figure 3b; Table II). However, despite of variability of the extent of isodisomy, the region of paternal UPD invariably includes the whole imprinted gene cluster at 11p15.5 (Figure 3b).

DISCUSSION

Paternal UPD of chromosome 11p is a relatively common and cancer prone mechanism in BWS patients [25] ; thus patients with this molecular subtype should be closely evaluated and prospectively followed-up. Regional or partial UPD are either caused by abnormal recombination between non-sister chromatids during mitosis or by deletion of a region and further duplication of the homologous region of the other chromosome.

From a total of 92 patients with confirmed molecular diagnosis of BWS only 9 patients (10%) had patUPD11p. This proportion is slightly lower than previous reports where close to 15-20% of the patients with BWS showed patUPD11p. This lower proportion may be due to the specific characteristics of our Registry with a high number of pregnancies obtained after assisted reproductive technologies, a procedure that it is hypothesized that predisposes to aberrant mehtylation of the centromeric imprinting center (IC2) [35] .

Percentage of 11p mosaicism and BWS phenotype

It has been demonstrated that phenotypic expression of BWS correlates with elevated levels of UPD in the organs and tissues that were tested [22] . However, it must be noted that the level of UPD has not been found to correlate with phenotypic expression. Our patients had clinical heterogeneity and lack of correlation with the percentage of mosaicism as well as the extension onto the short arm of chromosome 11. The only constant finding in all patients with patUPD11p in our series is hemihypertrophy. As an example of such lack of correlation, patient UPD 8 showed macrosomia at birth, hemihypertrophy and mild macroglossia and had 31 Mb of extension and 70 % of mosaicism and patient UPD 6 who has a smaller extension (6.6 Mb) and 64 % of mosaicism showed a more expanded phenotype including macrosomia, polyhydramnios, umbilical hernia, hemihypertrophy, macroglossia and

hypoglycaemia (Table IV). Thus, larger size of mosaic UPD and/or higher percentage of mosaicism do not directly imply a more florid manifestation of the disorder. This could be due to the fact that routine testing of a single tissue, such as blood, may not actually reflect the level of UPD in the tissues most susceptible to overgrowth in BWS [28] .

Comparison of mitotic and meiotic recombination breakpoints at 11p

Postfertilization errors such as nondisjunction with reduplication, mitotic recombination, or gene conversion might lead to complete or partial isodisomy. Mosaicism might be frequent with postfertilization errors, since there would be no selection against the original disomic line. The site of breakpoints were different in all patients suggesting that in contrast to some common breakpoints observed in meiosis there seem to be no common mitotic recombination regions [21,36] (Table II).

Correlation of the tests

We found that a combination of two tests (MS-MLPA + MS-HRMA or MS-MLPA + pyrosequencing) is the most useful approach for clinical diagnosis. SNP-arrays alone has been useful for diagnosing UPD11p, however it cannot discriminate between paternal and maternal contribution. The costs of the studies are also important since the SNP-arrays are still expensive and should be set up in experienced laboratories. The limitations of our study are that only 9 patients were evaluated and only one tissue (blood) has been tested in these patients. In contrast, we applied five different techniques to approach patUPD11p, including high density SNP-arrays, which in fact it would have been useful itself to solve most of the issues because it can interrogate not only the dosage but also can demonstrate the percentage of mosaicism and its extent through the short arm of chromosome 11.

Summing up, SNP-arrays have been useful and very informative for clinical diagnosis in patients with BWS with mosaic UPD11p and might be used as the unique

tool in clinical diagnostic laboratories, not only to evaluate mosaicisms but also to map the mitotic site and consequently the extent of UPD at the short arm of chromosome 11.

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Titles and legends to figures

Figure 1. (a) Schematic view of chromosome 11. Zoom of a part of 11p showing the location of genes and imprinting centres of 11p15.5 BWS locus and *WT1* gen. (b) Location of the 6 STR markers evaluated in patients and their parents.

Figure 2. (a) Example of microsatellite analysis; patient, mother and father are represented from top to bottom. Note the paternal origin of UPD and the different level of mosaicism denoted from the different contribution of paternal and maternal alleles in patients' results. (b) Methylation analysis by MS-MLPA in two representative patients. Note the different extent of epigenetic defect compared to controls' mean methylation. To higher level of mosaicism corresponds to higher hypermethylation of H19DMR and increased hypomethilation of KvDMR (and vice versa).

Figure 3. (a) SNP-array results for two UPD patients (UPD 4 and UPD 7). The B allele frequency is shown in the upper panel of each patient. The red zone represents the chromosome fragment with UPD and the level of mosaicism for each SNP. The graph Log R Ratio is the measure of genomic dosage (normal in both patients). Note the differences presented by these two cases on the extent of UPD fragment and their level of mosaicism which is higher for patient UPD 4 than UPD 7. (b) Graphic representation of the UPD extension for each patients and the latest SNP involved in the breakpoint.

Figure 4. (a) MS-HRM results of two patients (UPD 1 and UPD 7). In the first and in the second panel H19DMR and KvDMR curves are represented respectively. Note the different level of methylation between both patients; controls' DNA (C), 0% and 100% methylated synthetic DNAs are used to visually appreciate the curve variation. (b) Pyrosequencing image of the three first dinucleotidos CpG for H19DMR and KvDMR in

patients UPD 1 and UPD 7. Results of the methylation percentage for each CpG individually are similar to the MS-HRM results.

Figure 5. Scatter graph of Spearman's correlations and relatives R^2 values. (a) These two graphs represent the direct and inverse correlation between the percentage of mosaicism and the methylation level at H19DMR and KvDMR loci respectively. (b) Graphic representation showing that there is no correlation between the size of the UPD fragment and the methylation level of both imprinting centres and the percentage of mosaicism.

Table I. Estimated percentage of cells with UPD calculated for each STR markers (a); methylation index calculated by MS-MLPA (b), MS-HRM (c) and pyrosequencing (d) for each patient. For the methylation analysis it has been calculated the normal methylation index as controls' mean value and standard deviation, for each technique.

a

	STR markers					
Patient	D11S4046	D11S1318	D11S4088	D11S1338	D11S1346	TH01
UPD 1	85,77%	82,01%	77,77%	53,49%	-	89,38%
UPD 2	16,82%	47,91%	31,82%	48,47%	50,52%	37,52%
UPD 3	-	57,44%	21,49%	-	51,23%	41,39%
UPD 4	74,14%	73,08%	73,62%	51,26%	67,87%	64,08%
UPD 5	33,35%	45,58%	40,59%	-	74,83%	63,51%
UPD 6	81,70%	66,48%	31,26%	-	9,07%	36,31%
UPD 7	12,93%	28,35%	32,39%	22,79%	-	-
UPD 8	50,19%	-	63,11%	50,46%	53,73%	49,17%
UPD 9	58,78%	38,82%	73,84%	37,68%	45,54%	-

b **c** **d**

	MS-MLPA		MS-HRM		CpG Q-Pyro	
Patient	H19DMR 1	KvDMR	H19DMR	KvDMR	H19DMR	KvDMR
UPD 1	114,77%	8,89%	60,98%	24,19%	90,61%	10,96%
UPD 2	81,25%	31,70%	46,01%	48,91%	73,15%	27,94%
UPD 3	59,81%	42,76%	46,17%	53,24%	64,80%	28,31%
UPD 4	79,99%	21,38%	50,41%	40,36%	86,78%	20,50%
UPD 5	73,47%	23,22%	50,84%	36,46%	79,22%	18,59%
UPD 6	69,27%	33,98%	51,99%	50,68%	72,07%	26,05%
UPD 7	70,32%	42,05%	44,37%	48,52%	67,75%	29,13%
UPD 8	69,09%	34,47%	52,41%	45,96%	74,26%	28,34%
UPD 9	86,18%	35,85%	51,89%	50,48%	73,26%	22,99%
Controls' mean	50,26%	54,69%	39,64%	59,67%	52,11%	40,68%
Std. Desv.	7,18%	4,63%	2,07%	3,74%	2,02%	5,17%

Table II. SNP-array analyses results. The minimum and maximum size estimated for the UPD extent and the gap between these two values are shown. The breakpoints were estimated by means of the identification of the last SNP affected by UPD and the first SNP in heterozygosis. The lower and higher B allele frequencies for all SNPs were calculated. Taking into account that isodisomy is paternal, the percentage of mosaicism of UPD cells is considered to be represented by the frequency of superior B allele.

Patient	Latest UPD SNP	Position	First Het SNP	Position	Min UPD Size (Mbp)	Gap (Kbp)	Max UPD Size (Mbp)	Inferior B Allele Freq	Superior B Allele Freq	Stand dev
UPD 1	rs10838427	45,138,258	rs11038507	45,495,455	45,138	357,197	45,495	10,5%	89,5%	4,8%
UPD 2	rs10838591	46,230,719	rs8914	46,655,700	46,231	424,981	46,656	31,6%	68,4%	4,2%
UPD 3	rs35834377	41,774,946	rs11036703	42,105,122	41,775	330,176	42,105	35,5%	64,5%	4,2%
UPD 4	rs11038116	44,680,447	rs704664	44,743,777	44,681	63,330	44,744	23,1%	76,9%	4,6%
UPD 5	rs1061022	32,083,259	rs7105777	32,997,914	32,083	914,655	32,998	23,1%	76,9%	4,3%
UPD 6	rs2063082	6,624,904	rs11040978	6,690,629	6,625	65,725	6,691	35,7%	64,3%	6,5%
UPD 7	rs10832514	2,645,102	rs151288	2,742,878	2,645	97,776	2,743	39,1%	60,9%	3,3%
UPD 8	rs2177482	31,758,933	rs11031505	31,855,115	31,759	96,182	31,855	30,0%	70,0%	3,5%
UPD 9	rs2288249	46,290,815	rs876701	46,327,343	46,291	36,528	46,327	29,4%	70,6%	3,7%

Table III. Spearman's rank correlation coefficient (or Spearman's rho) is a non-parametric measure of statistical dependence between two variables. It assesses how well the relationship between two variables can be described using a monotonic function. In the ovals the correlation coefficients and the statistical significant of the correlation are highlighted. In the vertical ovals there is no correlation between the size of UPD fragment, the percentage of methylation at both imprinting centres and the level of mosaicism. In the horizontal oval are shown a direct correlation between the level of mosaicism and the percentage of methylation of H19DMR (r=+0.933) and an inverse correlation (r=-0.833) between the level of mosaicism and the percentage of methylation of KvDMR; both correlations are statistically significant.

Correlations

			IC I (H19KvDMR)CpG Q-Pyro	IC II (KvDMR)CpG Q-Pyro	Sup B Allele Freq	Min UPD Size (Mbp)
Spearman's rho	IC I (H19KvDMR)CpG Q-Pyro	Correlation Coefficient	1.000	-.767*	.933**	.367
		Sig. (2-tailed)	.	.016	.000	.332
		N	9	9	9	9
	IC II (KvDMR)CpG Q-Pyro	Correlation Coefficient	-.767*	1.000	-.833**	-.500
		Sig. (2-tailed)	.016	.	.005	.170
		N	9	9	9	9
	Sup B Allele Freq	Correlation Coefficient	.933**	-.833**	1.000	.583
		Sig. (2-tailed)	.000	.005	.	.099
		N	9	9	9	9
	Min UPD Size (Mbp)	Correlation Coefficient	.367	-.500	.583	1.000
		Sig. (2-tailed)	.332	.170	.099	.
		N	9	9	9	9

*. Correlation is significant at the .05 level (2-tailed).

**. Correlation is significant at the .01 level (2-tailed).

Table IV. (a) Molecular results and clinical findings of each UPD patient. (b) Summary table with frequency of main clinical signs in our group of patients.

a

Patient	**UPD 1**	**UPD 2**	**UPD 3**	**UPD 4**	**UPD 5**	**UPD 6**	**UPD 7**	**UPD 8**	**UPD 9**
Sex	Male	Male	Female	Female	Male	Male	Female	Male	Male
Age at diagnosis	3y 11m	4y 5m	2y 3m	2y 4m	2y 4m	1y 5m	2y 1m	3m	5m
% of mosaicism	89,5%	68,4%	64,5%	76,9%	76,9%	64,3%	60,9%	70,0%	70,6%
UPD extent	45,1Mbp	46,2Mbp	41,8Mbp	44,7Mbp	32,1Mbp	6,6Mbp	2,6Mbp	31,7Mbp	46,3Mbp
Pregnancy	Polyhydramnios	Polyhydramnios	-	-	-	Polyhydramnios	-	-	Polyhydramnios
Birth weight (g)	Increased (… g)	Increased (… g)	Increased (… g)	Increased (… g)	Increased (… g)	Increased (… g)	Increased (… g)	Increased (… g)	Increased (… g)
Hemihyperplasia	Hemihyperplasia	Left hemihyperplasia	Right hemihyperplasia	Right hemihyperplasia	Left hemihyperplasia	Hemihyperplasia	Right hemihyperplasia	Right hemihyperplasia	Hemihyperplasia
Craneofacial	Macroglossia; ear creases	Macroglossia	-	-	Macroglossia; ear creases	Macroglossia	Macroglossia	-	Macroglossia; ear creases
Cardiovascular	-	-	-	Atrial septal defect	-	-	-	-	-
Abdomen	Omphalocele	Umbilical hernia	Omphalocele	-	-	Umbilical hernia	-	-	Umbilical hernia
Skin	Capillary malformation	Capillary malformation	-	-	-	-	-	-	Capillary malformation
Wilms' tumor	-	-	-	-	-	-	-	-	-
Others	Hypoglycaemia	Hypoglycaemia	Hypoglycaemia	Pulmonary stenosis	Hypoglycaemia	Hypoglycaemia	-	-	Hypoglycaemia

b

Clinical Findings	
Male:Female	6:3
Polyhydramnios	4/9
Increased birth weight	9/9
Hemihyperplasia	9/9
Macroglossia	6/9
Ear creases	3/9
Omphalocele	2/9
Umbilical hernia	3/9
Capillary malformation	3/9
Wilms' tumor	0/9
Hypoglycaemia	6/9

Figure 1.

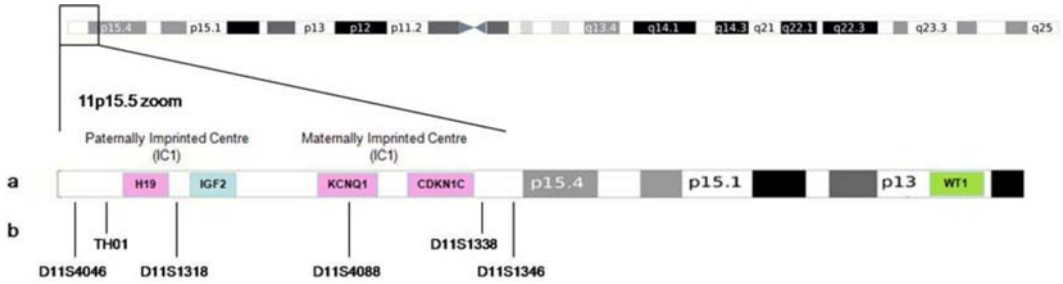

p15.4
p15.1
p13
p12
p11.2
q13.4
q14.1
q14.3
q21
q22.1
q22.3
q23.3
q25
11p15.5 zoom
Paternally Imprinted Centre (IC1)
Maternally Imprinted Centre (IC1)
a
H19
IGF2
KCNQ1
CDKN1C
p15.4
p15.1
p13
WT1
b
TH01
D11S4046
D11S1318
D11S4088
D11S1338
D11S1346

Figure 2.

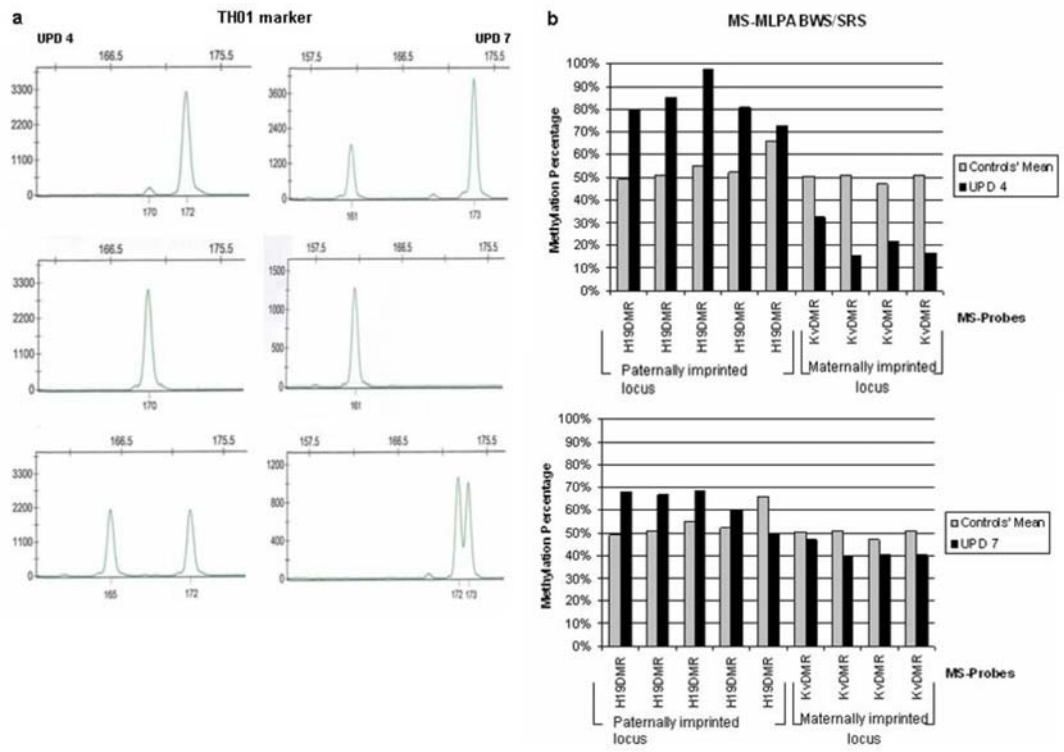

a
TH01 marker
UPD 4
UPD 7
b
MS-MLPA BWS/SRS
Methylation Percentage
Controls' Mean
UPD 4
UPD 7
H19DMR
KvDMR
MS-Probes
Paternally imprinted locus
Maternally imprinted locus

Figure 3.

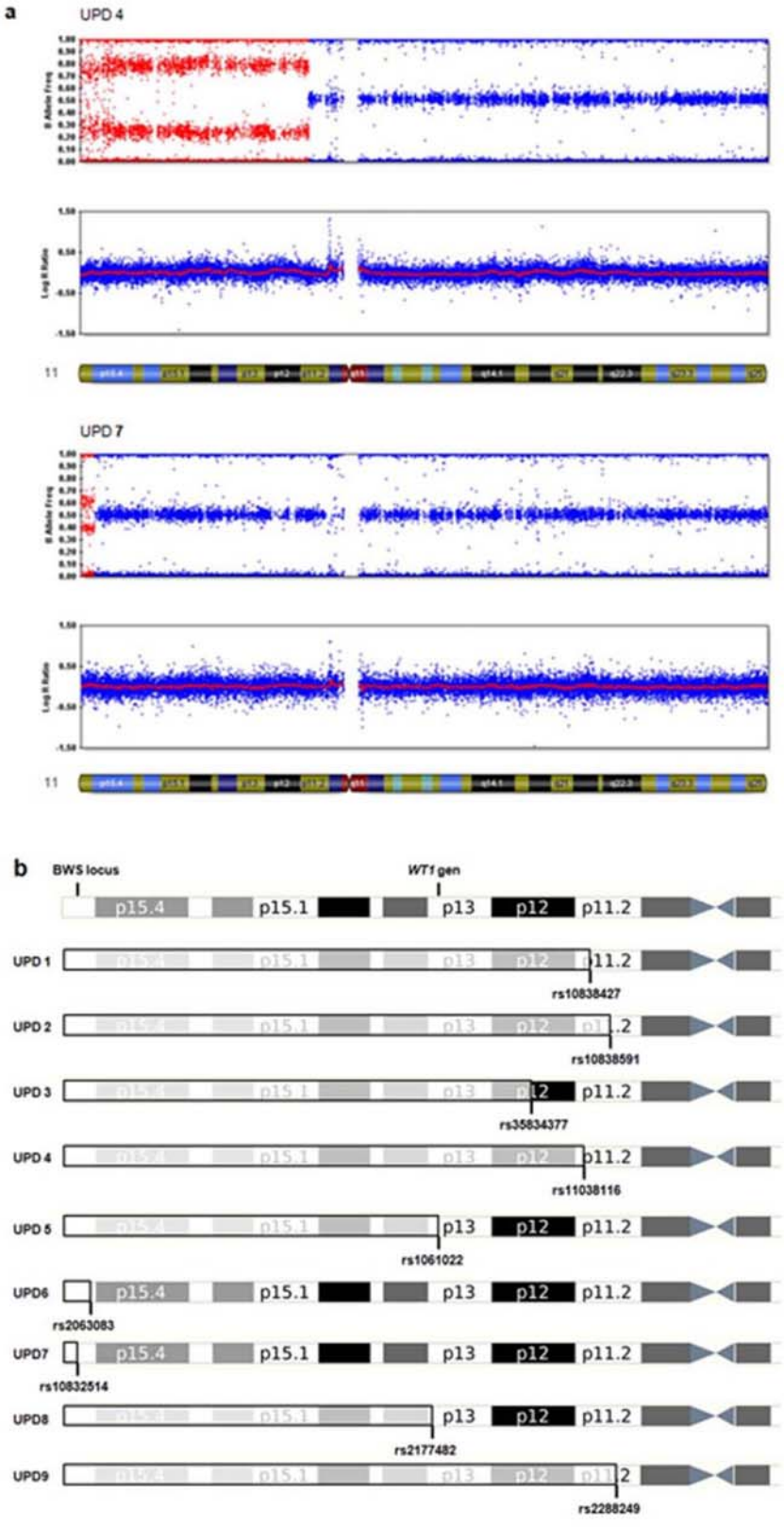

a
UPD 4
B Allele Freq
Log R Ratio
11
UPD 7
B Allele Freq
Log R Ratio
11
b
BWS locus
WT1 gen
p15.4
p15.1
p13
p12
p11.2
UPD 1
rs10838427
UPD 2
rs10838591
UPD 3
rs35834377
UPD 4
rs11038116
UPD 5
rs1061022
UPD6
rs2063083
UPD7
rs10832514
UPD8
rs2177482
UPD9
rs2288249

Figure 4.

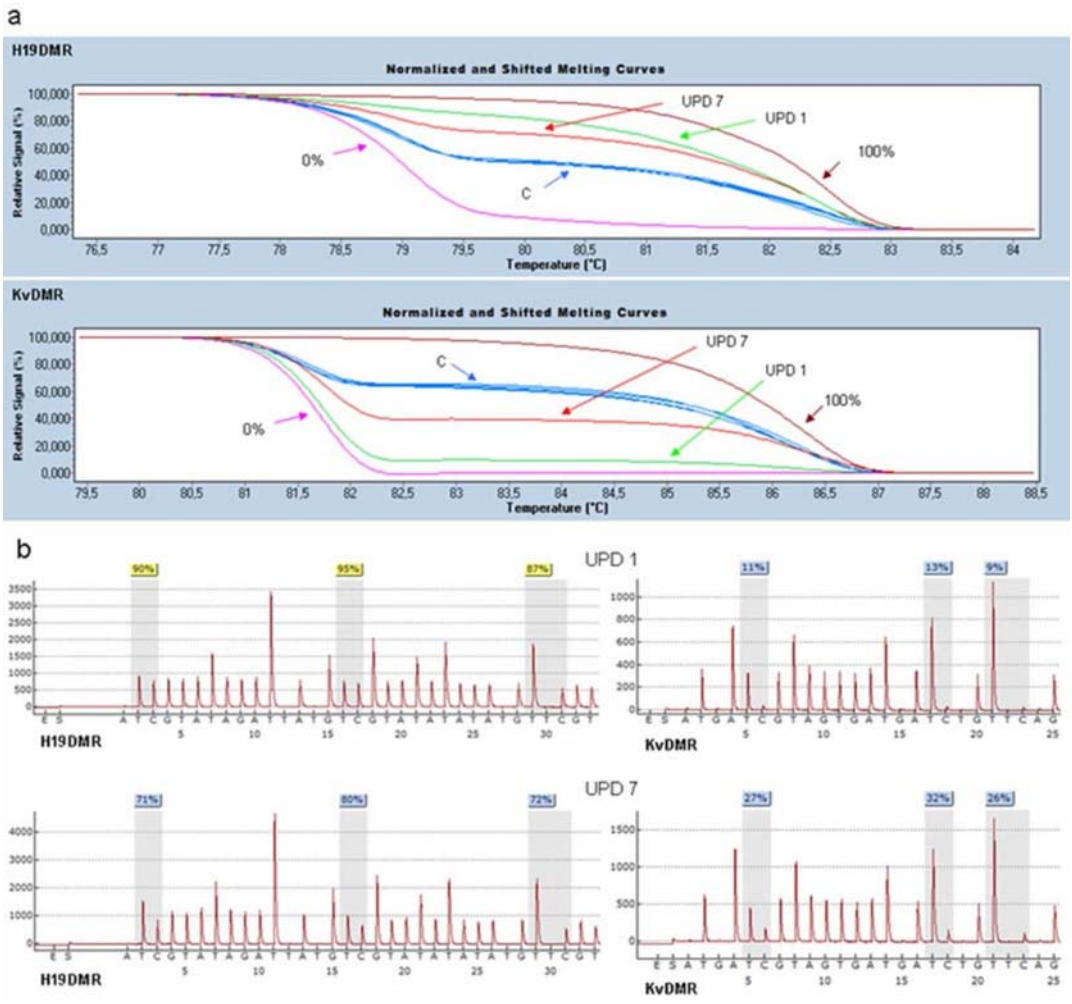
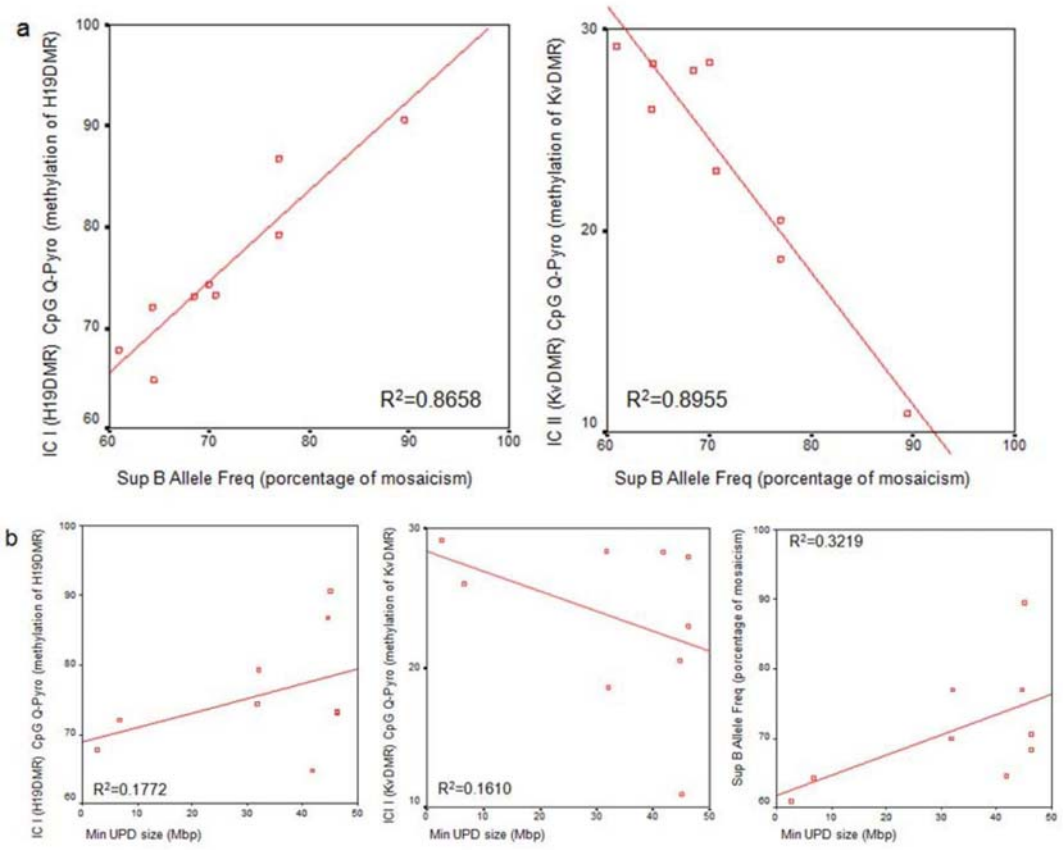

a
H19DMR
Normalized and Shifted Melting Curves
Relative Signal (%)
Temperature (°C)
UPD 7
UPD 1
100%
0%
C
KvDMR
Normalized and Shifted Melting Curves
Relative Signal (%)
Temperature (°C)
C
UPD 7
UPD 1
100%
0%
b
UPD 1
H19DMR
KvDMR
UPD 7
H19DMR
KvDMR

Figure 5.


a
IC I (H19DMR) CpG Q-Pyro (methylation of H19DMR)
Sup B Allele Freq (porcentage of mosaicism)
R²=0.8658
IC II (KvDMR) CpG Q-Pyro (methylation of KvDMR)
Sup B Allele Freq (porcentage of mosaicism)
R²=0.8955
b
IC I (H19DMR) CpG Q-Pyro (methylation of H19DMR)
Min UPD size (Mbp)
R²=0.1772
ICI I (KvDMR) CpG Q-Pyro (methylation of KvDMR)
Min UPD size (Mbp)
R²=0.1610
Sup B Allele Freq (porcentage of mosaicism)
Min UPD size (Mbp)
R²=0.3219

Constitutional Mosaic Genome-Wide Uniparental Disomy due to Diploidization: an Unusual Cancer-Predisposing Mechanism.

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Journal of Medical Genetics. 2010. Accepted.

Disomía uniparental paterna del genoma entero en mosaicismo constitucional debida a diploidización: un inusual mecanismo de predisposición al cáncer.

El síndrome de Beckwith-Wiedemann se caracteriza por un incremento de la frecuencia de tumores embrionarios. Este riesgo está elevado sobretodo en los casos debidos a disomía uniparental paterna (UPDp), pues a la presencia de dos copias derivadas del padre de la región 11p15 y a la baja o nula contribución materna para esta región.

La disomía uniparental lleva a la perdida de heterocigosidad (LOH, Loss Of Heterozygosity), mecanismo tumorigénico común, descrito en casi todos los tipos de cáncer, debido a la ausencia de un gen supresor de tumores en la región afectada. Una consecuencia frecuente de la LOH es la pérdida de imprinting (LOI, Loss Of Imprinting) localizada a los centros de imprinting involucrados, por la contribución parental no balanceada.

La paciente que presentamos en este trabajo tenía diagnostico de BWS debido a UPDp, dicho diagnóstico se hizo precedentemente con análisis de microsatélites y ahora se ha confirmado por MS-MLPA, pirosecuenciación y MS-HRM. Todas estas técnicas nos han permitido evaluar el origen de la disomía uniparental, su porcentaje de mosaicismo y el índice de metilación de los dos centros de imprinting del locus 11p15.

Además hemos reanalizado la paciente a través de la técnica de SNP-array, para hacer una evaluación más fina del porcentaje de mosaicismo y de la extensión de la UPD. Este análisis nos ha llevado a un resultado inesperado: todo el genoma presenta disomía uniparental paterna en un porcentaje de mosaicismo del 85%. El uso de array específicos de metilación nos ha permitido confirmar que más centros de imprinting, además de los del locus BWS/SRS, presentan defecto epigenético.

Al revisar la literatura hemos constatado que entre los pocos casos conocidos, el nuestro es el primero representado por un paciente adulto. Hemos entonces estudiado el mecanismo de generación de la UPD constitucional de todo el genoma y evaluado las posibles enfermedades y tumores debidos a la anormalidad de todos los centros de imprinting.

Disomia uniparentale paterna del genoma intero in mosaicismo costituzionale dovuta a diploidizzazione: un meccanismo insolito di predisposizione al cancro.

La sindrome di Beckwith-Wiedemann è caratterizzata da un aumento della frequenza di tumori embrionari. Questo rischio é elevato soprattutto nei casi dovuti a disomia uniparentale paterna (UPDp), ossia la presenza di due copie derivate dal padre della regione 11p15 e il basso o nullo contributo materno per questa regione.

La disomia uniparentale porta alla perdita di eterozigosità (LOH, Loss Of Heterozygosity), comune meccanismo tumorigenico, descritto in quasi tutti i tipi di cancro, dovuto all'assenza di un gene soppressore di tumori nella regione affetta. Una conseguenza frequente della LOH é la perdita di imprinting (LOI, Loss Of Imprinting) localizzata ai centri di imprinting coinvolti, per il contributo paternale non equilibrato.

La paziente che presentiamo in questo lavoro aveva diagnostico di BWS dovuta a UPDp, tale diagnostico si fece a suo tempo con analisi dei microsatelliti e adesso é stato confermato con MS-MLPA, pirosequenziamento e MS-HRM. Tutte queste tecniche ci hanno permesso valutare l'origine della disomia uniparentale, la sua percentuale di mosaicismo e lo statop di metilazione dei due centri di imprinting del locus 11p15.

Inoltre abbiamo rianalizzato la paziente con la tecnica di SNP-array, per fare una valutazione piú raffinata della percentuale di mosaicismo e della estensione della UPD. Quest'analisi ci ha portato a un risultato non atteso: tutto il genoma presenta disomia uniparentale paterna con una percentuale di mosaicismo del 85%. L'utilizzo di array specifici di metilazione ci ha permesso confermare che diversi centri di imprinting, oltre a quelli del locus BWS/SRS, presentano difetto epigenetico.

Revisionando la letteratura ci siamo resi conto che tra i pochi casi noti, il nostro é il primo rappresentato per un paziente adulto. Abbiamo quindi studiato il meccanismo di generazione della UPD costituzionale di tutto il genoma e valutato i possibili disordini e tumori dovuti alla anormalitá di tutti i centri di imprinting.

Constitutional Mosaic Genome-Wide Uniparental Disomy due to Diploidization: an Unusual Cancer-Predisposing Mechanism.

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KEY WORDS: Beckwith-Wiedemann syndrome; paternal uniparental disomy; diploidization; imprinting.

Summary

Molecular studies in a patient with Beckwith-Wiedemann syndrome phenotype who developed two different tumors revealed an unexpected observation of almost complete loss of heterozygosity of all chromosomes. We demonstrate, by means of numerous molecular methods that the absence of maternal contribution in somatic cells is due to high-degree (~85%) genome-wide paternal uniparental disomy. Our observations indicate the genome-wide UPD results from diploidization, and have important implications for genetic counseling and tumor surveillance for the growing number of UPD-associated imprinting disorders.

Beckwith-Wiedemann syndrome (BWS; [MIM 130650]), one of the commonest overgrowth syndromes, is characterized by pre and postnatal overgrowth, hemihyperplasia, macroglossia and an increased frequency of embryonic tumors [1-3]. It is caused, in up to 90% of patients, by a variety of genetic or epigenetic alterations within two imprinting sub domains located on human chromosome 11p15 [4;5]. The largest molecular subgroup (~60-70% of BWS cases) is comprised of patients carrying an epigenetic error at either the *H19*DMR (imprinting control region, ICR1) or *Kv*DMR (ICR2) [6], the regions of differential DNA methylation that regulate the appropriate allelic expression of the paternal *IGF2* and maternal *CDKN1C* genes respectively. Patients may also have paternal uniparental disomy (patUPD11p15) (~10-15%), *i.e*. two paternally derived copies of chromosome 11p15 and no maternal contribution, that results in over-expression of the potent mitogen *IGF2*, and absence of the cell cycle regulator *CDKN1C* [7]. Chromosome rearrangements comprising maternal translocations/inversions or paternal duplications are rare (~2-3% of cases) as are *CDKN1C* mutations (~5-7 %).

Loss of heterozygosity (LOH) may be due either to haploinsufficiency (deletion leading to the lack of contribution of one allele) or uniparental disomy (UPD) when both copies of a chromosome pair originate from one parent. LOH is a common mechanism of tumorigenesis where it indicates the absence of a functional tumor suppressor gene in the lost region. LOH has been described in almost all cancer types [8]. Localized loss of imprinting (LOI) is a common consequence of LOH due to the lack of balanced parental contribution in specific region with imprinting either by deletion/duplications or UPD. As stated above, LOH/LOI due to patUPD11p15 is relatively common in BWS and this molecular subgroup of individuals displays one of the highest risks of cancer [9].

Diploidization means the recovery of the normal diploid human chromosome number (2n=46) from an initially haploid cell (n=23) or triploid zygote (3n=69). Diploidization is a relatively common mechanism in plants and non-mammalian species but it has been unfrequently observed in humans [10-13].

We here report on a woman with BWS phenotype and tumors with almost complete LOH in all chromosomes, showing a near-complete contribution of the paternal genome for the diploid status. Postzygotic diploidization due to failure of maternal DNA replication followed by paternal genome endoreduplication would be the most plausible explanation for the observed findings. Thus, present patient would represent a further example of diplodization in humans.

The diagnosis of Beckwith-Wiedemann syndrome was established at birth in a girl of healthy and non-consanguineous parents because of macrosomia, overgrowth, hypoglycaemia and hemihypertrophy. The patient is included as part of a Research Program in the Spanish Overgrowth Syndrome Registry (IRB-HULP PI446). At birth she weighed 3750 g, and showed capillary malformations in legs, hands and thorax, contracture of fingers, facial asymmetry and hemihypertrophy of left hand and leg. She continued with hypoglycaemia and needed treatment with diazoxide for years. At 16 months she had episodes of convulsions needing valproic acid treatment. She started to walk at 19 months. Language was delayed and her school performance was lower than average for age. At age 9 years she underwent pancreatic resection due to intractable, symptomatic hypoglycaemias. At age 4 years 5 months a grade II mesenchymal Wilms tumor was diagnosed and received a combination of complete nephrectomy (including right adrenalectomy) and 9 months of chemotherapy. She also had renal stones (calcium) needing close follow-up for her unique kidney. Calcium, phosphate, alkaline phosphatase, and PTH were among normal ranges. She has an average height for Spanish standards (163.5 cm; 50^{th} centile). Skeletal surveys were normal except for the slight asymmetry due to hemihyperplasia. Her clinical outcome was then unremarkable until 20 years when an ectopic adrenocortical virilizing adenoma was suspected (and confirmed) due to hirsutism, secondary amenorrhea and the finding at MRI of an abdominal retroperitoneal mass, independent from the left adrenal gland. An excision surgery was followed by complete recovery. Six years later she remains asymptomatic, with normal adrenal function and regular menses. The patient is now 26 years old and works as administrative assistant.

Standard karyotyping along with single nucleotide polymorphism (SNP) and HumanMethylation 27 Infinium arrays, microsatellites, Methylation Specific-Multiplex Ligation dependent Probe Amplification (MS-MLPA), Methylation Sensitive-High Resolution Melting Assay (MS-HRMA), pyrosequencing, and array-CGH were performed on patient's samples (see Supplementary methods).

The diagnosis of BWS due to patUPD11p15 was suspected upon microsatellite analysis in the patient and her parents and confirmed through MS-MLPA (Fig. 1A,B). As expected, MS-HRMA and pyrosequencing of specific CpG dinucleotides in the *Kv*DMR and *H19*DMR, further delineated the degree of aberrant methylation reflecting the percentage of the patUPD11p15 (Fig. 1C-F).

SNP-arrays showed an abnormal, unforeseen, unusual pattern not only at chromosome 11 but also in all chromosomes. The Log R ratio (which evaluates the genomic dosage, e.g. deletions/duplications) was between normal ranges in all chromosomes but the allele frequency (evaluation of homozygosity/heterozygosity of each SNP) disclosed an almost complete drift (~85% LOH) to homozygosity in all chromosomes (Fig. 2A and Supplementary Fig. 1). Subsequent microsatellite analyses of several chromosomes confirmed in blood, skin, urine, buccal cells and some tissues (Wilms tumor, adrenal, ceccal appendix) that almost all of the genomic contribution was paternal with a minimum, low mosaic maternal contribution (Supplementary table 1). All tissue showed a similar pattern with different degree of mosaicism. Calculation of the percentage of mosaicism was applied to some tissues and showed an average contribution of ~85% from the paternal genome and ~15% of maternal genome (Supplementary table 1). Analysis of microsatellites of X chromosome in the patient indicated that in most cells both X chromosomes came from the only paternal X chromosome with only a minimum fluorescence of maternal markers (~15%) (Supplementary Fig. 2). SRY and amelogenin amplifications were indicative of absence of Y chromosome in the patient (data not shown).

Using MLPA targeting the Prader-Willi and Angelman syndrome (PW/AS) and Pseudohypoparathyroidism (PHP) loci, and the Illumina Infinium DNA-methylation BeadArray, we were able to clearly show the lack of appropriate allelic methylation in the patient at 14 DMRs located on 7 different autosomes. These included the maternally methylated *SNPRN, NDN, GNAS XL, GNAS EX1A, GRB10, MEST, PEG10, PEG3, ZAC/PLAGL1, L3MBTL, KvDMR/KCNQ1* and *DIRAS3*, as well as the paternally methylated *NESP* and *H19* DMRs (Fig. 2B). These results confirm the methylation profiles determined by both the MS-MLPA and MS-HRM assays.

There have been scant reports of genome-wide paternal UPD in the literature [14] and as far as we know our case is the only adult patient reported to date. Three patients with proved genome-wide paternal UPD developed pediatric tumors (pheochromocytoma, hepatoblastoma and pancreatic tumor) [13;15] and another two individuals without tumors have been recently reported [11;16]. These cases presented with combined features consistent in part with known imprinting disorders associated with paternal UPD of specific chromosomes, but the most pronounced phenotypic features in all cases resembled patUPD11/BWS. Other authors have described genome-wide paternal UPD confined to specific tissues but not present in blood [12;17] or exclusively confined to placenta and not to fetal tissues [18-21]. Although, the percentage of uniparental cells has not been calculated in all reports the patient presented herein showed one of the highest mosaicism (~85% in blood, 65-87% in other tissues).

In fitting with these previous reports, our patient showed clinical features of some classical paternal UPD syndromes (Supplementary Fig.4). She showed capillary malformation, hemihyperplasia, macroglosia, Wilms tumor and adrenocortical tumor (patUPD11), linear hypopigmentation, seizures and low-average mental development (patUPD15) and hypotonia (patUPD14). The observation of BWS phenotype in this woman is in agreement with our laboratory findings since patUPD11p15 is observed in about 15-20% of individuals with BWS. Furthermore, both tumors observed in the patient, Wilms tumor at 4 years and extra-adrenal

adrenocortical tumor at age 20, are also prevalent in BWS. Somatic pat11pUPD15 has been encountered in about one third of Wilms tumors [22] and such a tumor is the commonest neoplasia observed in BWS patients encompassing about half of tumors reported in this disorder [2].

Regional or partial LOH is observed in deletions or UPDs. In the present patient, deletion was ruled-out due to normal log R ratios in the CGH-array and SNP-array. In addition, almost complete LOH in all chromosomes observed by means of SNP-arrays, microsatellite analyses, MS-MLPA and MS-HRM is consistent with a mechanism of genome-wide UPD.

Potential mechanisms of genome-wide UPD are diploidization of an initial triploid foetus or a normal fertilization followed by a failure of replication and chromosome condensation of the maternal pronuclei with normal replication and segregation of the paternal chromosome (Supplementary Fig. 5). We discarded diploidization after dispermic triploidy as a possible mechanism because the microsatellite analysis in our patient revealed the involvement of a unique X-bearing sperm. We believe that the most probable and simplest explanation for our case is normal fertilization followed by failure of maternal DNA replication and paternal genome endoreduplication , resulting in androgenetic/biparental mosaicism, with over-representation of the genome-wide paternal UPD cell lines (Supplementary Fig. 5E). This diploidization, the recovery of the normal diploid human chromosome number (2n= 46) is common in plants and is in some ways similar to “trisomy rescue” observed in UPD, when trisomic cells (47 chromosomes) recover to normality loosing the extra chromosome in the next division. The pathological consequences of UPD are identical for a single chromosome as a results of trisomy rescue or for the entire genome due diploidization.

Differences in gene expression between the allele inherited from the mother and that inherited from the father are the result of genomic imprinting, and it is the most frequent molecular aberration in BWS. Thus, BWS patients carrying a mosaic paternal UPD of chromosome 11p15 have increased risk of cancer, mainly Wilms tumor, hepatoblastoma and

others embryonal tumors [2;23]. Further, the frequency of UPD detected in neoplastic tissues varies from 3 to 100% [8]. The patient reported herein developed two different neoplasias: Wilms tumor and adrenocortical neoplasia. Consistent with this observation, chromosome 11p15 UPD is observed in 30-40% and 32% of Wilms and adrenocortical tumors, respectively [22;24].

Aberrant allelic methylation at all loci analyzed was demonstrated in the patient by means of MS-MLPA, MS-HRMA, pyrosequencing and methylation array (Fig.1B-F and 2B). Analysis of the BWS, PW/AS and PHP regions revealed a methylation level partially abnormal due to the mosaic status; such an aberrant methylation led to abnormal imprinting of these loci. Thus, our patient showed typical clinical findings of BWS but not typical features of AS, PHP or transient neonatal diabetes mellitus (TNDM), demonstrating that partial loss of imprinting is sufficient for developing BWS but not sufficient for others disorders which usually requires total LOI to manifest the clinical features of the diseases. In addition, the lack of atypical BWS phenotypic features also suggests that inappropriate methylation at the *MEST*, *L3MBTL*, *GRB10*, *PEG3*, and *DIRAS3*, as revealed by the methylation array, is not associated with obvious dysmorphologies. However, the resultant increase in expression of the paternally expressed imprinted genes, due to biallelic expression, and the loss of maternally expressed transcripts maybe involved in tumorigenesis in this patient.

Because of the patient had two different cancers and she carried genome-wide paternal UPD, she may theoretically have increased risks for additional UPD-related tumors [8]. Thus, her prospective follow-up raised some important questions because there are no published recommendations for clinical evaluation schedule and survey for these individuals. Classic recommendations for cancer surveillance in general population may or may not apply for her, since the genetic background is quite different and any cancer surveillance schedule in this patient should involve the whole body. Finally we empirically opted to evaluate this woman with regular (twice a year) clinical evaluation together with annual body MRI (including breasts),

colonoscopy and a routine laboratory work- up including WBC and serological markers for cancer.

The demonstration of genome-wide paternal UPD has important implications for medical genetics, counseling and clinical oncology. Genomic technologies such as array-CGH, high-density methylation and SNP-arrays should be incorporated in diagnostic workflows of patients with suspected imprinting/UPD disorders presenting with dysmorphic features and/or cancer. Tumor surveillance in these patients is empiric since neither guidelines are available yet nor enough experience has been accumulated for guiding the follow-up. Finally, the mechanism of diploidization should be taken into account in patients with genetic disorders and cancer because diploidization of triploid or haploid fetuses is now a recognizable, emerging mechanism with potential risk of UPD and consequently, high risk of neoplasia.

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Figure 1. Microsatellites, MS-MLPA, MS-HRM and CpG pyrograms of patient's blood sample.

a) Genotypes of one microsatellite marker (THO1; 11p15 region), in peripheral blood from the patient, her mother and her father (from top to bottom). Note that in the patient, the maternal allele (157bp) is barely represented. b) MS-MLPA quantitative analysis of the 11p15 imprinting region in the patient. Black bars represent methylation values of the patient and grey bars represent the mean methylation values of the controls. Note hypermethylation of the 5 probes of *H19*DMR (mean methylation in controls 50.26% vs 89.68% in patient) and hypomethylation of the 4 probes of *Kv*DMR (mean methylation in controls 54.69% vs 11.43% in patient) in the patient, corresponding to patUPD11p15. c, d) Normalized melting curves of *H19*DMR and *Kv*DMR, respectively, with curves of controls (C), 0% and 100% methylated DNAs; e, f) CpG pyrogram of *H19*DMR and *Kv*DMR respectively, with methylation percentages calculation for each of the three CpG dinucleotides analyzed. Both analyses further demonstrate hypermethylation of the *H19*DMR and hypomethylation of the *Kv*DMR.

Figure 2. SNP-array of chromosome 11 and methylation profile using Illumina Bead arrays.

a) SNP-array. The Log R ratio was between normal ranges but the allele frequency showed an almost complete drift (~85%) to homozygosity (loss of heterozygosity: LOH). Upper panel: Black arrow showing no abnormal dose observed, ruling-out deletion/duplication. Lower panel: Blue arrows denote homozygous SNPs; red arrows: SNPs in mosaic state tending to homozygosity; green arrow: almost absence of heterozygosity in the sample. b) DNA Methylation profile of several imprinted genes evaluated through Illumina metylation BeadArray in lymphocytes of controls ('lymphocyte') and the patient ('pUPD'). The bar at the right grades from hypomethylation (green; 0.10) to hypermethylation (red; 0.90). Note that the majority of genes in the patient are extremely hypomethylated (green) and *H19*DMR and NESP are extremely hypermethylated (red). These results are in agreements with MS-MLPA and MS-HRM.

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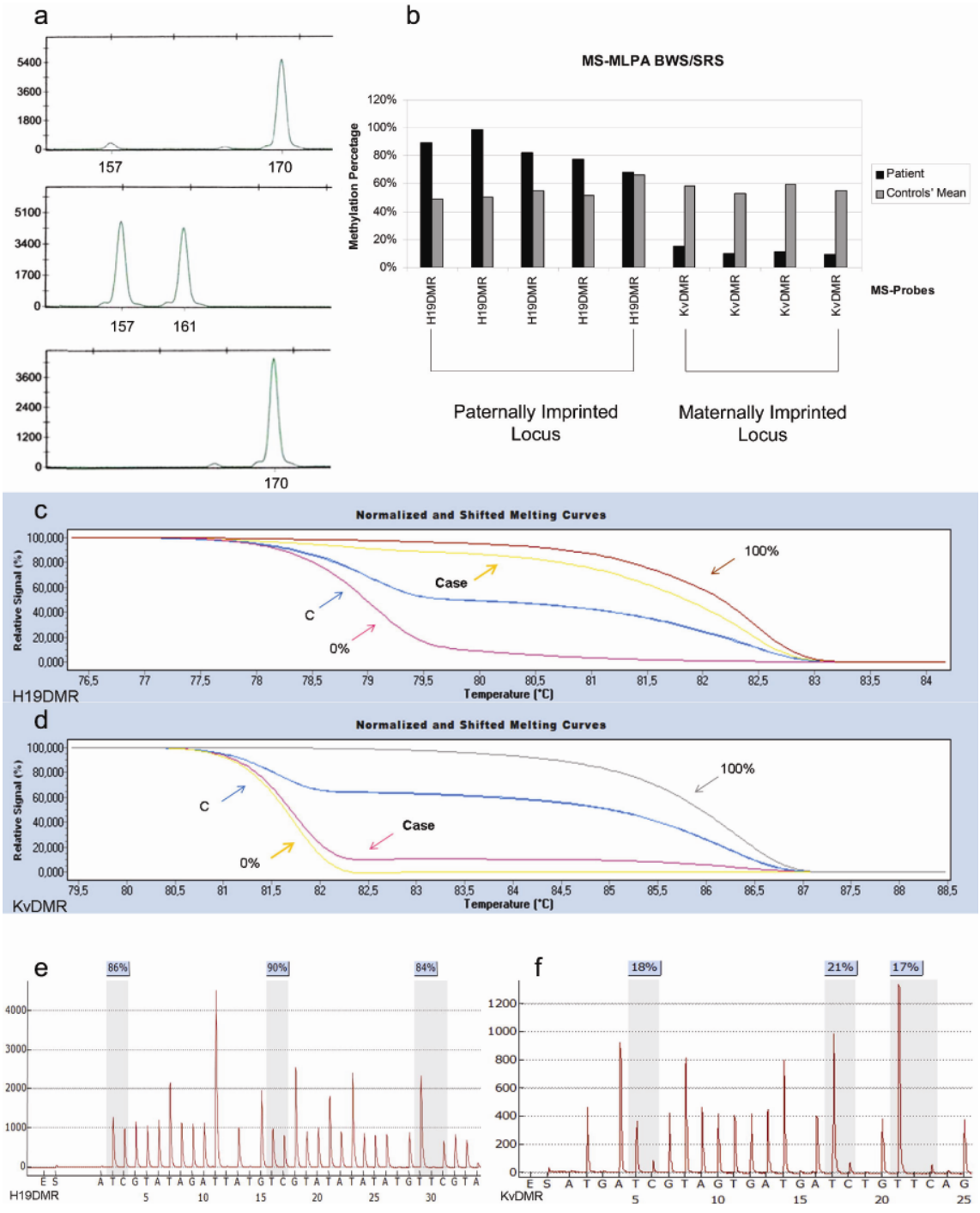
Competing Interests: None to declare

Figure 1.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60%
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Normalized and Shifted Melting Curves
Relative Signal (%)
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Temperature (°C)
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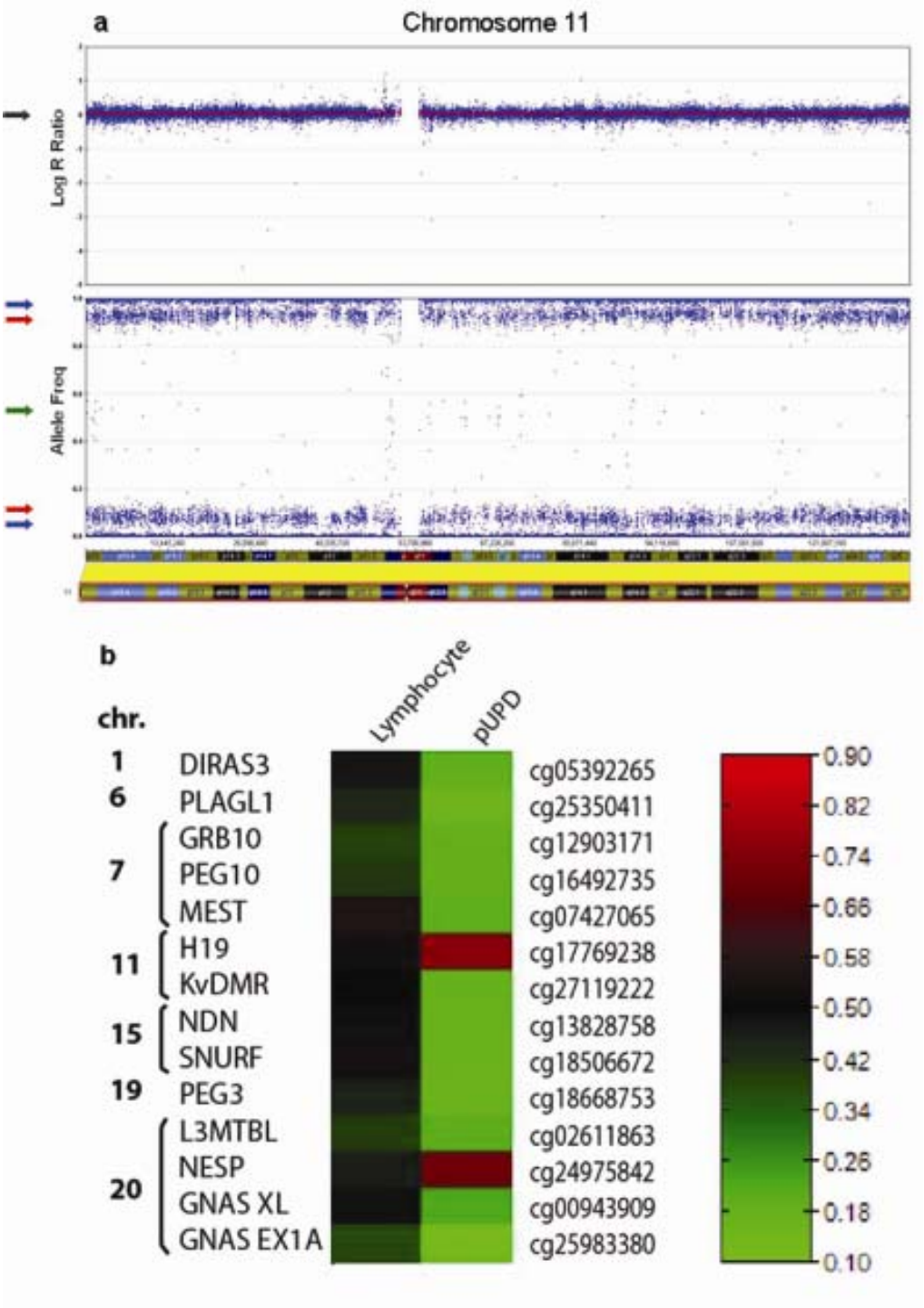
Figure 2.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Macrocephaly-Capillary Malformation:

Analysis of 13 Patients and Review of the Diagnostic Criteria

Martínez-Glez V, Romanelli V, Mori MA, Gracia R, Segovia M, González-Meneses A, López-Gutierrez JC, Gean E, Martorell L, Lapunzina P.

Am J Med Genet A. 2010. Accepted.

Macrocefalia-Malformación Capilar: análisis de 13 pacientes y revisión de los criterios diagnósticos.

El síndrome de Macrocefalia Malformación Capilar (M-MC) es un síndrome de etiología desconocida, caracterizado por circunferencia cefálica aumentada y malformaciones capilares.

La finalidad de este trabajo es reportar las características clínicas de nuestros 13 pacientes, realizar un análisis genómico con SNP-array y discutir los criterios diagnósticos.

Primero hay que destacar que incluiríamos el M-MC entre los síndromes de sobrecrecimiento por la frecuencia de los signos clínicos de sobrecrecimiento/asimetría, considerados además en el conjunto como un criterio mayor. Entre los criterios menores coincidimos con otros autores en considerar como tales el retraso mental y otros signos, pero proponemos que las alteraciones de la neuroimagen sean consideradas un criterio mayor.

El análisis molecular a través SNP-array de nuestros 13 pacientes no nos ha permitido identificar genes alterados ni regiones patológicas comunes a todos los casos y nos ha permitido descartar la hipótesis que alteraciones en el número de copias o amplias UPD pudieran ser las causas del síndrome.

Macrocefalia-Malformazione Capillare: analisi di 13 pazienti e revisione dei criteri diagnostici.

La sindrome di macrocefalia Malformazione Capillare (M-MC) é una sindrome di eziologia sconosciuta, caratterizzata da un aumento della circonferenza cefalica e malformazioni capillari.

Lo scopo di questo lavoro é di riportare le caratteristiche cliniche dei nostri 13 pazienti, analizzare il loro genoma con SNP-array e discutere i criteri diagnostici.

Innanzitutto c'é da evidenziare che includeremmo la M-MC tra le sindromi di ipercrescimento per la frequenza dei segni clinici di ipercrescimento/asimmetria, considerati inoltre nell'insieme come un criterio maggiore. Tra i criteri minori coincidiamo con altri autori nel considerare come tali il ritardo mentale e altri segni, mentre proponiamo che le alterazioni della nueroimmagine siano considerate un criterio maggiore.

L'analisi molecolare con SNP-array dei nostri 13 pazienti non ci ha permesso di identificare geni alterati né regioni patologiche comuni a tutti i casi e ci ha permesso scartare l'ipotesi che alterazioni nel numero di copio o amlpie UPD potessero essere cause della sindrome.

American Journal of Medical Genetics Part A

Macrocephaly-Capillary Malformation: Analysis of 13 Patients and Review of the Diagnostic Criteria

Journal:	*American Journal of Medical Genetics: Part A*
Manuscript ID:	10-0168.R1
Wiley - Manuscript type:	Clinical Report
Date Submitted by the Author:	08-Apr-2010
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Keywords:	Overgrowth syndrome, vascular malformations, enlarged head circumference, diagnostic criteria, neuroimaging alterations

Macrocephaly-Capillary Malformation:

Analysis of 13 Patients and Review of the Diagnostic Criteria

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Running title: M-CM: Review of Diagnostic Criteria

ABSTRACT

Macrocephaly-Capillary Malformation (M-CM) is a genetic syndrome of unknown etiology characterized by an enlarged head circumference and patchy, reticular capillary malformation. We describe the clinical features of 13 cases, report on the genome wide Copy Number Variation characterization of these patients, analyze the main clinical features of this syndrome and propose a modification of the current diagnostic criteria: the inclusion of both overgrowth/asymmetry and neuroimaging alterations as major criteria.

KEY WORDS: Overgrowth syndrome, capillary malformation, enlarged head circumference, diagnostic criteria, neuroimaging alterations

INTRODUCTION

Macrocephaly-Capillary Malformation (M-CM) [OMIM 602501], first described in 1997 [Clayton-Smith et al., 1997; Moore et al., 1997] as Macrocephaly-Cutis Marmorata Telangiectatica Congenita, is a genetic syndrome of unknown etiology characterized by an enlarged head circumference and patchy, reticular capillary malformations. Such capillary malformations frequently occur on the philtrum, upper lip and nose, and are often found on the limbs and trunk [Toriello and Mulliken, 2007]. Other characteristic features are neonatal hypotonia, developmental delay, hydrocephalus, partial/asymmetric overgrowth, syndactyly/polydactyly, asymmetry, and connective tissue defects [Giuliano et al., 2004; Lapunzina et al., 2004]. Neuroimaging abnormalities include white matter alterations, cerebral asymmetry, ventriculomegaly, cerebellar tonsillar herniation, cortical dysplasia, and polymicrogyria [Conway et al., 2007b]. The diagnostic criteria for this syndrome were proposed a decade ago by two different groups [Franceschini et al., 2000; Robertson et al., 2000] and were recently reviewed to adequately describe the capillary changes [Wright et al., 2009].

Here, we describe the clinical features in 13 cases of M-CM. We also report on the genome-wide SNP array characterization of these patients, analyze the main features of the syndrome and discuss the diagnostic criteria, suggesting the inclusion of both overgrowth/asymmetry and neuroimaging alterations as major criteria.

CLINICAL REPORTS

Biological samples, clinical-pathological data, and informed consent were obtained according to our institutional ethical committee's procedures (IRB-HULP PI446). The clinical features (Fig. 1) and data of the 13 patients are summarized in Table I. Cases 1 and 2 were clinically reported before as

Patients 3 and 1 in [Lapunzina et al., 2004]. In three cases (Patients 2, 8 and 11), we did not clinically assess the patients in our Genetic Service; data were provided by their pediatricians who filled out a detailed form designed to assess overgrowth syndromes. All patients are included in the Spanish Overgrowth Syndrome Registry (Madrid, Spain).

METHODS

Cytogenetic analysis was performed on blood from the probands according to routine procedures using GTG banding (550 bands). Genomic DNA was extracted from peripheral blood samples and quantified using PicoGreen (Invitrogen). As part of routine diagnosis in all patients (except cases 12 and 13), we have performed mutational analysis of the *NSD1* (Sotos syndrome) and *CDKN1C* (Beckwith Wiedemann syndrome) genes, and Methylation Specific-MLPA analysis of the 11p15 region (SALSA ME030 MRC-Holland. Amsterdam, The Netherlands).

A genome-wide scan of 620,901 tag SNPs was conducted on probands DNAs, using the Illumina Human610-Quad BeadChip according to the manufacturer's specifications (Illumina, San Diego, CA). DNA samples with GenCall scores <0.15 at any locus were considered "no calls". Image data was analyzed using the Chromosome Viewer tool contained in Beadstudio 3.2 (Illumina, San Diego, CA). The metric used was the log R ratio which is the log (base 2) ratio of the observed normalized R value for a SNP divided by the expected normalized R value [Simon-Sanchez et al., 2007]. In addition, an allele frequency analysis was applied for all SNPs. All genomic positions were based upon build 36 (dbSNP version 126). Copy Number Variation (CNV) analysis was performed using two different algorithms: cnvPartition 1.2.1 (Illumina, San Diego, CA) and PennCNV (www.openbioinformatics.org). cnvPartition algorithm identify regions of the genome that are aberrant in copy number using the log R ratio (LRR) and B allele frequency (BAF). cnvPartition models LRRs and BAFs for each of fourteen different copy number scenarios as simple bivariate Gaussian distributions. The second used algorithm, PennCNV, is a hidden Markov model (HMM) based approach, for kilobase-resolution detection of CNVs from Illumina high-density SNP genotyping data.

This algorithm also incorporates signal intensity and allelic intensity ratio at each SNP marker, the distance between neighboring SNPs and the allele frequency of SNPs.

RESULTS

Karyotypes from probands were normal at the 550-band resolution level. Mutational analyses for *NSD1* and *CDKN1C* genes and MS-MLPA analyses for 11p15 region were also normal. Array data from 13 M-CM DNA samples were analyzed using the BeadStudio software (Illumina, San Diego, CA). We used the combination of two CNV detection algorithms, PennCNV and Illumina cnvPartition 1.2.1, to call CNVs from the signal intensity data. We identified 360 CNVs, 179 with the cnvPartition and 181 with PennCNV algorithms. Combined data from the two algorithms, without overlapping CNVs, showed 242 CNVs (90 gains and 152 losses) among the 13 patients and scattered throughout 136 different regions of the genome. Of these CNVs regions, 67/136 fall into genic regions, involving 146 known RefSeq Genes according to the current release of curated gene entries from the NCBI RefSeq Project. The mean number of CNVs for each individual was 18.62, ranging from 10 to 38 (SD = 6.76). The mean size of the CNVs was 134.77 Kbp (Median = 59.01 Kbp), ranging from 0.7 to 9943 Kbp. Complete data can be found as a complementary material.

In the analysis of the different RefSeq genes we found 28 previously not reported as involved in CNV regions according to the Database of Genomic Variants (Toronto, Canada). From these genes, eight were discarded because of its RefSeq Project status (hypothetical, provisional or pseudogene). The analysis of the function of the remaining 20 genes (Table II), all of them coding sequences with reviewed or validated RefSeq status did not show any apparent relationship with the pathogenesis of M-CM. As there is a reported case of M-CM with a translocation between 2p11 and 17p13 [Stoll, 2003], these two regions were analyzed in detail using less restrictive parameters. We have found CNVs, present in several of the cases analyzed, in regions involving four genes located in 17p13: *ABR*, *YWHAE*, *SMYD4* and *TRPV3* (data not shown). Despite the use of less restrictive settings

in the CNV analysis and that the biological function of these genes does not suggest a possible causality; their involvement in the M-CM requires further verification.

DISCUSSION

Currently, more than 130 cases of M-CM have been reported. As its name suggests, the two main features of the syndrome are macrocephaly and capillary malformations. It is clear that the most characteristic cutaneous vascular anomalies are the capillary malformations rather than Cutis Marmorata Telangiectatica Congenita, as initially described [Toriello and Mulliken, 2007].

Clinical manifestations of M-CM have been discussed previously [Robertson et al., 2000; Lapunzina et al., 2004; Conway et al., 2007b; Katugampola et al., 2008; Gonzalez et al., 2009]. In our series, the most frequent features were neuroimaging alterations (100% - 11/11), macrocephaly (92%), overgrowth (92%), capillary malformation (85%), developmental delay (85%) and asymmetry (62%). Table I separates overgrowth and asymmetry, but they can be considered different forms of the same sign, and are present at 100% in our series and in more than 95% of reported cases in the literature. In the clinical criteria for diagnosis of M-CM proposed by Franceschini et al. [2000], these two features are considered separately and are included as minor criteria. The diagnostic proposal by Robertson et al. [2000] refers to overgrowth as a minor criterion, and the diagnostic scheme by Wright et al. [2009] groups them as a single feature, even in the minor criteria. We feel that M-CM is a condition that should be included as a true overgrowth syndrome [Lapunzina, 2005] and because of its frequency, overgrowth/asymmetry might be included among the major criteria together with macrocephaly and capillary malformations. Although developmental delay occurs in a large percentage of cases, this is a nonspecific characteristic and is therefore best classified in the group of minor features**.** **(**Table III.)

Among the minor features in our series we found hemangioma of the nose, lip and/or philtrum (54%), hydrocephalus (46%), hypotonia (46%), joint laxity (38%), tonsillar herniation/Chiari I (31%), syndactyly of the 2/3 toes (31%), hemimegalencephaly (31%) and polymicrogyria (31%). With respect to facial dysmorphism, the only feature that seems to be constant is frontal bossing. It occurs in 25% of

the cases in our series, in 55% of the cases reviewed by Lapunzina [2004], and is included as minor diagnostic criteria by both Robertson et al. [2000] and Wright et al. [2009]. Therefore, we agree with those authors in using the term frontal bossing instead of the vague term facial dysmorphism.

Neuroimaging alterations were reviewed and discussed *in extenso* by Conway et al. [2007] and include ventriculomegaly (76%), cavum septum pellucidum or cavum vergae (71%), cerebellar tonsillar herniation (69%), cerebral and/or cerebellar asymmetry (53%), thickened corpus callosum (44%), cortical dysplasia (24%) and polymicrogyria (12%). Reviewing 132 cases previously reported in the literature as M-CM and including our additional 11 cases, neuroimaging alterations were found in 82% (112/136) of the patients with a complete clinical description. Therefore, despite the fact that these features may overlap with other disorders, neuroimaging alterations should be considered among the major criteria, not only for its high frequency but also because it will reinforce the importance of MRI studies as part of the evaluation of patients with M-CM. It was recently described an overlap in the phenotypic characteristics of M-CM and MPPH (megalencephaly polymicrogyria-polydactyly hydrocephalus) syndromes [Gripp et al., 2009]. We agree with those authors in that the alterations, not only the neurological ones, used to distinguish between the two syndromes are not exclusive and that therefore could be grouped as a single entity with a possible common pathogenesis. The report of a greater number of cases might be useful to confirm this conclusion.

Using the diagnostic criteria proposed by Wright et al. [2009] and introducing the two changes we propose —the inclusion of overgrowth/asymmetry and neuroimaging alterations as major criteria, requiring three of the four major criteria and two minor criteria—, we were able to successfully diagnose 128 of 136 M-CM cases (94%), since we had access to their clinical descriptions [Lapunzina et al., 2004; Garavelli et al., 2005; Nyberg et al., 2005; Girard et al., 2006; Conway et al., 2007a; Conway et al., 2007b; Canham and Holder, 2008; Katugampola et al., 2008; Bagazgoitia et al., 2009; Franklin et al., 2009; Gonzalez et al., 2009; Mitha et al., 2009; Wright et al., 2009; Duenas-Arias et al., 2009; Gripp et al., 2009].

We are aware that some other overgrowth disorders as Proteus or Sotos syndromes may present much of the features and therefore meet the proposed diagnostic criteria. However, the typical gestalt of Sotos Syndrome patients and the natural history of individuals with Proteus Syndrome make diagnosis easy.

Although the clinical features of M-CM seem to be increasingly defined, the genetic cause(s) underlying the syndrome have yet to be identified. We performed a genome wide single nucleotide polymorphism genotyping on 13 patients diagnosed with M-CM. This platform is also useful for both typing copy number variants (CNVs) and for searching for uniparental disomy (UPD) [Altug-Teber et al., 2005; Yau and Holmes, 2008]. The results we obtained in the analysis of the SNP arrays did not identify any altered gene or region common to all patients. However, in our series, we were able to exclude copy number variations and large UPD as causes of the syndrome, but it is important to perform these experiments in a larger series of patients.

Copy number variation is a significant source of genomic variation that affects thousands of coding regions. Although CNVs can represent polymorphic variants contributing to phenotypic variability, they can also be the cause of Mendelian or sporadic traits, or can be involved in the pathogenesis of complex diseases. As an example, a recent CNV analysis of 512 patients with tetralogy of Fallot compared with 2,265 controls found that at least 10% of the sporadic cases can result from *de novo* CNVs [Greenway et al., 2009]. On the other hand, the inheritance of both copies of whole or partial chromosomes from a single parent (UPD) is a known cause of some genetic disorders, such as Beckwith-Wiedemann and Silver-Russell syndromes with aberrant genomic imprinting. The data obtained in our work, together with further assays, could be useful for further meta-analysis to obtain better insight of possible involvement of such abnormalities in the pathogenesis of M-CM.

In conclusion, we suggest a modification of the diagnostic criteria for M-CM presented by Wright et al. [2009]: the inclusion of both overgrowth/asymmetry and neuroimaging alterations as

major criteria (Table III). We also encourage the publication of genome-wide CNV studies to increase the number of analyzed M-CM affected patients.

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Table I. Clinical data on 13 MCM patients

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11	Case 12	Case 13
Sex	M	M	M	M	M	M	M	M	F	F	M	M	M
Age (years)	14	9	6	9	4	7	3	-	2	3	4	2	3
Gest. age (weeks)	40	37	33	34	41	37	34.3	40	40	36	37	41	38
Birth weight	4000 (>p97)	3980 (>p97)	3300 (>97)	3750 (>p97)	3960 (p90)	3600 (p97)	4080 (>p97)	4270 (p97)	2900 (p10)	3395 (>p97)	3498 (p75)	3800 (p90)	3920 (p90)
Birth length	51 (p75-p90)	51 (p75)	47.5 (>p97)	50 (>p97)	52 (p97)	49 (p75)	53 (>p97)	52 (p90)	49 (p50)	51 (p97)	49 (p75)	53.5 (p90)	52 (>p97)
OFC	37 (+2SD)	40.2 (+5.5SD)	39 (>97)	38 (>p97)	37 (p90)	36 (+2SD)	40.5 (+3SD)	41 (+3SD)	35 (+1SD)	37 (+3SD)	37 (>p97)	35 (p50)	36.5 (p90)
Macrocephaly	+	+	+	+	+	+	+	+	+	+	+	-	+
Macrosomia	+	+	+	+	+	+	+	+	-	+	+	+	+
Hydrocephaly	+	-	+	-	-	+	-	-	-	+	-	+	+
Midline facial capillary malformation	-	Philtrum, upper lip and and glabella	-	-	-	Philtrum	Upper lip and philtrum	ND	Upper lip and neck	Frontonasal, upper lip	-	Philtrum, glabella	Upper lip and philtrum
Capillary malformation, other	Right side of face	Extended	Abdominal	-	-	Extended	Extended	Extended	Chest and limbs	Shoulder	Soles, extended	neck	Extended
Head and Neck	Round face with mild right hemihypertrophy, broad nose, Thick lips and Astigmatism	Nystagmus	Frontal bossing, large fontanelle, broad nose, hypertelorism, downslanted palpebral fissures, low set ears, high vaulted palate	Dolichocephaly, frontal bossing, high vaulted palate	Face asymmetry	Cranial asymmetry	Tooth shape anomaly and spaced. Broad nose, anteverted nares, thick lower lip and abnormal helix	ND	Round face with right hemihypertrophy	Frontal bossing, large fontanelle, hypertelorism	-	Turricephaly	Round face with left hemihypertrophy
Thorax and abdomen	Mild asymmetry	Umbilical hernia	-	Umbilical hernia	Agenesis of right kidney	Pulmonary valve stenosis, arrhythmia, inguinal hernia	Patent ductus arteriosus. Eco: renal pelvis ectasia	-	-	diastasis recti and umbilical hernia	-	-	LIeft hemihypertrophy, dysplastic aorta without insufficiency
Limbs anomalies	Large hands, flat broad foot	Upper limb asymmetry, syndactyly of 2/3 toes	2/3 syndactyly	Large hands	Bilateral postaxial polydactyly on hands and rigth feet, triphalangeal thumbs	Right feet postaxial polydactyly, broad hands and foot, asymmetry	Broad large hands and foot, 2/3 syndactyly	Left postaxial polydactyly and syndactyly of toes	Broad large hands, upper limbs asymmetry	-	Right lower limb hemihypertrophy, large hands and feet	Lower limbs asymmetry	Large hands and feet
Skin	-	-	-	-	Hyperelastic skin	-	-	-	-	-	hyperelastic skin	-	-
Skeletal / Muscle anomalies	Hyperextensible joints	Hyperextensible joints	Hypotonia	Advanced bone age	Hypotonia	Hypotonia	Sever hypotonia and hyperextensible joints	ND	Mild hypotonia and hyperextensible joints	Mild hypotonia and hyperextensible joints	-	-	-
Developmental delay	+	+	+	+	-	+	+	+	+	+	+	-	+
Neuroimaging	Megalencephaly, Chiari I, Sylvius aqueduct stenosis, polymicrogyria and hipocampic nodular heterotopia	Septum pellucidum bIfida, asymmetry of cerebral hemispheres and hemimegalencephaly	Tonsillar herniation, Polymicrogyria, subependymal cyst	ND	Cavum septum pellucidum and thin corpus callosum	Left hemimegalencephaly, Chiari I, polymicrogyria, arachnoid cyst on left temporal fossa	Chiari I	ND	Asymmetry of cerebral hemispheres and periventricular white matter alterations	Megalencephaly	Ventriculomegaly	Hydrocephaly, cerebral cavernous malformation	Ventriculomegaly, periventricular white matter alterations and cerebral asymmetry
Other	-	-	Single umbilical artery, polyhydramnios, neonatal hyperglycemia	Undescended testes	-	Normal levels of alpha fetoprotein (AFP) and B-chorionic gonadotropin (BHCG)	Hypocalcemia, hypomagnesemia, hypophosphatemia, PTH deficit and normal levels of AFP and BHCG	-	-	-	-	-	-
karyotype	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XX	46,XX	46,XY	46,XY	46,XY

Table II. Genes with CNVs not described before

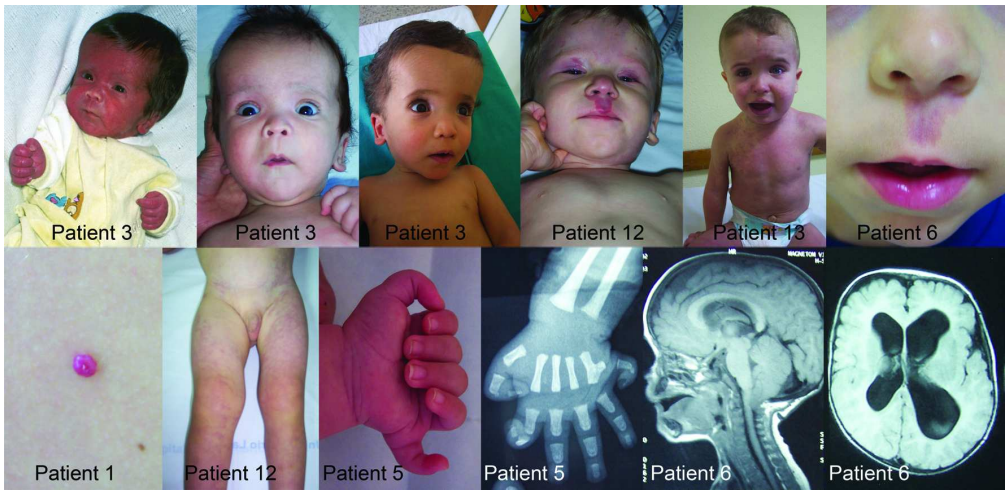
Gene Symbol	Name	Genomic Region	Affected Samples	Gain / Loss
DEFB126	defensin, beta 126	20p13	2	G
DEFB127	defensin, beta 127	20p13	2	G
AGAP1	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1	2q37	1	G
NCK1	NCK adaptor protein 1	3q21	1	G
TMEM22	transmembrane protein 22	3q22.3	1	G
ARID1B	AT rich interactive domain 1B (SWI1-like)	6q25.1	1	G
OCM2	oncomodulin 2	7q21.2	1	G
ASNS	asparagine synthetase	7q21.3	1	G
LMTK2	lemur tyrosine kinase 2	7q21.3	1	G
ADK	adenosine kinase	10q11-q24	1	G
UCP2	uncoupling protein 2 (mitochondrial, proton carrier)	11q13	1	L
UCP3	uncoupling protein 3 (mitochondrial, proton carrier)	11q13	1	L
C2CD3	C2 calcium-dependent domain containing 3	11q13.4	1	L
KCNJ1	potassium inwardly-rectifying channel, subfamily J, member 1	11q24	1	G
RICS	Rho GTPase-activating protein	11q24-q25	1	G
KCNJ5	potassium inwardly-rectifying channel, subfamily J, member 5	11q25	1	G
CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit	12p13.3	1	G
ZFYVE1	zinc finger, FYVE domain containing 1	14q22-q24	1	G
RBM25	RNA binding motif protein 25	14q24.3	1	G
SHCBP1	SHC SH2-domain binding protein 1	16q11.2	1	G

Table III. Proposed Diagnostic Criteria

		Frequency
Major Criteria (requires 3)	Macrocephaly	96%
	Capillary malformation	97%
	Overgrowth/Asymmetry	97%
	Neuroimaging alterations: ventriculomegaly (76%), cavum septum pellucidum or cavum vergae (71%), cerebellar tonsillar herniation (69%), cerebral and/or cerebellar asymmetry (53%)	82%
Minor Criteria (requires 2)	Developmental delay	85%
	Midline facial capillary malformation	76%
	Neonatal hypotonia	68%
	Syndactyly or polydactyly	63%
	Frontal bossing	59%
	Connective tissue abnormality (e.g., joint hypermobility, hyperelastic skin)	58%
	Hydrocephalus	50%

Figure Legends

Figure 1**.** Clinical features: Macrocephaly (3, 12, 13), frontal bossing (3), overgrowth/asymmetry (3, 12, 13), capillary malformations on philtrum/upper lip (6, 12, 13), limbs (12) and trunk/abdominal (1, 3, 12, 13), polydactyly (5), tonsillar herniation (6) and hemimegalencephaly (6). Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.



150x72mm (300 x 300 DPI)

The OFD1 (CXORF5) gene at Xp22 is not mutated in a subset of Simpson-Golabi-Behmel syndrome patients.

Romanelli V, Magano L, Segovia M, Cosentino V, González- Meneses A, del Campo M, Pérez Jurado LA, Giovannucci Uzielli ML, Soler V, Sanchis A, Arias P, Incera I, Gracia Bouthelier R, Lapunzina P.

Current Topics in Genetics. 2008. 3:45-47.

El gen *OFD1 (CXORF5)* en Xp22 no está mutado en un conjunto de pacientes con síndrome de Simpson-Golabi-Behmel.

El síndrome de Simpson-Golabi-Behmel (SGBS) es un síndrome de sobrecrecimiento ligado al X debido a alteraciones del gen *GPC3,* ubicado en la región Xq26. Los defectos descritos hasta ahora son deleciones o mutaciones que llevan a la ausencia de la proteína o a la generación de una forma truncada de la misma. De todas formas, no todos los individuos con diagnostico clínico de SGBS presentan mutaciones en *GPC3*, eso lleva a la hipótesis que exista otro locus en el cromosoma X responsable de este síndrome.

Recientemente han sido reportados algunos casos de una forma más severa de SGBS, denominada SGBS2. El estudio de estos pacientes llevó a la identificación de un nuevo gen responsable, el *OF*D1 de la región Xp22. Este gen ya ha sido definido como responsable del síndrome orofacial-digital de tipo I y codifica para una proteína de función desconocida.

En este trabajo hemos analizado 27 pacientes con SGBS a través de MLPA, para detectar deleciones/duplicaciones de *GPC3* y *GPC4* y secuenciación bidireccional del gen *GPC3*, para las mutaciones puntuales. Entre estos pacientes hemos encontrado 9 con anomalías de *GPC3* y hemos analizado los otros 18 por secuenciación bidireccional de *OFD1*. Los resultados obtenidos demuestran que ninguno de los pacientes con SGBS sin diagnostico molecular llevan defectos en *OFD1*, dejando abierta la posibilidad que exista otro locus ligado al X responsable en estos casos de la patología.

Il gene *OFD1 (CXORF5)* in Xp22 non é mutato in un gruppo di pazienti con sindrome di Simpson-Golabi-Behmel.

La sindrome di Simpson-Golabi-Behmel (SGBS) é una sindrome di ipercrescimento X-linked dovuta ad alterazioni del gene *GPC3*, localizzato nella regione Xq26. I difetti descritti finora sono delezioni e mutazioni che portano allįassenza della proteina o alla generazione di una forma tronca. In ogni modo, non tutti gli individui con diagnosi clinica di SGBS presentano mutazioni in GPC3, ció porta a ipotizzare l'esistenza di un altro locus nel cromosoma X responsabile di questa sindrome.

Recentemente sono stati riportati alcuni casi di una forma piú grave di SGBS, denominata SGBS2. Lo studio di questi pazienti portó all'identificazione di un nuovo gene responsabile, il *OFD1* della regione Xp22. Questo gene giá é stato defnito responsabile della sindrome orofaciodigital di tipo I e codifica per una proteina di funzione sconosciuta.

In questo lavoro abbiamo analizzato i nostri 27 pazienti con SGBS attraverso MLPA, per individuare delezioni/duplicazioni di *GPC3* e *GPC4* e sequenziamento bidirezionale del gene *GPC3*, per le mutazioni puntiformi. Tra questi pazienti abbiamo ne abbiamo trovati 9 con anomalie di *GPC3* e abbiamo analizzato gli altri 18 con sequenziamento bidirezionale di *OFD1*. I risultati ottenuti dimostrano que nessuno dei pazienti SGBS senza diagnosi molecolare presentano difetti in *OFD1*, quindi resta aperta la possibilitá che esista un altro locus X-linked responsable in questi casi della patologia.

Current Topics in Genetics

Original Communication

The *OFD1 (CXORF5)* gene at Xp22 is not mutated in a subset of Simpson-Golabi-Behmel syndrome patients

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ABSTRACT

Simpson-Golabi-Behmel syndrome (SGBS, MIM #312870) is an X-linked overgrowth disorder comprising multiple congenital anomalies and increased risk for embryonal tumors, mainly Wilms tumor, hepatoblastoma and neuroblastoma [1-6]. It is characterized by pre- and postnatal overgrowth, visceral and skeletal anomalies, coarse face, macroglossia, supernumerary nipples, congenital heart defects and hypotonia. In some SGBS patients mutations/deletions of *GPC3* localized at Xq26 have been observed [9-11]. We have evaluated 27 patients from the Spanish Overgrowth Syndrome Registry (who fulfil clinical criteria for SGBS) for both mutations and deletions of the *GPC3* and deletions of *GPC4* using a combination of MLPA and direct sequence analysis. Among them we found 9 patients with abnormalities on the *GPC3* gene and 18 patients who did not have any aberration on these genes. This result raised the possibility that those patients might have mutations of the *OFD1* gene at the Xp22 region, so we conducted a mutation analysis of this gene through direct bi-directional sequencing of the 23 exons using specific primers and comparing with the published sequence. We found that none of the 18 patient presented deleterious mutations of the *OFD1* gene. Our work confirms that a substantial proportion of patients with SGBS phenotype still remains without a molecularly proven cause of the disease.

KEYWORDS: overgrowth, MLPA, direct sequencing

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INTRODUCTION

Simpson-Golabi-Behmel syndrome (SGBS, MIM #312870) is an X-linked overgrowth disorder comprising multiple congenital anomalies and increased risk for embryonal tumors, mainly Wilms tumor, hepatoblastoma and neuroblastoma [1-6]. It is characterized by pre- and postnatal overgrowth, visceral and skeletal anomalies, coarse face, macroglossia, supernumerary nipples, congenital heart defects and hypotonia [7]. The

spectrum of clinical manifestations is broad, varying from very mild forms in carrier females to infantile lethal form in affected males [8]. Mutations/ deletions of *GPC3* localized at Xq26 have been observed in some SGBS patients [9-11]. Most reported *GPC3* mutations lead to the absence or truncation of the *GPC3* protein indicating that "classical" cases of SGBS are likely due to loss of function of *GPC3* [12], although with apparent lack of correlation between the genetic anomaly and the phenotypic expression of the disease, as noted by some authors [11].

However, the fact that not all individuals with clinical diagnosis of SGBS had abnormalities of the *GPC3* gene, raised the possibility that other loci on the X chromosome might be responsible for a proportion of individuals with this syndrome. Terespolsky *et al.* [8] presented 4 maternally-related male cousins with a severe variant of SGBS, characterized by multiple congenital anomalies, hydrops fetalis, and death within the first 8 weeks of life, and proposed that these cases may be genetically distinct to the classical SGBS. In 1999, Brzustowicz *et al.* [13] re-evaluated these patients using 25 tandem-repeat polymorphic markers spanning the X chromosome and localized the gene for this disorder to a ~6Mb region of Xp22. This finding leads to some authors to call this type of SGBS as the lethal form of the disorder or SGBS2 (MIM #300209). Budny *et al.* [15] recently reported a large Polish family in which 9 males presented phenotypic similarities to both the family originally described by Terespolsky *et al.* [1995] and the classical SGBS phenotype. The former authors found a 4-bp duplication in the *OFD1* gene in 2 affected males and in all obligate female carriers of this family. Mutations in this gene had been previously described in oral-facial-digital type I syndrome [15]. The *OFD1* gene encodes a predicted protein of unknown function containing a large number of coiled-coil domains, typically present in a variety of different molecules, from fibrous proteins to transcription factors. They showed that the *OFD1* cDNA is ubiquitously expressed, undergoes alternative splicing, and escapes X chromosome inactivation.

PATIENTS AND METHODS

We have evaluated 27 patients from the Spanish Overgrowth Syndrome Registry who fulfil clinical criteria for SGBS for both mutations and deletions of the *GPC3* and deletions of *GPC4* using a combination of MLPA (Multiplex Ligation dependent Probe Amplification; MRC-Holland, Kit P154) and direct sequence analysis. For those patients who did not present abnormalities on these genes we conducted a mutation analysis of the *OFD1* gene at the Xp22 region through direct bi-directional sequencing of the 23 exons using specific primers and comparing with the published sequence.

RESULTS AND DISCUSSION

Among the 27 SGBS patients evaluated, we found 9 patients with abnormalities on the *GPC3* gene [6, 16, 18] and 18 patients who did not have any aberration on these genes (Table 1, Figure 1). We

Table 1. Clinical findings in 18 SGBS patients negative for abnormalities in the *GPC3* or *GPC4* gene.

Clinical Findings	Number (%)
Overgrowth	18 (100)
Macrocephaly	16 (88)
Coarse face	16 (88)
Prominent forehead	14 (77)
Macroglossia	12 (66)
Hypotonia	12 (66)
Mental retardation	11 (61)
Grooved tongue	10 (55)
Crowded teeth	10 (55)
Extra nipples	10 (55)
Hepatosplenomegaly	8 (44)
Congenital heart defect	7 (38)
Broad, short hands	7 (38)
Polydactyly	6 (33)
Pectus excavatum	6 (33)
Herniae	5 (27)
Obesity	4 (22)
Autistic behavior	4 (22)
Enlarged kidneys	4 (22)
Abnormal columella	4 (22)
Cleft palate	4 (22)
Criptorchydism	3 (16)
Hydrocephalus	1 (5)
Capillary malformation	1 (5)

Figure 1. Phenotypic appearance of some patients with clinical diagnosis of SGBS.

hypothesized that these patients might have mutations of the *OFD1* gene at the Xp22 region so we conducted a mutation analysis of this gene.

We found that none of the 18 patients presented deleterious mutations of the *OFD1* gene. We observed two different intronic SNPs. One SNP (rs7878592; intron 9) was present in 17/18 patients and the other SNP (rs3815049; intron 11) was present in 8/18 patients. Both polymorphisms are located outside the codifying exons' sequence with no apparent pathogenic consequences.

Our findings demonstrate that the *OFD1* gene does not contribute to the pathogenesis of SGBS in our cohort of individuals with clinical diagnosis of this disorder and negative for *GPC3/GPC4* anomalies. This raises the possibility that other loci than *GPC3* and *OFD1* might be responsible of a proportion of patients with phenotype of SGBS or SGBS2.

Supported by Grants FIS 05/1217, ISCIII, Ministerio de Sanidad y Consumo, Spain, and the Fundación Mutua Madrileña del Automovilista (PL 2006).

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Germinal mosaicism in Simpson-Golabi-Behmel syndrome.

Romanelli V, Arroyo I, Rodriguez JI, Magano L, Arias P, Incera I, Gracia-Bouthelier R, Lapunzina P.

Clin Genet. 2007. 72: 384-386.

Mosaicismo germinal en el síndrome de Simpson-Golabi-Behmel.

El síndrome de Simpson-Golabi-Behmel (SGBS) es un síndrome de sobrecrecimiento ligado al X debido, en algunos pacientes, a mutaciones/deleciones del gen *GPC3* de la región Xp26.

En este trabajo reportamos una familia con una peculiar historia de casos de SGBS; los padres, jóvenes, sanos y no consanguíneos, tuvieron 4 embarazos. En el primero la madre dio a luz pre término a un niño con síntomas graves de SGBS que murió recién nacido (Paciente 1); luego nació un hermano sano; el tercer embarazo terminó con un aborto espontaneo y en el último nació otro hermano afecto de SGBS (Paciente 2) aunque con fenotipo menos severo que el primer hijo de la pareja.

Obtuvimos muestras de sangre periférica de los padres y del Paciente 2 y de tejidos en parafina del Paciente 1. Pudimos entonces descartar la presencia de deleciones de los genes *GPC3* y *GPC4* en los Pacientes 1 y 2 y detectar, por secuenciación bidireccional, la misma mutación sin sentido en *GPC3* en los dos hermanos. El hecho que la madre no presente la misma mutación de los dos hijos afectos, ni alguna otra, lleva a la conclusión que probablemente tenga un mosaicismo germinal para esta mutación.

Gracias a este estudio podemos añadir el SGBS al listado de síndromes ligados al X con mosaicismo germinal confirmado y evidenciar la importancia de tener en cuenta esta posibilidad en el consejo genético de las familias.

Mosaicismo germinale nella sindrome di Simpson-Golabi-Behmel.

La sindrome di Simpson-Golabi-Behmel é una sindrome di ipercrecimento X-linked dovuta, in alcuni pazienti, a mutazioni/delezioni del gene *GPC3* della regione Xp26.

In questo lavoro riportiamo una familia con una storia pculiare di casos di SGBS; i genitori, giovani, sani e non consanguinei, ebbero 4 gravidanze. Nella prima la madre ebbe un parto pretermine e diede alla luce un bamcon sintomi gravi di SGBS che morí nel periodo neonatale (Paziente 1); poi nacque un fratello sano; la terza gravidanza terminó con un aborto spontaneo e per ultimo nacque un altro fratello affetto da SGBS (Paziente 2) anche se con fenotipo meno grave del primo figlio della coppia.

Ottenemmo campioni di sangue periferico dei genitori del Paziente 2, del tessuto paraffinato del Paziente 1. Abbiamo cosí scartare lapresenzia di delezioni dei geni *GPC3* e *GPC4* nei Pazienti 1 e 2 e individuare, con sequenziamento bidirezionale, la stessa mutazione non-sense in *GPC3* nei due fratelli. Il fatto che la madre non presenti la stessa mutazione dei due figli affetti, né nessun'altra, ci porta alla conclusione che probabilmente abbia un mosaicismo germinale di questa mutazione.

Grazie a questo studio possiamo aggiungere la SGBS allal ista di sindromi X-linked con mosaicismo germinale confermato ed evidenziare l'importanza di tenere in conto questa possibilitá nella consulenza genetica delle famiglie.

Clin Genet 2007: 72: 384–386
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CLINICAL GENETICS
doi: 10.1111/j.1399-0004.2007.00871.x

Letter to the Editor

Germinal mosaicism in Simpson-Golabi-Behmel syndrome

To the Editor:

Simpson-Golabi-Behmel syndrome (SGBS, OMIM 312870) is an X-linked overgrowth disorder comprising multiple congenital abnormalities and increased risk for the development of embryonal tumours (1, 2), mainly Wilms' tumours, hepatoblastoma and neuroblastoma (1, 3–6). It is characterized by pre- and post-natal overgrowth, visceral and skeletal anomalies, coarse face, supernumerary nipples, congenital heart defects and hypotonia (7). The spectrum of clinical manifestations is broad, varying from very mild forms in carrier women to infantile lethal form in affected men (8). Mutations/deletions of *GPC3* localized at Xq26 have been observed in some SGBS patients (9, 10). Apparent lack of correlation between the extent of the deletions and the phenotypic expression of the disease was noted (11), leading to the conclusion that 'classical' cases of SGBS are likely due to loss of function of *GPC3* (12).

We report on two brothers with SGBS. Patient 1 was born pre-maturely (34 weeks) 23 years ago after a pregnancy complicated by polyhydramnios. Birth weight was 2050 g (75–90th centile). Parents were young, healthy and non-consanguineous. He had a coarse face, ambiguous external genitalia, abdominal distension, hypotonia, short fifth digits and a heart murmur [Fig. 1(**a**) and (**b**)]. Hydronephrosis, duodenal atresia and heart defect (atrial septal defect and tricuspid valve hypoplasia) were also found. The patient's clinical status worsened and finally died. Necropsy showed testes with adenomatous hyperplasia of the rete testis, Müllerian rests, bilateral hydronephrosis and nephroblastomatosis and adenomatous hyperplasia of rete testis. At the CNS, there was diffuse gliosis, isolated microcalcifications in the white matter and cerebellar cortical heterotopia. Karyotype was 46,XY. A diagnosis of multiple congenital anomalies/male pseudohermaphroditism of unknown cause and unknown recurrence risk was given to the family. Patient 2 is patient 1's brother and was the couple's fourth pregnancy. He had an older, healthy brother and his mother had had a spontaneous abortion in her third pregnancy. He was born by Cesarean section at 36 weeks after a pregnancy complicated by polyhydramnios and foetal macrosomia. Amniocentesis was 46,XY. Birth weight was 4500 g (>97th centile), length 54.5 cm (>97th centile) and OFC 36 cm (90th centile). He showed macrosomia, hypotonia, coarse face, macroglossia, macrostomia, short neck, single palmar crease, auricular tags, extra nipples, hypospadias, cryptorchidism, nephromegaly and anal atresia [Fig. 1 (**c**)–(**f**)]. After discharge, he was followed-up periodically by a paediatrician, who reported post-natal overgrowth, bilateral nephromegaly with cystic images in kidneys, liver and large, squared hands with short, stiff thumbs. His developmental milestones were normal, and he had no mental impairment.

DNA samples from peripheral blood were obtained for patient 2 and his parents from paraffin-embedded tissues from patient 1. Deletions of the *GPC3* and *GPC4* genes were evaluated through multiplex ligation-dependent probe amplification (13) using the SALSA P154 kit (MRC-Holland, Amsterdam, The Netherlands). Sequence analysis using the primers reported by Huber et al. (14) were performed by duplicate in different DNA extractions and experiments in all individuals.

Direct bi-directional sequencing of all *GPC3* exons in patient 2 showed a C → T transition (c.1605 C>T) in exon 4, leading to a CGA → TGA change (R387X). This change was also found in ancient DNA of patient 1, but was not observed in their mother (Fig. 2). Such evidence led us to the conclusion that she probably had germinal mosaicism for this mutation.

SGBS clinically resembles the Beckwith–Wiedemann syndrome (BWS) and Perlman syndrome (15). All three disorders display overgrowth, tumour predisposition and other occasional malformations. Patient 1 of this report showed coarse face, male pseudohermaphroditism with abnormal external genitalia and Müllerian rest, abdominal

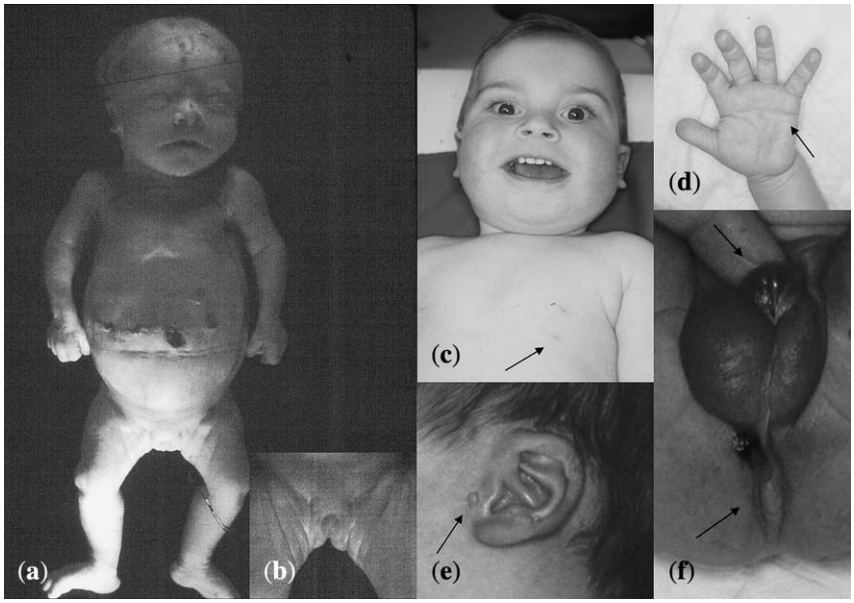

Fig. 1. Clinical appearance of patient 1 at birth. (**a**) Note coarse face, broad forehead, large mouth and ambiguous genitalia. (**b**) Detail of ambiguous genitalia. (**e**, **f**) Clinical findings in patient 2 at birth and (**c**, **d**) at 12 months. Note ear tag, coarse face, large mouth and tongue, hypospadias, anal atresia, single palmar crease and extra nipple (indicated by arrows).

distension, hypotonia, short fifth digits and heart disease; findings also observed in Perlman syndrome (15). Further, patient 1 and the child reported by Verloes et al. (16) were extremely similar with clinical overlap elements of SGBS, BWS and Perlman syndrome. Unfortunately, the latter patient had no molecular studies to confirm or rule out any disorder. A similar case has been reported by Li et al. (17) who studied a family with two maternal male cousins and their mothers, both men diagnosed as having SGBS with a 3–5 deletion of *GPC3*. However, no such deletion was detected in the grandparents, evidencing the possibility of germinal mosaicism of the grandmother for this deletion.

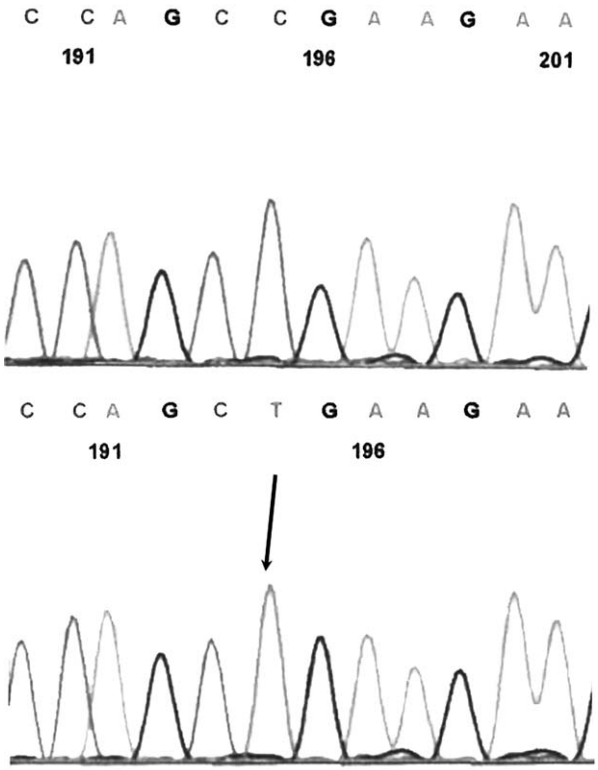

Fig. 2. Partial sequence analyses of the *GPC3* gene analysis in DNA from blood lymphocytes in the mother (normal, top), and from patient 2 (affected, bottom). The arrow shows the position of the C to T transition (c. 1605 C>T) in exon 4.

The family reported here adds another X-linked syndrome to the growing number of X-linked genetic disorders with proven germinal mosaicism (Duchenne, X-linked ichthyosis, Rett syndrome, Coffin–Lowry syndrome, X-linked agammaglobulinemia, Lowe syndrome, X-linked myotubular myopathy, Hunter syndrome, X-linked severe combined immunodeficiency and haemophilia). It also has important connotations for genetic counselling when assessing families with this rare overgrowth disorder, as the actual frequency of germinal mosaicism for SGBS is not known.

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Adults with Sotos syndrome: Case Report of the Oldest Person

and Review of 20 New Patients

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Am J Med Genet. Submitted.

Adultos con síndrome de Sotos: Informe del caso de la persona más mayor y revisión de 20 nuevos pacientes.

El síndrome de Sotos (SS) es un síndrome de sobrecrecimiento con anomalías congénitas múltiples bien caracterizadas, como macrocefalia, alta estatura, rasgos faciales distintivos y dificultad de aprendizaje de severidad variable. La causa genética de este síndrome se identifica en la haploinsuficiencia del gen *NSD1*.

El trabajo presentado es el informe clínico detallado de una paciente con SS diagnosticado genéticamente a los 63 años; se retiene que sea la persona más mayor descrita hasta la fecha con este trastorno. Para complementar las informaciones relativas a este caso, se han revisado los pacientes ya reportados en literatura y los con diagnostico clínico y molecular de SS del Registro de Síndromes de Sobrecrecimiento de nuestro hospital.

El análisis se ha limitado a 20 pacientes de los 27 totales, eligiendo sólo los con diagnostico molecular de mutación (14/20) o microdeleción (6/20) en el gen *NSD1*. La media de edad es de 25 años y las características más comunes encontradas son: dificultad de aprendizaje (18,90%), escoliosis (11,55%), problemas en los ojos (9,45%), problemas psiquiátricos (7,35%) y anomalías en las imágenes celébrales (6,30%).

Gracias a este preliminar estudio de casos de pacientes adultos, podemos sugerir modestos ajustes a sus generales necesidades de asistencia médica. Se aconseja entonces un examen oftalmológico anual justificado por la alta incidencia de enfermedades oculares y también un control regular de los trastornos psiquiátricos y de la depresión. Finalmente retenemos razonable una vigilancia del cáncer apropiada con la edad ya que no podemos confirmar un mayor riesgo de desarrollar tumores en los pacientes con síndrome de Sotos.

Adulti con sindrome di Sotos: Referto del caso de la persona piú anziana e revisione di 20 nuovi pazienti.

La sindrome di Sotos (SS) é una sindrome di ipercrecimiento con anomalie congenite multiple ben caratterizzre, come macrocefalia, elevata statura, lineamenti facciali distintivi e difficoltá di apprendimento di sveritá variabile. La causa genetica di questa sindrome si identifica nell'aploinsufficienza del gene *NSD1*.

Il lavoro presentato é il referto clinico dettagliato di una paziente con SS diagnosticado geneticamente ai 63 anni; si ritiene que sia la persona piú anziana descritta finora con questo disturbo. Per completare le informazioni relative a questo caso, sono stati revisionati i pazienti giá descritti in letteratura e quelli con diagnosi clinico e molecolare di SS del registro di Sindromi di Ipercrescimento del nostro ospedale.

L'analisi é stato limitato a 20 pazienti dei 27 totali, scegliendo solo quelli con diagnosi molecolare di mutazione (14/20) o microdelezione (6/20) del gene *NSD1*. L'etá media é di 25 anni e le caratteristiche piú comuni riscontrate sono: difficoltá di apprendimento (18,90%), scoliosi (11,55%), problemi agli occhi (9,45%), problemi psichiatrici (7,35%) e anomalie delle immagini celebrali (6,30%).

Grazie a questo studio preliminare di casi di pazienti adulti, possimo suggerire modesti adattamenti alla loro generale necessitá di assistenza medica. Si consiglia quindi un esame oftalmologico annuale giustificato dalla alta incidenza di malattie oculari e anche un controllo regolare dei disturbi psichiatrici e della depressione. Infine riteniamo ragionevole una vigilanza del cancro appropriata all'etá visto che non possiamo confermare un maggior rischio di sviluppare tumori nei pazienti con sindrome di Sotos.

Research Article

Adults with Sotos Syndrome: Case Report of the Oldest Person and Review of 20 New Patients

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ABSTRACT

Sotos syndrome is a well-described multiple anomaly syndrome characterized by overgrowth, distinctive craniofacial appearance and variable learning disabilities. The diagnosis of Sotos syndrome relied solely on these clinical criteria until haploinsufficiency of the *NSD1* gene was identified as causative. We describe a 63-year-old woman with classic features confirmed by a pathogenic *NSD*1 mutation who we believe is the oldest person reported with Sotos syndrome. She is notable for the diagnosis of Sotos syndrome late in life, mild cognitive limitation, and chronic kidney disease attributed to fibromuscular dysplasia for which she recently received a transplant. She has basal cell and squamous cell carcinoma which her lifetime of sun exposure and fair cutaneous phototype are viewed as risk factors. We also reviewed the literature (n = 10) about adults with Sotos syndrome, and studied patients ascertained in the Spanish Overgrowth Syndrome Registry (n = 15). Analysis was limited to 20/26 (77%) total patients who had molecular confirmation (14 with a mutation, 6 with a microdeletion). With a mean age of 25 years, the most common features were learning disabilities (90%), scoliosis (55%), eye problems (45%), psychiatric issues (35%) and brain imaging anomalies (30%). Learning disabilities were more severe in patients with a microdeletion than those with a point mutation. From this small study with heterogeneous ascertainment, we suggest modest adjustments to their general healthcare needs. Although this series includes neoplasia in 4 cases, this should not be interpreted as incidence. We think it is important that age-appropriate cancer surveillance should be maintained.

Keywords: adults with genetic disorders; familial gigantism; overgrowth syndrome; *NSD1*, Sotos syndrome; transitional care,

INTRODUCTION

In the 45 years since Sotos et al. [1964] reported five children at the Massachusetts General Hospital with a distinctive overgrowth syndrome, more than 400 cases of Sotos syndrome have been reported [Baujat et al., 2007; Tatton-Brown et al., 2009]. The cardinal features include rapid early growth, advanced bone age, with head circumference and height >2 SD above the mean, a typical craniofacial gestalt (macrocephaly, high, prominent forehead, sparse frontal hairline, inverted pear-shaped face, pointed chin), and learning disabilities ranging from mild to severe [Allanson and Cole, 1996; Cole, 2005; Tatton-Brown et al., 2009]. Although these features have been well-described throughout childhood, little is known about the identification, clinical manifestations and management of individuals diagnosed with Sotos syndrome later in life.

The diagnosis of Sotos syndrome relied solely on clinical criteria until haploinsufficiency of the *NSD1* (nuclear receptor SET domain-containing protein) gene was identified as causative [Kurotaki et al., 2002]. The *NSD1* gene is located at 5q35, the site of a common microdeletion in Japanese patients [Kurotaki et al., 2003], whereas non-Japanese patients are more likely to have a point mutation in *NSD1* [Douglas et al., 2003]. NSD1 is known to encode a histone methyltransferase involved in chromatin regulation whose epigenetic effects not only cause Sotos syndrome, but may be implicated in sporadic neuroblastoma [Berdasco et al., 2009]. The facial appearance changes with time - specifically, as the face lengthens the forehead becomes less prominent and the mandible and chin become the dominant facial features [Allanson and Cole, 1996]. This alteration in facial gestalt, tall stature, and lack of awareness of the disease among adult physicians, makes “possible Marfan syndrome” an occasional referring diagnosis [Maves et al., 2007]. The differential diagnosis of Sotos syndrome in adulthood also includes

Weaver syndrome, Simpson-Golabi-Behmel syndrome and Gorlin syndrome. Caring for adults with genetic syndromes is increasingly recognized as an important part of transitional care and internal medicine, but guidelines for screening and managing individuals with Sotos syndrome are not available [Cole, 2005].

We report a 63 year old woman who may be the oldest person with molecular confirmation of Sotos syndrome. In addition to this well-characterized individual, we provide information about patients in the literature and the Spanish Overgrowth Syndrome Registry. From this data we propose several recommendations for health supervision.

CLINICAL REPORT

A 63-year-old white woman with tall stature was referred by the nephrology service for evaluation of possible Marfan syndrome. The past medical history was provided by the patient's sister, her "guardian" since their mother had severe Alzheimer's disease, and their father was living out of state; over the period of this evaluation and manuscript submission, both parents have died. The patient was the first of four pregnancies (three female) delivered by cesarean-section and not known to be premature. She was described by her father as "spastic" at birth, referring to contractures. From early childhood, she was developmentally delayed, and had positional lower extremity deformities (pes planus) for which she wore braces. The patient had "fluid tapped from the brain," which may have been for presumed "hydrocephalus" since she had a pneumoencephalogram. Medical records refer to a "tumor in her chest" which was later found to be an enlarged thymus. She resided at a private school for persons with various learning disabilities, though she was aware they were more disabled than herself. During her childhood; she reports with insight and sadness that she was considered "retarded."

She eventually completed high school, attended a vocational school for three years, and lived in New England with her maternal aunt. In addition to this self-reported history from the patient and her sister, the family album (Fig. 1) provided a rich photographic natural history of a healthy appearing girl with a distinct facial appearance (large head, thin hair, high forehead, "pear-shaped" face, prominent chin, wide-spaced eyes) , tall stature and long limbs compared to siblings.

In her third decade of life the patient had a bowel obstruction. Surgery for a poorly defined indication included a hysterectomy and oophorectomy. The patient enjoyed many outdoor activities and lived in Bermuda for eight years prior to moving to Southern California where she was told that she had features of Marfan syndrome. When she was in her 30's, she was diagnosed with anemia and hypertension which led to a diagnosis of renal disease. She was evaluated at a New York City medical center where a renal artery biopsy showed fibromuscular dysplasia treated with a right renal artery bypass with a vein graft. Subsequent angiography showed a "diminutive left upper pole with fibromuscular dysplasia and patent right renal bypass." She underwent a cholecystectomy at that time as well. The patient transferred care to Massachusetts General Hospital in 2005 for treatment of renal insufficiency, which was viewed as chronic kidney disease, stage V. She underwent a successful deceased donor kidney transplant at age 63 years.

Her tall stature and habitus had been described by others as "Marfanoid". Subsequent echocardiogram showed a normal aortic diameter, without mitral valve prolapse, and mild aortic insufficiency. When examined in the genetics clinic, she was a well-groomed, tall and thin white female with stooped posture who moved slowly. She was quietly engaging, speaking in a soft voice. She appeared older than her stated age, frail, with mild jaundice.

Her weight was 64.1kg (75th percentile), height 177.4cm (>97th percentile), upper: lower segment ratio 1.03, BMI 20.4, head circumference 59 cm (>97th percentile). She was Fitzpatrick phototype 1 given her red hair, light eyes, fair skin, and history of easy sunburning without the ability to tan. Her skin was extensively photodamaged and significant for several hyperkeratotic erythematous papules and plaques over the dorsum of her hands, neck and face. Her facial appearance was striking for thin hair, receded frontal hairline, high forehead with prominent supra-orbital brow bone, large deep-set eyes, pear-shaped face, malar hypoplasia, small pointed chin, and a small nose with a tiny nasal tip. She had mild kyphoscoliosis, long hands and middle fingers without arachnodactyly, and mild diffuse contractures of the hands. There was diminished range of motion at the elbows and wrists, mild knee contractures, and pes planus. She had a mask-like facial appearance with reduced facial expression. She was oriented, conversant, and had a reasonable fund of knowledge and dry sense of humor.

Subsequent dermatology consultation diagnosed multiple basal cell and squamous cell cancinomas along with many actinic keratoses. Examination of the eyes did not show ectopic lentis. Chromosome analysis showed 46XX, and plasma homocysteine level was normal. Because of the compelling photographs from childhood supporting the physical examination, Sotos syndrome was considered a likely diagnosis. Subsequent *NSD1* testing performed at the University of Chicago detected a change at IVS9+3_+6delGAGT. The same mutation has been seen in two patients with Sotos syndrome [Tatton-Brown et al., 2005]. The test also revealed a known polymorphism, c.6903G>C which does not lead to an amino acid change (Gly2301Gly) [Douglas et al., 2003].

MATERIALS AND METHODS

Medline searches of the English language were completed using the terms “Sotos syndrome”, “Cerebral gigantism”, and “Adults and genetic disease”, and reviewed for adult (18 years and older) patients.

The Spanish Overgrowth Syndrome Registry (IRB number CEIC-HULP PI446), was established in 2003 as a national registry with all regions in Spain participating. At present, 236 of 1500 total entries in its database refer to “Sotos syndrome” , with confirmation by clinical information, pictures and molecular tests for *NSD1* in approximately half (n=112). Sixteen of 112 (14%) individuals were older than 18 years (one patient died at age 38 yrs).

RESULTS

Combining this new patient, 15 Spanish Overgrowth Registry patients and 10 literature cases [Halal, 1982; Cole, et al., 1992; Koenekoop, et al., 1995; Yen, et al., 2000; Inoue, et al., 2000; Cefle, et al., 2002; Compton, et al., 2004; Tatton-Brown, et al. 2005; Martinez-Glez, et al., 2007], there were a total of 26 cases who met clinical diagnostic criteria; 20/26 (77%) had molecular confirmation whose data form the basis of our analysis as presented in Table I and the text. There were more males than females (1.6:1) and the mean age was 26.6 years old (range 18-63). A “classic” presentation (at least three of the following characteristics: macrocephaly, tall stature, learning disabilities, advanced bone age and characteristic craniofacial appearance) was present in 70%

Growth and Development

In most (12/20, 60%) patients, the adult height was greater than the 90th percentile and all had head circumferences above the 97th percentile (with most measurements reported during adulthood). As expected, learning disabilities occurred in most patients, ranging from mild to

severe. Formal psychometric testing was not provided to define the degree of cognitive limitation.

Craniofacial

Photographs were available for most cases (5/25, 25% had serial photos) and were reviewed by M. R. F., P.L, and A E. L. to verify the facial appearance of Sotos syndrome. Seven patients met all four cardinal criteria as previously described [Cole and Hughes, 1996]. Formal anthropometric analysis of these non-standardized images was not possible, but limited qualitative analysis noted that the chin became less prominent, the malar hypoplasia and prominent forehead with a receded hairline persisted [Fig. 2].

Neuropsychiatric

Neuroimaging information on 30% of patients showed midline changes including cavum septum vergae and ventriculomegaly, two who were reported to have cerebral atrophy. Seizures were rare (2/20, 10%). A psychiatric diagnosis was reported in 35%, including affective disorders (2), hyperactivity (2), social isolation (2), and anxiety (1). One patient reported in the psychiatric literature [Compton, 2004] presented with psychosis.

Tumors

Three adults from the literature with cancer included retinoblastoma (*RB1* negative) [Martinez-Glez, 2007], small cell carcinoma [Cole, 1992] and acute lymphoblastic leukemia [Fryer, personal communication]. Our patient had numerous non-melanoma skin cancers and pre-cancers, the precise age of onset is unclear, approximately sixth decade. The skin cancers were excised without complication and the actinic keratoses were treated with cryotherapy. The formation of these lesions was attributed to her fair skin and history of extensive, intense sun exposure.

Other problems

Various eye problems occurred in almost half of the adults with Sotos syndrome, most commonly strabismus and myopia. Three cases from the ophthalmology literature [Koenekoop, et al., 1995; Yen, et al., 2000; Inoue, et al., 2000] reported more severe abnormalities including glaucoma, bilateral nuclear cataracts, optic disk pallor, retinal atrophy, megalocornea, and megalophthalmos, but the lack of strong clinical description or molecular confirmation makes the observations less reliable.

Musculoskeletal problems included scoliosis in 55%, in which the severity was not inconsistently reported, and pes planus in 2 patients. Three genotyped patients had with assorted renal anomalies (unilateral renal agenesis, nephrolithiasis and fibromuscular dysplasia) and one additional clinically diagnosed patient had with autosomal dominant polycystic kidney disease [Cefle, 2002]. Endocrine, pulmonary and cardiovascular problems were uncommon, occurring in fewer than three subjects each.

Genotype/Phenotype Correlations

Twenty subjects (one new patient, 15 from the Spanish Overgrowth Syndrome Registry, and 4 literature cases) had information about molecular analysis. Of those, there were 14 point mutations and 6 microdeletions. Most were males (60%) whose average age was 25 years old. Ninety percent (19/20) met at least two cardinal criteria and had a pathogenic *NSD1* alteration. Similar medical conditions were noted between the genotyped cohort and the entire sample (data not shown). However, Sotos syndrome patients with a microdeletion reported more severe learning disabilities than those with point mutation.

DISCUSSION

This woman in her 7th decade would be the oldest known person with Sotos syndrome adding valuable information to the natural history of this syndrome. We make several cautious observations about the clinical phenotype, acknowledging that this was a case report, with a cross-sectional physician reported survey and modest literature review...

Although behavioral problems are well characterized in childhood [Rutter and Cole, 1991] less is known about psychiatric disease in adults with Sotos syndrome [Cole in Allanson, 2005]. Further studies are needed to verify the incidence and define the types of these disorders. Some adults with Sotos syndrome have been noted to be "socially isolated" [Cole in Allanson, 2005 who noted limited data] due to social difficulties, medical illness, physical differences (overgrowth), or autism [Buxbaum, et al 2007, Morrow, 1990]. Further confounding may have occurred from misclassification as "retarded". Any of these features may lead to the reported social difficulties of withdrawal or withdrawing and isolating personality styles [Tantum et al., 1990]. We noted similar midline abnormalities as reported by Schaefer, et al [1997] and Horikoshi and Kato [2006]. Future research should correlate these to psychiatric diagnoses.

The only genotype-phenotype correlation seen in this study was the finding that patients with microdeletions have more severe learning disabilities than those with point mutations as described previously [Tatton-Brown et al., 2005]. Similar genotype/phenotype correlations are seen in the psychological profiles of children with Sotos syndrome: those with NSD1 point mutations have fewer total behavior problems, an easier temperament, and fewer internalizing behaviors [de Boer et al., 2005]. Our review did not have access to details about educational or vocational status. A previous survey using telephone interviews of adults in the Sotos Syndrome Support Association reported that adults with Sotos syndrome are often described as "socially

isolated" [Anderson and Schaefer, 2000]. Although most patients in this study were employed at least part time, 63% (10/16) lived with their parents and 31% (5/16) had significant psychiatric illness [Anderson and Schaefer, 2000].

Two of the patients in the Spanish Registry have unrelated renal anomalies, i.e. unilateral renal agenesis which can be due to an early embryonic defect, and nephrolithiasis which is usually metabolic in origin. Our new patient has fibromuscular dysplasia, which is viewed as a vascular anomaly rather than intrinsic renal disease. The additional clinically diagnosed case with autosomal dominant polycystic kidney disease would be attributed, theoretically, to the known genetic etiology of a mutation in polycystin-1 (*PKD1*, 16p13.3) and polycystin-2 (*PKD2*, 4q21). These disparate diagnoses imply that renal disease is unlikely due to the *NSD1* mutation, but additional cases will be needed to determine if a pattern exists.

The risk of neoplasia in Sotos syndrome has been a point of controversy for some time [Cohen, 1999; Tatton-Brown et al., 2009]. The four patients in this study have a variety of neoplasia including squamous and basal cell carcinoma, pre B-cell acute lymphoblastic leukemia, small cell lung carcinoma and retinoblastoma. Our patient has a lifelong history of sun exposure and an age-appropriate onset of cutaneous neoplasms with a high population incidence; therefore, it continues to be difficult to draw any firm conclusions regarding tumor risk in Sotos syndrome.

Guidelines for Adult Health Maintenance

Based on an apparent increased incidence of ophthalmologic disease, we recommend an ophthalmology exam at baseline for all adults with Sotos syndrome. Since nearly half of the subjects had psychiatric disease regular screening for depression and other common psychiatric disorders is advised. The risk of tumorigenesis remains unclear. In the absence of a predictable

timing or pattern of these tumors, a targeted health maintenance recommendation cannot be made. Paying rigorous attention to age-adjusted cancer screening is necessary. Attention throughout life should be given to postural scoliosis and contractures, since this appears to persist into adulthood.

LIMITATIONS AND STRENGTHS

This study is an initial survey of adults with Sotos syndrome. We attempted to interpret the information derived from the literature cases, acknowledging the bias inherent. Patients from the Spanish Overgrowth Syndrome Registry were reported by individual physicians, and they represent an inherent ethnic disposition, data which may not be generalizable to all patients with Sotos syndrome. Although the characterization of one well-described patient cannot be used as a guideline for all patients with this syndrome, our patient offers a positive glimpse into many aspects of the life of the older individual.

CONCLUSIONS

The transition of children with complex diseases to adult care is an important part of adult medicine. Older patients with Sotos syndrome have an distinctive appearance, neuropsychological issues, musculoskeletal features, and medical problems, but, the rarity of the condition, and the lack of information may make their care a challenge to practitioners. We hope that this preliminary report provides the foundation for larger, prospective studies.

ACKNOWLEDGEMENTS

We thank the parent and professional leaders of the Sotos Syndrome Support Association (SSSA) for their support. We also thank Drs. Rebecca Anderson, Bradley Schaefer, Trevor Cole, Alan Fryer, and Darrel Waggoner for thoughtful discussion. Finally, we dedicate this to the subject of the report and her supportive family.

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LEGENDS

Fig. 1. A photographic natural history of Sotos syndrome shows the new patient as a child, young girl and currently, at 63 years. She has features which are characteristic of Sotos syndrome including tall stature, typical facial appearance (high forehead, small chin), and those which can be associated with Sotos syndrome and aging, i.e. diffuse contractures, wrinkled skin.

Fig. 2. Serial photographs of a Spanish male with Sotos syndrome. Note that the chin becomes less prominent, while the high forehead with a receded hairline and the malar hypoplasia persist.

Figure 1.

Figure 1.

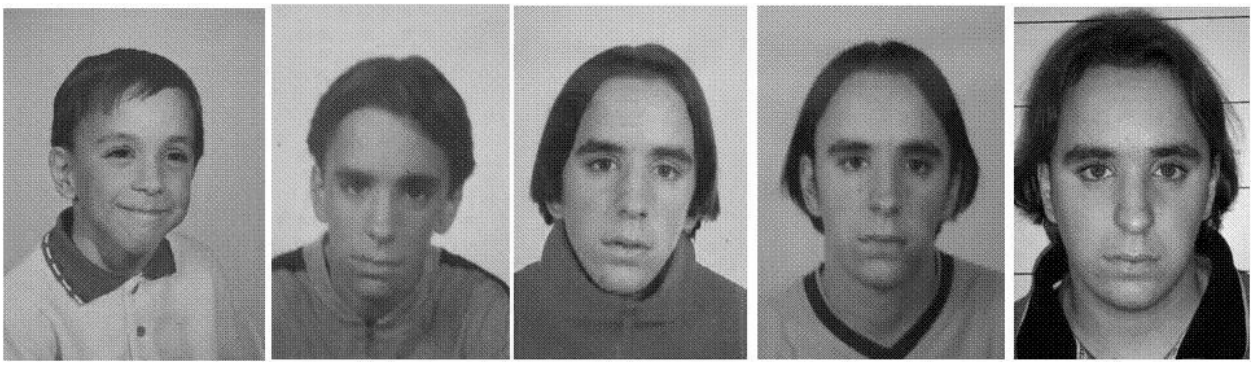

TABLE I. Clinical and Molecular Characteristics of 20 Adults (*NSD1* positive) with Sotos Syndrome (New Patient 1, Literature 4, Spanish Overgrowth Syndrome Registry 15) [1]

	Pathogenic Change			Personal Characteristics			Neurocognitive Features			Clinical Features			
Pt #	Nucleotide Change	Amino Acid Change	Micro-deletion 5q35	Sex	Age Yrs	Sotos Score[2]	CT/MRI findings	Learning Disability	Psychiatric diagnosis	Eye Problems	Renal Disease	Scoliosis	Tumors
Present Case	IVS9+3_+6delG AGT			F	63	1-2-3	VM Thinned corpus callosum	+ Mild	Social isolation	Cataract 63 yrs.	ESRD due to FMD. Deceased Donor Transplant	+ Kypho-scoliosis	BCC, SCC
A. Fryer in Lapunzina (2007)	2493de IG exon 5			M	30	1-2-3		+				+	ALL
Cole et al., [1992]	*			F	22	1-2-3-4		+		-			Small cell lung carcimoma
Study ID 709	c. 66605G>T	C2202S		F	63	1-3		Very mild	Depression			+	
Study ID 197	C6442delAGCG ACCA	K2151fs		M	31	1-4		-	-	Myopia, cataracts, retinal detachment	Nephro-lithiasis		
Study ID 55	c.2239insT	T747fs		M	22	1-2-3-4	CSV	Mild	Altered sociability	Strabismus		+	
Study ID 56	c.1318C>T	R440X		M	20	1-3-4		Moderate	-	-		+	
Study ID 140	2386del AAAG	E796fs		M	18	1-2-3-4		Very mild	-	-		+	
Study ID 166	c.5738A>G	N1913S		M		1-3-4	VM, CSV, left hemisphere atrophy	Moderate	Hyperactivity	-			
Study ID 583	c.4034insT	E1346fs		M	18	1-2-3-4		Moderate		-			
Study ID 54	c.4151insA			M	21	1-2-3-4	Cavum septum pellucidum	Moderate	Panic disorder	Strabismus, myopia		+	
Study ID 165	c.3680T>G	L1227X		M	24							+	
Study ID 233	c.3091C>T	R1031X		F	18	1-2-3		Moderate dyslexia				+	
Study ID 254	c.3546delCT	S1183fs		F	27	1-3	VM	Mild		Strabismus			

Tatton-Brown et al., [2005]		+	NS	20	1-3		"+++"		NS		+		
Tatton-Brown et al., [2005]		+	NS	24	1-3-4		"+++"		NS				
Study ID 606		+	F	19	2-3		Mild		Strabismus, cataract, retino-blastoma				Retino-blastoma[3]
Study ID 40		+	M	28	1-2-3-4		Severe	Depression, agoraphobia	Strabismus	Left renal agenesis	+		
Study ID 263		+	F	20	1-3-4		Severe	Hyperactivity	Proptosis				
Study ID 368		+	M	18	1-2-3-4	Brain atrophy	Severe		Myopia, strabismus				
Total N (%)	14/20 (70)	6/20 (30)	M 11/18 (61)	Mean 25	3 or more 14/20 (70)	6/20 (30)	18/20 (90)	7/20 (35)	9/20 (45)	3/20 (15)	11/20 (55)	4/20 (20)	

[1]Adapted from Baujat, et al. 2005. Clinical and Molecular Overlap in Overgrowth Syndromes. Am J Med Genet Part C 137C: 4-11.

[2]Sotos Score: 1) Characteristic craniofacial appearance/macrocephaly 2) overgrowth 3) learning disabilities 4) advanced bone age

[3]Martinez-Glez V, Lapunzina P. (2007). Sotos syndrome is associated with leukemia/lymphoma. Am J Med Genet Part A 143A: 1244-1245.

*Point mutation confirmed, nucleotide change not specified, personal correspondence with Trevor Cole, October 19, 2008

ALL, acute lymphoblastic leukemia; BCC, basal cell carcinoma; CSV, cavum septum vergae; ESRD, end stage renal disease; F, female; FMD, fibromuscular dysplasia; M, male; NS, not specified; SCC, squamous cell carcinoma; VM, ventriculomegaly

DISCUSIÓN

El trabajo desarrollado durante esta tesis doctoral ha permitido profundizar el conocimiento de algunos aspectos de cuatro de los Síndromes de Sobrecrecimiento (SSC) clásicos diagnosticados en el Instituto de Genética Médica y Molecular (INGEMM) del Hospital La Paz.

Los SSC son enfermedades raras pero siendo el INGEMM un centro de referencia para su estudio y confluyendo aquí pacientes de España, podemos disponer de un buen número de casos por analizar. En esta sección se discutirán por separado los hallazgos conseguidos en cada uno de los cuatro síndromes estudiados, siendo cuatro enfermedades clínica y etiológicamente distintas.

SÍNDROME DE BECKWITH-WIEDEMANN

Mutaciones puntuales en el gen *CDKN1C,* correlaciones fenotipo-genotipo y preeclampsia/HELLP

La búsqueda de mutaciones puntuales en el gen *CDKN1C* por secuenciación directa en pacientes BWS sin diagnostico molecular, nos ha permitido describir 7 nuevas mutaciones. Sumando estas a las ya conocidas y descritas en la literatura, contamos un total de 25 mutaciones a cargo de este gen.

El porcentaje de pacientes BWS con mutación en *CDKN1C*, respecto a la totalidad de nuestros casos, es un 6,2%, resultado similar al previamente descrito [Lee et al., 1997a; Li et al., 2001]. Analizando los padres de nuestros pacientes, notamos que una gran mayoría de casos (6/7) son de herencia materna, siendo el *CDKN1C* un gen con impronta paterna. Las madres aparentemente asintomáticas desarrollarían la mutación *de novo* en el cromosoma paterno o la heredarían del padre.

Entre los conocidos, sólo 2 cambios aminoacídicos se han encontrado en más de un pacientes. Estos cambios son los que afectan a los residuos Leu33 (en 2 pacientes, L33H y L33R) y Ser282 (en 4 pacientes, S282X y S282fsX) y se podrían considerar puntos calientes de mutaciones ya que representan el 23% de los descritos. No estando todavía disponible el modelo de la proteína CDKN1C, se ha construido por homología el modelo 3-D del dominio inhibidor CdK ligado a la ciclina A-CdK2. Este modelo sugiere que la pareja de aminoácidos Leu33 y Phe34 genere un pequeño bolsillo hidrofóbico en estrecho contacto con un surco hidrofóbico de la ciclina A. La introducción de un residuo de carga positiva, como la histidina, probablemente afecte a la estabilidad del complejo y modifique la interacción local proteína-proteína, como ya sugerido por el mutante L33R [Engel et al., 2000], causando una pérdida en la afinidad del contacto.

El estudio del gen *CDKN1C* ha sido realizado también en algunos casos de macroglosia, hemihipertrofia y onfalocele aislados y no nos ha permitido encontrar ningún cambio. Nos esperábamos este resultado negativo ya que nunca han sido descritas mutaciones en *CDKN1C* en esta clase de pacientes y nuestra población era muy reducida.

El análisis detallado de los signos clínicos de los casos BWS con mutación en *CDKN1C*, nos ha permitido delinear características mayormente representadas en correlación a este defecto molecular. Pudimos confirmar que, como ya descrito [Weksberg et al., 2010], muchos casos con mutación en *CDKN1C* presentan onfalocele y hernia umbilical. Detectamos la presencia de paladar hendido en 3 casos entre toda la población de BWS, todos en coincidencia con mutación del dominio QT de *CDKN1C*, eso nos lleva a sugerir un riesgo aumentado de dicha malformación en correlación con este defecto molecular, sugerencia reforzada por la evidencia de que se trate de una manifestación típica de los modelos de ratón cdkn1c -/- [Takahashi et al., 2000b]. Pudimos también describir hipospadias y criptorquidia en dos pacientes distintos, dato que nos lleva a considerar las malformaciones genitales más frecuentes de lo previamente descrito [Greer et al., 2008].

Finalmente hemos querido evaluar la correlación entre las mutaciones en *CDKN1C* y el aumento de riesgo oncológico, siendo este gen considerado un putativo supresor de cáncer de mama [Larson et al., 2008]. Podemos afirmar que hasta la fecha ninguno de nuestros pacientes BWS con mutación en *CDKN1C* ha desarrollado neoplasias y que sólo un caso de neuroblastoma ha sido reportado [Lee et al., 1997a]. El seguimiento de los pacientes continuará, debido a que algunos todavía no han alcanzado la edad recomendada de los 10 años [Lapunzina, 2005b], pero todas las evidencias sugieren que las mutaciones en *CDKN1C* no conlleven un aumento del riesgo de desarrollar tumor de Wilms ni otro tipo de neoplasia [Cooper et al., 2005].

De todos los pacientes que incluimos en nuestros estudios registramos informaciones sobre los padres, tales como la edad, historia de enfermedades anteriores, consanguinidad, origen étnico y acontecimientos durante el embarazo. Gracias a esta forma de recoger datos hemos podido apreciar un evento significativo que consiste en la identificación, entre las madres de todos los pacientes con BWS, de tres madres que desarrollaron preeclampsia /HELLP durante el embarazo. Coincidencia interesante fue que los tras niños nacidos presentaban BWS por mutación del gen *CDKN1C*.

La preeclampsia y el síndrome HELLP son enfermedades que afectan a dos individuos, la madre y el niño, de forma más o menos severa. La evaluación de esta dolencia se hace particularmente difícil por la potencial contribución de factores maternos y feto-placentarios a la patología, además de su heterogeneidad clínica y genética. Han sido mapeados algunos loci de susceptibilidad a la preeclampsia [Moses et al., 2000] y se han descrito factores de asociación epidemiológica/riesgo, pero la definición patológica de la enfermedad a nivel molecular en humano se ha visto obstaculizada por la contribución de los factores ambientales a la enfermedad [Knox and Baker, 2007].

A pesar de las dificultades encontradas en humanos, los mutantes de ratón han sido muy útiles y eficaces en definir la enfermedad. El modelo de ratón *cdkn1c -/-*, resume el espectro completo de los síntomas de la preeclampsia, mostrando no sólo un desarrollo anormal de la placenta, sino también parto prematuro, aumento de la presión sanguínea, proteinuria y lesiones glomerulares [Kanayama et al., 2002; Knox and Baker, 2007; Takahashi et al., 2000a].

Sorprendentemente las tres madres que desarrollaron preeclampsia/HELLP durante el embarazo, dieron a luz a niños con BWS debido a mutaciones en *CDKN1C* que llevan a la generación de formas truncadas de la proteína (Q78X, S282X and S282fsX). Además hay que destacar que de estos tres casos, dos son representados por madres portadoras de la mutación, mientras en uno de ellos la mutación se genera *de novo* en el feto. Este hallazgo es coherente con lo observado en ratón [Knox and Baker, 2007], pues evidencian que los efectos de la preeclampsia sean debidos a anomalías feto-placentarias más que a defectos aislados maternos. También hay coherencia entre lo observado en nuestros casos y la importancia de los factores ambientales en el desarrollo de preeclampsia/HELLP. A pesar de que la tres mujeres tengan una mutación que genera una forma truncada de la proteína, ellas manifiestan un curso clínico distinto de la enfermedad: una de ellas desarrolla el síndrome HELLP, mientras las otras dos presentan preeclampsia con distintos grados de severidad de los síntomas.

Estado de metilación de los dos centros de imprinting de la región 11p15

El síndrome de Beckwith-Wiedemann está generado, en un ~85% de casos, por alteraciones epigenéticas en uno o en los dos centros de imprinting de la región 11p15, H19DMR de imprinting paterno y KvDMR de imprinting materno [Henry et al., 1991; Maher and Reik, 2000; Weksberg et al., 2003]. Las alteraciones epigenéticas se distribuyen entre los casos de hipometilación materna

del KvDMR (~60%), hipermetilación paterna del H19DMR (~10%) y disomía uniparental paterna (~10-20%).

Resulta entonces evidente la necesidad de disponer de una técnica robusta y fiable que nos permita medir el nivel de metilación de los centros de imprinting del locus 11p15. Durante años, las técnicas utilizadas han sido el Southern Blot, para la evaluación de la metilación y el análisis de microsatélites para el diagnostico de la UPDp [Gaston et al., 2001; Gicquel et al., 2005].

En nuestro centro, desde hace tres años, el método adoptado para el diagnóstico molecular del BWS es el MLPA específico de metilación (MS-MLPA), técnica que nos permite evaluar dosis génica y estado de metilación del locus 11p15. También para una detección más rápida y una estimación finamente cuantitativa de los defectos epigenéticos, hemos puesto a punto la pirosecuenciación de los centros de imprinting H19DMR y KvDMR.

Recientemente se ha propuesto el uso de la novedosa técnica de High Resolution Melting (HRM) para el estudio de la metilación. Dicha técnica resulta ser aún más rápida y más rentable que la pirosecuenciación, aunque por definición no nos permita la cuantificación del defecto epigenético, sino sólo su identificación. A pesar de esta evidencia, hemos querido proponer un método de análisis de los resultados que nos permita traducir el perfil de la curva en valor cuantitativo del estado de metilación. Gracias a la estrecha colaboración con el Servicio de Bioestadística del Hospital La Paz, hemos podido evaluar la sensibilidad y la especificidad de la novedosa técnica de MS-HRM contra la de MS-MLPA, en pacientes cuyo defecto genético había sido ya estudiado y cuantificado por pirosecuenciación.

Nuestro trabajo resulta ser el primero que compare la fiabilidad del MS-HRM frente al MS-MLPA y que proponga un análisis cuantitativo y no sólo cualitativo de los resultados de MS-HRM. Los métodos estadísticos aplicados a nuestros resultados nos permiten afirmar que la técnica de MS-HRM detecta correctamente los defectos epigenéticos de los dos centros de imprinting implicados en el BWS y que el algoritmo desarrollado por nosotros permite cuantificar el estado de metilación de una forma tan fiable como si se utilizara una técnica cuantitativa como la pirosecuenciación.

Las ventajas de cuanto proponemos están en que el MS-HRM nos permite analizar hasta 96-384 muestras en un sólo ensayo y no se necesita mucha manipulación ni hibridación de las muestras. Finalmente otra ventaja es que el diseño y la puesta a punto de la técnica son muy sencillos y rápidos y eso permite poder diseñar experimentos para la evaluación de cualquier centro de imprinting.

Disomía uniparental paterna en BWS y enfoque a su importancia diagnóstica

El síndrome de Beckwith-Wiedemann es debido a disomía uniparental paterna en un 15-20% de casos; entre nuestros 92 pacientes BWS con diagnostico genético confirmado, encontramos 9 con UPD paterna (10%). La proporción obtenida resulta ser más baja de lo descrito, eso probablemente sea debido a las características de nuestro Registro de Sobrecrecimiento que comprende un elevado número de pacientes BWS nacidos por fecundación artificial, considerada causa de defectos en el centro de imprinting materno [Amor and Halliday, 2008].

Existe una correlación entre la expresión fenotípica de BWS y el grado de mosaicismo en los órganos y tejidos analizados [Itoh et al., 2000], pero hemos observado que no se puede encontrar tal correlación estudiando sólo un tejido como la sangre. Eso se puede explicar considerando que el nivel de mosaicismo de las células con UPD en sangre no refleja el representado por aquellos tejidos más susceptibles al sobrecrecimiento [Smith et al., 2007]. Pudimos también descartar una correlación entre severidad y expansión de los síntomas del síndrome y extensión de la UPD.

El defecto genético de disomía uniparental en BWS se presenta siempre en mosaicismo, evidencia que nos permite clasificarlo como un error postcigótico de recombinación mitótica. Evaluando los sitios de ruptura de nuestros 9 pacientes con SNP-array no pudimos encontrar ningún punto común de recombinación, así como se pudo observar para la recombinación meiótica [Cooper et al., 2007; Russo et al., 2006].

Finalmente hemos evaluado 5 técnicas distintas de diagnostico molecular y podemos sugerir que la forma más eficaz para la detección de la UPD paterna es la combinación de MS-MLPA + HRMA o MS-MLPA + pirosecuenciación. Podemos así establecer el origen de la disomía uniparental y el nivel de metilación de los dos centros de imprinting de la región 11p15, proporcional al grado de mosaicismo. El análisis de STR resulta obsoleto mientras la técnica de array de SNP, a pesar de permitirnos definir el punto de ruptura y el grado de mosaicismo, no nos informa sobre el origen parental, resulta ser más cara y necesita la ayuda de laboratorios de experiencia en su utilización.

El estudio molecular de los pacientes BWS con UPDp a través de la técnica de array de SNP, nos ha llevado a la inesperada observación en uno de ellos de disomía uniparental paterna de todo el genoma en elevado grado de mosaicismo. Revisando la literatura existente [Kotzot, 2008] resulta que hasta la fecha nuestra paciente represente el único caso descrito en edad adulta y ha

sido interesante notar en ella la presencia de rasgos que combinan las características clínicas de varios desordenes de imprinting conocidos, siendo sin embargo los del BWS los más pronunciados.

Distintos análisis moleculares se han llevado a cabo para poder entender el mecanismo molecular que genera tan peculiar desorden genético. Pudimos así excluir defectos del proceso de fertilización e indicar como responsable de la UPD de todo el genoma, un fallo de la replicación del genoma materno seguida por la endoreplicación del paterno. Eso llevaría a la generación de un mosaicismo biparental /androgénetico, con sobrerrepresentación de la línea celular con UPDp de todo el genoma [Golubovsky, 2003].

La UPD lleva a pérdida de heterocigosidad (LOH), mecanismo común de tumorigénesis descrito en casi todos los tipos de cáncer [Tuna et al., 2009]. Coherentemente con esta afirmación, nuestra paciente desarrolló dos diferentes tipos de neoplasias, tumor de Wilms y neoplasia adrenocortical y resulta ser un sujeto con elevado riesgo de tumores adicionales relacionados con la UPD.

Una consecuencia común de la pérdida de heterocigosidad (LOH) es la localizada pérdida de imprinting (LOI), desbalance de la contribución parental de las regiones cuyos genes están regulados por imprinting. Teniendo a disposición técnicas moleculares para evaluar el nivel de metilación de varios centros de imprinting, podemos afirmar un desarreglo del patrón normal de metilación en todos los analizados; aunque la mayoría no lleven a evidentes características clínicas, justificamos este fenómeno bien porque para algunos una pérdida parcial de la metilación no es suficiente para la expresión del fenotipo, bien porque otros no han sido todavía asociados a ninguna dismorfología en concreto.

La observación de este caso nos permite hacer hincapié sobre la importancia de un correcto diagnóstico de los pacientes con sospecha de síndromes de imprinting, sugiriendo el uso de tecnologías avanzadas como el array de SNP y de metilación.

SÍNDROME DE SIMPSON-GOLABI-BEHMEL

Caracterización molecular y mosaicismo germinal

El síndrome de Simpson-Golabi-Behmel (SGBS) es una enfermedad ligada al X y han sido descrito varios pacientes con mutaciones/deleciones del gen *GPC3* de la región Xq26 [Pilia et al., 1996; Weksberg et al., 1996; Xuan et al., 1994].

Tenemos a disposición en nuestro Registro de pacientes con sobrecrecimiento, 27 casos que cumplen los criterios clínicos de SGBS y hemos querido caracterizarlos a nivel molecular. El primer paso de nuestro estudio ha sido estudiar la presencia de mutaciones/deleciones en los genes *GPC3* y *GPC4* y mutaciones en *GPC3*, análisis que nos ha permitido diagnosticar anomalías en *GPC3* en 9 pacientes.

La imposibilidad de encontrar defectos en la primera región candidata Xq26 en 18 pacientes, eleva la posibilidad de que existan otros loci en el cromosoma X responsables del síndrome. Revisando la literatura encontramos publicaciones interesantes para proponer un nuevo gen candidato, como los 4 casos de la familia estudiada clínicamente por Terespolsky [Terespolsky et al., 1995] y luego a nivel molecular por Brzustowicz [Brzustowicz et al., 1999], los cuales definieron una forma más severa de SGBS, sucesivamente llamada SGBS2, y localizaron la anomalía genética en una región de ~ 6Mb en Xp22. Además de estas sugerencias, más recientemente [Budny et al., 2006] ha sido descrita una familia en la cual 9 miembros presentaban características clínicas similares bien a la forma clásica de SGBS bien a los descritos por Terespolsky. El estudio molecular de estos pacientes permitió encontrar en 2 de los casos una duplicación de 4bp en el gen *OFD1* de la región Xp22. Mutaciones en este gen habían sido previamente descritas como responsables del síndrome orofacial-digital de tipo I [Ferrante et al., 2001]. Elegimos entonces este gen como posible candidato responsable del SGBS en nuestros pacientes sin diagnostico molecular. El estudio nos llevó a un resultado negativo, permitiendo sólo la descripción de nuevos SNPs intronicos, aparentemente sin consecuencias patológicas.

El reestudio de todos los casos de SGBS de nuestro Registro de pacientes con sobrecrecimiento, nos ha llevado a la identificación de una familia con una peculiar historia de este desorden. La pareja de padres, jóvenes, sanos y no consanguíneos, tuvieron 4 embarazos. Del primero nació un niño con una forma severa de SGBS que falleció en el período perinatal (Paciente 1) y sucesivamente la madre dio a luz a un niño sano. El tercer embarazo se resolvió con un aborto espontaneo y, finalmente, de la cuarta gestación, nació un niño con una forma más leve de SGBS (Paciente 2).

Pudimos analizar a nivel molecular la familia, pudiendo obtener ADN de sangre periférica de los padres y del Paciente 2 y ADN de tejido parafinados del Paciente 1. En estas muestras evaluamos la presencia de deleciones de los genes *GPC3* y *GPC4* por MLPA y de mutaciones puntuales del gen *GPC3* por secuenciación directa bidireccional.

El hallazgo interesante de este estudio fue encontrar la misma mutación puntual (c.1605 C>T; R387X), que lleva a la generación de una forma truncada de la proteína, en los dos pacientes con SGBS (Pacientes 1 y 2) pero no poderla detectar en ADN de sangre periférica de la madre; aún más útil para nuestro estudio hubiese sido tener a disposición material del feto abortado. Aún así los resultados obtenidos nos llevan a proponer un mosaicismo germinal en la madre para esta mutación.

La familia descrita nos permite entonces añadir otro síndrome al número creciente de desordenes genéticos ligado al X con mosaicismo germinal comprobado y subrayar la importancia de tener en cuenta esta posibilidad en el consejo genético de las familias que presenten este raro síndrome de sobrecrecimiento.

SÍNDROME DE MACROCEFALIA-MALFORMACIÓN CAPILAR

Análisis clínico y molecular

Hasta la fecha han sido descritos unos 130 casos de M-CM; nuestro trabajo consistió en revisar clínicamente los casos reportado en literatura y analizar clínica y genéticamente a los 13 pacientes con M-CM incluidos en nuestro Registro de síndromes de sobrecrecimiento.

Las manifestaciones clínicas más frecuentes que hemos podido detectar son zonas de alteración estructural según neuroimagen, macrocefalia, sobrecrecimiento, malformación capilar, retraso del desarrollo y asimetría. Sugerimos considerar el sobrecrecimiento y la asimetría como formas diferentes del mismo síntoma que, por su frecuencia, nos permite incluir sin dudad la condición de M-CM entre los síndromes de sobrecrecimiento [Lapunzina, 2005b]. Además proponemos el sobrecrecimiento, junto con la macrocefalia y la malformación capilar, como criterio mayor del diagnostico de M-CM.

La revisión de los 132 casos descritos hasta la fecha, nos ha permitido estimar en un 82% la presencia de alteraciones en la neuroimagen; este dato refuerza la necesidad del estudio de RMN para evaluar los pacientes con M-CM y nos lleva a definir como un criterio mayor para el diagnóstico de estas alteraciones.

A pesar de que las características clínicas de los pacientes con M-CM se vayan definiendo siempre más finamente, todavía queda desconocida su causa etiológica. Por esa razón hemos querido realizar un análisis de todo el genoma por array de SNP, técnica útil para el estudio de

variaciones en el número de copias (CNV) y de disomía uniparental (UPD) [Altug-Teber et al., 2005; Yau and Holmes, 2008].

Los resultados obtenidos no nos permiten comunicar ninguna alteración en genes o regiones comunes a nuestros pacientes, pero sí podemos excluir variaciones en el número de copias y UPD extensas como causas del síndrome. Conscientes de que estas afirmaciones son relativas a nuestra pequeña serie de pacientes, sugerimos la necesidad de aplicar ese tipo de estudio a series más grandes.

SÍNDROME DE SOTOS

Caracterización clínica de pacientes adultos y protocolo de seguimiento

El síndrome de Sotos presenta signos clínicos bien descritos en la infancia pero aún se conoce poco sobre este síndrome en la edad adulta, incluyendo diagnóstico, problemas médicos y asesoramiento. Los rasgos faciales cambian mucho con el tiempo [Allanson and Cole, 1996] y estas alteraciones de la cara, la estatura elevada y la ausencia de información de este síndrome en adultos, lleva a diagnósticos diferenciales como síndrome de Marfan, Weaver y Simpson-Golabi-Behmel.

Gracias a la revisión de 20 pacientes adultos, elegidos por tener diagnóstico molecular confirmado de alteraciones en el gen *NSD1*, hemos podido notar que los problemas psiquiátricos son más frecuentes de lo que se sospechaba. Además varios pacientes presentan un comportamiento "socialmente aislado", posiblemente generado por dificultades sociales, enfermedades, diferencias físicas y retraso mental. Pudimos identificar una correlación genotipo-fenotipo, evidenciando que los pacientes con mutaciones puntuales de *NSD1* presentan dificultades de aprendizaje menos severas de los que presentan microdeleciones, así como menos problemas de comportamiento y temperamento más fácil. Aunque tengamos sólo datos preliminares, nos permitimos sugerir un control regular de la depresión y de otros desordenes psiquiátricos. Asimismo añadimos la recomendación de exámenes oftalmológicos en la edad adulta, vista la elevada incidencia de enfermedades oculares y sugerimos cuidar durante toda la vida la escoliosis postural y las contracturas, ya que estos defectos persisten en la edad adulta.

Finalmente entre los pacientes estudiados, 2 presentaron anomalías renales no relacionadas entre ellas; su diagnóstico tan desigual implica que no sean debidas a alteraciones de *NSD1*.

Reportamos también 4 neoplasias distintas entre los casos; la ausencia de patrones o tiempos de aparición comunes nos impide definir claramente el riesgo de tumorigénesis en el síndrome de Sotos aunque sigan siendo necesarias las exploraciones seriadas en estos pacientes.

CONCLUSIONES

CONCLUSIONI

Síndrome de Beckwith-Wiedemann

1. Se han identificado mutaciones puntuales en el gen *CDKN1C* en un porcentaje de pacientes similar a lo descrito en literatura.

2. No se han encontrado deleciones completas del gen *CDKN1C*, ni microdeleciones o microduplicaciones. No se han podido identificar puntos calientes de mutación, aunque existen algunas mutaciones descritas en más de 1 paciente.

3. Se pueden reconocer características fenotípicas en los pacientes con mutaciones puntuales en el gen *CDKN1C*. Confirmamos el riesgo aumentado de onfalocele y hernia umbilical y sugerimos una mayor frecuencia de paladar hendido y malformaciones genitales.

4. Se ha registrado un riesgo aumentado de desarrollar síndrome de preeclampsia/HELLP durante el embarazo en las madres de niños con mutaciones sin sentido en *CDKN1C*.

5. El ensayo de MS-HRM diseñado permite evaluar de forma cuantitativa los defectos epigenéticos de los centros de imprinting del locus BWS. Dicha técnica se puede además aplicar al estudio de la metilación de cualquier isla CpG.

6. En los pacientes con disomía uniparental paterna no se han podido establecer correlaciones entre la extensión de la UPD, el grado de mosaicismo y los signos clínicos.

7. Identificamos en el uso combinado de MS-MLPA + MS-HRMA o MS-MLPA + pirosecuenciación la mejor forma de detectar y caracterizar la UPD paterna.

8. Destacamos la eficacia de tecnologías avanzadas como el array de SNP y de metilación para un correcto diagnostico de los pacientes con sospecha de síndromes de imprinting.

Síndrome de Macrocefalia-Malformación Capilar

9. Incluimos entre los criterios mayores de diagnostico de M-MC sobrecrecimiento/asimetría y alteraciones de la neuroimagen. A pesar de las mejoras en la caracterización fenotípica de los pacientes, la causa etiológica del síndrome permanece sin conocerse.

Síndrome de Simpson-Golabi-Behmel

10. No se ha identificado alteraciones en el gen *OFD1* en los pacientes sin defectos en los genes *GPC3* y *GPC4* u otro diagnostico molecular.

11. Se describe un mecanismo de mosaicismo germinal en una familia con una peculiar historia de SGBS, añadiendo este desorden a los desordenes genéticos ligados al cromosoma X con mosaicismo germinal comprobado.

Síndrome de Sotos

12. Sugerimos un control regular de la depresión y de otros desordenes psiquiátricos que se han relevado más frecuentes de lo que se sospechaba; así mismo exámenes oftalmológicos en la edad adulta vista la elevada incidencia de enfermedades oculares.

Sindrome di Beckwith-Wiedemann

1 Sono state identificate mutazioni puntiformi nel gene *CDKN1C* in una percentuale di pazienti simile a quanto descritto in letteratura.

2 Non sono state trovate delezioni complete del gene *CDKN1C*, né microdelezioni o microduplicazioni. Non é stato possibile identificare hot spots, anche se esistono alcune mutazioni descritte in piú di un paziente.

3 Si possono riconoscere caratteristiche fenotipiche nei pazienti con mutazioni puntiformi del gene *CDKN1C*. Confermiamo l'aumento del rischio di onfalocele ed ernia ombelicale e suggeriamo una maggiore frequenza di palatoschisi e malformazioni genitali.

4 Si é registrato un aumento del rischio di sviluppare sindrome di preeclampsia/HELLP durante la gravidanza nelle madri di bambini con mutazioni senza senso in *CDKN1C*.

5 Il saggio di MS-HRM disegnato permette stimare in modo quantitativo i difetti epigenetici dei centri di imprinting del locus BWS. Tale tecnica si puó inoltre applicare allo studio della metilazione di qualunque isola CpG.

6 Nei pazienti con disomia uniparentale paterna non é stato possibile stabilire correlazioni tra la estensione della UPD, il grado di mosaicismo e i segni clinici.

7 Identifichiamo nell'uso combinato di MS-MLPA + MS-HRMA o MS-MLPA + pirosecuenciación la migliore forma di individuare e caratterizzare la UPD paterna.

8 Distacchiamo l'efficacia di tecnologie avanzate come l'array di SNP e di metilazione per la corretta diagnosi dei pazienti con sospetto di sindromi di imprinting.

Sindrome di Macrocefalia-Malformazione Capillare

9 Incudiamo tra i criteri maggiori di diagnosi di M-MC eccesso di crescita/asimmetria e alterazioni della neuroimmagine. Nonostante il miglioramento nella caratterizzazione fenotipica dei pazienti, la causa eziologica della sindrome rimane sconosciuta.

Sindrome di Simpson-Golabi-Behmel

10 Non sono state identificate alterazioni del gene *OFD1* nei pazienti senza difetti nei geni *GPC3* e *GPC4* o altra diagnosi moecolare.

11 Si descrive un meccanismo di mosaicismo germinale in una familia con una storia peculiare di SGBS, aggiungendo questa malattia alle malattie genetiche legate al cromosoma X con mosaicismo germinal comprovato.

Sindrome di Sotos

12 Suggeriamo un controllo regolare della depressione e di altri disordini psichiatrici che sono stati individuati piú frequentemente di quanto ci si aspettava; lo stesso vale per i controlli oftalmologici nell'etá adulta data la elevata incidenza di malattie oculari..

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