

The *KCNQ1OT1* imprinting control region and non-coding RNA: new properties derived from the study of Beckwith–Wiedemann syndrome and Silver–Russell syndrome cases

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A cluster of imprinted genes at chromosome 11p15.5 is associated with the growth disorders, Silver–Russell syndrome (SRS) and Beckwith–Wiedemann syndrome (BWS). The cluster is divided into two domains with independent imprinting control regions (ICRs). We describe two maternal 11p15.5 microduplications with contrasting phenotypes. The first is an inverted and *in cis* duplication of the entire 11p15.5 cluster associated with the maintenance of genomic imprinting and with the SRS phenotype. The second is a 160 kb duplication also inverted and *in cis*, but resulting in the imprinting alteration of the centromeric domain. It includes the centromeric ICR (ICR2) and the most 5' 20 kb of the non-coding *KCNQ1OT1* gene. Its maternal transmission is associated with ICR2 hypomethylation and the BWS phenotype. By excluding epigenetic mosaicism, cell clones analysis indicated that the two closely located ICR2 sequences resulting from the 160 kb duplication carried discordant DNA methylation on the maternal chromosome and supported the hypothesis that the ICR2 sequence is not sufficient for establishing imprinted methylation and some other property, possibly orientation-dependent, is needed. Furthermore, the 1.2 Mb duplication demonstrated that all features are present for correct imprinting at ICR2 when this is duplicated and inverted within the entire cluster. In the individuals maternally inheriting the 160 kb duplication, ICR2 hypomethylation led to the expression of a truncated *KCNQ1OT1* transcript and to down-regulation of *CDKN1C*. We demonstrated by chromatin RNA immunopurification that the *KCNQ1OT1* RNA interacts with chromatin through its most 5' 20 kb sequence, providing a mechanism likely mediating the silencing activity of this long non-coding RNA.

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INTRODUCTION

Imprinted genes are characterized by the gamete of origin-dependent expression. This is controlled by epigenetic modifications that are differentially established on the maternal and paternal alleles in the gametes or early embryo (1,2). The primary targets of these epigenetic modifications are *cis*-acting regulatory elements of the imprinted loci, also known as imprinting control regions (ICRs). The mechanisms involved in the establishment of imprinting are largely unknown. A large cluster of imprinted genes is located at chromosome 11p15.5 (3). The cluster is divided into two independent domains, each controlled by a separate ICR. The telomeric and centromeric ICRs (ICR1 and ICR2, respectively) work by different mechanisms and can be considered paradigms of different models of imprinting control (1,2). ICR2 is the promoter of the non-coding and imprinted *KCNQ1OT1* gene (4,5). This is contained into and has antisense orientation with respect to the protein-coding gene *KCNQ1*. The long non-coding *KCNQ1OT1* transcript silences *in cis* the imprinted genes of the centromeric domain on the paternal chromosome, although its mechanism of action is not fully understood (6,7). On the maternal chromosome, ICR2 is methylated, *KCNQ1OT1* is not transcribed and the flanking imprinted genes expressed.

Heterogeneous molecular defects affecting the 11p15.5 imprinted gene cluster are associated with the congenital growth disorders, Beckwith–Wiedemann syndrome (BWS) and Silver–Russell syndrome (SRS) (8,9). The BWS (OMIM 130650) is characterized by overgrowth, macroglossia, abdominal wall defects and predisposition to embryonal tumors in childhood. The SRS (OMIM 180860) is associated with growth restriction, hypotonia and characteristic dysmorphic features. Opposite DNA methylation defects have been found at ICR1 in BWS and SRS and associated with reciprocal alterations of IGF2-H19 expression (10). Loss of the maternal-specific ICR2 methylation is the most frequent defect in BWS, resulting in the bi-allelic activation of *KCNQ1OT1* and bi-allelic silencing of the centromeric domain genes, including the cell growth inhibitor *CDKN1C* (4,5,11). *In cis* mutations have been demonstrated in 20% of the BWS patients with gain of methylation at ICR1, and a loss-of-function defect of a *trans*-acting factor has been demonstrated in a familial case with multiple ICR hypomethylation (12–16). However, in most of the BWS cases with methylation abnormalities, no DNA sequence alteration has been identified so far. Uniparental disomy at 11p15.5 is frequent in BWS, but chromosome abnormalities are rarer and generally consist in paternal duplications, maternal deletions or balanced maternal translocations in BWS and maternal duplications in SRS (8,9). Mutations of *CDKN1C* account for 5% of the BWS cases (8).

Here, we describe two cases with 11p15.5 *in cis* microduplications of maternal origin and opposite phenotypes. The first case is a 1.2 Mb long inverted duplication encompassing the entire 11p15.5 imprinted gene cluster and associated with the SRS phenotype. The second is an exceptional case in which a 160 kb duplication including only ICR2 and the most 5' 20 kb of *KCNQ1OT1* co-segregates with the BWS phenotype in a three-generation pedigree. Maternal transmission of the 160 kb duplication is associated with ICR2 hypomethylation,

expression of a truncated *KCNQ1OT1* transcript and *CDKN1C* silencing. By showing that two close ICR2 sequences with opposite orientation display different DNA methylation status, our data demonstrate that this ICR sequence is not sufficient for imprinted methylation, and some other property, possibly orientation-dependent (e.g. transcription), is required. The results also indicate that the *KCNQ1OT1* RNA interacts with chromatin through its 5' sequence, indicating a possible mechanism mediating *CDKN1C* silencing in somatic tissues.

RESULTS

Clinical reports

SRS family. The SRS patient 1 (Fig. 1A) was a boy born to unrelated parents at 32 weeks of gestation. Intra-uterine growth restriction was observed, birth weight and length were below 3rd centile and occipitofrontal circumference around the 3rd centile. Physical examination at 14 months confirmed height and weight below the 3rd centile and demonstrated muscle hypotrophy, slight psychomotor delay and a characteristic face with triangular form and broad and prominent forehead. His mother had multiple miscarriages. No signs typical of SRS were evident in the other family members. Detailed clinical features of the affected individuals are shown in Table 1.

BWS family. The pedigree of the BWS family is presented in Figure 1B. The proposita (III-6, BWS patient 1, Fig. 1A), born to unrelated parents, was a female in a sibship including a miscarriage and a phenotypically normal sister (III-5). She was born at 29 weeks of gestation with adequate birth weight (1478 g, 50–75th centile), according to her gestational age. Delivery was carried out by cesarean section due to a large exomphalos diagnosed by pre-natal ultrasound. At clinical evaluation, facial *nevus flammeus*, ear creases, macroglossia, flat nasal bridge and bilateral post-axial hexadactyly at upper limbs were noted. A patent ductus arteriosus (PDA), requiring surgical correction, was present. Her mother (II-4, BWS patient 2, Fig. 1A) was born at term with elevated birth weight (>97th centile) and length (>97th centile) and an umbilical hernia that was surgically corrected. The proposita's father (II-5) was phenotypically normal. The maternal uncle (II-3, BWS patient 3) was born at term with elevated birth weight (>4000 g) and presented a 4 cm umbilical hernia that was surgically corrected. The maternal aunt (II-2) was born with elevated birth weight (90th centile) and had a miscarriage and two children, one of which (III-3) presented a large congenital umbilical hernia. Consent for genetic analysis of II-2 and III-3 was not obtained. The maternal grandmother (I-4) was phenotypically normal, and the grandfather, unavailable for sampling, was reported as phenotypically normal.

Analysis of DNA methylation and copy number at 11p15.5

SRS family. By employing Southern blotting hybridization and combined bisulfite restriction analysis (COBRA), slight ICR1 hypomethylation and slight ICR2 hypermethylation were demonstrated in the SRS patient 1, whereas normal 50% methylation at both loci was evident in his parents (Fig. 2A and B). This result is compatible with either maternal

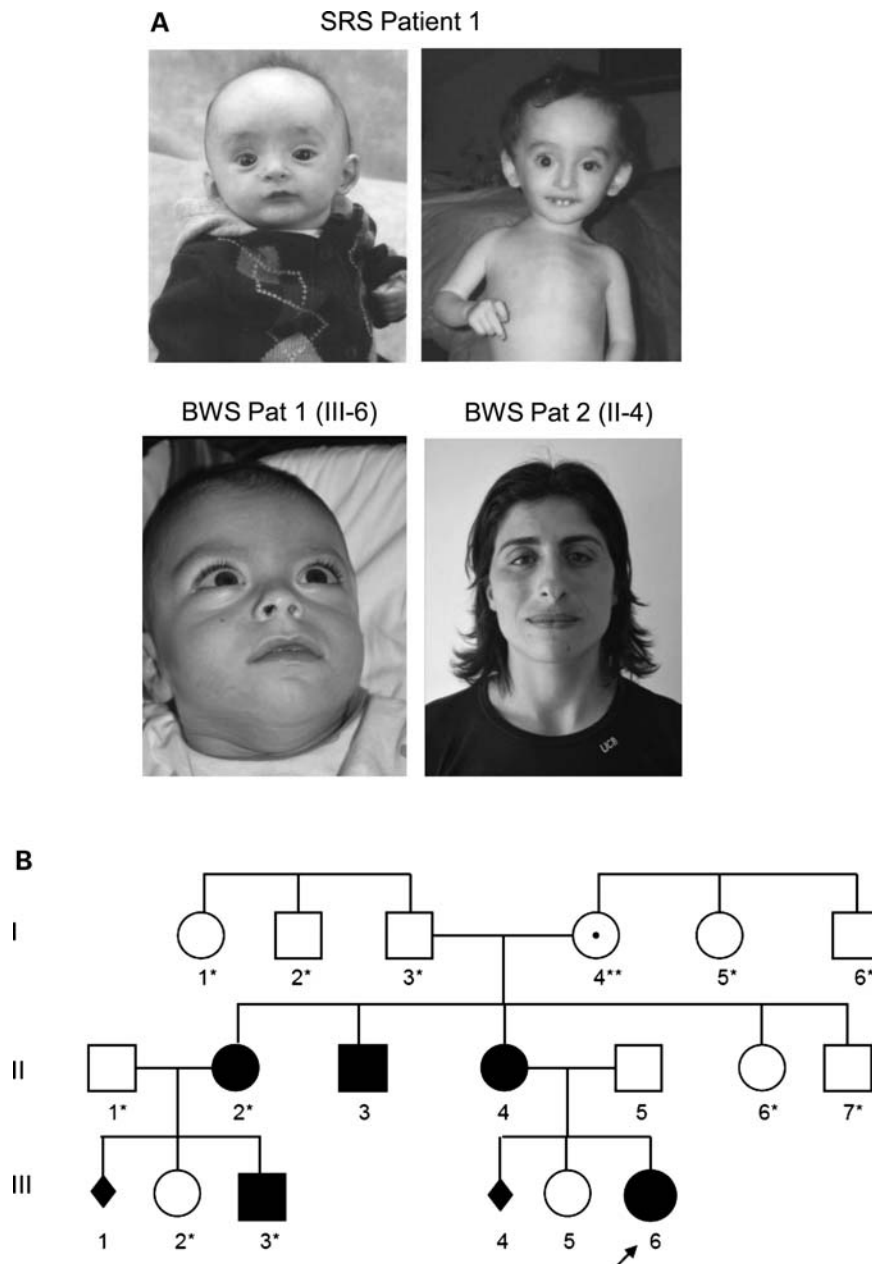


Figure 1. Clinical phenotype and pedigree of the patients. (A) SRS patient 1 at 5 months (top left) and 14 months (top right). BWS patient 1 at birth (bottom left) and BWS patient 2 (bottom right). (B) Three-generation pedigree of the BWS family. Carriers of the 160 kb microduplication are indicated. *Individuals unavailable to molecular analysis. **Paternal transmission of the microduplication in I-4 was inferred by allele-specific analysis of ICR2 methylation (see Results). Note that maternal transmission segregates with the BWS phenotype with complete penetrance.

uniparental disomy (UPD) or *de novo* maternal duplication at 11p15.5 in the proband (17,18). The allelic imbalances were further investigated by the analysis of 11p15.5 microsatellite markers and DNA sequencing. Typing for the TH and D11S4046 microsatellite markers and the rs11023840 single nucleotide polymorphism (SNP) demonstrated the presence of a *de novo* maternal duplication in the SRS patient 1 (Fig. 3A and B). The presence of germinal mosaicism in the mother could possibly explain her multiple miscarriages.

BWS family. In the BWS family, 11p15.5-specific methylation specific multiplex ligation-dependent probe amplification

assay (MS-MLPA) demonstrated hypomethylation at ICR2 and normal methylation at ICR1 in III-6 (BWS patient 1), II-4 (BWS patient 2), II-3 (BWS patient 3) and I-4 (Fig. 2C). Normal methylation at both ICR1 and ICR2 was found in II-5 and III-5. The other members of the family were unavailable to the analysis. The MS-MLPA also showed increased copy number (CN) of the *KCNQ1* exons 12–15 and ICR2 (located in *KCNQ1* intron 11) in all the individuals of family 2 displaying the ICR2 hypomethylation (Fig. 2D). The analysis of the D11S4088 microsatellite marker confirmed the allelic imbalance in patients 1 (III-6), 2 (II-4) and 3 (II-3) and in I-4 and showed maternal transmission of the 11p15.5 duplication from I-4 to II-3 and

Table 1. Clinical features in patients 1–4

Diagnosis	SRS patient 1	BWS patient 1	BWS patient 2	BWS patient 3
Present age	14 months	2 years, 9 months	28 years	32 years
Sex	Male	Female	Female	Male
Karyotype	46,XY	46,XX	46,XX	46,XY
Pregnancy and delivery				
Gestational age (weeks)	32	29	38	38
Polyhydramnios	No	No	No	No
Delivery	Cesarean	Cesarean	Spontaneous	Spontaneous
Growth pattern				
Pre-natal growth failure	Yes	No	No	No
Birth weight (g)	950 (<3rd centile)	1478 (75th centile)	~4300 (>97th centile)	~4500 (>90th centile)
Birth length (cm)	34.5 (<3rd centile)	38.3 (25–50th centile)	Unknown	Unknown
Post-natal growth failure	Yes	No	No	No
Present weight	5100 g (<3rd centile)	12.2 kg (25–50th centile)	75.2 kg	78.0 kg
Present stature (cm)	62 (<3rd centile)	95.2 (75–90th centile)	175 (>95th centile)	188 (>95th centile)
Characteristic face				
Triangular face	Yes	No	No	No
Frontal bossing	Yes	No	No	No
Micrognathia	Yes	No	No	No
Prognathism	No	Yes	No	Yes
Thin lips	Yes	No	No	No
Flat nasal bridge	No	Yes	No	No
Ear creases/pits	No	Yes ^a	No	No
Macroglossia	No	Yes	No	No
Abdominal wall defect				
Exomphalos	No	Yes	No	No
Umbilical hernia	No	No	Yes	Yes
Others				
Clinodactyly V	Yes	No	No	No
Brachydactyly V	Yes	No	No	No
Syndactyly	Yes	No	No	No
Hexadactily	No	Yes	No	No
Motor/neuropsychological delay	Yes	Yes ^b	No	No
Feeding difficulties	Yes	No	No	No
Muscle hypotrophy	Yes	No	No	No
Nevus flammeus	No	Yes	No	No
VSD/ASD/PDA shunt	No	PDA	No	No

ASD, atrial septal defect; PDA, patent ductus arteriosus; VSD, ventricular septal defect.

^aOnly ear creases.

^bProbably secondary to prematurity and perinatal isoposic injury.

II-4 and from II-4 to III-6 but not to III-5 (Fig. 3C). Overall, the results demonstrate the presence of maternal 11p15.5 duplications in both the SRS and BWS patients under study.

Structural analysis of the duplicated regions

To precisely assess the extension of the duplicated regions, the DNAs of the SRS family and BWS patients 1 and 2 were analyzed by array comparative genomic hybridization using the Affymetrix Genome Wide Human SNP Array 6.0. In the SRS patient, this study demonstrated a *de novo* 1.2 Mb duplication spanning from SNP_A-8649846 (Chr11:1 760 993 bp) to SNP_A-8525255 (Chr11:2 983 146 bp) probes and in BWS patients 1 and 2, a 160 kb duplication spanning from SNP_A-1859784 (Chr11:2 656 737 bp) to SNP_A-8584176 (Chr11:2 822 824 bp) probes, based on UCSC Genome Browser (<http://genome.ucsc.edu/>; March 2006 release) (see Fig. 4A and Supplementary Material, Fig. S1). The 1.2 Mb duplication covered the entire 11p15.5 imprinted gene cluster, whereas the 160 kb duplication encompassed only a portion of the *KCNQ1* gene (from exon 12 to exon 15), ICR2 and the

most 5' 20 kb of *KCNQ1OT1*. We then analyzed the chromosomal location of the duplications by fluorescence in situ hybridization (FISH) using bacterial artificial chromosome (BAC) and fosmid probes hybridizing within the duplicated regions: BACs RP11-81K4 (chr11:2 755 275–2 927 014), RP11-937011 (chr11:2 522 537–2 709 016) and RP11-876C12 (chr11:103 309 879–103 487 727) and fosmids G248P81952E2 (chr11:2 660 932–2 698 197) and G248P800755G6 (chr11:2 759 661–2 803 886). Metaphase FISH analysis of the patient lymphoblasts detected signals only at chromosome 11p in both the SRS and BWS cases, indicating that the duplications were *in cis* (Fig. 4B, left panels). FISH on interphase nuclei confirmed the 1.2 Mb and 160 kb duplications at 11p15.5 (Fig. 4B, middle panels) and demonstrated their inverted orientation (Fig. 4B, right panels). Mutations of the *CDKN1C* gene were excluded in BWS patient 1 by exon sequencing (data not shown).

Allele-specific methylation analysis of ICR2

To investigate further the effect of the duplications on imprinting, we analyzed the methylation of the maternal and

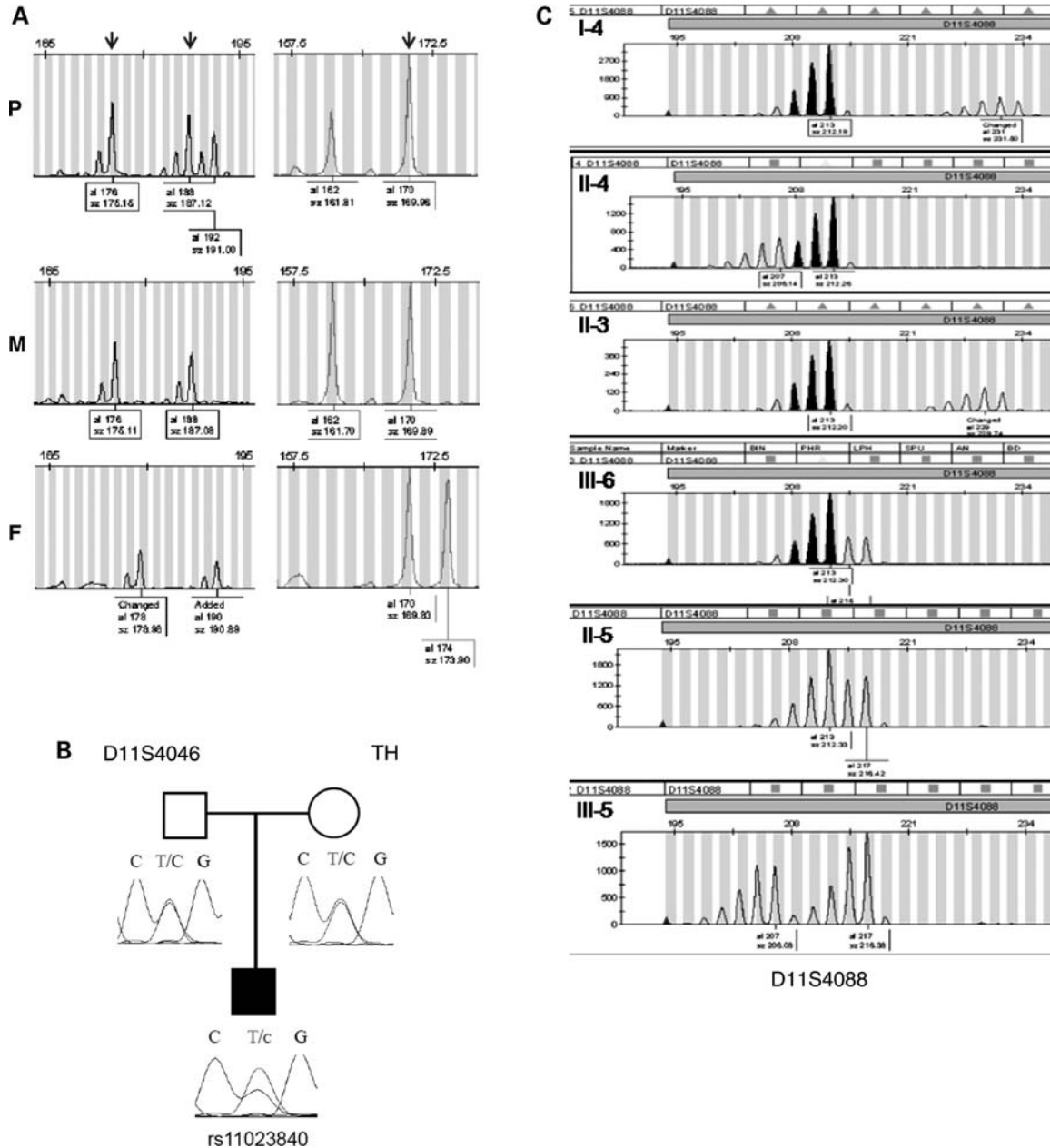


Figure 3. Analysis of microsatellite 11p15.5 markers. (A) Electropherograms obtained for microsatellite loci D11S4046 and TH in the SRS family. The arrows indicate the alleles that are maternally transmitted to the proband. F, father; M, mother; P, proband. Note the presence of two maternal alleles and one paternal allele at D11S4046 and an allelic imbalance at TH in the proband. (B) Electropherograms obtained by sequencing the rs11023840 SNP region in the SRS family. Note the increased dosage of the T allele in the proband. (C) Co-segregation of the 11p15.5 microduplication with the disease phenotype in the BWS family. Electropherograms obtained for the microsatellite locus D11S4088. Note that I-4, BWS patient 2 (II-4), BWS patient 3 (II-3) and BWS patient 1 (III-6) show increased dosage approximating a 2:1 ratio for the 213 bp allele (highlighted in dark gray).

of ICR2. A different picture was observed in the BWS family. Extensive hypomethylation at ICR2 was found in all the individuals carrying the 160 kb duplication (Fig. 5B; data not shown). However, the paternal allele maintained its non-methylated status and the maternal allele maintained its methylated status in I-4, whereas partial loss of the imprinted methylation of the maternal allele was evident in BWS patients 1 and 2. BWS patient 3 was not informative for SNPs in the ICR2 region (data not shown). The results obtained in I-4 indicate

that this individual has inherited the duplication from her father. Therefore, the 160 kb duplication consistently results in partial ICR2 hypomethylation, but leads to imprinting alteration and BWS phenotype only on maternal transmission. It is associated with normal parent of origin-specific methylation and normal phenotype on paternal transmission.

The partial hypomethylation we detected on the maternal chromosome in BWS patients 1 and 2 is compatible with two possible explanations. Either the methylation of both

ICR2 copies is mosaic or one copy is methylated and the other is non-methylated in all the cells. Since the duplicated ICR2 sequences were identical (data not shown), it was not possible to determine their individual methylation status by bisulfite sequencing. We sought to solve this problem by cloning the patient cells. To assess the validity of this approach, we analyzed DNA methylation in 10 clones of lymphoblasts derived from a BWS case with partial ICR2 hypomethylation due to mosaic UPD11. All the clones derived from the UPD11 case displayed complete loss of ICR2 methylation, whereas the clones derived from a control individual maintained 50% methylation, indicating that in the presence of mosaicism, the hypomethylated cells are selected during cell cloning (Fig. 5C). Significantly, the clones derived from BWS patient 1 did not show any selection and maintained the partial ICR2 hypomethylation similar to the peripheral blood lymphocytes (PBLs) from which they derived (Fig. 5C). Thus, the hypomethylation associated with the 160 kb duplication in BWS patients 1–3 is unlikely due to mosaicism, but rather due to lack of methylation of one of the two ICR2 copies present on the maternal chromosome.

Expression analysis of the imprinted genes

The aberrant ICR2 methylation we detected in BWS patients 1–3 suggested that the imprinted expression of the centromeric domain genes was also altered in these individuals. We therefore analyzed the expression of *KCNQ1OT1* and *CDKN1C* in the patients' cell lines. Since *KCNQ1OT1* was partially duplicated, we analyzed its transcription at different locations along the gene by reverse transcriptase–polymerase chain reaction (RT–PCR). The duplicated sequences present *in cis* were identical, but the *KCNQ1OT1* alleles present on the maternal and paternal chromosomes could be distinguished by SNPs. In both BWS patient 1 and BWS patient 2 lymphoblasts, we found bi-allelic expression of *KCNQ1OT1* with 1:1 allelic ratio in the duplicated (5') region of the gene, but normal monoallelic expression in the non-duplicated (3') region (Fig. 6A). These results demonstrate that the truncated *KCNQ1OT1* gene was expressed on the maternal chromosome carrying the 160 kb duplication. We then asked whether this aberrant expression of *KCNQ1OT1* was associated with silencing of the maternal *CDKN1C*. Since *CDKN1C* is poorly expressed in lymphoblasts (data not shown), we analyzed the expression of this gene in the skin fibroblasts by qRT-PCR. We found that the level of the *CDKN1C* RNA of BWS patient 2 was 20-fold lower than the average level found in eight age-matched normal control individuals and was comparable to that measured in BWS cases with loss of ICR2 methylation and no microduplication (Fig. 6B). This indicates that, upon maternal transmission of the microduplication, the full-length *KCNQ1OT1* gene retains its imprinted expression, whereas the truncated *KCNQ1OT1* is expressed and *CDKN1C* is silenced.

Interaction of *KCNQ1OT1* with chromatin

Previously, it has been shown that the mouse *Kcnq1ot1* RNA silences the flanking genes by interacting with chromatin and recruiting the chromatin-modifying machinery (19,20). Since

expression of the truncated *KCNQ1OT1* from the allele carrying the 160 kb duplication on the maternal chromosome correlates with silencing of *CDKN1C*, we thought that the truncated *KCNQ1OT1* RNA was responsible for the silencing activity. We therefore investigated whether *KCNQ1OT1* interacted with chromatin in the cells carrying the 160 kb duplication. To this end, we performed chromatin RNA immunoprecipitation (ChRIP), using antibodies raised against H3 histone and rabbit immunoglobulin G (IgG) (control antibody), on cross-linked chromatin obtained from four different lymphoblastoid cell lines: (i) normal control; (ii) BWS patient 2; (iii) BWS patient 1 and (iv) an unrelated BWS patient with ICR2 hypomethylation and no microduplication (BWS patient 4). Figure 7A shows that primers located 500 bp downstream of the transcription start site detected the *KCNQ1OT1* RNA in the immunoprecipitated chromatin twice more enriched in the BWS patients with the maternal microduplication (BWS P1 and BWS P2) or with ICR2 hypomethylation and no microduplication (BWS P4) than in the normal control (CTRL), indicating that, in addition to the *KCNQ1OT1* transcript derived from the paternal allele, the maternally derived *KCNQ1OT1* RNA interacted with chromatin in the BWS patients. To specifically address the allelic origin of the chromatin-associated transcript, we designed allele-specific primers to an SNP (rs463924) that is located 4 kb downstream of the *KCNQ1OT1* transcription start site (Supplementary Material, Figs S2 and S3). While the paternally derived RNA efficiently interacted with chromatin in all four cell lines, the enrichment of the maternally derived transcript in the immunoprecipitated chromatin was significantly higher in the BWS cell lines than in the control (Fig. 7B). To obtain a further confirmation, the ChRIP RNAs of the normal control, BWS P1, P2 and P4 cells were retrotranscribed, PCR-amplified and sequenced with primers around the SNP rs463924. The sequencing electropherograms clearly demonstrate the presence of both parental alleles (A/G) in the ChRIP RNAs of the BWS samples, but only one allele (G) in the control (Fig. 7C). Taken together, the data described earlier demonstrate that the human *KCNQ1OT1* interacts with chromatin, that this interaction is exerted at least in part through its most 5' 20 kb sequence and suggest that the silencing of *CDKN1C* by this long non-coding RNA (ncRNA) in the BWS cells occurs via interaction with chromatin.

DISCUSSION

Although rare, microdeletions/duplications at imprinted loci have often provided interesting information on how imprinting control mechanisms normally work and can be altered in human disease. In this study, we show how an *in cis* duplication of the entire 11p15.5 cluster is associated with the maintenance of genomic imprinting and the SRS phenotype, whereas a smaller duplication including the centromeric ICR results in imprinting alteration and BWS. The data are summarized in Figure 8. In patient 1, the microduplication originated *de novo* on the maternal chromosome and was 1.2 Mb long, present *in cis* and in inverted orientation. The methylated status of ICR2 and the non-methylated status of ICR1 were maintained in the duplicated region, and the SRS phenotype

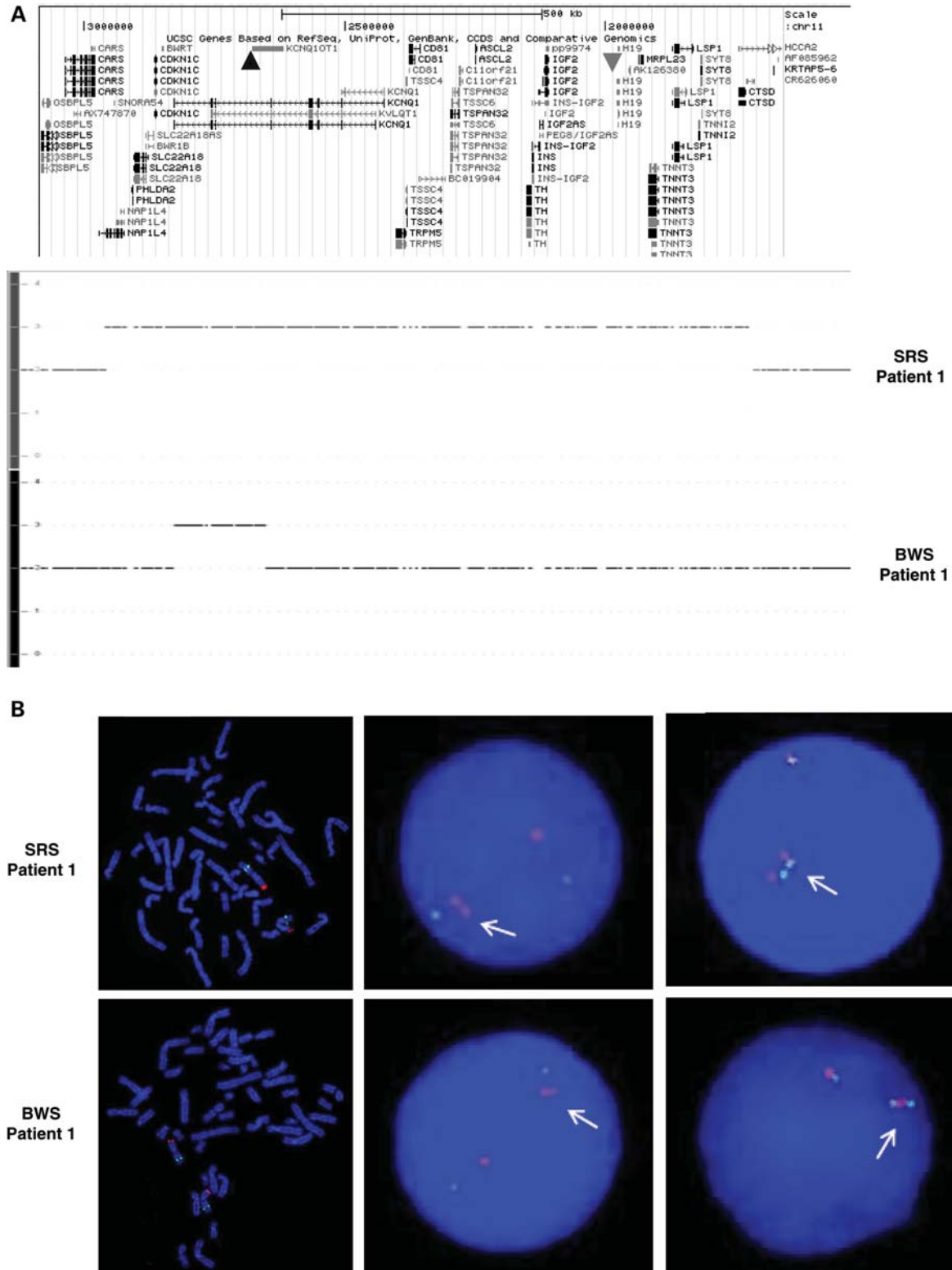


Figure 4. Structural characterization of the 11p15.5 microduplications. **(A)** Genomic profiles of SRS patient 1 and BWS patient 1 chromosome 11p15.5 as determined by SNP array-CGH. A screenshot of the UCSC Genome Browser showing that the relevant genomic region is present at the top and the normalized CN detected with the 11p15.5 probes is reported in the lower part of the figure. The analysis of BWS patient 2 produced results very similar to those of BWS patient 1 (data not shown). Note that the duplication of the SRS patient is 1.2 Mb long and includes the entire imprinted gene cluster, whereas the duplication of the BWS family is 160 kb long and includes the *KCNQ1* exons 12–15, ICR2 (dark gray triangle pointing upward) and the most centromeric 20 kb of *KCNQ1OT1*. ICR1 is indicated by a light gray triangle pointing downward. **(B)** Characterization by FISH of the 11p15 region duplicated in the patients under study. SRS patient 1, left panel: metaphase FISH using the BAC probes RP11-937011 (chr11:2 522 537–2 709 016, red) and RP11-876C12 (11q22.3, green). SRS patient 1, middle panel:

reflected the expected double dosage of the maternally expressed genes. A 160 kb long duplication was found in family 2. Similar to the larger one, the smaller duplication was inverted and present *in cis*. However, the 160 kb duplication was associated with loss of ICR2 methylation and co-segregated with BWS on maternal transmission. Analysis of cloned cells indicated that its maternal transmission results in the presence of both methylated and non-methylated ICR2 sequences on the same chromosome. We found that the 160 kb duplication did not affect the imprinted expression of the full-length *KCNQ1OT1*, but led to expression of a truncated *KCNQ1OT1* transcript and to *CDKN1C* silencing, when present on the maternal chromosome. Conversely, imprinting maintenance and normal phenotype were observed on paternal transmission. Finally, we obtained evidences that the truncated *KCNQ1OT1* interacted with chromatin through sequences present in the most 5' 20 kb of the transcript. These results imply the following: (i) the ICR2 sequence embedded in the 160 kb duplication is not sufficient for proper imprinting establishment; (ii) all features needed for correct imprinting are present in the larger duplication, including the entire 11p15.5 cluster and (iii) the first 20 kb of the *KCNQ1OT1* transcript has a functional role in the control of *CDKN1C* expression, likely mediated through interaction with chromatin.

In contrast to the maintenance of imprinting observed in the 1.2 Mb duplication, we found that the 160 kb duplication was associated with lack of ICR2 imprinted methylation. The absence of mosaicism in the BWS patient 2 cells suggests that the methylation defect arose at ICR2 during imprinting establishment in oocytes. Thus, two identical and closely located ICR2 sequences may acquire different methylation imprints, indicating that features, in addition to the ICR2 sequence itself, are necessary for imprinting establishment. The molecular basis for ICR recognition during imprinting establishment is still undefined (1,2). Through the analysis of mouse yeast artificial chromosome transgenes, we previously demonstrated that distantly located *cis*-acting sequences are needed for ICR2 imprinting (21). More recently, a transcription-based mechanism that would open chromatin for DNA methylation establishment at ICRs has been proposed, and transcripts derived from *Kcnq1* promoters and possibly serving this function have been identified in mouse oocytes (22). The reduced ICR2 methylation observed in BWS patients 1–3 could be explained by such models if the orientation of the ICR is important. ICR2 is the promoter of the *KCNQ1OT1* gene that is transcribed antisense to *KCNQ1*, and it is possible that the overlap of sense–antisense transcripts is necessary for imprinting establishment at this site. The inverted orientation of the 1.2 Mb duplication would be compatible with normal

imprinting because the *KCNQ1* promoter is included in this chromosomal imbalance. The lack of methylation of the paternal ICR2 in the individual I-2 (paternally inheriting the 160 kb duplication) of the BWS family argues against the possibility that the discordant ICR2 methylation results from convergent *KCNQ1OT1* transcription in BWS patients 1–3 (Fig. 8). Consistent with the hypothesis that the 5' end of the *KCNQ1* gene may control ICR2 methylation, a 50 kb duplication of the 5' portion of *KCNQ1* has been recently associated with a BWS case with ICR2 hypomethylation (16).

Long ncRNAs associated with imprinted gene clusters, such as *Kcnq1ot1*, silence protein-coding genes *in cis*, but their mechanism of action and in particular whether the long ncRNA is required at all for silencing or whether transcription itself induces silencing is not yet fully understood (7,23). Several evidences obtained in the mouse indicate that long ncRNAs control genomic imprinting by distinct mechanisms in embryonic and extra-embryonic tissues. *Kcnq1ot1* silences placental-specific imprinted genes via the recruitment of the histone methyltransferases EZH2 and G9a to promoters and ubiquitously imprinted genes via the recruiting of Dnmt1 to somatic differentially methylated regions (18,24). However, the location of the functional sequences in the 90 kb long *Kcnq1ot1* transcript is undefined. An 890 bp region in the 5'-region of the *Kcnq1ot1* RNA containing conserved sequence motifs has been shown to be required for imprinting maintenance (19,20). However, truncation of the *Kcnq1ot1* transcript to less than 3 kb but including the 890 bp causes loss of imprinted expression of most protein-coding genes in both embryonic and extra-embryonic tissues (6,25). Interestingly, *Cdkn1c* seems to be regulated by a different mechanism since 2–3 kb of *Kcnq1ot1* (or its transcription) is sufficient to silence *Cdkn1c* in many embryonic tissues (25). We found that *CDKN1C* was poorly expressed in the skin fibroblast of a BWS patient carrying the 160 kb duplication. In addition, the proposita's phenotype is typical of the BWS cases with loss of *CDKN1C* function (26,27). Furthermore, we observed that the truncated *KCNQ1OT1*, as well as the full-length transcript, interacts with chromatin. Therefore, our results indicate that *KCNQ1OT1* plays a direct role in *CDKN1C* silencing by interacting with chromatin through the 5'-part of its transcript. A recent study demonstrates that depletion of *Kcnq1ot1* ncRNA does not affect imprinting maintenance in stem cells, leaving open the possibility that this and other transcripts in the region play a role in imprinting establishment (28).

Chromosome imbalances at 11p15.5 have been described in both BWS and SRS. In BWS, duplications generally include the *IGF2* gene and are of paternal origin, whereas in SRS, duplications generally include *CDKN1C* and are of maternal origin (29,30). Indeed, BWS- and SRS-like phenotypes have

FISH analysis of interphase nuclei using the BAC probes RP11-937011 (red) and RP11-876C12 (green). The 11p15.5 probe detects a duplicated signal (arrowed), whereas the 11q22.3 probe shows normal signal. SRS patient 1, right panel: FISH analysis of interphase nuclei using the BAC probes RP11-937011 (green) and RP11-81K4 (chr11:2 755 275–2 927 014, red). Single and duplicated signals are detected on the two homologs. The order of the duplicated signal (arrowed): red-green-green-red indicates that the microduplication is inverted with the RP11-937011 probe in the middle. Patient BWS 1, left panel: metaphase FISH using the fosmid probe G248P81952E2 (chr11:2 660 932–2 698 197, red) and the BAC probe RP11-876C12 (11q22.3, green). Patient BWS 1, middle panel: FISH analysis of interphase nuclei using the fosmid probe G248P81952E2 (red) and the BAC probe RP11-876C12 (green). The 11p15.5 probe detects a duplicated signal (arrowed), whereas the 11q22.3 probe shows normal signal. Patient 2, right panel: FISH analysis of interphase nuclei using the fosmid probes G248P81952E2 (red) and G248P800755G6 (chr11:2 759 661–2 803 886, green). Single and duplicated signals are detected on the two homologs. The order of the duplicated signal (arrowed): green-red-red-green indicates that the microduplication is inverted with the G248P81952E2 probe in the middle.

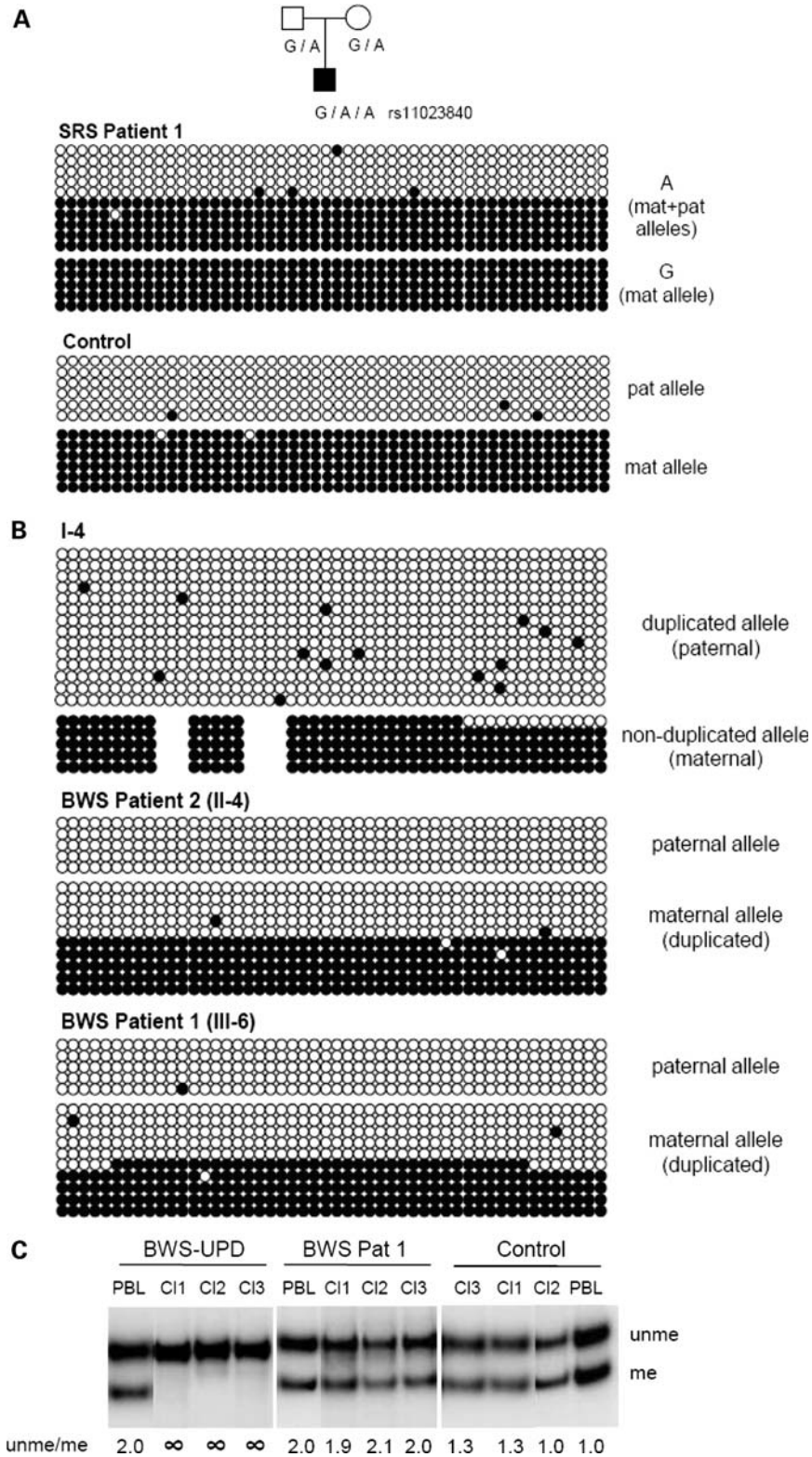


Figure 5. DNA methylation analysis of the ICR2 region. (A) Allele-specific DNA methylation analysis by bisulfite sequencing in SRS patient 1. DNA samples were treated with sodium bisulfite, amplified by PCR over the ICR2 region, cloned and sequenced. Each line corresponds to a single template DNA molecule, and each circle represents a CpG dinucleotide. Filled circles designate methylated cytosine and open circles correspond to unmethylated cytosines. The alleles were discriminated by typing for the SNP G/A rs11023840. Note that ICR2 is hypermethylated in patient 1 when compared with a control, but this is consistent with the microduplication of the methylated maternal allele. (B) Allele-specific DNA methylation analysis by bisulfite sequencing in BWS patients 1 and 2. DNA samples were analyzed as in (A). The maternal and paternal alleles were discriminated by typing for an insertion/deletion polymorphism and an SNP. Missing circles correspond to polymorphic CpGs. Although ICR2 is hypomethylated in all tested individuals, one allele is totally unmethylated and the other totally

been observed in the same family due to different parental origin of an unbalanced 11p15 translocation (17,18). However, exceptions to this rule have been recently described by Demars *et al.* (16). In this study, the authors report a maternal duplication in an SRS patient, which does involve only part of the IGF2/H19 domain and a 50 kb maternal duplication which does not involve CDKN1C in a BWS patient with loss of methylation at ICR2. Consistent with this study, here we demonstrate that *in cis* 11p15.5 maternal microduplications can be associated with both SRS and BWS. The unexpected BWS phenotype we observed is explained by the finding that maternal transmission of the microduplication leads to aberrant imprinting establishment at ICR2 and to *CDKN1C* silencing, linking a *cis*-acting mutation with a heritable imprinting defect of the centromeric BWS domain. Interestingly, no CN variation (CNV) similar to the 160 kb or overlapping the 5' 20 kb of *KCNQ1OT1* is reported in the Database of Genomic Variants (Supplementary Material, Fig. S4). Future investigation of 11p15.5 alterations in BWS and SRS cases with array- and deep seq-based methods may reveal further details of the regulatory mechanisms underlying genomic imprinting.

MATERIALS AND METHODS

Patients

DNAs from peripheral blood leukocytes from all the family members were extracted with Gentra Puregene Cell Kit (Qiagen). All the genetic analyses were performed after the informed consent had been obtained. The patients described in this study belong to a larger (more than 200 individuals) analyzed cohort, which has been analyzed by MS-MLPA and SNP array in order to detect non-polymorphic CN variants and CN-neutral loss of heterozygosity due to uniparental disomy.

MS-MLPA analysis

MS-MLPA was performed on genomic DNA with the SALSA MS-MLPA kit ME030-B2 lot 0309 for BWS–SRS (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer's instructions (<http://www.mrc-holland.com>). The SALSA MS-MLPA ME030 probemix is capable of rapidly detecting most causes of BWS and SRS, as both CNs and methylation status of the 11p15 region can be determined. The assay was validated on DNA samples from BWS and SRS patients and identified methylation defects as well as chromosome 11p15 duplications and deletions, as described (31,32). The ME030-B2 BWS/SRS probemix contains 26 probes specific for the BWS/SRS 11p15 region. Eleven of

these probes contain a *HhaI* recognition site and provide information about the methylation status of the target sequence. In addition, 19 MLPA probes located outside of the BWS–SRS locus are added for reference. These include three MS-MLPA control probes for complete *HhaI* digestion in the MS-MLPA reaction. Overall, the ME030-B2 BWS/SRS probemix contains 45 different probes with amplification products between 121 and 483 nt.

Data analysis

Copy number. The values of peak size were normalized as follows: the height of each peak (H_s) was divided by the sum of all 45 peak heights of that sample ($\sum H_s$). For each peak, this ratio ($nH_s = H_s/\sum H_s$) was divided by the relative peak height of the corresponding probe calculated as the average from two or three control DNA samples ($nH_c = H_c/\sum H_c$). A ratio (nH_s/nH_c) ranging from 0.8 to 1.1 was considered as a normal exon dosage; a deletion was suspected for ratio less than 0.7 and a duplication was suspected for ratio more than 1.2.

Methylation status. Quantification of the methylation status of a CpG site was done by dividing the peak area with the combined areas of the control probes lacking a *HhaI* site. The relative peak area of each target probe from the digested sample was compared with those obtained from the undigested sample. ICR2 hypomethylation was suspected when values were less than 0.40, whereas ICR1 hypermethylation was considered when values were greater than 0.65.

Southern blotting analysis

DNA methylation at ICR1 and ICR2 of 11p15.5 was analyzed by Southern blotting and COBRA, as described previously (12,33).

Microsatellite analysis

D11S4088 short tandem repeat (STR) marker mapping to the duplicated region was analyzed in I4, II3, II4, II5, III5 and III6 affected and non-affected family members. TH and D11S4046 STR mapping to the duplicated region was analyzed in patient 1 and his parents. Primers specific for the STR were obtained from NCBI Genome Database together with the PCR conditions. PCR amplification of 100 ng DNA was done using forward primer end labeled with Fam. Twenty-eight cycles of PCR products were run on the fluorescent capillary system ABI 3130XL. Data were analyzed using GeneMapper Software.

methyated in I-4, whereas the maternal allele is only partially methyated in BWS patients 1 and 2, indicating that the 160 kb duplication results in the alteration of the ICR2 imprinted methylation if maternally transmitted but does not affect imprinting when paternally inherited as likely happened in I-4. (C) Analysis of ICR2 methylation in lymphoblast cell clones as determined by COBRA. The DNAs derived from PBLs and lymphoblast clones of BWS patient 1, a BWS case with partial loss of ICR2 methylation due to mosaic UPD11, and a normal control were treated with sodium bisulfite, PCR-amplified and digested with restriction enzymes containing a CpG dinucleotide in their target sequence. The ratio (unme/me) between unmethylated and methylated alleles is indicated below each panel. Only three representative clones for each line are shown. The clones derived from the UPD11 case displayed complete loss of ICR2 methylation, whereas the PBLs from which they derived showed partial loss of methylation. The clones derived from a normal control maintained 50% methylation similar to the PBLs derived from the same individual. The clones derived from BWS patient 1 displayed partial ICR2 hypomethylation similar to their parental PBLs. The absence of selection indicates lack of epigenetic mosaicism in this patient.

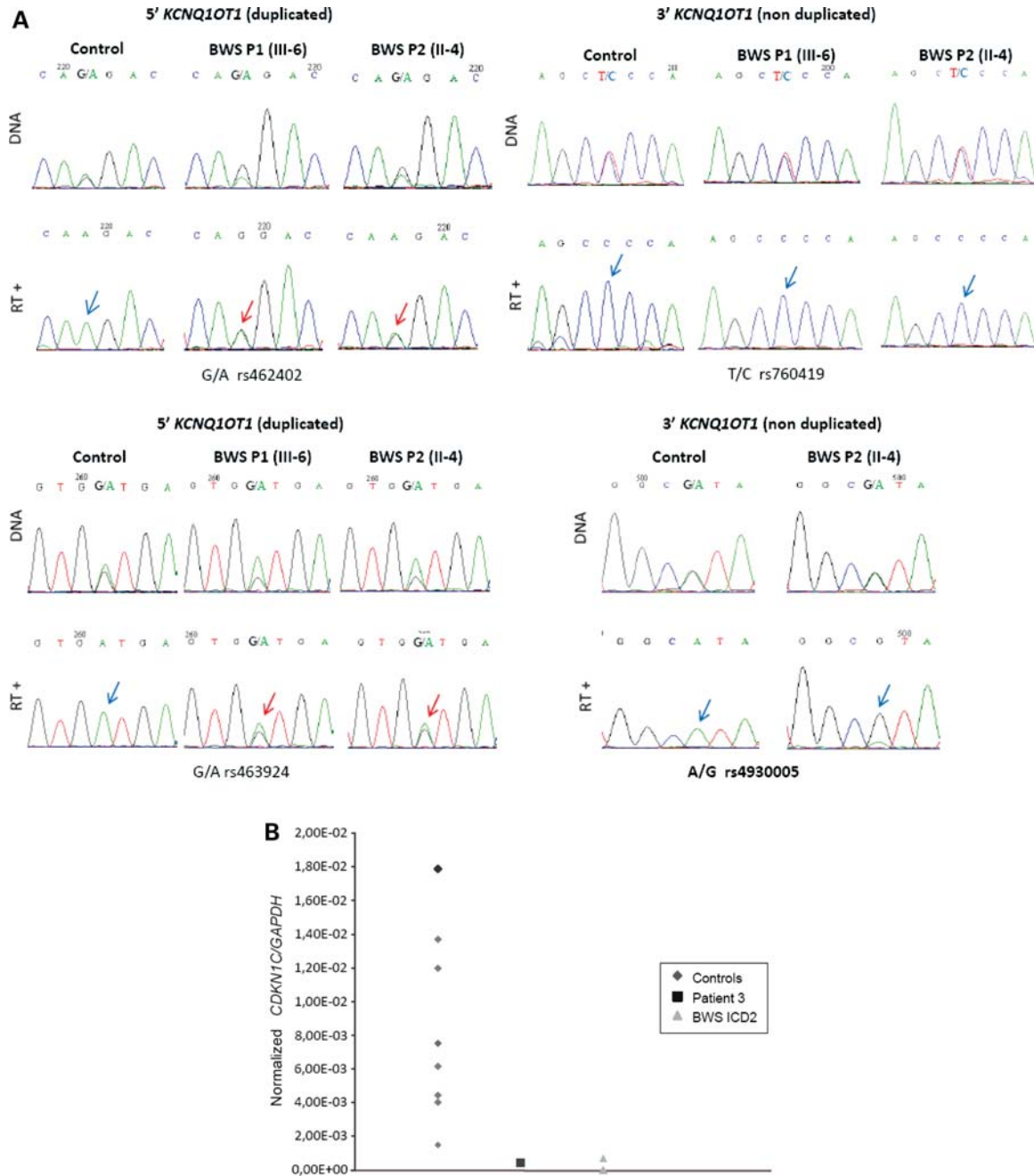


Figure 6. Expression analysis of the centromeric domain genes. **(A)** Allele-specific expression of *KCNQ1OT1*. DNAs and RNAs extracted from lymphoblast cells of BWS patient 1, BWS patient 2 and normal control individuals were analyzed by PCR and RT-PCR. The parental alleles of *KCNQ1OT1* were discriminated by typing for the SNPs G/A rs462402 and G/A rs463924 that were present in the duplicated (5') region of *KCNQ1OT1* and T/C rs760419 and A/G rs4930005 that were present in the non-duplicated (3') region of *KCNQ1OT1*. The electropherograms obtained from sequencing of the PCR products over the SNPs are shown. The red arrows indicate the presence of both parental alleles, and the blue arrows the presence of a single allele in the RT-PCR product. Note that the 5' part of *KCNQ1OT1* is expressed from both parental alleles, whereas the 3' part is expressed from only one allele in BWS patient 1 and BWS patient 2. **(B)** Analysis of *CDKN1C* expression. The level of *CDKN1C* RNA was assessed by quantitative real-time RT-PCR in the skin fibroblasts derived from eight age-matched normal control individuals (diamonds), BWS patient 2 (square) and two BWS patients with loss of ICR2 methylation (triangles). *CDKN1C* values were normalized to the expression of *GAPD*. Note that *CDKN1C* expression in BWS patient 2 is 20-fold lower than the average level found in normal control individuals and is comparable to that of BWS patients with ICR2 hypomethylation and no microduplication.

Array CGH

SNP array analysis was performed using the Affymetrix Genome Wide Human SNP Array 6.0, which includes over 906 600 SNPs and more than 946 000 probes for the detection

of CNVs. The median inter-marker distance taken over all 1.8 million SNP and CN markers combined is less than 700 bases. Sample preparation, hybridization and scanning were processed using GeneChip® Instrument System hardware,

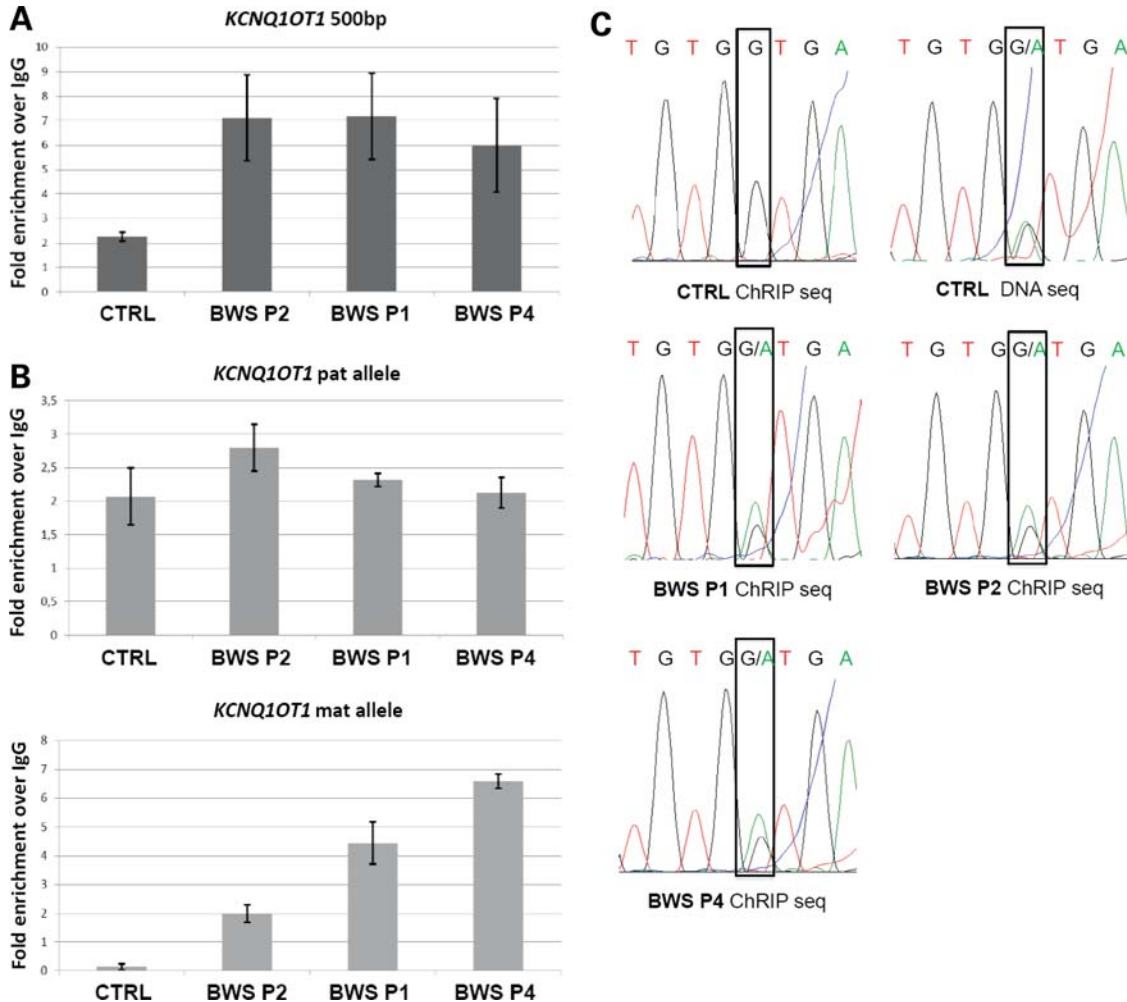


Figure 7. Interaction of the *KCNQ1OT1* RNA with chromatin. (A) qPCR analysis of ChRIP-purified RNA from four lymphoblast cell lines. The enrichment of the *KCNQ1OT1* RNA is measured with primers located 500 bp downstream of the transcription start site. The histogram represents fold enrichment of the H3 Ab over IgG. CTRL, normal control individual; BWS P1, BWS patient 1; BWS P2, BWS patient 2; BWS P4, BWS patient with ICR2 hypomethylation and no microduplication. (B) Allele-specific PCR analysis of ChRIP RNA. rs463924 allele-specific qPCR analysis of the cell lines analyzed in (A). The histograms report as enrichment over IgG the results obtained with primers specific for the paternal allele and primers specific for the maternal allele. All the above data represent mean \pm SD of at least two independent experiments. (C) Sequence analysis at SNP rs463924 (\pm 3 bp) of the cDNA obtained from the ChRIP RNA. The DNA sequence of the normal control individual (CTRL DNA seq) is shown to demonstrate the heterozygosity. Differently from the control cell line used in Figure 6A, the G allele is paternal in the control sample used for the ChRIP analysis. Note that both parental alleles of the *KCNQ1OT1* RNA interact with chromatin in the cells derived from the BWS patients carrying the 160 kb duplication.

according to manufacturer's specifications (Affymetrix, Santa Clara, CA, USA). The analysis was performed using Genotyping Console 3.0.2 (Affymetrix) software. Raw data files (.CEL) were imported to Genotyping Console and normalized using the default option. Unpaired analysis, using as baseline the 270 HapMap samples supplied by Affymetrix, was performed to obtain CN value from .CEL files, and the amplified and/or deleted regions were detected using hidden Markov model method.

Fluorescence in situ hybridization

FISH analysis using BAC and fosmid probes targeting the duplicated region in 11p15.5 was done on lymphoblasts to ascertain the mechanism of duplication (Fig. 3B). Chromosomal analyses were performed on lymphoblast cultures according to

standard methods [B-lymphocytes were immortalized by Epstein-Barr virus (EBV) extracted from B95.8 marmoset cells and cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS)]. The following BAC clones RP11-81K4, RP11-937011 and RP11-876C12 and fosmid clones G248P81952E2 and G248P800755G6 were used as probes for FISH analysis. Their location was defined according to the UCSC database, March 2006 assembly (University of California Santa Cruz, <http://genome.ucsc.edu/cgi-bin/hgGateway?db=hg18>). The clones belong to the RPCI library (<http://www.chori.org/bacpac/>; <http://bacpac.chori.org/hmale11.htm>) and were obtained from Resources for Molecular Cytogenetics (<http://www.biologia.uniba.it/rmc/>). BAC DNAs were extracted by conventional methods and labeled with green or red/orange fluorophores using a non-enzymatic nucleic acid labeling method (ULSTM, Kreatech Diagnostics,

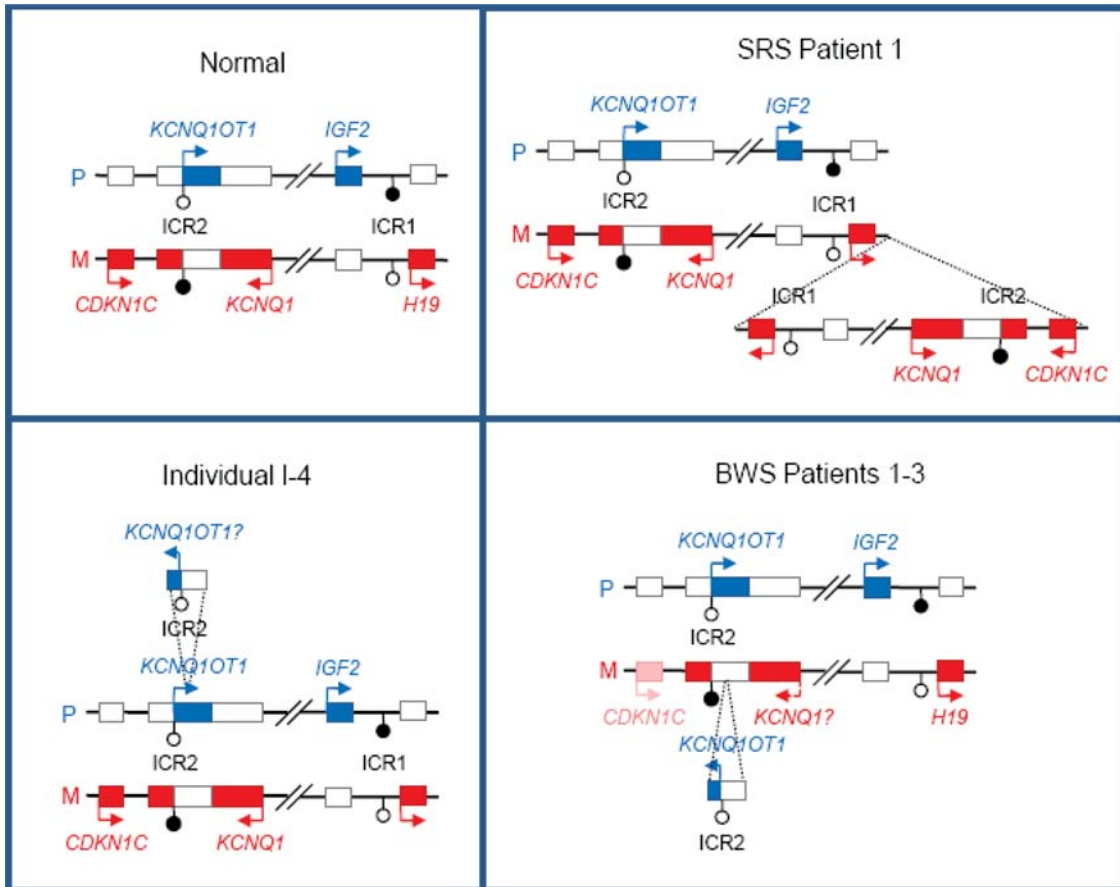


Figure 8. Diagram representing the observed and predicted methylation and expression patterns at chromosome 11p15.5 in the subjects under study. The duplicated regions are depicted separately from the chromosomes and connected to the breakpoint by dotted lines. The inverted orientation of the microduplications is shown. P, paternally derived chromosome; M, maternally derived chromosome. Representative imprinted genes are shown (those paternally expressed are in blue and those maternally expressed are in red). Gene transcription is indicated by arrows. Light red color for *CDKN1C* represents decreased expression. No quantitative data are available for the expression of *KCNQ1* in BWS patients 1–3 and *KCNQ1OT1* in the individual I-4 of the BWS family (indicated with ?). Filled and open circles represent methylated and non-methylated ICRs, respectively.

Amsterdam, The Netherlands). The chromosomes were counterstained with Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Hybridizations were analyzed using a Nikon Eclipse-1000 epifluorescence microscope (Nikon Instruments, Tokyo, Japan), and images captured and elaborated using the Genikon system v. 3.8.5 (Nikon Instruments S.p.a., Calenzano, FI, Italy).

Bisulfite sequencing

The methylation status of C-residues in DNA was determined by the bisulfite sequencing technique. In this procedure, DNA was treated with sodium bisulfite, PCR-amplified and the PCR products were cloned in Topo pCR2.1 vector (Topo-TA cloning kit, Invitrogen) and the clones sequenced. We also found an insertion/deletion polymorphism and an SNP (Accession No. AJ006345, dbSNP: rs11023840) that segregated with either the unmethylated or methylated C-residues. The SNP rs11023840 is a polymorphic CpG > TpG. To distinguish the parental origin after bisulfite conversion, we amplified and sequenced the lower strand (G > A).

Gene expression analysis

For expression analysis, we extracted RNA from lymphoblastoid and skin fibroblast cell lines, using the Trizol reagent (Invitrogen). About 1 μ g of total RNA was treated with RNase-free DNase, and first-strand cDNA was synthesized using Quantitech Reverse Transcription Kit (Qiagen), according to the protocol of the manufacturer. *CDKN1C* expression was examined by SYBR Green quantitative real-time RT-PCR (Applied Biosystems, Foster City, CA, USA). Reactions were run on ABI PRISM 7500 using the default cycling conditions. Relative expression was determined using the $\Delta\Delta C_T$ method, and gene expression values were normalized to the expression of the GAPDH reference gene. Primers, PCR conditions and polymorphisms used for the analysis are described in Table 2. PCR products were sequenced at PRIMM Sequencing Core (Naples).

CDKN1C sequencing

CDKN1C mutations were screened by means of direct sequencing using an automated sequencer (ABI PRISM 3100, PE

Table 2. Primers and PCR conditions

Locus	PCR primer pairs	Size (bp)	MgCl ₂ (mM)	Annealing temperature (°C)
<i>KCNQ1OT1</i> , G/A rs462402	5'-AATATGGATTCTAACTGAGCCC-3'; 5'-TGCCTTCTGCCAACACTTGGC-3'	567	1.0	50
<i>KCNQ1OT1</i> , T/C rs760419	5'-ACTCTCAAAGCATGAAAACAAGTGG-3'; 5'-CTATAAAACTGATTGTCAGGGCTGG-3'	535	2.5	64
<i>CDKN1C</i> ^a	5'-AGAGATCAGCGCTGAGAAG-3'; 5'-CACCTTGGGACCAGTGTACC-3'	336		58
<i>KCNQ1OT1</i> ^b 500 bp	5'-GACTGACGCACCGAGGAG-3'; 5'-CAGGGAGCTCCTCAGCAA-3'	143		55
<i>ACT1N</i> ^a	5'-CACACTGTGCCCATCTACGA-3'; 5'-CCATCTCTTGCTCGAAGTCC-3'	202		55
Rs463924 mat ^a	5'-AATATTTGTAAATGCCAACTGAACTT-3'; 5'-CTCTTCTGGGCCTCTGTCAT-3'	120		55
Rs463924 pat ^a	5'-AATATTTGTAAATGCCAACTGAACTT-3'; 5'-CTCTTCTGGGCCTCTGTCAC-3'	120		55
Rs463924 seq	5'-AATATTTGTAAATGCCAACTGAACTT-3'; 5'-CTTCTGAACAGTCCCCACTGA-3'	201	2.0	55

^aReal-time PCR (Power SYBR Green Master Mix by Applied Biosystems).

Applied Biosystems) using PCR and sequencing primers (34) with fluorescent dye terminators; PCRs for the GC-rich region were adapted using the GC-RICH PCR system (Roche-Applied Science, Indianapolis, IN, USA).

Lymphoblast cloning

Lymphoblastoid cell lines were established by EBV transformation of peripheral blood leukocytes. Cells were grown in the RPMI 1640 medium supplemented with 20% FBS, L-glutamine and antibiotics at standard concentration. Clonal isolation was performed separating cells with the help of an extended Pasteur pipette, serially diluting them to obtain a concentration of about 1000 c/ml. One hundred microliters of cell suspension was transferred to each well of a 96-well dish (under microscope there were about 20–50 countable cells). Conditioned media was added periodically to promote growth. Clones were detectable after 7 days and ready to score after 10–15 days. Growing cultures were expanded into larger vessels. A second time recloning of at least 10 clones for each sample was performed as described before to increase the likelihood that the cells originated from a single cell. ICR2 DNA methylation was determined by COBRA: 1.5 µg of DNA extracted from single-cell clones was treated with sodium bisulfite, PCR-amplified, the PCR product digested with a restriction enzyme containing a CpG dinucleotide in its target sequence and the fragments separated on a polyacrylamide gel. Primer sequences were: 5'-GYGTTATTTTATATTTAGTTAGTG-3' and 5'-TCTTACTAAAAAACTCCCTAAAAATC-3'.

Chromatin RNA immunoprecipitation

ChRIP was performed on the lymphoblastoid cell lines derived from BWS patients and normal individuals, as has been described previously (20,35) with antibodies raised against core histone H3 (Abcam) and rabbit IgG (Millipore). The immunoprecipitated RNA was purified using phenol–chloroform, DNase-treated and re-purified by the phenol–chloroform method. cDNA was synthesized by the ImProm II Reverse Transcriptase Kit (Promega). *KCNQ1OT1*, allele-specific *KCNQ1OT1* and β -*Actin* transcripts were detected by qPCR using the primers listed in Table 2.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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