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

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## 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<sup>1-4</sup>.

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<sup>5</sup> (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), microdeletions of KvDMR<sup>6</sup> or H19DMR<sup>7</sup> (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<sup>8-11</sup> and in approximately 40% of cases with a positive family history<sup>12</sup>.

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<sup>13,14</sup>. 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*<sup>15-17</sup>. 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<sup>18,19</sup>. 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<sup>9,12,20</sup>.

It has been demonstrated that BWS patients with paternal UPD always show mosaicism, suggesting that all cases had arisen as a postzygotic event<sup>19,21,22</sup>; a possible explanation is that lack of one or more chromosome 11 maternally expressed genes may lead to embryonic lethality<sup>23</sup>. 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<sup>24</sup>. 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*)<sup>19,21,23</sup>. 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<sup>23,25</sup>. 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<sup>26</sup>.

Clinically, a clear association between mosaic UPD and hemihyperplasia exists<sup>15,19,22,27</sup>. 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<sup>27-29</sup>. Moreover, it has been hypothesized that extremely high levels of UPD might drive severe phenotypic expression of BWS<sup>30</sup>; 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)<sup>31-33</sup>, 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. Among these patients we detected 71 with hypomethylation at KvDMR, 8 with point mutation on *CDKN1C* gene, 2 with hipermethylation of H19DMR and 2 with paternal duplication of 11p15.5 region. Finally, 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.<sup>34</sup>. The calculation was as follows:  $(K-1)/(K+1) \times 100$ ; 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 *HhaI* (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). To calculate the gene dosage, the analysis of results was based on the comparison between the signals of undigested samples and undigested controls. Calculation of the methylation level of probes containing the *HhaI* 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 normalized 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.



## **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 Biosystems). The primers amplify both methylated and unmethylated template, according to the principles set out to compensate for PCR bias<sup>35</sup>. 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 Software (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<sup>36</sup>. 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 (Supplementary Figure 1). We calculated the percentage of mosaicism by means of STRs and SNP-arrays (Figure 2a). The calculation of mosaicism using STRs has been done as recommended by Sasaki et al.<sup>34</sup> (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 methylation 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:  $R^2=+0.933$  for H19DMR and  $R^2=-0.833$  for KvDMR (Figure 3a; Supplementary Table I).

### **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 2a, 2b). To estimate the extent of pUPD we mapped the latest SNP in pUPD and the first SNP in heterozygosity 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 (Supplementary Figure 1b, 2a, 2b; 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 3b; Supplementary Table I).

### **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 2b; 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 2b).

### **DISCUSSION**

Paternal UPD of chromosome 11p is a relatively common and cancer prone mechanism in BWS patients <sup>27</sup> ; 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 methylation of the centromeric imprinting center (IC2) <sup>37</sup> .

### **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 <sup>24</sup> . 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 (Supplementary Table II). 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<sup>30</sup>.

#### **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<sup>23,38</sup> (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 mosaicism 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) 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 3.** 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.

**Supplementary Figure 1.** (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 hypomethylation of KvDMR (and vice versa).

**Supplementary Figure 2.** (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.

**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**

Patient	STR markers					
	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%	-

Patient	b MS-MLPA		c MS-HRM		d CpG Q-Pyro	
	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%

**Supplementary Table I.** 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).



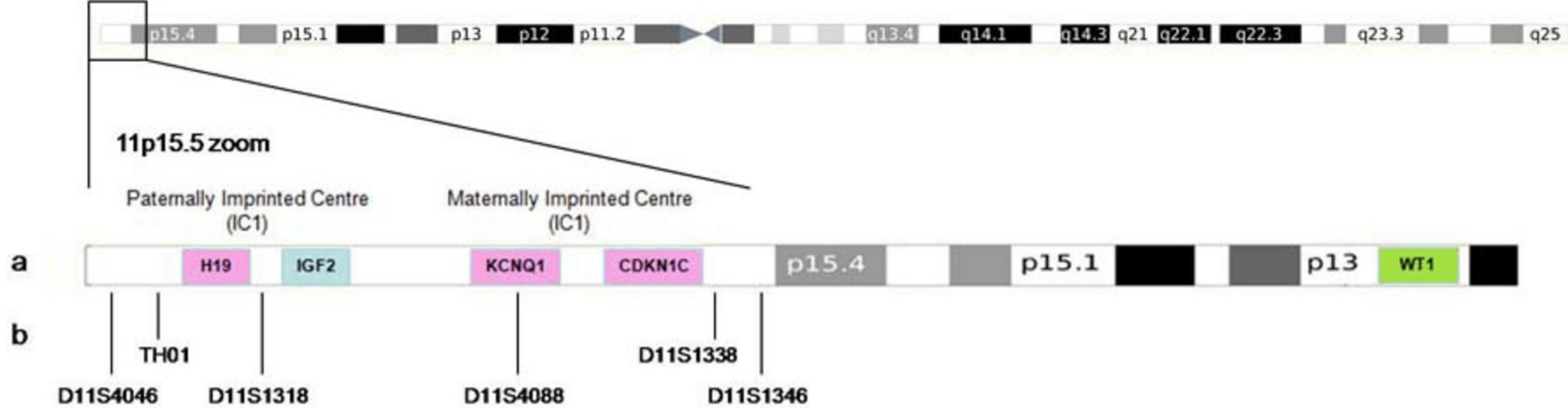
**Supplementary Table II.** (a) Molecular results and clinical findings of each patient. (b) Summary table with frequency of main clinical signs in our of patients.

**a**

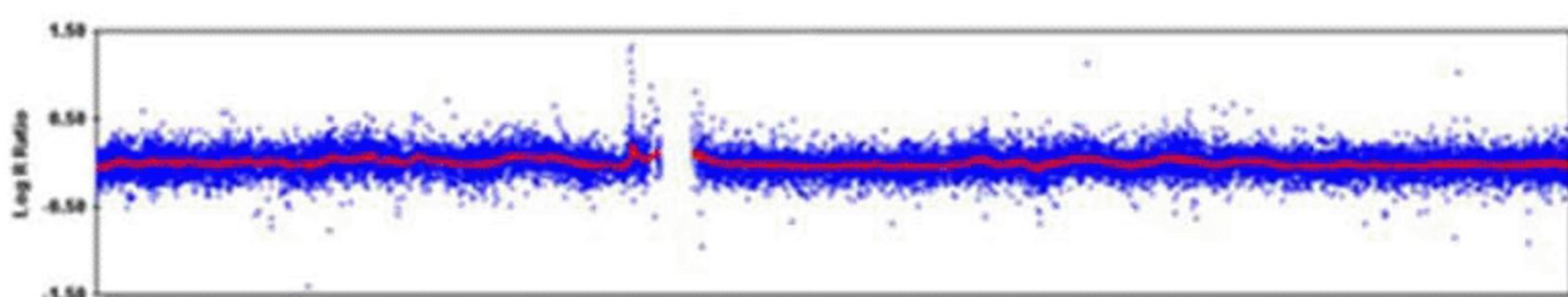
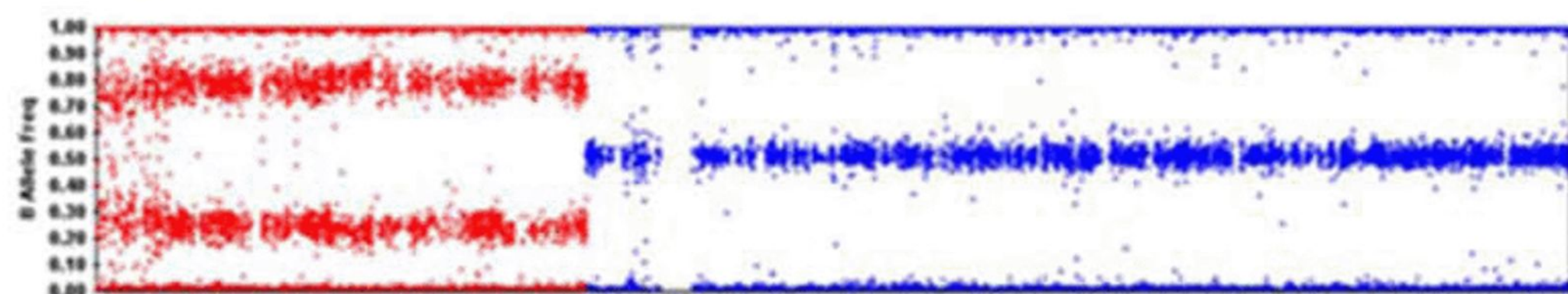
Patient	UPD 1	UPD 2	UPD 3	UPD 4	UPD 5	UPD 6	UPD 7	UPD 8	UPD 9
<b>Sex</b>	Male	Male	Female	Female	Male	Male	Female	Male	Male
<b>Age at diagnosis</b>	3y 11m	4y 5m	2y 3m	2y 4m	2y 4m	1y 5m	2y 1m	3m	5m
<b>% of mosaicism</b>	89,5%	68,4%	64,5%	76,9%	76,9%	64,3%	60,9%	70,0%	70,6%
<b>UPD extent</b>	45,1Mbp	46,2Mbp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

**b**

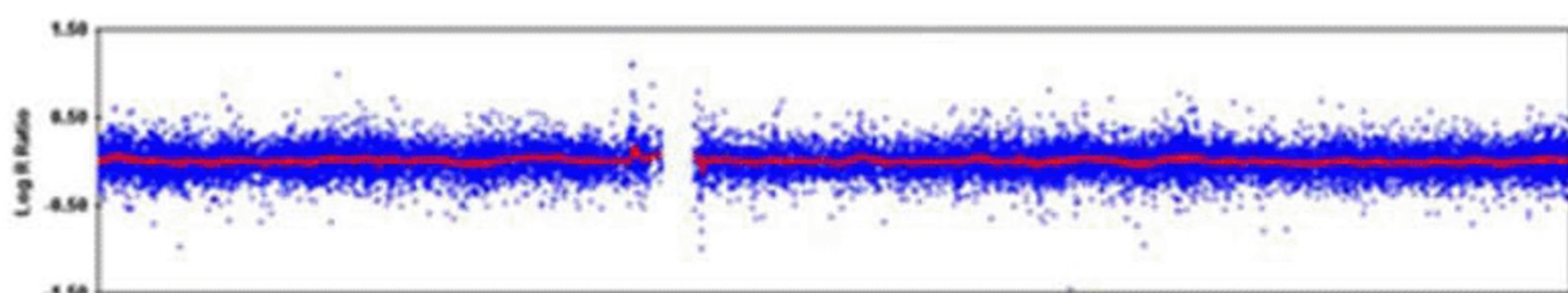
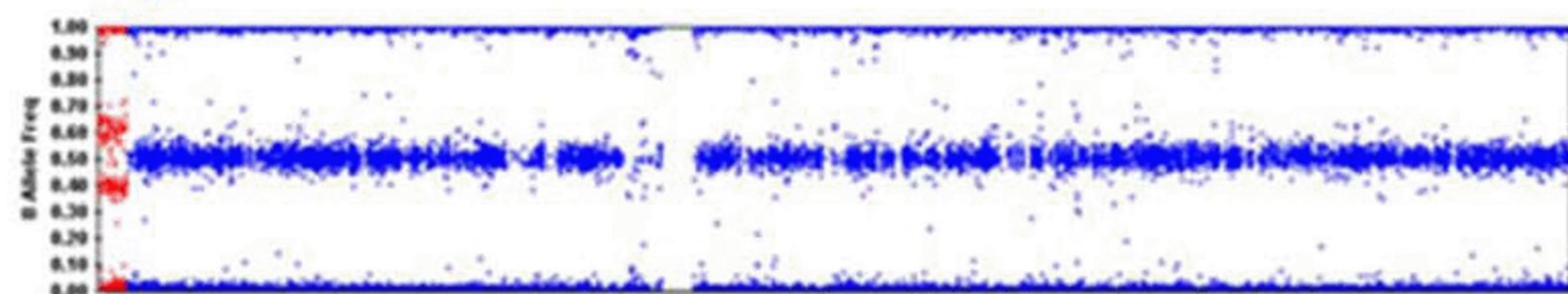
Clinical Findings	
Male:Female	6:3
Polyhydramnios	4/9
Increased birth weight	9/9
Hemihyperplasia	9/9
Macroglossia	6/9
Ear creases	3/9
Omphalocele	2/9
Umbilical hernia	3/9
Capillary malformation	3/9
Wilms' tumor	0/9
Hypoglycaemia	6/9





**a** UPD 4

## UPD 7

**b** BWS locus