Human Cytogenetics Case Report

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Detection of a novel familial deletion of four genes between BP1 and BP2 of the Prader-Willi/Angelman syndrome critical region by oligo-array CGH in a child with neurological disorder and speech impairment

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Abstract. Two common classes of deletions are described in the literature in individuals with Prader-Willi/Angelman syndrome (PWS/AS): one between breakpoint 1 (BP1) to BP3 and the other between BP2 to BP3 of the PWS/AS critical region on chromosome 15q11→q13. We present here a novel observation of an approximately 253-kb deletion between BP1 and BP2 on 15q11.2, in a 3½-year-old boy, who was referred to us with a clinical suspicion of having Angelman syndrome and presenting with mental retardation, neurological disorder, developmental delay and speech impairment. Karyotype and FISH results were found to be normal. The microdeletion between BP1 and BP2 includes four genes – *NIPA1*, *NIPA2*, *CYFIP1* and *TUBGCP5* which was detected by a high-resolution oligonucleotide array-CGH that was further validated by a Multiplex Ligation-dependent Probe Amplification (MLPA) assay. The same deletion was observed in the father who presented with similar but relatively milder clinical features as compared to the affected son. Methylation studies by methylation-specific MLPA (MS-MLPA) of the *SNRPN* imprinting center (IC) showed a normal imprinting pattern, both in the patient and the father. To our knowledge a microdeletion limited only to the BP1-BP2 region has not yet been reported. The familial genetic alteration together with the striking clinical presentation in this study are interesting, but from our single case study it is difficult to suggest if the deletion is causative of some of the abnormal features or if it is a normal variant. The study however further strengthens the fact that genome-wide analysis by array CGH in individuals with developmental delay and mental retardation is very useful in detecting such hidden interstitial chromosomal rearrangements.

The aetiology of mental retardation and dysmorphism is not well understood in over half of the affected individuals. Constitutional chromosomal abnormalities involving submicroscopic deletions or duplications are known to be important factors leading to the clinical conditions in this group of patients (Flint et al., 1995; Lupski, 1998; Knight et al., 1999; De Vries et al., 2001; Bailey et al., 2002; Kriek et al., 2004).

However, detection of such submicroscopic chromosomal aberrations is impossible due to the limitations of resolution of routine cytogenetic techniques. Recently matrix-based comparative genomic hybridization (array-CGH) appears to be a very promising tool for detecting such micro

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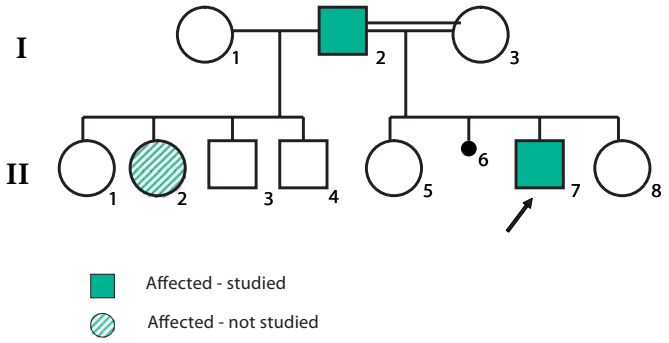

Fig. 1. Pedigree of the affected family. I2 and I3 = parents of the index patient. They are second cousins; II-5 = 6-year-old normal female; II-6 = spontaneous abortion at 2 months; II-7 = index patient; II-8 = 3-month-old female with cleft palate (corrected); II-1, II-3 and II-4 = normal children from first marriage; II-2 = affected 17-year-old female with severe mental retardation, developmental delay, cleft palate, seizures. Note: Only I-2, I-3, II-7 and II-8 were available for the present study.

deletions and/or duplications, where fluorescently labeled patient and control genomic DNA are cohybridized onto a genomic microarray (BACs or oligonucleotides). High-resolution screening of genomic copy number imbalances in mental retardation and dysmorphic patients by array-CGH has been reported by several groups of workers, demonstrating the usefulness of the technique (Antonarakis, 2001; Veltman et al., 2002; Vissers et al., 2003; Shaw-Smith et al., 2004). We present here a rare case of a 15q11.2 microdeletion between BP1 and BP2 of the Prader-Willi critical region, detected by oligo array-CGH in a child with mental retardation, developmental delay, neurological defects and speech impairment. The deletion was found to be about 253 kb in length, spanning four genes – *NIPA1*, *NIPA2*, *CYFIP1* and *TUBGCP5* – located within the BP1 and BP2 region of chromosome 15q11.2 which was further validated by a Multiplex Ligation-dependent Probe Amplification (MLPA) assay.

Case report

The patient is a 3½-year-old boy with developmental delay and several other abnormal features (II-7). He was born at full term after an uneventful third pregnancy of a 32-year-old mother and a 42-year-old father (Fig. 1). The parents are second cousins and the mother of the proband is the second wife of the patient's father. Their first child (II-5) of the second marriage, is a healthy 6-year-old girl, the second pregnancy (II-6) resulted in a spontaneous abortion, and the last child (II-8) is a healthy 3-month-old daughter that was born with a now corrected cleft palate. The proband had cleft palate at birth, which was corrected at the age of 9 months. The parents sought medical advice because of his delayed attainment of motor developmental milestones with crawling by the age of one year and walking without support by the age of three as well as delayed speech development, restricted to vocalizing. At the initial examination by a pediatrician, the patient was diagnosed as having developmental delay, attention deficit and hyperactivity. An amino acid chromatography and brain CT scan was performed and both were found to be normal. Finally, he was referred to a pediatric neurology clinic. Apart from the delayed developmental milestones, the examination showed several striking features including muscular hypotonia and brisk bilateral deep tendon reflexes. He can walk without support, but only with a wide base gait and uplifted, flexed and pronated fore arms, as well as an erratic tremulous movement of the limbs. His head circumference was below but weight and height were above the 50th percentile. In addition, he was found to have a typical happy puppet appearance with drooling and constantly smiling as well as hypermotoric (hyperactive) behavior and short attention span but no history of seizures. As the clinical phenotype was suggestive of Angelman syndrome (AS), the patient was referred to the Cytogenetics laboratory for chromosome and FISH studies. His father was also observed to be subnormal with very slow understanding, a peculiar smiling face and a childhood history of developmental delay. Family history suggested some ongoing medical conditions like mental retardation and developmental delay in other members of the extended family. A 17-year-old daughter (II-2) from his first marriage was reported to have severe mental retardation, developmental delay, cleft palate and seizures and she is institutionalized. Other affected family members including II-2 were not available for the present study. Clinical features of the three affected individuals (II-7, I-2 and II-2) are presented in Table 1.

Materials and methods

Cytogenetics

Cytogenetic studies were carried out on metaphase chromosome preparations from lymphocytes using routine tissue culture and banding methods. Fluorescence in situ hybridization (FISH) studies were undertaken using the LSI PWS/AS Region (D15S10) FISH probe from Vysis, specific for *UBE3A* locus on 15q11→q13. FISH labeling, hybridization and washing were carried out according to the manufacturer's protocol.

Oligonucleotide array-CGH

Human 44A microarray (Agilent) was used for the present study. It contains in situ synthesized 60-mer oligonucleotides representing a total of 44,290 features. The oligonucleotide probes span the human genome with an average spatial resolution of approximately 75 kb, including coding and non-coding sequences, providing sufficient coverage for a genome-wide survey of DNA aberrations.

Genomic DNA from peripheral blood of the index patient (II-7), father (I-2), mother (I-3), sister (II-8) and a normal male and female control was extracted by the routine Proteinase K method. Labeling of DNA, hybridization to oligonucleotide-array and post washing was carried out according to the protocol from Agilent Technologies. Briefly, 3 μg of genomic DNA of the index patient and the normal control were digested with the restriction enzymes *Alu*I (10 U/μl) and *Rsa*I (10 U/μl) at 37°C for 2 h. Patient DNA was labeled with Cy3-dUTP and control DNA with Cy5-dUTP, using the BioPrime CGH Genomic Labeling kit. Reverse labeling with Cy5-dUTP and Cy3-dUTP of the patient and control DNA respectively was also performed, which was used as an internal control of the experiment. Hybridiza-

Table 1. Clinical features seen in the three affected individuals

Index patient (II-7) with deletion	Affected father (I-2) with deletion	Affected half sister[a] (II-2)
Mental retardation	Mild sub-normality	Severe MR
Severe developmental delay	Mild dev. delay	Severe dev. delay
Speech impairment	Speaks less	Severe impairment
Receptive and non-verbal communication skills higher than verbal ones	–	
Movement/balance disorder: ataxia of gait and tremulous movement of limbs	Normal	
Behavioural uniqueness:		
– frequent laughter/smiling		
– apparent happy demeanor	Smiling face	
– easily excitable personality		
– often with hand-flapping movements		
– hypermotoric behaviour		
– short attention span	Short attention span	
Cleft palate	–	Cleft palate
Head circumference below 50th percentile	Normal	
Weight and height above 50th percentile	Normal	
No history of seizures	No history	History of seizures

[a] Note: Half sister was not available for clinical examination nor for the genetic study, only history was available, therefore not all features are known.

tion mix containing 50 μl of human Cot-1, 50 μl of Agilent 10× blocking agent , 250 μl of Agilent 2× hybridization buffer and 150 μl of the labeled patient and control DNA was heated to 95°C for 3 min and applied onto an Agilent 44A oligonucleotide array slide, and hybridized at 65°C for 40 h. After hybridization the slides were washed and air dried.

The slides were scanned using an Agilent Scanner and the data was analyzed using the FEATURE EXTRACTION software 8.1 (Agilent Technology). Data extraction of the tiff-images and normalization was done according to the preset standard parameters in the protocol for Agilent 44k CGH arrays. For visualization and comparison of the datasets the program CGH-Analytics 3.2 (Agilent Technologies) was used.

Multiplex Ligation-dependent Probe Amplification (MLPA) and methylation-specific MLPA assays

The deletion observed by array-CGH was validated by MLPA assay using EK1 kit supplemented with a novel chromosome 15q11→q13 MLPA probe mix (MRC-Holland, Amsterdam). This mix contains 22 probes of which 12 are specific for the chromosome 15q11→q13 region and ten probes specific for genes located on other chromosomes that are used for copy number determination. A list of the MLPA probes in this kit can be found in the supplementary information (www.mlpa.com). Ligation, PCR amplification and capillary electrophoresis were carried out according to Schouten et al. (2002). Fragment and data analysis was performed according to Nygren et al. (2005). A relative peak area lower than 0.7 was considered as deletion, and values above 1.3 as amplification.

Methylation Specific MLPA (MS-MLPA) was performed according to Nygren et al. (2005). The ME028 PWS/AS probe mix was used, which contains 25 probes specific for most of the genes in the PWS/AS critical region of chromosome 15q11→q13 and two probes for genes that are located outside this region. Among the probes in the PWS/AS critical region 13 probes are MS-MLPA probes, i.e. containing a *Hha*I recognition site in their target specific sequence. For methylation and copy number quantification, an additional 16 control probes are included that are not specific to genes on chromosome 15. Three of these probes are MS-MLPA probes and serve as digestion controls.

Results

G-band metaphase chromosome analysis at 500–550-band resolution as well as FISH studies did not show any apparent chromosomal rearrangement for any chromosome or deletion for the locus D15S10 specific for Angelman syndrome.

Further studies by oligonucleotide array-CGH showed a deletion of 12 Agilent probe sets at 15q11.2. This observation was confirmed by the reverse labeled array scan. The 12 deleted probe sets correspond to a 253-kb segment, spanning from nucleotide 20,384,943 to 20,637,977 (UCSC Genome Browser) on 15q11.2, which include genes *NIPA1, NIPA2, CYFIP1* and *TUBGC5* (Fig. 2a, b), within the BP1 and BP2 region of the Prader-Willi/Angelman critical region. The results were further validated by MLPA, which showed reduction in signal intensity for all four genes as compared to the control, but normal signal pattern for the gene *MKRN3* that is immediately outside the deletion region, suggesting that the breakpoint is centromeric to *MKRN3* (Fig. 3). The same deletion was confirmed by MLPA in the father but not seen in the mother or sister. Methylation studies showed no change in the methylation status of the imprinting centre surrounding the *SNRPN* promoter in the patient or in the father (data not shown).

Discussion

Chromosome 15q11→q13 is a highly unstable region that is prone to frequent deletions, duplications or even triplications including the well-described microdeletion syndromes Prader-Willi syndrome (PWS) and Angelman syn-

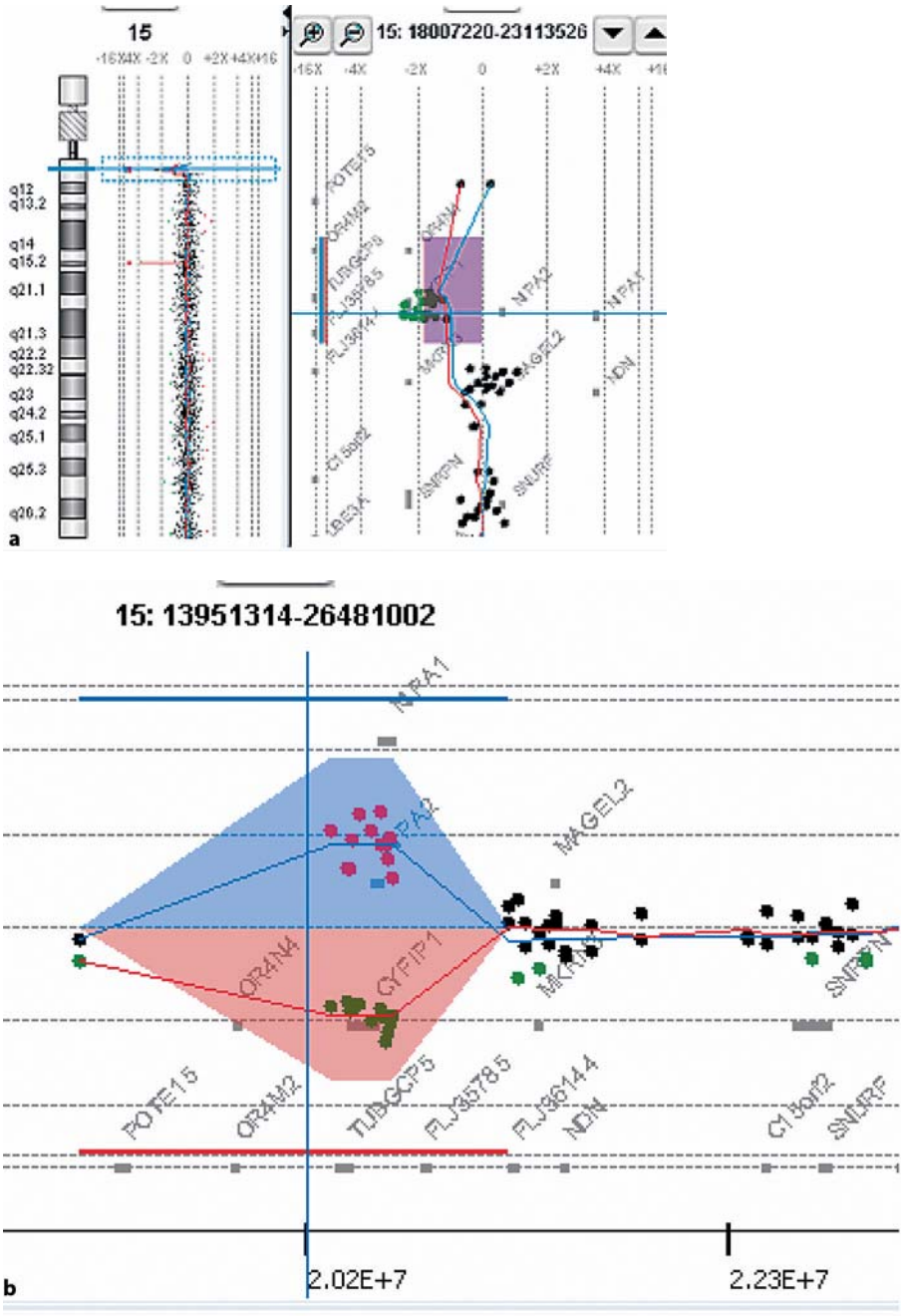

Fig. 2. Array-CGH showing deletion of four genes at the 15q11.2 region. The figure shows combined results of an experiment with patient DNA labeled with Cy3 and control DNA with Cy5 (red line, green probe sets) and reverse labeling – patient with Cy 5 and control with Cy 3 (blue line and red probe sets) hybridized to two separate 44A oligo arrays (Agilent Technologies) containing in situ synthesized 60-mer oligonucleotides representing a total of 44,290 features. Deletion of a cluster of 12 Agilent probe sets (green spots – direct view) was observed on 15q11.2 (**a**). Deletion of the four genes: *NIPA1*, *NIPA2*, *CYFIP1* and *TUBGCP5* on 15q11.2 (green spots on direct labeling and red spots on reverse labeling experiments) are seen in the magnified image of the same array viewed as an inverted image (**b**).

drome (AS). Both syndromes are distinct neurogenetic disorders characterized by deletions, uniparental disomy (UPD) or imprinting mutations resulting in an aberrant expression of genes located in the imprinted region on chromosome 15q11→q13 (Lossie et al., 2001; Butler et al., 2004). Approximately 70% of the PWS/AS cases are caused by a deletion of the PWS/AS imprinted domain located within this region. This genomic instability is mainly attributed to the presence of several low-copy repeat sequences in the common break point (BP) regions; BP1, BP2 and BP3 (Amos-Landgraf et al., 1999; Christian et al., 1999; Gimelli et al., 2003; Locke et al., 2004). It is reported that PWS patients with either Type I (BP1 to BP3) or Type II (BP2 to BP3) deletion are clinically heterogeneous (Butler et al., 2004; Milner et al., 2005). The region between BP1 and BP2 is comprised of four highly conserved genes, *NIPA1*, *NIPA2*, *CYFIP1* and *TUBGCP5* which are not imprinted (Chai et al., 2003). *NIPA1* and *NIPA2* function as either receptors or transporters. In addition, they mention that a deletion of these four genes may be associated with dosage-sensitive behavioral and psychological phenotypes. *NIPA1* missense mutation is shown to cause autosomal dominant hereditary spastic paraplegia (SPG6) (Rainer et al., 2003), thus haploinsufficiency, as seen in our patient, will not produce the

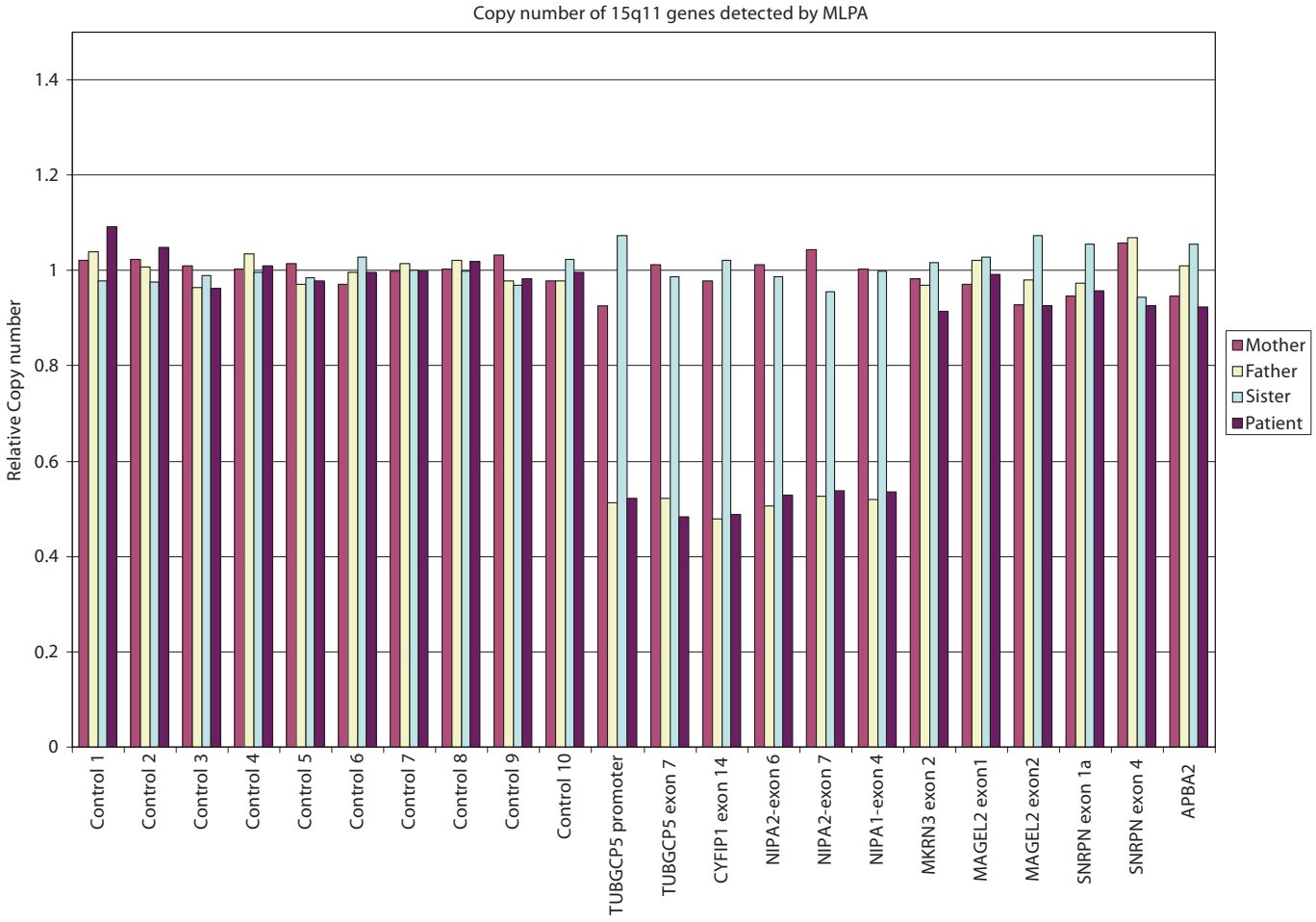

Fig. 3. Copy number of 15q11 genes detected by MLPA. A novel mix containing 22 MLPA probes was developed and tested on the affected family as well as control DNA. Of the probes included in this mix, 12 are specific for the chromosome 15q11→q13 region. An additional ten MLPA probes not specific to the region are used for copy number quantification. As can be seen the deletion in the patient spans the region including the genes *TUBGCP5*, *CYFIP1*, *NIPA2* and *NIPA1* but not the *MKRN3* gene located 800 kb q-telomeric of the region or any other chromosome 15q11→q13 gene tested.

phenotype. Deletion of the four genes are implicated in compulsive behavior and lower intellectual ability in Type I patients where these four genes are deleted in addition to the loss of the segment between BP2 and BP3 as in Type II deletion individuals (Butler et al., 2004; Milner et al., 2005). Phenotypic variability in AS was reported by Varela et al. (2004) where a comparison was made between different deletion classes and between deletions and UPD subjects. However, the authors did not find any major differences between the two main deletion classes except for the absence of vocalization, which is more prevalent in patients with BP1-BP3 deletion, suggesting that the genes between BP1 and BP2 may be involved in the degree of speech impairment. Our patient with a deletion restricted to the region between BP1 and BP2 presents with severe speech impairment and mental retardation and the father with the same deletion manifested with relatively milder features, such as slow understanding, low intelligence and a peculiar smiling face. Methylation studies showed normal methylation patterns for chromosome 15 IC surrounding the promoter of *SNRPN*, which implies that maternal deletion and methylation are unlikely putative causes leading to the AS-like phenotype. However, it should be mentioned that we tested DNA isolated from peripheral blood only, and the main gene responsible for AS is *UBE3A*, which is only imprinted in certain areas of the brain (Landers et al., 2005). Lossie et al. (2001) suggested that factors other than disruption of *UBE3A* such as other genes in the ubiquitin pathway may also be involved in causing AS.

High-resolution array-CGH is a very useful tool for the detection of microdeletions or duplications by a genome-wide analysis in patients with abnormal phenotype but with normal karyotype and FISH results. Such arrays are designed with the intention of studying the entire genome to its maximum extent. However, concerns such as the likelihood of a false-positive observation and or variants need to

be kept in mind (Bejjani et al., 2005; Vermeesch et al., 2005; Rickman et al., 2006). In our present study the likelihood of a false-positive result is ruled out by the fact that loss of a group of 12 probe loci was observed in both experiments with direct as well as reverse labeling, and this loss was further validated by another detection method (MLPA). Several large studies (Iafrate et al., 2004; Sebat et al., 2004; Feuk et al., 2006a, b) have identified copy number variations (CNVs) including inversions, insertions, deletions and other chromosomal rearrangements without apparent clinical consequences. To our knowledge this is the first report of a deletion spanning only in the region between BP1 and BP2 of the PWS/AS critical region at 15q11.2. Could this deletion possibly represent a new group of patients, or could it be a normal variant incidentally ascertained? Due to the non-availability of other affected family members from the extended family, including parents of the father (I-2) for the study, it is not possible at this stage to suggest if the deletion is causative of at least some of the presenting clinical features or not. Identification of more cases with a similar deletion will enable us to get a better understanding of the clinical manifestations due to this genetic alteration.

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