

Novel multilocus imprinting disturbances in a child with expressive language delay and intellectual disability

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ABSTRACT

Multilocus imprinting disturbances (MLID) have been associated with up to 12% of patients with Beckwith-Wiedemann syndrome (BWS), Silver-Russell syndrome (SRS), and pseudohypoparathyroidism type 1B (PHP1B). Single gene defects affecting components of the subcortical maternal complex (SCMC) have been reported in cases with multilocus hypomethylation defects. We present a patient with speech and language impairment with mild Angelman syndrome (AS) features who demonstrates maternal hypomethylation at 15q11.2 (*SNRPN*) as well as 11p15.5 (*KCNQ1OT1*) imprinted loci, but normal methylation at 6q24.2 (*PLAGL1*), 7p12.1 (*GRB10*), 7q32.2 (*MEST*), 11p15.5 (*H19*), 14q32.2 (*MEG3*), 19q13.43 (*PEG3*), and 20q13.32 (*GNAS* and *GNAS-AS1*). The proband also has no copy number nor sequence variants within the AS imprinting center or in *UBE3A*. Maternal targeted next generation sequencing (NGS) did not identify any pathogenic variants in *ZPF57*, *NLRP2*, *NLRP5*, *NLRP7*, *KHDC3L*, *PADI6*, *TLE6*, *OOEP*, *UHRF1* or *ZAR1*. The presence of very delayed, yet functional speech, behavioral difficulties, EEG abnormalities but without clinical seizures, and normocephaly are consistent with the 15q11.2 hypomethylation defect observed in this patient. To our knowledge, this is the first report of MLID in a patient with mild, likely mosaic, Angelman syndrome.

KEY WORDS

Multilocus imprinting disturbances, mosaic Angelman syndrome, maternal hypomethylation.

1 INTRODUCTION

Imprinting disorders are associated with disruption of parent-specific monoallelic expression at imprinted loci across the genome. Imprinting disorders are mostly caused by molecular changes including uniparental disomy (UPD), copy number variation within imprinted loci, epigenetic changes (epimutations or aberrant methylation) affecting imprinted loci, and pathogenic sequence variants within imprinted genes (Horsthemke, 2010; Monk et al., 2019). Genomic imprinting mainly through DNA methylation is a crucial step for epigenetic gene regulation throughout development, and it includes 3 major steps. The first step is erasure of all DNA methylation, generally across the genome and at imprinting centers (IC), in the primordial germ cells. The second step is reestablishment of gender-specific DNA methylation imprints in germ cells during gametogenesis (germline patterns) that are subsequently maintained through gamete maturation, fertilization, and zygote formation. The third step, occurring after fertilization, is zygotic genome activation leading to widespread demethylation of the germline methylation, but maintenance of parent-specific methylation patterns in the ICs. Somatic, tissue-specific DNA methylation imprints (somatic patterns) are then imparted beginning at the blastocyst stage and are subsequently maintained throughout the post implanted embryo and adult tissues (Eggermann et al., 2011; Elbracht et al., 2020; Mackay et al., 2015; Monk et al., 2019; Nazlican et al., 2004).

There are currently 12 imprinting disorders that have been identified (Elbracht et al., 2020; Soellner et al., 2017). Many are clinically recognizable, including Beckwith-Wiedemann syndrome (BWS, MIM 130650), Silver-Russell syndrome (SRS, MIM 180860, MIM 616489, MIM 618905), Prader-Willi syndrome (PWS, MIM 176270), Angelman syndrome (AS, MIM 105830), Temple syndrome (TS, MIM 616222), Kagami-Ogata syndrome (MIM 608149), transient neonatal diabetes (TNDM, MIM 601410), and pseudohypoparathyroidism type 1B (PHP1B, MIM 603233). Others display clinical overlap, yet all are distinguishable at the molecular level. Of note, multilocus imprinting disturbances (MLID), where patients carry primary epimutations at

multiple imprinted regions, have been reported mainly with BWS, SRS, TNDM, PHP1B, and TS (Elbracht et al., 2020). No patient with a clinical phenotype of Angelman syndrome has been reported with MLID. Generally, there are no epigenotype-phenotype correlations between the degree of hypomethylation or the number of affected loci and the severity of the clinical outcome in MLID patients (Boonen et al., 2013; Mackay et al., 2015; Monk et al., 2019). In addition, pathogenic variants in genes (*ZPF57*, *NLRP2*, *NLRP5*, *NLRP7*, *KHDC3L*, *PADI6*, *TLE6*, *OOEP*, *UHRF1* or *ZAR1*) that are important for reestablishing and maintaining the germline IC patterns have been reported in cases with MLID (Elbracht et al., 2020).

Angelman syndrome can be caused by chromosomal deletion, paternal UPD, IC mutation or deletion, *UBE3A* pathogenic variants, and by imprinting defects without DNA sequence alteration within 15q11.2 (Williams et al., 2006). Nazlican et al., 2004, showed that among Angelman syndrome patients with an imprinting defect due to a primary epimutation that hypomethylation of the maternal allele occurred postzygotically leading to somatic mosaicism. In 24 patients; normal cells constituted <1% to as much as 40% of the samples tested, and a higher percentage of normal cells generally correlated with milder symptoms (Nazlican et al., 2004).

In this paper we describe a patient with a mild or mosaic AS phenotype, who originally came to attention because of Methylation-sensitive Multiplex Ligation-dependent Probe Amplification (MS-MLPA) testing showing absent methylation of the PWS/AS IC representing the maternal allele. Further characterization of the proband disclosed a MLID involving not only PWS/AS, but also the BWS locus.

2 CLINICAL PRESENTATION

IRB-approved informed consent was obtained from the parents for participation in this research. The proband was the product of a naturally conceived, uncomplicated pregnancy, and was delivered at term. She weighed 8 pounds, 2 ounces (3.685 kg; 83rd percentile) at birth. Her early motor milestones were normal, with walking independently at 13 months. Concerns about expressive language developed around 1 year of age and neuropsychological evaluation at 2.75 years of age disclosed expressive language delay characterized as oral motor weakness and childhood apraxia of speech, with only 6-8 expressed words at ~3 years-old and some sign language, as well as impairment of receptive language skills (unable to identify simple action verbs in photos; did not recognize body parts; does not follow directions at home). She had no evidence for seizures and had normal steady gait. She was enrolled in Early On, speech, occupational and physical therapy and exhibited no regression. A nocturnal polysomnogram (at 2.5 years of age) showed no sleep disordered breathing. At ~3 years-old, she had only 20-25 expressed words and brief use of combinations. At 3.75 years of age, she had made developmental progress, being able to speak in 2-3 word phrases and she had an understandable expressive vocabulary of ~50 words and could count to 10 and name body parts. Notably, she participated in ballet. At 6 years of age, brain and spine MRI disclosed Chiari type I malformation with cerebellar tonsils extending 2.3 cm below the foramen magnum and the presence of a holocord. Otherwise, the brain parenchyma was normal. At 11 years, a leg length discrepancy (left lower extremity 1.3 cm longer than right) was identified requiring a shoe lift.

In terms of family history, the proband has one full sibling and one half-sibling through her mother, both with normal development. Her parents are nonconsanguineous, of mixed European ancestry and are without developmental concerns. Mother has a history of depression and anxiety.

The proband was originally evaluated in Pediatric Genetics at the University of Michigan at ~2 years of age for expressive language delay and because an outside laboratory MS-MLPA test of the PWS/AS locus showed complete loss of maternal-specific methylation, suggesting Angelman syndrome. Despite an epileptiform EEG, she had no history of clinically evident seizures and physical examination at 3 years of age revealed height 107.5 cm, and weight 20.2 kg, both at the 97th percentile and head circumference at the 44th percentile. Gait was steady. Renal ultrasound at that time was normal. Outside laboratory UPD15 testing including both parents, based on microsatellite marker genotyping, revealed normal biparental inheritance of chromosome 15. Peripheral blood karyotype, chromosomal microarray using Illumina HumanCytoSNP-12 (San Diego, California), and *UBE3A* sequencing were all normal.

3 MOLECULAR ANALYSIS

3.1 Evaluation of Imprinting at PWS/AS locus reveals hypomethylation, rather than complete absence of maternal methylation mark

Given the lack of clinical correlation with the original outside laboratory test result suggesting complete loss of methylation, we retrieved the outside laboratory sample and obtained another sample from the proband. We repeated the testing with a clinically validated sodium bisulfite genomic DNA modification (Frommer et al., 1992) and methylation-specific polymerase chain reaction (MS-PCR) assay (Kubota et al., 1997) for the PWS/AS loci. The results (Figure 1) showed hypomethylation of the maternal 15q11.2 imprinting center that could be due to reduced levels of methylation or somatic mosaicism for loss of methylation at the maternal 15q11.2 imprinting center. This result was technically repeated 3 times with new bisulfite preparations and a second set of primers with the same results (data not shown). Additionally, we repeated MS-MLPA testing, using the ME028-C1 PWS/AS kit (MRC-Holland, Amsterdam, The

Netherlands) per manufacturer instructions, as at the outside laboratory. The ME028-C1 PWS/AS kit has 4 probes, which all consistently showed 0% (complete loss) of methylation at 15q11.2 (*SNRPN*) in the proband samples, consistent with the results from the outside laboratory (Figures 2A and 2B). MS-MLPA data were analyzed using Coffalyser.Net software version v.140721.1958 (MRC-Holland, Amsterdam, The Netherlands). SNP chromosomal microarray testing (data not shown) and MLPA testing (Figures 2C and 2D) showed normal chromosome dosage at 15q11.2.

3.2 AS Imprinting Center lacks mutation

To determine if the hypomethylation observed was secondary to a sequence variant within the AS imprinting center, we performed Sanger sequencing across the genomic segment on (hg19) chr15:25199640-25200377 [primers: CGCACCAAATTTGAACAATG (Forward) and CAACAGCAAGCCTCTGAACA (Reverse)]. No variations were identified within the targeted region for the MS-PCR assay.

3.3 Maternal hypomethylation is observed at another imprinted locus

To explore whether disturbed methylation was confined to the PWS/AS locus, we examined the status of methylation at multiple known imprinting centers including 6q24.2 (*PLAGL1*), 7p12.1 (*GRB10*) and 7q32.2 (*MEST*), 11p15.5 (*H19* and *KCNQ1OT1*), 14q32.2 (*MEG3*), and 20q13.32. MS-MLPA, using ME034-B1 Multi-locus Imprint kit (MRC-Holland, Amsterdam, The Netherlands), at ten different imprinting loci (Figure 3) showed, using Coffalyser.Net software v.210604.1451, loss of methylation at the 15q11.2 (*SNRPN*) imprinting center, and hypomethylation at 11p15.5 (*KCNQ1OT1*) imprinting center 2 (Figure 3B), indicating a MLID in

the proband. In contrast, the methylation status at 6q24.2 (*PLAGL1*), 7p12.1 (*GRB10*), 7q32.2 (*MEST*), 11p15.5 (H19), 14q32.2 (*MEG3*), 19q13.43 (*PEG3*), and 20q13.32 (*GNAS* and *GNAS-AS1*) were normal compared with controls (Figures 3A and 3B).

Primary epimutations, as present in the proband, are often identified in individuals with a negative family history, and primary epimutations are not inherited. To test this, we applied the same assays to genomic DNA isolated from blood of the proband's parents. As expected, both showed normal methylation patterns (data not shown).

To estimate the sensitivity of our MS-PCR assay versus the MS-MLPA, we mixed DNA from an Angelman syndrome sample, showing complete loss of methylation at 15q11.2 by MS-PCR, and a normal DNA sample to approximate 30%, 20%, 15%, 10%, 5%, and 2.5% hypomethylation at 15q11.2. The MS-PCR results estimated the percentage of maternal methylation at 15q11.2 in our proband roughly between 5-10% (Figure 4). In contrast, testing with the newer ME034-B1 Multi-locus Imprint MS-MLPA kit (MRC-Holland, Amsterdam, The Netherlands), which includes only 2 probes within 15q11.2 (*SNRPN*), showed an average methylation index of 0.08 with one probe and an average methylation index of 0.00 (complete loss of methylation) with the second probe (Figure 3B). The ME034-B1 average methylation index for the 2 probes within 15q11.2 (*SNRPN*) is consistent with our MS-PCR estimation. Interestingly, the variable methylation signals detected by the same exact probe in ME028 (0%) and ME034 (~8%) suggest that the MS-MLPA results are not reliable with respect to peak height near the background threshold and therefore are not accurate for low level mosaicism. MS-PCR should be considered if clinically indicated, if low level mosaicism is suspected, and/or clinical features do not match the MS-MLPA results.

3.4 Analysis of genes implicated in MLID reveals no pathogenic variants

The SCMC is a multiprotein complex whose genes are highly expressed in the oocyte and are required for embryo development, however the exact biological roles of the components of the SCMC are largely unknown. Nevertheless, rare pathogenic variants have been identified in 9 genes (*NLRP2*, *NLRP5*, *NLRP7*, *KHDC3L*, *PADI6*, *TLE6*, *OOEP*, *UHRF1* or *ZAR1*) encoding some of the protein components of SCMC and in *ZFP57*, in MLID cases (Elbracht et al., 2020). To determine if the proband's methylation defects occurred secondary to a pathogenic variant(s) in any of these 10 genes, we performed targeted next generation sequencing (NGS) of the proband's mother. Briefly, DNA extracted from whole blood was biochemically fragmented to 250 bp, and the entire coding sequences (exons plus at least 15 bp upstream and 15 bp downstream of each coding exon) of the targeted genes were captured using the TruSight One Expanded Sequencing Panel (Illumina, San Diego, CA). Sequence reads were aligned to the human reference genome (GRCh37/hg19). A minimum NGS coverage of 20X was achieved for all coding exons. Variant analysis detected 21 variants in 8 of the genes that were classified, based on high allele frequency in the general population, as benign. No pathogenic variants were identified in any of these genes.

4 DISCUSSION

Angelman syndrome is a neurodevelopmental disorder mainly characterized by severe developmental delay or intellectual disability, severe speech impairment, among other clinical features (Williams et al., 2006). Our proband presented with speech and language delay with normal growth patterns. Molecular testing on our proband exhibited maternal hypomethylation at two imprinted loci (Figures 1 and 3). To our knowledge, this is the first report of MLID associated

with Angelman syndrome. Importantly, despite hypomethylation of IC2 at the BWS locus, our patient did not fulfill diagnostic criteria for BWS, and only later was found to have minimal leg length discrepancy, which is consistent with the literature with respect to clinical findings in patients with MLID (Elbracht et al., 2020).

Previously, a patient was reported with presumed mild Angelman syndrome, including speech delay; mild ataxia; happy demeanor; and EEG abnormalities, with maternal hypomethylation at the *SNRPN* locus, but at that time no other imprinted loci were tested (Brockmann et al., 2002). In addition, a 3 year-old girl with features of PWS and BWS was reported with MLID, including hypomethylation at 15q11.2 (*SNRPN*), and 11p15.5 (*KCNQ1OT1* and *H19*) (Baple et al., 2011).

In 2004, Nazlican and colleagues reported that of the 3-4% of patients with Angelman syndrome due to an imprinting defect without an IC sequence change (those with a primary epimutation), approximately one third have a faint methylated maternal band within the *SNURF-SNRPN* locus. Those patients tend to have milder AS manifestations (Nazlican et al., 2004). Quantitative methylation real-time PCR in 24 patients demonstrated a range of <1% to 40% of normal maternal methylation at 15q11.2, suggestive of somatic mosaicism for methylation at the maternal 15q11.2 imprinting center, which was proven with two patient samples. Imprinting defects at other imprinted loci were not tested (Nazlican et al., 2004). Additionally, 22 patients with mosaic Angelman syndrome were reported to mainly have global developmental delay without regression, and behavioral challenges. Other features included mild language impairment, microcephaly, EEG abnormalities, obesity, hyperphagia, ataxia, sleep disturbances, and facial dysmorphism (Carson et al., 2019).

The range of maternal methylation at 15q11.2 in our patient was estimated to be between 5-10% (Figures 3D and 4). Using the clinical score table provided by Nazlican and colleagues (Nazlican et al., 2004), we calculated a conservative score of ~6 for our patient. Based on their linear regression analysis (Nazlican et al., 2004), our patient would have the highest score for that degree of hypomethylation by comparison. It would be interesting to know if any of those 24 patients reported in Nazlican and colleagues (Nazlican et al., 2004) show MLID.

Epimutations originating in the germline are usually maintained in the somatic cells resulting in phenotypic outcomes later in development, while post-zygotic epimutations can result in somatic mosaicism (Monk et al., 2019). Although the mechanism is not well defined, it is tempting to speculate that somatic mosaicism is a result of a DNA methylation maintenance defect.

We did not find evidence for a microdeletion by SNP chromosomal microarray or MLPA (Figure 2), nor mutation within the AS imprinting center. There is no evidence of a parental imprinting defect, nor any sequence variant in any of the 10 known genes involved in imprinted methylation maintenance or regulation. None of the imprinted loci tested in our patient showed a paternal methylation defect. Thus, these hypomethylation findings are suggestive of a maternal specific re-methylation or maintenance defect occurring at a vulnerable time of development (Monk et al., 2019).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Drs. Tayeh and Innis designed the study and wrote the manuscript; Dr. Tayeh also interpreted data; Kristin LeSueur performed NGS sequencing, alignment and variant calling; Janean Devaul carried out molecular methylation analyses; Peedikayil Thomas carried out Sanger sequencing of initial gaps in NGS sequencing data and variant identification; Dr. Chen Yang interpreted methylation and NGS data; Drs. Mark Hannibal and Jirair Bedoyan made clinical diagnoses and collected clinical data.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Figure 1. MS-PCR at PWS/AS imprinting center. Sodium-bisulfite treated genomic DNA samples from the proband along with various controls were amplified using primers specific for the maternally methylated allele (174 bp; primers: TAAATAAGTACGTTTGCGCGGTC (Forward) and AACCTTACCCGCTCCATCGCG (Reverse)) and the paternally unmethylated allele (100 bp; primers: GTAGGTTGGTGTGTATGTTTAGGT (Forward) and ACATCAAACATCTCCAACAACCA (Reverse)) within 15q11.2.

Lanes: 1- 100 bp ladder, 2- Patient's 1st isolation, 3- Patient's 2nd isolation, 4- Patient's aliquot 1 (outside lab), 5- Patient's aliquot 2 (outside lab), 6- PWS positive control, 7- AS positive control, 8- Healthy control, 9- Untreated genomic DNA, and 10- No DNA template control.

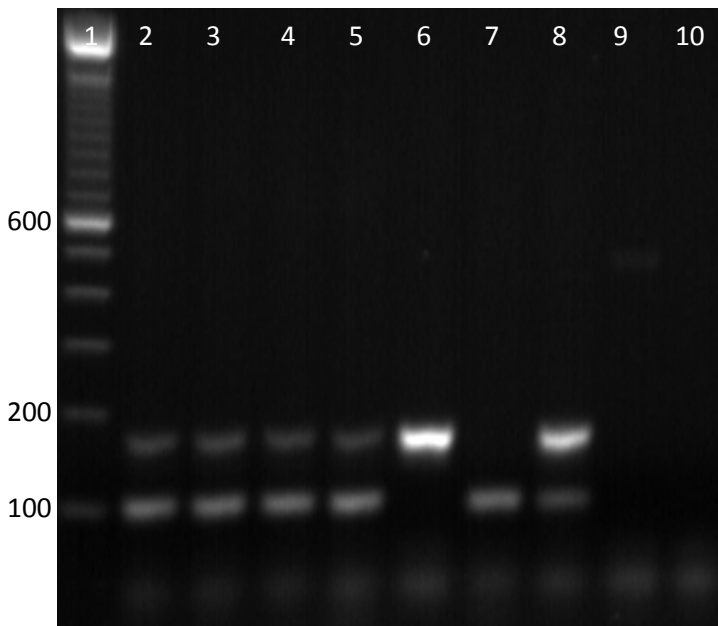


Figure 2. ME028-PWS/AS MLPA analysis within 15q11.2 imprinting center (*SNRPN*). **A** and **B**: MS-MLPA analysis at 15q11.2 imprinting center (*SNRPN*). Genomic DNA samples were digested with a methyl-sensitive restriction enzyme followed by loci-specific probe hybridization, amplification and fragment analysis to determine the methylation ratio. **A**: MS-MLPA analysis for a healthy control with an average methylation index (4 *SNRPN* probes) within normal range (0.41 – 0.61) at 15q11.2 (*SNRPN*). **B**: MS-MLPA analysis for the proband with average methylation index (4 *SNRPN* probes) of 0 at 15q11.2 (*SNRPN*). **C** and **D**: MLPA dosage analysis at 15q11.2 imprinting center (*SNRPN*). Genomic DNA samples were hybridized to loci-specific probes, followed by amplification and fragment analysis to determine chromosomal dosage. **C**: MLPA analysis for a healthy control. **D**: MLPA analysis for the proband showing normal chromosomal dosage at 15q11.2 (*SNRPN*) loci.

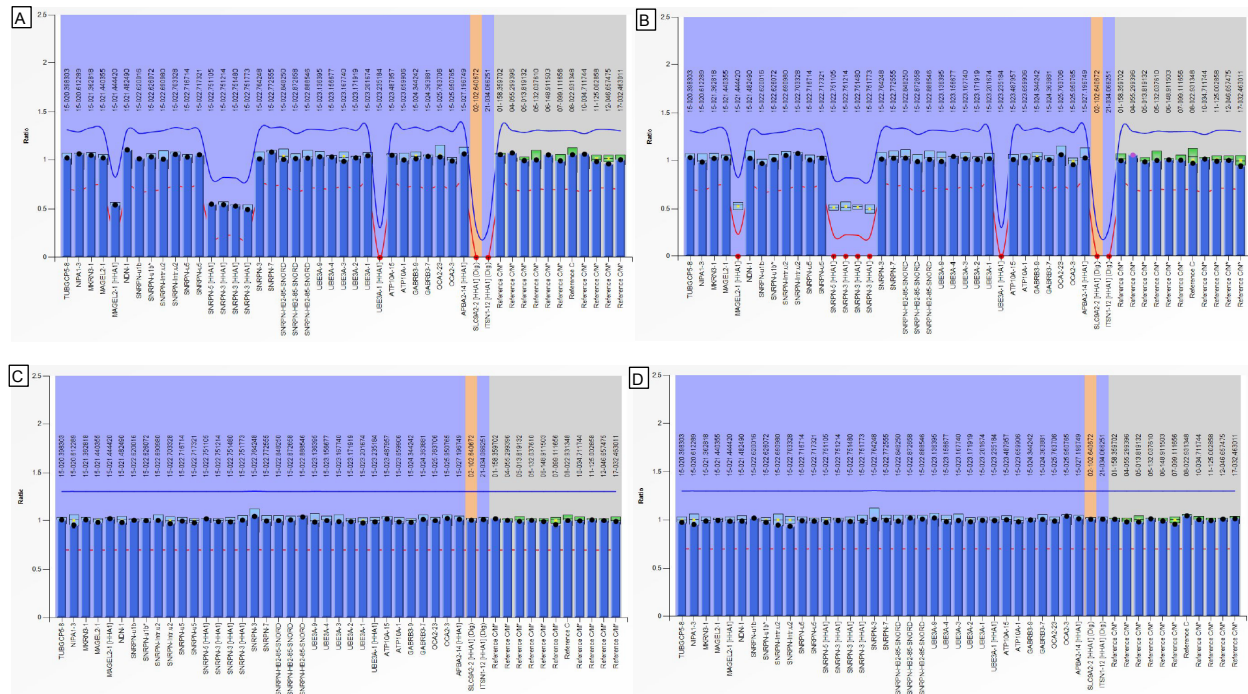


Figure 3. ME034-Multi-locus MS-MLPA analysis at multiple imprinting centers including 6q24.2 (*PLAGL1*), 7p12.1 (*GRB10*), 7q32.2 (*MEST*), 11p15.5 (H19 and *KCNQ1OT1*), 14q32.2 (*MEG3*), 15q11.2 (*SNRPN*), 19q13.43 (*PEG3*), and 20q13.32 (*GNAS* and *GNAS-AS1*). Genomic DNA samples were digested with a methyl-sensitive restriction enzyme followed by loci-specific probe hybridization, amplification and fragment analysis to determine the methylation ratio. **A:** MS-MLPA analysis for a healthy control with average methylation indices within normal range (0.41 – 0.61). **B:** MS-MLPA analysis for the proband with loss of methylation at 15q11.2 (*SNRPN*), hypomethylation at 11p15.5 IC2 (*KCNQ1OT1*), and normal methylation at the remaining loci. The average methylation index for the two methyl-sensitive probes within 15p11.2 IC is 0.03, and the average methylation index for the two methyl-sensitive probes within 11p15.5 IC2 is 0.26, which are greater than 3 standard deviations below the mean in normal control samples, while the average methylation index for all other methyl-sensitive probes were within normal range.

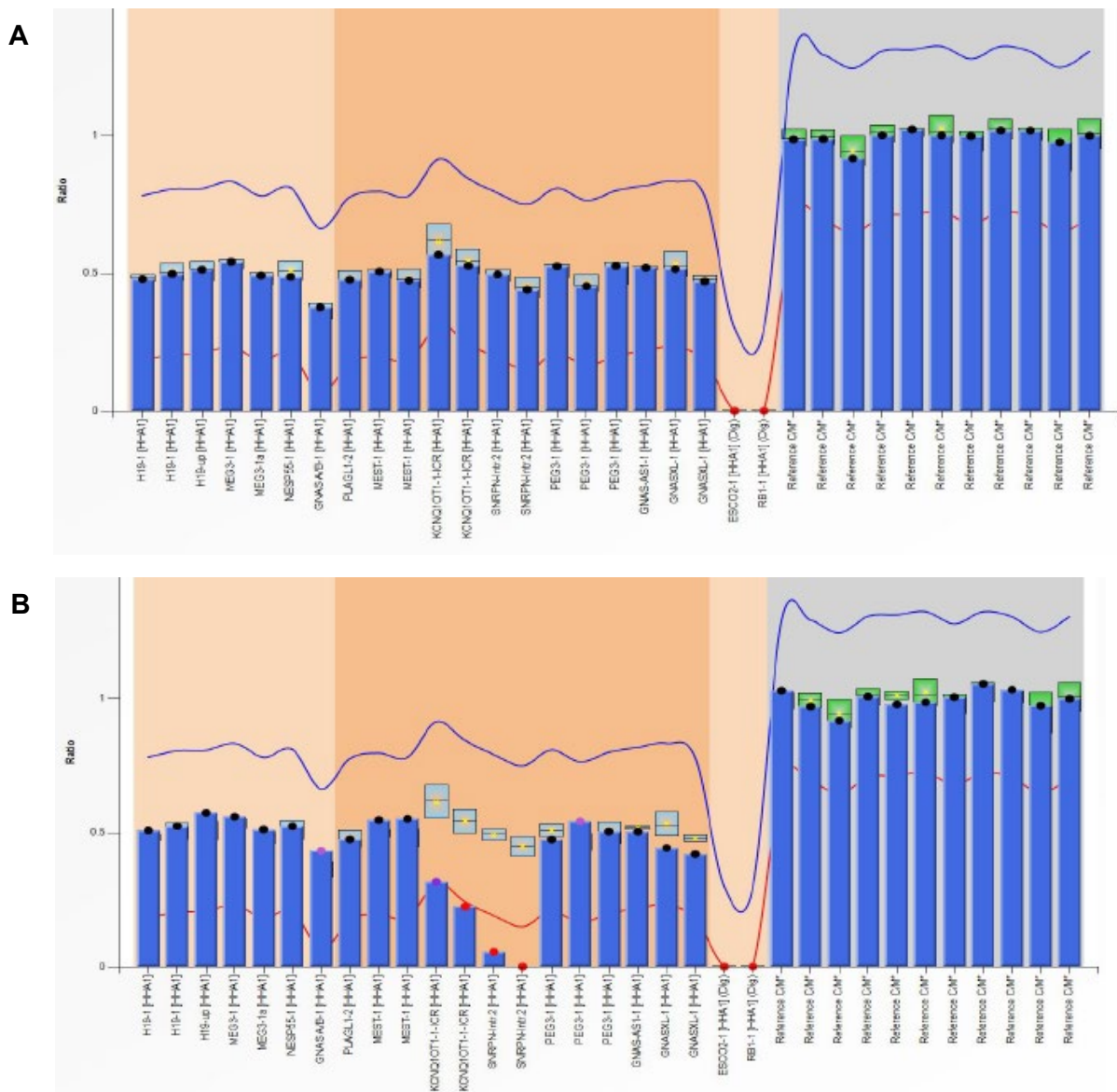


Figure 4. MS-PCR at PWS/AS imprinting center. Sodium-bisulfite treated genomic DNA samples from the proband along with a mixture of Angelman positive and a normal sample representing 30, 20, 15, 10, 5, and 2.5% maternal methylation at 15q11.2. Samples were amplified using primers specific for the maternally methylated allele (211 bp; primers: AGGGAGTTGGGATTTTTGTATTGC (Forward) and CCGACACAACCTTACCCG (Reverse)) and the paternally unmethylated allele (149 bp; primers: GTGGTGTGATAGGTTTTATTGT (Forward) and CCACATACCCTCACCACACA (Reverse)) within 15q11.2.

Lanes: 1- 100 bp ladder, 2- 30% maternal methylation, 3- 20% maternal methylation, 4- 15% maternal methylation, 5- 10% maternal methylation, 6- 5% maternal methylation, 7- proband, 8- 2.5% maternal methylation, 9- PWS positive control, 10- AS positive control, 11- Healthy control, 12- Untreated genomic DNA, and 13- No template control.

