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UNUSUAL SPECTRUM OF GENETIC PATHOLOGIES AND NOVEL MUTATIONS IN PWS AND AS PATIENTS DETECTED BY A WIDE CLUSTER OF METHODS

[1,2]Livia Kotysova, [1]Robert Petrovic and [1]Jan Chandoga

[1]Institute of Medical Biology, Genetics and Clinical Genetics,
Comenius University Faculty of Medicine and University Hospital Bratislava, Slovakia
[2]Department of Genetics, Faculty of Natural Sciences of Comenius University Bratislava, Slovakia

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ABSTRACT

Prader-Willi and Angelman syndromes are clinically distinct neurodevelopmental genetic disorders that map to 15q11.2-q13 locus. The common phenotypes are attributable to loss of expression of parentally specific imprinted genes inside this region, where the gene function is dependent on parental origin. Initial diagnosis was proved for the years by methylation pattern analyses of the *SNRPN* exon 1/promoter region within the PWS/AS critical domain. Apart from unifying methylation-specific PCR and allele specific real-time PCR with melt-curve analysis as the fundamental methods for suspected diagnosis confirmation, we combined several specifically methods used to clarify the molecular cause. In our study we had identified and genotyped 24 PWS and AS patients from 450 suspected. Applied cluster of methods-microsatellite analysis of SNPs within the chromosome 15, Methylation-specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) and *UBE3A* gene sequence analysis, enable us to determined atypical deletion that does not include common breakpoints, novel highly likely to be pathologic *UBE3A* mutation, uniparental heterodisomy together with partial isodisomy and epimutation without any deletions in the imprinting centre. We present genotype-phenotype correlation of all positive cases. In addition, we estimate the incidence for Slovakian population at 1 in 20,000 for PWS and 1 in 40,000 for AS.

Keywords: Prader-Willi and Angelman Syndromes, Unusual Cases, *UBE3A* Gene, 15q11.2-q13 Region's Deletion Breakpoints

1. INTRODUCTION

Prader-Willi (PWS; OMIM: 176270) and Angelman Syndromes (AS; OMIM: 105830) belong up to the present to the best known imprinting disorders. The common phenotypes of syndromes are attributed to loss of function of genes, which are under the control of a bipartite imprinting centre on chromosome 15 (15q11.2-q13). Their function depends on parental origin (Buiting *et al*., 1995; Nicholls *et al*., 1998). Imprinting centre spans over two regulatory regions defined by mapping of deletions in PWS and AS familial cases; the Shortest Region of Overlap (PWS-SRO)-a 4.3 kb sequence, that includes the *SNRPN* promoter/exon1 (Ohta *et al*., 1999) and AS-SRO an 880 bp sequence located 35 kb upstream to the *SNRPN* transcription start site (Buiting *et al*., 1999). The most important function of these regions, proposed by Nicholls and Knepper (2001), is realised in the gametogenesis. In oocytes AS-SRO mediates the establishment of the maternal epi-mark at the PWS-SRO as a cis-acting element. PWS-SRO on the other side controls the establishment of the paternal epi-mark during the spermatogenesis. In somatic cells, a

Corresponding Author: Livia Kotysova, Institute of Medical Biology, Genetics and Clinical Genetics, Comenius University, Faculty of Medicine and University Hospital Bratislava, Slovakia

cis-acting element from the paternal PWS-SRO maintains the paternal epigenotype in the PWS/AS imprinting domain, while the maternal PWS-SRO is methylated (Kantor *et al*., 2004).

PWS/AS critical region comprises ~4Mb of DNA and lies within a telomeric boundary in D15S174 locus (Greger *et al*., 1993) and a centromeric boundary in D15S1035 locus (Lee and Wevrick, 2000). Five Breakpoint (BP) sites have been identified as a restriction of 15q11-q14 region's boundaries, two proximal-BP1 (located between 18.68 and 20.22 Mb) and BP2 (located between 20.81 and 21.36 Mb), most common distal BP3 (located between 25.94 and 27.28 Mb) and less implicated BP4 and BP5 breakpoints (Varela *et al*., 2005). BPs can be implicated in mediating DNA recombination in recurrent deletions associated with PWS and AS. Related to the deletion extent as the leading syndromes cause; patients could be divided into four classes. Type I deletion is flanked by BP1-BP3 and encompasses genes from the *NIPA* family associated with deficits in adaptive behaviour (including mental-psychomotoric skills), autism spectrum, obsessive-compulsive behaviour and visual-motor integration. Patients with this type of deletion manifest more severe phenotype than patients with most common type II deletion (BP2-BP3) (Chai *et al*., 2003). Type III deletion (from BP1 to BP4) contributes only to 5% of all deletion cases and type IV deletion (from BP1 to BP5) has been reported only in inv dup (15) marker chromosomes or interstitial duplication and triplication cases (Roberts *et al*., 2003; Wandstrat *et al*., 1998).

PWS is characterised by clinical manifestations summarised in Holm's consensus diagnostic criteria (Holm *et al*., 1993). Generally, it is caused by disruption of paternally imprinted genes (*MKRN*3, *MAGEL*2, *NDN*, *PWRN*1, *C15orf*2, *SNURF-SNRPN* and snoRNAs) (Boccaccio *et al*., 1999; De los Santos *et al*., 2000; Jong *et al*., 1999; MacDonald and Wevrick, 1997; Özçelik *et al*., 1992), localised to the centromeric end of the region, as a result of either a paternally derived *de novo* deletion (~75%), maternal Uniparental Disomy (mUPD) (~20-30%), imprinting defects (~3%) and rare causes like duplication, chromosomal rearrangement or marker chromosomes (~2%). For example, two cases were found with the molecular-genetic background of inverted duplication of chromosome 15q11-q13. These patients showed strong autistic features, moderate motor delay, severe hypotonia and periods of moderate to severe lethargy after birth-phenotype similar to PWS (Gargus and Imtiaz, 2008). Loss of function of genes localised telomerically to the parental cluster of genes exclusively expressed from the maternal allele (*UBE3A*, *ATP10C*) (Kishino *et al*., 1997; Meguro *et al*., 2001; Rougeulle *et al*., 1998) leads to AS. Consensus diagnostic criteria were made by (Williams *et al*., 2006). The main causes of AS include a maternally derived *de novo* deletion (~68%), Paternal Uniparental Disomy (pUPD) (~7%), imprinting defects (~3%), point mutations in UBE3A gene (~11%), rare causes like duplication or translocation (~2%) and about 9% of cases is caused by the unknown etiology. *UBE3A* gene with complete coding sequence organised into 16 exons, encoding the E6-AP ubiquitin-protein ligase, is expressed in a maternally biased way in the brain. It´s biological activity is defined by its carboxyl terminal end, which is encoded by exons 9-16 (Kishino *et al*., 1997). To date, there is no evidence concerning any mutations in exons prior to exon 8.

Many types of diagnostic strategies were proposed. Apart from most common techniques used in the past such as Southern blot analysis, or recently used specific methylation sensitive restriction cleavage and MS-PCR, through alternatives like pyrosequencing and melt-curve analysis up to the new one-MS-MLPA. In present study thanks to wide scale of applied methods, we elucidated common molecular findings in majority of PWS and AS patients as so as very rare and unusual molecular changes. Likewise, in two AS patient we identified identical novel *UBE3A* hot spot mutation.

2. MATERIAL AND METHODS

2.1. Patients

A total of 450 patients from all regions of Slovakia were involved in the study. The samples were sent to our Institute due to the clinical indication supposed for Prader-Willi or Angelman syndrome between years 2007 and 2013. All subjects gave informed written consent. DNA was extracted from peripheral blood cells using MN NucleoSpin Blood-Mini (Macherey-Nagel).

2.2. Methylation Pattern Analyses

DNA for methylation analyses was treated with the Imprint® DNA Modification Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. All from 450 derived DNA samples were bisulfite modified and suspected diagnosis was proved or ruled out by methylation pattern analyses.

For *PCR-RFLP* the bisulfite modified DNA was amplified using primer sets common for both alleles (Zeschnigk *et al*., 1997). According to author's protocol we made two subsequent PCR reactions followed by methylation sensitive *HhaI* restriction analysis.

For *allele specific real-time PCR* the bisulfite modified DNA was amplified using SNRPN-M primer set for maternal allele (Kubota *et al*., 1997) and SNRPN-UNMET primer set for paternal allele (Kosaki *et al*., 1997) with assessment of melting curve profile in range from 68 to 90°C with temperature decreasing of 0.2°C each step.

2.3. Microsatellite Analysis

Genotyping was performed with $(CA)_n$ dinucleotide repeats microsatellite markers within the critical region 15q11-q13 and control marker outside located at the telomeric site of 15q24. Primer sequences were used according to Lee *et al*. (1998).

Diagnostics of an atypical uniparental disomy (where isodisomy and heterodisomy occurs on one chromosome pair) and of shorter deletion (out of breakpoints) was performed by Devyser UPD-15 kit (Devyser AB, Hägersten, Sweden) with tetra nucleotide STR markers along the 15.chromosome (**Fig. 1**) under the condition according to the manufacturer's protocol. Capillary electrophoresis was done on an ABI PRISM 310 Genetic Analyzer with GeneScan Analysis software (all from Applied Biosystems, Carlsbad, CA, USA).

2.4. Sequencing Analysis of *UBE3A* Gene

Exons 7-16 of *UBE3A* were amplified using primers flanking the intron-exon boundaries. The primers sequences based on the sequences published by (Malzac *et al*., 1998; Fang *et al*., 1999) and designed in Primer 3 software v. 0.4.0, checked by SNPCheck v3 and NCBI/Blast softwares (**Table 1**).

Primers with hybridisation sites inside the exon 9 were designed with resolution on distinguishing between gene and pseudogene sequence variants. 30-50 ng of nonconverted genomic DNA was amplified using 2×PCR Master Mix (Thermo Fisher Scientific), 1.5 mM $MgCl_2$ and 0.3 µM of each primer in a reaction volume of 25 µL for exons 7, 8, 9, 10, 11 and 15. For the rest exons PCR was performed in a 25 µL reaction volume using 10x buffer for Thermo Start polymerase (Thermo Fisher Scientific), 25 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs, 0.3 µmol L^{-1} of each primer, 0.625U of Thermo Start polymerase in addiction of BSA for exons 12 and 13+14. The amplification conditions are summarised in **Table 1**. PCR products were enzymaticaly purified using thermosensitive alkaline phosphatase fast AP™ and exonuclease I (Thermo Fisher Scientific) and directly sequenced. Sequencing analysis was performed with ABI 3100 Genetic analyser using Big Dye® Terminator v3.1 cycle sequencing kit, following the manufacturer's instructions except from the exon 16, in which 5% DMSO and 1M Betaine were added. Data were analysed by Chromas 2.2 (Technelysium Pty Ltd., Australia) and Vector NTI 11.5 (Informax); the sequences obtained were compared with the reference from GenBank (NM_130839.2). Mutation names were designed according to recommended nomenclature checked in HGMD® mutation biological database and LOVD v.2.0 variation database, with all nucleotide numbers based on cDNA from UBE3A-001 transcript (ENST00000232165, NM_130839.2). Novel mutations were analysed using web-based tools, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (Sorting Intolerant from Tolerant, http://sift.bii.a-star.edu.sg/) to assess their potential pathogenicity.

2.5. Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA)

MS-MLPA analysis was performed to distinguish between the deletion classes and to genotype positive patients whose parental blood samples absented. We used the ME028-B1 kit (MRC Holland, Amsterdam, Netherlands) according to the manufacturer's protocol. PCR products were analysed on a fluorescent capillary sequencer using Genescan (ABI 310, Applied Biosystems, Darmstadt, Germany). Runs were analysed using Peak Scanner Software v1.0 (Applied Biosystems). The Methylation Indexes (MIs) were calculated using the recommended Coffalyser version 8.0 directmethylation analysis method (MRC Holland) and were determined for each subject by the average of all MIs of target CpGs.

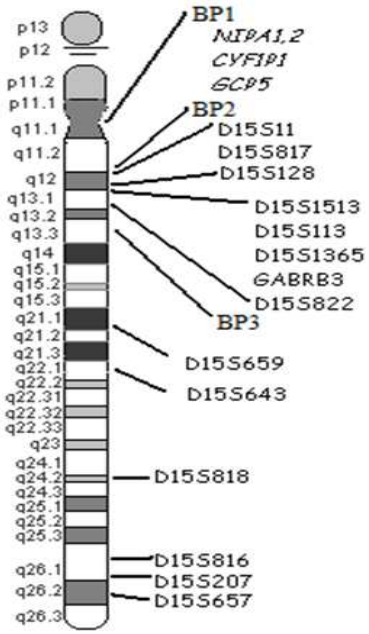

Fig. 1. Microsatellite markers location-chr. 15

Table 1. Primers for amplification and sequencing of genomic DNA; conditions for PCR amplification

Primer location	Primer sequence 5'→3'	Reference	Amplification conditions	Product size(bp)
7F	AGGTATGTTCCTATCTCCCATT	Fang *et al*. (1999)	95°C×5 min	
7R	ATTGTCTTCCTAAGTAATATGTCTA	*	[95°C×30 s; 57°C×30 s;	264 bp
8F	TTTGGCATATGATCTGCTTCTA	Fang *et al*. (1999)	72°C×30 s]×31 cycles	
8R	CACTGTGCTTATTGTTTGAATG	Malzac (1998)	72°C×10 min	537 bp
9aF	CTGGTGTAGACCCTTCTAAT	*		
9aR	TGCAACAGAGTAAACATACA	*		427 bp
9bF	GAAGCATCTTCCTCAAGGAT	*		
9bR	TAGATTTCGACTGTTAAATTCA	*		462 bp
9cF	GAGAATTTGTTCTCTGTTTC	*		
9cR	GTGGTCTAAATACAATGCAG	Malzac *et al*. (1998)		447 bp
9dF	CCCCATTATTAGGTTTTTAATCT	Malzac *et al*. (1998)		
9dR	ATTGTCGAAAACCACTTATC	Malzac *et al*. (1998)		343 bp
10F	GATACGACACCATAATCACATT	Fang *et al*. (1999)		
10R	GCAATCATCTTCTTTTCATGTT	Malzac *et al*. (1998)		221 bp
11F	GATAAGAGTATCAACAAAGATTCTA	Fang *et al*. (1999)		
11R	AGTCCTTAATAAAATACAAAAGT	Malzac *et al*. (1998)		373 bp
15F	TAAAAGTTTCCTCACACAATGACAG	*		
15R	ATGAATGCCAAACTGAAACCAG	Fang *et al*. (1999)		362 bp
12F	TTAATGAAGAGACAAAATGTGAC	Malzac *et al*. (1998)	95°C×15 min	
12R	TGTTGTATTTTGTAGTTCTATGG	Malzac *et al*. (1998)	[95°C×30 s;	258 bp
13+14F	CCTAGAGATAAAGGTCTGAAGCA	Fang *et al*. (1999)	60°C (ex12; 13+14)-	
13+14R	TGTTAAGAAGTAGGTGTAAAATTGA	Fang *et al*. (1999)	65°C (ex16)×30 s;	558 bp
16F	TTGTACTGGGACACTATCACCACCA	Fang *et al*. (1999)	72°C×30 s]×37 cycles	
16R	ACTGATGTCCTCTCTGTGGTTTTGT	Fang *et al*. (1999)	72°C×7 min	555 bp

3. RESULTS

3.1. Methylation Pattern Analysis

We found 22 patients to be positive, 16 with Presentation of maternal allele only (PWS) and 6 with presentation of paternal Allele only (AS) (**Table 2 and 3**). In every run, one patient with PWS and one with AS were used as a positive control (**Fig. 2 and 3**).

3.2. Fluorescent Multiplex PCR

Microsatellite analysis was performed in all patients with confirmed PWS and AS diagnosis and with the parental blood samples available. From 19 patients involved in this study, we identified ten patients with deletion, three with uniparental heterodisomy and three with uniparental isodisomy (**Fig. 4**). Patient 16, previously diagnosed as positive for Prader-Willi syndrome, showed only a single maternal allele on majority of loci from PWS/AS critical region, but both parental alleles on terminal D15S822 and GABRB3 loci. These results suggest a presence of shorter deletion, which does not include common breakpoint regions.

Patient 18 showed biparental inheritance for all chromosome 15 markers, but a methylation pattern characteristic for the Angelman syndrome was present (results from both methylation pattern analyses). Imprinting defect as the syndrome cause was presumed. According to the literature the majority of patients are sporadic cases without any detectable mutations in the ICR. Therefore we made also MS-MLPA analysis, in which no copy number changes were present; we assumed the imprinting defect without deletion in the IC.

Patient 6, in methylation pattern analysis diagnosed as Prader-Willi, showed the same pattern as his mother with presentation of both two alleles. On D15S659, D15S816, D15S657 and D15S207 loci (all outside the critical PWS/AS region) there were present signals identical with one maternal allele only. Compared with MS-MLPA analysis, where no copy number changes were present, syndrome cause was uniparental heterodisomy together with isodisomy on some loci.

3.3. Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA)

MS-MLPA had to be done in all deletion cases to determine deletion type, in all atypical cases and in all cases, in which the parental blood samples were missing.

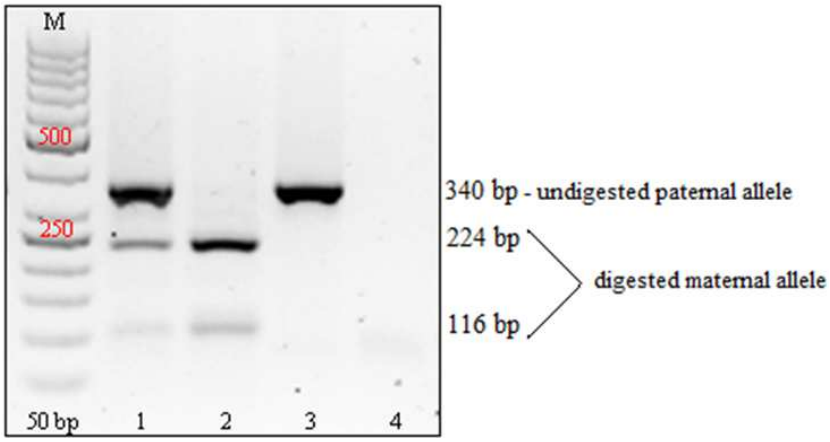

Fig. 2. Methylation PCR-RFLP analysis; the result of *HhaI* digestion (recognition sequence 5'-GCG▼C -3'). The cleavage occurred only at originally methylated (by bisulfite treatment nonconverted) allele, thus it allowed differentiation between parental alleles, digested maternally derived and undigested paternally derived. Lane 1, normal control with presence of both alleles; lane 2, PWS patient with maternal allele only; lane 3, AS patient with paternal allele only; lane 4, negative control without DNA

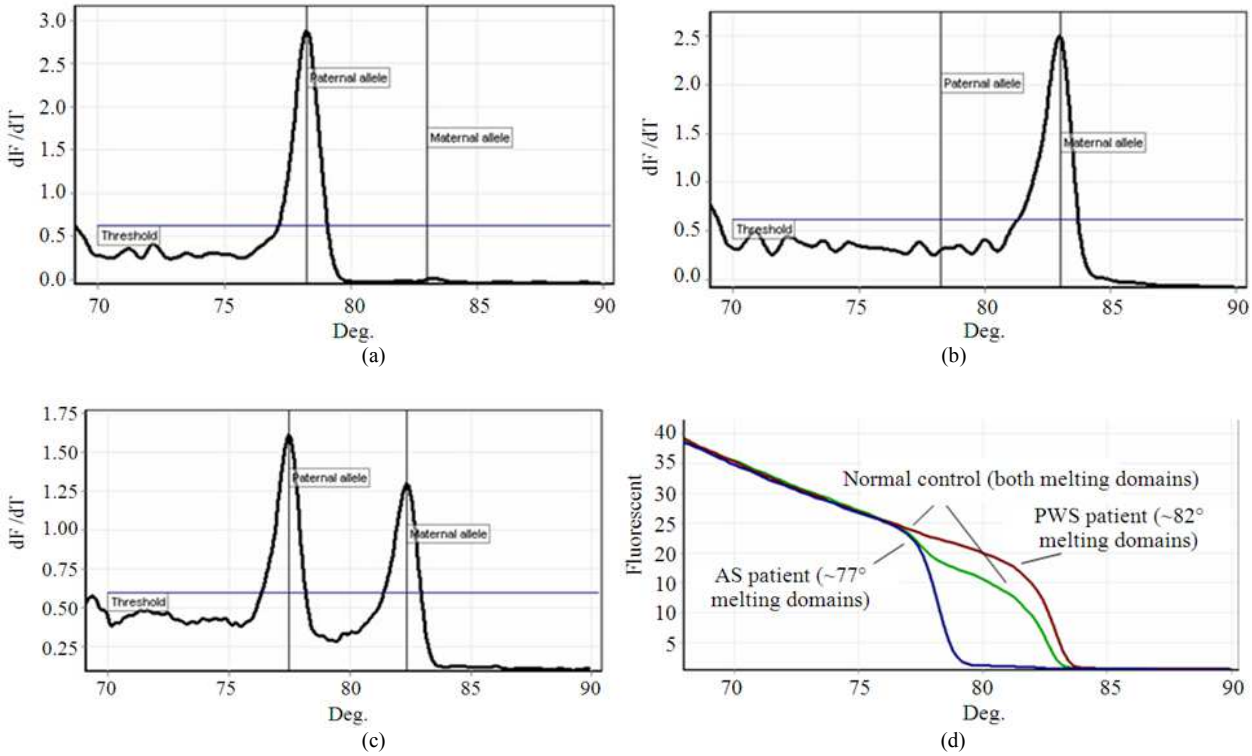

Fig. 3. Allele specific real-time PCR. Parent-of-origin specific methylation has been recognised according to the different GC content and therefore also different melting temperatures and size of the formed amplicons. PCR products derived from a bisulfite modified maternal (normally methylated) allele melted at ~82°C, compared to the paternal (normally unmethylated) allele, which melted at ~77°C. A: AS patient with presence of paternal allele only B: PWS patient with presence of maternal allele only C: Normal control with both alleles presentation D: Syndrome's distinguishing due to the melting domains differences

Table 2. PWS patients with specified genetic pathology and crucial symptoms

Cause of origin	No of cases	Age at evaluation (Patient = P.)	Main symptoms (from the clinical report)
Uniparental isodizomy	2	P. N°1: 2 months	infantile central hypotonia
		P. N°2: 11.5y	infantile central hypotonia, excessive central obesity, mental retardation, articulation difficulties
Uniparental heterodizomy	3	P. N°3: 5 months	infantile central hypotonia, hypogonadism, retention testes bilat.
		P. N°4: 2y	infantile central hypotonia, mental retardation
		P. N°5: 5y	infantile central hypotonia, hypogonadism, feeding problems/failure to thrive during infancy, hyperphagia
Uniparental	1	P. N°6: 5 months	infantile central hypotonia, hypogonadism, feeding problems/failure to iso/heterodizomy thrive during infancy, facial stigmatization
Unspecified methylation	2	P. N°7: 2 months	infantile central hypotonia and bradycardia, facial stigmatization, insuficiency clinodactylia
		P. N°8: 1.5y	infantile central hypotonia, hypogonadism, feeding problems/failure to thrive during infancy, facial stigmatization, psychomotor-mental retardation
Type I deletion (BP1-BP3)	4	P. N°9: 2 months	infantile central hypotonia, hypogonadism, feeding problems/failure to thrive during infancy, facial stigmatization
		P. N°10: 8 months	prenatal growth retardation, infantile central hypotonia, feeding problems/failure to thrive during infancy, facial stigmatization, hypopigmentation
		P. N°11: 3.5y †	infantile central hypotonia, hypogonadism, dyscrania, facial stigmatization, excessive central obesity Patient died in the 5thy due to cardiac failure.
		P. N°12: 14y	hypogonadism, dyscrania, facial stigmatization, subaortic ventricular septal defect, campodactyly
Type II deletion (BP2-BP3)	3	P. N°13: 1 month	infantile central hypotonia, facial stigmatization, hypopigmentation
		P. N°14: 5 months	infantile central hypotonia, hypogonadism, hypopigmentation, facial stigmatization, atrial septal defect
		P. N°15: 12y	infantile central hypotonia, hypogonadism, feeding problems/failure to thrive during infancy, behavioral disorder, mild mental retardation, excessive central obesity, facial stigmatization
Atypical deletion	1	P. N°16: 12y	infantile central hypotonia, feeding problems/failure to thrive during infancy, hyperphagia, excessive central obesity, facial stigmatization

In two PWS patients (7 and 8), from whom the parental blood samples were not available, we identified a methylation insuficiency. These patients possess two maternal copies of chromosome 15, both of which were methylated. *HhaI* does not digest the target templates and normalised peak areas were the same when comparing with control subjects.

In all deletion cases diagnosed previously by microsatellite analysis, we determined the deletion subtypes. In PWS patients with typical deletions the unmethylated paternal sequences were absent and therefore there was no change in peak areas when comparing *HhaI* digested versus undigested template DNA. We determined four patients with type I deletion (BP1-BP3), three patients with type II deletion (BP2-BP3) and one with atypical shorter deletion excluding common breakpoints. The mean normalised ratio from all deleted regions in the PWS/AS critical region was 0.53±0.08 in comparison to control cohort (1.01±0.09). Individuals with a type I deletion showed a deletion

involving *CYFIP1* and *TUBGCP5* genes with mean normalised ratio of 0.53±0.03 (control samples of 0.99±0.02). Patients with a type II deletion the copy numbers of *CYFIP1* and *GCPTUB5* were unaffected with mean normalised ratio of 1.01±0.05.

In patient 16 the atypical deletion range included genes from *NDN* to *ATP10A*. These results in combination with microsatellite analysis suggest that the proximal breakpoint was located between the *MAGEL2* and *NDN* genes at the centromeric end, while the distal breakpoint was located between the *ATP10A* and *GABRB3* genes at the telomeric end.

In AS patients, the copy number analysis was similar to that of PWS. In 2 patients we identified type I deletion (BP1-BP3) and only in one case the type II deletion (BP2-BP3). As in PWS patients, AS subjects with a type I deletion were hemizygous for all amplicons between BP1 and BP3, while those patient with a type II deletion was biallelic for *CYFIP1* and *TUBGCP5* genes and hemizygous for all sequences between BP2 and BP3 breakpoints. The mean normalised ratio for all deleted regions in the PWS/AS critical region in AS patients was 0.53±0.10.

3.4. Sequencing Analysis

All exons of *UBE3A* gene, encoding the major open reading frame for *E6-AP* ubiquitin-protein ligase, were sequenced in 15 subjects considered to have a highly likely clinical diagnosis of AS and normal results from methylation analyses. Among this group, the same mutation was identified in two patients (22 and 23) (**Table 3**). We detected a deletion of two base pairs between base pair 2566 and 2568 of UBE3A-001 transcript (ENST00000232165, NM_130839.2) in the hot spot region of exon 16. This c.2567_2568delAA (p.K856Gfs×24) affected open reading frame and predicted an elongated protein by changing the last 24 amino acids on its carboxyl terminal end. In both families, mothers´ samples of affected children were also sequenced, but they did not carry detected mutation. As the results shown, this mutation occurred *de novo* and in compliance with AS typical clinical phenotype it should be on the expressed maternal allele and is therefore highly likely to be disease-causing.

Found mutations were confirmed by PCR-RFLP analysis, in which the genomic DNA was amplified using primers for exon 16 from **Table 1** and since deletion creates the restriction site, PCR product was digested by *DdeI* restriction enzyme (**Fig. 5**).

Table 3. AS patients with specified genetic pathology and crucial symptoms

Cause of origin	No of cases	Age at evaluation (Patient = P)	Main symptoms (from the clinical report)
Uniparental isodizomy	1	P. N°17: 4y	mental retardation, hypersalivation, facial stigmatization
Epimutation-ID without	1	P. N°18: 3y	mental retardation, epileptic seizures, autistic features, hypotonia, deletion in the IC microcrania
Type I deletion (BP1-BP3)	2	P. N°19: 2y	developmental delay, mental retardation, epileptic seizures, hypopigmentation, frequent laughter/smiling, facial stigmatization
		P. N°20: 1y	developmental delay, feeding problems/failure to thrive during infancy, hypotonic-hyperkinetic syndrome, seizures
Type II deletion (BP2-BP3)	1	P. N°21: 2y	developmental delay, hypotonia, tremor
UBE3A mutation	2	P. N°22: 8y	developmental delay, balance disorder, ataxia, muscle fasciculations, severe speech impairment, microcephaly
		P. N°23: 3y	developmental delay, balance disorder, ataxia, muscle fasciculations, inappropriate laughter, severe speech impairment, microcephaly
Undefined	1	P. N°24: 4y	kvadruspastic syndrome, asymmetrical brain laterally chamber, foramen (MS-PCR only; 2007) ovale apertum, epileptic seizures

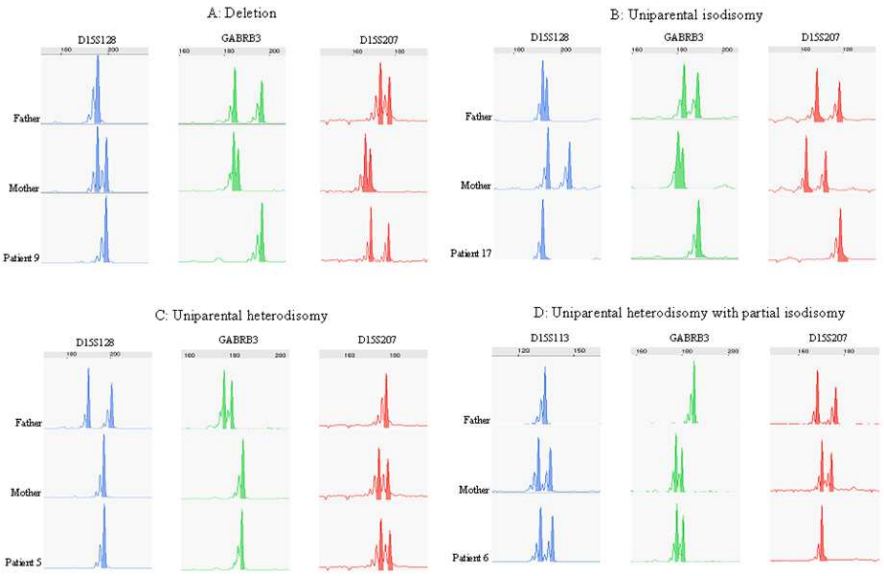

Fig. 4. Electropherograms of multiplex fluorescent PCR amplification from four families with different molecular background. In deletion cases, only a single parental allele (maternal in PWS and paternal in AS) was found on the critical region loci, while both parental alleles were detected on control loci. In uniparental disomy, proband sample showed the same pattern as parental one, according to the syndrome type. In heterodisomy two different paternal or maternal alleles were exhibited on all loci involving in PWS/AS critical region and likewise on control loci. In isodisomy the results indicated only single duplicated uniparental inheritance on all loci. Marked peaks represent the true allele peaks. The rulers above figures indicate the molecular sizes (bp), which were automatically computed with standard size marker

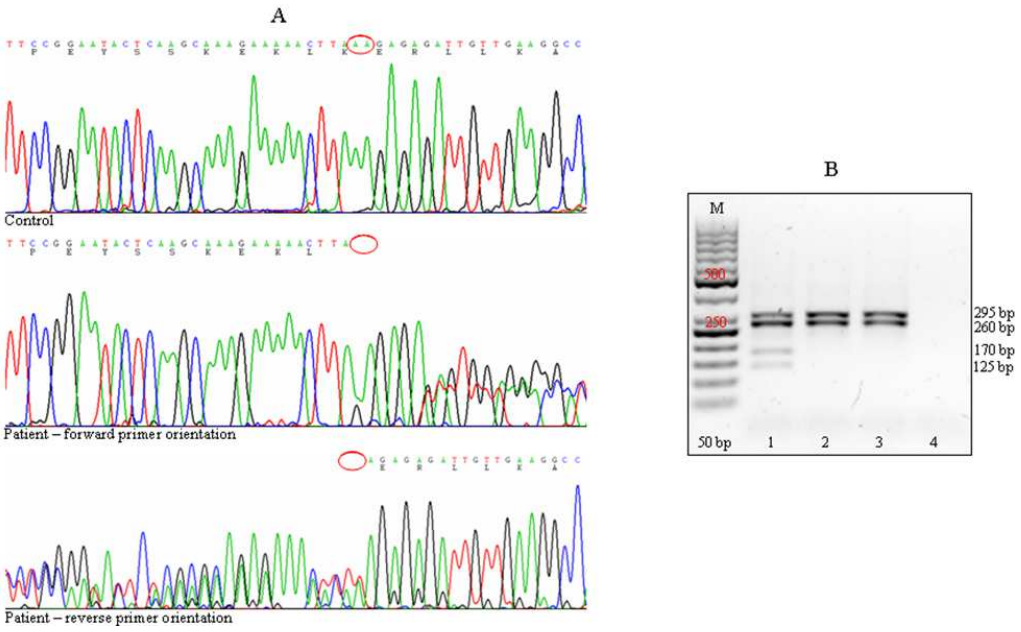

Fig. 5. A: c. 2567_2568delAA found in *UBE3A*. Sequences shown are the proportion of exon 16's chromatograms with corresponding amino acid single-letter codes; B: PCR-RFLP analysis with *DdeI* digestion. Lane 1, positive heterozygous sample for c.2567_2568delAA; lane 2, probands´ mother without mutation; lane 3, normal control; lane 4, negative control without DNA

4. DISCUSION

It has been reported frequently that there is a higher incidence of deletion cases (~75%) and lower mUPD cases (~25%) in PWS patients (Fan *et al*., 2009; Nicholls and Knepper, 2001). The similar proportion was also seen in AS patients, about 70% of deletion and 30% of nondeletion cases, from which about 10% are pUPDs (Fan *et al*., 2009; Malzac *et al*., 1998). Of the 8 AS patients determined in this study, 3 had deletions, 1 had pUPD, 1 had an imprinting defect and 2 patients had *UBE3A* mutation. Of the 16 PWS cases we identified 8 patients (50%) with 15q11-q13 deletion and 8 patients (50%) with methylation insuficiency. The reason for the difference between our data and those previously reported from other populations (Ramsden *et al*., 2010) remains unknown. The percentage discrepancy might arise due to the fact, that methods used in the past, like FISH analysis, were able to detect mainly deletions than the other causes. Comparison of average age at conception of our mUPDs patients´ mothers (36 years) with mothers of our PWS patients with deletion (26 years) suggests that errors during cell division correlate with maternal age, what can lead to increased proportion of UPDs.

The prevalence of PWS ranges from 1 in 25,000 (Butler, 1990) within the United States; through 1 in 16,000 (Ehara *et al*., 1995) in Asia; to 1 in 45,000 within the UK (Whittington *et al*., 2001). For AS the prevalence ranges from 1 in 20,000 in the United States (Buckley *et al*., 1998) to 1 in 12,000 in Sweden (Steffenburg *et al*., 1996). According to our study and to known natality, we estimate the minimal live birth prevalence for Slovakian population to 1 in 20,000 for PWS and to 1 in 40,000 for AS; showing that AS is 2 times less frequent than PWS.

This study, in addition, enables distinguishing between deletion subtypes. The deletion breakpoints aggregate due to the presence of hot spots, the large duplicated sequence with a size of 200-400 kb that are prone to non-homologous crossovers (Christian *et al*., 1999). Patient 16 has deletion lying outside the most common breakpoints region, which arose inside the critical region. The causal mechanism of this rearrangement could be the Nonhomologous End Joining (NHEJ), like in the human dystrophin gene, where in some deletion cases a short homology (2-4 bp) at the end of the junctions was found (Toffolatti *et al*., 2002). Patient 16 has probably the same typical PWS phenotype as patients with other causes of origin have. This finding suggests that the main clinical features depend on the *SNRPN* gene absence.

Approximately half of our PWS patients have UPDs. It could be the result of nondisjunction in the meiotic cycle and subsequent postzygotic correction of a trisomic embryo or duplication of the chromosome in a monosomic cell (Robinson *et al*., 2000). Heterodisomy could result from incorrect nondisjunction in the first meiotic cell division, while isodizomy may result from nondisjunction in the second meiotic cell division, but it must be followed by the loss of paternal chromosome 15. In the patient 6, we made quantitative analyses by MLPA, but there was no significant DNA dosage variation between the patient and controls. This finding suggests that DNA recombination leads to segmental isodisomy on the maternally derived heterodisomic chromosome.

From all reported AS cases imprinting defects occurred in ~3%. Very frequent cause is a microdeletion in the Imprinting Centre (IC), which is in familial form of disease associated with a 50% recurrence risk. In AS patients microdeletions occurred in the upstream sequences of the *SNRPN* gene and ultimately interrupt the rescue ability of the imprinting pattern. In general, microdeletions founded in PWS patients alter the *SNRPN* promoter methylation (Satapathy *et al*., 2014). Patient 18 did not show any IC deletions on MS-MLPA and therefore the methylation insuficiency is probably the result of a *de novo* defect in the imprinting process in PWS/AS critical region during the parental gametogenesis. In that families the recurrence risk of the siblings is less than 1% in comparison to those with IC microdeletions (50%). These rare cases must be precisely analysed to confirm the nature of the imprinting defect, what is necessary for prediction of the recurrence risk (Gabriel *et al*., 1999).

In our group of PWS patients, there is no significant correlation between the syndromes cause of origin and the clinical phenotype, except the mental retardation that occurred only in UPD cases. Our findings about genotype-phenotype correlation in AS patients are in agreement with previous reports. All deletion cases had typical characteristics of AS including developmental delay, seizures and hypopigmentation (Spritz *et al*., 1997). We identified only one patient with pUPD with clinical features more moderate in comparison to patients with deletion. He did not have seizures and his speech was better like it was published in similar case by (Smith, 2001). Mutations within the *UBE3A* gene give rise to a typical but more severe clinical presentation

without hypopigmentation. These findings led to the conclusion that this is the 15q11-q13 gene responsible for the AS phenotype (Kishino *et al*., 1997; Matsuura *et al*., 1997). Usually, patients with IC epimutation have microcephaly, hypopigmentetion and less frequently seizures (Ohta *et al*., 1999). Phenotype of patient 18 is atypical, but mental retardation and autistic features may suggest that the methylation insuficiency extends also to *NIPA1* and *NIPA2* genes in critical region, which are implicated in autistic disorders with moderate mental retardation (De Wolf *et al*., 2013).

Studying causal mechanisms of unique genomic rearrangement, recurrent and novel deletion breakpoints may help with disorders distinguishing and can be applied to genetic counselling of family members and in relevant cases also to prenatal testing. The main advantage of the early diagnosis is the possibility of the early initiation of growth hormone treatment in PWS.

5. CONCLUSION

We have implemented and combined several sensitive molecular-genetic methods what reduced the risk of false-negative or false-positive results. In that case, these methods are applicable also for the prenatal diagnostics. In a period of six years we have identified and genotyped 24 PWS and AS patients from 450 suspected cases. The wide variety of methods enabled us to determine atypical deletion that does not include common breakpoints, novel presumably pathological *UBE3A* mutation, uniparental heterodisomy together with partial isodisomy and epimutation without any deletions in the imprinting centre. We present genotype-phenotype correlation thanks to clinical data from all positive cases. The genotype-phenotype correlation is indispensable for clinical research and for correct and rapid clinical diagnostics. Early diagnosis determination is essential for the next patient's development and in some cases also for the rapid treatment.

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