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MOLECULAR-GENETIC DIAGNOSTICS OF ANGELMAN SYNDROME – THE BULGARIAN EXPERIENCE

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Abstract. Objective: *The aim of the study was to determine the molecular mechanisms of mutagenesis in Bulgarian patients with Angelman syndrome (AS). AS is a severe neurodevelopmental disorder caused by loss of expression in brain of the maternally inherited UBE3A gene as a result of various 15q11.2-q13 alterations.* ***Material and Methods:*** *In total 24 patients (11 boys, 13 girls) from 22 unrelated families with suspected clinical diagnosis AS were analysed. We used methylation specific PCR, multiplex ligation-dependent probe amplification, methylation sensitive MLPA, and direct sequencing of the UBE3A gene.* ***Results:*** *In 9 families (41%) pathogenic mutations were detected, which confirmed the clinical diagnosis on a molecular-genetic level. In 4 (44%) of these families we found 15q11-q13 region deletion with breakpoints BP1-BP3 or BP2-BP3. In 1 (11%) of the families we found imprinting defect: deletion of the AS-SRO regulatory region (part of the PWS-AS imprinting center). In 1 (11%) of the families we detected a rare finding – paternal uniparental disomy of chromosome 15. In 3 (33%) of the families different point mutations in the UBE3A gene were detected: two novel missence mutations c.488T > C; p.Leu163Ser and c.1832A > T; p.Gln611Leu, and one known frameshift mutation c.2576_2579delAAGA; p.Lys859Argfs*4.* ***Conclusion:*** *The obtained results helped us to develop a systematic diagnostic algorithm in order to provide proper diagnosis for the patients with AS. Combining excellent knowledge of the molecular mechanisms of mutagenesis and proper molecular-genetic testing approaches is a cornerstone in the management of AS patients, ensuring AS families would receive both adequate genetic counseling and prophylaxis of the disease in the future.*

Key words: *Angelman syndrome, UBE3A, imprinting center, CpG methylation, 15q11.2-q13, paternal UPD*

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INTRODUCTION

Angelman syndrome (OMIM#105830) is a rare and severe neurodevelopmental disorder, with prevalence of between 1:10 000 and 1:40 000 [1]. Clinically AS is characterized by developmental delay (evident by 6-12 months of age), motor dysfunction (patients do not walk until 3-4 years of age or do not achieve ambulation), severe intellectual and learning disability, speech impairment (none or minimal use of words), specific behavioural phenotype (waving movements, hypermotoric state, excessive laughter

and happy grimacing), epileptic seizures of various types (developed at 1-3 years of age), ataxia of gait and/or tremulous movement of the limbs, dysmorphic facies (prominent chin, deep set eyes, wide mouth with protruding tongue), microcephaly with a flat occiput (by the age of 3), sleep disturbances (night time awakenings, diminished total sleep time) [1, 2].

AS results from loss of expression of the *UBE3A* gene (MIM 601623), which encodes human papillomavirus E6-assocoated protein (E6-AP) that functions as both an ubiquitin-protein E3 ligase in the ubiquitin proteasome pathway and as a transcriptional coactivator [3, 4].

The gene *UBE3A* is located in 15q11.2-q13 chromosomal region where several imprinted genes are clustered (Fig. 1). Prader–Willi syndrome (OMIM 176270) is caused by loss of function of paternally expressed genes, whereas Angelman syndrome is caused by loss of function of the maternally expressed gene *UBE3A* in neuronal cells [5].

Methylation and gene expression in the imprinted region is regulated in cis by a bipartite imprinting centre located in the small nuclear ribonucleoprotein-associated protein N (SNRPN) upstream region [6]. Imprinted expression of *UBE3A* is regulated indirectly by small nucleolar RNA host gene 14 (SNHG14, formerly known as *UBE3A*ATS) whose product is a non-coding antisense transcript that is initiated at the paternal SNRPN promoter. Normally in neuronal cells the paternal region lacks methylation, resulting in transcription of SNHG14 and thus *UBE3A* gene is not expressed. The maternal region is methylated, SNHG14 is not expressed, resulting in *UBE3A* gene transcription [7, 8].

AS can result from various molecular mechanisms by which *UBE3A* can be disrupted including: 1) Deletions of the 15q11.2-q13 region (in 70%-75% of the cases) [1, 10]. In 90% of the cases the deletion spans the region between the breakpoins BP1-BP3 ot BP2-BP3, and rarely reaches BP4 or BP5 [11, 12]. 2) Paternal uniparental disomy of chromosome 15, wherein one inherits both chromosomes 15 from the father (in 1-3% of the cases) [1, 10]. 3) Imprinting center defects resulting from small deletions or disruption of DNA methylation in the imprinting center (in 3-6% of the cases) [13]. 4) *UBE3A* gene mutations (in 5-10% of the cases) [14].

The molecular-genetic diagnostics of AS requires application of different analytical methods [10, 15]. The methylation pattern within 15q11-q13 region could be assessed using methylation specific PCR (MS-PCR). Changes in the copy number of numerous genes within the target region could be detected by multiplex ligation-dependent probe amplification (MLPA). The simultaneous assessment of methylation status and changes in copy numbers across the 15q11-q13 region could be done by methylation sensitive multiplex ligation-dependent probe amplification (MS-MLPA). Direct sequencing for pathogenic variants in the *UBE3A* gene could be used as well.

The aim of the current work was to confirm on molecular-genetic level the suspected clinical diagnosis AS in Bulgarian patients in order to reveal the molecular mechanisms of mutagenesis in our sample and to develop a systematic diagnostic algorithm for proper diagnosis, providing opportunities for adequate genetic counseling and prophylaxis of the disease in the affected AS families.

MATERIAL AND METHODS

In total 24 patients (11 boys, 13 girls) from 22 unrelated families with suspected clinical diagnosis AS were

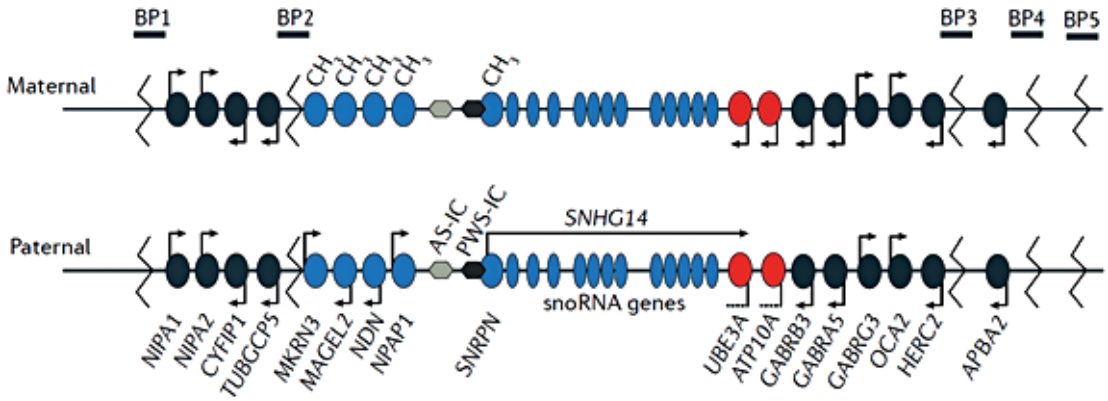

Fig. 1. Chromosomal region 15q11.2-q13. Paternally expressed genes are represented in blue, maternally expressed genes are marked in red, genes expressed from both alleles are given in grey. Transcription orientation is pointed by with arrows. AS-IC is the Angelman syndrome imprinting center. PWS-IC is the Prader-Willi syndrome imprinting center. BP1 to BP5 are the deletion breakpoints. CH3 indicates methylation [adapted from 9]

analysed, and where possible their parents were included in the research as well. DNA samples were obtained from peripheral blood after written informed consent was obtained. The study was approved by the Ethics Committee of Sofia Medical University.

In all patients we accessed the methylation pattern within the imprinted 15q11-q13 region by methylation specific PCR (MS-PCR). Two different primer pairs specific for differentially methylated sites within the SNRPN exon 1/promoter regions were used, according to the protocol of Kosaki et al. [16]. The DNA template for MS-PCR was chemically modified with sodium bisulfite, resulting in conversion of cytosine, but not 5-methyl-cytosine, to uracil, which permits to distinguish successfully the methylated maternal from the unmethylated paternal allele in the subsequent PCR. For the sodium bisulfite treatment we used EZ DNA Methylation–Gold ™ Kit (Zymo Research, USA) following the instructions of the manufacturer. Normal individuals have unmethylated paternal and a methylated maternal allele. In AS patients with deletion of 15q11-q13 region, paternal uniparental disomy or an imprinting defect, only a paternal unmethylated allele can be detected.

To access changes in the copy number in some patients we used MLPA with SALSA MLPA kit P245-A2 Microdeletion-1 probemix with 49 MLPA probes (MRC-Holland), which can detect 20 different microdeletion syndromes, including AS.

To access simultaneously the methylation status and changes in the copy number of some of the genes within the 15q11-q13 region we used SALSA MS-MLPA ME028-A1 PWS/AS probemix (MRC-Holland) with 43 MLPA probes or SALSA MS-MLPA ME028-B2 PWS/AS probemix (MRC-Holland) with 48 MLPA probes. The protocols for SALSA MLPA and SALSA MS-MLPA kits were performed strictly following the instructions of the manufacturer.

In patients with normal methylation status and no changes in the copy number within the 15q11-q13 region we proceeded with direct sequencing of the *UBE3A* gene in order to screen for pathological variants by BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The nucleotide changes in the *UBE3A* gene were described according to the coding DNA reference sequence of the longest isoform of the protein (*UBE3A* isoform II), where +1 nucleotide is the base adenine of the ATG start codon in the middle of exon 4 of the gene (https://databases.lovd.nl/shared/refseq/*UBE3A*_NM_000462.3_codingDNA.html).

The electrophoretic separation of the MLPA, MS-MLPA and direct sequencing fragments was performed on ABI Prism 3130 Sequence Genetic Analyzer (Applied Biosystems) with polymer POP7 (Applied Biosystems). The electrophoretic data were automatically analyzed with ABI3130 Data Collection Software (Applied Biosystems, Foster City, CA) for the direct sequencing or GeneMapper Software v.4.0 (Applied Biosystems, Foster City, CA) for the fragment analysis.

RESULTS

In 9 of the analyzed families (41%; 9/22) genetic abnormalities in the 15q11-q13 region were found, with which the clinically suspected diagnosis of AS was confirmed.

In 6 families the MS-PCR showed loss of the maternally methylated copy of the allele, but that test can't distinguish if AS is due to deletion of the 15q11-q13 region, paternal uniparental disomy or an imprinting defect (Fig. 2, Lines 2, 3 and 5). To determine the mechanisms of mutagenesis in these patients we proceeded further with MLPA and MS-MLPA. MS-PCR cannot prove the diagnosis of AS in patients with *UBE3A* gene mutation, because they have the same methylation profile as the normal control samples (Fig. 2, Lines 4 and 6). In cases of normal MS-PCR result *UBE3A* gene sequencing was performed.

MLPA and MS-MLPA results showed: 1) De novo deletion of the maternally inherited 15q11.2-q13 region in 4 of the 9 genetically confirmed AS cases (44%) (Fig. 3 and Fig. 4). Of note, mothers were not deletion carriers. 2) Paternal uniparental disomy in 1 of the 9 confirmed AS cases (11%) (Fig. 3 and Fig. 4). The parental chromosomes were normal. 3) Imprinting defect (deletion of the AS-SRO regulatory region in AS/PWS imprinting center) in 1 out of the 9 confirmed AS cases (11%) (data not shown), where the mother was a carrier of the mutation and was symptoms free.

In the profile of the patient with deletion the red arrows indicate the fragments with half of the intensity compared to the normal sample, which shows deletion of MGRN3, MAGEL2, NDN, SNRPN, *UBE3A*, ATP10A, GABRB3 and OCA2 genes (TUBGCP5, CYFIP1 and APBA genes are not included in the deletion, because the corresponding fragments are with the same intensity compared to the normal sample). In the profile of the patient with paternal uniparental disomy all fragments are with the same intensity compared to the normal sample, which shows that each of the genes in the 15q11-q13 region are presented in 2 allele copies.

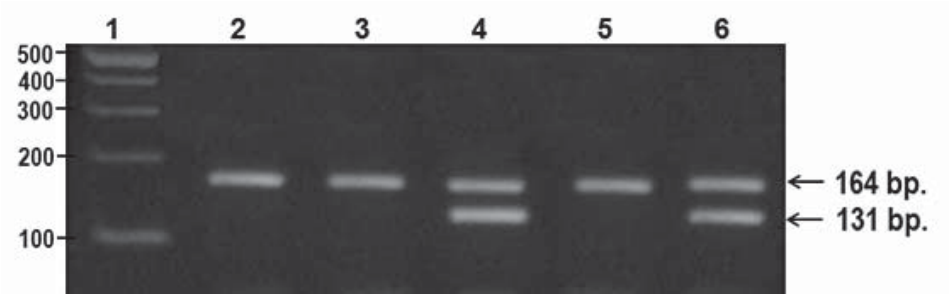

Fig. 2. Agarose gel electrophoresis of the fragments amplified by MS-PCR using two different primer pairs of primers in a multiplex reaction. Line 1 – 100 bp. ladder, Line 2 – patient with 15q11.2-q13 deletion, Line 3 – patient with paternal uniparental disomy, Line 4 – patient with *UBE3A* gene mutation, Line 5 – patient with imprinting defect, Line 6 – normal control sample. Normal individuals have unmethylated paternal allele (164 bp.) and a methylated maternal allele (131 bp.). In AS patients with deletion of 15q11-q13, paternal uniparental disomy or an imprinting defect only the paternal unmethylated allele (164 bp.) can be detected, while the maternal methylated allele is missing

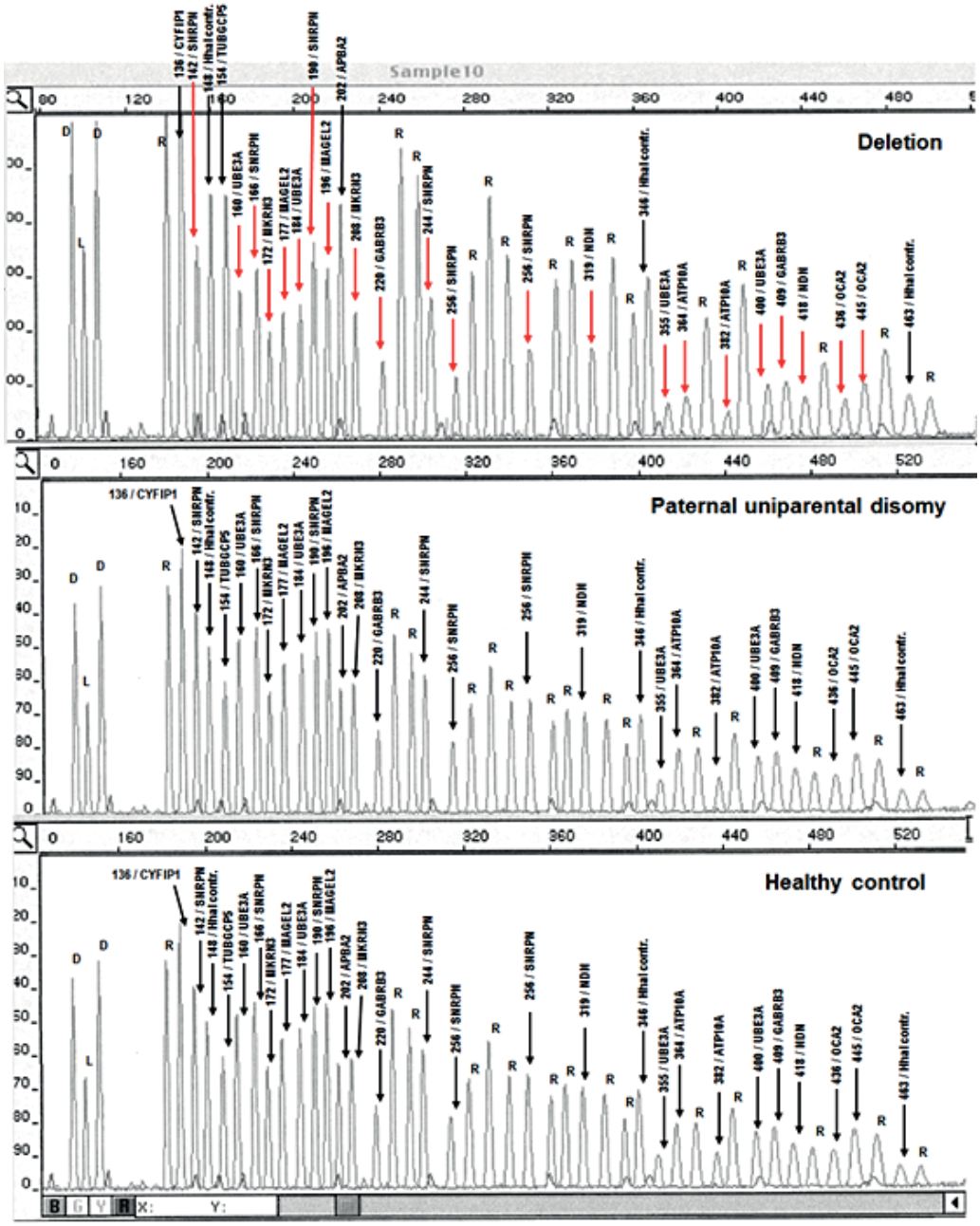

Fig. 3. MLPA results of a patient with 15q11-q13 deletion with breakpoints BP2-BP3, patient with paternal uniparental disomy, and healthy control sample with kit SALSA MS-MLPA ME028-A1. The length (in base pairs) of all specific fragments is provided with numbers. The control fragments "D" (two denaturation controls), "L" (one ligation control), "R" (15 reference probes on 10 chromosomes) are marked with letters. Black arrows indicate all fragments with normal intensity corresponding to 2 alleles (no deletion)

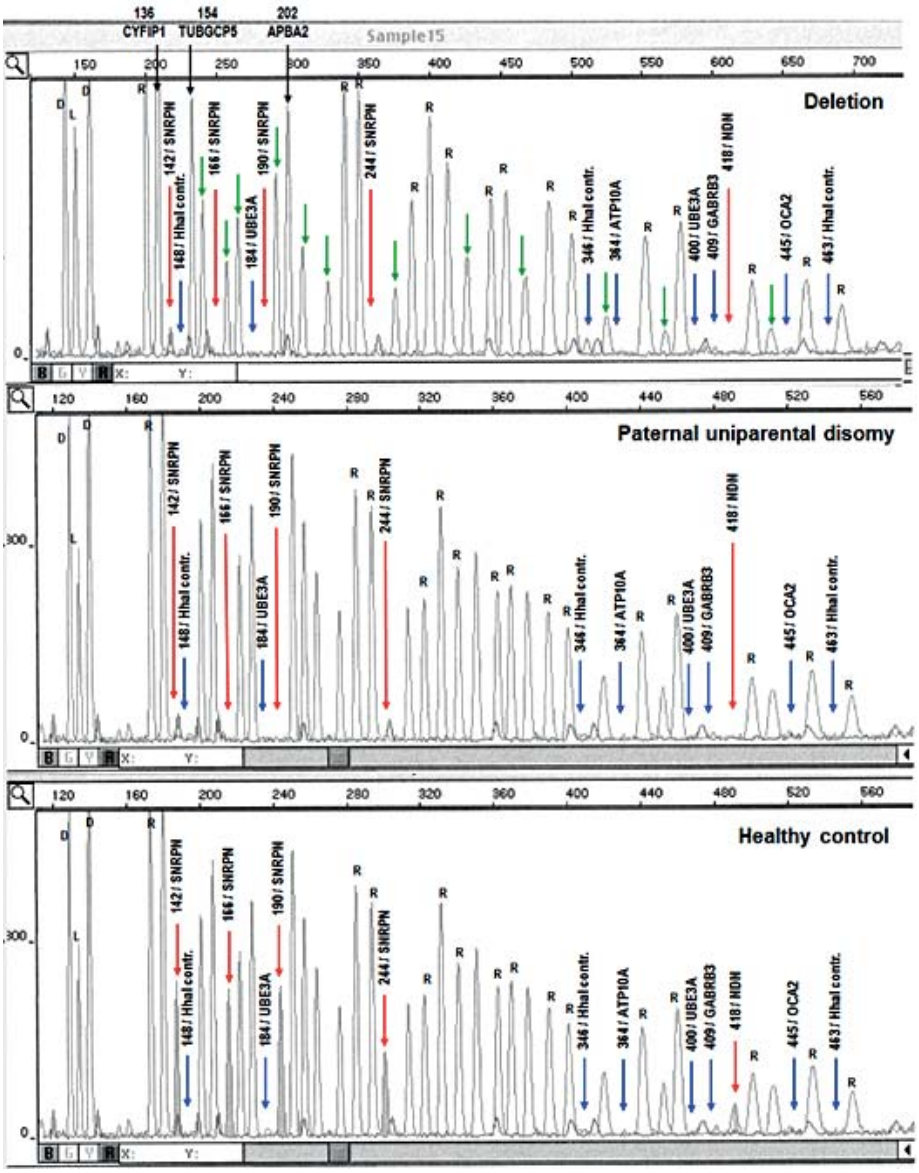

Fig. 4. MS-MLPA results of a patient with 15q11-q13 deletion with breakpoints BP2-BP3, patient with paternal uniparental disomy, and healthy control sample with kit SALSA MS-MLPA ME028-A1. The length (in base pairs) of some of the fragments is provided in numbers. The control fragments "D" (two denaturation controls), "L" (one ligation control), "R" (15 reference probes on 10 chromosomes) are marked by letters. Blue arrows indicate the eight fragments which obligatory are unmethylated, therefore cut by HhaI restrictase and the corresponding peaks are missing. Red arrows indicate the five methylation-specific sensitive to HhaI digestion fragments in CpG islands in SNRPN and NDN genes, which are missing in the patients either because of deletion of the maternal methylated copy of the 15q11-q13 region or because no maternal chromosomal 15q11-q13 15 region is inherited. In the profile of the patient with deletion the green arrows indicate the fragments with reduced intensity compared to the normal sample (corresponding to deletion of MGRN3, MAGEL2, NDN, SNRPN, *UBE3A*, ATP10A, GABRB3 and OCA2 genes), and with black arrows are marked the fragments with the same intensity compared to the normal sample (meaning the genes TUBGCP5, CYFIP1 and APBA are not included in the deletion). In the profile of the patient with paternal uniparental disomy the fragments are with the same intensity compared to the normal sample, which shows that each of the genes in the 15q11-q13 region is present in 2 alleles, but both of them are unmethylated and inherited from the father

UBE3A gene sequencing results showed three different point mutations in 3 of the 9 genetically confirmed AS cases (33%) – two previously unpublished missense mutations (c. 488T > C; p.Leu163Ser in exon 7 and c.1832A > T; p.Gln611Leu in exon 9), and one known frameshift mutation c.2576_2579delAAGA; p.Lys859Argfs*4 in exon 14 (Fig. 5). In one of the families with *UBE3A* gene mutation the mother was mutation carrier and had no clinical symptoms, while in the rest two families the mutations were de novo.

DISCUSSION

The current study revealed that all known mechanisms of mutagenesis (four types of mechanisms) resulting in AS were seen among 9 Bulgarian patients with AS.

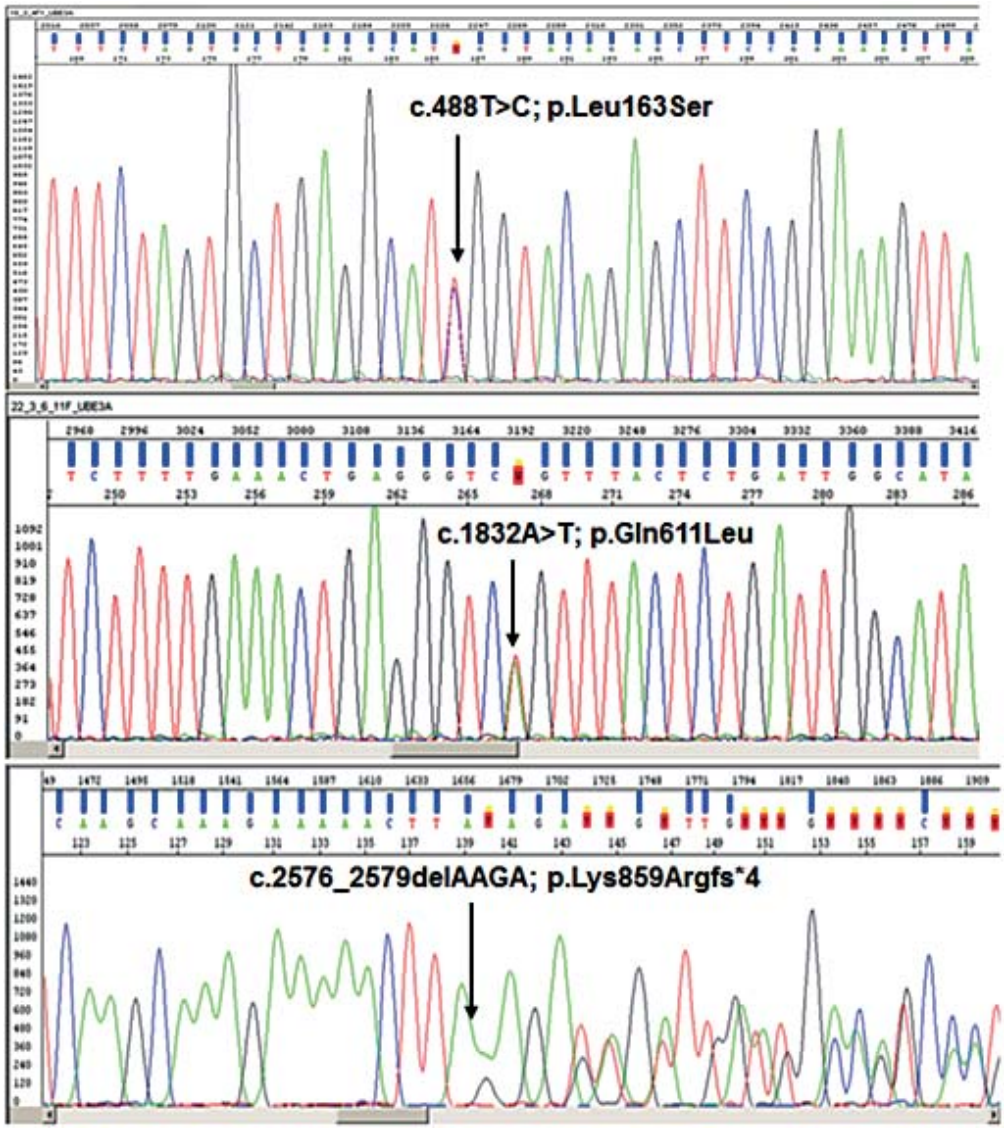

Fig. 5. Sequencing results of the *UBE3A* gene mutations c.488T > C; p.Leu163Ser in exon 7, c.1832A > T; p.Gln611Leu in exon 9, and c.2576_2579delAAGA; p.Lys859Argfs*4 in exon 14 in three AS cases

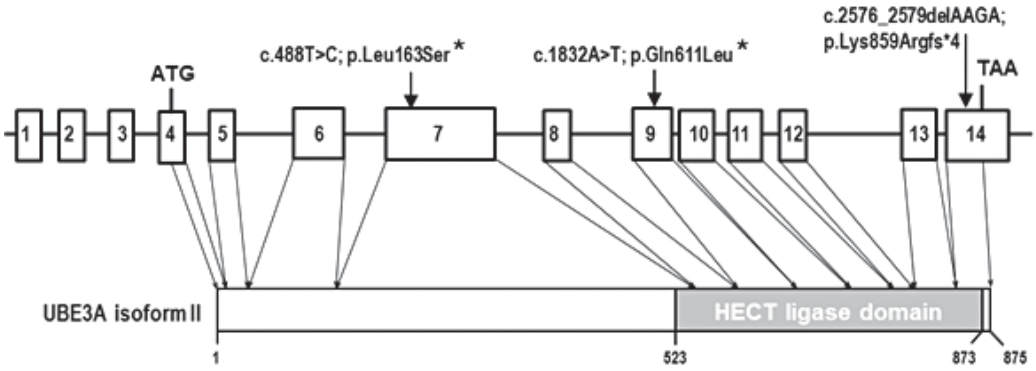

Fig. 6. Location of the Bulgarian point mutations along the *UBE3A* gene. The *UBE3A* gene exons are schematically represented by numbered rectangles – 1 to 14. The translation of the longest isoform of the protein (*UBE3A* isoform II composed of 875 amino acid residues) is initiated in exon 4 (the ATG starting codon). The stop codon (TAA) in exon 14 is marked. Part of exon 7 to exon 14 encodes the conservative catalytic HECT (homologous to the E6-AP carboxyl terminus) ubiquitine ligase domain which constitutes the last 350 amino acid residues of the protein. The three pathological variants found in our patients are marked with arrows above the affected exon. The two novel missense mutations c.488T > C; p.Leu163Ser in exon 7 and c.1832A > T; p.Gln611Leu in exon 9 are marked with asterisks

The most common mechanism of mutagenesis in our sample was deletion of the maternally inherited 15q11.2-q13 region – 44%, which is much lower than the percent published in the literature (70-75% of the cases) [1, 10]. The deletions described in our study fall between the most common breakpoints BP1-BP3 or BP2-BP3, and none of them spans to BP4 or BP5, which corresponds to the published data [11, 12].

The imprinting center defect in our AS patients was detected once, but having in mind our cohort was very small, this presumably resulted in a falsely high percent – 11%, which is twice as common compared to the literature (3-6%) [13]. The detected mutation belongs to the most common type of the imprinting center defects – microdeletion of the AS-SRO regulatory region in AS/PWS imprinting center resulting in loss of methylation in the SNRPN exon 1/promoter regions and inhibition of the *UBE3A* gene transcription [13].

We had the unique chance to find paternal uniparental disomy of chromosome 15 where the patient does not inherit *UBE3A* gene from the mother (one patient), which is considered to be the most rare mechanism of mutagenesis in AS (1-3% of the cases) [1, 10].

The percentage of *UBE3A* gene point mutations in our AS patients (33%) was more than three times higher than that published in the literature (5-10%) [14]. Our finding of two novel missense mutations (c.488T > C; p.Leu163Ser in exon 7 and c.1832A > T; p.Gln611Leu in exon 9) enriches the spectrum of missense mutation in AS. According to the published data, most of the mutations are insertions, deletions and nonsense mutations, while missense mutations are less common [14, 17].

The novel missense mutations c.488T > C; p.Leu163Ser in exon 7 is not a surprising finding, considering the large size of that exon (encodes 415 out of totally 875 amino acid residues) where about half of all *UBE3A* gene mutations are reported.

Our novel missense mutation in exon 9, together with the already published frameshift mutation c.2576_2579delAAGA; p.Lys859Argfs*4 in exon 14, which we also detected in one patient, fall in the highly conservative catalytic HECT (homologous to the E6-AP carboxyl terminus) ubiquitine ligase domain of the protein, probably resulting in severe impairment of the protein function (Fig. 6). Although exon 14 is 10 times smaller than exon 7 (encodes only 39 amino acid residues), a large number of small deletions and insertions were reported there [17].

Adequate genetic counseling requires proper assessment of the recurrence risk after molecular-genetic testing of all family members. In cases with uniparental disomy, when parental chromosomes are normal, the recurrence risk is very low (< 1%). In cases with de novo mutation (deletion, imprinting center mutation, or *UBE3A* gene mutation) the recurrence risk is very low (< 1%), but germ line mosaicism should be considered (about 10%). In cases when the mother is a carrier of a *UBE3A* gene mutation, there is a 50% recurrence risk, that is a 50% risk that the mother could have another child with AS.

CONCLUSION

Our results helped us to develop a systematic diagnostic algorithm for genetic evaluation of patients with AS in Bulgaria. Combining excellent knowledge of the molecular mechanisms of mutagenesis and proper molecular-genetic testing approaches is a cornerstone in the management of AS patients, ensuring AS families would receive both adequate genetic counseling and prophylaxis of the disease in the future.

Conflict of interests: *The authors declare no conflict of interests.*

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Disclosure Summary: *The authors have nothing to disclose.*

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