BJMG 13/2 (2010) 2-10
10.2478/v10034-010-0022-8

ORIGINAL ARTICLE

CLINICAL AND MOLECULAR DATA ON MENTAL RETARDATION IN BULGARIA

Todorov T[1#], Todorova A[1#], Avdjieva D[2], Dimova P[3], Angelova L[4], Tincheva R[2] and Mitev V[1]

***Corresponding author:** Tihomir Todorov, Department of Medical Chemistry and Biochemistry, Sofia Medical University, 2 "Zdrave" str., Sofia 1431, Bulgaria; Tel./Fax: +359 29530715; tisho.todorov@abv.bg

ABSTRACT

Mental retardation (MR) is clinically and genetically highly heterogeneous. We have done molecular genetic testing on 85 Bulgarian MR patients who were clinically classed as fragile X syndrome (FXS) (n = 32), Rett syndrome (RTT) (n = 36) and Prader-Willi/Angelman syndromes (PWS/AS) (n = 17). We tested for the fragile X MR 1 (FMR1), methyl-CpG binding protein 2 (MECP2), cyclin dependent kinase-like 5 (CDKL5), and Aristaless X (ARX) genes, and did methylation analyses of exon 1 of the small nuclear ribonucleoprotein polypeptide N (SNRPN), and multiplex ligation-dependent probe amplification (MLPA) analyses for large deletions/ duplications, and for the methylation status of FMR1 and SNRPN genes. In the FXS group we only found four mutations in the FMR1 gene (12.5%). In the RTT group we found nine mutations in the MECP2 gene (25.0%) but no CDKL5 gene mutations. In the PWS/AS group we found nine mutations in the 15q11-q13 region (53%). Thus, we clarified the molecular basis in 26.0% of the patients. The proportion of genetically proved diagnoses in our RTT patients (25.0%) is relatively high and all these cases are due to MECP2 mutations. Despite the type of mutation, all these cases are very similar from the clinical point of view and well recognized in Bulgaria.

Keywords: Fragile X syndrome (FXS), Mental retardation (MR), Multiplex ligation-dependent probe amplification (MLPA), Prader-Willi/Angelman syndrome (PWS/ AS), Rett syndrome (RTT)

INTRODUCTION

Mental retardation (MR) is clinically and genetically highly heterogeneous. Mental retardation and absent speech may be the only clinical finding, or may be associated with progressive neurodevelopmental deficits, seizures, autism and ataxia. A variety of genes, X-linked or autosomal, have been found to be involved in pathogenesis. The most common form of X-linked MR (XLMR) is the fragile X syndrome MR (FXS, OMIM #300624), which is due to an expansion of an unstable CGG repeat in the 5'UTR (5' untranslated region) of the FMR1 (fragile X MR 1) gene. The polymorphic CGG repeat numbers 6 to 54 ± 2 copies in normal individuals, 55 ± 2 to 200 copies in healthy carriers of premutation, and >200 copies in FXS patients [1,2].

[1] Department of Medical Chemistry and Biochemistry, Sofia Medical University, Sofia, Bulgaria
[2] University Pediatric Hospital, Department of Clinical Genetics, Sofia Medical University, Sofia, Bulgaria
[3] Neurological Hospital "St. Naum", Sofia, Bulgaria
[4] Laboratory of Medical Genetics, Varna Medical University, Varna, Bulgaria
[#]T.T. and A.T. contributed equally to this work.

One of the most common forms of severe MR in females is Rett syndrome (RTT, OMIM#312750), which is mostly due to point mutations or large deletions in MECP2 (methyl-CpG binding protein 2) gene, and rarely in CDKL5 (cyclin dependent kinase-like 5) gene [3,4]. On the other hand, retarded females with infantile spasms and other early onset seizures could be caused by mutations in the CDKL5 gene [5]. It has been expected that a number of mentally retarded males with epilepsy will be explained by the common mutation dup24 or other mutations in the Aristaless X (ARX) gene [6,7].

An autosomal gene mutation that gives rise to common clinical symptoms include Prader-Willi/Angelman syndromes (PWS/AS, OMIM#176270; 105830), due to a deletion of 15q11-q13 or uniparental disomy (UPD) 15. The CpG islands around exon 1 of the small nuclear ribonucleoprotein polypeptide N (SNRPN) gene are methylated on the maternal chromosome and completely devoid of methylation on the paternal chromosome [8]. This finding is used in developing diagnostic methods for PWS/AS. In case of male patients, PWS can have some clinical aspects of FXS, and in female patients, some clinical aspects of RTT. Here we report clinical/molecular data in a group of 85 Bulgarian patients, out of whom 32 were clinically diagnosed as FXS, 23 as classical RTT, 13 as atypical RTT, 14 as PWS, and three as Angelman syndrome (AS). Some of these patients were discussed elsewhere [9].

MATERIALS AND METHODS

In total, 85 unrelated patients with MR were included in the study. The clinical diagnoses were as follows: 32 FXS male patients (aged between 2 and 26 years), 36 RTT girls (aged between 2 and 20 years), of whom 23 had classical RTT and 13 atypical RTT [with concomitant diagnosis of autism, epilepsy, and West syndrome (OMIM# 308350)], PWS/AS, Pitt-Hopkins syndrome (OMIM# 610954), 14 PWS (aged between 1 and 11 years), 10 boys and four girls (one of the boys had a concomitant diagnosis of spinal muscular atrophy (SMA, OMIM#253300) and three AS (aged between 4 and 10 years, two girls and one boy). All patients are of Bulgarian ethnicity.

The research protocol was approved by The Ethics Committee for Research Investigations to the Medical University, Sofia, Bulgaria. Informed consent was obtained from the guardians of all patients.

DNA samples were extracted from peripheral blood, using DNA extraction kit (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany). The genetic tests covered the genes FMR1, MECP2, CDKL5, ARX, and methylation analysis of exon 1 of the SNRPN. In addition, multiplex ligation-dependent probe amplification (MLPA) analysis was used to look for large deletions/duplications and to clarify the methylation status of FMR1 and SNRPN genes. The MLPA analysis proved to be useful in clarifying mosaic FXS cases [10], in detecting large deletions on the X-chromosome genes, as well as in determining the type of mutation (deletion or UPD) in PWS/AS cases.

PROTOCOLS FOR FMR1 GENE ANALYSIS

Betaine-Based-PCR Protocol. The primers used to amplify the CGG repeat in the 5'UTR of the FMR1 gene, the polymerase chain reaction (PCR) mixture and cycling conditions have been described elsewhere [11]. This amplification permits detection of normal and of some premutated alleles. For the correct sizing, PCR products were subjected to electrophoresis on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in the presence of ROX500 size standard (Applied Biosystems).

Methylation Sensitive MLPA (MS-MLPA). The MS-MLPA (SALSA MS-MLPA ME029 probemix) was performed following the manufacturer's instructions (www.mlpa.com). DNA samples diluted in TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) were subjected to denaturation for 10 min. in denaturing buffer provided by the manufacturer. Hybridization with the FMR1-specific probes and 20 control probes was performed overnight at 60°C. Each sample was divided for the ligation reaction, which is the copy number test (CNT) for deletions/duplications detection, and for the ligation-digestion reaction, the methylation test (MT) for methylation assessment at CpG islands. In both tests, the hybridized probes were ligated by a specific ligase mix, provided

by the manufacturer. In the methylation, test the methylation-sensitive restriction enzyme *Hha*I (Pharmacia Biotech, Uppsala, Sweden) was added. The final step consisted of PCR amplification of the obtained fragments. The PCR buffer, PCR primers, the enzyme dilution buffer and the polymerase were provided in the kit.

The PCR products obtained were analyzed on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) in the presence of ROX500 size standard (Applied Biosystems). All patient samples were analyzed simultaneously with at least two normal male samples and a mutant male carrier of a full mutation.

Copy number changes were determined by comparison to the normal controls. The MT results were easy to interpret as the five FMR1 exon 1 methylation-sensitive fragments (see Figure 1) were absent in the normal controls after *Hha*I digestion, but were present, as a result of hypermethylation, in a patient carrying a full mutation.

PROTOCOLS FOR MECP2, CDKL5 and ARX GENE ANALYSIS

Sequencing. These X-chromosome genes were directly sequenced, as all the exons (including the first non coding one for the MECP2 gene) and intron/exon splice site borders were included/ covered. The sequence of primers used for PCR amplification was taken from the literature [6,12,13] or designed in our laboratory (the primer sequence is available upon request). Standard PCR conditions were applied: primer concentration 0.4 μM, 0.2 mM dNTPs, 0.4 M Betaine (Sigma, St. Louis, MO, USA), 4% DMSO; 1× supplied PCR reaction buffer (Genet Bio, Chungnam, Korea) and 0.5U Prime Taq (Genet Bio). The annealing temperature varied between 55°C and 68°C.

The PCR products were purified by PCR Product Pre-Sequencing Kit (USB, Affymetrix, Inc., Santa Clara, CA, USA) containing 4 U exonuclease I (10 U/μL) and 0.8 U shrimp alkaline phosphatase (2 U/μL). The sequencing reaction was performed

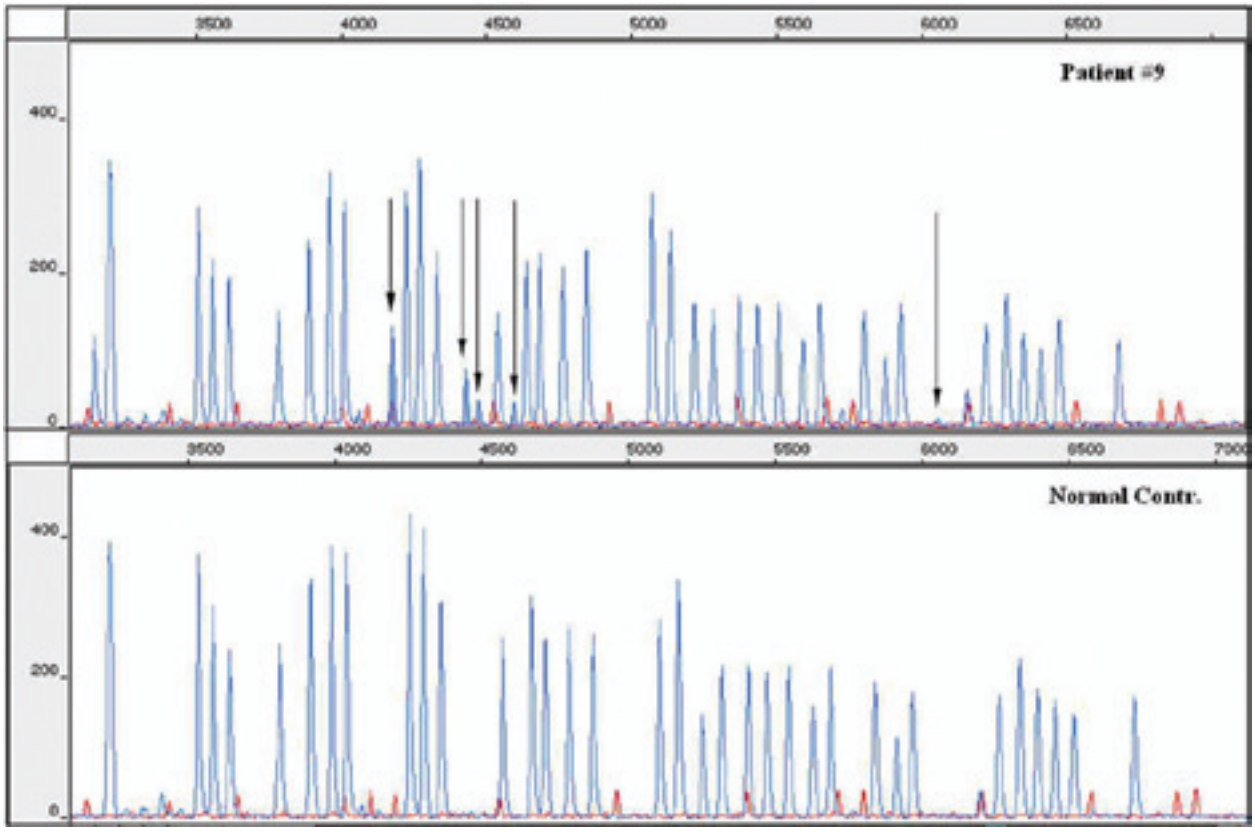

Figure 1. MLPA analysis (methylation test) of FMR1 gene. *Hha*I restriction enzyme cleaves unmethylated fragments, which disappear in the normal profile (bottom). Five hypermethylated fragments (pointed by arrows) are not digested by *Hha*I and thus appear in the patient's profile if carrier of a full hypermethylated mutation (top).

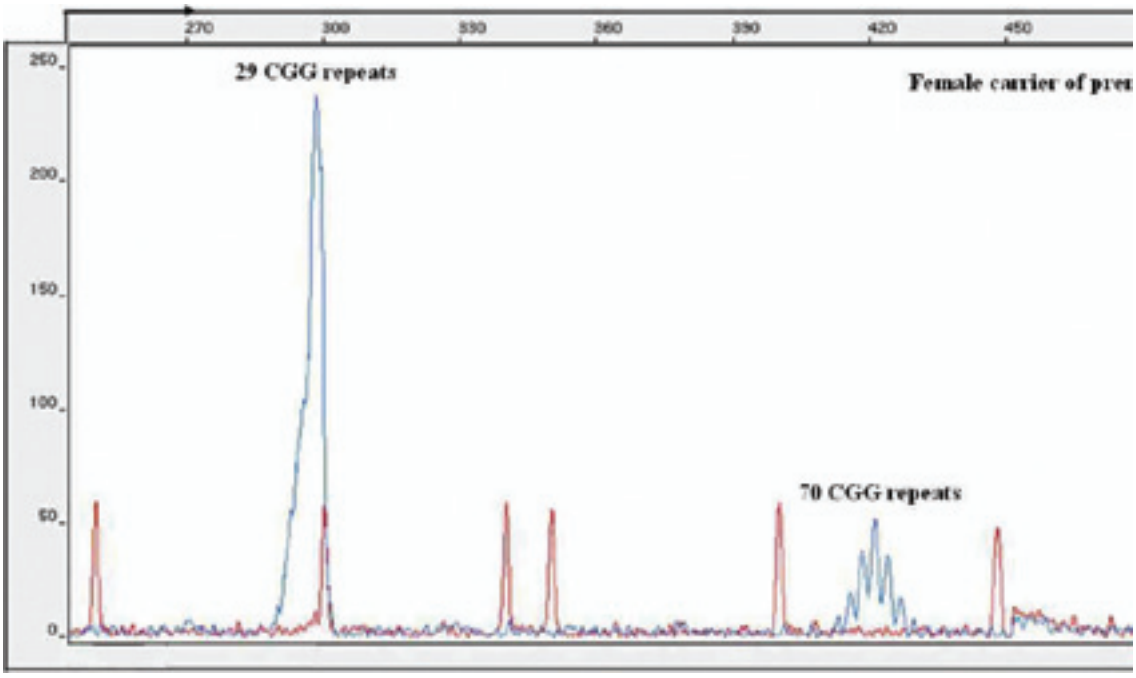

Figure 2. Amplification profile of a female carrier of 70 CGG repeats premutation.

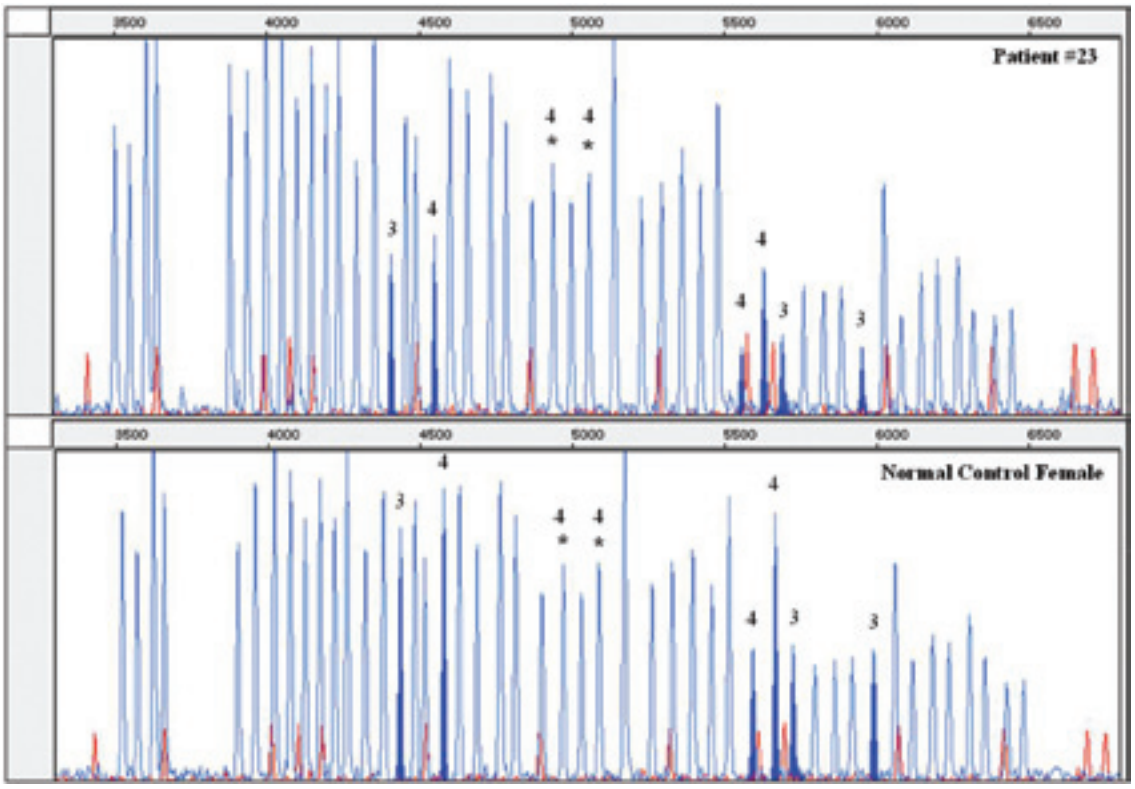

Figure 3. MLPA analysis for large deletion/duplication detection along the MECP2 gene. The patient #23 carries the MECP2 deletion of exon 3 and a part of exon 4. All three probes for exon 3 and 3 probes for exon 4 (the numbers of the exons are given above each peak) are deleted in the patient (top) in comparison to the control (bottom). The remaining 2 probes for exon 4 (marked by asterisks) are not deleted and these are the most 3' situated probes along the exon 4. The performed calculations for deletions assessment according to the formula given in "Materials and Methods" showed the following results: relative peak ratios for exon 3 probes were 0.54; 0.41 and 0.37 and for exon 4 probes – 0.48; 0.39 and 0.57. Both undeleted exon 4 probes, marked by asterisk showed relative peak ratios 1.04 and 0.94.

by ABI PRISM™ BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

MLPA analysis. The SALSA MLPA P015-D1 MECP2 probemix was used to screen for large deletions/ duplications along the genes MECP2, CDKL5 and ARX. The analysis was performed following the manufacturer's instructions [14]. Fifty to 200 ng of DNA was diluted in TE buffer to a total volume of 5 μL. The diluted samples were subjected to hybridization with the probes specific for the MECP2, CDKL5 and ARX genes, and 17 control probes. Hybridization was performed overnight at 60°C. Hybridized probes were ligated by a specific ligase mix, provided by the manufacturer. The final step represents PCR amplification of the obtained ligated fragments. The PCR buffer, PCR primers (6-FAM labeled), the enzyme dilution buffer and the polymerase were provided in the kit.

The obtained PCR products were analyzed on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) in the presence of ROX500 size standard (Applied Biosystems). Each patient sample was analyzed simultaneously with at least two normal controls. The relative peak ratios in order to assess deletions were calculated using the formula: $r = (\text{peak area}_{patient}/\text{mean peak area both neighbouring control peaks}_{patient})/(\text{peak area}_{control}/\text{mean peak area both neighbouring control peaks}_{control})$. Relative ratio of <0.6 corresponded to a deletion.

PROTOCOLS FOR SNRPN GENE ANALYSIS

Methylation-Specific PCR. DNA was modified by bisulfite treatment using MethylCode Bisulfite Conversion Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The standard protocol was used to amplify maternal methylated and paternal unmethylated alleles [15]. The amplicons were separated in 3% agarose gel and visualized after ethidium bromide staining. The lack of paternal unmethylated fragment (164 bp) leads to PWS, the lack of maternal methylated fragment (131 bp) leads to AS.

Methylation-Sensitive MLPA (MS-MLPA). The MS-MLPA (SALSA MS-MLPA ME028-A1 Prader-Willi/ Angelman probemix) was performed following the manufacturer's instructions [14]. This test is performed as a CNT and a MT in presence of methylation-sensitive restriction enzyme *Hha*I (Pharmacia Biotech). The protocol is as described above for FMR1 gene. The *Hha*I cleaves only unmethylated genomic DNA. If the CpG islands are methylated, the *Hha*I does not digest them; the fragment is amplified by PCR and detected by capillary electrophoresis. If the CpG sites are unmethylated, there is no PCR product in the electrophoretic profile. The CNT permits detection of deletions in PWS/AS cases, while the MT permits clarification of UPD in PWS, as two maternal methylated alleles are present.

RESULTS AND DISCUSSION

The clinical data, genetic tests performed and detected mutations are presented in Table 1 for FXS patients, Table 2 for RTT patients and Table 3 for PWS/AS patients.

FXS. Parents frequently stated that the disease onset has been provoked by severe illnesses, such as whooping cough, pneumonia, recurrent otitis. Sometimes, parents have noticed that their child's development regressed after an infectious disease.

Karyotype analysis showed normal results in all but patient 18 in whom the result was compatible with FXS: 46,fra(X)(q27.3)Y[18]/46,XY[22].

The PCR protocol failed to detect a normal fragment in three patients (9, 18 and 31 in Table 1),

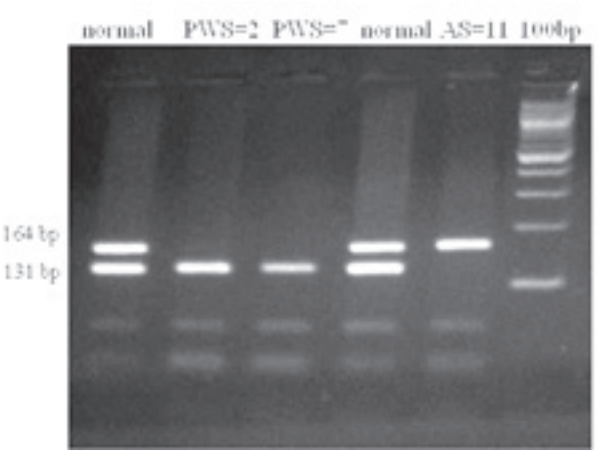

Figure 4. Methylation-specific PCR of the SNRPN gene for diagnosis of PWS/AS. The PCR fragment of 164 bp corresponds to the paternal unmethylated fragment (absent in PWS), the fragment of 131 bp corresponds to the maternal methylated fragment (absent in AS).

who were shown to have a hypermethylated CGG pattern by MLPA analysis (Figure 1). Their mothers showed single normal alleles and premutated expanded alleles of ~70, ~90 and ~180 CGG repeats, respectively (Figure 2) (the results were confirmed by Southern blotting, data not shown). In patient 31, a mosaic pattern normal/premutation/full mutation was detected as a smear located from the zone of normal fragments through the premutated zone to full expansion detected as hypermethylation.

Patient 3 showed a normal allele along the CGG repeated region, but this allele was three repeats longer than the normal allele of his mother, who also carried a premutation of about 90 repeats [10]. The hypermethylated MLPA profile of this patient confirmed a mosaic genotype of full mutation/ normal allele [10]. These results were confirmed by classical Southern blotting (data not shown).

The MLPA CNT did not show any deletion/ duplication on the FMR1 gene. In addition, the MLPA probemix contains 14 specific probes for the FMR2 gene (OMIM #300806), mutations which are associated with fragile X MR E (FRAXE) (OMIM #309584). Both FMR1 and FMR2 genes were simultaneously analyzed by CNT and MT and no pathological changes were detected on the FMR2 gene.

The remaining 28 FXS boys were subjected to genetic tests on the ARX and MECP2 genes. The reason is that mutations in the ARX gene have been associated with MR and epilepsy [6,7], but the results from a large series of screened patients have been disappointing [16]. On the other hand, mutations in the MECP2 gene can cause moderate MR and obesity [17]. We detected no mutations in the ARX gene or in the MECP2 gene.

RTT. Twenty-two of the mentally retarded girls in this group were born after a complicated pregnancy and delivery by Caesarean section. Various vaccinations or severe infections were reported by the parents as a triggering factor for disease onset.

Karyotype analysis gave normal results in all but patient 6 who carried a deletion of the short arm of the X chromosome 46,XX,del(X)(p1.22).

Sequencing of the MECP2 gene revealed seven mutations in this group (19.4%). Five different types of substitutions were detected on exon 4 of this gene: c.473C>T, p.Thr158Met; c.763C>T, p.Arg255Stop; c.808C>T, p.Arg270Stop (found in two girls); c.880C>T, p.Arg294 Stop, and c.916C>T, p.Arg306Cys. A deletion of 44 bp was also found by sequencing of exon 4 of this gene: c.1157_1200 del44, p.Leu386fs. These mutations have been reported in the MECP2 gene mutation database [18].

The other 29 patients were subjected to the MLPA analysis for large deletions of the MECP2, CDKL5 and ARX genes. Two large deletions, encompassing exon 3 and a part of exon 4 (c.27-?_*?del) of the MECP gene were detected in patients 23 and 35. This deletion is available at the MECP2 mutation database [18]. The MLPA profile is presented in Figure 3. The deleted fragments were assessed by the relative peak ratios, calculating the peak area in comparison to the mean peak area of both neighboring control peaks, and compared to the control sample. The relative ratios obtained for the probes in exon 3 and a part of exon 4 were <0.6, which we interpreted as a deletion. The calculated relative peak ratios are provided in the legend to Figure 3.

We screened for mutations in the CDKL5 gene in 27 patients without success. The PWS/AS as a differential diagnosis in some of the patients (5, 14, 24, 25, 34 and 36) were also excluded.

PWS/AS. Karyotype analysis showed normal results except in patient 17 in whom fluorescent *in situ* hybridization analysis showed results compatible with deletion: 46,XX,ishdel(15)(q11.2q11.2) (D15S10x1),15p11.2 (D15Z1x2),15q22(PMLx2). The methylation-specific PCR revealed that seven patients lacked the paternal unmethylated fragment (PWS) and that two patients lacked the maternal methylated fragment (AS) (Figure 4). To determine more precisely the mutation type (deletion or UPD), we analyzed the nine genetically confirmed cases by MS-MLPA and found six deletions and three to have UPD (Figures 5A and 5B). The calculated relative peak ratios in the deletion cases were less than 0.6 and are presented in the legend to Figure 5A.

Eight patients in this group remain with a still unclear molecular defect. The suspected diagnosis of FXS in the male patients was excluded by analysis of the FMR1 gene. The ARX gene was sequenced in the three PWS boys and no mutations were detected. Patient 1 (Table

3) was additionally sequenced for mutations in the MECP2 gene, but no pathological changes were detected.

Altogether, we were able to clarify the molecular basis in 22 of the 85 MR patients (26.0%), ~10.6% of whom were PWS/AS, the same percentage (~10.6%) were RTT and ~4.8 were FXS. The percentage of the genetically proved diagnosis in the group of FXS is relatively small (12.5%), which might be due to the clinical criteria for patient selection, including a number of autistic cases in this group. The test for FXS is always the first step in molecular diagnostics of boys with MR. It is worth mentioning, that genetically proved diagnosis among our RTT patients (25.0%) is relatively high and all these cases are due to MECP2 mutations. Despite of the type of mutation, all these cases are very similar from a clinical point of view and well recognized in Bulgaria.

ACKNOWLEDGMENTS

A fellowship from the Alexander von Humboldt Foundation to A. Todorova is gratefully acknowledged. The study was partially supported by grant No8-D/2008 and grant No32/2009, Medical University, Sofia, Bulgaria

Table 1. Clinical and molecular data of 32 fragile X syndrome patients

No. Diagnosis, Year Born	Symptoms, Degree of MR[a]	Physical Development	Epilepsy	EEG	Dysmorphic Features	Genetic Tests, Mutation
1. FXS, 2002	Speech delay, MR borderline	Normal	No	Normal	Broad face, big ears	FMR1, ARX, MECP2, no mutation
2. FXS/ epilepsy, 2001	No speech, behavioral disturbances, hyperactivity, auto-aggression, MR moderate	Normal	Yes	Slightly disorganized background activity	Broad face, big ears	FMR1, ARX, MECP2, no mutation
3. FXS, 1984	Intellectual deficit, behavioral problems, partial avoidance of eye contact, attention deficit, hyperactivity and aggression, impaired language skills, MR mild	Normal	No	N.D.	None	FMR1, mosaic full CGG expansion, normal allele
4. FXS, 2003	Speech delay, hyperactivity, MR moderate	Delay in motor development, head control at 4 months, walked at 18 months, calves' hypertrophy	No	Severely disorganized background activity	Broad face, squint, microglossia	FMR1, ARX, MECP2, no mutation
5. FXS/AS, 2003	Attention deficit, impaired language skills and communication activity, MR mild	Normal	No	N.D.	Discrete facial dysmorphism	FMR1, ARX, MECP2, no mutation
6. FXS, 2001	No speech, MR moderate	N.D.	N.D.	N.D.	N.D.	FMR1, ARX, MECP2, no mutation
7. FXS/ Autism, 2005	Speech delay with minimum vocabulary, hyperactivity, auto-aggression, no stabile eye contact, MR moderate	Delayed milestones, walked at 19 months only with help	No	N.D.	Broad face, low placed and backward rotated ears	FMR1, ARX, MECP2, no mutation
8. FXS, 2001	No speech, MR moderate	N.D.	N.D.	N.D.	N.D.	FMR1, ARX, no mutation

Table 1 / continue

9. FXS, 1993	Intellectual deficit, impaired language skills, behavioral problems, avoidance of eye contact, attention deficit, MR moderate	Tall stature, long arms and legs	No	N.D.	Dysmorphic face, big ears	FMR1, full CGG expansion
10. FXS, 2003	No speech, hyperactivity, MR severe	Delayed milestones	One single seizure	Disorganized background	Microcephaly	FMR1, ARX, no mutation
11. FXS/ Autism, 2003	No speech, no eye contact, hyperactivity, MR moderate	Stereotypic movements	No	N.D.	Facial dysmorphism, prognatia, short nasal base, large distance between teeth	FMR1, ARX, MECP2, SNRPN, no mutation
12. FXS/ PWS, 2004	No speech, MR mild	Muscular hypotonia, delayed milestones: sat up at 12 months, walked at 37 months, tremor, ataxia	No	N.D.	N.D.	FMR1, ARX, SNRPN, no mutation
13. FXS/ PWS, 2001	No speech, first degree obesity, MR mild	Normal	No	Normal	Facial dysmorphism, micorgenitalia	FMR1, ARX, SNRPN, no mutation
14. FXS/ Autism, 2003	No speech, MR moderate	Normal	No	N.D.	High forehead, big ears, dolichocephaly	FMR1, ARX, no mutation
15. FXS, 2004	No speech, MR moderate	N.D.	N.D.	N.D.	N.D.	FMR1, ARX, no mutation
16. FXS, 2002	Speech delay, cognitive and behavioral problems, MR mild	Normal	No	N.D.	Broad face, high palate	FMR1, ARX, no mutation
17. FXS/ Autism, 2002	Intellectual deficit, no speech, absent eye contact, MR moderate	Normal	One febrile seizure	Abnormal background with fast activity predominance	High palate, low placed ears, anatimongoloid slantet palpebral fissures, microgenitalia	FMR1, ARX, SNRPN, no mutation
18. FXS/ Autism, 2004	Auto-aggression, behavioral problems, speech delay, intellectual deficit, MR moderate	Normal	No	N.D.	Small nose, low placed ears, scaphocephaly	FMR1, full CGG expansion
19. FXS/ Autism, 2003	Speech delay, autistic behavior, MR moderate	Stereotypic hand movements	No	N.D.	Prominent ears, antimongoloid slanted palpebral fissures	FMR1, no mutation
20. FXS/ Atypical autism, 2005	No speech, MR moderate	Automatic hand movements	No	N.D.	Hypertelorism, low placed ears, antimongoloid slanted palpebral fisssures, wide spaced teeth, high palate, extraverted nostrils	FMR1, no mutation
21. FXS, 2004	No speech, hyperactivity, probable decline after infectious disease, MR moderate	Automatic hand movements	No	Normal	N.D.	FMR1, no mutation
22. FXS, 2006	No speech, MR moderate	N.D.	N.D.	N.D.	N.D.	FMR1, no mutation
23. FXS, 2005	Speech delay, hyperactivity, concentration deficit, aggression, MR moderate	Milestone delay: in-bed rotation at 6 months, sat up at 12 months, walked at 18 months	No	N.D.	Dolichocephaly, squint	FMR1, no mutation

Table 1 / continue

24. FXS, 1994	No speech, MR moderate	N.D.	N.D.	N.D.	N.D.	FMR1, no mutation
25. FXS, 1996	Speech delay, hyperactivity, aggression, autistic features, MR moderate	Milestone delay: walked at 32 months	One single seizure on second post partum	N..D.	Hypertelorism, antimongoloid slanted palpebral fissures, anteverted nostrils, squint, big prominent ears, high palate hypertrichosis, macroorchidism	FMR1, no mutation
26. FXS, 2004	No speech, hyperactivity, autistic behavior, MR moderate	Delayed milestones	No	Normal	Facial dysmorphism, big prominent ears	FMR1, no mutation
27. FXS, 2006	No speech, hyperactivity, autistic behavior, MR moderate	N.D.	N.D.	N.D.	N.D.	FMR1, no mutation
28. FXS, 1984	Intellectual deficit, MR borderline	Normal	No	Normal	Dysmorphic ears	FMR1, no mutation
29. FXS, 2003	No speech, MR moderate	N.D.	N.D.	N.D.	N.D.	FMR1, no mutation
30. FXS, 2001	Speech delay, cognitive and behavioral problems, intellectual deficit, MR mild/borderline	Normal	No	Normal	Discrete facial dysmorphism	FMR1, no mutation
31. FXS, 1998	Autoagression, behavioral problems, speech delay, intellectual deficit, MR moderate	Normal	N.D.	N.D.	Small nose, low placed ears, scaphocephaly	FMR1, mosaic normal/ premutation/ full CGG expansion
32. FSX, 2008	No speech, autistic behavior, MR moderate	N.D.	N.D.	N.D.	N.D.	FMR1, no mutation

Table 1 / end

EEG: Electroencephalograpy; N.D.: No data.

[a] Degree of MR: the degree of MR is assessed as profound (IQ <20), severe (IQ 20-34), moderate (IQ 35-49), mild (IQ 50-69), borderline (IQ 70-80), no speech equals to absent speech, just vocals.

Table 2. Clinical and molecular data of the 36 Rett syndrome patients

#. Diagnosis, Year Born	Age at Onset of Symptoms, Degree of MR[a]	Atypical Movement	Motor Development	Epilepsy	EEG	Facial Dysmorphism	Genetic Tests, Mutation
1. RTT, 2004	After 1 year, drooling, no speech, MR moderate	Hand stereotypies	Cannot stand up, unable to walk with help, ataxia	No	Excess of fast rhythms in EEG	Yes	MECP2, CDKL5, MLPA, no mutation
2. RTT, 2001	6 months, drooling, no speech, MR profound	Hand stereotypies	Cannot walk	Seizures	N.D.	N.D.	MECP2, c.473C>T, p.Thr158Met
3. RTT, 1999	After 20 months, no eye contact, autistic features, MR moderate	Hand stereotypies	Joint hyperflexibility, muscle hypotonia	No	Normal	Yes	MECP2, CDKL5, MLPA, no mutation
4. Atypical RTT, 2001	N.D., communication problems, MR mild	Hand stereotypies	Difficulties in running and standing up	Yes	Focal EEG abnormalities	None	MECP2, CDKL5, MLPA, no mutation
5. Atypical RTT/AS, 2003	N.D., MR moderate	N.D.	N.D.	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, SNRPN, no mutation
6. Autism, 2006	N.D., no speech, hyperactivity, aggression, MR moderate	N.D.	Can walk independently	No	Generalized background slowing	Discrete	MECP2, CDKL5, ARX, FMR1, MLPA, no mutation, 46,XX,del (X) (p1.22)
7. RTT, 2001	6 months, drooling, no speech, MR severe	Hand stereotypies, loss of purposeful hand use	Cannot walk	Seizures	N.D.	N.D.	MECP2, c.808C>T, p.Arg270Stop
8. Autism?, 2001	20 months, minimal speech, auto-aggression, MR moderate	N.D.	N.D.	N.D.	Excess of fast rhythm	N.D.	MECP2, CDKL5, MLPA, no mutation
9. RTT/ West, 2007	5 months, no eye contact, MR mild	Hand stereotypies	Minimal movement at 5 months	Infantile spasms	Hypsarithmia	Microcephaly	MECP2, CDKL5, MLPA, no mutation
10. RTT, 2005	N.D., no speech, avoids contact with children	N.D.	Walked at 18 months	Infantile spasms	Generalized EEG slowing	None	MECP2, CDKL5, MPLA, no mutation
11. RTT, 2003	N.D., no speech, MR moderate	N.D.	N.D.	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, no mutation
12. RTT, 2003	N.D., no speech, MR moderate	N.D.	N.D.	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, no mutation
13. RTT, 2000	2 years, no speech, auto-aggression, MR profound	Hand stereotypies	Walked at 20 months, not independently, lower limbs muscular hypotunia, locomotor ataxia	Focal epilepsy	Focal abnormalities right hemisphere	N.D.	MECP2, c.1557_1200 del144, p.Leu386fs

Table 2 / continue

14. RTT/ AS, 2002	2 years, no speech, aggression, MR moderate	Hand stereotypies	N.D.	N.D.	Diffuse background slowing in EEG	Yes	MECP2, CDKL5, MLPA, SNRPN, no mutation
15. RTT, 2006	3 months, no speech, MR moderate	Finger stereotypies	Stereotypical body movements (shaking), hypertonia, spasticity	Infantile spasms, focal seizures, vomiting	Generalized EEG discharges, multifocal	N.D.	MECP2, CDKL5, MLPA, no mutation
16. RTT, 2003	N.D., behavioral problems, MR, MR moderate	N.D.	Delayed milestones	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, no mutation
17. RTT, 2006	N.D., speech delay and regression, MR mild	Hand automatisms and stereotypies	Walked at 18 months, stereo-typic movements of body and lower limbs, locomotor ataxia, muscular hypotonia	Breath holding spells	Normal(?)	Squint	MECP2, c.808C>T, p.Arg270Stop
18. RTT, 2003	N.D., speech regression, MR profound	Hand stereotypies	Normal	Generalized tonic-clonic seizures	Moderate EEG dysrythmia	N.D.	MECP2, c.880C>T, p.Arg294Stop
19. RTT, 2001	N.D., single word speech, autistic behavior, MR moderate	Hand stereotypies	N.D.	N.D.	Moderate to severe EEG background slowing	Dolichocephaly, hypoplastic middle face, extraverted nostrils	MECP2, CDKL5, MLPA, no mutation
20. Autism, 1990	3-4 years, autistic behavior, no eye contact, no speech, MR mild	Hand stereotypies	Stereotypic body movements	N.D.	N.D.	Yes	MECP2, CDKL5, MLPA, no mutation
21. Autism/ AS, 2005	N.D., no speech, autistic behavior, MR mild	Hand stereotypies	Stereotypic body movements	N.D.	Normal	N.D.	MECP2, CDKL5, MLPA, SNRPN, no mutation
22. RTT, 1999	After birth, no speech, MR moderate	Extrapirimidal hyperkinesias, choreic and ballistic	Delayed early milestones, does not walk, mus-cular atrophy, kiphoscoliosis	Febrile seizures, epilepsy not clear	Generalized EEG background slowing	Microcephaly, hypertrichosis	MECP2, CDKL5, MLPA, no mutation
23. RTT, 2005	1 year, no speech, MR moderate	Hand stereotypies	Delayed motor development: sat, walked at 2 years, motor regression (cannot get up from bed, loco-motor ataxia)	No	Normal	Microcephaly, flat occiput	MECP2, MLPA, c.27-?_*del (deletion of exon 3 and part of exon 4)
24. RTT, 2007	3 months, MR moderate	Hand stereotypies	Delayed head control and turn-ing in bed, mus-cular dystrophy, ataxia, muscular hypotonia	N.D.	Abnormal α rhythm	Microcephaly	MECP2, CDKL5, MLPA, SNRPN, no mutation

Table 2 / continue

25. RTT/ AS/Pitt-Hopkins, 1998	After birth, aggression, auto-aggression, MR severe	Hand stereotypies, grimace stereotypies	Delayed turning in bed at14 months, walked at 28 months, ataxia, lumbal hyperlordosis, contractures	Epilepsy	Focal EEG abnormalities	Microcephaly, facial dysmorphism, hypertrichosis	MECP2, c.916C>T, p.Arg306Cis
26. RTT, 1994	N.D., MR mild	N.D.	N.D.	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, no mutation
27. RTT, 2005	After birth, delayed milestones, no speech, MR moderate	Hand stereotypies	Does not walk independently	Epilepsy with myoclonic astatic seizures	Generalized epileptiform EEG discharges	Microretrognathia high palate, big ears	MECP2, CDKL5, MLPA, no mutation
28. RTT, 2002	N.D., MR severe	Hand stereotypies	Delayed head control and turning in bed, muscular dystrophy, ataxia, muscular hypotonia	Epilepsy	N.D.	Microcephaly	MECP2, CDKL5, MLPA, no mutation
29. Autism, 2006	2 years, no speech	N.D.	N.D.	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, no mutation
30. RTT, N.D.	After birth, delayed milestones, no speech, MR severe	N.D.	N.D.	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, no mutation
31. RTT, 2006	N.D., MR severe	Hand stereotypies	N.D.	N.D.	N.D.	Microcephaly	MECP2, CDKL5, MLPA, no mutation
32. RTT, 2005	N.D., speech regression, MR profound	Hand stereotypies	N.D.	N.D.	N.D.	N.D.	MECP2, c.763C>T, p.Arg255Stop
33. Autism, 2006	N.D., no speech	N.D.	N.D.	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, no mutation
34. RTT/ AS, 2007	After birth, aggression, auto-aggression, MR severe	Hand stereotypies, grimace stereotypies	N.D.	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, no mutation
35. RTT, 2008	After birth, delayed milestones, no speech, MR severe	N.D.	N.D.	N.D.	N.D.	N.D.	MECP2, CDKL5, MLPA, no mutation, MECP2, MLPA, c.27-?_*?del (deletion of exon 3 and part of exon 4)
36. RTT/ AS, 2000	N.D., MR moderate	Hand stereotypies	N.D.	N.D.	N.D.	Microcephaly	MECP2, CDKL5, MLPA, SNRPN, no mutation

Table 2 / end

EEG: Electroencephalograpy; N.D.: No data.
[a] Degree of MR: the degree of MR is assessed as profound (IQ <20), severe (IQ 20-34), moderate (IQ 35-49), mild (IQ 50-69), borderline (IQ 70-80), no speech equals to absent speech, just vocals.

Table 3. Clinical and molecular data of the 17 Prader-Willi/Angelman syndrome patients

No. Diagnosis, Year Born	Age at Onset of Symptoms, Degree of MR[a]	Physical Development	Epilepsy/ EEG	Obesity	Dysmorphic Features	Genetic Tests, Mutation
1. PWS/ FXS, 2002	1 year, speech delay, MR mild	N.D.	N.D.	Yes	N.D.	SNRPN, FMR1, ARX, MECP2, no mutation
2. PWS 2000	5 months, speech and communication problems, MR mild	Muscular hypotonia, lumbal hyperlordosis	No	Yes	Bitemporal head narrowing, high palate, convergent squint	15q11-13 deletion of paternal unmethylated fragment
3. PWS, 2006	5-6 months, no speech, aggressive behavior, MR mild	Muscular hypotonia, limbs, kiphoscoliosis, no independent walk possible	No	No	Short and up-slanted palpebral fissures, high palate, big and low-set ears	15q11-13 deletion of paternal unmethylated fragment
4. PWS/ FXS, 1999	3 years, mild MR, communication problems, MR borderline	Thoracic kyphosis, muscular hypotonia, hand tremors	No	Yes	Big and low-set ears, thick lower lip, microgenitalia	SNRPN, FMR1, ARX, no mutation
5. PWS/ SMA?, 2003	2 months, mild MR, MR borderline	Muscular hypotonia, lumbal hyperlodosis	No	Yes	Dolichocephaly, low-set ears, high palate, corners of mouth down-turned, microgenitalia	1511-13 deletion of paternal unmethylated fragment
6. PWS, 1999	6 years, mild MR, MR moderate	Normal	No	Yes	Low-set ears, microgenitalia	SNRPN, FMR1, ARX, no mutation
7. PWS, 2007	At birth, MR, MR moderate	Motor delay: head control at 5 months, sat up at 10 months, walked at 17 months, muscular hypotonia	No	Yes	Microcephaly, broad face, open mouth, cryptorchism	15q11-13 UPD
8. PWS, 2003	At birth, mild MR, MR borderline	Muscular hypotonia	Seizures after vaccination	Yes	Dolichocephaly, narrow lips with up-slanted corners	15q11-13 deletion of paternal unmethylated fragment
9. PWS, 2008	At birth, MR mild	N.D.	N.D.	N.D.	N.D.	15q11-13 deletion of paternal unmethylated fragment
10. PWS, 2006	N.D., MR borderline	N.D.	N.D.	N.D.	N.D.	SNRPN, no mutation
11. PWS, 2005	N.D., MR mild	N.D.	N.D.	Yes	N.D.	SNRPN, FMR1, no mutation
12. PWS, 2009	At birth, MR mild	Muscular hypotonia	N.D.	Yes	Dolichocephaly, narrow lips with up slanted corners	15q11-13 UPD
13. PWS, 2007	At birth, MR mild	Normal	No	No	Normal	SNRPN, no mutation
14. PWS, 2008	N.D., MR borderline	N.D.	N.D.	Yes	N.D.	SNRPN, no mutation
15. AS, 2000	2 years, MR, aggression, auto-aggression, “happy puppet” appearance, MR severe	N.D.	Slow background EEG activity	N.D.	Prognathia, up-slanted mouth corners, big and low set ears	SNRPN, FMR1, no mutation

Table 3 / continue

16. AS, 2004	3 years, MR, single words' speech, MR severe	Delayed milestones: sat up at 12 months, walked at 17 months	Single epileptic febrile seizures during infection, disorganized EEG activity	NO	Brachicephaly, hyperterlorism macroglossia	15q11-13 UPD, lack of maternal methylated fragment
17. AS, 2006	At birth, "happy puppet" appearance, MR moderate	Delayed milestones: sat up at 10 months, walked at 18 months, muscular hypotonia, stereotypic hand movements	Epilepsy	No	Microcephaly	15111-13, deletion of maternal methylated fragment

Table 3 / end

EEG: Electroencephalograpy; N.D.: No data; SMA: spinal muscular atrophy; UPD: uniparental disomy.
[a] Degree of MR: the degree of MR is assessed as profound (IQ <20), severe (IQ 20-34), moderate (IQ 35-49), mild (IQ 50-69), borderline (IQ 70-80), no speech equals to absent speech, just vocals.

REFERENCES

1. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe J, Richards S, Verkert A, Holden J, Fenwick R, Warren S, Oostra B, Nelson D, Caskey C. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell. 1991; 67(6): 1047-1058.

2. Jin P, Warren ST. Understanding the molecular basis of fragile X syndrome. Hum Mol Genet. 2000; 9(6): 901-908.

3. Ravn K, Nielsen JB, Skjeldal OH, Kerr A, Hulten M, Schwartz M. Large genomic rearrangements in MECP2. Hum Mut. 2005; 25(3): 324-329.

4. Hardwick SA, Reuter K, Williamson SL, Vasudevan V, Donald J, Slater K, Bennetts B, Bebbington A, Leonard H, Williams SR, Smith RL, Cloosterman D, Christodoulou J. Delineation of large deletions of the MECP2 gene in Rett syndrome patients, including a familial case with a male proband. Eur J Hum Genet. 2007; 15(12): 1218-1229.

5. Archer HL, Evans J, Edwards S, Colley J, Newbury-Ecob R, O'Callaghan F, Huyton M, O'Regan M, Tolmie J, Sampson J, Clarke A, Osborne J. CDKL5 mutations cause infantile spasms, early onset seizures, and severe mental retardation in female patients. J Med Genet. 2006; 43(9): 729-734.

6. Stromme P, Mangelsdorf ME, Shaw MA, Lower KM, Lewis SME, Bruyere H, Lütcherath V, Gedeon AK, Wallace RH, Scheffer IE, Turner G, Partington M, Frints SGM, Fryns J-P, Sutherland GR, Mulley JC, Gécz J. Mutations in the human ortholog of Aristaless cause X-linked mental retardation and epilepsy. Nat Genet. 2002; 30(4): 441-445.

7. Mandel J-L and Chelly J. Monogenic X-linked mental retardation: is it as frequent as currently estimated? The paradox of the ARX (Aristaless X) mutations. Eur J Hum Genet. 2004; 12(9): 689-693.

8. Zeschnigk M, Schmitz B, Dittrich B, Buiting K, Horsthemke B, Doerfler W. Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. Hum Mol Genet. 1997; 6(3): 387-395.

9. Todorov T, Todorova A, Avdjieva D, Dimova P, Angelova L, Tincheva R, Mitev V. Molecular basis of mental retardation in a sample from Bulgaria. Genet Couns. 2010; 21(2): 257-262.

10. Todorov T, Todorova A, Kirov A, Dimitrov B, Carvalho R, Nygren AOH, Boneva I, Mitev V. Fragile X mosaic male full mutation/normal allele detected by PCR/MS-MLPA. BMJ Case Reports 2009; [doi:10.1136/bcr.06. 2008.0139].

11. Todorov T, Todorova A, Georgieva B., Mitev V. A unified rapid PCR method for detection of normal and expanded trinucleotide alleles of CAG repeats in Huntington chorea and CGG repeats in Fragile X syndrome. Mol Biotechnol. 2010; 45(2): 150-154.

12. Amir RE, Van den Veyver IB, Wan M, Tran ChO, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet. 1999; 23(2): 185-188.

13. Kato M, Das S, Petras K, Kitamura K, Morohashi K, Abuelo DN, Barr M, Bonneau D, Brady AF, Carpenter NJ, Cipero KL, Frisone F,

Fukuda T, Guerrini R, Iida E, Itoh M, Lewanda AF, Nanba Y, Oka A, Proud VK, Saugier-Veber P, Schelley SL, Selicorni A, Shaner R, Silengo M, Stewart F, Sugiyama N, Toyama J, Toutain A, Vargas AL, Yanazawa M, Zackai EH, Dobyns WB. Mutations of ARX are associated with striking pleiotropy and consistent genotype-phenotype correlation. Hum Mut. 2004; 23(2): 147-159.

14. MRC-Holland (www.mlpa.com).

15. Kosaki K, McGinniss MJ, Veraksa AN, McGinnis WJ, Jones KL. Prader-Willi and Angelman syndromes: diagnosis with a bisulfate-treated methylation-specific PCR method. Am J Med Genet. 1997; 73(3): 308-313.

16. Chaste P, Nygren G, Anckarsäter H , Råstam M, Coleman M, Leboyer M, Gillberg Ch, Betancur C. Mutation screening of the ARX gene in patients with autism. Am J Med Genet B Neuropsychiatr Genet. 2007; 144B(2): 228-230.

17. Kleefstra T, Yntema HG, Oudakker AR, Romein T, Sistermans E, Nillessen W,van Bokhoven H, de Vries BBA, Hamel BCJ. De novo MECP2 frameshift mutations in a boy with moderate mental retardation, obesity and gynaecomastia. Clin Genet. 2002; 61(5): 359-362.

18. International Rett Syndrome Foundation (IRSF) MECP2 Variation Database (www.mecp2.chw.edu.au).