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Research Article

Cytogenetic and fragile X testing in a group of Romanian autistic children

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Abstract. Background: Extensive literature data report abnormalities involving all chromosomes, especially in autistic individuals with dysmorphic features and low-functioning autism. Among the single gene disorders most frequently associated with autism is the fragile X syndrome (FraX). Our study aimed to identify the underlying chromosomal abnormalities in a group of autistic Romanian individuals, by focusing on karyotyping and chromosome X fragile sites analysis, since these methods are highly recommended as the initial step in the genetic diagnosis of autism spectrum disorders (ASDs). Patients and methods: The study group consisted of 40 children with ASDs, enlisted in several Autism Associations from Transylvania, Romania. Cytogenetic analysis was performed using an adapted protocol for the G banding technique with Trypsin and Giemsa. Analysis of the X chromosome was performed on isolated DNA samples, using the Methylation-Specific PCR (MS-PCR) and the Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA) techniques. Results: We report chromosomal abnormalities in 3 children: homogenous chromosome 9qh+ polymorphism and mosaicism 15q22-qter deletion in two male individuals and mosaic trisomy 8 in one female individual. All of the investigated children were negative for the fragile X sites analysis. Conclusions: This is the first genetic testing performed specifically on a group of autistic children in Romania and using both karyotyping and fragile X testing. Our results show that, by using these tests, the underlying genetic cause is apparent in only a small number of cases, suggesting that higher resolution molecular genetics techniques, if available, might be more useful for genetic diagnostic in selected cases.

Key Words: autism, cytogenetics, chromosomal abnormalities, karyotyping, fragile X syndrome.

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Introduction

Autistic disorders (ADs) are a heterogeneous group of complex neurodevelopmental disorders, defined by severe impairment in communication and social skills and repetitive patterns of behavior with onset between 18 and 24 months, that affect mostly the male population (average male-to-female ratio 4:1) (Xu et al 2012; Gurrieri 2012).

According to the American Psychiatric Association - Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV-TR), ADs include the following: autistic disorder, pervasive developmental disorder-not otherwise specified (PDD-NOS), Asperger's disorder, Rett's disorder and childhood disintegrative disorder (CDD) (American Psychiatric Association 2000). Recently, the definition and classification of ADs have changed by including autistic disorder, Asperger's disorder, PDD-NOS and CDD under the spectrum of autism spectrum disorders (ASDs) (American Psychiatric Association 2013).

According to some studies, the prevalence of ASDs has increased over the past decades, reaching today an alarming prevalence ranging from 0.07% to more than 2% and inflicting a serious burden on both families and society (Kim et al 2011; Xu et al 2012; American Psychiatric Association 2013; Lai et al 2014; CDC 2014; Talkowski et al 2014). Nevertheless, a more recent systematic review (Baxter AJ et al 2015) suggests there is no clear evidence for the increase of ASDs prevalence over the past two decades, but still reports an incidence of 1 in 132 individuals, making it very clear that, nowadays, ASDs represent a serious public health issue requiring attention.

Despite extensive investigations, the cause of ASDs remains elusive in most cases, many genetic as well as environmental factors being incriminated. In up to 90-95% of cases, autism is said to be idiopathic, since no causative or trigger factors can be identified (Reddy 2005). In this category, essential (70%) and complex (30%) autism can be differentiated by the absence and respectively, the presence of dysmorphic features, microcephaly or structural brain malformations (Barton & Volkmar 1998). In 5-10% of cases (Reddy 2005) or after some recent studies, in 15-20% of cases (Cozaru & Papari 2012) or even up to 30% of cases (Szczaluba 2014), ASDs are secondary to known environmental or genetic factors (i.e. chromosomal abnormalities or single gene disorders).

Extensive literature data report abnormalities involving all chromosomes especially (but not only) in individuals with dysmorphic features, low-functioning autism, seizures or neurological problems (Marshal et al 2008; Benvenuto et al 2009). Numerical (autosomal or heterosomal aneuploidies), structural (terminal or interstitial deletions, inversions, duplications, translocations) alterations and marker chromosomes have been identified in ASDs (Selvi et al 2010), involving chromosome 15q11-13 in approximately 1% of cases (Freitag et al 2010) or chromosome 16p11.2 in up to 1% of cases (Devlin & Scherer 2012).

Interestingly, common chromosomal polymorphisms such as 9qh+ and the variation in length of Yq have also been reported in autistic patients (Gillberg&Wahlström 1985; Vorsanova et al 2007; Vorsanova et al 2010). Due to all these chromosomal alterations that present autistic traits in the phenotype, cytogenetic analysis is nowadays recommended in all ASD patients, in some cases allowing genetic counseling and/or treatment/prevention of associated medical conditions (Freitag et al 2010). Single gene disorders most frequently associated with autism are the fragile X syndrome (FraX), tuberous sclerosis or the Rett syndrome (Reddy 2005; Benvenuto et al 2009; Abrahams et al 2010; Connolly&Hakonarson 2011). Since, Rett syndrome and tuberous sclerosis have very specific phenotypes and are easily diagnosed in most cases (Freitag 2007), several studies recommend only fragile X testing in ASDs, considering their overlapping symptoms such as intellectual disability and behavioral problems (Freitag et al 2010; Shen et al 2010) and the high frequency of autism diagnosis among FraX patients (up to 60-70%) (Winarni et al 2013; McCary 2013) or FraX cases among autistic individuals (2-6%) (Winarni 2013). Also, physical characteristics of FraX (e.g elongated face, large ears, macrocephaly, narrow high-arched palate, macroorchidism) are, in most cases, not visible until puberty (Hagerman et al 1999), so recent diagnostic protocols issued by the American Academy of Pediatrics recommend the analysis of CGG trinucleotide expansion in the *FMR1* gene (generating the FRAXA fragile site located on chromosome Xq27.3) among the first tests in ASDs, along with GTG-banded karyotyping or chromosomal microarray (CMA) (Freitag 2007; Martin & Ledbetter 2007; Lintas & Persico 2009; Shen et al 2010; Schaefer et al 2008; Chung BHY et al 2013). Another fragile site (FRAXE), located on chromosome Xq28, containing a CCG trinucleotide expansion in the *FMR2* (AFF2) gene is considered today as a second-tier genetic testing in ASDs, due to recent connection to autism (Shen et al 2010; Correia et al 2015), although its relation to mental impairment has been long known (Russo et al 1998).

To our knowledge, genetic testing in autistic patients in Romania included so far only the standard karyotyping and was performed either on small groups or on isolated cases (Buteica & Grigorescu 1999; Hertzog et al 2007; Cozaru & Papari 2012). In view of this data, our study aimed to perform the GTG-banded karyotype and fragile-X testing on a larger group of Romanian autistic children in order to evaluate the frequency of chromosomal aberrations and fragile X sites in our geographical area and the utility of these tests in diagnosing ASDs.

Material and methods

Our study group consisted of 40 children with ASD (32 males and 8 females; mean age 6.89±3.30 years; male-to-female ratio 4:1), enlisted in several Autism Associations from Transylvania (Romania) and diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-V) in the Child and Adolescent Psychiatry Hospital, Cluj-Napoca, Romania. Among these children, according to the previous DSM-IV, 28 children had autistic disorder, 3 children had Asperger's disorder and 9 children had PDD-NOS. Associated medical conditions for our study group were: mental retardation (20% of cases), epilepsy (5% of cases) and hyperkinetic disorder (22.5% of cases). A written informed consent was obtained from all the parents or legal tutors of children included in the study. The research protocol was developed in accordance with the WMA Declaration of Helsinki and was approved by the University Ethics Committee.

Cytogenetic analysis was performed on fresh heparinized peripheral blood, using an adapted protocol for the G banding technique with Trypsin and Giemsa (GTG banding) on two parallel lymphocyte cultures (450-550 band resolution) (Schreck & Distèche 2001). Briefly, 0.5-1.5 ml of heparinized whole blood were added to 7 ml of Lymphochrome complete medium (Lonza) and the lymphocytes were cultured for 70-72 h at 37◦C with 5% CO2. 25 minutes before the incubation was over, 10 µg/ml Demecolcine (Sigma) was added to arrest the lymphocytes in metaphase. After the incubation time was over, the contents were centrifuged at 1,200 rpm for 10 minutes. The supernatant was discarded and the pellet was treated with 8 ml of pre-warmed (38◦C) 0.075M KCl hypotonic solution (Sigma), then it was incubated at 37◦C for 25 minutes. The contents were again centrifuged at 1,200 rpm for 10 minutes. The supernatant was discarded and 8 ml of Carnoy fixative (methanol/acetic acid 3:1) was added drop wise while mixing the pellet on vortex to avoid clumping, then centrifuged at 1,200 rpm for 10 minutes. The second washing was performed identically, followed by 30 minutes at 4◦C and then by centrifugation at 1,200 rpm for 10 minutes. For the third washing, only 6 ml of fixative were used, followed again by centrifugation. The pellet was casted onto a clean glass slide, previously kept in distilled water at 4◦C. After the slides were dry, Giemsa staining was performed in order to check the quality of the pellet and the mitotic index. Then, for each sample, 4 more slides were made and aged between 2-10 days at room temperature. Afterwards, the slides were banded with 0.25% Trypsin (Lonza) and 1% Giemsa (Merck) and analyzed under the microscope. For each case, 32 metaphases were analyzed and the karyotype was finalized on 16 metaphases, using the IKAROS Metasystem software (version 5.3.1). If chromosomal abnormalities were detected, up to 50 metaphases were analyzed for each case.

Analysis of the X chromosome was performed on isolated DNA samples from all patients included in the study. DNA was isolated from 300 µl of whole peripheral blood, using the Promega reagents and protocol.

Methylation-Specific PCR (MS-PCR) technique was used to determine the methylation status of *FMR1* gene in female patients, using the Cells-to-CpGTM Bisulfite Conversion kit (Applied Biosystems) and followed by a "hot-start" polymerase chain reaction (PCR) amplification with specific primers designed to differentiate between methylated and unmethylated DNA sequences using CpG WIZ® Fragile X Amplification kit. Size of the PCR products was analyzed using a 2% agarose gel electrophoresis. Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA) technique and the SALSA MS-MLPA ME029-B2 *FMR1*/AFF2 probemix were used to assess the promoter methylation status and the presence of deletions or duplications in the *FMR1* and *AFF2* (*FMR2*) genes and to identify premutations (between 61-200 trinucleotide CGG repeats) or mutations (more than 200 trinucleotide CGG repeats) in the *FMR1* gene for male individuals.

Table 1. Identified chromosomal abnormalities in the study group

Gender	Age (years)	Chromosomal abnormality [number of metaphases]	Genetic diagnosis
male	7.7	46,XY,9qh+ [50]	Homogenous 9q heteromorphism
male	6.5	46,XY [48] / 46,XY,del(15)(q22;qter) [2]	15q terminal deletion in mosaic
female	6	46,XX [48] / 47,XX,+8 [2]	Trisomy 8 in mosaic

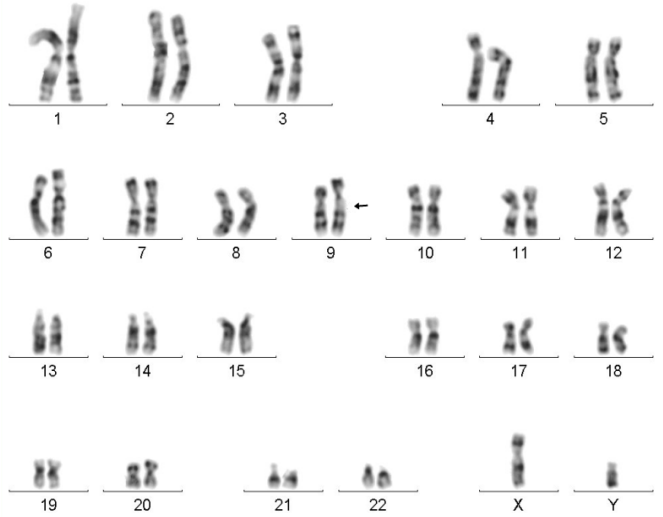

Figure 1. G-banded karyotype showing chromosome 9q heteromorphism in a male individual (450-550 band resolution)

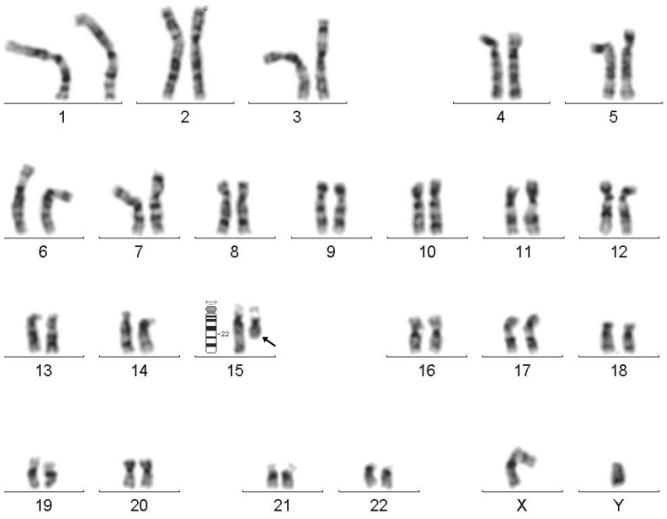

Figure 2. G-banded karyotype showing chromosome 15q terminal deletion in a male individual (450-550 band resolution)

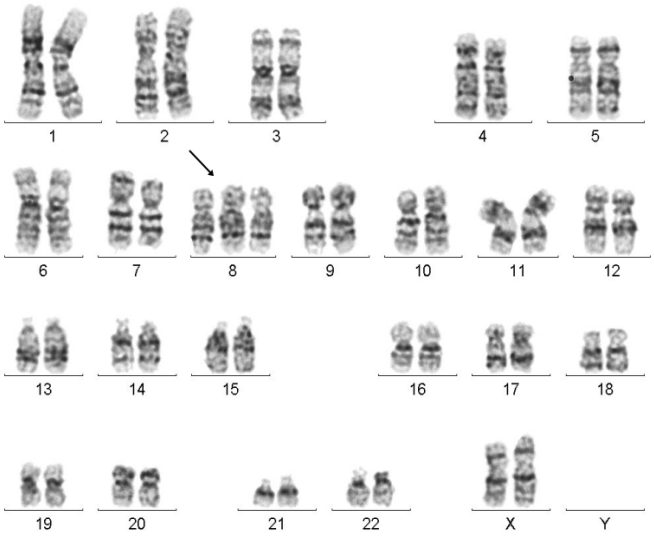

Figure 3. G-banded karyotype showing trisomy 8 in a female individual (450-550 band resolution)

Results

Our study revealed no chromosomal abnormalities on the GTG-banded karyotypes in 37 out of 40 cases. Three of the individuals (two males and one female), presented chromosomal abnormalities either in all or in only 2 of the 50 analyzed metaphases (Table 1), with the following genetic diagnoses: 46,XY,9qh+ [50] – heteromorphism (h+) of chromosome 9q in all analyzed metaphases in a male individual (Figure 1); 46,XY [48]/46,XY,del(15)(q22;qter) [2] – terminal deletion of 15q in 2 out of all analyzed metaphases in another male individual (Figure 2); 46,XX [48]/47,XX,+8 [2] – trisomy 8 in 2 out of all analyzed metaphases in a female individual (Figure 3).

The MS-PCR analysis revealed normal methylation status (normal *FMR1* gene) for all the individuals included in the study. The MS-MLPA testing showed no deletions, duplications or abnormal methylation status for both *FMR1* and *FMR2* genes.

Discussions and conclusion

In our study, the yield of cytogenetic abnormalities was 7.5%, two of the cases presenting a low-level (4%) mosaicism. According to literature data, chromosomal abnormalities can be detected by standard karyotyping with an average of up to 5% (Selvi et al 2010; Szczaluba 2014), in unselected ASD patients. The detection rate could increase in patients associating dysmorphic features, mental retardation, malformations or growth retardation (Szczaluba 2014). Our results confirm that in most cases, unselected autistic patients have no chromosomal abnormalities visible on a standard GTG-banded karyotype, probably due to its low resolution (Jeste & Geschwind 2014) or other etiological causes.

Mostly de novo, chromosomal abnormalities involving almost all chromosomes have been reported in ASDs, with a range varying from 0% to 54% (Xu et al 2004; Vorstman et al 2006; Marshal et al 2008; Selvi et al 2010), depending on sample size and characteristics and geographical factors. Chromosomal regions 1q21.1, 2q31-37, 7q21-36, 15q11-13, 15q24, 16p11.2, 16p13.11, 17q11.2-22, 22q11, 22q13.3, Xp22.3 and Xq13-28 are the most frequently cited (Wassink et al 2001; Martin & Ledbetter 2007; Rai 2011; Mefford et al 2012). Deletions and duplications of chromosome 15q11-13 have well been documented in autism (Mefford et al 2012), de novo isodic(15) or invdup(15) being specifically associated with more severe forms of ASDs (Dykens et al 2004; Martin & Ledbetter 2007; Miles 2011) and occurring in 1-3% of patients (Martin & Ledbetter 2007; Hogart et al 2010). Some studies associate deletions of 15q22, 15q23, 15q24 and 15q25 regions with autism (Smith et al 2000; Marshal et al 2008), and also with intellectual disability

and other recognizable features (Sharp et al 2007; Andrieux et al 2009; El-Hattab et al 2010; Mefford et al 2012). In our study, the 15q chromosome deletion appears to be terminal and to include the aforementioned regions q23-q25. Since this deletion was identified in a small percentage of cells (4%), it is difficult to say to which extent, if any, it contributed to autistic symptoms in our male patient. High-resolution karyotype or even CMA might be of use in this case both for the individual and his parents in order to identify more precisely the deleted region and genes, and to evaluate the degree of heritability for the purpose of genetic counseling.

Due to the very low percentage (4%) of mosaicism in the case of our female patient, the probability that the trisomy 8 was the trigger factor for autism is very low. Several studies have reported chromosome 8 abnormalities in autism (Veenstra-Vanderweele et al 2004; Wassink et al 2007). Trisomy 8 was previously cited in autism as partial trisomy 8p (Papanikolaou et al 2006; Fisch et al 2011; Tabarés-Seisdedos & Rubenstein 2009; Glancy et al 2009) or as a de novo mosaicism (Bailey et al 2008; Shen et al 2010). Apparently, the short arm of chromosome 8 contains several genes related to autism and related disorders (Tabarés-Seisdedos & Rubenstein 2009).

Some studies propose that low-level aneuploidy might be considered as a new genetic risk factor in autism, especially if it affects the structure and function of the developing brain (Yurov et al 2007), so this is worthy to consider in our two mosaic cases. The genes flanking the heterochromatin regions on chromosome 9 are involved in the normal development of the brain, making a possible connection to autism etiology (Vorsanova et al 2007) in the case of the other male patient. Some studies report a significant increase of variability in the heterochromatin regions on chromosomes 1, 9 and 16 (1phqh, 9qh+, 16qh-) in 60% of autistic patients with mild phenotypes, which may be explained by a gene position effect (Vorsanova et al 2007). Parents of autistic children (especially the mothers) carry the same heterochromatin variation, in approximately the same percentage (Vorsanova et al 2007). In our case, it would be interesting to do cytogenetic analysis of both parents in order to see if the 9q heteromorphism was inherited or de novo, mainly for the purpose of genetic counseling.

With respect to the FraX syndrome, literature data report both full mutation (> 200 CGG repeats) and premutation (61-200 CGG repeats) in exon 1 of the *FMR1* gene on chromosome Xq27.3 (generating the FRAXA fragile site), in approximately 1-3% (Gurrieri 2012) or even up to 5% (Freitag et al 2010) of autistic patients. Reversely, more than 60% of FraX patients exhibit autistic symptoms (McCary & Roberts 2012), reaching as high as 75% for male individuals or as low as 25% for female individuals (Klusek et al 2014). The CGG trinucleotide expansion causes the silencing of the gene (due to promoter methylation) resulting in the absence of FMRP that regulates or interacts with many autism candidate genes (Darnell & Klann 2013) and thus explaining symptoms such as mental retardation and autistic-like symptoms. Hypermethylation of the *AFF2* (*FMR2*) gene located on chromosome Xq28, due to CCG trinucleotide expansion (> 200 repeats creating the FRAXE fragile site) has been more frequently reported in cases of mental retardation (Stettner et al 2011), developmental and speech delay (Sahoo et al 2011). Recently, some studies revealed the possible association between *AFF2* mutations and autism (Stettner et al 2011; Mondal et al 2012; Correia et al 2015).

The negative result for *FMR1/AFF2* testing in our study group agrees with the low percentage of fragile X sites among autistic individuals and also could be explained by our small sample size. Some studies support the idea that fragile X testing on a general population of autistic patients, yields a positive result in less than 0.5%, suggesting that very strict selection criteria should be used (Roesser 2011). However, due to major implication for genetic counseling and the fact that the complete phenotypic expression of fragile X syndrome appears only after puberty (Freitag 2007; Shen et al 2010), it is prudent to do the testing in all autistic children, even in the absence of positive family history, dysmorphic features or mental retardation (Rousseau et al 2011).

The very low percentage of detected chromosomal abnormalities in our study group might be explained by the fact that in most cases the origin of autism is unknown, many genes and environmental factors interacting as triggers for the abnormal phenotype in autistic patients. The relatively small sample size could also contribute to the differences between our results and international studies.

These results are in agreement with worldwide genetic studies supporting the idea that for most patients, there are subtle genomic changes (submicroscopic deletions or duplications, or copy-number variants) involved. (Shen et al 2010) Higher resolution molecular genetics techniques such as CMA could offer a better detection of submicroscopic chromosomal alterations of up to 10% (Shen et al 2010), 30% (Szczaluba 2014) or up to 35% (Elsabbagh et al 2012) detection rate so wherever available it should be used as a first-tier genetic test for patients with ASDs, even if, it should be mentioned, the severity of ASD does not correlate with the presence of submicroscopic chromosomal abnormalities (Manning et al 2010; Miles 2011; Wisniowiecka-Kowalik B et al 2013). Low-resolution or targeted genetic testing will be probably used only for selected cases where there is a high suspicion of certain chromosomal or gene anomalies.

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Abbreviations

ADs Autistic Disorders
AFF2 / *FMR2* Fragile X Mental Retardation 2 gene
ASDs Autism Spectrum Disorders
CDD Childhood Disintegrative Disorder
CMA Chromosomal microarray

DSM-IV-TR Diagnostic and Statistical Manual of Mental Disorders IVth Edition – text revision
DSM-V Diagnostic and Statistical Manual of Mental Disorders Vth Edition
FMR1 Fragile X Mental Retardation 1 gene
FMRP Fragile X Mental Retardation Protein
FraX Fragile X syndrome
FRAXA Fragile X site A
FRAXE Fragile X site E
GTG banding G banding with Trypsin and Giemsa
h +/- duplication / deletion of heterochromatin
MS-MLPA Methylation-Specific Multiplex Ligation-Dependent Probe Amplification
MS-PCR Methylation-Specific PCR
p short chromosomal arm
PCR Polymerase Chain Reaction
PDD-NOS Pervasive Developmental Disorder Not-Otherwise Specified
q long chromosomal arm
rpm rotations per minute

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