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Original Article

Molecular Screening for Fragile X Syndrome in Children with Unexplained Intellectual Disability and/or Autistic Behaviour

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Abstract

Introduction: Fragile X syndrome (FXS, OMIM #300624) is the most common inherited form of intellectual disability and the leading monogenic cause of autism.

Aim: To present our experience with selective screening for FXS among high-risk children with intellectual disability/developmental delay/autistic behaviour and to further prove the importance of performing selective screening in a high-risk population.

Materials and methods: Fifty-two children (45 boys and 7 girls) hospitalized in pediatric clinics or referred to genetic counseling services were tested with triplet repeat primed PCR based commercial kit. The mean age of participants was 6 years (the youngest was 2 years old, the oldest - 15 years old). These patients were selected based on the presence of at least one of the following clinical features: developmental delay, intellectual disability, and autistic-like behaviour.

Results: All patients presented with developmental delay, including language delay. Intellectual disability and autistic-like behaviour were the most consistent features**.** Thirty-three children (63.4%) were with intellectual disability. Autism and autistic-like behaviour were observed in 22 patients (42.3%). Only 9 male patients (17.3%) presented with dysmorphic features typical for FXS. Three boys (5.7%) were found to be affected and two of their mothers - premutation carriers.

Conclusions: The present study is the first attempt for molecular genetic selective screening for FXS among high-risk groups in north-eastern Bulgaria. Screening for FXS helps in making a definitive diagnosis along with providing genetic counseling to the family which includes reproductive planning and risk assessment.

Keywords

developmental delay, FMR1 screening, genetic counseling

INTRODUCTION

Fragile X Syndrome (FXS, OMIM #300624) is the most common inherited form of intellectual disability (ID) and the leading monogenic cause of autism (ASD)[1] with an estimated incidence of 1 in 5000 males and 1 in 4000–8000 females.[2] FXS was first described in 1943 by Martin and Bell as a form of intellectual disability following an X-linked inheritance pattern.[3] Subsequently, in 1969 Lubs associated a cytogenetic marker with this syndrome, showing fragility at the terminal end of the long arm of the X chromosome (FRAXA) in a percentage of the metaphases, leading to its current name. In 1991, three groups working independently cloned the *FMR1* gene (fragile X mental retardation 1).[4]

Fragile X syndrome is predominately caused by an expansion of a trinucleotide (CGG)n repeat present in the 5′ untranslated (5′ UTR) region of exon 1 of the *FMR1* gene.[2] The (CGG)n repeat is highly polymorphic in the normal population. Normal alleles contain ≤44 CGG repeats that are stable from one generation to the next and intermediate alleles (also termed the "gray zone") of ≥45 to ≤54 CGG repeats, which may expand across future generations. The premutation (PM) allele is defined as containing ≥55 – 200 CGG repeats and may present with some phenotypic changes.[4] Nevertheless, the number of the repeats in PM carriers could expand in the next generation through maternal transmission[2] and the probability of this is directly dependent on the number of repeats.

If the number of the CGG repeats is above 200, this allelic constitution is called a full mutation (FM). It results in a hypermethylated state of the *FMR1* promoter, with consequent inhibition of FMR1 transcription and loss or heavy reduction of the protein product (FMRP). FMRP is an RNA binding protein and abundantly expressed in neurons.[2] The protein is reported to play a vital role in synaptogenesis and loss of this protein may affect synaptic plasticity, which results in brain dysfunction and could explain the symptoms from the nervous system in the affected individuals.[1] There are also other types of *FMR1* alterations (intragenic deletions/duplications, single-nucleotide variants), which are responsible for the remaining (<1%) molecular diagnoses of FXS.[3]

The term "fragile X syndrome" has been used to refer to the developmental disorder caused by CGG expansions and other *FMR1* mutations although another fragile site has been linked to expansions at the nearby FRAXE locus of the *AFF2* (*FMR2*) gene. It contains an unstable (CGG)n repeat whose expansions are responsible for a milder phenotype of non-syndromic ID in a small number of families.[5]

Although the severity and clinical manifestations of the disease vary, FXS has several characteristic symptoms: intellectual disability (mild to moderate), which may be accompanied by specific dysmorphic features such as a long face, large protruding ears, a large jaw, and macroorchidism. In many cases, FXS is also considered a behavioural disorder as patients present with attention deficit hyperactivity disorder (ADHD) or autism spectrum disorder (ASD). FXS is characterized by heterogeneous clinical penetrance. In almost all cases, men with full mutation in the *FMR1* gene have more severe clinical symptoms as compared to women. Approximately 50% of female carriers of the disease-causing mutation will have mild to moderate mental disabilities due to X-inactivation and cellular mosaicism.

Individuals with the premutation, especially males, also develop symptoms and are at risk for developing fragile X-associated tremor/ataxia syndrome (FXTAS). Females with the premutation have an increased likelihood of developing fragile X-associated primary ovarian insufficiency (FXPOI) before age 40.[7] Moreover, women who are PM carriers may have children affected with FXS due to the repeat expand during ovogenesis.[2]

Diagnosis of FXS is based on the determination of the precise CGG number and/or the methylation status of the CpG island. Identification of affected patients has considerably improved in the last years with the advancing technologies.[4] Initially, the diagnosis of FXS was based on the cytogenetic evaluation of the presence of FRAXA, induced by culturing cells in folic acid deficient medium. However, this method is no longer used because the procedure is time-consuming and less sensitive than molecular methods.[3] The gold standard for many years was the Southern blot, but the main disadvantage is that it requires a large amount of DNA and is rather laborious. This technique is being gradually replaced by the dTP-PCR (triplet repeat primed) approach. This type of PCR provides information about the exact CGG number and discriminates between normal, intermediate, premutation, and full mutation alleles, even in a mosaic fashion.[4,8]

Because clinical symptoms are neither specific, nor constant, testing for fragile X mutation is usually part of the basic genetic assessment in the cases of males or females who present with developmental delay, mental disabilities, and/or behavioural problems.[5]

AIM

The aim of this study was to present our experience with molecular genetic screening for FXS syndrome among children with intellectual disability / developmental delay / autistic behaviour and to further prove the importance of performing selective screening in a high-risk population.

MATERIALS AND METHODS

This study was conducted at the Laboratory of Medical Genetics at St Marina University Hospital, Varna. It was a part of a scientific project, funded by the Medical University in Varna, Bulgaria. The research protocol was approved by the Ethics Committee of the Medical University of Varna. The patient samples were collected between 2018 and 2019. The study included 52 children hospitalized in pediatric clinics

or referred to the genetic counseling office for unknown cause of intellectual disability / developmental delay / behaviour problems and clinically evaluated by a psychologist or psychiatrist. Patients were selected based on the presence of at least one of the following clinical features: developmental delay, intellectual disability, and autistic-like behaviour. Individuals with known etiology of these conditions (for example confirmed single gene or chromosomal pathology) were excluded. All of the parents signed an informed consent, because the children were minors.

The genomic extraction was done using the standard salting-out method and the genomic DNA was then subjected to molecular analysis.

For the purpose of our study and to identify CGG repeat expansion, the FastFrax *FMR1* Identification Kit (Biofactory Pre Ltd) was used. It is intended for distinguishing the expanded (a group of pre- and full mutation) from non-expanded (normal and high normal) *FMR1* alleles (≥55 rpts). This involved dTP-PCR, followed automatically by melting curve analysis (MCA) in a closed-tube reaction. The dTP-PCR assay was performed following the manufacturer's instructions. Two cut-off control DNA samples with 41 and 53 CGG repeats (NA20244, NA20230) (Coriell Cell Repositories) were used to establish threshold resumed baseline. Samples, which showed an expansion of the CGG repeats, were additionally confirmed by FastFrax *FMR1* Sizing Kit (Biofactory Pre Ltd) at the National Genetic Laboratory.

RESULTS

A total of 52 children, 45 (86.5%) boys and 7 (13.5%) girls, with unexplained intellectual disability and/or autistic behaviour were examined. The mean age of the participants was 6 years, the youngest one was 2 years old and the oldest one was 15 years old. Most of the patients (44, 84.0%) were recruited from the hospitalized children in the pediatric clinics of the hospital and almost all of them (96%) lived in the region of north-eastern Bulgaria, mainly in Varna (61.5%).

All patients presented with developmental delay, including language delay **(Fig. 1)**. Intellectual disability and autistic-like behaviour were the most consistent features. Thiry-three children (63.4%) were with intellectual disability: 29 with mild ID (6 girls and 23 boys) and 4 with moderate ID (1 girl and 3 boys). Another 12 (23%) were difficult to evaluate because of their age (2-3 years old). Autism and autistic-like behaviour were observed in 22 patients (42.3%) – 5 girls and 17 boys. Only 9 males (17.3%) presented with dysmorphic features typical for FXS.

From the molecular analysis: three samples (5.7%) were classified as expanded. These patients were males (6.6% of all tested males) at age 8 (case 1), 9 (case 2) and 11 (case 3) years. Their clinical features are summarized in **Table 1**. The mothers of case 1 and case 2 (case 3 not available) were additionally tested and their samples showed expansion. Conformation analysis showed that all three males carried a full mutation, and the two females were premutation carriers.

DISCUSSION

The frequency of fragile X-positive patients (5.7%) found in this study is consistent with the literature data reported for individuals with ID/developmental delay (2–9%).[3,9-11] Similar results are observed by L. Angelova[12] among Bulgarian school-age patients with ID, but using a cytogenetic screening method. She reported a 5.9% incidence of the syndrome in a screened group of 76 boys and 25 girls. Other non-population based studies of Bulgarian patients with ID, molecularly screened for mutation in the *FMR1* gene revealed an incidence of 1.3%[13], 11%[14], and 12.5%[15].

The clinical spectrum of FXS is wide, but the most important clinical abnormality is global developmental delay/ID. The psychomotor delay involves both walking age (mean=2.12 years) and age at first words (mean=2.43

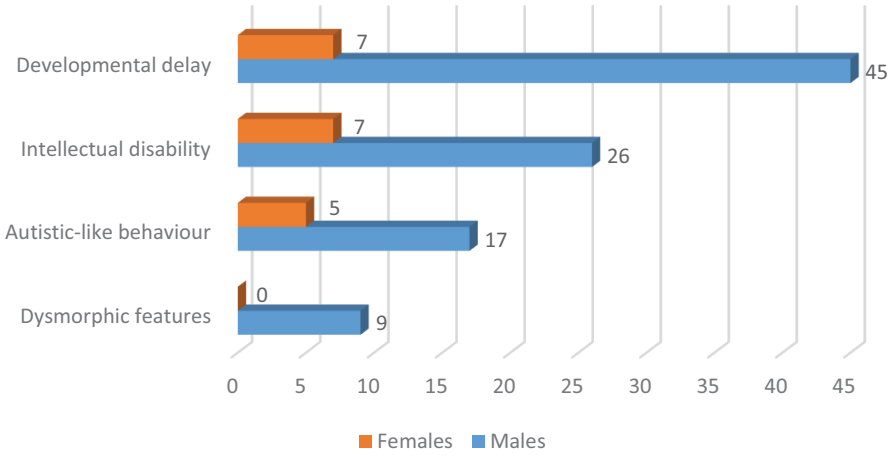

Figure 1. Clinical presentation of the selected patients based on the leading clinical criteria for inclusion in the study. Some of the patients presented with more than one feature so that the total number of the patients on the chart exceeds the number of selected group.

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Table 1. Clinical presentation of the patients with a full mutation

	Case 1	Case 2	Case 3
Age, yrs	8	9	11
CGG repeat size	>200	>200	>200
Intellectual disability	+ (moderate)	+ (mild)	+ (mild)
Developmental delay	+	+	+
Autism / autistic-like behaviour	+	-	+
Hyperactivity	+	+	+
Stereotypies	+	-	+
Facial dysmorphism	+	-	-
Macro-orchidism	+	-	+
Joint hypermobility	+	+	-
Obesity	-	-	+

years).[3] Both males and females with FXS present a wide range of learning disabilities in the context of normal, borderline IQ or mild to severe ID.[16] All three boys diagnosed with the syndrome in our study had developmental delay, especially language delay, mild (cases 2, 3) to moderate ID (case 1) and hyperactivity. They also presented with autistic-like behaviour and attention deficit (cases 1, 3), aggression and auto aggression (case 3). Hyperactivity and/or attention deficit especially in young boys were observed as the most common features in FXS in some studies.[17] FXS is the most common single-gene disorder associated with autism and it causes 1–6% of autism cases.[18] Autism spectrum disorder (ASD) evaluated by some authors[3,18] as a comorbidity of FSX is estimated in 30–50% of males and 25% of females with FXS. Individuals with FXS and ASD have a lower IQ and lower receptive and expressive language abilities compared to individuals having FXS alone.[19] Seizures and EEG findings consistent with epilepsy are another common features of FXS during childhood, with an incidence between 10% and 20% in boys and 5% in girls,[3] not manifested in our expanded or non-expanded samples.

Common physical characteristics of FXS patients include an elongated face, large and protruding ears and macroorchidism, but they are subtle during early childhood and may become more apparent with increasing age.[6] Case 1 had facial dysmorphism typical for FXS and macroorchidism even though he was 8 years old. The other two boys did not present with dysmorphic features typical for the syndrome and this shows the importance of conducting genetic screening among children (esp. boys) with DD/ID and no other symptoms.

FXS also shows an association of various medical problems that may or may not be present. It has been clear that the condition shares some features with the spectrum of connective tissue disorders.[3] These symptoms are related to the lack of FMRP on the structure of the elastin fibrils in the skin, heart, vessels and organs.[1] The skin can be soft and joint hypermobility is present in about half of the patients (case 1 in our study), affecting predominantly the small joints. Cardiac involvement in FXS (mitral valve prolapse and aortic root dilatation) are more prevalent in adults than children; connective tissue anomalies and hypotonia could contribute to gastrointestinal problems (gastro-oesophageal reflux, constipation, and diarrhea); recurrent otitis media, which may lead to conductive hearing loss.[1,3] None of the diagnosed boys had cardiac, gastrointestinal or ear problems.

Metabolic problems are common and well reported, with obesity and overweight being quite frequent in both sexes[3] and the presence of the Prader-Willi phenotype (seen in less than 10% of patients with FXS) leading to hyperphagia[20] may exacerbate the obesity problem. One of the diagnosed boys (case 3) had obesity (BMI 43.3) and Prader-Willi-like phenotype.

Due to the above mentioned subtle and varied features, many affected children remain undiagnosed. Diagnosis of fragile X syndrome is difficult when based on clinical evidence alone. Specific indications for testing for fragile X mutation include any male or female from positive family history with intellectual disabilities, developmental delay, speech and language delay, autism or learning disabilities of unknown cause.[5] We used these indications to select children (mostly boys), appropriate for molecular screening for FXS and a full mutation was found in 3 male patients.

In the era of molecular FXS diagnosis, FXS is still difficult to recognize and diagnose which could be attributable to the lack of an obvious phenotype at birth and the presence of only subtle phenotypes during the prepubertal period. A diagnosis of FXS is often made in young children of approximately 3 years of age, who show delayed or absent speech.[21] This timeline can be longer for girls and boys with milder symptoms. The results of various studies show a mean age at diagnosis between 3 and 6.3 years.[10,22,23] The mean age at diagnosis in our study is 9.3 years and this is higher than that reported in other studies and can be explained to some extent by the absence to date of molecular diagnostics for FXS routinely performed and reimbursed on a national level. A delay in diagnosis can reduce

access to early intervention, family support programs, and medical treatment. For a hereditary condition such as FXS, the delayed diagnosis of a first child reflects on insufficient genetic counseling for reproductive risk - approximately 30% of these parents have a second child with FXS before the first is diagnosed.[23]

The need for early diagnosis leads to the idea of newborn screening (NBS) for FXS, but its application remains controversial. FXS was not recommended for inclusion in the panel of conditions for NBS partly because there was no medical advantage for early detection/treatment.[21] To date, newborn screening for FXS is not performed in any country[3] though the technical feasibility of widespread testing and advances in treatment may change this.[21] Benefits of FXS NBS and testing may include early access to targeted therapies. Significant advances have been made in the development of treatments to help with behaviour problems, anxiety, and attention deficits in children with FXS (minocycline, lovastatin, metformin).[24-26] This illustrates the importance of genetic testing for FXS in all children with intellectual disability and autistic behaviour in order to provide the best care as soon as possible.

CONCLUSIONS

The present study was the first attempt for molecular genetic screening for *FMR1* gene mutations in a high-risk group of children with ID/developmental delay from north-eastern Bulgaria. Even though FXS is a well-known cause of intellectual disability, sometimes it could be difficult to be recognized, especially at an early age. This demonstrates the importance of screening for FXS, as it provides a definitive diagnosis for the family and facilitates genetic counseling of the affected individual and relatives.

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Молекулярный скрининг синдрома ломкой Х-хромосомы у детей с необъяснимой умственной отсталостью и/или аутичным поведением

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Резюме

Введение: Синдром ломкой Х-хромосомы (FXS, OMIM № 300624) является наиболее распространённой наследственной умственной отсталостью и ведущей моногенетической причиной аутизма.

Цель: Представить наш опыт селективного скрининга на FXS среди детей из групп высокого риска с умственной отсталостью, /отсталым/ аутистическим поведением и подтвердить важность селективного скрининга среди групп высокого риска.

Материалы и методы: Методом ПЦР с триплетным повторением было проведено обследование 52 детей (45 мальчиков и 7 девочек), госпитализированных в детские поликлиники или направленных на генетическое консультирование. Средний возраст участников составил 6 лет (самому младшему было 2 года, самому старшему – 15 лет). Эти пациенты были отобраны на основании наличия хотя бы одной из следующих клинических характеристик: задержка развития, умственная отсталость и аутистическое поведение.

Результаты: У всех пациентов наблюдалась задержка развития, включая задержку речевого развития. Основными признаками были умственная отсталость и аутистическое поведение. Умственно отсталыми были 33 ребёнка (63.4%). Аутизм и аутистическое поведение наблюдались у 22 пациентов (42.3%). Только у 9 пациентов мужского пола (17.3%) были обнаружены дисморфические черты, характерные для FXS. Выявлено, что трое мальчиков (5.7%) были поражены, а две их матери были носителями премутации.

Заключение: Настоящее исследование является первой попыткой молекулярно-генетического скрининга на FXS среди групп высокого риска из северо-восточной Болгарии. Скрининг FXS помогает поставить окончательный диагноз, а также провести генетическое консультирование семьи, которое включает репродуктивное планирование и оценку риска.

Ключевые слова

задержка развития, скрининг FMR1, генетическое консультирование