

Research Article



Mutation Screening and CGG-Repeat Distribution of the *FMR1* gene Among Mentally Retarded and Autistic Patients in Algeria

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ABSTRACT

Fragile X syndrome (FXS) is the leading cause of inherited mental retardation. The underlying molecular alteration consists of a CGG-repeat expansion within the *FMR1* gene. Therefore, searching for CGG expansion at the FXS locus among the mentally retarded should become a routine investigation in neuro-paediatric practice. This study was to establish a molecular diagnosis in Algerian patients exhibiting mental retardation with or without autistic features. It is important for the fragile X diagnosis to establish whether the range of allele distribution in Algerian patients are comparable to other populations, and if it is observed the same pattern of expansion associated with the disease. PCR was undertaken on 60 samples followed by Southern blot to analyze the CGG repeat number and methylation status. The molecular findings indicated in one young patient, diagnosed as autistic, mosaics mutations of 360 and 330 repetitions, while his mother was found carrying a premutated allele of 87 repetitions, and his two years old sister was found to have a contracted premutated allele of 63 repetitions of the CGG repeat. The distribution of the CGG repeats falls within the 30 repeats (17%), followed by a 31 repeat sizes (12%) instead of the 29 repeats as in the Caucasian population. The results of this study reconfirmed previous reports that the pattern of *FMR1* CGG repeat alleles is different regarding the racial/ethnic population studied. In conclusion, the detection of the FXS mutations has allowed us to offer more informed genetic counseling and reliable patient follow-up.

Keywords: autism, CGG repeats, *FMR1* gene, fragile X Syndrome, mental retardation.

INTRODUCTION

Fragile X syndrome (FXS) (OMIM n 300624) is the most common cause of inherited mental retardation and the second most common cause of genetically associated mental retardation following trisomy 21^{1,2}. FXS is caused by a dynamic mutation which involves an unstable expansion of a trinucleotide CGG repeat at the 5'-untranslated region (UTR) of the fragile X mental retardation 1 (*FMR1*) gene, located at Xq27.3^{3,4}. In the general population, individuals carry 6-52 CGG repeats and the triplet number is usually stably transmitted. Individuals carrying alleles 53-200 repeats are called premutated, the repeats are unstable through maternal transmission and tend to extend more often than contract^{3,5}. Affected individuals carry alleles > 200 repeats called full mutation which is generally associated with hypermethylation at the *FMR1* promoter and consequent transcriptional silencing⁶⁻⁹. The silencing of the *FMR1* gene leads to the deficiency of the fragile X mental retardation protein (FMRP) thus causes the classical FXS¹⁰. Moreover, there is a grey-zone with repeats between 45-55, these alleles called intermediate, are occasionally unstable and they are potential precursor of a premutation in subsequent generations¹¹. Size is not the only factor implicated in all instability. The number and position of AGG interspersions determining long uninterrupted CGG arrays at the 3' and 5' ends of the repeat¹², haplotype background and parental origin,

have been evoked as predisposing factors leading a common/intermediate allele to progress toward a mutation state^{13,14}.

The main clinical features in males are intellectual disability and behavioral problems including anxiety, aggression, hyperactivity, impulsivity, shyness, attention deficit disorder, and autism¹⁵⁻¹⁸ as well as macroorchidism, and a long narrow face with large averted ears¹⁹⁻²¹.

Numerous studies have found FXS in every ethnic group that has been evaluated with an incidence of 1 in 4500 males and 1 in 9000 females whereas the actual incidence of at-risk individuals is estimated at 1 in 1000 for males and 1 in 4000 for females²². The population-based prevalence study of full mutation of *FMR1* through DNA analysis was now estimated 1 in 4000 males and 1 in 8000 females²³.

Furthermore, there have been growing evidences of premutation-associated to clinical phenotypes such as milder forms of FXS including autistic features²⁴, developmental delay and latter phenotypes of fragile X-tremor ataxia syndrome in older premutated male carriers, and premature ovarian failure before the age of 40 in premutated females²⁵.

Considering the prevalence of full mutation and premutation as well as their consequences on children's



development and their latter phenotypes, the impact of FXS is thought to be much more enormous than what has earlier been thought.

There have been growing interests worldwide on the molecular diagnosis and clinical experiences on FXS. At present, polymerase chain reaction (PCR), and Southern blotting are considered as the golden standard for the diagnosis of FXS^{26,27}.

Although FXS has been identified in all the main ethnic groups, little is known about its prevalence with respect to ethnicity^{28,29}.

According to the literature and many reports, the screening of the FMR1 gene has never been conducted among MR (mentally retarded) patients neither on autistic patients in Algeria.

Therefore, this investigation is the first one done in the assessment of a molecular diagnosis on those specific patients.

MATERIALS AND METHODS

Patients and clinical assessments

Patients were recruited in three different psychiatric clinical practicing in Annaba (North-East of Algeria). Patients were recruited for participation if they were receiving special education services based on a cognitive disability (mental retardation or learning disability). This ongoing study was designed to collect families with one or more mental retarded (MR) offspring with or without autistic features. 55 patients were selected (15 females and 40 males), and 3 asymptomatic relatives (2 females and 1 male) for a total of 58 samples. The mean age of the sample was 08.46 ± 3.65 years and patients ranged from 02 to 20 years of age.

Dysmorphic features typical of the fragile X syndrome were not seen in any of the selected patients.

Collection of the samples was done according to the ethical of our laboratory guidelines. Legal representatives of all the patients gave their informed consent prior to molecular genetics research.

DNA samples

To study CGG transmission following FXS genotype, genomic DNA was extracted from peripheral blood leukocytes (5 to 10 ml of whole blood) according to standard procedures³⁰.

Fluorescent PCR analysis

PCR amplification (TECHNE 200) was carried out on both loci FRAXA³ and FRAXE³¹. Briefly, 200 ng of genomic DNA were used in a total volume of 20 μ l, containing: 5 pM of each primer FRAXA (Eurogentec): forward (**FAM**) 5' AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA 3' and reverse 5' GCT CAG CTC CGT TTC GGT TTC ACT TCC GGT 3' and 20 pM of each primer FRAXE (Eurogentec): forward (**HEX**) 5' GCG AGG AAG CGG CGG CAG TGG CAC TGG 3' and

reverse 5' CCT GTG AGT GTG TAA GTG TGT GAT GCT GCC G 3', and 200 μ M of each dNTP (Promega), 3% of MgSO₄ (Kit PLATINIUM[®]), 2 μ l of 10X PCR buffer, 40% of PCR enhancer (Kit PLATINIUM[®]) and 1.25 units of Platinum *pf*x DNA Polymerase.

Amplifications were initiated with a primary denaturation step at 95°C for 3 min, followed by 28 cycles of denaturation at 95°C for 15 sec, annealing at 64°C for 2 min and elongation at 72°C for 10 min. The final elongation was carried out at 72°C for 10 min.

Capillary electrophoresis

The PCR products (2 μ l) were mixed with 0.5 μ l size standard (ILS600, Promega) and 10 μ l deionized formamide (Hi-Di: Applied Biosystems) and heated for 5 min at 95°C. The DNA fragments were separated by automated capillary electrophoresis according to the manufacturer's instructions, in a polymer POP 7 3130 Genetic analyzer (Applied Biosystems) with a capacity of 16 samples per run.

Fragment analysis was performed with GenMapper v 4.0 software (Applied Biosystems).

Southern-blot

A total of 4 individuals (3 females and 1 male) were studied, 2 females and 1 male from one family case and 1 unrelated female, along with normal and premutated female controls. The protocol reported³² was followed with some modifications: 0.5 μ g of DNA were digested for 5 h at 37°C with 10 units of EagI (Biolabs) and 20 units of EcoRI (Biolabs) in a final volume of 40 μ l, including 1X Digestion Buffer and 100 μ g/ μ l BSA spermidine. 15 units of EagI and 20 units of EcoRI were added (after 5 h and 18 h); the final lap of digestion was left 8 h then stopped by the addition of 10% of blocking solution.

Electrophoreses was performed on a 0.8% Agarose gel overnight at 3.5 V/cm, and a molecular weight marker was run in parallel (2 Kb to 12 Kb). To deplete DNA fragments, gel was treated in 0.25 N HCl for 10 min. Denaturation was performed in 0.5 M NaOH and 1.5 M NaCl for 2 x 15 min, followed by neutralization in 0.5 M Tris-HCl and 0.5 M NaCl for 2 x 15 min, after which the gel was equilibrated in 20 X SSC for 10 min. All of the above steps were performed in room temperature with shaking. Upward capillary transfer of the DNA was performed overnight onto positively charged nylon membranes (HYBOND N⁺) in 20X SSPE. An StB12.3 probe (CREGEMES: Centre Régional de Génétique Médicale de Strasbourg), was radioactively labeled (Random Primed: InVitrogen). Pre-hybridization and hybridization were carried out in 40% formamide, 0.5X SSC, 0.1% SDS and 200 mg/ml of salmon sperm denatured DNA (10 min at 100°C) at 65°C for 1 and 16 h respectively, followed by the following washes: 0.5X SSC, 0.1% SDS for 4 x 15 min at 60°C.



(CGG) repetition distribution

In order to study the pattern of repeat size for the FMR1 gene in our Algerian panel, 66 chromosomes of unrelated individuals (28 from females and 38 from males) were analyzed and compared to published Caucasian and non-Caucasian distribution among MR patients and/or on normal population.

RESULTS AND DISCUSSION

The size of the fragments obtained by PCR and Southern-blot analyses and the classification of the alleles according to the degree of (CGG) repeats detected in males and females are shown in Table 1.

Fluorescent PCR analysis

PCR analysis of 60 individuals (19 females and 41 males) yielded the following results:

- i) Presence of two alleles of normal size (267-315 bp) in 11 females (78%);
- ii) one normal allele size (298 bp) in 1 female (5%);
- iii) one normal allele size (288-343bp) in 37 males (85%);
- iv) DNA sample was not amplified in 1 male (2%), and one normal allele size (295 bp) and the second premutated in 2 females (11%) from the same family (Fig.1).

Southern-blot

Southern-blot was carried out on samples showing one allele amplified in 3 females suggesting either they are normal homozygous or XFRA carriers, and on 1 autistic male, which DNA failed to amplify, suggesting a too large repeat to be amplified by PCR.

Patient (DM): Southern blot results showed that this patient is homozygous for the size of the CGG repeat.

Family (4): Southern blot results showed that the mother (I1), was a carrier of a premutation (Fig.2, 466 bp-fragments). The symptomatic proband DNA was not amplified, and thus he could be a carrier of a full mutation. His sister (II2) showed a unique band of 295 bp (normal allele size), suggesting homozygosity, but heterozygosity because of a full mutation cannot be ruled out, and thus, a Southern blot assay was carried out (Fig.2). The mother and the sister showed the typical Fragile-X-premutation carrier band pattern: 2.8 and 5.2 Kb bands denoted normal alleles of active and inactive X chromosome respectively, whereas 3.3 Kb bands indicated premutated allele. Therefore, the PCR results were confirmed in these cases.

The patient sister showed a contracted premutated allele of 63 (CGG) repeats while her mother showed a premutated allele of 87 (CGG) repeats.

The absence of the 2.8 Kb fragment and the presence of higher and different molecular weight bands in the patient (II1), denoted the presence of different full mutations (6 and 8 Kb), characteristic of a mosaic pattern.

(CGG) repeats distribution

Table 2 shows the distribution of allelic sizes in a total of 17 alleles, measured from 14 females and 38 males. Family members were discarded to avoid biasing results.

The number of trinucleotide repeats ranges from 18-46 CGG repeats (Tab.3).

Furthermore, we were able to characterize one allele with a repeat size of 46, which fall in the grey-zone (alleles between 45-55 CGG repeats).

Table 1: Patients and families with the corresponding CGG bands size

| Individuals | | Bands (bp) |
|---------------------------|------------------|---------------|
| Unrelated patients | | |
| - | 37 males | 258.11-343.82 |
| - | 11 females | 267.22-315.76 |
| - | 1 female (D.M.) | 298.3 |
| Families | | |
| - | Family 1 | |
| | Patient (male) | 272.69 |
| | Mother | 273.38-328.38 |
| | Brother | 272.57 |
| | Father | 290.35 |
| - | Family 2 | |
| | Patient (female) | 293-299 |
| | Sister | 294-300 |
| - | Family 3 | |
| | Patient (female) | 296-302 |
| | Brother | 296 |
| - | Family 4 | |
| | Patient (male) | FM mosaic |
| | Mother | 295.52/PM |
| | Sister | 295.66/PM |

FM, Full mutation PM, Premutation Bands in bold are in the grey zone

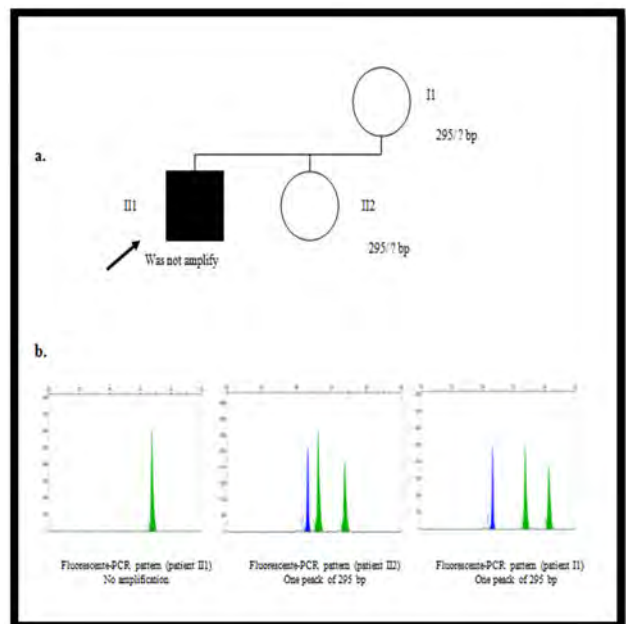


Figure 1: a-Pedigree of family (4) showing the molecular weight of PCR products with the corresponding (295 bp) normal alleles. b-Electrophoregrams of FRAXA (blue)/FRAXE (green) PCR products from family (4) members.

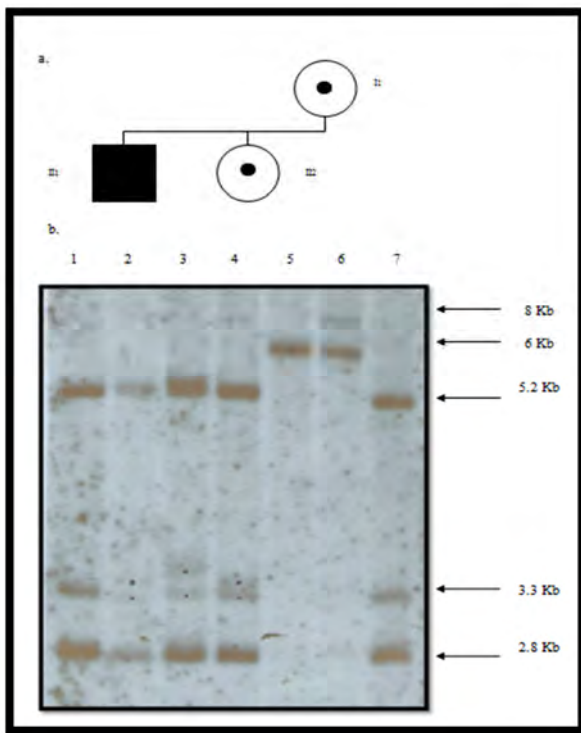


Figure 2: a. Pedigree of family (4) showing the carriers status of patients I1 and II2. b. Analysis of family (4) with StB12.3 hybridized to EcoRI/EagI double digest. Lanes 1 and 7: Normal control female showing two bands of 2.8 and 5.2 kb, Lane 2: Premutated control female, Lane 3: Mother (I1) showing the presence of one normal band of 2.8 Kb and one band from the premutated alleles, Lane 4: Sister (II2) showing the presence of one normal band of 2.8 Kb and one band from premutated allele, Lane 5: Mutated control male, Lane 6: Patient (II1) showing the presence of two bands of 6 and 8 Kb (full mutated alleles).

Table 2: Number of CGG repeats found in (66) analysed chromosomes

| Number of repeats | Number of chromosomes |
|-------------------|-----------------------|
| 18 | 1 |
| 20 | 6 |
| 22 | 1 |
| 23 | 3 |
| 25 | 1 |
| 26 | 2 |
| 28 | 1 |
| 29 | 7 |
| 30 | 17 |
| 31 | 12 |
| 32 | 7 |
| 33 | 3 |
| 34 | 1 |
| 37 | 1 |
| 40 | 1 |
| 41 | 1 |
| 46 | 1 |
| Total = 17 | Total = 66 |

Table 3: Polymorphism distribution of the CGG repeats among (66) chromosomes

| Sex | Frequency | Percentage | Cumulative Frequency | Cumulative Percentage |
|-----|-----------|------------|----------------------|-----------------------|
| F | 28 | 41.79 | 28 | 41.79 |
| M | 39 | 58.21 | 67 | 100.00 |

| Repeats nb | Frequency | Percentage | Cumulative Frequency | Cumulative Percentage |
|------------|-----------|------------|----------------------|-----------------------|
| 18 | 1 | 1.52 | 1 | 1.52 |
| 20 | 6 | 9.09 | 7 | 10.61 |
| 22 | 1 | 1.52 | 8 | 12.12 |
| 23 | 3 | 4.55 | 11 | 16.67 |
| 25 | 1 | 1.52 | 12 | 18.18 |
| 26 | 2 | 3.03 | 14 | 21.21 |
| 28 | 1 | 1.52 | 15 | 22.73 |
| 29 | 7 | 10.61 | 22 | 33.33 |
| 30 | 17 | 25.76 | 39 | 59.09 |
| 31 | 12 | 18.18 | 51 | 77.27 |
| 32 | 7 | 10.61 | 58 | 87.88 |
| 33 | 3 | 4.55 | 61 | 92.42 |
| 34 | 1 | 1.52 | 62 | 93.94 |
| 37 | 1 | 1.52 | 63 | 95.45 |
| 40 | 1 | 1.52 | 64 | 96.97 |
| 41 | 1 | 1.52 | 65 | 98.48 |
| 46 | 1 | 1.52 | 66 | 100.00 |

| Variable | Nb | Mean value | SD | Minimal | Maximal |
|-----------|----|------------|-----|---------|---------|
| Repeat Nb | 66 | 29.4 | 5.0 | 18.0 | 46.0 |

| Variable | Nb | Mean value | SD | Minimal | Maximal |
|----------|----|------------|------|---------|---------|
| Size | 66 | 292.5 | 18.9 | 197.2 | 343.8 |

DISCUSSION

Fragile X syndrome is a very subtle dysmorphic syndrome and it is difficult to diagnose clinically³³. Mental retardation (MR) in fragile X syndrome males varies from mild to profound with most affected males being moderately to severely retarded³⁴. The phenotype is subtle in young children and the features become prominent as the child grows. Many of these features suggest the possibility of autism, 20% (ranging from 5 to 53%) FXMR patients had autistic features³⁵. Furthermore, FXS is one of the most prevalent genetic causes of autism spectrum disorder²⁰.

We conducted the FMR1 screening on 55 individuals (17 MR patients) and (38 autistic patients). Southern blot analysis with probe StB 12.3 after double digestion with EcoRI and EagI in one individual (patient II1 from family 4) showed two bands of 6 and 8 kb corresponding to two different FMR1 mosaic full mutations. The mosaic pattern showed by Southern blot in our XFRA patient may be due to germ line and somatic instability of the (CGG)_n repeats thus, the patient in this case had a milder phenotype compared to those with full mutation. The FXS clinical diagnosis of the proband was then redefined as he was first diagnosed as autistic because the strikingly similar behavior to that of individuals with autism, including pronounced social awkwardness, repetitive actions, language impairment and a restricted range of interests.

Analysis of the family members showed that the mother (patient I1) and the sister (patient II2) had premutations

(87 and 63 CGG repeats, respectively), thereby allowing the carrier status determination of both mother and sister, which led to a genetic counseling session, regarding the risk of passing a dynamic mutation to offspring, and to contract ovarian premature failure (POF), condition which is highly linked to premutated allele of FMR1²³.

Fluorescent PCR amplification is useful for reliable assessment of Fragile X (CGG)_n alleles in normal and premutation range. Since full mutation failed to amplify, false positive results may arise from polymerase inhibitors (running in parallel a normal control sample), and false negative results may arise from allele specific amplification of the normal allele in the heterozygotes and mosaic. The problem of allele specific amplification can be overcome by reanalysis by Southern blotting of all male samples without peaks and female samples with one peak.

Thus, the usefulness of PCR as a diagnostic method was restricted to prescreening for normal alleles and for this reason the characterization of mutations was performed by Southern blot assay, a method that can identify all the expanded fragments including premutations and full mutations³⁶.

A normal Southern pattern does not imply an absence of Fragile-X Syndrome. Although the main causes of FXS are expansion of (CGG)_n other mutations may also generate FXS such as deletions in the FMR1 gene^{37,38}.

This is the first study on the distribution of the FMR1 size repeats in the Algerian population. It is important for the fragile X diagnosis to establish whether the range of allele distribution is comparable to other populations, and if it is observed the same pattern of expansion associated with the disease.

Our mode of distribution falls within the 30 repeats (17%), followed by a 31 repeat sizes (12%) instead of the 29 repeats as in the Caucasian population³⁹. As in other studies, there is a characteristic pile up around 20 and 30 repeats, and a dip at 21 repeats³.

The results of this study reconfirmed previous reports that the pattern of FMR1 CGG repeat alleles is different regarding the racial/ethnic population studied.

CONCLUSION

There are many reasons to make the diagnosis of FXS including genetic counseling and improved treatment. Once a child is diagnosed with FXS or the fragile X premutation, a genetic counseling is important because a variety of problems that can occur in other family members, such as a grandparent or uncle that may have tremor, ataxia or dementia or an aunt with POF or a sibling with emotional difficulties. Therefore all individuals with autism or autism spectrum disorders need to have fragile X DNA testing to identify premutations or full mutations.

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