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10 Years' Experience in Fragile X Testing Among Mentally Retarded Individuals in Greece: A Molecular and Epidemiological Approach

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Abstract. *Fragile X syndrome, the second most common genetic cause of mental retardation, is due to the expansion of a trinucleotide repeat (CGG)n within the first exon of the FMR-1 gene. Molecular genetic analysis provides accurate diagnosis and facilitates genetic counselling and prenatal testing. Screening for the fragile X mutation in a sample of 3,888 individuals in Greece is reported: 1,755 children with non-specific mental retardation, 1,733 parents and other family members and 400 normal individuals. Molecular analysis allowed for the identification and characterization of 52 fragile X families confirming the clinical diagnosis in 57 males and 4 females. Sixty-six female carriers (6 mentally retarded) and 4 normal transmitting males were also identified. Four severely retarded males and their mothers carried unmethylated premutations, while a moderately retarded girl had a deletion of ≈150 bp. Overall sizing of the CGG repeat produced an allele distribution of 6-58 CGG repeats (mean 28-30), similar to that in other Caucasian populations.*

Fragile X syndrome (FXS) accounts for almost 50% of the X-linked mental retardation (MR) and is estimated to affect approximately 1:4000 males and 1:7000 females (1-4). Patients present with mental retardation (IQ scores 20-60) associated with hyperactivity, hand flapping, avoidance of eye contact and "autistic-like" behavior. The syndrome is characterized by mild facial abnormalities including increased head circumference with typical elongated face, large protruding ears and coarsening of facial features. Macroorchidism is also noted in most post-pubertal males (5-7).

In the majority of cases, the molecular aberration underlying the disorder is caused by hyperexpansion of a polymorphic CGG repeat in the 5' UTR of the FMR-1 gene (8, 9). The repeat varies from 6 to 54 units, while mild expansions of 60-200 repeats, known as premutations (PM), are asymptomatic and characterize the carriers. Intermediate alleles of 41 to 60 repeats define a gray zone (GZ) and may or may not be unstably inherited. When transmitted by a female carrier, PMs may be further expanded to full mutations (FM) of more than 200 repeats (10, 11). FM is associated with abnormal hypermethylation of a CpG island upstream of the repeat, causing transcriptional shutting down of the gene (12). As a result, the FMR-1 protein (FMR-1P), an RNA-binding protein, is not expressed in the cells of FXS patients. In fewer than 1% of the cases, absence of the FMR-1P is not caused by the CGG expansion but is due to point mutations or deletions (13, 14).

Since the discovery of the gene, routine screening is feasible for the fragile X mutation, which can be offered to mentally handicapped individuals and their families.

This study reports on the results of a large-scale screening for fragile X syndrome among mentally retarded patients, as well as on the CGG distribution in the normal Greek population.

Patients and Methods

Since 1994, molecular analysis for fragile X has been performed in the Department of Medical Genetics of the Athens University, which is a referral centre in Greece. During this period, a total of 1,755 patients (1,426 males and 329 females, aged from 1-66

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years) and 1,733 parents or other family members were tested. In all cases, the reason for referral was mental retardation of unknown aetiology with or without other physical features. Cytogenetic studies had excluded other chromosomal anomalies. Most of the patients (57%), presented with borderline MR (IQ=70-85) often expressed as speech delay and learning difficulties. The remaining patients presented with mild (15%), moderate (16%), or severe (12%) mental retardation. During this period, 12 prenatal diagnoses were performed in mothers carrying pre- or full mutations.

In addition, 400 healthy individuals (350 females and 50 males), mainly blood donors or individuals undergoing DNA testing for thalassemia or cystic fibrosis carrier status, were also tested in order to determine the CGG distribution in the normal population. In all cases, informed consent was obtained.

DNA analysis. DNA was extracted from peripheral blood lymphocytes according to the Salting-out method (15). PCR analysis of the CGG repeat was performed according to a modified method based on the protocol by Fu *et al*. (10). Co-amplification of a CAG repeat from the androgen receptor gene was used as an internal control. Samples were preheated at 97˚C for 15 min. Thirty-five cycles were performed, composed of 45 s denaturation at 94˚C and 2 min annealing and extension at 68˚C. Finally, a last cycle of extension for two hours at 68˚C was performed. Fluorescent Texas Red (Qiagen, Hilden, Germany) or Cy5.5-labeled (Institute of Technology and Research, Heraklion, Greece) PCR primers were used and PCR products were electrophoresed on a 6% polyacrylamide denaturing gel using either a Vistra 725 automated sequencer (Molecular Diagnostics/Amersham Life Science, UK) or Visible Genetics Open Gene™ System automatic Sequencer with Gene Objects software (Visible Genetics, High Wycombe, UK). Data processing and CGG analysis was performed using either "FragmeNT" fragment analysis software (Visible Genetics, High Wycombe, UK) or Visible Genetics Open Gene™ System (Visible Genetics, High Wycombe, UK).

In all patients and all females individuals, without two distinct PCR products, Southern Blot analysis on EcoR1 or Hind III and Eag1-double-digested DNA was also performed, followed by hybridization with either StB12.3 or pP2 probes (16). Nonradioactive digoxigenin labeling and detection was applied for the hybridization probes as described in the protocols of the supplier using, PCR DIG probe synthesis kit (Cat No 1 636 090, Roche applied Science, Mennheim, Germany) and DIG luminescent DNA detection kit (Cat No1 363 514, Roche applied Science, Mennheim, Germany). The products were visualized after processing with the CSPD or CDP star chemiluminescence substrate (Cat No 1 685 627, Roche applied Science, Mennheim, Germany).

Comparative studies of patients and their parents allowed the elucidation of unresolved cases.

Results

Molecular analysis with sizing and characterization of the CGG repeat in 3888 individuals confirmed the diagnosis of FXS in 57 male and 4 female patients carrying methylated FMS, giving an overall frequency of ~3.5% of FXS among mentally retarded individuals in Greece. Mosaic patterns were revealed in four males carrying both methylated and unmethylated FMs, as well as both normal PM and FM alleles. One MR female patient carried a ~2.65 kbp HindIII/EagI fragment, which is 150 bp smaller than the normal 2.8 kbp allele. In addition, four males carried unmethylated premutations (60, 62, 75 and 74 CGG respectively). This expansion, however, cannot be definitely correlated with their phenotype.

Table I. *Correlation of MR status with molecular analysis results*.

Mental retardation	Molecular analysis		
	Normal (%)	Premutation (%)	Full mutation (%)
Borderline	100.00	-	0.00
Mild	97.90	-	2.10
Moderate	89.67	0.68	9.65
Severe	85.60	1.00	13.40

Molecular analysis for carrier status among 239 family members identified 8 women carrying methylated full mutations and 58 permutation carriers, 4 of which were normal transmitting males. In all families with FXS syndrome, the mother was a carrier of PM or FM and no *de novo* mutations were revealed. Among the 12 prenatal tests performed, 5 affected and 7 healthy embryos were diagnosed.

Table I shows the correlation of MR status with mutation analysis. No FXS patient was detected among individuals with borderline MR. Among individuals with mild MR 2.1% had FMs, among those with moderate MR 9.65% carried FMs, while among the severely MR 13.4% had FMs. Four premutation carriers were also detected, 2 with moderate and 2 with severe MR, although there is not enough evidence to correlate their phenotype with FXS.

CGG size variation and allele frequencies were determined in ~2,500 X chromosomes analysed. Forty different alleles (6-58 repeats) were observed with the most common allele carrying 28 CGG (27.9%) (Figure 1). The majority of these alleles (94.8%) were less than 39 repeats. As shown in Table II, a total of 145 alleles carrying 39-58 repeats were detected and were characterized as intermediate, or GZ. Extended studies in three families of interest proved that these GZ alleles were unstable and underwent a mutation leading to the detection of different repeat sizes between family members. In the first family, a maternal 39 CGG allele increased in size to 58 CGG when transmitted from a grandmother to her daughter and grandson. In the second family, a paternal 54 CGG allele was altered when inherited from father to daughters carrying 52 and 58 repeats respectively and in the third, a maternal 42 CGG allele was transmitted as a 45 CGG allele to her son.

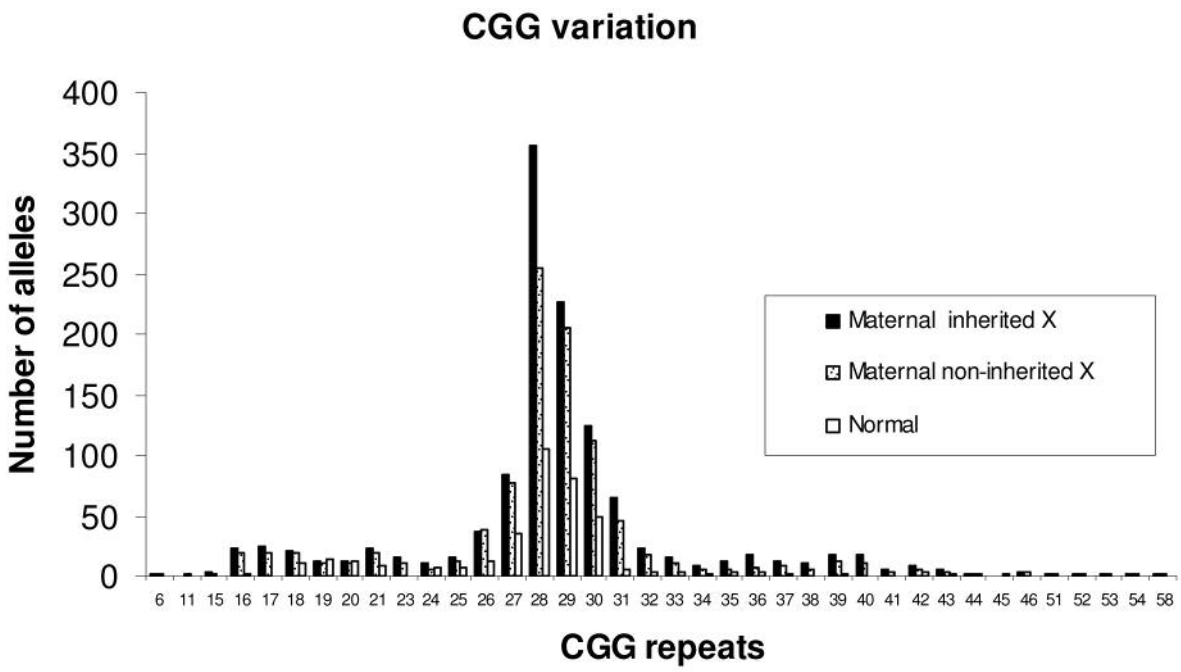

Figure 1. *Distribution of CGG alleles among Greek individuals.*

Discussion

The overall frequency of ~3.5% FXS patients found in this study among Greek mentally retarded individuals is markedly lower when compared with the previous epidemiological study on FXS in Greece, which, based on cytogenetic findings, reported an incidence of 6.5% (17). On the other hand, two molecular surveys concerning Greek patients reported FXS rates of 1.15% (18) and 0.9% (19) respectively. This discordance is probably due to differences in the techniques used and selection of samples analyzed. In the first study, the overestimation of FXS is possibly due to the cytogenetic detection of the fragile site, which does not allow the distinction between the X-fragile site (*FRAXA*) and other fragile sites located in the same region (20). In the two molecular studies, samples either consisted of patients with nonspecific MR and without typical phenotypic features of the syndrome or positive family history (18), or concerned a selected population based on a protocol that excluded obvious clinical syndromes, chromosomal abnormalities and metabolic disorders (19). Although the present study surveyed a large MR population and estimated the occurrence of FXS among MR patients (3.5%), an exact calculation of the frequency of FXS in the Greek population is not possible, since this would require larger screening of general unselected individuals such as newborns or elementary school students across the country.

It is generally accepted that every male with nonspecific mental retardation is entitled to fragile X testing. However, since mental retardation especially in younger patients could be differently scored, there was a difference between clinical overdiagnosis and molecular underdiagnosis of FXS. This could possibly be due to socioeconomic differences of the families, leaving a large percentage of FXS patients undiagnosed, either because they were institutionalized and never clinically examined, or because their symptoms were too mild to be correlated to a genetic syndrome. In addition, since recent studies among severely retarded institutionalized individuals detected no FXS patients, it is possible that other, until now understated, molecular aberrations, such as subtle chromosomal rearrangements, could be a more common cause of severe MR (21).

Table II. *Gray zone alleles detected in the individuals tested.*

	39-44 CGG	45-50 CGG	50-55 CGG	55-60 CGG
Maternal X				
Inherited	77	8	6	6
Not inherited	30	4	1	1
General population	8	2	-	2

As far as the GZ alleles are concerned, this survey's findings support the significant role of the stability of the repeat and indicate that the risk of expansion is not necessarily related to the size of the repeat. At present, it is believed that stability of the CGG sequence is maintained by

the presence of an AGG triplet – every 32-34 CGG triplets – which prevents the formation of hairpins and loops leading to the CGG expansion. Although loss of AGG interspersions has been related to the instability of the repeat region (22, 23), recent studies report that this may not be the case at least for the transition from intermediate to premutation alleles (24). Further studies of the GZ repeats are expected to provide information concerning a possible mechanism involved in the transition from normal to unstable alleles. In our study, an excess of intermediate/premutation alleles was detected among the maternal X transmitted to their affected children (~5.2%) in comparison with the noninherited X (2.75%) and the control group (2.8%). Other studies have also reported the same observation, suggesting that the presence of GZ/PM alleles is somehow correlated with the FXS phenotype (25-27). More studies, however, with larger sample sizes are needed to elucidate this. The detection of GZ unstable alleles in the Greek population implies a possible need for carrier screening among females of a reproductive age, especially since recent studies report variable frequencies, ranging from 1:250 to 1:69 female carriers of a 50-100 CGG allele (16 and 28-31). In the present report, the frequency of female carriers with intermediate alleles of 50-60 CGGs is approximately 1:130 among Greek women. Although this incidence is not as high as that reported by Berkenstadt *et al*. (32), further studies should be performed in order to evaluate the true carrier status among the general Greek population.

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