SHORT REPORTS

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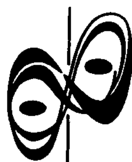

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Combined Molecular and Cytogenetic Analysis for the Rapid Diagnosis of Fragile X Syndrome

C. Bussani Mastellone[1], M.L. Giovannucci Uzielli[1], M. Grasso[2], P. Chiurazzi[3], G. Neri[3], Q. Wang[4]

[1] *Human Genetics Service, University of Florence, Florence, Italy;* [2] *Genetics Centre, Galliera Hospital, Genoa, Italy;* [3] *Institute of Medical Genetics, Catholic University, Rome, Italy;* [4] *Division of Medical and Molecular Genetics, Paediatric Research Unit, Guy's Hospital, London, England*

Abstract. The fragile X mutation is the result of an abnormal expansion of a CGG repeat sequence in the FMR-1 gene.

Molecular techniques enable the detection of the mutation and also of the exact length of this DNA sequence, allowing the classification of the tested subjects as normal, carrier or affected.

We propose a protocol of analysis that combines a method of non-radioactive PCR, Southern blotting and cytogenetic testing.

This protocol can be used for screening programme of selected groups of mentally retarded individuals and for prevention studies in families at risk.

Key words: Fragile X syndrome, FRAXA locus, Non-radioactive PCR

INTRODUCTION

The fragile X syndrome is the most frequent cause of inherited mental retardation. The high prevalence of the disease (1 in 1300 males and 1 in 2500 females) urgently requires technical methods that allow rapid analysis of many individuals suspected of fragile X syndrome and subjects at risk because of a family history.

The fragile X syndrome is associated with a folate-sensitive fragile site on chromosome Xq27.3. However, the limit of the cytogenetic analysis is the variable percentage of the chromosome marker, ranging from 2-3 up to about 60% in affected subjects. Only about 50% of carrier females express the fragile site; moreover, such expression takes place in a very low number of metaphases. Transmitting males are cytogenetically normal.

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The molecular defect consists of the abnormal expansion of a repetitive trinucleotide DNA sequence (CGG)n, contained within the fragile X mental retardation gene 1 (FMR1), in the FRAXA locus at Xq27.3 [1]. In normal individuals, the number of CGG repeats is variable, ranging from 5 up to 52. Phenotypically normal carriers have up to approximately 200 copies (premutation), while affected individuals have DNA amplification consistent with several hundred to several thousand copies of the CGG repeat (full mutation) [2, 3].

Analysis at the molecular level provides a good diagnostic tool and must be considered the first choice for investigation alone or in combination with cytogenetic testing.

We present an analytic protocol that utilises different combinations of a non-radioactive PCR method, Southern blotting and cytogenetics.

MATERIALS AND METHODS

Cytogenetic analysis was performed according to standard procedures (TC 199 medium, 6% FCS, 3.7% phytohaemagglutinin).

DNA was obtained from peripheral blood leucocytes, using sarkosyl and proteinase K, with a saline extraction (NH_4 acetate 7,5 *M*).

Aliquots of 10 μg DNA were double-digested overnight at 37 °C with 50 u *Eco*RI and 50 u *Eag*I, separated in 0,8% agarose gel, with a 20-cm migration of bromophenol blue. Southern blotting was performed using Hybond N+ membrane (Amersham) [4]. Probe StB 12.3 (kindly provided by Dr. Jean-Louis Mandel, Strasbourg) was used for hybridisation.

PCR amplifications were carried out in a PTC-100 (MJ Research) termocycler, with a non-radioactive protocol, which yields a product of 311-base pairs for a normal 30-repeat allele.

100 ng genomic DNA were mixed with 30 pmol of each primer (FXc: 5'-GCT CAG CTC CGT TTC GGT TTC ACT TCC GGT-3'; FXf: 5'-AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA-3') [2], in a total volume of 50 μl containing Taq polymerase buffer (33.5 m*M* Tris-HC1 ph 8.8, 8.3 m*M* $(NH_4)_2SO_4$, 1 m*M* $MgCl_2$ and 85 μg/ml BSA) and 200 μ*M* each of dATP, dCTP and dTTP, 150 μ*M* dGTP, 50 μ*M* 7-deaza-dGTP, 10% dimethylsulphoxide. The samples were heated to 95 °C for 10 min (hot start procedure). Following the addition of 2.5 U Taq polymerase, the samples were subjected to 30 cycles of amplification (95 °C, 1 min; 63 °C, 1.5 min; 72 °C, 2 min) with a final extension of 5 min at 72 °C. Products were analysed directly under UV light after electrophoresis in a 3% agarose gel (Metaphore, FMC), containing ethidium bromide.

When duplex PCRs were performed, the CAG repeat in the androgen receptor was simultaneously amplified, using 10 pmol of each primer (ARa: 5'-ACC AGG TAG CCT GTG GGG CCT CTA CGA TGG GC-3'; ARc: 5'-CCA GAG GCC GCG AGC GCA GCA C-3').

This method allows visualisation of all normal alleles and also premutations up to about 100 repeats. Affected males are indirectly deduced from the lack of amplification due to the large (1,000-2,000 bp) expansion present in these subjects.

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RESULTS

We analysed 375 subjects, including individuals clinically suspected of fragile X syndrome, subjects previously ascertained as affected or as being carriers through cytogenetics, and relatives at risk.

Using a non-radioactive PCR method, we found 35 premutated carriers (4 males and 31 females), 45 full-mutated patients (37 males and 8 females) and 295 individuals (199 males and 96 females) with alleles in the normal range.

All subjects were first tested by the PCR method. Southern blotting was performed in 150 of the 375 individuals. Cytogenetic testing was carried out in all cases, both for confirmation of PCR results and for exclusion of other chromosomal abnormalities.

Following PCR analysis, normal males showed a single allele of 5-52 repeats for the FRAXA locus. All affected males were characterised by the absence of amplification.

Diagnosis of normal transmitting males was achieved, using PCR, in 4 individuals, with the visualisation of an allele in the premutation range. A male with mental retardation who presented a premutated allele was ascertained to be mosaic after Southern blotting.

Using PCR amplification, normal females presented one or two fragments in the normal range. Carrier females showed only one normal allele. In most cases, the expanded allele in the premutation range was visible. Full mutated females showed only a single normal allele.

Southern blotting is necessary for the exact interpretation of all cases with only one normal allele in females and also in males with a lack of amplification. Southern blotting is also useful for the identification of mosaic subjects.

DISCUSSION

Our protocol could be very useful for a programme of diagnosis and prevention of fragile X syndrome. In fact, it is important to emphasise that only 10% of all referred individuals suspected of fragile X syndrome will actually be affected.

The first step of the protocol, non-radioactive PCR, compared to Southern blotting, limits the use of radioactive substances and requires a minimal amount of DNA.

Because of the small length of the amplified products, the size resolution between the alleles is improved, which has the advantage of detecting alleles in the "intermediate" zone between the normal and the premutation range.

However, Southern blotting must be performed to confirm full mutations, the largest premutation alleles and also apparently homozygous females.

Our protocol reduces the time-consuming cytogenetic search for the fragile X marker, with a global cost, which is lower than that of Southern blotting and cytogenetics combined.

The protocol we propose is suitable for a screening programme of families at risk.

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Correspondence: Dr. Cecilia Bussani Mastellone, Human Genetics Service, University of Florence, Via Masaccio 209, Florence 50132, Italy.

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