Original articles

Evaluation of a mutation screening strategy for sporadic cases of ATR-X syndrome

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Received 3 July 1998
Revised version accepted for publication 20 November 1998

Abstract

We report on the evaluation of a strategy for screening for XNP/ATR-X mutations in males with mental retardation and associated dysmorphology. Because nearly half of the mutations in this gene reported to date fall into a short 300 bp region of the transcript, we decided to focus in this region and to extend the mutation analysis to cases with a negative family history. This study includes 21 mentally retarded male patients selected because they had severe mental retardation and a typical facial appearance. The presence of haemoglobin H or urogenital abnormalities was not considered critical for inclusion in this study. We have identified six mutations which represents a mutation detection rate of 28%. This figure is high enough for us to propose this strategy as a valid first level of screening in a selected subset of males with mental retardation. This approach is simple, does not require RNA preparation, does not involve time consuming mutation detection methods, and can thus be applied to a large number of patients at a low cost in any given laboratory.
(*J Med Genet* 1999;**36**:183–186)

Keywords: mental retardation; ATR-X; mutation; zinc finger

Mutations in the zinc finger helicase XNP/ATR-X are responsible for an X linked mental retardation syndrome, the ATR-X syndrome (MIM 301040).[1] This syndrome was first identified by clinical observations of mentally retarded patients with mild α thalassaemia, but without rearrangements or mutations at the α globin locus on chromosome 16. The pedigrees of familial forms of this syndrome further indicated that the ATR-X locus was located on the X chromosome.[2] Careful clinical observation of patients established a core of clinical signs, which were present in the majority of the patients. These included severe mental retardation, characteristic facial dysmorphism, microcephaly, anomalies of the urogenital tract, and α thalassaemia.[3]

The discovery of the gene involved in this disorder enabled a search for mutations in patients with similar but not identical clinical phenotypes. We have subsequently identified mutations in this gene in families affected with Juberg-Marsidi syndrome, Carpenter-Waziri syndrome, and males with mental retardation without α thalassaemia.[4–7] These studies have shown that the phenotype associated with XNP mutations can be extended and that only the severe mental retardation and the facial dysmorphism were constant features.

Sporadic cases constitute the majority of the mentally retarded population. Among them, there are probably males affected with an X linked mental retardation disorder. However, the absence of a conclusive X linked inheritance pattern and the impossiblity of drawing a consensus clinical picture based on other affected subjects in a family render the diagnosis less than definitive. However, it is of great importance for genetic counselling to screen for mutations in these sporadic cases in order to determine the risk of transmission of a recurrent mutation in the family. As mutations in the XNP gene lead to a severe phenotype, and because of the great demand for genetic counselling from families, we decided to screen for XNP mutations in mentally retarded males who had a negative family history.

As the most characteristic features, α thalassaemia and genital abnormalities, were not consistently present in ATR-X patients, we screened patients on the basis of less selective criteria, severe mental retardation associated with hypotonic facial features. Concerning the screening strategy, a detailed mutation screening approach in a large number of patients, although feasible in theory, would be expensive and time consuming because of the large size of the XNP transcript (10.5 kb[8]). Moreover, the complex organisation of the gene (35 exons[8]) renders the analysis unrealistic when only genomic DNA is available.

To date, 27 different mutations in 43 pedigrees have been described in the XNP/ATR-X gene. Out of these known mutations, 11 are located within the region coding for the three zinc finger domains of the protein (exons 7, 8, and the beginning of exon 9[8 9]). This

represents 41% of the known mutations localised in 4% of the coding region. This striking observation led us to investigate whether a mutation screening strategy based on the analysis of the 300 bp zinc finger coding region would be of interest in the detection of sporadic cases of ATR-X. This approach has the advantage of being quick and inexpensive, and the ability to be used in a larger scale screen for mutations.

We have performed a systematic screen for mutations of the zinc finger region in 21 sporadic cases fitting our criteria (severe mental retardation and facial dysmorphism). We found that in 28% of the cases a mutation was present in this region of the transcript. We thus propose that systematic analysis of exons 7-9 of XNP in males who have severe mental retardation associated with a hypotonic facies should be considered.

Methods

PRIMER PAIRS

Two primer pairs were used in order to sequence the zinc finger coding region from the XNP transcript. The first primer pair was 5'Di (5'-GGA TTA TGA GGT ATT TCA TGT CT-3')/5'DRi (5'-CTC AAA GG CCT GGT ATA TGG-3'), which amplifies exon 7 and its flanking intronic boundaries. The second primer pair was 5'Ci (5'-CAG TGT CCT GGA GAT TTT CC-3')/5'R (5'-GTG CGG AAT AAG AGT AGG TTA C-3') which amplifies the 3' boundary of intron 7, exon 8, intron 8, and 370 bp at the beginning of exon 9.

PCR AND SEQUENCING REACTIONS

PCR reactions were performed using 200 ng total genomic DNA in a final volume of 50 µl. Annealing temperature was 58°C for all primer pairs; 32 cycles of 50 seconds denaturation, 50 seconds annealing, and one minute extension were performed using 2.5 U of *Taq* polymerase. The PCR products were directly sequenced using primers 5'Di for the product obtained with primers 5'Di and 5'DRi, and primers 5'Ci and 5'R2 (5'-TTT GAA CTA GTC TTC TTT GGA G-3') for the product obtained using primers 5'Ci and 5'R.

Results

PATIENTS

A summary of the phenotypic traits of the patients is presented in table 1. The patients were selected on the basis of their having severe mental retardation as well as dysmorphic features, although the extent of the dysmorphology was variable (fig 1). The presence of genital anomalies or Hb H was not considered to be valid for inclusion in this study. The original diagnosis on first referral varied among the patients. For some of them, ATR-X was initially considered. One patient was first diagnosed as affected with Coffin-Lowry syndrome (patient 3) and the phenotype of another one was considered to be the result of birth trauma (patient 1). All the clinical signs were re-evaluated by trained clinical geneticists in order to obtain the present subset of patients.

MUTATION SCREENING

For each patient, the zinc finger coding region (exons 7, 8, and the beginning of exon 9) was amplified from genomic DNA. Each sequence was compared with the reference sequence

Table 1 Phenotype of the 21 patients studied

	Patient																				
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*
Age at assessment (y)	1.3	2.3	o	4.5	6.5	18	o	19	5	o	3	5	10	4	6	7	7	10.5	3	1	8
Birth weight (g)	2700	3100	o	2600	o	3200	o	3400	o	o	o	2400	2900	3100	3900	3300	2400	4000	3400	3200	3100
Facial																					
Microcephaly	+	+	+	+	+	+	−	−	+	o	+	−	+	+	+	−	−	+	+	+	−
Epicanthic folds	+	+	+	+	+	+	o	−	+	−	+	−	−	+	+	+	−	+	+	+	−
Flat nasal bridge	+	+	+	+	+	+	+	−	−	+	+	+	−	+	−	+	+	+	−	o	−
Midface hypoplasia	+	o	+	−	+	+	−	+	−	+	+	o	+	o	+	+	+	+	+	+	+
Small, triangular nose	+	+	+	+	−	+	+	−	−	−	+	o	−	o	−	−	−	+	+	o	−
Anteverted nostrils	+	+	+	+	+	+	+	−	−	−	+	o	+	o	−	−	−	+	+	o	−
Triangular mouth	+	+	+	+	+	+	+	−	o	−	+	o	+	o	−	+	+	+	+	o	+
Widely spaced incisors	+	+	+	+	+	+	+	+	−	−	−	o	+	o	+	+	o	−	+	o	+
Abnormal ears	+	+	+	+	+	−	+	+	−	+	+	o	−	+	+	−	−	+	+	+	−
Astigmatism/strabismus	−	o	o	+	+	+	o	−	+	−	+	+	+	+	o	+	o	+	o	o	o
Genital																					
Cryptorchidism or high lying testes	−	+	−	+	−	+	+	−	+	−	−	+	−	−	+	−	−	+	+	+	+
Hypospadias	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−
Skeletal																					
Clino-/camptodactyly	+	+	−	o	+	−	o	−	−	−	−	+	−	+	+	+	o	−	−	o	+
Syndactyly	−	−	−	o	+	−	o	−	−	−	+	−	−	o	−	−	o	+	−	o	−
Brachydactyly	−	−	−	o	−	−	o	−	−	−	−	+	−	o	o	−	o	−	−	+	−
Polydactyly	−	−	−	−	−	−	o	−	−	−	−	−	−	o	−	−	o	−	−	+	−
Joint hyperlaxity	−	o	+	+	+	−	o	−	−	−	−	−	o	o	−	o	o	−	o	o	−
Hb H inclusions	+	+	+	−	+	+	−	o	−	o	−	−	−	+	−	−	+	−	−	−	o
Neurological																					
Severe mental retardation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Seizures	−	−	−	+	o	+	−	+	−	−	−	+	+	o	+	−	−	−	o	−	+
Cortical atrophy	+	−	+	o	−	−	−	−	−	o	−	−	−	o	−	+	−	−	+	o	−
Hypotonia	+	+	+	+	+	+	+	+	+	o	+	+	+	o	+	+	o	+	+	+	o
Miscellaneous																					
Vomiting/reflux	+	+	−	+	o	o	o	−	−	−	−	+	+	o	+	+	−	−	o	o	−
Severe constipation	+	+	−	−	o	o	o	−	o	−	−	−	+	o	+	−	−	−	+	o	−
Hearing loss	−	−	−	+	−	−	−	−	+	−	−	−	+	o	o	o	o	+	o	o	−
Mutation	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+ = present, − = absent, o = data not available.

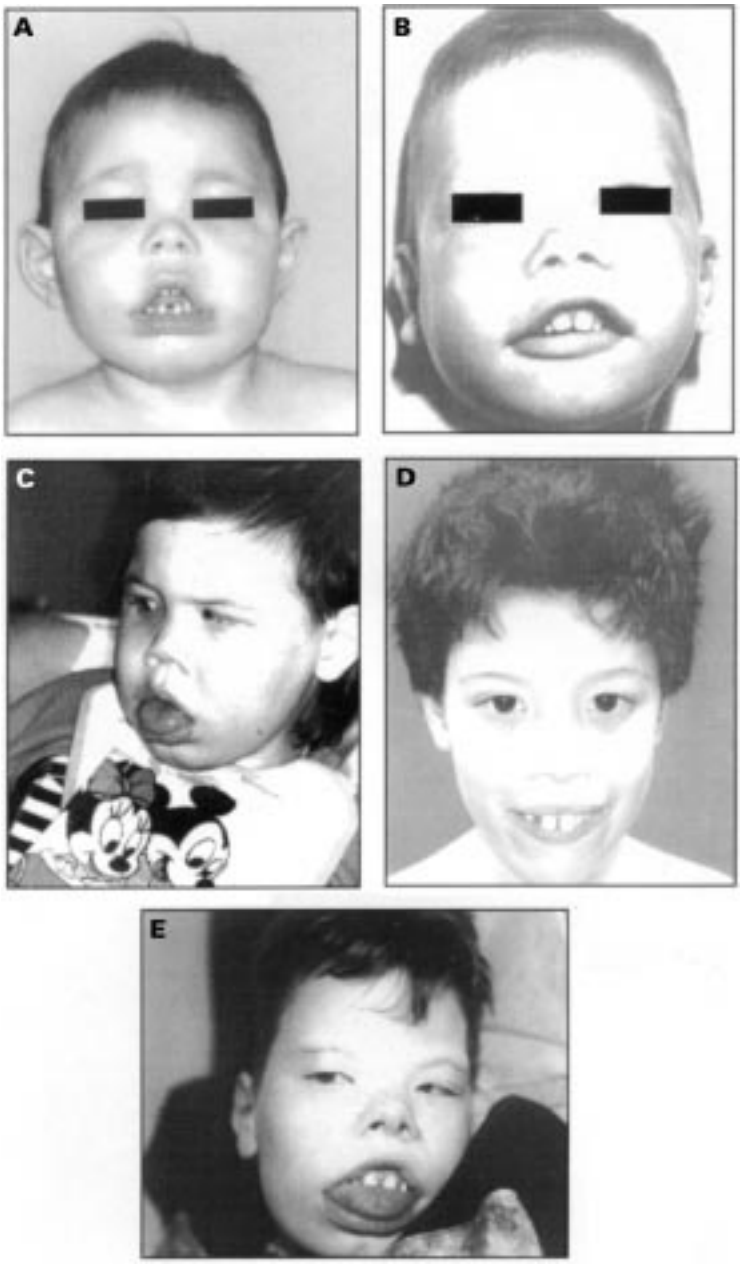

Figure 1 Pictures of five out of six patients in whom a mutation was identified. The dysmorphic features of the different patients are variable, being more pronounced in patients 2, 4, and 6 (detailed in table 1). (A) Case 2. (B) Case 3. (C) Case 4. (D) Case 5. (E) Case 6. (Photographs reproduced with permission.)

(GenBank accession number U75653). A mutation was identified in six patients (table 2). In each case, enzymatic digestion confirmed that the mutation was indeed present in the genomic DNA and absent from 100 unrelated X chromosomes (data not shown).

Table 2 Summary of the mutations identified in the patients analysed. In each case, the nucleotide and the amino acid change are indicated together with the number of the corresponding exon. Nucleotide and amino acid numbers are given according to GenBank entry U75653. The status of the mother's DNA is indicated when known

Patient ID	*Nucleotide change*	*Amino acid change*	*Exon*	*Mother's status*
1	C775T	Pro190Ser	7	Unknown
2	G731A	Gly175Glu	7	Carrier
3	A863C	Gln219Pro	8	Carrier
4	A743G	Δ (Val178-Lys198)	7	Carrier
5	G943T	Arg246Leu	9	Carrier
6	G952T	Gly249Cys	9	Not carrier

The first mutation, identified in patient 2, is located in exon 7 and causes the replacement of a glycine by a glutamic acid residue (G175E). It is the most N-terminal mutation identified to date (fig 2). The second mutation, identified in patient 3, is located at the 3' end of exon 8 and changes a glutamine to a proline (Q219P). The third mutation, identified in patient 6, is located in exon 8 and causes the replacement of a glycine by a cysteine residue (G249C). The fourth mutation, identified in patient 1, is located in exon 7 and changes a proline into serine (P190S). The fifth mutation, identified in patient 4, is located in exon 7. It causes the splicing out of 63 nucleotides (confirmed by RT-PCR on the patient's lymphocyte RNA, data not shown) coding for 21 amino acids (from V178 to K198) and the maintenance of the open reading frame thereafter, potentially giving rise to the production of a protein lacking 21 amino acids within itself. The sixth mutation, identified in patient 5, is located in exon 9 and causes the replacement of an arginine residue by a leucine (R246L). For mutations 1, 5, and 6, another mutation has already been reported at the same residue but involves another non-conservative change (P190A, R246C, and G249D respectively[9]).

The mutations were looked for in the patients' mother's DNA when possible. For patients 2, 3, 4, and 5, the mutation was present in their mother's DNA. In the case of patient 6, the mutation was absent from his mother's DNA indicating that it could be a de novo mutation or the result of gonadal mosaicism, which may occur in ATR-X syndrome.

Discussion

The only constant features in ATR-X syndrome patients are the severe mental retardation and facial dysmorphism. It has been documented that neither the α thalassaemia nor the urogenital anomalies are constant signs.[4–7] Furthermore, the reliability of the method used to detect Hb H inclusions is highly variable among laboratories. Thus, the number of patients affected by mutations in the XNP/ATR-X gene could be underestimated because of a bias in selection. A systematic screening of the XNP/ATR-X gene in severely mentally retarded sporadic male cases with ATR-X-like dysmorphology was thus undertaken. For speed and economy, we decided first to screen for mutations in the zinc finger domain, in which more than 40% of mutations have been described so far.[9]

The screening of 21 patients, selected by trained clinical geneticists, led us to identify six mutations (table 2). This represents a mutation detection rate of 28%. Although based on a relatively small number of people, this figure is high enough for us to propose this strategy as a valid first level of screening. Using this approach systematically will have many advantages. First of all, it is very simple, can be conducted on any DNA sample, does not require RNA preparation, and does not involve time consuming mutation detection methods. Furthermore, because it is simple, it can be applied

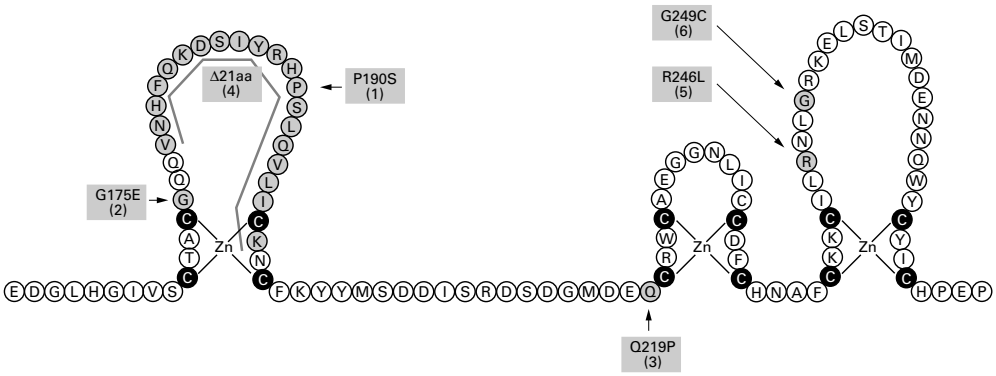

Figure 2 Schematic representation of the three C_2-C_2 zinc fingers of the XNP protein. Exon 7 is coding for R160 to K198, exon 8 for N199 to R221, and exon 9 for W222 to S1245 (the diagram stops at P272). The six mutations reported in this study are indicated in shaded boxes together with the corresponding patient ID. Amino acid residues affected by a mutation are shaded except for the zinc coordinating cysteines which are shown in black.

to a large number of patients at a low cost in any given laboratory.

The phenotypes of the patients studied (table 1, fig 1) do not allow one to make clear genotype-phenotype correlations. However, it seems that the most important diagnostic criteria are hypotonic facial features and the presence of associated microcephaly. We would recommend that these two clinical signs be considered when selecting patients for mutation screening in this region of the XNP/ATR-X gene. Although five out of six patients in whom a mutation was identified have Hb H inclusions, we think the absence of inclusion bodies should not be considered as an exclusion sign, because several ATR-X mutations have been reported[4–7] (this study) in patients where no inclusions could be detected. In the present subset of patients, the Hb H inclusion tests gave highly variable results when performed. For instance, patient 6 was positive when the test was performed for the third time. However, when the Hb H inclusion test is negative, we would suggest paying particular attention to the dysmorphic features before initiating the mutation screening.

Regarding the location of the mutations (fig 2), it is of interest to note that a hot spot for mutations (nucleotide change C942T and corresponding amino acid change R246C) was reported in the zinc finger coding region of the gene.[9] None of the mutations described in this paper affect the nucleotide transition (C942T) described in that report. Surprisingly, one of the families reported by Gibbons *et al*[1 9] (pedigree 16) has been described both with a "hot spot" mutation (R246C) and with a nonsense mutation at the end of the coding region (R2386*) supposed to confer the phenotype. It is thus legitimate to wonder if the so-called hot spot is the cause of the observed phenotype. In the light of these observations, we would recommend screening of the whole zinc finger coding region instead of only looking for the few residues affected with known mutations, and to check for the presence of an eventual rare polymorphism in 100 unrelated X chromosomes originating from the same population.

A question still unanswered is what about the rest of the patients without a "zinc finger mutation"? A review of the clinical presentation cannot distinguish them from those in which a mutation was identified; no obvious clinical criteria discriminated between patients with or without a mutation in this study. Two explanations are possible: either some of them are affected with a phenocopy of ATR-X (possibly not of genetic origin) or they have a mutation elsewhere in the XNP gene.

For this reason, and owing to the size of the gene (350 kb, 35 exons, and an ORF of 7 kb), a robust mutation detection aproach will be needed in order to screen the whole transcript, in sporadic and familial ATR-X patients, as well as in different phenotypic presentations where one can expect to find mutations elsewhere in the gene. We are in the process of setting up such an approach in the laboratory.

We thank Dr R Curtis Rogers, Gail Anderson, and Debbie Bealer for their assistance, Ms Judy Franklin, UAB, for assistance in obtaining photographs of patient 4, Ms Gail Anderson for assistance with patients 8 and 9, and Dr L Colleaux for helpful discussion. The work was supported in part by INSERM program PARMIFR. Funding to CS was by a NICHD grant (2RQ1HD26202) and the South Carolina Department of Disabilities and Special Needs.

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