Exclusion of CAG repeat expansion as the cause of disease in autosomal dominant retinitis pigmentosa families

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Abstract

The involvement of genes with expanded tracts of (CAG)_n in some neurodegenerative diseases is well established. Whether genes containing these motifs could also have a role in degenerative diseases affecting the retina, which is also neural in origin, is unknown. We investigated (CAG)_n expansions as a cause of disease in a panel of eight autosomal dominant retinitis pigmentosa (ADRP) pedigrees, including families known to map to the RP9, RP11, and RP13 loci, using the technique known as "repeat expansion detection" (RED). An expansion was detected in one of the unlinked families, but it did not segregate with the disease and was thus non-pathogenic. Expansions were not detected in any other families. In conclusion, expanded (CAG)_n repeats are not the cause of disease in the families we have studied, but given the high level of heterogeneity in RP and in retinal degenerations in general they remain strong candidates for involvement in other forms of retinal dystrophy.

(J Med Genet 1997;34:130-132)

Keywords: retinitis pigmentosa; CAG; repeat expansion detection.

Expansion of (CAG)_n triplet repeats within the coding sequence of genes cause at least five dominant neurodegenerative diseases, each of which is characterised by the loss of specific subpopulations of neurones.¹ A common feature of some neurodegenerative and psychiatric disorders is a progressive increase in the severity of the condition through successive generations. This phenomenon, known as anticipation, has been shown to be indicative of the expansion of such repetitive elements, underlying the pathological process.²⁻³ In some triplet repeat expansion (TRE) diseases anticipation is not observed, but even in these the size of an expansion is known to correlate well with the clinical severity.⁴⁻⁵

Retinitis pigmentosa is a progressive, inherited retinal degeneration characterised by restriction of the visual field (tunnel vision), night blindness, and abnormal pigmentation in the peripheral retina.⁶ A range of modes of inheritance have been observed, including autosomal dominant, autosomal recessive, and X linked forms,⁷ as well as more complex digenic RP.⁸ At least 17 different loci have been

identified. Of these, six are associated with mutations in known photoreceptor specific genes,⁹ while the remainder are defined only by linkage to anonymous loci. In about one third of autosomal dominant cases, mutations in the genes coding for rhodopsin¹⁰ or RDS-peripherin¹¹⁻¹² are associated with the disease. This leaves the underlying cause in most cases of dominant RP unaccounted for. This is therefore an extremely heterogeneous disease with a complex pathology. Since the retina is essentially a neural tissue, it would be reasonable to speculate that some forms of RP could be caused by triplet repeat expansions.

We therefore investigated the possible involvement of expanded CAG/CTG repeats in a panel of autosomal dominant retinitis pigmentosa families using the technique of "repeat expansion detection".¹³ With this technique the presence of triplet repeat expansions anywhere in a person's genome is shown as a series of ligated (CTG)₁₇ oligomers. These are generated in a thermal cycling reaction containing genomic DNA, the (CTG)₁₇ oligomer, and a thermally stable DNA ligase. Extended tracts of CAG in the genomic DNA allow (CTG)₁₇ oligomers to align themselves adjacent to each other, making them a substrate for the ligase. A single ligation product generates a (CTG)₃₄ band, this being the upper limit of non-pathogenic expansions at triplet expansion loci associated with dominant disease phenotypes. However, shorter expansions have been shown to cause X linked spinal and bulbar muscular atrophy.⁵ The (CTG)₃₄ ligation product also serves as a positive control for the reaction, though it is not known if all expanded CAG loci are equally detectable. Larger expansions (to 51, 68, 85, and more CAGs) have been seen in a proportion of normal controls in several studies. These presumably derive from one or several other (CAG)_n tracts in the genome, at which expansions are not in themselves pathogenic.

Eight ADRP families were studied, all of which had been screened for rhodopsin or RDS mutations and found negative. In three of the families studied, the disease mapped to known ADRP loci on chromosomes 7p (RP9),¹⁴ 17p (RP13),¹⁵ and 19q (RP11),¹⁶ for which the genes involved have not yet been identified. For the remaining five families, the chromosomal location of the disease loci was unknown. Among the group of unlinked families there was one in which there was an apparent increase in severity of the disease in the

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Received 24 June 1996
Revised version accepted for
publication
4 September 1996

Figure 1 The pedigree of the ADRP family potentially displaying anticipation. Members of the family who carry the expanded (CAG)_n allele are indicated by an asterisk. Numbers below the symbols denote alleles for D18S499.

lower generations (fig 1). Also, with both RP9 and RP11 there exists considerable variability in the disease phenotype, both in its severity and the age of onset.^{17 18} Such variability could be explained by dynamic variation of an expanding and contracting CAG repeat.

Methods

Affected and normal members of the ADRP families used in this study were assessed as follows. First an ophthalmic history was taken to include details of visual acuity loss and age of onset of night blindness, then visual fields were determined. In addition, a brief general medical history and physical examination were undertaken to exclude the possibility of other defects cosegregating with RP in these families. Specifically, no members of these families gave a history indicating any neurological disease. Pupils were then dilated to allow fundus examination. Some patients also underwent more detailed psychophysical and electrodiagnostic tests.^{17 18} In the light of the apparent partial penetrance of the disease in the family shown in fig 1, key subjects were re-examined fundoscopically three years after their initial assessment to look for signs of disease progression, but they remained normal on re-examination.

Genomic DNA was prepared from peripheral blood lymphocytes of RP patients and their relatives. The RED technique was then performed essentially as it was originally described¹³ incorporating some slight modifications.¹⁹ The ligation mixture consisted of 50 ng of 5'-phosphorylated (CTG)₁₇, 4 U *Pfu* ligase (Stratagene), and 5 µg of genomic DNA in 20 mmol/l Tris-HCl (pH 7.5), 20 mmol/l KCl, 10 mmol/l MgCl₂, 0.1 mmol/l ATP, 1 mmol/l DTT, and 0.1% NP40 made up to a final volume of 20 µl. Thermal cycling of the mixture was carried out in a Perkin Elmer GeneAmp PCR System 9600 at an annealing ligation temperature of 77°C for 45 seconds and a denaturing temperature of 95°C for 10 seconds over 198 cycles. At that point each reaction was replenished with 4 U *Pfu* ligase, 5 µg genomic DNA, and subjected to a further 198 thermal cycles. The reaction products were fractionated on a denaturing, 6% poly-

Figure 2 Autoradiograph of the ligation products generated in a "repeat expansion detection" experiment. The samples are arranged in pairs of affected subjects from ADRP families. Lanes 9 and 10 contain products generated from the two severely affected sibs (IV.2 and IV.3) from the pedigree in fig 1.

acrylamide gel, then electroblotted onto Hybond-N+ positively charged nylon membrane. To visualise the RED products, the membrane was probed using a (CAG)₁₀ oligonucleotide end labelled with α-³²P dATP using terminal deoxynucleotidyl transferase. The hybridisation was carried out at 58°C in a standard phosphate buffer for 16 hours, followed by two stringency washes in a buffer containing 0.5% SSC and 0.5% SDS at 58°C. The autoradiograph was exposed overnight at -80°C using Kodak X-Omat film and two intensifying screens.

Results

The repeat expansion detection method (RED) was carried out as previously described^{13 19} (fig 2). Two affected subjects were studied from each of the eight families. In the families where the disease penetrance was variable (RP9 and RP11), severely affected subjects were studied to maximise the chance of observing an expansion if one was present. Ligation products of 255 bp (five oligos) were seen in two sibs from the family that appeared to display anticipation. On the strength of this result the rest of the available members of the pedigree were tested. However, subsequent analysis showed that the source of the expanded allele in the family was a normal person who had married into it (fig 1). The triplet expansion had been inherited by both normal and affected descendants. Ligation products that would be indicative of a CAG expansion were not seen in any of the other families, though in all cases the single ligation product was present indicating that the oligomer ligation reaction had occurred with that DNA sample.

Discussion

If triplet repeat expansion was the underlying cause of RP at the RP9, 11, or 13 loci or in a substantial proportion of the unmapped ADRP families tested, we would have expected to find that all affected members of such a family would have a repeat expansion above the normal range, consistent with a dominant mode of inheritance. The negative results from this study therefore suggest that expanded (CAG)_n repeats are not the cause of disease in this group of ADRP families. The detection of a non-pathogenic expansion makes us confident that we would have detected expansions had they been present. However, while (CAG)_n expansions do not cause RP in the families we have studied, they remain strong candidates to underlie some forms of inherited retinal degeneration.

Expansions beyond the (CAG)₃₄ level occur in the normal population in about 25% of people.¹⁹ In the original report describing the RED technique, an anonymous (CAG)_n expansion (RED-1) was genetically linked in a CEPH family to a microsatellite marker on chromosome 18. Analysis of this marker (D18S499) in the family described here shows at least one crossover between the marker and the expansion. Subjects III.3 and III.4 inherited different alleles from the father carrying the expansion, yet neither inherited the expansion. While this result argues against the expansion observed here being at the same location as RED-1, such tentative data cannot be said to have excluded the RED-1 locus. It remains to be seen whether there is a single active locus where non-pathogenic expansions occur or if such expansion events occur throughout the genome.

One further limitation in the interpretation of this study is that it was not possible to analyse DNA from the tissue affected, namely the retina. It has recently been suggested that tissue specific variation in the extent of repeat expansion can affect the presentation of the phenotype. In the case of dentatorubral-pallidoluysian atrophy it appears that there is a correlation between the degree of variation of repeat size and age at death, though an expansion was observed in all tissues examined.²⁰

In this study we have assumed that fully dominant expression would be the most likely model for the effect of an expanded (CAG)_n repeat in a retinal gene. While this has been the mode of expression in the neurodegenerative diseases that have been characterised so far, recent reports have also implicated (CAG)_n repeats as risk factors in schizophrenia^{19 21} and

bipolar affective disorder.²² It might thus also be appropriate to study the relative occurrence of TREs in a multifactorial retinal disease such as age related macular dystrophy.

We thank the Wellcome Trust (grant numbers 035535/Z/92 and 037331/Z/93) for funding our work and two anonymous reviewers for their helpful comments.

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