RP1 Protein Truncating Mutations Predominate at the RP1 adRP Locus

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PURPOSE. Recent reports have shown that the autosomal dominant retinitis pigmentosa (adRP) phenotype linked to the pericentric region of chromosome 8 is associated with mutations in a gene designated RP1. Screening of the whole gene in a large cohort of patients has not been undertaken to date. To assess the involvement and character of RP1 mutations in adRP, the gene was screened in a panel of 266 unrelated patients of British origin and a Pakistani family linked to this locus.

METHODS. Patients exhibiting the adRP phenotype were screened for mutations in the four exons of the RP1 gene by heteroduplex analysis and direct sequencing. Linkage of the Pakistani family was achieved using microsatellite markers. Polymerase chain reaction (PCR) products were separated by nondenaturing polyacrylamide gel electrophoresis. Alleles were assigned to individuals, which allowed calculation of LOD scores. Microsatellite marker haplotyping was used to determine ancestry of patients carrying the same mutation.

RESULTS. In the 266 British patients and 1 Pakistani family analyzed, 21 loss-of-function mutations and 7 amino acid substitutions were identified, some of which may also be disease-causing. The mutations, many of which were deletion or insertion events, were clustered in the 5′ end of exon 4. Most mutations resulted in a premature termination codon in the mRNA. Haplotype analysis of nine patients carrying an R677X mutation suggested that these patients are not ancestrally related.

CONCLUSIONS. RP1 mutations account for 8% to 10% of the mutations in our cohort of British patients. The most common disease-causing mechanism is deduced to be one involving the presence of a truncated protein. Mutations in RP1 have now been described in adRP patients of four ethnically diverse populations. The different disease haplotype seen in the nine patients carrying the same mutation suggests that this mutation has arisen independently many times, possibly due to a mutation hot spot in this part of the gene. (Invest Ophthalmol Vis Sci. 2000;41:4069–4073)

Retinitis pigmentosa (RP) is the term used to define a clinically and genetically heterogeneous group of retinal degenerations primarily affecting the rod photoreceptors. RP, which is one of the more common causes of genetic blindness, is characterized by progressive loss of vision, initially manifesting as night blindness and reduction in the peripheral visual field, and later involving loss of central vision.1 Affected individuals often have abnormal electroretinograms (ERGs) in the early stages, and significant visual impairment is usually present by middle age. Ophthalmoscopy typically reveals pigmentary disturbances of the midperipheral retina.

RP may be inherited as an autosomal recessive, autosomal dominant, digenic, or X-linked trait. Autosomal dominant RP (adRP) accounts for 20% to 25% of all cases.2 Mutations causing adRP have so far been identified in only five genes: rhodopsin, localized on chromosome 3q,3 peripherin-RDS on 6p21,4 CRX on 19q13.3,5 NRL on 14q11,6 and most recently the RP1 gene on 8q11-13.7,8 In addition, there are six mapped adRP loci (1cen, 7p, 7q, 17p, 17q, and 19q) in which the responsible disease gene remains unknown.9

The RP1 gene was localized to the pericentric region of chromosome 8 by Blanton et al.,10 after the initial identification of the locus by Heckenlively et al.11 and Field et al.12 and further refinement positioned the locus to a 4-cM interval between D8S601 and D8S285.13 The gene was identified using positional cloning techniques and differential display analysis with the mouse model of oxygen-induced retinal neovascularization, as a gene that undergoes significant change in expression in response to retinal hypoxia. The RP1 mRNA is expressed only in the photoreceptor cell bodies and inner segments of the retinas shown by in situ hybridization experiments. There is an increase in RP1 mRNA expression during development of photoreceptor outer segments in mice.7

The RP1 gene has four exons giving rise to an mRNA of approximately 7 kb in length encoding a protein of 2156 amino
acids with partial sequence homology to the human doublecortin gene (DCX).\textsuperscript{13} Mutations, most of which are located in codons 663-777, have been found in 21 families by various groups on screening only a small part of the gene.\textsuperscript{7,8,15} To date, mutation screening of the whole RP1 gene has not been undertaken in a large cohort of patients.

**METHODS**

**Ascertainment of Patients**

All 266 patients were identified through the retinal dystrophy clinics at Moorfields Eye Hospital, London, and Addenbrooks Hospital, Cambridge, United Kingdom. Patients were collected with a clinical diagnosis of RP made on the basis of clinical symptoms of progressive night blindness and constriction of the peripheral visual field, without loss of central vision. Fundus changes included progressive atrophy in the retinal periphery, with or without macular involvement, with intraretinal pigment migration, attenuation of the retinal blood vessels, and some degree of pallor of the optic disc. In most of the patients, additional electrodiagnostic tests were performed, including full-field flash ERG, pattern ERG, and electro-oculography, all following International Society for Clinical Electrophysiology of Vision (ISCEV) guidelines. The results showed moderate to severe rod photoreceptor involvement in all patients tested, with additional cone involvement ranging from very mild to severe. Patients were considered to have autosomal dominant disease if at least two family members were equally affected in at least two successive generations.

Patients were residents of the United Kingdom and were of a variety of ethnic origins (European White and Asian from the Indian subcontinent). Patients were selected to serve as control subjects for allele frequencies and mutation screening.

Informal consent for genetic studies in adherence to the Declaration of Helsinki was obtained by the examining clinicians. Peripheral blood samples were collected from patients and control subjects and used for genetic analysis. Genomic DNA was extracted using an extraction kit (Nucleon II; Scotlab Biocience; Strathclyde, Scotland, UK).

**Linkage Analysis**

Genetic analysis in the 24RP family was performed with markers corresponding to the 10 known adRP loci.\textsuperscript{9} Primers were synthesized from the MapPairs set (Research Genetics, Huntsville, AL) or synthesized commercially according to data from Genome Database (Johns Hopkins University, Baltimore, MD).

Nonradioactive polymerase chain reaction (PCR) was performed in a 10-μl reaction with 300 ng of genomic DNA, according to previously published protocols.\textsuperscript{16} The amplified products were then separated by electrophoresis on 6% to 8% nondenaturing polyacrylamide gels (Protogel, National Diagnostics) and stained with ethidium bromide.

**Mutation Detection**

Primers were synthesized from the genomic sequence of the RP1 gene. The primer sequences are given in Table 1. PCR was performed in a 50-μl reaction with 1 μg of genomic DNA, according to previously published protocols.\textsuperscript{16} The resulting product was allowed to cool slowly to room temperature to maximize the formation of heteroduplexes.\textsuperscript{17} Once detected, sequence variants were sequenced using a kit (FS; Perkin-Elmer, Warrington, UK), in both the forward and reverse directions on an automated fluorescent sequencer (model 373; ABI Biosystems; Foster City, CA). All the PCR fragments obtained from the Pakistani family were sequenced.

**TABLE 1. RP1 Primer Pairs Used in Screening**

<table>
<thead>
<tr>
<th>Exon (PCR Fragment)</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GTCTGGTGATTAAGCATCACCATGTTG</td>
<td>CTAACAGTTAAAGCAGAACACCAG</td>
</tr>
<tr>
<td>2(1)</td>
<td>CTTGGAATATCCTGGAAGTGTGG</td>
<td>GGCACCCATTCCCTGCTGAGG</td>
</tr>
<tr>
<td>2(2)</td>
<td>GAGCTGAGGAGGAGGAGGATCC</td>
<td>GCCACAGATTCCATATCCACAAC</td>
</tr>
<tr>
<td>3</td>
<td>GCATCTGACAGCTAAATATGTCGCG</td>
<td>GTCTCCTTTTATCTTGTAGTCC</td>
</tr>
<tr>
<td>4(1)</td>
<td>CATAGAGCTCAGAAGACATAT</td>
<td>CTTCTCCTTTTATCTTGTAGTCC</td>
</tr>
<tr>
<td>4(2)</td>
<td>CAAGAGCAGCATTGAGCAGTGG</td>
<td>GAGCATGGTTGAATATGACAG</td>
</tr>
<tr>
<td>4(3)</td>
<td>CACAGGAGGAAGCAAGAACAGC</td>
<td>GCTTAAATCATCCTCCTTGG</td>
</tr>
<tr>
<td>4(4)</td>
<td>CAGATGCTAGAAGACAGGAGT</td>
<td>GCCACAGATTCCATATCCACAAC</td>
</tr>
<tr>
<td>4(5)</td>
<td>TCAGCAGAGCAGAGTAAAT</td>
<td>GCCACAGATTCCATATCCACAAC</td>
</tr>
<tr>
<td>4(6)</td>
<td>TTTGACAGAGCAGAGTAAAT</td>
<td>GCCACAGATTCCATATCCACAAC</td>
</tr>
<tr>
<td>4(7)</td>
<td>CAGGCTGACAGCTAAATATGTCGCG</td>
<td>GTCTCCTTTTATCTTGTAGTCC</td>
</tr>
<tr>
<td>4(8)</td>
<td>GCTGAAAATTAGCAGGACAGG</td>
<td>GCCACAGATTCCATATCCACAAC</td>
</tr>
<tr>
<td>4(9)</td>
<td>GCCAGTACCATCAGAAGGAG</td>
<td>GCCACAGATTCCATATCCACAAC</td>
</tr>
<tr>
<td>4(10)</td>
<td>GAGGAGGTCTGTGTTACTACTG</td>
<td>CTTCTCCTTTTATCTTGTAGTCC</td>
</tr>
<tr>
<td>4(11)</td>
<td>GATATGGAAGCGAAGCAGGACT</td>
<td>ACCAATCAGGATGACTGAGAG</td>
</tr>
<tr>
<td>4(12)</td>
<td>GTTATTCAGAGTCCTCTCCTG</td>
<td>GTACAGGCTCCTCTTGG</td>
</tr>
<tr>
<td>4(13)</td>
<td>GTATGTTATGCTGAGGAGGAC</td>
<td>GTGACATGCTAGTACCCCTTGG</td>
</tr>
<tr>
<td>4(14)</td>
<td>CGAACATGAAATCCTGCTGAC</td>
<td>GTGACATGCTAGTACCCCTTGG</td>
</tr>
<tr>
<td>4(15)</td>
<td>CAATAGACAGTAGTACAGAC</td>
<td>CCGAGGCTCCTACCTTGGCAATATC</td>
</tr>
<tr>
<td>4(16)</td>
<td>ATGCATACCGGTAGAC</td>
<td>GCTTACGAGATTGGATATC</td>
</tr>
<tr>
<td>4(17)</td>
<td>CAATTACAGACCGGTCTCTG</td>
<td>CTTAGGAGGATGACTGAGAG</td>
</tr>
</tbody>
</table>

Patients were residents of the United Kingdom and were of a variety of ethnic origins (European White and Asian from the Indian subcontinent). For comparison, 100 ethnically matched individuals with no personal or family history of retinopathy were selected to serve as control subjects.

In addition a two-generation Pakistani family (24RP) in which adRP affected six living members was identified through eye clinics in Islamabad, Pakistan. One hundred unrelated normal individuals of Pakistani origin were used as control subjects for allele frequencies and mutation screening.
RESULTS

Linkage Analysis of Pakistani Family (24RP)

Twenty-four members of the family were typed for all the known adRP loci. Linkage was obtained between adRP and markers at 8q11-13, with a maximum lod score of 3.61 (at θ = 0.00) for the markers D8S285, D8S260, and D8S1752. Recombination events were observed involving the markers D8S1815 and D8S1771.

Mutation Analysis of RP1

For mutation screening of the RP1 gene, 266 unrelated adRP patients, along with the Pakistani family described above were selected. Of the 266 British patients screened, 17 different types of mutation and possible disease-causing variants were found. These are described in Tables 2 and 3, respectively, along with a T373I mutation in exon 4 identified in the Pakistani family. Of the 266 British patients with adRP, 21 had definite RP1 mutations (some mutations occurred in more than one patient, Table 2) indicating an 8% incidence in the British adRP population. This frequency is second only to the incidence of rhodopsin mutations, estimated at between 20% and 25%. As highlighted in previous studies, the most common mutation was the R677X change identified in 9 and 266 (or 3%) of our cohort of patients.

We detected eight deletions and insertion mutations, all of which caused a frame shift leading to a premature termination codon downstream of the mutation. Indeed, the majority (19/21) of British RP1-associated families carried mutations, which ultimately resulted in a premature termination codon, caused either by a frame shift or as a result of a nonsense mutation. This finding correlates with that of Bowne et al. in 17 of 19 of their RP1-carrying patients mutations that result in a premature termination.

A relatively small number of probable disease-causing variants (n = 7) were detected. All were located in the 5' end of

Table 2. RP1 Mutations Found in Patients with adRP

<table>
<thead>
<tr>
<th>Exon (PCR Fragment)</th>
<th>Base Change</th>
<th>Amino Acid Change</th>
<th>Patient Families of 266 Screened (Affected Members Tested from Each Family)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4(3)</td>
<td>1498-1499insGT</td>
<td>Frame shift from codon 500 leading to a premature termination codon</td>
<td>1</td>
</tr>
<tr>
<td>4(4)</td>
<td>2029C → T</td>
<td>R677X†‡</td>
<td>9 (at least 2)*</td>
</tr>
<tr>
<td>4(5)</td>
<td>2098G → T</td>
<td>Q700X‡</td>
<td>1</td>
</tr>
<tr>
<td>4(5)</td>
<td>2168-2181del</td>
<td>Frame shift from codon 723 leading to a premature termination codon†</td>
<td>1</td>
</tr>
<tr>
<td>4(5)</td>
<td>2206-2207insT</td>
<td>Frame shift from codon 736 leading to a premature termination codon</td>
<td>1# (2)</td>
</tr>
<tr>
<td>4(5)</td>
<td>2280-2284del</td>
<td>Frame shift from codon 763 leading to a premature termination codon‡</td>
<td>2 (at least 2)*</td>
</tr>
<tr>
<td>4(5)</td>
<td>2232T → A</td>
<td>C744X‡‡</td>
<td>1 (3)</td>
</tr>
<tr>
<td>4(5)</td>
<td>2284-2289del</td>
<td>Frame shift from codon 762 leading to a premature termination codon</td>
<td>1</td>
</tr>
<tr>
<td>4(5)</td>
<td>2171-2186del</td>
<td>G724Q and 725-729del</td>
<td>2 (at least 2)*</td>
</tr>
<tr>
<td>4(6)</td>
<td>2594-2596del</td>
<td>Frame shift from codon 865 leading to a premature termination codon</td>
<td>1</td>
</tr>
<tr>
<td>4(6)</td>
<td>2608-2609insA</td>
<td>Frame shift from codon 870 leading to a premature termination codon</td>
<td>1</td>
</tr>
</tbody>
</table>

Of the 100 control subjects screened, none had any of the listed mutations.

* Evidence for cosegregation with disease through two or more generations.
† Previously reported by Pierce et al., Sullivan et al., and Bowne et al.
‡ Previously reported by Bowne et al. in this cohort of patients.

Table 3. Possible Disease-Causing RP1 Variants in Patients with adRP

<table>
<thead>
<tr>
<th>Exon (PCR Fragment)</th>
<th>Base Change</th>
<th>Amino Acid Change</th>
<th>Species Conservation</th>
<th>Patient Families of 266 Screened (Affected Members Tested from Each Family)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4(2)</td>
<td>1118C → T</td>
<td>T373I</td>
<td>Conserved residue in human and mouse proteins</td>
<td>1 (Pakistani) (7)</td>
</tr>
<tr>
<td>4(4)</td>
<td>1989G → T</td>
<td>K663N‡</td>
<td>Nonconserved residue in human and mouse proteins</td>
<td>1* (2)</td>
</tr>
<tr>
<td>4(5)</td>
<td>2376A → C and 2393G → C</td>
<td>K792Q and R798N</td>
<td>K is a conserved residue, R is a nonconserved in human and mouse proteins</td>
<td>1* (3)</td>
</tr>
<tr>
<td>4(6)</td>
<td>2700A → C</td>
<td>K900T</td>
<td>Conserved residue in human and mouse proteins</td>
<td>1</td>
</tr>
<tr>
<td>4(12)</td>
<td>4784A → G</td>
<td>R1595Q</td>
<td>No comparable region in the mouse protein sequence available on database</td>
<td>1</td>
</tr>
<tr>
<td>4(13)</td>
<td>5378C → T</td>
<td>P1793S</td>
<td>No comparable region in the mouse protein sequence available on database</td>
<td>1</td>
</tr>
<tr>
<td>4(16)</td>
<td>6358C → A</td>
<td>T2113N</td>
<td>No comparable region in the mouse protein sequence available on database</td>
<td>1</td>
</tr>
</tbody>
</table>

Of the 100 control subjects screened, none had any of the listed mutations. Symbols as in Table 2.
 exon 4 (not coding for the doublecortin homologous domain, Table 3). These base changes were classified as probable disease-causing variants, if not present in our control group. We were unable to demonstrate segregation with disease in all families, because other family members were not available in every case. (Those in which it was possible are marked with an asterisk in Tables 2 and 3.) Two missense sequence changes were identified in one patient. Which of these is the major disease-causing mutation remains to be elucidated; however, the lysine residue at 792 is a conserved residue, and the arginine 798 is not. Because both segregated with disease in the family through two generations, both of the changes are presumed to be present on the same allele. It was interesting that the Pakistani family had a missense mutation associated with disease, discovered during sequencing the entire coding region of RP1.

We identified eight polymorphisms and sequence variants of which c.884-68T → C in PCR fragment 4(1), N985Y in 4(7), and A1670T and S1691P in 4(16) were the most prevalent (Table 4).

Haplotype analysis of the nine patients with the R677X mutation indicated that these patients do not share a common disease haplotype (data not shown). This is in agreement with the findings of Bowne et al., who also showed that independent mutation events probably accounted for the numerous R677X mutations in their population.

**DISCUSSION**

Previous studies have identified 10 different RP1 mutations all located between codons 663 and 777, except one identified in codon 1808, with five families carrying the R677X mutation. This clustering of mutations could be due to this region of the gene’s being a mutational hotspot, or it could be because only a limited number of patients (n = 56) had been screened for mutations in the whole gene. (A cohort of 603 had been screened for mutations in the region of the gene between codons 650 and 796 only.)

We have shown that in the British population more mutations exist, many of which are insertions or deletions. Of note, in our patient population, the majority of mutations resulted in a premature termination of the protein. This phenomenon therefore does not allow speculation about which residues of the protein are important for its functioning, because most of the mutant proteins expressed in our patients would be truncated. We therefore suggest that the adRP phenotype in these patients is due either to haploinsufficiency, or to interaction of the truncated proteins with other proteins during its malfunctioning, perhaps sequestering the latter.

The small number of missense mutations and probable disease-causing variants detected were located primarily in the 5’ end of exon 4, suggesting that these residues, if they can be shown to be disease-causing, have an important functional role. Alternatively, should these changes be non-disease-causing sequence variants, it would suggest that these residues are unimportant. The T373I mutation detected in the Pakistani family has been reported as a sequence variant by Grimsby et al. in a small family.

Although we cannot be certain this is the disease-causing mutation without performing biochemical analysis of the mutant protein, because we found segregation of the mutation with disease giving an LOD score greater than 3, there was no other change in the gene sequence, it is absent from 100 ethnically matched control subjects, and if this residue is conserved between species, we have to consider it as a possible mutation.

The frequency of the R677X mutation does not appear to be due to a founder effect. Marker analysis did not reveal a shared haplotype between families. The high frequency of this mutation may be due to a CpG dinucleotide, which is likely to be methylated and therefore to be prone to mutation of CpG to TpG. The presence of many deletion and insertion mutations in the 5’ end of exon 4 could be explained by the presence of a series of multiple A residues in the DNA sequence immediately surrounding the mutation sites. This could cause DNA slippage due to mismatch of the two DNA strands during replication a mechanism known to contribute to mutations.

The identification of RP1 mutations in patients from Australia, the United Kingdom, the United States, and Pakistan indicate that mutations in this gene are present in patients of diverse ethnic origins.

Sequence variants were detected along with disease-causing mutations. Although these variants were not enough to cause disease on their own, they may modify the severity of the phenotype. This phenomenon, known as “complex” alleles (where two mutations/sequence variants occur in the same allele of a gene), has been described in patients with cystic fibrosis (CF). The phenotype at the RP1 locus is reported to be variable. The presence of polymorphisms in cis, as in the case of complex alleles, or in trans, in the case of the mutated alleles.
allele, or in possible modifier genes in other parts of the genome should be investigated as a possible cause of phenotypic variability.

Future protein expression studies along with greater knowledge of the function of this protein are ultimately required to demonstrate the effect of the mutations found in this and other studies. Knockout animal models may provide insights into the effect of an absence of this protein on eye development and functioning.

References