Evaluation of the Human Gene Encoding Recoverin in Patients With Retinitis Pigmentosa or an Allied Disease

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Purpose. To determine whether defects in the human recoverin gene cause retinitis pigmentosa (RP) or an allied disease such as Usher syndrome, Leber congenital amaurosis, or the Bardet–Biedl syndrome.

Methods. Single-strand conformation polymorphism analysis and direct genomic sequencing techniques were used to screen 596 unrelated patients, comprising 167 patients with dominant RP, 168 with recessive RP, and 261 with an allied disease.

Results. Four sequence variants were discovered. The first was a missense change (Ala200Thr) found in one family with autosomal dominant RP and in one family with autosomal recessive RP; it did not segregate with disease. The second was a silent, single-base variation affecting codon Ser24 with a minor allele frequency of approximately 0.5%. The third was a silent, single-base variation affecting codon Val1122. The fourth was a single-nucleotide substitution in intron 2, 11 bp upstream of exon 3.

Conclusions. The authors found no evidence that mutations in the recoverin gene are a cause of RP or another of the hereditary retinal diseases studied. The human phenotype associated with mutations of the recoverin gene remains unknown. Invest Ophthalmol Vis Sci. 1997;38:704-709.

Retinitis pigmentosa (RP) and allied diseases are clinically and genetically heterogeneous.1–5 Among families with RP, ophthalmologists have observed autosomal dominant, autosomal recessive, X-linked, digenic, and mitochondrial inheritance patterns. Even among families with the same inheritance pattern, there is nonallelic heterogeneity. Specifically, research studies have implicated at least eight loci causing dominant RP (assigned to chromosomes 3q,6 6p,5,8 8q,7p,10 7q,17q,13 and 19q14,15), 14 loci causing autosomal recessive RP (assigned to chromosomes 1q,16,17 1q,18 3q,19 3p,4p,4cen,42 5q,6p,7p,11p,11q,11q,11q,14q,15q,15q,16q,30), at least 3 distinct loci causing X-linked RP,31 and two unlinked loci causing digenic RP (assigned to chromosomes 6p and 11q). Additional loci are being implicated during the course of ongoing candidate gene studies and linkage studies in various laboratories, so that it is likely that this set of chromosomal assignments for RP genes will soon be incomplete, if it is not already.

Of the RP genes known to exist, fewer than 10 have been identified precisely. Four of these encode members of the phototransduction cascade. These are rhodopsin,6 the α and β subunits of rod phosphodiesterase21,23 and the α subunit of the cyclic guanosine monophosphate-gated channel.22 These findings suggest that defects in genes encoding other members of the phototransduction cascade also may cause some forms of RP or an allied disease.

Recoverin is a member of the phototransduction cascade. It is thought to modulate the sensitivity of photoreceptors to varying levels of light intensity.33 Because mutations in genes encoding other members of the phototransduction cascade also may cause some forms of RP or an allied disease. Recoverin is a member of the phototransduction cascade. It is thought to modulate the sensitivity of photoreceptors to varying levels of light intensity.33 Because mutations in genes encoding other members of the cascade cause RP, we decided to analyze the recoverin gene for mutations in patients with RP or an allied disease. This article describes our analysis.
Analysis of Recoverin in Retinitis Pigmentosa

2653

---GCTGCCCACTCTTCTCACTC

AGT//Coding Region Exon 1//-ATG

2677

tgcagtttcccctctcccctttctggctgggcggtgcggggt---

2678

2680

2663

---GCTGCCCACTCTTCTCACTC

AGT//Coding Region Exon 2//-ATG

2655

gtgaattccttttccttcattgcctttgatttttcaaccatgg---

2656

2645

---GCTGCCCACTCTTCTCACTC

AGT//Coding Region Exon 3//-ATG

2646

FIGURE 1. Genomic sequence of the human recoverin gene. Arrows indicate positions of oligonucleotide primers used for the polymerase chain reaction. Numbers are laboratory designations of the polymerase chain reaction primers. Upper case letters represent the complementary DNA sequence, and lower case letters represent the intron sequence.

METHODS

Recruitment of Patients

This research involving human subjects was done in accordance with the Declaration of Helsinki and was approved by the Human Studies Committees of the Massachusetts Eye and Ear Infirmary and Harvard Medical School. After informed consent was obtained, 10 to 50 ml of venous blood was drawn from each patient using disodium ethylenediaminetetraacetic acid as an anticoagulant. DNA was purified and stored in 10 mM Tris, pH 7.7, 1 mM ethylenediaminetetraacetic acid at 1°C to 10°C for up to 6 years before analysis. Almost all patients were evaluated clinically in this center. Most resided in the United States or Canada. Those with RP had electroretinograms with decreased amplitudes and delayed b-wave implicit times. Patients with sector RP with normal implicit times were not included. Blood samples of some relatives were obtained for cosegregation analyses in selected cases. We excluded patients with known mutations in another RP gene (rhodopsin, peripherin/RDS, the α or β subunits of rod phosphodiesterase, the α subunit of the rod cyclic guanosine monophosphate-gated channel, or ROM1).

Single-strand Conformation Polymorphism Analysis and Direct Genomic Sequencing

The human recoverin gene contains three exons. Exons 1, 2, and 3 have 127, 38, and 35 codons, respectively. Intron sequences flanking the exons were determined using previously published cloned genomic fragments as templates and standard dideoxy sequencing methods (Fig. 1).

To analyze each patient included in our study, the coding regions contained within the exons were amplified from genomic DNA by the polymerase chain reaction using oligonucleotide primers based on the intron sequences flanking the exons or based on the sequences in the 5' or 3' untranslated regions (Table 1). Because of the large size of the coding region in exon 1 (381 base pairs), it was divided into 3 overlapping segments to increase the sensitivity of the single-strand conformation polymorphism technique. Included in the analysis were the splice acceptor and donor sites of each intron.

Each amplification by the polymerase chain reaction was performed in the well of a microtiter plate in a volume of 20 μl containing 20 to 50 ng of leukocyte DNA, 20 pmol of each oligonucleotide primer, 20 mM Tris–HCl (pH 8.4 or 8.6, Table 1), 0.02 mM deoxyadenosine triphosphate, 0.02 mM deoxyguanosine triphosphate, 0.02 mM deoxythymidine triphosphate, 0.002 mM deoxycytidine triphosphate labeled at the α position with 0.6 μCi of 32P, 0.5 U of Taq DNA polymerase, 0.1 mg/ml bovine serum albumin, 50 mM potassium chloride, 0 or 10% dimethyl sulfoxide (Table 1), and magnesium chloride at the concentration listed in Table 1. The polymerase chain reaction was performed in a thermal cycler (MJ Research, Watertown, MA) programmed for 22 to 35 cycles. Each cycle consisted of 30 seconds at 94°C for denaturation, 30 seconds at an annealing temperature as specified in Table 1, and 30 seconds at 71°C for polymerization. Amplified DNA was heat denatured, and the single-stranded fragments were separated by electrophoresis at room temperature through each of two
TABLE 1. Oligonucleotides and Polymerase Chain Reaction Conditions for Screening the Recoverin Gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers</th>
<th>Temperature* (°C)</th>
<th>Mg2+ (mM)</th>
<th>pH</th>
<th>% Dimethyl Sulfoxide</th>
</tr>
</thead>
</table>
| 1A   | 5'-GCTGCCCACTCTTCTCTG  
     | 5'-TGGAACTGCTGGGTGAT  | 58        | 0.25 | 8.6 | 10 |
| 1B   | 5'-GACGTACTCCTTGAAGTCCA  
     | 5'-CGATTCCAACCTCGACGGCA  | 58        | 0.50 | 8.6 | 10 |
| 1C   | 5'-ACCCCGCACCGCCCAGCCAG  
     | 5'-TTAAAGTTCCTCCTAACGAG  | 54        | 1.5  | 8.4 | 10 |
| 2    | 5'-ATGGTTGGAAATCAAAGGCG  
     | 5'-GTATATATTTTTCACAGGAC  | 54        | 1.25 | 8.4 | 0  |
| 3    | 5'-AGGACACGTGACAGTTGCC  | 54        | 1.25 | 8.4 | 0  |

* Annealing temperature.

6% acrylamide gels, one with and one without 10% glycerol. Patients with variant bands were evaluated further by direct sequencing of the appropriate region using techniques described previously.36

RESULTS

We investigated 596 unrelated patients including 167 patients with dominant RP, 168 patients with recessive RP, and 261 patients with an allied disease (Table 2). Few sequence variants were discovered. A missense variant was found to be heterozygous in exon 3 in patient 001-104 with dominant RP from family #6255 and in patient 003-084 with recessive RP from family #5702. The change affected codon 200 (GCC to ACC) and was designated Ala200Thr (Fig. 2A). It did not segregate with disease in family #6255 (Fig. 2B). A mutation in the homologous allele could not be found in patient 003-084. Based on these findings, the allele was deemed a nonpathogenic, rare variant with an estimated allele frequency of 0.2% (2 Ala200Thr alleles of 1192 alleles [596 individuals] examined). One silent variant affected codon Ser24 (TCG to TCC) and had a minor allele frequency of approximately 0.5% (6 TCC alleles of 1192 alleles examined). Another single-base substitution affected codon Val122 (GTG to GTA) in one allele of patient 060-008 with generalized choroidal sclerosis from family #7710. A single nucleotide substitution in intron 2, 11 bp upstream of exon 3 (C to G) was present heterozygously in patient 184-046 with Usher syndrome type II from family #E042.

DISCUSSION

Recoverin is an abundantly expressed, retina-specific protein that contains two calcium-binding domains.38 It serves as a calcium sensor in photoreceptors and is believed to affect the termination of the phototransduction cascade by affecting the ability of rhodopsin kinase to phosphorylate photoactivated rhodopsin.39-41 The exact mechanism of action of recoverin remains to be identified.

Because recoverin is expressed in a variety of retinal cell types, including rod and cone photoreceptors, bipolar cells, and ganglion cells, we could not predict in advance what retinal phenotype, if any, might be caused by defects in the recoverin gene. Therefore, the evaluation of recoverin as a candidate gene included patients with a variety of photoreceptor degenerations and patients with presumed inner retinal disease such as autosomal dominant optic atrophy. No
convincingly pathogenic mutations were discovered. These results make it unlikely that mutations in the recoverin gene are a frequent cause of retinal degeneration or dysfunction.

The human recoverin gene is within a region on chromosome 17p13.145,46 linked to loci for autosomal dominant RP in one family.45,46 Leber congenital amaurosis in five families,46 and autosomal dominant cone degeneration in two families.46,47 However, based on meiotic recombinations and DNA sequence analyses, the responsible loci in the families with autosomal dominant RP and autosomal dominant cone degener-
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Key Words

Bardet–Biedl syndrome, Leber congenital amaurosis, recoverin, retinitis pigmentosa, Usher syndrome

References

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