11-cis Retinol dehydrogenase mutations as a major cause of the congenital night-blindness disorder known as fundus albipunctatus

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Purpose: Patients with fundus albipunctatus uniformly experience difficulty with vision at night. Their retinas are spotted with characteristic light yellow flecks of unknown composition that typically spare the macula. A defect in the transport or utilization of visual cycle retinoids is thought to underlie this recessive disorder with variable clinical expression. To elucidate the molecular defect we considered the genes for interphotoreceptor retinoid-binding protein (RBP3) and 11-cis retinol dehydrogenase (RDH5) as candidates for this disease.

Methods: We examined two unrelated families with fundus albipunctatus. The diagnosis was determined clinically and RBP3 and RDH5 were analyzed by molecular screening methods and direct genomic sequencing.

Results: Each family had two affected members with typical fundus albipunctatus. The affected members were siblings born to unaffected parents who were seventh cousins in the first family and unrelated in the second family. The probands from both families were clinically similar except for the fundus dots that were more extensive in the second family to the point of involving the parfoveal region. In the initial phase of genetic screening, RDH3 defects were ruled-out as the cause of the disease in both families. In contrast, RDH5 mutations were found in the affected siblings in both families. The proband in one had a homozygotic Gly238Trp missense mutation (GGG → TGG) involving exon 4 and in the other carried compound heterozygotic changes Arg280His (CGC → CAC) and Ala294Pro (GCC → CCC) in exon 5. The disease phenotype was only manifested in family members with two abnormal RDH5 alleles consistent with autosomal recessive inheritance in both pedigrees.

Conclusions: These findings strongly implicate defects of RDH5 as the cause of fundus albipunctatus and point to a heterogeneity of RDH5 mutations in this form of congenital stationary night blindness with variable expressivity.

Fundus albipunctatus cum hemeralopia is a stationary form of congenital night blindness recognized since the late 1800’s [1]. Patients with this autosomal recessive disorder typically experience visual difficulty only in low lighting conditions. Yellow-white lesions that spare the macula and a reduced rate of rhodopsin regeneration characterize the retinopathy. The dots appear to be located within the retina or the retinal pigmented epithelium (RPE) although their exact location and composition is unknown.

The resemblance of the retinal dots to those found in vitamin A deficiency [2-4] and abetalipoproteinemina [5] has raised the possibility that a defect in vitamin A transport or metabolism may underlie the pathogenesis of this disorder. However, serum retinol-binding protein, serum retinoid and lipoprotein levels are all normal in fundus albipunctatus [1,6]. Furthermore, vitamin A supplementation does not alter the dark-adaptation rate or appearance of the fundus lesions in this disease [1]. Therefore, any defect in vitamin A transport or metabolism in fundus albipunctatus, if present, would have to be attributed to the processing of retinoids locally in the retina during the vitamin A cycle. This notion is supported by fundus reflectometry studies showing that the major physiologic defect in patients with fundus albipunctatus is a marked reduction in the rate of rhodopsin regeneration [7].

A reduced rate of rhodopsin regeneration could be caused by a number of defects in the vitamin A cycle. The cycle begins with the photosomerization of 11-cis retinal to all-trans retinal bound to rhodopsin. Rhodopsin then passes through a number of transient intermediates leading to the release of all-trans retinal. This aldehyde is reduced to all-trans retinol by a photoreceptor short chain alcohol dehydrogenase referred to as all-trans retinol dehydrogenase [8]. This reaction represents the rate-limiting step in the visual cycle [9]. All-trans retinol, possibly in conjunction with interphotoreceptor retinoid-binding protein (RBP3), then enters the RPE where it is esterified with long chain fatty acids [10] thought to be the substrate for an isomerase that converts all-trans retinal ester to 11-cis retinol [11]. 11-cis retinol is then oxidized to 11-cis retinal by an RPE 11-cis retinol dehydrogenase (RDH5) [12]. The 11-cis retinal then returns to outer segments to regenerate rhodopsin. RBP3 is believed to promote the release of 11-cis retinal from the RPE and facilitate its transfer through the interphotoreceptor matrix to regenerate rhodopsin [13,14]. It is plausible that a defect in a number of the above reactions could result in delayed dark-adaptation similar to that seen in fundus albipunctatus.

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Recently mutations in RDH5 were reported to cause fundus albipunctatus in two families [15]. The defects were a Gly238Trp and a Ser73Phe substitution both of which led to marked reduction in the specific activities of the encoded RPE enzymes in vitro. Besides these mutations, no others have been reported to cause fundus albipunctatus to date.

To determine whether mutations in RDH5 and RBP3 could be a major cause of fundus albipunctatus, we examined two new pedigrees for defects in these genes. We ruled out RBP3 mutations as the cause of fundus albipunctatus but uncovered defects in RDH5 in both families. Our findings extend the spectrum of known RDH5 mutations in fundus albipunctatus and help establish RDH defects as a major cause of classic fundus albipunctatus.

METHODS

Patient Families and Ophthalmological Examinations: Two families with fundus albipunctatus were identified. Both families currently reside in the United States. The clinical features of these families have not been previously described in the literature. Standard ophthalmologic examination was carried out on the affected members. Dark adaptation tests, full-field electroretinograms, and fluorescein angiography were performed on selected patients. The studies followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects before blood was donated for this research.

Mutation Analysis: The genes for interphotoreceptor retinoid-binding protein (RBP3) and 11-cis retinol dehydrogenase (RDH5) were analyzed by sequencing the leukocyte DNA extracted from venous blood specimens. Oligonucleotide primers (summarized in Table 1), were generally selected using the PrimerSelect program of the GCG package.

Interphotoreceptor retinoid-binding protein gene (RBP3): Southern blot analysis of restriction enzyme digested total genomic DNA and PCR amplified RBP3 segments were performed using standard methods. The heteroduplex analysis of RBP3 intron A was carried out as previously described [16]. The entire coding region was sequenced in both directions from overlapping PCR amplified regions in proband of family 1006. The primers (not shown) were selected from the human genomic sequence under GenBank accession number J05253[17]. Fluorescence sequencing was carried out as described below for the 11-cis retinol dehydrogenase gene.

11-cis retinol dehydrogenase gene (RDH5): The RDH5 coding exons 2 through 5 with flanking intron sequence were analyzed by single stranded conformation-polymorphism analysis (SSCP) [18] and direct genomic sequencing. Primers were selected from the flanking intron sequences obtained from GenBank accession numbers U43559 and AF037062 [19]. Exons 4 and 5 were sequenced both by autoradiographic and fluorescence sequencing methods. Fragments were amplified by PCR and used in a cycle-sequencing protocol with radiolabeled primers (Thermo Sequenase; Amersham Life Science, Cleveland OH). For automated fluorescence sequencing the exons were amplified separately by nested PCR and subjected to DNA sequencing using magnetic beads and dye-terminator chemistry as previously described [20]. The sense primer of the inner pair was biotinylated at its 5' terminus and contained the M13(-21)-forward sequence. The antisense

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<th>Exon</th>
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<tr>
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<td>CCGGACACATATTGCTG</td>
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<tr>
<td>3</td>
<td>CAGCATCCTTTCTCTCCC</td>
<td>TGTTAGCTCGGAGCCCCA</td>
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<tr>
<td>4</td>
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<td>GTTGCCCTCATGCCCCCT</td>
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<td>5</td>
<td>CTGATGCGACACCTTAGG</td>
<td>CAATCTCTTGTGAGGCT</td>
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<th>Exon</th>
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<td>2</td>
<td>TTTCCTTTGTACAGATGCC</td>
<td>GATGCGGAGATGAAAAAGTG</td>
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<td></td>
<td>M13=GCGACATATTGCTGCAACAC&quot;</td>
<td>M13=GCTTCTCGCTAACATCTCC&quot;</td>
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<td>3</td>
<td>TCCGTATCCTCGACGACC</td>
<td>TGCGCAGACGACGACCTCC</td>
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<td>M13=GCTACAAGAGTCTACGAGG</td>
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<td>4</td>
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<td>TTTCCCTCTGACTCCAGCC</td>
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<td>M13=AAACCTCTGCCCCGCTGCTG</td>
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<td>5</td>
<td>TGAAGAGCAGAGATGGTTAC</td>
<td>GCCAATCTCTCCTCGAG</td>
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<td>M13=ATGGAAGCGCCTTGATCC</td>
<td>M13=GTAGATGGAGAGGCTGCC</td>
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*The second primer pair for each exon region corresponds to the nested set.
*For sense M13 = TGGAAAAGCGGCGCCAGT (M13 forward sequence). The nested sense primer is biotinylated at the 5' end.
*For antisense M13 = CAGGAAAAGCTATGACC (M13 reverse sequence).

Figure 1. The pedigrees of two unrelated families with fundus albipunctatus. Both families show an autosomal recessive inheritance pattern with two affected siblings. Family 1002 (A) is of Greek descent. The parents of the effected daughters are seventh cousins in family 1002. Family 1006 (B) is of German descent. The asterisks indicate family members with known RDH5 genotype. The arrow indicates the proband in each family.
primer contained the M13-reverse sequence. The individual DNA strands were purified by avidin coated magnetic beads (Dynal AS, Oslo, Norway). DNA sequencing was performed using a PE-Applied Biosystems PRISM 377 automated fluorescence DNA sequencer (Foster City, CA).

RESULTS

Clinical findings: Two families with fundus albipunctatus were studied to determine the molecular genetic basis of the disease. Figure 1 shows the two pedigrees with two affected siblings in each born to unaffected parents. The parents in family 1002, who are of Greek descent, are seventh cousins. The parents in family 1006 with largely German ancestry, live in the Southeastern United States, and have no history of consanguinity. In early childhood the affected members in both families had difficulty adapting to the dark. There was no other associated ocular or systemic disorders in either family. The above historical data are consistent with autosomal recessive inheritance of the eye disease in our families.

Clinical evaluation established the diagnosis of fundus albipunctatus in both pedigrees. Figure 2A shows the fundus of the proband from family 1002 at age 26. Overall, the findings include typical yellow-white dots, which spare the macula and radiate from the perimacular region. The dots are sharply demarcated and appear to be deep to the retinal vessels. A well-defined foveal reflex, absent pigmentary changes and normal vascular caliper further characterize the fundus appearance of the proband in this family. The fundus photograph of the family 1006 proband at age 28 is shown in Figure 2B. Multiple light yellow scattered discrete lesions are present in the retina of both siblings. The photograph shows that the dots tend to enlarge in size from the central to peripheral regions. Electrophysiologic studies supported the clinical diagnosis by demonstrating normal dark adapted thresholds, absence of decline in the retinal function over several years and the typical delay in dark adaptation in both probands. Ongoing detailed clinical studies suggest considerable variation in the phenotype of the affected patients in families with known RDH5 mutations (unpublished data).

Molecular Genetic Studies: We examined and excluded RBP3 as a candidate gene for fundus albipunctatus in our pedigrees. The entire coding region of RBP3 from the family 1006 proband was PCR amplified and sequenced. The only deviation from the known sequence is an ACG → ACA change of codon 257. The sequence variant, which does not change the threonine coded for at this position, is not among those previously reported [21]. Southern blot analysis of total genomic DNA and PCR amplified regions of RBP3 was not informative in this pedigree using known polymorphic markers Msp I, Sty I and Bgl I [22,23] (data not shown). Heteroduplex analysis showing a polymorphic variation in intron A among the two affected siblings was used further to rule out the linkage of RBP3 with the disease in family 1006. In family 1002, the RBP3 locus was excluded as a candidate by demonstrating variance in the intragenic restriction fragment-length pattern among the affected siblings (D.A. Saperstein and J.M. Nickerson personal communication).

Next, we used SSCP to screen for mutations in the RDH5 locus in our pedigrees. The coding segment of the RDH5 gene from various family members were amplified in the presence of single stranded conformation-polymorphism (SSCP) analysis of exons harboring mutations in the RDH5. Amplified 32P-labeled DNA fragments from the affected individuals and their relatives in each pedigree was denatured and subjected to electrophoresis on nondenaturing polyacrylamide gels with or without 10% glycerol followed by autoradiography. The position of the major bands are indicated by the arrows. Lane 1 contains amplified DNA from normal control. A. SSCP analysis of Exon 2 in family 1002. B. SSCP analysis of Exon 5 in family 1006.

Figure 2. Fundus photograph of the proband in family 1002 (A) at age 26 and the proband of family 1006 (B) at age 28. The foveal regions are designated by asterisks.

Figure 3. Single stranded conformation-polymorphism (SSCP) analysis of exons harboring mutations in the RDH5. Amplified 32P-labeled DNA fragments from the affected individuals and their relatives in each pedigree was denatured and subjected to electrophoresis on nondenaturing polyacrylamide gels with or without 10% glycerol followed by autoradiography. The position of the major bands are indicated by the arrows. Lane 1 contains amplified DNA from normal control. A. SSCP analysis of Exon 2 in family 1002. B. SSCP analysis of Exon 5 in family 1006.
ence of $\alpha$-32P-dCTP, denatured and electrophoresed on a nondenaturing gel. Figure 3 shows the DNA band patterns in both pedigrees compared to normal controls (lane 1). The affected members in pedigree 1002 (Figure 3A, lanes 2 and 3) display similar shifts in exon 4 upper and lower DNA bands compared to the unaffected brother (lane 6) and the control (lane 1). The unaffected parents (lanes 4 and 5) display shifted bands in addition to a normal complement (Figure 3A, middle band) consistent with their heterozygosity. In pedigree 1006 (Figure 3B, lanes 2 and 3) similar band shifts were observed in exon 5 among the affected siblings. No other shifts were detected elsewhere in the $RDH5$ coding sequence in either pedigree. The above data show abnormal appearing $RDH5$ alleles in members of both pedigrees. The affected members appear to be homozygotes or compound heterozygotes for mutations involving exon 4 in pedigree 1002 and exon 5 in pedigree 1006.

To pinpoint the $RDH5$ defect in family 1002, direct genomic sequencing was performed on the amplified exon 4 fragment from the proband. This individual was found to be homozygous for $GGG \rightarrow TGG$ mutation substituting tryptophan for glycine at codon 238 (Figure 4). The radiographic and fluorescence sequencing shown in Figure 4 reveal the presence of this mutation on both alleles. The proband’s affected sister was found to have the same mutation. No other mutation was detected elsewhere in the gene.

In family 1006, sequence analysis showed the proband to be a compound heterozygote with mutations involving exon 5 of $RDH5$ (Figure 5). The proband has a $CGC \rightarrow CAC$ mutation in one allele leading to a Arg280His substitution and a $GCC \rightarrow CCC$ mutation in the other allele leading to an Ala294Pro substitution. The proband’s affected sister is also heterozygotic with these same two mutations (data not illustrated). The proband’s unaffected mother carries only the mutant Arg280His allele. The unaffected daughter carries the other mutant allele, Ala294Pro which was presumably inherited from the proband’s deceased father. The above findings clearly demonstrate independent assortment of the two $RDH5$ mutations consistent with the allelic nature of the mutations in family 1006.

**DISCUSSION**

Insights gained into the physiological and biochemical processes underlying visual transduction and visual pigment regeneration have helped clarify the etiology of the various types of congenital stationary night blindness. Each disorder has a characteristic defect in the rate of dark adaptation, extent of involvement of the cones compared to rods, and rate of visual pigment regeneration [7]. In fundus albipunctatus both the cone- and rod-mediated dark adaptation and visual pigment regeneration rates are markedly delayed. However the kinetics of the photoreceptor recovery following brief light flash are unaffected [7,24].

To search for visual cycle defects that correlate with the physiologic abnormalities seen in fundus albipunctatus, we began by examining the locus for interphotoreceptor retinoid-binding protein ($RBP3$) as a candidate gene. The encoded extracellular glycolipoprotein is thought to shuttle 11-cis retinal and all-trans retinol between the RPE and photoreceptors and thus may appear a logical candidate. However, $RBP3$ was excluded as a disease causing locus by sequence and linkage analysis in our pedigrees. Interestingly, targeted disruption of $RBP3$ in mice likewise does not appear to interfere with the recovery of 11-cis-retinal and of regeneration of rhodopsin [25]. Taken together the above suggests that a defect in $RBP3$ does not cause fundus albipunctatus.

In contrast, evidence supports a causal role for $RDH5$ mutations in fundus albipunctatus. The Gly238Trp substitution has been found in three unrelated probands one in this and two in the study by [15]. The substitution leads to marked reduction in dehydrogenase activity and stability in vitro [15].
The other missense change (Ser73Phe) found in one of the pedigrees examined by [15] also leads to a marked reduction in enzyme activity consistent with its pathogenicity. The novel mutations Arg280His and Ala294Pro found in family 1006 in our study, are also likely to be pathogenic based on statistical and cosegregation analysis. With ~1% allele frequency calculated from the data reported by Yamamoto et al. [15], we would expect the frequency of the unaffected homozygotes and compound heterozygotes with silent polymorphic changes to be in the order of 1 in 10,000 in the population assuming Hardy-Weinberg equilibrium. Thus the finding of nonconservative mutations in homozygotic and heterozygotic state in family 1006 as well as in all other three unrelated probands with fundus albipunctatus examined to date provides compelling evidence for the pathogenicity of RDH5 mutations.

Little is known about the role of Arg280 and Ala294 in the normal function of RDH5. RDH5 is one of the members of the short chain dehydrogenae/reductase superfamily that is thought to be an integral membrane protein [19,26]. RDH5 is anchored to membranes by N- and C-terminal hydrophobic peptide segments. The Ala294Pro substitution reported here may therefore disrupt the C-terminal anchor spanned by residues 289-310 [27]. Between the anchors is the catalytic domain in the luminal compartment where Arg280 resides. The specific role of this presumably critical residue in the catalytic domain is unknown.

The identification of RDH5 defects in fundus albipunctatus may be helpful in distinguishing this nonprogressive disease from a degenerative retinopathy it closely mimics. Retinal dots similar to those seen in fundus albipunctatus may also be found in retinitis punctata albescens, a disease distinguished classically by its progressive course [28]. It has been argued that the two diseases fall on a continuum [29], while others consider fundus albipunctatus as a distinct entity [30-32]. Recently, a variety of mutations in the cellular retinaldehyde-binding protein [33,34] peripherin/RDS [35,36], and rhodopsin [37] have all been shown to cause retinitis punctata albescens. However, no RDH5 defects have been reported in patients with retinitis punctata albescens to date. In contrast, only RDH5 mutations have been found in the fundus albipunctatus patients studied to date [15]. Thus fundus albipunctatus and retinitis punctata albescens may have distinct molecular etiologies. Furthermore, retinitis punctata albescens appears to have a more heterogenous genetic basis in contrast to fundus albipunctatus.

Despite the relatively homogenous genetic basis, there does appear to be significant clinical variability among patients and pedigrees of fundus albipunctatus. Electrophysiologic studies on unrelated probands with different RDH5 mutations reported have revealed differences in the dark adaptation rate [15]. A patient with fundus albipunctatus has also been described that is intermediate between the typical fundus albipunctatus and normal [38]. This 14-year-old girl presented with nystagmatia, good vision and retinal lesions characteristic of fundus albipunctatus. Although the spots appeared to involve more of the retina than is usually the case in fundus albipunctatus, the kinetics were only four times longer than normal and therefore markedly faster than what has been reported for fundus albipunctatus. A family with an autosomal dominant form of fundus albipunctatus has also been described [39]. Members of this family had typical nystagmatia, fundus features and electrophysiologic abnormalities of fundus albipunctatus during childhood, but showed improvement in their night blindness, disappearance of their fundus flecks and normalization of electrophysiological responses by the end of the second decade of life. The above observations suggest that there is variability in the funduscopy, progression and genetics within fundus albipunctatus.

The genetic and environmental basis for variation in the clinical appearance and course of this disease remains of considerable interest to our group and others. Some differences are evident from the comparison of fundus photographs (Figure 2A,B). Contributing factors in addition to the genetic variability may include dietary factors and vitamin A intake. Even though Marmor et al. [1] have shown no change in the physiology by vitamin A supplementation, it is not clear whether these and other forms of dietary intervention might nonetheless alter the course of disease over time in a subset of patients leading to their symptomatic improvement. The answers to these and other questions await further investigation. Ongoing studies in our laboratories focus on correlating the location of the mutations to the structure and function of RDH5 and the clinical features within the specific families.

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