Extending the Spectrum of EYS-Associated Retinal Disease to Macular Dystrophy

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Purpose. To assess the phenotypic variability and natural course of inherited retinal diseases (IRDs) caused by EYS mutations.

Methods. Multiethnic cohort study (N = 30) with biallelic EYS variants from a clinical IRD database (retinitis pigmentosa [RP], N = 27; cone-rod dystrophy [CRD], N = 1; and macular dystrophy, N = 2). In vitro minigene splice assay was performed to determine the effect on EYS pre-mRNA splicing of the c.1299+5_1299+8del variant in macular dystrophy patients.

Results. We found 27 different EYS variants in RP patients and 7 were novel. The rate of visual field loss of the V4e isopter area was −0.84 ± 0.44 ln(deg2) per year, and the rate of visual acuity loss was 0.75 Early Treatment Diabetic Retinopathy Study letters per year. Ellipsoid zone width was correlated with area of the hyperautofluorescent ring, with r2 = 0.78 and P < 0.001. Rate of decline in ellipsoid zone width was −57 ± 17 μm per year (P < 0.01) (n = 14) or −3.69% ± 0.51% from baseline per year (P < 0.001). An isolated CRD patient carried a homozygous EYS variant (c.9405T>A), previously identified in RP patients. Two siblings with macular dystrophy carried compound heterozygous EYS variants: c.1299+5_1299+8del and c.6050G>T. The former was novel and shown to result in skipping of exon 8, and the latter was a known RP variant.

Conclusions. We report on EYS-associated macular dystrophy, extending the spectrum of EYS-associated IRDs. We observed heterogeneity between RP patients in age of onset and disease progression. Identical EYS variants were found in cases with RP, CRD, and macular dystrophy. Screening for EYS variants in CRD and macular dystrophy patients might increase the diagnostic yield in previously unsolved cases.

Keywords: retinitis pigmentosa, cone-rod dystrophy, macular dystrophy, inherited retinal disease, EYS

Inherited retinal diseases (IRDs) are a heterogeneous group of genetic eye diseases characterized by progressive degeneration of photoreceptor and/or retinal pigmented epithelium (RPE) cells, leading to severe visual impairment and blindness. Retinitis pigmentosa (RP), a rod-cone dystrophy, is the most common subtype of IRD with an estimated prevalence of 1 in 4000 individuals.1 Patients report night blindness and visual field (VF) constriction from early adolescence and gradually decreasing visual acuity later in life. Over 250 genes have been described to be mutated in IRD, of which several can be mutated in different clinical subtypes of IRD.2

Eyes shut homolog (EYS; OMIM: 612424) was first reported in 2008 by two independent groups,3,4 and both described this gene as the human ortholog of Drosophila “eyes shut” (eys), also known as Spacemaker (spam). Mutations in EYS account for ~5% to 35% of European and Asian autosomal recessive retinitis pigmentosa cases5-11 but have also been described in three patients with autosomal recessive cone-rod dystrophy (CRD).4,12,13 EYS is located on chromosome 6p12 (RP25 locus), spans over 2 Mb, and consists of 44 exons that together code for a protein that is predicted to harbor 27 epidermal growth factor (EGF)-like domains and 5 laminin G-like domains. There are at least four isoforms, all of which are expressed in the human retina.14 The Drosophila ortholog plays an important role in retinal morphogenesis and architecture.15 In zebrafish, Eys is expressed in the outer segments and connecting cilium/transition zone (CC/TZ) of both rod and cone photoreceptors.16-18 Functional studies in zebrafish suggest Eys helps to maintain the stability of the ciliary axoneme in both rods and cones and the integrity of the ciliary pocket in cones.16-18 Eys knockout zebrafish showed a cone-rod pattern of retinal degeneration,16 and the Eys protein...
was assumed to be essential for the structural integrity of photoreceptor cells. However, the exact function of the EYS protein and the role of the different isoforms in the human retina still remains unclear.

We have assessed the spectrum of retinal disease and the course of visual function in our multiethnic cohort of 30 patients carrying biallelic EYS mutations to improve patient counseling on prognosis and to provide guidance for the timing of therapeutic intervention if available. Twenty-seven patients were diagnosed with RP, one patient was diagnosed with CRD, and two patients with macular dystrophy. To find an explanation for the generalized versus more localized retinal dystrophy among our subjects, we performed functional testing of a novel splice site variant and bioinformatically assessed the nature of other (presumed) pathogenic variants.

MATERIALS AND METHODS

Study Subjects

We gathered all available DNA testing results from IRD patients from two tertiary care hospitals (The Rotterdam Eye Hospital and Erasmus Medical Center) and selected patients with biallelic EYS mutations. In total, we included 30 IRD patients from 25 families. Four patients were previously described by Littink et al. Eighteen patients were isolated cases with a negative family history of inherited retinal dystrophies. Twenty-seven patients were diagnosed with RP, one patient with CRD, and two patients with macular dystrophy based on clinical characteristics, retinal imaging, and visual function testing. The study protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board and the Ethics Committee of the Erasmus Medical Center (Rotterdam, The Netherlands).

Molecular Diagnosis

The molecular diagnosis of EYS variants (NM_001142800.1) was made using Sanger sequencing (9 patients), autosomal recessive RP APEX genotyping array (3 patients), and targeted analysis of 286 IRD-associated genes (Supplemental data) after exome sequencing (18 patients). To determine the effect of the c.1299+5_1299+8del variant on EYS pre-mRNA splicing, an in vitro minigene splice assay was performed. For this, we generated a wild-type minigene and a mutant minigene harboring the c.1299+5_1299+8del variant, which each contain exon 8 and parts of the flanking introns of EYS (Fig. 1A). To investigate if the c.1299+5_1299+8del variant leads to alterations in splicing, HEK293T cells were transfected with the wild type or mutant minigene constructs, followed by RT-PCR analysis. A detailed description of the applied techniques is provided in the Supplementary data.

Clinical Work-Up

Data were collected from our own medical charts, and historic data were retrieved from referring ophthalmologists to maximize the follow-up period. Ophthalmologic examination included best-corrected visual acuity (BCVA), Goldmann kinetic VF testing, full-field electroretinogram (ERG) according to the International Society for Clinical Electrophysiology of Vision standards (Diagnosys, Lowell, MA, USA), multifocal electroretinogram (DRE) analysis, dilated fundus examination, color fundus photography (D300 [Nikon, Tokyo, Japan]; TRC-NW65; Topcon, Tokyo, Japan; and Zeiss FF 450 Plus Fundus Camera [Carl Zeiss Meditec, Jena, Germany]), spectral-domain optical coherence tomography (SD-OCT) (Spectralis; Heidelberg Engineering GmbH, Dossenheim, Germany), and 30° field fundus autofluorescence (FAF) (Spectralis). Quantitative image analysis was performed with Heidelberg Eye Explorer Software. The area inside the hyperautofluorescent ring was measured from the outer border by using a free-hand drawing tool (Supplementary Fig. S1A). The width of the ellipsoid zone was measured in the horizontal foveal B-scan with a distance measurement tool (Supplementary Fig. S1B). VFs were digitized using a method described by Dagnelie. We measured the retinal area of the V4e target because this target was consistently used in all examinations.

Statistical Analysis

Visual impairment was defined as either low vision (BCVA worse than 0.50 logMAR but equal or better than 1.30 logMAR and/or central VF radius of the V4e target smaller than 20 but equal or larger than 10° in the better eye) or blindness (BCVA worse than 1.50 logMAR and/or central VF radius of the V4e target smaller than 10° in the better eye) in accordance with the World Health Organization criteria. We used the Spearman correlation coefficient to analyze the strength and the direction of the association between variables. For analysis of the VF area, the ellipsoid zone width, and the area of the hyperautofluorescent ring on FAF, we used the mean of the right and the left eye as they were significantly correlated (ellipsoid zone \( r_s = 0.95, \ P < 0.001 \); and hyperautofluorescent ring \( r_s = 0.96, \ P \leq 0.001 \)). To calculate the annual rate of decline in visual function, we used mixed effects linear regression modeling with visual acuity in logMAR and with log-transformed area of the V4e isopter expressed in degrees squared (deg\(^2\)) for VF and corrected for repeated measurements by entering a fixed effect. Patients with a single visit were excluded from longitudinal analysis. We used a Student’s t-test to compare differences in age of onset, age at diagnosis, and age at last examination between patients with and without constricted VFs at last examination. To calculate the annual rate of decline in ellipsoid zone width, we used mixed effects linear regression modeling and corrected for repeated measurements by entering a fixed effect. Additionally, we calculated the rate of decline of the ellipsoid zone width as a percentage of baseline allowing comparison with other studies.

RESULTS

Cohort Characteristics

We have collected clinical data from 30 patients with biallelic EYS variants from 25 families with a median follow-up of 7 years (range, 0–24 years) (Table 1). Twenty-seven patients were diagnosed with RP, two siblings had macular dystrophy, and one isolated patient was diagnosed with CRD. Our multiethnic cohort consisted of 14 patients from European, 13 from Asian, 2 from African, and 1 from mixed European-Asian descent. Nine patients had a history of consanguinity. The current mean age was 45 years (range, 19–75 years), and sex distribution was equal, with 16 patients (53%) being male.

Molecular Diagnosis

Of 27 RP patients, 15 carried compound heterozygous variants (Table 2) and 12 patients had homozygous EYS variants, of which 8 reported a history of consanguinity. We found 27 different variants: 8 frame shift, 8 nonsense, 8 indels, 3 missense, and 1 splice site variant (Table 3). Seven of these variants were novel. All missense variants were classified as pathogenic by SIFT and Polyphen2 algorithms and were
located in conserved residues of EYS protein. According to the American College of Medical Genetics (ACMG) classification, one missense variant, p.(Gly2186Glu), was classified as likely pathogenic and two variants, p.(Arg2604His) and p.(Ile2995Asn), were classified as being of uncertain significance (Table 3).

In patient XXV, diagnosed with CRD, a homozygous EYS variant, p.(Tyr3135*) was detected using whole-exome sequencing. Besides this change, no other pathogenic variants were found. The p.(Tyr3135*) variant was previously identified homozygously in two Dutch siblings, namely, one with CRD and one with RP,4 and three Spanish siblings with RP.6 In our cohort, two RP patients were heterozygous carriers of this variant.

For patient XXIV-1, diagnosed with macular dystrophy, ABCA4 was initially screened with Sanger sequencing, but no variants were found. Subsequently, targeted whole-exome sequencing identified two variants in EYS, c.1299+5_1299+8del and p.(Gly2017Val).23 Using Sanger sequencing, we detected these EYS variants in his brother, patient XXIV-2. Segregation analysis of the offspring of patient XXIV-1 confirmed that both variants were located on different alleles. The first variant (c.1299+5_1299+8del) was novel, whereas the second variant (p.(Gly2017Val)) was previously found homozygously in an RP patient.6 This missense variant was predicted to be pathogenic by in silico prediction tools (Table 3).

**Minigene Splice Assay in HEK293T Cells**

To investigate potential splice defects associated with the novel c.1299+5_1299+8del variant, wild-type and mutant minigenes harboring this change were generated and transfected into HEK293T cells. RT-PCR analysis showed that transfection of the mutant minigene resulted in skipping of EYS exon 8, whereas transfection of the wild-type minigene resulted in normal splicing (Fig. 1B). Skipping of EYS exon 8 for the mutant minigene was validated by Sanger sequencing (Fig. 1C).

**Phenotype and Visual Function of Patients With EYS-Associated RP**

Most RP patients developed symptoms of night blindness and VF constriction in the second and third decade of life, and the mean age at diagnosis was 30 years (range, 11–56 years). Demographic information and clinical features are available in
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Current Age, y, Sex</th>
<th>Age at Diagnosis, y</th>
<th>Age at First Visit, y</th>
<th>Initial Symptoms</th>
<th>Ophthalmic History</th>
<th>Visual Acuity at First Visit, LogMAR</th>
<th>Refractive Error, SE, D</th>
<th>Ophthalmoscopy at Baseline</th>
<th>ffERG Rod Derived</th>
<th>ffERG Cone Derived</th>
<th>ffERG Age Phenotype</th>
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<tbody>
<tr>
<td>I</td>
<td>61, M</td>
<td>56</td>
<td>56</td>
<td>Night blindness, decreased visual acuity (high myopia)</td>
<td>Amblyopia LE, CE BE</td>
<td>0.10 0.52</td>
<td>−7.75 −12.25</td>
<td>Tilted, waxy optic disks with peripapillary atrophy, severe attenuation of the retinal vessels, and bone spicules in the periphery.</td>
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<td>NR</td>
<td>56 RP</td>
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<td>Refractive surgery BE for myopia</td>
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<td>−5.25 −4.25</td>
<td>Waxy optic disks, attenuated retinal vessels, normal aspect of the macula, intraretinal pigmentations in the periphery.</td>
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<td>NR</td>
<td>35 RP</td>
</tr>
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<td>II-2</td>
<td>42, F</td>
<td>23</td>
<td>24</td>
<td>Night blindness, decreased visual acuity</td>
<td></td>
<td>0.4 0.3</td>
<td>−4.00 −3.75</td>
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<td>NR</td>
<td>24 RP</td>
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<td></td>
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<td>−1.50 −0.50</td>
<td>Pallor of the optic disc, attenuated retinal vessels, and peripheral bone spicules and pigment alterations.</td>
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<td>NR</td>
<td>22 RP</td>
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<td>19</td>
<td>19</td>
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<td>Somatostatin analogue treatment for CME</td>
<td>0.0 0.05</td>
<td>−0.25 −0.50</td>
<td>Pale aspect of the optic disk, attenuated retinal vessels, CME, and peripheral atrophy and bone spicules.</td>
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<td>IV</td>
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<td>26</td>
<td>45</td>
<td>Night blindness</td>
<td></td>
<td>0.3 0.3</td>
<td>+2.00 NA</td>
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<td>NP</td>
<td>NA RP</td>
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<td></td>
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<td>Normal aspect of the optic disk, attenuated vessels, absent foveal reflex, and peripheral RPE alterations.</td>
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<td>NP</td>
<td>21 RP</td>
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<td>ffERG Cone Derived</td>
<td>ffERG Age</td>
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<td>−1.25</td>
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<td>NA</td>
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<td>−4.25</td>
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<td>XI</td>
<td>40, F</td>
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<td>Night blindness, decreased visual acuity</td>
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<td>−1.00</td>
<td>−1.00</td>
<td>NR</td>
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<td>Ophthalmoscopy at Baseline</td>
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<td>ffERG Cone Derived</td>
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<tr>
<td>XII</td>
<td>34, F 14 26</td>
<td>Night blindness</td>
<td>Refractive laser surgery BE</td>
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<td>-1.00 -1.25</td>
<td>Pale aspect of the optic disk, severely attenuated retinal vessels, small island with intact RPE in the macula, peripapillary creasing of the inner limiting membrane (ILM), extensive lobular atrophy of the RPE in the posterior pole and to periphery, and intraretinal bone spicules on the nasal side.</td>
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<tr>
<td>XIII</td>
<td>53, F 53 52</td>
<td>Night blindness, visual field constriction</td>
<td>0.1 0.05 +0.25 +0.5</td>
<td>Normal aspect of the optic disk, mildly attenuated vessels, macula coarsely pigmented, tapetal reflex temporally, RPE atrophy in the far periphery with bone spicule pigmention.</td>
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<tr>
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<td>0.0 0.4 NA NA</td>
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<tr>
<td>XVI</td>
<td>46, F 26 26</td>
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<td>Acetazolamide treatment for CME</td>
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<td>Moderate pallor of the optic disk, attenuated vessels, CME, RPE atrophy, and bone spicules in the periphery.</td>
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<td>53, F 11 52</td>
<td>Night blindness, visual field constriction</td>
<td>Cuba therapy BE, CE BE</td>
<td>0.5 0.7 NA NA</td>
<td>Pale optic disks with peripapillary atrophy, attenuated retinal vessels, normal aspect of the macula, midperipheral, and peripheral bone spicules.</td>
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<td>Patient ID</td>
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<td>43, F</td>
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<td>30</td>
<td>Night blindness</td>
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<td>0.05</td>
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<tr>
<td>XXI</td>
<td>31, F</td>
<td>28</td>
<td>30</td>
<td>Night blindness</td>
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<td>NA</td>
<td>NA</td>
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<tr>
<td>XXII</td>
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<td>Night blindness</td>
<td>CE LE, postoperative steroid-induced glaucoma LE</td>
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<td>−1.50</td>
<td>−2.00</td>
<td>NA</td>
<td>36</td>
</tr>
</tbody>
</table>

Mild pallor optic disk, vessels near normal caliber, preserved RPE in the posterior pole with mild epiretinal membrane formation, RPE changes in the macula with intraretinal crystals. Midperipheral and peripheral atrophy of the RPE.

Normal optic disks, attenuated retinal vessels, wrinkling and fibrosis of the ILM, and bone spicules in the far periphery.

Pink aspect of the optic disks, attenuated vessels, preserved RPE in the posterior pole with mild wrinkling of the ILM LE>RE. Midperipheral mottling of the RPE with intraretinal bone spicule pigmentations.

Moderate pallor of the optic disk, attenuated blood vessels with sheathing, normal aspect of the RPE in the posterior pole, wrinkling of the ILM, and pigment alterations and bone spicule pigmentations in the periphery.
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Current Age, y, Sex</th>
<th>Age at Diagnosis, y</th>
<th>Age at First Visit, y</th>
<th>Initial Symptoms</th>
<th>Ophthalmic History</th>
<th>Visual Acuity at First Visit, LogMAR</th>
<th>Refractive Error, SE, D</th>
<th>ffERG Rod &amp; Cone Derived</th>
<th>Ophthalmoscopy at Baseline</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXIV-1</td>
<td>38, M</td>
<td>32</td>
<td>28</td>
<td>Decreased visual acuity, color vision disturbances</td>
<td>0.8 0.8</td>
<td>+0.25 +0.25</td>
<td>Normal aspect of the optic disk, normal retinal vessels, positive foveal reflexes, bull’s-eye maculopathy with pigment alterations and tiny white dots.</td>
<td>NL NL</td>
<td>32 MD</td>
<td></td>
</tr>
<tr>
<td>XXIV-2</td>
<td>34, M</td>
<td>18</td>
<td>22</td>
<td>Decreased visual acuity</td>
<td>1.0 0.8</td>
<td>-1.00 -0.50</td>
<td>Normal aspect of the optic disk, normal caliber of the retinal vessels, RPE alterations in the fovea with subfoveal atrophy OD, normal appearance of the peripheral retina.</td>
<td>NL NL</td>
<td>22 MD</td>
<td></td>
</tr>
<tr>
<td>XXV</td>
<td>55, M</td>
<td>55</td>
<td>55</td>
<td>Decreased visual acuity, night blindness</td>
<td>0.5 0.3</td>
<td>-8.00 -9.00</td>
<td>Pale aspect of the optic disk, attenuated vessels, yellow dots in the macula, peripheral mottling of the RPE.</td>
<td>NR NR</td>
<td>55 CRD</td>
<td></td>
</tr>
</tbody>
</table>

BE, both eyes; CE, cataract extraction; CME, cystoid macular edema; DM II, diabetes mellitus type 2; F, female; ID, identifier; LE, left eye; M, male; MD, macular dystrophy; IM, intramuscular; NA, not applicable; NL, normal; NP, not performed; NR, no response; RE, right eye; SE, spherical equivalent.
## Table 2: Genotype of Patients With EYS-Associated Inherited Retinal Dystrophies (NM_001142800.1)

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Nucleotide Change 1</th>
<th>Protein Effect 1</th>
<th>Nucleotide Change 2</th>
<th>Protein Effect 2</th>
<th>Phenotype</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>c.2308C&gt;T</td>
<td>(Gln770*)</td>
<td>c.2308C&gt;T</td>
<td>(Gln770*)</td>
<td>RP</td>
<td>Iraq - Asian†</td>
</tr>
<tr>
<td>II-1</td>
<td>c.(331+1_332-1)_(1056+1_1057_1)del</td>
<td>p.?</td>
<td>c.(331+1_332-1)_(1056+1_1057_1)del</td>
<td>p.?</td>
<td>RP</td>
<td>Turkish - Asian†</td>
</tr>
<tr>
<td>II-2</td>
<td>c.(331+1_332-1)_(1056+1_1057_1)del</td>
<td>p.?</td>
<td>c.(331+1_332-1)_(1056+1_1057_1)del</td>
<td>p.?</td>
<td>RP</td>
<td>Turkish - Asian†</td>
</tr>
<tr>
<td>III</td>
<td>c.4350_4356del</td>
<td>(Ile1451Profs*3)</td>
<td>c.4350_4356del</td>
<td>(Ile1451Profs*3)</td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>IV</td>
<td>c.6714del</td>
<td></td>
<td>c.6714del</td>
<td></td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>V</td>
<td>c.4350_4356del</td>
<td>(Ile1451Profs*3)</td>
<td>c.5319_5342del</td>
<td>(Asn1773_Val1781delinslys)</td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>VI-1</td>
<td>c.6799_6800del</td>
<td></td>
<td>c.7095T&gt;G</td>
<td></td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>VI-2</td>
<td>c.6799_6800del</td>
<td></td>
<td>c.7095T&gt;G</td>
<td></td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>VII-1</td>
<td>c.5928-5_5928-1del</td>
<td></td>
<td>c.5928-5_5928-1del</td>
<td></td>
<td>RP</td>
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<td>VII-2</td>
<td>c.5928-5_5928-1del</td>
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<td>c.5928-5_5928-1del</td>
<td></td>
<td>RP</td>
<td>Pakistan - Asian†</td>
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<tr>
<td>VIII</td>
<td>c.5167-5168del</td>
<td></td>
<td>c.6424-1_6425+1_(6751+1_6752-1)del</td>
<td>p.</td>
<td>RP</td>
<td>Dutch - European†</td>
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<tr>
<td>IX</td>
<td>c.4350_4356del</td>
<td>(Ile1451Profs*3)</td>
<td>c.7811G&gt;A</td>
<td></td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>X</td>
<td>c.1673G&gt;A</td>
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<td>c.2811C&gt;A</td>
<td></td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>XI</td>
<td>c.4955C&gt;G</td>
<td>(Ser1652*)</td>
<td>c.8984T&gt;A</td>
<td></td>
<td>RP</td>
<td>Dutch - European†</td>
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<tr>
<td>XII</td>
<td>c.7919G&gt;A</td>
<td>(Trp2640*)</td>
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<td></td>
<td>RP</td>
<td>Turkish - Asian</td>
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<tr>
<td>XIII</td>
<td>c.9063del</td>
<td>(Leu1720Glys*6)</td>
<td>c.9405T&gt;A</td>
<td></td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>XIV</td>
<td>c.4350_4356del</td>
<td>(Ile1451Profs*3)</td>
<td>c.6714del</td>
<td></td>
<td>RP</td>
<td>Turkish - Asian</td>
</tr>
<tr>
<td>XV</td>
<td>c.32dup</td>
<td>(Met12Glyfs*6)</td>
<td>c.532dup</td>
<td></td>
<td>RP</td>
<td>Turkish - Asian</td>
</tr>
<tr>
<td>XVI</td>
<td>c.1161del</td>
<td>(Ile387Asns*54)</td>
<td>c.2137-1_22137+1_(2259+1_2260-1)dup</td>
<td>p.</td>
<td>RP</td>
<td>Turkish - Asian</td>
</tr>
<tr>
<td>XVII</td>
<td>c.4350_4356del</td>
<td>(Ile1451Profs*3)</td>
<td>c.6079-2A&gt;G</td>
<td></td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>XVIII</td>
<td>c.4712C&gt;G</td>
<td>(Ser571*)</td>
<td>c.6557G&gt;A</td>
<td>(Gly2186Glu)</td>
<td>RP</td>
<td>South Korea-USA</td>
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<tr>
<td>XIX</td>
<td>c.6714del</td>
<td>(Ile2239Serfs*17)</td>
<td>c.1185T&gt;C&gt;T</td>
<td>(splice)</td>
<td>RP</td>
<td>Turkish - Asian</td>
</tr>
<tr>
<td>XX</td>
<td>c.1376del</td>
<td>(Cys459Serfs*56)</td>
<td>c.331+1_332-1_(862+1_863-1)dup</td>
<td>p.</td>
<td>RP</td>
<td>Dutch - European†</td>
</tr>
<tr>
<td>XXI</td>
<td>c.4045C&gt;T</td>
<td>(Arg1349*)</td>
<td>c.4045C&gt;T</td>
<td>(Arg1349*)</td>
<td>RP</td>
<td>Turkish - Asian†</td>
</tr>
<tr>
<td>XXII</td>
<td>c.8258_8260del</td>
<td>(Leu2752_Asn2754delinsTyr)</td>
<td>c.8258_8260del</td>
<td>(Leu2752_Asn2754delinsTyr)</td>
<td>RP</td>
<td>Turkish - Asian†</td>
</tr>
<tr>
<td>XXIII</td>
<td>c.4350_4356del</td>
<td>(Ile1451fs)</td>
<td>c.6050G&gt;T</td>
<td>(Tyr265*)</td>
<td>MD</td>
<td>Morocco – African</td>
</tr>
<tr>
<td>XXIV-1</td>
<td>c.1299+5_1299+8del</td>
<td>p.?</td>
<td>c.6050G&gt;T</td>
<td>(Tyr265*)</td>
<td>MD</td>
<td>Morocco – African</td>
</tr>
<tr>
<td>XXIV-2</td>
<td>c.1299+5_1299+8del</td>
<td>p.?</td>
<td>c.6050G&gt;T</td>
<td>(Tyr265*)</td>
<td>MD</td>
<td>Morocco – African</td>
</tr>
<tr>
<td>XXV</td>
<td>c.9405T&gt;A</td>
<td>(Tyr3135*)</td>
<td>c.9405T&gt;A</td>
<td>(Tyr3135*)</td>
<td>CRD</td>
<td>Dutch - European</td>
</tr>
</tbody>
</table>

Novel variants are displayed in bold font.
† Consanguinity.
Table 3. Missense EYS Variants (NM_001142800.1) Identified in This Study and Their In Silico Functional Analyses

<table>
<thead>
<tr>
<th>Exon</th>
<th>cDNA Change</th>
<th>Protein Change</th>
<th>PolyPhen-2 Score</th>
<th>PolyPhen-2 Prediction</th>
<th>SIFT Score</th>
<th>SIFT Prediction</th>
<th>AF (gnomAD)</th>
<th>AC (gnomAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>c.6050G&gt;T</td>
<td>p.(Gly2017Val)</td>
<td>1.60</td>
<td>Deleterious</td>
<td>0</td>
<td>Deleterious</td>
<td>0.000000002904</td>
<td>5</td>
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<tr>
<td>32</td>
<td>c.6557G&gt;A</td>
<td>p.(Gly2186Glu)</td>
<td>2.44</td>
<td>Deleterious</td>
<td>0</td>
<td>Deleterious</td>
<td>0.00000003471</td>
<td>5</td>
</tr>
<tr>
<td>40</td>
<td>c.7811G&gt;A</td>
<td>p.(Ile2995Asn)</td>
<td>6.32</td>
<td>Deleterious</td>
<td>0</td>
<td>Deleterious</td>
<td>0.0000000598</td>
<td>1</td>
</tr>
<tr>
<td>43</td>
<td>c.8984T&gt;A</td>
<td>p.(Thr3021Met)</td>
<td></td>
<td></td>
<td>0</td>
<td>Deleterious</td>
<td>0.000000006598</td>
<td></td>
</tr>
</tbody>
</table>

Classification was assessed according to the ACMG guidelines. AC, allele count; AF, allele frequency.

We gathered 146 visual acuity measurements, with a mean of 6 measurements per patient (range, 1–22 measurements). Four patients became visually impaired (BCVA worse than 0.3 logMAR) during follow-up at ages 41, 43, 64, and 70 years and already had constricted VFs (<20°) at earlier examinations. Mixed effects linear regression modeling showed an overall increase in logMAR visual acuity of 0.015 ± 0.002 in the best performing eye per year, which corresponds to a loss of 0.75 Early Treatment Diabetic Retinopathy Study (ETDRS) letters per year (P < 0.001) (Fig. 2A).

Fifty-seven Goldmann VF examinations were available, ranging from one to eight measurements per patient. At first examination, 11 patients were visually impaired (central VF radius of the V4a target, <20°) and 5 were blind (central VF radius, <10°). One patient became visually impaired during follow-up at age 39. Ten patients had a central VF larger than 20° at last examination. They did not significantly differ from patients that were visually impaired in terms of age of onset (P = 0.2262), age at diagnosis (P = 0.259), or age at last examination (P = 0.136). All 10 patients carried 2 truncating EYS variants, of which 9 were located in the N-terminal part of the protein, and 11 in the C-terminal part. Forty-five VFs from 16 patients, were eligible for longitudinal analysis, and the rate of VF loss of the V4a isoper area was −0.84 ± 0.44 ln(deg²) per year (P < 0.001) (Fig. 2B).

In 23 patients, ffERG was performed: in 15 patients (mean age, 40 years) no scotopic or photopic responses could be elicited, in 5 patients (mean age, 43 years) both scotopic and photopic responses were severely reduced, and in 3 patients (mean age, 29 years) there were no scotopic responses and severely reduced photopic responses.

OCT scans of 24 patients and autofluorescence imaging of 23 patients were available for analysis. In 19 patients, the ellipsoid layer length was shortened but continuous in the foveal area, 16 of them had a hyperautofluorescent ring (Fig. 3B), and 3 had a crescent shape hyperautofluorescence pattern (I, VI-1, and VII-2) (Fig. 3F). In a single patient (VII-3B), the ellipsoid layer was continuous over the width of the 4-mm single line scan, accompanied by a crescent shape pattern on autofluorescence imaging. The horizontal ellipsoid zone width in the foveal scan ranged between 0.43 and 5.22 mm (median width, 2.14 mm; n = 62) (Fig. 2D). The area of the hyperautofluorescent ring ranged between 0.96 and 15.36 mm² (median area, 4.69 mm²; n = 24). The ellipsoid zone width was significantly correlated with the area of the hyperautofluorescent ring, with r² = 0.78 and P < 0.001 (Fig. 2C). The rate of decline in ellipsoid zone width was −0.057 ± 0.017 mm per year (P < 0.01) (n = 14). When calculating the rate of decline as a percentage of baseline, the rate was −3.69% ± 0.51% per year (P < 0.001).

In patients with a distinguishable hyperautofluorescent ring, the length of the ellipsoid zone was shorter than in patients with a “crescent” autofluorescence pattern, as described by Sengillo et al.24 (Fig. 3). Four patients (II-2, XV, XVI, and XX) had foveal abnormalities in at least one eye (Fig. 3I, 3J), and it became impossible to discriminate between the different outer retinal layers because retinal architecture in the macula, including the fovea, appeared severely distorted. The majority of RP patients had epiretinal membranes, but there were no patients with macular holes or tractional proliferations.
macular edema. Four patients developed cystoid maculopathy during follow-up, one patient was treated with acetazolamide tablets, and two with somatostatin analogue injections.

**Phenotype and Visual Function of a Patient With EYS-Associated CRD**

One isolated patient (XXV) noted both a decrease in visual acuity and night blindness as first symptoms, and he previously underwent refractive surgery to correct for high myopia. At first examination, visual acuity was 0.3 logMAR in the best eye. Goldmann VF revealed a central relative scotoma with moderate peripheral constriction (Fig. 4D). On fundoscopy, the optic disks appeared pale, vessels were thin, the macular area displayed thinning of the RPE with a tiny preserved foveal island surrounded by patchy atrophy, and there were subtle RPE changes in the periphery (Fig. 4A). OCT imaging showed atrophy of the outer retinal layers in the posterior pole (Fig. 4B), and fundus autofluorescence showed a hyperautofluorescent ring around the macula and optic nerve, with hypoautofluorescence inside the ring (Fig. 4C).

**Phenotype and Visual Function of Patients with EYS-Associated Macular Dystrophy**

The proband (XXIV-2) was the youngest in a family of five siblings; he suffered from decreased visual acuity from the age of 7. He was first examined at age 12, and his binocular visual acuity was 1.0 logMAR with eccentric fixation. At age 22, he was diagnosed with juvenile macular dystrophy, visual acuity was 0.8 logMAR, and funduscopy revealed a bull’s eye maculopathy. Central VF testing with Humphrey Field Analyzer 10-2 showed a central scotoma, and color vision testing with Lanthony’s desaturated 15-Hue test revealed a tritan defect. ffERG showed normal scotopic and photopic responses. At age 23, pronounced macular atrophy was seen on OCT imaging (Fig. 5G). His older brother (XXIV-1) noted a decrease in visual acuity and color vision problems from the age of 18. At age 32, visual acuity was 0.3 logMAR and funduscopy revealed normal foveal reflexes, perimacular RPE alterations, and very tiny white dots, with normal aspect of the peripheral retina. OCT imaging showed atrophy of the ellipsoid zone in the fovea, and fundus autofluorescence revealed a hyperautofluorescent ring (Figs. 5A–C). At age 36, ffERG rod and cone responses were within normal range, and multifocal electroretinogram showed...
decreased foveal responses (Supplementary Fig. S3). At age 38, visual acuity was 0.7 logMAR and OCT imaging showed atrophy of all outer retinal layers and thinning of the RPE. Fundus autofluorescence revealed a larger hyperautofluorescent ring surrounding the hypoautofluorescent macula (Figs. 5D and 5F).

**DISCUSSION**

In this study, we have assessed the spectrum of retinal disease and the course of visual function in 30 patients carrying biallelic *EYS* variants. Twenty-seven patients had RP, one patient had CRD, and two patients had macular dystrophy, based on clinical characteristics, functional testing, and retinal imaging. *EYS* mutations are one of the most common causes of autosomal recessive retinitis pigmentosa in Asia and Europe. Novel findings included the presence of homozygous *EYS* mutations in CRD patients and compound heterozygous *EYS* mutations in patients with macular dystrophy.

The age of onset in RP patients ranged from 7 to 51 years, and all had one or more typical fundus features corresponding with RP (Supplementary Fig. S1). Four patients became visually impaired (BCVA worse than 0.3 logMAR) during follow-up at ages 41, 43, 64, and 70 years. Mixed effects linear regression

**FIGURE 3.** Optical coherence tomography, fundus autofluorescence, fundus photography, and visual field of three RP patients with different RP phenotypes. Patients II-3 (age 23) shows a typical RP phenotype with (A) shortening of the ellipsoid layer length and (B) a hyperautofluorescent ring surrounding the fovea. (C) the optic disk appears pale and vessels are narrow. Visual field (D) is severely constricted (>10°). Patient VII-2 (age 41) has a milder phenotype, (E) the ellipsoid layer length is longer, and there is (F) a “crescent-shaped” hyperautofluorescent pattern visible. (G) The optic disk appears pink and vessels are narrow. There is decreased sensitivity of the peripheral visual field (H), with an absolute scotoma nasally. Patient XX (age 73) has end-stage RP, and (I) the ellipsoid zone and other outer segment layers are no longer discernible. There is a (J) small relatively hyperautofluorescent patch in the perifoveal area, surrounded by hypoautofluorescence, and the fovea itself appears hypoautofluorescent as well. In fundo (K), the optic disk appears pale, vessels are very narrow, and there is extensive atrophy of the RPE and choriocapillaris with bone spicule pigmentation. A small island of RPE remains in the macula with RPE alterations. The visual field (L) is severely constricted (>10°).

**FIGURE 4.** Autofluorescence, SD-OCT, and fundus pictures of patient with CRD. (A) Fundus autofluorescence of patient XXV shows a hyperautofluorescent ring within the vascular arcade, surrounding the macular area, which appears patchy, with diffuse hypoautofluorescent lesions. The fovea appears hypoautofluorescent. (B) On OCT, there is a foveal empty space, the outer retinal layers are distorted, and the ellipsoid zone can no longer be distinguished. (C) Atrophy of the posterior pole with discernable choroidal vasculature, and the retinal vessels are attenuated. (D) Goldmann visual field reveals a relative central scotoma with mild to moderate peripheral constriction.
modeling showed an overall increase in logMAR visual acuity of 0.015 in the best performing eye per year, which corresponds to a loss of 0.75 ETDRS letters per year \((P < 0.001)\). One study in a heterogeneous group of RP patients found a decline in visual acuity of 2.3 ETDRS letters per year. This would place EYS-associated RP in the milder spectrum of disease. Based on VF constriction, 11 out of 27 RP patients were visually impaired (41%) and 5 were blind (19%) according to World Health Organization criteria at first examination in our centers. The rate of peripheral VF loss for the V4e target was 0.84 \(\text{ln(deg}^2)\) per year in our cohort. McGuigan et al. reported the rate as 23% of normal per year. Ten RP patients had a central VF larger than 20 \(\mu m\) at last follow-up (mean age, 39 years). They did not differ from RP patients with VF constriction in age of onset or age at last examination. All 10 of these patients carried compound heterozygous or homozygous truncating variants.

Differences in disease manifestation and progression are often attributed to a difference in underlying causal variants (e.g. missense versus truncating variants). Three out of 27 RP patients carried 1 heterozygous missense EYS variant, and 1 truncating EYS variant. There were no patients with homozygous missense EYS variants; therefore, we could not study whether missense variants were associated with milder retinal disease in our cohort. Siblings with the same EYS genotype can differ substantially in age of onset, disease presentation, and rate of disease progression. Our study was not suited to determine genotype-phenotype correlations in detail, as many variants were observed only once. The estimated decline in ellipsoid zone width was 57 \(\pm 17 \mu m\) per year, which was less than in previous publications where yearly decline varied between \(-76.4 \mu m\) to \(-248 \mu m\). However, these estimates were based on RP patients with different underlying genetic defects and inheritance modes. We also calculated the rate of shortening as a
percentage from baseline to enable comparison with two studies in EYS patients. Our estimate was $-3.69\% \pm 0.51\%$, which corresponds with the findings of two previous studies, reporting a rate of $-4.65\% \pm 2.89\% (n = 12)${superscript}21$ and $-5.6\% \pm 2.6\%$ per year ($n = 10$).\textsuperscript{21}

Two-thirds of RP patients in our cohort had typical hyperautofluorescent rings, and one-third had a crescent shape hyperautofluorescent pattern, as described by Sengillo et al.\textsuperscript{24} The crescent pattern was associated with larger VFs and, therefore, milder disease progression. Three out of four patients had two mutations near the C-terminal domain, which could have a less detrimental impact on protein structure or function. Additional studies would be most helpful to study the effect of these mutations on the different isoforms.

Three cases of EYS-associated CRD have been published so far; the first case was a Dutch patient that carried the same homozygous variant as our CRD patient, p.(Tyr3135*)\textsuperscript{4}. The second case was a Japanese patient, carrying compound heterozygous EYS variants, p.(Tyr2935*) and p.(Ser1653fs). Segregation analysis confirmed that these variants were located on different alleles. Each separate variant homozygous, as well as the identical compound heterozygous combination of these variants, were previously identified as causal in RP patients,\textsuperscript{4,5} including in two RP patients of this cohort.\textsuperscript{3,4,30–32} The third case was a French patient with double homozygous variants, p.(Trp558*) and p.(Asn745Ser). The pathogenicity of the missense variant was questionable, as it proved to be not conserved, and the pathogenicity is uncertain according to the ACGM classification.\textsuperscript{18} The homozygous nonsense variant p.(Tyr3135*) that our CRD patient as well as the first mentioned CRD case was also detected in RP patients,\textsuperscript{4,5} including in two RP patients of this cohort. The variant is located in the last EYS exon and leads to a premature stop codon. Functional assays might reveal whether the variant leads to nonsense-mediated decay or the formation of a C-terminal-truncated EYS protein. It is unclear why identical genotypes can result in both RP and CRD phenotypes. The EYS variants found in CRD patients did not cluster in a specific domain. Of the four known EYS isoforms, only the two long isoforms (isofrom 1 and 4) are predicted to be affected by the mutations, as all CRD-associated variants are all located after the 594th amino acid. Unfortunately, this does not help explain the difference in phenotype because some of the underlying genotypes were also found in RP patients. The presence of genetic and epigenetic modifiers, or environmental factors, could also play a role in the observed differences in phenotype in these patients.

Macular dystrophy is an IRD in which the central retina is primarily affected and peripheral photoreceptor function is spared. Patients XXIV-1 and XXIV-2 presented with an isolated macular dystrophy, with a normal fERG. Targeted whole-exome sequencing identified compound heterozygous EYS variants, c.1299G$\rightarrow$C and c.6050G$\rightarrow$T. To assure the functional effect of the splice site variant, we generated a mutant minigene. RT-PCR analysis showed that transfection of the mutant minigene resulted in skipping of EYS exon 8, suggesting that similar missplicing events can occur in the retina. This variant has not been described in other IRD patients; therefore, it is not clear whether this variant is solely associated with macular dystrophy or can also lead to other phenotypes. Exon 8 codes for the fifth EGF-like domain that is part of all four known EYS isoforms. EGF-like domains are usually located in the extracellular domain of membrane-bound proteins, such as EYS, and are important for the structural integrity of the protein. The second variant, c.6050G$\rightarrow$T, was previously found homozygous in an RP patient.\textsuperscript{4} EYS is expressed in the outer segments of both rods and cones and is thought to be crucial for the stability of the ciliary axonema and photoreceptor homeostasis in humans. In several mammal lineages, such as rodents, EYS is not expressed, which limits the availability of animal models that better mimic human anatomy and physiology. EYS zebrafish knockout studies showed CRD pattern of retinal degeneration,\textsuperscript{25} and RP or macular dystrophy phenotypes have not been described in animals that are mutant for Eys.

Because molecular testing can uncover pathogenic variants in genes that have not been associated with the phenotype of interest, it remains crucial to perform segregation analysis and to keep looking for variants in other genes. Reassessing the phenotype might be worthwhile, especially in patients with end-stage disease in whom discriminating between different subtypes of IRD might prove difficult, as both central and peripheral retinal architecture are frequently severely distorted. To better understand the pathophysiology of EYS-associated IRDs, it is essential to perform functional studies that focus on the effect of mutations on the different EYS isoforms and their effect within the retina.

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**References**


