Copy-Number Variations in *EYS*: A Significant Event in the Appearance of arRP

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PURPOSE. Autosomal recessive retinitis pigmentosa (arRP) has recently been associated with mutations in a novel gene, *EYS*, which is a major gene for this disease. All published mutations so far are based on conventional PCR and are not adequate to identify mid-sized DNA rearrangements. This study was conducted to establish the prevalence of copy-number variations (CNVs) in the *EYS* gene in a cohort of arRP patients, including individuals in whom only one pathogenic change was detected by PCR-based sequencing.

METHODS. A multiple ligation-dependent probe amplification (MLPA) was used for the molecular genetic analyses of CNVs by a novel *EYS*-specific kit. PCR-based direct sequencing was used in families where a pathogenic deletion or duplication was identified in one allele. Bioinformatics analyses were undertaken to study the effect of the mutations on protein structure and function.

RESULTS. Six novel pathogenic CNVs were identified. Also, the presence of four mid-sized deletions was confirmed in patients

previously identified. Mid-sized genomic rearrangements in *EYS* are disease causing in ~4% of the families with no reported mutations and constitute the second pathogenic variation in ~15% of cases where a mutation has been detected by direct sequencing.

CONCLUSIONS. This is the first report of a systematic CNV screening of *EYS* gene in a cohort of arRP patients. Results suggest that mid-sized genomic rearrangements in *EYS* gene would be a common event in the appearance of RP phenotype. An efficient and cost-effective strategy validating a novel MLPA kit as a complementary diagnostic method for *EYS* pathogenic evaluation has been demonstrated. (*Invest Ophthalmol Vis Sci*. 2011;52:5625–5631) DOI:10.1167/iovs.11-7292

Retinitis pigmentosa (RP [MIM 268000]) is a generic name for inherited retinal dystrophies with loss of photoreceptor cells and retinal pigment deposits at midperiphery of the retina, which are visible on fundus examination. RP is characterized by primary degeneration of the rod photoreceptors followed by secondary degeneration of cones. This degeneration initially causes night blindness, followed by constriction of the visual field, abnormal color vision, and progressive loss of visual acuity in the later life.¹ Prevalence of RP is approximately 1/4000 births with over 1 million individuals affected worldwide.² This disease can be inherited as an autosomal dominant, autosomal recessive, or an X-linked trait. All genes identified to date are believed to account for roughly 50% of all retinal dystrophy cases.³ The autosomal recessive form of RP is the commonest worldwide, accounting for approximately 50%–60% of cases.² To date, a total of 32 genes have so far been implicated in autosomal recessive RP (arRP; <http://www.s-ph.uth.tn.edu/Retnet/>). However, all together these genes appear to account for only 35%–45% of total arRP cases.²

Recently we have identified a new gene, *EYS*, corresponding to the *RP25* locus,⁴ encoding an ortholog of *Drosophila* space-maker (SPAM), as a commonly mutated gene in arRP.⁵ Spanning over 2 Mb within the *RP25* locus (6q12.1–6q15), *EYS* is the largest eye-specific gene identified so far. The longest *EYS* isoform encodes a protein of 3165 amino acids. Immunohistochemical data reveal its localization in the outer segment of the photoreceptor layer. Although its role in visual function has not yet been established, the evolutionary data, the new extensive bioinformatic analyses, and the known function of the *drosophila* ortholog support a structural role for this new protein contributing to the human retinal morphogenesis and architecture.^{6,7} The initial identification of six independent mutations in our cohort of Spanish families linked to *RP25*, together with the presence of different mutations in arRP families from different origins, supports *EYS* as the first major gene for arRP.^{5,7–12}

The identification of *EYS* causative mutation is important for clinical diagnosis, predictions of the clinical course of the disease, genetic counseling, and future gene-targeted therapies. Majority

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of mutations identified so far include missense, nonsense, microdeletions and insertions, and putative pathogenic changes in the 5'UTR region. However, no duplications and few mid-sized deletions affecting *EYS* (to a few Kb) have been reported to date. This low rate of identified copy-number variations (CNVs) leading to arRP may be explained by the fact that the techniques usually used, based on conventional PCR, are not adequate to identify such rearrangements. Due to the technical difficulties in determining gene dosage in this size range, it is possible that the proportion of mutations that are deletions or duplications has been underreported, and the presence of mid-sized rearrangements undetectable by PCR-based procedures would also explain the failure to detect the second mutant allele in a significant number of arRP families.^{7,9,10} In fact, only three studies have investigated mid-sized insertions/deletions in *EYS* using comparative genomic hybridization (CGH), CNVs analysis (Genechip 6.0 Affymetrix array; Affymetrix, Santa Clara, CA) and/or self-designed multiple ligation probe-dependent amplification (MLPA) probes.^{5,7,9}

Thus, the availability of a rapid accurate molecular diagnostic complementary method to identify mid-sized rearrangements could enhance acute clinical management as well as genetic counseling. Here we report the pathogenic implication of *EYS* CNVs in Spanish and French arRP populations using MLPA. For this purpose we have tested and validated a novel *EYS* MLPA kit (MRC-Holland, Amsterdam, the Netherlands) in 95 arRP patients, including those where only one pathogenic change had previously been identified by PCR-based direct genomic sequencing. We have identified novel *EYS* rearrangements in six arRP cases. In addition, we have confirmed the presence of CNVs in four families with mid-sized deletions previously detected by CGH and self-designed MLPA.

MATERIALS AND METHODS

Subjects and Clinical Data

The study cohort comprises 95 Spanish and French unrelated patients affected by arRP, comprising 78 Spanish and 17 French patients. A full ophthalmic examination was performed, and the clinical diagnosis was based on visual acuity, fundus photography,

TABLE 1. arRP Families with One Likely Pathogenic Change Included in MLPA Studies in Order to Find a Second Mutant Allele

Family ID	DNA Change Previously Reported by Direct Sequencing	Reference of the Variation	DNA Change Identified by MLPA
F51	p.Trp558X	Audo et al. ⁹	—
F228	p.Cys1001X	Audo et al. ⁹	c.(340-?_748+?del)
F444	p.Trp2783X	Audo et al. ⁹	—
F618	p.Cys183AlafsX74	Audo et al. ⁹	c.(1300-?_1459+?del)
F311	p.Pro2265GlnfsX46	Audo et al. ⁹	—
F360	p.Ser1610PhefsX46	Audo et al. ⁹	c.(6572-?_6725+?del)
F109	p.Asp1682Tyr	Audo et al. ⁹	—
F393	p.Leu2189Pro	Audo et al. ⁹	—
F481	p.Gly2907Glu	Audo et al. ⁹	—
F116	p.Cys1176Arg	Audo et al. ⁹	—
F649	p.Cys2139Tyr	Audo et al. ⁹	—
F715	p.Asp1682Tyr	Audo et al. ⁹	—
F123	p.Asn745Ser	Audo et al. ⁹	—
F85	c.2023+1G>C	Audo et al. ⁹	—
F221	p.Pro1739Leu	Audo et al. ⁹	—
RP60	p.Gln27ArgfsX16	Barragán et al. ⁷	—
RP81	p.Asn745Ser	Barragán et al. ⁷	—
RP33	p.Trp1484X	Barragán et al. ⁷	—
RP383	p.Glu2503Lys	Barragán et al. ⁷	—
VRP8	p.Trp1484Arg	Barragán et al. ⁷	—

TABLE 2. List of Probes to Detect CNVs in the *EYS* Gene

EYS Exon	Ligation Site NM_001142800.1	Distance to Next Probe (Kb)	
Exon 1	6 nt after exon 1	66.8	
Intron 1	415 nt before exon 2	144.1	
Exon 2	—		No probe
Exon 3	173 nt before exon 3	0.8	
Exon 4	553-554	1.2	
Intron 4	463 nt after exon 4	92.1	
Exon 5	—		No probe
Exon 6	—		No probe
Intron 7	419 nt after exon 7	17.6	
Exon 8	1806-1807	30.9	
Exon 9	1935-1936	18.4	
Exon 10	—		No probe
Exon 11	2201-2202	39.2	
Exon 12	2509-2510	0.2	
Exon 12	177 nt after exon 13	238.1	
Exon 13	2673-2674	60.0	
Exon 14	2743-2742 reverse	51.8	
Exon 15	2889-2890	33.2	
Exon 16	3007-3008	9.9	
Exon 17	233 nt before exon 17	0.3	
Exon 17	3262-3263	15.7	
Exon 18	—		No probe
Exon 19	3503-3504	64.0	
Exon 20	3632-3633	1.0	
Exon 21	3743-3744	8.1	
Exon 22	3810-3811	187.6	
Exon 23	132 nt after exon 23	8.5	
Exon 24	4146-4147	24.4	
Exon 25	4415-intron 25	2.0	
Exon 26	5328-5329	153.5	
	1.6Kb after exon 27	0.9	
Intron 27	1.3Kb before exon 28		
Intron 27	367 nt before exon 28	0.4	
Exon 28	6398-6399	47.5	
Exon 29	6540-6541	80.5	
Exon 30	6710-6731	76.2	
Exon 31	6798-6799	164.5	
Exon 32	—		No probe
Exon 33	28 nt after exon 33	67.2	
Exon 34	1 nt after exon 34 reverse	14.5	
Exon 35	7445-7444 reverse	120.3	
Exon 36	7735-7736	58.0	
Exon 37	8 nt after exon 37	17.1	
Exon 38	12 nt after exon 38	0.9	
Exon 39	8208-8209	10.1	
Exon 40	8425-8426	15.4	
Exon 41	8488-8489	35.8	
Exon 42	143 nt before exon 42	5.2	
Exon 43	8931-8932		
Reference probe	18q21.1		
Reference probe	9q21.31		
Reference probe	4p16.3		
Reference probe	2q36.1		
Reference probe	17q11.1		
Reference probe	19p13.2		
Reference probe	22q12.3		
Reference probe	6q23.3		

The eight reference probes were used as controls.

computerized testing of central and peripheral visual fields, and electroretinography (ERG) findings. RP was defined as bilateral visual loss, initial hemeralopia, restriction of visual field, gradual increased bone spicule pigmentation and decrease of visual acuity, attenuation of retinal vessels, reduced or undetectable electroretinogram (ERG), and waxy disc pallor. The pattern of inheritance in all participating patients was diagnosed based on the pedigree

FIGURE 1. MLPA profiles for control individuals (red) and for arRP patients with homozygous deletions (blue). The absence of the peak of *EYS* was observed for exons 12, 13, 14, 15, 16, 17, 19, 33 and introns 12 and 17, indicated by arrows.

structure, characterized by more than one affected member and the nonaffected progenitors in arRP families.

Globally, our cohort included 71 arRP patients with no known mutation, 20 patients with a single mutation detected by PCR-based direct sequencing, and four positive controls for CNVs. Also three negative controls were included for technique normalization.

The Spanish cohort included five carriers of a pathogenic variant, previously identified in a heterozygote state by PCR-based direct sequencing.⁷ (Table 1). Additionally, two mid-sized *EYS* deletion carriers previously identified were included as positive controls.⁵ (Table 1).

The French cohort was composed of 15 patients with a previously identified heterozygous *EYS* mutation,⁹ (Table 1) and included two other homozygous individuals with mid-sized deletions as positive controls for the validation of MLPA.

In addition, available samples of probands' family members were tested for cosegregation studies. An informed consent was obtained from all participants for clinical and molecular genetic studies. The study conformed to the tenets of the Declaration of Helsinki.

MLPA Analysis

Gene dosage variation in *EYS* were analyzed by MLPA technology using a novel MLPA kit especially designed for *EYS*. The P328-X1 *EYS* MLPA Kit (MRC-Holland, Amsterdam, the Netherlands) contains 50 MLPA probes designed to detect alterations in the copy number of one or more exons in the *EYS* gene that can be implicated in arRP. In addition, this kit also includes eight reference control probes that hybridize to sequences in different regions of the genome (Table 2). The MLPA

TABLE 3. Copy-Number Variations and Mutations Identified by PCR-Based Direct Sequencing of *EYS* Gene in Spanish and French Families

Family ID	Exon	DNA Change	Protein Change	Type of Change	Reference for the Variation
F228	3-4	Deletion	c.(−340-?_748+?del)*	Heterozygous	This study
	20	c.3003T>A	p.Cys1001X	Heterozygous	Audo et al. ⁹
RP1	3-4	Duplication	c.(−340-?_748+?dup)*	Heterozygous	This study
	43	c.8897G>A	p.Gly2945Glu	Heterozygous	This study
F618	4	c.547delT	p.Cys183AlafsX74	Heterozygous	Audo et al. ⁹
	9	Deletion	c.(1300-?_1459+?del)†	Heterozygous	This study
RP73	12	Deletion c.(1767-?_2023+?del)	p.Cys590TyrfsX4	Heterozygous	Abd-El Aziz et al. ⁵ /This study
	28	c.5857G>T	p.Glu1953X	Heterozygous	Abd-El Aziz et al. ⁵
F735	12	Deletion c.(1767-?_2023+?del)	p.Cys590TyrfsX4	Homozygous	Audo et al. ⁹ /This study
RP3	13-14	Deletion c.(2024-?_2259+?del)	p.Gly676GluX9del	Homozygous	This study
RP5	15-19	Deletion c.(2260-?_2992+?del)	p.Ser754AlafsX6	Homozygous	Abd-El Aziz et al. ⁵ /This study
RP2	16-19	Deletion c.(2382-?_2992+?del)	p.Cys795HisfsX4	Heterozygous	This study
F360	26	c.4827_4830delTTCA	p.Ser1610PhefsX7	Heterozygous	Audo et al. ⁹
	33	Deletion	c.(6572-?_6725+?del)‡	Heterozygous	This study
F115	32-33	Deletion	p.Asp2142AlafsX14	Homozygous	Audo et al. ⁹ /This study
		c.(6425-?_6725+?del)			

* There are no probes to detect possible deletions/duplications in exons 5 and 6.

† There are no probes to detect possible deletions in exon 10.

‡ There are no probes to detect possible deletions in exon 32.

reaction was performed in a thermal cycler (Biometra TGRADIENT; Biometra, Goettingen, Germany), following the manufacturer's recommendations (<http://www.mlpa.com>).

Capillary electrophoresis analysis was performed in an analyzer (3730 DNA analyzer; Applied Biosystems, Foster City, CA), and commercially available software (GeneMarker v. 1.75; Softgenetics, State College, PA) was used for data analysis. We normalized the samples comparing the peak heights of patients versus controls. In addition, we included positive controls harboring three homozygous arRP *EYS* deletion patients (F115, F735, and RP5) and one patient with a heterozygous deletion (RP73).^{5,9} The MLPA analysis criteria were as follows: (1) normal if the individual dosage quotient values are within 0.8–1.0; (2) deletions or duplications if the dosage quotient values are around 0.5 or 1.5, respectively; and (3) the mean SD of all samples for each peak should be below 10%.

PCR-Based Direct Genomic Sequencing of *EYS*

To find the second pathogenic variation in those families where a new deletion/duplication was identified in one of the alleles by MLPA, we sequenced *EYS* by PCR-based direct sequencing. Moreover, these results helped to discard the presence of allelic variants in the probe target sequences that may have led to false positive results. PCR and sequencing procedures follows the protocols previously described in Barrágan et al.⁷

To evaluate the pathogenicity of the novel variants, software (NetPhos2.1; NetPhos2.0 Server, <http://www.cbs.dtu.dk/services/NetPhos/>) was used to predict the alteration in phosphorylation.¹³

All the changes were assigned a nucleotide number starting at the first translation base of *EYS* isoform according to the GenBank Reference Sequence Version FJ416331; GI: 212675237; Transcript Reference Sequence: NM_001142800.1 (ensemble entry ENST00000503581).

RESULTS

Validation of *EYS* P328-X1 MLPA Kit

Using a novel MLPA P328-X1 Kit, we first confirmed the presence of four mid-sized deletions in patients previously identified by CGH and self-designed MLPA.^{5,9} They consisted of three mid-sized homozygous deletions and one patient with a heterozygous deletion affecting exons 15–19, 32–33, and 12, in control families RP5, F115, F735, and RP73, respectively (Fig. 1, Table 3).

Identification of Novel Very Likely Pathogenic CNVs

MLPA analyses led to the identification of six novel *EYS* rearrangements. The sequence variants were designated in accordance with the Human Genome Variation Society recommendations (<http://www.hgvs.org/mutnomen/>) (Table 1).

In the *RP1* family, we found a heterozygous duplication of exons 3 and 4 in the affected individual (Fig. 2). It is the first

FIGURE 2. MLPA profiles for control individuals (red) and for arRP patients with heterozygous deletions (blue). For the RP patients a decrease of dosage was observed for *EYS* exons 3, 4, 12, 16, 17, 19, 33 and introns 4, 12, and 17, indicating a heterozygous deletion. In the case of RP1 family, an increment for the dosage of *EYS* exons 3 and 4 indicates a heterozygous duplication for these exons.

FIGURE 3. (A) Electropherogram of *EYS* mutation at nucleotide position c.8897G>A in the proband of family RP1. (B) Alignment of the SPAM peptide sequence in human and in other species using CLUSTAL W (1.82) multiple sequence alignment.

time a duplication in *EYS* is reported. Direct sequencing of this patient's DNA revealed a c.8897G>A (p.Gly2945Glu) as the second pathogenic change (Fig. 3). The amino acid substitution locates in an EGF domain of SPAM. Turning to evolutionary conservation, this glycine is present in this position in *EYS* homologues previously reported by our group.⁷ The substitution of glycine to glutamate in

position 2945 introduces an acidic polarity in a previously hydrophobic position. In addition, this change is not tolerated according to computational predictions, since it implies the loss of one phosphorylation site (NetPhos2.1). In the *RP3* family index patient, we found a novel homozygous deletion including exons 12 and 13. The segregation analysis in the family was consistent with the recessive pattern of inheritance (Table 4). Index patients in *F225*, *F618*, and *F360* families had been previously described as carrying truncating mutations identified by PCR-based direct sequencing (Table 1). After MLPA screening, we have identified three mid-sized heterozygous deletions, affecting exons 3–4, 9, and 33, respectively, as the second pathogenic change in the *EYS* gene. In addition, family segregation in available members (*F618* and *F360*), supported the pathogenic nature of those mutations (Table 4). The screening of the index patient from family *RP2* revealed a heterozygous deletion involving exons 16–19. However, in this case, PCR-based direct sequencing failed to identify the second mutant allele.

DISCUSSION

Molecular diagnosis of RP is a challenging task given the important genetic heterogeneity of these groups of diseases. *EYS* represents a major arRP gene, as can be appreciated in view of the high number of *EYS* mutations detected by PCR-based direct genomic sequencing reported in different arRP patients and the diverse ethnic origins of these families.^{5,7–12} However, mutations due to mid-sized deletions (a few kilobases) in *EYS* have seldom been reported. Typical screening protocols using conventional PCR-based sequencing methods are only able to detect small duplications/deletions (a few base pairs) and are dependent on the size of the amplified fragment in one reaction (~1 Kb maximum). On the other hand, cytogenetic techniques can exclusively detect large alterations ranging several megabases in size. None of the

TABLE 4. Segregation of Pathogenic *EYS* Mutations in arRP Patients and Family Members

Index Patient, Family Members	Family	Exon	Nucleotide Exchange	Protein effect	Allele State	Reference
CIC01001	F618	4	c.547delT	p.Cys183AlafsX74	Het	Audo et al. ⁹
Unaff. spouse		9	Deletion	c.(1300-?_1459+?del)**	Het	This study
CIC03487		4	—	—	—	Audo et al. ⁹
Aff. sister		9	—	—	—	This study
CIC03499		4	c.547delT	p.Cys183AlafsX74	Het	Audo et al. ⁹
Unaff. mother	RP3	9	Deletion	c.(1300-?_1459+?del)**	Het	This study
CIC03605		4	c.547delT	p.Cys183AlafsX74	Het	Audo et al. ⁹
03/2522b		9	—	—	—	This study
Unaff. daughter		13–14	Deletion	p.Gly676GlufsX9del	Hom	This study
03/2523b		13–14	Deletion	p.Gly676GlufsX9del	Het	This study
Unaff. son	F360	13–14	Deletion	p.Gly676GlufsX9del	Het	This study
03/2524b		13–14	Deletion	p.Gly676GlufsX9del	Het	This study
Unaff. niece 12227		13–14	Deletion	p.Gly676GlufsX9del	Het	This study
Unaff. nephew 12226		13–14	Deletion	p.Gly676GlufsX9del	Het	This study
CIC00529		26	c.4827_4830delTTCA	p.Ser1610PhefsX7	Het	Audo et al. ⁹
Unaff. father	F360	33	Deletion	c.(6572-?_6725+?del)***	Het	This study
CIC00794		26	c.4827_4830delTTCA	p.Ser1610PhefsX7	Het	Audo et al. ⁹
Unaff. mother		33	—	—	—	This study
CIC00528		26	—	—	—	Audo et al. ⁹
Unaff. sister		33	Deletion	c.(6572-?_6725+?del)***	Het	This study
CIC00710	F360	26	—	—	—	Audo et al. ⁹
Unaff. sister		33	Deletion	c.(6572-?_6725+?del)***	Het	This study
CIC00766		26	c.4827_4830delTTCA	p.Ser1610PhefsX7	Het	Audo et al. ⁹
		33	—	—	—	This study

two approaches are powerful enough and adequate to detect CNVs affecting specific regions corresponding to a few kilobases. There are some traditional techniques used to detect mid-sized deletions/duplications such as southern blot or quantitative/semiquantitative PCR, but they are expensive, time consuming, and not suitable for high-throughput results. For example, only a few samples can be run per gel by southern blot, and tests may take several days; using real-time PCR, the number or targets that can be interrogated in a reaction is limited by the number of fluorophores available. Also, the cost of buying fluorescently labeled probes for every intended target can be prohibitive.

For these reasons we sought to perform CNV screening using MLPA technology (see Supplementary Material and Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7292/-DCSupplemental>) in arRP patients, including the ones where only one pathogenic change had been identified by PCR-based direct sequencing.

The major advantages of MLPA are ease of use, high throughput, and being cheaper than other techniques such as real-time PCR or CGH. The MLPA method allows the detection of mid-sized insertions/deletions ranging from the probe size (~40 bases) to a few Kb (depending on the length of tested gene) in a large number of individuals within a short time period. This technique is being used extensively in the molecular diagnosis of a wide range of diseases caused by deletions or duplications of one or more exons in specific genes.^{14,15} In this case the novel MLPA kit has a maximum detection range of ~2 Mb, corresponding to the *EYS* genomic interval.

First, to evaluate the novel MLPA kit, we confirmed the presence of four mid-sized deletions in patients previously identified by CGH and self-designed MLPA.^{5,9} Therefore, validation of the novel kit used was successfully achieved.

As a result of the MLPA screening for CNVs, we have identified six novel *EYS* rearrangements in six out of 91 unrelated patients with arRP (see Supplementary Fig. S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7292/-DCSupplemental>). Majority of identified CNVs are predicted to generate a frameshift leading to a premature stop codon, and probably, most of these altered mRNA transcripts will be degraded through nonsense mediated decay.¹⁶ In the case of F228, RP1, and F360 families, we cannot

predict the putative protein effect of the mutation, since the extent of the CNVs is unknown, due to the absence of probes for the adjacent exons (see Supplementary Fig. S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7292/-DCSupplemental>). It is very likely that they would lead to a frame shift generating a novel stop codon, or the loss/gain of a significant portion of the protein, rich in important functional domains. In both cases the outcome would be sufficient to be pathogenic.

The identification of different CNVs in *EYS* in three out of 71 families with no known mutations, reveals a probable mutation frequency of ~4% of the cases, indicating that this kind of genomic rearrangements in *EYS* gene would be a significant possibility in the appearance of the RP phenotype. Also, three out of 20 families carrying a mutation in one allele detected by PCR-based direct sequencing were found to have a rearrangement in the second mutated allele in *EYS*. This indicates that, at least, 15% of families with one heterozygous change identified by PCR-based direct sequencing would be a carrier of a mid-sized pathogenic deletion/insertion. However, it is still possible that some of the 17 remaining cases with only one pathogenic mutation also carry deletions/duplications affecting the *EYS* gene, as the current P328-X1 probemix does not contain probes for exons 2, 5, 6, 10, 18, and 32. Therefore, the P328-X1 probemix must be updated to improve both the accuracy and detection rate of rearrangements in *EYS* by MLPA.

In summary, this is the first report of a systematic CNV screening of *EYS* gene in a large cohort of arRP patients. Our results show that mid-sized genomic rearrangements in *EYS* are a common event in the development of the RP phenotype. They are responsible for the disease in about 4% (3/71) of the families with no reported mutations and constitute the second pathogenic variation in approximately 15% (3/20) of cases where a mutation has been detected by PCR-based direct sequencing. Globally, ~5% (5/91) of all arRP families included in this study bear a pair of *EYS* mutations as the likely cause of the disease, and ~1% (1/91) present a heterozygous mutation. Of the mutations, ~7% (6/91) are mid-sized rearrangements, whereas ~2% (2/91) are single-nucleotide substitutions, and ~2% (2/91) are other variants detectable by PCR-based sequencing (Fig. 4).

FIGURE 4. Distribution of *EYS* mutations along the domain structure of SPAM. Large rearrangements are indicated in red. Blue is used for depicting small deletions and single nucleotide changes. Mutations previously identified by PCR-based direct sequencing are indicated by an asterisk.

Determination of the underlying genetic defects in a patient is a prerequisite for gene or mutation-specific therapy; thus, this knowledge is crucial for future therapies design. Therefore, the development of efficient and cost-effective strategies to detect mutation is mandatory. We have demonstrated that MLPA is an efficient and cost-effective strategy as a complementary diagnostic method for *EYS* pathogenic evaluation in arRP families.

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