Design and Validation of a New MLPA-Based Assay for the Detection of *RS1* Gene Deletions and Application in a Large Family with X-Linked Juvenile Retinoschisis

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Aims: X-linked juvenile retinoschisis (XLRS) is a severe ocular disorder that can evolve to blindness. More than 200 different disease-causing mutations have been reported in the *RS1* gene and approximately 10% of these are deletions. Since transmission is X-linked, males are always affected and females are usually carriers. The identification of female carriers is always important and poses a technical challenge. Therefore, we sought to develop a multiplex ligation dependent probe amplification (MLPA)-based method to identify deletions or duplications in this gene. We then used our assay to study a large XLRS family.

Methods: We designed six probes specific for each *RS1* exon and then optimized and validated our method using control samples with known gene deletions. In the XLRS family, *RS1* gene copy number variation was assessed by “home-made” MLPA analysis and by single nucleotide polymorphism (SNP) array analysis using the Cyto-Scan HD Array. Direct sequencing was used for deletion breakpoint mapping.

Results: Our assay detected all deletions in control samples. All affected males of the family were positive for a deletion of exon 2 of the *RS1* gene (RS1:NM_000330:c.53-?_78+?del). Carrier females were also identified.

Conclusion: Our method is easily replicated, reliable, and inexpensive and allows female carriers to be detected. This is the first report of deep characterization of a whole exon deletion in the *RS1* gene.

Keywords: retinoschisis, MLPA, RS1, XLRS

Introduction

X-LINKED JUVENILE RETINOSCHISIS (XLRS OMIM* 312700) is a vitreoretinal dystrophy characterized by early onset cystic macular dystrophy that can evolve into central atrophy, thus leading to blindness (The Retinoschisis Consortium, 1998). Normally, visual acuity decreases in the first or second decade of life, mainly affecting central vision, however, after the third decade, a significant decline in visual acuity typically occurs (George *et al.*, 1995). Clinical diagnosis is based on retinal instrumental examination and can be confirmed by molecular genetic testing. Early identification of the disease, patient education, and long-term follow-up are critical for preserving vision against associated complications, such as retinal detachment, vitreous hemorrhage, neovascular glaucoma, cataract, and strabismus that may occur at disease onset or in later stages of the pathology (George *et al.*, 1995, 1996), and may require surgical treatment.

The gene responsible for the disease is *RS1*, which encodes retinoschisin, a secretory discoidin-domain protein that takes part in the maintenance of retinal structural and functional integrity (Sikkink *et al.*, 2007). The disease is inherited in an X-linked fashion, so that males with *RS1* mutant allele in hemizygosity are affected and females with the mutant allele in heterozygosity are usually healthy carriers, although some heterozygous females with full clinical phenotype have also been reported. At least one affected female heterozygous for an *RS1* gene mutation has been described in the literature, probably due to skewed X-inactivation (Saldana *et al.*, 2007), whereas homozygosity in affected females has been described in related families (Mendoza-Londono *et al.*, 1999). About 200 disease-causing mutations have been reported in the *RS1* gene, ~10% of which are deletions (including exons) and the phenotype does not seem to be correlated with the severity of the mutation (The Retinoschisis Consortium, 1998; Kim and Mukai, 2013).

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Full gene or partial gene deletions and duplications spanning one or more exons as well as gene copy number variation (CNV) are relatively frequent disease-causing mutations. For X-linked genes, the identification of these genetic variations in males is often feasible by polymerase chain reaction (PCR), but for female carriers it may only be possible by specific methods. Detection of CNV is mostly carried out by Southern blot and multiplex PCR. Southern blot is a labor-intensive and time-consuming method, whereas multiplex PCR has proven to be a method for rapid detection of deletions in affected males, but is not effective for precise identification of heterozygous deletions and duplications in females. Multiplex ligation-dependent probe amplification (MLPA) analysis has proven to be a method of choice for the detection of CNV (Sellner and Taylor, 2004).

For prenatal diagnosis, the availability of a rapid genetic test to identify these variations is of particular importance in the detection of female carriers. We, therefore, decided to optimize and validate an MLPA-based method that enables identification of deletions and duplications in the *RS1* gene in affected males and heterozygous female carriers. We also described the molecular characterization of an XLRS family with four affected males and a large pedigree suggesting X-linked inheritance.

We first analyzed the family for *RS1* gene mutations by PCR and direct sequencing of six coding exons and boundary regions in the proband. The exon 2 fragment of the *RS1* gene failed to be amplified in repeated efforts and proper positive controls, suggesting the possibility of exon 2 deletion. To confirm this hypothesis and to map deletion boundaries, single nucleotide polymorphism (SNP) array analysis was performed. To confirm the array results and to detect the exon 2 deletion in other family members, MLPA analysis was performed using our home-made single probes, specific for the *RS1* gene.

Patients and Methods

The proband and eight relatives were examined and monitored in different eye clinics before coming to our observation in 2011 (Neurophthalmology Unit, IRCCS Fondazione Bietti, Roma, Italy) when the genetic test was proposed for all family members. After signing an informed consent form on the ophthalmological procedures, the proband and his affected siblings underwent further comprehensive ophthalmic examination, including visual acuity, fluorescein angiography, optical coherence tomography (OCT), manual visual field, and full-field scotopic and photopic electroretinograms. Results of all examinations performed in our and other clinics, as well as information on personal and family medical history, were collected and are briefly summarized below.

The proband (subject III:3, date of birth 1983) is a male who currently refuses to be seen. From his mother we know that he is suspected to be autistic and we, therefore, also tried to find links with the gene adjacent to *RS1* that codes for Rett Syndrome. The last available medical records reported a best corrected visual acuity of 0.125 Snellen for the right eye (RE) and 0.1 Snellen for the left eye (LE) at 17 years of age.

The proband's younger brother (patient III:6, date of birth 1993) has had exotropia of both eyes (convergent strabismus) since early infancy (5 months of age); a severe form of juvenile retinoschisis was diagnosed at 7 months of age on the basis of massive vitreous hemorrhage (situated between

the retinoschisis and posterior hyaloid or in the retinoschisis itself) of the RE and retinoschisis extending from the optic papilla to the temporal periphery and just under the macula (not detached) in the LE. At 1 year, he underwent vitrectomy with silicon oil for retinal detachment in RE followed by cataract extraction at 1.5 years of age. At 2 years, he underwent a second vitrectomy due to total retinal detachment with vitreoretinal tractions in aphakia in RE. The visual acuity measurement revealed negligible light perception.

The LE showed moderate vitreous hemorrhage and intraschisis hemorrhage, treated with vitrectomy followed by injection of gas into the posterior segment of the globe, inner-wall retinectomy, membrane peeling, endolaser photocoagulation, perfluorocarbon liquid, and silicone oil exchange. Visual acuity measurement revealed good light response and good vision up to 0.3 Snellen in LE. At 4.5 years of age, retinal detachment and vitreoretinal proliferation of LE were diagnosed. The patient currently has neurosensory nystagmus, ectopic pupil, and many synechiae at 360° in both eyes (more severe in RE), as well as no light perception.

The proband's middle brother (patient III:4, date of birth 1985) showed the first symptoms of reduced visual acuity at 4 years of age. The anterior segment of RE was within normal limits, whereas slight opacity of the lens (posterior cataract) was found in LE. Fundus examination showed spoke wheel maculopathy in both eyes, with retinoschisis in the inferior and nasal sectors and posterior holes that extended almost to the inferior macular arcades only in LE (stable since 1990).

TABLE 1. DATA OF THE PROBES UTILIZED IN THE *RS1* MLPA KIT

Length (nt)	Gene	Gen Bank exon	Chr. pos.	Chr coordinates (hg19)
88	CARM1	1	19p13.2	10982569–10982616
92	IL1B	4	02q13	113590971–113591021
108	ATP8A1	10	04p13	42583682–42583747
130	SLC9A2	2	02q12.1	103274243–103274299
148	ITK	15	05q33.3	156672906–156672972
172	PISRT1	01	03q23	138951973–138952034
178	ITSN1	12	21q22.11	35144381–35144440
184	SETX	7	09q34.13	135210049–135210124
191	NODAL	02	10q22.1	72195489–72195569
196	GFRA3	8	05q31.2	137588485–137588551
208	AMOT	4	Xq23	112058654–112058714
214	PARD3	20	10p11.21	34606158–34606232
220	LAMA2	24	06q22.33	129635840–129635912
226	SPAST	12	02p22.3	32362177–32362250
232	PPHLN1	8	12q12	42787408–42787483
239	UTY	15	Yq11.221	15467146–15467209
246	EVC2	15	04p16.2	5620347–5620413
252	JPH3	up	16q24.2	87635471–87635527
258	SATB2	09	02q33.1	200233348–200233429
265	EYS	27	06q12	65300933–65301003
274	GRIN2A	14b	16p13.2	9853569–9853647
115	RS1	1	Xp22.13	18690172–18690244
125	RS1	2	Xp22.13	18675773–18675849
120	RS1	3	Xp22.13	18674704–18674781
154	RS1	4	Xp22.13	18665449–18665560
141	RS1	5	Xp22.13	18662488–18662586
135	RS1	6	Xp22.13	18660193–18660285

Unbold: control probes as indicated in the data sheet of SALSA MLPA probemix P300-B1 reference; bold: *RS1* gene exon probes. MLPA, multiplex ligation-dependent probe amplification.

TABLE 2. SAMPLES WITH DELETION OF *RS1* GENE PREVIOUSLY IDENTIFIED

No.	Patient ID	Family relationship	RS1 gene exon deletions	Chromosome coordinates (Hg19)
1	R479	Proband	1	Xp22.13 (18,689,722–18,690,832)×0
2	R480	Mother		
3	R481	Proband	4–5	Xp22.13 (18,661,426–18,665,836)×0
4	R482	Mother		
5	R477	Proband	1–4	Not yet characterized
6	R476	Mother		

OCT scans showed cystic macular edema. Goldmann Visual Field revealed slight constriction of peripheral isopters in the superior sector of RE and slight constriction of peripheral isopters in the superior and nasal sectors of LE. Of the three brothers, this patient currently has the best clinical condition, with a best corrected visual acuity of 0.3 Snellen (−2.75 sph) in RE and 0.2 Snellen (−1 sph −1 cyl 90) in LE.

The proband's cousin (patient III:7, date of birth 1986) presented a typical common XLRS phenotype without complications. Visual acuity measurement revealed 0.1 Snellen in RE, but only perception of dark and light in LE. The patient also has Daltonism, like his maternal uncle (subject II:5).

All females (subjects II:4, III:5, III:8, and III:9) are healthy and clinical examination did not reveal any sign of the disease.

Molecular characterization of proband

Genetic testing was performed at MAGIs Laboratory (MAGI Non-Profit Human Medical Genetics Institute, Rovereto, Italy). All patients were enrolled after genetic counseling to explain the risks and benefits of genetic testing. They were invited to sign specific consent to make their anonymized clinical and genetic data available for research and publication. Genomic DNA of the proband and family members was extracted from peripheral blood using a commercial kit (the Blood DNA Kit E.Z.N.A.; Omega Bio-Tek, Inc., Norcross, GA).

In the proband, *RS1* (Xp22.2; OMIM*300839 NM_000330.2) gene point mutation screening was performed by PCR and direct sequencing of the six coding exons using a CEQ 8000 sequencer (Beckmann Coulter, Milan, Italy). All primer sequences and PCR conditions are available from the corresponding author.

The electropherograms of amplified fragments were analyzed using a ChromasPro 1.5 instrument (Technelysium Pty. Ltd., Australia).

Design and validation of *RS1* gene MLPA assay

A new MLPA-based assay for the detection of exon deletions in the *RS1* gene was designed and validated using previously characterized samples with exonic deletions. Six probes specific for the *RS1* exons were designed according to the guidelines for designing an MLPA assay (www.mrc-holland.com); as indicated in the protocol, the six probes were mixed with control probes (SALSA MLPA P300 Human DNA reference-2 probemix) (Table 1). The Salsa MLPA Kit thus obtained was used as indicated in standard MLPA assay procedure. Samples then underwent capillary electrophoresis in a CEQ 8000 sequencer (Beckman Coulter) and raw data were analyzed with coffalyser.net software (Mrc Holland) to determine the copy number.

The Home-Made MLPA Kit for the *RS1* gene was first validated with control samples (three female samples and three male samples) to test if the *RS1* gene probes were working correctly. Then six DNA samples with known deletions were analyzed, three boys and their mothers (Table 2), to test *RS1* gene deletion detection (samples collected at the National Eye Institute, National Institutes of Health, Bethesda, MD) (D'Souza *et al.*, 2013).

CytoScan HD array

Variations in *RS1* gene copy number were assessed by SNP array analysis (Medical Genetics Unit, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy) using a CytoScan HD Array (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. Briefly, genomic DNA was digested, bound to an adapter, and PCR amplified. After DNase I digestion, PCR products were labeled and hybridized. Washing and staining were then performed using a Fluidics

FIG. 1. Results of segregation study in the family. Deletion of exon 2 is reported according to ISCN (array) and HGVS (MLPA) nomenclature. *Documented clinical evaluation; E1, CytoScan HD array result: arr[hg19] Xp22.13 (18,674,960×2,18,675,273–18,681,809×1,18,681,810×2) for females and arr[hg19] Xp22.13 (18,674,960×1,18,675,273–18,681,809×0,18,681,810×1) mat for males; E2, MLPA assay result; E+ and E−, positive and negative to test, respectively; “•,” Obligate carrier. MLPA, multiplex ligation-dependent probe amplification.

FIG. 2. Sequence of PCR product (in light gray) in FASTA format aligned (ClustalW2) with Alu repeats (www.repeatmasker.org) adjacent to breakpoint; arrow indicates the probable junction between AluJr and AluSz. PCR, polymerase chain reaction.

Station 450. The array was scanned with a GeneChip® Scanner 3000 7G, and quality control and copy number analysis were performed using Chromosome Analysis Suite Software: (1) the raw data file (CEL) was normalized using default options; (2) unpaired analysis was performed using 270 HapMap samples as baseline to obtain copy numbers from CEL files, whereas the amplified and/or deleted regions were detected by the standard Hidden Markov Model method. The Database of Genomic Variants (www.projects.tcga.ca/variation/) was used to compare findings with those of previously reported studies. Coordinates of copy number variants are based on the UCSC GRCh37/hg19 assembly.

After deletion mapping with SNP, array-specific primers around deletion breakpoints were designed and used to obtain a PCR fragment corresponding to the *RS1* exon 2 deleted allele (the wild-type allele failed to amplify due to its large size). This fragment then underwent direct sequencing to characterize deletion breakpoints.

Genetic characterization of *XLRS* family

Finally, the validated MLPA-based assay was used to perform segregation of the *RS1* exon 2 deletion in the *XLRS* family, where a total of nine individuals (four males and five females) were genetically characterized using this method.

To draw this large *XLRS* pedigree (Fig. 1), we used HaploPainter software (HaploPainter; Thiele and Nürnberg, 2005).

Results

CytoScan HD array characterization of the *XLRS* family

The exon 2 fragment of the *RS1* gene failed to amplify in the proband. To further confirm exon 2 deletion and to map deletion boundaries, SNP array analysis was performed: Log2 ratio values of probes mapping the *RS1* gene showed a deletion from C-7FYTD (18,675,273 bp) to C-6OYLC (18,681,809 bp) of the telomere (first and last deleted oligonucleotides, respectively). The flanking normal probes were C-5IHRT (18,674,960 bp) and C-5ILYC (18,681,810 bp). SNP array analysis was then performed in all females of the family to identify carriers of exon 2 deletion; the results are reported in ISCN format in Figure 1.

Thus, the deletion was restricted to a region of ~6500 bp spanning from intron 1 to intron 2. This made it possible to design PCR primers around the breakpoint that produced a fragment of 1200 bp corresponding to the deleted allele in all affected males and all carrier females. This fragment was sequenced over the predicted deleted nucleotides of a few

FIG. 3. Scheme of deletion mechanism (NAHR). Arrows, position of Alu sequences; numbered boxes, position of *RS1* gene exons. NAHR, nonallelic homologous recombination.

hundred bases in 5' and few tens of bases in 3', because despite the high resolution of this type of SNP array, the accuracy does not exceed 1 kb. Sequences around the breakpoint showed the presence of three Alu repeats that were probably involved in the deletion mechanism.

The sequence obtained corresponds to AluJr, except for a nucleotide at 3' that may indicate the junction with AluSz (Fig. 2). Unfortunately, the presence of polyA in both senses prevents sequencing beyond the junction to confirm it. According to this hypothesis, the deletion mechanism is probably nonallelic homologous recombination at two complementary Alu sequences (Fig. 3).

However, we cannot exclude the presence of a sequence inserted at the breakpoint, impossible to sequence, due to the presence of polyA. In this case, the mechanism responsible for exon 2 deletion would be a more complex rearrangement including a deletion/insertion event.

Validation of MLPA-based assay

RS1 MLPA assay was first tested in normal controls. The results showed that the six probes, corresponding to the six *RS1* gene exons, worked correctly; exon copy number (dosage quotient [DQ]) was $0.85 < \text{DQ} < 1.15$ as expected for normal dosage. Then RS1 MLPA assay was tested in deleted controls and identified the deletion in all samples: affected males proved to be hemizygous ($\text{DQ}=0$) and their mothers proved to be heterozygous ($0.35 < \text{DQ} < 0.65$) for the expected deletions (Fig. 4). Although we did not have samples specifically bearing the exon 6 deletion, analysis based on normal male and female controls suggested it would be as sensitive as other exons.

Genetic characterization of XLRS family

Finally, the validated RS1 MLPA Kit was used to extend segregation analysis to all available family members. It confirmed the preliminary SNP array results (Fig. 1).

FIG. 4. MLPA results in female carriers and female control. y-axis: copy number in DQ; x-axis: *RS1* gene exons. Normal: $0.85 < \text{DQ} < 1.15$ (female control); heterozygous deletion: $0.35 < \text{DQ} < 0.65$; patient no. 2 (ID R480) shows the heterozygote deletion in exon 1; patient no. 4 (ID R482) shows deletions in exon 4 and 5; patient no. 6 (ID R476) shows deletions in exon 1, 2, 3, and 4. DQ, dosage quotient.

Discussion

Intragenic deletions and duplications are relatively frequent disease-causing mutations. Compared with single nucleotide variations, they are likely to be more deleterious for protein function. While these mutations in X-linked genes can be rapidly and efficiently detected in male patients by simple PCR-based methods, the detection of heterozygous female carriers is a real challenge by the same protocols. Given the importance of having a rapid test for female carriers, we decided to develop and validate an MLPA-based assay for the detection of possible deletions in the six exons of the *RS1* gene. MLPA is the technique of choice, because it is easy to reproduce when optimized and validated, besides being fast and relatively cheap.

In this article, we described the designing and validation of the MLPA-based assay for detection of deletions in the *RS1* gene responsible for X-linked juvenile retinoschisis. We also reported its application to the molecular diagnosis of a family affected with X-linked juvenile retinoschisis. The full exon 2 deletion found with our method explains the severe clinical phenotype: three out of four affected members of the last generation are legally blind. The *RS1* gene exon 2 deletion causes loss of corresponding protein residues in the exon (aa 18–26) and alteration of the coding frame, leading to a truncated protein p.(Ala18Glyfs*2). It can therefore be considered a null mutation. The exon 2 deletion is reported in other XLRS1 patients, and is the most frequent exonic deletion in the *RS1* gene (The Retinoschisis Consortium, 1998) due to the numerous repeated regions in intron 1 (61% of the entire sequence).

Variable genotype–phenotype correlations were documented in XLRS1. Some of the cases with exon deletions are associated with a very severe phenotype (Khan *et al.*, 2001; Chan *et al.*, 2004; Bowles *et al.*, 2011; D'Souza *et al.*, 2013) as we found in the proband and his brothers of this family. However, comparative study of the phenotype of these patients is necessary to further clarify this aspect.

In conclusion, we developed an assay for characterizing CNV in the *RS1* gene. It is a valuable test and can save time and expense. To our knowledge, there are no commercial kits available for this purpose. Female heterozygous carriers are easily detected by MLPA analysis and this has a significant impact on the clinical management of families with X-linked juvenile retinoschisis.

Acknowledgments

The contribution by Fondazione Bietti in this article was supported by the Ministry of Health and Fondazione Roma. The authors are grateful to Helen Ampt for revising the article.

Author Disclosure Statement

No competing financial interests exist.

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