Screening for mutations in *RPGR* and *RP2* genes in Jordanian families with X-linked retinitis pigmentosa

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ABSTRACT. Retinitis pigmentosa (RP) is a clinically and genetically heterogeneous disease causing progressive degeneration of retinal photoreceptor cells. X-linked RP (XLRP), in which photoreceptor degeneration begins in early childhood and complete blindness often occurs by the fourth decade of life, constitutes the most severe form of this disease. Two genes commonly associated with XLRP have previously been cloned: retinitis pigmentosa GTPase regulator (*RPGR*) and retinitis pigmentosa 2 (*RP2*). We sought to identify mutations in these genes in Jordanian families suffering from this disease. Five unrelated Jordanian families with confirmed XLRP were screened for such mutations using direct sequencing. Three mutations were identified in the ORF15 exon of *RPGR*. The silent g. ORF15+470G>A substitution and the g. ORF15+1822insA insertion in the 3'-untranslated region were found in both normal and affected male family members at comparable frequencies, and thus were considered normal variants. The third mutation, g. ORF15+588G>A, in which alanine is substituted by threonine, was found in all affected men and one unaffected man

in the two families harboring this variant. Thus, this mutation may be pathogenic, but with incomplete penetrance. No *RP2* mutations were found among the examined families. Mutation screening of RP patients is essential to understand the mechanism behind this disease and develop treatments. A complete family history is required to identify its inheritance pattern and provide genetic counseling for patients and their families.

Key words: X-linked; Retinitis pigmentosa; Mutation; *RPGR*; *RP2*

INTRODUCTION

Retinitis pigmentosa (RP) represents a clinically and genetically heterogeneous group of inherited retinal dystrophies characterized by progressive retinal degeneration. Typical symptoms include night blindness (nyctalopia) followed by a gradual decrease in the visual field starting from the periphery, leading to tunnel vision and eventually complete blindness (Hamel, 2006). RP predominantly impacts rod photoreceptors, although in later stages cone cells are also affected. Apoptosis appears to be the principal cause of photoreceptor degeneration in this disease (Marigo, 2007).

RP demonstrates various modes of inheritance, including autosomal-dominant, autosomal-recessive, X-linked recessive, and sporadic patterns. Rare forms, such as X-linked dominant, mitochondrial, and digenic RP also exist. X-linked RP (XLRP) accounts for 10 to 15% of all cases (Ferrari et al., 2011), and represents the most severe inherited form in terms of early onset and rapid progression of retinal degeneration in men (Aldred et al., 1994; García-Hoyos et al., 2006). According to a recent report by Shifera and Kay (2015), female carriers of XLRP may also manifest an early-onset, severe form of the disease, although the reason for this remains unclear. Patients with XLRP may become completely blind by their third or fourth decade of life, in some cases even earlier (Ji et al., 2010).

Two genes have been implicated in the pathogenesis of XLRP: retinitis pigmentosa GTPase regulator (*RPGR*) and retinitis pigmentosa 2 (*RP2*). Mutation of *RPGR* is considered the most common cause of XLRP, accounting for 70 to 75% of all XLRP cases (Pelletier et al., 2007; Jayasundera et al., 2010; Churchill et al., 2013). The *RPGR* gene has been shown to be responsible for four phenotypes, related to each other by their effects on photoreceptor cells. These include cone-rod dystrophy 1 (Mendelian Inheritance in Man No. [MIM] 304020), X-linked atrophic macular degeneration (MIM 300834), X-linked retinitis pigmentosa 3 (MIM 300029 or 312610), and X-linked retinitis pigmentosa with sinorespiratory infections (MIM 300455). *RPGR*, located on Xp21.1 (Roepman et al., 1996) and spanning 70 kb, consists of 19 exons encoding a protein of approximately 815 amino acids (Meindl et al., 1996). Open-reading frame 15 (ORF15) is the most commonly mutated *RPGR* exon in XLRP, accounting for 60 to 80% of cases (Vervoort et al., 2000; Pierrottet et al., 2014). ORF15 encodes a sequence of 567 amino acids rich in glutamic acid and glycine at the C-terminus of the *RPGR* protein (Vervoort et al., 2000; Yokoyama et al., 2001). Mutations in ORF15 have also been identified in X-linked cone-rod dystrophy, which belongs to the same group of retinal dystrophies as typical RP (Ebenezer et al., 2005).

RP2, mutations of which are the second most common genetic cause of XLRP, is located on Xp11.3. This gene comprises five exons and encodes a protein of 350 amino acids

that is weakly expressed in various body tissues (Schwahn et al., 1998). The RP2 N-terminal domain demonstrates homology with cofactor C, involved in tubulin folding (Evans et al., 2006), while the C-terminal domain shares similarity with nucleoside diphosphate kinase (Pelletier et al., 2007). RP2 is presumed to function as a chaperone protein (Jin et al., 2005).

The present study aimed to investigate mutations in the coding sequence of *RP2* and ORF15 exon of *RPGR* in five Jordanian families with XLRP.

MATERIAL AND METHODS

Subjects

Ethical approval for clinical study was obtained from the institutional review board of Jordan University of Science and Technology. Five Jordanian families confirmed as having XLRP based on inheritance patterns were recruited for this study. Of these five, three were enrolled following referral from King Abdullah University Hospital, and two were referred by collaborating clinicians in private ophthalmology clinics. XLRP diagnoses were confirmed by ophthalmic examination and pedigree analysis. Participants included both affected and non-affected family members. The latter were used as controls for mutation analysis. Informed consent was obtained from patients and healthy subjects after the nature of the study had been fully explained.

RPGR ORF15 and *RP2* gene analysis

Whole blood (3 mL) was collected from each affected and non-affected subject and stored in ethylenediaminetetraacetic acid-containing blood collection tubes at -20°C. Genomic DNA was extracted from blood samples using a QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA) following the manufacturer protocol. The polymerase chain reaction (PCR) primers used to amplify DNA fragments are shown in Table 1. ORF15 and *RP2* exons were amplified using the conditions described by Sheng et al. (2010) and Neidhardt et al. (2008), respectively. PCR products were then purified using a PCRquick-spin Kit (iNtRON Biotechnology, Seongnam, Korea), which uses silica-membrane technology. Purified PCR products were sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 310 DNA sequencer (Applied Biosystems). DNA sequencing was carried out in one direction for all exons, using either the forward or reverse primer. These results were then confirmed using the other primer of the pair.

Sequencing results were analyzed using the ChromasPro software (<http://www.technelysium.com.au/ChromasPro.html>), and ORF15 and *RP2* exon reference sequences were obtained from the Ensembl Genome Browser (<http://www.ensembl.org/index.html>).

RESULTS

The families were screened for mutations in *RPGR* ORF15 and all *RP2* exons. The X-linked pattern of inheritance was determined from pedigrees, in which only men were affected. In addition, no male-to-male transmission of the disease was observed. From these families, 16 affected males aged between 7 and 40 years were screened for mutations in the sequences of interest. Upon detecting a mutation, all family members were screened for the sequence variant identified. Sequencing analysis revealed three mutations in the ORF15 exon.

Of these, the missense mutation g. ORF15+588G>A (Figure 1), causing the amino acid alanine to be replaced by threonine, was found in two families.

Table 1. Sequences of primers used for screening of *RP2* exons and *RPGR* ORF15, and sizes of amplified fragments (Neidhardt et al., 2008; Sheng et al., 2010).

Primer	Forward (5'-3')	Reverse (5'-3')	Size (bp)
ORF15 1	AGGAAGGAGCAGAGGATTCA	CCCTCTCTTCCATTCTTCC	348
ORF15 2	GGGGAGAAAGACAAGGGTAG	TCCTTTCCCTCCTCTACTT	444
ORF15 3	GGAAGAAGGAGACCAAGGAG	CCCATTCCCTGTGTGTTAG	982
ORF15 4	GCAGGATGGAGAGGAGTACA	GAGAGAGGCCAAAATTACCA	415
RP2-1	GGGGTCCCAGGGTTCAC	TATCCGCGTCAAGAGAGTG	301
RP2-2 (5'-region)	CTGGCAGCCAATAGTCCTTT	TTTGAGGAAGACTCAATGATGG	401
RP2-2 (3'-region)	TTTCCGGAATTGCAGAGATT	TGAGGGTTGCCTGTATTCC	575
RP2-3	TCAGTGTGCTGTTGTGCAT	CAAATGAGAAAGAGAAGGCAATG	330
RP2-4	CCCTCAAAGAAGATGCTGGA	TCCAAGCAACATTAGGGACA	294
RP2-5	CTGTGGAGAACATGGGCTTT	AAACCATTGCTAATACACAAAAGTG	251
RP2-5s	CTGAAGTACTTGGTACTGATT	Sequencing primer	251

Figure 1. Results of DNA sequencing of the *RPGR* g. ORF15+588G>A mutation. **A.** Healthy son lacking the mutation; **B.** heterozygous carrier mother; and **C.** affected son with the mutation. Arrows show the affected nucleotide.

The first family (family A) consisted of three generations, including four affected men, 10 unaffected men, and 12 unaffected women. The second family (family B) comprised two affected men, three unaffected men, and four unaffected women. Screening all members of these two families revealed the presence of this missense mutation in all affected men and their mothers, but its absence in their fathers and unaffected men (with one exception in family A).

We also identified the insertion of a single adenine residue into an adenine stretch in the 3'-end of ORF15 (g. ORF15+1822insA; Figure 2). This was detected in one family (family C) consisting of three generations, with four affected men, 12 unaffected men, and 13 unaffected women, and was equally present among affected and unaffected family members.

Figure 2. Results of DNA sequencing of the *RPGR* g. ORF15+1822insA mutation, showing **A.** a healthy son carrying the insertion, and **B.** the carrier mother. The arrow in A shows the insertion position, while those in B highlight the displacement of the other abnormal X-chromosome copy.

Finally, the silent mutation g. ORF15+470G>A, in which the encoded glutamate residue is unaffected, was distinguished in one family (family D; Figure 3) comprising three generations, including five affected men, 17 unaffected men, and 14 unaffected women. This mutation was identified among affected and unaffected family members in equal measure. With respect to the last family (family E, made up of one affected man, three unaffected men, and three unaffected women), no mutations were detected in *RPGR* ORF15. Direct sequencing of all exons of *RP2* failed to reveal any mutations in the five participating families.

Figure 3. Results of DNA sequencing of the *RPGR* g. ORF15+470G>A mutation. **A.** Unaffected son with the mutation; **B.** affected son lacking the mutation; and **C.** heterozygous carrier mother. Arrows show the mutation locus.

DISCUSSION

RP is the most common form of inherited retinal degeneration (Sheng et al., 2010), and a high percentage (40-50%) of cases present a sporadic inheritance pattern (Kim et al., 2011). In XLRP, which is responsible for approximately 5 to 15% of all RP cases (Siemiakowska et al., 2011), most mutations occur in the *RPGR* gene (around 50% of cases) (Zito et al., 1999; Pusch et al., 2002; Neidhardt et al., 2008). *RP2* is also implicated in between 10 and 20% of XLRP cases, depending on the ethnic group under consideration (Zito et al., 1999; Pusch et al., 2002; Sheng et al., 2010). XLRP represents the most severe RP form, and different mutations within the same gene lead to clear variations in phenotype (Sheng et al., 2010).

In the present study, five families with XLRP were screened for mutations in *RP2* and the ORF15 exon of *RPGR*. No mutations in *RP2* were discerned in the families under examination. However, three mutations in ORF15 were identified. The g. ORF15+470G>A variant was classified as a silent mutation, since the resulting amino acid sequence was not affected. The 3'-terminal insertion mutation g. ORF15+1822insA was considered a normal variation, owing to its presence in affected and healthy family members at equal frequencies.

Finally, we detected a missense mutation involving a guanine-to-adenine substitution (g. ORF15+588G>A), resulting in the replacement of alanine with threonine (p. Ala196Thr) in the encoded protein sequence. This mutation was found in all affected male members and one unaffected male control subject in the two families harboring the variant. In addition, it was absent in male parents and present in heterozygous form in female parents in these families. This mutation is expected to be pathogenic and to affect protein function, since, although both neutral, the original and substituted amino acids differ in their side chains. The presence of this mutation in one unaffected man may indicate incomplete penetrance. This phenomenon is widely observed in genetic diseases, as the pathogenic locus may be affected by modifying genes and environmental factors. In accordance with this finding, this mutation was reported previously by Bader et al. (2003) in XLRP patients and some normal subjects (13%), and has also been documented by others (Roepman et al., 1996; Yokoyama et al., 2001). Alternatively, this mutation may be non-pathogenic but in linkage disequilibrium with another, as yet unknown, pathogenic *RPGR* sequence variation.

The absence of mutations in the other families studied suggests that further loci may be involved. In the present study, only the ORF15 exon of *RPGR* was screened. However, mutations in other exons of this gene have been reported as being linked to XLRP. For example, Neidhardt et al. (2008) and Vervoort and Wright (2002) reported pathogenic *RPGR* mutations, in exons other than ORF15, implicated in XLRP in patients from Germany, the Netherlands, Denmark, and Switzerland. In the current study, we were unable to screen all exons of this gene due to budget limitations. Thus, testing the remaining exons in future studies may uncover the genetic factors responsible for XLRP in the families for which no such variations were identified here.

CONCLUSIONS

Three mutations in the *RPGR* ORF15 exon were identified among Jordanian families with XLRP. g. ORF15+470G>A and g. ORF15+1822insA were considered normal variants. The g. ORF15+588G>A mutation is thought to be pathogenic with incomplete penetrance, or in linkage disequilibrium with an unidentified disease-causing sequence variation. In

agreement with previous reports, this study has shown that the ORF15 exon of the *RPGR* gene constitutes a mutation hotspot in Jordanian families diagnosed with XLRP. Future study will include screening and analysis of other *RPGR* exons in Jordanian families with XLRP.

Conflicts of interest

The authors declare no conflict of interest.

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