Molecular genetics of retinitis pigmentosa in two Romani (Gypsy) families

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Purpose: To identify the disease-causing mutations in two large Bulgarian Romani (Gypsy) pedigrees: one with autosomal dominant retinitis pigmentosa (adRP) with partial penetrance and the other with severe X-linked RP (xIRP).

Methods: Detailed clinical investigations were undertaken and genomic DNA was extracted from blood samples. DNA was analyzed by PCR amplification with gene-specific primers and direct genomic sequencing.

Results: Analysis of the complete coding sequence of PRPF31 in the adRP family led to the identification of a new heterozygous splice site mutation IVS6+1G>T. RPGR mutation screening in affected male individuals in the X-linked RP family identified a hemizygous c.ORF15+652_653delAG mutation. Interestingly this mutation was found in a homozygous state in one severely affected female from the family.

Conclusions: In this first report of molecular genetic analysis of retinitis pigmentosa in Romani families, we describe a novel PRPF31 mutation and present the first case of a homozygous mutation in the RPGR gene in a female individual.

Retinitis pigmentosa (RP) is a clinically and genetically heterogeneous disorder with incidence of almost 1 in 3500 or a total of 1.8 million people worldwide [1,2]. Affected individuals suffer from a progressive degeneration of the photoreceptors, eventually resulting in severe visual impairment. The disease is characterized by bone spicule-like pigmented deposits and reduced or absent electroretinogram (ERG). RP may be transmitted as an autosomal dominant (adRP), familial recessive (arRP) or X-linked trait [3,4]. To date, at least 12 causative genes for adRP have been identified, namely the genes for rhodopsin (3q), peripherin-RDS (6p), PIM1K (7p), IMPDH1 (7q), RP1 (8q), ROM1 (11q), NRL (14q), CA4 (17q23), FSCN2 (17q25), and the human homologues of yeast pre-mRNA splicing factors PRPF3 (1q), PRPF8 (17p) and PRPF31 (19q; RetNet). The products of these genes are associated with either photoreceptor structure, cellular function including the phototransduction cascade, or gene expression (e.g., transcription and mRNA splicing) [5]. Recently, two new loci for adRP have been reported RP31 on chromosome 9p22-p21.1 and (RP33 on chromosome 2cen-q12.1) but the genes remain to be identified [6,7].

X-linked RP (xIRP) is a genetically heterogeneous and relatively severe inherited retinal degeneration. Family-based linkage studies have identified five loci for xIRP: RP2 (Xp11.23), RP3 (Xp11.4), RP6 (Xp21.3-p21.2), RP23 (Xp22), and RP24 (Xq26-q27), and two genes (XRP2 for RP2 and RPGR for RP3) cloned using a positional cloning approach (RetNet). RP3 is the major subtype of xIRP accounting for over 70% of affected families [8,9]. The gene known as Retinitis Pigmentosa GTPase Regulator (RPGR) encodes several distinct alternatively spliced transcripts that are widely expressed [10,11]. Mutations in the constitutive RPGR protein of 815 amino acids are detected in approximately 20% of xIRP patients [12]. Subsequent studies revealed an unusual exon, ORF15 (immediately following exon 15) encoding a Gly- and Glu-rich carboxyl-terminal domain of 567 amino acids; mutations in this exon accounted for an additional 50% of xIRP. It is worth noting that 25% of RP males with no family history of the disease also have mutations in RPGR [9,13,14].

In this paper we describe the first report of the genetic basis for RP in Romani families. Romas are believed to be a genetically isolated founder population of Indian origin who left India during the 5th-10th century A.D. and spread throughout Europe by the end of the 15th century [15-19]. A large group of the initial migrant population, called “Balkan Gyp-
“Gypsies,” settled permanently in the Balkans south of the Danube in Bulgaria. Interestingly the various geographically, socially, and linguistically divergent Romani groups have been shown to share unique Mendelian disorders and founder mutations due to their common origin [19]. Our purpose was to search for mutations causing retinal degenerations in the Hungarian Romani families who have been an interesting resource for Mendelian genetics. Over a period of time an extensive clinical database of retinal degeneration patients has been established, and through detailed pedigree information and genealogical studies two large multigeneration families with RP have been constructed. One family displayed a dominant mode of inheritance with partial penetrance and the other family showed a severe form of xLRP. Partial penetrance associated with the chromosome 19q adRP locus (RP11) led us to undertake mutation screening of PRPF31. We screened XRP2 and RPGR since majority of xLRP families have mutations in these two genes.

METHODS

Family data and ophthalmologic examination: A five-generation Hungarian family of Romani origin with an autosomal dominant form of RP with partial penetrance was ascertained (Figure 1A). After informed consent, a detailed medical history was obtained and a full ophthalmological examination performed on 14 members of the family. A second, six-generation family from the same origin, showing X-linked mode of inheritance was recruited and 27 members clinically examined (Figure 1B). This included corrected visual acuity, Goldmann kinetic perimetry (V-4 and I-4 white test target), static threshold perimetry using the automated perimeter DICON TKS 5000 (Division of Vismed Inc., San Diego, CA), direct and indirect ophthalmoscopy, color fundus photography and fluorescein angiography, slit lamp biomicroscopy and full-field ERG.

Polymerase chain reaction and sequence analysis: Total genomic DNA was extracted from blood samples using Nucleon Genomic DNA Extraction Kit (Tepnel, Life Science). PRPF31 coding sequence of 14 exons, including their intron-exon boundaries, were amplified by polymerase chain reaction (PCR) from each individual to identify a mutation in Family A. The same methods were applied for the mutation screening of Family B, amplifying the whole coding sequence and splice sites of XRP2 (5 exons) and RPGR (19 exons), respectively, in both genes known to cause X-linked RP.

The mutation hot-spot within RPGR, ORF15 [20], was amplified and sequenced in four overlapping fragments. All exons and their respective acceptor and donor splice sites were directly analyzed from the PCR products using the ABI Prism® BigDye™ dGTP Terminator Ready Reaction Cycle Sequencing Kit, Foster City, CA. All sequences were analyzed in both forward and reverse directions on an ABI3100 fluorescent sequencer. Primer sequences and reaction conditions are available on request.

Figure 1. Romani pedigrees with retinitis pigmentosa from Bulgaria. A: Structure of a Romani family from Sofia with autosomal dominant retinitis pigmentosa. Asterisks mark all clinically phenotyped individuals. Normal individuals are shown as clear circles (female) or squares (male), and affected individuals are shown as solid symbols. Shaded circles denote asymptomatic obligate carriers of the mutation in PRPF31.

B: A Romani pedigree from Peshtera (Western Bulgaria) with X-linked retinitis pigmentosa. Filled circle indicates the female was found to be homozygous for the c.ORF15+652_653delAG mutation.
Reverse transcriptase polymerase chain reactions: Total RNA was isolated with an RNA extraction kit (Qiagen RNasy Midi kit; Qiagen Ltd., Crawley, UK). cDNA was made from RNA using SuperScript™ II Reverse Transcriptase (Invitrogen, UK). PRPF31 specific primers (forward 5'-CGA GCT GAA CA T CA T CCA TAA G-3' spanning the boundaries of exons 4 and 5 and reverse 5'-GGC CAC ACC CAT GAT CTT G-3' spanning the boundaries of exons 8 and 7) were designed to amplify a product of 391 nt of cDNA and a second product of 579 nt containing intron 6 of PRPF31.

RESULTS

Mutation analysis of PRPF31: Analysis of the mutation screening of the whole coding sequence of PRPF31 in Family A led to the identification of a heterozygous splice site mutation IVS6+1G>T (Figure 2A,B). The mutation is predicted to retain intron 6 in the splicing process and should result in a truncated protein of 186 amino acids (G176fsX186). The mutation was found in six affected individuals in Family A, who showed retinal degeneration, and also in three subjects, who appear to be asymptomatic. The retention of intron 6 in the affected groups were confirmed by the analysis of cDNAs made from lymphocyte RNAs (Figure 3).

Mutation analysis of RPGR: Screening of XRP2 revealed no mutations. RPGR mutation screening in affected individuals in Family B confirmed the presence of c.ORF15+652_653delAG mutation (GenBank accession number AF286472). This mutation is predicted to cause a frameshift in the protein product and has previously been reported to cause X-linked RP in different ethnic populations [9]. Interestingly this mutation was found in a homozygous state in one female member (marked with filled circle on Figure 1B) of the family with a severe form of RP (Figure 2C-E).

Clinical assessment of Family A (adRP): Fourteen members of Family A were clinically evaluated. Through ophthalmological examination and ERG testing, nine were found to be affected (age range between 12-48 years) and five appeared completely normal. Of the nine affected patients, six were symptomatic and three were asymptomatic with reduced scotopic b-wave amplitude on ERG as the only pathological finding. In the six symptomatic patients, onset of night blindness ranged from 8-11 years and the onset of subjective visual field

Figure 2. Sequence analysis for PRPF31 and RPGR. Comparison of electropherograms of (A) the wild-type sequence of the PRPF31 gene with (B) the novel single nucleotide change (G>T) at position +1 of intron 6 in the affected individuals in the autosomal dominant retinitis pigmentosa (adRP) Romani family from Sofia. C: Normal male control for RPGR. D: Positive male control for c.ORF15+652_653delAG mutation. E: Affected homozygous female for c.ORF15+652_653delAG mutation.

Figure 3. cDNA analysis by RT-PCR. Gel electrophoresis of PCR products obtained with specific cDNA primers of one unaffected and two affected individuals from Family 1A. In the affected individuals, a heterozygous pattern of bands migrating at 391 and 579 nt is seen due to unspliced intron 6 in one of the alleles. The unaffected individual shows only one band of 391 nt amplified from cDNA containing exons 5, 6, and 7.
loss was from 13-15 years. Visual acuity group in the affecteds ranged between 0.02-0.9 (metric). Two patients had refractive errors and only one patient (a 48-year-old male) had mild bilateral posterior subcapsular cataract.

Ophthalmoscopy disclosed typical signs of RP including narrowing of retinal vessels, scattered bone-spicule pigmentation in the midperipheral retina, RPE atrophy, choroidal sclerosis and optic disc pallor (Figure 4A). Cystoid macular edema was noted in one patient on fluorescein angiography. The youngest (a 12-year-old male) symptomatic patient had minimal fundus changes with narrowing of the retinal arterioles and loss of pigment from the pigment epithelium. Visual field examination (by kinetic Goldmann perimetry and automated static perimetry) revealed severe restriction of the visual field with intact central island (10-30°) of vision in 5/6 patients with the youngest showing only a discrete pericentral (“ring”) scotoma (Figure 4C). The rod and cone ERGs were nondetectable in almost all symptomatic patients with the three asymptomatics showing detectable but reduced scotopic b-wave amplitude (120 µV-140 µV).

Clinical assessment of Family B (xIRP): Twenty seven individuals from this family, which contains more that 150 members, were clinically examined, and 22 (14 males and 8 females) were found to be affected. The ages the male patients ranged from 22 to 78 years. Their initial symptom of night blindness was noted in early childhood (5-8 years) and the disease rapidly progressed to a severe loss of vision by the age 30-35 years in most of the men. Visual acuity exams were as follows: only light perception in three patients; light perception and projection in four patients; hand movement in two patients; counting fingers in two patients; visual acuity of both eyes was 0.01 in two patients; and only one patient (22 years old) had visual acuity of both eyes of 0.04. Low visual acuity prevented a precise visual field examination in most of the patients. Posterior subcapsular cataract was found in two patients. Ophthalmoscopy revealed signs of typical RP including attenuation of retinal vessels, RPE atrophy, choroidal sclerosis, scattered pigment deposits with bone-spicule configuration and pallor of the optic disc with relative preservation of the macular area (Figure 4B). Twelve patients had

![Figure 4](http://www.molvis.org/molvis/v12/a103/)

Figure 4. Clinical information for RP patients. A: Fundus photographs of right eye of a 34-year-old affected patient with IVS6+1G>T mutation in PRPF31. B: Fundus photographs of right eye of a 45-year-old affected patient heterozygous for c.0RF15+652_653delAG in RPGR. C: Pericentral (ring) scotoma in patient with autosomal dominant retinitis pigmentosa (adRP; left eye).
The four female patients (24-47 years) were found to have RP on the basis of medical history and ophthalmological examination. Compared to male patients, three of four of the female patients had more discrete symptomatology. Their visual acuity test results ranged between 0.08 and 0.6, reported later onset of night blindness (in their twenties to thirties) and in all three the disease progressed slowly. Ophthalmoscopy revealed minimal fundus changes, consisting of discrete diffuse dust-like intraretinal pigmentation but without pigmentary clumping or narrowing of the retinal vessels or RPE atrophy or optic disc pallor. Visual field was found to be constricted up to 30° with incomplete pericentral (“ring”) scotoma in one of the females and the amplitude of the scotopic b-wave was markedly reduced (60 µV in the right eye and 80 µV in the left eye). The fourth female patient (shown with an asterisk in Figure 1B) displayed typical signs of RP and was found to be as severely affected as a male in the pedigree. Generally this is unusual, however, quite uniquely a homozygous RPGR mutation was identified in this individual and may explain the very interesting clinical findings reported here.

**DISCUSSION**

This is the first report of the molecular basis of RP in Bulgarian Romani (Gypsy) families. Romas are a genetically isolated founder population. Many interesting Mendelian disease genes have been cloned previously due to the unique possibility for linkage mapping in this population [21]. In this report, we have identified a novel splice-site mutation (IVS6+1G>T) in the gene for pre-mRNA splicing factor PRPF31. Mutation cosegregates with the RP phenotype in all affected individuals in the X-linked pedigree. The substitution of nucleotide (G>T) occurs at the splice donor of intron 6, leading to the retention of this intron (Figure 3). It causes a frameshift that is predicted to lead to the formation of a truncated protein of 186 amino acids (G176fsX186). Previous research has shown mutation in PRPF31 that affects the third base in the donor site of intron 6 (IVS6+3A>G) results in the formation of the same truncated protein of 186 amino acids [22]. To the best of our knowledge approximately 30 mutations in PRPF31, comprising point mutations, splice site mutations, deletions, insertions, and indels have been detected. All adRP families with mutations in the PRPF31 gene show incomplete penetrance as a special characteristic. The Bulgarian Romani family also has this characteristic and has symptomatic and asymptomatic individuals within the pedigree.

The second mutation (c.ORF15+652_653delAG) was identified in a large X-linked kindred and appeared to be in the ORF15 of the RPGR gene. So far 97 mutations have been identified in the gene (hgmd). It was interesting to note and perhaps as expected, the female with the homozygous mutation is more severely affected compared to carrier females and is clinically on par with the affected male patients. The discovery of the mutation in the X-linked pedigree is not only important to the family in its own right, but also leads to a number of issues relating to possible diagnostic and predictive applications. The scope for testing for carrier status in females and possible pre-symptomatic testing in younger male offspring may allow the expansion of diagnostic services especially considering that this family has already been extensively worked up and possible new branches of the family may well be revealed in the future. The results of this study confirm the previous findings that the majority of RPGR mutations reside in ORF15 in families with definite X-chromosome assignment. Together with previous studies [23], our investigation confirms the RPGR gene to diagnostically be the most important single genetic locus for RP and this may also turn out to be the case in families of Romani origin.

Data provided by the social sciences as well as through genetic research suggest that the 10 million Romas who currently live in Europe can be best described as a conglomerate of genetically isolated founder populations. To date, the following sixteen Mendelian disorders are believed to be caused by private (i.e., confined to this population) “Roma” mutations: congenital glaucoma, galactokinase deficiency, hereditary motor and sensory neuropathy-Lom, hereditary motor and sensory neuropathy-Russe, congenital cataracts, facial dysmorphism neuropathy, limb-girdle muscular dystrophy type 2C, congenital myasthenia, Gitelman syndrome, neuronal ceroid lipofuscinoses, Glanzmann thrombasthenia, ataxia-te langiectasia, Von Willebrand disease, CD8 T-cell deficiency, congenital renal proximal tubular dysplasia, and kidney disease and hyperparathyroidism-jaw tumor syndrome [21]. Some of these are with documented founder effect and others have been reported in a small number of families with limited mutation frequency data so far. Recent population studies, initiated and designed to answer the questions raised by medical genetics, have made Romas one of the most interesting founder populations and valuable contributors to European genetic diversity [21].

Our study aimed to investigate large Roma families in Bulgaria with inherited retinal degenerations and to identify the molecular basis with particular reference to RP in this unique population. The 800,000 Romas in Bulgaria that comprise a large number of smaller groups represent approximately 10% of the Bulgarian population. Moreover, the major lesson learned from Mendelian genetics so far is that the identification of a small number of affected Roma families sharing the same private mutation usually signals a widespread problem, involving a larger number of patients in many countries. Currently available frequency data show that an average of one in eight subjects in the general Roma population is a carrier of one of five “private” mutations tested. Within individual Roma groups, carrier rates for specific mutations often exceed 5% and can be as high as 16% [21].

Our findings support the addition of two novel private mutations to the list of mutations associated with Mendelian disorders in the Romani (gypsy) population. This information opens up a new field of ophthalmic genetics and in particular for retinal degenerations in this newly discovered founder population.
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