Detection of alterations in all three exons of the peripherin/RDS gene in Swedish patients with retinitis pigmentosa using an efficient DGGE system

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Abstract

***Aims*—To develop a sensitive mutation screening procedure suitable for routine analysis of the peripherin/RDS gene, and to estimate the nature and prevalence of peripherin/RDS gene mutations in Swedish patients with autosomal dominant retinitis pigmentosa.**

***Methods*—To make the method as sensitive as possible, as many as eight segments, covering the three exons and the flanking intron sequences of the peripherin/RDS gene, were analysed by denaturing gradient gel electrophoresis. A group of 38 Swedish patients with a clinical diagnosis of autosomal dominant retinitis pigmentosa were screened for mutations in the peripherin/RDS gene.**

***Results*—Three point mutations were found in four of the patients and five polymorphisms were defined. One mutation in exon 1, R172W, has been described previously in other ethnic groups as causing a macular degeneration. Another mutation, in exon 2 and causing the substitution F211L, was found in two unrelated patients. A third mutation, resulting in the likely non-pathogenic substitution S289L, as well as a polymorphism not reported previously, was found in exon 3.**

***Conclusions*—The screening procedure described allows detection of mutations in all of the exons, including the polymorphic 5' and 3' ends of the gene, and is therefore suitable for routine screening of peripherin/RDS gene defects in patients with autosomal dominant retinitis pigmentosa. The frequency of mutations found in the Swedish patient group indicates that defects in the peripherin/RDS gene might be a more common cause of autosomal dominant retinitis pigmentosa than was thought previously.**

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Retinitis pigmentosa is a heterogeneous entity with constriction of the visual fields, moderate reduction of visual acuity, and pigmentation of the retina as the common features; these characteristics have been the main diagnostic criteria in the past. The overall incidence of retinitis pigmentosa is about one in 4000, without any apparent ethnic or racial differences.[1] Retinitis pigmentosa unassociated with other abnormalities is inherited as an autosomal dominant, as an autosomal recessive or, less frequently, as an X-linked recessive disease. In recent years, multiple different defects in the genes encoding a number of photoreceptor associated proteins have been reported for patients with retinitis pigmentosa, including rhodopsin,[2] peripherin/RDS,[3 4] ROM-1,[5] rod PDE-β,[6] rod cGMP gated channel,[7] and rod PDE-a,[8] displaying the heterogeneity of the molecular basis for the disease.

In our study, we have examined 38 Swedish patients with the clinical diagnosis of autosomal dominant retinitis pigmentosa, looking for sequence variations in the peripherin/RDS gene. We used denaturing gradient gel electrophoresis (DGGE) analysis[9] of polymerase chain reaction (PCR) amplified DNA fragments,[10] which contained a 40 base pair (bp) long GC clamp at one end,[11] and covered all three exons and flanking intron sequences of the peripherin/RDS gene. The sensitivity of the screening procedure was optimised by dividing the three exons of the gene into eight fragments according to the MELT87 computer algorithm (kindly supplied by L Lerman and W Fripp, Cambridge, Massachusetts, USA).

Material and methods

SUBJECTS

Thirty eight patients from the department of ophthalmology were included in our study. The patients were diagnosed clinically as having retinitis pigmentosa, and family history indicated that the disease was inherited. The ophthalmological examination included visual acuity, ophthalmoscopy, slit lamp examination, and fundus photography. Visual field testing was performed with Goldmann kinetic perimetry and the final dark adapted rod thresholds were obtained with a Goldmann-Weeker adaptometer.

ELECTRORETINOGRAPHY

Full field electroretinograms were recorded in a Nicolet Compact Four analysis system (Nicolet Biomedical Instruments, Madison, Wisconsin, USA), as described previously.[12] Both eyes were tested after maximal pupil dilatation with topical 10% phenylephrine hydrochloride and 1% cyclopentolate hydrochloride, and after 40 minutes of dark adaptation. A Burian-Allen bipolar contact lens electroretinogram electrode was applied on the topically anaesthetised cornea, together with a

Table 1 Primers and conditions for PCR and DGGE of peripherin/RDS exon fragments

Fragment	*Primer*	*Oligonucleotide sequence (5′ to 3′)*	*PCR annealing temperature*	*DGGE (%)*
1A	UE171	GTT TGG GGT GGG AGC TGT GC	63°C	50–70%
	UE172	GC-AG TTG GGC ACA AAA TGG CTC TC		
1B	UE173	GC-TT CCT GAA GAT TGA ACT CCG AAA GA	63°C	50–70%
	UE174	GCA GAG AGC CAC AAG GAA GAG		
1C	UE175	GC-CT GAA GCC GTA CCT GGC TAT C	63°C	50–70%
	UE176	GTG TCC CGG TAG TAC TTC ATG C		
1D	UE177	GCT CGC TGG AGA ACA CCC TG	63°C	50–70%
	UE178	GC-CC CCA ATA TAT TCA TAG CTC TGA C		
2A	UE179	GC-GG GAA GCC CAT CTC CAG CTG T	60°C	50–75%
	UE180	TGG TGA TCT GAT ACT GGA TGC AG		
2B	UE181	GC-TC CCT TTC AGC TGC TGC AAT C	63°C	50–75%
	UE182	GGA GGC TCT CCT TAC CCT CTA		
3A	UE183	GC-CT CCC AGC GAT TCT CCC AGA T	60°C	50–75%
	UE184	GGC ACG CTC TTC TCC AGC AG		
3B	UE185	GC-TG GAT GGT GTG TCC AAC CCC	63°C	65–90%
	UE186	GAG TGC ACT ATT TCT CAG TGT TC		

For each fragment, the nucleotide sequence of the primers used, the PCR annealing temperature, and the DDGE gradient used for analysis are given.
GC-, GC clamp (GCG CCG CGC CCG TCC CGC CGC CGC CCG CCG CCG GCG CCC G).

ground electrode on the forehead. Responses were obtained with a wide band filter (3 dB at 1 Hz and 500 Hz), stimulating with single, full field flashes (100 μsecond) with dim blue light (Wratten filters numbers 47, 47A, and 47B combined; 25 cd/m^2), and with white light (7500 cd/m^2) (0.75 cd•second/m^2). Cone responses were obtained with 30 Hz flickering white light (7500 cd/m^2) averaged from 20 sweeps. The referred luminances of the three different light stimuli were measured on the light reflected from the Ganzfeld sphere.

MUTATION SCREENING

Blood samples from the subjects were collected in tubes containing ethylenediaminetetra acetic acid (EDTA) as anticoagulant and genomic DNA was isolated from the leucocyte fraction.[13] Melting maps of PCR fragments corresponding to each of the three exons of the peripherin/RDS gene with a 40 bp GC clamp attached to either the 5'-end or the 3'-end of the fragments were calculated using the MELT87 computer algorithm. Because of exon length and composition, exon 1 was divided into four overlapping fragments (denominated 1A–D) and exons 2 and 3 were divided into two overlapping fragments each (2A and B, 3A and B, respectively). The design of the primers used was based on the genomic exon 1, 2, and 3 sequences deposited in the EMBL database (entries U07147–9). To optimise detection of splice site mutations, the primers were constructed so that at least 15 bp of the flanking intron sequences were included in the amplified fragments. PCR was performed in a thermal cycler (Perkin Elmer-Cetus, Norwalk, Connecticut, USA) using reagents in the GeneAmp PCR reagent kit (Perkin Elmer-Cetus). Amplification was performed using one minute denaturation at 95°C, one minute annealing, and one minute extension at 72°C, repeated for 32 cycles. The cycles were preceded by five minutes denaturation at 95°C and followed by a six minute extension at 72°C. Table 1 gives annealing temperatures and the sequences of the primer pairs used.

DGGE was performed on dedicated equipment from CBS Scientific Co (Del Mar, California, USA) according to the protocol described by Myers *et al*.[9] The amplified GC clamped fragments were analysed on a gel with a denaturating gradient ranging from 50% to 90% denaturant (100% denaturant is 7 M urea and 40% (vol/vol) deionised formamide), with the exact range depending on the fragment screened (table 1). The buffer temperature was 60°C. Electrophoresis was carried out for 22 hours at 100 V (5 V/cm). The gels were stained with ethidium bromide (3 mg/ml) and the band patterns visualised by UV light.

PCR fragments indicated by DGGE to contain sequence alterations were sequenced using the dideoxy chain termination method.[14] Fragments for sequencing were prepared by PCR of the indicated fragment according to the PCR

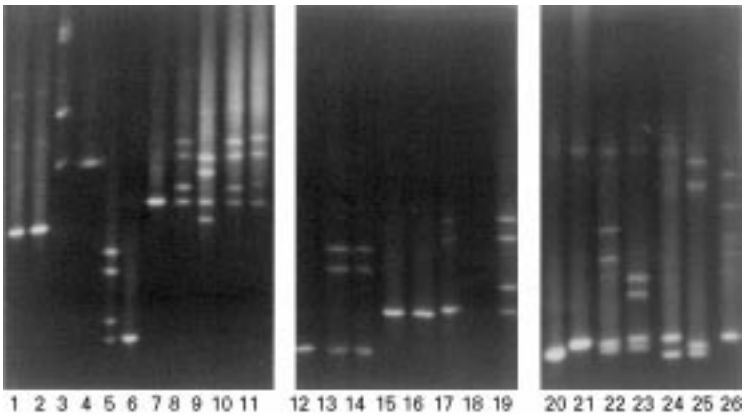

Figure 1 DGGE patterns. The amplified GC clamped fragments were analysed on a gel with a denaturating gradient ranging from 50% to 90% denaturant (100% denaturant is 7 M urea and 40% (vol/vol) deionised formamide), depending on the the screened fragment. Electrophoresis was carried out for 22 hours. The gels were stained with ethidium bromide and the band patterns visualised by UV light. The photographs are from three different gel runs with different denaturing gradients: lanes 1–11, 50–70%; lanes 12–19, 50–75%; and lanes 20–26, 65–95%. A sequence alteration gives rise to additional bands compared with the single band that is seen for the wild-type homoduplex in the electropherogram. In the description below, fragments with mutations are denominated with the resulting amino acid substitution in the case of control DNA samples with known mutations or, in the case of the patient DNA samples analysed in our study, by patient number. Exon 1A (promotor, codon 55), lanes 1 and 2: wt (wild-type fragments). Exon 1B (codons 49–111): lane 3, poly (fragments with polymorphisms); lane 4, wt. Exon 1C (codons 105–136): lane 5, poly; lane 6, wt. Exon 1D (codon 131, intron 1): lane 7, wt; lane 8, RP13; lane 9, L185P; lane 10, R172W; lane 11, R172Q. Exon 2A (intron 1, codon 220): lane 12, wt; lane 13, RP6; lane 14, RP37. Exon 2B (codon 217, intron 2): lane 15, wt; lane 16, wt. Exon 3A (intron 2, codon 306): lane 17, poly; lane 18, empty; lane 19, RP27. Exon 3B (codon 297–3' non-translated region), seven different genotypes—different combinations of four polymorphisms in fragment 3B (in codons 304, 310, and 338, and in the 3' non-translated region (nucleotide 13), respectively): lane 20, RP6 (E/E304, K/K310, G/G338, C/C); lane 21, RP2 (E/E304, K/K310, G/G338, T/T); lane 22, RP11 (Q/E304, K/K310, D/G338, C/C); lane 23, RP8 (E/E304, K/K310, G/G338, C/T); lane 24, RP14 (Q/E304, R/K310, D/G338, C/T); lane 25, RP13 (Q/E304, R/K310, D/G338, C/C); lane 26, RP7 (Q/E304, K/K310, D/G338, C/T).

Table 2 Sequence alterations found in patients with autosomal dominant retinitis pigmentosa

Identity	*Type*	*nt change*	*Protein change*	*Location*	*Comments*
Disease causing mutations					
RP13	Missense R172W	C→T	Arg→Trp	Exon 1	[a]
RP6	Missense F211L	C→G	Phe→Leu	Exon 2	[b]
RP37	Missense F211L	C→G	Phe→Leu	Exon 2	[b]
Sequence alteration of unclear significance					
RP27	Missense S289L	C→T	Ser→Leu	Exon 3	Novel[c]
Polymorphisms					
V106V		C→T	None	Exon 1	[d]
E304Q		G→C	Glu→Gln	Exon 3	[e]
K310R		A→G	Lys→Arg	Exon 3	[e]
G338D		G→A	Gly→Asp	Exon 3	[e]
3′NT, nt 13		C→T	None	Exon 3	Novel[c] (Apa I site)

[a]Wroblewski *et al*.[16]
[b]EH Souied *et al*, the association for research in vision and ohpthalmology meeting, Fort Lauderdale, Florida, USA, 1995 (abstract).
[c]No reports found in the Medline database, as of June 1997.
[d]Farrar *et al*.[3]
[e]Jordan *et al*.[15]
nt, nucleotide; 3′NT, 3′ non-translated region.

protocol described above. Both strands of the PCR fragments were sequenced to cover the entire sequence, and the mutations were determined by sequencing both strands. Sequence reactions were performed according to the Sequenase PCR product sequencing kit (United States Biochemical, Cleveland, Ohio, USA).

Results

The peripherin/RDS gene fragments giving aberrant band patterns on DGGE (fig 1) were analysed and divided into two groups. Identical aberrant band patterns seen on DGGE of an exon fragment from several patients was denominated a polymorphism. Such polymorphisms were also found when screening a set of 90 individuals with non-autosomal dominant retinitis pigmentosa. Table 2 gives details of the polymorphisms found, as defined by sequencing. Five different polymorphisms were detected. The four located in the coding parts of the exons have been described previously.[3 15] The fifth polymorphism is unreported and is situated just downstream from the stop codon in exon 3, leading to a novel restriction site for Apa I. The other group of exon fragments showing aberrant patterns on DGGE, patterns unique for one or two patients but not found in the group of non-autosomal dominant retinitis pigmentosa individuals, was suspected of carrying potentially disease causing mutations. Three different sequence alterations, detected in four of the 38 patients investigated, belonged to this group (table 2). The patients were heterozygous for the gene alterations.

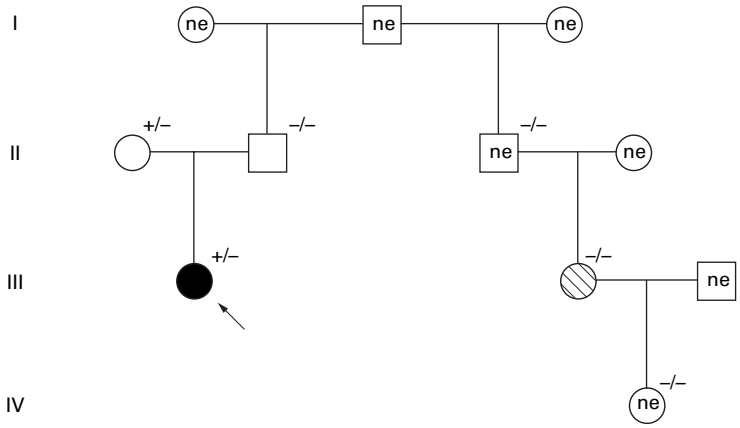

Figure 2 Pedigree for the family of patient RP27 with the S289L substitution. Circles, women; squares, men; open symbols, patients were examined and had a normal phenotype; closed symbol, patient was examined and had the retinitis pigmentosa phenotype; hatched symbol, patient was not examined but had the retinitis pigmentosa phenotype by patient history; NE, not examined and unavailable for investigation; +/−, presence or absence of the S289L mutation; the arrow indicates the index case.

One of the mutations, found in exon 1 of patient RP13, results in the amino acid substitution R172W, and has been described previously in at least four unrelated families.[16–18] The clinical picture of this patient was that of macular degeneration.

An exon 2 mutation was found in patients from two apparently unrelated families (RP6 and RP37). This mutation gives rise to the substitution F211L, which has been detected previously, but without segregation information (EH Souied *et al*, the association for research in vision and ophthalmology meeting, Fort Lauderdale, Florida, USA, 1995 (abstract)). Investigation of members in three generations of one of the affected families demonstrated a co-segregation of the F211L mutation and a disease phenotype of a rather atypical form of retinitis pigmentosa.[19]

Another previously undescribed sequence alteration, in exon 3, was found in patient RP27, causing the substitution S289L. The patient showed a retinitis pigmentosa phenotype with typical clinical characteristics and electroretinograms demonstrated typical findings for retinitis pigmentosa. The visual field was constricted to 10° with stimuli I_{4e} and to 20° with stimuli V_{4e}. Best corrected visual acuity was 20/30 and ophthalmoscopy revealed attenuated vessels and spicular pigmentation in all four quadrants. Final dark adapted rod thresholds were raised by at least 2–3 log units. Full field electroretinography revealed no rod responses to dim blue light and that responses to white light were greatly reduced, indicating loss of rod and cone function. The isolated cone response to 30 Hz flickering light was reduced and delayed. Further investigation of the patient's family showed that the mutation was present in DNA from the patient's mother, but not in that of the patient's older relative who suffered from retinitis pigmentosa (fig 2). However, the investigation also revealed that both of the patient's parents had normal vision, in spite of the initial patient history, incorrectly

indicating dominant inheritance at the time when the patient was included in our study. Nonetheless, the substitution S289L could not be detected in any of the other 37 patients with autosomal dominant retinitis pigmentosa or in any of the 90 non-autosomal dominant retinitis pigmentosa patients who were screened. Thus, it represents an uncommon sequence alteration, which could be phenotype modulating and hence possibly affect the expression of the disease in patient RP27.

Discussion

In patients with autosomal dominant retinitis pigmentosa, several pathogenic alterations in the perpherin/RDS gene have been described. There are many reasons for analysing the occurrence of such possible mutations. Defining the gene defects of individual patients will help to elucidate the relation between the loci of the defects and the clinical expression of autosomal dominant retinitis pigmentosa. In addition, such information will be of importance for the understanding of the normal functions of the peripherin protein. Furthermore, the clinical diagnosis of autosomal dominant retinitis pigmentosa can be difficult in some patients, especially in children, who have not yet developed the characteristic criteria of the disease. In such cases, mutation analysis can be decisive for correct diagnosis, thereby achieving an early assessment of the visual handicap of the patient and the prognosis of the disease. Molecular biochemical analyses will therefore be of increasing importance.

A number of different screening approaches can be used to narrow down the gene region that needs to be sequenced to reveal a disease causing gene defect. None of the common screening methods detects every possible mutation in a given gene fragment; methods include those based on single strand conformation polymorphism analysis (SSCP), temperature gradient gel electrophoresis, the RNAase cleavage method, heteroduplex analysis, the chemical cleavage method, or DGGE. DGGE and SSCP, the two most widely used screening methods, are thought to detect more than 80% of single base pair substitutions in given fragments,[20 21] but the methods probably do not detect the same mutations if applied to the same gene material. DGGE as a screening method for detection of point mutations and other minor gene defects has proved to be helpful in the mutation screening of an array of different genes, including the peripherin/RDS gene.[17 22] In our efforts to optimise the sensitivity of the method, guided by theoretical calculations with the computer program MELT87, we divided the three exons into as many as eight overlapping DNA fragments. We also used a long DGGE run time (22 hours). We found mutations in all three exons of the gene with this optimised DGGE system. Notably, those in exons 1 and 3 were both located in the vicinity of polymorphic loci in the gene, but could still be readily distinguished by their unique and clear DGGE patterns (fig 1). In our previous analysis of DNA from the 38 patients with SSCP under standard conditions (1996, unpublished), we could only detect the exon 2 mutation, whereas using the optimised DGGE system we could detect mutations in all three exons.

Four of the 38 patients with retinitis pigmentosa included in the our study had sequence alterations in the peripherin/RDS gene. In three of these patients, the mutations are potentially disease causing for the following reasons. The R172W and F211L mutations are both located in a segment of the polypeptide chain that is thought to be located on the intradiscal side, when the molecule is inserted in the disk membrane. These mutations also affect amino acid residues that are wholly conserved in the human, mouse, rat, and bovine peripherin/RDS sequences.[23] In addition, co-segregation of the R172W and F211L mutations with a retinitis pigmentosa disease phenotype has been shown.[16–19]

The S289L mutation affects a residue probably located on the cytoplasmic side of the membrane which, in contrast to the mutations above, is unique for the human sequence (A289 in the mouse, rat, and bovine sequences). The mutation could not be detected in any of the other 127 individuals tested and should thus be regarded as a rare sequence alteration. The lack of co-segregation of the mutation with the disease in the patient's family makes it possible that an alteration in a gene other than the peripherin/RDS gene is the disease causing event. Nevertheless, the fact that the family of the patient carrying the S289L mutation had a history of eye disease could mean that the S289L mutation is one of the mutations responsible for a digenic retinitis pigmentosa in which the other disease gene has not been identified yet. A digenic cause of retinitis pigmentosa has been reported, with concomitant mutations in the peripherin/RDS and ROM-1 genes found in affected individuals.[24]

In earlier studies, less than 5% of the autosomal dominant retinitis pigmentosa cases have been ascribed to mutations in the peripherin/RDS gene.[4 25] However, in some of these studies, a part of the polymorphic region of the gene was not screened for mutations. Although our material is limited, our finding that at least three of 38 patients exhibited disease causing mutations indicates that peripherin/RDS gene defects might be more frequent than thought previously. Earlier reports have also indicated that mutations in the rhodopsin gene are more common in patients with autosomal dominant retinitis pigmentosa than peripherin/RDS gene mutations. For comparison, our earlier studies of the same patient group that was investigated here have revealed rhodopsin gene defects in only three of the 38 families.[26–28]

In conclusion, the DGGE screening described here has proved to be reliable and highly sensitive, making peripherin/RDS gene analysis by DGGE suitable as a routine procedure in the management of families with retinitis pigmentosa. Mutations in the peripherin/RDS gene as the underlying cause of

autosomal dominant retinitis pigmentosa seem to be at least as common among Swedish patients as in populations examined previously.

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