Assessment of the interphotoreceptor matrix proteoglycan-1 (IMPG1) gene localised to 6q13-q15 in autosomal dominant Stargardt-like disease (ADSTGD), progressive bifocal chorioretinal atrophy (PBCRA), and North Carolina macular dystrophy (MCDR1)

Andrea Gehrig, Ute Felbor, Rosemary E Kelsell, David M Hunt, Irene H Maumenee, Bernhard H F Weber

Abstract

We have recently characterised the genomic organisation of a novel interphotoreceptor matrix proteoglycan, IMPG1, and have mapped the gene locus to chromosome 6q13-q15 by fluorescence in situ hybridisation. As the interphotoreceptor matrix (IPM) is thought to play a critical role in retinal adhesion and the maintenance of photoreceptor cells, it is conceivable that a defect in one of the IPM components may cause degenerative lesions in retinal structures and thus may be associated with human retinopathies. By genetic linkage analysis, several retinal dystrophies including one form of autosomal dominant Stargardt-like macular dystrophy (STGD3), progressive bifocal chorioretinal atrophy (PBCRA), and North Carolina macular dystrophy (MCDR1) have previously been localised to a region on proximal 6q that overlaps the IMPG1 locus. We have therefore assessed the entire coding region of IMPG1 by exon amplification and subsequent single stranded conformational analysis in patients from 6q linked multigeneration families diagnosed with PBCRA and MCDR1, as well as a single patient from an autosomal dominant STGD pedigree unlinked to either of the two known STGD2 and STGD3 loci on chromosomes 13q and 6q, respectively. No disease associated mutations were identified. In addition, using an intragenic polymorphism, IMPG1 was excluded by genetic recombination from both the PBCRA and the MCDR1 loci. However, as the autosomal dominant Stargardt-like macular dystrophies are genetically heterogeneous, other forms of this disorder, in particular STGD3 previously linked to 6q, may be caused by mutations in IMPG1.

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The positional candidate approach relies on the mapping of a disease locus and a candidate gene to a common chromosomal region and the

assessment of the functional properties of the gene product with regard to the disease pathology. Several criteria, such as tissue specificity of gene expression, cellular localisation of the transcript, immunohistochemical data, possibly in combination with interspecies nucleotide/protein sequence comparison or protein pattern database searches, may provide helpful information on the probable function of a candidate gene. With an increasing number of mapped genes and expressed sequence tags (ESTs) available through the international efforts of the Human Genome Project, the positional candidate strategy has become the method of choice for the identification of disease genes.¹

Recently, a novel gene, IMPG1, encoding a major proteoglycan of the interphotoreceptor matrix (IPM), was identified and shown to be preferentially expressed in retina by both rod and cone photoreceptor cells.²⁻³ The IPM occupies the space between the neurosensory retina and the retinal pigment epithelium (RPE) and is largely composed of insoluble glycoconjugates that appear to form specific structures around the rod and cone photoreceptor cells, commonly referred to as cone matrix sheaths and rod matrix domains.⁴⁻⁷ The IPM glycoconjugates, which are primarily chondroitin sulphate proteoglycans, have been proposed to mediate interactions among various cell types, including the RPE, the photoreceptors, the glial Müller cells, and the choriocapillaris⁸⁻⁹ and may be vital for retinal adhesion and maintenance of photoreceptor cells.¹⁰⁻¹¹

More recently, we have determined the genomic organisation of IMPG1 and have mapped the gene locus to chromosomal region 6q13-q15¹² (fig 1). Interestingly, several retinal dystrophies including autosomal dominant Stargardt-like macular dystrophy (STGD3),¹³ progressive bifocal chorioretinal atrophy (PBCRA),¹⁴ and North Carolina macular dystrophy (MCDR1)¹⁵ have been localised to an overlapping region on the proximal long arm of chromosome 6 by genetic linkage analysis (fig 1). In order to assess whether mutations in IMPG1 may be associated with one of these macular dystrophies, we have now analysed the 17 coding exons of the gene for disease causing mutations in a family with autosomal dominant Stargardt-like macular dystrophy (ADSTGD),

Institut für
Humangenetik,
Biozentrum,
Universität Würzburg,
Am Hubland, D-97074,
Germany
A Gehrig
U Felbor
B H F Weber

Augenklinik,
Universität Würzburg,
D-97074 Würzburg,
Germany
U Felbor

Institute of
Ophthalmology,
University College
London, London
EC1V 9EL, UK
R E Kelsell
D M Hunt

The Wilmer Eye
Institute, The Johns
Hopkins Center for
Hereditary Eye
Diseases, Baltimore,
MD 21287-9237, USA
I H Maumenee

Correspondence to:
Dr Weber.

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Figure 1 Idiogram of chromosome 6 and genetic map of proximal 6q illustrating the relative locations and flanking DNA markers of gene loci for autosomal dominant Stargardt-like macular dystrophy (STGD3),¹³ North Carolina macular dystrophy (MCDR1),¹⁵ and progressive bifocal chorioretinal atrophy (PBCRA).¹⁴ Below, the genomic organisation of the gene encoding the interphotoreceptor matrix proteoglycan-1 (IMPG1) and its chromosomal localisation is shown.¹²

so far unlinked to STGD2 or STGD3,¹⁶ in three MCDR1 pedigrees of German descent recently linked to proximal 6q^{17, 18} and in a five generation British PBCRA pedigree previously mapped to proximal 6q.¹⁴ Our results definitively exclude IMPG1 as a candidate gene for MCDR1 and PBCRA on the basis of SSCA and the analysis of recombinant chromosomes. Furthermore, direct sequencing has not shown any disease associated mutations in our patient with ADSTGD. However, as autosomal dominant Stargardt-like macular dystrophies are known to be genetically heterogeneous,^{13, 16, 19} an involvement of IMPG1 in one form of ADSTGD cannot be ruled out at present and must await the mutational analyses

Figure 2 Pedigrees segregating autosomal dominant Stargardt-like macular dystrophy (ADSTGD),¹⁶ North Carolina macular dystrophy (MCDR1),¹⁵ and progressive bifocal chorioretinal atrophy (PBCRA).¹⁴ The closed symbols indicate affected status, while open symbols indicate subjects who were examined and found to be clinically normal. Affected subjects analysed for mutations in the IMPG1 gene are marked by an arrow.

of the IMPG1 gene in STDG3 previously linked to proximal 6q.¹³ The availability of the exon/intron structure will allow the further assessment of the potential role of IMPG1 in various retinopathies of unknown aetiology.

Materials and methods

PATIENTS AND FAMILIES

The clinical, histopathological, and ultrastructural phenotype of a three generation pedigree with autosomal dominant Stargardt-like macular dystrophy (also known as fundus flavimaculatus-like macular dystrophy) and late onset atrophic macular degeneration has been described in detail elsewhere.¹⁶ In brief, mild blurring of central vision was first noted by the index patient at the age of 38. A paracentric scotoma was present in both eyes. Ophthalmoscopy showed a reddish foveal appearance surrounded by small yellowish-white, atrophic subretinal flecks and a reticular pattern of subretinal pigmentation in the midperiphery of both eyes. Multiple focal and confluent areas of choroidal hyperfluorescence were detected by fluorescein angiography. Bilateral retinal pigment epithelium (RPE) window defects were present in the fovea. There were areas of silent or dark choroid. The photopic electroretinogram (ERG) was subnormal in both eyes whereas scotopic ERG and electro-oculogram (EOG) were within the normal range. Inspection of the pedigree indicates autosomal dominant inheritance (fig 2). For mutational analysis, a DNA sample from a single affected subject was available (arrowed in fig 2).

In a recent study, the North Carolina macular dystrophy (MCDR1) phenotype was linked in three multigeneration families of German descent to DNA markers derived from chromosome 6q14-q16.2,^{17, 18} a region known to harbour the MCDR1 gene.¹⁵ The family members underwent comprehensive ophthalmological examination. The affected subjects in families L, A, and W presented with fundus abnormalities consistent with MCDR1 and are described in detail elsewhere.^{17, 18} The DNA from one affected subject per family was used for the mutational analyses (arrowed in fig 2).

Genetic linkage analysis on a five generation British pedigree expressing the progressive bifocal chorioretinal atrophy (PBCRA) phenotype has mapped the gene locus by two point linkage to an interval between D6S249 and D6S301 on proximal chromosome 6q.¹⁴ Detailed descriptions of ophthalmological findings in individual members of the PBCRA family are given in Douglas *et al*²⁰ and Godley *et al*.²¹ Briefly, PBCRA is a progressive disease characterised by two distinct foci of atrophy, a temporal focus which is present at birth and a nasal focus which appears in the second decade of life. In addition, it is associated with nystagmus, myopia, and a significant reduction in visual acuity and colour vision. Photopsia and retinal detachment are complications of the disease.

MUTATIONAL ANALYSIS

DNA was isolated from leucocyte nuclei by standard extraction methods. Based on the genomic exon/intron sequences of IMPG1,¹²

Table 1 Exon/intron oligonucleotide primers for IMPG1

Exon No	Exon size (bp)	Forward primer	Reverse primer	Fragment size (bp)	Annealing (°C)
1	67	74: 5'-AGATTTGAGGTTGTTCTGTG-3'	74R: 5'-TCGGTAGATTTTATAGATAAGC-3'	173	54
2	234	Ex2F: 5'-TATTCACCAGGATATCAATC-3'	5'R: 5'-ATAACACAAGGCTAAACGG-3'	303	51
3	167	429F: 5'-GACCTCTTAGGCATGGCC-3'	429R: 5'-AACTGTTTCGTGCAGTGTTC-3'	254	58
4	29	605F: 5'-TCTTCGGTTGGCATTATCTC-3'	605R: 5'-GAATAGCTTAGTTTCACTCC-3'	165	53
5	65	672F: 5'-TCTCTTTCAACACCTCTTCC-3'	672R: 5'-TTGAATGAAAGTTGAGAAGC-3'	150	51
6	104	789F: 5'-CTCCATTCTCATTTTTGTGTC-3'	789R: 5'-ATGAAGAGCTAGATCAACTC-3'	182	53
7	141	4F: 5'-GTTTAGGTTAACAAGAAGTCC-3'	4RII: 5'-CACCTCTCAGTCACAGCTTG-3'	235*	54
8	59	947F: 5'-AGCAGAACCAAAGAGTATT-3'	947R: 5'-AATTGAGAAGAGTTTGATTG-3'	132	49
9	21	993-1113F: 5'-TTGAATGTCTCAATAAATGTC-3'	993-1113R: 5'-ATATAAATTTGGGGGAGACG-3'	151	51
10	248	Ex8F: 5'-TTTGGGTGGGGAATGTGTC-3'	Ex8R: 5'-TAAACTCAGAAGCAATC-3'	313	53
11	77	1328F: 5'-AGGAAAGATACATTCAAATC-3'	1328R: 5'-GGTTTTGAGACTTTGTTC-3'	167	49
12	79	1399F: 5'-ATAGCCTTTCTCAACATCTG-3'	1399R: 5'-CATAGACTTTGTGAGGGGAA-3'	146	53
13	533	1F: 5'-TTGGGGAATACTTGGACATA-3'	1880R: 5'-GAATTGTGAGCAGTGCCC-3'	600†	54
14	220	ExBF: 5'-AACACAGTTAAGAGGAAACC-3'	2R: 5'-CACTTTCTGGGTTGGATTCT-3'	280	53
15	199	2214F: 5'-GCTATTCATACATGCGTTAA-3'	3R: 5'-AATGAGTCTCTTGAGTCTGTG-3'	281	51
16	73	2373F: 5'-AAGCCCAAGAAACAAGATAC-3'	2373R: 5'-TGCAACCAACTAGAAAGTA-3'	145	51
17	78	3F: 5'-CCTTGGGTGGCATTCTTA-3'	2779R: 5'-AGAATTTACTGGTTGCCAAG-3'	295	54

*For SSCA: digestion with *HinfI* results in two fragments of 100 and 135 bp.
†For SSCA: digestion with *NlaIV* results in three fragments of 250, 210, and 140 bp.

oligonucleotide primer pairs were designed to amplify the 17 coding exons of the gene using the polymerase chain reaction (PCR) (table 1). Reaction mixtures contained approximately 50 ng template DNA, 15 pmol of each primer, 100 µmol/l dNTPs, 0.1 µl [α -³²P]dCTP (3000 Ci/mmol), 1 × PCR buffer supplemented with 1–2 mmol/l MgCl₂, and 0.5 units of *Taq* DNA polymerase. The reaction mixture was denatured once at 94°C for five minutes and was subsequently subjected to 32 cycles of 30 seconds at 94°C, 30 seconds at an annealing temperature optimised for each primer pair (55–60°C), and 30 seconds at 72°C, followed by a final extension at 72°C for five minutes. Five microlitres of the 1:9 diluted PCR samples were subsequently added to 95% formamide, 5 mmol/l NaOH, 0.1% xylene cyanol, and 0.1% bromophenol blue. The samples were heat denatured for three minutes, immediately placed on ice, and electrophoretically separated at 4°C in 6% non-denaturing polyacrylamide gels which were run once with and once without 5% glycerol. The forward and reverse strands of PCR products corresponding to polymorphic mobility shifts were directly sequenced using the Thermo Sequenase radio-

labelled terminator cycle sequencing kit (Amersham).
For the segregation analysis of the exon 13 polymorphism in the PBCRA and MCDRI families, SSCP was carried out using the CleanGel-System (Pharmacia).²² To increase the sensitivity of the SSCP analysis, the exon 13 PCR fragment was digested with restriction enzyme *NlaIV* (New England Biolabs) before loading on a 15% non-denaturing polyacrylamide gel. This resulted in three fragments of 250, 210, and 140 bp in size. Electrophoresis was done at 150 V, 6 W for 30 minutes followed by 550 V, 12 W for 60 minutes. The DNA was detected by silver staining as previously described.²³
Results
MUTATIONAL ANALYSIS
For mutational analyses, DNA samples from one patient with late onset autosomal dominant Stargardt-like macular dystrophy,¹⁶ three patients diagnosed with North Carolina macular dystrophy,^{17, 18} and two patients with progressive bifocal chorioretinal atrophy from a single family¹⁴ were used (fig 2). The 17 exons of the IMPG1 gene were amplified using the

Figure 3 Single stranded conformational polymorphism (SSCP) in exon 13 of the IMPG1 gene. (A) SSCP of controls shows the polymorphic mobility shifts. (B) Sequencing of homozygous mobility shift as seen in controls 5 and 6 shows a C to G transversion at the first nucleotide of codon 518 resulting in a His/Asp protein polymorphism.

Figure 4 Segregation of C/G polymorphism in a branch of the PBCRA family. Affected subjects II.1, III.2, IV.1, and IV.2 are heterozygous for the "C" and "G" allele, respectively. Affected subjects IV.1 and IV.2 have inherited the "G" allele from their affected father III.2. In contrast, affected subjects II.4, II.5, and III.3 are homozygous for the "C" allele. This analysis shows a recombination event between PBCRA and IMPG1 in this pedigree. ds=double strand.

polymerase chain reaction (PCR) and intronic primers flanking the respective exons including the splice consensus acceptor and donor sequences (table 1). Subsequently, the PCR fragments were subjected to single stranded conformational analysis (SSCA) by using two gel running conditions to increase the sensitivity of mutation detection. Except for polymorphic mobility shifts in exon 13 (fig 3A), no altered migration patterns were identified in the remaining exons. Sequencing of exon 13 showed a G to C transversion in the first nucleotide of codon 518 changing the amino acid histidine (His) to aspartic acid (Asp) (fig 3B).

SEGREGATION ANALYSIS OF INTRAGENIC POLYMORPHISMS IN THE MCDR1 AND PBCRA FAMILIES

The polymorphic mobility shifts in exon 13 (His/Asp polymorphism) were used to analyse the segregation of IMPG1 alleles in the three MCDR1 and the single PBCRA families (fig 2). The polymorphism was not informative in the three MCDR1 families (data not shown). In contrast, segregation of the G/C alleles in exon 13 in the PBCRA family excludes linkage between the IMPG1 gene and the disease phenotype (fig 4). While the two affected children, IV.1 and IV.2, inherited the G allele from their affected father III.2, affected subjects II.4, II.5, and III.3 are homozygous for the C allele indicating a recombination event between IMPG1 and the disease (fig 4).

Discussion

The application of the positional candidate approach has led to the identification of several genes associated with retinal dystrophies. For example, mutations in the rhodopsin gene have been shown to cause chromosome 3q linked

autosomal dominant retinitis pigmentosa.²⁴ Similarly, the peripherin/RDS gene was found to be mutated in autosomal dominant retinitis pigmentosa linked to chromosome 6p.²⁵ Also, one form of Leber's congenital amaurosis was shown to be caused by mutations in the retinal specific guanylate cyclase on chromosome 17p.²⁶ Colocalisation of Sorsby's fundus dystrophy (SFD) and the tissue inhibitor of metalloproteinases-3 (TIMP3) on chromosome 22q13-qter has led to the identification of TIMP3 as the gene underlying SFD pathology.²⁷ Finally, functional aspects and chromosomal localisation to 1p has identified the rod photoreceptor cell specific ABC transporter as the gene causing autosomal recessive Stargardt's disease.²⁸

Based on the chromosomal localisation of the interphotoreceptor matrix (IPM) proteoglycan gene, IMPG1, to 6q13-q15¹² and the potential role of IPM molecules in mediating interactions between retinal cells, the RPE and the choriocapillaris,^{8,9} we reasoned that IMPG1 represents an excellent candidate for chromosome 6q linked retinopathies, including autosomal dominant Stargardt-like macular dystrophy (STGD3),¹³ North Carolina macular dystrophy (MCDR1),^{15,17,18} and progressive bifocal chorioretinal atrophy (PBCRA)¹⁴ (fig 1). However, SSCP analysis has not shown any aberrant mobility shifts in the affected subjects. Furthermore, using an intragenic IMPG1 polymorphism in exon 13 we have identified a recombination event between the IMPG1 gene and the PBCRA locus.

Extended haplotype analysis has previously been performed in the PBCRA family using DNA markers spanning a genetic distance of approximately 50 cM and has excluded a location of the PBCRA locus centromeric to D6S249 and telomeric to D6S268 in recombinant subject II.1¹⁴ (fig 2). The present IMPG1 allelotyping in the PBCRA family in combination with the previous findings in recombinant subject II.1 excludes the IMPG1 gene from an interval flanked by markers D6S249 and D6S268. As this genetic interval contains the region harbouring the MCDR1 locus (fig 1), the PBCRA recombination data similarly exclude the IMPG1 gene as the cause of MCDR1 as well as PBCRA.

Autosomal dominant Stargardt-like macular dystrophy was analysed in a single affected member of a three generation family with clear autosomal dominant transmission of the Stargardt-like phenotype.¹⁶ Similarly to MCDR1 and PBCRA, SSCP analysis of the IMPG1 gene in the ADSTGD patient has not identified a disease causing mutation. However, it should be noted that such a mutation may have remained undetected owing to a well established detection sensitivity of the SSCP system for single base changes in the order of 75 to 85%. Since DNA samples from additional members of the family were not available, we have not been able to perform genetic linkage analysis on this family. Therefore, the chromosomal position of the gene locus in this family is still unknown. However, it is well established that the autosomal

dominant Stargardt-like macular dystrophies are genetically heterogeneous with at least two independent gene loci on chromosome 6q¹³ and chromosome 13q.¹⁹ Thus, our results cannot rule out that mutations in the IMPG1 gene are involved in one form of autosomal dominant Stargardt-like macular dystrophy. To resolve this issue additional patients diagnosed with autosomal dominant Stargardt-like macular dystrophy need to be analysed. In particular, the mutational testing of IMPG1 in affected members of an autosomal dominant Stargardt-like macular dystrophy family previously mapped to proximal chromosome 6q (designated STGD3)¹³ will be useful.

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