Genomewide Homozygosity Mapping and Molecular Analysis of a Candidate Gene Located on 22q13 (Fibulin-1) in a Previously Undescribed Vitreoretinal Dystrophy

Maike Weigell-Weber, MD; Gian-Marco Sarra, MD; Dieter Kotzot, MD; Lodewijk Sandkuijl, PhD†; Elmar Messmer, MD; Martin Hergersberg, PhD

Objectives: To localize the gene that causes an autosomal recessively inherited vitreoretinal dystrophy that has not been described, to our knowledge, and to analyze a candidate gene mapped to 22q13 (fibulin-1 [FBLN1]).

Methods: Homozygosity mapping with 500 microsatellite markers spread over the whole genome (mean distance, 7.2 centimorgans [cM]) and mutation analysis of the complete coding region of FBLN1.

Results: Homozygosity for all analyzed markers was found in the 4 affected siblings in a region on chromosome 22 encompassing 12 cM from D22S444 (centromeric) to D22S1170 (telomeric). Lod scores were between 0.017 and 2.36 (theta=0). A mutation analysis of the complete coding region of FBLN1, which encodes interacting extracellular matrix proteins, revealed 4 previously undescribed single nucleotide polymorphisms.

Conclusions: A genomewide homozygosity mapping analysis supported the hypothesis that the gene responsible for a unique vitreoretinal dystrophy is located on chromosome 22q13. No obviously pathogenic mutation was found in the candidate gene, FBLN1.

Arch Ophthalmol. 2003;121:1184-1188

HEREDITARY vitreoretinal dystrophies are potentially blinding disorders characterized by an abnormal vitreous gel, associated retinal changes, and a heterogeneous origin. The phenotype is extremely variable in both syndromic and nonsyndromic types. Some of them are associated with axial myopia of different extents and retinal detachment. Disease-causing mutations have been identified in various genes that encode components of the extracellular matrix (ECM).1 Syndromic types of vitreoretinal dystrophies are Knobloch syndrome (Online Mendelian Inheritance in Man [OMIM] *120328) and the rather heterogeneous Stickler syndrome (OMIM #108300, OMIM #604841, and OMIM #184840). Nonsyndromic types are the Wagner syndrome (OMIM *143200), Goldmann-Favre dystrophy (OMIM #268100), and the allelic enhanced S-cone syndrome. In addition, similar ophthalmologic findings have been described in families with X-chromosomal, autosomal dominant, and autosomal recessive inheritance. As reported by Sarra et al.,2 the chromosomal loci of some of these disorders (high myopia on 18p11, 12q21-23, and 7q36, Stickler syndrome [COL1A1 on 12q13, COL1A1 on 1q21, COL1A2 on 6p21.3], Wagner syndrome on 5q13-14, Knobloch syndrome on 21q22.3, Goldmann-Favre [enhanced S-cone] syndrome on 15q23, and multiple vitreoretinopathies on 11q13) were excluded by linkage analysis.

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Here, we report on a genomewide homozygosity mapping approach that found a 12-centimorgan (cM) homozygous region on chromosome 22q13 containing, among other genes, fibulin-1 (FBLN1). This attractive candidate gene was further analyzed for mutations.

METHODS

SUBJECTS

The family originated from eastern Switzerland. Informed consent and medical history were obtained from 3 healthy and 4 affected siblings aged 68 to 77 years at the time of investigation. The eighth sibling as well as both parents were not affected and died prior to this investigation. The clinical data have been reported in detail.3 Briefly, in the 4 affected siblings, the onset of visual disturbance started in early childhood, followed by progressive night blindness and visual field impairment during...
the third to fifth decade of life. Clinical evaluation revealed a peculiar vitreoretinal degeneration characterized by vitreous liquefaction and strand formation along with diffuse retinal pigment epithelium atrophy and pigment clumping of the retina, and high myopia with deep posterior staphyloma. The mean axial length of the eyes was 31.4 mm (range, 28.49-33.41 mm). The depth of the posterior staphylomata was 0.8 mm to 2.7 mm (mean, 1.75 mm). Visual acuity ranged from no light perception to 20/70. Kinetic perimetry revealed severe constrictions of the isopters, with remaining small central islands. Electrophysiologic investigations showed severe retinal damage, suggestive of a superimposed widespread retinal dystrophy.

A genealogic analysis of 5 generations revealed no evidence for parental consanguinity (Manuel Aicher, Genetologische Forschung, Dietikon, Switzerland). However, the origin of most of the ancestors from a relatively isolated population and several consanguinities in the different parental pedigrees were indicated.

### GENOTYPING

Blood samples were obtained from all 7 living siblings after written informed consent was obtained. DNA was extracted using standard salting out procedures. For homozygosity mapping, the DNA-pooling method was applied (affected vs unaffected). The mean distance between the 500 highly polymorphic markers used was 7.2 cM. Microsatellites chosen for maximum informativeness were obtained from the Genethon human genetic linkage map and from the Marshfield map. Polymerase chain reaction (PCR) primers were from Research Genetics (Huntsville, Ala) or were synthesized with the available sequence information. Polymerase chain reaction was performed using standard conditions. Products were electrophoretically separated on 6% polyacrylamide sequencing gels and visualized by silver staining. Linkage was calculated using the LINKAGE (version 5.1) program package (Laboratory of Statistical Genetics at Rockefeller University, New York, NY), assuming autosomal recessive inheritance, 100% penetrance in both sexes, and a gene frequency of 0.001. The same assumptions were used for the lod score calculation for the homozygous region with the MAPMAKER/HOMOZ algorithm. For this calculation, each marker allele that showed homozygosity in the 4 patients was assumed to be the most frequent allele of the corresponding marker.

### MUTATION ANALYSIS OF FBLN1

The PCR primers, annealing temperatures, and PCR product sizes for the amplification of all 20 exons of FBLN1, including the exon-intron boundaries, were derived from the gene structure as described by Pan et al. and are available on request. Heteroduplex analysis of the PCR products was performed by denaturing high-performance liquid chromatography on a Wave system (Transgenomic Inc, Omaha, Neb). The column used was a DNasep Cartridge (Transgenomic Inc). After heteroduplex formation, DNA fragments were run, with column temperatures between 50°C to 68°C. The column temperature was changed in incremental steps of 2°C. An elution time of 14 minutes was used: the linear acetonitrile–trihetyl ammonium acetate–buffer gradient ratio went from 43%/57% to 68%/32% during 10 minutes, with a buffer flow of 0.9 mL/minute, followed by a 100% acetonitrile wash for 30 seconds, and in an equilibration step, was reduced to 43%/57% for the last 4 minutes. For sequence analysis, PCR products were purified with Quiaquick spin columns (Qiagen, Hilden, Germany). Sequencing was done on an ALF Express sequencing automate (Amersham Pharmacia Biotech, Uppsala, Sweden), using the respective Cy5-labelled PCR primers as sequencing primers with the thermostable-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech). Alternatively, the PCR primers were used for sequencing of the PCR amplification products with the Applied Biosystems Dye terminator sequencing kit (Foster City, Calif), followed by sequence analysis on an ABI 3700 capillary sequencer (Micosynth, Balch, Switzerland). RNA was isolated from EDTA anticoagulated blood using a QiAamp RNA Blood Mini Kit (Qiagen). Reverse transcriptase (RT)-PCR analysis was performed with the Titan RT-PCR kit (Roche Diagnostics, Rotkreuz, Switzerland), using the experimental conditions provided by the supplier. The primers used for RT-PCR were A4F, TCTGCACTGACACCTCAAG, and A4R, GGATTTCGCAACACACAT (nucleotides 2141-2160 and 2321-2344, respectively, of the complementary DNA sequence of variant A of FBLN1). The RT reaction was performed at 60°C for 30 minutes, followed by 15 minutes at 95°C. The denaturation, annealing, and extension temperatures were 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds, respectively, for 40 cycles.

### HAPLOTYPING OF FBLN1

Assays were developed for the 4 single nucleotide polymorphisms (SNPs) identified during mutation analysis of FBLN1. The allele frequencies of the different SNPs and the haplotypes arising from the different allele combinations were analyzed in the DNA of 30 families (parents and at least 1 child) not affected by vitreoretinal dystrophy or severe myopia. These families had given written informed consent that their anonymous DNA samples could be used for research.

### HOMOZYGOSITY MAPPING

A homozygous region on chromosome 22q13 was identified, which extends from the proximal marker, D22S444, to the distal marker, D22S1170 (Figure 1). The calculated lod score for the polymorphic markers that are homozygous in this region was between 0.017 and 2.18 (theta = 0). This region contains FBLN1 (base pairs 29/341-390-29/439/641 on chromosome 22), which was analyzed further. The highest possible lod score of 2.18 was found for the microsatellite marker D22S444, which is located in the largest intron (intron 14) of FBLN1. This lod score was also found for the markers D22S1170 and D22S928. The latter polymorphism is located outside the homozygous region and is heterozygous in the 4 affected persons. This is additional evidence that the 4 pa-
The PCR primers for the allele-specific amplification of the polymorphism in exon 9 are available on request.

ommendations of Antonarakis et al, beginning with the

2218C

3

SNPs were located in intron 5 (543+17C) exchange at amino acid position 318 in exon 9. The 3 other

sis of the PCR products of the 20 exons of

fribulin-1.

Table 1. Single-Nucleotide Polymorphisms Identified in the FBLN1 Candidate Gene and Techniques for Their Detection*

<table>
<thead>
<tr>
<th>Base change</th>
<th>Location</th>
<th>Detection technique</th>
<th>Allele frequency 1</th>
<th>Allele frequency 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>543 + 17C&gt;G</td>
<td>Intron 5</td>
<td>Acyl</td>
<td>0.34/0.67</td>
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<tr>
<td>963C&gt;T, P318I</td>
<td>Exon 9</td>
<td>Allele-specific amplification</td>
<td>0.58/0.42</td>
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<tr>
<td>2147C&gt;T</td>
<td>Exon 17</td>
<td>Hinfl</td>
<td>0.39/0.61</td>
<td></td>
</tr>
<tr>
<td>2228C&gt;G</td>
<td>Exon 17</td>
<td>Tsel</td>
<td>0.33/0.67</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: FBLN1, fibulin-1.

*The polymorphisms in exons/introns 5 and 17 were detected by digest of the exon-specific polymerase chain reaction (PCR) products with the indicated restriction enzymes. The PCR primers for the allele-specific amplification of the polymorphism in exon 9 are available on request.

Figure 2. Results of reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of the FBLN1 (fibulin-1) transcript. In lane 1, there is a 100-base-pair ladder, and in lane 2 to lane 7, the products of the RT-PCR analysis performed with total RNA isolated from whole blood of 3 affected (II-3, II-5, II-6) and 3 unaffected (II-1, II-2, II-7) siblings were loaded. No difference was detectable.

Table 2

<table>
<thead>
<tr>
<th>Candidate Gene and Techniques for Their Detection</th>
<th>Base change</th>
<th>Location</th>
<th>Detection technique</th>
<th>Allele frequency 1</th>
<th>Allele frequency 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBLN1</td>
<td>543 + 17C&gt;G</td>
<td>Intron 5</td>
<td>Acyl</td>
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<td>FBLN1</td>
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<td>FBLN1</td>
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<td>Tsel</td>
<td>0.33/0.67</td>
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</tr>
</tbody>
</table>

**AN FBLN1 HAPLOTYPE SEGREGATING WITH THE DISEASE PHENOTYPE**

The SNPs identified in FBLN1 were used to determine the frequency of the different haplotypes of this gene in the DNA of 30 unrelated families (both parents and at least 1 child). Genotyping of these families allowed the identification of 10 of the 16 possible haplotypes in these families (Table 2). The homozygous haplotype 16 identified in the 4 patients was also found in 1 control subject with normal vision. It is therefore unlikely that the observed haplotype per se is responsible for the described phenotype of the eye.

**COMMENT**

We report on a genomewide homozygosity mapping approach and analysis of a candidate gene located on 22q13 (FBLN1) in a nonsyndromic and previously undescribed vitreoretinal dystrophy. This vitreoretinal dystrophy is characterized by excessive early-onset axial myopia with deep and sharply demarcated posterior staphylomata, pronounced vitreous liquefaction, retinal degeneration with diffuse retinal pigment epithelium atrophy, and premature cataract formation. As described in Sarra et al,2 the clinical picture and the striking electrophysiologic and visual field defects suggested the presence of a primary vitreoretinal dystrophy rather than just late sequelae of a pathologic myopia.

The rarity of the disease phenotype in siblings, and the origin of their parents from a relatively isolated population in neighboring villages, led us to the hypothesis of a hidden consanguinity. By a genomewide homozygosity mapping approach with more than 500 polymorphic markers, homozygosity for a 12-cM region located on 22q13 was found. The maximal lod score of 2.18 was identified for the markers D22S1170 and D22S444. Both markers were either heterozygous or homozygous for other alleles in the unaffected siblings (Figure 1). The affected persons were heterozygous for the marker D22S928, which was adjacent to the homozygous region and which also had a lod score of 2.18 due to an identical genotype in the 4 affected siblings and different genotypes in the 3 non-affected siblings. Recently, evidence has been published that homozygous or autozygous genomic regions can occur with a higher then expected percentage, either through underestimation of the extent of inbreeding or by

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Table 2. Sixteen Possible Haplotypes of 4 Different Intragenic SNPs in FBLN1 and Frequency of Their Occurrence in 30 Families (Parents and 1 Child)*

<table>
<thead>
<tr>
<th>Haplotype</th>
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<th>4</th>
<th>5</th>
<th>6</th>
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<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
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<td>C</td>
<td>C</td>
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<td>T</td>
</tr>
<tr>
<td>No. of haplotypes</td>
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<td>1</td>
<td>8</td>
<td>1</td>
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<td>15</td>
<td>1</td>
<td>...</td>
<td>...</td>
<td>11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: SNP, single-nucleotide polymorphism; FBLN1, fibulin-1; and ellipses, not found.

*The identified number of haplotypes is determined by the number of informative families. Haplotype 16 was identified in the affected probands.

chance. On one hand, this allows the interpretation that homozygosity is possible in a family despite lack of evidence for parental consanguinity due to a relatively small and inbreeding population. The genealogic analysis discovered 3 consanguinities between ancestors of the proband's father. On the other hand, it has to be mentioned that the observed association between the phenotype and the homozygous region can result from chance only. However, no other polymorphic marker used in the genome scan showed the same allele distribution pattern, ie, homozygosity in the 4 affected siblings and different genotypes in the 3 unaffected siblings. It is therefore unlikely that the occurrence and distribution of these homozygous segments is a chance event resulting from a generally low genetic heterogeneity in this family. If we assume that parental consanguinity exists, their closest connection must be at least 6 generations ago (a result of the genealogic investigation). A statistical analysis with MAPMAKER/HOMOZ, assuming the existence of such a distant connection, yielded an overall lod score of 2.36 for linkage to this same region. This lod score represents a very conservative estimate of the evidence for linkage because a conservative estimate for the frequency of the marker alleles for which the patients are homozygous was used: for each marker, the frequency of the most frequent allele was specified.

The homozygous region on chromosome 22 in the 4 affected persons contains at least 11 genes predicted by computer searches, and 16 genes identified both by bioinformatic analysis and by transcript analysis. From these 27 genes, FBLN1 is the most interesting candidate gene. FBLN1 encodes a calcium-binding ECM protein and is characterized by a complex splicing pattern, resulting in 4 protein variants, FBLN1 A–D, which differ in their carboxy termini (Figure 3). These variants differ in function and tissue expression. FBLN1 expression has been detected in a wide range of tissues in a complex temporal-spatial pattern, including the fetal eye and adult retinal pigment epithelium sheets. It can therefore be concluded that FBLN1 is expressed in the tissues of the family described here (Unigene database). Recently, disruption of FBLN1 was reported in a family with a t(12;22) associated with a complex type of synpolydactyly. This translocation specifically interferes with the synthesis of variant FBLN1-D. No ocular symptoms were reported.

FBLN1 is a particularly interesting candidate gene for the following reasons: (1) in the allelic retinopathies, Malattia leventinese and Doyne honeycomb retinal dystrophy (OMIM #126600), a mutation has been identified in the gene that encodes the epidermal growth factor–containing fibulin-like extracellular matrix protein 1 (EFEMP1), formerly designated SI-5, or FBLN3. (2) The FBLN1 protein interacts with a number of ECM proteins, including fibronectin, nidogen, aggrecan, and versican. In particular, FBLN1 interacts with α-I (XVIII) collagen, which was mutated in several families with Knobloch syndrome. (3) The 2 ECM proteins, aggrecan and versican, to which FBLN1 binds, are expressed in the sclera with dynamic expression patterns during growth and aging. Because the versican gene is a candidate gene for Wagner disease, it is tempting to speculate that the similar phenotype of the affected family members described here with Wagner syndrome on one side and Knobloch syndrome on the other side is the consequence of a functional connection between the protein products of the genes that mutated in these diseases. (4) A further member of the fibulin gene family, FBLN4 (also designated EFEMP2), was recently identified in the multiple retinopathy critical region on 11q13 and considered as a candidate gene for the different vitreoretinopathies mapped to this region. Other members of the fibulin gene family in addition to FBLN1 are FBLN2 on 3p25, and EVECDANCE/FBLN5 on 14q32.1.

Figure 3. Scheme of the fibulin-1 gene (FBLN1) with all exons according to the description of Pan et al and the 4 protein variants FBLN1 A–D due to different splicing sites.
which is often mutated at a retina-specific exon in RP3. Mutations were therefore not detectable in DNA and RNA from other tissues. This hypothesis is also in agreement with the finding that the homozygous knockout null mutation of \( \text{FBNL1} \) was found to be perinatally lethal in the \(-/\) mouse, and that a (12;22) disrupting the variant \( \text{FBNL1-D} \) is associated with a complex type of synpolydactyly. This indicates that the described ophthalmologic disease cannot result from a null mutation. Planned experiments will aim at the quantification of the transcript levels of \( \text{FBNL1} \) in patients and probands to test the idea that a subtle difference in the transcription rate of the allele causes the ophthalmologic phenotype.

Sertie et al. discussed the idea that yet to be identified alleles of \( \text{COL18A1} \) encoding the \( \alpha 1 \) (XVIII) collagen could be susceptibility alleles for high myopia. In light of the finding that some clinical aspects of the new vitreoretinal dystrophy described here are similar or identical to findings in patients with high myopia, this argument could also be applied for the functionally related \( \text{FBNL1} \). This would allow a better understanding of the molecular processes leading to the development of myopia.

**Sources**

Ensemble Project (http://www.ensembl.org/Homo_sapiens/) for genes and markers located in 22q13.

Foundation Jean Dausset CEPH (http://www.ceph.fr/cephdb/) for allele frequencies of microsatellite markers.

Genbank (http://www.ncbi.nih.gov/Genbank/) for sequences of the different \( \text{FBNL1} \) messenger RNA sequences: \( \text{FBNL1-A, NM_006487 (X53741); FBNL1-B, NM_006485 (X53742); FBNL1-C, NM_001996 (X53743); and FBNL1-D, NM_006486 (U01244).} \)

Genome database (http://gdbwww.gdb.org/) for primer sequences of polymorphic sites in candidate genes, and for allele frequencies of microsatellite markers.

Marshfield Medical Center for Medical Genetics (http://research.marshfieldclinic.org/genetics/) for the order and genetic distances of microsatellite markers.

Online Mendelian Inheritance in Man OMIM (http://www.ncbi.nlm.nih.gov/Omim/) for Knobloch syndrome (OMIM *120328), Stickler syndrome type I (OMIM #108300), Stickler syndrome type II (OMIM #604841), Stickler syndrome type III (OMIM #184840), Wagner disease (OMIM *143200), Goldmann–Favre dystrophy (OMIM #268100), MYP2 (OMIM *160700), MYP3 (OMIM *603221), Marfan syndrome (OMIM #154700), and Doyle honeycomb retinal dystrophy (OMIM * #126600).

The Sanger Institute (http://www.sanger.ac.uk/HGP/Chr22/) for genes and markers located in 22q13.

Unigene (http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi) (Gene Cluster Hs.79732) for the expression pattern of \( \text{FBNL1} \).

Submitted for publication July 26, 2002; final revision received March 10, 2003; accepted March 20, 2003.

We gratefully acknowledge the support of the Hartmann-Müller-Stiftung (No. 737/1998), Zurich, the Schweizerischer Fonds zur Verhütung und Bekämpfung der Blindheit, Zurich, and the Schweizerischen Nationalfonds (No. 31-59267.99), Berne, Switzerland.

We thank Prof Andreas Huber, MD, for support, and Ms I. Moro for technical help. We thank the family members for their participation and continued interest in this study.

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**References**


