Genetic Linkage of Snowflake Vitreoretinal Degeneration to Chromosome 2q36

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PURPOSE. To identify the chromosomal location of the gene causing snowflake vitreoretinal degeneration (SVD), an autosomal dominant retinal degeneration characterized by small yellow-white dots in the retina, fibrillar anomaly of the vitreous humor, and retinal detachment.

METHODS. Clinical data were collected on 31 family members by history and examination. Thirteen family members underwent prospective examination. Genotyping was performed using microsatellite markers spaced at approximately 10 cM intervals. Two-point and multipoint linkage analysis was performed (FASTLINK version of the MLINK program and the VITESSE algorithm, both available at http://linkage.rockefeller.edu/soft/list.html). Direct DNA sequencing of amplified genomic DNA and mRNA was performed for candidate gene analysis.

RESULTS. The SVD locus was linked to markers in a region of chromosome 2q36 defined by D2S1958 and D2S2202, based on meiotic breakpoint mapping of affected individuals. A maximum two-point lod score of 5.5 was obtained with marker D2S172 at θ = 0 within this region. Direct DNA sequencing of all 52 exons of the COL4A3 gene revealed no potentially pathogenic coding sequence variation or evidence for deletion.

CONCLUSIONS. The genetic locus for SVD lies in a 9 Mb region flanked by D2S1958 and D2S2202. Localization of SVD to a genomic region distinct from both Wagner disease and the Stickler syndromes indicates that SVD is a distinct genetic entity. The absence of coding sequence variation in the only collagen gene within the disease-region, suggests a novel pathogenesis for vitreoretinal degeneration. Snowflake vitreoretinal degeneration should be considered in the differential diagnosis of families with fibrillar anomaly of the vitreous. (Invest Ophthalmol Vis Sci. 2004;45:4498–4503) DOI: 10.1167/iovs.04-04722

The vitreoretinal degenerations are a group of retinal degenerations characterized by early-onset cataract, liquefaction of the vitreous humor, variable degeneration of the retina, and abnormalities of the interface between the vitreous and retina leading to increased traction on the retina and retinal detachment. Most of the described conditions, including snowflake vitreoretinal degeneration (SVD), are autosomal dominant diseases with ocular and systemic manifestations, variable expressivity, and nearly complete penetrance of the vitreoretinal degeneration.

The most common vitreoretinal degeneration is the condition originally described by Stickler et al.5 Mutations leading to haploinsufficiency of the collagen 2A1(COL2A1) gene usually cause Stickler syndrome type I (STL1, MIM 108300).6 These patients have a vitreous anomaly characterized by vestigial vitreous gel occupying the immediate retrolental space and minimal to no discernable gel in the central vitreous cavity.7 The expression of systemic features exhibits variability both between and within families.8–12 Mutations within exon 2 are associated with a predominantly ocular phenotype,10,11,13,14 presumably due to tissue-restricted expression of this exon to the vitreous in adults.15,16 The remaining exons are expressed both in the vitreous and cartilage,17 and mutations introducing premature stop codons lead to typical Stickler syndrome type 1.17 Because mutations in exon 2 of the COL2A1 gene lead to a predominantly ocular phenotype, some families have been reported as having Wagner disease.10,14,18 The diagnostic confusion that led to these diseases being lumped together as Wagner–Stickler disease has been clarified through careful clinical and molecular genetic investigation.

Mutations in the collagen 11A1 (COL11A1) gene cause Stickler syndrome type II (STL2, MIM 604841).19 Unlike COL2A1 disease, such COL11A1 mutations lead to a fibrillar vitreous anomaly with limited and random fibrils throughout the vitreous cavity.20 Mutations altering intron–exon splicing of the COL11A1 gene lead to Marshall syndrome that is distinguished from Stickler syndrome by more pronounced facial dysmorphism and less frequent retinal detachment.21 Mutations in the related collagen 11A2 gene lead to systemic features of Stickler syndrome without ocular features since this gene (COL11A2) is not expressed in the eye. There is evidence that mutations in other, as yet unknown, genes lead to Stickler syndrome.22

The gene for Wagner disease23 (MIM 143200) has been localized to chromosome 5.24 The vitreous findings in Wagner disease include an empty vitreous cavity with avascular strands.25 Features distinguishing Wagner disease from Stickler syndrome include anterior chamber angle abnormalities, marked chorioretinal atrophy, and tractional retinal detachment. There are no known systemic manifestations.

The advances in our understanding of Wagner disease and the Stickler syndromes led us to reevaluate snowflake vitreoretinal degeneration (MIM 193230).26,27 Although this condition was first reported in 1973, only one other family with a clearly identical phenotype has been reported.26,27 Snowflake vitreoretinal degeneration is an autosomal dominant condition characterized by fibrillar anomaly of the vitreous, peripheral retinal abnormalities including minute crystalline deposits resembling...
FIGURE 1. Pedigree of members of the original snowflake vitreoretinal degeneration (SVD) family used for the genome wide scan is shown. The haplotypes of 16 markers from chromosome 2q36 localize the SVD gene between markers D2S2158 and D2S2202 based on meiotic breakpoints in affected individuals.
snowflakes, and early-onset cataracts. Systemic abnormalities such as hearing loss, cleft palate, midface hypoplasia, and arthropa thy seen in Stickler syndrome are absent. We previously reported a prospective review of the clinical features and exclusion of the Stickler and Wagner loci for SVD. Here we describe a novel locus for vitreoretinal degeneration in the original snowflake family on chromosome 2q36 and our initial evaluation of candidate genes within this region.

Materials and Methods

Family and DNA Specimens

The original SVD family containing 31 individuals (13 affected individuals, 14 unaffected individuals, and 4 unaffected spouses) was enrolled in this study (Fig. 1). This American family is of European extraction. The thirteen subjects diagnosed with SVD ranged from 12 to 85 years of age. No obligatory carriers of the snowflake trait were found to be normal. The inheritance pattern was autosomal dominant. The institutional review boards of the University of Texas Southwestern Medical Center and the National Eye Institute approved this study, and informed consent was obtained from all participating family members, consistent with the Declaration of Helsinki. The examination and diagnostic criteria have been described previously. Briefly, comprehensive eye and focused physical examinations were performed by a vitreoretinal specialist and internist prospectively for 13 subjects. Clinical histories and medical records were obtained from these and other subjects and reviewed. Blood was obtained from each participant for isolation of genomic DNA. Before the initiation of genotyping, subjects with SVD were designated as affected based on the presence of early-onset cataract and severe fibrillar vitreous anomaly (Fig. 2).

Genotyping

DNA was extracted from blood using a purification kit (Master Pure Genomic DNA purification kit; Epicentre Technologies, Madison, WI). A genome-wide scan was performed with 385 fluorescently labeled short tandem repeat markers (Prism Linkage Mapping Set MD10, panels 1–27; Applied Biosystems, Inc. [ABI], Foster City, CA). Each multiplexed polymerase chain reaction (PCR) was carried out in a 5-μL mixture containing 40 ng genomic DNA, various combinations of 10 μM fluorescent dye-labeled primer pairs, 0.5 μL 10X PCR Buffer II (GeneAmp; ABI), 250 μM dNTP mix (GeneAmp; ABI), 2.5 mM MgCl₂, and 0.2 U Taq DNA polymerase (AmpliTaq Gold Enzyme; ABI). Amplification was carried out in an ABI 800 Catalyst Molecular Biology Labstation (ABI). Initial denaturation was carried out for 12 minutes at 95°C, followed by 10 cycles of 15 seconds at 94°C, 15 seconds at 55°C, and 30 seconds at 72°C, and then 20 cycles of 15 seconds at 89°C, 15 seconds at 55°C, and 30 seconds at 72°C, finishing with a 20-minute extension cycle at 72°C and a final hold at 4°C. PCR products from each DNA sample were pooled and mixed with a loading cocktail containing formamide, Gs-100HD ROX standards (PE Applied Biosy stems), and loading dye. The product was loaded onto a 5% acrylamide gel and run in a 577 Prism DNA sequencer (ABI). Data were analyzed by using ABI GeneScan 3.1 and ABI Genotyper 2.1 software. Two independent masked individuals read all gels, with conflicts being resolved by a third independent reader. Data producing conflicts that could not be unambiguously resolved were discarded or repeated as needed.

Linkage Analysis

Initially a genome-wide linkage screen was carried out by using the Medium Density Marker panel (ABI) with short tandem repeat markers distributed with an average of 10-cM intervals. Standard two-point linkage analysis was then carried out for SVD family by using all markers in the ABI MD10 panels 1–27. Two point linkage analysis was performed using the FASTLINK version of MLINK from the LINKAGE Program Package. Maximum lod scores were calculated using LINK. SVD was analyzed as a 95% penetrant autosomal dominant trait. The gene frequency of SVD was set at 0.0001. For screening, equal allele frequencies were assumed for all markers, while fine mapping was carried out with marker allele frequencies estimated by counting alleles of 25 unrelated individuals (50 chromosomes) of European ethnicity. Fine mapping on chromosomes 2, 10, and 17 was carried out using markers and intermarker distances from the Genethon database (Table 1). Multipoint mapping was carried out using the VITESSE algorithm for rapid exact multilocus linkage analysis. Association between marker alleles was not taken into consideration in calculation of multipoint lod scores.

Candidate Gene Analysis

Exons 2 through 52 of the COL4A3 were located within the disease-gene interval. Each of these exons along with flanking DNA sequence was amplified and subjected to direct DNA sequencing as previously described. In addition, the mRNA was amplified in overlapping segments from total human RNA isolated from lymphoblastoid cell lines by reverse transcriptase, PCR, and directly sequenced as previously described. Primers and conditions are available on request.

Results

Genome-Wide Screen and Fine Mapping

Ten markers yielded lod scores of 1 or greater in the genome wide scan. Of this group, D2S2211, D4S2935, D12S83, and D13S265 were closely linked to adjacent markers, yielding large negative lod scores. Given that SVD was not consistent with linkage across chromosome 10 and 17, showing results consistent with true linkage. Fine mapping was consistent with linkage across the D2S2158–D2S2202 interval (Table 1) and did not require double recombinants to allow linkage of markers showing significantly positive lod scores (Fig. 1). Two-point linkage analysis with markers from this interval gave lod scores greater than 3.0 for eight contiguous markers (Table 1). Multipoint linkage was not carried out in an ABI 800 Catalyst Molecular Biology Labstation (ABI). Initial denaturation was carried out for 12 minutes at 95°C, followed by 10 cycles of 15 seconds at 94°C, 15 seconds at 55°C, and 30 seconds at 72°C, and then 20 cycles of 15 seconds at 89°C, 15 seconds at 55°C, and 30 seconds at 72°C, finishing with a 20-minute extension cycle at 72°C and a final hold at 4°C. PCR products from each DNA sample were pooled and mixed with a loading cocktail containing formamide, Gs-100HD ROX standards (PE Applied Biosystems), and loading dye. The product was loaded onto a 5% acrylamide gel and run in a 577 Prism DNA sequencer (ABI). Data were analyzed by using ABI GeneScan 3.1 and ABI Genotyper 2.1 software. Two independent masked individuals read all gels, with conflicts being resolved by a third independent reader. Data producing conflicts that could not be unambiguously resolved were discarded or repeated as needed.

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The analysis showed lod scores greater than 3 for all points between D2S2158 and D2S2348, and lod scores greater than 6 for markers in the D2S2185 to D2S206 interval. The maximum lod score was 2.05. The linkage analysis showed lod scores greater than 3 for all points between D2S2158 and D2S2185, and this was confirmed in individual 21. A proximal recombinant occurred between D2S336 and D2S2202 in affected individual 46, and more proximal recombinants occurred between D2S336 and D2S2348 in unaffected individuals 17 and 32.

**Screening COL4A3 for Mutations**

COL4A4 lies just proximal to D2S2158 and is excluded from the linked interval. However, D2S2158 lies in intron 1 of the COL4A3 gene. Thus, mutations in most of this gene would fall within the linked interval. Although 7 SNPs in or near the 3′ untranslated region of the COL4A3 gene were genotyped, they were all uninformative in the SVD family (data not shown). Consequently, the COL4A3 gene was sequenced by amplifying each exon as well as by RT-PCR of COL4A3 mRNA, followed by direct sequencing. No variation in coding or exon flanking sequence consistent with a mutation was found by either approach in two affected individuals from the SVD family. No shift in overlapping amplification products of the COL4A3 from retinal mRNA was identified, thereby excluding alternative use of exons. Synonymous polymorphic DNA variation was identified, thereby excluding alternative use of exons. Synonymous polymorphic DNA variation was identified, thereby excluding alternative use of exons. Synonymous polymorphic DNA variation was identified, thereby excluding alternative use of exons. Synonymous polymorphic DNA variation was identified, thereby excluding alternative use of exons. Synonymous polymorphic DNA variation was identified, thereby excluding alternative use of exons.

**DISCUSSION**

Linkage of snowflake vitreoretinal degeneration to markers on chromosome 2q36 is reported. The evidence for linkage to the
9 Mb region on chromosome 2q36 includes statistically significant lod scores for eight contiguous markers (Table 1). These markers include from D2S2362 through D2S2348 with a maximum two-point lod score of Z_{max} = 5.5 at θ = 0 with D2S172. Further, haplotype analysis was consistent with a critical region for the disease gene defined from D2S2158 through D2S2202 (Fig. 1).

The clinical appearance of the vitreous in patients with vitreoretinal degeneration can be predictive of the underlying genetic cause. Snowflake vitreoretinal degeneration should be considered in the differential diagnosis of patients with fibrillar vitreous anomaly. Before the present study, the only known locus for fibrillar vitreous anomaly was the COL11A1 gene associated with Stickler syndrome type 2 and Marshall syndrome. The other known vitreoretinal degenerations have membranous anomaly of the vitreous. The central vitreous cavity in patients with membranous anomaly is notable for the relative absence of vitreous fibers giving rise to an optically empty appearance.

The phenotypic overlap of SVD with Stickler syndrome type I and type II is considerable and includes early onset cataract, severe anomaly of the vitreous, lattice degeneration, radial paravascular retinal degeneration, and retinal detachment. Although SVD can be distinguished by corneal guttae, fibrillar vitreous anomaly, optic nerve head dysmorphism, crystalline deposits within the retina, and low incidence of retinal detachment, these findings can be subtle and not present in all affected subjects. It is possible that previously identified families with fibrillar vitreous anomaly may have SVD. Thus, our finding of a new locus for fibrillar vitreous anomaly demonstrates that SVD is genetically distinct from other diseases with fibrillar vitreous anomaly and may enable identification of other families linked to chromosome 2q36. The chromosome 2q36 locus for SVD contains approximately 200 genes and potential genes. The only known gene associated with fibrillar vitreous anomaly is COL11A1. The COL4A3 and COL4A4 genes lie head-to-head on chromosome 2q36-q37, sharing a common promoter. These two genes encode the α3 and α4 chains of Type IV collagen, both of which are components of basement membranes. Mutations in either can result in benign familial hematuria or Alport syndrome. Alport syndrome is characterized by progressive renal impairment and often hearing loss, lenticulons, and a flesched retinopathy. Part of the COL4A3 gene is located within the SVD critical region and is expressed in ocular tissues affected by the SVD mutation. Alport syndrome patients have not been reported to have vitreoretinal degeneration, including one highly myopic patient who developed a retinal detachment. Although we hypothesized that a different class of mutations in COL4A3 might be associated with SVD, no mutations were identified on sequencing this gene. Snowflake vitreoretinal degeneration also has dysmorphogenic features that share some overlap with Wagner disease. For example, anterior segment abnormalities and optic nerve head dysmorphism are seen in both disorders. Thus, with COL4A3 excluded and the available recombinant breakpoints in the original SVD family well localized, the selection of candidate genes for analysis must rely on such clinical observations and ocular expression patterns. Candidate genes include predicted genes related to α-kinase anchoring protein, and an ankyrin repeat protein as well as kinesin and sp24.

In summary, we have demonstrated that snowflake vitreoretinal degeneration is a genetically and clinically distinct disease. The disease gene was localized to a novel 20 cm (9 Mb) interval on chromosome 2q36. This finding will enable genetic diagnosis of other families with snowflake vitreoretinal degeneration and help to dissect the genetic causes of fibrillar anomaly of the vitreous. Studies to refine this interval and screen other candidate genes in this chromosomal region for mutations are underway.

**ElectRONIC DATABASE INFORMATION**


Genethon database: http://www.genethon.fr/

Stanford Human Genome Center: http://shgc-www.stanford.edu/

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**References**


