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Identification of the genetic defect in the original Wagner syndrome family

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Purpose: The aim of the present study was to determine the genetic defect in Wagner syndrome, a rare disorder belonging to the group of hereditary vitreoretinal degenerations. This disease has been genetically mapped to chromosome 5q14.3.
Methods: Molecular analysis was performed in the progeny of the original pedigree described by Wagner in 1938. We searched for pathogenic mutations and their effects in two candidate genes, *CSPG2* and *EDIL3*, which locate to the critical chromosomal interval. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis was used to investigate potential splice defects of CSPG2 transcripts.
Results: While no alterations were detected in the exons of *EDIL3*, several changes were identified in the *CSPG2* gene. Only one of the novel changes, a heterozygous G to A substitution of the first nucleotide in intron 8, cosegregates with the disease phenotype. This change disrupts the highly conserved splice donor sequence. In blood cells of an index patient, we found CSPG2 transcripts with normally spliced exon 8/9 junction but also two additional CSPG2 transcripts, which were not detected in the control. One lacks the entire exon 8, while the other is missing only the last 21 bp of exon 8.
Conclusions: *CSPG2* encodes versican, a large proteoglycan, which is an extracellular matrix component of the human vitreous and participates in the formation of the vitreous gel. The splice site mutation described here may lead to a complete lack of exon 8 in CSPG2 transcripts, which shortens the predicted protein by 1754 amino acids and leads to severe reduction of glycosaminoglycan attachment sites.

Wagner syndrome (OMIM 143200) is a rare vitreoretinal degeneration inherited as an autosomal dominant trait. This potentially blinding disease was first described in 1938 in a large Swiss pedigree and has been identified subsequently in several other families [1-5]. Penetrance in Wagner syndrome is complete, and the disease manifests in childhood or adolescence with a progressive course. Affected individuals usually present an “empty” vitreous cavity with fibrillary condensation or avascular strands and veils. Additional features include chorioretinal atrophy with loss of the retinal pigment epithelium, lattice degeneration of the retina, complicated cataracts, mild myopia and peripheral traction retinal detachment [6]. Rod and cone electroretinography (ERG) shows reduced b-wave amplitude and correlates with severe chorioretinal pathology [6]. These additional findings are extremely variable and are age-dependent (Table 1). The pathogenic processes leading to the complex eye phenotype of Wagner syndrome are poorly understood. Most likely, however, liquefaction of the vitreous initiates a degenerative cascade. Whether the additional features evolve secondary to the degeneration of the vitreous or represent independent features still remains to be determined.

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The chromosomal location of this disease (*WAG1*) had initially been mapped in the original Wagner family to a 35-cM region on chromosome 5q13-14 [7]. This region was later reduced to 2 cM between markers D5S626 and CRTL1 [3,7]. Within this region several known genes can be found. One of them is *CSPG2*, which encodes within 15 exons the large (3396 amino acids) chondroitin sulfate proteoglycan, versican [8,9]. Differential splicing of the two largest exons, 7 and 8, yields four isoforms, which accumulate in a tissue-specific manner [10,11]. Variant V0 contains both exons and can bind 17 to 23 glycosaminoglycan (GAG) chains, V1 lacks exon 7 and can bind 12 to 15 GAGs, V2 lacks exon 8 and binds only 5 to 8 GAGs and V3 lacks both exons and hence has no GAG attachment sites. Versican participates as a structural component of the human vitreous. Both the chromosomal location and its function make it a likely candidate for a pathogenic factor causing the Wagner syndrome.

Perveen and colleagues analyzed 18 families with Wagner syndrome. They found a single nucleotide exchange causing an amino acid exchange within exon 7 of *CSPG2* in only one of the families and therefore did not consider versican the pathogenic cause [3]. Recently, Miyamoto et al. [2] identified a sequence alteration in intron 7 of *CSPG2* leading to aberrant RNA splicing, which co-segregated with Wagner syndrome in a Japanese family.

Here we report on sequence analyses in the original Wagner family of two candidate genes, *CSPG2* and *EDIL3*, which locate to the 2 cM region identified by Perveen et al.

[3] and provide data that implicate a role of versican in the development of Wagner syndrome.

METHODS

Patients: All patients studied belong to the single large pedigree originally described by Wagner in 1938 (updated pedigree shown in Figure 1). Forty-six family members and 11 spouses were genetically analyzed. All family members had received a complete clinical eye examination, which resulted in unequivocal determination of the disease status. The clinical follow-up of this cohort has been described in a previous publication [6].

Mutation screening by DNA sequencing: Between 50 and 100 ng of genomic DNA from the index patient IV/49 (Figure 1) was amplified by polymerase chain reaction (PCR) and subjected to DNA sequence analysis using the ABI Prism 3100 or 377 system (Applied Biosystems, Rotkreuz, Switzerland). Primers, spanning the 10 exons and flanking introns of *EDIL3* (GenBank accession number NM_005711) and the 15 exons and flanking introns of *CSPG2* (GenBank accession number NM_004385) were designed using Primer3 software. Primer sequences and PCR conditions are available upon request. The large exons 7 and 8 of *CSPG2* were amplified in 5 and 12 overlapping fragments, respectively. All other exons could be analyzed from a single PCR reaction. PCR fragments were electrophoretically separated in 1 or 2% agarose, either excised and purified with a QIAquick Gel Extraction Kit (Qiagen; Hombrechtikon, Switzerland) or cleaned with the EXOSAP enzyme treatment (USB, Cleveland, OH) followed by sequencing. For most sequence alterations found in the index patient, the corresponding sequence of the affected father and the unaffected mother was also determined. Sequence of the splice donor in intron 8 was determined from 47 family members.

Reverse transcriptase polymerase chain reaction experiments: RNA was extracted from venous blood of the index patient IV/49 (Figure 1) using the Pre-Analytix System (Qiagen), and its integrity and concentration was determined with the Agilent system (Agilent, Basel, Switzerland). cDNA was synthesized from 1 μg total RNA using the Superscript III kit (Invitrogen, Basel, Switzerland) and 1 μl was subjected to reverse transcriptase polymerase chain reaction (RT-PCR; Table 1, Figure 2). Amplification with HotStar polymerase in the presence of 2 mM $MgCl_2$ (Qiagen) occurred in 35 cycles of 1 min incubations each at 95 °C, 59 °C, and 72 °C in a reaction volume of 25 μl. Reverse transcription and PCR reactions were obtained from two separate blood samples of the

Table 1. Clinical features in Wagner syndrome

Features	Ocular symptoms	Observation in younger patients	Observations in older patients
Hallmark	Empty vitreous with fibrillary condensations	Frequent	Not observed anymore
	Avascular strands and veils	Frequent	Almost always
Additional	Chorioretinal atrophy	Frequent	Always present
	Rhegmatogenous retinal detachment	Rare	Not observed
	Peripheral tractional retinal detachment	Rare	Common
	Cataracts	Frequent	Does not apply
	Visual acuity	Normal to subnormal	Severely affected
	Refractive error	Frequent mild to moderate myopia	Does not apply
	Visual field	Minor defects	Marked field loss
	Abnormal pattern of the central retinal vessels	Rare	Frequent
	Optic atrophy	Not observed	Frequent if advanced chorioretinal atrophy
	Electroretinography (ERG) dark adaptation	Frequently pathologic rod threshold frequently elevated	Progressively pathologic

Patients of the original Wagner syndrome family were assigned to the older or younger group based on the age of approximately 30 years. This age limit should be considered of gliding nature. The division into hallmark and additional features reflects our hypothesis that hallmark features represent the primary defect while additional features may be of secondary, consequential nature. More detailed information about the clinical observations can be found in the literature [4,6].

index patient. The following primers were used: RTexon7f (5'-CTC CTG CTA CAC AGC CAA CA-3'), RTexon8f (5'-AGG TTG CAA CAC CAC CAT TT-3'), RT exon9r (5'-TCA CAC TGG TCT CCG CTG TA-3'), and RTexon10r (5'-GCT CAC AAA GTG CAC CAA CA-3'). Products were electrophoretically separated on 1% agarose or 3% Nusieve agarose (Agarose 3:1, Eurobio, Brunschwig, Basel, Switzerland). DNA sequence was determined using the ABI 3100 sequencer as previously described. Potential splicing events were investigated using the NNSPLICE software (Berkeley Drosophila Genome Project, Berkeley, CA) [12].

RESULTS

DNA sequence analysis: The search for disease-causing genes led us to two potential candidates located in the region of 5q13-14, to which the Wagner syndrome had been mapped. One of them was *EDIL3* (epidermal growth factor-like repeats and discoidin I-like domains 3), an extracellular matrix protein. The coding region of *EDIL3* did not carry any mutation (data not shown).

The other candidate gene was *CSPG2*. DNA sequence of the index patient revealed 16 changes: ten of them in exons, one in the promoter region, and four in introns. Most of them are known single nucleotide polymorphisms (SNPs), except the promoter and three intron changes (Table 2). Most of the regions that showed known SNPs or novel changes in the index patient were also analyzed in the affected father and the unaffected mother but did not segregate with the phenotype

Figure 1. Pedigree of original Swiss family with dominantly inherited Wagner syndrome. Filled symbols represent affected individuals. Family members of generations III, IV, and V, who have been tested for the splice site mutation, are numbered. An arrow points to the index patient. Clinical information on the eye phenotype of generation I is not available.

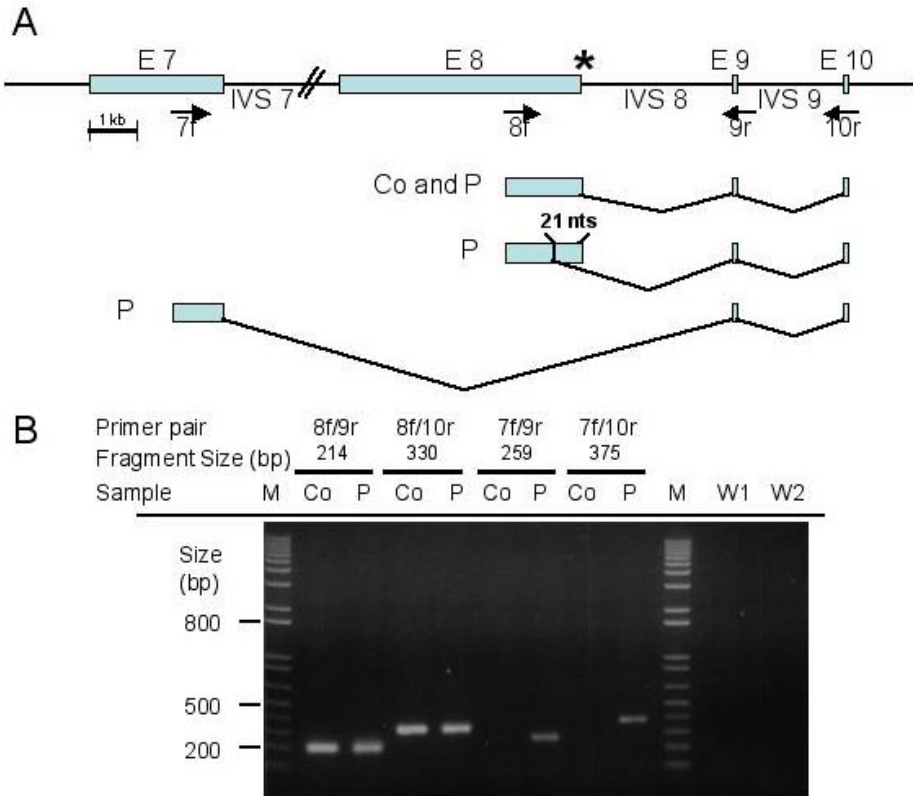

Figure 2. CSPG2 transcript analysis. Schematic diagram (**A**) and gel electrophoresis (**B**) show the accumulation of normal and aberrant splice products of *CSPG2* in control and patient venous blood RNA as determined by RT-PCR analysis. **A**: The size of exon (E) 7 through 10 and intron (IVS) 7 through 9 is given in base pairs (bp). IVS7 and primers 7f, 8f, 9r, and 10r are not drawn to scale. Primer arrows point to 3' direction. Asterisk (*) symbolizes the c.9265+1G>A splice site mutation. To the left of the diagrammed splice products is indicated the sample in which the respective fragment was found. **B**: Ethidium bromide stained agarose gel shows migration of the 1 kb molecular weight marker (M) and RT-PCR products from control (Co) and patient (P) RNA samples. Expected fragment sizes are noted below each primer combination. Water templates (W) did not show any amplification products.

(Table 2). Of the novel changes only the G to A substitution in intron 8 segregated with the disease status within this nuclear family. Consequently, this mutation was tested in 47 members of the entire Wagner family, and it segregated with the phenotype in 21 affected and 26 unaffected individuals (Figure 1). It destroys the highly conserved splice donor sequence of intron 8 of one allele (Figure 3). In 104 normal alleles, this G residue was not mutated.

Transcript analysis: Four different isoforms of versican are regulated in a tissue-specific pattern [8-10]. In RT-PCR experiments with RNA from venous blood from a normal control person, no fragments were amplified using primer pairs 7f/9r or 7f/10r (Figure 2). This is expected if exon 8 (5262 bp) is present in the transcript and PCR conditions were not optimized for amplification of larger products. In contrast, RNA from the index patient IV/49 (Figure 1) with the splice donor site mutation IVS8+1G>A resulted in amplification of a 259 bp fragment with primer pair 7f/9r and a 375 bp fragment with primer pair 7f/10r (Figure 2). DNA sequence was determined from both RT-PCR fragments and showed splicing of exon 7 to exon 9. To investigate the splice events of intron 8 further, primers 8f/9r and 8f/10r were used. In the control sample, one fragment of 214 bp and one of 330 bp in size was amplified with primer pairs 8f/9r and 8f/10r, respectively. DNA sequence of these fragments revealed splicing of exon 8 to exon 9. In RNA samples from the index patient, the same size RT-PCR fragments yielding the same sequence were obtained, as expected due to the heterozygous mutation. In addition, both primer pairs resulted in the amplification of a slightly shorter fragment, which was only visible as a faint band after electrophoresis in 3% agarose Nusieve gel (data not shown). We determined the sequence of this fragment, which was revealed to lack the last 21 base pairs of exon 8 (sequence not shown), and which may lead to an in frame deletion of seven amino acid residues.

DISCUSSION

We have shown that a heterozygous mutation of the highly conserved splice donor site in intron 8 of the *CSPG2* gene segregates with the dominant disease status in the original Wagner family. Moreover, we confirmed that the mutation produces aberrantly spliced CSPG2 transcripts.

Argument for pathogenic mutation: Genetically, Wagner syndrome had been mapped to the 5q13-14 chromosomal interval. Within this region, eight known and three hypothetical genes are located (Genome Browser, version 107). Two of the known genes, *EDIL3* and *CSPG2*, encode proteins with functions in the extracellular matrix and seemed likely candidates to affect the vitreous gel. Neither we nor others [2] found a mutation in *EDIL3* and therefore excluded this gene from being involved in the development of Wagner syndrome. In contrast, the destruction of the highly conserved splice donor site of intron 8 of the *CSPG2* gene perfectly co-segregates with the disease phenotype and was not found in normal alleles. Recently, a mutation in *CSPG2*, also causing a splicing defect, was reported to segregate in a Japanese family with Wagner syndrome [2]. Together with an earlier finding of a nucleotide transition mutation in exon 7 of *CSPG2* in a differ-

TABLE 2. *CSPG2* SEQUENCE ALTERATIONS

			Sequence results			
Location	SNP/DNA change	Protein change	Index	Affected father	Unaffected mother	SNP reference
Promoter	-648A>G		G			novel
Exon 3	c.587C>T	p.T116T	T			rs12332199
Exon 5	c.884G>A	p.V215V	A	G/A	G/A	rs4470745
Exon 8	c.1601G>A	p.Q454Q	A	G/A	A	rs2548541
Exon 8	c.1825A>G	p.K529R	G	A/G	A/G	rs309559
Exon 8	c.2755G>A	p.R839H	A	A	A	rs188703
Exon 8	c.3086T>C	p.G949G	C	T/C		rs309557
Exon 8	c.4001A>G	p.R1254R	G	A/G	A/G	rs160279
Exon 8	c.4180T>A	p.F1314Y	A	T/A	T/A	rs160278
Exon 8	c.6087G>T	p.D1950Y	T	G/T	G/T	rs160277
Exon 14	c.7160C>T	p.V2307V	T	T	T	rs308365
Intron 8	c.9265+1G>A		G/A	G/A	G	novel
Intron 9	c.9380-181G>A		A			rs7728751
Intron 9	c.9380-75insACT		ACT	---/ACT	---/ACT	novel
Intron 10	c.9513-63T>A		A			rs6873404
Intron 11	c.9653-149A>G		G	A/G	A/G	novel

Sequence alterations in *CSPG2* of index patient IV/49, affected father III/22 and unaffected mother II/23 (Figure 1). Nucleotide position based on cDNA and protein changes are listed. The disease-causing mutation in intron 8 is printed in bold. Substitutions (>) and insertions (ins) are indicated. In the index patient, all alterations except the disease-causing change in intron 8 appear homozygous. Known single nucleotide polymorphisms (SNPs) are listed by their identification number (dbSNP). Reference sequence for CSPG2 is from GenBank accession number NM_004385.

ent Wagner family [3], these data provide strong evidence that mutations in the *CSPG2* gene are the underlying genetic defect in Wagner syndrome.

Hypothesis of the disease mechanism: CSPG2 encodes a large extracellular chondroitin sulfate proteoglycan, versican, which is 3396 amino acids in length with about a 370 kDa mass [8,9]. The protein is secreted and found in many extracellular matrices, including the vitreous gel. It forms large aggregates via bound hyaluronan at the N-terminus and GAG at specific attachment sites (GAG α and β), which reside in the middle section of the protein, and it most likely participates in the establishment and maintenance of supramolecular structures. The middle section of the protein is encoded by two large exons, 7 and 8, whose presence or absence determines the identity of four different variants of versican. Variant V0 contains both exons, V1 contains exon 8, V2 contains exon 7, and V3 lacks both exons. Accordingly, the number of GAG side chains varies (V0 has 17-23, V1 has 12-15, V2 has 5-8, and V3 has none). The four splice variants accumulate at rather strikingly different levels in a tissue-dependent manner. Within the eye, transcripts of all four splice variants have been found in the retina, the trabecular meshwork and the ciliary muscle [2,10,13]. The retina expresses mainly the V3 transcript [2], while in the trabecular meshwork, and the ciliary muscle, the V1 mRNA prevails [13]. It is not clear which of these tissues contributes versican to the mostly acellular vitreous gel [14]. In our Swiss Wagner family, destruction of the splice donor site of intron 8 leads to ectopic expression of versican V2 mRNA in leukocytes. It seems possible that upregulation of the V2 variant and haploinsufficiency of the V0 and V1 variant may also occur in the tissues, which contribute to vitreous development and maintenance.

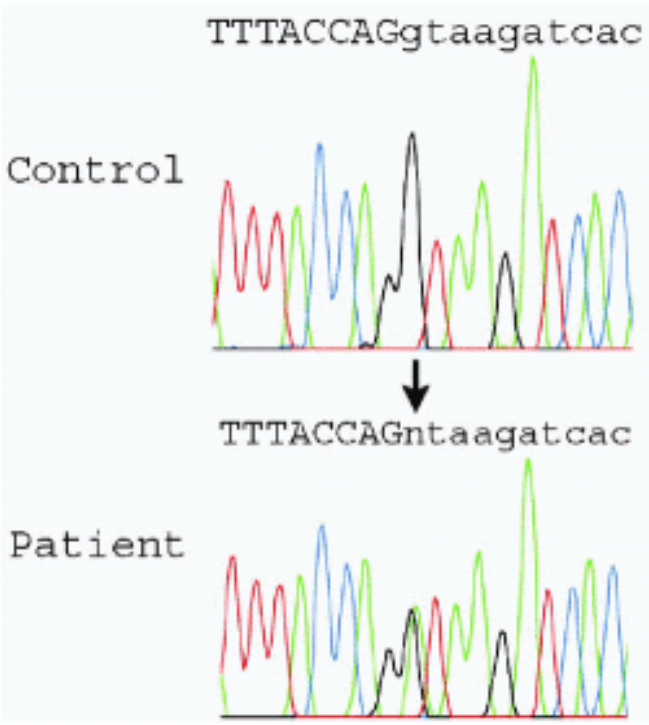

Figure 3. *CSPG2* splice mutation. Electropherogram displays 18 nucleotides surrounding the heterozygous splice donor mutation (c.9265+1G>A). Capital letters designate exon 8 sequences; lowercase letters represent intron 8 sequences. The "n" in the sequence depicts the heterozygous sequence mutation of the index patient.

The effect and the mechanism by which the second aberrant transcript, which contains almost all of exon 8 with the exception of the last 21 base pairs, could have been generated are not clear. Most likely the activation of a cryptic donor site within exon 8 is involved although a search for such sites did not reveal obvious sequence candidates within reasonable vicinity. Translation of this aberrant transcript would result in a protein lacking the last seven amino acids of exon 8. No known function can be assigned to these skipped seven amino acids. Whether this transcript participates in the pathogenicity of the Wagner syndrome remains to be examined.

Defective splicing of CSPG2 transcripts is also discussed as the pathogenic cause of Wagner syndrome in a Japanese family [2], in which alteration of the splice acceptor site of intron 7 was found to be mutated, leading to haploinsufficiency of V0 and V1. This deficiency of V0/V1 and hence of glygosaminoglycane attachment sites is common to both the original Swiss and the Japanese Wagner families, supporting the hypothesis that splicing defects of *CSPG2* may lead to the vitreoretinopathy.

As versicans interact through their C-terminal globular domain with fibrillin-1 [15], they may be responsible for maintaining the spatial separation of hyaluronan, the microfibrillar structures and the collagen fibers. In this model, the GAG containing middle portions are likely to play a crucial role in keeping the fibrillar structures apart, preventing tangling and aggregation of the collagen fibers. Relative overproduction of variants V2 or V1 and deficiency of V0 in Wagner syndrome would lead to altered core protein size, thereby affecting interactions with other extracellular matrix components and consequently disturbing the ultrastructural organization of the vitreous gel. This could cause accelerated liquefaction of vitreous gel and finally manifesting in the "empty" vitreous observed in Wagner patients.

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