Erosive Vitreoretinopathy and Wagner Disease Are Caused by Intronic Mutations in *CSPG2/Versican* That Result in an Imbalance of Splice Variants

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Purpose. Linkage intervals for erosive vitreoretinopathy (ERVR) and Wagner disease previously were found to overlap at 5q14.3. In a Japanese family with Wagner disease, a *CSPG2/Versican* splice site mutation (c.4004-2A→G) was recently reported that resulted in a 39-nucleotide exon 8 in-frame deletion. We investigated whether *CSPG2/Versican* was mutated in six Dutch families and one Chinese family with Wagner disease and in a family with ERVR.

Methods. In all families, extensive ophthalmic examinations, haplotype analysis of the 5q14.3 region, and sequence analysis of *CSPG2/Versican* were performed. The effects of splice site mutations were assessed by reverse transcription–polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR (QPCR).

Results. Three novel intron 7 sequence variants (c.4004-5T→C, c.4004-5T→A, c.4004-1G→A) were identified in seven families. The c.4004-5T→C variant was identified in four families with Wagner disease and a family with ERVR. The families were shown to carry the same 5q14.3 haplotype, strongly suggesting that this is a common Dutch founder variant. All three changes segregated with the disease in the respective families and were absent in 250 healthy individuals. In patients with the c.4004-5T→A and c.4004-1G→A variants, RT-PCR analysis of *CSPG2/Versican* showed activation of a cryptic splice site resulting in a 39-nt exon 8 in-frame deletion in splice variant V0. QPCR revealed a highly significant ($P < 0.0001$) and consistent increase of the V2 (>38-fold) and V3 (>12-fold) splice variants in all patients with intron 7 nucleotide changes and in a Chinese Wagner disease family, in which the genetic defect remains to be found.

Conclusions. Wagner disease and ERVR are allelic disorders. Seven of the eight families exhibit a variant in intron 7 of *CSPG2/Versican*. The conspicuous clustering of sequence variants in the splice acceptor site of intron 7 and the consistent upregulation of the V2 and V3 isoforms strongly suggest that Wagner disease and ERVR may belong to a largely overlooked group of diseases that are caused by mRNA isoform balance shifts, representing a novel disease mechanism. (*Invest Ophthalmol Vis Sci.* 2006;47:3565–3572) DOI:10.1167/iovs.06-0141

Patients with Wagner disease (OMIM 143200; Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/Omim/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), a dominantly inherited vitreoretinopathy, show vitreal syneresis with optically empty vitreous and equatorial avascular vitreous veils. Additional features include mild to moderate myopia, typical dot-like cortical cataracts, abnormal retinal vessel architecture (inverted papilla), ectopic fovea, perivascular pigmentation and sheathing, retinal thinning, and slowly progressive chorioretinal atrophy. Most patients under the age of 20 enjoy normal vision. However, with advancing age, cataract, retinal detachment, optic atrophy, and chorioretinal atrophy may cause visual loss.[1–3] Erosive vitreoretinopathy (ERVR; OMIM 143200) was first reported as a new clinical entity in 1995.[4] ERVR also displays an autosomal dominant pattern of inheritance and in addition to the clinical features of Wagner disease, reveals progressive nyctalopia (night blindness), visual field constriction, and chorioretinal atrophy. Fluorescein angiography reveals a conspicuous loss of retinal pigment epithelium (RPE) and choriocapillaris. Electroretinography (ERG) studies show a rod–cone abnormality.[4] Typically, systemic abnormalities are not present in Wagner disease and ERVR, in contrast to Stickler syndrome.

Linkage studies assigned the Wagner disease gene to a 2-cM interval at 5q14.3,[4,5] but until recently, no definite causal mutations were identified. The critical region for a genetic defect underlying ERVR was found to overlap the critical region for Wagner disease, suggesting that these diseases are allelic.[4] Recently, a heterozygous splice site change in *CSPG2/Versican* (c.4004-2A→G) was reported in a Japanese family with Wagner disease.[6] This variant was shown to result in deletion of 13 amino acids from the beginning of the glycosaminoglycan (GAG)-β domain of the mature protein.[6]

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CSPG2/Versican has been an attractive candidate for Wagner disease and ERVR based on its genomic location and function. It encodes a large extracellular matrix proteoglycan (chondroitin sulfate proteoglycan type 2) that is present in virtually every human tissue, including different parts of the eye.[7] CSPG2/Versican is believed to maintain the structure of the vitreous body in the human eye by keeping the collagen molecules apart.[8] It has a tridomain structure, and the amino-terminal end binds to hyaluronan. The carboxyl-terminal domain has a C-type lectin domain adjacent to two epidermal growth factor domains and a complement regulatory region. The central area of CSPG2/Versican is encoded by two large exons (exons 7 and 8) and contains chondroitin sulfate attachment sites. The chondroitin sulfate chains of CSPG2/Versican are highly negatively charged and contribute to its antiadhesive properties. Alternative splicing of these two exons results in four different isoforms of the mature protein (see Fig. 6). V0 contains exons 7 and 8, V1 lacks exon 7 but contains exon 8, V2 contains exon 7 but lacks exon 8, and V3 lacks both these exons.[7] Accordingly, the potential number of glycosoaminoglycan (GAG) attachment sites for each form are V0, 17 to 23; V1, 12 to 15; V2, 5 to 8; and V3, 0.[7]

In this study, we show that most Dutch families with Wagner disease and a family with ERVR share a common 5q14.3 founder haplotype and a *CSPG2/Versican* splice site variant. In two additional unrelated families with Wagner disease we found two other sequence variants in the same intron 7/exon 8 splice junction. We found a qualitative effect on *CSPG2/Versican* mRNA splice isoforms (i.e., a 39-nt in-frame truncation, in two families with Wagner disease). In addition, in all families, we found a significant increase of *CSPG2/Versican* mRNA isoforms V2 and V3 which leads us to propose a novel mechanism for disease pathogenesis.

Methods

Sample Collection

This study was conducted in accordance with the tenets of the Declaration of Helsinki. We ascertained seven multigenerational families with Wagner disease and one family with ERVR (Fig. 1). The clinical diagnosis of Wagner disease or ERVR was based on detailed ophthalmic examinations, including Snellen visual acuity testing, slit lamp anterior segment and vitreous examinations, indirect funduscopy, slit lamp biomicroscopy, and color fundus photography. In selected cases, fluorescein angiography, kinetic and static perimetry, electroretinography, and electro-oculography were also performed.

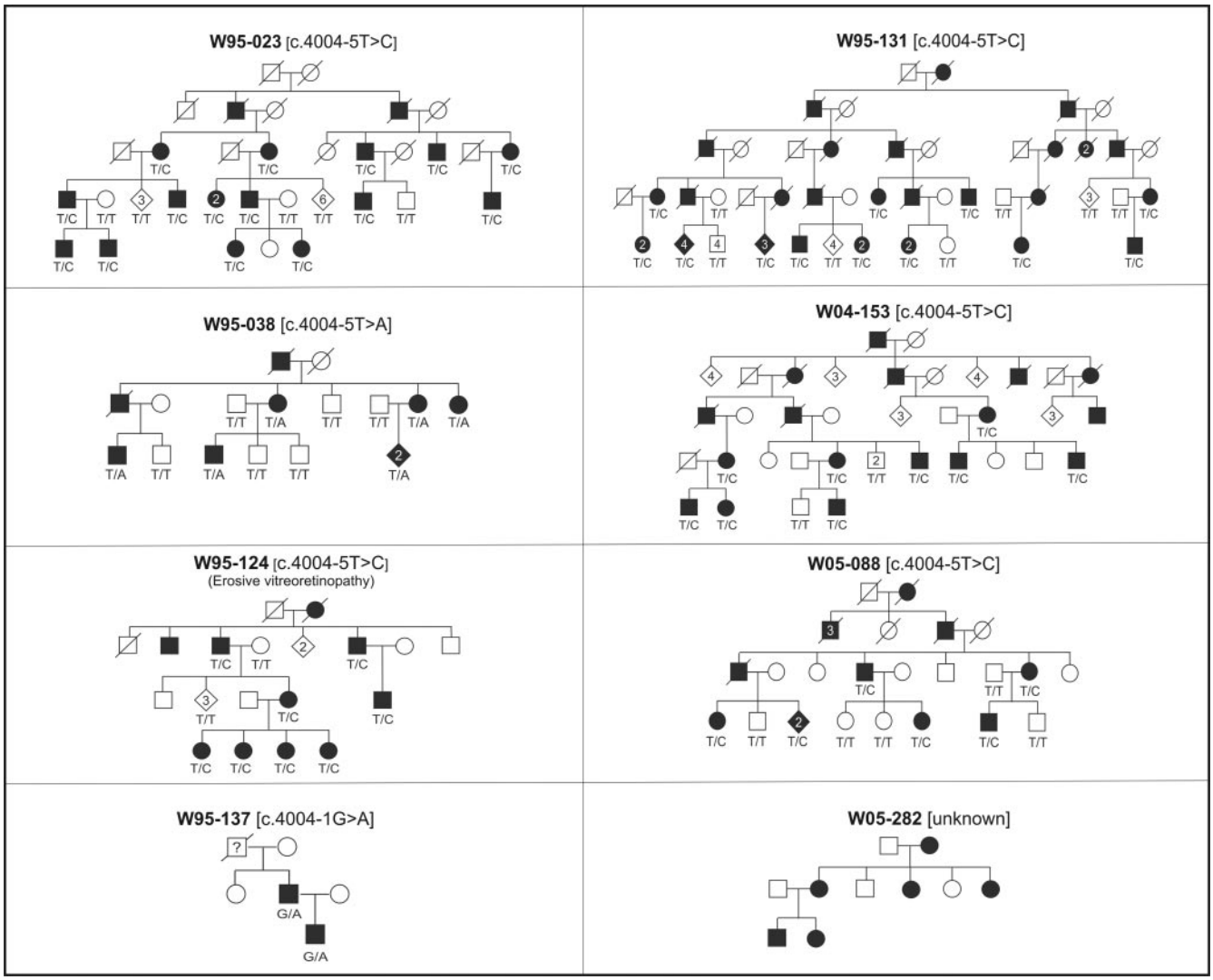

Figure 1. Wagner disease and ERVR pedigrees and segregation of *CSPG2/Versican* sequence variants. Although the overall number of patients and nonaffected siblings are accurate, the pedigrees were anonymized for privacy.

EDTA-anticoagulated venous blood was collected after obtaining the informed consent from the participating individuals. Genomic DNA[9] and total RNA (Qiagen RNeasy Midi Kit; Qiagen, Venlo, The Netherlands) were isolated from EDTA blood using standard procedures. A panel of 250 anonymous healthy Dutch control subjects was tested for the presence of intron 7/exon 8 splice junction variants. For family W95-137, a fibroblast cell line was generated from skin biopsy of one patient. Typically, cells were grown in flasks detached to the bottom. Dulbecco's modified Eagle's medium was used containing 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were split into two equal portions every 3 days, and RNA was isolated from 80% confluent cultures.

Genotyping and Linkage Analysis

Genotyping at the Wagner disease/ERVR locus on chromosome 5 was performed by selecting 13 microsatellite markers from the Genome Database (www.gdb.org/ provided by RTI International, NC) covering a region of approximately 3 cM. For all the markers except D5S626, genotyping PCR was performed with primers with a fluorescent label and the results were analyzed (GeneMapper; Applied Biosystems, Inc. [ABI], IJssel, The Netherlands). For D5S626, ^{32}P end-labeled primers were used. Haplotypes were constructed by Cyrillic 2.1 (Cyrillic Software, Wallingford, UK), and checked manually for confirmation of cosegregation of the marker alleles with the disease phenotype.

Sequence Analysis

The open reading frame (ORF) and the exon–intron junctions of *XRCC4* was analyzed for a proband of family W95-131, as described previously.[5] The ORF and the exon–intron junctions of the 15 exons of *CSPG2/Versican* were sequenced in a proband from each of the eight families (primer sequences available on request). Polymerase chain reaction (PCR) was performed according to the standard protocols.[10] PCR products were purified (QiaQuick columns; Qiagen), either directly or after excision from the gel and were directly sequenced using dye termination chemistry (model 3700; ABI).

RT-PCR and Real-Time Quantitative RT-PCR Analysis

Total RNA was isolated from 10 mL of EDTA-anticoagulated venous blood within 4 hours after sampling (RNeasy minikit; Qiagen) according to the manufacturer's protocol. cDNA was synthesized from 2.0 μg total RNA using random hexamers (GE Healthcare, Roosendaal, The Netherlands), and M-MLV reverse transcriptase (Invitrogen, Groningen, The Netherlands) in a total volume of 85.0 μL. After the synthesis, the cDNA was purified on PCR purification columns (QIAquick; Qiagen) and eluted in 30 μL sterilized water (milliQ; Millipore, Bedford, MA).

RT-PCR was performed using primer pairs that specifically amplify each of the four mRNA splice variants (see Fig. 6).[6] In addition, we used a common primer pair that amplifies all mRNA variants, termed V_t for total expression (Supplementary Table S1, available online at http://www.iovs.org/cgi/content/full/47/8/3565/DC1).

For QPCR, 10× dilutions of the cDNA were made and 5.0 μL from the diluted stock was used for each PCR reaction. Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Amplicon lengths were typically kept between 80 to 100 bp (Supplementary Table S1, http://www.iovs.org/cgi/content/full/47/8/3565/DC1). Quantitation was performed using relative quantification strategies (SYBR Green), according to the manufacturer's protocol (Bio-Rad, Hercules, CA). PCR reactions were performed on a thermocycler (iCycler MyiQ Single-Color Real-Time Detection System; Bio-Rad). Initially, the primer conditions were optimized and a standard curve was determined for six different dilution points (10×–320×) until for each of the primers PCR efficiencies close to 100% were obtained. For calculation purpose all the PCR efficiencies were assumed to be exactly 100%. For each sample triplicate PCR reactions were performed to ensure reproducibility. The differences in expression between patients and controls were calculated by using the $2^{\Delta\Delta Ct}$ method.[11,12] By definition, C_t is the required number of amplification cycles to reach the threshold fluorescence level (automatically determined); ΔC_t implies the difference in the C_t between the reference gene (*GUSB*) and the gene of interest (*CSPG2/Versican*) and $\Delta\Delta C_t$ represents the difference between two ΔC_ts. *GUSB* was used as a reference gene, because of its stable expression in lymphocytes.[13,14] $\Delta\Delta C_t$ is the difference between the mean ΔC_t of eight control subjects and the ΔC_t for individual patients. For statistical analysis of the QPCR data, the probabilities were calculated in the *P*-value calculator (www.graphpad.com/quickcalcs/PValue1.cfm/ GraphPad, Inc., San Diego, CA) available in the public domain.

Splice-Site Score

The predicted effect of the identified intronic variants on splicing were estimated at the server of the Children's Mercy Hospitals and Clinics at the University of Missouri-Kansas City (https://splice.cmh.edu).[15]

Results

Ocular Phenotypes

The clinical features of at least two patients from each of the four unrelated families with Wagner disease, as well as from four patients with ERVR from one family are summarized in Table 1. Patients from Wagner disease families W95-023, W95-131, W04-153, and W05-088, based on molecular genetic studies (see below), are considered to belong to one superfamily. Patients with ERVR, in addition to the features observed in patients with Wagner disease (vitreous abnormalities, cataract, retinal thinning, ectopia of the macula, and abnormal retinal blood vessel architecture), consistently show a progressive and severe choriocapillaris and RPE atrophy, resulting in a progressive nyctalopia and a marked visual field constriction.

Representative fluorescein angiography pictures are depicted in Figure 2. A 50-year-old patient with Wagner disease (W95-131P3) showed almost no chorioretinal atrophy (Fig. 2A). In contrast, a patient with ERVR (W95-124P2) at a somewhat younger age (44 years) shows a severe midperipheral chorioretinal atrophy with macular sparing (Fig. 2C). In a 32-year-old patient with ERVR (W95-124P3), the chorioretinal atrophy is restricted to the peripapillary region (Fig. 2B).

Identification of a Founder Haplotype in Dutch Families with ERVR and Wagner Disease

We determined the 5q14.3 haplotypes in ~120 individuals of the families W95-023, W95-038, W95-124, W95-131, W04-153, and W05-088, most of which are depicted in Figure 1, employing 13 microsatellite markers. The phenotypes in these families fully cosegregated with marker alleles of the 5q14 region (data not shown) suggesting genetic homogeneity for Wagner disease. The haplotypes of patients carrying critical recombinations in 5q14 are depicted in Figure 3. Recombinations observed in the affected individuals from families W95-023 and W95-131 restricted the region to approximately 850 kb with *D5S626* and *D5S107* as the flanking markers. All patients from four Wagner disease families (W95-023, W95-131, W04-153, W05-088) and from the ERVR family (W95-124) showed the same haplotype for the critical region, suggesting the presence of a founder allele in The Netherlands.

Identification of Three Novel Intronic Mutations in *CSPG2/Versican* in Patients with Wagner Disease and ERVR

The two genes present in the critical interval—namely, *CSPG2/Versican* and *XRCC4*—were analyzed in a patient of family W95-131. No variants were identified, except for a c.4004-5T→C change in *CSPG2/Versican* (Fig. 4). The same variant was identified in the DNA of all patients from the three other pedigrees (W95-023, W04-153, and W05-088) belonging to the

TABLE 1. Ocular Phenotypes of Wagner Disease and Erosive Vitreoretinopathy Patients

Patient Number (gender)	*CSPG2/ Versican* Variant	Age at Diagnosis (y)	Age at Examination (y)	Lens (ODS)	Corpus Vitreum (ODS)	Preretinal Membrane	Situs Inversus	Retina	RD
Patients with Wagner disease									
W95-038P1 (F)	c.4004-5T→A	5	23	Moderate cortical cataract	Optically empty, fibrillary vitreous	OS	ODS	Thin retina (OD), foveal ectopia (ODS)	ODS
W95-038P2 (F)	c.4004-5T→A	3	20	Clear	Optically empty	OD	ODS	?	No
W95-131P1 (F)	c.4004-5T→C	<19	19	Clear	Optically empty	No	No	Peripheral pigmentations and retinal holes (ODS)	ODS
W95-131P2 (M)	c.4004-5T→C	3	12	Clear	Optically empty, fibrillary vitreous	ODS	ODS	Lattice degenerations and retinal holes (ODS)	OS
W95-131P3 (F)	c.4004-5T→C	4	4	Posterior cortical cataract	Optically empty, fibrillary vitreous	ODS	No	Peripheral pigmentation, peripheral retinal defects, vitreous ridge inferonasal (ODS)	?
W95-137P1 (M)	c.4004-1G→A	11	35	Posterior subcapsular cataract	Optically empty, vitreous cords	No	ODS	Lattice degeneration and peripheral chorioretinal atrophy (ODS), peripheral pigmentations (OD), end arteries ending in temporal midperiphery (OD), vitreous ridge temporal area (OD)	No
W95-137P2 (M)	c.4004-1G→A	6	6	Mild posterior cortical cataract	Optically empty, vitreous cords	ODS	ODS	Vitreous ridge temporal area (ODS), lattice degeneration and preretinal membrane (ODS)	No
W05-282P1 (F)	Unknown	19	43	Anterior and posterior cortical cataract, sublaxation of lens	Syneresis	ODS	No	RP, occluded vessels on papil, sheathing of vessels	No
W05-282P2 (M)	Unknown	12	18	Mild posterior subcapsular cataract	Syneresis	No	?	Sheathing of vessels	No
Patients with erosive vitreoretinopathy									
W95-124P1 (M)	c.4004-5T→C	?	69	Cataract extraction at 45 y	Optically empty	ODS	No	Extensive chorioretinal atrophy with sparing of macular area, retinal pigmentations, ectopic macula (ODS)	No
W95-124P2 (M)	c.4004-5T→C	34	72	Afakia (lens extraction) at 51 y	Optically empty, vitreous cords	No	No	Extensive chorioretinal atrophy, some pigment clumps in retina, ectopic macula	No
W95-124P3 (M)	c.4004-5T→C	32	44	Cataract operation at 34 y	Optically empty	No	No	Demarcated chorioretinal atrophy, ectopic macula, paravascular retinal pigmentations ODS	No
W95-124P4 (F)	c.4004-5T→C	27	37	Cortical opacities	Optically empty	No	No	Demarcated chorioretinal atrophy ectopic macula	No

F, Female; M, Male; OD, Right eye; OS, Left eye; ODS, Both eyes; RD, Retinal detachment; RP, Retinitis pigmentosa; ?, unknown.

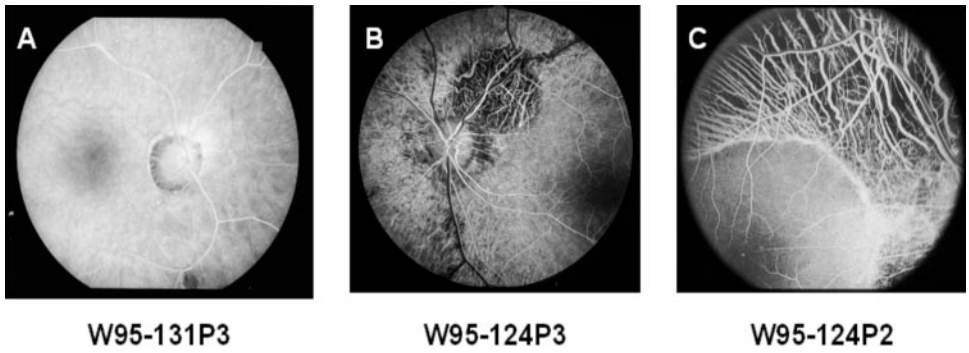

FIGURE 2. Fluorescence angiography (FAG) pictures of patients with erosive vitreoretinopathy and Wagner disease. (**A**) FAG of Wagner disease patient W95-131P3, at the age of 50 years. (**B**) FAG of peripapillary region of ERVR patient W95-124P3 at age 32. (**C**) FAG of posterior pole of ERVR patient W95-124P2 at age 44. Note the widespread atrophy of the choriocapillaris and RPE which visualizes the larger choroidal blood vessels.

Wagner disease superfamily, and none of the unaffected individuals (Fig. 1). In addition, we found a c.4004-5T→A change in the proband of family W95-038 and a c.4004-1G→A change in a proband from family W95-137 (Fig. 4) which also showed cosegregation with the disease in the respective families (Fig. 1).

As expected from the haplotype data, all patients and none of the unaffected individuals from the ERVR family (W95-124) showed the same T→C change as observed in the other related families with Wagner disease (Fig. 1). Sequence analysis of the intron 7–exon 8 splice junction in 250 ethnically matched Dutch healthy individuals did not reveal any of these changes. These results strongly suggest the presence of a founder allele (c.4004-5T→C) in most of the Dutch patients with Wagner disease and in a family with ERVR, a clinically related phenotype. The causal variant in the Chinese family with Wagner disease has not yet been identified.

Effect of Intron 7 Variants on *CSPG2/Versican* Transcripts

The c.4004-1G→A variant affects one of two completely conserved nucleotides of the canonical 3′ splice site of intron 7 and therefore is predicted to have a major effect on *CSPG2/Versican* splicing. The c.4004-5T→C and c.4004-5T→A variants are predicted to retain 64% and 14% of the original binding capacity, respectively (https://splice.cmh.edu). To investigate their effect on splicing, both qualitative and real-time quantitative RT-PCR analyses were subsequently performed.

The c.4004-1G→A and c.4004-5T→A nucleotide variants result in the activation of a cryptic downstream splice acceptor site of exon 8. V0 shows the wild-type and the 39-nt shortened cDNA fragments (Fig. 5, lanes 1, 2, and 4), mimicking the previously described result.[6] For V1, this effect is faintly visible. The c.4004-5T→C variant has only a small effect on the downstream splice acceptor (Fig. 5, lane 3). RNA from both lymphocytes and fibroblasts show the same effect on V0 and V1 for c.4004-1G→A mutation (Fig. 5, lanes 1 and 2). RT-PCR analysis of RNA from a patient of the Chinese family with Wagner disease (W05-282) did not show an activation of the downstream cryptic splice acceptor site (Fig. 5, lane 5). For the ERVR family the RT-PCR result was similar to that of W95-131 (data not shown).

Quantitation of the *CSPG2/Versican* mRNA Isoforms

To quantitate the expression levels for different *CSPG2/Versican* isoforms in patients, QPCR was performed on blood cell RNA using a relative quantification method (SYBR Green; Bio-Rad). The expression levels of each splice variant and the total expression (V_t) of *CSPG2/Versican* were analyzed for two patients from each of the Wagner disease families with different intron 7 sequence variants, as well as two patients from a Chinese family with Wagner disease (W05-282) and the ERVR family, and compared to the mean expression values obtained from eight control subjects. Initially, we used total RNAs isolated from EBV-immortalized lymphocytes of control subjects and patients, but results were very variable (data not shown). Therefore, we collected new venous blood samples from controls and patients and extracted RNA within 4 hours of venipuncture. The total expression level of *CSPG2/Versican* in eight controls now was robust, enabling us to use the mean values for further analyses (Table 2). Tables 3 and 4 show the ΔC_t and the *x*-fold changes ($2^{\Delta\Delta Ct}$), respectively, for the patients with Wagner disease and ERVR. The V2 isoform was upregulated between 38- and 478-fold ($P < 0.0001$), and the V3 isoform was upregulated between 12- and 52-fold ($P < 0.0001$). A 40-fold upregulation in patients indicates that the threshold fluorescence is reached approximately five amplification cycles earlier than that in control subjects. The patients from family W95-131 (Wagner disease) and W95-124 (ERVR), carrying the c.4004-5T→C change, showed comparable upregulation for V2 and V3 (Table 4). Patients W95-137P1 and P2,

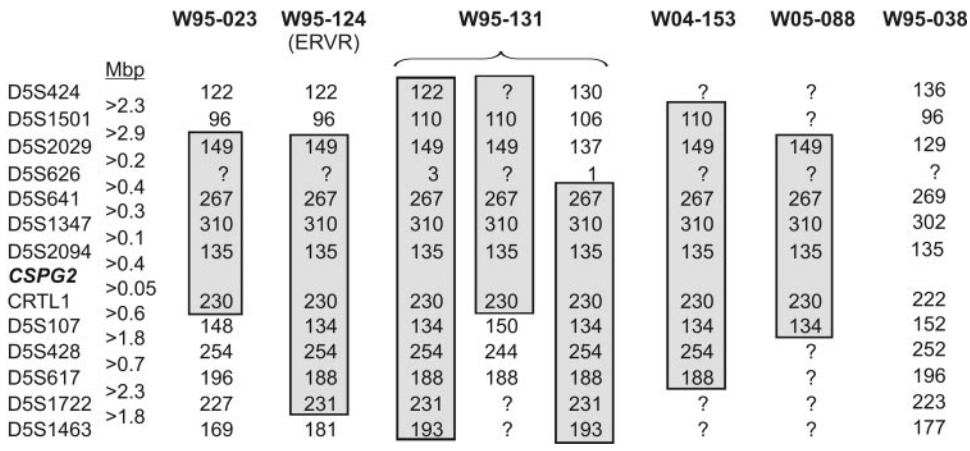

FIGURE 3. Haplotype analysis of the 5q14.3 region in Dutch families with Wagner disease and erosive vitreoretinopathy. The disease haplotypes are shown for one or a few patients that show recombination events that position the critical region between *D5S626* and *D5S107*. The physical distances between the markers are given as obtained from the UCSC genome browser. All families, except W95-038, share part of the same haplotype for the critical region (▨), suggesting that the c.4004-5T→C variant represents a founder variant in The Netherlands. *Question marks*: markers not tested or not scored.

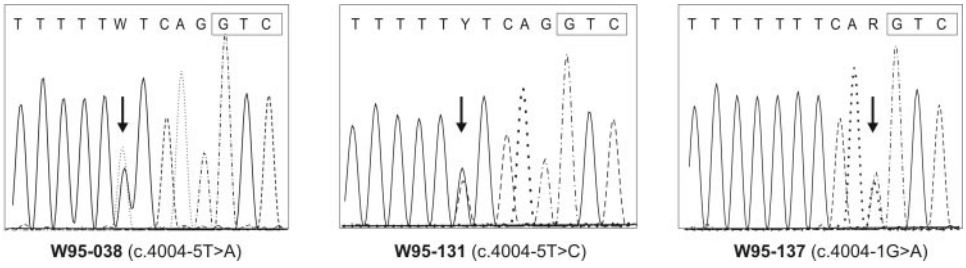

FIGURE 4. *CSPG2/Versican* sequence variants at the intron 7/exon 8 junction identified in patients with Wagner disease. Chromatograms showing the sequence variants (*arrows*). *Box* around GTC: 5′ end of exon 8. PCR and sequencing primers are depicted in Supplementary Table S1, http://www.iovs.org/cgi/content/full/47/8/3565/DC1.

carrying the c.4004-1G→A change, as well as the two Chinese patients with Wagner disease (W05-282P1 and -P2), showed the highest upregulation of V2 and V3. The V0 and V1 isoforms showed a tendency to be downregulated (Table 4).

DISCUSSION

In this study, we narrowed the critical region for Wagner disease to a 850-kb interval on 5q14.3 and also identified a founder haplotype in four large Dutch families with Wagner disease which, to our surprise, was also present in a family with ERVR. Affected ancestors of these five families were traced to the same (southern/middle) geographical region of The Netherlands. These families harbor >90% of all Dutch patients with Wagner disease. On identification of a *CSPG2/Versican* intron 7 sequence variant (c.4004-5T→C) in one of the probands we showed that this variant was present in all affected individuals of these four families with Wagner disease and the ERVR family. In addition, we found c.4004-5T→A and c.4004-1G→A variants in two unrelated families with Wagner disease.

Detailed RNA analysis of patients from these families, as well as from a Chinese Wagner disease family in which a sequence variant was not yet found, revealed two putatively important effects on *CSPG2/Versican* mRNA isoforms. First, we identified a 39-nt deletion in mRNA isoform V0 in patients carrying c.4004-1G→A (W95-137) and c.4004-5T→A (W95-038) variants. The same 39-nt deletion was found previously in a Japanese family with Wagner disease, carrying a c.4004-2A→G mutation.[6] Because the deleted 13 amino acids of V0 does not harbor a GAG attachment site, this truncation is likely to have little or no effect on the function of the protein, although changes on secondary or tertiary structure cannot be ruled out. The c.4004-5T→C variant in the four Dutch families with Wagner disease and the ERVR family and the unknown mutation in the Chinese family were not associated with a clear-cut structural effect on the V0 isoform. However, we assume that all the identified intron 7 variants lead to a decrease of isoforms V0 and V1. Possibly, patients with the c.4004-5T→C variant harbor an additional, as yet unidentified, intron 7 sequence variant that contributes to the skipping of exon 8. The reduction in mRNA isoforms V0 and V1 is difficult to quantify, but is probably reflected in the second effect we observed on *CSPG2/Versican* mRNA, a significant increase in isoforms V2 (>38-fold) and V3 (>12-fold), which have five to eight or no GAG side chains, respectively.

Given the relatively low V2 and V3 splice isoform abundances in normal blood samples (Table 2), even a small decrease in the V1 isoform is predicted to have a dramatic effect on the expression of the V2 and V3 isoforms (Table 3). In view of the unknown absolute levels of CSPG2/Versican isoforms in different compartments of the human eye, it is difficult to predict the functional effect of the observed mRNA isoform changes. Recently, Zhao and Russell reported on the expression of *CSPG2/Versican* in human trabecular meshwork and ciliary muscle with V1, V0, V3, and V2 in a decreasing order of expression levels,[16] which is similar to the expression levels we found in peripheral blood and skin fibroblasts. Of note, as described in the ocular phenotype section, the patients with ERVR clearly had a more severe eye phenotype than did patients with Wagner disease, which suggests that other genetic factors play a role. A more severe retinal phenotype in patients

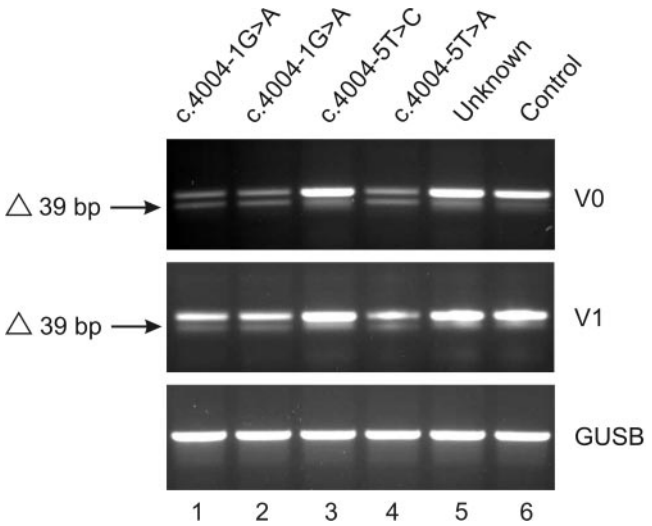

FIGURE 5. RT-PCR results for the V0 and V1 *CSPG2/Versican* splice variants in patients with Wagner disease. Total RNAs were isolated within 4 hours after venopuncture, except for patient W95-137P1 (*lane 1*), for which RNA was isolated from cultured fibroblasts. *Lanes 1* and *2*: patient W95-137P1; *lane 3*: patient W95-131P1; *lane 4*: patient W95-038P1; *lane 5*: patient W05-282P1; and *lane 6*: control. The V0 variant in patients W95-137P1 and W95-038P1 (*lanes 1*, *2*, and *4*) shows approximately equal quantities of wild-type and 39-nt deleted cDNA fragments; patients W05-282P1 and W95-131P1 (*lanes 3* and *5*) show no or almost no deleted V0 cDNA. This effect is faintly visible for splice variant V1.

TABLE 2. Real-Time Quantitative mRNA Analysis of *CSPG2/Versican* in Eight Control Samples

		CSPG2/Versican Splice Variant			
Control Individual	**V_t**	**V0**	**V1**	**V2**	**V3**
1	3.8	−0.9	3.4	−10.1	−4.0
2	2.9	−2.2	2.7	−10.8	−4.2
3	2.8	−2.5	2.3	−11.0	−3.5
4	3.0	−1.9	2.6	−11.7	−3.8
5	3.1	−1.4	3.0	−11.2	−3.4
6	3.9	−1.3	3.4	−9.9	−2.8
7	3.3	−1.4	3.0	−10.2	−4.0
8	3.2	−1.3	2.9	−10.5	−5.0
Mean	3.2	−1.6	2.9	−10.7	−3.8
Standard deviation	0.42	0.52	0.38	0.62	0.66

ΔCt ($Ct_{GUSB} - Ct_{CSPG2}$) for *CSPG2/Versican* total mRNA (V_t) and splice variants (V0–V3).

TABLE 3. Real-Time Quantitative mRNA Analysis of *CSPG2/Versican* in Patients with Wagner Disease and Erosive Vitreoretinopathy

Patient	Sequence Variant	V_t	*CSPG2/Versican* Splice Variant			
			V0	V1	V2	V3
W95-038P1	c.4004-5T→A	2.3	−2.3	1.7	−5.0	0.0
W95-038P2	c.4004-5T→A	2.9	−1.9	2.0	−5.2	0.0
W95-124P1	c.4004-5T→C	2.2	−1.6	2.3	−5.4	−0.1
W95-124P2	c.4004-5T→C	3.2	−0.6	2.9	−4.5	0.2
W95-131P1	c.4004-5T→C	2.9	−1.9	2.3	−5.2	−0.2
W95-131P2	c.4004-5T→C	3.2	−2.0	2.7	−5.4	0.1
W95-137P1	c.4004-1G→A	3.0	−1.9	2.3	−3.6	0.7
W95-137P2	c.4004-1G→A	3.4	−2.0	2.0	−2.6	1.3
W05-282P1	Unknown	3.6	−1.8	2.3	−1.8	1.9
W05-282P2	Unknown	3.7	−1.5	2.2	−1.9	1.4

ΔCt ($Ct_{GUSB} - Ct_{CSPG2}$) for *CSPG2/Versican* total mRNA (V_t) and splice variants (V0–V3) in the patients with Wagner disease or erosive vitreoretinopathy. All the samples are from families with Wagner disease except W95-124, which is the family with erosive vitreoretinopathy.

with ERVR is not reflected in the QPCR results of patients with ERVR (W95-124P1 and -P2) versus Wagner disease patients with the same intron 7 variant (W95-131P1 and -P2; Table 4), as no consistent difference in upregulation of the V2 and V3 isoforms was observed. We hypothesize that insufficient quantities of isoforms V0 and V1 may underlie both Wagner disease and ERVR. Whether subtle quantitative differences of V0 and/or V1 isoform can explain the difference in clinical severity of Wagner disease and ERVR remains to be investigated. The method used is not accurate enough to quantitate these subtle differences. Possibly, expression levels of the normal *CSPG2/Versican* gene also play a role.

Natural variation of *CSPG2/Versican* isoform expression may also explain the relatively high upregulation we identified in the Chinese family. If the normal expression of *CSPG2/Versican* isoforms V0 and V1 in the Asian population is higher than in the white population, or if isoforms V2 and V3 expression levels are lower in the Asian population than in the white population, disruption of V0 and V1 splicing would lead to higher relative upregulation of V2 and V3, as we observed. We propose that the causal variant resides in intron 7 or 8 causing similar balance shifts as found in the Dutch families with Wagner disease. The efforts to identify such variants are currently ongoing.

The absence of disease-causing mutations in the sizeable ORF (10,200 bp) of *CSPG2/Versican* in the families included in this study and in at least five other unrelated patients with Wagner disease (Black GCM, unpublished results, 1999), the conspicuous clustering of putative causal variants in the splice acceptor site of intron 7 found in this study and in the previously reported Japanese family,[6] and the consistent V2 and V3 mRNA isoform upregulation lead us to believe that Wagner disease and ERVR are caused by an imbalance of the *CSPG2/Versican* isoforms, mediated by intronic variants. It is estimated that as much as 15% of point mutations causing human diseases affect the canonical splice sites,[17] but this may be an underestimate. In most cases, the invariant AG or GT sequences of the 3′ and 5′ splice sites are mutated. The polypyrimidine stretch of 3′ splice sites often harbor benign sequence variants. However, in a family with X-linked mental retardation, an −11C→T change of intron 1 of the *ARHGEF6* gene resulted in skipping of the second exon.[18] To the best of our knowledge, the only other inherited disorder that is exclusively caused by intronic mutations and exon skipping is autosomal dominant sensorineural deafness type 5.[19]

In conclusion, in this study the pathologic role of *CSPG2/Versican* mutations in Wagner disease has been significantly strengthened. We provide strong evidence that, in our cohort

TABLE 4. Relative Quantities ($2^{\Delta\Delta Ct}$) of *CSPG2/Versican* mRNA Splice Variants in Patients with Wagner Disease or Erosive Vitreoretinopathy Compared with Control Subjects

Patient	Sequence Variant	V_t	*CSPG2/Versican* Splice Variant			
			V0	V1	V2	V3
W95-038P1	c.4004-5T→A	0.54	0.62	0.44	52.0*	13.9*
W95-038P2	c.4004-5T→A	0.81	0.81	0.54	45.3*	13.9*
W95-124P1	c.4004-5T→C	0.49	1.02	0.64	38.3*	12.8*
W95-124P2	c.4004-5T→C	1.0	1.95	0.98	71.2*	16.3*
W95-131P1	c.4004-5T→C	0.81	0.81	0.66	45.3*	12.1*
W95-131P2	c.4004-5T→C	1.00	0.76	0.87	39.4*	14.9*
W95-137P1	c.4004-1G→A	0.87	0.81	0.66	137.2*	22.6*
W95-137P2	c.4004-1G→A	1.15	0.76	0.54	274.4*	34.3*
W05-282P1	Unknown	1.32	0.87	0.66	477.7*	52.0*
W05-282P2	Unknown	1.41	1.07	0.62	445.7*	36.8*

$2^{\Delta\Delta Ct}$ represents the *x*-fold change of the respective splice variant in the patient compared with the mean expression level of the splice variant in eight healthy control subjects. ($\Delta\Delta Ct = \Delta Ct_{patient} - \Delta Ct_{control\ mean}$).

* $P < 0.0001$. The probabilities were calculated in *P*-value calculator (http://www.graphpad.com/quickcalcs/PValue1.cfm) from the *Z* value, based on the standard deviations obtained from the control group (Table 2). All the samples are from families with Wagner disease except W95-124, which is the family with erosive vitreoretinopathy.

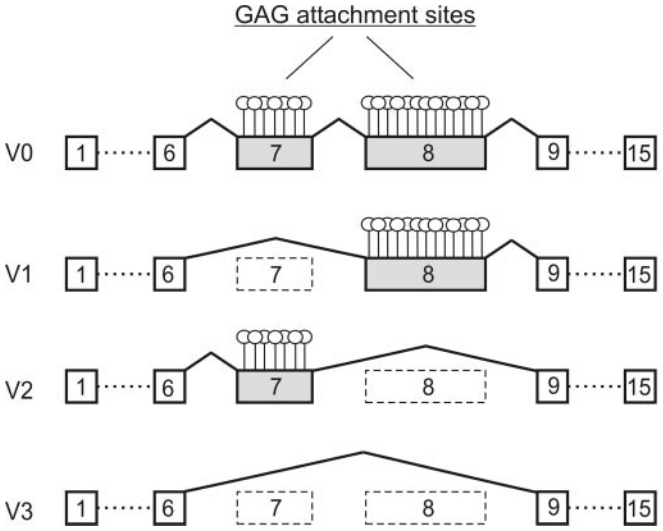

FIGURE 6. *CSPG2/Versican* splice variants and their relative quantities in healthy individuals versus patients with Wagner disease. The four major isoforms are schematically depicted. The skipping of exon 7 and/or exon 8 are shown as appropriate. The constitutive used exons 2 to 5 and 10 to 14 are not shown. The GAG attachment sites on exons 7 and 8 are indicated. At the *right*, the relative expression levels of different isoforms are shown for normal individuals versus patients with Wagner disease. Please note that for each isoform a different scale was used for normalization. The question marks indicate that the exact reductions for V0 and V1 could not be determined.

of patients, Wagner disease is associated with *CSPG2/Versican* intron 7 variants which result in a 39-nt truncation of isoform V0 in at least two of seven families. In addition, we show that Wagner disease and ERVR are allelic disorders and that they can be associated with the same intron7–exon 8 splice site variant. The intron 7 variants consistently result in a dramatic upregulation of the V2 and V3 isoforms, most likely due to the suppression of the V0 and V1 mRNA isoforms (Fig. 6). Both a V2/V3 overexpression model or a partial V0/V1 loss-of-function model could underlie Wagner disease and ERVR, though we favor the latter model. The pathogenic mechanism of the disease likely involves a reduction of chondroitin sulfate side chains of CSPG2/Versican. It can be predicted that other Wagner disease mutations can be found deep in intronic regions, most likely introns 7 (14,697 nt) or 8 (3,268 nt). Hence, Wagner disease and ERVR seem to belong to a largely overlooked group of diseases that are caused by a novel disease mechanism based on mRNA isoform balance shifts. Recent studies showed how splicing modulation may restore the function of the cystic fibrosis transmembrane conductance regulator.[20] If such an approach becomes feasible in a therapeutic setting, Wagner disease may be amenable to this treatment.

Note Added in Proof

During the revision of this manuscript, the *CSPG2/Versican* defect in the original Wagner disease was published in: Kloeckener-Gruissem B, Bartholdi D, Abdou MT, Zimmermann DR, Berger W. Identification of the genetic defect in the original Wagner syndrome family. *Mol Vis.* 2006;12:350–355.

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