

Evaluation of the Gene Encoding the Tissue Inhibitor of Metalloproteinases-3 in Various Maculopathies

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Purpose. Mutations in the gene encoding the tissue inhibitor of metalloproteinases-3 (TIMP3) have been shown previously to cause Sorsby's fundus dystrophy, an autosomal-dominant disorder characterized by extracellular matrix irregularities in Bruch's membrane. To assess the involvement of TIMP3 in a variety of other macular dystrophies, the authors have screened this gene for disease-causing mutations in age-related macular degeneration (AMD), adult vitelliform macular dystrophy (AVMD), central areolar choroidal dystrophy (CACD), syndrome-associated macular dystrophies, cone-rod dystrophy, and a group with unspecified macular degeneration.

Methods. Single-stranded conformational analysis of the entire coding region was performed using the polymerase chain reaction and oligonucleotide primers flanking the five exons of the TIMP3 gene as well as the putative promotor region and a highly conserved fragment of the 3'-untranslated region. The authors analyzed a total of 217 patients, including 143 patients with AMD, 28 patients with AVMD, 21 patients with CACD, and 25 patients with other forms of macular dystrophy.

Results. In the 217 patients analyzed, the authors have identified one sequence alteration (a G-to-C base change) in the 5'-untranslated region in a patient with AMD. However, the functional consequences of this mutation are not clear. No other disease-causing mutations were found. The authors have characterized a frequent intragenic polymorphism in exon 3 of the TIMP3 gene (heterozygosity = 0.57) that will be useful for genetic linkage or allele sharing analyses or both.

Conclusions. The authors' results suggest that TIMP3 is not a major factor in the cause of AMD, AVMD, and CACD. Thus far, Sorsby's fundus dystrophy appears to be the only phenotype known to be associated with mutations in TIMP3. *Invest Ophthalmol Vis Sci.* 1997;38:1054-1059.

Despite the continuous success in the mapping of macular dystrophies to specific subchromosomal regions, thus far only two genes, peripherin-retinal degeneration slow (RDS) and the tissue inhibitor of metalloproteinases-3 (TIMP3), have been identified and shown to be directly involved in the molecular pathogenesis of some forms of macular degeneration.

Originally, the peripherin-RDS gene was shown

to be the cause of a slow form of retinal degeneration in the RDS mouse.¹ Subsequently, mutations in the homologous human gene were found to be responsible for a variety of retinal degenerations presenting with a broad spectrum of clinical phenotypes, including autosomal-dominant retinitis pigmentosa,² retinitis punctata albescens,³ adult vitelliform macular dystrophy (AVMD),⁴ macular dystrophy,⁴ and pattern dystrophy of the fovea.⁵ The striking clinical heterogeneity associated with mutations in the peripherin-RDS gene was further underscored by the finding of a single amino acid deletion at codon 153/154 in a family in which affected members presented either with retinitis pigmentosa, pattern dystrophy, or fundus flavimaculatus.⁶ At present, the mechanisms underlying the extensive clinical heterogeneity as well as the

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TABLE 1. Number of Unrelated Patients Analyzed in TIMP3 According to Diagnosis

<i>Diagnosis</i>	<i>Number of Patients</i>	<i>Patients With Positive Family History</i>	<i>Mode of Inheritance</i>
Age-related macular degeneration	143	12	Unclear*
Adult vitelliform macular dystrophy	28	3	AD
Central areolar choroidal dystrophy	21	3	AD
Unclassified macular degeneration†	17	8	AD
Syndromic forms‡	5	5	AD
Cone-rod degeneration	3	3	AD
Total	217	34	

TIMP3 = metalloproteinases-3; AD = autosomal dominant.

* In four cases, an additional sibling was affected. In eight cases, the father or mother were affected.

† Patients had unclear chorioretinal dystrophies.

‡ Including patients with various retinopathies in addition to other phenotypic features.

molecular pathology of the various peripherin-RDS mutations are not well understood.

One member of the family of tissue inhibitors of metalloproteinases, TIMP3, was identified as the disease-causing gene in Sorsby's fundus dystrophy (SFD)⁷ based on its chromosomal localization to 22q13.1^{8,9} and its known functional properties in the homeostasis of the extracellular matrix (ECM).¹⁰ The ECM irregularities in Bruch's membrane appear to be one of the key features in SFD¹¹ and precede loss of central vision caused by choroidal neovascularization or geographic atrophy.¹²

Primarily two considerations have prompted us to investigate the possible involvement of TIMP3 in the pathogenesis of a variety of macular dystrophies. First, some phenotypic similarities at the level of the RPE and Bruch's membrane, in particular between age-related macular degeneration (AMD) and SFD, have

been noted frequently.^{13,14} Second, allelic TIMP3 mutations may be responsible for various forms of macular degeneration similar to the observation of extensive clinical heterogeneity associated with peripherin-RDS mutations.

MATERIALS AND METHODS

Recruitment of Patients

Ten-milliliter ethylenediaminetetraacetic acid blood samples were obtained from 217 unrelated outpatients of primarily German descent, including 143 patients with AMD, 28 with AVMD, 21 with central areolar choroidal dystrophy, and 25 with other forms of macular dystrophies (Table 1). The study was conducted in accordance with the rules and regulations of the local ethics committee and approval was granted. The ten-

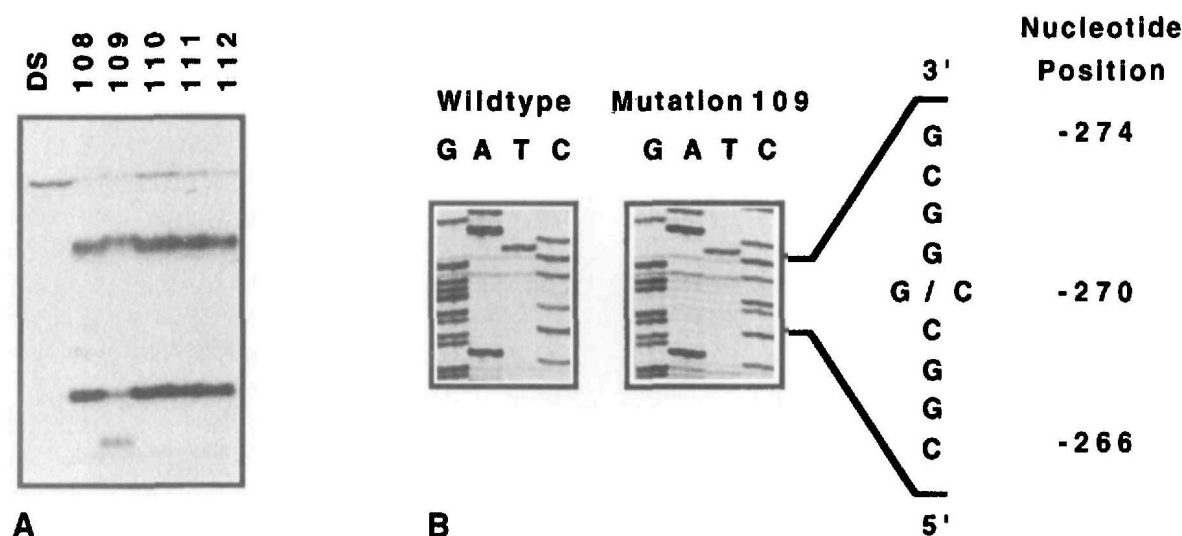


FIGURE 1. (A) Single-stranded conformational analysis of the 5'-untranslated region fragment showing a heterozygous mobility shift in age-related macular degeneration (patient 109). (B) Sequencing of the mutant allele shows a G-to-C transversion at position-270 (numbering starts with +1 at the first base of the translation initiation codon ATG).

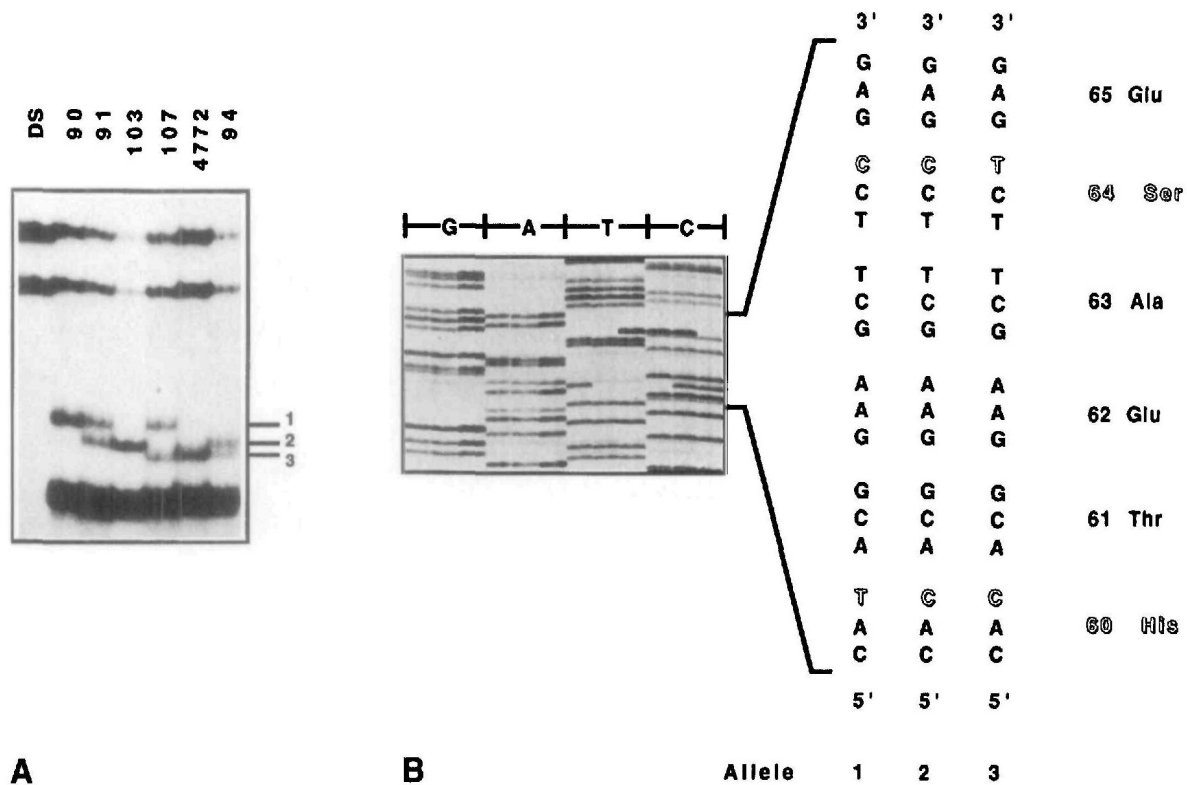


FIGURE 2. (A) Single-stranded conformational analysis of exon 3 of the metalloproteinase-3 (TIMP3) gene showing three mobility shifts (alleles 1, 2, and 3), each in the homozygous (patients 90, 103, and 4772) and heterozygous (patients 91, 107, and 94) form. DS = nondenatured double strand control. (B) Sequence analysis of the allelic variants identified in exon 3. The T-to-C transition in codon 60 (corresponding to allele 2) and the C-to-T transition in codon 64, in addition to the codon 60 alteration (corresponding to allele 3), occur in the third base positions, respectively, and do not affect the codon specificities.

ets of the Declaration of Helsinki were followed. All patients were informed of the purpose of the study, and written consent was obtained. In total, 34 patients disclosed a positive family history, and ophthalmologic examination of at least one relative of each patient was performed (Table 1). The ophthalmoscopic diagnosis was confirmed by fluorescein and indocyanine green angiography in most cases. In the AMD group, the average age of the patients was 69.7 years (range, 48 to 91 years). Approximately 16% of the patients with AMD had early nonexudative signs (RPE changes, soft confluent drusen) or geographic atrophy, whereas 84% had progressed to an exudative stage of the disease, which was bilateral in 49. All 28 AVMD probands analyzed in this study had been tested in the peripherin-RDS gene, and five presumably pathogenic alterations were identified in patients with no known family history of AVMD.¹⁵

Mutational Analysis

The DNA was isolated from leukocyte nuclei by standard extraction methods. Based on the genomic exon-intron sequences of the TIMP3 gene,¹⁶ oligonucleotide primers were designed to polymerase chain reaction (PCR) amplify the five coding exons of

TIMP3 as well as a 516-bp fragment, including part of the 5'-untranslated region (UTR) and the potential promoter region. We also examined an evolutionarily conserved 130-bp fragment within the 3'-UTR that shows a significant sequence identity among chicken, mouse, and human TIMP3.¹⁷ The oligonucleotide primer sequences as well as the conditions for the PCR amplification of the five coding exons have been published elsewhere.¹⁸ The 5'-UTR fragment was assessed using primers PR2-F (5'-AGG GGT AGC AGT TAG CAT TC-3') and PR1-R (5'-AGG AGG AGG AGA AGC CGT C-3') and an annealing temperature of 56°C. The 3'-UTR fragment was amplified using oligonucleotide primers 3'-TIMP-F (5'-ACC TCA CTT CCC TCC CTT C-3') and 3'-TIMP-R (5'-GAC AGC ATA GAC CTT TCT TTA A-3') and an annealing temperature of 57°C.

To increase sensitivity of the single-stranded conformational analysis,¹⁹ the PCR products of exons 1 to 5 were digested with appropriate restriction enzymes that generated DNA fragments ranging in size between 65 bp and 172 bp.¹⁸ The 516-bp PCR fragment was digested with *Sma* I, yielding restriction fragments of 228 bp, 194 bp, and 94 bp in size. Five microliters of the 1:5 diluted samples subsequently were

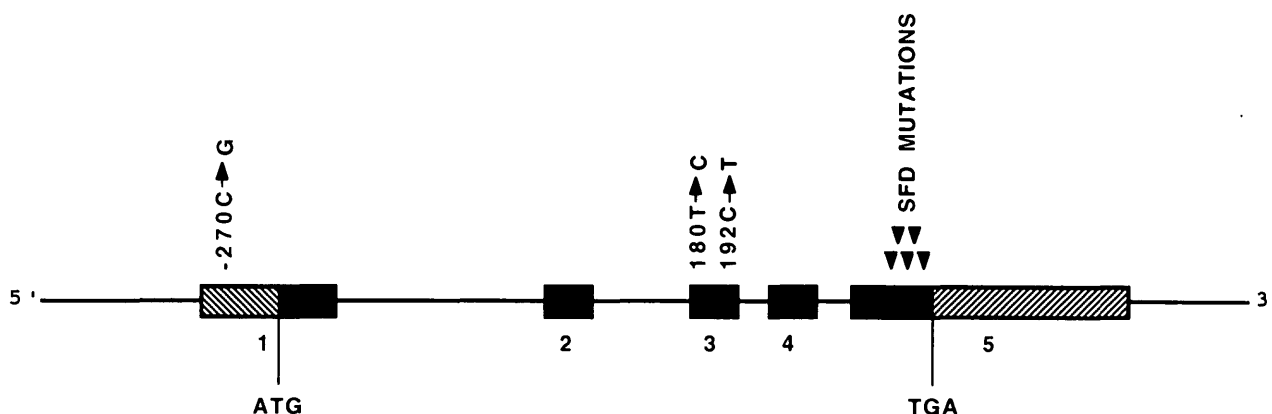


FIGURE 3. Location of sequence alterations within the metalloproteinase-3 (TIMP3) gene. Exons are indicated by solid boxes with the respective numbers below. Introns are shown by thin lines. The varying size of exon 5 is indicated by a hatched box downstream the termination codon, TGA. The G-to-C transversion identified in age-related macular degeneration (patient 109) is located 270 nucleotides upstream of the ATG initiation codon within the 5'-untranslated region (hatched box). The polymorphic alterations T₁₈₀→C and C₁₉₂→T (Fig. 2) were identified in exon 3. For completeness, the known Sorsby's fundus dystrophy mutations are indicated in exon 5 (arrowheads).^{7,18,25-27}

added to 95% formamide, 5 mM sodium hydroxide, 0.1% xylene cyanol, and 0.1% bromophenol blue. The samples were heat-denatured for 3 minutes, immediately placed on ice, and separated electrophoretically at 4°C in 6% nondenaturing polyacrylamide gels that were run at two conditions, one with and one without 5% glycerol.

The PCR products corresponding to altered mobility shifts were cloned into the cloning kit (pCR II vector; TA Cloning Kit; Invitrogen, Leek, The Netherlands). Plasmid DNA of recombinant clones was isolated by the alkaline lysis method²⁰ and sequenced using the Sequenase Version 2.0 Sequencing Kit (United States Biochemical, Cleveland, OH) and primers flanking the cloning site (M13-5: 5'-CGC CAG GGT TTT CCC AGT CAC GAC-3' and M13-6: 5'-AGC GGA TAA CAA TTT CAC ACA GGA-3') as given in the manufacturer's protocol.

RESULTS

Single-stranded conformational analysis was used to assess the TIMP3 gene in a total of 217 patients affected with various macular dystrophies (Table 1).

A mobility shift was seen in the 5'-UTR fragment in one patient (patient 109) who had an exudative form of AMD in her left eye at the age of 82 years (Fig. 1A). Sequence analysis showed that the mobility shift was caused by a heterozygous G-to-C transversion at position -270 upstream of the first base of the initiation codon antithymocyte globulin (numbering according to material published elsewhere)¹⁶ (Fig. 1B). This alteration introduced a novel *Eag*I endonu-

lease restriction site that was used to establish a simple enzymatic assay to screen for the mutational change in the remaining 216 patients. Although *Eag*I cleaves a 516-bp PCR product into two fragments of 445 bp and 71 bp in the wild-type allele, digestion of the mutant allele results in three fragments of 445 bp, 44 bp, and 27 bp. The G-to-C transversion was found exclusively in patient 109.

Single-stranded conformational analysis of exons 1, 2, 4, 5, and the 3'-UTR fragment of the TIMP3 gene showed no further band shifts in the 217 patients studied.

In exon 3, we identified polymorphic mobility shifts representing three different alleles (Fig. 2A). Sequencing of the respective homozygous alleles showed a silent T-to-C transition in the third position of codon 60 and a silent C-to-T transition in the third position of codon 64 (Fig. 2B). Combinations of the two sequence alterations give rise to the three observed mobility shifts with *CAT* (codon 60). . . *TCC* (codon 64) representing allele 1, *CAC*. . . *TCC* representing allele 2, and *CAC*. . . *TCT* representing allele 3 (Fig. 2A). The fourth possible combination *CAT*. . . *TCT* (allele 4) was not found in our sample. The frequencies of the three alleles were determined separately for each subgroup of patients (Table 2) but did not differ significantly ($\chi^2 = 8.3$ [df = 6]; $P = 0.22$).

DISCUSSION

Two considerations have led us to analyze the tissue inhibitor of metalloproteinases-3 in a variety of macu-

TABLE 2. Frequencies of Polymorphic Alterations in TIMP3 Exon 3 Listed Independently for the Various Patient Subgroups

Subgroup of Patients	Haplotype Codon 60. . . Codon 64	Number of Haplotypes Analyzed	Frequency*
AMD (<i>n</i> = 143)	1†	143	0.5
	2‡	126	0.44
	3§	17	0.06
AVMD (<i>n</i> = 28)	1	27	0.48
	2	24	0.43
	3	5	0.09
CACD (<i>n</i> = 21)	1	19	0.45
	2	16	0.38
	3	7	0.17
Others (<i>n</i> = 25)	1	19	0.38
	2	26	0.52
	3	5	0.1
Total (<i>n</i> = 217)	1	208	0.48
	2	192	0.44
	3	34	0.08

TIMP3 = metalloproteinases-3; AMD = age-related macular degeneration; AVMD = adult vitelliform macular dystrophy; CACD = central areolar choroidal dystrophy.

* No significant differences in allele frequencies between subgroups [$\chi^2 = 8.3$ (*df* = 6); *P* = 0.22].

† CAT. . . TCC (cDNA sequence according to Apte et al, 1994).

‡ CAC. . . TCC.

§ CAC. . . TCT.

lar dystrophies. First, mutations in TIMP3 have been shown to cause SFD, an autosomal-dominant disorder of the macula with onset in the third or fourth decade of life.⁷ In the early stages, SFD is characterized by an abnormal accumulation of yellowish material within the inner part of Bruch's membrane¹¹ that can be seen clinically as yellow dots or drusen. Later in life, sudden loss of central vision may occur either because of choroidal neovascularization with subretinal hemorrhage and the development of a disciform scar or, in the minority of patients, because of atrophic changes.¹² At present, the physiological consequences of a dysfunctional TIMP3 in the pathogenesis of SFD remain unclear, although the abnormal deposition of the lipofuscin-like material within Bruch's membrane may be a direct consequence of an imbalance in the TIMP3-matrix metalloproteinase system. Nevertheless, some early morphologic similarities primarily in the ECM between SFD and other maculopathies, such as AMD and AVMD, raise the possibility of a common pathogenic pathway directly or indirectly involving TIMP3.

Second, genetic heterogeneity seems to be a common feature in retinal dystrophies. For example, mutations in the genes for the α - and the β -subunits of the cyclic guanosine monophosphate phosphodiesterase^{21,22} as well as the α -subunit of the rod cyclic guano-

sine monophosphate-gated channel²³ can cause autosomal-recessive RP. In addition, mutations in a single gene, such as peripherin-RDS, have been shown to result in a wide range of phenotypes, including features of retinitis pigmentosa, retinitis punctata albescens, macular degeneration, or fundus flavimaculatus.²⁴

To date, mutations in the TIMP3 gene have been associated exclusively with the SFD phenotype.^{7,18,25,26} It appears that only a single type of mutation affecting the C-terminus of the protein causes the clinical features of SFD, because all known mutations have been identified in exon 5 of the TIMP3 gene and lead to an additional cysteine residue in the mature protein (Fig. 3). This raises the possibility that another type of mutation in TIMP3 qualitatively different from the known one could result in a clearly distinct phenotype.

Our extensive mutational analyses have shown only a single base change in one patient with AMD (Fig. 1). At present, the consequences of this alteration within the 5'-UTR of the TIMP3 gene are unknown, particularly because mRNA expression cannot be tested as a result of the unavailability of additional blood samples. Therefore, it cannot be ruled out that this alteration represents a rare but silent polymorphism. Because no other changes were identified either in the putative promotor region nor in the coding sequences nor in the evolutionarily highly conserved 3'-UTR, we conclude that mutations in the TIMP3 gene are not a major factor in the cause of a significant portion of AMD, AVMD, and central areolar choroidal dystrophy. However, our findings do not rule out the possibility that other genes or gene products essential for the homeostasis of the extracellular matrix (e.g., other metalloproteinases or their inhibitors) may participate in the pathogenesis of macular degeneration. The growing understanding of the biochemical processes underlying ECM metabolism and the increasing knowledge of the genes involved will make it feasible to evaluate further the role of the ECM in the cause of macular degenerations.

Key Words

extracellular matrix, macular degeneration, retinal pigment epithelium, Sorsby's fundus dystrophy, TIMP3

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