

Matrix Metalloproteinase-2 Is Expressed in Melanoma-Associated Spongiform Scleropathy

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PURPOSE. To correlate the expression of matrix metalloproteinases (MMPs) with melanoma-associated spongiform scleropathy (MASS) and scleral tumor invasion in eyes with uveal melanoma.

METHODS. Eleven specimens with MASS and 11 eyes without MASS were investigated. Sections were examined for MMP-1, -2, -9, and -13 mRNA expression by in situ hybridization with ³⁵S-radiolabeled riboprobes. Immunohistochemical studies of the same specimens were conducted with MMP-2-specific antibodies. For double-labeling experiments, primary MMP-2-specific antibodies and antibodies binding to fibroblasts and macrophages were used.

RESULTS. MMP-2 mRNA expression was detected in 10 (91%) of 11 eyes with MASS and scleral tumor invasion. In eight (73%) of these cases, the expression signals were seen in numerous scleral fibroblasts. In melanoma cases without MASS, MMP-2 mRNA expression was detected in four (36%) cases, and only one (9%) showed numerous positive cells. Tumor-infiltrating macrophages were found to harbor MMP-2, shown by a double-labeling experiment. The MMP-2 expression by immunostaining coincides with MMP-2 expression by in situ hybridization. No MMP-2 expression was detected in the tumor cells.

CONCLUSIONS. MASS is considered a tumor-induced scleral degradation process. There is a significantly higher expression of MMP-2 in MASS-positive areas, indicating that MMP-2 is involved in the development of MASS and that MMP-2 is produced by scleral fibroblasts under the influence of tumor cells and/or tumor-infiltrating macrophages. These changes may represent a step in the invasion of uveal melanoma by facilitating the spread of tumor cells through the sclera. (*Invest Ophthalmol Vis Sci.* 2008;49:2806–2811) DOI:10.1167/iovs.07-1436

The functional integrity of the sclera plays a major role as a mechanical barrier against the direct spread of intraocular tumors. This integrity is created by fibroblasts embedded in a matrix of densely packed collagen bundles.^{1,2} Degradation or

remodeling of scleral collagen is an essential step in melanoma invasion and metastasis.

Different proteolytic enzymes, including matrix metalloproteinases (MMPs), are involved in this degradation process. The role of MMPs in tumor progression and invasion has been well established.^{3,4} Proteolytic enzymes have been reported to be released from stromal fibroblasts in the vicinity of the tumor under the influence of tumor cells, from tumor-infiltrating macrophages, or from the tumor cells themselves.^{5–8} Little is known about the MMP profile of uveal melanoma, but MMP-2, and to a lesser extent MMP-9, have been reported to be associated with the invasion of uveal melanoma and to correlate with a poor prognosis.^{9–12}

Melanoma-associated spongiform scleropathy (MASS) has been characterized and described by light microscopy as areas within the sclera adjacent to a choroidal-ciliary body melanoma where collagen bundles appear disintegrated into loose fibers¹³ (Fig. 1A). A significantly higher incidence of MASS has been reported in eyes with scleral tumor invasion.¹⁴

We performed a biochemical analysis of scleral tissue with MASS. The results of the analysis showed a significant reduction in collagen content manifesting as decreased amino acids and total proteins, indicating a collagen degradation process.¹⁴ MASS could therefore be tumor-induced matrix changes caused by MMPs secreted by tumor or peritumor stromal cells. In the present study, we investigated the expression of MMP-1, -2, -9, and -13 in primary uveal melanoma.

We report the results of MMP-2 upregulation in scleral fibroblasts and tumor-infiltrating macrophages and correlate its expression to MASS and its possible role in tumor invasion.

MATERIALS AND METHODS

Twenty-two formalin-fixed and paraffin-embedded whole-eye specimens with primary choroidal melanoma were selected. Eleven specimens demonstrated MASS and tumor invasion into or through the sclera. Eleven specimens were without MASS changes and without tumor invasion of the sclera.

The tissue was obtained from the files of the Section of Eye Pathology, Institute of Neuroscience and Pharmacology, University of Copenhagen. All studies were performed with the informed consent of the subjects and were adherent to the tenets of the Declaration of Helsinki.

Methods

cDNA and In Vitro Transcription. A 180-bp human MMP-1 probe was generated by RT-PCR on cDNA from the monocytic U937 cell line (American Type Culture Collection, Manassas, VA) with a cDNA kit (ThermoScript; Invitrogen, Carlsbad, CA). One microgram of total RNA was used for each reaction with poly-T primers and amplified (AmpliTaq Gold; Applied Biosystems, Inc. [ABI], Foster City, CA). Amplification was performed by using primers according to Yamanaka et al.¹⁵ in an automated thermal cycler (model 2400; Perkin Elmer, Wellesley, MA) with a 5-minute denaturing step at 95°C followed by 30

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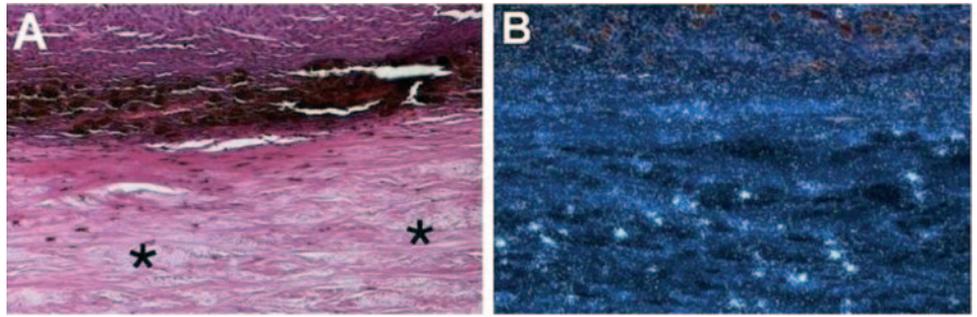
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FIGURE 1. A section of choroidal malignant melanoma and extensive scleral MASS changes showing MMP-2 mRNA expression in scleral fibroblasts visualized by dark-field illumination. (A) MASS changes (★) in the sclera adjacent to the choroidal melanoma. (B) Same area, showing MMP-2 expression by in situ hybridization in scleral fibroblasts seen as white dots with dark-field illumination. Magnification: (A) $\times 150$; (B) $\times 300$.



cycles of 30 seconds at 95°C, 45 seconds at 60°C, and 45 seconds at 72°C. The specific sequence of the MMP-1 cDNA (base pairs 191–370, GenBank accession number X90925; <http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) was confirmed by DNA sequencing (MWG Biotech, Risskov, Denmark).

Plasmids containing MMP-2 cDNA (pCol7201, base pairs 647–1284), MMP-9 cDNA (pCOL9201), and MMP-13 cDNA (phMMP13-730) have been described elsewhere.^{16,17} All plasmids were linearized before incorporation of ³⁵S-labeled UTP by in vitro transcription of sense and antisense strand for 2 to 3 hours with either T7 or Sp6 RNA polymerase.

In Situ Hybridization. Formalin fixed, paraffin-embedded eye specimens were sectioned (4 μ m) and mounted on slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). Before hybridization, the sections were treated with proteinase K (5 μ g/mL in 50 mM Tris, 5 mM EDTA and pH 8) and dried after dehydration. The probes were denatured at 80°C for 3 minutes before addition of the hybridization mix (1 \times SALTS, 40% deionized amide, 10% dextran sulfate, t-RNA [1 mg/mL], and 0.1 M DTT). The sections were incubated overnight at 55°C with the sense or antisense probes in high humidity. After hybridization, excess probe was removed by washing under increasing stringency at 55°C in 0.1% SDS and 10 mM DTT with 2 \times SSC, 1 \times SSC, and 0.2 \times SSC, and the sections were RNase A treated (20 μ g/mL in 1 NTE buffer) at 44°C before stringency washing and dehydration with 0.3 M ammonium acetate in ethanol. Sections were air dried and dipped in NTB2 emulsion (VWR Scientific, Herlev, Denmark) and exposed for 7 days. After development, slides were counterstained with hematoxylin, air dried, and coverslipped.

Hybridization signals from antisense probes were compared with sense probes obtained from the same plasmids. Sections of small intestine were used as the positive control.

Slides were examined for MMP mRNA expression by in situ hybridization under light- and dark-field illumination microscopy, with particular attention to areas of contact between the main bulk of the tumor and the sclera in areas with MASS changes (Fig. 1B).

Immunohistochemistry

Immunohistochemical staining for MMP-2 was performed with two different antibodies to human MMP-2. A mouse monoclonal antibody

to human MMP-2 (1:100, clone MAB 13431; Chemicon International Inc.-AH Diagnostics, Aarhus, Denmark), and rabbit polyclonal antibody to human MMP-2 raised against MMP-2 peptide (undiluted; catalog no. PA1 21074; Affinity Bioreagents-AH Diagnostics). For the negative control, slides were incubated with bovine serum albumin (catalog no. P-0834; Sigma-Aldrich, St. Louis, MO).

For immunoperoxidase staining, sections were deparaffinized, boiled for 20 minutes in a microwave oven in TE buffer (pH 9.5) for antigen retrieval, and incubated with 3% H₂O₂ for 8 minutes at room temperature, to inhibit endogenous peroxidase activity. The sections were incubated with MMP-2-specific antibodies and washed twice with PBS, and the primary antibodies were detected with biotinylated anti-mouse and anti-rabbit IgG, incubated with dilution of streptavidin peroxidase complex reagent, and finally visualized by the use of AEC chromogen (Dako Inc., Copenhagen, Denmark), according to the manufacturer's instructions.

For immunofluorescence double labeling, tissue sections were pre-treated as described and incubated for 30 minutes with rabbit polyclonal antibodies against MMP-2 (undiluted), together with a mouse monoclonal antibody against macrophages, CD68, (1:100) or together with a monoclonal antibody against vimentin (1:100; Dako Inc.).

Furthermore, immunofluorescence double labeling was performed for scleral fibroblasts by using mouse monoclonal antibody against MMP-2 (1:100) together with a rabbit polyclonal antibody against S100A4, which recognizes fibroblasts (1:100; Dako Inc.). The immunohistochemical signals were detected by using Cy 2-conjugated goat anti-mouse (Jackson ImmunoResearch, West Grove, PA) and rhodamine-red-conjugated goat anti-rabbit (Jackson ImmunoResearch). Sections were coverslipped with fluorescence-preserving mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence was visualized by a conventional fluorescence microscope (Diaphot 200; Nikon, Tokyo, Japan) equipped with rhodamine and FITC filter sets (Figs. 2, 3).

Evaluation

The expression of MMP-2 by in situ hybridization was scored in three categories: negative (–), no expression signals in the sclera (Fig. 4A); few (+), some scattered positive cells in the sclera (Fig. 4B); and numerous (++) , many positive cells in the sclera (Fig. 4C).

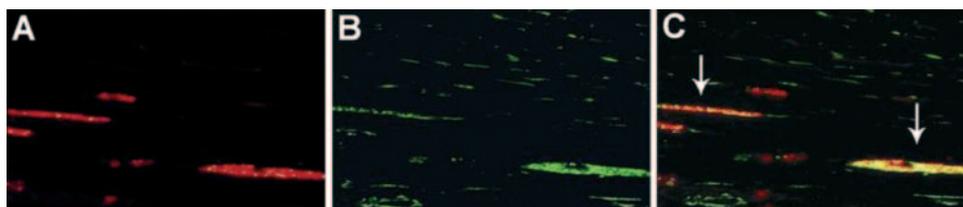


FIGURE 2. Double-immunofluorescence experiment showing colocalization between MMP-2 and vimentin in scleral fibroblasts. Cells stained with (A) the primary MMP-2-specific antibody (red) and (B) the fibroblast-specific antibody, anti-vimentin (green). (C) Colocalization between MMP-2- and vimentin-positive cells (yellow fluorescence). Magnification, $\times 600$.

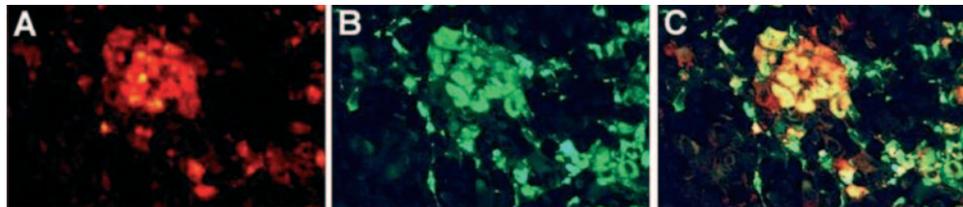


FIGURE 3. Double-immunofluorescence experiment showing colocalization between MMP-2 and CD68 in tumor macrophages. Cells stained with (A) the primary MMP-2-specific antibody and (B) the macrophage-specific antibody, anti-CD68 (green). (C) Colocalization between MMP-2 and CD68-positive cells (yellow fluorescence). Magnification, $\times 600$.

The immunostaining of both scleral fibroblasts and tumor macrophages was also scored in three categories: negative (–), no stained cells; few (+), 1 to 10 positive-stained cells per high-power field ($\times 400$); and numerous (++) , >10 cells per high-power field ($\times 400$).

Masked cross evaluation was conducted by three of the authors, and an average of the scores of the three authors was considered the final score. The Fisher exact test was used for statistical analysis and $P < 0.05$ was considered significant.

RESULTS

In Situ Hybridization

A distinct hybridization signal with MMP-2 antisense probes was scattered in the sclera adjacent to the tumor, mainly in areas of MASS changes. No signals were detected in the tumor cells.

MMP-2 mRNA positive signals were identified in the sclera, mainly in areas of MASS (Fig. 1B). Expression signals were detected in 10 (91%) of 11 eyes with MASS (Table 1). In eight (73%) of the 10 positive cases, expression signals were from numerous cells (++) in the sclera (Table 1; Fig. 4C). In the 11 eyes without MASS, MMP-2 mRNA expression signal was detected in four (36%) cases. Only one of these showed numerous positive (++) fibroblasts in the sclera (Table 1). No MMP-2 mRNA expression was detected in the main bulk of the tumors in any of the cases studied.

A potential positive signal in melanin-positive tumor cells and tumor-infiltrating macrophages was not possible to resolve by this technique, because the fields were masked by the heavy pigmentation of the tumors in most of the cases. However, nonpigmented and slightly pigmented tumor cells demonstrated no signals (Fig. 5B).

No hybridization signals were detected with the sense probe. In situ hybridization analyses for MMP-1, -9, and -13 revealed no specific signals in the sclera or the tumors in any of the cases.

Immunohistochemistry

Expression of MMP-2 immunostaining was seen in scleral fibroblasts and tumor infiltrating macrophages (Fig. 5). Similar staining results were detected with the two different MMP-2 antibodies used (MAB 13431 and PA1 21074). No staining was detected in sections incubated with the negative control.

The results of scoring of MMP-2 immunoreactivity of the two different antibodies in scleral fibroblasts were identical. This MMP-2 immunoreactivity was in agreement with the MMP-2 mRNA localization by in situ hybridization (Tables 1, 2).

To verify that cells harboring MMP-2 detected by immunoperoxidase staining were scleral fibroblasts, double-immunofluorescence analysis of three cases of MASS and positive MMP-2 mRNA expression was performed. Vimentin was used as the cell marker for fibroblasts. MMP-2 and vimentin were colocalized in the MMP-2-positive scleral cells (Fig. 2) verifying that these cells are fibroblasts. Similarly tumor infiltrating MMP-2-positive cells were shown to be macrophages by double-immunofluorescence analysis, since these cells showed colocalization with the macrophage-specific antibody CD68 (Fig. 3).

Positively stained macrophages were seen in 8 (73%) of 11 eyes with MASS (Table 3). In seven of these eight cases, the stained macrophages were numerous (++ ; Table 3). In the 11 eyes without MASS, positively stained macrophages were detected in five (45%) cases, and, in two of these cases, the MMP-2-positive macrophages were numerous (++ ; Table 3).

No MMP-2 staining was detected in tumor cells by immunoperoxidase (Fig. 5B) or immunofluorescence staining.

The results of in situ hybridization (Table 1) showing the expression of MMP-2 mRNA in scleral fibroblasts coincide with the results of immunostaining of scleral fibroblasts and tumor-infiltrating macrophages (Tables 2, 3). Both showed a significantly higher expression of MMP-2 in eyes with melanoma and MASS changes than in eyes without MASS ($P < 0.05$).

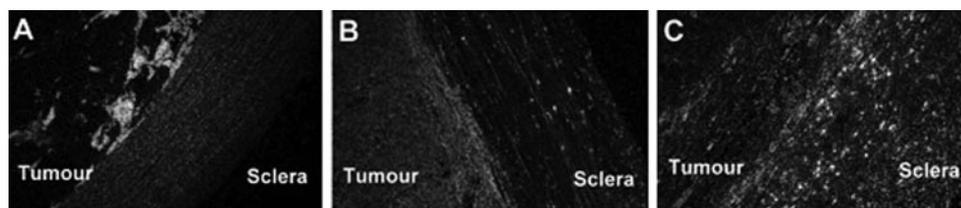


FIGURE 4. Expression of MMP-2 mRNA by in situ hybridization in sections of three different eyes with choroidal malignant melanoma. Sections were visualized by dark-field illumination. Examples of (A) no (–) hybridization signals in the sclera without MASS changes in an eye with uveal melanoma and (B) a few (+) scattered signals from scleral fibroblasts in an eye with MASS changes. (C) An example of numerous (++) signals from scleral fibroblasts in an eye with severe MASS changes. Magnification, $\times 50$.

TABLE 1. MMP-2 Expression in Scleral Fibroblasts Identified by In Situ Hybridization in Eyes with and without MASS Changes

Intensity of MMP-2 Expression	MASS ⁺ n (%)	MASS ⁻ n (%)	P
-	1 (9)	7 (64)	0.003
+	2 (18)	3 (27)	0.631
++	8 (73)	1 (9)	0.001

MASS⁺, eyes with MASS changes; MASS⁻, eyes without MASS changes; -, negative; +, few; ++, numerous.

DISCUSSION

The essential step in tumor invasion and metastasis is a degradation of the extracellular matrix. Such a degradation process is mainly conducted by proteolytic enzymes, among which MMPs play an important role.⁴

In our previous studies, we showed that sclera with MASS contains less collagen than sclera with a normal morphology.¹⁴ We found a significant reduction of the main amino acids of type 1 collagen and of total proteins.¹⁴

Recent studies have suggested an interaction between tumor cells, stromal fibroblasts, and inflammatory cells such as macrophages and monocytes in the invasion process of tumors. This interaction is mediated through MMP production.^{18,19}

The production of MMPs by fibroblasts in the extracellular matrix surrounding or in the vicinity of tumors have been demonstrated in several human carcinomas.²⁰⁻²² This production is induced either by humoral factors produced by tumor cells^{23,24} or by direct interaction between tumor cells and fibroblasts.^{25,26}

Among the MMP family, MMP-2 is the most frequently demonstrated enzyme expressed in melanomas.^{10,27,28} This MMP-2 expression is restricted to fibroblasts adjacent or close to invading tumor cells.^{22,29} Other studies have suggested that it is the interaction between tumor cells and fibroblasts that enhances fibroblast production of pro-MMP-2, which is activated by cancer cells.^{30,31} In both mechanisms, stromal fibroblasts could have the ability to synthesize a metalloproteinase that, together with stromal macrophages and neoplastic cells, may participate actively in extracellular matrix remodeling and disruption of the basement membrane's integrity, features that characterize tumor invasion.³²

Our findings demonstrated that MMP-2 is expressed in the vicinity of uveal melanoma. This expression is seen in scleral fibroblasts and tumor-infiltrating macrophages. We identified scleral fibroblasts based on their morphology, pattern of distribution, and immunocytochemical staining characteristics with

TABLE 2. MMP-2 Expression in Scleral Fibroblasts Identified by Immunostaining in Eyes with and without MASS Changes

Intensity of MMP-2 Expression	MASS ⁺ n (%)	MASS ⁻ n (%)	P
-	2 (18)	7 (64)	0.016
+	3 (27)	3 (27)	1.000
++	6 (55)	1 (9)	0.012

Abbreviations are as defined in Table 1.

anti-vimentin and anti-S100A4 antibodies. Double labeling of eyes with anti-MMP-2 and anti-vimentin identified fibroblasts as the cells expressing MMP-2.

MMP-2 cleaves soluble, triple helical type I collagen and also degrades type I collagen fibrils.³³ Scleral collagen consists mainly of type I collagen,^{1,34} which may lead to the assumption that MMP-2 produced by scleral fibroblasts is bound to scleral collagen fibers that are eventually degraded. This MMP-2-induced scleral collagen degradation process could be the mechanism behind the development of the histopathologic picture of MASS.

In the present study, the MMP-2 expression demonstrated by in situ hybridization was supported by immunohistochemical studies. The expression is seen both in eyes with MASS and in those without MASS. The results of both studies showed that the expression was significantly higher in frequency and intensity in eyes with MASS than in eyes without MASS (Tables 1-3). This finding indicates a close correlation between the expression of MMP-2, MASS, and scleral collagen degradation.

Tumor cells use MMPs produced by neighboring stromal cells like fibroblasts and macrophages rather than MMPs produced by the tumor cells themselves for tumor progression, invasion, and metastasis.^{23,35} This finding in prior studies is supported by the findings in the present one, in which tumor cells did not show MMP-2 expression either by in situ hybridization or by immunostaining.

A correlation between MMP-2 expression and hematogenous metastasis has been described,^{9,11} and MMP-2 has been suggested as a prognostic marker in both skin and uveal melanoma.^{28,10} We have shown in previous studies a significant correlation between MASS and extrascleral tumor extension.¹⁴ MASS was seen in more than 90% of eyes with scleral tumor invasion.¹⁴ This significant relation between MASS and scleral tumor extension, together with the significant correlation between MASS and MMP-2 expression indicates a possible correlation between MMP-2 production by scleral fibroblasts and local tumor invasiveness. In addition in the same study,¹⁴ eyes

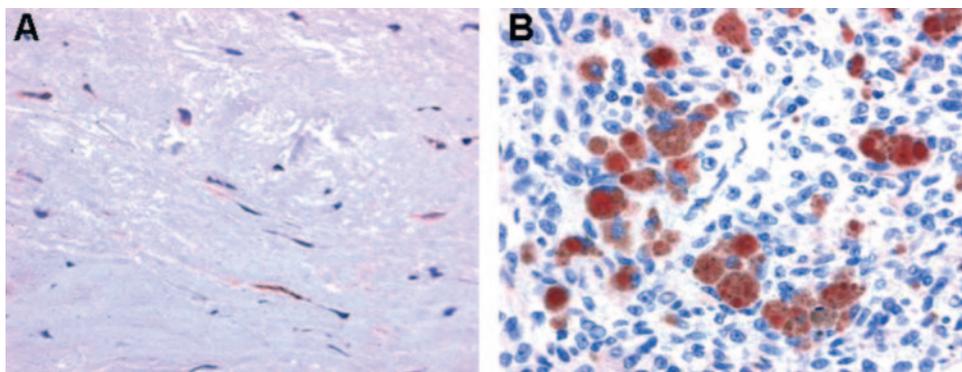


FIGURE 5. Immunostaining for MMP-2 in scleral fibroblasts and tumor-infiltrating macrophages. (A) The sclera in an area of MASS showing MMP-2-positive scleral fibroblasts (red). (B) Section of the same eye showing MMP-2-positive tumor-infiltrating macrophages (red). The tumor cells were MMP-2 negative. Magnification, $\times 400$.

TABLE 3. MMP-2 Expression in Tumor Macrophages Identified by Immunostaining in Eyes with and without MASS Changes

Intensity of MMP-2 Expression	MASS ⁺ n (%)	MASS ⁻ n (%)	P
-	3 (27)	6 (55)	0.106
+	1 (9)	3 (27)	0.293
++	7 (64)	2 (18)	0.015

Abbreviations are as defined in Table 1.

receiving pre-enucleation brachytherapy demonstrated significantly less MASS in the sclera outside the tumor despite the tumor's invasiveness, probably because radiation induced a reduction in scleral fibroblasts.

We may assume that tumor cells in invasive choroidal melanoma, either directly or via infiltrating macrophages, induce adjacent scleral fibroblasts to produce MMP-2, which results in scleral degradation seen as MASS. We suggest that MMP-2 plays a role in facilitating the local invasion of uveal melanoma into and through the sclera. Further studies will be conducted to investigate the relation between MMP-2 expression in the sclera with MASS changes in eyes with uveal melanoma and patients' survival.

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