

Osteopontin Expression and Serum Levels in Metastatic Uveal Melanoma: A Pilot Study

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PURPOSE. This was a pilot study conducted to examine the expression of osteopontin in uveal melanoma and to determine whether serum osteopontin can be used in detecting metastatic uveal melanoma.

METHODS. Osteopontin mRNA was measured in three uveal melanoma cell lines of various invasive potential by real-time PCR. Tissue sections of primary and metastatic uveal melanomas were stained for osteopontin. Serum osteopontin levels were measured by ELISA assays in 15 patients with metastatic uveal melanoma and in 37 patients who were disease-free for at least 10 years after treatment of the primary tumor. Paired serum samples drawn from eight patients before and after development of metastasis were analyzed.

RESULTS. By real-time PCR, highly invasive primary and metastatic uveal melanoma cells expressed 6- and 250-fold excess osteopontin mRNA, respectively, compared with poorly invasive primary uveal melanoma cells. Tissue sections of primary uveal melanomas lacking looping vasculogenic mimicry patterns either did not stain for osteopontin or exhibited weak, diffuse staining. In primary melanomas containing looping vasculogenic mimicry patterns, strong osteopontin staining was detected in the tumor periphery where patterns were located. Diffuse strong expression of osteopontin was detected in eight samples of uveal melanomas metastatic to the liver. Serum osteopontin levels were significantly higher in patients with metastatic uveal melanoma than in patients who had been disease free for at least 10 years after treatment ($P = 0.0001$) or in age-matched control subjects. Serum osteopontin levels were significantly higher ($P = 0.008$) after metastasis than before the detection of metastasis in eight patients. When a cutoff of 10 ng/mL was used, the sensitivity and specificity of serum osteopontin in detecting metastatic melanoma was 87.5%, and the area under the receiver operator characteristic curve was 96%.

CONCLUSIONS. Osteopontin is expressed diffusely in tissue sections of hepatic metastases from uveal melanoma, and in-

creased serum osteopontin levels correlate with melanoma metastasis to the liver with high specificity and sensitivity. (*Invest Ophthalmol Vis Sci.* 2006;47:802-806) DOI:10.1167/iovs.05-0422

Uveal melanoma tends to spread first and preferentially to the liver.¹ Currently, there is no effective treatment for metastatic uveal melanoma, in part because the metastatic tumor burden is typically high when liver metastases are detected by abnormalities in liver function test results or imaging studies.²⁻⁴ Our laboratory⁴ recently reported that the sensitivity and specificity of each of seven different liver function tests in detecting hepatic metastases from uveal melanoma was unsatisfactory for clinical use. Therefore, we continue to seek sensitive and specific biomarkers that can be used to detect metastatic uveal melanoma.

Osteopontin is a 314-amino-acid phosphoglycoprotein that is a component of the noncollagenous bone matrix.⁵ Osteopontin has been described in the context of diverse physiological roles such as chemotaxis, cell migration, cell adhesion, angiogenesis, apoptosis, cell-extracellular matrix interactions, immune regulation and tumor metastasis.⁶

Osteopontin actively promotes the tumorigenic phenotype and contributes to metastasis. Increased osteopontin expression is associated with aggressive behavior and metastasis in breast, colon, prostate, lung, liver, and ovarian cancers.⁷ Osteopontin is secreted into the blood where it can be detected by ELISA. Elevated serum osteopontin levels in the blood have been observed in patients with advanced or metastatic cancers.⁸⁻¹⁴ Recently, osteopontin expression has been shown to increase the invasive behavior of cutaneous melanoma cells.¹⁵ In this pilot study, we investigated the expression of osteopontin in uveal melanoma cell lines, in tissue sections of primary and metastatic uveal melanomas, and in the serum of patients treated for uveal melanoma, to determine its potential as a biomarker that correlates with metastasis to the liver.

MATERIALS AND METHODS

Cell Lines

Cell lines were derived from primary and metastatic uveal melanomas (M619 and MUM2B), as described previously.¹⁶ The OCM1a cell line¹⁷ was a generous gift from June Kan-Mitchell (Karmanos Cancer Institute, Wayne State University, Detroit, MD). These cell lines have been shown repeatedly to model the behavior of primary and metastatic uveal melanoma in vivo.^{16,18-21} The primary uveal OCM1a melanoma cell line is poorly invasive in membrane invasion culture system (MICS)²² assays and does not form vasculogenic mimicry patterns in three-dimensional cultures. By contrast, the M619 primary uveal melanoma cell line and the metastatic MUM2B uveal melanoma cell line are highly invasive in MICS assays and form vasculogenic mimicry patterns in three-dimensional cultures.¹⁶ Melanoma cells were plated in DMEM (BioWhittaker, Inc., Walkersville, MD) and supplemented with 10% fetal bovine serum (Fisher Scientific, Ottawa, Ontario, Canada) without the addition of exogenous extracellular matrix molecules.

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Real-Time PCR Quantitation of Osteopontin mRNA

Osteopontin mRNA was quantified in the three uveal melanoma cell lines with different tumorigenic phenotypes. Total RNA was extracted (RNeasy Mini Kit; Qiagen, Valencia, CA), according to the manufacturer's instructions. Osteopontin mRNA was quantified by one-step RT-PCR in a thermocycler (icycler iQ; Bio-Rad, Hercules, CA, using the Quantitect SYBR Green RT-PCR kit; Qiagen, Valencia, CA) with 2 ng RNA used as a template. The following primers were used: forward, 5'-TGG CCG AGG TGA TAG TGT G-3', and reverse, 5'-CGG GGA TGG CCT TGT ATG-3'. Each 50- μ L reaction contained 1 \times buffer with dNTPs, RT enzyme, DNA polymerase (HotStarTaq; Qiagen), 2.5 mM MgCl₂, 20 pM of each primer, and 10 nM fluorescein dye. Reverse transcription was at 50° for 45 minutes followed by 95° for 13.5 minutes, to inactivate the RT enzyme and activate the DNA polymerase. Subsequently, 42 cycles of PCR were performed: Each cycle consisted of 95° for 20 seconds and 59° for 35 seconds. Fluorescence data was acquired during the combined annealing-extension phase of PCR. Finally, a melting-curve analysis was performed after PCR to confirm specificity. A standard curve was constructed with five 10-fold serial dilutions of in vitro transcribed osteopontin RNA (2 \times 10⁶ copies to 2 \times 10² copies). All standards and samples were run as two replicates.

Demonstration of Osteopontin in Histologic Sections of Primary and Metastatic Uveal Melanomas

In multiple independent studies, the detection of vasculogenic mimicry patterns in histologic sections has been associated with death caused by metastatic uveal melanoma.²³⁻²⁷ Looping vasculogenic mimicry patterns include arcs, arcs with branching, loops, and networks, and each of these patterns is associated with metastatic behavior.²⁸ The parallel with cross-linking pattern is also associated with metastatic behavior.²⁹ Vasculogenic mimicry patterns are associated with the presence of epithelioid cells²⁹ and with monosomy 3,³⁰ another highly reliable marker of metastatic behavior in uveal melanoma. Recently, an almost perfect association was shown between a gene expression profile that identified primary uveal melanomas at risk for metastasis and the presence of vasculogenic mimicry patterns.³¹ We therefore used the absence and presence of vasculogenic mimicry patterns as a histologic marker for aggressive behavior in primary uveal melanomas.

We stained for osteopontin the formalin-fixed, paraffin-embedded tissue sections of 4 primary uveal melanomas that lacked any of the vasculogenic mimicry patterns associated with metastasis, 13 primary uveal melanomas that contained vasculogenic mimicry patterns, and 8 hepatic metastases from uveal melanoma. Tissue sections were hydrated to distilled water and were then placed in phosphate-buffered saline (PBS) for 15 minutes followed by enzyme blocking for 10 minutes using peroxidase (Dako Peroxidase block; DakoCytomation, Carpinteria, CA), according to the manufacturer's instructions. Slides were rinsed with buffer and then treated with protein block for 10 minutes. Tissue sections were blotted and incubated with a polyclonal antibody against osteopontin (ab8448, 1:400; Abcam Inc, Cambridge, MA) for 30 minutes, rinsed, and treated with a polymer (EnVision Plus; DakoCytomation) for 30 minutes at room temperature. After sections were rinsed in buffer, the reaction product was detected by 3,3'-diaminobenzidine (DAB Plus; DakoCytomation) for 10 minutes. Slides were rinsed in distilled water, counterstained, rinsed again in distilled water, dehydrated through an alcohol gradient, and mounted (Permount; Fisher Scientific). In negative control experiments, primary antibody was replaced by buffer.

Serum Samples

Fifty-two serum samples were obtained from the Ocular Oncology Serum Bank at Hadassah-Hebrew University Medical Center: 1 sample

from each of 15 patients with metastatic uveal melanoma, 37 patients who had been disease free for at least 10 years after treatment of the primary tumor, and 30 age- and sex-matched control subjects. Among the 15 patients with metastatic melanoma, there were eight samples for which paired premetastatic sera were also available. Blood (7 mL) was obtained from patients at the time of diagnosis of the primary tumor, before treatment and at least every 6 months after treatment. After collection, blood was centrifuged for 10 minutes at 1200 rpm. Serum was collected after centrifugation and was stored at -20°C.

Assay for Serum Osteopontin Levels

Serum levels of osteopontin were evaluated with an enzyme linked immunosorbent assay (ELISA) kit from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. The kit is a 4.5-hour solid-phase ELISA designed to measure human osteopontin in serum and plasma. Results obtained with natural human osteopontin showed linear curves that were parallel to the standard curves obtained when the kit standards were used. These results indicated that the ELISA kit could be used to determine relative mass values for naturally occurring osteopontin.

The ELISA kit used employs a quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for osteopontin was precoated onto a microplate. Standards and samples were pipetted into the wells, and any osteopontin present was bound by the immobilized antibody. After any unbound substances were washed away, an enzyme-linked polyclonal antibody specific for osteopontin was added to the wells, and color was developed in proportion to the amount of osteopontin bound in the initial step. The color development was stopped and the intensity of the color was measured with a microplate reader at 450 nm. Results are calculated according to the standard curve created by the standards of osteopontin.

Human Subjects

The use of human tissue samples in these studies was approved by the Institutional Review Board of the University of Illinois at Chicago. The use of patient's sera was approved by the Hadassah-Hebrew University Medical Center Helsinki Committee.

RESULTS

Osteopontin Expression in Uveal Melanoma Cell Lines

Osteopontin is secreted and may bind to tumor cells. Therefore, to determine whether melanoma cells are capable of generating osteopontin, we compared the expression of osteopontin mRNA in three uveal melanoma cell lines that manifest various invasive and metastatic behavior. Real-time PCR quantitation showed that the highly invasive and metastatic uveal melanoma cell lines (M619 and MUM2B) expressed 6- and 250-fold excess of osteopontin mRNA, respectively, compared with OCM1a cells.

Osteopontin Expression in Primary and Metastatic Uveal Melanomas Tissue Sections

In three of four primary uveal melanomas lacking any of the vasculogenic mimicry patterns of prognostic significance, there was no evidence of staining for osteopontin; in one of these cases, diffuse, weak cytoplasmic staining was noted. In 8 (62%) of 13 cases of primary uveal melanomas containing looping vasculogenic mimicry patterns associated with metastatic behavior, cytoplasmic staining was intense, was confined to the periphery of the tumors, and tended to highlight looping patterns. In 5 of these 13 primary uveal melanoma cases, cytoplasmic staining for osteopontin was diffuse and weaker. Because macrophages are known to secrete osteopontin^{32,33} and because macrophages tend to be distributed in primary

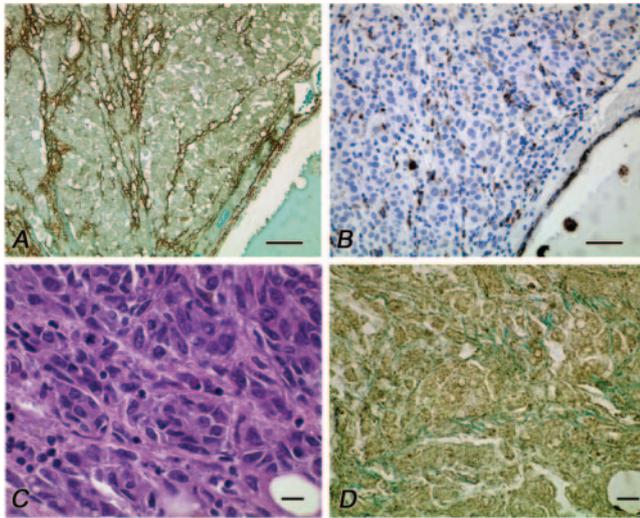


FIGURE 1. Distribution of osteopontin in primary and metastatic uveal melanoma. (A) Osteopontin is distributed along looping vasculogenic mimicry patterns at the periphery of a primary uveal melanoma. Note the cytoplasmic staining of tumor cells and the absence of nuclear staining. (B) Section adjacent to that illustrated in (A), stained for CD68, a macrophage marker. Although macrophages are identified within vasculogenic mimicry patterns, the distribution of CD68⁺ cells does not correspond to the diffuse staining of patterns that are present when the tissue is labeled for osteopontin (A). (C) Uveal melanoma metastasis to the liver. (D) Section adjacent to (C) stained for osteopontin. Note the diffuse distribution of osteopontin in contrast with a primary uveal melanoma (A) and the presence of both nuclear and cytoplasmic staining. (A) Osteopontin is counterstained *light green*; (B) CD68 counterstained with hematoxylin; (C) hematoxylin-eosin; (D) osteopontin counterstained *light green*. Magnification bars: (A, B) 50 μ m; (C, D) 10 μ m.

uveal melanomas within looping vasculogenic mimicry patterns,^{3,4} sections adjacent to tumors in which osteopontin decorated looping patterns were stained for the macrophage marker CD68. CD68-positive cells were detected within some areas of the patterns, but did not match the uniform distribution of osteopontin in these areas. Tissue sections of each of eight deposits of uveal melanoma metastases to the liver obtained from different patients all showed diffuse staining (cytoplasmic and nuclear) for osteopontin (Fig. 1).

Serum Osteopontin Levels

Serum osteopontin levels in patients with metastatic uveal melanoma were first compared with levels from patients who were disease free for at least 10 years after treatment of the primary uveal melanoma. The mean serum osteopontin

level in patients with metastatic uveal melanoma was 17.62 ng/mL ($n = 15$, SE = 3.59, SD \pm 13.89), whereas the mean level in patients with uveal melanoma patients who had been disease free for at least 10 years was 7.15 ng/mL ($n = 37$, SE = 0.49, SD \pm 2.96; Kruskal-Wallis $\chi^2 = 21.1296$, $df = 1$, $P = 0.0001$). Serum osteopontin levels were measured at least 6 months before the development of metastases in 8 of the 15 patients in who had uveal melanoma metastasis to the liver. In these eight patients, the mean premetastatic serum level of osteopontin was 6.19 ng/mL (SE = 1.05, SD \pm 2.69), and the mean level of osteopontin at the time of the detection of metastasis was 19.66 ng/mL (SE = 6.39, SD \pm 18.07; sign test, $P = 0.008$).

Serum levels of osteopontin in a group of 30 age- and sex-matched normal patients were compared with levels in patients with uveal melanoma metastasis to the liver and with levels in patients who had been disease-free for at least 10 years. With adjustment for age and sex, the serum level of osteopontin in patients with uveal melanoma metastasis to the liver was significantly higher than those in normal control subjects ($F = 15.33$, $df = 1$, $P = 0.0004$). There was no significant difference between serum osteopontin levels in the control group and those in the group of patients who had survived disease free for at least 10 years after treatment of the primary tumor ($F = 0.23$, $df = 1$, $P = 0.6321$). However, there was a significant difference between serum osteopontin levels in the group of long-term survivors and levels in those patients who had uveal melanoma metastasis to the liver, when controlled for age and sex ($F = 24.94$, $df = 1$, $P < 0.0001$). There were no significant differences in age (contingency $\chi^2 = 3.6140$, $df = 6$, $P = 0.7287$) or distribution of gender (contingency $\chi^2 = 1.0520$, $df = 2$, $P = 0.5910$) between the three populations studied (normal control subjects, long-term survivors, and patients with uveal melanoma metastasis to the liver, compared by three-way analysis of variance). Controlling for age and sex, we calculated the least-square means of serum osteopontin levels: control patients, 6.74 ng/mL, long-term surviving patients, 6.89 ng/mL, and patients with uveal metastatic melanoma, 18.25 ng/mL. These data are summarized in Table 1.

With a cutoff value of 10 ng/mL, the specificity of serum osteopontin for detecting metastasis was 83.7% in patients who remained disease free for more than 10 years. With the same cutoff, both the specificity and sensitivity were 87.5% for detection of metastasis in the patients with paired pre- and postmetastasis samples. Receiver operator characteristic (ROC) curve analysis showed that the area under the curve was 96%, with a cutoff of 10 ng/mL when pre- and postmetastatic osteopontin levels were analyzed in the paired samples.

TABLE 1. Comparison of Serum Osteopontin Levels by Group

	Control				Metastatic Melanoma				Long-Term Survivors			
	<i>n</i>	Mean	SD	SE	<i>n</i>	Mean	SD	SE	<i>n</i>	Mean	SD	SE
Age (y)												
<50	6	5.42	4.47	1.82	4	9.93	2.86	1.43	9	6.09	2.01	0.67
50–59	10	4.82	3.41	1.08	6	15.97	7.68	3.13	11	6.43	2.33	0.70
60–69	8	11.50	10.26	3.62	2	19.00	5.66	4.00	5	6.42	2.40	1.07
\geq 70	6	4.77	4.65	1.90	3	30.33	28.30	16.34	12	7.55	2.97	0.86
Sex												
Male	15	8.74	8.23	2.13	7	14.71	3.95	1.49	14	6.90	3.19	0.85
Female	15	4.68	3.95	1.02	8	20.19	18.86	6.67	23	6.59	1.99	0.41
Overall	30	6.71	6.70	1.22	15	17.63	13.88	3.58	37	6.70	2.47	0.41

DISCUSSION

In this study, we used a progressive series of experiments to investigate the potential role of serum osteopontin in the detection of metastatic uveal melanoma. We first detected an increase in the expression of osteopontin mRNA in aggressive primary M619 uveal melanoma cells relative to nonaggressive primary OCM1a uveal melanoma cells, and an even greater expression of mRNA in the metastatic MUM2B uveal melanoma cell line compared with the two primary melanoma cell lines. These data are important for two reasons: First, it is known that macrophages synthesize osteopontin,^{32,33} and the infiltration of macrophages into primary uveal melanomas has been associated with increased metastatic behavior in primary tumors.³⁴ The high expression of osteopontin mRNA by metastatic uveal melanoma cells compared with that in aggressive and nonaggressive uveal melanoma cells suggests that the tumor cell itself may be a source of serum osteopontin. Second, the progressive increase in mRNA between highly invasive primary uveal melanoma cells (a 6-fold increase over that in noninvasive primary uveal melanoma cells) and metastatic uveal melanoma cells (a 250-fold increase over noninvasive primary uveal melanoma cells) suggests that we should prioritize the study of patient serum samples for the use of serum osteopontin levels to detect hepatic metastases. It may be worthwhile in a future study to investigate the role of osteopontin in stratifying patients with primary uveal melanocytic lesions into risk categories for metastasis by using this simple blood test, because a sixfold change in mRNA expression in highly invasive melanoma cells compared with poorly invasive cells is significant.

Using immunohistochemistry, we next confirmed differential osteopontin mRNA expression in cell lines by detecting osteopontin in tissue sections of primary uveal melanomas (with and without vasculogenic mimicry patterns) and in metastatic uveal melanoma. In hepatic metastases from uveal melanoma, osteopontin expression was diffuse and strong (Figs 1C, 1D). In primary uveal melanomas, strong staining for osteopontin was confined to vasculogenic mimicry patterns at the periphery of the tumor (Fig. 1A). This finding is consistent both with the tendency of these patterns to be localized at the tumor periphery³⁵ and the tendency of osteopontin to be expressed at the peripheral tumor-stromal interface in highly malignant neoplasms.³⁶ Cytoplasmic staining for osteopontin confirmed the observation from mRNA studies from cultured cells suggesting that melanoma cells generated osteopontin. CD68-positive cells (macrophages) were detected within these patterns as reported previously,³⁴ but the distribution of these cells was scattered (Fig. 1B), indicating that although tumor-infiltrating macrophages may contribute to elevations in serum osteopontin, the greater contribution to serum osteopontin levels is most likely from melanoma cells.

It is also of interest that in histologic sections of primary uveal melanomas lacking vasculogenic mimicry patterns, osteopontin was either not detected anywhere in the tumor or was expressed as weak diffuse cytoplasmic staining. These data suggest that it may be worthwhile to design a study to investigate the sensitivity and specificity of serum osteopontin levels in discriminating primary uveal melanomas lesions at low and high risk for metastasis.

This study focused on the potential use of serum osteopontin levels to detect metastatic uveal melanoma. Consistent with observations that blood levels of osteopontin are useful markers of metastatic behavior in a variety of cancers,⁸⁻¹⁴ we observed that patients with metastatic uveal melanoma had significantly higher levels of serum osteopontin when compared with patients who remained disease free for at least 10 years. Both sensitivity and specificity of serum osteopontin in detecting metastasis in patients with uveal melanoma after

treatment of the primary lesion is 87.5%, and analysis of the area under the ROC curve indicates that the probability of a correct diagnosis of metastasis based on a serum osteopontin level is 96%. Recent data from the authors' laboratories⁴ indicate that the sensitivity and specificity of six liver function tests including bilirubin, gamma glutamyl transpeptidase (γ GT), alkaline phosphates (ALK), lactate dehydrogenase (LDH), aspartate-aminotransferase (AST), and alanine-aminotransferase (ALT) are low compared with the sensitivity and specificity of serum osteopontin in the detection of hepatic metastases reported in this study. For example, at the time of the diagnosis of hepatic metastasis, the sensitivity of ALT was 40%, the sensitivity of AST and γ GT tests was 50%, and the sensitivity of ALK and bilirubin tests was 60%. The sensitivity of LDH was 80% at the time of the detection of hepatic metastasis, but elevated LDH is not specific for hepatic metastasis. Data on liver function tests from our laboratory are consistent with reports of other investigators who discovered that the sensitivity of γ GT is 21%, the sensitivity of ALK is 25%,³⁷ and the sensitivity of LDH is 67%.^{3,38} Thus, based on this pilot study, serum osteopontin levels appear to be both more specific and more sensitive than any currently available hepatic function test.

It is especially significant that the serum level of osteopontin appears to increase as metastases develop, consistent with the positive expression of osteopontin in tissue sections of uveal melanoma metastasis to the liver (Fig. 1). Thus, it may be useful to obtain blood to establish the serum level of osteopontin before treatment of the primary lesion and to follow these serum levels sequentially. Elevation of serum osteopontin over a period, analogous to the monitoring of elevations in blood prostate-specific antigen to identify patients at risk for metastatic prostate carcinoma,^{39,40} may be useful in identifying early metastasis of uveal melanoma.

In this study, the association between elevated serum osteopontin levels in patients with early metastatic uveal melanoma is consistent with the increased osteopontin expression by different types of highly invasive and metastatic tumor cells.⁸⁻¹⁴ By contrast, an increased expression of osteopontin mRNA from primary uveal melanoma tissues was associated with improved rather than decreased survival in two gene-expression array studies of primary uveal melanoma tissue.^{41,42} Because osteopontin is expressed preferentially in the periphery of primary uveal melanomas, the inhomogeneous distribution of tumor cells expressing osteopontin may result in a systematic sampling bias if tissue for gene expression studies is extracted from the center of the tumor.

Additional studies are needed with larger patient samples, to validate the results of this pilot study. Also, it may be useful to include serum osteopontin levels in a panel of serum biomarkers, including serum melanoma inhibitory activity (MIA).⁴³ MIA, secreted by the melanoma cell line HTZ-19,⁴⁴ has been detected in histologic sections of primary uveal melanoma and uveal melanoma metastasis to the liver by immunohistochemistry. In addition, serum levels of MIA detected by ELISA increased significantly when metastases to the liver developed, thus demonstrating the feasibility of using serum levels of molecules overexpressed in melanoma to detect metastatic uveal melanoma.⁴⁵ By using a panel of serum biomarkers, including osteopontin, it may be possible to achieve an outstanding sensitivity and specificity in detecting early metastasis from uveal melanoma. Discovery of early metastasis while the hepatic tumor burden is relatively small may be helpful in designing strategies to treat patients at high risk of dying of metastatic uveal melanoma.

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