Using the Direct-Injection Model of Early Uveal Melanoma Hepatic Metastasis to Identify TPS as a Potentially Useful Serum Biomarker

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PURPOSE. To develop a method to screen for serum biomarkers of early hepatic metastasis from uveal melanoma.

METHODS. Cytokeratin 18 (TPS) was identified from gene expression profiles as protein generated by highly invasive uveal melanoma cells. Sera were collected from two groups of 15 SCID mice 2 weeks after injection of either tissue culture medium or MUM2B human metastatic uveal melanoma cells into the mouse liver. Serum TPS levels were assayed in 53 healthy human controls, 61 uveal melanoma patients who were disease free for at least 10 years, and 37 patients with metastatic uveal melanoma.

RESULTS. After 2 weeks, small hepatic nodules (0.1–2.8 mm; mean, 0.80 mm) developed in 11 of 15 mice injected with MUM2B cells. Serum TPS levels in media-injected mice (84.7 U/L) were substantially lower than levels in MUM2B-injected mice (601 μg/L). TPS levels were significantly higher (P < 0.0001) in patients with metastatic uveal melanoma (139.63 ± 22.20) than in healthy controls (54.23 ± 9.76). Significant differences were found between TPS levels before and after the development of hepatic metastases (P < 0.01), and serum TPS levels became elevated in four patients at least 6 months before the detection of hepatic metastases by abdominal ultrasonography.

CONCLUSIONS. The direct-injection model of uveal melanoma in the mouse liver may be used to screen for potential serum biomarkers of metastatic uveal melanoma. (Invest Ophthalmol Vis Sci. 2007;48:4399 - 4402) DOI:10.1167/iovs.07-0552

The tendency for uveal melanoma to spread first and preferentially to the liver is well documented.1 For many years, oncologists have monitored liver enzyme levels on sequential visits after the treatment of the primary tumor to detect metastatic uveal melanoma. However, the sensitivity and specificity of liver enzyme levels in detecting metastasis is disappointingly low,2 and patients may have significant hepatic replacement by tumor despite normal liver enzymes.3 The sensitivity of hepatic ultrasonography in detecting metastases from uveal melanoma is high, but the specificity is low.4 One clinical investigation team concluded that ultrasound screening did not contribute to improved outcomes and called for the development of “better screening tests and more effective multimodality treatments. . . to improve survival in uveal melanoma patients with hepatic metastases.”5

Serum osteopontin,6,7 S100p protein,8 and melanoma inhibitory activity9 have all been shown to be sensitive markers of metastatic uveal melanoma. Recently, Barak et al.10 showed that receiver operator characteristic analysis for all three markers in combination revealed an area under the curve of 0.91. Nevertheless, these sera biomarker tests are benchmarked to abdominal ultrasonography—they identify patients who have metastatic melanoma as detected ultrasonographically. One immediate translational research goal is the discovery of serum biomarkers that are sensitive in detecting very small metastases, even before detectability by ultrasonography.

Genomic11,12 and proteomic13–15 profiles developed from uveal melanoma cell lines have identified a large number of secreted proteins that might serve as useful clinical biomarkers of uveal melanoma metastasis. Screening banked human sera repositories for large numbers of putative biomarkers of clinical relevance is wasteful of sera, expensive, and therefore impractical.

Directly implanting human uveal melanoma cells into the livers of severe combined immunodeficiency (SCID) mice makes it possible to generate very small human uveal melanoma nodules in relatively short periods of time.16 We hypothesized that it should be possible to assay mouse sera in these animals to detect human proteins secreted by these very small human uveal melanoma xenografts and to select candidate proteins for testing across samples in a uveal melanoma patient serum repository to detect early hepatic metastases.

To test the feasibility of this experimental strategy, we screened a comprehensive uveal melanoma gene expression profile12 for secreted proteins. Among a variety of candidate proteins, we noticed that MUM2B cells differentially express cytokeratin 18. We had shown previously that coexpression of cytokeratin 18 with vimentin was associated with the invasive uveal melanoma phenotype.17 Serum levels of tissue polypeptide–specific (TPS) antigen cytokeratin 18 have never been tested as biomarkers in patients with uveal melanoma, though TPS is an effective serum biomarker for identifying the lead time to early metastases from breast cancer18,19 and lung can-

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cer.20 In these patients, adding serum TPS to established markers such as CA15–3 or CEA (for breast cancer) and CEA or CYFRA 21–1 (for lung cancer) increases the sensitivity of the test to more than 90%.20

We therefore pursued the strategy of testing MUM2B culture supernatants for the presence of TPS, followed by the expression of TPS by very small human MUM2B xenografts to the mouse liver. We observed that TPS levels increased with tumor burdens as small as 1 mm in diameter. Based on these experimental data, we screened for the TPS levels in sera from patients with uveal melanoma who had been without disease for at least 10 years, in uveal melanoma patients with metastatic disease, and in healthy age-matched controls. We discovered that, as predicted, serum TPS levels become significantly elevated in patients with uveal melanoma and that, in some patients, elevated serum TPS levels precede the ultrasonographic detection of hepatic metastases by 6 months.

MATERIALS AND METHODS

Direct Injection Model of Uveal Melanoma in the Liver

MUM2B cells were developed from a focus of metastatic uveal melanoma in the liver; the biological properties of this line have been described.21 This cell line has been authenticated as human22 and is free of mycoplasma and common viral pathogens. These cell lines have been shown repeatedly to model the behavior of primary and metastatic uveal melanoma in vivo.23–25 Five million cells were grown in 1% agar-coated 60-mm tissue culture dishes containing 15% heat-inactivated fetal bovine serum, glutamine, and MEM/EBSS media (Hyclone, Logan, UT) until spheroids formed (typically within 2 days). When the spheroids grew to 200 to 500 µm, they were injected directly into the livers of SCID mice, as described recently.16

Briefly, each mouse was anesthetized before surgery with ketamine (100 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection. A small horizontal abdominal incision (1 cm) was made in the left upper quadrant such that the left lobe of the liver was exposed. Spheroids of melanoma cells were drawn into a Hamilton syringe with a 29-G needle creating a 15-µL slurry that was injected into the parenchyma of the exposed liver with the assistance of a stereomicroscope (American Optical Company, Buffalo, NY), roughly according to a protocol described previously to establish models of colon cancer in the liver.24 It had been shown previously that the use of cell slurries fashioned from tumor cell spheroid was particularly effective in establishing animal models of primary uveal melanoma in the rat.25 Gentle pressure was applied to the hepatic injection site for 1 minute with a cotton-tipped applicator. After tumor cell injection, the left lobe of the liver was repositioned into the peritoneal cavity, and the abdominal wall was then closed with 6.0 absorbable sutures (Ethicon, Somerville, NJ).

In these studies, 15 mice were injected with MUM2B cells, as described, and 15 mice were injected with an equivalent volume of tissue culture media. All animal protocols were approved by the Animal Care Committee of the University of Illinois at Chicago and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Human Subjects

One of us (JP) monitored patients treated for uveal melanoma by abdominal ultrasonography when the primary tumor was diagnosed and every 6 months after treatment of the primary lesion. Positive results of ultrasonography were confirmed by tissue biopsy. Blood was drawn as part of each 6-month follow-up visit, which included an ultrasound examination, assays for liver function tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALK), gamma glutamyl transpeptidase (γGT), and lactate dehydrogenase (LDH), and serum was banked as part of a research protocol approved previously by the Helsinki Committee of the Hadassah–Hebrew University Medical Center (Declaration of Helsinki Compliance).5

Three patient populations were studied: 64 patients who were treated for primary uveal melanoma and who had been without disease for at least 10 years, 37 patients with metastatic uveal melanoma, and 53 healthy age-matched controls. Blood (7 mL) was drawn from patients at the time of diagnosis of the primary tumor before and at least every 6 months after treatment. After collection, blood was centrifuged for 10 minutes at 1200 rpm, and serum was stored at −20°C. All collected samples were assayed. Among the 37 patients with metastatic uveal melanoma, sera were assayed before and after the clinical appearance of metastases.

Biomarker Assay Methods

Supernatant from MUM2B metastatic uveal melanoma cells grown in 3D culture conditions, as described previously, was assayed for osteopontin (R&D Systems, Minneapolis, MN) and TPS (IDL, Bioteck AB, Bromma, Sweden) by ELISA, as was pooled serum from four untreated SCID mice. Sera were then collected from two groups of 15 SCID mice 2 weeks after injection of either tissue culture medium (control) or human metastatic uveal melanoma (MUM2B) into the mouse liver, as described. For each group of mice, sera were pooled to measure TPS because the volume of blood in each mouse did not generate a sufficient volume of serum for the ELISA assays. For human studies, serum levels of TPS were assayed by ELISA, as described, according to the manufacturers’ instructions.

Statistical Methods

The Student’s t-test was used to compare marker serum levels among the three patient groups, and the Sign test was used to compare serum biomarker levels before and after the development of metastatic uveal melanoma; P < 0.05 was considered significant.

RESULTS

Results are summarized in Table 1. Briefly, we detected 335 U/L TPS in the supernatants of MUM2B cells grown in 3D culture conditions. After 2 weeks, small hepatic nodules (0.1–2.8 mm; mean, 0.80 mm) developed in 11 of the 15 mice injected with MUM2B cells. Serum TPS levels in normal untreated mice (50.6 U/L) and media-injected mice (84.7 U/L) were substantially lower than levels in MUM2B-injected mice (601 U/L). TPS levels in the control mice that did not receive MUM2B cells (50.6 U/L and 84.7 U/L) most likely resulted from cross-reactivity of the assay with the mouse TPS protein (Endo B), which has 89.7% amino acid sequence identity to the human TPS protein.26 Thus, the sharp elevation in the level of TPS indicated that TPS may be a clinically sensitive marker for detecting hepatic metastases in patients with uveal melanoma.

<table>
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<tr>
<th>TPS (U/L)</th>
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<tr>
<td>Supernatant from MUM2B 3D tissue cultures</td>
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<td>Sera from normal SCID mice (n = 4)</td>
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<td>Sera from media-injected SCID mice (n = 15)</td>
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<tr>
<td>Sera from MUM2B-injected SCID mice (n = 15)</td>
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* TPS levels in the control mice that did not receive MUM2B cells most likely resulted from cross-reactivity with mouse TPS protein (Endo B), which has 89.7% amino acid sequence identity to the human TPS protein.
patients and their pre-metastatic stage \( (P < 0.01) \). In four patients, elevations in serum TPS levels preceded the detection of hepatic metastases by abdominal ultrasonography by 6 months.

**Discussion**

By directly injecting human uveal melanoma cells into the livers of immunosuppressed mice, it is possible to faithfully reproduce the histology of human uveal melanoma metastatic to the liver. This model was designed to study the biology of uveal melanoma in the liver \(^6\) and can be used to investigate novel therapeutic strategies to prevent the emergence of clinically significant metastases from microdeposits of tumor and to treat established hepatic nodules of uveal melanoma in the liver.

Many oncologists rely on liver function tests to detect the emergence of hepatic metastases. However, recent data from the authors’ own laboratories \(^2\) indicate that the sensitivity and specificity of six liver function tests—bilirubin, \( \gamma \)GT, ALK, LDH, AST, and ALT—are low compared with the sensitivity and specificity of serum osteopontin to detect 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 for AST and \( \gamma \)GT was 50%, and the sensitivity of ALK and bilirubin 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 from other investigators, who discovered that the sensitivity of \( \gamma \)GT is 21%, the sensitivity of ALK is 25%, \(^2\) and the sensitivity of LDH is 67%. \(^2\) and with data from the Collaborative Ocular Melanoma Study suggesting that the sensitivity and specificity of liver function tests are suboptimal for guiding clinical management. \(^30\)

In this study, we expand the use of this model to test for the sensitivity of a biomarker new to uveal melanoma, TPS, to detect very small quantities of tumor. Rising levels of TPS in very small human uveal melanoma deposits in the mouse liver suggested that the kinetics of serum TPS in patients treated for primary uveal melanoma may be a sensitive marker for the early detection of very small subclinical metastases in the liver when combined with abdominal ultrasonography. As predicted from the model data, TPS is significantly elevated in patients with metastatic uveal melanoma compared with age-matched controls and patients who are disease free. In some patients, TPS serum levels become elevated before abdominal ultrasonography is positive for metastatic uveal melanoma. Because serum TPS can be elevated in patients with alcoholic liver disease—keratin 18 is a component of Mallory bodies \(^31\)—thorough knowledge of the patient’s general health status is required before this test is introduced to detect subclinical hepatic metastases from uveal melanoma.

Recently, investigators have shown that serum osteopontin \(^6\) \(^7\) \( \text{S100}\beta \) protein, \(^8\) and melanoma inhibitory activity \(^9\) are sensitive markers of metastatic uveal melanoma. Unpublished data from our laboratory suggest that when TPS is added to these markers, the area under the receiver operator characteristic curve exceeds 91%, suggesting that these markers provide a sensitive means to diagnose hepatic metastases.

The major purpose of the study was to describe the use of a novel animal model to screen for putative serum biomarkers that may be helpful in detecting metastatic uveal melanoma. TPS is illustrated as an example of a marker that was identified using this method. The potential clinical usefulness of TPS was illustrated in a pilot study, and additional studies are necessary to assess the relative value of TPS to other markers of metastasis. Therefore, a detailed analysis of the sensitivity and specificity of rising TPS levels is under way in our laboratories on a larger cohort of patients with primary uveal melanoma.

This study illustrates another application of the direct-injection animal model of uveal melanoma in the liver. This model can be applied to screening a large library of secreted proteins to identify potential clinically relevant serum biomarkers of very early metastatic uveal melanoma for testing in large patient sera repositories.

**References**


