Association Between NM23-H1 Gene Expression and Metastasis of Human Uveal Melanoma in an Animal Model

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Purpose. To evaluate the role of nm23 gene expression in the development of metastases of human uveal melanomas in an animal model.

Methods. Seven human uveal melanoma cell lines and two murine skin melanoma cell lines were subjected to Northern blot analysis for the detection of nm23-H1 mRNA and to immunohistochemistry to detect nm23 antigen. Each tumor cell line was transplanted intracameraly into nude mice, and the metastatic behavior was evaluated by histopathologic analysis of the livers and by determining host survival times.

Results. There was a strong inverse correlation between the levels of nm23 mRNA expression and nm23 antigen expression and the development of metastases of all seven human uveal melanomas and both murine skin melanomas transplanted intracameraly. Host survival time also was correlated with the degree of nm23 gene expression.


Uveal melanoma is the most common intraocular malignancy in adults.1 As in most neoplasms, metastasis is the leading cause of death in patients with uveal melanoma.2–4 Considerable effort has focused on identifying risk factors and prognostic markers for predicting the malignant potential of uveal melanomas. Several studies have suggested that morphometric characteristics, such as the standard deviation of nucleolar area and the mean of the largest nuclear diameter, can be correlated with malignant potential and patient survival time.5,6 Others suggest that the morphologic phenotype of a uveal melanoma provides the most consistent indication of metastatic potential and overall malignancy.7,8 In other words, uveal melanomas are classically categorized by the morphology of their cellular components. The Callender classification scheme categorizes uveal melanoma cellular components as either spindle A, spindle B, or epithelioid.9 Although uveal melanomas can be composed of all three types, a predominance of epithelioid components carries significantly greater malignant potential and a shorter patient survival time than melanomas comprised largely of spindle cellular elements.7,8 There are no molecular markers, however, that have been shown to correlate with the malignant potential of human uveal melanomas.

At least 10 different oncogenes have been shown to augment the metastases of a variety of tumors in experimental animals.10 Of the potential metastasis suppressor genes suggested, the nm23 gene has received the most attention.11 NM23 was discovered by differential colony hybridization experiments with murine K1735 melanoma cell lines.12 Since its original description in murine melanoma, nm23 gene expression has been associated with reduced metastasis.
sis in several categories of human cancers, such as breast cancer\textsuperscript{13} and cutaneous melanoma.\textsuperscript{14} However, in cancers such as colon carcinomas\textsuperscript{15} and lung adenocarcinomas,\textsuperscript{16} there does not seem to be any correlation between nm23 gene expression and metastasis. The role of the nm23 gene in human uveal melanomas is unknown.

In the current study, we examined nm23 gene expression in several human uveal melanoma cell lines and evaluated the metastatic potential of the respective melanoma cells after intracameral transplantation in athymic nude mice.

MATERIALS AND METHODS

Mice

We have found that the source of athymic mice can have a significant effect on the metastasis of intraocular melanomas. In particular, we have reported\textsuperscript{17} that OCM-1 melanoma cells do not form progressively growing metastases in athymic nude mice purchased from Simonsen Laboratories (Gilroy, CA). However, OCM-1 melanoma cells spontaneously metastasize from the eye and form progressive liver metastases in nude mice purchased from Jackson Laboratories (Bar Harbor, ME). Accordingly, female athymic nude BALB/c (H-2\textsuperscript{b}) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Accordingly, female athymic nude BALB/c (H-2\textsuperscript{b}) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and were incorporated into experiments at 8 to 10 weeks of age. The use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Lines and Culture

Seven human uveal melanoma cell lines, designated OCM1, OCM3, OCM8, EOM5, 92-1, OM431, and MEL202, were used. OCM1, OCM3, and OCM8 were generously provided by Dr. June Kan-Mitchell (University of California, La Jolla, CA). OCM1 has a predominantly spindle morphology, whereas OCM3 and OCM8 are predominantly epithelioid.\textsuperscript{18,19} The EOM3 cell line displays an epithelioid morphology in vitro but was derived from a posterior choroidal melanoma of mixed cell morphology.\textsuperscript{20} MEL202 was kindly provided by Dr. Bruce Ksander (Schepens Eye Institute, Boston, MA) and is comprised predominantly of spindle cell elements.\textsuperscript{21} OM431 was generously provided by Dr. Daniel Albert (University of Wisconsin, Madison) and is predominantly epithelioid.\textsuperscript{22} 92-1 is comprised of both epithelioid and spindle elements. Its origin and characterization have been described.\textsuperscript{23} OCM1, OCM3, OCM8, and OM431 cells were cultured in Ham's F-12 medium containing 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% vitamin solution, and 1% antibiotic–antimycotic solution. EOM3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 1% Hepes buffer, and 1% antibiotic–antimycotic solution. OCM1 cell line was established from a liver metastasis in a nude mouse with an intraocular OCM1 tumor and was cultured in Ham's F-12 medium. Two murine cutaneous B16 melanoma cell lines, designated Queen's and D5.1G4, were used as controls. The highly metastatic Queen's melanoma was a generous gift from Dr. J. Szalay (Queens College, Queens, NY). This cell line readily metastasizes when injected intravenously or when transplanted intracamerally.\textsuperscript{24,25} The origin, characterization, and cultivation of the D5.1G4 melanoma cell line has been described by Knisely and Niederkorn.\textsuperscript{26} This mutant cell line possesses low metastatic activity when injected intravenously or intracameral into euthymic C57BL/6 mice. A human keratoocyte line, HK/10, was generously provided by Dr. YuGuang He (University of Texas Southwestern Medical Center, Dallas, TX) and was cultured in Dulbecco's modified Eagle's medium as previously described.\textsuperscript{27}

RNA Analysis

Total RNA was extracted from $10^7$ to $10^8$ cells by the TRI Reagent (Molecular Research Center, Cincinnati, OH) RNA isolation following the instructions of the manufacturer. RNA concentrations were determined spectrophotometrically. Total RNA (20 μg per lane) was electrophoresed in 6.2% formaldehyde–1.0% agarose gels and transferred onto a Hybond (Amer sham, Amersham, UK) nylon membrane. After ultraviolet cross-linkage (UV Stratalinker 1800; Stratagene, La Jolla, CA), the membranes were hybridized sequentially to either the $^{32}$P-labeled nm23 probe or the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. Hybridizations were performed at 42°C in 50% formamide, 5 × SSC buffer, 5 × Denhardt's solution, 1% sodium dodecyl sulfate, and salmon sperm DNA (ssDNA) 0.1 mg/ml for 6 hours. The membranes were removed from the hybridization buffer and washed three times for 30 minutes each in 0.1 × SSC buffer and 1% sodium dodecyl sulfate at 60°C. Blot membranes were exposed on Kodak XAR film (Eastman Kodak, Rochester, NY) with an intensifying screen.

Probes for RNA Analyses

The DNA probes used in the analyses were a ~0.8-kb BamH1 restriction endonuclease fragment of human nm23-H1 cDNA from the plasmid pnm23-H1 (generously provided by Dr. Patricia Steeg, National Insti-
Densitometric Quantitation

The autoradiographic signal was quantified by a Computing Densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA). A complete digital image of each sample was scanned and measured as units of optical density \( \times \text{cm}^2 \) (OD \( \times \text{cm}^2 \)). Quantitative analysis was performed measuring the nm23-H1 and GAPDH autoradiographic signal of each sample; the ratio between these two values was taken as the expression level of nm23-H1 gene per unit of mRNA loaded onto the membrane.

Intracameral Transplantation and Hepatic Metastasis Determination

A modified quantitative technique for the orthotopic intracameral transplantation of precise numbers of tumor cells into the mouse eye has been described. Mice were deeply anesthetized with 0.66 mg of ketamine hydrochloride (Vetalar; Parke-Davis, Detroit, MI) administered intramuscularly. Tumor cells (10^5/5 \( \mu l \)) were inoculated intracameraly using a 1-ml Hamilton syringe fitted with a 35-gauge glass needle. Metastatic hepatic tumor foci were readily demonstrable by histopathologic examination of the liver and were scored as previously described. Severity of metastases was scored as: clear (0 = no discernible foci); minimal involvement (1+ = metastatic tumors involved <10% of the liver); moderate (2+ = metastatic tumors involved 10% to 25% of the liver); or extensive (3+ = metastatic tumor mass involved \( \geq \)25% of the liver).

Immunoperoxidase Staining for NM23 Antigen

The presence of the nm23 antigen (i.e., nucleoside diphosphate kinase A [NDPK-A]) was detected in tumor-bearing tissues by immunoperoxidase staining with a mouse monoclonal antibody specific for the human NDPK-A protein (Novocastra Laboratories, Newcastle, UK). The specificity of the Novoceastra anti-nm23–NDPK-A antibody has been demonstrated by Western blot and immunohistochemistry. This antibody does not react with other cytoplasmic proteins and forms a single 20 kDa band on immunoblots using a variety of tumor cells. Tumor-bearing eyes or liver specimens from all seven human uveal melanoma cell lines, were embedded in paraffin, cut in 4 \( \mu \)m sections, and mounted on superfrost-plus glass slides (Fisher Scientific, Pittsburgh, PA). Slides were deparaffinized with Hemo-de solution (Fisher Scientific) and rehydrated with gradient alcohols and distilled water. Slides were incubated with 0.4% trypsin for 30 minutes at 37°C, washed in phosphate-buffered saline (PBS)–Tween 20, and treated with hydrogen peroxide in absolute methanol (1 ml 30% hydrogen peroxide/9 ml methanol for 10 minutes) to inactivate intrinsic peroxidase activity. The slides were rinsed three times in PBS and incubated with serum blocking solution for 10 minutes at 37°C, followed by incubation for 60 minutes at 37°C with a 1:100 dilution of mouse monoclonal antibody directed against nm23/NDPK-A protein. The tumor slides were washed in PBS and exposed to biotin-labeled, horse anti-mouse immunoglobulin G (Zymed Laboratories, San Francisco, CA) for 30 minutes at 37°C. Slides were washed three times in PBS, exposed to substrate-chromogen mixture for 5 minutes at 37°C, washed three additional times in PBS, counterstained with hematoxylin, dehydrated, and mounted with a coverslip. Slides were examined microscopically, and at least 100 tumor cells were scored per X40 field of five random fields selected for each specimen. Immunoreactivity was graded as: 0 = no positive cells; 1+ = 5% tumor cells positively stained; 2+ = 6% to 30% tumor cells demonstrated moderate immunoreactivity; 3+ = >30% tumor cells showed strong immunoreactivity.

Statistics

The Pearson’s correlation coefficient was used to evaluate the correlation between the paired values of the nm23 RNA expression and the metastatic potential. The Wilcoxon test was used for survival analysis.

RESULTS

NM23-H1 mRNA Expression

Northern blot hybridization detected a 0.8 kb mRNA that corresponded to transcripts of nm23-H1 in specimens from all seven human uveal melanoma cell lines, the cell line from liver metastasis (L-OCM1), the normal keratocyte cell line, and both murine cutaneous melanoma cell lines (Fig. 1). Blots were rehybridized to GAPDH, which served as an internal standard for the amount of mRNA loaded per lane on the blot. The nm23:GAPDH ratio in L-OCM1 cell line was arbitrarily assigned a value of 1.00, and the corresponding ratios in each human uveal melanoma cell line were adjusted against the value for the L-OCM1 cell line. Densitometric quantitation concluded that seven human uveal melanoma cell lines expressed nm23 RNA levels ranging from 1.15 to 7.39 (Table 1). nm23-H1 expression in the normal human keratocyte cell line was very low, approximately the same as in the uveal melanoma.
NM23-H1 Expression Correlates With Liver Metastasis

Animal experiments were used to analyze the metastatic potential of the various human uveal melanoma cell lines. Uveal melanoma cells were transplanted intracamerally into nude mice, and the growth of the transplanted tumors was monitored. All transplanted tumors, except EOM3, grew progressively and filled the entire anterior chamber between 14 and 30 days after transplantation. EOM3 melanomas grew transiently in the anterior segments of nude mice and resolved by day 14. All mice were necropsied on day 50, and the liver metastatic foci were detected histopathologically. The incidence of hepatic metastases ranged from 0% to 80% (Table 2). Extensive liver metastases were detected in 80% of mice bearing OCM1 and 92-1 intraocular melanomas. Liver metastases were small and infrequent (<20% of the animals) in mice with OCM3, OCM8, and MEL202 intraocular melanomas. No metastatic liver foci were detected in hosts bearing intraocular EOM3 and OM431 tumors and were necropsied on day 50. Data in Tables 1 and 2 demonstrate an inverse relationship between the development of liver metastases and nm23-H1 gene expression. The close relationship between nm23-H1 gene expression and reduced metastatic incidence was confirmed by Pearson's correlation coefficient (Fig. 2). The correlation coefficients between the nm23-H1 expression and reduced metastatic incidence was -0.975 (P < 0.01).

Immunohistochemical Analysis of Human Uveal Melanoma Using mAb nm23—NDPK-A

The presence of the nm23 gene product was confirmed by immunohistochemistry. Examination of intraocular melanomas in nude mice revealed that nm23-H1 antigen was expressed diffusely in the cytoplasm (Fig. 3). OCM1 and 92-1 expressed low levels of nm23-H1 mRNA in vitro and comparably low quantities of the nm23 antigen in vivo. Similarly, OCM3, OCM8, OM431, and MEL202 had comparatively high nm23 mRNA and displayed intense positive staining for the nm23 antigen on the intraocular melanomas (Table 3). Thus, the results reveal a close correlation between transcription and translation of the nm23-H1 gene in human uveal melanomas and reduced metastatic potential. The relationship between low nm23

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**TABLE 2. Incidence and Severity of Liver Metastases in Athymic Nude Mice**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Severity of Metastatic Foci*</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCM1</td>
<td>3+ 3+ 3+ 1+ 0</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td>OCM3</td>
<td>0 1+ 0 0 0</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>OCM8</td>
<td>0 0 1+ 0 1+</td>
<td>2/5 (40)</td>
</tr>
<tr>
<td>EOM3</td>
<td>0 0 0 0 0</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>92-1</td>
<td>3+ 0 2+ 2+ 3+</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td>OM431</td>
<td>0 0 0 0 0</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>MEL202</td>
<td>0 0 1+ 0 0</td>
<td>1/5 (20)</td>
</tr>
</tbody>
</table>

1+ = minimal; 2+ = moderate; 3+ = extensive.
* Scores are for individual animals.

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NM23 Gene Expression and Uveal Melanoma Metastasis

expression and metastasis was demonstrated further by the faint and sparse immunoreactivity of liver metastases (data not shown).

NM23-H1 Expression and Survival of Intraocular Melanoma-Bearing Mice

The ultimate test as to whether nm23 expression influences metastatic disease is to evaluate survival times in hosts harboring low and high nm23 expressing intraocular melanomas. This was tested by transplanting high and low nm23-expressing melanomas into the eyes of athymic nude mice. Mice were observed for a 90-day period, and the survival times for each group were plotted as a Kaplan-Meier survival graph (Fig. 4). Results demonstrate a clear demarcation between the high and low nm23 expression groups (P < 0.005). Hosts bearing low nm23-expressing tumors (OCM1 and 92-1) had a mean survival time of 67 days, and none survived beyond day 85. Death was presumed to be caused by liver metastasis because both OCM1 and 92-1 melanomas produce extensive liver (but not pulmonary) metastasis after intracameral transplantation (data not shown).

DISCUSSION

Mounting evidence suggests that nm23 may act as a metastasis suppressor gene in some forms of cancer.11 Studies on biopsies from various human tumors have shown a positive correlation between nm23 mRNA levels and clinical parameters, indicative of a good prognosis.15,16 Moreover, the diminution in the intensity of immunohistochemical staining of the nm23 antigen in primary tumors was found to be correlated closely with tumor progression.59,60 Similar observations were made in the current study using a murine model of human uveal melanoma. Uveal melanomas expressing high levels of nm23 RNA developed fewer and smaller liver metastases compared to low nm23-expressing uveal melanomas. Moreover, the degree of immunohistochemical staining of the nm23 antigen in the primary intraocular melanomas was inversely correlated with the development of liver metastases. Immunohistochemical staining of liver metastases confirmed the prediction that metastatic foci would express only low levels of the nm23 antigen. Results from the survival study provided the most convincing demonstration of the importance of nm23 in limiting the metastasis of uveal melanoma. Two uveal melanoma cell lines that expressed low levels of nm23 RNA were highly malignant, leading to the death of 100% of the experimental mice by day 85. By contrast, 60% of the hosts bearing the high nm23-expressing OM431 melanomas survived beyond 90 days, even though the intraocular tumors had perforated the globes and were growing progressively.

The mechanism whereby nm23 suppresses metastasis remains unclear. The nm23 gene product is identical to the NDP kinase A in human erythrocytes.28 It has been suggested that NDPK activity contributes to the antimetastatic effect of nm23 through its role in signal transduction.83 NDP kinases also influence microtubule assembly and disassembly.84 However, it appears that the putative antimetastatic effects of nm23 are not caused by antiproliferative effects on the primary tumor because transfection of highly metastatic cutaneous murine melanomas with nm23-H1 cDNA significantly reduced metastases without affecting tumor cell growth rate.85 Moreover, the level of nm23 mRNA expression in biopsies from patients with skin melanoma did not correlate with tumor size or location.14 Results from the current study reinforce this conclusion. 92-1 uveal melanoma cells expressed low levels of nm23 RNA and produced extensive metastases, yet they grew slowly as primary intraocular tumors. Conversely, OM431 uveal melanoma cells expressed high levels of nm23 RNA and did not metastasize, even though the tumors grew rapidly within the eye. A similar relationship was found with two sublines of B16 murine melanoma. The highly metastatic Queens...
melanoma expressed low levels of nm23 RNA compared to the nonmetastatic D5.1G4 melanoma, even though both tumors grow at approximately the same rate within the eye.

The time-honored association between morphologic features of uveal melanomas and malignant potential suggests that epithelioid melanomas display greater metastatic behavior and express lower levels of nm23. However, this was not the case. There was no clear-cut correlation between uveal melanoma cell
TABLE 3. Correlation Between nm23 Expression and Uveal Melanoma Metastasis

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>RNA Expression</th>
<th>Metastasis Incidence (%)</th>
<th>nm23 Immunoreactivity*</th>
<th>Day Tumor Occupied 100% of AC†</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCM1</td>
<td>1.15</td>
<td>80</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td>OCM3</td>
<td>5.6</td>
<td>20</td>
<td>++</td>
<td>30</td>
</tr>
<tr>
<td>OCM8</td>
<td>4.59</td>
<td>40</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td>EOM3</td>
<td>7.39</td>
<td>0</td>
<td>ND</td>
<td>No growth</td>
</tr>
<tr>
<td>92-1</td>
<td>1.77</td>
<td>80</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>OM431</td>
<td>5.94</td>
<td>0</td>
<td>++</td>
<td>20</td>
</tr>
<tr>
<td>MEL202</td>
<td>5.21</td>
<td>20</td>
<td>+++</td>
<td>30</td>
</tr>
</tbody>
</table>

ND = not determined.
* Immunoreactivity of primary intraocular tumors as determined by immunoperoxidase.
† Tempo of intraocular tumor growth scored by biomicroscopy. The time required for each tumor to occupy 100% of the anterior chamber of the eye is listed in days and represents the mean for five mice.

morbidity and metastatic potential. In fact, one of the most metastatic uveal melanoma cell lines, OCM1, was comprised predominantly of cells with a spindle morphology, whereas a low-metastasizing melanoma cell line, OCM3, displayed an epithelioid morphology. However, caution must be exercised when using morphologic characteristics alone in predicting the malignant potential of uveal melanomas. McLean and co-workers reappraised 192 specimens in the Registry of Ophthalmic Pathology at the Armed Forces Institute of Pathology and found that most tumors, originally classified as spindle A, contained a significant number of spindle B cells. In some cases, the percentage of spindle B cells was as high as 50%. Although spindle cell uveal melanomas are less malignant than tumors comprised predominantly of epithelioid elements, spindle cell melanomas can be metastatic and fatal. In one retrospective study, 11 of 75 (i.e., 15%) patients diagnosed with spindle cell uveal melanomas died of metastases. Moreover, spindle cells frequently are observed in metastatic uveal melanoma foci, and there is at least one report of liver metastasis comprised of pure populations of spindle A cells. Thus, predicting the metastatic behavior of uveal melanomas should incorporate a variety of parameters, including Callender classification of the histologic type, morphometric criteria such as standard deviation of nucleolar area and the mean largest nuclear diameter, and even a criterion as simple as the diameter of the tumor in situ. Results from the current study suggest that molecular tools can be useful in predicting the metastatic potential of uveal melanomas, especially in tumors that exhibit highly malignant behavior in spite of expressing morphologic features suggestive of a benign phenotype.

The results reported here strongly suggest that nm23 expression correlates with reduced metastasis of uveal melanomas and can be used as a prognostic tool for predicting metastatic potential. Although this association has been made with other tumors, such as breast cancer and skin melanoma, a similar correlation was not found in colon cancer or lung cancer. However, caution must be exercised in interpreting nm23 antigen expression in uveal melanoma cell lines and biopsy specimens. Although we found only weak immunohistochemical expression of the nm23–NDPK-A antigen in intraocular 92-1 uveal melanomas in nude mice, Luyten et al (manuscript in preparation) have observed intense immunohistochemical staining of the same cell line in vitro. This discordance in immunologic staining of nm23 antigen may be the result of the use of different anti-nm23–NDPK-A antibodies for immunohistochemistry. In the current study, we used a mouse monoclonal antibody directed against nm23–NDPK-A, whereas Luyten et al...
used a rabbit polyclonal anti-nm23–NDPK-A antiserum that may have reacted with more epitopes than our monoclonal antibody, thereby producing stronger immunoreactivity. It is also possible that the 92-1 melanoma cell cultures underwent phenotype drift in vitro. Because the 92-1 melanoma cell line originally contained both epithelioid and spindle elements, it is possible that our in vitro culture techniques led to the selection of a highly malignant subpopulation of cells with a low nm23 phenotype, whereas the culture methods of Luyten and co-workers selected for a high nm23 expressing cell population. Nonetheless, the results from the current study demonstrate a strong correlation between nm23 expression and reduced metastatic behavior in an experimental model of human uveal melanoma.

Key Words
melanoma, metastasis, NM23, tumor suppressor gene, uvea

References
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