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

Ding Ma,* Gregorius P. Luyten,† Theo M. Luider,† Martine J. Jager,‡ and Jerry Y. Niederkorn*  

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 immuno-histochemistry to detect nm23 antigen. Each tumor cell line was transplanted intracamerally 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 intracamerally. Host survival time also was correlated with the degree of nm23 gene expression.

Conclusions. The expression of nm23 mRNA and nm23 antigen in human uveal melanomas is correlated closely with reduced metastatic behavior in experimental animals and may serve as a sensitive prognostic indicator of malignancy and survival in patients with uveal melanomas.


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 with high and low metastatic potential.12 Murine melanoma cell lines with high metastatic potential expressed 10 times less nm23 mRNA than low metastatic cell lines.12 Since its original description in murine melanoma, nm23 gene expression has been associated with reduced meta-

From the *Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas; the †Department of Ophthalmology, Erasmus University Rotterdam; and the ‡Department of Ophthalmology, Leiden University Hospital, Leiden, Netherlands.

Supported by National Institutes of Health grants CA30276 and by an unrestricted grant from Research to Prevent Blindness, New York, New York.

Submitted for publication February 12, 1996; revised May 6, 1996; accepted June 26, 1996.

Proprietary interest category: N.

Reprint requests: Jerry Y. Niederkorn, Department of Ophthalmology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-9037.
We have found that the source of athymic mice can
from the eye and form progressive liver metastases in
growing metastases in athymic nude mice purchased
from Simonsen Laboratories (Gilroy, CA). However,
Mice
provided by Dr. Bruce Ksander (Schepens Eye Institute,
MATERIALS AND METHODS
The role of the nm23 gene in human uveal melano-
somes. In particular, we have reported17 that
OCM-1 melanoma cells do not form progressively
mentastases in athymic nude mice purchased from Jackson Laboratories (Bar
Habor, ME). Accordingly, female athymic nude
Mice
at 8 to 10 weeks of age. The use of animals con-
son) and is predominantly epithelioid.
OCM1, OCM3, OCM8, and OM431, 22 92-1,  OM431,  and
OM431 was generously provided
23 OCM1, OCM3, OCM8, and OM431 cells were cul-
OM431 was generously provided
by Dr. Daniel Albert (University of Wisconsin, Madi-
ogenerously provided by Dr. June Kan-Mitchell (Uni-
MAL1 melanoma cell lines and evaluated the metastatic potential of the re-
pective melanoma cells after intracameral trans-
planted by Dr. YuGuang He (University of Texas Southwestern Medi-
cellular differentiation. OCM1, OCM3, OCM8, and OM431, and
MEL202 were used. OCM1, OCM3, and OCM8 were
generously provided by Dr. June Kan-Mitchell (Uni-
University of California, La Jolla, CA). OCM1 has a pre-
ominantly spindle morphology, whereas OCM3 and
OCM8 are predominantly epithelioid.18,19 The EOM3
cell line displays an epithelioid morphology in vitro
but was derived from a posterior choroidal melanoma
of mixed cell morphology.20 MEL202 was kindly pro-
duced by Dr. Bruce Ksander (Schepens Eye Institute,
Boston, MA) and is comprised predominantly of spin-
Cell Lines and Culture
Seven human uveal melanoma cell lines, designated
OCM1, OCM3, OCM8, EOM3, 92-1, OM431, and
MEL202, were used. OCM1, OCM3, and OCM8 were
merged from Dr. J. Szalay (Queens College, Queens, NY).
This cell line readily metastasizes when injected intra-
evously or when transplanted intracameral.
This cell line readily metastasizes when injected intrave-
vously or when transplanted intracameral.24,25 The
 origin, characterization, and cultivation of the D5.1G4
melanoma cell line has been described by Knisely and
Niederkorn.26 This mutant cell line possesses low met-
astatic activity when injected intravenously or intra-
acameral into euthymic C57BL/6 mice. A human ker-
atocyte line, HK/10, was generously provided by Dr.
RNA Analysis
Total RNA was extracted from 107 to 108 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–agarose gels and transferred onto a Hybond
(Amersham, Amersham, UK) nylon membrane. After ultravi-
oret cross-linkage (UV Stratallinker 1800; Strategene,
La Jolla, CA), the membranes were hybridized sequen-
tially to either the 32P-labeled nm23 probe or the glyc-
eraldehyde 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 mem-
RNA Analysis
The DNA probes used in the analyses were a ~0.8-kb
BamHI restriction endonuclease fragment of human
nm23-H1 cDNA from the plasmid pnm23-H1 (gener-
ously provided by Dr. Patricia Steeg, National Insti-
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.

**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.
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+ 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>

* Scores are for individual animals.

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
NM23 Gene Expression and Uveal Melanoma Metastasis

NM23 Gene Expression and Uveal Melanoma Metastasis

NM23 RNA Expression Index

Liver Metastasis (%)

FIGURE 2. Direct linear relationship between the levels of nm23-H1 mRNA expression and the incidence of hepatic metastases after intracameral transplantation of seven human uveal melanoma cell lines and one liver metastasis (L-OCM1) cell line into athymic mice. The nm23-H1 expression index and the liver metastatic incidence were plotted from data shown in Tables 1 and 2. The nm23-H1 gene expression levels were related directly to the liver metastatic incidence as evaluated by Pearson’s correlation coefficient test ($r = 0.975; P < 0.001$; regression line $Y = 99.65 - 14.75 X$). There were five mice in each experimental group. Values for mRNA expression are derived from Table 1, and percent liver metastasis are derived from Table 2. $\bullet = OCM1$; $\bigcirc = 92-1$; $\square = OCM8$; $\Box = MEL202$; $\Delta = OCM3$; $\triangle = OM431$; and $\blacktriangledown = EOM3$.

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). By contrast, 60% of the hosts harboring the high nm23 expressing OM431 melanoma were alive at the end of the 90-day observation period.

DISCUSSION

Mounting evidence suggests that nm23 may act as a metastasis suppressor gene in some forms of cancer. Studies on biopsies from various human tumors have shown a positive correlation between nm23 mRNA levels and clinical parameters, indicative of a good prognosis. 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. 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. It has been suggested that NDPK activity contributes to the antimetastatic effect of nm23 through its role in signal transduction. NDP kinases also influence microtubule assembly and disassembly. 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. Moreover, the level of nm23 mRNA expression in biopsies from patients with skin melanoma did not correlate with tumor size or location.

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
mRNA expression and uveal melanoma metastasis

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 ACf†</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>7.99</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.

mRNA expression 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 coworkers reappraised 132 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-NDPKA 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-NDPKA antibodies for immunohistology. In the current study, we used a mouse monoclonal antibody directed against nm23-NDPKA, 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 921 melanoma cell cultures underwent phenotype drift in vitro. Because the 921 melanoma cell line originally contained both epithelioid and spindle elements, it is possible that 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**

NM23 Gene Expression and Uveal Melanoma Metastasis


