Expression Level of the \textit{nm23} Gene in Clonal Populations of Metastatic Murine and Human Neoplasms\textsuperscript{1}

Robert Radinsky, Herschel Z. Weisberg, Alexander N. Staroselsky, and Isaiah J. Fidler\textsuperscript{2}

Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

The purpose of this study was to determine whether \textit{nm23} steady-state mRNA expression levels correlate with metastatic potential of mouse K-1735 melanoma cells, human KM12 colon cancer cells, and human SN12 renal cancer cells. Since neoplasms are heterogeneous and contain subpopulations of cells with different metastatic potentials, we analyzed multiple sets of nonmetastatic and metastatic clones isolated from each neoplasm. In addition, we also examined nine somatic cell hybrids produced by the fusion of nonmetastatic and metastatic K-1735 clones. In the mouse melanoma, we found heterogeneity in \textit{nm23-Hl} steady-state expression levels among the clones and hybrids that did not correlate with their metastatic phenotype. Clones isolated from human colon or renal carcinomas expressed similar levels of \textit{nm23-Hl} regardless of metastatic potential in nude mice. All of the human tumor cells were heterozygous for the \textit{nm23-Hl}-specific allelic DNA fragments, with no allelic deletions or gross alterations detected. Since the failure of tumor cells to produce metastasis can be due to multiple deficiencies, these data stress the importance of using independent clones with different metastatic potentials for the analysis of gene regulation of this process.

Introduction

To produce clinically relevant metastases tumor cells residing in a primary neoplasm must complete a complex series of linked sequential steps (1). Continuous processes such as tumor progression and each distinct step in metastasis are likely to be regulated by transient or permanent alterations in different genes at the DNA, mRNA, and/or protein levels. Moreover, specific types or combinations of gene products may differ among tumors of different etiology and histological origin (2–7).

The identification and characterization of genes (or their protein products) that regulate metastasis have been facilitated by advances in molecular techniques and the availability of tumor cell clones and lines with distinct metastatic capabilities isolated from heterogeneous rodent and human neoplasms (8–17). One such tumor system is the K-1735 melanoma syngeneic to C3H/HeN mice (18). This tumor is biologically heterogeneous and contains clonal populations with qualitative differences in metastatic capacity in syngeneic mice (18–21). In this tumor, the metastatic phenotype predominated in the majority of somatic cell hybrids produced by fusing various nonmetastatic and metastatic clones (22). Whether this was due to the activation of dominant genes or to the deactivation of suppressor genes or both is unclear.

Differential hybridization of nonmetastatic and metastatic K-1735 clones identified \textit{nm23-I} as a candidate suppressor gene for metastasis (8, 23). mRNA levels of the \textit{nm23-I} gene were found to be approximately 10-fold higher in two low-metastatic-potential K-1735 clones (C-16 and C-19) compared with two highly metastatic clones (M4, M2, and three variant cell lines selected from M2) (8, 24). Analyses of additional rodent tumor cell lines and human breast carcinomas have also correlated reduced \textit{nm23} mRNA expression with high metastatic potential (25–30). These findings cannot be generalized, since human colon carcinomas were found to exhibit enhanced \textit{nm23-Hl} mRNA expression compared with normal mucosa (31), and increased \textit{nm23-Hl} protein levels were found in advanced-stage neuroblastoma (32). Regardless of clinical staging, increased \textit{nm23-Hl} protein activity (i.e., NDP\textsuperscript{3} kinase A) was reported for most human tumors, including noninvasive and invasive ductal breast carcinomas, colon, and cervix carcinomas (33–35).

A possible explanation for the discrepancy in correlations of \textit{nm23-I} with metastasis could be the differences in the heterogeneous neoplasms used. To avoid the problem of intertumoral heterogeneity (1), we assessed \textit{nm23-I} steady-state mRNA expression levels in multiple nonmetastatic and metastatic clones and somatic cell hybrids of the K-1735 melanoma. We also examined \textit{nm23-Hl} mRNA expression and allelic deletion in the well-characterized human SN12 renal carcinoma (36–39) and human KM12 colon carcinoma (40–43). Both tumors exhibit metastatic heterogeneity, and clonal populations isolated \textit{in vitro} or \textit{in vivo} (in nude mice) are available. In all three tumor systems, \textit{nm23} steady-state expression levels did not invariably correlate with the metastatic or nonmetastatic phenotype.

Materials and Methods

K-1735 Marine Melanoma. The K-1735 melanoma, induced in a C3H/HeN mouse by exposure to UV light followed by painting with croton oil, was a gift of Dr. Margaret L. Kripke (M. D. Anderson Cancer Center) (18). The parental tumor was cloned \textit{in vitro} by a double-dilution cloning method (19). Clones 3, 19, and 23 (designated C-3, C-19, and C-23) are nonmetastatic or not very metastatic in immune-competent or immune-suppressed mice (18, 20). Clone 4 (designated C-4) is highly metastatic and produces melanotic foci in the lungs and lymph nodes of syngeneic mice. K-1735, M-2, and X-21 lines were derived from solitary spontaneous lung metastases produced by the K-1735 parental line growing subcutaneously (20); both are clonal in origin (44, 45) and highly metastatic in recipient mice (44).

K-1735 Somatic Cell Hybrids. Cell hybrids were produced as described previously (22). Briefly, drug resistance markers (G418 sulfate or hygromycin-B) were introduced into three nonmetastatic (C-3, C-19, C-23) and human KM 12 colon carcinoma (40–43). Both tumors exhibit metastatic heterogeneity, and clonal populations isolated \textit{in vitro} or \textit{in vivo} (in nude mice) are available. In all three tumor systems, \textit{nm23} steady-state expression levels did not invariably correlate with the metastatic or nonmetastatic phenotype.

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\textsuperscript{2}To whom requests for reprints should be addressed, at the Department of Cell Biology, Box 173, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030.

\textsuperscript{3}The abbreviations used are: NDP, nucleoside diphosphate; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; \textit{nm23-I}, murine gene; \textit{nm23-Hl}, human gene; cDNA, complementary DNA.
C-23) and three metastatic (C-4, M-2, X-21) clones. The somatic cell hybrids were generated by fusing nonmetastatic to metastatic cells. All hybrids were nearly tetraploid, containing a nearly complete chromosomal complement by both fusion partners (22). The metastatic capacity of these cell hybrids was characterized in both syngeneic and nude mice (23) and is summarized in Table 1.

**SN12 Human Renal Carcinoma.** The human SN12C cell line was isolated from a primary renal cell carcinoma that was granular in cell type with extensive invasion of periurethral fat (36). The highly metastatic SN12PM6 cell line was established from a spontaneous lung metastasis produced in a nude mouse by SN12C parental cells growing in the kidney (36-38). Clones 2, 4, and 8, which have different metastatic potentials, were isolated from the parental tumor by a double-limiting dilution method as described (38, 39).

**KM12 Human Colon Carcinoma.** The human KM12C cell line was isolated from a primary colon carcinoma classified as Dukes' stage B2 (40, 41). The highly metastatic KM12SM cell line was isolated from a spontaneous liver metastasis produced by parental KM12C cells growing in the cecal wall of a nude mouse. The highly metastatic KM12L4 cell line was selected by 4 cycles of intraperitoneal injections that lead to the production of experimental hepatic metastases. Low-metastatic-potential clones 1, 3, and 6 were isolated from the parental tumor by a double-limiting dilution method as described (42, 43).

### In Vitro Culture Conditions
All tumor cell lines were maintained in tissue culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, and 2-fold vitamin solution (Gibco, Grand Island, NY). Cell cultures were maintained on plastic and were incubated in 5% CO2/95% air at 37°C. Cultures were free of *Mycoplasma* and the following murine viruses: reovirus type 3; pneumonia virus; K virus; Theiler's encephalitis virus; Sendai virus; mouse adenovirus; mouse hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). Cultures were maintained for no longer than 4 weeks after recovery from frozen stocks.

**mRNA Analyses.** Total cellular RNA was extracted from 107-108 tumor cells growing in culture using a guanidinium thiocyanate/hot phenol method as previously described (46). For Northern blot analyses, polyadenylated RNA was purified by oligodeoxythymidine-cellulose chromatography, fractionated on 1% denaturing formaldehyde/agarose gels, electrotransferred to 0.6 A to GeneScreen nylon membrane (DuPont Co., Boston, MA), and UV cross-linked with 120,000 μJ/cm2 using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Hybridizations were performed as described (46, 47). Filters were washed three times at 65°C-68°C with 30 mM NaCl/3 mM sodium citrate, pH 7.2/sodium dodecyl sulfate (0.1% w/v).

**DNA Analyses.** DNA was extracted from 3-5 × 106 tumor cells growing in culture as previously described (47). Briefly, the cell pellet was resuspended in 630 μl of buffer: 55 mM Tris (pH 8.0), 110 mM EDTA (pH 8.0), and 110 mM NaCl. Proteinase K (0.5 mg/ml) and sodium dodecyl sulfate (0.5% w/v) were added to the cell suspension for incubation at 50°C for 8-18 h. The suspension was extracted once with phenol and multiple times with a phenol/chloroform solution and dialyzed against 30 mM Tris (pH 8.0), 10 mM EDTA, and 10 mM NaCl overnight at 4°C. RNAase A (70 μg/ml) was added for 1 h at 37°C. The digest was extracted once with phenol and then multiple times with a phenol/chloroform solution, with final dialysis against 10 mM Tris (pH 8.0), 1 mM EDTA for 18 h at 4°C. DNA digestions with the BglII restriction endonuclease were conducted at 37°C for 8 h, and Southern blot analysis/hybridization was performed as described (46, 48). Nytan nylon blots (Schleicher and Schuell, Inc., Keene, NH) were washed two times at 60°C with 15 mM NaCl, 1.5 mM sodium citrate (pH 7.2), sodium dodecyl sulfate (0.1% w/v).

**Probes for DNA and mRNA Sequences.** The DNA probes used in these analyses were: (a) either a 0.9-kilobase BamHI restriction endonuclease DNA fragment, a 0.45-kilobase *Hpall* DNA fragment, or the 0.6-kilobase *PstI*-BamHI gene fragment, each from the plasmid pmn23-1, containing the murine nm23-1 cDNA (8); (b) a ~0.9-kilobase BamHI restriction endonuclease fragment of human nm23-H1 gene from the plasmid pmn23-H1 (49); (c) a 1.8-kilobase BamHI cDNA fragment corresponding to the full-length human wild-type p53 gene (50); and (d) a 1.3-kilobase *PstI* gene fragment corresponding to rat GAPDH (51). Each DNA fragment was purified by agarose gel electrophoresis, recovered using GeneClean (BIO 101, La Jolla, CA), and radiolabeled by the random primer technique using [γ-32P]deoxyribonucleotide triphosphates (52).

### Northern Blot Analysis
nm23 mRNA expression was quantitated on an LKB ultrascan XL laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden) by averaging three separate measurements in the linear range of the film of the left, middle, and right areas of each nm23 transcript band as well as control GAPDH transcripts (46). Each sample measurement was calculated as the ratio of the average areas between the 0.8-kilobase nm23 transcript and the 1.3-kilobase GAPDH transcript.

### Results
**nm23-1 mRNA Expression in K-1775 Murine Melanoma Clones.** In the first set of studies, we determined whether nm23-1 gene expression inversely correlated with the metastatic potential of K-1775 clones and somatic cell hybrids. Northern blot analysis of three nonmetastatic K-1775 clones (C-3, C-19, and C-23) demonstrated expression of a ~0.8-kilobase nm23-specific transcript as well as a weak hybridizing transcript approximately 1.0 kilobase in size (Fig. 1A, *Lanes A, B, and E*). These two mRNA transcripts were reproducibly found using three different murine nm23-1 DNA fragments as probes (see "Materials and Methods") and after deletion of xylene cyanol from the sample gel-loading dye (8) (data not shown). Comparison of the nm23-1 expression level among the

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*Table 1 Expression level of nm23-1 mRNA in murine K-1775 melanoma clones and somatic cell hybrids with different metastatic potential*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Metastatic potential</th>
<th>Lung metastasis Median (range)</th>
<th>nm23-1 mRNA expression index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-19</td>
<td>Low</td>
<td>2 (0-3)</td>
<td>1.0</td>
</tr>
<tr>
<td>C-19N</td>
<td>Low</td>
<td>2 (0-6)</td>
<td>1.4</td>
</tr>
<tr>
<td>C-19H</td>
<td>Intermediate</td>
<td>2 (0-1)</td>
<td>1.3</td>
</tr>
<tr>
<td>C-19C</td>
<td>Low</td>
<td>2 (0-0)</td>
<td>0.8</td>
</tr>
<tr>
<td>C-3</td>
<td>Low</td>
<td>2 (0-2)</td>
<td>0.4</td>
</tr>
<tr>
<td>C-4</td>
<td>High</td>
<td>200 (180-280)</td>
<td>1.1</td>
</tr>
<tr>
<td>X-21</td>
<td>High</td>
<td>119 (100-163)</td>
<td>1.5</td>
</tr>
<tr>
<td>M-2</td>
<td>High</td>
<td>270 (180-350)</td>
<td>0.6</td>
</tr>
<tr>
<td>Somatic cell hybrids*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>Low</td>
<td>2 (0-2)</td>
<td>0.4</td>
</tr>
<tr>
<td>C-3H/C-4N</td>
<td>High</td>
<td>90 (73-107)</td>
<td>0.7</td>
</tr>
<tr>
<td>C-3N/X-21H</td>
<td>High</td>
<td>195 (130-210)</td>
<td>0.8</td>
</tr>
<tr>
<td>C-3MM/N-2N</td>
<td>High</td>
<td>84 (8-90)</td>
<td>0.3</td>
</tr>
<tr>
<td>C-19</td>
<td>Low</td>
<td>2 (0-3)</td>
<td>1.0</td>
</tr>
<tr>
<td>C-19H/C-4N</td>
<td>Intermediate</td>
<td>24 (2-59)</td>
<td>0.9</td>
</tr>
<tr>
<td>C-19N/X-21H</td>
<td>High</td>
<td>65 (24-120)</td>
<td>0.7</td>
</tr>
<tr>
<td>C-19H/MM-2N</td>
<td>Intermediate</td>
<td>34 (18-53)</td>
<td>0.8</td>
</tr>
<tr>
<td>C-23</td>
<td>Low</td>
<td>2 (0-6)</td>
<td>0.5</td>
</tr>
<tr>
<td>C-23H/C-4N</td>
<td>Low</td>
<td>0 (0-1)</td>
<td>0.2</td>
</tr>
<tr>
<td>C-23NN/X-21H</td>
<td>High</td>
<td>99 (72-104)</td>
<td>0.5</td>
</tr>
<tr>
<td>C-23MM/L-2N</td>
<td>Low</td>
<td>4 (2-5)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Quantiﬁcation of nm23-1 mRNA expression. The ratio of the areas between the 0.8-kilobase nm23-1 transcript and the 1.3-kilobase GAPDH transcript was compared for each case (see "Materials and Methods") with the low-metastatic-potential K-1775 C-19 cells deﬁned as 1.0. Low-metastatic-potential cells (2 × 106) were injected i.v. into syngeneic C3H/HeN mice as described. The mice were killed 45 days after injection, and the number of lung tumor colonies was determined (22). Somatic cell hybrids (2 × 106) were injected i.v. into syngeneic C3H/HeN mice as described. Moribund mice were killed 19-30 days after injection, and the number of lung tumor colonies was determined (22).*
three nonmetastatic clones revealed clone-specific variation (Fig. 1A; Table 1). Densitometric quantitation concluded that C-19 and C-23 expressed similar levels of nm23-1 transcripts, whereas C-3 had an approximate 60% reduction in steady-state levels compared with C-19 (see Table 1). GAPDH, a reference gene that is refractory to transcriptional induction by various agents and relatively constant among most tissues (53, 54), served as an internal standard for the amount of mRNA loaded per lane on the blot. Following transfection and stable integration of either pSV2Neo or pSV2Hygro nonmetastatic C-19 expressed slightly higher levels of nm23-1 mRNA transcripts as compared to untransfected C-19 (Fig. 1A, compare Lanes C and D with Lane B; Table 1). Similar results were observed with the other transfected nonmetastatic or metastatic K-1735 clones (data not shown).

Northern blot analyses of the three highly metastatic clones (C-4, X-21, and M-2) also revealed clone-specific differences in levels of nm23-1 mRNA expression (Fig. 1A, Lanes F–I; Table 1). For example, a 40% reduction in levels of nm23-1-specific transcripts was observed in highly metastatic clone M-2 as compared with nonmetastatic C-19 (Fig. 1A, compare Lane H with Lane B; Table 1). This result confirms a previous report of lower nm23 expression in M-2 than C-19 (8). However, highly metastatic C-4 and X-21 had similar or even increased levels of nm23-1 expression as compared with C-19 (Fig. 1A, compare Lanes F and G with Lane B; Table 1). Further analyses of nm23-1 mRNA expression in the metastatic clones at different in vitro passages confirmed these results (data not shown).

**nm23-1 mRNA Expression in K-1735 Somatic Cell Hybrids.** Previous studies from our laboratory indicated that the metastatic capacity of K-1735 cells predominate in somatic cell hybrids between nonmetastatic and metastatic K-1735 clones (22). To determine whether this predominance extends to the expression level of nm23-1, we analyzed multiple somatic cell hybrids produced by the fusion of nonmetastatic (C-3, C-19, and C-23) with metastatic (C-4, X-21, and M-2) clones. Fusion of nonmetastatic C-3 with metastatic C-4, X-21, or M-2 resulted in metastatic hybrids (Table 1). Densitometric quantitation demonstrated 1.75- and 2.0-fold increases in nm23-1 expression in metastatic hybrids C-3H/C-4N and C-3N/X-21H, respectively, as compared to nonmetastatic C-3 (Fig. 1B, compare Lanes B and C with Lane A; Table 1), whereas the level of nm23 expression in the metastatic hybrid C-3H/M-2N was similar to that of C-3 (Fig. 1B, compare Lane D with Lane A; Table 1). These results indicate that in somatic cell hybrids consisting of C-3 as the nonmetastatic partner, reduced nm23 expression did not correlate with increased metastatic potential.

We next analyzed six additional somatic cell hybrids produced by the fusion of nonmetastatic C-19 or C-23 with metastatic C-4, X-21, or M-2. All hybrids containing C-19 were metastatic, and their expression of nm23-1 was slightly reduced relative to C-19 (Fig. 1B, compare Lanes F–H with Lane E; Table 1). Analysis of C-23H/C-4N, C-23N/X-21H, and C-23H/M-2N hybrids also showed a reduction in nm23-1 levels as compared with C-19 (Fig. 1B, compare Lanes J–L with Lane E; Table 1) or the hybrids containing C-19 (Fig. 1B, compare Lanes J–L with Lanes F–H; Table 1). However, in this group of hybrids, C-23H/C-4N and C-23H/M-2N expressed the lowest levels of nm23-1-specific mRNA, and the metastatic C-23N/X-21H hybrid had a slightly higher level of nm23-1 expression (Table 1). These results confirm the intratumoral heterogeneity observed among individual clones of the K-1735 melanoma and lack of correlation between nm23-1 expression and production of metastasis.
nm23-H1 Expression Levels in Nonmetastatic and Metastatic Human Renal and Colon Carcinoma Cells. Northern blot analysis of SN12C renal carcinoma cells with high and low metastatic potential demonstrated similar expression levels of nm23-H1 transcripts (Fig. 2A, Lanes C–F). Densitometric quantitation of the 0.8-kilobase nm23-H1 transcript (utilizing GAPDH gene transcripts as an indicator of mRNA amount per lane) showed almost identical levels between SN12C parental, SN12PM6 (highly metastatic), and low-metastatic-potential SN12 Cl-4 or Cl-8 (Table 2). Regardless of their ability to produce metastasis in nude mice, all SN12 cells expressed 3-fold higher levels of nm23-H1 transcripts as compared with human placental tissue and 5-fold higher levels than an allogeneic normal kidney tissue (Fig. 2A, compare Lanes C–F with Lanes A and B; Table 2).

Human colon cancer cells with different metastatic behavior in nude mice had similar patterns of nm23-H1 mRNA expression (Fig. 2B, Lanes A–F). Highly metastatic liver-colonizing KM12SM and KM12L4 cells expressed identical levels of nm23-H1 gene transcripts that were higher than those observed for low-metastatic-potential KM12C cell line (Fig. 2B, compare Lanes B and C with Lane A; Table 2). Low-metastatic-potential Cl-1 and Cl-3 showed no detectable difference in steady-state expression levels of the nm23-H1 gene as compared with parental cells, whereas Cl-6 did (Fig. 2B, compare Lanes D, E, and F with Lane A; Table 2).

Analysis of Somatic Allelic Deletion of nm23-H1 in Human SN12 Renal and KM12 Colon Carcinoma Cells. A recent report has suggested that somatic allelic deletion of nm23-H1 gene sequences in primary human colorectal carcinoma can be associated with the formation of distant metastases and that alterations at or near the nm23-H1 locus localized at chromosome 17q21 were significant in this process (55, 56). To examine this possibility in our human tumor systems, Southern blot analysis of a BgII restriction fragment length polymorphism of chromosomal DNA isolated from SN12 renal carcinoma cells was carried out. The expected 2.3- and 7.6-kilobase nm23-H1 allelic fragments were identified (Fig. 3A, Lanes C–G). Similarly, both allelic bands were present in an allogeneic normal kidney tissue sample, whereas a human placental tissue sample was homozygous for the 7.6-kilobase allelic band (Fig. 3A, Lanes B and A). Rehybridization of the identical blot with a p53-specific cDNA probe (chromosome localization at chromosome 17p13) showed that each of the renal carcinoma cells and control tissues was homozygous for

Table 2 Quantitation of nm23-H1 mRNA expression in human SN12 renal carcinoma and KM12 colon carcinoma cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Metastatic potential</th>
<th>nm23-H1 mRNA expression index</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN12 (parental)</td>
<td>Intermediate</td>
<td>1.0</td>
</tr>
<tr>
<td>SN12PM6</td>
<td>High</td>
<td>1.0</td>
</tr>
<tr>
<td>SN12 clone 4</td>
<td>Low</td>
<td>1.0</td>
</tr>
<tr>
<td>SN12 clone 8</td>
<td>Low</td>
<td>1.2</td>
</tr>
<tr>
<td>Normal kidney</td>
<td>Not applicable</td>
<td>0.2</td>
</tr>
<tr>
<td>Human placenta</td>
<td>Not applicable</td>
<td>0.3</td>
</tr>
<tr>
<td>KM12C (parental)</td>
<td>Low</td>
<td>1.0</td>
</tr>
<tr>
<td>KM12SM</td>
<td>High</td>
<td>1.2</td>
</tr>
<tr>
<td>KM12L4</td>
<td>High</td>
<td>1.2</td>
</tr>
<tr>
<td>KM12 clone 1</td>
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<tr>
<td>KM12 clone 3</td>
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<td>1.1</td>
</tr>
<tr>
<td>KM12 clone 6</td>
<td>Low</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*a Determined subsequent to orthotopic implantation in nude mice (36–43).

*b Quantitation of nm23-H1 mRNA expression (see "Materials and Methods"). The ratio of the areas between the 0.8-kilobase nm23-H1 transcript and the 1.3-kilobase GAPDH transcript was compared in each case with the parental KM12C or SN12C cells defined as 1.0.
the 12.0-kilobase \( p53 \) allelic DNA fragment, which occurs 10 times more frequently than a 9.0-kilobase allelic fragment in both normal and malignant tissues (57, 58) (Fig. 3A, Lanes A'–G).

Southern analysis of the \( Bgl \) II restriction fragment length polymorphism in chromosomal DNA isolated from the KM12 colon carcinoma cells also demonstrated that, regardless of their metastatic potential, the cells were heterozygous for the \( nm23-H1 \) allelic fragments, i.e., all cells contained the 2.3- and 7.6-kilobase allelic DNA fragments (Fig. 3B, Lanes B–G). Rehybridization of this identical blot for \( p53 \)-specific gene sequences showed all cell types were homozygous for the 12.0-kilobase \( p53 \) allelic DNA fragment (Fig. 3B, Lanes A'–G).

Discussion

We assessed the steady-state mRNA levels of the \( nm23 \) gene previously implicated in the suppression of metastasis in some (8, 24–28) but not all (32–35) tumors. We reasoned that the well-documented biological heterogeneity of neoplasms (1) could contribute to the above discrepancy and that the use of clonal populations of cells with different metastatic properties isolated from heterogeneous neoplasms could overcome this problem. In particular, the present studies used K-1735 murine melanoma clones and somatic cell hybrids with different metastatic properties, and metastatic and nonmetastatic variant cells isolated from a human renal carcinoma and human colon carcinoma. In the K-1735 murine melanoma system, we found heterogeneity with respect to steady-state mRNA levels of the \( nm23-1 \) gene that did not correlate with their metastatic potential. In agreement with the original report (8), we also found that low-metastatic-potential C-19 expressed higher steady-state levels of the 0.8-kilobase \( nm23-1 \) mRNA transcripts than the highly metastatic clone M-2 (8, 24).

Since the process of metastasis consists of sequential, selective events, the inability of tumor cells to produce metastases could be due to different and independent deficiencies (1–3,21). For this reason, the validity of data showing that a factor can suppress or enhance metastasis must be ascertained in multiple clonal populations isolated from a heterogeneous malignant neoplasm (1, 3). Indeed, when we examined six different clones and nine different somatic cell hybrids of the K-1735 melanoma, we found that the cells expressed low or high levels of

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**Fig. 3. Southern blot analyses of human renal and colon carcinoma cell types.** \( Bgl \) II-digested chromosomal DNA isolated from either SN12 (A) or KM12 (B) cells was first probed with a 0.9-kilobase \( BamH\) human \( nm23-H1 \) DNA fragment (Lanes A–G), where allelic bands of 7.6 and 2.3 kilobases are identified as well as a 21-kilobase constant band (55, 56). The second probe, a human \( p53 \)-specific DNA fragment, was rehybridized to the identical stripped blots (Lanes A'–G). This probe detects allelic bands of 12.0 and 9.0 kilobases, with the former allelic band occurring 10 times more frequently in both normal and malignant tissues (see "Results"), as well as a nonallelic constant band at 3.2 kilobases (57, 58). Left, DNA fragment sizes estimated by using \( HindIII \)-digested \( \lambda \) DNA; right, size in kilobases of allelic DNA fragments. A, analysis of SN12 renal carcinoma cell types. Lanes: A, human placental tissue; B, unmatched normal human kidney tissue; C, SN12 parental cells; D, SN12PM6 highly metastatic cells; E, SN12 clone 2 cells; F, SN12 clone 4 low-metastatic-potential cells; G, SN12 clone 8 low-metastatic-potential cells. Lanes A'–G are identical to Lanes A–G. B, analysis of KM12 colon carcinoma cell types. Lanes: A, human placental tissue; B, KM12 parental cells; C, KM12L4 highly metastatic cells; D, KM12SPM highly metastatic cells; E, KM12 clone 1 low-metastatic-potential cells; F, KM12 clone 3 low-metastatic-potential cells; G, KM12 clone 6 low-metastatic-potential cells. Lanes A'–G are identical to Lanes A–G.
clones found hybrid-specific heterogeneity in nm23-l expression by the fusion of these nonmetastatic and highly metastatic cells regardless of their metastatic potential. For example, cently been described in human colorectal carcinomas (55, 56).

Transcripts. Similar analyses of somatic cell hybrids produced with a low protein level. This possibility has been ruled out by on nm23-l mRNA-expressed patterns (8). Since the nm23 gene properties, all cells demonstrated similar expression levels of the metastatic process must await more rigorous analysis.

levels were, suggesting independent regulation (59). Few pub nm23-H2 levels were not reduced to the extend that nm23-H1 levels were, indicating that protein levels correlate with NDP kinase A activity of the protein product. Recent evidence, however, indicated that a mutation in the nm23 gene affects the down-modulation of mRNA may not be associated with a low protein level. This possibility has been ruled out by studies in both the K-1735 melanoma and human ductal breast carcinoma, demonstrating that nm23 mRNA levels directly correlate with nm23 protein levels (49). These data therefore argue against translational control of the nm23 gene and support the utilization of steady-state mRNA levels as a measurement of the nm23 gene as presented in this report.

Data analyzing nm23-HI expression levels in human breast carcinomas suggested an inverse relationship between levels of mRNA expression and metastatic potential (26, 27) and that low nm23 protein expression in infiltrating ductal breast carcinoma correlated with reduced survival (28, 29). In contrast, recent analyses of NDP kinase level activity in human breast carcinomas concluded that this enzyme increases in all cancer cells (as compared with normal stroma) without correlation with lymph node metastasis (33, 34). Similarly, in human neuroblastoma, high levels of the nm23-HI protein homologue correlated with advanced-stage disease (32). Since the above studies were carried out in tumors of different patients and different histological origin, we analyzed two well-characterized human neoplasms: SN12 renal carcinoma (36-39) and KM12 colon carcinoma (40-43). Regardless of their metastatic properties, all cells demonstrated similar expression levels of nm23-HI mRNA transcripts. We did not find clonal heterogeneity in nm23-HI expression levels and metastatic potential. Similar to the report that colon cancers express more nm23-HI than normal colon mucosa (31), we found that all SN12 renal carcinoma cells expressed 3- to 5-fold higher levels of nm23-HI transcripts than normal kidney or placental tissue. Additionally, a second human gene has been identified, nm23-H2 (59). RNA blot analyses of human breast tumors indicated that nm23-H2 levels were not reduced to the extent that nm23-HI levels were, suggesting independent regulation (59). Few published reports have analyzed nm23-H2; thus its putative role in the metastatic process must await more rigorous analysis.

Another possible factor that can influence the interpretation of the data is that a mutation in the nm23 gene affects the activity of the protein product. Recent evidence, however, indicates that the amino acid sequence of NDP kinase A isolated from human erythrocytes is indistinguishable from the primary structure derived from the cDNA encoding the nm23-HI protein in tumors (60), suggesting that the nm23-HI gene is not mutated in tumor as compared with normal tissue (33, 34). Furthermore, an examination of 39 human tumors (of different histology) concluded that they contained higher levels of nm23-HI/NPD kinase A protein than did corresponding normal tissues, indicating that protein levels correlate with NDP kinase A activity levels (33, 34).

An association of somatic allelic deletions at or near the nm23-HI locus at chromosome 17q21 with metastasis has recently been described in human colorectal carcinomas (55, 56). Since recent data described higher nm23-HI mRNA expression in invasive colorectal carcinomas than paired normal colon mucosa (31), we tried to determine whether somatic allelic deletion of nm23-HI gene sequences could be found in the human colon and renal carcinoma systems. Parental, metastatic, and nonmetastatic cells contained no allelic deletions or gross alterations in nm23-HI sequences detectable by this analysis.

In summary, we did not find an association between nm23 expression level and metastatic potential of mouse melanoma, human renal carcinoma, and human colon carcinoma cells. Each discrete step of metastasis is likely to be regulated by transient or permanent changes at the DNA or RNA level in different genes that vary among tumors of different histological origin (1-6). The present data demonstrate a heterogeneity of nm23-1 mRNA expression in mouse tumor cells that is unrelated to their metastatic capability and emphasize that in order to determine a genetic regulation of metastasis, multiple, independent, well-characterized clones of any given tumor must be analyzed.

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References

nm23 GENE IN CLONAL METASTATIC MURINE AND HUMAN NEOPLASMS


Expression Level of the \textit{nm23} Gene in Clonal Populations of Metastatic Murine and Human Neoplasms


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