Elevation of Breast Carcinoma Nm23-H1 Metastasis Suppressor Gene Expression and Reduced Motility by DNA Methylation Inhibition

Melanie T. Hartsough,1,2 Susan E. Clare,1,3 Michael Mair, Abdel G. Elkahloun, Dennis Sgroi, C. Kent Osborne, Gary Clark, and Patricia S. Steeg

INTRODUCTION

Metastasis suppressor genes are defined by their inhibition of tumor cell line metastatic capability in vivo on transfection, without the inhibition of primary tumor size. Several of these genes are thought to affect the non-angiogenesis-dependent colonization of tumor cells at the metastatic site and, therefore, constitute interesting translational targets (1, 2). The nm23 gene family, currently containing eight members, exhibits metastasis suppressor activity. Transfection of nm23 cDNAs into metastatically competent breast carcinoma (3–5), melanoma (6–9), colon carcinoma (10), and oral squamous cell carcinoma (11) cell lines resulted in 40–98% decreased metastatic potential in vivo. In the MDA-MB-435 breast carcinoma model system, overexpression of nm23-H1 resulted in a 50–90% inhibition of metastasis in vivo, reduced colonization in soft agar under both normal and transforming growth factor-β stimulated conditions (3), inhibition of motility to a variety of attractants (12–14), and morphological and biosynthetic evidence of differentiation (15).

Tumor cohort studies indicate that reduced Nm23 expression is associated with aggressive breast carcinomas, although it is not an independent prognostic factor. Reduced Nm23 expression has been correlated with poor patient survival, nodal metastases, or aggressive histopathological criteria in 18 of 24 recent breast cancer cohort studies (reviewed in 16). In the only large node-negative cohort study reported, low Nm23-H1 expression was a significant predictor of poor survival in univariate and multivariate analyses (17), and comparison with other metastasis-associated proteins indicated that loss of Nm23-H1 is an early event (18). In another cohort study, side-by-side analysis of allelic deletion at nm23-H1, Nm23-H1 expression and patient survival revealed that Nm23-H1 expression, but not allelic deletion, was a predictor of poor patient clinical course. Moreover, mutations in the nm23-H1 coding sequence were not observed (19).

Because low nm23-H1 RNA and protein expression correlate with poor clinical course, we have focused on the regulation of nm23-H1 at the transcriptional level. One notable mechanism of transcription regulation that has been defined in cancer is aberrant methylation of genes. DNA methylation occurs at CpG dinucleotides on cytosine residues. Regions of a gene that contain a high frequency of CpG dinucleotides are defined as CpG islands. These islands are normally unmethylated except in particular instances such as X-chromosomal inactivation and genomic imprinting (20, 21). In the neoplastic process, hypermethylation of CpG islands within tumor and metastasis suppressor genes has been reported to silence a host of these genes by down-regulation of expression (reviewed in Refs. 22, 23). DNA methylation inhibitors, such as 5-Aza-CdR4, have been shown to demethylate these regions, thereby causing an increase in gene expression (22).

In this report, we examined the contribution of DNA methylation to the regulation of Nm23-H1 expression, based on the finding that the nm23-H1 promoter contains two CpG islands. We present evidence herein that the DNA methylation inhibitor 5-Aza-CdR increases the Nm23-H1 expression of 5 of 11 human breast carcinoma cell lines, including 3 of 3 with metastatic competence in vivo. Increased Nm23-H1 expression was accompanied by minimal growth inhibition, but significantly lower motility in vitro.

MATERIALS AND METHODS

Cell Lines, Tissue Specimens, and Reagents. RPMI 1640 supplemented with 10% FCS and glutamine (RPMI complete medium) and DMEM supplemented with 10 mm HEPES, 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (DMEM serum-free medium) was purchased from Life Technologies. Oligonucleotides were synthesized by Lofstrand Labs and Life Technologies. TSA and 5-Aza-CdR were obtained from Sigma. MDA-MB-231, MDA-MB-435, ZR-75–1, MCF7, HBL-100, MDA-MB-157, BT-474, MDA-MB-468, SK-BR-3 and MDA-MB-157 cell lines were obtained from American Type Culture Collection. MDA-MB-231 and MDA-MB-435 cells were also a generous gift of Dr. Janet Price (M.D. Anderson, Houston, TX), and equivalent results were obtained. The LCC15-MB cell line was a generous gift from Dr. Robert Clarke (Georgetown University, Washington D.C.). All of the cells were grown in RPMI complete medium at 5% CO2.

2 To whom requests for reprints should be addressed, at Cancer Genetics Branch, National Human Genome Research Institute, Bethesda, Maryland 20892 [A. G. E.]
3 Present address: Department of Surgery, Northwestern University School of Medicine, 300 East Superior Street, Chicago, IL 60611-3095.

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4 The abbreviations used are: 5-Aza-CdR, 5-aza-2’-deoxycytidine; ATRA, all-trans-retinoid acid; RAR, retinoic acid receptor; TSA, trichostatin A; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescence.

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2] To whom requests for reprints should be addressed, at National Cancer Institute, NIH, Building 10, Room 2A33, Bethesda, MD 20892. Fax: (301) 402-8910; E-mail: hartsou@box-h.nih.gov.

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were obtained from the Baylor University SPORE National Breast Cancer Tissue Resource. These tumors were designated exempt from further review by the NIH Office of Human Subjects Research. For the 20-tumor cohort, mean estrogen receptor expression was 109 fmol/mg protein (range, 0–763), progesterone receptor expression was 215 fmol/mg protein (range, 0–1270), and S phase was 7.7% (range, 0.9–21.9%). A direct correlation of estrogen receptor and progesterone receptor expression was observed in the cohort (Spearman correlation coefficient, 0.465; P = 0.039) as was an indirect correlation of progesterone receptor expression and S phase (Spearman correlation coefficient, 0.490; P = 0.028).

5-Aza-CdR Treatment. MDA-MB-231, MDA-MB-435, ZR-75-1, MC7, HBL-100, MDA-MB-157, BT-474, MDA-MB-468, LCC15-MB, SK-BR-3, and MDA-MB-157 cells were plated at densities of 2 × 10^4, 1 × 10^4, 5 × 10^3, 2.5 × 10^3, 4.0 × 10^2, 1.0 × 10^2, 2.0 × 10^1, 1 × 10^1, 3 × 10^0, and 4 × 10^0 cells/100 mm dish, respectively (three plates per cell line), in RPMI complete media. The medium was changed to fresh RPMI complete media on day 3. On day 4, one plate of each cell line was treated with RPMI complete media containing 0, 0.075, or 0.75 μM 5-Aza-CdR. The medium was changed on day 6, maintaining 5-Aza-CdR treatment. On day 7, the cells were harvested and counted via hemacytometer. The cells were then centrifuged at 1500 rpm, 4°C for 1 min, washed with cold PBS, reconstituted and resuspended in cold lysis buffer [50 mM Tris-HCl (pH 7.6), 5 mM EDTA, 150 mM NaCl, 0.1% NP40, 10 mM 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide], 1 mM PMSF, 10 mM NaF, 0.1 mM NaVO_3, and 10 μg/ml Pepstatin A]. The cells were incubated on ice for 10 min and then frozen at −80°C until needed.

For reversal experiments, MDA-MB-231 cells were plated at densities of 2 × 10^3, 1 × 10^3, 5 × 10^2, 2.5 × 10^2, and 2 × 10^1 cells/100-mm plate and incubated with 0.75 μM 5-Aza-CdR for 3 days (treatment started on day 2). On day 5, the media was changed to fresh RPMI complete media without 5-Aza-CdR. Cells were harvested on days 5, 7, 9, 11, and 13, respectively, resulting in the withdrawal of 5-Aza-CdR for 0, 2, 4, 6, and 8 days. Harvested cells were counted and lysed, as described previously, and frozen at −80°C until needed.

For immunoblotting, lysates were thawed and centrifuged at 13,000 rpm, 4°C, for 15 min to remove particulate matter. One × 10^6 cells/sample were separated on 15% SDS/PAGE gels, transferred to PVDF, and immunoblotted with anti-Nm23-H1 (Ab1; Ref. 24) or anti-E-cadherin (H-108; Santa-Cruz) for 1 h at room temperature or 4°C, overnight, respectively, followed by a secondary incubation with IgG-horseradish peroxidase conjugate for 1 h at room temperature. Nm23-H1 and E-cadherin proteins were visualized by ECL (Amersham). Protein densities of Nm23-H1 were determined by densitometry (AlphaEase 5.1; Alpha Innotech Corp.).

TSA Treatment. MDA-MB-231 and MC7 cells were plated, as described above, except that the cells were either untreated or treated with TSA (0.3 μM) or 5-Aza-CdR (0.75 μM), alone or in combination. 5-Aza-CdR was added to the cells on day 4, and TSA was added on day 6. On day 7, the cells were harvested and counted. Nm23-H1 protein expression was then determined as described for 5-Aza-CdR treatment.

Tumor Protein Analysis. Tumor tissue (50–100 mg) was lysed in 1% SDS, vortexed vigorously, boiled for 3 min, vortexed, and spun at room temperature for 10 min at 14,000 × g. Protein concentration of the supernatant was determined by the BCA method (Pierce). Fifty μg of protein were loaded on a 15% SDS-PAGE gel and transferred to PVDF. Nm23 (Ab11) protein expression was determined by ECL. Data were analyzed by densitometry of the Nm23-H1 band (AlphaEase 5.1).

Cell Motility Assay. Cell motility was determined using 48-well Boyden chambers (Neuro Probe; Ref. 13). The chemoattractant, FCS (0.01%, 0.5%, and 0.1%), was placed in the lower wells of the chamber and the chambers were sandwiched between the upper and lower wells of the chamber. MDA-MB-231 and MDA-MB-435 cells were plated and treated with 5-Aza-CdR, as described above. After a 3-day incubation, cells were trypsinized, rinsed once in PBS, and resuspended in DMEM serum-free media at a density of 2.0 × 10^5 cells/ml. Fifty thousand cells were added to the upper wells of the chamber, and the chambers were incubated for 3 h at 37°C in a humidified 5% CO_2 incubator. The filters were then stained with Diff Quick (Baxter Healthcare Corp.), and the migrating cells counted by light microscopy in 6–9 fields at ×20. Data for a particular concentration represents the mean of three replicate wells.

Proliferation Assay. Cell viability was determined by using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega), as per manufacturer’s instructions. Briefly, cells were plated in a 96-well plate at 5000 cells/well in 75 μl and were incubated overnight at 37°C, 5% CO₂. The next day, the cells were treated, in triplicate, with 0, 0.075, or 0.75 μM, 5-Aza-CdR (final volume of 100 μl) and incubated for 3 days at 37°C, 5% CO₂. On the 3rd day, 15 μl of dye solution were added to each well, and the cells were incubated at 37°C, 5% CO₂ for 3 h. Solubilization/stop solution (100 μl) was then added to each well, and the plates were incubated overnight at room temperature for complete color development. Cell viability was then determined by spectrophotometry (562 nm) kinetic microplate reader (Molecular Devices). Cell proliferation was calculated as mean percentage of control (no 5-Aza-CdR); data represent the mean of three replicate wells.

Sodium Bisulfite Treatment of DNA. Genomic DNA was extracted from one 150-mm plate of cells treated with or without 0.75 μM 5-Aza-CdR (as described above) using the Nucleon 8Acc-2 Kit (Pharmacia) and from 25 mg of tumor tissue using the DNAeasy Tissue Kit (Qiagen), as per instructions. One μg of DNA was then subjected to sodium bisulfite modification using the CpGenome DNA modification kit (Intergen), as per manufacturer’s instructions. Universal methylated DNA was used as the positive control (Intergen). Treated DNAs were stored at −20°C until needed.

Sodium Bisulfite DNA Sequencing. Bisulfite converted DNA (∼50 ng) was amplified with 5’ primer: tattgggctggtatggaggg (−3658 bp to −3634 bp) and 3’ primer: cccaaacactccaaactaat (−3337 bp to −3358 bp). These primers encompass a region within the most 5' CpG island (−3634 bp to −3337 bp) of the promoter, generating a 321-bp PCR product. Conditions for the PCR were 1 cycle at 94°C for 10 min; 25 cycles at 94°C for 30 s, at 60°C for 1 min, and at 72°C for 2 min, and 1 cycle at 72°C for 7 min. The PCR product was cloned into the TA vector pCR2.1 (Invitrogen). Ten subclones were confirmed by restriction analysis and sequenced using the M13 reverse primer (Invitrogen) with a Prism 377 DNA Sequencer (Perkin-Elmer Applied Biosystems).

Microarray Analysis of MDA-MB-231 Cells. Poly(A) RNA was extracted from two 150-mm plates of MDA-MB-231 cells (treated or untreated with 5-Aza-CdR for 3 days) using the FastTrac2.0 mRNA isolation kit (Invitrogen). Microarray hybridization was then performed with 1 μg of DNAse-treated poly(A) RNA, as described previously (25). Briefly, the RNA was reverse-transcribed in the presence of 50 μCi of [3P]dCTP or 50 μCi [3P]dATP, 500 ng of oligo-dT, and 200 units of Superscript II RT (Life Technologies, Inc). The second strand was synthesized in the presence of 50 μCi [3P]dCTP or 50 μCi [3P]dATP, 500 ng of random hexamers, and 2500 units of large DNA polymerase I (Life Technologies, Inc). The labeled DNA was denatured and hybridized to the GF211 GeneFilter as described previously (25). The filters were then exposed on a Packard screen and scanned at 50-μm resolution using a phosphorimager instrument (Cyclone Storage Phosphor Imager; Packard Inc.). The tiff images resulting from the phosphorimager were directly imported into the image analysis software Pathways and analyzed (Research Genetics, Inc).

Statistical Analysis. Student’s t test was used for the analysis of motility data.

RESULTS

The DNA Methylation Inhibitor 5-Aza-CdR Elevates the Nm23-H1 Expression of Human Breast Carcinoma Cell Lines in Vitro. A schematic of the nm23-H1 promoter is shown in Fig. 1. The initiating ATG is located in exon 2, preceded by a large intron. A small exon 1 contains the transcriptional initiation site. The frequency of CpG dinucleotides through the nm23-H1 promoter is shown in Fig. 1. Two areas of high CpG dinucleotide frequency occur: (a) bases, −1672 to −2343 (island B), which includes the translation initiation site; and (b) additional 5’ between bases, −3090 and −3922 (island A). CpG islands were not present in the Nm23-H1 coding sequence (data not shown). The presence of these CpG islands suggested the hypothesis that DNA methylation may contribute to the regulation of Nm23-H1 expression levels.
To determine whether alterations in DNA methylation could influence Nm23-H1 expression, cells from the human breast carcinoma cell line MDA-MB-231 were incubated for 3 days in 5-Aza-CdR, and a Western blot was processed for Nm23 proteins. Low nanomolar (≤7.5 nm) concentrations of 5-Aza-CdR elevated Nm23-H1 expression ~4-fold by densitometry; elevated Nm23-H1 expression was maintained at higher 5-Aza-CdR concentrations. Nm23-H2 expression was coordinately induced (see Fig. 2). To determine the generality of this finding, cultures from 11 human breast carcinoma cell lines were incubated with or without 5-Aza-CdR for 3 days, and the Nm23 expression levels were determined (Fig. 3). A dose-dependent increase in Nm23-H1 and -H2 expression was observed in 5 of 11 cell lines, including all 3 of the in vivo metastatically competent cell lines (26, 27). The data are grouped by the extent of change in Nm23-H1 expression in response to 5-Aza-CdR. In Fig. 3A, two low-Nm23-H1-expressing, in vivo metastatically competent cell lines, MDA-MB-231 and LCC15-MB, exhibited a 4- to 5- and 6- to 9-fold increase in Nm23-H1 in response to 75 nm and 0.75 μM 5-Aza-CdR, respectively. The remaining low-Nm23-H1-expressing, in vivo metastatically competent cell line, MDA-MB-435 (26), was present in the moderately increased expression group (Fig. 3B). Nm23-H1 overexpression in this group ranged from 1.5- to 2.5-fold and 2- to 3-fold for 75 nm and 0.75 μM 5-Aza-CdR, respectively. The cell lines shown in Fig. 3C showed no repeatable elevation of Nm23 proteins in response to 5-Aza-CdR. Among these cell lines are two high-Nm23-expressing, low-to-nonmetastatic lines (MCF7 and ZR-75–1; Ref. 28), as well as lines thought to be more aggressive because of specific molecular alterations (Her-2 and EGFR amplification in SKBR3 and MDA-MB-468, respectively).

Several additional aspects of 5-Aza-CdR elevation of Nm23-H1 expression were investigated. In the experiment shown in Fig. 4, MDA-MB-231 cells were incubated with 0.75 μM 5-Aza-CdR for 3 days; the culture medium was then removed and replaced with medium lacking 5-Aza-CdR (shown as day 0). Elevated Nm23-H1 and -H2 levels were observable after 2 and 4 days of culture without drug, respectively. The potential interaction between 5-Aza-CdR and TSA, a histone deactylase inhibitor, was determined (Fig. 5). A representative cell line from the high-, moderate-, and no-induction groups from Fig. 2 was incubated with 5-Aza-CdR, TSA, or combinations thereof, and Nm23 expression was determined on Western blots. In contrast to other reported genes, the combination of 5-Aza-CdR and TSA was not synergistic in inducing Nm23 expression.

It has been estimated that the genome contains thousands of CpG islands that could participate in gene regulation through altered DNA methylation. Thus, any potential phenotypic effect caused by elevation of Nm23 expression in response to DNA methylation inhibitors could be offset by numerous other changes. To ascertain the degree to
which other gene expression changes occurred in response to DNA methylation inhibition, MDA-MB-231 cells were treated with or without 0.75 μM 5-Aza-CdR for 3 days; mRNA was then extracted and hybridized to a Genefilter array. Of the 4000 genes that were quantitated, the expression of 22 (0.6%) changed. All showed a modest (2- to 3-fold) increase in the 5-Aza-CdR-treated cells (Table 1). One of the genes identified was Nm23-H1 (NDPKA), confirming our protein observations at the mRNA level. The listed genes have been reported to have a wide variety of functions in vitro and in vivo, including three involved in general transcription and translation, two that suppress the transformed phenotype in vitro, PECAM-1 and glutathione S-transferase activity, and two that suppress the transformed phenotype in vitro, PECAM-1 and glutathione S-transferase activity.

### Table 1: Array analysis of gene expression changes in 5-Aza-CdR-treated MDA-MB-231 human breast carcinoma cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold-induction</th>
<th>Known functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connective tissue growth factor</td>
<td>3.0</td>
<td>Insulin-like growth factor binding protein family (52); induces apoptosis in breast cells (53)</td>
</tr>
<tr>
<td>Elastase IIA precursor</td>
<td>2.7</td>
<td>Family of serine proteases</td>
</tr>
<tr>
<td>M-phase phosphoprotein, mpp9</td>
<td>2.5</td>
<td>Golgi localized protein (54)</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>2.4</td>
<td>Reservoir for phosphorylated β-catenin (55); regulated by DNA methylation (56)</td>
</tr>
<tr>
<td>Macrophage stimulating factor 1</td>
<td></td>
<td>Transcription factor family; expressed in breast cancer cells (57)</td>
</tr>
<tr>
<td>POU homeobox protein</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>ATP synthetase H+ transporting Mitochondrial F1 complex, β polyypeptide</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Human bdk region fip-3</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Nucleoside diphosphate kinase A (Nm23-H1)</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Hepatoma-derived growth factor</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Laminan</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>2.1</td>
<td>Endothelial growth factor (58)</td>
</tr>
<tr>
<td>Farnesyl diphosphate farnesyltransferase</td>
<td>2.1</td>
<td>Suppresses colony formation in transformed fibroblasts (59); correlated with high grade in breast carcinomas (60); regulation of collagen fibril assembly (61)</td>
</tr>
<tr>
<td>EIF3</td>
<td>2.0</td>
<td>Metabolism of carcinogens; regulated by DNA methylation (62, 63)</td>
</tr>
<tr>
<td>Doc-1</td>
<td>2.0</td>
<td>Sterol biosynthesis (64)</td>
</tr>
<tr>
<td>Extracellular matrix protein-1</td>
<td>2.0</td>
<td>Translation initiation (65)</td>
</tr>
<tr>
<td>Elongation factor-1-γ</td>
<td>1.9</td>
<td>Suppresses in vitro transformation, associates with DNA polymerase (66)</td>
</tr>
<tr>
<td>Retinoblastoma (Rb) binding protein 3</td>
<td>1.8</td>
<td>Secreted protein (67)</td>
</tr>
</tbody>
</table>

- Based on hybridization to array filters.
- Only a partial literature search is presented because of space limitations.
induction of Nm23 protein expression by 5-Aza-CdR correlated with reduced biological function.

**DNA Sequencing of CpG Islands in the nm23-H1 Promoter.** The two CpG islands in the nm23-H1 promoter were examined in human breast carcinoma cell lines for DNA methylation patterns by bisulfite sequencing. Briefly, DNA extracted from each cell line was bisulfite treated, amplified by PCR, and cloned; 10 clones per line were sequenced. The percentage of clones exhibiting methylation at CpGs in the island, as evidenced by lack of conversion of a cytosine to uracil, was determined. For CpG island B (21672 bp to 22343 bp), no CpG methylation was apparent at 53 CpGs in the MDA-MB-231, MDA-MB-435, or MCF7 breast carcinoma cell lines, nor in two infiltrating ductal carcinomas (data not shown). For CpG island A (23090 bp to 23922 bp), 19 CpGs were analyzed by bisulfite sequencing (Table 2). The low-Nm23-H1-expressing MDA-MB-231 cell line was highly methylated (80–100% methylation of all CpGs) throughout the island in a manner similar to that in MDA-MB-231 cells. High-Nm23-H1-expressing MCF7 and ZR-75-1 breast carcinoma cells exhibited only minor and focal reductions in DNA methylation. The same question was asked of 20 infiltrating ductal breast carcinomas. The Nm23-H1 content of the tumors was determined on Western blots normalized by total protein loaded, and divided into high-, medium-, and low-expression groups. CpG island A was highly methylated in all of the tumors examined except in one medium-Nm23-expressing tumor (T-15). Tumor 15 exhibited low but positive estrogen receptor expression (8 fmol/mg protein) and was progesterone receptor negative, aneuploid (DNA index 2.08), and high in S-phase (11.5%). Minor and focal differential methylation was occasionally observed at other sites.

**DISCUSSION**

The use of DNA methylation inhibitors to increase gene expression is an attractive model system for the study of several cancer-related genes. In breast cancer cell lines or tumor specimens, altered expression of a host of genes has been associated with aberrant DNA methylation, including BRCA1 (31, 32), RAR-β (33), 14-3-3 (34), Wilms’ tumor 1 (35), p16 (36, 37), uPA (38), estrogen receptor (39), E-cadherin (40), maspin (41), and others. Whereas the potential re-expression of genes thought to be inhibitory to tumorigenesis and metastasis is of interest, the clinical utility of DNA methylation inhibitors will require that these genes actually alter cellular phenotypes, such as growth, metastasis, apoptosis, and so forth. We report...
Four cell lines and 20 anonymized infiltrating ductal breast carcinomas, numbered T-1 through T-20 were used. In addition, MDA-MB-231 cells were treated with 0.75 μM 5-Aza-CdR for 3 days and analyzed.

Nm23-H1 expression determined from densitometric analysis of Western blots, normalized by β-actin total protein loaded. Cell line data shown in Fig. 3. Tumor Nm23-H1 expression was divided into three groups: Low, 0 – 400 densitometric units/μg protein loaded (L); Medium, 400 – 600 densitometric units/μg protein loaded (M); High, >600 densitometric units/μg protein loaded (H).

Data extracted from each sample was treated with sodium bisulfite and described in “Materials and Methods.” The percentage of 10 independent clones exhibiting DNA methylation at each CpG island is listed.

herein that nanomolar concentrations of 5-Aza-CdR elevated expression of the Nm23-H1 metastasis suppressor gene in vitro in 5 of 11 human breast carcinoma cell lines tested, including the 3 in vivo metastatically competent breast carcinoma cell lines available. When the growth phenotype was investigated, minimal inhibition of anchorage-dependent proliferation was observed in response to 5-Aza-CdR. In contrast, 5-Aza-CdR treatment of breast cells significantly reduced their in vitro motility in Boyden chamber assays, an in vitro assay for one part of the tumor metastatic process. These data suggest that DNA methylation inhibitors may impact the breast cancer metastatic process in vivo, for which experiments are under way in animal models in our laboratory.

Several aspects of the in vitro data are noteworthy. Among the five responding breast carcinoma cell lines, a 1.5- to 5-fold increase in Nm23-H1 was observed on incubation with 75 nM 5-Aza-CdR, and 2- to 9-fold increased Nm23-H1 at 0.75 μM 5-Aza-CdR). For the MDA-MB-231 cell line, induction of Nm23-H1 expression was observed at ≥1 nM 5-Aza-CdR. Thus, the elevation of Nm23-H1 appears sensitive to low concentrations of 5-Aza-CdR in vitro. Other breast-related genes have been tested only at higher concentrations of DNA methylation inhibitors, 0.5–0.75 μM for 14-3-3, Wilms’ tumor 1, and estrogen receptor (34, 35, 39), and >1 μM for maspin and RAR-β (33, 41). Elevation of Nm23-H2 expression at comparable levels was also observed. We have not yet examined the nm23-H2 promoter for CpG islands. The high level of Nm23-H1 expression in MDA-MB-231 cells induced by 5-Aza-CdR was maintained for 2 days of culture without agent, and was still 2.5-fold induced over controls at 4 days without agent.

Estimates of the abundance of CpG islands within the human genome vary from ~60% of all genes (42) to 45,000 (43). In a study of 1,184 unselected CpG islands in 98 tumors of various origins, an average of 600 CpG islands (range, 0–4,500) were estimated to be aberrantly methylated. Both heterogeneous and tissue-specific patterns of methylation were found (43). These data prompt the question of the relative specificity of DNA methylation inhibitors. To test this question, we performed DNA array analysis of MDA-MB-231 breast carcinoma cells that were treated with and without 5-Aza-CdR. Surprisingly, only a handful of the 4,000 genes analyzed were elevated in expression, including nm23-H1 and two other genes previously reported to be methylation sensitive. All were induced 2- to 3-fold at the mRNA level, the limit of sensitivity for this assay. Further validation of these results are required. It remains likely that other genes are induced by 5-Aza-CdR in this cell line, for instance, E-cadherin over a longer time course. These data, as well as ongoing proteomics evaluations,5 suggest that only a limited number of alterations in gene expression accompany 5-Aza-CdR treatment of a breast cell line in vitro, which indicates that specificity in phenotypic alterations may be observed.

In general, limited data concerning the phenotypic consequences of DNA methylation inhibition is available in the literature. The most compelling experiments are in vivo; these include the inhibition of intestinal neoplasia in the Apcmin mouse by 5-Aza-CdR (44), the inhibition of tumorigenicity of an adrenocortical tumor in LAFl mice by antisense to DNA methyltransferase (45), and the reduction of T24 tumor size in response to 5-Aza-CdR (46). Our examination of the biological phenotypes induced by 5-Aza-CdR included anchorage-dependent proliferation and motility in vitro. Minimal (<25%) inhibition of proliferation was observed in 10 of 11 breast carcinoma cell lines. None of the 10 lines exhibited a >10% inhibition of proliferation at the lower 75-μM dose. Inhibition of proliferation by 5-Aza-CdR, to the extent that it occurred, was not correlated with the induction of Nm23-H1 expression. These data stand in general agreement with Bovenzi et al., who found that 1–1000 ng/ml (~4 nm–4 μM) 5-Aza-CdR inhibited the growth of MDA-MB-231 cells by <20–69% on day 3 of culture (47). Other reports have used nonbreast...
cell lines: a 1.5- to 2-fold extension of bladder and colon carcinoma and melanoma cell line doubling times was reported after 9 days of treatment with 0.5 μM 5-Aza-CdR (46). An antisense oligonucleotide to DNA methyltransferase (48) also inhibited the growth of T24 cells. The relationship of DNA methylation to proliferation is complex in fields other than cancer, because embryonic stem cells from DNA methyltransferase knockout mice proliferated normally (49), but the knockout mice exhibited histological evidence of reduced proliferation (50).

In contrast to the proliferation data, both high-responder MDA-MB-231 and moderate-responder MDA-MB-435 cells exhibited reduced motility in vitro to multiple doses of attractant after preincubation with 5-Aza-CdR. Motility was suppressed several-fold by 75 nM 5-Aza-CdR, confirming the sensitivity observed at the Nm23-H1 protein expression level. Nonresponder MCF7 breast carcinoma cells were nonmotile and remained nonmotile after 5-Aza-CdR treatment (data not shown). Although the Western blot and array data suggest that increased Nm23-H1 expression is participatory in this phenotype, other molecular events may also contribute. Although limited by the number of breast carcinoma cell lines that exhibit motile behavior in vitro, the data to date suggest a degree of uniformity in the phenotypic consequences of DNA methylation inhibition, and that the metastatic process may be a sensitive target.

Our data showed that 5-Aza-CdR inhibited the methylation of one of the two CpG islands in the MDA-MB-231 nm23-H1 promoter coincident with increased protein expression. No synergy with TSA was observed in cell lines, which suggests that the global remodeling of the nm23-H1 promoter associated with histone organization is not operative. However, it is apparent that aberrant methylation of this CpG island is not a mechanism used frequently to maintain high Nm23-H1 expression, in either cell lines or tumors. Only 1 of 12 moderate-to-high-expression tumors exhibited uniform low methylation within this CpG island. Several possible explanations may underlie these data: (a) high Nm23-H1 expression may occur independently of DNA methylation through regulation by transcription factors that act independently of CpG island methylation; (b) elevation of Nm23-H1 may occur in a DNA-methylation-sensitive manner in nature, but indirectly. A fascinating possibility was recently suggested was observed in cell lines, which suggests that the global remodeling consequences of DNA methylation inhibition, and that the metastatic phenotype in the metastatic site, and growth factor-independent proliferative activity in culture. Cancer Res., 55: 2359–2365, 1995.


Elevation of Breast Carcinoma Nm23-H1 Metastasis Suppressor Gene Expression and Reduced Motility by DNA Methylation Inhibition

Melanie T. Hartsough, Susan E. Clare, Michael Mair, et al.

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