Inactivation of the MAL Gene in Breast Cancer Is a Common Event That Predicts Benefit from Adjuvant Chemotherapy

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Abstract

Dysregulation of MAL (myelin and lymphocyte protein) has been implicated in several malignancies including esophageal, ovarian, and cervical cancers. The MAL protein functions in apical transport in polarized epithelial cells; therefore, its disruption may lead to loss of organized polarity characteristic of most solid malignancies. Bisulfite sequencing of the MAL promoter CpG island revealed hypermethylation in breast cancer cell lines and 69% of primary tumors analyzed compared with normal breast epithelial cells. Differential methylation between normal and cancer DNA was confined to the proximal promoter region. In a subset of breast cancer cell lines including T47D and MCF7 cells, promoter methylation correlated with transcriptional silencing that was reversible with the methylation inhibitor 5-aza-2'-deoxycytidine. In addition, expression of MAL reduced motility and resulted in a redistribution of lipid raft components in MCF10A cells. MAL protein expression measured by immunohistochemistry revealed no significant correlation with clinicopathologic features. However, in patients who did not receive adjuvant chemotherapy, reduced MAL expression was a significant predictive factor for disease-free survival. These data implicate MAL as a commonly altered gene in breast cancer with implications for response to chemotherapy. (Mol Cancer Res 2009;7(2):199–209)

Introduction

Cancer can be caused by the accumulation of both genetic and epigenetic alterations frequently leading to downstream changes in gene expression patterns. Epigenetic changes do not alter the DNA sequence, and therapeutics targeted at reversing epigenetic modifications hold the potential to reactivate expression of previously silenced genes, potentially altering the malignant phenotype (1, 2). Furthermore, these epigenetic changes can be used as markers for detection of malignant cells in bodily fluids (3). In the present study, we identify MAL (myelin and lymphocyte protein) as a novel epigenetically regulated gene in breast cancer.

Although 70% to 80% of CpGs in human cells are normally methylated, cytosines within CpG islands are protected from methylation (4). Even as CpG islands are typically unmethylated, the areas flanking the islands are methylated and act as barriers protecting against aberrant promoter methylation (5, 6). In neoplasia, the barriers protecting the promoter CpG islands are commonly overridden with de novo methylation believed to begin at the distal ends of the island and then progressively spreading into the core (5). Therefore, cancer-associated hypermethylation is a dynamic process that may change with time, disease state, or treatment.

MAL was first identified in a screen for genes differentially expressed during T-cell development (7). The MAL gene encodes a 17 kDa transmembrane protein selectively found in glycolipid-enriched membrane microdomains or lipid rafts (8–10). In this context, MAL appears to function in glycolipid-enriched membrane-mediated apical sorting of membrane and secretory proteins in polarized epithelial cells (11, 12). The MAL protein is expressed on the apical face of most types of human epithelia consistent with its role in polarized secretion (13). Moreover, its location in membrane microdomains suggests that it may have a role in cell signaling (9, 14).

Interest in this gene in our laboratory arose from a microarray study showing that MAL is the most differentially expressed transcript between serous ovarian cancers with good versus poor outcome (15). This finding led us to examine MAL transcriptional regulation in ovarian6 and breast cancer. Using bisulfite genomic sequencing, we examined the methylation profile of the MAL promoter region in both benign and breast cancer specimens. Our results show that hypermethylation of the MAL promoter is common in primary breast cancer and that, in some cases, this methylation appears to impair gene transcription. Further, we describe a cancer-specific methylation

Received 7/3/08; revised 10/22/08; accepted 10/23/08; published OnlineFirst 02/10/2009.

Grant support: NIH grant CA84955 (J.R. Marks) and National Institute of General Medical Sciences grant F31GM092048 (H.N. Horne).

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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6 P.S. Lee., in preparation.
pattern that should guide future epigenetic studies at this locus. Finally, we begin to analyze the functional consequences of MAL protein expression in cell culture and in primary breast tumors.

Results
MAL Promoter Hypermethylation in Breast Cancer Cell Lines Is Associated with Gene Silencing

Interest in MAL gene regulation arose from our microarray profiling study showing that it was the most differentially expressed gene between two groups of advanced serous ovarian cancers matched for stage: cancers from patients with >7-year survival versus <3-year survival after initial diagnosis (15). Although we observed evidence of promoter methylation in ovarian cancers cell lines and in primary ovarian tumors, the reactivation by DAC was also highly variable (Fig. 1D). The three primary HMEC cultures had nearly identical responses to DAC reactivation and methylation inhibition. The partial methylation observed in these two lines indicates that epigenetic control is only one factor regulating expression of this gene in breast cells.

Methylation of the MAL Promoter in Primary Breast Tumors

To determine if the pattern of methylation seen in the cancer cell lines was also present in breast tumors, we analyzed a series of 36 primary breast cancer specimens and matched constitutive DNA (from peripheral blood lymphocytes) by bisulfite sequencing. We observed varying degrees of methylation and classified the cancers into three categories based on the average percent methylation quantitated by phosphorimaging of the sequencing gels: heavily methylated (defined as an average methylation level >40%), partially methylated (5-40%), and unmethylated (<5%) (Fig. 2A). Of 36 primary cancers analyzed, 7 of 36 (19%) were heavily methylated, 18 of 36 (50%) exhibited partial methylation, and the remaining 11 (31%) had no evidence of methylation. In summary, we detected MAL promoter methylation in 25 of 36 (69%) of the primary tumors analyzed. All but one of the matched lymphocyte DNA samples were completely unmethylated in this region.

The breast cancer specimens used in this study are typical of the disease and contained a heterogeneous admixture of cells. To confirm that hypermethylation of MAL was derived from the cancer epithelium, we analyzed DNA extracted from laser capture microdissected cancer cell nuclei and normal breast epithelium (from reduction mammoplasty specimens with no evidence of cancer). DNA suitable for bisulfite sequencing was obtained from 10 primary cancers and 4 normal specimens. Comparing DNA extracted from bulk tumor versus the same tumor subjected to laser capture microdissection indicates that the methylation signal was derived from the malignant epithelial cells (Fig. 2B). In the example shown, the cancer scored initially as partially methylated (28% as measured by phosphorimaging) appears to be completely methylated in this region after microdissection of the tumor epithelia. Importantly, DNA from microdissected normal breast epithelia exhibited no methylation in this region (Fig. 2B). Although only primary cancers that contained at least 50% cancer cells were used in the analysis of bulk tumor DNA, it is likely that our scoring of partial methylation underestimates the degree of methylation in these cases. Absence of methylation in normal breast epithelium suggests that hypermethylation in cancer is associated with the oncogenic process. Furthermore, methylation analysis of pure ductal carcinoma in situ samples (with no coexisting invasive component) showed hypermethylation in three of five specimens, indicating that this epigenetic event can occur early during neoplastic progression (Supplementary Fig. S1).

Differential Methylation in the MAL Promoter Is Confined to the Proximal Promoter Region

In designing a methylation-specific PCR assay for this region, we noticed that placement of the upstream primer was critical in obtaining results consistent with the sequence
FIGURE 1. Methylation of the MAL promoter region in breast cancer and reactivation of expression by DAC. A. Diagram of the MAL gene indicating the four alternatively spliced exons (hatched boxes) and the promoter CpG island (solid bar above the sequence). The complete sequence of the region analyzed by bisulfite sequencing is shown (top) with the CpG dinucleotides (bold), the transcription start site (+1), and the ATG translation initiation codon (underlined). B. Representative bisulfite sequencing gels for primary HMECs (HMEC1), immortalized cell lines (26NC), and breast cancer cell lines (T47D). The region was sequenced using the reverse primer R3; the complement of the methylated C is evident in the G lane. Right, positions of the top and bottom nucleotides relative to the start of transcription. C. A schematic of bisulfite sequencing of the MAL promoter region in the complete panel of benign and cancer cells showing unmethylated CpGs in HMECs, partial methylation in immortalized cell lines (IM), and hypermethylation in the breast cancer cells (CA). Top, position of the first and last CpG dinucleotides relative to the start of transcription. D. Reactivation of MAL expression specifically in methylated cancer cell lines and the immortalized cell line MCF10A. Expression was measured by TaqMan RT-PCR. Numbers at the bottom, basal level (log2) of expression measured by quantitative RT-PCR. The status of three of the most differentially methylated residues in the MAL promoter is also shown, corresponding to cytosines 2 to 4 (underlined in C).
analysis described above. This led to additional sequencing of the promoter region from a series of specimens that revealed an abrupt transition in the methylation profile ~350 bp upstream of the transcription start site. We analyzed 4 microdissected normal breast epithelial specimens, 4 benign immortalized mammary epithelial cell lines, and 36 normal lymphocyte samples (from breast cancer patients) to determine the position and frequency of constitutive methylation in this region (Supplementary Fig. S2). Upstream of this transition zone, the majority of normal breast epithelia, normal lymphocytes, and tumors were partially or fully methylated. Figure 2C shows the position of the transition point in both a normal breast and a breast cancer sample taken from two separate patients, showing the appearance of partial methylation upstream of the transition point in the normal specimen. This non-disease-associated methylation extends to at least -621 by our sequence analysis (Fig. 2D). Therefore, the region of differential or disease-associated methylation is confined to a relatively small region of the proximal promoter.

Expression of MAL Results in Altered Raft Composition and Reduced Motility

To our knowledge, expression of the MAL protein in breast epithelial cells has yet to be shown. By immunohistochemistry using a well-characterized monoclonal antibody (mAb; refs. 8, 9, 13), we were able to detect MAL in normal breast epithelium from reduction mammoplasty specimens (Supplementary Fig. S3). In other epithelial cell types, the MAL protein has been shown to localize to cholesterol-enriched membrane microdomains characterized by their resistance to detergent solubilization. We examined this localization in breast epithelial cell lines expressing endogenous (HCC1937) or exogenous (MCF10A stably transfected with a V5-tagged construct) MAL protein. Fractionation of detergent-extracted cells by sucrose gradient velocity sedimentation followed by immunoblot analysis showed endogenous MAL protein in HCC1937 cells predominantly in the insoluble fraction (Fig. 3A, left). Detection of the lipid raft resident protein flotillin-1 (16) in this fraction confirmed the biochemical localization.

To begin to investigate the consequences of MAL expression, we next established a MCF10A line stably expressing MAL with a COOH-terminal V5 tag. Parental MCF10A cells are hypermethylated at the MAL promoter and express low levels of mRNA (Fig. 1D) and protein (data not shown). Fractionating these cells indicated that the exogenous MAL protein localized to both the soluble and the insoluble compartments. Interestingly, probing these same fractions for flotillin-1 indicated that MAL expression induces a compartmental change in this protein. Whereas flotillin-1 was found entirely in the soluble fraction in the vector control line (under G418 selection), introduction of MAL produced a dramatic
that observed in those lacking methylation (468 ± 62, expression normalized to β2-microglobulin) being lower than that observed in those lacking methylation (468 ± 171); however, this difference did not reach statistical significance (P = 0.23; Supplementary Fig. S5). This finding suggests that MAL promoter methylation, although common in breast cancer, cannot exclusively account for the observed differences in MAL expression. Further, of the cancers that exhibited methylation (either partial or heavy), the majority were nuclear hormone receptor positive, whereas all of the unmethylated cancers for which data were available were ER/progesterone receptor-negative (Supplementary Table S1).

Our earlier data showed that the level of methylation is not an absolute predictor for basal mRNA expression, specifically in the HCC1937 and BT474 cell lines, which contain promoter methylation but have high levels of MAL mRNA (Fig. 1D). Consequently, we examined MAL protein expression as a more direct measurement to determine the dynamics of MAL in breast cancer. We evaluated MAL expression by immunohistochemical analysis in a representative sample of 122 banked frozen breast cancers resected at Duke University Medical Center between 1990 and 1998. In all staining runs, controls included a normal breast specimen and a normal kidney (distal and collecting tubules express high levels, whereas glomeruli and proximal tubules are negative) as positive and negative controls. MAL protein was detected in the vector control (C)-containing cell lines and actin was only detected in the soluble fractions. Representative images from an in vitro wound-healing assay done on MCF10A cells stably transfected with either vector control (top) or V5-tagged MAL (bottom). Images were taken at 0, 24, and 48 h after wounding. Original magnification, ×10. C. Quantitation of the rate of closure for the wound-healing assay in both control and MAL-expressing cell lines. Statistical significance was calculated using a two-factor ANOVA test.

FIGURE 3. Role of MAL in lipid raft composition and cell motility. A. Detection of either endogenous MAL in HCC1937 or exogenous COOH-terminal V5-tagged MAL in MCF10A cells after extraction with 1% Triton X-100 and centrifugation to equilibrium. Aliquots from either detergent-soluble (S) or insoluble (I) lipid raft-containing fractions were analyzed with anti-MAL 6D9 mAb (top left blot), anti-VS (top right blot), flotillin-1, or actin antibodies. No MAL protein was detected in the vector control (C)-containing cell lines and actin was only detected in the soluble fractions. B. Representative images from an in vitro wound-healing assay done on MCF10A cell lines stably transfected with either vector control (top) or V5-tagged MAL (bottom). Images were taken at 0, 24, and 48 h after wounding. Original magnification, ×10. C. Quantitation of the rate of closure for the wound-healing assay in both control and MAL-expressing cell lines. Statistical significance was calculated using a two-factor ANOVA test.

Correlation of MAL Protein Expression with Clinicopathologic Features and Outcome

Because promoter methylation has a direct effect on gene transcription levels, we initially looked for a relationship between MAL promoter methylation and mRNA expression. Analyzing 36 primary tumors by quantitative RT-PCR, we observed varying levels of mRNA expression with the average MAL expression in the methylated tumors (288 ± 62, expression normalized to β2-microglobulin) being lower than that observed in those lacking methylation (468 ± 171); however, this difference did not reach statistical significance (P = 0.23; Supplementary Fig. S5). This finding suggests that MAL promoter methylation, although common in breast cancer, cannot exclusively account for the observed differences in MAL expression. Further, of the cancers that exhibited methylation (either partial or heavy), the majority were nuclear hormone receptor positive, whereas all of the unmethylated cancers for which data were available were ER/progesterone receptor-negative (Supplementary Table S1).

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that reacted with the antibody. We observed no significant relationship between MAL protein expression and common clinicopathologic variables (Table 1). Disease-free survival, with an average follow-up of 96 months, trended toward better outcome in patients with MAL-expressing cancers but did not reach statistical significance (Fig. 4C).

Whereas MAL expression does not have strong prognostic value, predictive value is of equal or greater importance as a determinant of who will derive benefit from adjuvant chemotherapy. Overall, we observed no difference in disease-free survival based on the delivery of chemotherapy alone.
MAL staining did not identify a difference in the subpopulation of patients that received adjuvant cytotoxic therapy (Fig. 5B). However, in the group of patients who were not treated, absence of MAL staining was a significant predictor of disease progression (Fig. 5C). MAL-positive and MAL-negative tumors from patients who did not receive adjuvant chemotherapy were not significantly different based on age, nodal status, tumor size, or hormone receptor status (Supplementary Table S2), indicating that the MAL protein may be an independent predictor of benefit from chemotherapy.

Discussion

Hypermethylation of gene promoters leading to transcriptional silencing is associated with the onset and progression of cancer. Identification of genes that are epigenetically regulated in cancer can provide targets for early detection and therapeutics. In this study, we identified MAL as a novel epigenetically regulated gene in breast cancer. MAL, a component of lipid rafts, plays a central role in the reorganization of these membrane microdomains for signaling and/or intracellular protein transport in epithelial cells (11, 12, 14). Martin-Belmonte et al. (17) showed that RNA interference directed against MAL impaired apical transport leading to the accumulation of secretory proteins in the Golgi and loss of polarity in Madin-Darby canine kidney cells. Loss of polarized growth is a common hallmark of malignant progression; loss or reduced MAL expression through epigenetic mechanisms may prove to be an important early event in the transformation process. Our observation of MAL hypermethylation in ductal carcinoma in situ samples strongly supports this possibility.

We observed hypermethylation of the MAL promoter in all of the breast cancer cell lines (6 of 6) and 69% of primary tumors analyzed relative to cultured primary breast epithelial cells, patient matched lymphocytes, and normal breast tissue from healthy donors. When attempting to correlate the levels of MAL expression to promoter methylation, we found that this association could be made for some but not all cell lines or primary tumors. The consequences of promoter methylation on MAL expression could be shown in breast cell culture in that MAL mRNA expression was inducible by DAC only in hypermethylated cell lines. In a series of cell lines, we observed

![Figure 4](image-url)
a correlation between the degree of hypermethylation and the induction of the MAL transcript, specifically in MCF7, T47D, ZR75-1, and MCF10A cells. MAL expression was not affected in primary mammary epithelial cells that have no detectable methylation. Further, methylated cell lines that responded to DAC had basal levels of MAL transcripts that were 10- to 40-fold below that found in benign HMEC cultures. Inhibiting methylation in these cells induced MAL to a level comparable with primary breast epithelia. Notably, two breast cancer cell lines did not follow this trend, HCC1937 and BT474. The MAL promoter is hypermethylated, yet both lines exhibit high basal levels of expression that were not further induced by DAC treatment. The presence of completely unmethylated residues, for example, in CpG sites 1 and 3 of HCC1937 (Fig. 1C), may indicate that the critical CpG residues for the transcriptional regulation of this gene are not sufficiently methylated in these lines. Alternatively, these cell lines may contain high levels of certain transcription factors allowing for the elevated expression of MAL. Ultimately, high basal expression in the presence of partial methylation in these two lines indicates that promoter methylation is not the only mechanism regulating MAL expression in breast cells. This study is the first describing the epigenetic regulation of MAL in breast cancer; however, Mimori et al. (18) showed that treatment of esophageal cancer cell lines with DAC and trichostatin A up-regulated MAL gene expression in 12 of 13 cell lines examined. More recently, a genome-wide search (19) found that MAL was frequently hypermethylated in colon cancer correlating with reduced expression. These reports and our current data suggest that epigenetic silencing of MAL may be a common event involved in the initiation and progression of epithelial cancers. The pattern and amount of promoter hypermethylation can be very heterogeneous leading to varying degrees of gene silencing in tumor cells. The level of promoter methylation can vary not only within a given cell population but also between alleles of a given gene and from one CpG site to another in a single CpG island (20). Given the heterogeneous nature of promoter methylation, we thought it necessary to consider not only the presence or absence of methylation in a few CpG dinucleotides within the MAL promoter but also the pattern of methylation over a relatively large portion of the promoter region. We surveyed >800 bp of sequence, which included the

<table>
<thead>
<tr>
<th>Table 1. Clinicopathologic Features of Patients by MAL Immunohistochemistry</th>
<th>MAL−, n (%)</th>
<th>MAL+, n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>35 (29)</td>
<td>87 (71)</td>
<td>0.44</td>
</tr>
<tr>
<td>Age (y)</td>
<td>≤50</td>
<td>18 (51)</td>
<td>38 (44)</td>
</tr>
<tr>
<td>Race</td>
<td>White</td>
<td>28 (80)</td>
<td>66 (76)</td>
</tr>
<tr>
<td>Non-White</td>
<td>7 (20)</td>
<td>21 (24)</td>
<td>0.7</td>
</tr>
<tr>
<td>ER status</td>
<td>Positive</td>
<td>17 (49)</td>
<td>49 (56)</td>
</tr>
<tr>
<td>Progesterone receptor status</td>
<td>Positive</td>
<td>16 (55)</td>
<td>45 (56)</td>
</tr>
<tr>
<td>Node involvement</td>
<td>Yes</td>
<td>16 (48)</td>
<td>28 (34)</td>
</tr>
<tr>
<td>Stage</td>
<td>1 or IIA</td>
<td>22 (63)</td>
<td>47 (54)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>T1 A-C</td>
<td>15 (45)</td>
<td>32 (39)</td>
</tr>
<tr>
<td>Histologic grade</td>
<td>1</td>
<td>1 (3)</td>
<td>3 (4)</td>
</tr>
<tr>
<td>2</td>
<td>5 (17)</td>
<td>25 (34)</td>
<td>0.17</td>
</tr>
<tr>
<td>3</td>
<td>24 (80)</td>
<td>45 (62)</td>
<td>0.17</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td>Yes</td>
<td>22 (63)</td>
<td>47 (54)</td>
</tr>
<tr>
<td>Hormonal therapy</td>
<td>Yes</td>
<td>15 (45)</td>
<td>49 (56)</td>
</tr>
<tr>
<td>No</td>
<td>13 (37)</td>
<td>40 (46)</td>
<td>0.18</td>
</tr>
</tbody>
</table>

NOTE: Statistical significance was calculated using χ² analysis.
methylation status of 71 CpG dinucleotides. From this sequence analysis, we observed a transition point in the methylation profile. Differential methylation between normal and cancer cells was confined to a region within 356 bp upstream of the start of transcription. This type of sharp transition in methylation has been observed in other CpG islands including those associated with the β-glucuronidase (6), E-cadherin, and von Hippel-Lindau genes (5). A distinct definition of boundaries between these regions was correlated with the presence of SP1 binding sites and multiple Alu repeats of both E-cadherin and von Hippel-Lindau genes in benign breast and kidney cells, respectively. The transition point we have defined may represent the 5'-boundary of the functional MAL promoter CpG island. Analysis of this region did not reveal any Alu repeats; however, MAL contains several SP1 binding sites in the 5'-flanking proximal promoter region (~110 bp upstream of the transcriptional start site; ref. 21).

Hypermethylation of MAL was readily detectable in primary breast cancer specimens and microdissection confirmed that these events occur in the malignant epithelial cells within the tumor. Similar to what was seen in certain breast cancer cell lines (HCC1937 and BT474), promoter methylation may explain down-regulation of MAL gene expression in some but not all primary breast cancers. On average, lower MAL mRNA expression was seen in the methylated tumors compared to unmethylated tumors; however, this difference did not reach statistical significance (Supplementary Fig. S5). We observed a strong correlation of MAL hypermethylation with ER-positive (P = 0.003) and progesterone receptor-positive (P = 0.006) cancers (Supplementary Table S1). This was consistent with our cell line data showing that the hormone receptor-positive MCF7, T47D, and ZR75-1 lines are all methylated and MAL expression could be reactivated by DAC. This is not the first finding that links ER/progesterone receptor status with promoter methylation in breast cancer. A recent study by Wei et al. (22) showed that methylation of the BRCA1 promoter was more frequently observed in women with high-grade ER-negative and progesterone receptor-negative tumors. If reduced MAL expression is associated with loss of polarity, then it is possible that the higher percentage of ER-positive cancers that exhibit MAL hypermethylation is indicative of a greater selection for this property during the neoplastic progression of luminal type cancers compared with ER-negative cancers more likely to be of the basal subtype. Whether this reflects the varied etiology of these cancers or a cell lineage phenomenon is not known.

From available gene expression array data (23), we found that MAL is contained within a group of coordinately regulated genes (metagene) that are immune cell related. MAL was first discovered as a T-cell-specific protein. Therefore, it was formally possible that the bulk of MAL transcription in breast cancer could be attributed to infiltrating inflammatory cells. To address this, we compared levels of MAL expression with the expression of T-cell-restricted genes (CD2 and CD27) by quantitative RT-PCR and array analysis (data not shown). Although some cancers with very high levels of MAL also coexpressed T-cell markers, this association was not invariant. Together with microdissection data, immunohistochemistry, immunoblotting, and cell culture results, we are confident that breast epithelial cells can and do express MAL mRNA and protein. However, elevated expression of MAL mRNA seen in some cancers that are bulk extracted may be more indicative of the presence of immune cells in these tissues than an up-regulation of the gene in malignant epithelia.

Disruption of MAL expression has been implicated in the etiology of several human cancer types. Down-regulation of MAL expression was observed in colon (19), cervical (24), renal, and thyroid (13, 25) neoplasms when compared with their respective benign epithelium. In esophageal cancer, MAL was suggested to be a tumor suppressor gene based on studies showing that MAL expression repressed the formation of tumors induced by TE3 cells in nude mice, inhibited cell motility, and induced apoptosis via the Fas signaling pathway (18). Furthermore, MAL expression was indicative of disease outcome and prognosis for patients with T-cell lymphoma (26), Hodgkin’s lymphoma (27), and serous ovarian cancer (15). The function of MAL in normal or neoplastic breast epithelial cells is unknown; however, its location in the detergent-insoluble fraction in HCC1937 cells suggests that, similar to other cell types, it plays an integral role in membrane microdomains. Exogenous expression of the MAL protein in the MCF10A cell line resulted in its accumulation in the detergent-insoluble fraction. Further, flotillin-1, another lipid raft component, was redistributed from the soluble to the insoluble fraction when MAL was expressed in these cells, suggesting that MAL may be critical for organizing the composition and likely the function of these microdomains in breast epithelial cells. Similar to an esophageal cancer cell line (18), expression of MAL in breast cells also reduced motility. However, we did not observe a similar effect on growth or cell cycle resulting from MAL expression in these cells.

Given the complexity of the methylation pattern at this locus and the availability of a high-quality mAb, we examined MAL expression at the protein level in a series of primary frozen breast specimens. Consistent with mRNA expression of cultured HMEC, we found that benign breast epithelia expressed easily detectable MAL protein by immunohistochemical staining. Categorizing 122 primary cancers based on the absence or presence of staining in malignant epithelial cells, no significant correlations were found with standard clinicopathologic variables including age, stage, nodal status, tumor size, hormone receptor status, or disease-free survival. Over 40% of the patients in this study did not receive adjuvant cytotoxic chemotherapy. In this subgroup, absence of MAL protein expression was highly associated with shorter disease-free survival (P = 0.003). In patients who were treated with chemotherapy, MAL staining was not predictive. As expected, patients who did not receive chemotherapy were older, had smaller tumors, and were more frequently node-negative and hormone receptor-positive (Supplementary Table S2). These findings suggest that lack of MAL protein expression might identify a subgroup of breast cancer patients that would benefit from receiving adjuvant chemotherapy. We are currently investigating whether loss of MAL protein expression makes breast cancer cells more responsive to the standard cytotoxic chemotherapies used in our cohort of patients.

To our knowledge, this is the first article implicating MAL expression in the development of breast cancer. Our data, and...
that of others, support the importance of the proper regulation of MAL expression in a variety of cancers. The role of MAL in maintaining proper cell polarity, secretion, signaling, and motility may define a new functional class of tumor suppressor genes and thus warrants further study with respect to the development, progression, and treatment of cancer.

Materials and Methods

Methylation Analyses

Sodium bisulfite modification of DNA was done based on a protocol by Grunau et al. (28) with additional modifications to accommodate a 96-well format (29). Briefly, 1 µg genomic DNA was denatured with 3 mol/L NaOH for 20 min at 42°C followed by deamination in saturated sodium bisulfite/10 mmol/L hydroquinone (Sigma) solution for 4 h at 55°C. Nuclease-free water was added to the samples in bisulfite solution and transferred to a Montage PCR96 96-well filtration plate (Millipore). All remaining steps of the protocol were done in the Montage PCR96 96-well filtration plate using a vacuum plate (Millipore). All remaining steps of the protocol were done in the Montage PCR96 96-well filtration plate using a vacuum manifold (Millipore Multiscreen Vacuum Manifold) and an in-house vacuum source. The DNA was desalted with nuclease-free water three times followed by desulfonation with 0.1 mol/L NaOH and a final wash step with water. The DNA was recovered in 50 µL of 10 mmol/L Tris-HCl (pH 8.0) by using a plate shaker to release the DNA from the filtration matrix for 10 min (Vortex Genie 2) followed by transfer to individual tubes and storage at 4°C.

For sequencing, bisulfite-treated genomic DNA was PCR amplified with primers specific for bisulfite-converted sequences. Primer sequences (5'-3') include F1 GGGAGTAATTTTTT-TATTTTAGTGA (forward), F2 ACCAAAAACACTCAAA-ACTC (reverse), R3 AACACTAAACAAAAATCTACC (reverse), R4 CCAAACTAAAATCTACTTACAC (reverse), and R5 CAAAACACACACATTACAA (reverse). The PCR products were resolved on agarose gels and purified using Sigma GenElute spin columns (Sigma) followed by cycle sequencing (Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit; Amersham Biosciences). Following resolution of the sequencing reactions on denaturing polyacrylamide gels, dried gels were exposed to radiographic film (Kodak X-OMAT MR) and/or a Phosphorimager screen followed by a quantitative determination of relative band intensity using the Storm PhosphorImager System and ImageQuant Software (GE Healthcare Life Sciences). Specimens with average methylation ranging from 40% to 100% were classified as heavily methylated, those exhibiting methylation levels between 5% and 40% were partially methylated, and specimens with methylation levels <5% were designated as unmethylated.

Cell Culture and Treatment

The human breast cancer cell lines MCF7, MDA-MB-468, T47D, ZR75-1, BT474, and HCC1937 were obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies). Benign breast epithelial cultures and lines were also used in these studies and were cultured in DFCI medium (30). Primary HMECs were obtained from women undergoing reduction mammoplasty with no evidence of cancer. The 26NC cell line is a chemically immortalized (dimethylbenzanthracene) derivative of the 26N primary culture and has been maintained in our laboratory for >10 years (31). The BE20E6 line was immortalized by stable transfection of a plasmid expressing the human papillomavirus E6 gene (provided by Ray White, University of Utah) and MCF10A are spontaneously immortalized adherent mammary epithelial cells obtained from the Michigan Cancer Foundation. DU99 cells are telomerase-immortalized HMECs derived at Duke University Medical Center.

Both benign and cancer cells were treated with 5 µmol/L DAC (decitabine/DAC, Sigma) from a 200 µmol/L stock dissolved in 50% acetic acid for 24 h. All treatments were carried out in complete medium with cells in logarithmic growth phase. Control cultures were treated with the vehicle only. Following treatment, cells were harvested and total RNA was extracted using the Qiagen RNeasy Mini Kit. Total RNA (1 µg) was then reverse transcribed using Transcriptor RT (Roche). Subsequent RT-PCRs (TaqMan Assays-on-Demand; Applied Biosystems, MAL, Hs00242748_m1) were done using a 1:15 dilution of the cDNA according to the manufacturer’s recommendation on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems), with the exception that a 25 µL reaction volume was used with 50 total cycles. The relative expression level of MAL was obtained for each sample by normalization to the expression level of the human β2-microglobulin gene (Applied Biosystems). All MAL and B2M expression analyses were done in parallel. Results are from two separate experiments done in triplicate.

Generating Stable Transfectants

For stable transfection of MAL, the MAL coding sequence (bases 60-518; accession no. NM002371) was PCR amplified, sequence verified, and inserted into the Gateway entry vector pDONR 221 (Invitrogen). Once in pDONR 221, the coding region was subsequently cloned into pDEST 40, a COOH-terminal V5-tagged Gateway expression vector (Invitrogen) using the LR recombination reaction following the manufacturer’s protocol. Transfection of pDEST 40-MALV5 into MCF7 or MCF10A cells was done using Lipofectin (Invitrogen) or
In vitro (Perkin-Elmer). Developed using the Western Lightning Chemiluminescence Kit coupled to horseradish peroxidase, washed extensively, and membranes were incubated for 1 h with goat anti-mouse IgG. After several washings, antibodies raised in the presence of anti-MAL 6D9 mAb (8, 9), anti-V5 (Invitrogen), and anti-flotillin-1 (Transduction Laboratories) or 2 h in the presence of anti-MAL 6D9 mAb (8, 9) or mouse IgG (Vector Laboratories) were done on all specimens. Human kidney was used as a positive control in all staining runs, and lymphocytes, which express MAL, were used as internal positive control cells. Binding of the antibody was visualized using the ABC (Vector Laboratories) immunoperoxidase system according to the manufacturer’s recommendations. Cancers were considered positive for MAL protein if >20% of the malignant epithelial cells had detectable cytoplasmic staining.

**Statistical Analysis**

For comparison of average gene expression levels between samples, two-tailed t tests were used to calculate significance. Significant differences between clinicopathologic features of patient subgroups (methylated versus unmethylated, MAL+ versus MAL−, and Chemo+ versus Chemo−) were calculated using χ² analysis or Fisher’s exact test when groups contained n < 5. Survival curves were generated for primary breast cancer patients with the Kaplan-Meier estimate and log-rank test using the Prism statistical software (GraphPad).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

Inactivation of the *MAL* Gene in Breast Cancer Is a Common Event That Predicts Benefit from Adjuvant Chemotherapy

Hisani N. Horne, Paula S. Lee, Susan K. Murphy, et al.


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Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-08-0314

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