Published in final edited form as:

Mol Cancer Res. 2009 February ; 7(2): 199–209. doi:10.1158/1541-7786.MCR-08-0314.

Inactivation of the MAL gene in breast cancer is a common event that predicts benefit from adjuvant chemotherapy

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Abstract

Dis-regulation 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 to 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 clinico-pathologic 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.

Keywords

myelin and lymphocyte protein; breast cancer; hypermethylation; lipid rafts; chemotherapy

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 as a novel epigenetically regulated gene in breast cancer.

Although 70–80% 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 (myelin and lymphocyte protein) 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 (GEM) microdomains or lipid rafts (8–10). In this context, MAL appears to function in GEM 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 demonstrating 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 ovarian (P.L., manuscript in preparation) 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 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 greater than 7 year survival versus less than 3 year survival after initial diagnosis (15). While we observed evidence of promoter methylation in ovarian cancers cell lines and in primary ovarian tumors (P.L., in preparation), in parallel we also examined breast cancer cell lines. *MAL* has a promoter CpG island of approximately 1500 bp that contains 116 CpG dinucleotides and extends into the first intron. Our initial bisulfite sequence analysis covered 22 CpG dinucleotides spanning the region from –48 to +82 relative to the start of *MAL* transcription (Fig. 1A). Comparing a series of cultured mammary epithelial cells ranging from benign primary cells derived from breast reductions (HMEC), immortalized cell lines from breast reductions, and cancer cell lines, we found that the methylation status in this region was highly variable (Fig. 1B and C). HMEC cultures from four different reduction specimens (ages ranging from 15–43) showed no evidence of methylation in this region. Four immortalized cell lines each had detectable methylation with the MCF10A line showing the highest and the telomerase immortalized DU99 line the lowest level of methylation. Of the six cancer lines analyzed, the ER positive MCF7 and T47D cells had the highest levels of methylation. It is notable that even in these long-cultured cell lines, methylation at any given CpG was not necessarily uniform over the 22 dinucleotide pairs

assayed. These data indicate that the *MAL* promoter region may be a common target for hypermethylation in breast cancer.

To assess the effects of promoter methylation on *MAL* mRNA expression we treated a series of cultured benign and breast cancer cells with the methylation inhibitor 5-aza-2'-deoxycytidine (DAC) and then measured mRNA expression by quantitative RT-PCR. We observed induction (10–40 fold) of *MAL* mRNA transcription in the cancer cell lines T47D, MCF7, and ZR751 but either very little or no increase in the benign DU99 line and primary mammary epithelial cells (Fig. 1D). *MAL* expression was also strongly induced by DAC in the immortalized cell line MCF10A. Comparing methylation status to reactivation by DAC (Fig. 1D), we found that only the cell lines with the highest degree of promoter methylation induced *MAL* expression after treatment with the methylation inhibitor. The status of three of the most differentially methylated residues located at –46, –44, and –42 relative to the start of transcription correlate most closely with reactivation by DAC. Basal expression of *MAL* in these cells was also highly variable (Fig. 1D). The three primary HMEC cultures had nearly identical *MAL* mRNA levels whereas each of the four cell lines with DAC inducible expression had very low basal levels (MCF10A, T47D, MCF7 and ZR75–1). Two of the cancer lines (BT474 and HCC1937) expressed particularly high levels of *MAL*, approximately 15 and 150 fold higher than the HMEC cultures, respectively. 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 upon 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 was 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 (LCM) cancer and normal breast epithelia (from reduction mammoplasty specimens with no evidence of cancer). DNA suitable for bisulfite sequencing analysis was obtained from 10 cancers and 4 normal specimens. Comparing DNA extracted from bulk tumor versus the same tumor subjected to LCM 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). While 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* (DCIS) samples (with no co-existing invasive component) demonstrated hypermethylation in 3 out of 5 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 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 approximately 350 bp upstream of the transcription start site. We analyzed four microdissected normal breast epithelial specimens, four 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 breast cancer sample taken from two separate patients, demonstrating 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 demonstrated. By immunohistochemistry using a well-characterized monoclonal antibody (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 panels). 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 an MCF10A line stably expressing MAL with a C-terminal V5 tag. Parental MCF10A cells are hypermethylated at the MAL promoter and express low levels of mRNA (Fig. 1D) and protein (not shown). Fractionating these cells indicated that the exogenous MAL protein localized to both the soluble and 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 redistribution of this protein into the insoluble fraction (Fig. 3A, right panels). This suggests that decreased MAL expression may impact the protein composition of membrane microdomains.

To determine if expression of MAL induces a phenotypic effect related to cancer progression, we monitored cell motility using a wound-healing assay in stably transfected cells expressing either MAL or a vector control plasmid. MAL expressing MCF10A cells demonstrated a significant decrease in cell motility assessed by the ability to completely close a wound in the cell monolayer (Fig. 3B and C). In the transformed MCF7 and MD468 cells, a similar trend was observed in which MAL expression caused a reduction in motility (Supplementary Fig. S4). However, this decrease did not reach statistical significance. Reduced motility was not attributable to changes in proliferation or cell cycle as measured by propidium iodide staining and flow cytometry (data not shown).

Correlation of MAL protein expression with clinico-pathologic features and outcome

Since promoter methylation has a direct effect on gene transcription levels, we initially looked for a relationship between *MAL* promoter methylation and mRNA expression. Analyzing thirty-six 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 $\beta 2M$) 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, while all of the unmethylated cancers for which data was available were ER/PR negative (Supplementary Table 1).

Our earlier data demonstrated 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 (IHC) 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 while glomeruli and proximal tubules are negative) as positive and negative controls and to normalize for inter-assay variability in intensity. In addition, 20 cancers were included (in a blinded fashion) in more than one staining run to assure reproducibility. Within the invasive cancer samples we observed highly variable *MAL* protein levels, from undetectable to abundance similar to the kidney distal tubule epithelium (Fig. 4). Tissues were categorized as negative for *MAL* staining in the malignant breast epithelium (Fig. 4A) or positive for *MAL* (Fig. 4B). Many of the cancers contained infiltrating inflammatory cells that reacted with the antibody. We observed no significant relationship between *MAL* protein expression and common clinico-pathologic parameters (Table 1). Disease-free survival, with an average follow-up of 96 months, trended towards better outcome in patients with *MAL* expressing cancers but did not reach statistical significance (Fig. 4C).

While *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 (Fig. 5A). *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 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 2) 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 and colleagues (17) demonstrated that RNAi 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 (6/6) 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 demonstrated 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 a correlation between the degree of hypermethylation and 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 10–40 fold below that found in benign HMEC cultures. Inhibiting methylation in these cells induced *MAL* to a level comparable to 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) demonstrated that treatment of esophageal cancer cell lines with DAC and trichostatin A upregulated *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 not only consider the presence or absence of methylation in a few CpG dinucleotides within the *MAL* promoter, but the pattern of methylation over a relatively large portion of the promoter region. We surveyed over 800 bp of sequence, which included the 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 beta-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 the 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) (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 estrogen ($p = 0.003$) and progesterone receptor ($p = 0.006$) positive cancers (Supplementary Table 1). 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/PR 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 and PR negative tumors. If reduced *MAL* expression is associated with loss of polarity, then it is possible that the higher percentage of ER+ cancers that exhibit *MAL* hypermethylation is indicative of a greater selection for this property during the neoplastic progression of luminal type cancers compared to ER- 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 to the expression of T cell restricted genes (*CD2* and *CD27*) by qRT-PCR and array analysis (data not shown). While some cancers with very high levels of *MAL* also co-expressed 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 upregulation 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 to 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 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 re-distributed 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 monoclonal antibody, 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 upon the absence or presence of staining in malignant epithelial cells, no significant correlations were found with standard clinico-pathologic parameters 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 2). 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 paper implicating MAL expression in the development of breast cancer. Our data, and that of others, supports 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 performed based on a protocol by Grunau *et al.* (28) with additional modifications to accommodate a 96-well format (29). Briefly, 1 μ g of genomic DNA was denatured with 3 M NaOH for 20 min at 42°C followed by deamination in saturated sodium bisulfite/10mM hydroquinone (Sigma; St. Louis, MO) 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; Billerica, MA). All remaining steps of the protocol were performed 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 M NaOH and a final wash step with water. The DNA was recovered in 50 μ L of 10 mM 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 GGGAGTAATTTTTATTTTATAGGTAGA (forward), F3 GTTAGATTTATAGTTTTAGTTTTGG (forward), R2 ACCAAAAACCACTCACAACTC (reverse), R3 AAACCACTAAACAAAATACTACCC (reverse), R4 CCAAACTAAAACTATAAATCTAAC (reverse), and R5 CAAAACAAAACCACTTTAATCAAA (reverse). The PCR products were resolved on agarose gels, purified using Sigma GenElute spin columns (Sigma; St. Louis, MO) followed by cycle sequencing (Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit; Amersham Biosciences, Piscataway, NJ). Following resolution of the sequencing reactions on denaturing polyacrylamide gels, dried gels were exposed to radiographic film (Kodak X-OMAT MR; New Haven, CT) 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; Piscataway, NJ). Specimens with average methylation ranging from 40–100% were classified as heavily methylated, those exhibiting methylation levels between 5 and 40% partially methylated, and specimens with methylation levels <5% were designated as unmethylated. Methylation of DCIS samples was analyzed by fluorescence-based cycle sequencing using Big Dye Terminator Cycle Sequencing Reagents (Applied Biosystems; Foster City, CA).

Laser Capture Microdissection

The Veritas LCM system (Arcturus Engineering, Mountain View, CA) was used to obtain pure populations of ductal carcinoma in situ (DCIS), normal or malignant breast epithelial cells. The normal breast tissue specimens were obtained from women undergoing reduction mammoplasty with no evidence of cancer. Seven-micron sections were stained with hematoxylin and areas to be captured were selected based upon cytologic morphology (Supplementary Fig. S6). DNA was extracted using the Puregene DNA extraction kit (Gentra Systems; Minneapolis, MN), followed by sodium bisulfite modification and sequence analysis as described above, with the exception that the DNA was recovered in 25 μ L of 10 mM Tris/HCl (pH 8.0).

Cell culture and treatment

The human breast cancer cell lines MCF7, MDA-MB468, T47D, ZR75–1, BT474, and HCC1937 were obtained from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc.; Grand Island, NY). Benign breast epithelial cultures and lines were also used in these studies and were cultured in DFCI medium (30). Primary human mammary epithelial cells (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 over 10 years (31). The BE20E6 line was immortalized by stable transfection of a plasmid expressing the human papillomavirus (HPV) 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 human mammary epithelial cells derived at Duke University Medical Center.

Both benign and cancer cells were treated with 5 μ M DAC (Decitabine/5-Aza-2'-deoxycytidine, Sigma) from a 200 mM 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 extracted using the Qiagen RNeasy Mini Kit (Qiagen; Valencia, CA). Total RNA (1 μ g) was then reverse transcribed using Transcriptor RT (Roche; Basel, Switzerland). Subsequent real-time 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 μ L 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 β -2 microglobulin (*B2M*) gene (Applied Biosystems, 4326319E). All *MAL* and *B2M* expression analyses were done in parallel. Results are from two separate experiments performed in triplicate.

Generating Stable Transfectants

For stable transfection of *MAL*, the *MAL* coding sequence (bases 60–518, accession #NM002371) was PCR amplified, sequence verified and inserted into the Gateway entry vector

pDONR 221 (Invitrogen; Carlsbad, CA). Once in pDONR 221 the coding region was subsequently cloned into pDEST 40, a C-terminal V5 tagged Gateway expression vector (Invitrogen) using the LR recombination reaction following the manufacturer's protocol. Transfection of pDEST40-MALV5 into MCF7 or MCF10A cells was performed using Lipofectin (Invitrogen) or GenePORTER (Genlantis, Inc.; San Diego, CA) respectively, following the supplier's protocol. Control cell lines were established by transfecting an empty expression plasmid, pcDNA 3.1(+) (Invitrogen), into the respective parental cell lines. The efficiency for each transfection was analyzed by a GFP-containing plasmid. The selection of positive clones was carried out with 500 µg G418 sulfate (CellGro; Manassas, VA). After 3 weeks, several clones of MCF7 or MCF10A cells expressing stably MAL-V5 were isolated by cloning cylinders and expanded under continued selection.

Detergent Extraction Procedures

Glycolipid-enriched membranes (GEMs) were isolated by standard procedures (32). Cells were grown to confluence in 100-mm dishes, rinsed with phosphate-buffered saline and lysed for 20 min in 1 ml of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 at 4°C. The lysate was scraped from the dishes with a rubber cell scraper, the dishes were rinsed with 1 ml of the same buffer at 4°C, and the lysate was homogenized by passing the sample through a 22-gauge needle. The extract was finally brought to 40% sucrose in a final volume of 4 ml and sequentially overlaid with 6 ml of 30% sucrose and 2 ml of 5% sucrose. Gradients were centrifuged for 18 h at 39,000 rpm at 4°C in a Beckman SW40 rotor. The opalescent band at the 5–30% sucrose interface, containing rafts, was collected as the Triton X-100 insoluble fraction, whereas the 40% sucrose layer containing the load was harvested as the Triton-X soluble fraction. Aliquots were then subjected to immunoblot analysis.

Immunoblot Analyses

For immunoblot analysis, samples were subjected to SDS-PAGE in 8–16% gradient acrylamide gels under reducing conditions and transferred to Whatman nitrocellulose membranes. After blocking with 10% (w/v) nonfat dry milk, 0.1% (v/v) Tween-20 in phosphate-buffered saline for 1 h, membranes were incubated for 2 h in the presence of anti-MAL 6D9 monoclonal antibody (8,9), anti-V5 (Invitrogen), anti-flotillin-1 (Transduction Laboratories; Lexington, KY), or 1 h with an anti-actin Ab (Sigma). After several washings, membranes were incubated for 1 h with goat anti-mouse IgG coupled to horseradish peroxidase, washed extensively, and developed using the Western Lightning Chemiluminescence Kit (PerkinElmer; Boston, MA).

In Vitro Wound-Healing Assay

Cell motility was measured using an *in vitro* wound-healing assay. Cells were seeded on six-well tissue culture plates and grown to 100% confluence. Wounds were created by scraping the monolayer with a sterile pipette tip (1mm O.D.). The wounded monolayers were washed twice with phosphate-buffered saline to remove cell debris then incubated in fresh medium, imaged through a Nikon Diaphot inverted microscope and photographed with a Nikon D50 digital camera at 0, 12, 24, 36 and 48 hours. Replicate areas were wounded and measurements of the wound closure were made on the digital images.

Immunohistochemical Analysis

Tissue specimens were obtained under an IRB approved protocol from patients undergoing breast surgery at Duke University Medical Center between 1990 and 1998. Data for the survival analysis and clinico-pathologic features listed in Table 1 were obtained from 122 invasive breast cancer patients. Percent disease free survival was measured from the date of initial diagnosis to the date of first recurrence or the date of last follow-up. Non-cancerous tissue

samples were obtained from reduction mammoplasties. These specimens were flash frozen and maintained at -135°C . Sections of $5\text{ }\mu\text{m}$ thickness were cut, air dried, and fixed in acetone for 5 min. Immunologic detection using the anti-MAL 6D9 monoclonal antibody (8,9) or mouse IgG (Vector Laboratories; Burlingame, CA) was performed 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 sub-groups (methylated vs. unmethylated; MAL+ vs. MAL-; Chemo+ vs. Chemo-) were calculated using chi squared analysis or Fisher's exact test when groups contained an $n < 5$. Survival curves were generated for primary breast cancer patients with the Kaplan-Meier estimate and log-rank test using the Prism statistical software (GraphPad; San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Nancy Glover for assistance with the immunohistochemical studies.

Grant support: NIH CA84955 (J.R. Marks) and NIGMS F31GM082048 (H.N. Horne).

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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 by hatched boxes and the promoter CpG island with a solid bar above the sequence. The complete sequence of the region analyzed by bisulfite sequencing is shown on top with the CpG dinucleotides in bold, the transcription start site indicated with +1, and the ATG translation initiation codon underlined. **B.** Representative bisulfite sequencing gels for primary human mammary epithelial cells (HMEC1), immortalized (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. Positions of the top and bottom nucleotide relative to the start of transcription are

indicated to the right of the sequencing gel. **C.** A schematic of bisulfite sequencing of the *MAL* promoter region in the complete panel of benign and cancer cells showing unmethylated CpGs in human mammary epithelial cells (HMEC), partial methylation in immortalized cell lines (IM), and hypermethylation in the breast cancer cells (CA). The position of the first and last CpG dinucleotide relative to the start of transcription is indicated above the diagram. **D.** Graph shows reactivation of *MAL* expression specifically in methylated cancer cell lines and the immortalized cell line MCF10A. Expression was measured by TaqMan real-time PCR. The numbers below the graph indicate the basal level ($\log_2$) of expression measured by qRT-PCR and the status of three of the most differentially methylated residues in the *MAL* promoter is also shown, corresponding to cytosines 2, 3, and 4 underlined in panel C.

FIGURE 2.

Methylation of the *MAL* promoter region in primary breast tumors. Methylation analysis of the *MAL* promoter region by bisulfite sequencing in a total of 36 primary breast tumors and matched lymphocytes revealed three distinct patterns of methylation in the tumors including: **A.** 7/36 tumor samples exhibited heavily methylated cytosine residues, 18/36 tumor samples showed partial methylation of their cytosine residues, and 11/36 tumor samples were unmethylated while matched lymphocyte DNA revealed the absence of methylation in all but one of the samples analyzed. The region was sequenced using the reverse primer R3, the complement of the methylated C is evident in the G lane. Positions of the top and bottom nucleotide relative to the start of transcription are indicated to the right of the sequencing gel.

B. The methylation status obtained from the whole tumor samples was confirmed by bisulfite sequencing of DNA from laser microdissected (LCM) cells. Bisulfite sequencing of LCM normal breast epithelial cells revealed the absence of methylation. The region was sequenced using the reverse primer R5, the complement of the methylated C is evident in the G lane. Positions of the top and bottom nucleotide relative to the start of transcription are indicated to the right of the sequencing gel. **C.** Examples of bisulfite sequencing of normal and breast cancer DNA indicating CpG dinucleotides by asterisks. The arrow indicates the point where the methylation profile transitions, located at -356 relative to the start of transcription. Positions of the top and bottom nucleotide relative to the start of transcription are indicated to the right of the sequencing gel. **D.** Diagram showing the *MAL* promoter CpG Island and flanking regions with arrowheads indicating the positions of the sequencing primers and the area where the methylation profile transitions from differential methylation (DM) to universal methylation (UVM). Vertical lines indicate CpG dinucleotides and the start of transcription is designated by the thick black arrow.

FIGURE 3.

Role of MAL in lipid raft composition and cell motility. **A.** Detection of either endogenous MAL in HCC1937 or exogenous C-terminal V5-tagged MAL in MCF10A cells after extraction with 1% Triton-X and centrifugation to equilibrium. Aliquots from either the detergent soluble (S) or insoluble (I), lipid raft containing fractions were analyzed with anti-MAL 6D9 mAb (top left blot), anti-V5 (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 performed on MCF10A cell lines stably transfected with either vector control (top panels) or V-5 tagged MAL (bottom panels). The images were taken at 0, 24, and 48 hours after wounding. Original

magnification X10. **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 4.

MAL protein expression and survival analysis in breast cancer patients. **A.** Representative IHC of a breast tumor negative for MAL expression stained with either MAL6D9 monoclonal antibody, mouse IgG, or H&E. **B.** Representative IHC of a breast tumor positive for MAL expression. Original magnification X20. **C.** Kaplan-Meier plot of percent disease survival for MAL- and MAL+ breast cancer patients.

FIGURE 5.

Survival analysis of breast cancer patients by adjuvant chemotherapy treatment. Kaplan-Meier plot of percent disease free survival for: **A.** all patients, **B.** those who received adjuvant chemotherapy, and **C.** those who did not receive chemotherapy.

Table 1
Clinico-pathologic Features of Patients by MAL IHC

	MAL- n (%)	MAL+ n (%)	P
Patients (n)	35 (29)	87 (71)	
Age			0.44
≤50	18 (51)	38 (44)	
>50	17 (49)	49 (56)	
Race			0.62
White	28 (80)	66 (76)	
Non-White	7 (20)	21 (24)	
<i>Black</i>	7	16	
<i>Am Indian</i>	0	2	
<i>Asian</i>	0	3	
Estrogen receptor status			0.47
Positive	18 (55)	50 (62)	
Negative	15 (45)	31 (38)	
Progesterone receptor status			0.7
Positive	17 (52)	45 (56)	
Negative	16 (48)	36 (44)	
Node involvement			0.14
Yes	16 (48)	28 (34)	
No	17 (52)	55 (66)	
Stage			0.19
I or IIA	22 (63)	60 (75)	
IIB and higher	13 (37)	20 (25)	
Tumor stage			0.49
T1 A, B, C	15 (45)	32 (39)	
T2 and higher	18 (55)	51 (61)	
Histologic Grade			0.17
1	1 (3)	3 (4)	
2	5 (17)	25 (34)	
3	24 (80)	45 (62)	
Adjuvant chemotherapy			0.37
Yes	22 (63)	47 (54)	
No	13 (37)	40 (46)	
Hormonal therapy			0.18
Yes	15 (43)	49 (56)	
No	20 (57)	38 (44)	

NOTE: Statistical significance calculated using chi squared analysis