MECHANISM AND CONSEQUENCE OF THE HYPERMETHYLATOR PHENOTYPE IN HUMAN BREAST CANCER

Jacqueline Devon Roll

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology and Laboratory Medicine.

Chapel Hill
2008

Approved by:

William B. Coleman, Ph.D.

Lisa A. Carey, M.D.

Frank C. Church, Ph.D.

William K. Funkhouser, M.D., Ph.D.

Alisa S. Wolberg, Ph.D.

ABSTRACT

JACQUELINE DEVON ROLL: Mechanism and Consequence of the Hypermethylator Phenotype in Human Breast Cancer
(Under the direction of William B. Coleman, Ph.D.)

DNA hypermethylation events and other epimutations occur in many neoplasms, producing gene expression changes that contribute to neoplastic transformation, tumorigenesis, and tumor behavior. Some human cancers exhibit a hypermethylator phenotype, characterized by concurrent DNA methylation-dependent silencing of multiple genes. To determine if a hypermethylation defect occurs in breast cancer, the expression profile and promoter methylation status of 66 methylation-sensitive genes were evaluated among 16 breast cancer cell lines. The relationship between gene expression (assessed by RT-PCR and quantitative real-time PCR), promoter methylation (assessed by MSP, bisulfite sequencing, and 5-aza-2'deoxycytidine treatment), and the DNA methyltransferase machinery (total DNMT activity, DNMT1, DNMT3a, and DNMT3b proteins) were examined. Unsupervised cluster analysis of the expression of methylation-sensitive genes revealed two groups of cell lines that possess distinct methylation signatures: (i) hypermethylator cell lines, and (ii) low-frequency methylator cell lines. The hypermethylator cell lines are characterized by high rates of concurrent methylation of nine genes (*CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*), whereas the low-frequency methylator cell lines typically do not methylate these genes. Hypermethylator cell lines coordinately overexpress total DNMT activity and DNMT3b protein compared to

normal breast cells. In contrast, most low-frequency methylator cell lines possess DNMT activity and protein levels that are indistinguishable from normal. Mining of microarray expression data from primary breast cancers identified groups of tumors that express a hypermethylation signature defined by loss of gene expression of seven to nine indicator genes. On average, the hypermethylator breast cancers represent ~23% of tumors, with ~79% of hypermethylator tumors belonging to the basal subtype, and ~58-81% of all basal tumors exhibiting this hypermethylation defect, suggesting that the hypermethylator defect cosegregates with poor prognosis breast cancers. Methylation analysis of 26 primary breast tumors revealed extensive methylation of genes of interest among basal tumors, but low levels of methylation in tumors of other molecular subtypes. RNAi knockdown of DNMT3b in hypermethylator MDA-MB-453 and BT549 cells resulted in reexpression of methylation-silenced indicator genes. These results strongly suggest that overexpressed DNMT3b protein drives aberrant methylation of a concurrent set of epigenetically-regulated genes and typifies a novel hypermethylator phenotype in human breast cancer.

ACKNOWLEDGEMENTS

I would like to begin by thanking my advisor and mentor, Bill Coleman, for his never-failing patience, optimism, and advocacy. Thank you for creating a learning environment that provides supportive guidance whenever needed but also the independence to help me develop my skills as a scientist. I would especially like to thank my committee members—Drs. Lisa Carey, Frank Church, William Funkhouser, and Alisa Wolberg—for their guidance, advice, and insight regarding my project. You have each been instrumental in shaping this document and my graduate career. I truly appreciate the time and thought that each of you has put into helping me make this project the best that it can be. I'd also like to acknowledge Dr. Wendell Jones for his collaboration on the microarray data mining aspects of this project.

I would like to thank my family—my mom, aunt, and grandmother—for their love, encouragement, and for attempting to understand the science that I spend my days pursuing. Here I must not only acknowledge the family that has watched me grow through the years and nurtured my abilities, but also the family I have recently joined—that of David and Sue Roll. Thank you for celebrating my successes as your own and for welcoming me into your hearts as well as your family! I feel truly blessed to have such fantastic in-laws!

I also want to thank many past and present lab members who have taught me not only about science, but also about life. To my current labmates—Kristen White and Rupninder Sandhu—thank you for helping me face the ups and downs of graduate school with humor and determination. I am truly grateful for having had the chance to work with you and call

you my friends. To my past labmates—Hunter Best, Jen Jahn, Robyn Mercer, and Ashley Rivenbark—I want to thank you for paving the way and showing me the light at the end of the tunnel.

Most of all I want to express my love and deepest gratitude to my best friend and loving husband, Rob. Thank you for taking a leap of faith, uprooting your life, and following me to North Carolina four years ago (and for doing so, once again, in a few more weeks)! You have been beyond supportive and knowing that I can always count on you has gotten me through every tough time and has made even the smallest victory that much sweeter. This is every bit your accomplishment as it is mine. Thank you for embarking on this adventure with me! I love you very much!

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LIST OF ABBREVIATIONS

$\Delta\Delta C_T$	Comparative method for real-time RT-PCR analysis
5-aza	5-aza-2'-deoxycytidine
ANOVA	Analysis of variance
bp	Basepairs
BSA	Bovine serum albumin
CDH1	E-cadherin
cDNA	Complementary DNA
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
CIMP	CpG island methylator phenotype
CK18	Cytokeratin 18
CST6	Cystatin E/M
DCIS	Ductal carcinoma *in situ*
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleotide-triphosphate
GNA11	Guanine nucleotide binding protein, alpha 11
Her2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
LOH	Loss of heterozygosity
miR	MicroRNA
mRNA	Messenger ribonucleic acid

μg	Microgram
ml	Milliliter
mM	Millimolar
MMP	Matrix metalloproteinase
MSP	Methylation-sensitive PCR
MUC1	Mucin 1
MYB	v-myb myeloblastosis viral oncogene homolog
ng	Nanogram
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA-interference
RT-PCR	Reverse transcription polymerase chain reaction
SCNN1A	Sodium channel, nonvoltage-gated 1 alpha
S.E.M.	Standard error of the mean
shRNA	Small hairpin RNA
TFF3	Trefoil factor 3
TMA	Tissue microarray
TMI	Total methylation index

INTRODUCTION

BREAST CANCER: INCIDENCE AND MORTALITY

Worldwide, over 1.3 million women are diagnosed with breast cancer each year, of which over 460,000 will die from their disease (1). The lifetime probability of developing breast cancer in developed countries is about 4.8%, and in developing countries the probability is 1.8% (1). In industrialized nations like the United States, breast cancer is the most frequently occurring cancer among women, with approximately 180,000 new cases annually (2). Breast cancer also represents the second most common cause of cancer-related mortality (over 40,000 deaths annually) in American women, a mortality rate that is second only to lung cancer (2).

In the United States, advances in detection, therapeutic treatments, as well as a better understanding of how breast cancer develops, has resulted in more successful management of the disease. These gains have contributed to a current overall 5-year survival rate of 89% (Table 1) and a breast cancer mortality rate that has been in decline since 1990 (3). As with many cancers, the earlier stage at which breast cancer is detected, the better the outcome and overall survival (Table 2). The majority (77%) of breast cancers occur in women over 50 years of age, making age an important risk factor for the development of breast cancer (1). However, while breast cancer is less common in women of a young age, younger women who develop the disease tend to have more aggressive breast cancers than older women. Thus, the five-year survival rate is 81% for women under 45, 85% for women aged 45-64,

Table 1. Expected 5-year survival rates for breast cancer, by stage[1].

Stage	5-year Survival
0	100%
I	100%
IIA	92%
IIB	81%
IIIA	67%
IIIB	54%
IV	20%

[1] Adapted from the American Cancer Society Website (www.cancer.org).

Table 2. Expected overall survival rates for breast cancer, by time from diagnosis[1].

Time	Overall Survival Rate
5 years	89%
10 years	81%
15 years	73%

[1] Adapted from the American Cancer Society Website (www.cancer.org).

and 86% for women aged 65 and older (1). Paradoxically, while breast cancer mortality has declined in Western countries, breast cancer rates have risen about 30% over the last 25 years (1). This may be attributable to improved screening techniques such as mammography and its increasingly widespread use, although the precise cause for the increasing incidence remains unknown. In the United States, breast cancer incidence increased sharply in the 1980s and continued to rise, although less rapidly, in the 1990s (3). After continuously increasing for more than two decades, U.S. breast cancer incidence began to decline, decreasing by 3.5% per year from 2001 to 2004 (1). This slight decline in incidence has been suggested by some to be attributable to the reduced use of hormone replacement therapy after the discovery that these hormones significantly raise the risk of developing breast cancer (3). While these trends have been closely monitored in the U.S. and around the world, debate still continues on what the changing trends in breast cancer incidence and mortality mean and what factors contribute to these rate fluctuations.

RECOGNIZED RISK FACTORS FOR THE DEVELOPMENT OF BREAST CANCER

The risk of developing breast cancer is not the same for all women. Currently, a number of risk factors are known to contribute to the development of this disease. While age and sex are considered the chief risk factors for breast cancer development, it is generally recognized that it is the combination of numerous factors, rather than a single factor, that drives the initiation and progression of the majority of breast tumors. These additional risk factors include lifetime estrogen exposure, race, family history, and environmental factors (carcinogen exposure, diet, smoking, exercise, and obesity), all of which are important in assessing a woman's relative breast cancer risk. However, it is important to note, that while

having numerous risk factors increases the likelihood that a woman will develop breast cancer, simply having these risk factors in no way guarantees that cancer will ever develop.

Risk Factors Related to Estrogen Exposure

Epidemiologic studies have identified a number of risk factors for breast cancer that are associated with reproductive history, suggesting a role for lifetime exposure to estrogens in breast carcinogenesis. The importance of lifetime estrogen exposure was first suggested by the observation that nuns had a relatively higher incidence of breast cancer compared to women who bore children, an association later confirmed by scientific study (4). As more than two-thirds of breast cancers are fueled by estrogen at some point during the course of cancer progression, this association between estrogen exposure and breast cancer risk is an important one. For example, factors such as younger age at menarche, older age at menopause, infertility, having children later in life, and nulliparity, each increases a woman's risk of breast cancer, while lactation decreases her risk (5).

Exposure to environmental compounds that mimic estrogen (such as organochlorine pesticides) may also increase a woman's risk of developing breast cancer (6), although there is some disagreement about the relative contributions of these exposures to breast cancer incidence (7). This observation highlights the importance of understanding the carcinogenic potential of these chemicals to which people in industrialized nations are commonly exposed. Since industrialized countries have higher incidences of breast cancer than non-industrialized nations, environmental exposure to chemical carcinogens may account for some of the incidence discrepancy between these two regions (8). For example, after studies showed that certain organochlorines such as PCBs, DDT, kepone, chlordane, lindane, and benzene hexachloride (BCH) were carcinogenic, use of these chemicals was banned in the United

States. However, the possible carcinogenic roles of other related chemicals that are still widely used (such as endosulfan, methoxychlorine, triazine, nonylphenol, phthalates, vinyl chloride, and bisphenol A), are under investigation, with some studies suggesting that exposure to these compounds may also be linked to a person's risk of developing breast cancer (6).

Diet and Exercise

There are additional factors that may account for the higher incidence of breast cancer in Western countries. These include a high fat diet and/or a diet low in fruits and vegetables, as well as low levels of physical activity and obesity (9, 10). Evidence that a Western diet has a role in breast cancer development comes from studies that followed the immigration of Asian women (who typically have low rates of breast cancer) to Western countries. Within one generation, women of Asian-descent born in Western countries have breast cancer rates similar to those of Caucasians (11). In fact, Asian-American women born in the West have a breast cancer risk that is 60% higher than Asian-Americans born in the East (11). Another study found that women in China who ate a more Westernized diet (high in meat, white bread, milk, and puddings) had twice the risk of developing breast cancer compared to Chinese women who ate a more traditional Eastern diet (high in vegetables) (12). Concurrent with a high fat diet, low levels of physical activity and obesity are also linked with an increase in breast cancer risk (1). Obesity itself, in addition to being indicative of lifestyle, may contribute to breast cancer directly since fat cells can produce excess estrogens. Obesity is known to affect prognosis through numerous pathways, including associated adverse disease features and comorbidities that can interfere with treatment (13).

Race and Breast Cancer Risk

Incidence data suggests that race is a major factor in the development of breast cancer (Table 3). In the United States, Caucasian, Hawaiian, and African-American women have the highest incidence of invasive breast cancer, which is approximately four times higher than that of Korean, American Indian, and Vietnamese women (8). Additionally, African-American women have the highest death rate from breast cancer (Table 4) and are more likely to be diagnosed with a later stage of breast cancer than Caucasian women (8). Reasons for these ethnic differences in breast cancer incidence are likely multifactorial and may be attributable to socioeconomic factors, access to care, different cultural practices, dietary habits, and/or subtle genetic variations.

Family History and Breast Cancer Risk

Family history of breast cancer is a significant risk factor for developing the disease. Women with an affected first-degree relative (such as a mother, sister, or daughter) have a two-fold to four-fold increased risk of developing breast cancer (5). The contribution of family history to breast cancer incidence suggests that genetic factors can play a strong role in the development of breast cancer.

Several genes have now been shown to be mutated in familial forms of breast cancer. The elucidation of the roles genetic mutations play in heritable breast cancer was significantly advanced with the discovery that the *BRCA1* gene was often mutated in individuals with family history of breast cancer (14). Similar studies later revealed the role of a second 'breast cancer gene," *BRCA2,* in inherited breast cancer (15). The *BRCA* genes are tumor suppressors which control cell growth and cell death via mechanisms such as DNA

Table 3. Breast cancer incidence for American women, by ethnicity[1].

Ethnicity	Incidence (per 100,000)
All Ethnicities	127.8
Caucasian	132.5
African-American	118.3
Asian / Pacific Islander	89.0
American Indian / Alaskan Native	69.8
Hispanic	89.3

[1] Adapted from National Cancer Institute, SEER Cancer Statistics Review, 2007 (www.seer.cancer.gov).

Table 4. Breast cancer deaths for American women, by ethnicity[1].

Ethnicity	Death Rate (per 100,000)
All Ethnicities	25.5
Caucasian	25.0
African-American	33.8
Asian / Pacific Islander	12.6
American Indian / Alaskan Native	16.1
Hispanic	16.1

[1] Adapted from National Cancer Institute, SEER Cancer Statistics Review, 2007 (www.seer.cancer.gov).

damage repair. A woman with a mutated *BRCA1* gene has a 65% risk of developing breast cancer by the age of 70, while a woman with a mutated *BRCA2* gene has a 45% risk (1). Heritable mutations such as those in *BRCA1, BRCA2, p53,* and *pTEN* genes are responsible for 5-10% of all breast cancer cases, with *BRCA1* and *BRCA2* mutations accounting for approximately half of all inherited breast cancers. Women with mutations in the *BRCA1* gene not only have an increased risk of developing breast cancer, but also of developing ovarian cancer (16). Women with this condition, which is known as hereditary breast ovarian cancer syndrome, are likely to be diagnosed at a younger age (under 50), have a family history of breast and/or ovarian cancer, and have bilateral cancer (independent tumors arising in both breasts or ovaries). Women with inherited mutations in the *p53* tumor suppressor gene, such as those with Li-Fraumeni Syndrome, are at risk for many forms of cancer, including breast cancer (17). As the gene plays an integral role in cell cycle regulation, DNA repair, and apoptosis, mutations in *p53* result in unchecked tumor growth and cellular proliferation. Likewise, women with inherited mutations in the *PTEN* gene, which is normally responsible for regulating cell division and apoptotic signaling, like those with Cowden syndrome, have increased risk of developing breast, thyroid, and/or uterine tumors (18). In fact, women with Cowden syndrome have a 25-50% chance of developing breast cancer in their lifetime (19). Other inherited mutations which lead to a significant increase in breast cancer risk include those including the *CHEK2, ATM, NBS1, RAD50, BRIP1,* and *PALB2* genes (20).

It is important to note that inherited mutations in these genes do not guarantee tumor development. Loss of heterozygosity (LOH), by which normal function of one copy of a gene is lost after the other allele was already inactivated (by mutation), explains why this is the case (21). Individuals born with an inherited mutation in one allele of these key genes are at a

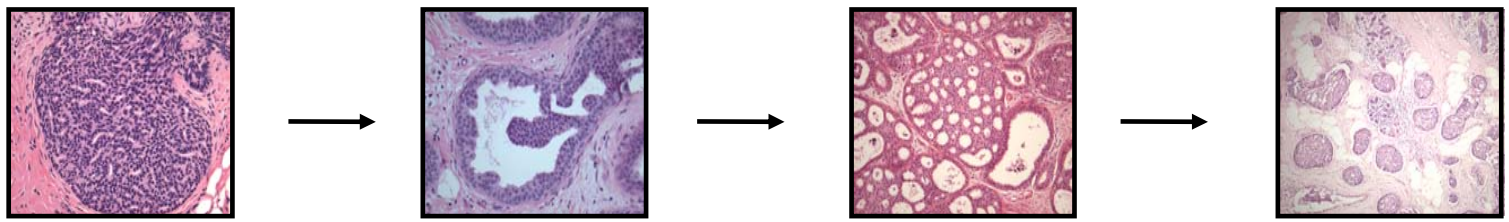
higher risk of developing breast cancer because only one event is needed to inactivate the second allele, the so-called "second hit" of Knudson's two hit hypothesis (22). Other factors are needed for tumor development beyond a single gene mutation, such as additional genetic or epigenetic changes at other loci, as well as lifestyle factors such as obesity, alcohol consumption, and smoking history.

BREAST CANCER: NATURAL HISTORY AND PATHOGENESIS

Natural History of Human Breast Cancer

The majority of breast cancers are thought to arise along the anatomic boundary of the terminal duct-lobular unit. The primary components that make up the breast are luminal epithelial cells, myoepithelial cells, adipose tissue, and mammary stroma. Luminal epithelial cells form the inner glandular components of breast epithelial compartments, while myoepithelial cells reside in an outer location (23). Debate exists as to whether these different cell types give rise directly to different cancer types or if progenitor cells give rise to tumors that progress through specific paths of differentiation. However, most breast cancers are believed to pass through well-defined histologic stages, including a progression from normal to hyperplastic, hyperplasia to atypical hyperplasia, followed by ductal carcinoma *in situ* (DCIS), and finally invasive ductal carcinoma (IDC), which can metastasize (Figure 1). In breast hyperplasia (the first precursor lesion), epithelial cells of the normal breast tissue become hyperplastic, proliferating beyond what is normal. In the next stage, atypical breast hyperplasia, there is an atypical proliferation of breast cells, rather than a simple proliferation of otherwise typical breast cells. If these atypical cells accumulate certain genetic or epigenetic lesions that allow them to acquire neoplastic characteristics,

Figure 1. Histological stages associated with breast cancer development. Breast cancer typically evolves in a step-wise manner that is accompanied by a series of histological changes including: ductal hyperplasia, atypical ductal hyperplasia, DCIS, and IDC. Images provided by Dr. Chad A. Livasy (Department of Pathology and Laboratory Medicine, UNC).



Ductal Hyperplasia → **Atypical Ductal Hyperplasia** → **Ductal Carcinoma *in situ*** → **Invasive Ductal Carcinoma**

they can become dysplastic, gaining abnormal characteristics which may, in turn, allow them to form a breast tumor. If neoplastic ductal epithelial cells have multiplied within the duct but have not yet breached the basement membrane, the lesion is known as a DCIS. Between 30-50% of women whose DCIS is treated with surgery alone will go on to develop invasive breast cancer within 10 years (24). Approximately 62,000 cases of DCIS are diagnosed in the United States each year, most commonly through mammography (1). DCIS often appears in mammograms as a cluster of microcalcifications and is generally treated by lumpectomy (21). There are a number of subsets of DCIS depending on their histological appearance including comedo, solid, cribiform, papillary and micropapillary (21). Comedo DCIS, which is marked by necrosis, is generally more aggressive than other forms of the disease (21). If additional genetic and epigenetic changes occur, the cells may be able to breech the basement membrane. In this case, the lesion is known as invasive breast carcinoma and begins to infiltrate the surrounding breast tissue. The majority of invasive breast cancers (80%) are IDC in histology, while lobular carcinomas constitute 5-10%, and the remainder is divided into rare histologic subtypes including tubular, medullary, and mucinous (21). These invasive breast cancer cells can enter the blood or lymph system and metastasize to various organs including the lung and brain, resulting in metastatic breast cancer.

Clinical outcome is strongly influenced by the stage of the breast lesion at time of diagnosis, with the lower stage corresponding to better survival rates. The staging system takes into account tumor size, tumor spread to lymph nodes (known as node positivity), as well as distant metastasis. Stage 0 breast cancer is defined as carcinoma *in situ*, Stage I as a tumor $\leq$2 cm with negative axillary nodes, Stage II as tumors between 2-5 cm and/or the

presence of positive axillary nodes, Stage III as a tumor >5 cm and/or fixed axillary nodes, while Stage IV as the presence of distant metastases beyond axillary nodes (21).

Molecular Pathogenesis of Breast Cancer

In order for a normal breast epithelial cell to become neoplastic, a number of genetic and/or epigenetic changes that confer various neoplastic properties are required. Typically, these changes allow the cancer cell to escape normal control mechanisms that would typically result in cell death, that is—escape from apoptosis, self-sufficiency in growth signals, insensitivity to antigrowth signals, limitless reproductive potential, sustained angiogenesis, and the ability to invade tissue and metastasize (25). A cell may acquire these properties in a number of ways—genetic mutations, chromosomal changes, and epigenetic changes—that result in the loss of tumor suppressor gene expression and/or the overexpression (or inappropriate expression) of oncogenes.

In many instances of breast carcinogenesis, LOH explains how both alleles of a tumor suppressor gene become lost (or sustain 'two hits'), resulting in both sporadic and hereditary breast cancers. Many sites of chromosomal deletion marked by LOH correspond to regions containing known tumor suppressor genes (21). Genes involved in cancer induction and progression often play integral roles in certain key categories: growth factor receptors, intracellular signaling molecules, cell cycle regulators, regulators of apoptosis, cell adhesion molecules, and matrix metalloproteinases (21). Growth factor receptors, such as the *EGFR* family of genes that includes the Her2/*neu* oncogene, encode transmembrane proteins with tyrosine kinase activity. Genes involved in kinase activity, which normally serve to phosphorylate downstream signaling targets, can become oncogenic when overexpressed or amplified, leading to deregulated growth and proliferation. Approximately 20-40% of breast

cancers have overexpression of at least one *EGFR* family member (21), suggesting an integral role for this gene family in breast carcinogenesis. Genes involved in intracellular signaling, such as the *ras,* c-*myc,* and c-*src* proto-oncogenes, have been found to play roles in numerous signaling pathways whose dysregulation has been implicated in breast cancer. Cell cycle genes, such as *p53, RB, TGFβ,* and numerous cyclin proteins, participate in regulating passage through the cell cycle. As such, any aberration in genes involved in this process can result in uncontrolled cell growth and neoplastic transformation. Additionally, since properly functioning cell cycle checkpoint regulation prevents damaged (unrepaired) DNA from being replicated, unchecked progression through the cell cycle can result in the generation of mutations and/or accumulation of mutations in abnormal cells. Similarly, apoptosis is normally triggered by stimuli (such as lack of growth factors, DNA damage, and *p53* expression) which prevent damaged cells from replicating. Thus, genetic or epigenetic changes in the genes which normally trigger apoptosis, such as *bcl-2*, and *p73*, can contribute to the unchecked growth and proliferation of breast cancer cells. During tumor initiation, neoplastic cells must circumvent normal adhesion mechanisms in order to breach the basement membrane and invade surrounding tissues. Thus, changes in cell adhesion molecules, such as cadherin family proteins, are necessary for tumor progression and metastasis. Typically, cell-cell and cell-matrix interactions keep cells anchored within the tissue normal architecture. The finding that the *CDH1 (E-cadherin)* gene is abnormally expressed in 60-70% of breast cancers, suggests an integral role for this protein in tumor progression (21). Similarly, extracellular proteins are important for regulating normal cell-matrix interactions and can lead to tumor cell invasion when dysregulated, resulting in basement membrane degradation. This is demonstrated by the overexpression of matrix

metalloproteinases (MMPs) in many tumors. MMPs are found overexpressed in some 20-80% of breast cancers (21).

Dysregulation of genes by genetic, chromosomal, and/or epigenetic changes that function in any of these pathways can set the stage for breast carcinogenesis. Mutations in tumor suppressors like *BRCA1, p53,* and *RB* can effectively deactivate cell cycle and DNA repair checkpoints, leading to unrestricted cell growth and replication (21). Though some mutations are inherited, such as a familial *BRCA1* mutation, most breast cancers occur spontaneously, as a result of a lifetime of accumulation of mutations. Chromosomal changes such as gains or losses in chromosome number (which can result in changes in gene copy number) and translocations (in which chromosomes are improperly rearranged) have important implications for tumor development (26). These aberrant chromosomal patterns can cause tumor suppressors to become mutated and/or deleted and oncogenes to become activated and/or amplified. In contrast, epigenetic mechanisms, such as DNA methylation and chromatin modification, are heritable changes in gene expression that do not alter the DNA itself. Since DNA methylation can silence gene expression, improper hypermethylation of tumor suppressor genes can contribute to neoplastic transformation (27). Alternatively, improper demethylation of proto-oncogenes can result to their activation, contributing to cancer induction and progression (28). Post-translational modifications to histone proteins (including acetylation, methylation, and ubiquitination) can also result in epigenetic changes in the transcriptional states of DNA within functional units of chromatin (29). Thus, epigenetic mechanisms can lead to dysregulation of segments of DNA resulting in the silencing of tumor suppressors or activation of oncogenes.

BREAST CANCER SUBTYPES

Molecular Subtypes of Breast Cancer

Breast cancer has long been known to be a heterogeneous disease. Our understanding of the various breast cancer subtypes and the implications that these distinctions hold for patient outcome has changed substantially over the last four decades and continues to evolve rapidly. In the late 1960s the importance of lymph node involvement (30) and tumor size (31) in predicting patient outcome was first recognized. With the discovery of the importance of estrogen receptor (ER) in determining response to endocrine therapy and the advent of the anti-estrogen drug tamoxifen, hormone receptor typing (which also includes progesterone receptor status) was added to traditional histological grading and staging of breast tumors (32). Microarray technology has revolutionized the traditional, histologic-based classification systems that were primarily concerned with tumor morphology and elucidated the value of a molecular-based system of breast tumor subtyping. When microarray data from 42 breast cancer patients was analyzed, researchers found that human breast cancers cluster into at least four subtypes based on expression differences in an 'intrinsic' gene set: an ER+ luminal group, and the (typically) ER- groups including a normal-like breast group, a Her2+ group, and a basal-like group (33). Intrinsic genes exhibit large-scale expression variations in tumors from different individuals, but show little expression variation within an individual (34). Various other molecular classifications of different datasets have revealed additional subtypes, including the luminal A, luminal B, luminal C, and claudin-low groups. These breast cancer subtypes represent clinically distinct groups which appear to possess differences in risk of metastasis and patient survival (35). Evidence has emerged that the metastatic potential of breast tumors appears to be an inherent feature of breast tumors, thus

metastatic potential is likely acquired earlier in cancer progression than initially believed (36, 37). Luminal breast tumors tend to have expression of keratin proteins similar to breast duct epithelial cells and are generally less aggressive than ER- subtypes. However, a poorer prognosis luminal subset, known as luminal B (as opposed to good prognosis luminal A breast tumors) was subsequently identified (35). These poorer outcome luminal tumors tend to be higher grade and have a higher expression of proliferation genes, as compared to luminal A tumors (35, 38, 39). Most investigators agree that luminal A tumors represent approximately 40-60% of all breast cancers and tend to have the best outcome, followed by luminal B tumors, which account for 15-20% of breast tumors. Importantly, these ER-positive groups generally have better outcomes than the ER-negative Her2+ subtype (which accounts for ~7-12% of breast cancers) or the basal subtype (which accounts for ~13-25% of all breast cancers) (40-43).

Triple-Negative Breast Cancer

Approximately 7-20% of breast cancers fall into the "triple-negative" category. This subset of breast cancers is characterized as ER-negative, PR-negative, and Her2-negative (ER-/PR-/Her2-) (44). The majority (~90%) of triple-negative breast cancers are classified as basal subtype (23), which is defined immunohistochemically as being ER-/PR-/Her2- and positive for CK5/6 and/or Her1. Basal breast tumors are so named because they tend to express basal cytokeratins (such as CK5/6, CK14, and CK17) and other basal markers including integrin, laminin, vitmentin, p-cadherin, αβcrystalline, fascin, and caveolins 1 and 2 (45). While the majority of basal breast cancers are triple-negative, a small percentage do express one or more of these proteins to some degree, meaning that not all basal breast cancers are triple-negative and vice versa (46). Also contributing to triple-negative breast

cancer are tumors of the claudin-low subtype, which are almost exclusively ER-negative, tend to be of higher grade, and are marked by low levels of tight and adherens junction genes such as claudin 3 and *CDH1* (47).

Since triple-negative breast cancers typically lack normal expression of the hormone receptors ER and PR (which are often utilized in targeted therapy) and do not display amplification of Her2 (thus not treatable with trastuzumab), targeted therapeutic options are limited for these breast tumors. In addition to lack of targeted therapeutics, this subset of breast cancers tends to display aggressive phenotypic characteristics such as poor differentiation, high proliferation index, high mitotic index, central necrosis, a pushing boarder of invasion, and a stromal lymphocytic response (45, 46). These factors, combined with the fact that these tumors tend to have a high incidence of *p53* mutations, *EGFR* overexpression, increased likelihood of *BRCA1* mutation, and are associated with a younger age of onset, elucidate possible reasons that basal and triple-negative breast tumors have been linked to poor relapse-free survival and shortened overall survival times (35, 45, 48, 49). Basal breast cancers are known to be overrepresented in premenopausal, African-American women whose incidence of this subtype of breast cancer approaches 40% (compared to approximately 15% in all other patient groups) (40). Intriguingly, basal breast tumors are reported to have different metastatic characteristics compared to other breast tumors: they are less likely to metastasize to axillary lymph node or bone, and are more likely to spread hematogenously and metastasize to the brain and lungs (50). Patients with basal and/or triple-negative cancer appear to have significantly shorter survival times after a metastatic event than do patients with non-triple negative metastatic breast cancers (51).

Currently, the only systemic therapy option to treat triple-negative and basal breast cancers is chemotherapy. Although triple-negative tumors show high rates of response to neoadjuvant therapies (particularly anthracycline-based and taxane-based chemotherapy), if patients do not achieve pathological complete response with treatment, they are more likely to have significantly poorer prognosis than patients with tumors of other molecular subtypes (52). This combination of aggressive tumor characteristics and lack of targeted therapeutics may be responsible for the disproportionately high number of breast cancer deaths attributable to this subtype each year, as well as the shorter overall survival times associated with triple-negative breast cancer (35).

ROLE OF EPIGENETICS IN HUMAN CANCER

DNA Methylation and Cancer

Epigenetics has gained a principal role in cancer biology as evidence mounts that non-sequence altering modifications to the genome make a substantial contribution to the carcinogenic process (53). As opposed to genetic changes, epigenetic changes (such as methylation, genomic imprinting, and histone modification) are frequent and reversible (54). DNA methylation, which occurs at CG dinucleotides (CpGs), is one epigenetic event of major importance in neoplasia, as it serves as a non-mutational mechanism for inactivating tumor suppressors and other negative regulators of cell growth (55). Numerous genes have CpG-rich regions known as CpG islands (defined as ≥200 bp with ≥50% G+C content and ≥0.6 CpGs observed/CpGs expected) (56) in their promoter sequences proximal to their transcriptional start sites. Such CpG islands in regulatory regions of active genes are typically unmethylated, while those of transcriptionally silent genes are often methylated (57). Recent

evidence suggests that genes lacking typical CpG islands are also susceptible to methylation-dependent silencing (58), adding a new importance to understanding individual CpG methylation events within promoters. This relationship between DNA methylation and gene silencing suggests that alteration of normal methylation patterns can result in the perturbation of gene expression. Importantly, a substantial number of genes that are involved in the hallmarks of cancer (25) are subject to methylation-dependent silencing (59). Moreover, epigenetic gene inactivation is believed to be at least as frequent, if not more so, than mutation in carcinogenesis (60-62), making the elucidation of methylation mechanisms in carcinogenesis an important target of further research.

DNA methylation is catalyzed by DNA methyltransferase enzymes (DNMTs) which transfer methyl groups from S-adenosyl-methionine donors to the fifth carbon of a cytosine in a CpG dinucleotide. Two kinds of DNA methyltransferase activity are known to occur *in vivo*: (i) *de novo* methylation, which establishes the methylation pattern of a given DNA segment, and (ii) maintenance methylation, which ensures patterns are faithfully copied to newly replicated daughter strands. To date, five human DNA methyltransferases have been identified: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L, although DNMT2 (identified by sequence similarity alone) and DNMT3L possesses no recognized methyltransferase activity (63). *De novo* methylation is typically attributed to DNMT3a and DNMT3b, which are highly expressed during embryogenesis, but not in normal adult tissues (64), while maintenance methylation is usually attributed to DNMT1 because of its significant preference for hemi-methylated substrates (65). However, recent findings suggest that the roles of individual DNMTs are not so easily delineated and evidence of cooperativity and partial redundancy among these enzymes has emerged (66). For example, DNMT1 has

been found to exhibit *de novo* methylation activity towards unmethylated substrates which surpasses that of DNMT3a and DNMT3b (65). Additionally, some studies suggest that DNMT3a and DNMT3b are jointly necessary for maintenance methylation of specific sequences (67-69).

Several DNMT abnormalities have been detected in various cancer cell types, but questions persist regarding the roles of individual DNMTs in the neoplastic process, owing to the tissue-specific nature of many findings. Generally, cancer cells exhibit higher methylation capacities than their normal counterparts, but the extent of DNMT overexpression appears to be highly variable, with rates ranging from 4-fold to 3000-fold in one study alone (70). For example, DNMT1 is increased in nearly one-half of leiomyomas, while DNMT3a and DNMT3b are decreased in three-fourths of these tumors (71). Another recent study of bladder, colorectal, renal, and pancreatic tumors demonstrated significant overexpression of DNMT3b, but not DNMT1 or DNMT3a (72). Thus, tissue type appears to determine important differences in which and to what extent individual DNMTs are aberrantly expressed in cancer cells.

In an attempt to discern the roles that DNA methyltransferases play in the methylation abnormalities observed in human cancer, many studies have examined the effect of experimentally modulating DNMT levels individually or in combination. The results of these studies appear to be dependent upon the method of DNMT inhibition, the cell type, the target genes examined for methylation changes, as well as the methods of detecting DNA methylation changes (73, 74). Some studies, like those in bladder (75), colon (76), breast (77, 78), and lung cancer cells (78) find that knocking down DNMT1 levels is sufficient to cause re-expression of methylation-silenced genes. In contrast, other studies find that decreasing

DNMT1 alone is insufficient and that DNMT3b must also be targeted in order for methylation-silenced tumor suppressors to become re-expressed (79-81). Concurrent loss of both DNMT1 and DNMT3b (but not individual loss) eliminates nearly all methyltransferase activity in colorectal cancer cells (79), indicating that these enzymes cooperatively maintain methylation in this tissue. DNMT3b may play a prominent role in the methylation abnormalities of breast cancers, as it was found to be overexpressed in a greater percentage of breast tumors than DNMT1 or DNMT3a, and was significantly related to more aggressive tumors and poorer prognosis in patients receiving adjuvant hormone therapy (82). Such tumors were also more likely to be ER- and demonstrate increased proliferation, suggesting that overexpression of certain DNMTs may result in important differences in tumor biology. Regardless of whether DNMTs act alone or in concert, DNMT elevation appears to be an early event in carcinogenesis. For example, DNMT1 expression is low in normal colonic mucosa cells, elevated in premalignant polyps ~60-fold, and increased in colon cancers more than 200-fold (83). Importantly, DNMT activity is elevated in the early stages of colorectal and lung cancers, indicating that these enzymes may constitute an important biomarker for early detection of neoplasia (84).

Methylator Phenotypes in Human Cancer

The CpG island methylator phenotype (CIMP) was first used to describe a distinct subset of colorectal tumors that had high rates of concordant methylation of specific genes (85). CIMP has since been detected in many other neoplasms including: ovarian (86), bladder (87), prostate (87), gastric (88), hepatic (89), pancreatic (90), esophageal (91) and renal cancers (92), as well as leukemia, lymphoma, and neuroblastoma (93-95). While many of these methylator phenotypes methylate genes indicative of colorectal CIMP (*MLH1, MGMT,*

MINT1-MINT33, CDKN2A, THBS1), they also demonstrate methylation of tissue-specific genes, such as *RIZ1* in gastric tumors (96), *CALCA* in leukemia (97), *COX2* in liver (89), *CACNA1G* in pancreatic (90), *PCDHB* in neuroblastoma (95), and *VHL* in renal cancers (92). These findings suggest that tissue type is important in determining which genes are indicative of CIMP in a given tumor system, and implies that the definition of CIMP may vary somewhat between tumor systems. However, the core of the CIMP concept is that tumors exhibit methylation-dependent silencing of a specific panel of genes, which are lost to Type C (or cancer-specific) methylation rather than Type A (age-specific) methylation. Genes affected by Type A methylation reflect an age-dependent mechanism of expression loss—small levels of methylation are detected in normal mucosa of younger patients and methylation increases with time, as a normal part of the aging process. In contrast, genes affected by Type C methylation become methylated in response to the carcinogenic process. Type C methylation is found exclusively in tumors, rather than normal mucosa, and is independent of age.

In colorectal cancer, CIMP status correlates with poor prognosis in patients treated with surgery alone and is associated with various clinical features including: poor histologic grade (98), family history (99), location in the colon, and microsatellite instability status (100). However, patients with CIMP-positive tumors respond better to 5-flourouracil treatment than those with CIMP-negative tumors (98). Thus, in cases where patients receive both surgery and chemotherapy, CIMP status predicts better survival. As in colorectal cancers, CIMP status has important consequences in neuroblastoma and esophageal tumors, with CIMP-positive tumors predicting poor prognosis (95, 101). In neuroblastoma, CIMP-positive tumors carry a significantly increased risk of death compared to their CIMP-negative

counterparts (95). A recent study of acute lymphoblastic leukemias (ALL) revealed that CIMP-positive patients experience significantly higher relapse and mortality rates (102). However, methylation abnormalities do not always correspond with bleak prognostic outlook. In gastric carcinomas CIMP is associated with increased survival rates (103). Paradoxically, these CIMP-positive gastric carcinomas exhibit increased DNMT1 protein levels and display poor tumor differentiation regardless of cell type of origin (104). This suggests not only that DNMT abnormalities might underlie the gastric CIMP phenotype but also that this methylation defect has an important impact on the clinicopathological characteristics of the tumors in question. The possible explanations that account for these survival differences are unknown, but may be tied to the roles of the tissue-specific CIMP genes which become methylated in various tumor types.

To date, breast cancers have been examined for CIMP in a single study which failed to find support for a methylator phenotype amongst histologically distinct tumor classes (105). While researchers determined that hypermethylation of the twelve genes examined was frequent, they found a unimodal distribution of methylation events, rather than two distinct methylation classes. An important consideration of this study design is the number and choice of genes chosen for methylation analysis. With dozens of genes reported to be methylated in breast carcinogenesis and many more likely to be discovered, it is quite possible inclusion of additional genes would alter the findings of such an investigation.

DNA METHYLATION AND HUMAN BREAST CANCER

Recent evidence suggests that epigenetic mechanisms play a major role in breast carcinogenesis (53). In addition to contributing to widespread genetic instability (106),

aberrant DNA methylation contributes to the silencing of specific genes. A number of genes are inactivated in breast cancer through methylation-dependent silencing (54). Genes that have been determined to be directly silenced by DNA methylation in breast cancer include cell cycle control genes (*APC, RASSF1, TFAP2A*), steroid receptor genes (*ESR1, RARα*), tumor suppressor genes (*CST6, PRDM2*), metastasis-associated genes (*CDH1, CEACAM6, LGALS3BP*), and many others (107-109). For example, the *adenomatous polyposis coli (APC)* gene, which exerts cell cycle control by regulating β-catenin-induced proliferation, is methylated in ~36-49% of primary breast tumors (110, 111). Intriguingly, *APC* is often methylated concurrently with other biologically important genes such as *RASSF1A* (112). As the frequency of *APC* methylation appears to increase with tumor size and stage (113), methylation of this gene represents an independent marker of poor prognosis in breast cancer patients (114). *Estrogen receptor (ESR1*) is a nuclear hormone receptor that activates transcription of cell growth genes and is perhaps the most important methylation sensitive gene in terms of breast cancer prognosis. Loss of this gene is an important prognostic factor in breast cancer and is associated with poor differentiation, insensitivity to hormonal therapy, and poor clinical outcome (115). While a significant percentage of breast cancers lack expression of the estrogen receptor (and other steroid receptors), loss of *ESR1* expression is usually caused by methylation (116, 117). *Cystatin M (CST6)* is a protease inhibitor with tumor suppressor properties and known to be methylated in both breast cancer cell lines and primary breast tumors (58, 118). Treatment with demethylating agents results in the re-expression of *CST6* in breast cancer cell lines (119). Additionally, ectopic expression of *CST6* suppresses neoplastic phenotype in MDA-MB-435S breast cancer cells, reducing proliferation, migration, and invasion (120). *E-cadherin* (*CDH1*) is a well-known suppressor

of invasion, as it plays a vital role in the maintenance of cell-to-cell adhesion (121). It is one of the most commonly methylated genes in breast cancer, with the frequency of methylation estimated to be ~60% for primary breast tumors and up 90% for lymph node metastases (111, 122). Methylation of *CDH1* often marks aggressive breast tumors and is associated with a higher incidence of metastasis, poor differentiation, and decreased patient survival (123). These examples illustrate the large number of genes which are reported to be silenced by methylation in breast cancer. Due to the cellular activities in which these genes participate, their aberrant methylation is likely to have a substantial impact on breast tumor biology.

SUMMARY AND SIGNIFICANCE

Recent studies have begun to unravel the complexities of methylation events in cancer, including the contributions of individual DNMTs to the methylation states of cancer cells as well as the concurrent methylation of specific genes that can indicate a hypermethylator phenotype. Strong evidence for CIMP has been found in a number of different tumor systems, although the genes involved and the impact of such a phenotype on tumor biology appears to differ by tumor type. The primary goals of this study are to determine if a hypermethylator phenotype exists in breast cancer, and if so, how this phenotype can be expected to impact patient outcome. In the following studies, we address basic questions related to mechanisms governing the establishment of a hypermethylator phenotype in breast cancer cells (including direct investigation of the methylation machinery), explore the consequences of decreasing DNMT transcript levels in cells with aberrant methylation, and strive to determine the clinicopathological relevance of such a hypermethylator phenotype in human breast cancer.

Unraveling the complexities of this hypermethylation defect in neoplastic breast disease holds important implications for cancer diagnosis, identification of new targets for therapy, and development of new strategies for clinical management. Since aberrant methylation-dependent silencing is thought to be an early event in carcinogenesis, elevated detectable levels of methylation in genes characterizing the hypermethylator phenotype may constitute an important biomarker for early detection in patients developing breast tumors. Furthermore, the various proteins and enzymes of the DNA methylation machinery may represent novel targets for breast cancer therapy. It follows that if a hypermethylator phenotype tumor is detected, such patients may benefit significantly from a targeted demethylation treatment as an adjunct to standard chemotherapeutic regimens. Epigenetic chemosensitization has been used to improve the efficacy of standard chemotherapeutics against tumor cells with known methylation defects, and evidence suggests that chemotherapeutic resistance can be overcome with demethylating treatment in certain cases. Thus, if a subset of breast cancer cells are found to possess aberrant DNA methylation and other epimutations that are potentially reversible, better understanding the methylation defects of such cells will hold promise for better diagnosis and improved treatment of such tumors.

EXPERIMENTAL PROCEDURES

EXPERIMENTAL PROCEDURES RELATED TO EXPRESSION ANALYSIS

Cell Lines

Human breast cancer cell lines BT20 (ATCC# HTB19), BT549 (HTB122), HS578T (HTB126), MCF7 (HTB22), MDA-MB-231 (HTB26), MDA-MB-415 (HTB128), MDA-MB-435S (HTB129), MDA-MB-436 (HTB130), MDA-MB-453 (HTB131), MDA-MB-468 (HTB132), SKBR3 (HTB30), and ZR-75-1 (CRL-1500) were obtained from the Tissue Culture Core Facility of the University of North Carolina Lineberger Comprehensive Cancer Center (Chapel Hill, NC). Human breast cancer cell lines HCC1937 and SUM149 were obtained as a kind gift from the laboratory of Dr. William K. Kaufmann (Department of Pathology and Laboratory Medicine, UNC School of Medicine). Likewise, human breast cancer cell lines SUM102 and SUM185 were a kind gift from the laboratory of Dr. Carolyn I. Sartor (Department of Radiation Oncology, UNC School of Medicine). The normal breast epithelial cell line MCF12A (CRL-10782) was obtained from the American Type Culture Collection (ATCC, www.atcc.org). In the figures and tables that follow, some cell lines names are designated using the following abbreviations: 231 (for MDA-MB-231), 415 (MDA-MB-415), 435S (MDA-MB-435S), 436 (MDA-MB-436), 453 (MDA-MB-453), 468 (MDA-MB-468), 1937 (HCC1937), 102 (SUM102), 149 (SUM149), 185 (SUM185), HS (for HS578T), SK (SKBR3), and ZR (ZR-75-1).

Cell Culture

Each breast cancer cell line was grown in the appropriate medium, as recommended by the ATCC. BT20, MCF7, and MDA-MB-231 cells were cultured in minimal essential medium (MEM) with Earle's salts and the following additives: 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 μg/ml insulin (GIBCO/Invitrogen Life Technologies, Carlsbad, CA), 10% fetal bovine serum (Hyclone, Logan, UT), and 1% Antibiotic-Antimycotic (Gibco/Invitrogen Life Technologies). HS578T, MDA-MB-415, MDA-MB-435S, MDA-MB-436, and MDA-MB-453 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with: 4 mM L-glutamine, 10 μg/ml insulin (GIBCO/Invitrogen Life Technologies), 10% fetal bovine serum (Hyclone), and 1% Antibiotic-Antimycotic (Gibco/Invitrogen Life Technologies). BT549, ZR-75-1, and HCC1937 cells were cultured in RPMI-1640 medium containing: 10% fetal bovine serum (Hyclone), and 1% Antibiotic-Antimycotic (Gibco/Invitrogen Life Technologies). MDA-MB-468 cells were cultured in Leibovitz's L-15 medium supplemented with: 10% fetal bovine serum (Hyclone), and 1% Antibiotic-Antimycotic (Gibco/Invitrogen Life Technologies). SKBR3 cells were cultured in McCoy's 5A medium containing: 10% fetal bovine serum (Hyclone), and 1% Antibiotic-Antimycotic (Gibco/Invitrogen Life Technologies). MCF12A, SUM102, SUM149, and SUM185 cells were cultured in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12, Gibco) medium supplemented with: 5% horse serum (Gibco/Invitrogen Life Technologies), and 1% Antibiotic-Antimycotic (Gibco/Invitrogen Life Technologies) in accordance with previous studies (124). Cell cultures were routinely provided with fresh growth medium, three times weekly.

Treatment with 5-aza-2'-deoxycytidine

Cell lines selected for treatment with the demethylating agent 5-aza-2'-deoxycytidine (5-aza, Sigma Chemical Company, St. Louis, MO) were propagated in the appropriate ATCC-recommended growth medium (described above) containing 250 nM 5-aza, with feeding of fresh growth medium three times weekly, for a total of three weeks (58). Two treatment groups were established from a single founding population of each cell line of interest: (i) control medium, and (ii) 5-aza containing medium. Cells were plated at an approximate density of 5,000 cells/cm^2 in 150 mm polystyrene dishes (Corning Inc., Corning, NY). After three weeks of demethylating treatment, cells were allowed to recover for five weeks in control growth medium, with weekly subcultivations. As described previously (58), the concentration of 5-aza used in this study is 4-6-fold lower than traditional methods which allows for long-term 5-aza exposure without the typically encountered cytotoxic effects (125, 126).

RNA isolation from Cell Lines

Confluent cell cultures were harvested for RNA preparation according to the method of Chomczynski and Sacchi (127), utilizing TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. For cell lines subjected to demethylating treatment, cells were harvested for total RNA isolation after three weeks of 5-aza treatment and at week eight, corresponding to the end of the five week recovery period. Cells were washed with Hank's Balanced Salt Solution (Cellgro, Lawrence, KS) and RNA was isolated using 5 ml of TRIzol reagent per 150 mm polystyrene dish. After a five minute room temperature incubation, the cell lysate solution was collected and centrifuged at 10,000 g for 20 minutes at 4°C. The resulting aqueous phase was transferred into a 15 ml conical

tube containing 5 ml 100% isopropanol and incubated at room temperature for 10 minutes to precipitate the RNA. Precipitated RNA was collected by centrifugation at 10,000 g for 40 minutes at 4°C. Subsequently, the supernatant was removed, 10 ml of 75% ethanol was added, and the solution vortexed for 30 seconds. Isolated total RNA was stored at -20°C as an ethanol precipitate prior to use for RT-PCR. Immediately before use, RNA was centrifuged and re-dissolved in 200 µl RNase-treated water and quantitated by UV spectroscopy.

Semi-Quantitative RT-PCR Analysis

Sixty-six genes were selected for analysis in this study. These genes (listed in Table 5) represent marker genes for a CpG island methylator phenotype (CIMP) in other tumor systems or genes that have been shown to be methylated in breast cancer (58, 90, 92, 94, 111, 117, 118, 128-250). Total RNA (2 µg) collected from each cell line was reverse-transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA) and oligo(dT) as the primer, according to standard methodology. Gene-specific oligonucleotide primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were synthesized by the UNC Oligodeoxynucleotide Synthesis Core Facility (Chapel Hill, NC) based upon the known cDNA sequences (Genbank, www.ncbi.nih.gov) for selected mRNAs of interest. The RT-PCR primer sequences for gene-specific primers are given in Table 6. Verification of equal cDNA template concentrations between samples was accomplished using *β-actin* primers (forward 5'-AGA-GAT-GGC-CAC-GGC-TGC-TT and reverse 5'-ATT-TGC-GGT-GGA-CGA-TGG-AG). PCR reactions were performed in a 50 µl total volume of buffer containing

Table 5. Methylation-sensitive genes.

Gene Designation	Gene Title	Unigene Number	Role in Carcinogenesis	Methylated in the following cancers (reference)
ADAM23	A desintegrin and metallo-protease domain 23	Hs.591643	Adhesion	Brain (128)
APBA1	Amyloid beta A4 precursor protein-binding	Hs.592974	Adhesion	Breast (129), colon (130), esophageal (131), gastric (132)
APBA2	Amyloid beta A4 precursor protein-binding 2	Hs.525718	Adhesion	Breast (129), colon (133), esophageal (131), gastric (134), leukemia (135), oral (136), pancreatic (90)
APC	Adenomatous polyposis coli	Hs.158932	Cell cycle check-point, proliferation	Breast (110), head/neck (137), gastric(138)
BARD1	BRCA1 associated rong domain 1	Hs.591642	Cell growth	Breast (129)
BF	B-factor	Hs.69771	Unknown	Breast(58)
BRCA1	Breast cancer 1, early onset	Hs.194143	DNA repair	Breast/ovarian (139)
C8ORF4	Chromosome 8, open reading frame 4	Hs.591849	Regulator of Wnt-mediated growth	Breast (58)
CCND2	Cyclin D2	Hs.376071	Proliferation	Prostate (140), breast (141)
CDH1	E-cadherin	Hs.461086	Adhesion and metastasis; proliferation	Breast/gastric/leukemia (142), cervical (143), colon (144), lung (145), nasopharyngeal (146), ovarian (147) , prostate (148), thyroid (149), renal (150)
CDKN1A	Cyclin-dependent kinase inhibitor 1A	Hs.370771	G1 checkpoint; apoptosis	Lung (151)
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Hs.512599	G1 checkpoint	Bladder (152), breast (153), colon (154), gastric (155), head/neck (156), lung (157), lymphoma (158), ovarian (159), pancreatic (160)
CDKN2B	Cyclin-dependent kinase inhibitor 2B	Hs.72901	G1 checkpoint	Lymphoma (161), leukemia (162), oral (163), ovarian (164)
CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5	Hs.220529	Adhesion; anti-apoptotic	Breast (58)
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6	Hs.466814	Adhesion; proto-oncogene	Breast (58)
CST6	Cystatin E/M	Hs.139389	protease inhibitor	Breast (58, 118), glioma (165)
CTCF	CCCTC-binding factor	Hs.368367	Cell cycle regulator	Head/neck (166)
CYP1B1	Cytochrome p450, family 1, subfamily B, polypeptide 1	Hs.154654	Metabolizes procarcinogens	Breast (167)
DAPK1	Death-associated protein kinase 1	Hs.380277	Pro-apoptotic	Breast (168), gastric (169), liver (170), lymphoma (171), lung (172), uterine/ovarian (173)
ESR1	Estrogen receptor 1	Hs.208124	Cell growth transcription factor	Breast (174), cervical (176), colon (176), gastric (177), head/neck (137), lung (178), ovarian (179)
ESR2	Estrogen receptor 2	Hs.443150	Cell growth transcription factor	Breast (180), prostate (181)
G1P3	Interferon, α-inducible protein 6	Hs.523847	Anti-apoptotic	Breast (58)
GADD45A	Growth arrest and DNA-damage inducible, α	Hs.80409	DNA repair	Breast (182)
GJB2	Gap junction protein β2	Hs.591234	Cell-cell signaling	Breast (183), lung (184)
GNA11	Guanine-nucleotide binding protein α11	Hs.73797	Negative regulator of growth	Breast (185)
GPC3	Glypican-3	Hs.567276	Growth regulation	Breast (168)

Table 5. Methylation-sensitive genes (continued)

Gene Designation	Gene Title	Unigene Number	Role in Carcinogenesis	Methylated in the following cancers (reference)
GSTP1	Glutathione s-transferase π1	Hs.523836	Metabolizes carcinogens	Breast(142), colon (186), gastric (138), liver (187), lung (188), ovarian (179), prostate (189), renal (150)
HIC1	Hypermethylated in cancer1	Hs.72956	Cell cycle regulator	Brain (190), breast (191), ovarian (192)
HOXD11	Homeobox D11	Hs.421136	transcription factor	Breast (183), melanoma (193)
HS3ST2	Heparin sulfate 3-O-sulfotransferase-2	Hs.622536	Unknown	Breast, colon, lung, pancreatic (194), esophageal (195)
IFI27	Interferon α-inducible protein 27	Hs.532634	Negative regulator of proliferation	Breast (58)
IGFBP5	Insulin-like growth factor binding protein 5	Hs.369982	Growth inhibitor and proapoptotic	Breast (58)
ISG15	ISG15 ubiquitin-like modifier	Hs.458485	Proteosome pathway	Breast (58)
ISGF3G	Interferon-stimulated transcription factor 3	Hs.1706	Transcription regulator	Breast (58)
KRTHB1	Keratin, hair, basic 1	Hs.584773	Cell-cell signaling	Breast (58)
LCN2	Lipocalin 2	Hs.204238	Anti-metastatic	Breast (58)
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein	Hs.514535	Adhesion / metastasis	Breast (58)
MGMT	O(6)-methylguanine-DNA methyltransferase	Hs.501522	DNA repair	Bladder (196), breast (197), cervical (198), colon (186) esophageal (199), gastric (200), liver (201), ovarian (202), pancreatic (203), prostate (204), renal (92)
MINT31	Methylated in tumors 31	N/A	Unknown	Breast (129), colon (205), esophageal (131), gastric (132), lymphoma (94), oral (136), pancreatic (206)
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2	Hs.195364	DNA repair	Breast (206), colon, endometrial (142) gastric (138)
MUC1	Mucin 1, cell surface associated	Hs.89603	Cell signalling	Breast (208)
MYB	v-myb myeloblastosis viral oncogene homolog	Hs.531941	Transcription regulator	Melanoma (209)
PARP12	poly (ADP-ribose) poly-merase family, member 12	Hs.12646	Unknown	Breast (58)
PCDHGB6	Protocadherin γ, B6	Hs.368160	Adhesion	Breast (183)
PER1	Period 1	Hs.445534	Transcription	Breast (210), endometrial (211)
PGR	Progesterone receptor	Hs.368072	Cell growth	Breast (117), endometrial (212), prostate (213)
PRDM2	PR domain-containing 2, with ZNF domain	Hs.371823	Transcription regulator	Breast, colon, liver, lung (214)
PRKCDBP	Protein kinase C, delta binding protein	Hs.434044	Unknown	Breast and lung (215)
RARα	Retinoic acid receptor α	Hs.137731	Growth regulation	Breast (216), leukemia (217)
RARβ	Retinoic acid receptor β	Hs.536687	Growth regulation	bladder (218), breast (219), cervical (220), colon (221), head/neck (222), gastric (223)
RASSF1	Ras association domain, family 1 isoform A	Hs.476270	Cell cycle arrest	Bladder (224), breast/ovarian (225), lung (226), pancreas, kidney, liver, cervix, prostate (227)
RB1	Retinoblastoma 1	Hs.408528	Cell cycle regulator	Brain (228), breast (197)

Table 5. Methylation-sensitive genes (continued)

Gene Designation	Gene Title	Unigene Number	Role in Carcinogenesis	Methylated in the following cancers (references)
SASH1	SAM and SH3 domain containing 1	Hs.193133	Cell cycle regulator	Breast (229)
SAT	Spermidine / spermine N1-acetyltransferase	Hs.28491	Apoptosis	Breast (58)
SCNN1A	Sodium channel, non-voltage-gated 1α	Hs.591047	Ion transport	Breast (58)
SERPINB5	Serpin peptidase inhibitor, clade B, member 5	Hs.55279	Pro-apoptotic	Breast (230), endometrial (231), gastric (232), ovarian (233), pancreas (234), thyroid (235)
SFN	Stratifin	Hs.523718	G2/M checkpoint	Breast (236), lung (237), oral (238), ovarian (239)
SIM1	Single-minded homolog 1	Hs.520293	Cell differentiation	Breast (183)
ST18	Suppression of tumorigenicity	Hs.147170	Transcription regulator	Breast (240)
STK11	Serine/threonine kinase 11	Hs.515005	Growth regulation	Cervical, colon, testicular (241), pancreatic (242)
TFAP2A	Transcription factor activator protein 2α	Hs.519880	Cell cycle arrest, apoptosis	Breast (243)
TFF3	Trefoil factor 3	Hs.82961	Unknown	Liver (244)
THBS1	Thrombospondin 1	Hs.164226	Angiogenesis	Brain (228), colon (186), esophageal (199)
TMEM45A	Transmembrane, 45A	Hs.126598	Membrane protein	Breast (58)
TP73	Tumor protein p73	Hs.192132	DNA repair; apoptosis	Colon (205), head/ neck (245), leukemia/ lymphoma (246), ovarian (247), pancreatic (203)
WT1	Wilms tumor 1	Hs.591980	Anti-proliferative	Breast (248), colon (249), ovarian (250)

Table 6. Primer sequences and reaction conditions for RT-PCR.

Gene	Forward Primer	Reverse Primer	Product Size, bp	TM	Cycles
ADAM23	GTC-AGT-GCC-CAC-CAA-ATC-TT	GCA-GTT-TCC-CTT-CTC-AGT-GC	188	62	35
APBA1	CAG-CCC-ACG-TCA-TTA-AGG-TT	TTG-CCC-AAG-GCA-GTT-ATT-TC	186	58	30
APBA2	CAT-CCA-CTT-CTC-AAA-CTC-GG	GCC-CAG-CTG-GTA-CTT-GAG-GT	345	60	30
APC	GTC-TGT-TCA-GGC-TGG-TGG-AT	CTC-GAG-GAA-GGG-ATG-ATG-AA	191	60	30
BARD1	AGC-TCG-TCA-CTG-CAG-GTG-GG	TTC-CAG-ACT-TTG-CCC-TGC-CG	133	58	35
BF	GGC-AGC-AAC-AAA-AGG-AAG-AG	GCA-AGT-ATT-GGG-GTC-AGC-AT	242	57	30
BRCA1	ACA-GCT-GTG-TGG-TGC-TTC-TGT-G	CAT-TGT-CCT-CTG-TCC-AGG-CAT-C	100	62	30
C8ORF4	TTT-CAA-ACA-GGT-TGC-ACA-AA	GTT-GCA-TGA-CAT-TTG-CCA-GT	229	58	30
CCND2	TTC-CCT-GCA-GTC-TAG-CAC-CT	ATT-TCT-TCT-CCC-AGC-CCT-GT	160	60	30
CDH1	TCT-TGC-TGT-TTC-TTC-GGA-GG	TGA-CTC-TGA-GGA-GTT-CAG-GG	380	60	30
CDKN1A	CAG-ACA-TTT-TAA-GAT-GGT-GG	TGG-TCC-CTG-CCC-TCG-AGA-GG	268	58	35
CDKN2A	CAC-ATT-CAT-GTG-GGC-ATT-TC	CTT-TGG-TTC-TGC-CAT-TTG-CT	142	62	30
CDKN2B	GCG-GAT-TTC-CAG-GGA-TAT-TT	AGT-GGG-AGA-TTC-ATC-CAT-CG	138	62	35
CEACAM5	CAT-CGT-GAA-ACC-CCA-TCT-CT	TCT-GTT-GCC-AGA-CTG-GAG-TG	169	60	30
CEACAM6	TGA-GCC-AGT-GGT-GCT-AAA-TG	TGG-AAC-AAG-GAA-ACA-GAA-CCA	235	58	30
CST6	AAG-ACC-AGG-GTC-ACT-GGA-GA	CGG-GGA-CTT-ATC-ACA-TCT-GC	163	58	30
CTCF	AGC-CAG-CAT-TTG-AAC-CCT-GT	CCA-GCT-TAT-AAG-GGC-TGC-TG	154	62	30
CYP1B1	CCC-TCA-TTG-TGT-TTC-TAC-CG	GGC-TAA-GTT-CTG-GGA-CAT-GAA	222	59	35
DAPK1	CCC-CGT-CTC-ATT-CCG-TTG-TC	CCC-TGG-AGG-AGG-ATT-CCC-TT	564	61	35
ESR1	TTG-TCC-CAT-GAG-CAG-GTG-CC	GTA-TGC-ATC-GGC-AAA-AGG-GC	201	58	30
ESR2	GAT-GAG-GGG-AAA-TGC-GTA-GA	GGG-ACC-ACA-TTT-TTG-CAC-TT	365	62	30
G1P3	CTC-GCT-GAT-GAG-CTG-GTC-T	TGC-TGG-CTA-CTC-CTC-ATC-CT	181	60	30
GADD45A	GCT-CCT-GCT-CTT-GGA-GAC-CG	TCC-ATG-TAG-CGA-CTT-TCC-CG	162	58	30
GJB2	CAC-AGT-ACC-ATT-TAA-TGG-GG	ACC-ATG-TCA-AGC-ATA-ATG-GC	282	60	30
GNA11	CCA-CTG-CTT-TGA-GAA-CGT-GA	GCA-GGT-CCT-TCT-TGT-TGA-GG	185	60	30
GPC3	TGC-TCT-TAC-TGC-CAG-CGA-CT	GCT-TTC-CTG-CAT-TCT-TCT-GC	238	60	30
GSTP1	GGA-GGA-CCT-CCG-CTG-CAA-AT	GGA-AGG-CAA-ACT-CTG-CCT-CC	384	61	35
HIC1	ATC-TGC-GGG-AAG-AAG-TTC-AC	GCA-TCT-TCA-TGT-GGC-TGA-TG	223	58	35
HOXD11	AAA-TGC-AAA-CGT-CCC-GTT-AC	CAT-TCA-CCG-AAG-AGG-AAG-GA	181	58	35
HS3ST2	AGT-CGG-TCC-CTG-TCA-TTG-TC	TCA-CCA-AAG-GGT-CTG-TCT-CC	206	60	35
IFI27	TCC-TCC-ATA-GCA-GCC-AAG-AT	CCT-GGC-ATG-GTT-CTC-TTC-TC	221	60	35
IGFBP5	TTC-ACA-GAC-TCT-GGC-CTC-CT	TGT-GCT-ATC-CAT-GTG-GGC-TA	185	59	30
ISG15	CAC-CTG-AAG-CAG-CAA-GTG-AG	CTT-TAT-TTC-CGG-CCC-TTG-AT	228	59	30
ISGF3G	GAG-CTC-TTC-AGA-ACC-GCC-TA	GGC-TCT-ACA-CCA-GGG-ACA-GA	226	62	35
KRTHB1	TAG-GCA-CCC-CAA-CTC-AAG-TC	AAG-TGG-GGG-ATC-ACA-CAG-AG	162	59	30
LCN2	ACG-CTG-GGC-AAC-ATT-AAG-AG	CGA-AGT-CAG-CTC-CTT-GGT-TC	162	59	30
LGALS3BP	ACC-AAC-AGC-TCG-AAG-AGC-AC	GGT-CAT-TGC-AGA-GAG-GAA-GG	202	59	30
MGMT	ACG-AAA-TAA-AGC-TCC-TGG-GC	CCA-GGG-CTG-CTA-ATT-GCT-GG	275	60	30
MINT31	CCG-GCC-TCA-TTT-ACA-CAA-CT	GCG-TTG-TTC-ACT-CCC-CTA-AG	203	60	35
MLH1	TTG-CCC-AAA-AAC-ACA-CAC-CC	CCC-GGG-AAT-CTG-TAC-GAA-CC	318	60	30
MUC1	TCT-CTT-ACA-CAA-ACC-CAG-CA	AGA-AGT-GTC-CGA-GAA-ATT-GG	211	56	30
MYB	GAG-ATG-GAG-GAG-TGG-TCT-GC	GGT-TCG-GAT-TTG-GCT-TGT-TA	247	58	30
PARP12	CTT-ATT-GGC-ACC-AGG-GAC-AG	GTG-TCA-GAG-CAA-CAG-GCA-GA	191	59	30
PCDHGB6	CCC-TTG-GGA-AAC-AGA-AAC-AA	GGC-CTG-ATT-GAT-TTG-GAA-GA	208	60	30
PER1	GGC-TAT-GGA-GGA-GGA-GGA-AG	TTC-CCA-CTG-GTT-GGT-CTA-GC	174	62	30
PGR	CTA-CAA-ACA-CGT-CAG-TGG-GC	ATA-GAA-ACG-CTG-TGA-GCT-CG	314	60	35
PRDM2	GCG-TAA-TGG-AGA-GGA-AAC-CA	ATC-TGT-ACA-GGC-CTG-GGA-TG	186	60	30
PRKCDBP	CTT-GTG-CCT-TGT-CCC-AAA-AT	TTA-TTG-ATG-GTG-AGC-GCA-AG	173	58	30
RARα	TGA-CCG-CCC-ACG-CCA-CAT-GG	CCT-CTG-TCA-CCA-ACC-GAG-GC	275	60	35
RARβ	CGT-AGC-ATC-AGT-GCT-AAA-GG	GAC-TGA-CCC-CAC-TGT-TTT-CC	196	60	35
RASSF1	GTG-CTG-GCT-CAC-AGT-ACA-GC	GCC-AAT-TCT-CTC-AGG-CCC-CA	256	60	35
RB1	CCC-TCC-TTA-ATT-TGG-GAA-GG	TGC-CTA-ACC-CAT-AAT-GAC-CC	131	60	35
SASH1	CTG-TCA-CCC-CTT-CAG-TGT-TT	CTG-CTC-TTT-TTG-GCA-GGA-AC	215	61	35
SSAT	CCG-TGG-ATT-GGC-AAG-TTA-TT	TCC-ACC-CCT-CTT-CAC-TGG-AC	217	60	30
SCNN1A	GCC-CCC-TTT-GTT-ACT-TAG-GC	AAA-GAC-ACA-GGG-CAG-AGG-TG	153	60	30
SERPINB5	TCC-ATA-GAG-GTG-CCA-GGA-GC	TGG-CGG-CTT-CCT-GAT-CCA-GC	258	60	30
SFN	TTG-CAG-CTG-TTG-AGC-GCA-CC	CAT-GCT-TTC-CCT-CAG-TCT-CG	268	60	30
SIM1	TTG-CCA-ACA-CTT-CAC-CAT-GT	TGG-TCT-CCT-GCT-GTC-TGA-TG	202	62	35
ST18	GCA-CTC-ACA-AAA-GCA-CAG-GA	GGA-TAC-GAG-TTG-CCA-ACG-AT	217	60	30
STK11	TGT-GAG-GGG-TGT-TTG-GGA-GC	GCG-ATG-GCG-TTT-CTC-GTG-TT	238	60	35
TFAP2A	GGA-GAC-GTA-AAG-CTG-CCA-AC	GGT-CGG-TGA-ACT-CTT-TGC-AT	216	60	30
TFF3	GAG-TGC-CTT-GGT-GTT-TCA-AG	GGA-GCA-TGG-GAC-CTT-TAT-TC	230	60	30
THBS1	TTC-TAC-GAG-CTG-TGG-CAA-TG	TTT-CTT-GCA-GGC-TTT-GGT-CT	286	62	30
TMEM45A	GGA-GAA-CAG-CTG-GCT-AAG-GA	TTC-ATA-GTG-TGG-GCA-TCC-AA	203	59	35
TP73	GAC-CGA-AAA-GCT-GAT-GAG-GA	TCA-GCT-CCA-GGC-TCT-CTT-TC	232	60	30
WT1	CTC-CTT-GCA-CAA-ATG-GAG-GG	ACA-GTA-ATT-TCA-AGC-AAC-GG	339	60	30

50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each dNTP (EasyStart Micro 50 PCR-mix-in-a-tube, Molecular BioProducts, San Diego, CA), 0.4 μM of each primer, and 2.5 units AmpliTaq enzyme (Perkin Elmer/Cetus, Foster City, CA). Reactions were carried out in an Eppendorf Mastercycler Thermocycler as follows: 30-35 cycles at 94°C for denaturing (1 minute), 58-65°C for annealing (1.5 minutes), and 72°C for extension (2 minutes) (Table 6). PCR products were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA and visualized by ethidium bromide staining.

Quantitative Real-time PCR Analysis

Total RNA samples (2 μg) from cell lines of interest were DNAse treated (Promega, Madison, WI), purified using the Qiagen RNeasy mini-kit (Qiagen, Valencia, CA), and reversed transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Real-time primers and probes for *CDH1* (Assay ID: Hs00170423_m1), *CEACAM6* (Hs00366002_m1), *CST6* (Hs00154599_m1), *ESR1* (Hs00174860_m1), *SCNN1A* (Hs00168906_m1), and *β-actin* (Hs99999903_m1) were purchased from Applied Biosystems. Reactions were carried out using TaqMan Universal PCR Master Mix (Applied Biosystems) and the following amplification conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression levels were normalized using *β-actin* for each cell line and differences in gene expression were determined using the comparative Ct method described in the ABI Prism 7700 User Bulletin #2 (Applied Biosystems).

Breast Cancer Tissue Samples

Formalin-fixed, paraffin-embedded human breast tissues were immunostained according to standard methods. Two tissue microarrays containing 92 and 83 invasive primary human breast tumors, respectively, were constructed at the Dartmouth-Hitchcock Medical Center (courtesy of Dr. Gregory J. Tsongalis and Dr. Wendy A. Wells, Dartmouth-Hitchcock Medical Center, Lebanon, NH). Combined, these tissue microarrays contain 122 luminal A-like tumors (ER+/PR+/Her2-), 30 triple-negative tumors (ER-/PR-/Her2-), 17 Her2+ tumors (ER-/PR-/Her2+), as well as 15 unclassified (ER+/PR-/Her2-) primary breast tumors. Additionally, a breast cancer cell line tissue microarray was constructed at the UNC Anatomical Pathology Translational Core Lab (Chapel Hill, NC) containing samples of each of the 17 breast cancer cell lines utilized in these studies.

Immunohistochemistry

Tissue sections were incubated at 60°C for 30 minutes, deparaffinized using Slide Brite (S&S Company, Albany, GA), and incubated with 3% hydrogen peroxide in methanol prior to rehydration in a series of alcohol washes. Antigen retrieval was accomplished by steaming in citrate buffer (Dako, Inc., Carpinteria, CA) for 30 minutes. Tissues were incubated with a protein block (Dako, Inc.) for 10 minutes, followed by a 2 hour incubation with the appropriate mouse monoclonal primary antibody: anti-CEACAM6 antibody (Covance, Berkeley, CA) diluted 1:40, 2.5 μg anti-cystatin E/M antibody (R&D Systems, Minneapolis, MN), anti-DNMT3b (Imgenex, San Diego, CA) diluted 1:100, anti-E-cadherin (Zymed Laboratories, Carlsbad, CA) diluted 1:50, predilute anti-Her1 (Invitrogen Corp., Carlsbad, CA), predilute anti-HER2/neu antibody (BioGenex, San Ramon, CA), anti-pancytokeratin (Abcam, Cambridge, MA) diluted 1:25, or anti-SCNN1A (Proteintech Group,

Chicago, IL) diluted 1:100. Subsequently, tissues were washed and incubated with a two-step universal secondary antibody containing a biotintylated link and streptavidin-conjugated horseradish peroxidase (Dako, Inc.) for 10 minutes each and counterstained in filtered hematoxylin. Negative control staining followed the same procedure except that tissues were incubated for 2 hours with Dulbecco's phosphate buffered saline (Sigma) instead of a primary antibody.

Unsupervised Cluster Analysis of Gene Expression in Cell Lines

Expression levels for genes of interest were analyzed by RT-PCR using cDNA templates derived from 12 breast cancer cell lines (BT20, BT459, HS758T, MDA-MB-231, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MDA-MB-468, MCF7, SKBR3, and ZR-75-1) and normal MCF12A breast epithelial cells. RT-PCR results for breast cancer cell lines were expressed on a discrete scale (none, low, medium, high) relative to the expression levels of MCF12A cells. Genes from the original panel of 66 that were not expressed in MCF12A cells (*APBA2, CDKN2B, CDKN2A, GADD45A, IGFBP5, PGR, PRKCDBP, RB1, SERPINB5, SIM1, STK11, THBS1, TMEM45A,* and *WT1*) were omitted from subsequent cluster analyses, to ensure that cancer-specific methylation events were captured. The expression data were mapped to a quantitative scale (0, 1, 2, 3) for clustering purposes. For some analyses, a combined expression score was generated for each cell line by adding the quantitative RT-PCR expression levels of each of the top nine genes of interest. Clustering of cell lines was carried out with SAS/STAT PROC CLUSTER (SAS Institute, Cary, NC) using complete linkage and no squaring of distance. Kernel density estimation for trimming used the 5 nearest neighbors.

Data-mining of Breast Cancer Gene Expression Signatures

In order to determine if a hypermethylation signature was detectable in primary human breast tumors, publicly available microarray data was mined for a concurrent loss of expression of genes determined to define the hypermethylator phenotype *in vitro*. Analysis of microarray datasets was performed by Dr. Wendell D. Jones (Expression Analysis, Durham, NC). Clustering of transcripts was carried out with SAS (PROC CLUSTER) based on distance of the log ratio values using complete linkage with 5% trimming. The kernel density estimation for trimming used the 10 nearest neighbors. After an unsupervised clustering analysis was carried out on a subset of tumors from the UNC microarray database, a rule was generated to identify individual tumors that exhibit the hypermethylation signature corresponding to a lack of normal expression (less than the median level of expression for the dataset) of at least seven of the nine genes (*CEACAM6, CDH1, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*) that are associated with the hypermethylator phenotype. This rule was then applied to other mircoarray datasets, including: (i) the UNC microarray dataset at https://genome.unc.edu/pubsup/breastGEO/ that includes gene expression data for primary breast tumors analyzed in previous studies (34, 37, 251, 252), (ii) the Hess *et al.* dataset (253), (iii) the Wang *et al.* dataset (254), and (iv) the van de Vijver *et al.* dataset (255).

EXPERIMENTAL PROCEDURES RELATED TO METHYLATION ANALYSIS

Human Breast Tissue

This study included analysis of 26 paraffin-embedded human primary breast tumors. These archival human tissues were obtained from the University of North Carolina Lineberger Comprehensive Cancer Center (Chapel Hill, NC) with the assistance of Dr. Chad

A. Livasy (Department of Pathology and Laboratory Medicine, UNC School of Medicine). The tumors tissues analyzed included 15 basal breast cancers, three Her2+ tumors, six luminal A tumors, and two luminal B tumors. Protection of patient privacy and handling of specimens followed strict policies of the Institutional Review Board of the University of North Carolina.

DNA Isolation from Breast Cancer Cell Lines

Genomic DNA from $2x10^6$ cultured cells was isolated using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, PA). Cells were washed with Hank's Balanced Salt Solution (Cellgro, Lawrence, KS), trypsinized with 0.05% trypsin-ETDA (Sigma, St. Louis, MO) and pelleted at 1,000 g for 5 minutes. After excess medium was aspirated, cells were combined with 300 µl Cell Lysis Solution (Puregene DNA Isolation Kit, Gentra Systems, Minneapolis, MN), and incubated with proteinase K at 55°C, overnight. RNase A solution (1.5 µl) was added and samples were incubated at 37°C for five minutes before addition of the Protein Precipitation Solution (Gentra Systems). The lysate was incubated on ice for 5 minutes prior to centrifugation at 20,000 g for 5 minutes. DNA was precipitated with 100% isopropanol, washed in 70% ethanol and hydrated by incubation at 65°C for 30 minutes. Isolated DNA was stored at -20°C before use.

DNA Isolation from Primary Human Breast Tumors

Paraffin-embedded breast tumor specimens were microdissected using a clean razor blade using the H&E staining as a guide. Sections composed of ≥ 80% tumor were selected for microdissection, deparaffinized, and genomic DNA was isolated utilizing the QIAamp DNA Micro Kit (Qiagen, Inc., Valencia, CA), according to manufacturer's instructions.

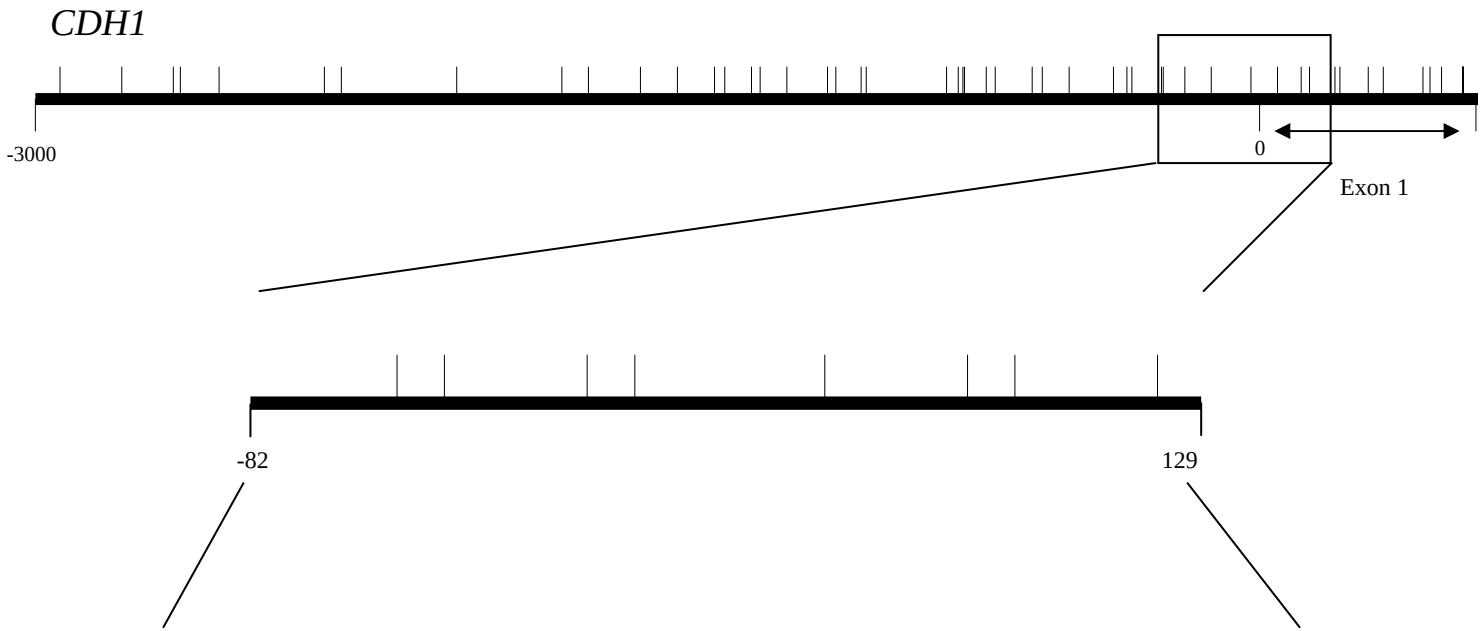
Slides were incubated at 56°C for 5-10 minutes before microdissection, and tissue was transferred to a solution containing buffer ATL (QIAamp DNA Mini Kit, Qiagen, Valencia, CA) and proteinase K and incubated with rocking at 56°C for 1.5 hours. DNA was precipitated, washed, and eluted with 35 µl of elution buffer.

Bisulfite Modification of Breast Cancer Cell Line DNA

Bisulfite modification of genomic DNA was performed using a procedure adapted from Grunau *et al*. (256) and provided by Dr. Randy L. Jirtle (Department of Radiation Oncology, Duke University Medical Center, Durham, NC). Three micrograms of genomic DNA was digested with *XhoI* (New England Biolabs, Ipswich, MA) overnight before heat inactivation at 65°C for 20 minutes. Approximately 1.5 µg of DNA was denatured with 5 µl freshly-prepared, 3 M sodium hydroxide at 42°C for 20 minutes, and incubated with 450 µl of freshly prepared, saturated sodium bisulfite solution (pH 5.0) at 55°C for four hours. Bisulfite-modified DNA was purified with the Wizard DNA Clean-up Kit (Promega, Madison, WI), according to manufacturer's instructions. DNA was reconstituted with 50 µl Tris-Cl (pH 8.0) and desulfonated with 5.5 µl 3 M sodium hydroxide. DNA was precipitated using 40 µl of 7.5 M ammonium acetate and 300 µl of 100% ethanol at -20°C for 30 minutes. The resulting DNA pellet was washed with 70% ethanol and resuspended in 20 µl of 1 mM Tris-Cl.

Bisulfite-converted DNA was amplified using primers directed to specific segments of the promoter and/or exon I region of specific genes of interest (Figs 2-10). PCR conditions for amplification of bisulfite converted DNA varied by gene and are given in Table 7. PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

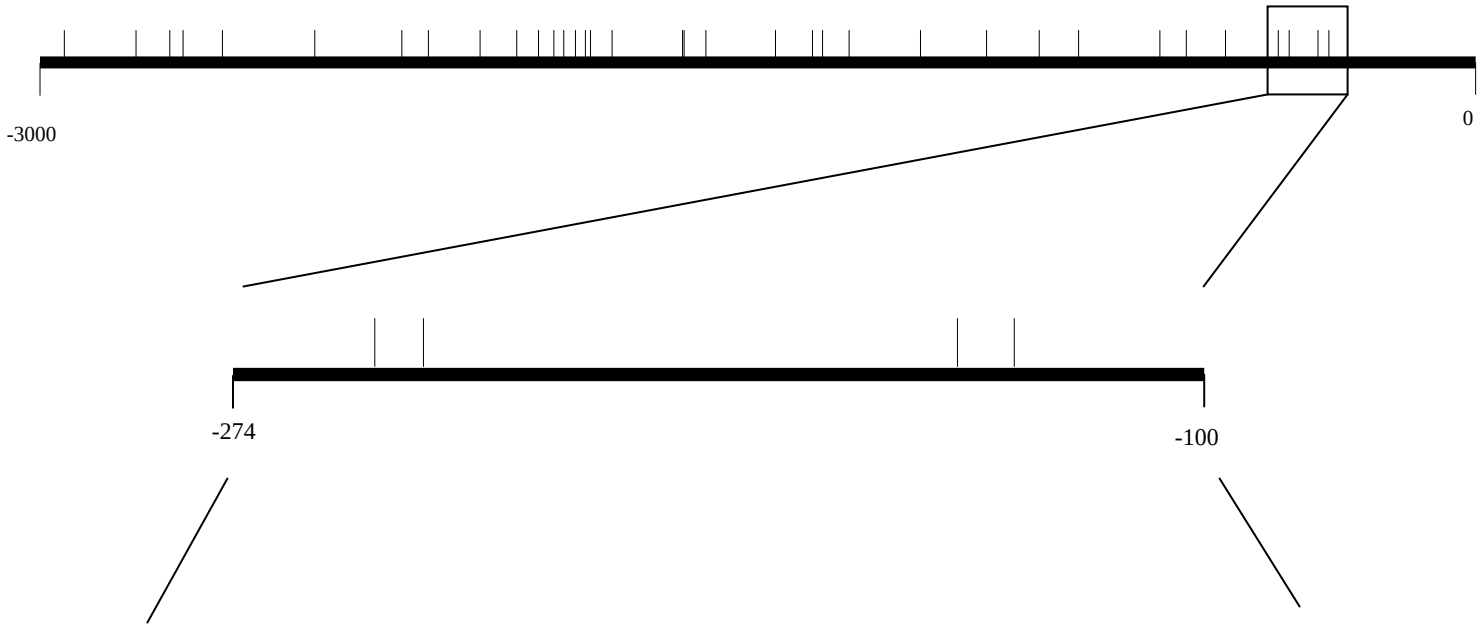
Figure 2. CpG features associated with the proximal promoter and exon 1 region of *CDH1*. Diagrams depicting the promoter and exon 1 regions of the methylation-sensitive gene *CDH1*. CpG dinucleotides are represented by vertical thin lines. The region analyzed by bisulfite sequencing is shown in detail and the sequence provided.



…**G**gcaggtgaaccctcagccaatcag**CG**gta**CG**gggggg**CG**gtgcctc**CG**gggctcacctggct
gcagcca**CG**caccccctctcagtgg**CG**t**CG**gaactgcaaagcacctgtgagcttg**CG**gaagtcagtt
cagactccagcc**CG**ctccagcc**CG**gccc**G**acc**CG**ac**CG**cacc**CG**g**CG**cctgccc**CG**ct**C**
Gg**CG**tccc**CG**gccagccatggg…

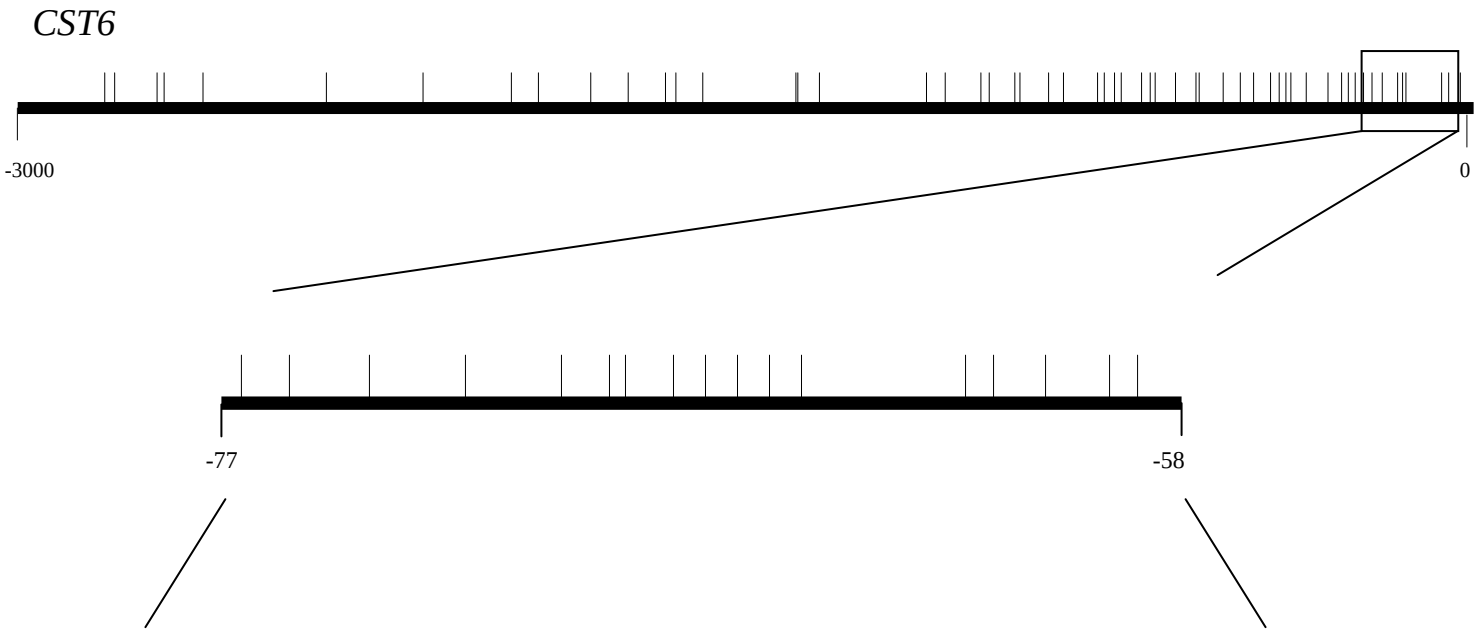
Figure 3. CpG features associated with the proximal promoter region of *CEACAM6*. Diagrams depicting the promoter region of the methylation-sensitive gene *CEACAM6*. CpG dinucleotides are represented by vertical thin lines. The region analyzed by bisulfite sequencing is shown in detail and the sequence provided.

CEACAM6



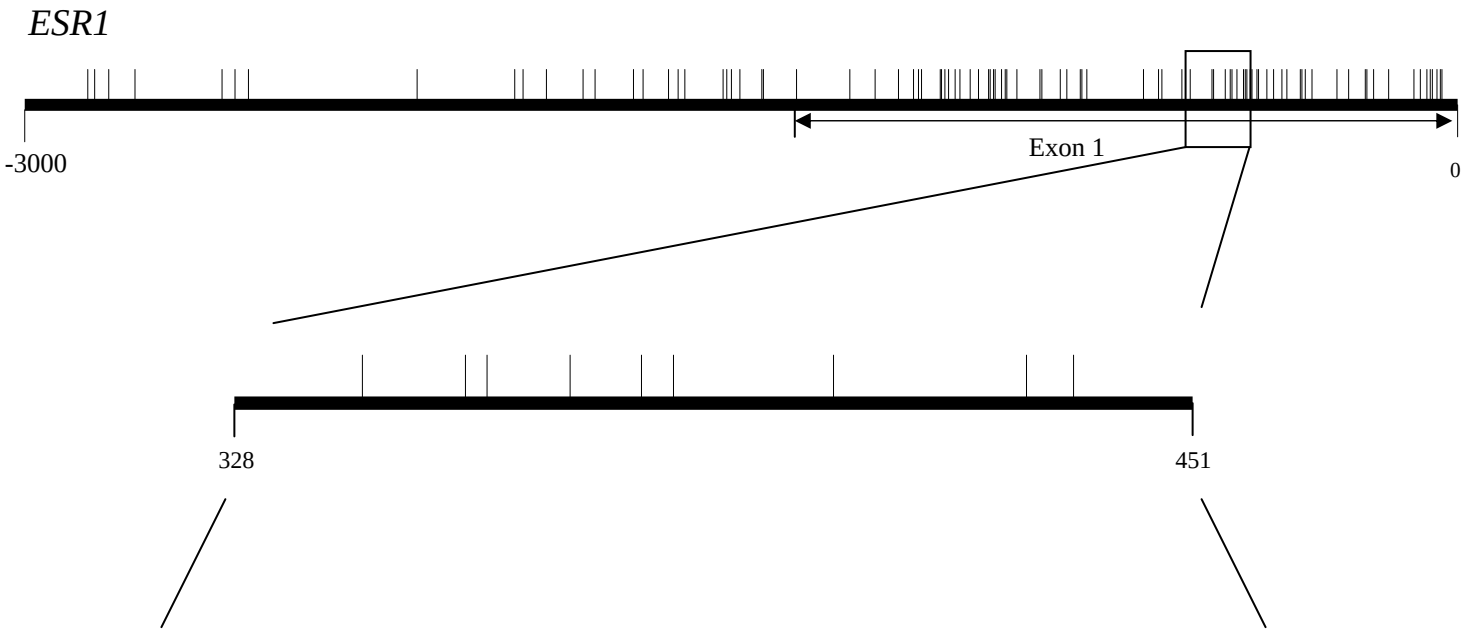
…aggg**CG**ggt**CG**tcctgttatggaacaggggtccaaacaagcttgcttctcagagcatcttctggggaac
tgaatataaacagaaagggaagaggaggagggacaaaagagacagaaatgagaggggaggggatagagg
attcctgaacagagac**CG**cacccatgaccca**CG**tga…

Figure 4. CpG features associated with the proximal promoter region of *CST6*. Diagrams depicting the promoter region of the methylation-sensitive gene *CST6*. CpG dinucleotides are represented by vertical thin lines. The region analyzed by bisulfite sequencing is shown in detail and the sequence provided.



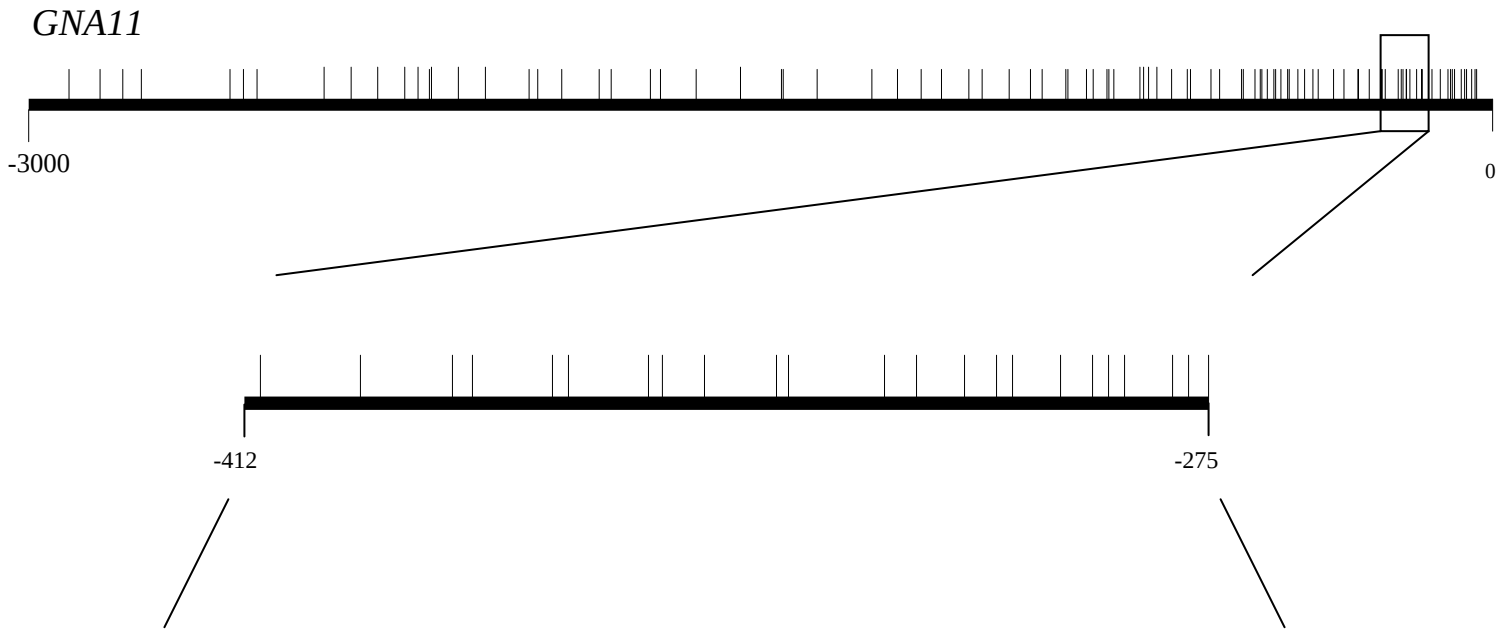
…t**CG**agccc**CG**ccccagctccaggc**CG**cgggggg**CG**cat**CGCG**gg**CG**t**CG**gg**CG**ggg
CGgcccag**CG**ggtaaaagctgcg**CG**gc**CG**caagct**CG**gcactca**CG**gctctgagggctc**CG**
a**CG**gcactgacggccatgg…

Figure 5. CpG features associated with the proximal promoter and exon 1 region of *ESR1*. Diagrams depicting the promoter and exon 1 regions of the methylation-sensitive gene *ESR1*. CpG dinucleotides are represented by vertical thin lines. The region analyzed by bisulfite sequencing is shown in detail and the sequence provided.



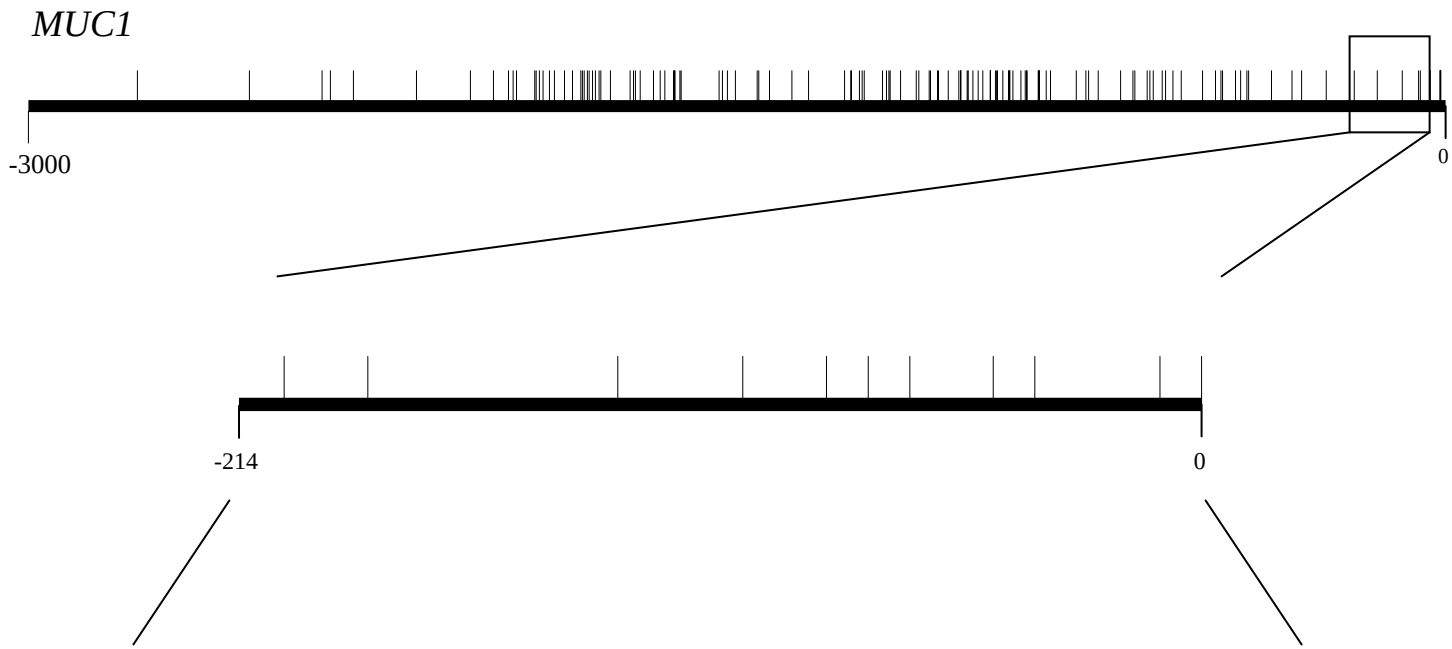
…c**CG**gtttctgagccttctgccctg**CG**gggaca**CG**gtctgcaccctgcccg**CG**gcca**CG**gaccat
gaccatgaccctccacaccaaagcatctgggatggccctactgcatcagatccaagggaa**CG**agctggagc
ccctgaac**CG**tc**CG**c…

Figure 6. CpG features associated with the proximal promoter region of *GNA11*. Diagrams depicting the promoter region of the methylation-sensitive gene *GNA11*. CpG dinucleotides are represented by vertical thin lines. The region analyzed by bisulfite sequencing is shown in detail and the sequence provided.



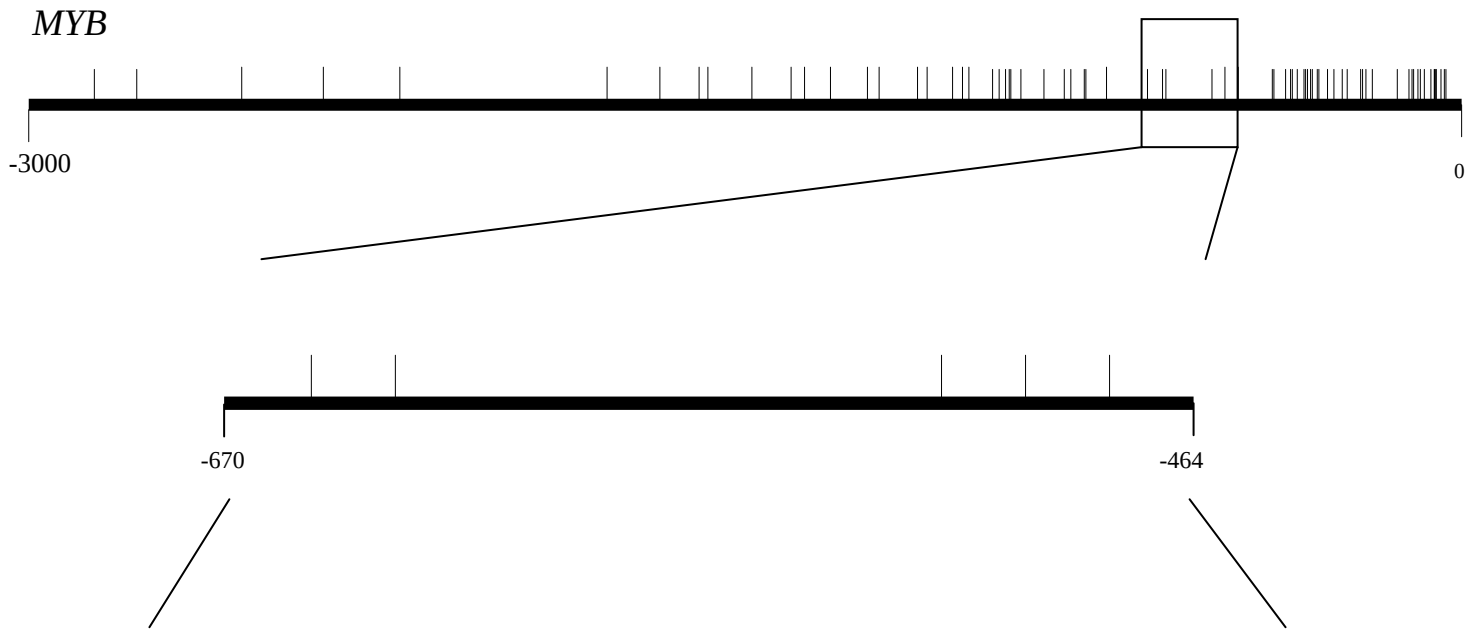
…gcacctggagacccagagag**CG**cag**CG**gc**CGCG**tg**CGCG**cctc**CG**ccac**CGCG**cct
gggccgagc**CG**ag**CG**ggac**CG**ag**CG**gggc**CG**aa**CG**gagc**CG**ag**CG**gagc**CG**agcct
ggc**CG**ggc**CG**agtc**CG**ccacattcc…

Figure 7. CpG features associated with the proximal promoter region of *MUC1*. Diagrams depicting promoter region of the methylation-sensitive gene *MUC1*. CpG dinucleotides are represented by vertical thin lines. The region analyzed by bisulfite sequencing is shown in detail and the sequence provided.



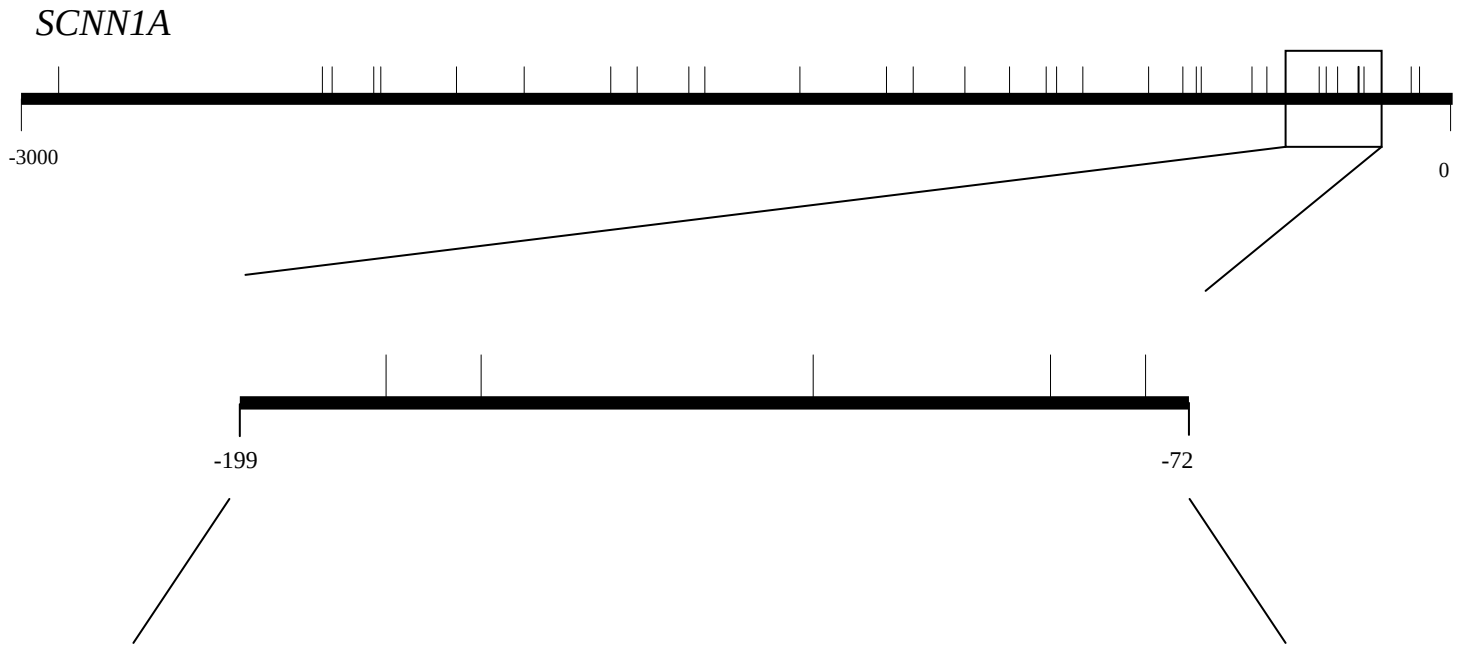
…gggaggccagctggagaacaaa**CG**ggtagtcaggggttgag**CG**attagagcccttgtaccctaccc
aggaatggttggggaggaggaggaagaggtaggaggtaggggaggggg**CG**gggttttgtcacctgtcac
ctgctc**CG**gctgtgcctaggg**CG**gg**CG**gg**CG**gggagtgggggac**CG**gtataaag**CG**gtagg
CGcctgtgcc**CG**ctcc…

Figure 8. CpG features associated with the proximal promoter region of *MYB*. Diagrams depicting the promoter region of the methylation-sensitive gene *MYB*. CpG dinucleotides are represented by vertical thin lines. The region analyzed by bisulfite sequencing is shown in detail and the sequence provided.



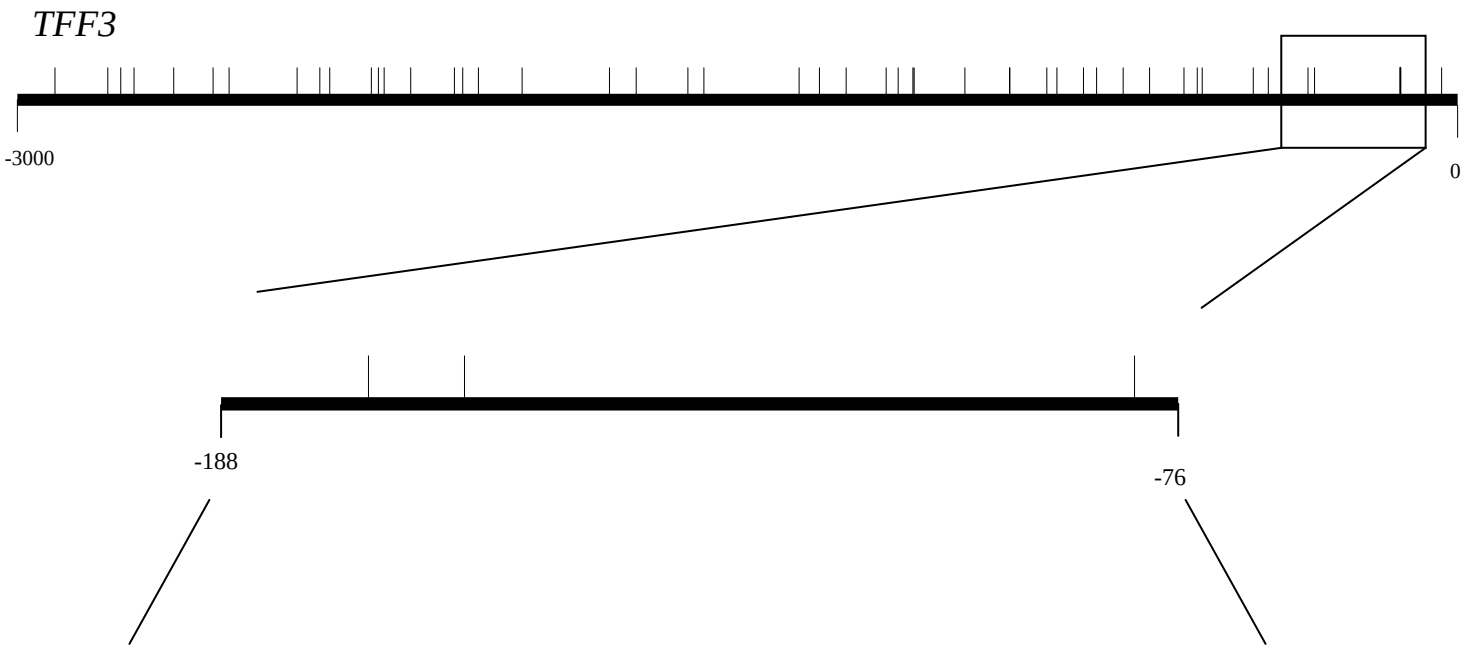
…gatggttgc**CG**cccacttgtattgaag**CG**tcctttgtcactaacaagttaaattagagatgttatttatttaa
gaagaaggaaaaaaaaccctagccaaacagcctatgaatacatatgctcacatcccctactcctccaactcct
aatttccc**CG**tctccagagggcacagttgtaaaccttga**CG**aaaatccaatcttctgtg**CG**…

Figure 9. CpG features associated with the proximal promoter region of *SCNN1A*. Diagrams depicting the promoter region of the methylation-sensitive gene *SCNN1A*. CpG dinucleotides are represented by vertical thin lines. The region analyzed by bisulfite sequencing is shown in detail and the sequence provided.



…ccagcctccttgcctgtctg**CG**tctaaagcccctgcccagagtc**CG**ccttctcaggtccagtactcccag
ttcacctgccct**CG**ggagccctccttcctt**CG**gaaaactcc**CG**gctctgactcctcc…

Figure 10. CpG features associated with the proximal promoter region of *TFF3*. Diagrams depicting the promoter region of the methylation-sensitive gene *TFF3*. CpG dinucleotides are represented by vertical thin lines. The region analyzed by bisulfite sequencing is amplified and the sequence provided.



…actcagagctgcctgtctc**CG**aggc**CG**atctgggatgaagcagcctggggctctcttgtcatgggacca
ggggtgttctgagggcttctggctgggaggctgagatggaa**CG**gacaccacaccctggtcctgcca…

A portion (2-5µl) of the PCR product was cloned into the pGEM-T Easy Vector system (Promega). Five to ten colonies were selected per gene segment and expanded in liquid culture. DNA was purified using the Wizard Plus Miniprep kit (Promega) and a portion was digested with *NcoI* and *NdeI* (New England Biolabs) in order to confirm the presence of the insert. Validated clones were sequenced using the universal M13R3 primer at the UNC Genome Analysis Facility (Chapel Hill, NC) utilizing an Applied Biosystems automated sequencer. The results of the methylation analysis were expressed as total methylation index (TMI), which corresponds to the number of methylated CpGs observed divided by the total number of CpGs analyzed for each sequence of interest, and is expressed as percent methylation (257).

Bisulfite Modification of Primary Human Breast Tumor DNA

Genomic DNA was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research Corp., Orange, CA). Between 200 and 500 ng of DNA was treated with a conversion reagent and incubated at 98° C for 10 minutes, 53°C for 30 minutes, before a step cycle program consisting of 8 cycles of 53°C for six minutes and 37°C for 30 minutes. Samples were transferred to columns, washed, desulfonated, and eluted with 20 µl of elution buffer. Two microliters of converted DNA was used in subsequent MSP reactions. A portion of the PCR product was cloned, expanded in liquid culture, and the DNA was purified as described above.

Methylation-sensitive PCR Analysis

MSP reactions were carried out in EasyStart Micro 50 PCR-mix-in-a-tube (Molecular BioProducts, San Diego, CA) using bisulfite-converted DNA template (described above).

The primers and thermocycling conditions for *CDH1, CST6,* and *ESR1* genes have been described previously (118, 258, 259). MSP primers directed against methylated and unmethylated alleles of *CEACAM6, GNA11, MUC1I, SCNN1A,* and *TFF3* and cycling conditions are given in Table 7. PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining. For some analyses, MSP results were converted from a discrete scale (unmethylated product only, both methylated and unmethylated products, or methylated product only) to a quantitative scale (0, 1, 2) in order to generate a methylation score for each cell line that reflects the combined methylation status of select genes of interest.

EXPERIMENTAL PROCEDURES RELATED TO DNMT ANALYSIS

DNA Methyltransferase Activity Analysis

Total DNA methyltransferase activity was measured using EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit (Epigentek, Brooklyn, NY) as previously described (260), using nuclear extracts from 16 human breast cancer cell lines (BT20, BT549, HCC1937, HS578T, MCF7, MDA-MB-231, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MDA-MB-468, SKBR3, SUM102, SUM149, SUM185, and ZR-75-1) and MCF12A cells. Nuclear extracts were isolated using the EpiQuik Nuclear Extraction Kit (Epigentek, Brooklyn, NY) and 3 µl of nuclear extract was added to each reaction well, according to manufacturer's protocol. The final volume of nuclear extract yield was used to normalize the assay results for differences in cell number. Nuclear extracts were incubated with methylation substrate for one hour at 37°C, and then exposed to the

Table 7. MSP primers and PCR reaction conditions for select genes of interest.

Gene Segment	Methylated	Unmethylated	Product size	PCR Conditions
CDH1	F: GGTGAATTTTTAGTTAATTAGCGGTAC R: CATAACTAACCGAAAACGCCG	F: GGTAGGTGAATTTTTAGTTAATTAGTGGTA R: ACCCATAACTAACCAAAAACACCA	211	TM = 60° 35 cycles
CEACAM6	F: AGGGCGGGTCGTTTTGTTAT R: TCACGTAAATCATAAATACGATCTCT	F: AGGGTGGGTTGTTTTGTTAT R: TCACATAAATCATAAATACAATCTCT	174	U: TM = 55° 35 cycles M: TM = 58° 35 cycles
CST6	F: TCGAGTTTCGTTTTAGTTTTAGGTC R: CATAACCGTCAATACCGTCG	F: TGAGTTTTGTTTTAGTTTTAGGTT R: CCATAACCATCAATACCATCAA	135	U: TM = 55° 38 cycles M: TM = 60° 38 cycles
ESR1	F: GATACGGTTTGTATTTTGTTCGC R: CGAACGATTCAAAAACTCCAACT	F: GGATATGGTTTGTATTTGGTTTGT R: ACAAACAATTCAAAAACTCCAACT	123	TM = 58° 35 cycles
GNA11	F: GATTACGGGCGTGTATTATTAC R: CCAACACTTTAAAAAACCGAAACGAA	F: TTGGGATTATGGGTGTGTATTATTAT R: ATCCCAACACTTTAAAAAACCAAAACAAA	137	TM = 59° 35 cycles
MUC1	F: GGAGGTTAGTTGGAGAATAAAC R:AACAAATAACAAATAACAAAACCCCG	F: GGAGGTTAGTTGGAGAATAAATG R: ACAAATAACAAATAACAAAACCCCAC	139	TM = 58° 35 cycles
MYB	F: TAGAGGGTATAGTTGTAAATTTTGAC R: CTCACTATCGCGAAAACGAC	F:AGAGGGTATAGTTGTAAATTTTGATGA R:CTCCCACTCACTATCACAAAAA	90	TM = 58° 35 cycles
SCNN1A	F: TTTTTTAGTTTTTTTGTTTGTTTGC R: AAAACCGAAAATTTTCCGAA	F: TTAGTTTTTTTGTTTGTTTGTGT R: AAAATCAAAACCAAAAATTTTCCA	127	TM = 53° 35 cycles
TFF3	F: TTAGAGTTGTTTGTTTTCGAGGTC R: AACAAAACCAAAATATAATATCCGTTCCA	F: ATTTAGAGTTGTTTGTTTTTGAGGTTGA R: AACCAAAATATAATATCCATTCCATCTCA	132	TM = 59° 35 cycles

capture antibody for 60 minutes and the detection antibody for 30 minutes, at room temperature. Absorbance was determined using a microplate spectrophotometer at 450 nm, and DNMT activity (O.D./h/ml) was calculated according to the following formula: (sample OD - blank OD) / (sample volume x 1000), according to manufacturer's instructions. Results are given in activity units expressed relative to the activity level detected in MCF12A cells.

DNA Methyltransferase Protein Analysis

Nuclear extracts were assayed for individual DNMT proteins of interest (DNMT1, DNMT3a, or DNMT3b) using the Epiquik DNMT1, DNMT3a, and DNMT3b assay kits, respectively (Epigentek). Protein standards of known concentration (30 ng, 20 ng, 10 ng, and 2 ng) were included to generate a standard curve. The amount of DNMT protein was calculated as follows: DNMT protein (ng/ml) = (Sample OD - blank OD / standard slope) x sample dilution, according to the manufacturer's instructions, and are expressed relative to the protein levels of MCF12A cells.

RNA-interference Mediated Knockdown of DNMT3b

In order to determine if knockdown of DNMT3b protein induces re-expression of methylated genes in hypermethylator cell lines, shRNA was employed to target the overexpressed DNMT3b protein associated with the hypermethylation defect. PLVTHM plasmid containing DNMT3b-specific oligos for shRNA were a kind gift from the laboratory of Dr. P.P. Jagodzinski (Poznan University of Medical Sciences, Poznan, Poland). The construction of the shRNA was described previously (261). The PLVTHM plasmid was reconstituted in 50 µl Tris-EDTA (TE) buffer (pH 8.0) then centrifuged at 700 g for 2 minutes. Three microliters of plasmid were used to transform 50 µl of JM109 cells according

to conventional methods. Five to ten colonies were selected, expanded in liquid culture, and the DNA was purified using the Wizard Plus Miniprep kit (Promega). Diagnostic restriction enzyme digest using *FspI* and *NdeI* (New England Biolabs) produced one 8,041 nucleotide fragment and a 3,044 nucleotide fragment, confirming the plasmid size of 11,085 nucleotides.

Transfection of Human Breast Cancer Cell Lines

One microgram of PLVTHM plasmid was digested with *SalI* and *XbaI* (New England Biolabs) at 37°C for 2.5 hours before use in transfection, producing one 3,857 nucleotide fragment and a 7,228 nucleotide fragment. The linearized section of the plasmid (3,857 nucleotides), containing the H1 promoter, shRNA sequence, and GFP tag, was confirmed by gel electrophoresis. MDA-MB-453 and BT549 cells were grown in a six-well polystyrene plate to 80% confluency in 1 ml of medium, according to ATCC recommendations. In a 1.5 ml tube, 7.5 µl of TransIT-LT1 Transfection reagent (Mirus, Madison, WI) was added to 250 µl of Opti-MEM serum free medium (Gibco/Invitrogen Life Technologies), mixed by pipetting, and incubated at room temperature for 15 minutes. One microgram of DNA was added to the dilute TransIT and incubated at room temperature for 20 minutes. The TransIT/DNA complex was added dropwise to the wells. After 48 hours the presence of GFP+ cells was assessed. Clonal selection of GFP+ cells was accomplished using 3 mm sterile cloning discs saturated in 0.25% trypsin (Gibco) to select and isolate clones of interest.

Verification of DNMT3B Knockdown

Western blots were performed by Dr. Ashley G. Rivenbark (UNC Lineberger Comprehensive Cancer Center, Department of Biochemistry and Biophysics, UNC School of

Medicine). For analysis of DNMT3b protein levels, MDA-MB-453 breast cancer cells transfected with scrambled or RNAi constructs were lysed with 1x phosphate buffered saline containing 0.1 mM phenylmethanesulphonylfluoride (PMSF), 1 ug/ml pepstain A, leupeptin, and aprotinin, 5 μg/ml phosphatase inhibitor cocktail, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, and 0.1% Triton X-100. The anti-DNMT3b mouse monoclonal antibody was obtained from Imgenex (San Diego, CA) and used at a dilution of 1:500. Protein lysates were resolved on 8% SDS-PAGE gels, followed by transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore; Billerica, MA). PVDF membranes were incubated with the primary antibody for 2 hours at room temperature in Tris-Buffered Saline Tween-20 (TBST) containing 5% milk and 0.05% sodium azide. Subsequently membranes were washed with TBST 3 times for 5 minutes, and then incubated with a sheep anti-mouse (1:5000) horseradish peroxidase-conjugated secondary antibody (GE Healthcare; Piscataway, NJ) in TBST containing 5% milk for 1 hour at room temperature. The membranes were washed with TBST 3 times for 5 minutes, and bound primary antibody was detected using ECL-Plus substrate (GE Healthcare; Piscataway, NJ).

Assessment of Target Genes

For RT-PCR analysis of target gene expression in DNMT3b-knockdown cells, RNA was isolated as previously described (above) from MDA-MB-453 and BT549 cells for each of the following treatments: parental control (untreated) cells, scrambled control shRNA, and DNMT3b-targeted shRNA. Total RNA (2 μg) collected from each cell line was reverse-transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA) and oligo(dT) as the primer, as previously described and the

expression levels of target genes (*CEACAM6, CDH1, CST6, ESR1, GNA11, MUC1,* and/or *SCNN1A*) were assessed by semi-quantitative RT-PCR (PCR conditions, Table 6).

STATISTICAL ANALYSIS

The values for the mean and S.E.M. were calculated using the statistical function of KaleidaGraph Version 3.5 (Synergy Software, Essex Junction, VT). Statistical significance was determined using an unpaired t-test (KaleidaGraph). Error bars depicted represent S.E.M. P values for correlation coefficients (R values) were calculated using VasserStats Significance of Correlation Coefficient Calculator (http://faculty.vassar.edu/lowry/rsig.html). The Bayesian analysis was performed as described previously (262) and the percentage of correct assignments, as well as sensitivity, specificity, and positive and negative predictive values were calculated.

RESULTS

EXPRESSION OF METHYLATION-SENSITIVE GENES AMONG HUMAN BREAST CANCER CELL LINES AND PRIMARY BREAST TUMORS

Semi-quantitative RT-PCR was performed on a panel of 66 methylation-sensitive genes in each of 12 breast cancer cell lines (BT20, BT549, HS578T, MCF7, MDA-MB-231, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MDA-MB-468, SKBR3, and ZR-75-1), as well as the normal breast epithelial cell line MCF12A (Figure 11). Epigenetically-regulated genes that are predictive of the CpG island methylator phenotype (CIMP) in other tumor systems, as well as genes known to be aberrantly methylated in breast cancer, were selected for expression analysis (Table 5). In instances where RT-PCR expression was detectable, levels of expression for each gene in the breast cancer cell lines were scored relative to the levels of expression in MCF12A cells: low (detectable, but <MCF12A), normal (equivalent to MCF12A), or high (>MCF12A). A number of patterns of gene expression were observed (Figure 12). Some genes lacked differential expression, including those expressed in the majority of breast cancer cell lines (such as *BARD1, CDKN1A*, and *CEACAM5*), while others lacked expression in the majority of cell lines examined (such as *ADAM23, ESR2,* and *ST18*). Other genes (*APBA2, C8orf4, CDKN2B, CDKN2A, GADD45A, IGFBP5, PARP12, PGR, PRKCDBP, RB1, SERPINB5, SIM1, STYK11, THBS1, TMEM45A,* and *WT1*) were not expressed in MCF12A cells and were

Figure 11. Expression analysis of methylation-sensitive genes in human breast cancer cell lines. Representative agarose gels of RT-PCR products from *CST6, CDH1, CEACAM6, ESR1,* and *SCNN1A*. The source of cDNA template is identified for each lane. Normal breast epithelial MCF12A cells represent the positive control cell line. *β-actin* was amplified as a positive control.

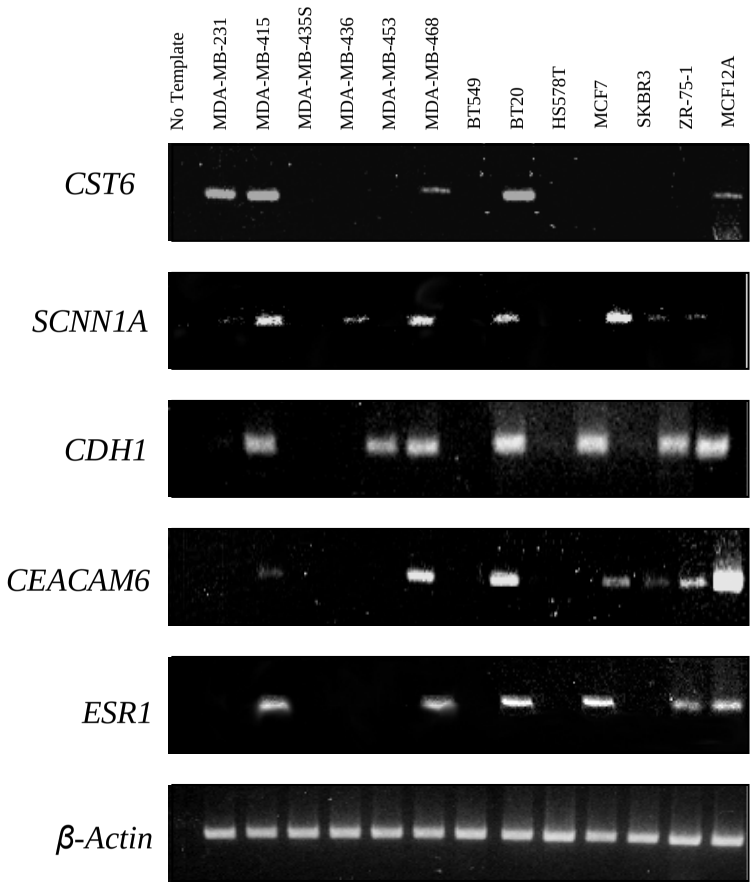
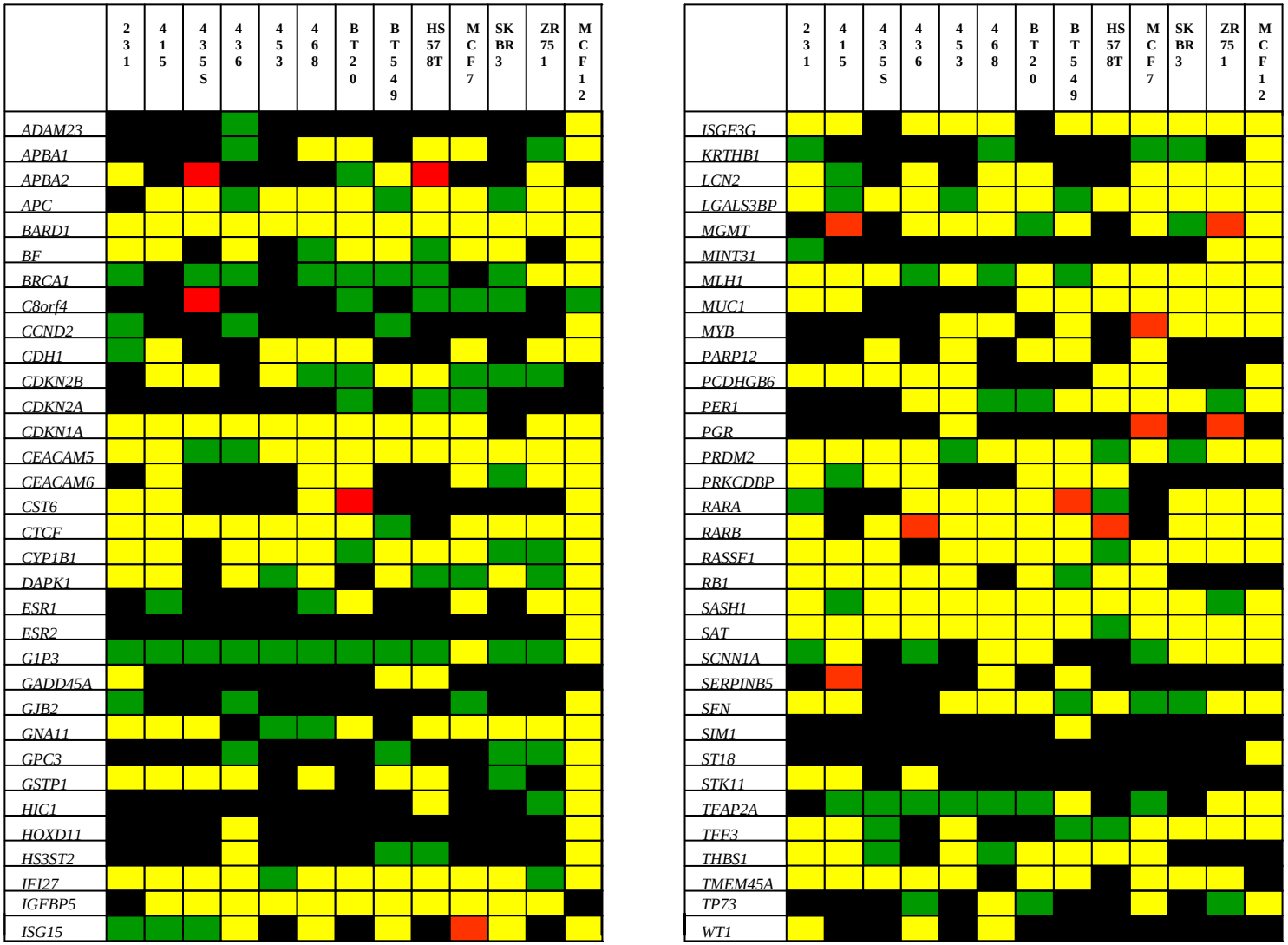

No Template
MDA-MB-231
MDA-MB-415
MDA-MB-435S
MDA-MB-436
MDA-MB-453
MDA-MB-468
BT549
BT20
HS578T
MCF7
SKBR3
ZR-75-1
MCF12A
CST6
SCNN1A
CDH1
CEACAM6
ESR1
β-Actin

Figure 12. Expression analysis of 66 methylation-sensitive genes in a panel of 12 breast cancer cell lines. Heatmap showing RT-PCR results from 66 methylation-sensitive genes examined for differential expression among 12 breast cancer cell lines and the non-neoplastic breast epithelial cell line MCF12A. For instances of detectable expression, results were scored relative to the expression level of MCF12A cells. Red boxes indicate genes with high level expression (compared to MCF12A), yellow boxes indicate those with normal level expression (similar to MCF12A), green boxes indicate low level expression (compared to MCF12), and black boxes indicate instances of no detectable gene expression.


231
415
435S
436
453
468
BT20
BT549
HS578T
MCF7
SKBR3
ZR751
MCF12
ADAM23
APBA1
APBA2
APC
BARD1
BF
BRCA1
C8orf4
CCND2
CDH1
CDKN2B
CDKN2A
CDKN1A
CEACAM5
CEACAM6
CST6
CTCF
CYP1B1
DAPK1
ESR1
ESR2
G1P3
GADD45A
GJB2
GNA11
GPC3
GSTP1
HIC1
HOXD11
HS3ST2
IFI27
IGFBP5
ISG15
ISGF3G
KRTHB1
LCN2
LGALS3BP
MGMT
MINT31
MLH1
MUC1
MYB
PARP12
PCDHGB6
PER1
PGR
PRDM2
PRKCDBP
RARA
RARB
RASSF1
RB1
SASH1
SAT
SCNN1A
SERPINB5
SFN
SIM1
ST18
STK11
TFAP2A
TFF3
THBS1
TMEM45A
TP73
WT1

therefore excluded from further analysis (n=16 genes) in order to ensure that only cancer-specific methylation was reflected in the dataset. Quantitative real-time PCR was performed on a subset of epigenetically-regulated genes (n=5) to confirm the RT-PCR expression results (Figure 13). This analysis revealed a statistically significant correlation ($R=0.76$, $p<0.0001$) between the quantitative real-time PCR and RT-PCR results. The gene expression results for the remaining 48 genes with detectable expression in MCF12A cells from the 12 breast cancer cells of interest were subjected to an unsupervised cluster analysis (*MUC1* and *TFF3* were not included in this analysis). This analysis indentified two distinct groups of six cell lines that differ in their expression of methylation-sensitive genes: cluster I is composed of cell lines that express a putative hypermethylator phenotype (MDA-MB-436, BT549, MDA-MB-453, MDA-MB-435S, HS578T, and MDA-MB-231), and cluster II consists of cell lines that express a putative low-frequency methylator phenotype (ZR-75-1, MDA-MB-468, SKBR3, BT20, MDA-MB-415, and MCF7) (Figure 14). The separation of these two groups is driven predominately by the differential expression of nine methylation-sensitive genes (*CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A*, and *TFF3*), which are largely unexpressed by the cell lines in cluster I (putative hypermethylator group), and typically expressed by the cell lines in cluster II (putative low-frequency methylator group) (Figure 14). As these nine genes (*CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A* and *TFF3*) are excellent predictors of methylator status in the panel of breast cancer cell lines, they were termed indicator genes for the purposes of the subsequent analyses.

We next analyzed the expression of these nine indicator genes in four additional breast cancer cell lines—HCC1937, SUM102, SUM149, and SUM185. With the exception

Figure 13. Hypermethylator cell lines lack expression of methylation-sensitive genes. Quantitative real-time PCR results for *CST6, SCNN1A, CDH1, CEACAM6,* and *ESR1*. Black bars correspond to hypermethylator cell lines, cross-hatched bars correspond to low-frequency methylator cell lines, and the white bar (far right) corresponds to MCF12A (index control cell line). The expression level of each gene is depicted relative to that of MCF12A cells. Error bars represent S.E.M. Instances of no detectable level of quantitative real-time PCR expression are indicated by an asterisk (*).

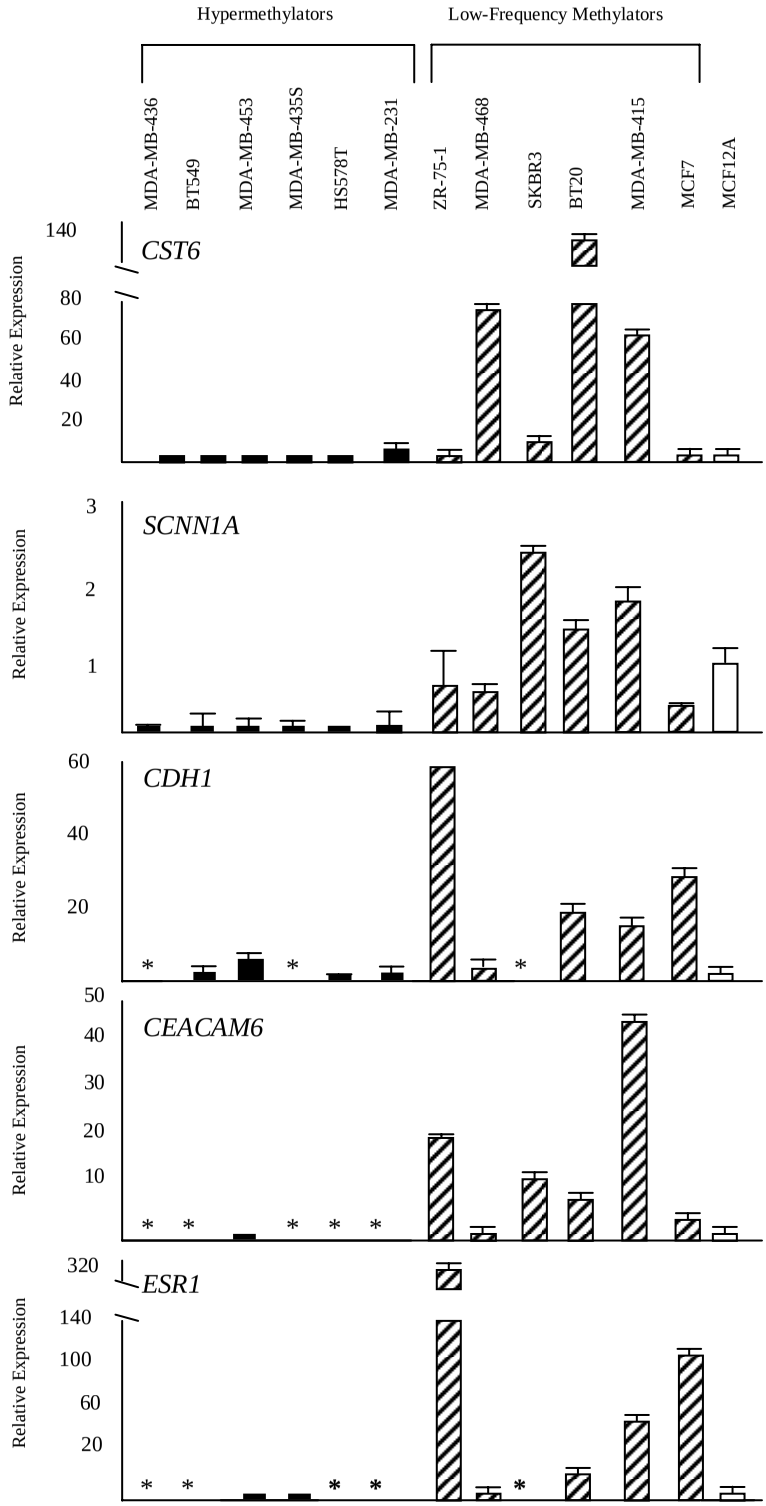
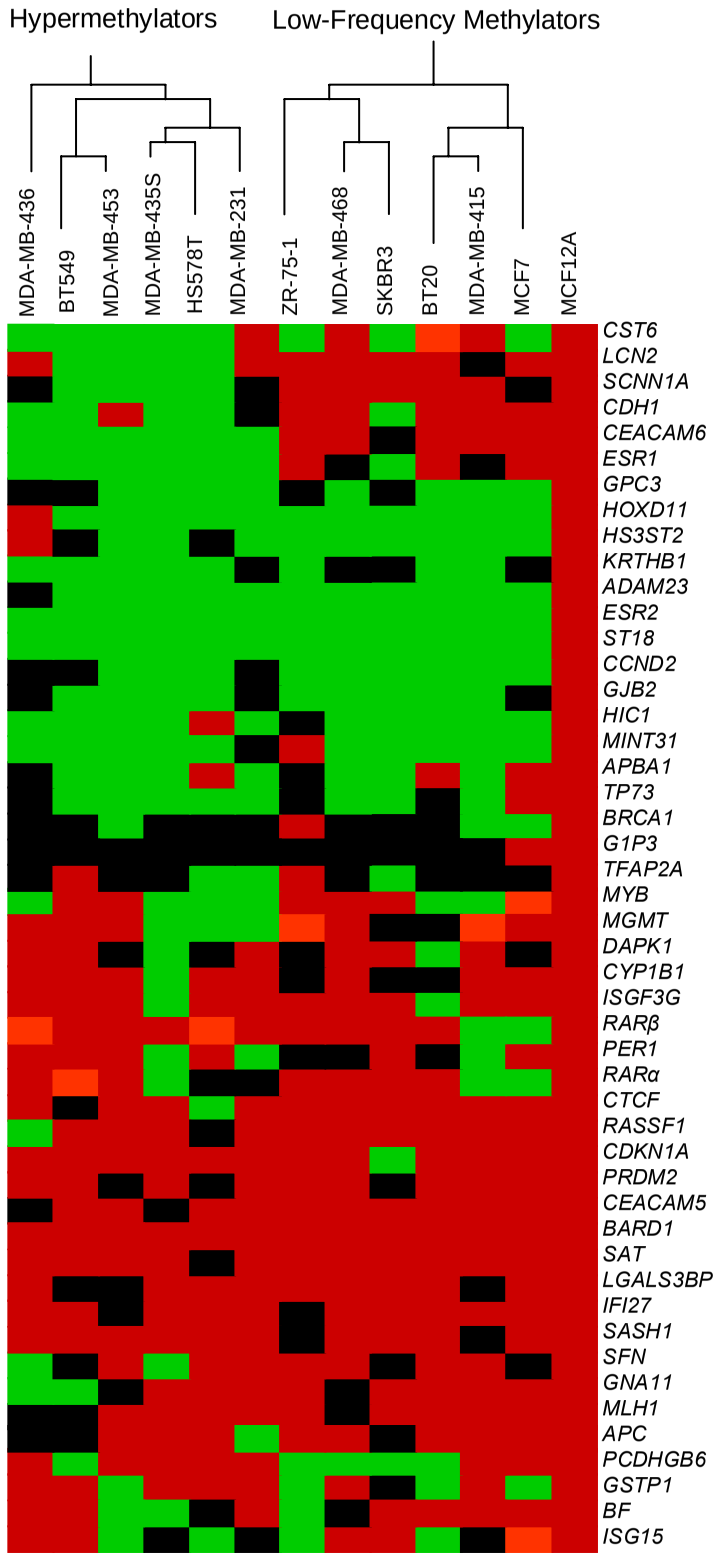

Hypermethylators
Low-Frequency Methylators
MDA-MB-436
BT549
MDA-MB-453
MDA-MB-435S
HS578T
MDA-MB-231
ZR-75-1
MDA-MB-468
SKBR3
BT20
MDA-MB-415
MCF7
MCF12A
CST6
SCNN1A
CDH1
CEACAM6
ESR1
Relative Expression

Figure 14. Expression analysis of methylation-sensitive genes in human breast cancer cell lines reveals two distinct clusters. Unsupervised cluster analysis for the 48 methylation-sensitive genes that are expressed at a detectable level in MCF12A cells. The 12 breast cancer cell lines group into two distinct clusters, one cluster corresponding to hypermethylator cell lines and a second cluster corresponding to low-frequency methylator cell lines. Orange boxes indicate high levels of expression (compared to MCF12A), red boxes indicate normal levels of expression, black boxes indicate low levels of expression, and green boxes indicate instances of no detectable expression.


Hypermethylators
Low-Frequency Methylators
MDA-MB-436
BT549
MDA-MB-453
MDA-MB-435S
HS578T
MDA-MB-231
ZR-75-1
MDA-MB-468
SKBR3
BT20
MDA-MB-415
MCF7
MCF12A
CST6
LCN2
SCNN1A
CDH1
CEACAM6
ESR1
GPC3
HOXD11
HS3ST2
KRTHB1
ADAM23
ESR2
ST18
CCND2
GJB2
HIC1
MINT31
APBA1
TP73
BRCA1
G1P3
TFAP2A
MYB
MGMT
DAPK1
CYP1B1
ISGF3G
RARβ
PER1
RARα
CTCF
RASSF1
CDKN1A
PRDM2
CEACAM5
BARD1
SAT
LGALS3BP
IFI27
SASH1
SFN
GNA11
MLH1
APC
PCDHGB6
GSTP1
BF
ISG15

of SUM185 (which has been classified as Her2+), these cell lines have been reported to be basal-like (263, 264). RT-PCR expression of these breast cancer cell lines revealed that SUM102 cells were most similar to other hypermethylator cell lines, expressing only 2 of the 9 genes at normal levels (Figure 15). SUM149 cells lacked expression of 5 genes of interest, SUM185 lacked expression of 3, and HCC1937 lacked expression of 4 such genes.

Predictive Value of Nine Indicator Genes in Determining Methylator Status

A Bayesian analysis was performed to evaluate the value of each gene for predicting correctly which of the two clusters a given cell line was sorted (Table 8). Nine genes emerged as excellent individual indicators (predictors) of cluster assignment, having correct assignment values of 67% or greater: *CDH1* (CA= 83%), *CEACAM6* (92%), *CST6* (67%), *ESR1* (75%), *GNA11* (67%), *MUC1* (67%) *MYB* (67%), *SCNN1A* (92%) and *TFF3* (67%). These genes individually display excellent sensitivity (range: 63-100%) and specificity (range: 63-86%). Furthermore, these genes display good positive predictive value (range: 50-83%) and negative predictive value (range: 50-100%). Cell lines of the hypermethylator phenotype frequently do not express these genes (5/6 hypermethylator cell lines express $\leq$ 0-2 genes at normal levels) (Figure 15). In contrast, the cell lines belonging to the low-frequency methylator group frequently express the majority of these genes at normal levels, with low-frequency methylator cell lines retaining some level of expression of at least 6 of 9 genes (Figure 15).

A cell line tissue microarray was constructed containing the 16 breast cancer cell lines (BT20, BT549, HCC1937, HS578T, MCF7, MDA-MB-231, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MDA-MB-468, SKBR3, SUM102, SUM149,

Figure 15. Expression analysis of select genes among breast cancer cell lines. RT-PCR results for nine methylation-sensitive genes of interest in a panel of 16 breast cancer cell lines. Gene expression levels are expressed relative to that of MCF12A cells. Yellow boxes indicate the same level of expression as MCF12A, red boxes indicate high level expression, green boxes indicate low level expression, and black boxes indicate no expression relative to MCF12A cells.

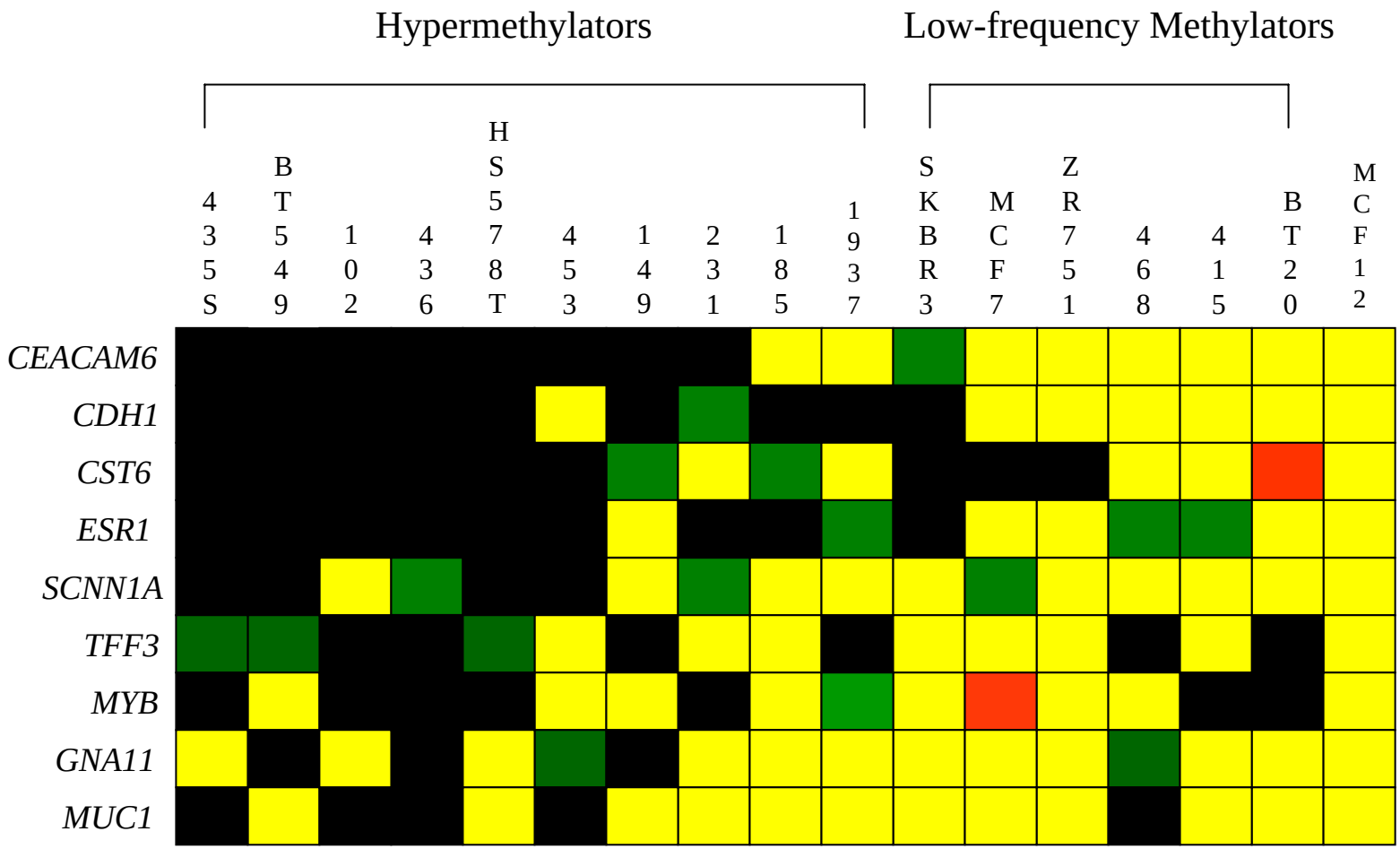

Hypermethylators
Low-frequency Methylators
435S
BT549
102
436
HS578T
453
149
231
185
1937
SKBR3
MCF7
ZR751
468
415
BT20
MCF12
CEACAM6
CDH1
CST6
ESR1
SCNN1A
TFF3
MYB
GNA11
MUC1

Table 8. Bayesian values for methylation-sensitive genes of interest[1].

Gene	Correct Assignment	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
CEACAM6	0.92	1.00	0.86	0.83	1.00
CDH1	0.83	0.83	0.83	0.83	0.83
CST6	0.67	0.75	0.63	0.50	0.83
ESR1	0.75	1.00	0.67	0.50	1.00
SCNN1A	0.92	1.00	0.86	0.83	1.00
TFF3	0.67	0.67	0.67	0.67	0.67
MYB	0.67	0.67	0.67	0.67	0.67
GNA11	0.67	0.63	0.75	0.83	0.50
MUC1	0.67	0.63	0.75	0.83	0.50

[1] Bayesian analysis reflects the ability of a given gene to correctly predict the methylator (hypermethylator versus low-frequency methylator) status of a given cell line.

SUM185, and ZR-75-1) and the normal breast cell line MCF12. Six of these cell lines were previously characterized as hypermethylators (BT549, HS578T, MDA-MB-231, MDA-MB-435S, MDA-MB-436, and MDA-MB-453) and six were previously characterized as low-frequency methylators (BT20, MCF7, MDA-MB-415, MDA-MB-468, SKBR3, and ZR-75-1). The others (HCC1937, SUM102, SUM149, and SUM185) have been classified differently by different investigators. SUM102 cells are reported to be basal-like (263, 265), while SUM149 cells have been reported to be basal-like (263, 264, 266) and also to be isolated from a patient with inflammatory breast cancer (267, 268). SUM185 cells are reported to be Her2+ (269) or luminal-like (266), while HCC1937 (which are Her2- and BRCA1 defective) have been classified as basal-like (266, 270) or as a luminal/basal intermediate cell type (271). Given that the ER and PR status of these cell lines have been previously reported, the cell line TMA was stained for Her2, Her1, and cytokeratin 5/6, and the results scored to facilitate classification of the cell lines. Cell lines that were ER+/PR+/Her2- were classified as luminal A, cell lines which were ER+/PR+/Her2+ were classified as luminal B, cell lines which were ER-/Pr-/Her2+ were classified as Her2+, and cell lines that were ER-/PR-/Her2- with positive staining of either Her1 and/or cytokeratin 5/6 were classified as basal-like, in keeping with previous studies (40). The results of the immunohistochemical analysis are shown in Table 9. Of the six hypermethylator cell lines, five display a basal-like IHC signature, while none of the low-frequency methylators appeared basal-like, suggesting a substantial overlap between the hypermethylator cell lines and the basal-like subtype. The majority of low-frequency methylators were luminal A-like.

Table 9. IHC analysis of breast cancer cell lines.

Cell Line	ER[1]	PR[1]	Her2+	Her1	Cyto 5/6	Designation[2]	Subtype[3]
MDA-MB-436	-	-	-	+	Low	HM	Basal
BT549	-	-	Low	+	-	HM	Basal
MDA-MB-453	-	+	+	-	Low	HM	Her2
MDA-MB-435S	-	-	-	-	Low	HM	Basal
Hs578T	-	-	-	Low	-	HM	Basal
MDA-MB-231	-	-	-	+	Low	HM	Basal
SUM102	-	-	-	+	+	HM	Basal
SUM149	+	-	-	+	Low	HM	Luminal A
SUM185	-	-	-	Low	Low	HM	Basal
HCC1937	Low	-	-	+	-	HM	Luminal A
ZR751	+	+	Low	-	Low	LFM	Luminal A
MDA-MB-468	Low	-	+	+	Low	LFM	Luminal B
SKBR3	-	-	+	-	-	LFM	Her2
BT20	+	-	-	+	Low	LFM	Luminal A
MDA-MB-415	Low	-	Low	-	Low	LFM	Luminal A
MCF7	+	+	-	-	Low	LFM	Luminal A
MCF12	+	+	-	Low	Low	Normal[4]	Normal

[1] ER and PR designations were determined from ATCC factsheet for each cell line or the SUM cell line fact sheet (263).

[2] HM= hypermethylator, LFM= low frequency methylator, designations are based on expression of 9 indicator genes.

[3] Basal = ER-/PR-/Her2- and either Her1 or Cytokeratin 5/6 positive; Her2+ = ER-/PR-/Her2+; Luminal A = ER+/PR+/Her2-; Luminal B = ER+/PR+/Her2+

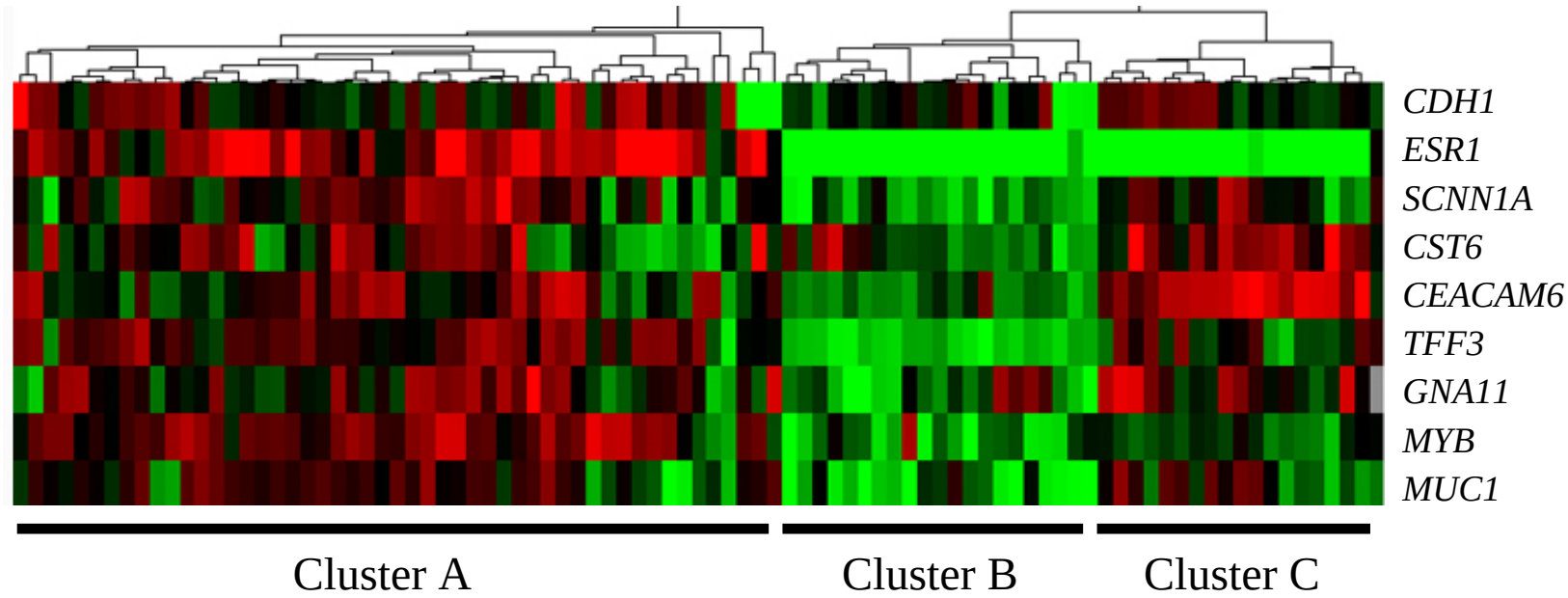
[4] MCF12A is a normal non-neoplastic breast cell line (256).

Two of the basal-like cell lines (SUM102 and SUM185) had a basal-like immunohistochemical signature, while SUM149 and HCC1937 exhibited a more luminal A-like immunostaining pattern (Table 9).

mRNA-based Expression Analysis of Indicator Genes in Primary Human Breast Tumors

In order to determine if the putative hypermethylator phenotype occurs in primary breast cancers as well as breast cancer cell lines, microarray gene expression data from 91 primary breast tumors from the UNC Microarray Database (34, 37, 251, 252) were analyzed for expression of the nine genes (*CEACAM6, CDH1, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*) whose loss characterizes the hypermethylator phenotype among breast cancer cell lines. Unsupervised cluster analysis of these data identified three strong clusters (Figure 16). Of the 91 tumors included in this database, 90 clustered successfully and one was excluded from further analysis (due to the 5% trimming guideline). The 90 breast cancers in this analysis which clustered reflect the following molecular classification: 33/90 (37%) luminal A, 24/90 (27%) basal-like, 17/90 (19%) Her2+, and 16/90 (18%) luminal B. Three major clusters emerged from this analysis (designated A-C). Cluster B is composed of 21 tumors (23% of all the tumors in the dataset) that express a hypermethylation signature, characterized by low expression of at least seven of the nine genes analyzed. Strikingly, 100% (21/21) of these putative hypermethylator tumors are of the basal subtype, and this hypermethylator cluster contains 88% (21/24) of all basal tumors in the dataset. This observation suggests that expression of the hypermethylator phenotype represents a major biological property of basal-like breast cancers. As shown in Figure 16, Cluster A (n=51) is composed primarily of luminal A and luminal B breast tumors (65% and 29%, respectively),

Figure 16. Microarray analysis of 91 primary breast tumors reveals significant overlap between basal breast cancers and neoplasms expressing a hypermethylation signature. Unsupervised cluster analysis of microarray expression data from the UNC microarray database for nine indicator genes. Cluster analysis performed by Dr. Wendell Jones (Expression Analysis, Durham, NC). Red indicates high level expression, green designates low level expression, and black indicates normal expression levels for genes of interest. Cluster B represents the putative hypermethylator cluster and exhibits concurrent downregulation of the selected genes. Tumors in cluster A are predominately of the luminal A and luminal B subtypes, tumors in cluster B are predominately of the basal subtype, while tumors in cluster C are predominately of the Her2+ subtype.


CDH1
ESR1
SCNN1A
CST6
CEACAM6
TFF3
GNA11
MYB
MUC1
Cluster A
Cluster B
Cluster C

with one basal and two Her2+ tumors also within this cluster. Cluster C (n=19) is composed primarily of Her2+ breast tumors (16/19, 84%), but also contains 2 basal and 1 luminal B breast tumor(s) (Figure 16). The results of this unsupervised cluster analysis led to development of a rule that was used to classify breast cancers based upon their gene expression patterns in subsequent analyses of microarray datasets. This rule classifies a tumor as a hypermethylator if it expresses at least seven of the nine indicator genes at levels less than the median for the dataset. When this rule is applied to the training set, it captures 25/90 (28%) tumors as hypermethylators, a group which includes the original 21 hypermethylator tumors.

When the rule was applied to an expanded UNC dataset containing 272 tumors, a similar pattern was detected, demonstrating significant overlap between tumors expressing a hypermethylation signature and tumors of the basal subtype (Figure 17). The 272 breast cancers in this analysis reflect the following molecular classification: 103/272 (38%) tumors are of the basal subtype, 68/272 (25%) are luminal A, 49/272 (18%) are luminal B, 37/272 (14%) are Her2+, and 15/272 (6%) are classified as claudin-low. In this expanded analysis, a large group of hypermethylator tumors is discernable (Figure 17). This group of hypermethylator tumors is composed of 80 (29%) tumors that express a hypermethylation signature (expression scores lower than the mean for at least 7 of 9 indicator genes). Strikingly, 81% (65/80) of these putative hypermethylator tumors are of the basal subtype, and this putative hypermethylator group contains 63% (65/103) of all basal-like tumors in the dataset (Table 10). Fifteen non-basal tumors were classified as hypermethylators, including, 1/80 (1%) of luminal B tumors group with the hypermethylators, as do 1/80 (1%) Her2+

Figure 17. Microarray analysis of 272 primary breast tumors suggests a linkage between basal breast tumors and the hypermethylator phenotype. Supervised analysis of microarray expression data from the UNC microarray database for nine indicator genes. Gene expression patterns for individual tumors were analyzed to determine the number of genes that were expressed at a level less than the median for the dataset. Tumors with ≥7 genes below the median expression level were classified as hypermethylators. Microarray data mining analysis was performed by Dr. Wendell Jones (Expression Analysis, Durham, NC). Tumors with no genes underexpressed are shown on the left and those with 9 genes underexpressed are shown on right. Red indicates high level expression, green designates low level expression, and black indicates normal expression levels for the genes of interest. The original training set of 91 primary breast tumors was excluded from this set. The hypermethylator cluster is magnified. This cluster exhibits concurrent downregulation of genes indicative of the hypermethylator phenotype and is predominately composed of basal-like tumors.

Hypermethylator Cluster

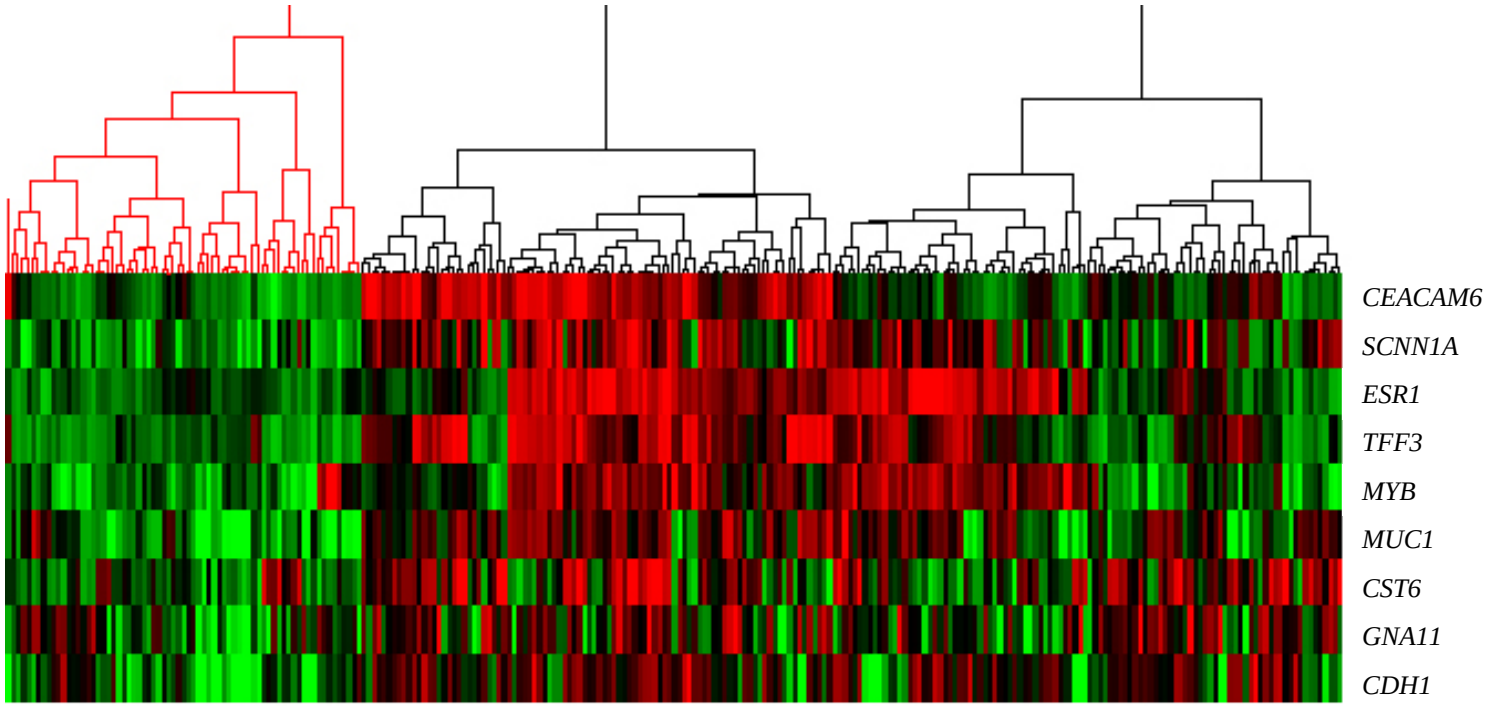


Table 10. Microarray analysis of primary breast tumors

Dataset[1]	Number of Tumors	Total % HM[2]	Composition of HM[3]	% of basals that are HM
Expanded UNC	272	80/272 (29%)	65/80 (81%) basal 1/80 (1%) LB 1/80 (1%) Her2+ 13/80 (16%) CL	65/103 (63%)
Hess *et al.*	133	33/133 (25%)	26/33 (79%) basal 4/33 (12%) Her2+ 2/33 (6%) LA/B 1/33 (3%) NL	26/32 (81%)
Wang *et al.*	295	59/295 (20%)	44/59 (75%) basal 12/59 (20%) LA/B 3/59 (5%) Her2+	44/76 (58%)
Van de Vijver *et al.*	246	48/246 (20%)	39/48 (81%) basal 7/48 (15%) LA/B 2/48 (4%) Her2+	39/66 (59%)
Total	946	220/946 (23%)	174/220 (79%) basal 22/220 (10%) LA/B 6/220 (3%) Her2+ 13/220 (6%) CL 1/220 (0.4%) NL	174/277 (63%)

[1] References for the datasets as follow: UNC microarray dataset (which does not contain the 91 training set) from (34, 37, 251, 252); the Hess *et al.* dataset (253), Wang *et al.* dataset (254); and the van de Vijver *et al.* dataset (255).

[2] HM = hypermethylators.

[3] Subtypes abbreviated as follows: luminal A (LA), luminal B (LB), claudin-low (CL), normal-like (NL).

tumors, and 13/80 (16%) claudin-low tumors.

A second microarray dataset containing 133 primary breast tumors was obtained from Hess *et al*. (253) and subjected to tumor sorting using the hypermethylator rule in order to determine if the relationship between the hypermethylator signature and basal breast tumors was consistent among various datasets. Tumors in this dataset included 30/133 (23%) luminal A, 39/133 (29%) luminal B, 32/133 (24%) basal, 20/133 (15%) Her2+, and 12/133 (9%) normal-like primary breast tumors. Of these 133 tumors, 33 (25%) exhibited a hypermethylator gene expression signature characterized by lower than median expression of at least 7 indicator genes (Figure 18, Table 10). Of these 33 hypermethylator tumors, 26 (79%) were basal-like, 4/33 (12%) Her2+, 1/33 (3%) luminal A, 1/33 (3%) luminal B, and 1/33 (3%) were normal-like (Figure 18). Consistent with the results obtained with the UNC dataset, the vast majority (26/32, 81%) of basal tumors within this dataset exhibit the hypermethylator signature (Table 10).

A third microarray dataset composed of 295 primary breast tumors (254) was analyzed to identify tumors that express the hypermethylator signature. This dataset consists of: 171/295 (58%) luminal A/B, 76/295 (26%) basal, 36/295 (12%) Her2+, and 12/295 (4%) normal-like tumors. A subset of tumors were classified hypermethylators (Figure 19), reflecting 59/295 (20%) tumors contained in the dataset (Table 10). Of these hypermethylator breast tumors, the majority are of the basal subtype: 44/59 (75%) of hypermethylator tumors are basal, while 12/59 (20%) are luminal A/B and 3/59 (5%) are of the Her2+ subtype. Furthermore, the majority of basal tumors (44/76, 59%) contained in this dataset were classified as hypermethylators (Table 10).

Figure 18. Microarray analysis of 133 primary breast tumors suggests a linkage between basal breast tumors and the hypermethylator phenotype. Supervised analysis of microarray expression data from Hess *et al.* (253) for nine indicator genes. Gene expression patterns for individual tumors were analyzed to determine the number of genes that were expressed at a level less than the median for the dataset. Tumors with ≥7 genes below the median expression level were classified as hypermethylators. Microarray data mining analysis was performed by Dr. Wendell Jones (Expression Analysis, Durham, NC). Tumors were sorted in accordance with the hypermethylator rule developed by the UNC database analysis. Red indicates high level expression, green designates low level expression, and black indicates normal expression levels for genes of interest.

Hypermethylator Cluster

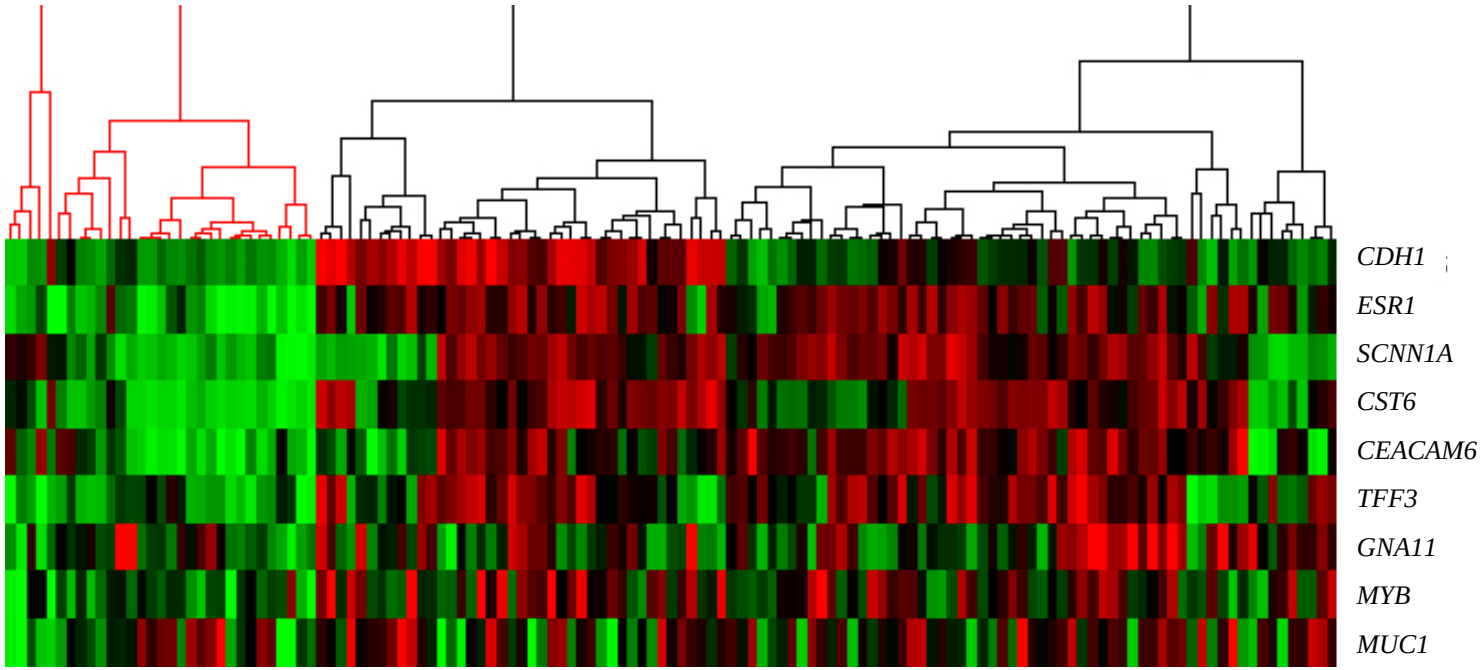


Figure 19. Microarray analysis of 295 primary breast tumors suggests a linkage between basal breast tumors and the hypermethylator phenotype. Analysis of microarray expression data from Wang *et al.* (254) for nine indicator genes. Gene expression patterns for individual tumors were analyzed to determine the number of genes that were expressed at a level less than the median for the dataset. Tumors with ≥7 genes below the median expression level were classified as hypermethylators. Microarray data mining analysis was performed by Dr. Wendell Jones (Expression Analysis, Durham, NC). Tumors were sorted in accordance with the hypermethylator rule developed by the UNC database analysis. Red indicates high level expression, green designates low level expression, and black indicates normal expression levels for genes of interest. The hypermethylator cluster is magnified. This cluster exhibits concurrent downregulation of genes indicative of the hypermethylator phenotype and is composed predominately of basal-like tumors.

Hypermethylator Cluster

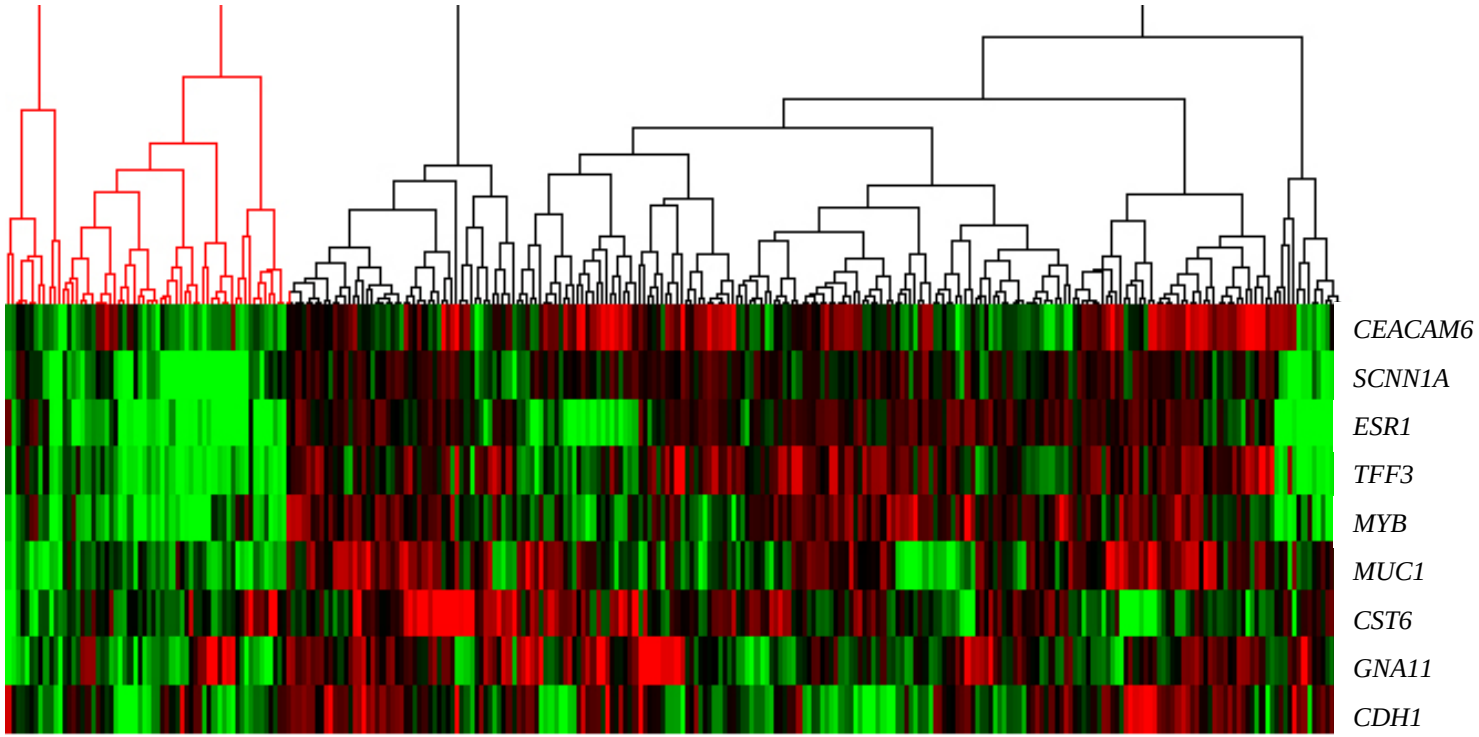
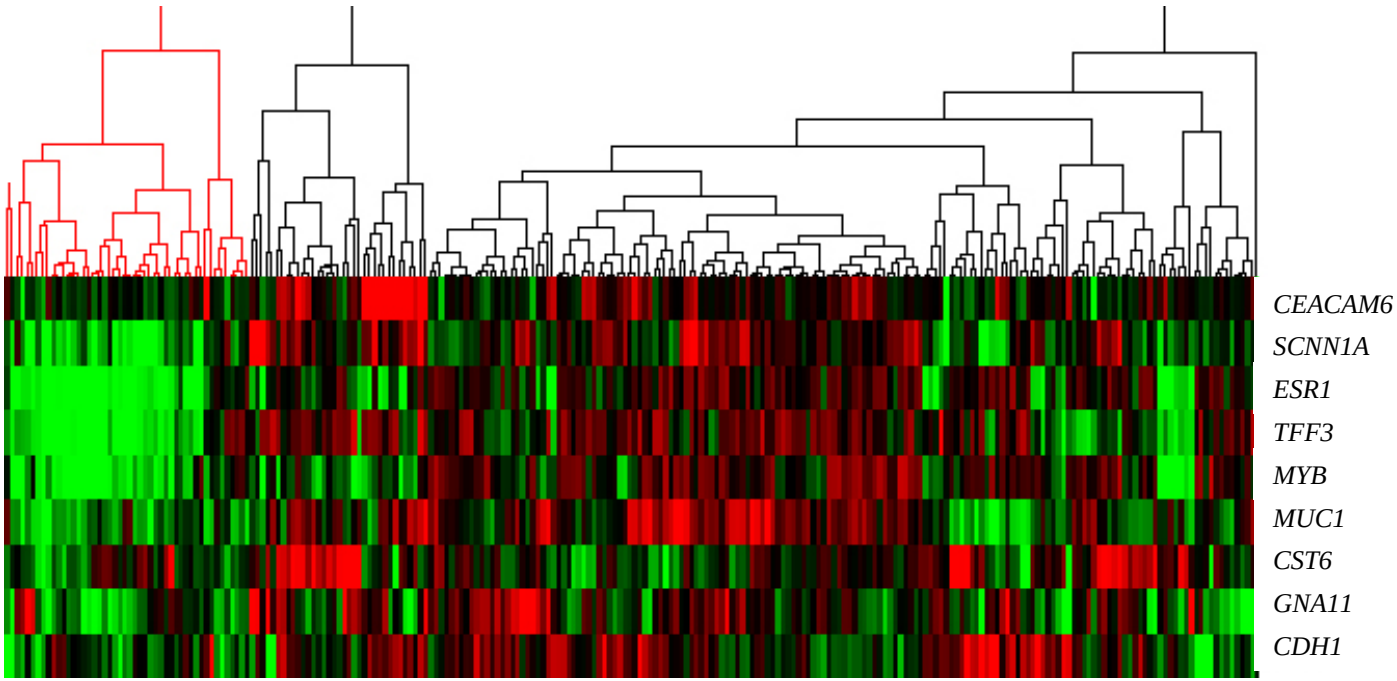


Figure 20. Microarray analysis of 246 primary breast tumors suggests a linkage between basal breast tumors and the hypermethylator phenotype. Supervised analysis of microarray expression data from van de Vijver *et al.* (255) for nine indicator genes. Gene expression patterns for individual tumors were analyzed to determine the number of genes that were expressed at a level less than the median for the dataset. Tumors with ≥7 genes below the median expression level were classified as hypermethylators. Microarray data mining analysis was performed by Dr. Wendell Jones (Expression Analysis, Durham, NC). Tumors were sorted in accordance with the hypermethylator rule developed by the UNC database analysis. Red indicates high level expression, green designates low level expression, and black indicates normal expression levels for genes of interest. The hypermethylator cluster is magnified.

Hypermethylator Cluster



A fourth microarray dataset, consisting of 246 primary breast tumors (255), was mined to identify hypermethylator tumors. The breast tumors in this dataset include in this dataset were: 143/246 (58%) luminal A/B, 66/246 (27%) basal, and 37/246 (15%) Her2+. Application of the rule revealed that a subset of tumors 48/246 (20%) from this dataset classify as hypermethylators (Figure 20). Of these hypermethylator tumors, the majority are of the basal subtype: 39/48 (81%) are basal, while 7/48 (15%) are luminal A/B, and 2/48 (4%) are of the Her2+ subtype (Table 10). Once again, the majority of basal tumors (39/66, 59%) within the dataset were classified as hypermethylators, suggesting that a significant majority of basal breast cancers can be expected to exhibit the hypermethylation signature.

In total, microarray data from 942 primary breast tumors was analyzed to determine if hypermethylator breast tumors occurred *in vivo*. We found that 220/946 (23%) of all tumors examined displayed a hypermethylator signature (defined as having 7 or more indicator genes with expression levels below the median) (Table 10). Of hypermethylator tumors, the majority 174/220 (79%) were basal, while 22/220 (10%) were luminal A/B, 6/220 (3%) were Her2+, 13/220 (6%) were claudin-low, and 1/220 (0.4%) were normal-like (Table 10). This finding suggests a large degree of correspondence between basal-like tumors and hypermethylator breast tumors. In fact, of all basal tumors examined, 174/277 (63%) were also hypermethylators.

Immunohistochemical Analysis of Primary Human Breast Tumors

Two tissue microarrays comprised of 137 tumors with known ER, PR, and Her2 status were analyzed for CDH1, CEACAM6, CST6, and SCNN1A protein expression. Of these primary breast tumors, 96 were classified as luminal A (ER+/PR+/Her2-), 23 were basal-like (ER-/PR-/Her2-), and 18 were Her2+ (ER-/PR-/Her2+). In general, luminal A and

Her2+ tumors retained expressed of a majority of these four proteins at normal levels, while basal tumors were more likely to have decreased expression of one or more of these genes (Figure 21). Of the basal-like tumors, 21/23 (91%) lost normal protein expression of at least two protein products, with 11/23 (48%) of basal-like tumors exhibiting diminished or lost expression of 3-4 of these proteins (Figure 22). In contrast, only 1/18 (6%) Her2+ and 27/96 (28%) luminal A tumors lost expression of 3-4 protein products (Figure 22). Average expression scores were calculated for each protein of interest for each of the three groups: basal, Her2+, and luminal A (Figure 23). This revealed that CEACAM6 is the protein product most often lost by basal-like tumors, with an average expression score of 0.83 compared to 1.61 for Her2+ and 1.80 for luminal tumors. In fact, tumors of the basal-like subtype had a trend toward lower expression scores, on average, for each protein compared to either Her2+ or luminal A breast tumors (Figure 23).

METHYLATION ANALYSIS OF A SUBSET OF BREAST CANCER CELL LINES AND PRIMARY HUMAN BREAST TUMORS

Methylation Analysis of Breast Cancer Cell Lines

To confirm that lack of gene expression of known methylation-sensitive genes among breast cancer cell lines reflects true methylation-dependent epigenetic silencing, a number of methods were employed to assess gene promoter methylation: (i) methylation-specific PCR (MSP), (ii) bisulfite sequencing, and (iii) response to 5-aza-2'-deoxycytidine (5-aza) treatment. MSP analysis of nine genes (*CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*) that are differentially expressed between hypermethylator and low-frequency methylator cell lines revealed differences in the methylation status of specific

Figure 21. Basal breast tumors lack expression of protein products of select methylation-sensitive genes of interest, but show increased DNMT3b expression. Representative immunohistochemistry for five protein products of interest (CEACAM6, CDH1, CST6, SCNN1A, and DNMT3b) in tumors of the basal, Her2+, and luminal A subtypes at 20X magnification. Positive control staining is represented by cytokeratin 18 (CK18).

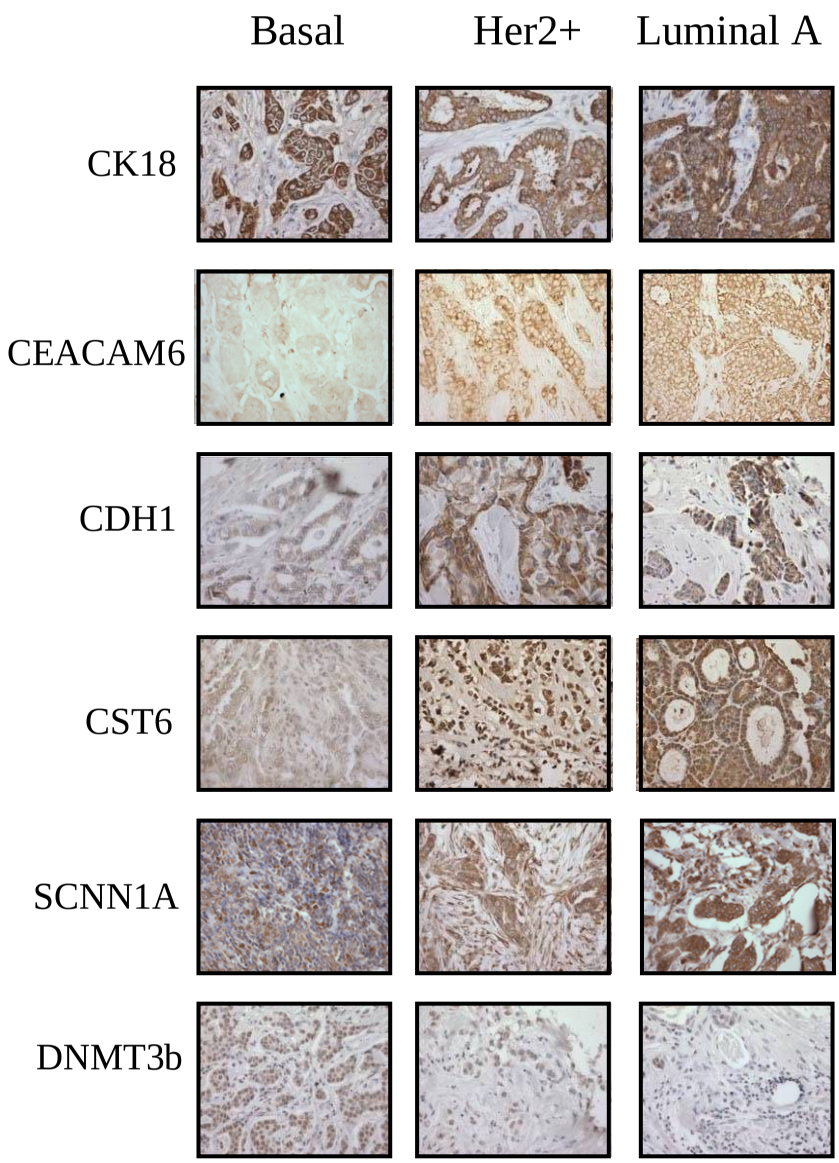

Basal
Her2+
Luminal A
CK18
CEACAM6
CDH1
CST6
SCNN1A
DNMT3b

Figure 22. Loss of protein expression corresponding to methylation-sensitive genes in primary breast tumors. Percentage of tumors of each subtype lacking expression of gene products corresponding to 4/4 genes of interest (*CEACAM6, CDH1, CST6,* and *SCNN1A*), 3/4, 2/4, 1/4, or 0/4 genes, respectively. The results for basal tumors are shown in red, Her2+ tumors are shown in green, and luminal A tumors are shown in blue.

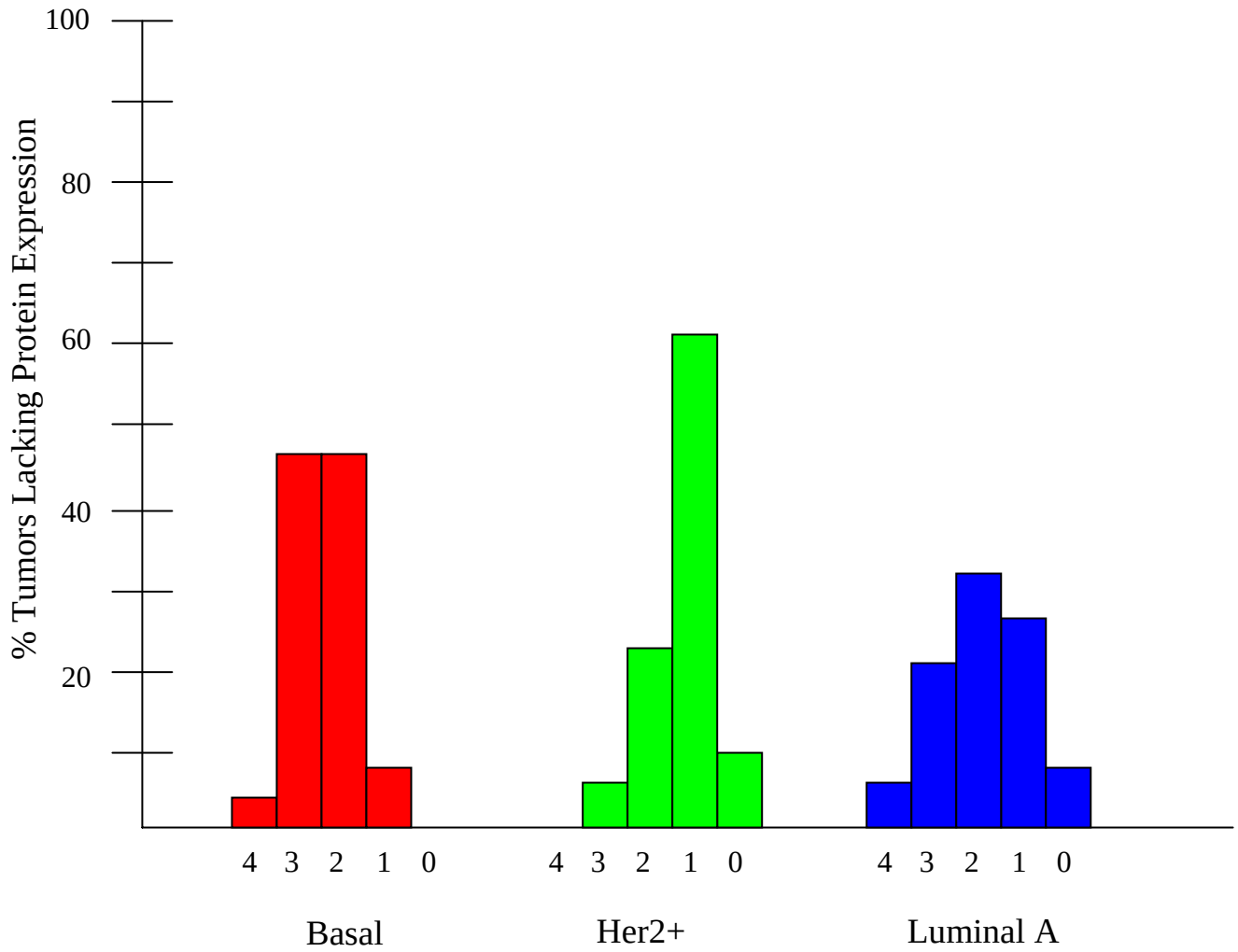
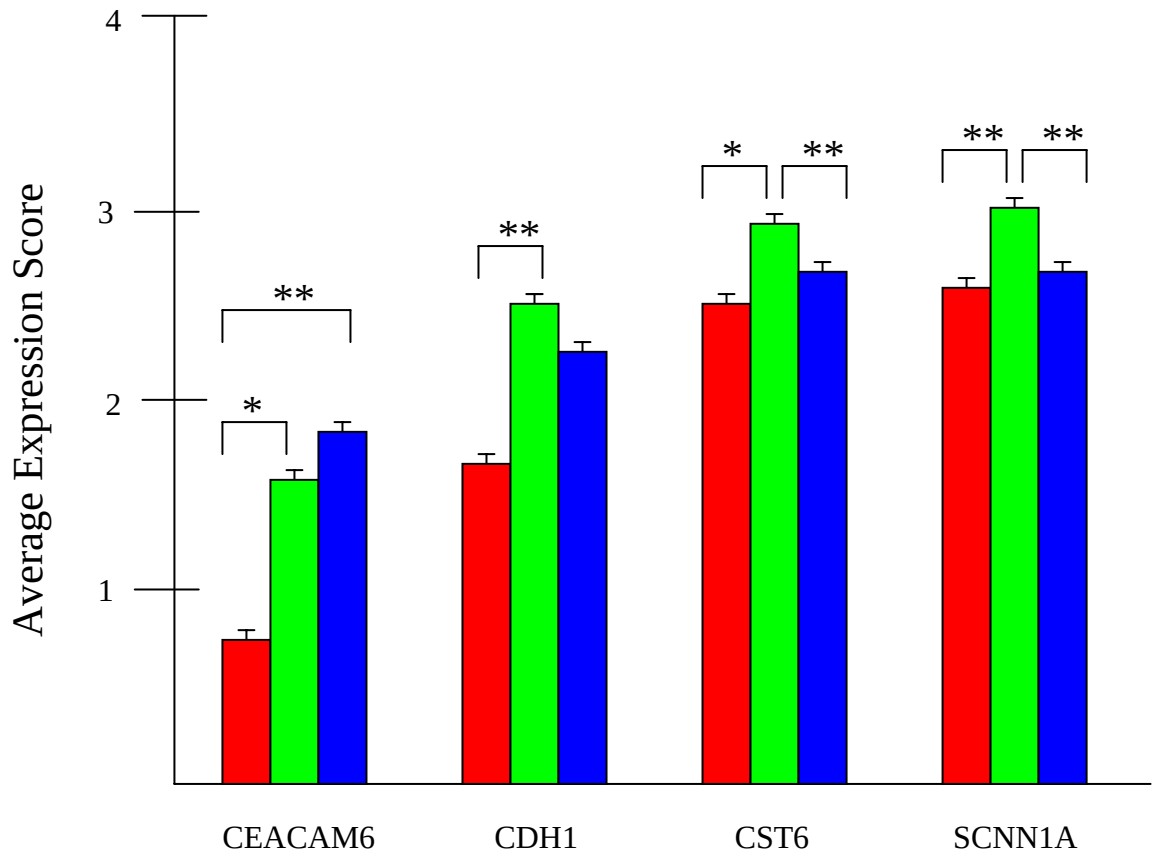

% Tumors Lacking Protein Expression
100
80
60
40
20
4 3 2 1 0
4 3 2 1 0
4 3 2 1 0
Basal
Her2+
Luminal A

Figure 23. Expression of the protein products corresponding to select methylation-sensitive genes of interest vary by tumor type. Protein expression score is based on immunohistochemical analysis of primary human breast tumors of various subtypes: 23 basal tumors (shown in red bars), 18 Her2+ tumors (green bars), and 96 luminal A tumors (blue bars). Protein expression was scored on a discrete scale with 4 = high expression, 3 = average level expression, 2 = low expression, 1 = weak but detectable expression, and 0 = no detectable expression. Statistical significance is indicated by asterisk, where * corresponds to $p<0.05$ and ** corresponds to $p<0.009$.


Average Expression Score
4
3
2
1
*
**
**
*
**
**
**
CEACAM6
CDH1
CST6
SCNN1A

CpGs within regulatory regions of each gene promoter, in accordance with the methylator status of given cell line. The relationship between gene promoter methylation (as assessed by MSP) and loss of gene expression is strong across all hypermethylator cell lines for the genes examined. For example, the hypermethylator cell lines express *SCNN1A* at undetectable or diminished levels (Figure 15), and MSP analysis of this gene revealed that 5/6 (83%) of these cell lines produce only a methylated MSP product, while MSP analysis of *SCNN1A* in MDA-MB-231 cells produced both unmethylated and methylated products. Conversely, all of the low-frequency methylator cell lines (of which 5/6, 83% express *SCNN1A* at normal levels) produced an unmethylated *SCNN1A* MSP product, and only two of these cell lines (BT20 and MDA-MB-468) produced a detectable methylated MSP product (Figure 24). Methylated MSP products were detected for at least 55% (5/9) of the genes examined in each of the hypermethylator cell lines, with 4/6 hypermethylator cell lines exhibiting detectable methylation at 7 or more of the 9 genes. In contrast, unmethylated MSP products were detected for at least 89% (8/9) of the genes examined in each of the low-frequency methylator cell lines, with 67% (4/6) of low-frequency methylator cell lines exhibiting unmethylated products for each of the genes examined (Figure 24).

Selected MSP products were sequenced to examine the methylation status of a greater number of CpGs within regulatory regions of selected genes of interest and to evaluate promoter methylation for genes that produced both unmethylated and methylated MSP products in some of the cell lines examined. The results of the bisulfite sequencing analysis supports a direct association between gene promoter methylation and gene expression status in this panel of methylation-sensitive genes (Figure 25). For example, hypermethylator cell

Figure 24. Hypermethylator cell lines exhibit high levels of gene-specific methylation. MSP results for 16 breast cancer cell lines and MCF12A for each of nine genes of interest. Red boxes indicate the presence of a methylated MSP product (even in cases where both methylated and unmethylated products were present), white boxes indicate the presence of an unmethylated MSP product only. Hs578T cells are abbreviated as Hs, ZR-75-1 cells as ZR, and SKBR3 cells as SK.

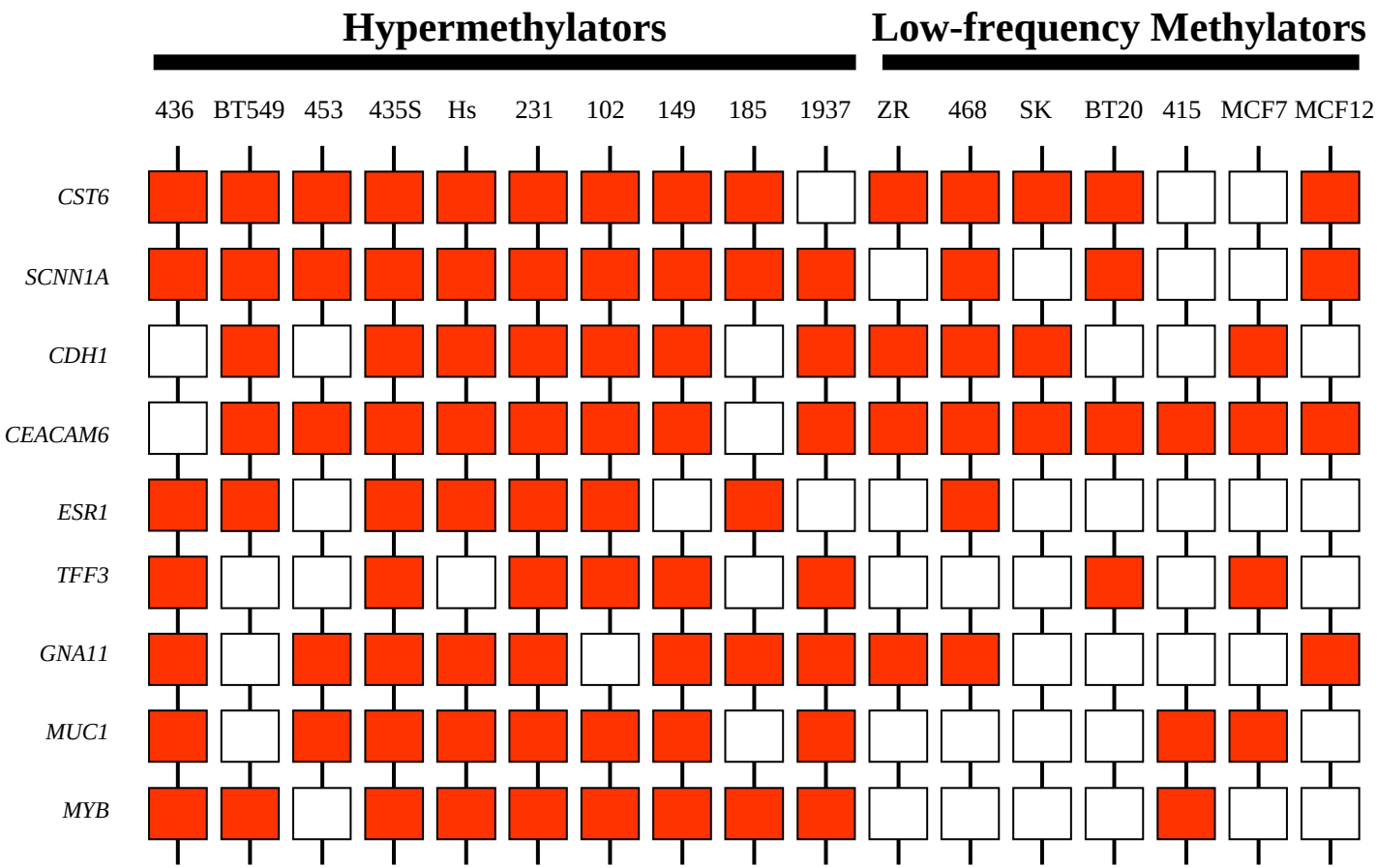
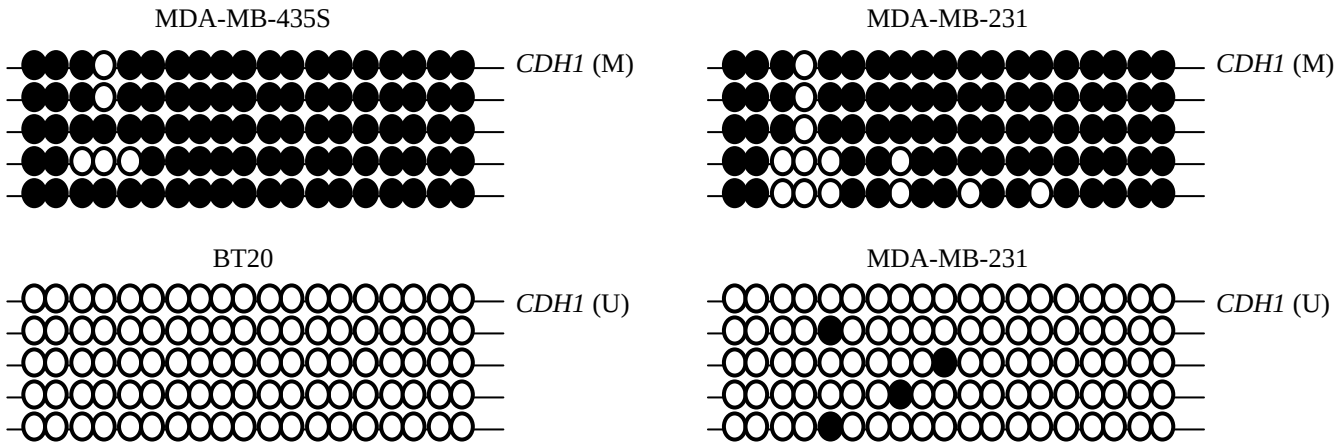

Hypermethylators
Low-frequency Methylators
436
BT549
453
435S
Hs
231
102
149
185
1937
ZR
468
SK
BT20
415
MCF7
MCF12
CST6
SCNN1A
CDH1
CEACAM6
ESR1
TFF3
GNA11
MUC1
MYB

Figure 25. *CDH1* is differentially methylated between hypermethylator and low-frequency methylator cell lines. Summary of bisulfite sequencing for breast cancer cell lines of MSP products for *CDH1*. Methylated CpGs are designated by closed circles, unmethylated CpGs are designated by open circles for MDA-MB-435S, BT20, and MDA-MB-231 cell lines (5 replicates each). M = methylated MSP product, U = unmethylated MSP product.


MDA-MB-435S
CDH1 (M)
MDA-MB-231
CDH1 (M)
BT20
CDH1 (U)
MDA-MB-231
CDH1 (U)

line MDA-MB-435S lacks detectable expression of *CDH1* (Figure 15) and MSP shows that the *CDH1* promoter is methylated (Figure 24). Bisulfite sequencing of the intervening CpGs within the MSP product demonstrated that the majority of CpGs in this region of the *CDH1* promoter are methylated (TMI = 95%) (Figure 25). Sequencing of the same region of the *CDH1* promoter in low-frequency methylator BT20 cells (which express *CDH1*) revealed that all 19 CpGs are unmethylated (TMI = 0%). Additionally, bisulfite sequencing of the *CDH1* promoter in hypermethylator MDA-MB-231 cells (which display low level expression of *CDH1* and exhibit both a methylated and unmethylated *CDH1* MSP product) revealed that the methylated PCR product is highly methylated (TMI = 84%), while the unmethylated PCR product is sparsely methylated (TMI = 4%) (Figure 25). One explanation for instances where MSP analysis for a cell line produces both a methylated and an unmethylated product is that these cells possess one unmethylated and one methylated allele.

Six hypermethylator cell lines (BT549, HS578T, MDA-MB-231, MDA-MB-435S, MDA-MB-436, and MDA-MB-453) were treated with the demethylating agent 5-aza-2'-deoxycytidine (5-aza), and changes in methylation and expression patterns for five genes (*CEACAM6, CDH1, CST6, ESR1, SCNN1A*) were examined. Representative RT-PCR results are shown in Figure 26. Whereas these genes are not expressed in the majority of hypermethylator cell lines (Figure 15), treatment with 5-aza results in robust expression in each case (Figure 26). Bisulfite sequencing of *CDH1* and *ESR1* confirmed that promoter demethylation following 5-aza treatment coincided with gene expression for these genes (Figures 27-28). For example, specific CpGs within the promoter region of the *ESR1* gene are 75% methylated at control levels which decreases to 42% methylation during the three

Figure 26. Hypermethylator cell lines are 5-aza responsive. Representative agarose gels of RT-PCR products for *CST6, SCNN1A, CDH1, CEACAM6,* and *ESR1* demonstrating 5-aza induction of gene expression in hypermethylator cell lines. RT-PCR results using cDNA template from untreated (-) and 5-aza treated (+) are shown.

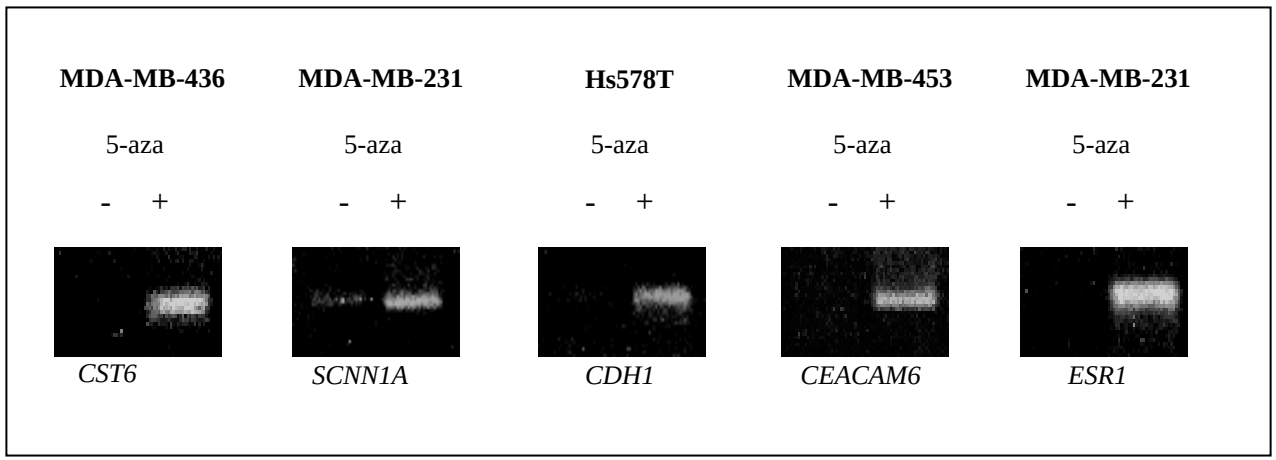

MDA-MB-436
MDA-MB-231
Hs578T
MDA-MB-453
MDA-MB-231
5-aza
5-aza
5-aza
5-aza
5-aza
- +
- +
- +
- +
- +
CST6
SCNN1A
CDH1
CEACAM6
ESR1

Figure 27. Methylation analysis of *CDH1* in Hs578T cells. A 435 bp segment (containing 22 CpGs) within the CpG island proximal to the *CDH1* promoter was selected for bisulfite analysis in hypermethylator cell line Hs578T. The majority of the demethylation / remethylation events in response to the demethylating agent 5-aza-2'-deoxycytidine occur within a cluster of three CpGs (shown center). Red balls designate significantly methylated CpGs (>75%), blue balls designate significantly unmethylated CpGs (<25%), and yellow balls designated partially methylated CpGs (50%). Results shown are averages of 5-8 replicates for each condition.

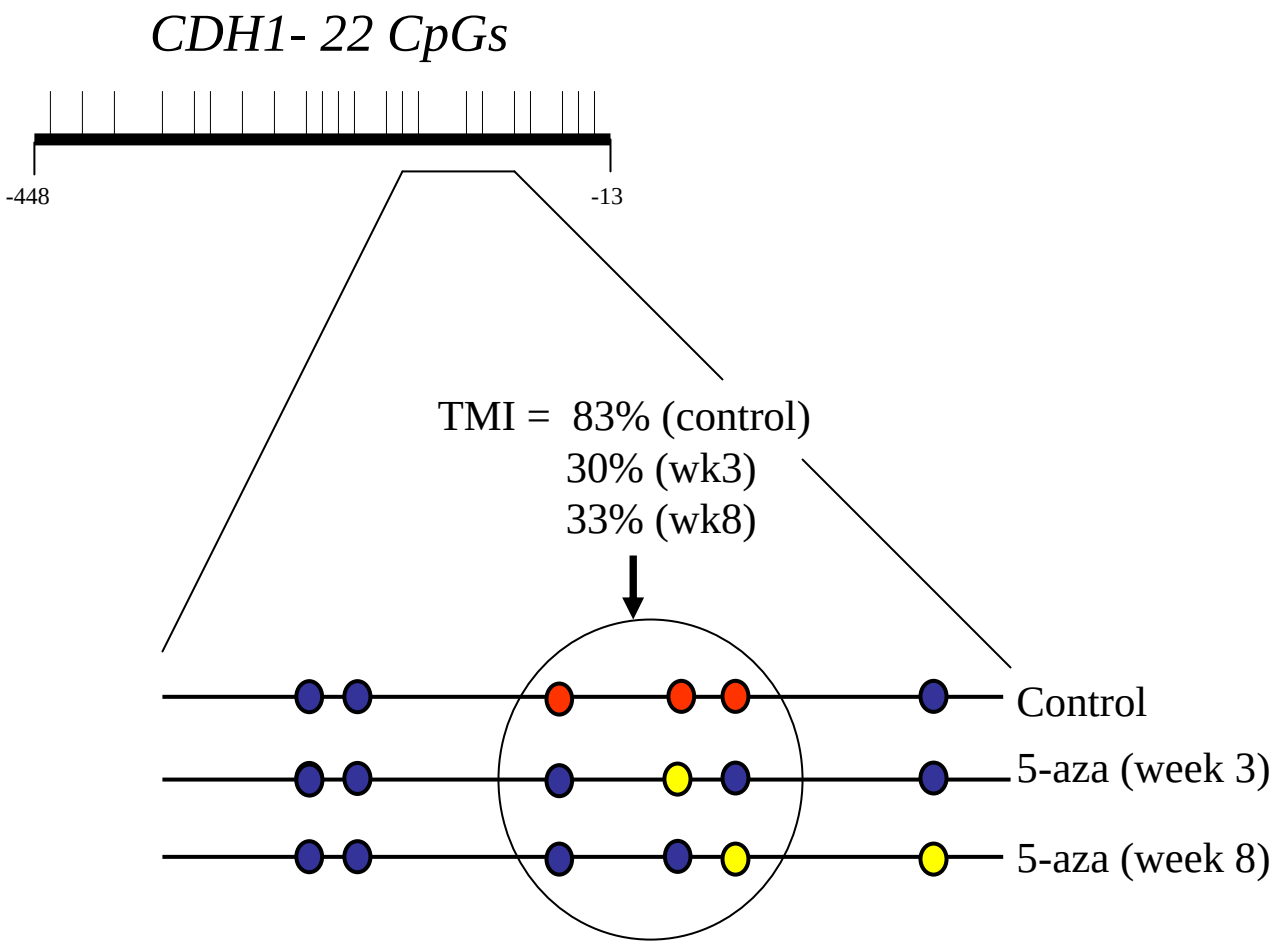
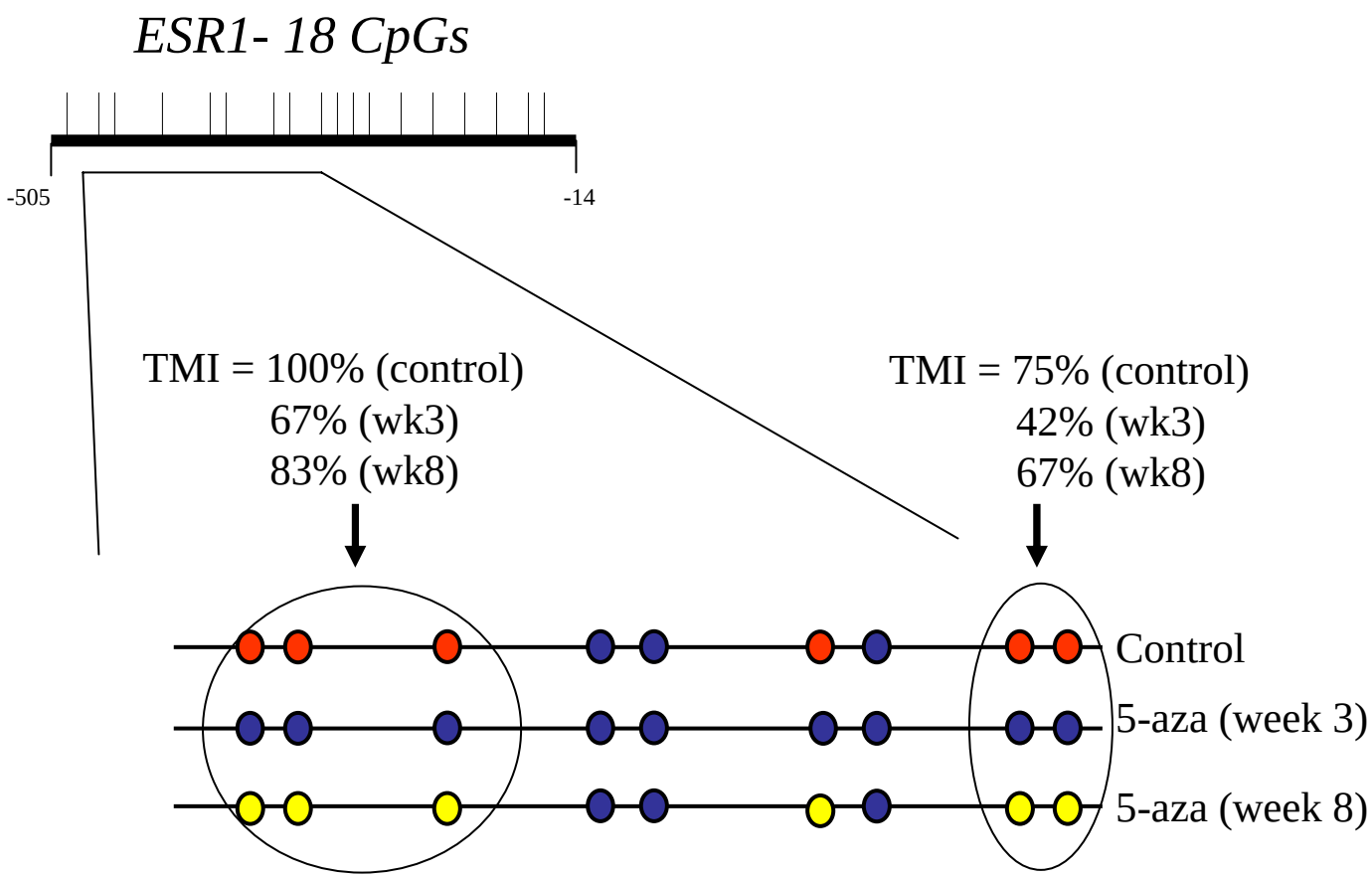

CDH1- 22 CpGs
-448
-13
TMI = 83% (control)
30% (wk3)
33% (wk8)
Control
5-aza (week 3)
5-aza (week 8)

Figure 28. Methylation analysis of *ESR1* in MDA-MB-435s cells. A 491 bp segment (containing 18 CpGs) within the CpG island proximal to the *ESR1* promoter was selected for bisulfite analysis in the hypermethylator cell line MDA-MB-435s. The majority of the demethylation / remethylation events in response to the demethylating agent 5-aza-2'-deoxycytidine occur within two clusters of three and two CpGs, respectively (shown center). Red balls designate significantly methylated CpGs (>75%), blue balls designate significantly unmethylated CpGs (<25%), and yellow balls designated partially methylated CpGs (50%). Results shown are averages of 5-8 replicates for each condition.


ESR1- 18 CpGs
-505
-14
TMI = 100% (control)
67% (wk3)
83% (wk8)
TMI = 75% (control)
42% (wk3)
67% (wk8)
Control
5-aza (week 3)
5-aza (week 8)

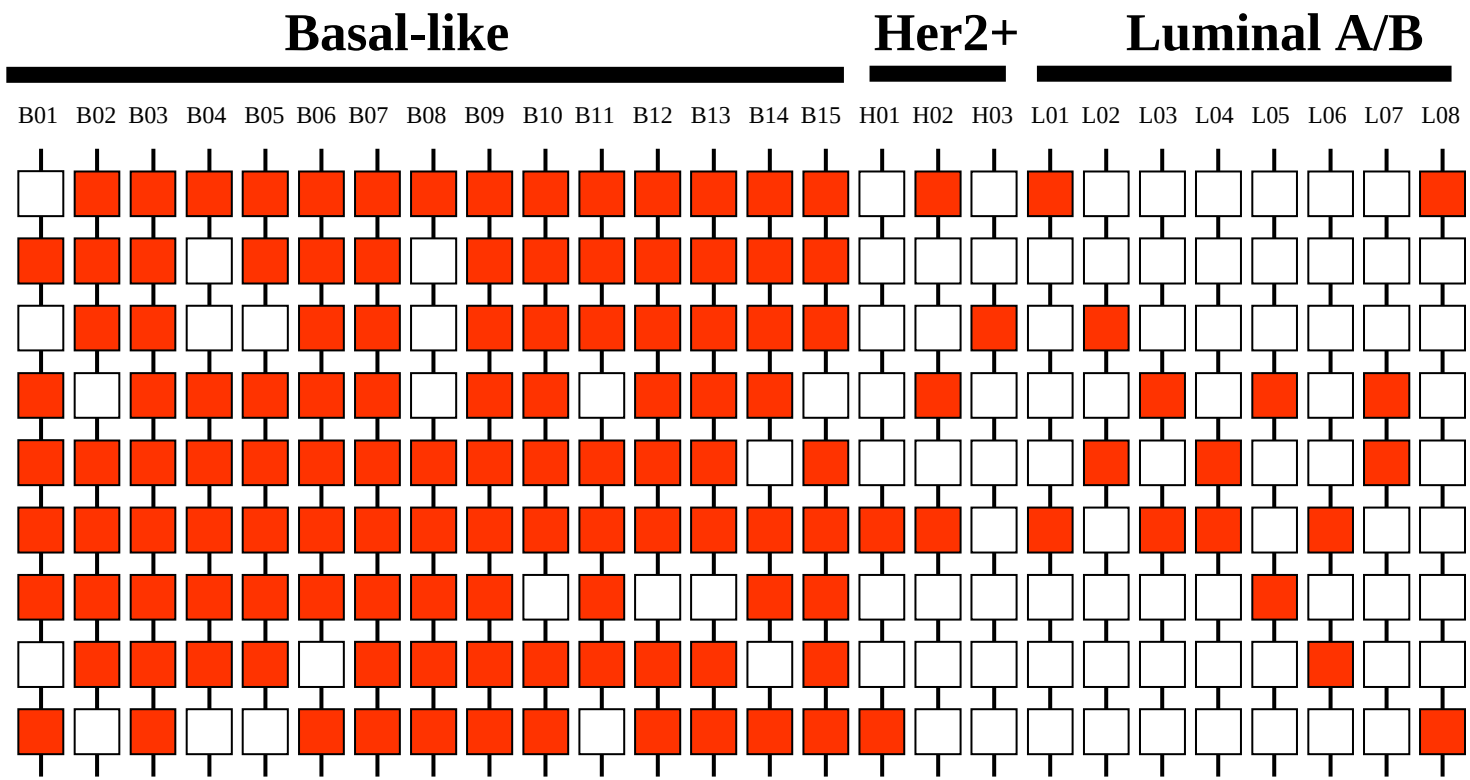
week 5-aza treatment period, indicating effective demethylation. Additionally this same region begins to remethylate after treatment is withdrawn, resulting in a TMI of 67% by week 8 (Figure 28).

Methylation Analysis of Primary Human Breast Tumors

Twenty-six primary breast tumors (15 basal-like, 8 luminal A/B, and 3 Her2+) were utilized for MSP analysis of the nine genes found to be indicative of a hypermethylator phenotype in the microarray studies (*CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*). MSP analysis of these nine genes that are differentially expressed between hypermethylator and low-frequency methylator tumors revealed differences in the methylation status of specific CpGs within regulatory regions of each gene promoter (Figure 29). Methylated MSP products were detected for at least 67% (6/9) of the genes analyzed in each of the basal breast cancers examined. Among this cohort of basal tumors, 80% (12/15) met the criteria for the hypermethylator tumor category (detectable methylation at 7 or more of the 9 indicator genes). In contrast 0% (0/11) of non-basal tumors were hypermethylators, indicating a strong correlation between the hypermethylator phenotype and basal-like breast tumors (Figure 29). For example, basal tumors B03, B07, and B09 produced methylated MSP products for each of the nine of the genes analyzed (Figure 29). In contrast, unmethylated MSP products were detected for at least 66% (6/9) of the genes examined in each of the luminal A/B and Her2+ tumors, with 91% (10/11) of these non-basal tumors exhibiting only 1-2 methylated products out of the nine examined (Figure 29).

Selected MSP products were sequenced to examine the methylation status of a greater number of CpGs within regulatory regions of selected genes of interest and to evaluate

Figure 29. Basal breast tumors exhibit high levels of concurrent gene-specific methylation of epigenetically-regulated genes of interest. Red boxes indicate presence of a methylated MSP product (even when an unmethylated product was also detected), white boxes indicate presence of an unmethylated MSP product only. Primary breast tumors of the basal-like subtype are designated B01-B15, Her2+ as H01-H03, or luminal A/B as L01-L08. Tumors L01-L06 are luminal A, while tumors L07 and L08 are of the luminal B subtype.


Basal-like
Her2+
Luminal A/B
B01 B02 B03 B04 B05 B06 B07 B08 B09 B10 B11 B12 B13 B14 B15 H01 H02 H03 L01 L02 L03 L04 L05 L06 L07 L08
CST6
SCNN1A
CDH1
CEACAM6
ESR1
TFF3
GNA11
MUC1
MYB

promoter methylation for genes that produced both unmethylated and methylated MSP products. Representative bisulfite sequencing results are shown for *CDH1*, *CST6*, and *GNA11* for six basal-like and three non-basal (one Her2+, one luminal A, and one luminal B) breast tumors (Figures 30-32). Bisulfite sequencing of the 19 CpGs spanning the region 82 bp upstream of the promoter to 129 bp into exon 1 of *CDH1* reveals extensive promoter methylation among basal tumors, with TMIs ranging from 92-97% (Figure 30). In contrast, TMIs for the three non-basal tumors range from a low of 9% to 50%, revealing many fewer methylation events in the promoter region of this gene among these tumors (Figure 30). These six basal-like tumors also exhibit extensive methylation of the promoter region of the *CST6* and *GNA11* genes, while the luminal or Her2+ tumors examined do not (Figures 31-32). Similar results were seen for each of the other six indicator genes (*CEACAM6, ESR1, MUC1, MYB, SCNN1A* and *TFF3*) examined by bisulfite sequencing. For example, the region analyzed in the promoter region of *CEACAM6* included four CpGs and had a TMI of 100% in all six basal tumors examined, and remained completely unmethylated (TMI = 0%) in the luminal A/B and Her2+ tumors subject to bisulfite analysis. The *ESR1* gene was substantially methylated in 6/6 basal tumors (ranging from 71%-100% TMI), but substantially unmethylated in the three non-basal tumors examined (0-29% TMI). In the 5/6 basal tumors where *MUC1* exhibited methylation, sequencing reveals partial methylation, with TMIs ranging from 50-58%. However in the three non-basal tumors, *MUC1* exhibited 0-5% methylation. Similar trends were seen in the rest of the genes examined, with extensive promoter methylation (ranging from 50%-100% TMI) of basal tumors and less frequent methylation (TMIs ranging from 0%-29%) for non-basal tumors breast tumors.

Figure 30. Basal breast tumors exhibit extensive methylation of CpGs within the *CDH1* promoter. Summary of bisulfite sequencing results for the *CDH1* gene in primary breast tumors. 100% methylated CpGs are represented by red balls, CpGs where the majority (>50%) of clones were methylated are represented in yellow, CpG sites where clones were <50% methylated are represented in green, and 100% unmethylated CpG sites are shown in blue. CpG sites that fall within the MSP primers are delineated by the vertical lines. Methylation status is representative of 3-5 sequenced clones. The Total Methylation Index (TMI) for each tumor analyzed was calculated based on intervening CpGs only.

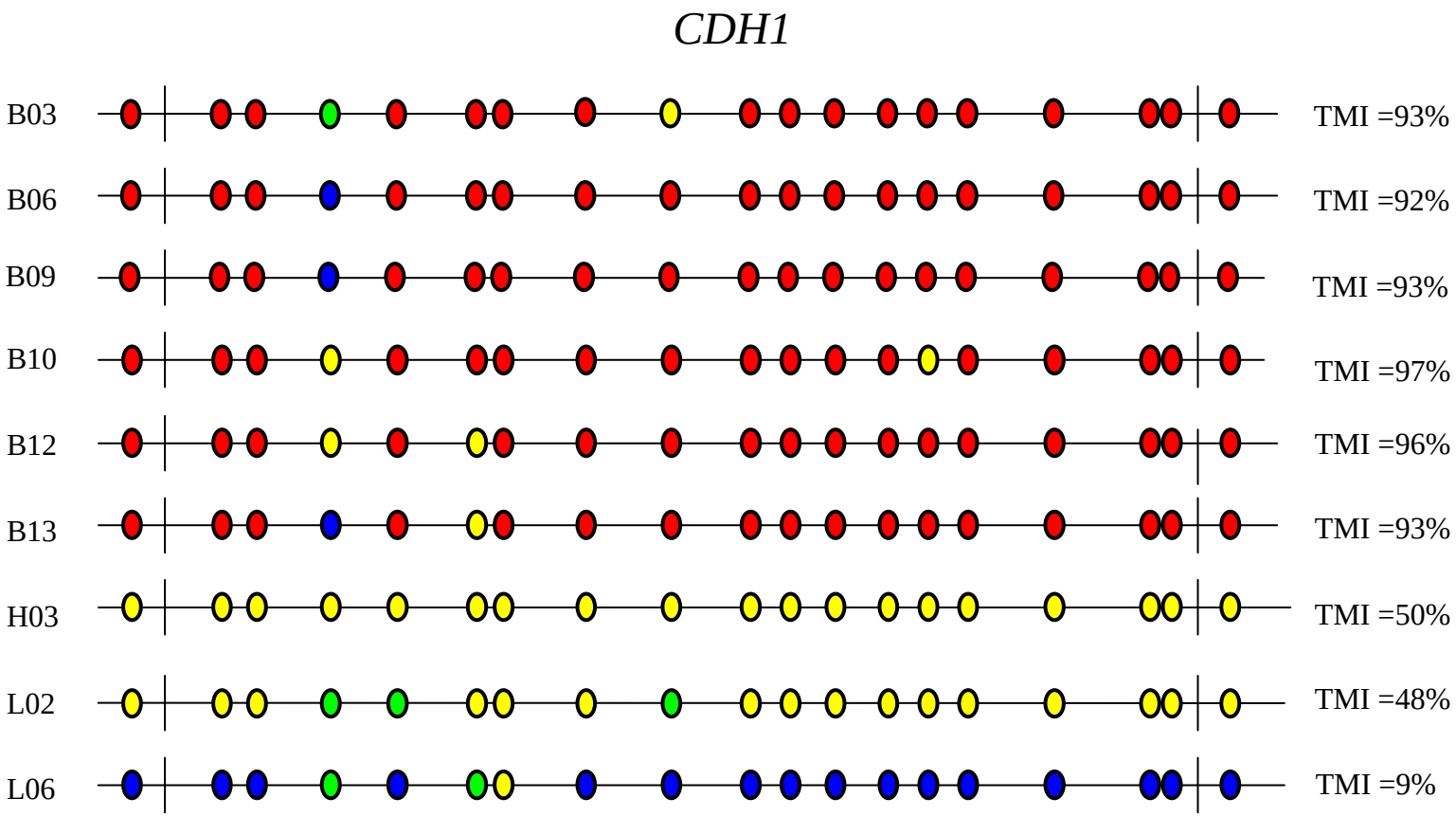

CDH1
B03
TMI =93%
B06
TMI =92%
B09
TMI =93%
B10
TMI =97%
B12
TMI =96%
B13
TMI =93%
H03
TMI =50%
L02
TMI =48%
L06
TMI =9%

Figure 31. Basal breast tumors exhibit extensive methylation of CpGs within the *CST6* promoter. Summary of bisulfite sequencing results for the *CST6* gene in primary breast tumors. 100% methylated CpGs are represented by red balls, CpGs where the majority (>50%) of clones were methylated are represented in yellow, CpG sites where clones were <50% methylated are represented in green, and 100% unmethylated CpG sites are shown in blue. CpG sites that fall within the MSP primers are delineated by the vertical lines. Methylation status is representative of 3-5 sequenced clones. The Total Methylation Index (TMI) for each tumor analyzed was calculated based on intervening CpGs only.

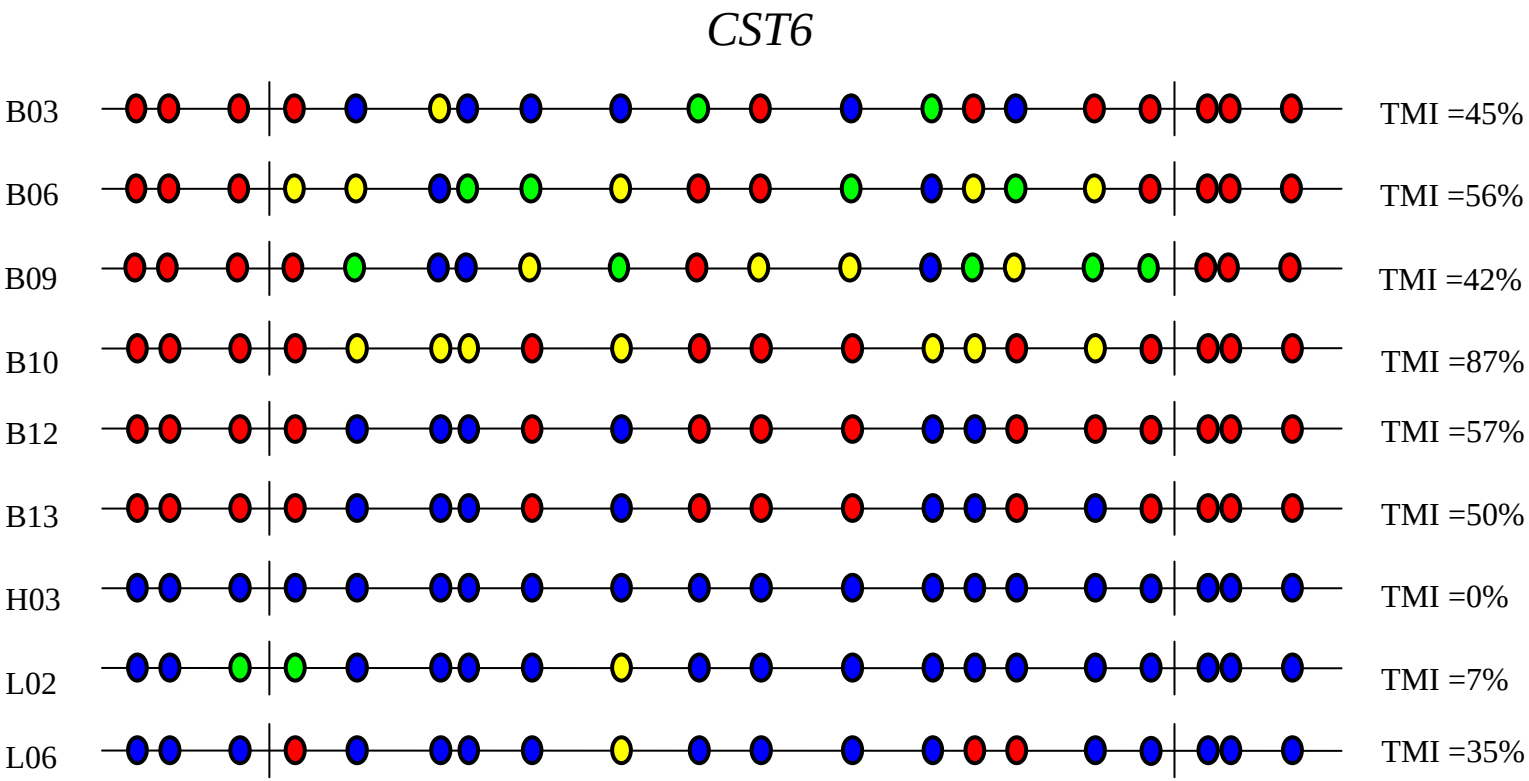
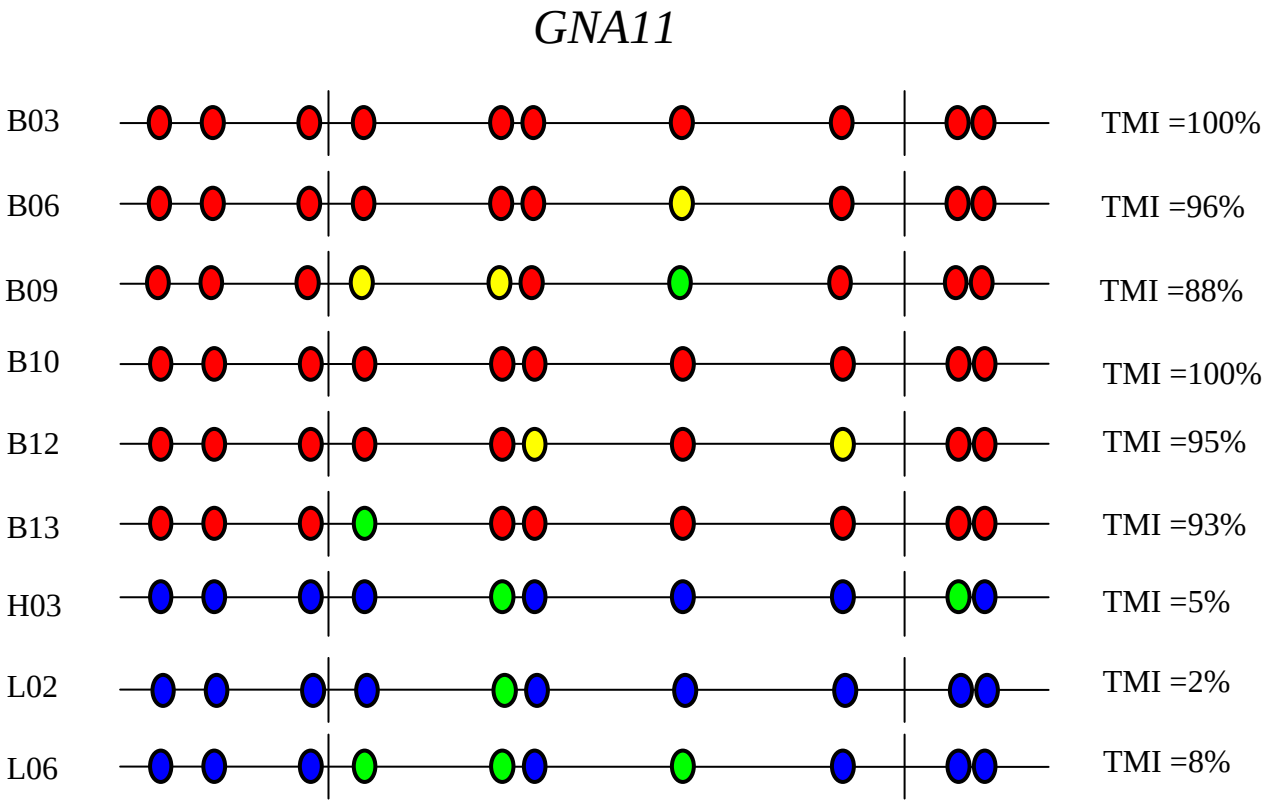

CST6
B03
B06
B09
B10
B12
B13
H03
L02
L06
TMI =45%
TMI =56%
TMI =42%
TMI =87%
TMI =57%
TMI =50%
TMI =0%
TMI =7%
TMI =35%

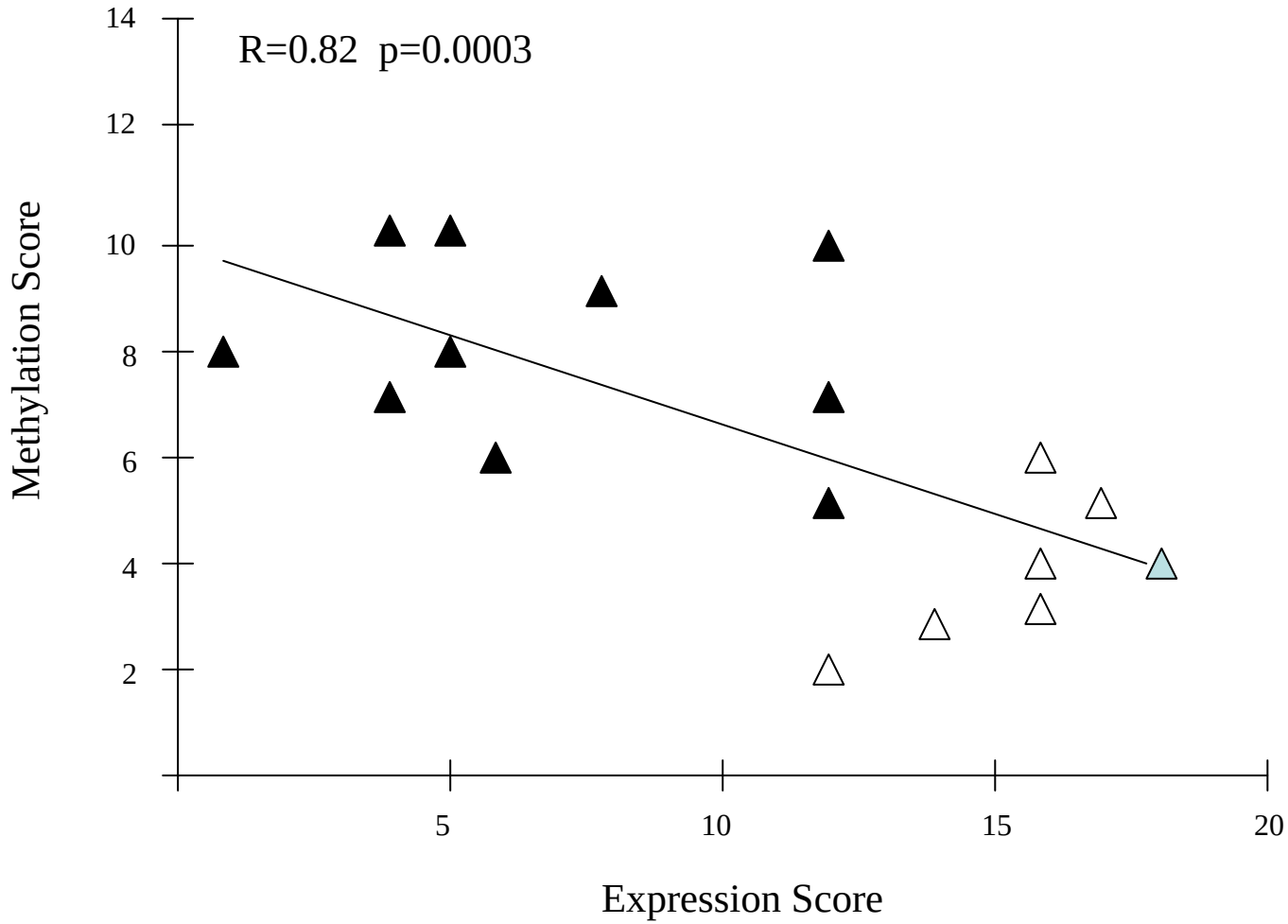
Figure 32. Basal breast tumors exhibit extensive methylation of CpGs within the *GNA11* promoter. Summary of bisulfite sequencing results for the *GNA11* gene in primary breast tumors. 100% methylated CpGs are represented by red balls, CpGs where the majority (>50%) of clones were methylated are represented in yellow, CpG sites where clones were <50% methylated are represented in green, and 100% unmethylated CpG sites are shown in blue. CpG sites that fall within the MSP primers are delineated by the vertical lines. Methylation status is representative of 3-5 sequenced clones. The Total Methylation Index (TMI) for each tumor analyzed was calculated based on intervening CpGs only.


GNA11
B03
B06
B09
B10
B12
B13
H03
L02
L06
TMI =100%
TMI =96%
TMI =88%
TMI =100%
TMI =95%
TMI =93%
TMI =5%
TMI =2%
TMI =8%

Correlation of Gene Expression and Promoter Methylation

To examine the relationship between gene expression status and promoter methylation for each of the nine indicator genes, an expression score and a methylation score were generated for each cell line. These scores reflect the combined relative expression and the combined relative methylation status for these genes of interest (*CEACAM6, CDH1, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*) for a given cell line. A strong inverse correlation (R=0.82, p=0.0003) exists between these two parameters: cell lines with low expression scores tend to have higher methylation scores, and those with high expression scores tend to have low methylation scores (Figure 33). Hypermethylator cell lines exhibit an average expression score of 5.5 $\pm$ 1.5, while low-frequency methylator cell lines exhibit an average expression score of 15.2 $\pm$ 0.8. This difference in average expression scores between the two groups was significant (p=0.0005). Likewise hypermethylator cell lines produced an average methylation score that was significantly higher than that of the low-frequency methylator cell lines (8.2 $\pm$ 0.7 versus 3.8 $\pm$ 0.6, p=0.0007). These results suggest that the loss of gene expression observed in hypermethylator cell lines is a direct consequence of aberrant promoter methylation for the genes of interest. Interestingly, the scores of the basal-like cell lines are similar to those of the hypermethylators, with an average expression score of 9.0 $\pm$ 1.9, and an average methylation score of 7.8 $\pm$ 1.1. When these basal-like cell lines are included in the hypermethylator group, the average expression score is 6.9 $\pm$ 1.2, significantly lower than that of the low-frequency group (p=0.0001). Similarly, when the basal-like cell lines are included in the hypermethylator group, the methylation score becomes 8.0 $\pm$ 0.6, still significantly different from that of the low

Figure 33. Gene expression status correlates with promoter methylation status among breast cancer cell lines. Association between RT-PCR expression and MSP methylation status of nine genes of interest for 16 breast cancer cell lines. Hypermethylator cell lines (black triangles) and low-frequency methylator cell lines (white triangles) demonstrate a statistically significant relationship between gene expression status and promoter methylation status. MCF12A is shown in pale blue. Expression scores are generated based on the number of genes a given cell line expresses at normal levels (via RT-PCR), and methylation scores are calculated based on the MSP results for a given cell in genes of interest.


R=0.82 p=0.0003
Methylation Score
Expression Score
14
12
10
8
6
4
2
5
10
15
20

frequency methylators (p=0.0003). While the methylation score of these basal-like cell lines is nearly equivalent to that of the six hypermethylators alone (7.8 versus 8.2, respectively), the expression score of the basal cells is somewhat higher than the six hypermethylator cell lines (9.0 versus 5.5, respectively). This suggests that while the expression of indicator genes is more common among these basal-like lines, it is still less than the levels of low-frequency methylator cells. Additionally, these basal-like cell lines reflect methylation levels that are quite comparable to hypermethylators, suggesting that they may be expected to have the same methylation defects as hypermethylator cell lines.

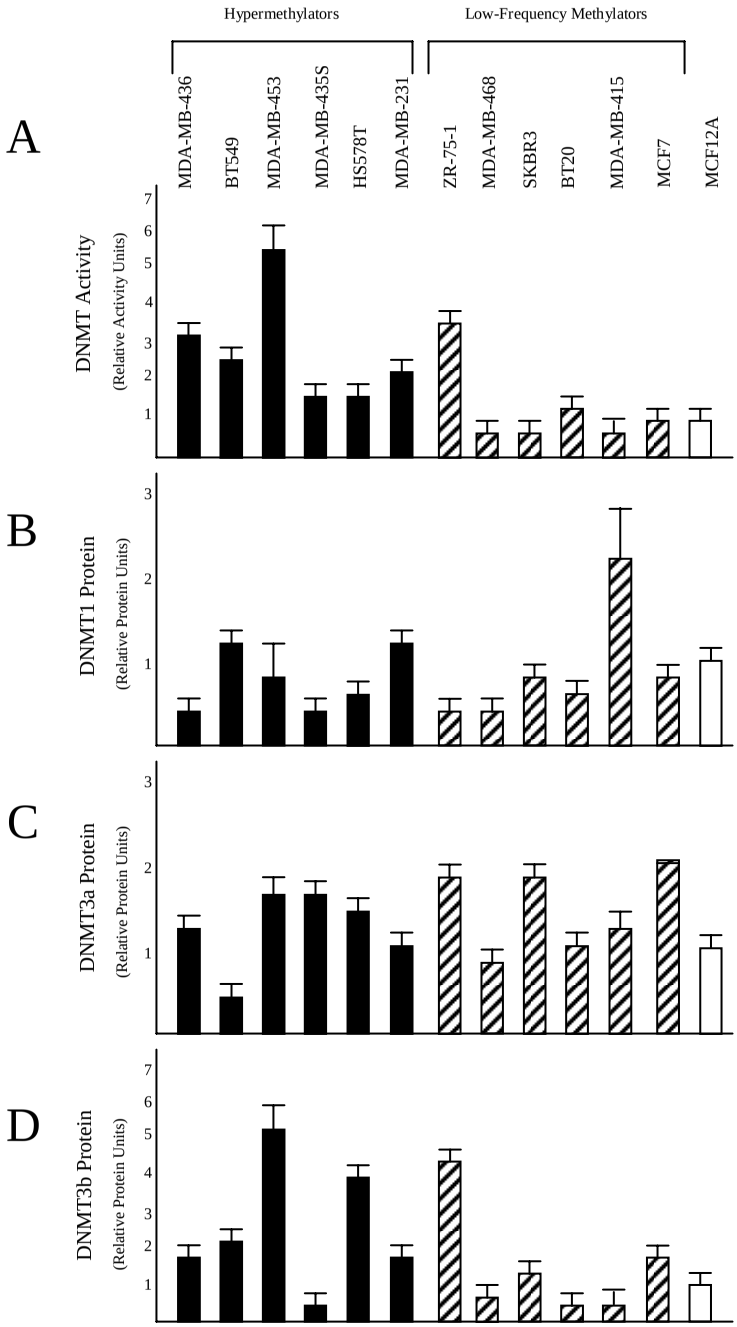
RESULTS RELATED TO DNMT ANALYSIS

In order to determine if the high levels of methylation displayed by the hypermethylator cell lines in the concurrent silencing of the nine epigenetically-regulated indicator genes was due to a defect in methylation machinery, we examined the three functional human DNMT enzymes (DNMT1, DNMT3a, and DNMT3b) in these cells. We also examined the total DNMT activity in hypermethylator cell lines in comparison to both low frequency methylators and the non-neoplastic breast cell line MCF12, in order to determine if certain aberrations in the methylation machinery may account for the different methylation signatures between these two subsets of breast cancer cell lines.

DNMT Analysis of Breast Cancer Cell Lines

Hypermethylator cell lines exhibit total DNMT activity levels that are higher than that of low-frequency methylator cell lines and non-neoplastic MCF12A cells (Figure 34A).

Figure 34. **DNA methyltransferase activity and enzyme levels are aberrant in hypermethylator cell lines.** Results from triplicate determination of total DNMT activity and individual DNMT protein assays are shown. Hypermethylator cell lines are represented by black bars, low-frequency methylators are represented by cross-hatched bars, and MCF12A cells are represented by a white bar. Error bars represent S.E.M. One unit of DNMT activity or DNMT protein level corresponds to the equivalent amount of activity or protein expressed in MCF12A cells. **(A)** Total DNMT enzymatic activity; **(B)** DNMT1 protein; **(C)** DNMT3a protein; and **(D)** DNMT3b protein.


Hypermethylators
Low-Frequency Methylators
MDA-MB-436
BT549
MDA-MB-453
MDA-MB-435S
HS578T
MDA-MB-231
ZR-75-1
MDA-MB-468
SKBR3
BT20
MDA-MB-415
MCF7
MCF12A
A
DNMT Activity
(Relative Activity Units)
B
DNMT1 Protein
(Relative Protein Units)
C
DNMT3a Protein
(Relative Protein Units)
D
DNMT3b Protein
(Relative Protein Units)

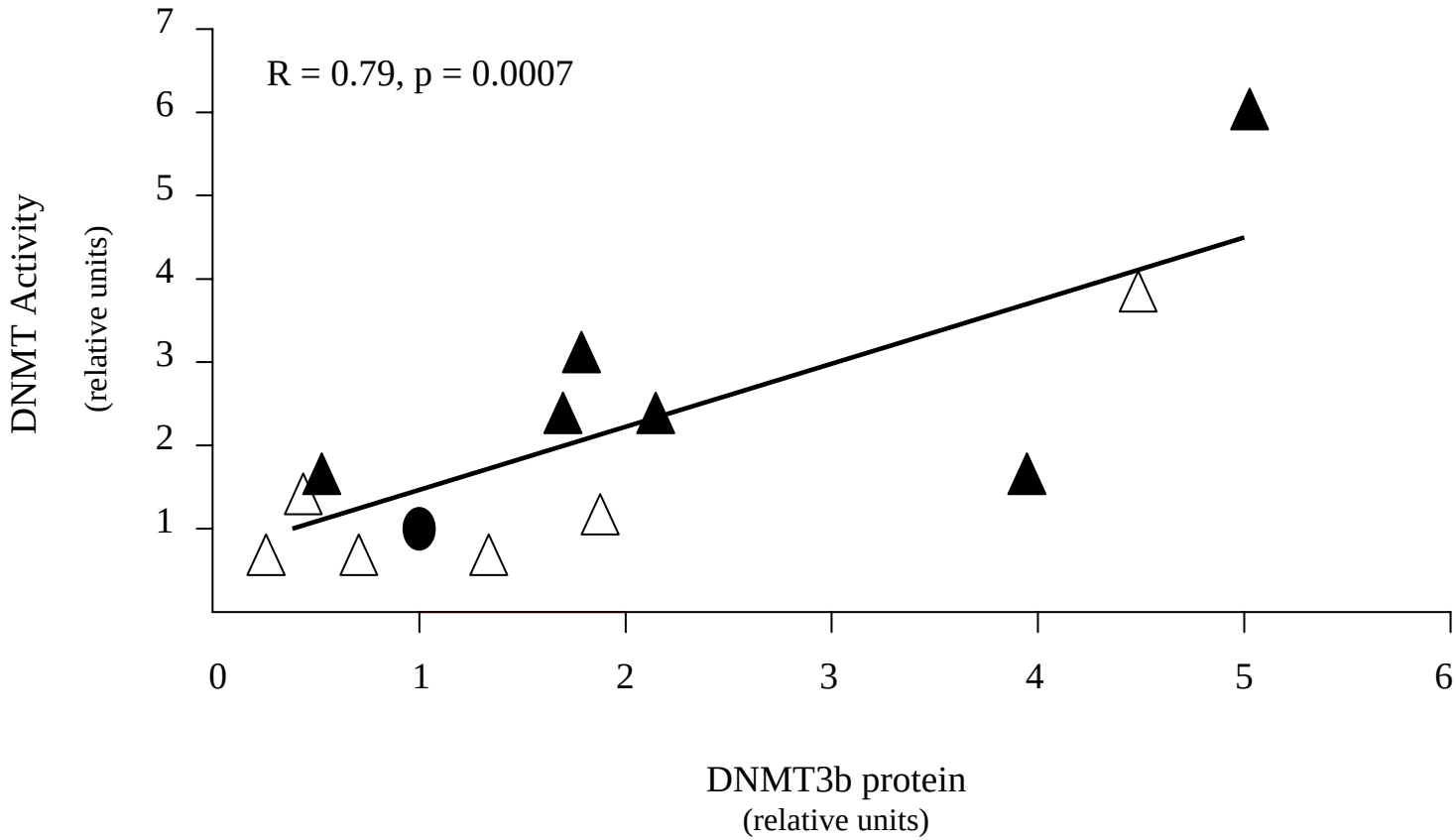
Each of the hypermethylator cell lines exhibit DNMT activity levels that are ≥1.7-fold higher than that of MCF12A cells (Figure 34A), whereas 5/6 (83%) low-frequency methylator cell lines (MDA-MB-468, SKBR3, BT20, MDA-MB-415, and MCF7) exhibit DNMT activity levels that are ≤1.4-fold that of MCF12A cells (Figure 34A). The average DNMT activity level for the hypermethylator cell lines (2.9 $\pm$ 0.6) is greater than that of the low-frequency methylator cell lines (1.4 $\pm$ 0.5), but the difference does not reach significance (p=0.095, NS). This is due to the level of DNMT activity in ZR-75-1 cells (3.8 $\pm$ 0.2), which is much higher than MCF12A cells, making it unlike the other five cell lines in the low-frequency methylator group. When ZR-75-1 cells are excluded, the average DNMT activity level of the low-frequency methylator group becomes indistinguishable from that of MCF12A cells and significance emerges between the total DNMT activity levels of the hypermethylator and low-frequency methylator groups (p=0.027).

No significant differences were detected for DNMT1 or DNMT3a protein levels between hypermethylator cell lines, the low-frequency methylator cell lines, and MCF12A cells (Figure 34B-C). The average DNMT1 protein level for the hypermethylator cell lines (0.8 $\pm$ 0.15) and the low-frequency methylator cell lines (0.88 $\pm$ 0.29) are indistinguishable from those of MCF12A cells (p=0.82, NS) (Figure 34B). MDA-MB-415 cells overexpress DNMT1 (2.3-fold compared to MCF12A), but the other cell lines exhibit a DNMT1 protein level of 1.3-fold or lower regardless of their methylation status (Figure 34B). Likewise, the average DNMT3a protein level for the hypermethylator cell lines (1.24 $\pm$ 0.17) and the low-frequency methylator cell lines (1.39 $\pm$ 0.2) are indistinguishable from that of MCF12A cells (p=0.59, NS) (Figure 34C). In contrast to DNMT1 and DNMT3a, the average DNMT3b protein levels for the hypermethylator cell lines are much higher (2.5 $\pm$ 0.67) than those of

the low-frequency methylator cell lines (1.5 $\pm$ 0.64) (Figure 34D), but this difference was not statistically significant. Among the hypermethylator cell lines, 5/6 (83%) express ≥1.7-fold MCF12A levels of DNMT3b protein. In contrast, among the low-frequency methylator cell lines, only ZR-75-1 cells (which also display high DNMT activity) exhibit an elevated level of DNMT3b protein level expression (Figure 34D). While ZR-75-1 cells display elevated DNMT3b protein and total DNMT activity, they fail to silence the methylation-sensitive genes that are methylated in the hypermethylator phenotype cell lines. Thus, ZR-75-1 cells are more similar to the low-frequency methylator cell lines with respect to gene expression and methylation of the six indicator genes. When the cell line ZR-75-1 is excluded from the low-frequency methylator group, the average DNMT3b protein level for the low-frequency methylator cells is 0.91-fold that of MCF12A cells, approaching significance when compared to the hypermethylator cell lines (p=0.069).

A correlation analysis was performed to identify significant relationships between DNMT protein levels and DNMT activity among the hypermethylator and low-frequency methylator cell lines. No significant association was found between DNMT activity and DNMT1 or DNMT3a protein levels (R<0.3, NS). However, a strong association (R=0.79, p=0.0007) between DNMT activity and DNMT3b protein levels was observed (Figure 35). The correlation coefficients for the relationship between DNMT3b protein and DNMT activity for both hypermethylator cell lines (0.71, p=0.0036), and the low-frequency methylator cell lines (R=0.90, p=0.0028) were statistically significant. This observation suggests that DNMT3b significantly contributes to total DNMT activity among breast cancer cell lines. Consistent with this suggestion, in cell lines with DNMT activity ≥1.8-fold higher

Figure 35. **DNMT activity levels in breast cancer cell lines correlate with DNMT3b expression.** Association between DNMT total activity and DNMT3b protein levels for 12 breast cancer cell lines and MCF12A cells. Hypermethylator cell lines (black triangles), low-frequency methylator cell lines (white triangles), and MCF12A cells (black circle) demonstrate a statistically significant relationship between DNMT total activity and DNMT3b protein levels.


R = 0.79, p = 0.0007
DNMT Activity
(relative units)
DNMT3b protein
(relative units)
0
1
2
3
4
5
6
7

than MCF12A cells (n=7), 86% (6/7) exhibit elevated (≥1.7-fold higher than MCF12A) DNMT3b levels. With the exception of ZR-75-1 cells, all of these cell lines belong to the hypermethylator group (MDA-MB-436, BT549, MDA-MB-453, HS578T, and MDA-MB-231). Significant associations were recognized between DNMT activity and the additive values of (i) DNMT1 and DNMT3b (R=0.74, p=0.002), (ii) DNMT3a and DNMT3b (R=0.74, p=0.002), and (iii) DNMT1, DNMT3a, and DNMT3b (R=0.70, p=0.004). However, these relationships primarily reflect the contribution of DNMT3b to DNMT activity rather than a true additive effect of the various DNMT enzymes. These findings combine to demonstrate significant correlation between hypermethylator status, elevated total DNMT activity, and overexpression of DNMT3b protein.

DNMT3b Protein Analysis Primary Human Breast Tumors

We examined the DNMT3b protein expression in the 137 primary breast tumors which were included in the TMA analyzed above. Basal tumors on average have a slightly higher expression score for DNMT3b (2.70 $\pm$ 0.17) compared to both Her2+ (2.22 $\pm$ 0.24) and luminal A (2.35 $\pm$ 0.09) breast tumors. This immunohistochemical analysis revealed that, while detectable overexpression of DNMT3b protein by IHC was rare, 3/23 (13%) of basal tumors exhibited very high expression of DNTM3b, compared to 1/96 luminal (1%) and 0/18 (0%) of Her2+ tumors.

DNMT Activity and DNMT3b Protein Analysis of Basal-like Breast Cancer Cell Lines

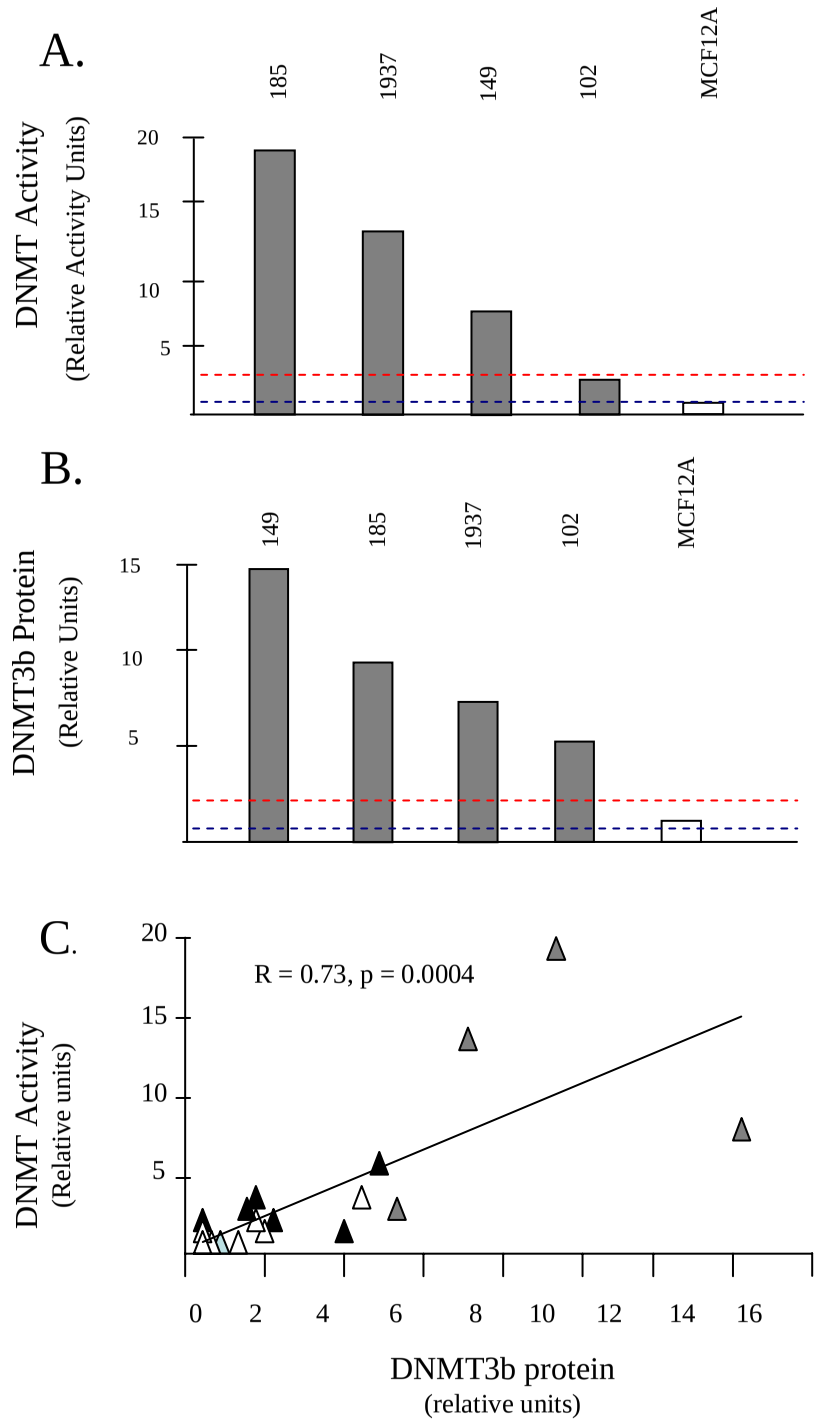
Basal-like breast cancer cell lines SUM102 and SUM185 and luminal A-like SUM149 and HCC1937 cell lines were assessed for the total DNMT activity as well as DNMT3b protein levels to determine how this subset of cell lines compared to the

hypermethylator cell lines in respect to DNMT-based abnormalities. Analysis of the total DNMT activity in these four breast cancer cell lines revealed elevated total activity in each cell line, compared to that of MCF12A cells: SUM102 cells exhibit 2.8-fold higher total DNMT activity than MCF12A cells, SUM149 cells exhibit 7.9-fold higher total activity, SUM185 cells exhibit 19.4-fold elevated total activity, while HCC1937 exhibit total activity levels that were 13.5-fold higher than MCF12A cells (Figure 36A). Subsequent DNMT3b protein analysis revealed elevated DNMT3b protein in each of these cell lines: SUM102 cells exhibit DNMT3b protein levels that are 5.4-fold higher than MCF12A, SUM149 cells exhibit 14.3-fold higher DNMT3b protein, SUM185 exhibit 9.4-fold increased DNMT3b protein, while HCC1937 cells exhibit DNMT3b protein levels that are 7.1-fold higher than non-neoplastic MCF12A cells (Figure 36B). When we include these cell lines in our analysis we see a strong correlation between DNMT3b protein and total DNMT activity in a panel of 16 breast cancer cell lines (Figure 36C) persists, as described previously ($R=0.73$, $p=0.0004$).

DNMT3B Knockdown of Hypermethylator Cell Lines

In order to determine if overexpression of DNMT3b protein is directly responsible for methylation-dependant silencing of the genes associated with the hypermethylation signature, we treated MDA-MB-453 and BT549 cells with GFP-tagged shRNA targeted to DNMT3b. The RNA-interference construct employed was previously utilized to knockdown DNMT3b expression in MCF7 cells (261). Populations of GFP+ MDA-MB-453 and BT549 cells were established following transfection. These cell populations were utilized to examine expression of the methylation-sensitive genes which were not expressed in these cell lines. A pure GFP+ cell population was not isolated during these experiments due to the lack of a

Figure 36. DNMT analysis of basal-like cell lines. Basal-like cell lines shown in grey bars and triangles, hypermethylators shown in black triangles, low-frequency methylators shown in white triangles, and MCF12A shown in blue triangles and white bars. Average activity / DNMT3b level for hypermethylators is shown by red dashed lines, while average levels for low-frequency methylators is shown by blue dashed lines. **(A)** Total DNMT activity; **(B)** DNMT3b protein analysis; **(C)** DNMT activity correlates with DNMT3b protein.


A.
185
1937
149
102
MCF12A
DNMT Activity
(Relative Activity Units)
20
15
10
5
B.
149
185
1937
102
MCF12A
DNMT3b Protein
(Relative Units)
15
10
5
C.
R = 0.73, p = 0.0004
DNMT Activity
(Relative units)
20
15
10
5
0
2
4
6
8
10
12
14
16
DNMT3b protein
(relative units)

positive selectable marker on the pLVTHM vector construct. These cell populations were found to be composed of ~75% GFP+ cells for the MDA-MB-453 cell populations and ~85% GFP+ cells for the BT549 cell populations. GFP+ cells exhibited various features distinct from non-transfected control cells, including a decreased growth rate and a higher proportion of cells that are larger, but otherwise morphologically similar to the parental tumor cell line. In contrast, MDA-MB-453 and BT549 cells that were transfected with the control scrambled sequence vector exhibited none of these features. Western blot analysis revealed that the shRNA-transfected population of MDA-MB-453 cells contained detectably lower levels of DNMT3b protein compared to either the parental or scrambled control MDA-MB-453 cells, suggesting effective shRNA-mediated knockdown (Figure 37). The hypermethylator cell line MDA-MB-453 lacks expression of *CEACAM6*, *CST6, ESR1, MUC1*, and *SCNN1A,* and expresses *GNA11* at very low levels (compared to normal MCF12A cells) (Figure 15). RT-PCR analysis revealed that stable RNAi-mediated knockdown of DNMT3b in MDA-MB-453 cells results in reexpression or increased expression of all six genes (Figure 38). In the case of *CEACAM6, CST6, ESR1*, and *GNA11*, the DNMT3b knockdown cells express these genes at levels equal to or slightly higher than MCF12A cells (Figure 38). Methylated genes *MUC1* and *SCNN1A* were also expressed after DNMT3b knockdown, but at levels slightly lower than normal (Figure 38). These results demonstrate that diminished levels of DNMT3b results in reexpression of these methylation-sensitive genes in hypermethylator MDA-MB-453 cells, and suggest strongly that *CEACAM6*, *CST6, ESR1, GNA11, MUC1*, and *SCNN1A* are gene targets for epigenetic regulation by DNMT3b. The hypermethylator cell line BT549 lacks expression of *CEACAM6, CDH1, CST6, ESR1, GNA11,* and *SCNN1A*. RT-PCR

Figure 37. Western analysis of shRNA-transfected MDA-MB-453 cells reveals evidence of effective knockdown of DNMT3b protein. Western analysis of DNMT3b protein in MDA-MB-453 cells treated with shRNA targeted to DNMT3b reveals lower levels of detectable protein. The molecular weight of the DNMT3b protein is approximately 110 kDa. Templates loaded as follows: lane 1, parental MDA-MB-453 cells (control); lane 2, MDA-MB-453 scrambled control; lane 3, MDA-MB-453 knockdown. There is a detectable knockdown of shRNA-transfected MDA-MB-453 cells, but no change in the level of MDA-MB-453 cells transfected with the scrambled control. β-actin blot is shown.

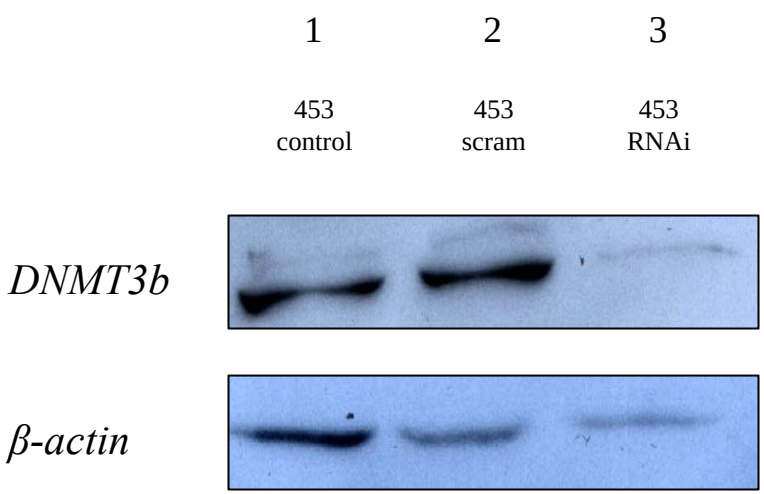
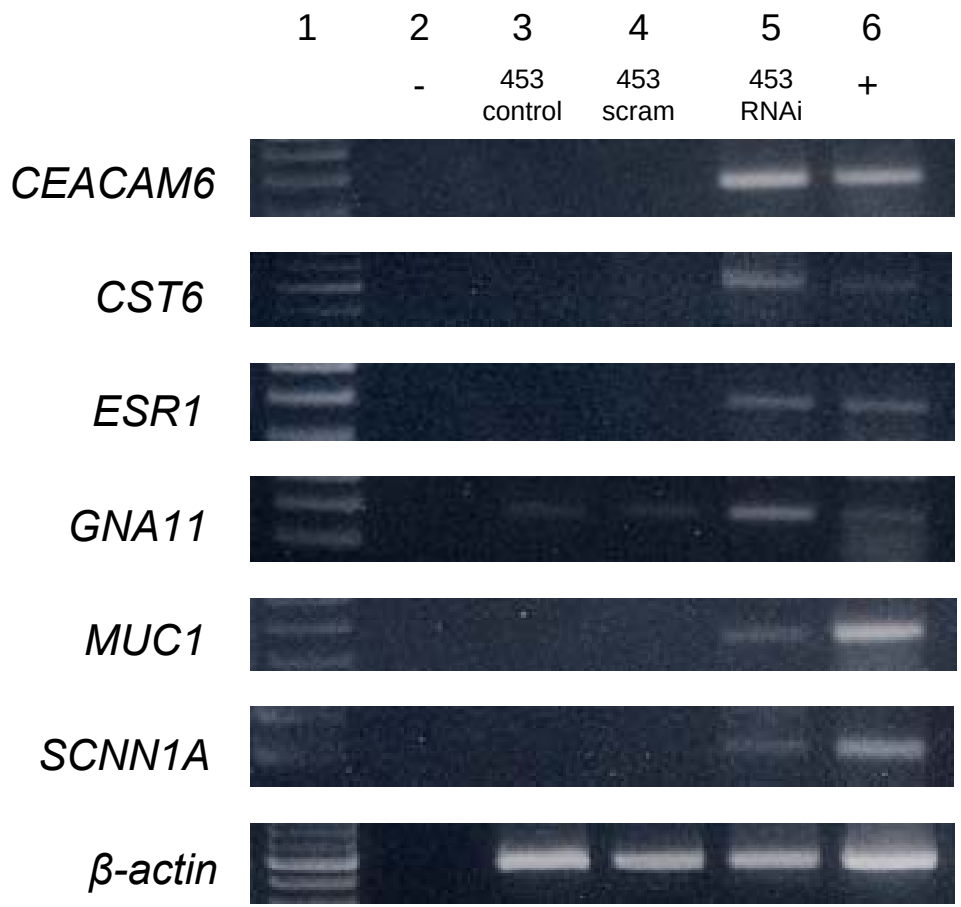
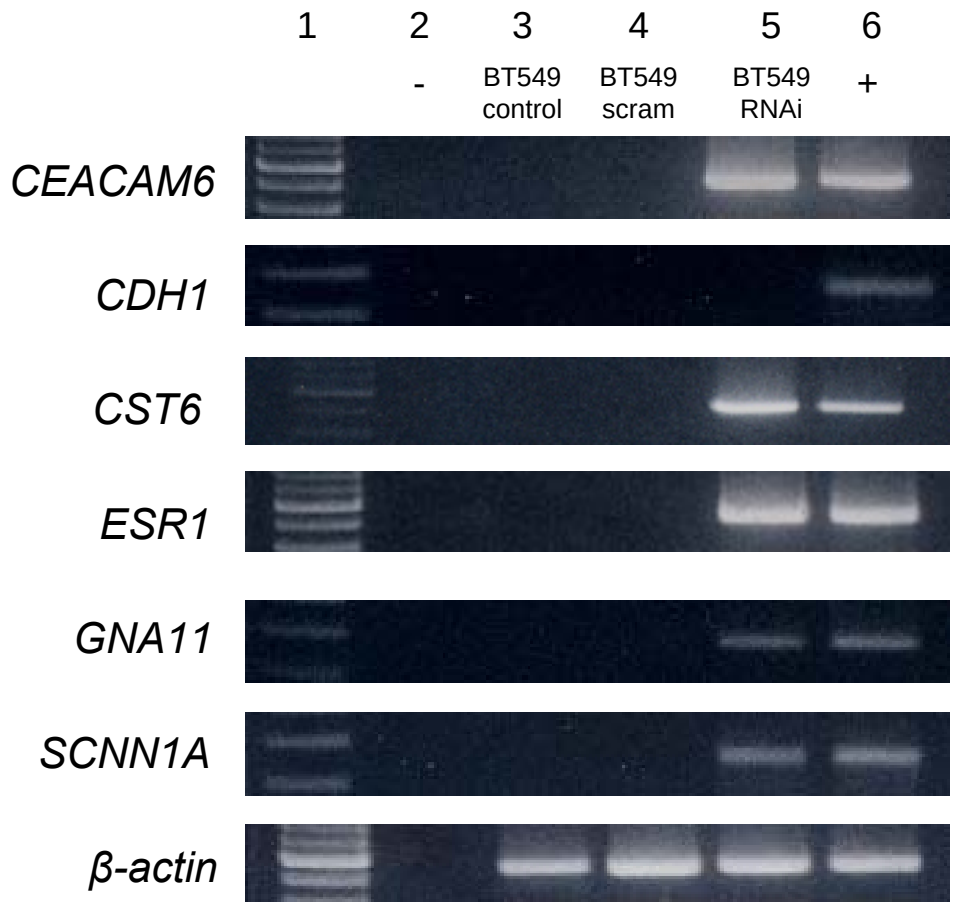

1
2
3
453
control
453
scram
453
RNAi
DNMT3b
β-actin

Figure 38. RNA-interference mediated knockdown of DNMT3b restores expression of methylated genes in MDA-MB-453 cells. Agarose gels showing RT-PCR products for six indicator genes which lack normal expression in the hypermethylator cell line MDA-MB-453. Templates loaded as follows: lane 1, molecular size standard; lane 2, no template (negative control); lane 3, parental MDA-MB-453 cells (control); lane 4, MDA-MB-453 scrambled control; lane 5, MDA-MB-453 knockdown; lane 6, MCF12A (non-neoplastic index cell line). MDA-MB-453 cells lack expression of *CEACAM6, CST6, ESR1, MUC1*, and *SCNN1A*, while expressing *GNA11* at low levels (compared to MCF12A). RNAi-treated MDA-MB-453 cells (lane 5) exhibit re-expression or induced expression of these genes. *β-actin* was amplified as a positive control.


1
2
3
4
5
6
-
453 control
453 scram
453 RNAi
+
CEACAM6
CST6
ESR1
GNA11
MUC1
SCNN1A
β-actin

analysis showed that RNAi-mediated knockdown of DNMT3b in BT549 cells results in the reexpression of five of these six genes (Figure 39). Thus, these results indicate that overexpression of DNMT3b significantly contributes to the methylation defect exhibited by hypermethylator cell lines which, in turn, results in the concurrent methylation of multiple genes.

Figure 39. RNA-interference mediated knockdown of DNMT3b restores expression of methylated genes in BT549 cells. Agarose gels showing RT-PCR products for six indicator genes which lack normal expression in the hypermethylator cell line BT549. Templates loaded as follows: lane 1, molecular size standard; lane 2, no template (negative control); lane 3, parental BT549 cells (control); lane 4, BT549 scrambled control; lane 5, BT549 knockdown; lane 6, MCF12A (non-neoplastic index cell line). BT549 cells lack expression of *CEACAM6, CST6, ESR1, MUC1*, and *SCNN1A*, while expressing *GNA11* at low levels (compared to MCF12A). RNAi-treated BT549 cells (lane 5) exhibit re-expression or induced expression of these genes. *β-actin* was amplified as a positive control.


1
2
3
4
5
6
-
BT549 control
BT549 scram
BT549 RNAi
+
CEACAM6
CDH1
CST6
ESR1
GNA11
SCNN1A
β-actin

DISCUSSION

THE CHALLENGE OF POOR-PROGNOSIS BREAST CANCER

The inherent heterogeneity associated with breast cancer makes determination of tumor characteristics that greatly impact patient outcome (such as aggressiveness, ability to metastasize, response to various chemotherapeutic agents) difficult or impossible, especially at the time of initial diagnosis. Given that a number of different genetic, chromosomal, and epigenetic changes contribute to the development of breast cancer, every tumor is intrinsically complex and potentially distinct. Because the aberrant cells that give rise to these tumors accumulate numerous molecular changes during neoplastic transformation and tumorigenesis, the response to treatment and eventual progression for a given tumor is determined by the combined influence of numerous genes (and other factors). Thus, understanding the molecular mechanisms that affect numerous genes concurrently may hold the key for better clinical management of breast cancer.

Recent advances in our understanding of the gene expression patterns that characterize different classes of breast cancer and the links between gene expression, tumor behavior, and patient outcome promise to provide better prognostication for individual patients, as well as guides for clinicians in choosing appropriate treatment strategies (33, 35, 37). The development of targeted therapies for some subsets of breast cancer (such as ER+ and Her2+ breast tumors) has greatly reduced the cancer burden and mortality rates associated with these breast tumors, and extended the life expectancy for many women.

While these advances have improved breast cancer treatment, several classes of tumors remain difficult to treat effectively. Tumors types that lack targeted therapies (such as basal breast cancer) are more difficult to treat and clinicians are left with cytotoxic chemotherapeutics as the only means of treatment. As a result, these tumors account for a disproportionate number of breast cancer deaths each year. Thus, there is an urgent need not only for better targeted treatment strategies, but also for an improved understanding of the molecular basis of these poor-prognosis breast tumors in order to improve the long-term outcome of women with these tumors. Better understanding, rapid identification, and improved treatment of basal-like tumors will result in substantial alleviation of the overall breast cancer burden since the poorest outcome tumors account for a large portion of breast cancer deaths.

Challenges of Basal Breast Cancer

Women with triple negative (ER-/PR-/Her2-) breast cancer, of which basal tumors make up a large subset, are among those with the poorest overall survival rates (35, 40, 50). Breast tumors of the basal subtype make up ~25% of all breast cancers and tend to display more aggressive tumor characteristics, such as increased size, rapid tumor growth, increased rate of metastasis to other organ sites, higher incidence of relapse, and lower overall patient survival (40, 49, 272, 273). The genetic and epigenetic events that lead to development of these cancers is incompletely understood, and, represents the focus of much scientific inquiry in the hopes of uncovering the molecular basis of these neoplasms (46). In addition to possessing a number of aggressive characteristics, basal breast cancers have been reported to exhibit a number of unique features that are not well understood. For example, basal breast cancer appears to occur at a higher incidence in pre-menopausal African-American women

(compared either pre-menopausal Caucasians or post-menopausal women of either ethnicity), complicating the understanding of incidence of this subtype (40). In fact, a recent study of 148 Nigerian women with breast cancer revealed that 59% of these women had basal-like breast cancer, a rate more than double what would be expected (reviewed in 46). Although the true cause remains unclear, the factors that account for this overrepresentation of poor-prognosis basal tumors in women of African ancestry may include genetic predisposition, environmental factors, cultural practices, socioeconomic factors (access to care), or a combination of all of these. Additionally, several studies have reported findings that seem to indicate that basal breast cancers may metastasize in unique ways, being less likely to metastasize to the bone than the viscera, and having higher rates of brain metastases compared to metastatic breast cancers of other subtypes (46, 50, 274). Better understanding of the unusual features of basal-like breast cancer may lead to the development of new therapies and more rapid molecular diagnostics.

Current Treatment Options for Basal Breast Cancer

Basal breast tumors display a general resistance to most currently available targeted treatment options for breast cancer, including hormonal therapies (as they are typically ER- and PR-), as well as trastuzumab (as they are typically Her2-). Whereas numerous studies have shown that basal-like breast cancers generally respond well to preoperative chemotherapy (52, 275), patients who do not achieve pathologic complete response with such treatment face a higher likelihood of relapse than patients with breast cancers representing the other molecular subtypes. Additionally, basal breast cancers tend to be highly proliferative (46), which may partially explain this tendency towards initial response to treatment followed by relapse. The higher proliferation rates of these tumors may result in

more rapid death of a high percentage of tumor cells after drug treatment, but leaving a few resistant tumor cells which could cause to eventual relapse. The genetic instability exhibited by many basal breast cancers may also confer an increased likelihood toward development of resistance to whichever treatment regime is administered (46). For example, many basal breast cancers are *p53* mutant. The *p53* status of these breast cancers impact significantly on drug treatment, since *p53* mutant tumors have been reported to be resistant to certain anthracycline-based chemotherapeutic regimens (276).

Currently a number of signaling molecules are under investigation as potential targets for basal-like breast cancers. For example, *EGFR* signaling has been inhibited successfully in other cancers and *EGFR* is frequently overexpressed in basal-like breast cancers (46). However the development of an IHC-based assay that accurately measures EGFR protein levels has proven difficult to achieve (277), slowing the progress on this front. Drugs that target the growth factor receptor *c-KIT* (such as imatinib) are already used to treat many cancers. However, *c-kit* is only expressed by ~30% of basal breast cancers, limiting the potential usefulness of imatinib. Furthermore, imatinib was found to have no activity against metastatic breast cancers in a recent phase II clinical trail (278). Many other signaling targets are currently under investigation for development of drugs for use against breast malignancies, including inhibitors of *Ras, Raf, MEK, MTOR,* and *Src* (46), but until the unique mechanisms underlying basal breast cancer are better understood, progress in treating these malignancies will be incremental at best.

IDENTIFICATION OF A HYPERMETHYLATOR PHENOTYPE IN HUMAN BREAST CANCER

Identification of a Novel Hypermethylation Signature

Our findings suggest that a hypermethylator phenotype occurs in a subset of human breast tumors, and, like methylator phenotypes in other tumor systems, this subset of breast tumors tends to methylate tissue-specific genes more often than 'classical' colorectal CIMP genes (such as the *MINT* gene family and *hMLH1*). In breast cancer, this hypermethylator signature is defined by concurrent methylation of numerous genes of interest, including *CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*. This hypermethylation defect corresponds to elevated DNMT activity secondary to overexpression of DNMT3b protein. The CpG island methylator phenotype (CIMP) was first used to describe a distinct subset of colorectal tumors that display high rates of concordant methylation of specific genes (85). Subsequently, similar epimutational phenomena have been described in a wide range of neoplasms (86-95). In our study, we examined not only genes with conventionally defined CpG islands, but also those with atypical CpG features (such as *CEACAM6*), which have recently been reported as epigenetically-regulated despite lacking typical CpG islands (58). Thus, we use the term "hypermethylator phenotype" rather than "CpG island methylator phenotype" to describe the hypermethylation defect in breast cancer cell lines since the targets of aberrant methylation are not restricted to genes with large CpG islands.

A previous study that examined methylation patterns of primary breast tumors in search of a hypermethylator phenotype found frequent but essentially equally distributed methylation events at 12 genes among different histologic subsets of neoplasms (105). The authors concluded that a CpG island methylator phenotype does not occur in breast cancer.

The difference in conclusions about the existence of a hypermethylator phenotype in breast cancer between the current study and the earlier published report is likely attributable to the number and choice of genes examined in the two studies. The previous study did not examine many of the genes that we found to be highly predictive of a hypermethylator phenotype (*CEACAM6, CST6, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*), but did include several genes (including *GSTP1, RARβ, RB,* and others) which we found to be less useful for predicting the hypermethylator phenotype in breast cancer. Thus, our results are consistent with the previous findings: when the set of genes analyzed by Bae *et al* (105) are used as criteria for detecting a methylator phenotype, no distinct hypermethylator phenotype is detectible. It is only through a survey of numerous methylation-sensitive genes that evidence for a hypermethylator phenotype emerges.

The subset of human breast cancer cell lines and primary breast tumors determined in our study to express a hypermethylator phenotype are characterized by concurrent methylation-dependent silencing of a number of genes, including a specific set of genes with excellent predictive power (*CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*) that are involved in a wide range of neoplastic processes. *E-cadherin* (*CDH1*) is a well-known suppressor of invasion and metastasis that functions in the maintenance of cell-cell adhesion (121). Methylation of *CDH1* (estimated to occur in 40-90% of breast tumors) is associated with lymph node metastasis, loss of differentiation, and ER negativity in primary breast cancers (111, 115, 279). *CDH1* and *ESR1* are frequently concurrently methylated in breast tumors (115), a relationship also supported by the results of the present study. *CEACAM6* is a tumor-related gene that is involved in adhesion, migration, invasion, metastasis, apoptosis, and chemoresistance (280, 281), although the implications of

CEACAM6 loss in breast cancers are not well understood. The majority of what is known about the role of this gene in cancer comes from studies of tumors in which *CEACAM6* is overexpressed, which tends to correspond to more aggressive cancer phenotypes, including those of the colorectum (282) and pancreas (283). *Cystatin M* (*CST6*) is a recognized breast cancer tumor suppressor gene (284) that was recently reported to be silenced due to promoter hypermethylation in numerous breast cancer cell lines, as well as primary breast tumors (58, 118). *CST6* is estimated to be methylated in ~27% of primary and ~67% of metastatic breast cancers (285). A cysteine protease inhibitor, *CST6* is involved in regulation of signaling molecules, extracellular matrix components, kinases, phosphatases, and transcription factors (286). *ESR1*, which is silenced by methylation in the majority of estrogen-negative breast tumors (115), is a very important methylation-sensitive gene in breast carcinogenesis, holding important implications for sensitivity to hormone therapy and clinical outcome. *ESR1* is a nuclear hormone receptor which can activate transcription of cell growth genes. While many breast cancers are fueled by estrogen, some become estrogen-independent and can proliferate even in the absence of this hormone. Such ER- cancers tend to have worse prognosis than ER+ breast tumors, in part because they are unresponsive to anti-estrogen therapy. Loss of *ESR1* gene expression is an important prognostic factor in breast cancer and is associated with poor differentiation, insensitivity to hormonal therapy, and poor clinical outcome (115). While a significant percentage of breast cancers lack expression of the estrogen receptor (and other steroid receptors), loss of *ESR1* expression is only rarely due to deletion or mutation (287). Rather, methylation-dependent silencing of the *ESR1* gene is responsible for the loss of expression in as many as 46% of breast tumors (116, 117). *GNA11*, a guanine nucleotide binding protein, is involved in gonadotropin-releasing hormone

receptor signaling, which in turn, negatively regulates cell growth in normal settings (185). In cases where *GNA11* expression is lost, as is the case in ~63% of breast cancers (185), aberrant cell growth can proceed unchecked, contributing significantly to neoplastic potential. The protein products of *MUC1*, a transmembrane mucin, are generally highly expressed in breast cancer tissue, and as such, serve as markers for tumor progression and metastasis in epithelial cancers, with high expression correlating with malignant potential (208, 288, 289). Paradoxically, other studies have correlated *MUC1* overexpression with lower grade tumors as well as ER-positivity, both of which would suggest *MUC1* expression may be an indicator of better outcome breast tumors (290). Previous studies demonstrated that hypomethylation of a specific tandem repeat sequence within the *MUC1* gene is necessary for its overexpression (291) and that *MUC1* gene expression is further epigenetically regulated by DNA methylation and histone H3K9 modification (208). The primary function of mucins (including *MUC1*) is in hydrating and lubricating the epithelium. However, these proteins have also been implicated in regulation of growth factor signaling and cell adhesion (292). *MYB*, an oncogene which promotes cell cycle progression and is targeted by estrogen signaling, is generally expressed in ER+ breast tumors and generally not expressed by ER- tumors (293). As *MYB* appears to be required for proliferation of certain ER+ breast cancer cell lines (294), it is paradoxical that it is not expressed in hypermethylator breast cancer cells, yet breast tumors that express the hypermethylation defect (and that are MYB negative) are more likely to be highly proliferative and have very poor prognosis. The role of ion transport gene *SCNN1A* in breast carcinogenesis is not well understood, although its epigenetic regulation in MCF7 cells has previously been noted (58). In general, sodium content of cancerous breast epithelium is higher than its normal

counterpart, and recent studies have shown a correlation between increased sodium content and increased breast tumor invasion. As such, there is some evidence that proliferation of breast tumors can be prevented by treatment with inhibitors to *SCNN1A* and its family members (295). Paradoxically, *SCNN1A* is generally not expressed in hypermethylator tumors which also tend to be of poor prognosis, suggesting that sodium concentration may not be what is driving the proliferation of these breast cancers. In pulmonary adenocarcinomas high expression of *SCNN1A* is associated with a good prognosis (296), while in testicular germ cell tumors *SCNN1A* overexpression corresponds to tumors that are less differentiated (297). Thus, tissue type and context appear to be important for determining whether *SCNN1A* expression correlates with better or worse prognosis. *TFF3* is a trefoil peptide which functions to guard the luminal epithelium from injury and is involved in repairing damage and preventing inflammation. *TFF3* has been reported to be overexpressed in ER+ breast tumors and can be upregulated by estrogen (292). As such, *TFF3* is recognized to contribute to a luminal epithelial signature and is associated with well-differentiated, low-grade breast tumors (292). These methylation-sensitive indicator genes function in a wide variety of aspects of the normal biology of the breast epithelium. Therefore, concurrent methylation-dependent silencing of multiple genes in neoplastic breast epithelium (as observed in hypermethylator cell lines) is likely to significantly contribute to tumor biology and behavior.

DNMT Abnormalities Associated with the Hypermethylator Phenotype

The results of the current studies suggest that the mechanism that accounts for the hypermethylator phenotype in human breast cancer cell lines is related to elevated DNMT activity secondary to overexpression of DNMT3b. The hypermethylator cell lines exhibit

aberrantly increased DNMT activity (and overexpress DNMT3b) and display correspondingly high rates of methylation-dependent gene silencing compared to both low-frequency methylator cells and non-neoplastic breast cells. Consistent with the suggestion that overexpression of DNMT3b results in a hypermethylation defect in these cells, RNAi-mediated knockdown of DNMT3b results in the expression of methylation-silenced genes. These results are in agreement with those of other recent studies, in which DNMT3b overexpression was implicated in the methylation abnormalities of breast cancers (82) and other cancers (298). Tumor cells exhibiting DNMT3b overexpression would be expected to exhibit aberrant gene expression patterns related to primary DNA methylation events (gene silencing) and secondary gene expression changes. One study showed that breast tumors that overexpress DNMT3b are more likely to be *ESR1*-negative, display increased proliferation, and be associated with poor patient prognosis (82). These observations are supported by our findings. Thus, it seems reasonable to expect that aberrant expression of DNMT3b protein may produce significant differences in tumor biology for breast tumors of the hypermethylator phenotype. In addition to the hypermethylator cell lines which had elevated DNMT3b protein and total DNMT activity, one low-frequency methylator cell line (ZR-75-1) exhibited overexpressed DNMT3b and high levels of total DNMT activity. However ZR-75-1 cells retain expression of a number of epigenetically-regulated genes, making it functionally similar to other low-frequency methylator cell lines. A number of explanations may account for this apparent discrepancy: ZR-75-1 cells may methylate other epigenetically-regulated genes which were not surveyed in the present study. Alternatively, ZR-75-1 cells may possess the same functional defect in the DNMT machinery as cells of the

hypermethylator phenotype but express additional repressor proteins which block the methylation capacity of the overabundant DNMT3b protein.

Based upon results of our *in vitro* studies, we posited that a subset of primary breast cancers may express a hypermethylator phenotype characterized by expression of a hypermethylator signature of multiple methylation-sensitive genes. Further, we speculated that these tumors would have distinct characteristics reflecting important differences in tumor biology/behavior and patient outcome. This is the case in colorectal cancer, where CIMP status is associated with various clinical features (98-100). CIMP-positive neuroblastomas, esophageal tumors, and leukemias tend to have poorer prognosis and are associated with significantly higher relapse and mortality rates (95, 101, 102). The protective and tumor suppressor-like normal functions of the genes *CDH1, CST6, ESR1, GNA11,* and *TFF3* would suggest that concurrent loss of these methylation-sensitive genes in hypermethylator tumors might drive neoplastic transformation, progression, and invasion. However, because hypermethylator cells also silence genes which act as oncogenes (*MYB*) and genes whose overexpression is known to correlate with poor prognosis in some tumors (*CEACAM6, MUC1,* and *SCNN1A*), predicting hypermethylator behavior is more complex. In fact, our microarray data mining studies revealed that the hypermethylator phenotype is a fundamental biological property of the majority of basal breast cancers, a poor prognosis molecular subtype of breast cancer. It is tempting to hypothesize that some of the aggressive features of basal tumors may be due to the concurrent methylation-dependent silencing of tumor suppressors such as *CDH1, CST6, ESR1, GNA11, TFF3* and others.

DNMT3b Knockdown in Hypermethylator Phenotype Cell Lines

Given that our *in vitro* studies showed that hypermethylator cell lines are

characterized by high levels of DNMT activity which correlate with overexpression of DNMT3b protein, we decided to target this protein by shRNA in order to determine if reducing DNMT3b levels in hypermethylator cells would result in the reexpression of methylation-sensitive genes. DNMT3b is one of two catalytically active DNMT enzymes that are chiefly responsible for *de novo* methylation in human cells, and as such, would be capable of catalyzing the aberrant methylation that characterizes the hypermethylator phenotype. Previous studies have implicated DNMT3b in breast carcinogenesis, finding that DNMT3b is the methyltransferase enzyme overexpressed in the greatest percentage of breast tumors, and is likely to be overexpressed in aggressive, poor-prognosis, ER- tumors (82). Our findings that basal breast tumors tend to exhibit methylation defects and expression signatures of the hypermethylator phenotype are consistent with the implications of this previous study.

RNA interference-mediated knockdown of DNMT3b in the hypermethylator cell lines MDA-MB-453 and BT549, resulted in the reexpression of methylation-silenced indicator genes (Figure 38-39). This strongly suggests that the methylation defect of hypermethylator cells which causes extensive concordant methylation-dependent silencing of a specific subset of genes is mediated by aberrant DNMT3b expression. Further, knockdown of DNMT3b alleviates this defect, allowing for the reexpression of improperly silenced genes. Overexpression of DNMT3b has been reported in MCF7 breast cancer cells, where it was determined that RNAi knockdown of DNMT3b also resulted in promoter demethylation and reexpression of *CXCL12* (261). However, adding complexity to the issue is the detection of six transcriptional variants of DNMT3b (DNMT3b1-DNMT3b6) which result from alternative pre-mRNA splicing (67). Overexpression of transcript variant DNMT3b1 (but not

DNMT3a) promoted colon tumor formation in mouse models and resulted in the methylation and silencing of the tumor suppressors *Sfrp2, Sfrp4,* and *Sfrp5* (299). Another study showed that RNAi-mediated knockdown of alternative splice variant *ΔDNMT3b4* resulted in the promoter demethylation and reexpression of the *RASSF1A* tumor suppressor in lung cancer cells (300). However, *ΔDNMT3b4* targeting was shown to have promoter specific and tissue specific effects — while knockdown of *ΔDNMT3b4* resulted in reexpression of *RASSF1A* in lung cancer cells, it did not result in the reexpression of tumor suppressor, $p16^{INK4A}$. Additionally, knockdown of *ΔDNMT3b4* resulted in an increase, rather than decrease, in *RASSF1A* promoter methylation in bronchial epithelial cells (300). Thus, the effects of overexpressed DNMT3b appear to be both target and tissue specific. Interestingly, a recent study linked DNMT3b overexpression in liver tissues with both age and sex. In this study, DNMT3b was found to be expressed at significantly higher levels in older individuals as compared to younger ones, and in women as compared to men, indicating that both age and gender influence the methylation machinery (301). While the exact mechanisms responsible for these trends remain unknown, there is some evidence that hormones can affect DNMT expression (302-304). Hormones can interact with transcription factors known to regulate DNMT expression, such as c-*Jun, fos, Sp1,* and *Sp3* (305-307). These observations may explain, to some extent, why women might be at an increased risk of developing tumors related to aberrant DNMT3b expression.

IDENTIFICATION OF A HYPERMETHYLATOR PHENOTYPE AMONG A SUBSET OF PRIMARY HUMAN BREAST TUMORS

Our findings suggest that breast cancer cells that express the hypermethylation defect tend to be ER-, suggesting that the hypermethylator phenotype cosegregates with a subset of

breast cancers that tend to have poor prognosis (308). A number of molecular subtypes of breast cancer have been described (including luminal A, luminal B, HER2+, and basal-like), and these different subtypes correlate with important differences in tumor biology, clinical behavior, and patient survival (33, 35). Luminal A and luminal B tumors are ER-positive and respond better to treatment resulting in better long-term patient outcome compared to the ER-negative basal-like and HER2+ subtypes (33). Our microarray data mining analysis of primary breast cancer gene expression suggests that the hypermethylation defect observed in breast cancer cell lines can also be identified in primary tumors. Preliminary investigation of a limited dataset (n=91 tumors) identified a strong cluster of tumors that express the hypermethylator signature (Figure 16), with low levels of expression of the nine genes of interest (*CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*). Strikingly, all of the tumors in this cluster were classified as basal-like, and 88% of the basal-like tumors in the dataset expressed the hypermethylation signature. Subsequent investigation of additional datasets reflected many consistent trends. In total, the additional microarray datasets contained expression information on 946 primary breast tumors. Our analysis revealed a strong hypermethylator group in each dataset which was predominately basal-like (75-81%) in molecular subtype. On average, 68% (range 59-81%) of all basal breast tumors exhibited a hypermethylator signature characterized by lack of normal expression of at least 7 of 9 indicator genes. Thus, the majority of hypermethylator breast tumors are basal-like, and the majority of basal tumors are hypermethylators. In agreement with these relationships, our methylation analysis reveal that 80% of basal tumors were hypermethylators, with detectable promoter methylation of at least 7 of 9 indicator genes. Thus, it appears that this hypermethylation defect may be a fundamental property of many basal breast cancers, a

clinical subtype of particular importance given their overall poor survival outcomes (35, 43, 50, 272). For the first time, this finding suggests the presence of a breast cancer hypermethylator phenotype *in vivo*. This strong correlation between hypermethylator signature and the basal subset suggests that the hypermethylation defect is a novel and fundamental feature of basal-like breast cancer. While the majority of hypermethylator tumors are of the basal subset, the rare instances of luminal or Her2+ breast cancers that are also hypermethylators may represent cases where tumors otherwise expected to be of good-prognosis actually result in poor prognosis and shorter survival than what would be predicted based on their molecular subtype.

IMPLICATIONS FOR HYPERMETHYLATOR BREAST CANCER ON THE TREATMENT OF POOR-PROGNOSIS BREAST CANCER

Targeting the Hypermethylator Defect for Improved Therapeutics

DNA methylation is a reversible process, making the existence of methylation defects within tumor cells an enticing target for therapeutic intervention. Such "epigenetic therapy" would seek to target genes which are inappropriately methylated in cancer cells (such as tumor suppressors) and restore their expression (309). Restoration of tumor suppressor gene expression could result in apoptosis of tumor cells or sensitize them to cell killing by chemotherapeutic agents. When this concept was tested in MCF7 breast cancer cells, it was discovered that treatment of these cancer cells with demethylating agents prior to chemotherapy enhanced cell killing (310). Clinical trials are now underway which will examine this combined effect of demethylating drugs and cytotoxic agents (311). Our studies showed that treatment with the demethylating agent 5-aza resulted in the reexpression of genes silenced by methylation in hypermethylator cell lines. This suggests that the genes

inappropriately targeted by methylation in hypermethylator cell lines, particularly the tumor suppressors *CDH1, CST6, ESR1, GNA11,* and *TFF3*, should be achievable with demethylating agents that are already on the market and in clinical use (such as Vidaza).

Hypermethylator tumor cells also exhibit overexpression of DNMT3b, which suggests another candidate for therapeutic intervention in breast tumors with this defect. We have shown that targeted inhibition of DNMT3b via RNAi in MDA-MB-453 and BT549 cells results in promoter demethylation and reexpression of indicator genes (Figures 38-39). Thus, if targeted alleviation of overexpression of this protein could be achieved in primary breast tumors of the of hypermethylator phenotype, it would be expected to result in the reexpression of tumor suppressor genes *CDH1, CST6, ESR1, GNA11,* and *TFF3*. This, in turn, may prime hypermethylator tumors for cell killing via apoptosis or by sensitization to chemotherapeutics. One means of this would be targeting DNMT3b with small molecule inhibitors in hypermethylator tumors, lowering DNMT3b levels and, by extension, reexpressing methylated tumor suppressors. Demethylating agents already in use (such as 5-aza) may actually work in this way, by combating the effects of overabundant DNMT3b protein.

Additional Implications of the Hypermethylation Defect

While our studies demonstrate a role for DNMT3b overexpression in breast carcinogenesis, the mechanism that accounts for DNMT3b overexpression in hypermethylator cells remains unknown. Recent studies have implicated microRNAs (miRs) in the regulation of DNMT3b, specifically members of the miR29a and/or miR148 families (312, 313). These small, noncoding RNAs are known to regulate gene expression by binding to the 3'-UTR of target mRNAs and blocking translation or inducing mRNA degradation

(314). Recent studies have implicated miRs in carcinogenesis and revealed that miRs tend to be expressed at lower levels in tumor cells then normal cells (312). The miR29 family (consisting of miR29a, 29b, and 29c) is known to directly target DNMT3b, and miR29 expression is inversely correlated with DNMT3b expression in lung cancers (312). Additionally, enforced expression of miR29 in lung cancer cells *in vitro* resulted in the reestablishment of normal methylation patterns, including the expression of methylated tumor suppressors *FHIT* and *WWOX,* and inhibited tumorigenicity (312). However, another study revealed that the expression of ~10% of miRs was regulated by DNA methylation (315). This observation raises the possibility that miRs might contribute to methylation abnormalities (via DNMT3b modulation), which may in turn regulate miR expression. Further studies are needed to elucidate the mechanisms underlying this complex system. Another possible mechanism of DNMT3b overexpression may be naturally-occurring genetic polymorphisms. New evidence suggests that genetic polymorphisms may play a role in aberrant DNMT expression. A single base C → T transition located -149 bp from the transcriptional start site of DNMT3b has recently been reported, and was found to confer a two-fold increase in the risk of developing lung cancer (316). This polymorphism may result in increase DNMT3b activity, and has been associated with decreased survival in patients with small-cell carcinoma of the head and neck (317). Thus, various mechanisms may account for the aberrant expression of DNMT3b which characterizes cells of the hypermethylator phenotype; further studies should elucidate our understanding of this complex system which increasingly appears central to carcinogenesis.

SUMMARY AND SIGNIFICANCE

The results of this study suggest that a novel hypermethylator phenotype is detectable in breast cancer (both *in vitro* and *in vivo*), and that this phenotype can be expected to significantly impact patient outcome, with hypermethylator breast tumors being among those with the worst prognosis. Hypermethylator breast cancer cells tend to exhibit concurrent downregulation of numerous methylation sensitive genes, typified by expression loss of at least seven of the following nine genes: *CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A*, and *TFF3*. Additionally, methylation of these specific genes appears to be attributable to overexpressed DNMT3b protein within these cells, as RNAi-mediated knockdown of DNMT3b in hypermethylator cells restores normal expression of previously methylated genes. More investigation is needed to determine what causes the dysregulation of the methylation machinery in these cells that results in aberrantly overexpressed DNMT3b protein. Unraveling the complexities of this hypermethylation defect in neoplastic breast disease holds important implications for cancer diagnosis, identification of new targets for therapy, and development of new strategies for clinical management. Since aberrant methylation-dependent silencing is thought to be an early event in carcinogenesis, elevated detectable levels of methylation in genes characterizing the hypermethylator phenotype may constitute an important biomarker for early detection in patients developing hypermethylator breast tumors. Furthermore, the various proteins and enzymes of the DNA methylation machinery (such as overabundant DNMT3b) may represent novel targets for breast cancer therapy for women with hypermethylator breast tumors. Such patients may benefit significantly from a targeted demethylation treatment as an adjunct to standard chemotherapeutic regimens.

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