Experimental and Molecular Pathology 95 (2013) 276–287

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Dysregulation of the epigenome in triple-negative breast cancers: Basal-like and claudin-low breast cancers express aberrant DNA hypermethylation

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ARTICLE INFO

Article history:
Received 9 September 2013
Available online 14 September 2013

Keywords:
Triple-negative breast cancer
Basal-like breast cancer
Claudin-low breast cancer
Aberrant DNA hypermethylation

ABSTRACT

A subset of human breast cancer cell lines exhibits aberrant DNA hypermethylation that is characterized by hyperactivity of the DNA methyltransferase enzymes, overexpression of DNMT3b, and concurrent methylation-dependent silencing of numerous epigenetic biomarker genes. The objective of this study was to determine if this aberrant DNA hypermethylation (i) is found in primary breast cancers, (ii) is associated with specific breast cancer molecular subtypes, and (iii) influences patient outcomes. Analysis of epigenetic biomarker genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*) identified a gene expression signature characterized by reduced expression levels or loss of expression among a cohort of primary breast cancers. The breast cancers that express this gene expression signature are enriched for triple-negative subtypes — basal-like and claudin-low breast cancers. Methylation analysis of primary breast cancers showed extensive promoter hypermethylation of epigenetic biomarker genes among triple-negative breast cancers, compared to other breast cancer subclasses where promoter hypermethylation events were less frequent. Furthermore, triple-negative breast cancers either did not express or expressed significantly reduced levels of protein corresponding to methylation-sensitive biomarker gene products. Together, these findings suggest strongly that loss of epigenetic biomarker gene expression is frequently associated with gene promoter hypermethylation events. We propose that aberrant DNA hypermethylation is a common characteristic of triple-negative breast cancers and may represent a fundamental biological property of basal-like and claudin-low breast cancers. Kaplan–Meier analysis of relapse-free survival revealed a survival disadvantage for patients with breast cancers that exhibit aberrant DNA hypermethylation. Identification of this distinguishing trait among triple-negative breast cancers forms the basis for development of new rational therapies that target the epigenome in patients with basal-like and claudin-low breast cancers.

Introduction

Breast cancer is a heterogeneous disease characterized by variant pathological features, disparate response to therapeutics, and substantial differences in long-term patient survival. DNA microarray-based transcription profiling of invasive breast cancer identified distinct and reproducible molecular subtypes that are associated with different clinical outcomes (Perou et al., 2000; Sorlie et al., 2001, 2003; Sotiriou et al., 2002, 2003; van de Vijver et al., 2002; van 't Veer et al., 2002). Among these molecular subtypes are estrogen receptor-negative (ER−) cancers (designated as basal-like, claudin-low, and HER2+/ER−), estrogen receptor-positive (ER+) cancers (designated as luminal A and luminal B), and normal breast-like cancers (Perou et al., 2000; Prat et al., 2010; Sorlie et al., 2001). Together the basal-like and claudin-low molecular subtypes represent subsets of “triple-negative”

Abbreviations: DNMT, DNA methyltransferase; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; HER2, gene encoding the epidermal growth factor receptor 2; miR, microRNA; PR, progesterone receptor; TIC, tumor initiating cell; TMI, total methylation index.

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http://dx.doi.org/10.1016/j.yexmp.2013.09.001

breast cancers (classified by immunohistochemistry), lacking expression of the estrogen and progesterone receptors (ER−/PR−) and amplification of HER2 (HER2−) (Elias, 2010). The basal cell phenotype of breast cancer was first described in studies based upon immunohistochemistry (Wetzels et al., 1989). This subtype of breast cancer re-emerged through more recent microarray analyses of gene expression patterns (Perou et al., 2000; Sorlie et al., 2001, 2003; Sotiriou et al., 2002, 2003; van de Vijver et al., 2002; van 't Veer et al., 2002). The basal-like subtype is typically HER2-negative and displays some characteristics of breast myoepithelial cells (Nielsen et al., 2004; Perou, 2011; Prat et al., 2013). Basal-like breast cancers have high rates of cell proliferation and extremely poor clinical outcomes (Sorlie et al., 2001, 2003). These cancers are associated with distinct risk factors, including early-onset menarche, younger age at first full-term pregnancy, high parity combined with lack of breast feeding, and abdominal adiposity (Millikan et al., 2008). Basal-like breast cancers have been shown to be over-represented in patients of certain age and ethnic groups, specifically young African-American women (Carey et al., 2006), but these cancers affect women of every age and ethnic group (Millikan et al., 2008). Basal-like breast cancers generally respond to preoperative chemotherapy (Carey et al., 2007; Rouzier et al., 2005). However, despite the observation of pathologic complete response in some patients with basal-like breast cancer, these patients have a very poor prognosis and dismal long-term survival, perhaps related to a higher likelihood of relapse in those individuals where pathologic complete response is not achieved (Carey et al., 2007). Claudin-low breast cancers are enriched for markers of epithelial-to-mesenchymal transition (EMT), and stem cell-like and/or tumor initiating cell (TIC) features (Prat et al., 2010). Similar to the basal-like cancers, claudin-low breast cancers respond to some chemotherapeutic agents, but patients exhibit poor recurrence-free and overall survival outcomes. This observation may reflect the fact that these cancers display mesenchymal properties and may not exhibit sensitivity to standard chemotherapy treatment (Prat et al., 2010).

Neoplastic transformation is associated with epigenetic alterations involving both changes in gene promoter DNA methylation and post-translational modification of histone proteins. The majority of DNA methylation reflects 5-methylcytosine occurring at CpG dinucleotides, 70–80% of which is methylated in a cell type-specific manner in human cells, and the CpG methylation pattern is faithfully transmitted to daughter cells during cell division (Bird, 2002; Klose and Bird, 2006). In cancer cells, epigenetic alterations that include both global hypomethylation of the genomic DNA and gene-specific promoter hypermethylation have been extensively documented (Baylin, 2001; Baylin et al., 1998; Herman and Baylin, 2003). Hypomethylation of cancer cell genomes is associated with loss of methylation in CpG-depleted regions where most CpG dinucleotides are typically methylated in normal cells (Feinberg and Vogelstein, 1983a, 1987; Goelz et al., 1985). The loss of methylation in these regions of the genome may be associated with aberrant or inappropriate expression of genes that contribute to neoplastic transformation, tumorigenesis, or cancer progression (Feinberg and Vogelstein, 1983b). In addition, genome-wide demethylation can contribute to chromosomal instability by destabilizing pericentromeric regions of certain chromosomes (Eden et al., 2003; Gaudet et al., 2003; Narayan et al., 1998). DNA methylation is catalyzed by DNA methyltransferase enzymes DNMT1, DNMT3a, and DNMT3b. DNMT1 maintains existing DNA methylation patterns, whereas DNMT3a and DNMT3b are mainly involved in the de novo establishment of DNA methylation marks (Jin et al., 2011; Jurkowska et al., 2011). Gains in DNA methylation in cancer cells typically reflect hypermethylation of regions of CpG density (including CpG islands) located in gene promoter regions, which can lead to gene silencing (Herman and Baylin, 2003). DNA methylation-dependent gene silencing is a normal mechanism for regulation of gene expression (Momparler, 2003). However, in cancer cells methylation-dependent epigenetic gene silencing represents a mutation-independent mechanism for inactivation of tumor suppressor-like genes (Jones and Laird, 1999). A significant number of cancer-related genes have been identified that are subject to methylation-dependent silencing (Tsou et al., 2002; Widschwendter and Jones, 2002), and many of these genes contribute to the hallmarks of cancer (Hanahan and Weinberg, 2000). In breast cancer, significant alteration of gene expression patterns can result from aberrant DNA methylation (Asch and Barcellos-Hoff, 2001; Baylin, 2005). Some cancers exhibit concurrent hypermethylation of numerous genes, a phenomenon known as the CpG island methylator phenotype (CIMP) (Abe et al., 2005; Dulaimi et al., 2004; Eads et al., 2000; Kaneko et al., 2003; Liang et al., 1998; Melki et al., 1999; Shen et al., 2002; Strathdee et al., 2001; Toyota et al., 1999a, 1999b; Ueki et al., 2000). Our previous studies suggest that a subset of breast cancer cell lines and primary breast cancers (primarily basal-like breast cancers) exhibit aberrant DNA hypermethylation (similar to CIMP) that is characterized by promoter methylation-dependent silencing of multiple epigenetic biomarker genes (Roll et al., 2008). Further, this aberrant DNA hypermethylation is associated with overexpression of DNMT3b protein and elevated DNMT activity in breast cancer cell lines leading to concurrent methylation-dependent silencing of numerous genes (Roll et al., 2008). In our initial studies, aberrant DNA hypermethylation was found to be strongly associated with triple-negative primary breast cancers (basal-like breast cancers), but rarely with other molecular subtypes (Roll et al., 2008). The mechanism for overexpression of DNMT3b in breast cancer cell lines that exhibit aberrant DNA hypermethylation is related to concurrent loss of microRNAs (miRs) that post-transcriptionally regulate DNMT3b mRNA, including miR29c, miR148a, miR148b, mir26a, miR26b, and miR203 (Sandhu et al., 2012b).

In the current study, 946 primary breast cancers (from publicly available databases) were classified for aberrant DNA hypermethylation status based upon microarray-based gene expression patterns for nine epigenetic biomarker genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*). This analysis identified a strong association between aberrant DNA hypermethylation and triple-negative breast cancer subtypes (basal-like and claudin-low breast cancers based upon intrinsic gene expression patterns). Methylation analysis of primary breast cancers using methylation-specific PCR (MSP) and bisulfite sequencing verified extensive promoter hypermethylation of epigenetic biomarker genes among triple-negative breast cancers, compared to other breast cancer subtypes where promoter hypermethylation events were less frequent. Immunostaining analysis of 137 primary breast cancer tissues demonstrated selective loss of expression of methylation-sensitive gene products in ER−/PR−/HER2− (triple-negative) breast cancers, but not in ER+/PR+/HER2− (luminal A-like) or ER−/PR−/HER2+ (HER2-enriched) breast cancers. These observations combine to strongly suggest that expression of aberrant DNA hypermethylation is a common characteristic of triple-negative breast cancers and may represent a fundamental biological property of the majority of basal-like and claudin-low breast cancers. Subsequently, 779 primary breast cancers from the UNC Microarray Database were classified for aberrant DNA hypermethylation status and subjected to Kaplan–Meier analysis of recurrence-free survival which revealed a disadvantage for patients with breast cancers that exhibit aberrant DNA hypermethylation. The identification of this distinguishing trait may form the basis for development of new rational therapies that target the epigenome in patients with basal-like and claudin-low breast cancers (and other triple-negative breast cancers).

Materials and methods

Mining of microarray gene expression data

To identify primary breast cancers that display the gene expression signature associated with aberrant DNA hypermethylation, publicly available microarray gene expression data was mined for a concurrent loss of expression of methylation-sensitive genes that were previously associated with aberrant DNA hypermethylation status in vitro (Roll

et al., 2008). 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 breast cancers from the UNC Microarray Database, a rule was generated to identify individual cancers that display the aberrant DNA hypermethylation-associated gene expression signature. Individual cancers that lack expression or show reduced expression levels (less than the median level of expression for that gene among all cancers in the individual dataset) for at least seven of nine (≥7) epigenetic biomarker genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*) are classified as exhibiting aberrant DNA hypermethylation. Breast cancers that express normal levels (greater than the median level of expression for that gene among all cancers in the individual dataset) of three or more (≥3) of the nine epigenetic biomarker genes are classified as lacking aberrant DNA hypermethylation. This rule was applied to additional microarray-based gene expression datasets, including: (i) the expanded UNC Microarray Database (https://genome.unc.edu/pubsup/breastGEO/) that includes gene expression data for primary breast cancers analyzed in previous studies (N =272 cancers) (Hu et al., 2006; Oh et al., 2006; Perreard et al., 2006; Weigelt et al., 2005), (ii) the Hess et al. dataset (N = 133 cancers) (Hess et al., 2006), (iii) the Wang et al. dataset (N = 295 cancers) (Wang et al., 2005), and (iv) the van de Vijver et al. dataset (N =246 cancers) (van de Vijver et al., 2002). The molecular classifications of the breast cancers contained in these datasets are described in Table 1.

Isolation and bisulfite conversion of genomic DNA from human breast tissue

Archival human primary breast cancers (N = 26 total) were obtained from the paraffin archives of the UNC Cancer Hospital and the UNC Lineberger Comprehensive Cancer Center (Chapel Hill, NC), including 15 basal-like breast cancers, 3 HER2+ breast cancers, 6 luminal A breast cancers, and 2 luminal B breast cancers. Clinical classification of primary breast cancers into molecular subtypes was accomplished based upon surrogate immunostaining patterns for ER, PR, and HER2. Breast cancers were classified as luminal A-like (ER+/PR+/HER2−), luminal B-like (ER+/PR+/HER2+), HER2-enriched (ER-/PR-/HER2+), or triple-negative (ER−/PR−/HER2−). Protection of patient privacy and handling of specimens followed strict policies of the Institutional Review Board of the University of North Carolina School of Medicine. The current study was determined to be exempt following a review by the Institutional Review Board based upon the use of existing data and existing tissue specimens that were stripped of all identifying information. Hence, patient consent was not required and was not sought. Paraffin-embedded breast cancer specimens were microdissected with a clean razor blade using a serial H&E-stained section as a guide. Regions of breast tissue sections composed of ≥80% cancer cells were selected for microdissection and deparaffinized. Genomic DNA was isolated as described previously according to manufacturer's instructions (Rivenbark et al., 2007). Briefly, DNA was extracted utilizing the QIAamp DNA Micro Kit (Qiagen, Inc., Valencia, VA). Slides were incubated at 56 °C for 5–10 min before microdissection, and tissue was transferred to a solution containing buffer ATL (QIAamp DNA Mini Kit, Qiagen) and proteinase K followed by incubation with rocking at 56 °C for 1.5 h. DNA was precipitated, washed, and eluted with 35 μl of elution buffer. For DNA methylation analysis, 200–500 ng of genomic DNA was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research Co., Orange, CA) according to the manufacturer's protocol, as described previously (Rivenbark et al., 2007). In general, 2 μl of modified DNA was used in subsequent PCR reactions.

Methylation-specific PCR, cloning, and sequencing

Methylation-specific PCR (MSP) reactions were carried out in EasyStart Micro 50 PCR-mix-in-a-tube (Molecular BioProducts, San Diego, CA) using bisulfite converted DNA template. The primers and thermocycling conditions for *CDH1*, *CEACAM6*, *CST6*, *ESR1*, and *SCNN1A* genes have been described previously (Ai et al., 2006; Lapidus et al., 1998; Rivenbark et al., 2006b; Roll et al., 2008; Yuecheng et al., 2006). MSP primers directed against methylated and unmethylated alleles of *GNA11*, *MUC1*, *MYB*, and *TFF3* are as follows: methylated *GNA11* forward primer 5′-GATTACGGGCGTGTATTATTAC, reverse primer 5′-CCAACA CTTTAAAAAACCGAAACGAA; unmethylated *GNA11* forward primer 5′-TTGGGATTATGGGTGTGTATTATTAT, reverse primer 5′-ATCCCAACACT TTAAAAAACCAAAACAAA (59 °C, 35 cycles, 137 bp product); methylated *MUC1* forward primer 5′-GGAGGTTAGTTGGAGAATAAAC, reverse

Table 1
Gene expression patterns reveal a strong association between basal-like and claudin-low breast cancers and expression of the aberrant DNA hypermethylation-associated gene expression signature.

Dataset	Total breast cancers	Breast cancers expressing the aberrant DNA hypermethylation-associated gene expression signature	Composition of breast cancers expressing the aberrant DNA hypermethylation-associated gene expression signature	Basal-like and claudin-low breast cancers that express the aberrant DNA hypermethylation-associated gene expression signature
Expanded UNC	272	29% (80/272)	65/80 (81%) basal-like 1/80 (1%) luminal B 1/80 (1%) HER2+ 13/80 (16%) claudin-low	66% (78/118)
Hess et al.	133	25% (33/133)	26/33 (79%) basal-like 2/33 (6%) luminal A/B 4/33 (12%) HER2+ 1/33 (3%) normal-like	81% (26/32)
Wang et al.	295	20% (59/295)	44/59 (75%) basal-like 12/59 (20%) luminal A/B 3/59 (5%) HER2+	58% (44/76)
van de Vijver et al.	246	20% (48/246)	39/48 (81%) basal-like 7/48 (15%) luminal A/B 2/48 (4%) HER2+	59% (39/66)
Total	946	23% (220/946)	174/220 (79%) basal-like 22/220 (10%) luminal A/B 6/220 (3%) HER2+ 13/220 (6%) claudin-low 1/220 (0.4%) normal-like	64% (187/292)

The datasets described in this table are from (i) expanded UNC (Hu et al., 2006; Oh et al., 2006; Perreard et al., 2006; Weigelt et al., 2005), (ii) Hess et al. (Hess et al., 2006), (iii) Wang et al. (Wang et al., 2005), and (iv) van de Vijver et al. (van de Vijver et al., 2002).

primer 5′-AACAAATAACAAATAACAAAACCCCG; unmethylated *MUC1* forward primer 5′-GGAGGTTAGTTGGAGAATAAATG, reverse primer 5′-ACAAATAACAAATAACAAAACCCCAC (58 °C, 35 cycles, 139 bp product); methylated *MYB* forward primer 5′-TAGAGGGTATAGTT GTAAATTTTGAC, reverse primer 5′CTCACTATCGCGAAAACGAC; unmethylated *MYB* forward primer 5′-AGAGGGTATAGTTGTAAAT TTTGATGA, reverse primer 5′-CTCCCACTCACTATCACAAAAA (58 °C, 35 cycles, 90 bp product); methylated *TFF3* forward primer 5′-TTAGAGTTGTTTGTTTTCGAGGTC, reverse primer 5′-AACAAAACCAA AATATAATATCCGTTCCA; unmethylated *TFF3* forward primer 5′-ATTTAGAGTTGTTTGTTTTTGAGGTTGA, reverse primer 5′-AACCAAA ATATAATATCCATTCCATCTCA (59 °C, 35 cycles, 132 bp product). PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining. A portion of each PCR product (2 to 5 μl) was cloned into pGEM-T Easy Vector (Promega, Madison, WI) and 5–10 colonies were selected per gene segment and expanded in liquid culture (Rivenbark et al., 2006b). Plasmid DNA was purified using the Wizard Plus Miniprep DNA Purification Kit (Promega, Madison, WI). Sequencing of validated clones was accomplished using the universal M13R3 primer with an Applied Biosystems automated sequencer at the UNC Genome Analysis Facility (Chapel Hill, NC). The bisulfite conversion efficiency was calculated for each sequenced clone based upon the ratio of converted Cs (non-CpG) to the total number of Cs (non-CPG) in a given gene segment. Only clones determined to have a conversion efficiency >95% were included in the analyses presented. The results of methylation analyses are expressed as total methylation index (TMI). This measure of methylation can be applied to single CpG dinucleotides, select groups of CpG dinucleotides, or to continuous groups of CpG dinucleotides in a given gene segment. TMI was calculated for each cancer and clone by dividing the number of methylated CpGs observed by the total CpGs analyzed and expressed as percent methylation (Rivenbark et al., 2006a). For instance, in an analysis containing 19 CpG dinucleotides and 10 clones sequenced, TMI would be calculated based upon 190 possible CpG methylation events (10 × 19). Epigenetic biomarker genes that display TMI >40% are considered hypermethylated.

Immunohistochemistry

A tissue array containing 137 invasive primary human breast cancers was constructed at the Dartmouth-Hitchcock Medical Center (courtesy of Dr. Gregory J. Tsongalis and Dr. Wendy A. Wells, Dartmouth-Hitchcock Medical Center, Lebanon, NH). This tissue array contains 96 ER+/PR+/HER2− breast cancers, 23 ER−/PR−/HER2− breast cancers, and 18 ER−/PR−/HER2+ breast cancers. Immunohistochemical staining was performed as described previously (Rivenbark et al., 2007). Briefly, tissue sections were incubated at 60 °C for 45 min, deparaffinized in Slide Brite (Biocare Medical, Concord, CA), incubated with 3% H_2O_2 in methanol to block endogenous peroxidase activity, and rehydrated through a series of ethanol washes. Antigen retrieval was accomplished by steaming in 1X citrate buffer (Dako Inc., Carpinteria, CA) for 30 min. After incubation with serum-free protein block (Dako Inc.) for 10 min, tissues were incubated for 2 h at room temperature with monoclonal mouse antibodies diluted as follows: 1:100 for CEACAM6 (product #SIG-3750, Signet Laboratories, Dedham, MA); 1:100 for CDH1 (product #135700, Invitrogen, Grand Island, NY), 25 μg/ml for CST6 (product #MAB1286, R&D Systems, Minneapolis, MN); and 1:50 for SCNN1A (product #10924-2-AP, Proteintech Group Inc., Chicago, IL). Subsequently, tissue arrays were washed and layered with a two-step secondary antibody, incubated with HRP substrate, and counterstained with hematoxylin. Control immunostaining reactions were performed at room temperature with mouse monoclonal anti-cytokeratin 18 antibody (product #SC-6259, Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:1000. Normal breast tissue (from reduction mammoplasty cases) was used as a positive control for antibody staining.

Kaplan–Meier survival analysis

Gene expression data from a combined cohort of 855 breast cancers (Harrell et al., 2012) was used to evaluate clinical characteristics in breast cancers that exhibit aberrant DNA hypermethylation. From this patient cohort, 779 breast cancers were classified as luminal A, luminal B, HER2+, basal-like, or claudin-low (Harrell et al., 2012). This subset of primary breast cancers was utilized for the subsequent Kaplan–Meier survival analysis. Classification of individual breast cancers as exhibiting aberrant DNA hypermethylation was assigned when ≥7 of 9 methylation-sensitive target genes displayed expression values less than the median expression for the entire dataset. Aberrant DNA hypermethylation status was related to breast cancer subtype using the subtypes assigned (Harrell et al., 2012). Categorical survival analyses were performed using a log-rank test and visualized with Kaplan–Meier plots.

Results

Analysis of microarray gene expression data reveals a strong association between basal-like and claudin-low breast cancers and aberrant DNA hypermethylation

In previous studies (Roll et al., 2008), unsupervised cluster analysis of microarray gene expression data for six methylation-sensitive genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *SCNN1A*, and *LCN2*) among 88 primary breast cancers (UNC Microarray Database) identified a cluster of 18 cancers that displayed an aberrant DNA hypermethylation-associated gene expression signature (Roll et al., 2008). These 18 breast cancers displayed either greatly reduced or lack of expression of this set of methylation-sensitive biomarker genes compared to other cancers in the dataset. Strikingly, 18/18 (100%) breast cancers that exhibit the aberrant DNA hypermethylation-associated gene expression signature were classified as basal-like, and 18/23 (78%) basal-like cancers in the dataset displayed the aberrant DNA hypermethylation-associated gene expression signature (Roll et al., 2008). In the current study, a similar analysis was performed with a refined set of nine methylation-sensitive epigenetic biomarker genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*) among primary breast cancers from the UNC Microarray Database. Lack of or reduced expression of this panel of nine methylation-sensitive biomarker genes provides a more stringent classification of cell lines that display aberrant DNA hypermethylation. Unsupervised cluster analysis of these gene expression data among 90 primary breast cancers identified three strong clusters (Fig. 1): (i) a luminal A/B-enriched cluster containing 51 breast cancers (48/51, 94%, are luminal A or B breast cancers), (ii) a basal-like-enriched cluster containing 21 breast cancers (100% are basal-like breast cancers), and (iii) a HER2+-enriched cluster containing 18 breast cancers (15/18, 83%, are HER2+ breast cancers). Among the basal-like-enriched cluster, 21/21 (100%) breast cancers displayed an aberrant DNA hypermethylation-associated gene expression signature with ≥7/9 epigenetic biomarker genes expressed at low or negligible levels (Fig. 1). Overall, 24/90 (27%) breast cancers exhibited an aberrant DNA hypermethylation-associated gene expression signature, and 22/24 (92%) of breast cancers that exhibit an aberrant DNA hypermethylation-associated gene expression signature were from the basal-like molecular subtype (with the remaining two belonging to the luminal B subtype). Further, 22/24 (92%) basal-like breast cancers were found to express the aberrant DNA hypermethylation-associated gene expression signature (Fig. 1). The subset of breast cancers that were classified as basal-like in this dataset most likely also contains the more recently described claudin-low cancers (Prat et al., 2010). However, the molecular subtype classifications of these primary breast cancers were determined before the claudin-low subtype was recognized. These observations strongly suggest that expression of the aberrant DNA hypermethylation-

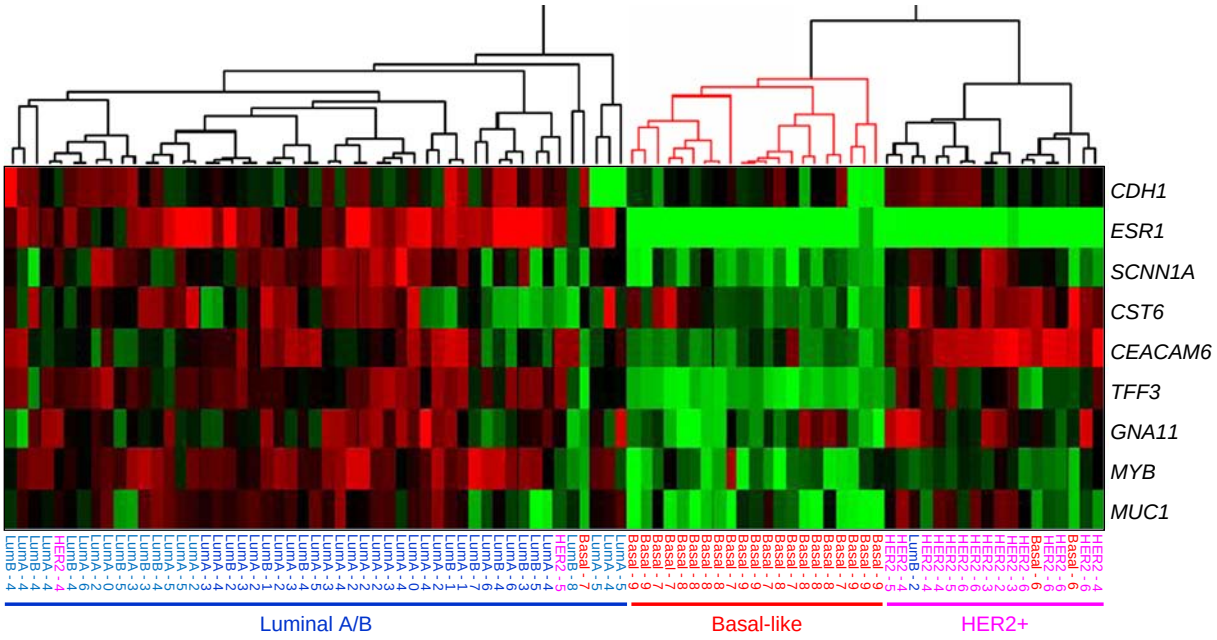

Fig. 1. The aberrant DNA hypermethylation-associated gene expression signature is closely associated with the basal-like breast cancer molecular subtype. Gene expression data from 90 primary human breast cancers from the UNC Breast Cancer Microarray Database were subjected to unsupervised cluster analysis based upon the mRNA expression of nine genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*) which characterize the aberrant DNA hypermethylation-associated gene expression signature in breast cancer cell lines. Gene designations are depicted vertically and cancer subtype designations and number of genes down-regulated in each cancer are shown horizontally. The expression level for each gene is shown relative to the median expression of that gene across all samples, with high expression shown in red and low expression shown in green, while genes with median expression are shown in black. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

associated gene expression signature is closely associated with the basal-like breast cancer molecular subtype and may represent a major biological property of this molecular subtype of breast cancer.

This analysis was expanded to include 946 primary breast cancers from publicly available databases, including the UNC Breast Cancer Microarray Database (N = 272 cancers), and published studies by Hess et al. dataset (Hess et al., 2006) (N = 133 cancers), Wang et al. dataset (Wang et al., 2005) (N = 295 cancers), and van de Vijver et al. dataset (van de Vijver et al., 2002) (N = 246 cancers). Among the breast cancers found in the expanded UNC Microarray Database, 80/272 (29%) exhibited an aberrant DNA hypermethylation-associated gene expression signature with ≥7/9 epigenetic biomarker genes expressed at low or negligible levels (Fig. 2A). The majority of these breast cancers are of the basal-like subtype (65/80, 81%) and this subset of breast cancers contains 63% (65/103) of all basal-like breast cancers in the dataset (Table 1). Of note, the majority of claudin-low breast cancers (13/15,

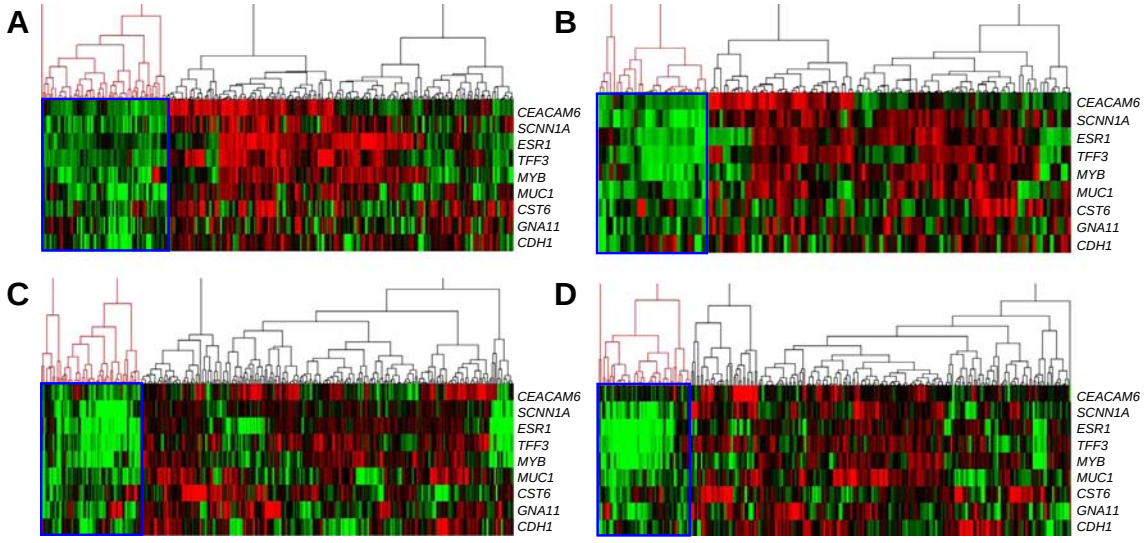

Fig. 2. Basal-like breast cancers express the aberrant DNA hypermethylation-associated gene expression signature. Gene expression data from 946 primary human breast cancers from publically available databases including (A) UNC Breast Cancer Microarray Database (N = 272 cancers) (Hu et al., 2006; Oh et al., 2006; Perreard et al., 2006; Weigelt et al., 2005), (B) Hess et al. dataset (Hess et al., 2006) (N = 133 cancers), (C) Wang et al. dataset (Wang et al., 2005) (N = 295 cancers), and (D) van de Vijver et al. dataset (van de Vijver et al., 2002) (N = 246 cancers). Shown is the unsupervised cluster analysis based upon the mRNA expression of nine genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*) which characterize the aberrant DNA hypermethylation-associated gene expression signature in breast cancer cell lines. Gene designations are depicted vertically. The expression level for each gene is shown relative to the median expression of that gene across all samples, with high expression shown in red and low expression shown in green, while genes with median expression are shown in black. Clusters of breast cancers expressing the hypermethylation phenotype-associated gene expression signature are indicated by a blue box beneath the red dendrogram. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

87%) displays an aberrant DNA hypermethylation-associated gene expression signature with ≥7/9 epigenetic biomarker genes expressed at low or negligible levels. These results demonstrate significant overlap between breast cancers exhibiting an aberrant DNA hypermethylation-associated gene expression signature and specific molecular subtypes of breast cancer — specifically, the basal-like and claudin-low subtypes. This observation strongly supports the idea that aberrant DNA hypermethylation is a defining feature of these molecular subtypes of breast cancer. In total, 78/118 (66%) basal-like and claudin-low breast cancers exhibit the aberrant DNA hypermethylation-associated gene expression signature.

Three additional datasets from other institutions were mined to verify the relationship between triple-negative breast cancers and the aberrant DNA hypermethylation-associated gene expression signature. This analysis specifically examined the relationship between basal-like breast cancers and the aberrant DNA hypermethylation-associated gene expression signature since the classification employed for these datasets did not identify the claudin-low molecular subtype. Of the 133 breast cancers contained in the Hess et al. dataset (Hess et al., 2006), 33/133 (25%) exhibited an aberrant DNA hypermethylation-associated gene expression signature with ≥7/9 epigenetic biomarker genes expressed at low or negligible levels (Fig. 2B), with the majority of these breast cancers (26/33, 79%) representing the basal-like molecular subtype (Table 1). Consistent with the results from the UNC dataset, most basal-like breast cancers in this dataset (26/32, 81%) expressed the aberrant DNA hypermethylation-associated gene expression signature (Table 1). Among the 295 breast cancers from the Wang et al. dataset (Wang et al., 2005), 59/295 (20%) exhibited an aberrant DNA hypermethylation-associated gene expression signature (Fig. 2C), and 44/59 (75%) of these breast cancers were basal-like (Table 1). Furthermore, a substantial fraction of basal-like breast cancers (44/76, 58%) contained in this dataset displayed an aberrant DNA hypermethylation-associated gene expression signature (Table 1). From the 246 breast cancers contained in the van de Vijver et al. dataset (van de Vijver et al., 2002), 48/246 (20%) exhibit the aberrant DNA hypermethylation-associated gene expression signature (Fig. 2D). Most of these breast cancers (39/48, 81%) represent the basal-like molecular subtype, and most basal-like breast cancers (39/66, 59%) expressed this gene expression signature (Table 1). In summary, among 946 breast cancers, 220/946 (23%) exhibit the aberrant DNA hypermethylation-associated gene expression signature; 174/220 (79%) of breast cancers expressing this gene expression signature are basal-like and 174/277 (63%) of basal-like breast cancers express the hypermethylation phenotype-associated gene expression signature. This analysis confirms a strong association between aberrant DNA hypermethylation and basal-like breast cancers.

Methylation analysis reveals extensive promoter hypermethylation in triple-negative breast cancers

To confirm that lack of gene expression of methylation-sensitive genes related to the aberrant DNA hypermethylation-associated gene expression signature in primary human breast cancers reflects true DNA methylation-dependent gene silencing, methylation-specific PCR (MSP) and bisulfite sequencing were employed to examine promoter methylation status. Twenty-six primary breast cancers (15 triple-negative, 8 luminal A/B, and 3 HER2+) were utilized for methylation analysis. MSP of the nine genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*) that constitute the aberrant DNA hypermethylation-associated gene expression signature revealed differences in the methylation status of specific CpGs within regulatory regions of each gene promoter in accordance with the aberrant DNA hypermethylation status of a given breast cancer (Fig. 3A). Methylated MSP products were detected for at least six of the nine genes analyzed in each of the triple-negative breast cancers examined. Among this cohort of breast cancers, 80% (12/15) met the criteria for the aberrant DNA hypermethylation category (detectable promoter hypermethylation in ≥7 epigenetic biomarker genes) (Fig. 3A). For example, triple-negative cancers TN03, TN07, and TN09 produced methylated MSP products for each of the nine of the genes analyzed (Fig. 3A). All 15 triple-negative breast cancers showed hypermethylation of the *TFF3* promoter, and 14/15 (93%) exhibited hypermethylation of the *CST6* and *ESR1* promoters (Fig. 3A). In contrast, luminal A/B and HER2+ cancers exhibited fewer methylated gene promoter sequences (Fig. 3A). Among 11 luminal A/B and HER2+ cancers, individual cancers were found to be positive for promoter hypermethylation by MSP for one to three genes (Fig. 3A). *SCNN1A* was not found to be hypermethylated in any cancer in this cohort and *TFF3* was methylated most frequently (6/11, 55%) (Fig. 3A). These results indicate a strong association between aberrant DNA hypermethylation and triple-negative breast cancer subtype.

MSP products were sequenced to examine the methylation status of a greater number of CpGs within the promoter/exon 1 region of select genes (*CDH1*, *CST6*, *GNA11*). Bisulfite sequencing results are shown for *CDH1*, *CST6*, and *GNA11* for six triple-negative and three ER+ breast cancers (one luminal A, one luminal B, and one HER2+) (Fig. 3B–D). Bisulfite sequencing of 19 CpGs spanning the transcriptional start site of *CDH1* reveals extensive promoter methylation among triple-negative breast cancers (all containing a methylated MSP product, Fig. 3A), with a total methylation index (TMI) ranging from 92 to 97% (Fig. 3B). The HER2+ breast cancer H03 contained a methylated MSP product for *CDH1* and when sequenced exhibited a TMI of 50% (Fig. 3B). Likewise, luminal cancer L02 contained a methylated MSP product for *CDH1* and when sequenced demonstrated a TMI of 48%. However, bisulfite sequencing revealed a TMI of 9% for *CDH1* in luminal cancer L06, which was shown to be unmethylated by MSP. Triple-negative breast cancers exhibit extensive methylation of the promoter region of *CST6* (TMI = 42–87%) and *GNA11* (TMI = 88–100%), while the luminal A/B and HER2+ cancers examined have low levels of CpG methylation for *CST6* (TMI = 0–35%, Fig. 3C) and *GNA11* (TMI = 2–8%, Fig. 3D). Similar results were generated for each epigenetic biomarker gene (*CEACAM6*, *ESR1*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*) (data not shown). For example, the promoter region analyzed in *CEACAM6* included 4 CpGs and had a TMI of 100% in all triple-negative breast cancers examined, but was unmethylated (TMI = 0%) in the luminal A/B and HER2+ breast cancers. The *ESR1* gene (8 CpGs evaluated) was substantially methylated in triple-negative breast cancers (TMI = 71–100%), but substantially unmethylated in the luminal A/B and HER2+ breast cancers examined (TMI = 0–29%). *MUC1* (11 CpGs examined) exhibited partial methylation in triple-negative breast cancers (TMI = 50–58%). However, in luminal A/B and HER2+ breast cancers, *MUC1* exhibited very little 0–5% methylation (TMI = 0–5%). Similar trends were seen in *MYB* (5 CpGs evaluated), *SCNN1A* (5 CpGs evaluated), and *TFF3* (3 CpGs evaluated) with extensive promoter methylation (TMI = 50%–100%) in triple-negative breast cancers and less frequent methylation (TMI = 0%–29%) in luminal A/B and HER2+ breast cancers. These results suggest that the loss of epigenetic biomarker gene expression observed in hypermethylator breast cancers is a direct consequence of aberrant promoter CpG hypermethylation.

Loss of methylation-sensitive gene products in triple-negative breast cancers

137 breast cancers with known ER, PR, and HER2 status were analyzed for CDH1, CEACAM6, CST6, and SCNN1A protein expression. Of these primary breast cancers, 96 were ER+/PR+/HER2−, 23 were ER−/PR−/HER2−, and 18 were ER−/PR−/HER2+. In general, the majority of ER+/PR+/HER2− and ER−/PR−/HER2+ breast cancers expressed normal levels of CDH1, CEACAM6, CST6, and SCNN1A protein, while ER−/PR−/HER2− (triple-negative) breast cancers were more likely to have decreased expression of one or more of these gene products (Fig. 4). Of the triple-negative cancers, 21/23 (91%) displayed

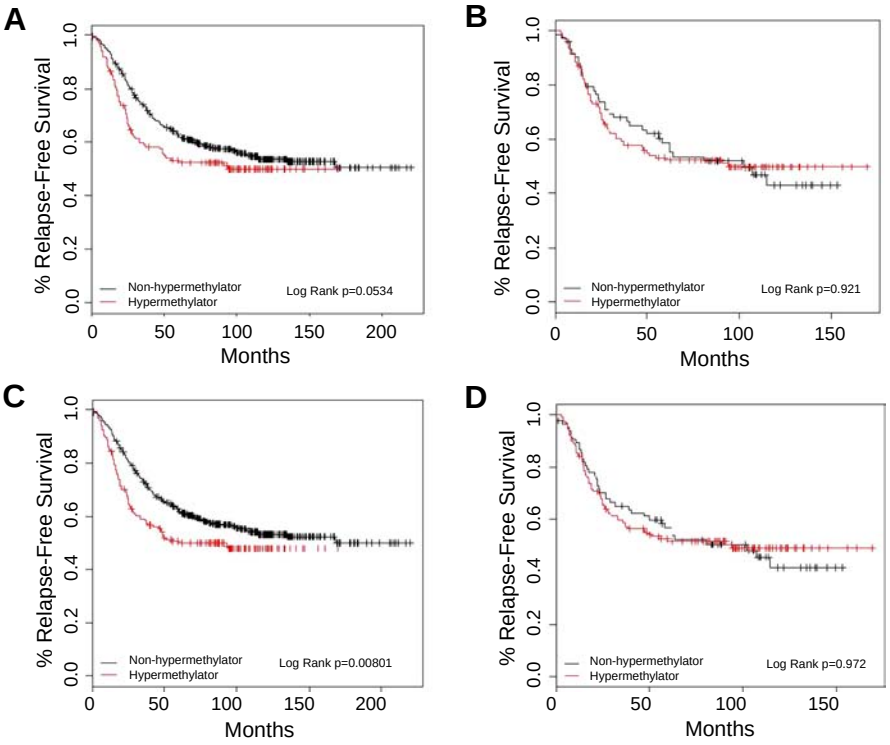

Fig. 3. Breast cancers with aberrant DNA hypermethylation exhibit poor long-term survival. Shown are Kaplan–Meier plots and log rank tests for 779 primary human breast cancers (Harrell et al., 2012) with documented first site of relapse for cancers that exhibit aberrant DNA hypermethylation (red) or lack aberrant DNA hypermethylation (black). (A) All breast cancer subtypes, all cancer stages; (B) Basal-like and claudin-low breast cancers, all cancer stages; (C) All breast cancer subtypes, stages I and II; (D) Basal-like and claudin-low negative breast cancers, stages I and II. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

diminished expression of at least two methylation-sensitive gene products, with 11/23 (48%) triple-negative breast cancers exhibiting decreased or lack of expression of 3 or 4 of these proteins (Fig. 4 and data not shown). In contrast, only 27/96 (28%) ER+/PR+/HER2− and 1/18 (6%) ER−/PR−/HER2+ breast cancers lost expression of 3–4 protein products (Fig. 4 and data not shown). These results are consistent with the suggestion that methylation-sensitive genes are frequently silenced in triple-negative breast cancers resulting in loss of protein product expression.

Breast cancers exhibiting aberrant DNA hypermethylation are associated with poor long-term survival

To address the possibility that expression of the hypermethylation phenotype is associated with long-term patient outcomes and survival, we combined four public microarray datasets with distance weighted discrimination (Benito et al., 2004) to generate a cohort of 855 breast cancers with known molecular subtype and documented the first site of relapse (Harrell et al., 2012). The breast cancers contained in this combined dataset were classified for aberrant DNA hypermethylation status based upon the expression pattern of nine methylation-sensitive epigenetic biomarker genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*), as described above. Among this cohort of breast cancers, 174/855 (20%) exhibited an aberrant DNA hypermethylation-associated gene expression pattern (with reduced or lack of expression of these methylation-sensitive genes). The breast cancers exhibiting aberrant DNA hypermethylation-associated gene expression signatures consisted of 140 basal-like (80%) and 34 claudin-low (20%). Breast cancers lacking the aberrant DNA hypermethylation-associated gene expression signature (681/855, 80%) consisted of 243 luminal A (36%), 162 luminal B (24%), 144 HER2-enriched (21%), 0 basal-like (0%), 56 claudin-low (8%), and 76 normal-like (11%). Of note, 140/140 (100%) basal-like breast cancers in this cohort displayed an aberrant DNA hypermethylation-associated gene expression signature, and 34/90 (38%) claudin-low breast cancers displayed an aberrant DNA hypermethylation-associated gene expression signature. Overall, 174/230 (76%) basal-like and claudin-low breast cancers exhibited the aberrant DNA hypermethylation-associated gene expression signature. Immunohistochemical staining data was available for 766 breast cancers from this dataset — 111/766 (14%) represent triple-negative (ER−/PR−/HER2−) breast cancers and 94/111 (85%) were classified as basal-like (67/94, 71%) or claudin-low (27/94, 29%) (Harrell et al., 2012). Among these triple-negative breast cancers, 73/111 (66%) exhibit an aberrant DNA hypermethylation-associated gene expression pattern, while 38/111 (34%) did not. Among the triple-negative breast cancers that exhibit the aberrant DNA hypermethylation-associated gene expression signature, 70/73 (96%) were classified as basal-like or claudin-low, whereas 24/28 (63%) of triple-negative breast cancers lacking the aberrant DNA hypermethylation-associated gene expression signature were classified as basal-like or claudin-low. These results strongly support the suggestion that aberrant DNA hypermethylation is closely associated with basal-like and claudin-low breast cancers, as well as other triple-negative breast cancers.

Kaplan–Meier analysis of relapse-free survival in this cohort of patients (779 breast cancers representing luminal A, luminal B, HER2+, basal-like and claudin-low subtypes) revealed a significant ($P = 0.05$) disadvantage for patients with breast cancers exhibiting the aberrant DNA hypermethylation-associated gene expression signature (Fig. 5A). For all breast cancer molecular subtypes and stages, 40% of breast cancers lacking aberrant DNA hypermethylation-associated gene expression signatures relapses within approximately 70 months, whereas the time to relapse for breast cancers exhibiting the aberrant DNA hypermethylation-associated gene expression signature is much earlier (40% relapse within approximately 30 months) (Fig. 5A). The relationship between aberrant DNA hypermethylation status and relapse-free survival persists when only basal-like and claudin-low breast cancers are analyzed (Fig. 5B). As observed with all breast cancers, 40% of

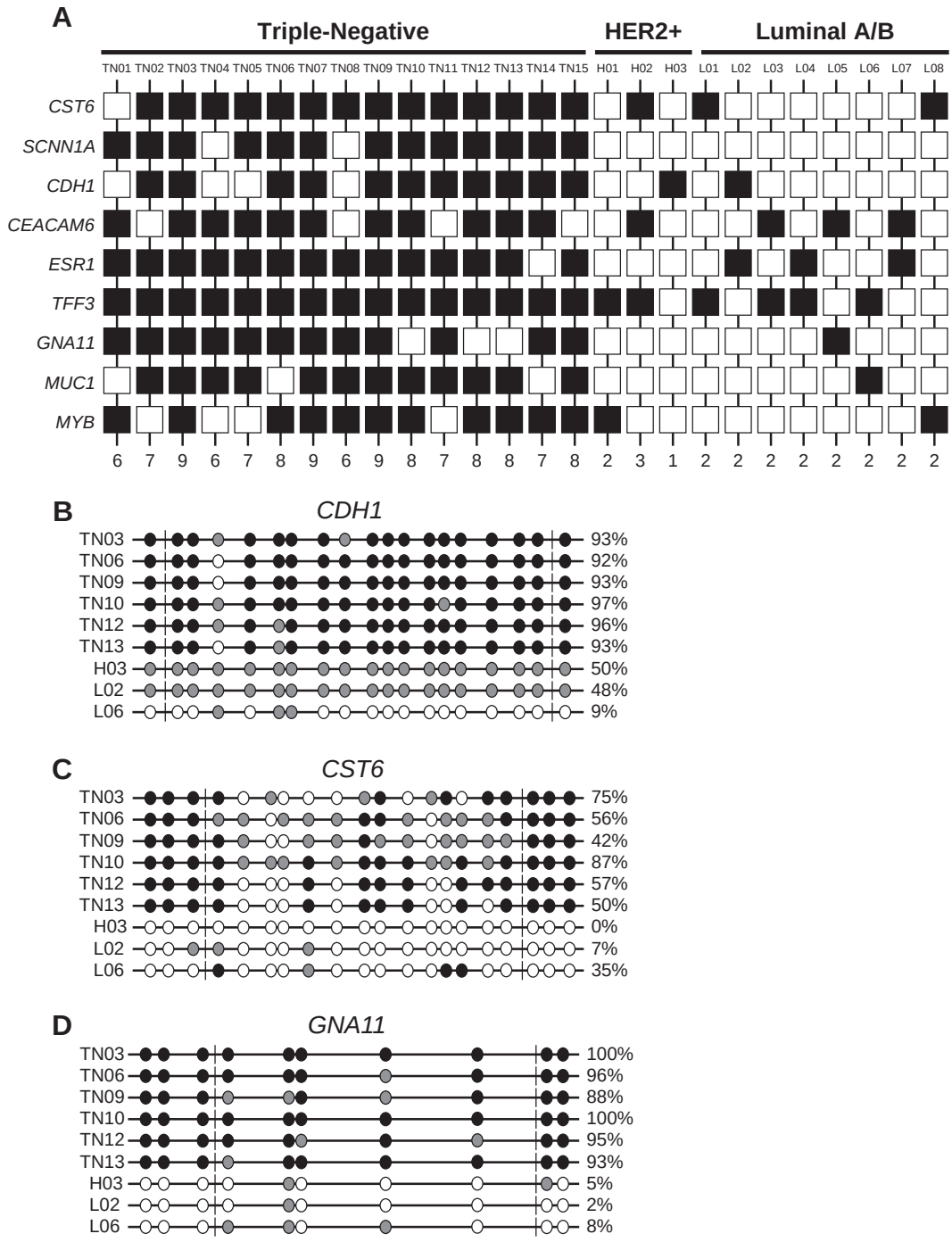

Fig. 4. Methylation analyses reveal methylation-dependent gene silencing in triple-negative primary breast cancers that express the aberrant DNA hypermethylation-associated gene expression signature. (A) Summary of MSP data examining the nine epigenetic biomarker genes that characterize the hypermethylation phenotype-associated gene expression signature in breast cancer cell lines (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*) among a subset of primary human breast cancers. Black boxes indicate the presence of a methylated MSP product (even when an unmethylated product was also detected); white boxes indicate the presence of an unmethylated MSP product only. Primary breast cancers of the triple-negative subtype are designated as TN01–TN15, HER2 + breast cancers are designated as H01–H03, and luminal A/B breast cancers are designated as L01–L08. L01–L06 correspond to luminal A breast cancers, while L07 and L08 are luminal B breast cancers. The numbers under the boxed columns indicate the number of genes where a methylated MSP product was detected. (B–D) Summary of bisulfite sequencing results for select genes: (B) *CDH1*, (C) *CST6*, and (D) *GNA11* in primary breast cancers. 100% methylated CpGs are represented by black circles, <100% methylated CpGs are shown in gray, and 100% unmethylated CpG sites are shown in white. MSP primer boundaries are delineated by the vertical dashed lines. The total methylation index (TMI) corresponding to these genes in individual breast cancers was calculated based on intervening CpGs only. The methylation results reflect 3–5 sequenced clones for each gene and primary cancer evaluated.

basal-like and claudin-low breast cancers that lack aberrant DNA hypermethylation-associated gene expression signatures relapses within approximately 60 months and the time to relapse for basal-like and claudin-low breast cancers that display the aberrant DNA hypermethylation-associated gene expression signature is within approximately 30 months (Fig. 5B). However, there was no statistically significant relapse-free survival disadvantage associated with aberrant DNA hypermethylation status among the basal-like and claudin-low breast cancers. stage I and II patients (all subtypes) were examined for relapse-free survival and Kaplan–Meier plots show a highly significant ($P = 0.008$) disadvantage for patients with stage I or II breast cancers that exhibit the aberrant DNA hypermethylation-associated gene

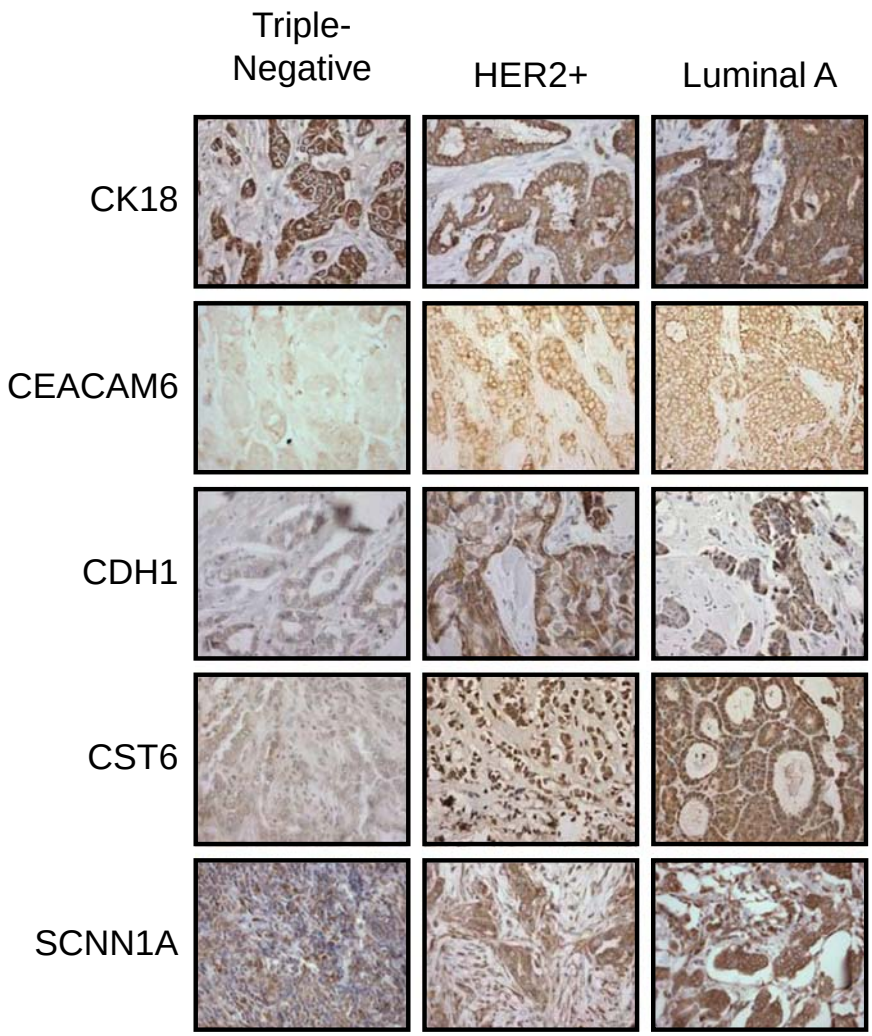

Fig. 5. Immunohistochemical analysis correlates with gene expression status and aberrant DNA hypermethylation status among breast cancers. Representative immunohistochemistry for selected protein products (CEACAM6, CDH1, CST6, and SCNN1A) in triple-negative (ER−/PR−/HER2−), HER2-enriched (ER−/PR−/HER2+), and luminal A-like (ER+/PR+/HER2−) breast cancers. Images were captured at 20× magnification. Positive staining is represented by cytokeratin 18 (CK18).

expression signature (Fig. 5C). 50% of patients with breast cancers displaying aberrant DNA hypermethylation-associated gene expression signatures relapsed within 50 months, while only 35% of patients with breast cancers lacking aberrant DNA hypermethylation-associated gene expression signatures relapsed within a similar time-frame (Fig. 5C). However, aberrant DNA hypermethylation status does not appear to influence relapse-free survival among stage I or II basal-like and claudin-low breast cancer patients (Fig. 5D). This observation likely reflects the observation that basal-like and claudin-low breast cancers (irrespective of aberrant DNA hypermethylation status) are typically at elevated risk for recurrence. These data suggest that aberrant DNA hypermethylation status is a good predictor of relapse-free survival without respect to molecular subtype, especially in patients who have stage I or II cancers (Fig. 5A and C). In contrast, the presence of aberrant DNA hypermethylation does not confer a worse relapse-free survival in basal-like and claudin-low breast cancers, irrespective of stage (Fig. 5B and D).

Discussion

Breast cancer is well known to be a heterogeneous clinical disease where therapeutic options and treatment strategies (and associated success rates) are dictated by fundamental biological properties of the disease subsets. From the perspective of the oncologist, breast cancers are managed based upon three major disease subsets: (1) ER-positive cancers that are treated with anti-estrogen therapy (such as tamoxifen) and chemotherapy, (2) HER2-positive cancers that are treated with HER2-directed therapy (such as Herceptin) and chemotherapy, and (3) triple-negative cancers where chemotherapy represents the only treatment modality available due to lack of targeted therapies (Rakha et al., 2008; Reis-Filho and Tutt, 2008). Triple-negative breast cancers include the basal-like breast cancers (Badve et al., 2011) and the more recently characterized claudin-low breast cancers (Prat et al., 2010). Women with triple-negative breast cancer exhibit poor overall survival rates, reflecting the relatively greater aggressiveness of this form of breast cancer. Breast cancers of the basal-like subtype make up ~20–25% of all breast cancers and display aggressive phenotypic characteristics, such as large size, rapid growth characterized by high mitotic index, high rate of metastasis to distant sites, high incidence of relapse, and lower overall patient survival (Banerjee et al., 2006; Carey et al., 2006; Nielsen et al., 2004; van de Rijn et al., 2002). In addition to possessing a number of aggressive characteristics, basal-like breast cancers have been reported to exhibit unique features that are not well understood. For example, basal-like breast cancer appears to occur at a higher incidence in pre-menopausal African-American women (Carey et al., 2006). Claudin-low breast cancers account for 7–14% of all breast cancers and display aggressive clinical characteristics similar to those observed in other triple-negative breast cancers (Prat et al., 2010).

While patients with triple-negative breast cancers respond very well to some chemotherapeutic regimens (often with pathologic complete response), long-term patient survival is poor due to high rates of cancer recurrence and progression (Carey et al., 2007). Hence, great efforts are now directed towards identification of therapeutic targets and novel treatment strategies for triple-negative breast cancers (Carey, 2010a, 2010b, 2011).

Epigenetic alterations in cancer cells include both global hypomethylation of the genomic DNA and gene-specific promoter hypermethylation. Global DNA methylation analyses in breast cancer suggest that triple-negative cancers (specifically basal-like breast cancers) exhibit less overall methylation than the other molecular subtypes (luminal A, luminal B, HER2+) (Bediaga et al., 2010; Network, 2012). Concurrent with global hypomethylation of the genome, numerous studies have characterized hypermethylation of promoter sequences associated with specific gene targets. Cancer-related gains in DNA methylation typically reflect hypermethylation of CpG islands in gene promoter regions, which can lead to gene silencing (van Engeland et al., 2003). However, methylation-dependent gene silencing events may involve genes that lack CpG islands in their promoter regions (Rivenbark et al., 2006b). While methylation-dependent gene silencing is a normal mechanism for regulation of gene expression (Momparler, 2003), in cancer cells, it represents a mutation-independent mechanism for inactivation of tumor suppressor genes (Jones and Laird, 1999). Many genes have been shown to be inactivated in breast cancer through DNA methylation-dependent gene silencing — some through a direct effect of DNA methylation, and others via indirect mechanisms. Some cancers exhibit concurrent hypermethylation of numerous genes, a phenomenon known as the CpG island methylator phenotype (CIMP) (Abe et al., 2005; Dulaimi et al., 2004; Eads et al., 2000; Kaneko et al., 2003; Liang et al., 1998; Melki et al., 1999; Shen et al., 2002; Strathdee et al., 2001; Toyota et al., 1999a, 1999b; Ueki et al., 2000). Our previous studies suggest that a subset of breast cancer cell lines and primary breast cancers (primarily basal-like breast cancers) exhibit aberrant DNA hypermethylation (similar to CIMP) that is characterized by promoter methylation-dependent silencing of epigenetic biomarker genes (Roll et al., 2008). Further, this aberrant DNA hypermethylation is associated with overexpression of DNMT3b protein and elevated DNMT activity in breast cancer cell lines leading to concurrent aberrant hypermethylation of numerous genes (Roll et al., 2008). The mechanism accounting for overexpression of DNMT3b in hypermethylator breast cancer cell lines is related to concurrent loss of microRNAs (miRs) that post-transcriptionally regulate *DNMT3b* mRNA (Sandhu et al., 2012b). The results from the current study strongly suggest that the aberrant DNA hypermethylation observed in breast cancer cell lines is readily identifiable among primary breast cancers, as we suggested previously (Roll et al., 2008). The robust association of the aberrant DNA hypermethylation-associated gene expression pattern with triple-negative breast cancers across multiple datasets suggests strongly that we have identified a fundamental biological feature of the majority of breast cancers of this type. The identification of this biological property of triple-negative breast cancers suggests that targeting the epigenetic machinery of these cancers may benefit treatment. In a cell culture model system, we showed that breast cancer cell lines with aberrant DNA hypermethylation can be sensitized to chemotherapy through 5-aza-2′-deoxycytidine-mediated inhibition of the DNA methyltransferase enzymes (Sandhu et al., 2012a). Likewise, we showed that direct genetic targeting of *DNMT3b* using siRNA resulted in increased sensitivity of breast cancer cells to chemotherapy (Sandhu et al., 2012a). Hence, pharmacologic and genetic targeting of the DNMT enzymes may enhance treatment in triple-negative breast cancers that express the hypermethylation phenotype.

Given that aberrant DNA methylation and epigenetic silencing of gene expression are now well recognized as hallmarks of cancer (Bachman et al., 2001; Baylin, 2001, 2005; Baylin et al., 1998), numerous investigators have suggested that cancer should be treated with "epigenetic therapy" (Brueckner and Lyko, 2004; Brueckner et al., 2007; Yoo et al., 2004). The goal of epigenetic therapy is to effect changes in gene expression, including reexpression of silenced genes (like tumor suppressor genes), that alter the clinical behavior of the cancer or the response of the cancer to other therapeutic modalities (such as chemotherapy). This concept has been tested in a breast cancer cell model system based upon the MCF7 cell line using known demethylating drugs (Chavez-Blanco et al., 2006; Segura-Pacheco et al., 2006). These studies provide strong evidence for enhancement of chemotherapeutic effect in MCF7 cells following demethylation of genomic DNA. Investigators have also initiated clinical trials with combination therapy using demethylating and cytotoxic drugs (Arce et al., 2006; Zambrano et al., 2005). These studies strongly suggest that this epigenetic therapy will benefit breast cancer patients. Additional evidence for the use of epigenetic therapy in the treatment of chemoresistant cancers can be found in a recent paper by Juergens et al. that describes a phase I/II trial of epigenetic therapy combined with cytotoxic chemotherapy in patients with advanced non-small cell lung cancer that was refractory to standard chemotherapy (Juergens et al., 2011). This study showed a remarkable improvement in overall survival among patients that received epigenetic therapy in combination with cytotoxic chemotherapy after failure of standard chemotherapy, and that reversal of epigenetic alterations (and reexpression of silenced tumor suppressor genes and other genes encoding negative mediators of cancer cell growth) can sensitize cancer cells to standard drug therapies (Juergens et al., 2011). Demonstration of this important clinical finding in lung cancer suggests that other cancers that are difficult to treat due to the biology of aberrant DNA hypermethylation (like triple-negative breast cancer) will also benefit from this therapeutic approach. Further unraveling the complexities of aberrant DNA hypermethylation holds important implications for identification of new targets for therapy and development of new strategies for clinical management of triple-negative breast cancer.

Conflict of interest statement

The authors declare that there are no conflicts of interests.

Acknowledgments

The authors thank Dr. Gregory J. Tsongalis and Dr. Wendy A. Wells (Dartmouth-Hitchcock Medical Center, Lebanon, NH) for constructing the tissue array utilized for this study, and Dr. Charles M. Perou (University of North Carolina School of Medicine, Chapel Hill, NC) for providing access to microarray-based gene expression datasets and for providing critical comments on this manuscript.

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