Distinct Hypermethylation Profile of Primary Breast Cancer Is Associated with Sentinel Lymph Node Metastasis

Masaru Shinozaki,[1] Dave S.B. Hoon,[1] Armando E. Giuliano,[2] Nora M. Hansen,[2] He-Jing Wang,[3] Roderick Turner,[4] and Bret Taback[1,2]

[1]Department of Molecular Oncology; [2]Joyce Eisenberg Keefer Breast Center; Divisions of [3]Biostatistics and [4]Surgical Pathology, John Wayne Cancer Institute and Saint John's Health Center, Santa Monica, California

ABSTRACT

Purpose: **Gene promoter region hypermethylation is a significant event in primary breast cancer. However, its impact on tumor progression and potential predictive implications remain relatively unknown.**

Experimental Design: **We conducted hypermethylation profiling of 151 primary breast tumors with association to known prognostic factors in breast cancer using methylation-specific PCR for six known tumor suppressor and related genes: *RASSF1A*, *APC*, *TWIST*, *CDH1*, *GSTP1*, and *RAR-β2*. Furthermore, correlation with sentinel lymph node (SLN) tumor status was assessed as it represents the earliest stage of metastasis that is readily detected. Hypermethylation for any one gene was identified in 147 (97%) of 151 primary breast tumors. The most frequently hypermethylated gene was *RASSF1A* (81%).**

Results: **Hypermethylation of the *CDH1* was significantly associated with primary breast tumors demonstrating lymphovascular invasion ($P = 0.008$), infiltrating ductal histology ($P = 0.03$), and negative for the estrogen receptor ($P = 0.005$), whereas *RASSF1A* and *RAR-β2* gene hypermethylation were significantly more common in estrogen receptor–positive ($P < 0.001$) and human epidermal growth factor receptor 2–positive ($P < 0.001$) tumors, respectively. In multivariate analysis, hypermethylation of *GSTP1* and/or *RAR-β2* was significantly associated with patients having macroscopic SLN metastasis compared with those with microscopic or no sentinel node metastasis (odds ratio, 4.59; 95% confidence interval, 2.02-10.4; $P < 0.001$). In paired SLN metastasis, *CDH1* was the most frequently methylated gene (90%) and provides evidence in patients corroborating its role in the clinical development of metastasis.**

Conclusion: **Hypermethylation profiling of primary breast tumors is significantly associated with known pathologic prognostic factors and may have additional clinical and pathologic utility for assessing patient prognosis and predicting early regional metastasis.**

INTRODUCTION

Improved access to mammography and increased patient awareness in breast cancer screening have resulted in a dramatic increase in the detection of early breast cancers (1, 2). It is important, at the time of breast cancer diagnosis, to identify concurrent metastatic disease for accurate patient staging and therapeutic decision making. Axillary lymph node dissection has provided an invaluable approach to assess for the presence of tumor cell metastasis, particularly in early disease states where standard radiographic imaging is less sensitive. However, axillary lymph node dissection can be associated with considerable morbidity including lymphedema and reduced shoulder mobility (3, 4).

Sentinel lymph node (SLN) biopsy provides an effective alternative approach to the identification of regional nodal metastasis, and is associated with reduced morbidity when compared with standard axillary lymph node dissection (5, 6). This procedure, although less invasive, is not entirely risk-free as it still requires an axillary incision and general anesthesia, it subjects patients to lymphatic mapping reagents, and its success is dependent on the skill of the surgeon (7, 8). Furthermore, it remains controversial whether axillary surgery offers clinically node-negative patients any survival advantage (9–11). Therefore, alternative minimally invasive methods that could identify/predict disease progression are being investigated, such as the assessment of blood and bone marrow for the presence of occult circulating tumor cells (12–14). These assays have limited sensitivity when assessing the earliest stage patients where minimal residual disease is extremely low or nonexistent. Others are investigating primary tumor gene expression profiles as predictors of clinical outcome (15, 16). However, without correlation to other known prognostic factors in breast cancer or intermediate end points, long-term clinical trials with patient outcome will be needed to validate these preliminary results. Nevertheless, to date, in clinical practice, axillary lymph node status remains the single most important prognostic factor for patients (17, 18).

Breast cancer development is a consequence of a serial accumulation of genetic alterations ultimately resulting in the ability of epithelial cells to proliferate uncontrollably, invade tissues, and avoid apoptosis. These genetic events lead to gene activation/inactivation through the mechanisms of mutation, amplification, and deletion. More recently, it has been shown that different cancers show significant CpG island hypermethylation in the promoter regions(s) of specific tumor-suppressor and

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Requests for reprints: Dave Hoon, Department of Molecular Oncology, John Wayne Cancer Institute, 2200 Santa Monica Boulevard, Santa Monica, CA 90404. Phone: 310-449-5267; Fax: 310-449-5282; E-mail: hoon@jwci.org.

related genes when compared with normal cells (19, 20). As these genes regulate normal cellular processes and their methylation in tumor tissue contributes to transcriptional silencing, it is conceivable that they may be involved with breast tumor progression (21). Currently, many of these studies remain descriptive and lack association with known clinicopathologic risk factors affiliated with breast cancer metastasis. Identifying methylation profiles in primary tumors that are associated with metastasis would not only elucidate those epigenetic events involved with disease progression but may aid in the development of a genomic prediction marker panel for patient outcome that can readily be assessed from paraffin-embedded tissue specimens. Because lymph node metastasis remains a critical benchmark in breast cancer and is often the earliest sign of tumor progression, we sought to determine whether promoter hypermethylation of a marker panel comprising six tumor suppressor and cancer-related genes implicated in early-stage regional nodal breast cancer progression: RAS association domain family protein 1A protein (*RASSF1A*), adenomatous polyposis coli (*APC*), *TWIST* gene a basic-helix-loop-helix family of transcription factors, E-cadherin (*CDH1*), glutathione *S*-transferase *pi* 1 (*GSTP1*), and retinoic acid–binding receptor-β2 (*RAR-β2*), was associated with the presence of SLN metastasis. To date, majority of the studies on hypermethylation of tumor-related genes in breast cancer have been descriptive and none have shown relation to prognostic factors and to early stage metastasis in a large series of patients. We showed that hypermethylation of *GSTP1* and *RAR-β2* was significantly associated with prognostic factors of the primary tumor and level of disease in the SLN.

PATIENTS AND METHODS

Patients. A total of 151 patients were identified from the Breast Cancer Database at the John Wayne Cancer Institute who underwent surgery for their primary breast cancer with SLN biopsy alone or followed by axillary lymph node dissection, if the SLN contained metastasis, from August 1992 to May 2001. Two thirds of the patients were postmenopausal and mean patient age was 55 years (range, 27-86 years) with a mean tumor size of 3.1 cm (range, 0.1-10 cm). Additional primary tumor characteristics are listed in Table 1. The study was approved by the joint Saint John's Health Center/John Wayne Cancer Institute's institutional review board with all patients providing informed written consent.

In addition, 29 patients had readily available paraffin-embedded SLN tissue blocks containing large metastasis identified on H&E. These were evaluated in tandem with the primary tumor to determine whether gene methylation status correlated with the matched-paired SLN metastasis.

The estrogen receptor (ER), progesterone receptor, and human epidermal growth factor receptor 2 (HER2) receptor status were evaluated on the primary tumor specimen by standard immunohistochemistry. ER was assessed with the anti-ER monoclonal antibody 6F11 (Ventana, Tuscon, AZ) and considered positive if ≥1% of cells showed nuclear staining. Progesterone receptor was assessed with the anti–progesterone receptor monoclonal antibody 16 (Ventana) and considered positive if ≥1% of cells stained. The HER2 receptor status was evaluated with the anti-HER2 polyclonal antibody c-erb-B2 (DAKO, Carpenteria, CA) and tumors were considered to have overexpression with a staining intensity of 3+; scores of 0 or 1 were negative and scores of 2+ were indeterminant and further evaluated with fluorescence *in situ* hybridization (FISH) by a commercial pathology service (IMPATH, Los Angeles, CA).

DNA Extraction and Methylation-Specific PCR. Paraffin-embedded primary tumor specimen blocks (and 29 paired SLNs with metastasis) were sectioned at 10 μm and deparaffinized in 100% xylene, followed by 100% ethanol incubation, and stained with H&E. Tumor tissue was microdissected compared with a similarly stained and coverslipped reference slide cut in sequence from each tissue block. The samples were incubated in buffer containing SDS-proteinase K for 48 hours at 50°C with an additional 1 μg proteinase K added twice within each 24-hour period. DNA was extracted and bisulfite modification was done using the agarose bead technique as previously described (22). Briefly, following extraction, DNA was quantified using Picogreen (Molecular Probes, Eugene, OR) and 1 μg genomic DNA was mixed with 0.3 mol/L NaOH, 2 volumes 2% LMP agarose (BioWhittaker Molecular Applications, Rockland, ME) dissolved in molecular grade

Table 1 Patient characteristics

Clinicopathologic factors ($N = 151$)	n(%)
Menopausal	
Pre	51 (34)
Post	100 (66)
T stage	
T_{1a}	1 (1)
T_{1b}	4 (3)
T_{1c}	13 (9)
T_2	118 (78)
T_3	15 (10)
N stage	
N_0	71 (47)
N_1	74 (49)
N_2	6 (4)
M stage	
M_0	147 (97)
M_1	4 (3)
American Joint Committee on Cancer Stage	
I	1 (1)
IIa	86 (57)
IIb	43 (29)
IIIa	17 (11)
IV	4 (3)
Histology	
Ductal	118 (78)
Lobular	33 (22)
Differentiation	
Well	30 (20)
Moderate	62 (42)
Poor	57 (38)
Unknown	[2]
Invasion	
No	102 (68)
Yes	43 (29)
Unknown	[6]
SLN status	
Negative	70 (46)
Micro	40 (27)
Macro	41 (27)

water, heated at 80°C for 10 minutes, and then added to two to three drops of chilled mineral oil to create an agarose bead. Sodium bisulfite conversion of DNA suspended in the agarose bead was achieved by adding 2.5 mol/L sodium metabisulfite and 125 mmol/L hydroquinone and incubating at 50°C for 14 hours. Subsequently, desulfonation was done by removing residual mineral oil and adding 0.2 mol/L NaOH ×2 for 15 minutes each, followed by neutralization with 1/5 volume 1 mol/L HCl for 5 minutes, and then the bead was washed in Tris-EDTA buffer and stored in molecular grade water at 4°C until analysis. A panel of six genes was assessed for their methylation status: *RASSF1A*, *APC*, *TWIST*, *CDH1*, *GSTP1*, and *RAR-β2*. Methylation-specific PCR was done on each bead in a 100 μL reaction containing 200 μmol/L each of deoxynucleotide triphosphate and AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT) and 50 pmol of each forward (F) and reverse (R) primer set for methylated (M) and unmethylated (U) sets as follows: *RAR-β2*, (M) F-GAACGCGAGCGATTCGA GT and R-GACCAATCCAACCGAAACG, (U) F-GGATTGGGATGTTGAGAATGT and R-CAACCAATCCAACCAAAACAA; *CDH1*, (M) F-TTAGGTTAGAGGGTTAT CGCGT and R-TAACTAAAAATTCACCTACCGAC, (U) F-TAATTTTAGGTTAGA GGGTTATTGT and R-CACAACCAATCAACAACACA; *APC*, (M) F-TATTGCGG AGTGCGGGTC and R-TCGACGAACTCCCGACGA, (U) F-GTGTTTTATTGTGGA GTGTGGGTT and R-CCAATCACAAACTCCCAACAA; *RASSF1A*, (M) F-GTGTT AACGCGTTGCGTATC and R-AACCCCGCGAACTAAAAACGA, (U) F-TTTGGTT GGAGTGTGTTAATGTG and R-CAAACCCCACAAACTAAAAACAA; *GSTP1*, (M) F-TTCGGGGTGTAGCGGTCGTC and R-GCCCCAATACTAAATCAC GACG, (U) F-GATGTTTGGGGTGTAGTGGTTGTT and R-CCACCCCAATACTAAATCACAA CA; *TWIST,* (M) F-TTTCGGATGGGGTTGTTATCG and R-GACGAACGCGAAACG ATTTC, (U) F-TTGGATGGGGTTGTTATTGT and R-ACCTTCCTCCAACAAACA CA. PCR was done after optimizing annealing temperatures for each primer set to include 40 timed cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds, and extension at 72°C for 30 seconds. Post-methylation-specific PCR product analysis was done using capillary array electrophoresis (CEQ 8000XL Genetic Analysis System, Beckman Coulter, Fullerton, CA) as previously described (22). Briefly, each well of a 96-well microplate is loaded with 1 μL methylated and 1 μL unmethylated post-PCR product mixed with 40 μL loading buffer and 0.5 μL dye labeled size standard (Beckman Coulter). Forward methylated and unmethylated specific primers labeled with different Beckman Coulter WELLRED Phosphoramidite-linked dyes permit differentiation of the respective post-PCR amplicons during multiplex analysis (Fig. 1). Methylated/unmethylated peak patterns for each gene were compared with cell line controls as previously described (23). Those samples demonstrating peaks at the corresponding size marker only for the unmethylated DNA were considered unmethylated for the gene, whereas samples demonstrating peaks at the corresponding size marker for methylated DNA alone or in combination with peaks for unmethylated DNA were considered as positive for methylation of the target gene.

Sequencing Analysis. Sixteen primary breast tumor samples were randomly selected and analyzed by sequencing to validate the accuracy of the methylation-specific PCR assay for individualized genes. Briefly, PCR was done on bisulfite-modified DNA in 40 μL reactions with forward and reverse primers for specific genes as previously described (22, 23). Fifteen microliters of post-PCR products were resolved on 2% Tris-borate EDTA-agarose gels and target bands were isolated and purified using the Qiagen Gel purification kit (Qiagen Inc., Valencia, CA). Sequencing reactions were done with the dye terminator cycle sequencing kit on the CEQ 8000XL.

Statistical Analysis. Descriptive statistics, such as mean, SD, median, frequency, and percentage were used to summarize patient's characteristics and gene hypermethylation status. Student t test (for continuous variables) and χ^2 test (for categorical variables) were used for comparing clinical factors between tumors demonstrating hypermethylation versus no hypermethylation.

A logistic regression model was developed to investigate the correlation of gene methylation status with lymph node metastasis status, whereas the effects of clinical factors on node metastasis were taken into account. First, a stepwise procedure was used to select clinical factors that significantly related with lymph node metastasis status. Tumor size and ER status were selected in the model, a stepwise procedure was used again to select genes that predict node metastasis status. The statistical analysis was done using SAS software (SAS, Cary, NC) and all tests are two-sided with significance at $P \leq 0.05$. The α level was not adjusted for multiple testing.

RESULTS

Primary Breast Tumor Hypermethylation Profiles. Promoter region CpG hypermethylation was identified in 147 (97%) of 151 primary breast tumors when evaluated for any one marker using the following panel of genes: *RASSF1A*, *APC*, *TWIST*, *CDH1*, *GSTP1*, and *RAR-β2*. The most frequently hypermethylated gene detected was *RASSF1A* occurring in 122 (81%) patients' tumors; this was followed by *CDH1* (53%), *APC* (49%), *TWIST* (48%), *RAR-β2* (24%), and *GSTP1* (21%). Forty-five (30%) of 151 tumors showed hypermethylation for three genes, 43 (28%) tumors for two genes, 25 (17%) for four genes, 20 (13%) for one gene, 10 (7%) for five genes, and four (3%) for all six genes. In only four patient's tumors, hypermethylation was not detected for any of the six genes assessed. Sequence analysis was done on 16 randomly selected primary tumors to verify their hypermethylated or unmethylated status. In all cases, direct sequencing of the PCR product correlated with the methylation status as initially detected by methylation-specific PCR. Ten normal breast tissue samples showed no promoter hypermethylation for any of the genes assessed under the optimal conditions used for tumor assessment.

Methylation Status between Primary Tumor and Matched-Paired Sentinel Lymph Node. Among SLN metastasis, the most frequently methylated gene was *CDH1* occurring in 26 (90%) patients; this was followed by *RASSF1A* (59%), *RAR-β2* (48%), *APC* (34%), *TWIST* (28%), and *GSTP1* (24%). Correlation between primary tumor and SLN metastasis for gene methylation status (methylated/methylated and unmethylated/unmethylated) was common in most instances occurring in 90% of patients for *GSTP1*, 79% for *TWIST*, 72% for *RASSF1A*, 69% for *APC*, 66% for *RAR-β2*, and 55% for *CDH1* (Table 2).

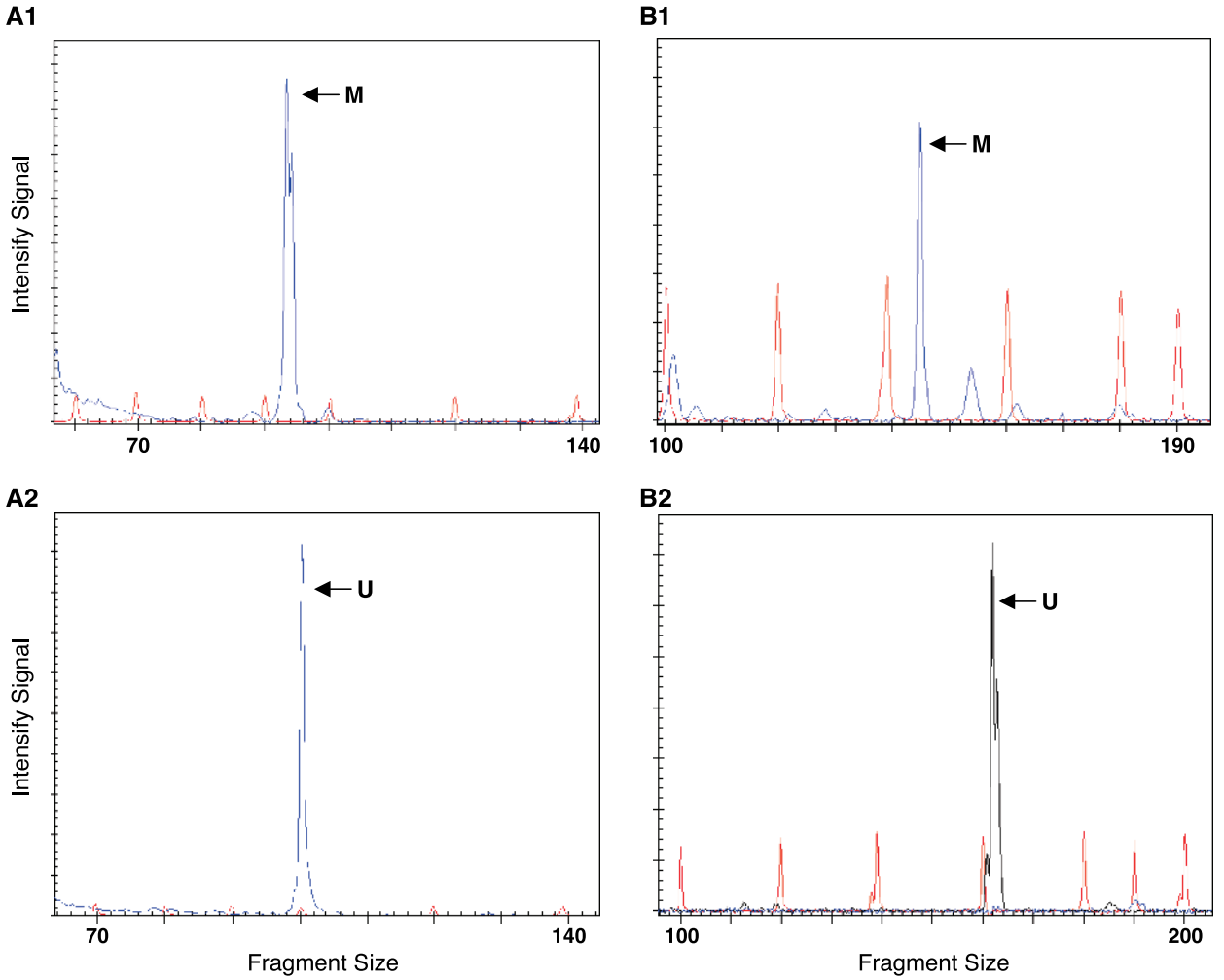

Fig. 1 Representative results from capillary array electrophoresis evaluation of gene methylation status in primary breast tumors. *A,* two different patients' tumor: *A1,* methylated (*M*); *A2,* unmethylated (*U*) GSTP1 promoter region. *B,* two different patients' tumors: *B1,* methylated (*M*); *B2,* unmethylated (*U*) *RAR-β2* promoter region.

Correlation of Primary Tumor Hypermethylation Profiles with Clinicopathologic Prognostic Parameters. The individual gene hypermethylation status for each patient's tumor was assessed to determine whether any clinical or pathologic correlation could be identified for any of the following prognostic parameters associated with breast cancer: patients' age, menopause status, tumor size, histology, degree of differentiation, DNA index, the presence of lymphovascular invasion, T stage, nodal involvement, American Joint Committee on Cancer stage, hormone receptor (ER and progesterone receptor) status, and HER2 receptor presence. *GSTP1* methylation was significantly more frequent in primary breast tumors demonstrating lymph node metastasis occurring in 22 (28%) of 81 patients, compared with 10 (14%) of 70 patients without evidence of lymph node involvement ($P = 0.044$; Table 3). Hypermethylation of the *CDH1* was more frequent in primary tumors demonstrating lymphovascular invasion, 31 (72%) of 43 patients versus 49 (48%) of 102 patient tumors without lymphovascular invasion ($P = 0.008$); those with an infiltrating ductal histology, 68 (58%) of 118 tumors compared with infiltrating lobular histology, 12 (36%) of 33 tumors ($P = 0.03$); and in ER-negative tumors, 27 (73%) of 37 patients' tumors versus 53 (47%) of 114 patients'

Table 2 Methylation status of primary tumor and lymph node metastasis

Methylation status of primary tumor → LN metastasis	*APC*	*GSTP1*	*RASSF1A*	*RAR-β2*	*CDH1*	*TWIST*
M → M	8	7	16	9	14	7
M → U	7	3	7	5	1	5
U → M	2	0	1	5	12	1
U → U	12	19	5	10	2	16

NOTE. M, methylated; U, unmethylated; →, methylated/unmethylated status for each DNA marker in 29 paired primary and lymph nodes assessed.

Table 3 Analysis of gene hypermethylation and prognostic factors

Gene hypermethylation and tumor histopathology	*P*
GSTP1	
Lymph node (+)	0.044
CDH1	
Lymph node (+)	0.008
Infiltrating ductal histopathology	0.03
Estrogen receptor (+)	0.005
RASSF1A	
Estrogen receptor (+)	<0.001
RAR-β2	
HER2 receptor (+)	<0.001
GSTP1 and/or *RAR-β2*	
Lymph node (+)	<0.02
HER2 receptor (+)	0.001

NOTE. Correlations of hypermethylation of genes were made to known prognostic factors. Only significant values are shown.

ER-positive tumors ($P = 0.005$). In contrast, *RASSF1A* hypermethylation was more frequently associated with ER-positive tumors occurring in 99 (87%) of 114 patients versus 23 (62%) of 37 patients with ER-negative tumors ($P < 0.001$). *RAR-β2* hypermethylation was more common in HER2-positive than HER2-negative tumors, 15 (48%) of 31 cases versus 21 (19%) of 112 cases, respectively ($P < 0.001$). No clinical or pathologic correlations that included lymph node metastasis were identified for *APC* or *TWIST* hypermethylation.

In a similar manner, the combination of hypermethylated genes was assessed to determine whether there was any predictive correlation. The presence of hypermethylation for *GSTP1* and/or *RAR-β2* was more frequently associated with the presence of lymph node metastasis and HER2 receptor−positive tumors. Thirty-six (44%) of 81 primary tumors with corresponding lymph node involvement showed hypermethylation for one or both of these markers, whereas this event was only detected in 18 (25%) of 70 primaries without lymph node metastasis ($P < 0.02$). Additionally, hypermethylation for either one or both of these genes was more often found in HER2-positive breast cancers than those that were HER2 negative: 19 (61%) of 31 primary tumors versus 33 (30%) of 112 primary tumors, respectively ($P = 0.001$).

Correlation of Primary Tumor Hypermethyation Profiles and Size of Sentinel Lymph Node Metastasis. It has been suggested that the amount of regional lymph node involvement is associated with a worse patient prognosis (24, 25). To determine whether hypermethylation profiling of the primary tumor was associated with lymph node tumor burden, patients were categorized according to the size of the SLN metastasis as accepted by the American Joint Committee on Cancer Staging Manual sixth edition (26): macro, >2.0 mm ($n = 41$); micro, ≤2.0 mm but >0.2 mm ($n = 40$); and none ($n = 70$), absence of tumor identification following H&E and immunohistochemistry staining. Among these three groups, there was a statistically significant association between increasing SLN tumor burden and larger primary tumor size ($P < 0.015$). Correlation with primary tumor hypermethylation status found a greater frequency of *GSTP1* hypermethylation associated with macro-SLN metastasis, 13 (32%) of 41 patients, compared with those without tumor cells in the SLN, 10 (14%) of 70 patients ($P < 0.029$). *RAR-β2* hypermethylation was more common in those primary tumors having macro-SLN metastasis, 17 (42%) of 41 patients, versus micro-SLN metastasis, 6 (15%) of 40 patients, or no SLN metastasis, 13 (19%) of 70 patients ($P < 0.009$ for each, respectively). Similarly, the presence of either *GSTP1* hypermethylation, *RAR-β2* hypermethylation, or both was more frequently observed in primary tumors having macro-SLN metastasis, 23 (56%) of 41 patients, than micro-SLN metastasis, 12 (30%) of 40 patients, or no SLN metastasis, 18 (26%) of 70 patients, $P < 0.018$ and 0.002, respectively (Table 4).

Multivariate Analysis. A logistic regression model was developed to investigate the correlation of gene methylation status with SLN tumor status, whereas the effects of clinical factors on node metastasis were taken into account. Only tumor size and *RAR-β2* gene hypermethylation were significantly associated with a greater risk for a macro-SLN metastasis compared with micro-SLN or no SLN involvement (odds ratio, 1.595%; confidence interval, 1.16-1.93; $P < 0.002$) and 3.86 (95% confidence interval, 1.65-9.00; $P < 0.002$). Similarly, in multivariate analysis, the presence of either *GSTP1* hypermethylation, *RAR-β2* hypermethylation, or both markers in primary tumors correlated with an increased risk of having a macroscopic SLN metastasis (odds ratio, 4.59; 95% confidence interval, 2.02-10.4; $P < 0.001$). Increasing primary tumor size was also associated with a greater risk for macro-SLN metastasis (odds ratio 1.57, 95% confidence interval, 1.21-2.05; $P < 0.001$). No clinical, pathologic, or hypermethylation gene marker variables could discriminate between microscopic SLN metastasis or histologically tumor-free SLN.

DISCUSSION

This study provides the largest series to date with correlation to known prognostic factors in breast cancer to determine the role of gene promoter hypermethylation status as a molecular predictor of disease progression. We found *GSTP1* methylation to correlate strongly with increasing tumor size and a greater likelihood of SLN metastasis. This finding is important as GSTs are a family of enzymes that detoxify hydrophobic electrophiles, which include carcinogens that have been implicated in a variety of cancers (27). Because diet has been implicated in breast cancer (28), *GSTP1* loss may result in impaired cellular defenses leading to increasing genome damage and cancer progression (29). A multicenter study using large-scale populations will better identify these risks and characterize the potential impact of gene-environment interactions.

Table 4 Correlation between gene hypermethylation and SLN histopathology status

Gene: SLN metastasis histopathology status	*P*
GSTP1	
Macro vs none	<0.015
RAR-β2	
Macro vs micro	<0.009
Macro vs none	<0.009
GSTP1 and/or *RAR-β2*	
Macro vs micro	<0.018
Macro vs none	<0.002

NOTE. Macro, metastasis >2 mm; micro, metastasis <2 mm but >0.2 mm; none, no metastasis detected by H&E and immunohistochemistry.

Hypermethylation of *RAR-β2* was shown to correlate more frequently with HER2-positive tumors, which is overexpressed in 25% to 30% of all breast cancers and when identified is associated with a poorer patient prognosis. Retinoids have been shown to inhibit the growth of breast cancer cell lines in culture and breast tumors in animal models (30–32). *RAR-β2* has been proposed as a tumor suppressor gene and loss of expression has been found in variety of tumors as well as premalignant lesions resulting in uncontrolled cellular proliferation (33, 34). Detection of *RAR-β2* hypermethylation may identify additional therapeutic targets of interest in these groups of patients with more aggressive tumors. Current trials are under way to evaluate the effect of administering retinoids in patients with breast cancer (35, 36). Pretreatment assessment of *RAR-β2* methylation status may more accurately identify patients likely to respond to therapy. The correlation of *RAR-β2* with the presence of macroscopic SLN metastasis was significant. Tumor burden in the lymph nodes is a significant prognosticator of patient outcome (37). However, the clinical implication of occult tumor cells in lymph nodes remains a controversial issue (38, 39). We have shown that patients with SLN micrometastasis (≤2.0 mm but >0.2 mm) have equivalent overall survival rate as those without SLN metastasis and both groups have a better outcome than those with SLN macrometastasis (>2.0 mm; ref. 40). Genetic markers that predict for lymph node metastasis may avoid further surgery in patients with clinically insignificant disease in their axilla and better identify those more likely to benefit from the addition of systemic therapy.

We found *CDH1* hypermethylation to be highly associated with ER-negative tumors and those demonstrating lymphovascular invasion. *CDH1* is involved in maintaining cell-to-cell adhesion and is regarded as suppressor of cellular invasion (41). Furthermore, loss of CDH1 expression in primary tumors has been associated with decreased patient survival (42). These findings are consistent with our results demonstrating *CDH1* methylation predominates in primary tumors with a more aggressive phenotype. Loss of E-cadherin protein expression is most frequent for infiltrating lobular tumor types and is often a biallelic event resulting from any combination of gene promoter hypermethylation, mutation, or allelic loss, whereas ductal histology often presents with varying levels of expression (43, 44). In our series, *CDH1* methylation was more common in tumors with infiltrating ductal histology and may show a different preferential pathway for gene inactivation compared with lobular cancers. Alternatively, this may be reflective of sample selection as most of our cases were infiltrating ductal tumors, which is the most common type at diagnosis and was not a discriminating factor at the study onset.

In patients with paired primary tumors and SLNs available for methylation-specific PCR analysis, the majority showed agreement between the methylation status in the tumor and its corresponding metastasis, suggesting a predominance of epigenetic clonality among cells comprising metastasis to the SLN. We found *CDH1* to be the most frequently methylated gene in SLN metastasis (frequency, 90%). Furthermore, although infrequent, when there was a lack of concordance between gene methylation of the primary tumor and its corresponding SLN metastasis, the tendency was for the primary tumor. The sole exception was for *CDH1*, which favored hypermethylation in the SLN metastasis. These findings provide vital clinical evidence in patients that supports *in vitro* and animal models implicating alterations of *CDH1* in the metastatic process (45). It has been previously reported that a higher frequency of hypermethylation exists in breast cancer metastasis (46). However, correlation between gene hypermethylation in the primary tumor and the associated lymph node metastasis were limited. Because our report is the first to evaluate SLNs, which is the first site of lymphatic spread, it represents the earliest stage of regional metastasis and, therefore, may have fewer epigenetic alterations than those associated with more advanced disease. Overall, recurrence in patients with positive SLN in our experience is low and requires long-term follow-up for evaluation (40).

RASSF1A was the most frequently methylated gene detected in this study and our results are consistent with other studies (47). Together, these findings suggest a likely importance of *RASSF1A* gene silencing in breast cancer. Additionally, correlating gene promoter hypermethylation profiles with known pathologic prognostic factors, including SLN metastasis, provides improved understanding of the biology of breast cancer. Molecular events associated with the primary tumor that predict for metastasis and patient outcome offers the desired opportunity to avoid additional surgical interventions for staging and will prove more suitable in this new era of earlier cancer detection.

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