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Prognostic relevance of promoter hypermethylation of multiple genes in breast cancer patients

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Abstract. *Background*: Methylation-mediated suppression of detoxification, DNA repair and tumor suppressor genes has been implicated in cancer development. This study was designed to investigate the impact of concurrent methylation of multiple genes in breast tumors on disease prognosis.

Methods: Methylation specific PCR was carried out to analyze the methylation status of seven genes in archived breast tissues and determine the effect of aberrant methylation of multiple genes on disease prognosis and patients' survival.

Results: Promoter hypermethylation was observed in *PRB* 67%, *ER*α 64%, *RASSF1A* 63%, *p16INK4A* 51%, *RAR*β*2* 22%, *GSTP1* 25% and *BRCA1* 27% of the breast cancers, respectively. Concurrent methylation of *BRCA1*, *ER*α, *GSTP1* and *RAR*β*2*, was observed in a large proportion of breast cancers analyzed, suggesting that these genes do not appear to be methylated alone. Patients with high methylation indices had poor prognosis ($p < 0.001$, Hazards ratio $= 14.58$). Cox regression analysis showed *RAR*β*2* promoter methylation to be an independent important determinant of breast cancer prognosis.

Conclusion: Our results suggest that methylation of multiple genes plays an important role in prognosis of breast cancer. Our study not only describes the association of methylation mediated silencing of multiple genes with the severity of disease, but also drives to speculate the molecular crosstalk between genes or genetic pathways regulated by them individually.

Keywords: Breast cancer, methylation, prognosis, *RAR*β, *GSTP1*, *BRCA1*

1. Introduction

Breast cancer is the most commonly prevailing malignant disease in women, and tumor recurrence is responsible for the majority of cancer-related deaths. The prognoses of the extensively heterogeneous breast tumors are very different. The 10-year distant recurrence rate is less in lymph node negative and ERα-positive patients even without adjuvant chemotherapy [22]; in contrast, a significant proportion of patients have poor prognosis and will develop recurrence even if given adjuvant chemotherapy [22]. This necessitates a need for more sensitive and specific prognostic indicators. The clinical and biological significance of molecular alterations in breast cancer are under intense investigation [1,21,25,31,35,39,45,50,52]. To ensure better characterization and treatment of breast tumors, new approaches are needed to complement the classical clinicopathological analysis. In particular, tools that exploit the most recent molecular biology knowledge and technological advances are required to overcome this challenge.

Aberrant DNA methylation is now recognized as one of the most common molecular abnormalities in cancer (reviewed in [13] and references therein). This

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epigenetic modification occurs at the cytosines of CpG dinucleotides, which often exist in clusters called CpG islands. Methylation of these sites in the promoter region of a gene can result in chromatin condensation and gene silencing. In cancer cells, aberrant methylation has frequently been reported in tumor suppressor genes, DNA repair genes, and genes related to cancer metastasis and invasion [13]. The silencing of these functionally important genes leads to a shift of cells from a normal cellular cycle to a state of high proliferation that favors tumor development and progression [12,14,38]. It has been observed that promoter methylation of specific genes in cancer occurs in both tissue-specific and cell specific manner, making the identification of methylation patterns a potentially useful tool for cancer management [8]. This may be especially important for patients with breast cancer, because early detection and accurate prediction of disease prognosis can improve survival. We and others have detected DNA methylation in different biological materials such as tissue biopsy specimens, serum, nipple aspirates and other body fluids and shown the potential of DNA hypermethylation in early detection and disease management [3,20,34,36].

Recent research has focused on identification of groups of genes with consistent, concurrent methylation (methylator profiles or phenotypes) in cancers of different organ sites including colorectal [51], cervical [18], ovarian [54] and breast [17] cancers. However, there remains a great deal of uncertainty regarding the presence and classification of these methylator phenotypes, due to the relatively small number of genes analyzed and differences in selection of genes and analytical methods in various studies. What remains particularly unclear is which genes should be used to identify a methylator phenotype that best describes the process of either tumorigenesis or progression in a tissue-specific manner. Genes that are aberrantly methylated in specific tumors may be treated as potential molecular signatures for tumor diagnosis and prognosis. Specific DNA methylation signatures vary in different cancers, as almost all tumor-suppressing genes are involved at different stages of all cancers [7,11]. The pathological features of breast cancer follow a sequential progression from transition of a normal cell to benign proliferative hyperplasia, hyperplasia with atypia, in situ carcinoma and, eventually, to invasive and metastatic disease [6]. However, the time-course of epigenetic alterations during this progression is little understood. Thus, there is an urgent need for stage specific evaluation of different cancers [37].

In the current study, we analyzed 101 breast cancer patients with invasive ductal carcinoma for concurrent methylation of a panel of 7 genes known to be important in development and progression of breast cancer: *estrogen receptor-α* (*ERα*), *progesterone receptor B (PRB), Retinoic acid receptor β2 (RARβ2), p16INK4A, Breast cancer gene 1 (BRCA1), Rass Associated Family 1 A (RASSF1A) glutathione S-transferase P 1 (GSTP1)*. Suppression of *ERα*, *PRB*, *RARβ2*, *p16INK4A*, *BRCA1*, *RASSF1A* expression by methylation has been reported in several malignancies, including breast cancer using freshly collected breast tumors in prospective studies by our laboratory and others [4,5,34,43,44]. The present retrospective study was undertaken to determine the prognostic potential of these genes individually and in a panel. Further, in view of the high proportion of triple negative breast cancers in Indian women we determined the correlation of methylation of this panel of genes with expression of estrogen receptor α(ERα), progesterone receptor (PR) and Her2/*neu*.

2. Materials and methods

2.1. Study population

This study was approved by the Human Ethics Committee of All India Institute of Medical Sciences (AIIMS), New Delhi, India. In this retrospective study, 101 breast cancer patients, who underwent definitive surgical resection in the Department of Surgical Disciplines, AIIMS, between May 1996 and December 2005, and for whom the tissue blocks as well as follow up data were available in the breast cancer follow up clinic data bank were included. All available paraffin blocks were reviewed by a pathologist and were histologically confirmed to be invasive ductal breast carcinomas. No patient had received chemotherapy and/or hormonal therapy prior to surgery. After completion of the primary treatment the patients underwent physical examination, blood test and chest X-ray examination every 3 months for 2 years post-operatively and subsequently every 6 months. Overall survival (OS) is defined as the time from the date of surgery to the date of death or last contact if the patient was still alive and ranged from 0.83 months to 131.3 months (median, 33.6 months). Disease free survival (DFS) is defined as the time from the date of surgery to the date of local recurrence or metastasis; ranged from 0.33 months to 131.3 months (median, 36.05 months).

2.2. DNA extraction from paraffin embedded tissues

Formalin-fixed, paraffin embedded tissue blocks were cut into 10 μm thick tissue sections. Serial tissue sections from each paraffin block were placed on slides prior to DNA extraction and stained with H&E and sections which showed 75–80% tumor cells were collected in 15 ml centrifuge tubes, deparaffinized overnight at 63°C in xylene, and vortexed vigorously. Supernatants were removed by centrifugation; ethanol was added to remove the residual xylene, and centrifuged. After ethanol evaporation, tissue pellets were resuspended in lysis buffer ATL (DNeasy Tissue Kit, Qiagen, Hilden, Germany) and the genomic DNA was isolated using a DNeasy Tissue kit according to the manufacturer's instruction.

2.3. Methylation-specific PCR

The methylation status of the promoter region of *RASSF1A*, *p16INK4A*, *RARβ2*, *GSTP1*, *ERα*, *BRCA1* and *PRB* genes was determined by methylation-specific PCR, as described by Herman et al. [23]. Two sets of primers were designed for each gene, one specific for DNA methylated at the promoter region and the other specific for unmethylated DNA. Primer sequences and annealing temperatures for the methylation-specific PCR of these 7 genes are given in Supplementary Table 1 (see: http://www.qub.ac.uk/isco/JCO) and were previously published [15,16,23, 27,42,53] and used in our earlier studies [5,34,43,44]. Briefly, 1 μg of genomic DNA was denatured by incubation with 0.2 mol/l NaOH for 10 min at 37°C. Aliquots of 3 mol/l sodium bisulfite (pH 5.0) and 10 mmol/l of hydroquinone (both from Sigma Chemical, Co., St. Louis, MO, USA) were then added, and the solution was incubated at 50°C for 16 h. The modified DNA was purified using Wizard DNA purification system (Promega Corp., Madison, WI, USA), followed by ethanol precipitation. Modified DNA was stored in aliquots at –20°C until required. For positive and negative controls of the MSP, a breast cancer cell line (MCF-7, MDA-MB-231, MDA-MB-157 and T47D) or tumor with known hypermethylation as a positive control, normal lymphocyte and normal breast tissue DNA as negative controls and water with no DNA template as a control for contamination were included in each experiment. After amplification, each PCR product was electrophoresed using a 2–2.5% agarose gel, stained with ethidium bromide and visualized under UV illumination.

2.4. Immunohistochemistry

Monoclonal antibodies were purchased against PR (A0098) from Dako Cytomation (Glostrup, Denmark) and ERα (sc-8005), Her2/neu (sc-08) and p53 (sc-126) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Immunohistochemical analysis was carried out using paraffin embedded tissue sections as described by us [43]. Briefly, tissue sections were deparaffinized in xylene, hydrated and incubated with 0.3% (v/v) H_2O_2 in methanol for 45 min, to inactivate the endogenous peroxidases. Antigen was retrieved by microwave treatment for 15 min in 0.01 M citrate buffer (pH 6.0), sections were incubated with primary antibody (1:50 dilution) at 4°C overnight. Thereafter, sections were incubated with biotinylated anti-mouse anti-serum and subsequently with horse-radish peroxidase-streptavidin conjugate (Dako Cytomation, Glostrup, Denmark), followed by color development using 3,3′-diaminobenzidine hydrochloride (DAB) as chromogen, counterstained with Mayer's hematoxylin and mounted for evaluation using microscope (NIKON microphot-FXA, Japan). In the negative control, primary antibody was replaced by isotype-specific IgG. In case of ERα, PR and p53 only nuclear staining was considered as immunopositive. The slides were scored as follows: negative, $<10\%$ tumor cells showing immunoreactivity; positive, $>10\%$ tumors cells showing with nuclear immunoreactivity were considered [40]. For Her2/*neu* protein expression, membrane immunostaining was considered as positive. The slides were scored following the criteria: no staining or membrane staining in fewer than 10% of tumor cells, 0; faint, barely perceptible membrane staining in more than 10% of tumor cells, the cells are stained only in part of the membrane, 1+; weak to moderate membranous staining observed in more than 10% of tumor cells, 2+; and strong membranous staining in more than 10% of tumor cells, 3+ [41].

2.5. Statistical analysis

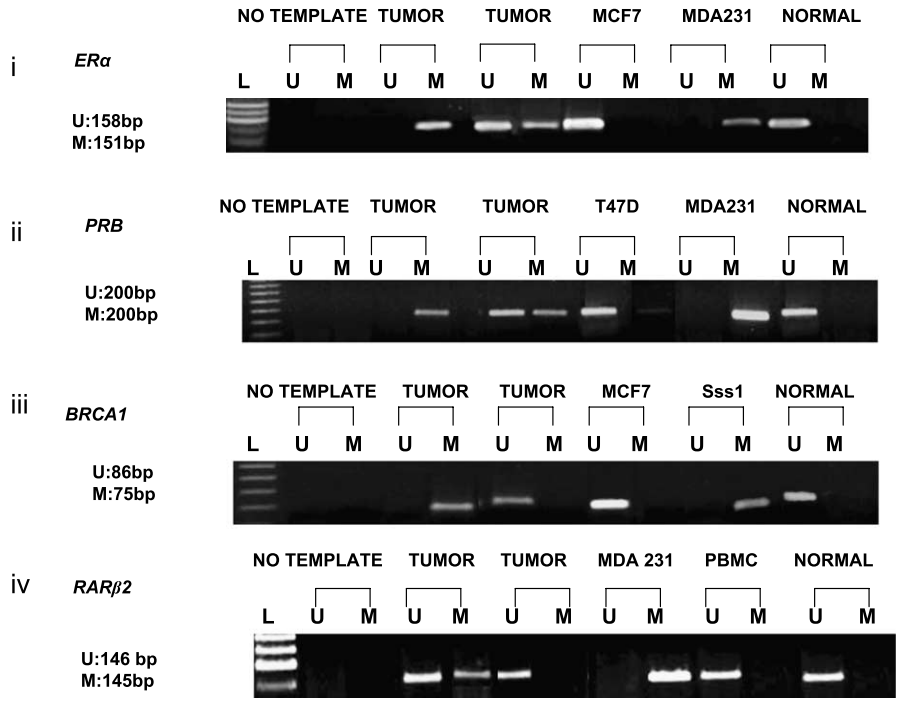
Associations between clinical and pathologic characteristics and individual gene promoter methylation status were examined by Chi-square tests. To examine the combined effect of methylation of multiple gene promoters, methylation index (MI = total number of genes methylated/total number of genes analyzed) was calculated. This index was further divided into 4 categories (0/7–1/7 (reference), 2/7–3/7, 4/7–5/7,

6/7–7/7) based on total number of gene methylation in Cox regression models.

Disease free survival (DFS) and overall survival (OS) curves were calculated with the Kaplan–Meier method. Univariate Cox regression models were used to compute hazard ratios for DFS and OS, 95% confidence intervals and p-values for gene methylation and clinicopathological characteristics. In terms of adjusting the effect of several clinicopathological characteristics, the multiple Cox regression models were used by adding these indicators to the univariate Cox regression models. The multiple Cox regression with backward selection was also used to select important effects for DFS as well as OS among gene methylation and clinicopathological characteristics. A p-value <0.05 was considered as statistically significant. All of the statistical analyses were performed using SAS software (version 9.12; SAS Institute, Cary, NC, USA).

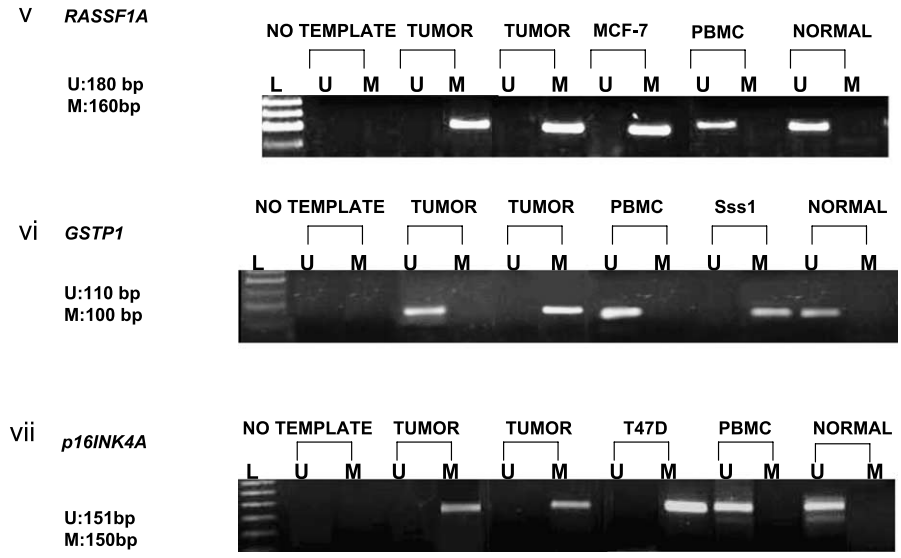
3. Results

Promoter methylation of 7 genes (*RASSF1A*, *p16INK4A*, *RARβ2*, *GSTP1*, *BRCA1*, *ERα* and *PRB)* was evaluated in 101 patients with invasive ductal breast carcinoma. Figure 1a and b shows representative methylation status of *RASSF1A*, *p16INK4A*, *RARβ2*, *GSTP1*, *ERα*, *BRCA1* and *PRB* in invasive ductal breast carcinomas. Promoter methylation occurred frequently in *PRB*, *ERα*, *RASSF1A* and *p16INK4A* in



(a)

Fig. 1. (a) MSP analysis of *ERα*, *PRB*, *BRCA1* and *RARβ2* genes in archival breast tumors. (i) *ERα* panel viewed from left to right shows a 50-bp ladder as molecular weight marker, a water control for contamination in the PCR reaction, patient 1 shows presence of methylated DNA in tumor, patient 2 shows presence of both methylated and unmethylated DNA. Breast cancer cell line MDA-231 used as a positive control shows methylated DNA. MCF-7 used as a positive control for unmethylated DNA, normal breast tissue used also shows unmethylated DNA. (ii) *PRB* panel viewed from left to right shows a 50-bp ladder as molecular weight marker, a water control for contamination in the PCR reaction, patient 1 methylated DNA is detected in tumor, patient 2 shows presence of both methylated and unmethylated DNA. Breast cancer cell line MDA-231 used as a positive control shows methylated DNA. T47D used as a positive control for unmethylated DNA, normal breast tissue used also shows unmethylated DNA. (iii) *BRCA1* panel viewed from left to right shows a 50-bp ladder as molecular weight marker, a water control for contamination in the PCR reaction, patient 1 methylated DNA is detected in tumor, patient 2 shows presence of unmethylated DNA in tumor. Breast cancer cell line MCF7 used as a positive control shows unmethylated DNA whereas Sss1 treated PBMC cells were used as a positive control for methylated DNA, normal breast tissue used also shows unmethylated DNA. (iv) *RARβ2* panel viewed from left to right shows a 50-bp ladder as molecular weight marker, a water control for contamination in the PCR reaction, patient 1 shows presence of methylated and unmethylated DNA in tumor, patient 2 shows presence of unmethylated DNA detected in tumor. PBMC used as a positive control shows unmethylated DNA.MDA-231 used as a positive control for methylated DNA, normal breast tissue also shows unmethylated DNA.



(b)

Fig. 1. (Continued.) (b) MSP analysis of *RASSF1A*, *GSTP1* and *p16INK4A*. (v) *RASSF1A* panel viewed from left to right shows a 50-bp ladder as molecular weight marker, a water control for contamination in the PCR reaction, patient 1 shows presence of methylated DNA in tumor, patient 2 also shows presence of methylated DNA detected in tumor. Breast cancer cell line MCF7 used as a positive control shows methylated DNA. PBMC was used as a positive control for unmethylated DNA, normal breast tissue also shows unmethylated DNA. (vi) *GSTP1* panel viewed from left to right shows a 50-bp ladder as molecular weight marker, a water control for contamination in the PCR reaction, patient 1 unmethylated DNA is detected in tumor, patient 2 shows presence of methylated DNA in tumor. PBMC used as a positive control shows unmethylated DNA. Whereas Sss1 treated PBMC cells were used a positive control for methylated DNA, normal breast tissue used as shows unmethylated DNA. (vii) *p16INK4A* panel viewed from left to right shows a 50-bp ladder as molecular weight marker, a water control for contamination in the PCR reaction, patient 1 shows presence methylated DNA in tumor, patient 2 also shows presence of methylated DNA detected in tumor.

67%, 64%, 63% and 51% of breast cancers, respectively (Table 1). *BRCA1*, *GSTP1* and *RARβ2* showed methylation in 27%, 25% and 22% cases respectively. Figure 1c summarizes the methylation patterns of the panel of 7 genes in the set of invasive ductal breast carcinomas analyzed.

3.1. Correlation of hypermethylation of individual gene with clinicopathological characteristics

The methylation status of each gene was correlated with clinicopathological parameters to determine their clinical relevance (Table 1). *BRCA1* and *GSTP1* hypermethylation correlated significantly with tumor size ($p = 0.018$ and <0.001, respectively). *PRB*, *ERα* and *BRCA1* were significantly more frequently methylated in primary breast tumors with lymph node metastasis ($p = 0.042$, 0.049 and 0.028, respectively). *p16INK4A, BRCA1, GSTP1* and *RARβ2* hypermethylation was significantly associated with higher tumor stage ($p = 0.019$, 0.002, 0.001 and 0.018, respectively).

The methylation index (computed by the number of genes methylated divided by total number of genes analyzed) was significantly associated with tumor size and stage ($p = 0.024$ and <0.001).

3.2. Relationships between hypermethylation of individual genes with ERα, PR, HR and Her2/neu status

ERα and *PRB* hypermethylation was associated with loss of their respective protein expression (Table 1). *ERα* hypermethylation was observed in 44 of 57 (77%) ERα-negative tumors; in comparison 21 of 44 (48%) patients with ERα-positive breast tumors showed *ERα* hypermethylation ($p = 0.002$). Similarly, *PRB* hypermethylation occurred in 44 of 52 (85%) PR-negative breast tumors, compared to 24 of 49 (49%) patients with PR-positive breast tumors ($p < 0.001$). Similarly, *ERα* hypermethylation was significantly associated with loss of PR protein expression in breast tumors ($p < 0.001$) and *PRB* methylation was significantly associated with ERα-negative tumors ($p < 0.001$).

p16INK4A and *BRCA1* hypermethylation was significantly associated with ERα and PR protein status. Specifically, breast cancer patients with negative

(c)

Fig. 1. (Continued.) (c) CpG island methylation profile of 101 invasive ductal breast cancer patients. Each column represents one gene. Each row is a primary breast tumor. Methylated genes are represented as dark rectangles and unmethylated genes are displayed as bright rectangles.

ERα and PR status had higher methylation frequencies of *p16INK4A* ($p = 0.004$ and 0.002, respectively) and *BRCA1* ($p = 0.002$ and 0.001, respectively).

Further, there was significant association between hypermethylation of *p16INK4A* and *BRCA1* and negative hormone receptor status (HR; ERα and PR-negative; $p < 0.001$ and <0.001, respectively).

Her2/*neu* amplification correlated positively with hypermethylation status of *GSTP1* and *PRB* ($p = 0.004$ and 0.022, respectively). Both the genes were more frequently hypermethylated in breast tumor tissues harboring Her2/*neu* amplification as compared to those which did not show Her2/*neu* amplification. We observed significant association of triple negative tumors with *PRB*, *ER*α, *p16INK4A* and *BRCA1* hypermethylation ($p = 0.009$, 0.008, 0.006 and 0.048, respectively).

The higher methylation index was significantly associated with negative ERα and PR status, positive Her2/*neu* status, negative HR and triple negative status ($p < 0.001$, 0.001, 0.008, <0.001 and 0.024, respectively).

3.3. Correlation of methylation status between tumor related genes

The methylation status of each gene was correlated with methylation status of other genes in the panel to determine any associations among promoter methylation of all these genes (Table 2). *PRB* methylation was significantly associated with *ER*α methylation (88% vs. 15%, $p < 0.001$). In addition, *PRB*, *ER*α methylation was also associated with *BRCA1* (35% vs. 11%, $p = 0.008$). Similarly, the methylation of *RASSF1A* was associated with methylation of *BRCA1* ($p = 0.023$), *GSTP1* ($p < 0.001$) and *RAR*β*2* ($p < 0.001$). Importantly, *BRCA1* methylation was associated with methylation of 5 of these 6 genes (*ER*α, *RASSF1A*, *p16INK4A*, *GSTP1* and *RAR*β*2*) examined in our panel, the only exception being *PRB*, which was of borderline significance ($p = 0.06$). In addition, the gene methylation of *RASSF1A, BRCA1, GSTP1* and *RAR*β*2* were all significantly associated with each other (Table 2). The patients were further subdivided based on their total numbers of methylated genes. For those patients with only one gene methylated ($n = 9$), 22% ($n = 2$) were *PRB*, 33% ($n = 3$) were *RASSF1A* and 44% ($n = 4$) were *p16INK4A*. The *ER*α, *BRCA1*, *GSTP1* and *RAR*β*2* did not appear to be methylated alone.

3.4. Correlation with methylation of a panel of genes and disease prognosis

The methylation status of all the genes in our panel was evaluated as a prognostic variable by univariate analysis (Table 3A and B, Cox regression analysis). All the genes in our panel, except *ER*α were significantly associated with disease recurrence (hazards ratio, HR = 2.11–3.88). The methylation of *BRCA1* (HR = 5.06, 95% CI = 1.58 − 16.22, $p = 0.006$), *GSTP1* (HR = 6.61, 95% CI = 1.99 − 21.98, $p = 0.002$) and *RAR*β*2* (HR = 9.26, 95% CI = 2.76 − 31.05, $p < 0.001$) were significantly associated with the overall survival rates. Breast cancer patients with high MI

Table 1
Association of gene methylation with clinicopathological characteristics

Variables (n, # of patients)	*PRB* n (%)	*ERα* n (%)	*RASSF1A* n (%)	*p16INK4A* n (%)	*BRCA1* n (%)	*GSTP1* n (%)	*RARβ2* n (%)	Methylation index[1]		
								Mean	SD	t-test
Total (101)	68 (67)	65 (64)	64 (63)	51 (51)	27 (27)	25 (25)	22 (22)	0.46	0.25	
Age group										
>45 years (51)	36 (71)	32 (63)	32 (63)	29 (57)	14 (28)	12 (24)	10 (20)	0.46	0.24	ns
<45 years (50)	32 (64)	33 (66)	32 (64)	22 (44)	13 (26)	13 (26)	12 (24)	0.45	0.27	
Menopausal status										
Pre (45)	28 (62)	28 (62)	32 (71)	24 (53)	14 (31)	12 (27)	11 (24)	0.47	0.25	ns
Post (56)	40 (71)	37 (66)	32 (57)	27 (48)	13 (23)	13 (23)	11 (20)	0.44	0.25	
Tumor size										
$T_1 + T_2$ (57)	40 (70)	37 (65)	35 (61)	24 (42)	10 (18)	7 (12)	9 (16)	0.41	0.24	0.024
$T_3 + T_4$ (44)	28 (64)	28 (64)	29 (66)	27 (61)	17 (39)	18 (41)	13 (30)	0.52	0.26	
					$p = 0.018$	$p < 0.001$				
Node involvement										
Negative (32)	26 (81)	25 (78)	17 (53)	14 (44)	4 (13)	5 (16)	4 (13)	0.42	0.19	ns
Positive (69)	42(61)	40 (58)	47 (68)	37 (54)	23 (33)	20 (29)	18 (26)	0.47	0.28	
	$p = 0.042$	$p = 0.049$			$p = 0.028$					
Stage										
I + II (59)	39 (66)	36 (61)	34 (58)	24 (41)	9 (15)	7 (12)	8 (14)	0.38	0.22	<0.001
III (42)	29 (69)	29 (69)	30 (71)	27 (64)	18 (43)	18 (43)	14 (33)	0.56	0.25	
				$p = 0.019$	$p = 0.002$	$p < 0.001$	$p = 0.018$			
p53										
Positive (30)	21 (70)	21 (70)	18 (60)	18 (60)	10 (33)	7 (23)	7 (23)	0.49	0.24	ns
Negative (71)	47 (66)	44 (62)	46 (65)	33 (47)	17 (24)	18 (25)	15 (21)	0.44	0.26	
ERα status										
Positive (44)	20 (46)	21 (48)	28 (64)	15 (34)	5 (11)	11 (25)	8 (18)	0.35	0.24	<0.0001
Negative (57)	48 (84)	44 (77)	36 (63)	36 (63)	22 (39)	14 (25)	14 (25)	0.54	0.24	
	$p < 0.001$	$p = 0.002$		$p = 0.004$	$p = 0.002$					
PR status										
Positive (49)	24 (49)	23 (47)	33 (67)	17 (35)	6 (12)	12 (25)	8 (16)	0.36	0.23	<0.001
Negative (52)	44 (85)	42 (81)	31 (60)	34 (65)	21 (40)	13 (25)	14 (27)	0.55	0.24	
	$p < 0.001$	$p < 0.001$		$p = 0.002$	$p = 0.001$					
Her2/*neu* status										
Positive (23)	20 (87)	17 (74)	17 (74)	11 (48)	9 (39)	11 (48)	8 (35)	0.58	0.29	0.008
Negative (78)	48 (62)	48 (62)	47 (60)	40 (51)	18 (23)	14 (18)	14 (18)	0.42	0.23	
	$p = 0.024$					$p = 0.004$				
HR status										
Positive (51)	25 (49)	24 (47)	33 (65)	17 (33.3)	6 (12)	12 (24)	8 (16)	0.35	0.23	<0.001
Negative (50)	43 (86)	41 (82)	31 (62)	34 (68)	21 (42)	13 (26)	14 (28)	0.56	0.23	
	$p < 0.001$	$p < 0.001$		$p < 0.001$	$p < 0.001$					
ERα/PR/Her2/*neu* status										
Positive (61)	35 (57)	33 (54)	41 (67)	24 (39)	12 (20)	17 (28)	13 (21)	0.41	0.27	0.024
Negative (40)	33 (83)	32 (80)	23 (58)	27 (68)	15 (38)	8 (20)	9 (23)	0.53	0.21	
	$p = 0.009$	$p = 0.008$		$p = 0.006$	$p = 0.048$					

Notes: [1]Methylation index – total number of genes methylated divided by total number of genes analyzed; ns – nonsignificant; ERα, estrogen receptor α; PR – progesterone receptor.

Table 2
Correlation of hypermethylation of a panel of genes

Genes	Methylation status (n)	*PRB* n (%)	*ERα* n (%)	*RASSF1A* n (%)	*p16INK4A* n (%)	*BRCA1* n (%)	*GSTP1* n (%)	*RARβ2* n (%)
PRB	U (33)		5 (15)	22 (67)	16 (49)	5 (15)	8 (24)	6 (18)
	M (68)		60 (88)	42 (62)	35 (52)	22 (32)	17 (25)	16 (24)
			$p < 0.001$					
ERα	U (36)	8 (22)		23 (64)	16 (44)	4 (11)	8 (22)	6 (17)
	M (65)	60 (92)		41 (63)	35 (54)	23 (35)	17 (26)	16 (25)
		$p < 0.001$				$p = 0.008$		
RASSF1A	U (37)	26 (70)	24 (65)		19 (51)	5 (13)	2 (5)	1 (3)
	M (64)	42 (66)	41 (64)		32 (50)	22 (34)	23 (36)	21 (33)
						$p = 0.023$	$p = 0.001$	$p < 0.001$
p16INK4A	U (50)	33 (66)	30 (60)	32 (64)		9 (18)	13 (26)	9 (18)
	M (51)	35 (69)	35 (69)	32 (63)		18 (35)	12 (24)	13 (26)
						$p = 0.049$		
BRCA1	U (74)	46 (62)	42 (57)	42 (57)	33 (45)		11 (15)	7 (10)
	M (27)	22 (82)	23 (85)	22 (82)	18 (67)		14 (52)	15 (56)
			$p = 0.008$	$p = 0.023$	$p = 0.049$		$p < 0.001$	$p < 0.001$
GSTP1	U (76)	51 (67)	48 (63)	41 (54)	39 (51)	13 (17)		8 (11)
	M (25)	17 (68)	17 (68)	23 (92)	12 (48)	14 (56)		14 (56)
				$p < 0.001$		$p < 0.001$		$p < 0.001$
RARβ2	U (79)	52 (66)	49 (62)	43 (54)	38 (48)	12 (15)	11 (14)	
	M (22)	16 (73)	16 (73)	21 (96)	13 (59)	15 (68)	14 (64)	
				$p < 0.001$		$p < 0.001$	$p < 0.001$	
Total # of	1 (9)	2 (22)	0 (0)	3 (33)	4 (44)	0 (0)	0 (0)	0 (0)
methylated	2 (26)	12 (46)	11 (42)	16 (62)	7 (27)	1 (4)	3 (12)	2 (8)
genes in each	3 (17)	16 (94)	16 (94)	7 (41)	10 (59)	0 (0)	2 (12)	0 (0)
patient[1]	4 (21)	19 (91)	19 (91)	17 (81)	15 (71)	7 (33)	5 (24)	2 (10)
	5 (11)	9 (82)	8 (73)	10 (91)	7 (64)	8 (73)	5 (46)	8 (73)
	6 (6)	5 (83)	6 (100)	6 (100)	3 (50)	6 (100)	5 (83)	5 (83)
	7 (5)	5 (100)	6 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.003$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Notes: [1]The patients were further divided by their total numbers of methylated genes. For those patients with only one gene methylated ($n = 9$), 22% ($n = 2$) were *PRB*, 33% ($n = 3$) were *RASSF1A* and 44% ($n = 4$) were *p16INK4A*. The *ERα*, *BRCA1*, *GSTP1* and *RARβ2* do not appear to be methylated alone.

(6/7–7/7) had significantly shorter DFS (Table 3A; HR = 14.58, 95% CI = 3.19–66.73, $p < 0.001$) and OS (Table 3B; HR = 12.16, 95% CI = 1.44–102.77, $p = 0.022$). Figure 2 shows Kaplan–Meier survival curves for methylation of each of these genes and also for the combined effect of the methylated genes.

To determine whether the prognostic value of gene methylation was independent of other risk factors associated with clinical outcome, we examined the prognostic significance of gene methylation by adjusting for age, menopausal status, and tumor stage, ERα, PR and Her2/*neu* status. The methylation of *PRB*, *BRCA1* and *RARβ2* was significantly associated with DFS when adjusted for age; menopausal status and tumor stage (Table 3A). However, only the *RARβ2* methylation remained significant for both DFS and OS when adjusted for potential confounding factors such as age, menopause, stage, ERα, PR and Her2/*neu* (HR = 3.19, 95% CI = 1.49–6.83, $p = 0.003$; HR = 9.22, 95% CI = 1.63–52.15, $p = 0.012$). The effect of total number of genes methylated was examined on DFS and OS. The multiple Cox regression with backward selection was used to select important effects for DFS and OS among gene methylation and clinicopathological characteristics (Table 4). Tumor stage was an important factor for both outcomes (HR = 3.51

Table 3A
Hazard ratios for disease free survival

Variable	Univariate			Adjusted by age, menopause, stage			Adjusted by age, menopause, stage, ERα, PR, Her2/*neu*		
	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI	*p*-value
PRB methylation		1.07–5.50	0.034	3.14	1.35–7.29	0.008	2.07	0.81–5.30	
ERα methylation	1.68	0.82–3.45		1.67	0.81–3.43		1.18	0.55–2.56	
RASSF1A methylation	2.14	1.02–4.51	0.045	1.80	0.82–3.93		1.80	0.79–4.09	
p16INK4A methylation	2.11	1.10–4.06	0.024	1.92	0.98–3.76		1.49	0.73–3.05	
BRCA1 methylation	3.88	2.05–7.34	<0.001	2.83	1.45–5.55	0.002	2.03	0.96–4.29	
GSTP1 methylation	2.79	1.47–5.26	0.002	1.92	0.99–3.76		1.76	0.87–3.59	
RARβ2 methylation	3.87	2.04–7.34	<0.001	3.24	1.66–6.33	<0.001	3.19	1.49–6.83	0.003
MI[1]: (2–3)/7 vs. (0–1)/7	1.44	0.31–6.67		1.14	0.24–5.38		1.08	0.23–5.16	
MI[1]: (4–5)/7 vs. (0–1)/7	4.67	1.08–20.27	0.040	3.85	0.88–16.92		3.02	0.66–13.90	
MI[1]: (6–7)/7 vs. (0–1)/7	14.58	3.19–66.73	<0.001	7.32	1.49–36.00	0.014	6.25	1.01–38.60	0.048
ERα-negative	2.32	1.15–4.66	0.018	2.33	1.15–4.71	0.018			
PR-negative	1.90	0.99–3.66		2.30	1.17–4.52	0.015			
Her2/*neu*-positive	1.93	0.99–3.77		1.88	0.92–3.82				

Notes: [1]MI (Methylation index) – total number of genes methylated divided by total number of genes analyzed; ERα – estrogen receptor α; PR – progesterone receptor.

Table 3B
Hazard ratios for overall survival analysis

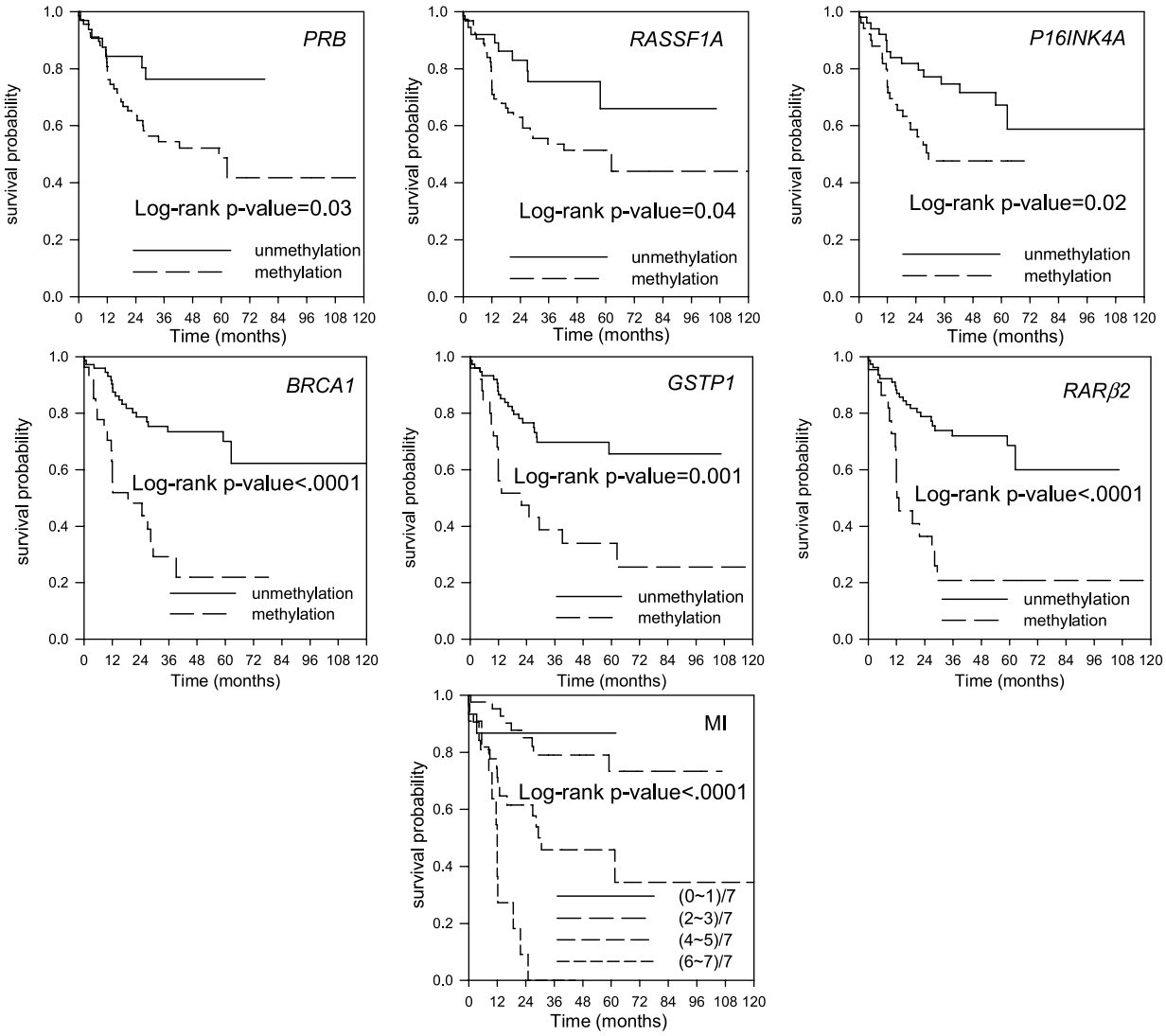
Variable	Univariate			Adjusted by age, menopause, stage			Adjusted by age, menopause, stage, ERα, PR, Her2/*neu*		
	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI	*p*-value
PRB methylation	2.51	0.55–11.45		2.96	0.60–14.70		1.08	0.16–7.14	
ERα methylation	1.75	0.47–6.49		1.43	0.38–5.38		0.68	0.15–3.05	
RASSF1A methylation	6.13	0.79–47.55		5.62	0.69–45.55		4.05	0.47–34.92	
p16INK4A methylation	1.25	0.40–3.92		1.02	0.31–3.38		0.66	0.18–2.39	
BRCA1 methylation	5.06	1.58–16.22	0.006	3.10	0.90–10.69		2.12	0.47–9.63	
GSTP1 methylation	6.61	1.99–21.98	0.002	3.05	0.88–10.60		4.90	1.10–21.88	0.037
RARβ2 methylation	9.26	2.76–31.05	<0.001	6.03	1.69–21.45	0.006	9.22	1.63–52.15	0.012
MI[1]: (2–3)/7 vs. (0–1)/7	0.30	0.02–4.83		0.17	0.01–3.03		0.13	0.01–2.62	
MI[1]: (4–5)/7 vs. (0–1)/7	1.91	0.21–17.14		1.28	0.14–11.99		1.02	0.08–12.48	
MI[1]: (6–7)/7 vs. (0–1)/7	12.16	1.44–102.77	0.022	3.00	0.31–28.93		5.68	0.22–150.18	
ERα-negative	4.46	0.98–20.38		4.81	1.01–22.79	0.048			
PR-negative	2.18	0.65–7.27		2.58	0.72–9.26				
Her2/*neu*-positive	3.55	1.14–11.02	0.028	2.50	0.76–8.25				

Notes: [1]MI (Methylation index) – total number of genes methylated divided by total number of genes analyzed; ERα – estrogen receptor α; PR – progesterone receptor.

and 14.37, respectively). The *RARβ2* hypermethylation was significant for both DFS (HR = 3.29, 95% CI = 1.72–6.28, $p < 0.001$) and OS (HR = 6.71, 95% CI = 1.93–23.29, $p = 0.003$). In addition, *PRB* hypermethylation was only significant for DFS (HR = 2.40, 95% CI = 1.06–5.46, $p = 0.037$).

4. Discussion

This study was designed to evaluate the prognostic significance of promoter methylation of a panel of genes in Invasive ductal carcinomas of breast. The most salient findings of our study are (i) the hyper-

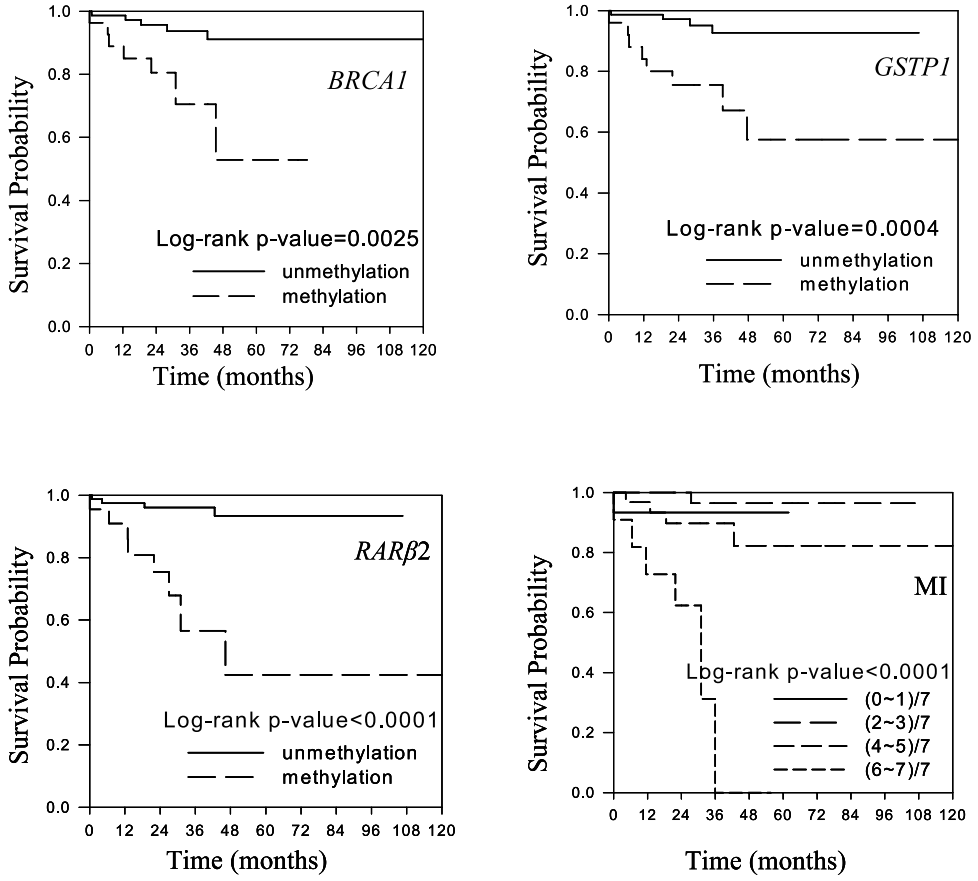


(a)

Fig. 2. Kaplan–Meier survival curves to demonstrate a relationship between each gene methylation and methylation index (MI) with the probability of disease free survival (a) and overall survival (b) among all the breast cancer patients.

methylation of *p16INK4A*, *BRCA1*, *ER*α and *PRB* was associated with negative ERα, PR, HR status and triple negative breast cancers; (ii) there was significant correlation between hypermethylation of *BRCA1*, *ER*α, *GSTP1* and *RAR*β*2*; (iii) hypermethylation of *GSTP1* and *PRB* was significantly associated with positive Her2/*neu* status; (iv) hypermethylation of *p16INK4A*, *BRCA1* and *GSTP1* was significantly more in advanced stages of breast cancer; (v) combined effect of hypermethylation of multiple gene promoters was evaluated by using MI. Higher MI was significantly associated with higher tumor stage and negative ERα, PR, HR and triple negative tumors; (vi) *RAR*β*2* hypermethylation was significantly associated with reduced DFS and OS; (vii) patients with higher MI showed adverse disease prognosis (reduced DFS and OS).

Overall, the evaluation of hypermethylation in a panel of genes indicated that promoter hypermethylation does not occur randomly in breast cancer. Indeed, cancer related genes are targeted in a specific manner with a direct correlation among *ER*α, *GSTP1*, *RAR*β*2* and *BRCA1*. Furthermore, patients with higher stage tumor, negative ERα, PR status and triple negative cancers were likely to harbor higher methylation



(b)

Fig. 2. (Continued.)

Table 4
Multiple Cox regression analysis with backward selection for disease free survival and overall survival

Variables	Disease-free survival			Over all survival		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
PRB methylation	2.40	1.06–5.46	0.037			
RARβ2 methylation	3.29	1.72–6.28	<0.001	6.71	1.93–23.29	0.003
Stage III	3.51	1.77–6.95	<0.001	14.37	1.80–114.78	0.012

index. Among all these genes, *RARβ2* hypermethylation emerged as the most important prognostic marker for breast cancer.

The methylation frequencies in breast tumors in this study were similar to those reported previously for most of the genes in candidate gene methylation studies including the earlier work from our own group in independent sets of breast cancer patients [10,15,27, 28,43]. In the earlier reports, methylation data have been correlated with hormone receptor status to clarify the existence of an interaction between DNA methylation and hormone receptor status biology in breast cancer cells [19,47]. Consistent with these investigations, we observed a positive association between hypermethylation of *BRCA1*, *p16INK4A* and *ERα* and negative ERα, PR and HR status [48]. Previously, on the basis of the microarray profiling of invasive breast carcinomas, five distinct subtypes of tumors (luminal A, luminal B, normal breast-like, HER-2/neu overexpressing, and basal) associated with different clinical outcomes

have been identified [46]. The basal subtype is associated with poor clinical outcome and is the subtype observed in BRCA1-related breast cancers. Mostly basal-like subtypes are triple-negative (that is to say, negative for ER, PR and HER-2/*neu* expression) and poorly differentiated tumors [49]. Hence, our study supports the previous studies that phenotypical, immunohistochemical characteristics and molecular features are shared by basal-like breast cancers and tumors that exhibit BRCA1 loss either by mutation or promoter hypermethylation.

Another important finding of our molecular survey was the correlation between promoter hypermethylation of tumor related genes. Methylation status of *BRCA1* was significantly correlated with *ER*α, *GSTP1* and *RAR*β*2*. The molecular significance of this concordance remains to be determined, but *BRCA1* gene, originally cloned as the gene responsible for familial breast cancers [33], encodes a multifunctional protein involved in DNA repair, cell cycle check point control, protein ubiquitinylation and chromatin remodeling [9]. Therefore, we hypothesize that *BRCA1* may be functionally related to *ER*α and *RAR*β*2*. Previous studies suggest that all nuclear receptors and *BRCA1* require coactivator proteins such as p300 and its close relative CREB-binding protein (CBP) to activate target gene transcription [9]. CBP/p300 interacts with *ER*α and *RAR*β in their ligand-bound conformation to induce gene expression. Further, *BRCA1* has been shown to interact with and inhibit the transcriptional activity of *ER*α and also regulate *PR* signaling in mammary epithelial cells [29,30]. This apparent non-random distribution of promoter hypermethylation of some genes suggests the existence of specific factors causing selective promoter region hypermethylation of tumor-related genes. The association between increasing levels of DNA methylation and poor prognosis is a recurrent observation in oncology, consistent across multiple tumor types that include liver cancers, esophageal cancers, lung cancers and various leukemias [24]. A plausible hypothesis is that tumors with high degrees of methylation are more likely to inactivate genes critical for tumor progression and response to chemotherapy.

We also observed that breast cancer patients with a higher MI were more likely to have poor prognosis compared with patients who had a low MI. To our knowledge this is one of the first few studies to examine the prognostic relevance of multiple gene methylations in breast cancer using a candidate gene approach. The most salient finding of our study is that methylation of a panel of genes, *BRCA1*, *GSTP1* and *RAR*β*2* is an important determinant of breast cancer prognosis. After adjustments for confounders such as age, menopausal status, tumor stage, ERα, PR and Her2/*neu* status. The emergence of *RAR*β*2* as the most important adverse prognosticator is cognizant with its role in regulation of gene expression and its retinoid-mediated antiproliferative, differentiative, immuno-modulatory and apoptosis-inducing properties. Retinoids have been shown to inhibit the growth of breast cancer cell lines in culture and breast tumors in animal models [26]. RARβ2 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 [2,32]. Detection of *RAR*β*2* 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 [26]. Pretreatment assessment of *RAR*β*2* methylation status may more accurately identify patients likely to respond to therapy.

The current results underscore the importance of methylation of a panel of genes in the development and prognosis of breast cancer. Further, MI composed of important tumor suppressor and DNA repair genes is likely to have clinical implications in the prognosis for patients with breast cancer. Consequently, methylation of these important genes may also serve as potential therapeutic targets for future studies examining the effect of demethylating agents.

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