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

Gayatri Sharma a, Sameer Mirza a, Yi-Hsin Yang d,e, Rajinder Parshad b, Priya Hazrah b, Siddartha Datta Gupta c and Ranju Ralhan a,*

a Department of Biochemistry, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India
b Department of Surgery, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India
c Department of Pathology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India
d Department of Oral Hygiene, Kaohsiung Medical University, Kaohsiung, Taiwan
e Division of Statistical Analysis, Department of Clinical Research, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan

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%, ERα 64%, RASSF1A 63%, p16/INK4A 51%, RARβ 2 22%, GSTP1 25% and BRCA1 27% of the breast cancers, respectively. Concurrent methylation of BRCA1, ERα, GSTP1 and RARβ2, 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 RARβ2 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, RARβ, 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
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 genes were 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. 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) H2O2 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 antimouse 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 invasive ductal breast carcinomas. The MSP analysis of ERα, PRB, BRCA1 and RARβ2 genes in archival breast tumors is shown in Figure 1a. (i) ERα panel viewed from left to right shows a positive control shows methylated DNA in tumor, patient 1 shows presence of methylated and unmethylated 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 positive control for unmethylated DNA, normal breast tissue used also shows 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 positive control for methylated DNA, normal breast tissue used also shows unmethylated DNA. 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 positive control for methylated DNA, normal breast tissue used also shows unmethylated DNA. 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.
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, 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.

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.

3.2. 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 ($p = 0.024$). 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
mors with PRB observed significant association of triple negative tumors which did not show Her2/neu amplification correlated positively with 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 ERα methylation (88% vs. 15%, p < 0.001). In addition, PRB, ERα methylation was also associated with BRCA1 (35% vs. 11%, p = 0.008). Similarly, the methylation of RASSFIA was associated with methylation of BRCA1 (p = 0.023), GSTP1 (p < 0.001) and RARβ2 (p < 0.001). Importantly, BRCA1 methylation was associated with methylation of 5 of these 6 genes (ERα, RASSFIA, p16INK4A, GSTP1 and RARβ2) examined in our panel, the only exception being PRB, which was of borderline significance (p = 0.06). In addition, the gene methylation of RASSFIA, BRCA1, GSTP1 and RARβ2 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 RASSFIA and 44% (n = 4) were p16INK4A. The ERα, BRCA1, GSTP1 and RARβ2 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 ERα 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 RARβ2 (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             | PRB n (%) | ERα n (%) | RASSF1A n (%) | p16INK4A n (%) | BRCA1 n (%) | GSTP1 n (%) | RARβ2 n (%) | Methylation index† | 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            |           |           |               |                |             |             |             |                   |       |     |        |
| T1 + T2 (57)          | 40 (70)   | 37 (65)   | 35 (61)       | 24 (42)        | 10 (18)     | 7 (12)      | 9 (16)      | 0.41              | 0.24  | 0.024 |        |
| T3 + T4 (44)          | 28 (64)   | 28 (64)   | 29 (66)       | 27 (61)        | 17 (39)     | 18 (41)     | 13 (30)     | 0.52              | 0.26  |     |        |
| p = 0.018             |           |           |               |                |             |             |             |                   |       |     |        |
| 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 |           |               |                |             |             |             |                   |       |     |        |
| 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 |           |               |                |             |             |             |                   |       |     |        |
| 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 |           |               |                |             |             |             |                   |       |     |        |
| 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 |           |               |                |             |             |             |                   |       |     |        |
| 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             |           |           |               |                |             |             |             |                   |       |     |        |
| 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 |           |               |                |             |             |             |                   |       |     |        |
| 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 |           |               |                |             |             |             |                   |       |     |        |
| Notes: †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

| Genes     | Methylation status (n) | PRB n (%) | ERα n (%) | RASSF1A n (%) | p16INK4A n (%) | BRCA1 n (%) | GSTP1 n (%) | RAR/32 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 = 0.008              |           |           |               |               |             |             |              |
| 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              |           |           |               |               |             |             |              |
| 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              |           |           |               |               |             |             |              |
| 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              |           |           |               |               |             |             |              |
| RAR/32    | 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              |           |           |               |               |             |             |              |
| Total # of methylated genes in each patient | 1 (9) | 2 (22) | 0 (0) | 3 (33) | 4 (44) | 0 (0) | 0 (0) | 0 (0) |
|          | 2 (26)                 | 12 (46)   | 11 (42)   | 16 (62)       | 7 (27)        | 1 (4)       | 3 (12)      | 2 (8)       |
|          | 3 (17)                 | 16 (94)   | 16 (94)   | 7 (41)        | 10 (59)       | 0 (0)       | 2 (12)      | 0 (0)       |
|          | 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)     |              |
|          | 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: 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 RAR/32 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 = 4.4–102.77, 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 RAR/32 was significantly associated with DFS when adjusted for age; menopausal status and tumor stage (Table 3A). However, only the RAR/32 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

| 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 |         |
| RAR/β2 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   |
| MI1: (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 |         |
| MI1: (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 |         |
| MI1: (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: 1MI (Methylation index) – total number of genes methylated divided by total number of genes analyzed; ERα – estrogen receptor α; PR – progesterone receptor.

Table 3B

| 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   |
| RAR/β2 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   |
| MI1: (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 |         |
| MI1: (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 |         |
| MI1: (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: 1MI (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-
methylation of p16INK4A, BRCA1, ERO and PRB was associated with negative ERα, PR, HR status and triple negative breast cancers; (ii) there was significant correlation between hypermethylation of BRCA1, ERO, GSTP1 and RARβ2; (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; (vii) RARβ2 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 ERα, GSTP1, RARβ2 and BRCA1. Furthermore, patients with higher stage tumor, negative ERα, PR status and triple negative cancers were likely to harbor higher methylation.
Table 4

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

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 ERα, GSTP1 and RARβ2. 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 checkpoint control, protein ubiquitinylation and chromatin remodelling [9]. Therefore, we hypothesize that BRCA1 may be functionally related to ERα and RARβ2. 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 ERα and RARβ in their ligand-bound conformation to induce gene expression. Further, BRCA1 has been shown to interact with and inhibit the transcriptional activity of ERα 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 RARβ2 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 RARβ2 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 uncontrollable cellular proliferation [2,32]. Detection of RARβ2 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 RARβ2 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 of future studies examining the effect of demethylating agents.

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