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PRECLINICAL STUDY

Estrogen receptor α, *BRCA1*, and *FANCF* promoter methylation occur in distinct subsets of sporadic breast cancers

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Abstract *Estrogen receptor* α (*ER*) and its ligand estrogen play vital roles in the development, progression and treatment of breast cancer. An increasing number of studies have also provided evidence linking disruption of the Fanconi anemia/BRCA cascade to breast cancer. Our objectives were to examine the methylation status and expression profiles of *ER*, correlate the findings with *BRCA1* and *FANCF* methylation and map the critical CpGs for ER expression. We found that the CpG islands in the 5′ region of the *ER* gene are methylated in 59 of 120 (49.2%) primary breast cancers, including 45 of 59 ER-negative tumors (76.3%, $P < 0.00001$). In addition, we observed a strong correlation between *ER* promoter and *BRCA1* promoter methylation (odds ratio 3.12, 95% confidence interval 1.10–9.68, $P = 0.02$). In contrast, *FANCF* methylation was rare in breast tumors: one of 120 (0.8%). *ER* methylation was associated with high tumor grade (60.4% methylated vs. 39.6% unmethylated in grade 3 tumors, $P = 0.04$) and tumor subtype ($P = 0.03$). Though small in number, all tumors of the medullary subtype were *ER* methylated. In contrast, the lobular subtype had the least methylation (23.1% methylated vs. 76.9% unmethylated). After treatment of MDA-MB-231 cells with 5-aza-cytidine (5-aza-dC) and trichostatin, which resulted in re-expression of *ER* mRNA, we localized dramatic demethylation effects to CpG islands in positions +68, +165, +192, +195, +337, +341 and +405 from transcription start site of the *ER* promoter. These data suggest that unlike *FANCF*, both *ER* and *BRCA1* are specifically targeted for methylation in sporadic breast cancers, a phenomenon that should be explored for development of novel diagnostic and therapeutic approaches.

Keywords Breast cancer · *ER* · *BRCA1* · *FANCF* · Methylation

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Introduction

Estrogen receptor α (*ER*) and its ligand estrogen play critical roles in breast cancer pathogenesis, progression and treatment. Hormonal therapy via estrogen depletion or selective estrogen receptor modulators is widely used to block the action of estrogen in women with hormone-responsive breast cancers [1]. A potential mechanism for hormone resistance is the acquired loss of *ER* gene expression at the transcriptional level during disease progression [2, 3]. Methylation of the CpG islands in the 5′ regulatory region of the *ER* gene has been associated with loss of *ER* gene expression in ER-negative breast cancers [4, 5]. Thus, *ER* promoter methylation may be used as a marker for breast cancer detection, prognosis, and treatment outcome prediction.

Methylation of the *BRCA1* promoter has previously been linked to reduced mRNA expression in primary breast cancer samples, with proportions ranging from 11 to 31% [6]. It has been reported that *BRCA1*-associated breast cancers, which predominantly occur in premenopausal women, are more frequently of the ER-negative phenotype [7]. We previously demonstrated that inactivation of *BRCA1* by promoter methylation is associated with reduced transcripts, decreased gene copy number and chromosome 17 aneusomy, as observed in tumors from *BRCA1* mutation carriers [8]. Furthermore, an increasing number of studies have provided evidence linking disruption of Fanconi anemia/BRCA cascade in sporadic cancers [9]. *FANCF*, a Fanconi anemia gene encodes a protein required for DNA damage-inducible monoubiquitination of FANCD2, and for targeting of FANCD2 to DNA repair nuclear foci [10]. A previous study suggested that inactivation of *FANCF* in ovarian tumors resulted from methylation of its CpG islands, and acquired cisplatin resistance during tumor progression was correlated with demethylation of *FANCF* [11]. It is not clear whether methylation of *FANCF* would have similar effects as *BRCA1* inactivation, for which gene either promoter methylation or inherited mutation can serve as a "first hit" in a model of breast tumor progression [12]. To test this hypothesis, we analyzed the *FANCF* promoter in the same panel of primary breast tumor samples and correlated our findings with *ER* and *BRCA1* methylation. To our knowledge, this is the first study to analyze these three critical genes. While demonstrating a strong association between *ER* methylation and *BRCA1* methylation, we found no association with *FANCF* methylation.

Materials and methods

Cell lines

Human breast cancer cell lines MCF-7, MDA-MB-231, HCC-1937 and SK-BR3 were obtained from ATCC (Rockville, MD, USA). UACC3199 was obtained from the University of Arizona Cancer Center. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA). MDA-MB-231, HCC1937 and UACC3199cells were grown in RPMI 1640 medium (Invitrogen), and SK-BR3 cells were cultured in McCoy's 5a medium containing 1.5 mM of L-glutamine, 3.0 g/l glucose and 2.2 g/l sodium bicarbonate. All media were supplemented with 10% FBS. Medium for the HCC1937 cell line was also supplemented with 0.5 μg/ml insulin. All cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Patient materials

The study was conducted under research protocols approved by the University of Chicago Institutional Review Board. Primary breast tumor tissues were obtained by surgical resection at the University of Chicago and stored in liquid nitrogen as previously described [13]. Tissue sections containing >80% tumor cells were selected after microscopic examination. Diagnoses were confirmed by review of medical records, and data were collected on clinic-pathological features including race, age, tumor size, histological type, tumor grade, hormone receptor status, nodal status and tumor stage.

DNA extraction and bisulfite modification

Genomic DNA was extracted from cultured cells with the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN, USA). To extract DNA from frozen breast tissue, the samples were digested overnight at 55°C in a 50-mM Tris–HCl buffer containing 0.5% SDS and 0.3μg/ml Proteinase K (Invitrogen) followed by phenol/chloroform extraction and ethanol precipitation. Sodium bisulfite reactions were carried out as described [14]. Approximately 1 μg of alkali-denatured DNA was incubated in 3 M $NaHSO_3$ and 0.5 mM hydroquinone for 16 h at 54°C. This bisulfite-treated DNA was then desalted with the Wizard DNA Clean-up System (Promega, Madison, WI, USA) and eluted into sterile water. The DNA was subsequently precipitated by 0.5 M ammonium acetate with ethanol after desulfolation and resuspended in TE.

Analysis of *ER* promoter methylation by methylation specific PCR

Promoter methylation was determined by methylation specific PCR (MSP) with bisulfite-converted DNA. For *ER*, we selected *ER1*, *ER3*, *ER4*, and *ER5* for MSP from the six primer pairs previously described [5] because these covered the most significantly methylated loci. PCR was carried out in a total volume of 20 μl containing 0.5 U of AmpliTaq Gold II (Roche, Nutley, NJ, USA). Each PCR reaction underwent initial denaturation at 95°C for 10 min, and 40 cycles of the following profile: 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. Each reaction completed its PCR cycle profile with a 10-min extension at 72°C. The PCR products were then electrophoresized on a 2% agarose gel or 6% acrylamide gel, stained with ethidium bromide and visualized by UV transillumination. Placental DNA treated in vitro with *SssI* bacterial methylase was used as a

positive control and DNA from normal lymphocytes or normal breast tissue was used as a negative control.

BRCA1 and *FANCF* promoter methylation analyses

Methylation specific PCR of *BRCA1* was done using primer sequences reported previously for the methylated reaction [15] and unmethylated reaction [8]. *FANCF* methylation was analyzed as previously described [11].

Demethylation of MDA-MB-231 cells with 5-aza-dC and TSA

MDA-MA-231 cells were seeded at a density of 5×10^5 cells in 100-mm plate. After 48 h, the cells were treated with 10, 50 or 100 ng/ml of 5-aza-dC (Sigma, St Louis, MO, USA) or with 10, 50 or 100 ng/ml of trichostatin (TSA; Sigma). To assess the effect of a combination of 5-aza-dC and TSA on the above cells, we treated cells with 50 ng/ml of 5-aza-dC and 50 ng/ml of TSA. The medium was changed after 48 h of treatment and the cells were cultured for another 48 h before harvesting. The 5-aza-dC was dissolved in PBS and TSA was reconstituted in absolute ethanol.

RNA isolation and reverse transcriptase PCR

Total cellular RNA was extracted from cultured breast cancer cells using the Trizol reagent (Invitrogen). Reverse transcription reactions were performed with the SUPERSCRIPT™ One-Step RT-PCR System (Invitrogen), using 2 μg of DNase-treated RNA and 1 μl of oligo (dT) 12–18 primer. For the *ER* gene (NM_000125), the primers were: 5′ CAC CCT GAA GTC TCT GGA AG 3′ (forward; 1752–1771) and 5′ GGC TAA AGT GGT GCA TGA TG 3′ (Reverse; 2200–2219). The housekeeping ribosomal protein gene *36B4* was used as an internal control. Primers for *36B4* were: 5′ GAT TGG CTA CCC AAC TGT TGC A 3′ (forward) and 5′ CAG GGG CAG CAG CCA CAA AGG C 3′ (reverse).

Sodium bisulfite genomic sequencing of the *ER* promoter

The *ER* promoter was amplified from the bisulfite-modified DNA by two rounds of PCR using previously described primers [16]. The resultant 642 bp PCR product includes 55 CpG dinucleotides. The product was gel purified and cloned into TOPO TA Cloning vector (Invitrogen). Ten recombinant clones were isolated using a Qiaprep spin plasmid miniprep kit (Qiagen) and sequenced on an ABI automated DNA sequencer. The methylation status of individual CpG sites was determined by comparison with the sequence from known *ER* sequences. The number of methylated CpGs at each specific site was divided by the number of clones analyzed ($n = 10$), to yield a value that represents the percentage of methylation for each site as previously described [8].

Statistical analysis

Summary statistics were computed for patient demographic and disease characteristics expressed on a continuous scale, and compared between *ER*-methylated and unmethylated tumors using the two-sample *t*-test. For characteristics classified into discrete categories, frequency distributions by methylation status were compared using Fisher's exact test. The odds ratio was used as a measure of association between *BRCA1* and *ER* methylation status.

Results

Methylation of the *ER* promoter in breast cancer cell lines

We first analyzed the methylation status of five breast cancer cell lines using methylation-specific PCR. The five breast cancer cell lines studied included one ER-positive cell line, MCF-7, and the four ER-negative cell lines: MDA-MB-231 (231), HCC1937 (1937), SK-BR3 (BR3) and UACC3199 (3199). As shown in Fig. 1, MCF-7 cells were unmethylated across all four regions analyzed, *ER1* through *ER5*. In contrast, MDA-MB-231 cells were methylated across all four regions examined. SK-BR3 cells were methylated at *ER1*, *ER3* and *ER4*, but not at *ER5*. UACC3199 cells were methylated at *ER1* and *ER3* regions. HCC1937 cells were methylated in the *ER3* and *ER4* regions of the *ER* promoter.

ER promoter methylation in primary breast carcinomas and correlation with clinico-pathologic features

The clinico-pathologic characteristics of the 120 unselected primary breast cancer cases are described in the supplementary data. Bisulfite-treated DNAs were amplified with primers for *ER1*, *ER3*, *ER4* and *ER5* (Fig. 2a and data not shown). *ER* methylation was not observed in genomic DNA from normal breast tissues, but was observed in in vitro methylated DNA. In agreement with data obtained

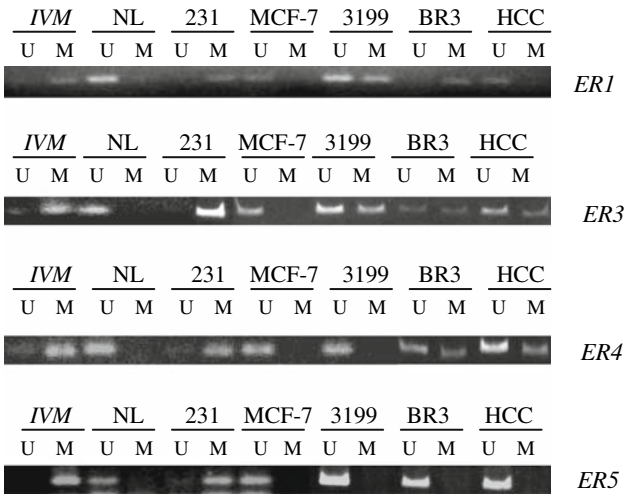

Fig. 1 Methylation specific PCR (MSP) analysis of *ER* promoter methylation in breast cancer cell lines. Breast cancer cell lines, including the ER-positive cell line MCF-7 and ER-negative cell lines MDA-MB-231 (231), HCC1937 (1937), SK-BR3 (BR3) and UACC3199 (3199), were analyzed by MSP using four pairs of unmethylated (U) and methylated (M) sequence-specific primers for *ER*. In vitro methylated DNA (*IVM*) was used as a positive control. DNA from normal lymphocytes (NL) was used as a negative control

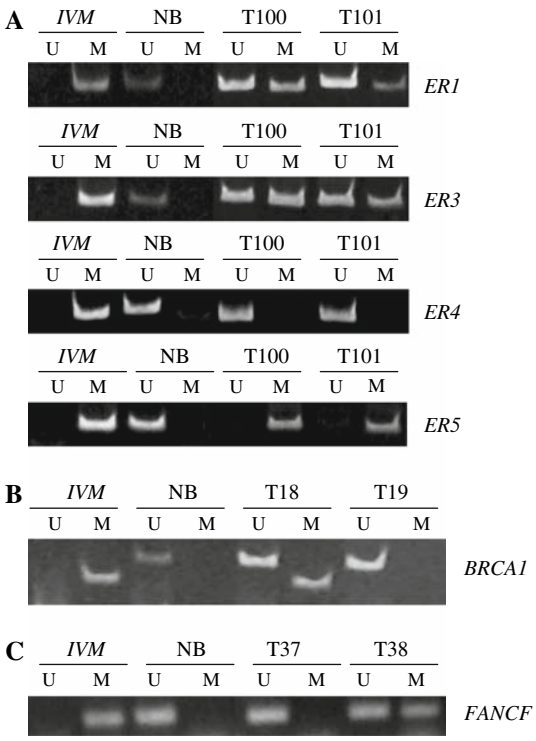

Fig. 2 Methylation of genes in primary breast tumors. Representative results of MSP assay of *ER* (**a**), *BRCA1* (**b**) and *FANCF* (**c**). *Lane M*, methylated product; *lane U*, unmethylated product. In vitro methylated DNA (*IVM*) was used as a positive control. DNA from normal breast tissue (NB) was used as a negative control

from cell lines, analysis of methylation patterns in the primary tumors demonstrated a concordance between ER-negativity by IHC and MSP-positivity in two or more regions of the promoter ($P < 0.0001$). Therefore tumors were classified as methylated if two or more regions were positive by MSP. Using this definition, the *ER* promoter was methylated in 59 of 120 primary breast tumors (49.2%) by the MSP assay. We next explored the relationship between *ER* methylation and clinicopathological characteristics of the primary breast tumors (Table 1). *ER* promoter methylation was not associated with age at diagnosis, race, tumor size, number of positive nodes, or tumor stage. However, methylated cases tended to be of higher grade (60.4% methylated vs. 39.6% unmethylated in grade 3 tumors, $P = 0.04$). Methylation was also associated with tumor subtype ($P = 0.03$). Though small in number, all tumors of the medullary subtype were methylated. In contrast, the lobular subtype had the least methylation (23.1% methylated vs. 76.9% unmethylated). A strong correlation was found with ER-negativity in *ER* methylated cases, with 77.6% of methylated cases being ER-negative vs. 22.4% of methylated cases being ER-positive ($P < 0.00001$). Methylation status of the *ER* promoter was also highly correlated with PR-negativity ($P = 0.0002$).

ER promoter methylation correlated with *BRCA1* promoter methylation

To determine if there is an association between *ER* and *BRCA1* promoter methylation patterns, we analyzed the methylation status of the *BRCA1* promoter in the same subset of primary breast cancers (Fig. 2b; Table 2). *BRCA1* promoter methylation was identified in 24 tumors (20.0%). Among these 24 *BRCA1*-methylated cases, 17 cases were also *ER* promoter-methylated. The relationship between *ER* methylation and *BRCA1* methylation is shown in Table 2. The *ER*-methylated cases were three times more likely to be *BRCA1*-methylated than unmethylated cases (odds ratio = 3.12, $P = 0.02$).

FANCF promoter methylation in primary breast carcinomas

To determine if *FANCF* might serve as a substitute for *BRCA1* methylation, we analyzed the methylation status of the *FANCF* promoter in the same 120 primary tumors by MSP, using a primer set from the *FANCF* CpG islands. Methylation of the *FANCF* gene was detected in only one of the 120 primary tumors (Fig. 2c).

Table 1 Association between *ER* promoter methylation and clinicopathological features of sporadic breast cancer ($N = 120$)

Feature	*ER* methylated, $n = 59$ (49.2)[a]	*ER* unmethylated, $n = 61$ (50.8)	p-Value[b]
Age at diagnosis (years)	$n = 54$	$n = 60$	0.31
≤55	33 (61.1)	29 (48.3)	
>55	21 (38.9)	31 (51.7)	
Race	$n = 51$	$n = 51$	0.55
African American	25 (49.0)	29 (56.9)	
Caucasian	25 (49.0)	22 (43.1)	
Hispanic	1 (2.0)	0 (0.0)	
Tumor size (cm)	$n = 52$	$n = 58$	0.25
Mean ± SD	3.76 ± 3.29	3.84 ± 2.48	
Nodes involved	$n = 52$	$n = 55$	0.79
0	23 (44.2)	21 (38.2)	
1–3	11 (21.2)	15 (27.3)	
4–9	14 (26.9)	13 (23.6)	
10+	4 (7.7)	6 (10.9)	
Tumor type	$n = 54$	$n = 57$	0.03
Ductal	48 (89.0)	47 (82.5)	
Lobular	3 (5.5)	10 (17.5)	
Medullary	3 (5.5)	0 (0)	
Tumor stage	$n = 51$	$n = 56$	0.81
I	11 (21.6)	8 (14.3)	
II	26 (50.9)	34 (60.7)	
III	11 (21.6)	12 (21.4)	
IV	3 (5.9)	2 (3.6)	
Tumor grade	$n = 50$	$n = 50$	0.04
1	1 (2.0)	6 (12.0)	
2	20 (40.0)	25 (50.0)	
3	29 (58.0)	19 (38.0)	
Estrogen receptor status	$n = 58$	$n = 60$	<0.00001
Negative	45 (77.6)	14 (23.3)	
Positive	13 (22.4)	46 (76.7)	
Progesterone receptor status	$n = 53$	$n = 57$	0.0002
Negative	40 (75.5)	23 (40.3)	
Positive	13 (24.5)	34 (59.7)	

[a] Numbers in parentheses are percentages

[b] Test of association between methylation status and the factor indicated

Table 2 Correlation between *ER* methylation and *BRCA1* methylation

Odds ratio = 3.12, 95% confidence interval = 1.10–9.68. Fisher's exact test $P = 0.02$

Promoters	*ER* methylated (%)	Unmethylated (%)	Total
BRCA1			
Methylated (%)	17 (28.8)	7 (11.5)	24 (20)
Unmethylated (%)	42 (71.2)	54 (88.5)	96 (80)
Total	59 (49.2)	61 (50.8)	120 (100.0)

Restoration of *ER* expression by 5-aza-dC and TSA in MDA-MB-231 cells

To map the critical CpGs involved in *ER* expression, we examined *ER* expression by RT-PCR after drug exposure. No *ER* mRNA was detectable in untreated MDA-MB-231 cells. Treatment of MDA-MB-231 cells with 5-aza-dC or TSA resulted in re-expression of *ER* mRNA in a dose-dependent manner (Fig. 3a). Bisulfite sequencing confirmed partial demethylation of *ER* promoter with drug exposure. After drug exposure, 24 out of 55 CpG islands located in the 642 bp region were partially demethylated in

MDA-MB-231 cells (Fig. 3b), with dramatic demethylation at positions +68, +165, +192, +195, +337, +341, and +405 relative to the transcription start site.

Discussion

It is now increasingly clear that molecular alterations occur at both the genetic and epigenetic levels, leading to tumor formation and progression. DNA methylation that results in gene silencing during tumorigenesis has been observed in numerous genes, including *E-cadherin*, *RB*, *p16*, *p15*, *MLH1*, and *PTEN* [17–20]. In this study, we demonstrated that methylation of the *ER* gene occurred in nearly half of the breast cancer cases and is highly correlated with ER-negativity ($P < 0.00001$) and *BRCA1* methylation (odds ratio 3.12, 95% confidence interval 1.10–9.68, $P = 0.02$). In contrast, we showed that *FANCF* promoter methylation was rare in breast tumors. Of note, *ER* methylation was associated with grade 3 tumors ($P = 0.04$) and tumor subtype ($P = 0.03$) with all three medullary breast cancers being *ER*-methylated.

Interpreting MSP results obtained from primary tumor samples can be complicated due to the heterogeneity of tumor tissue and sample source, e.g. fresh-frozen or paraffin-embedded [5]. Beyond that, the *ER* promoter is more complex than the promoter of other genes [21]. We first devised a classification rule that MSP-positivity in two or more regions would be counted as *ER* methylated based on: (1) Four established ER-negative breast cancer cell lines showing MSP-positivity in at least two of four regions of the *ER* promoter and (2) Statistical analysis of MSP data from primary tumors showing that there was a strong concordance between ER-negativity by IHC and MSP-positivity in two or more regions ($P < 0.0001$). Using this stringent classification rule, we detected *ER* promoter methylation in 59 of 120 (49.2%) primary breast tumors. The correlation between *ER* promoter methylation and reduction of ER protein expression was high, as 76.3% of tumors that were histologically classified as ER-negative were methylated at the *ER* promoter. Conversely, only 22% of tumors that were histologically ER-positive showed *ER* promoter methylation. This correlation is of high statistical significance, with $P < 0.00001$. This classification rule is

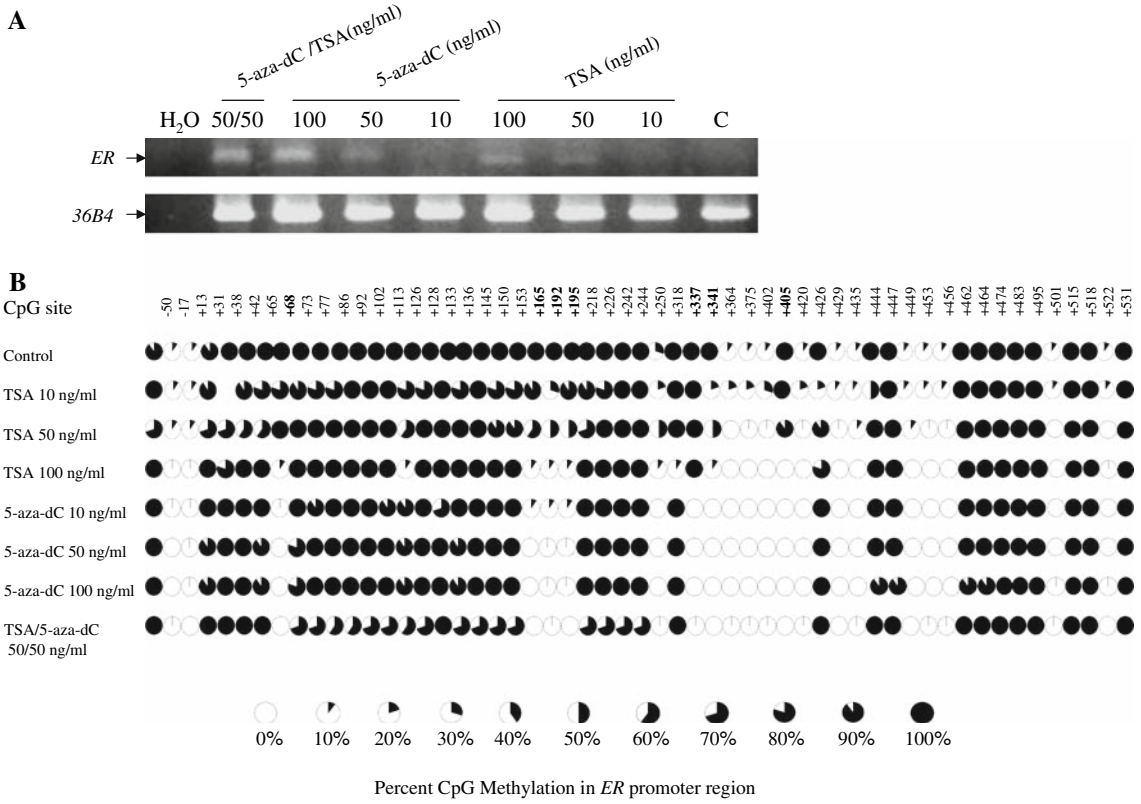

Fig. 3 Demethylation of *ER* promoter in MDA-MB-231 breast cancer cells. (**a**) RT-PCR analysis of *ER* mRNA expression in MDA-MB-231 cells after 5-aza-dC and TSA treatment. No *ER* mRNA was detectable in untreated MDA-MB-231 cells, while 5-aza-dC and TSA induced re-expression of *ER* mRNA. Amplification of *36B4* cDNA was used as an internal control. (**b**) Bisulfite-modified genomic DNA sequencing of the *ER* promoter in MDA-MB-231 cells. The treatment of 5-aza-dC and TSA led to dramatic CpG island demethylation for positions +68, +165, +192, +195, +337, +341 and +405

in concordance with bisulfite sequencing data, since demethylated CpG islands that were associated with re-expression of *ER* in MDA-MB-231 cells were located in three separate regions of the *ER* promoter. Thus, promoter methylation is the predominant mechanisms for down-regulating *ER* in ER-negative tumors.

We found that *ER* methylation was also correlated with several clinicopathological characteristics of the primary breast cancers. Methylated cases tended to have a higher tumor grade. The same phenomenon has been described for *BRCA1*-mutated and *BRCA1*-methylated tumors [8, 22]. This finding suggests that methylation of *ER* or *BRCA1* could serve as a biomarker for aggressive histologic tumor phenotype. In agreement with a previous report [23], *ER* methylation was also associated with particular histological types. Lobular tumors had the least methylation among the histological subtypes, while all three medullary tumors were *ER*-methylated. Interestingly, *BRCA1*-mutated tumors and *BRCA1* promoter-methylated tumors also have an excess of medullary subtype [24]. While we are unable to draw a definitive conclusion as the numbers are small, this finding underscores the need for larger studies of specific subtypes of breast cancer, as the etiologic risk factors and pathogenesis may vary.

Concordant methylation of *CDH1* and *ER* has been reported [23, 25, 26]. We report here that there is a correlation between *ER* promoter methylation and *BRCA1* promoter methylation. Previous studies have shown that hereditary *BRCA1*-associated tumors are more frequently ER-negative than sporadic tumors [22, 27, 28]. Many studies, including ours, show that epigenetic inactivation of *BRCA1* may also play an important role in a subset of breast tumors. Indeed, many tumors that do not carry *BRCA1* mutations have been designated as "*BRCA1*-like," both by histopathological criteria and by analysis of distinctive genome-wide transcription patterns [29, 30]. It is not currently known what triggers the genetic and epigenetic events that result in the "*BRCA1*-like" phenotype but it is plausible that dysregulation of DNA methylation inactivates multiple susceptible genes simultaneously during tumorigenesis, including *BRCA1* and *ER*. Our observations suggest that *ER* and *BRCA1* may be targeted by the same mechanisms in breast cancer while *FANCF* is not. This observed link at least in part explains why *BRCA*-like tumors are mostly ER-negative. Interestingly, while most "*BRCA1*-like" tumors are ER-negative by definition, only a fraction are *BRCA1*-methylated, as demonstrated in this study.

The FA-BRCA1 pathway plays a crucial role in DNA damage response, and inactivation of this pathway leads to cancer susceptibility [31]. The FA-BRCA pathway is disrupted in a subset of ovarian tumors by *FANCF* promoter methylation, and the tumors acquired cisplatin resistance during progression after demethylation of *FANCF* [11]. Promoter methylation of *FANCF* has been observed in 31% of cervical tumors [32]. However, in another study, no methylation was observed for *FANCF* in 106 ovarian tumors analyzed by MSP [33]. We were able to confirm *FANCF* promoter methylation by MSP in only one of the 120 tumors. The MSP assay could have missed the critical CpGs in breast cancer and we did not perform bisulfite sequencing for *FANCF*. Nonetheless, it is possible that *FANCF* does not play a major role in breast cancer, as no *FANCF* mutations have been identified in breast cancer [34].

MDA-MB-231, which shows *ER* methylation at all four sites, is transcriptionally inactive for the *ER* gene. We confirmed previous observation that treatment of these cells with the DNMT inhibitor 5-aza-dC and the HDAC inhibitor TSA leads to re-expression of the *ER* gene [35]. In addition, we observed that demethylation does not occur homogenously in the whole promoter region of treated MDA-MB-231 cells, but rather causes dramatic demethylation of CpG islands in positions +68, +165, +192, +195, +337, +341 and +405 relative to the transcription start site. This could reflect differences in accessibility of each island due to chromatin configuration or site-specific secondary effects of global demethylation. Nevertheless, these CpG sites are crucial in regulating re-expression of *ER* in cancer cells, and future work will examine how methylation of these CpG islands affects transcription factor binding.

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