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Research Article

BRCA1 Promoter Methylation in Sporadic Breast Cancer Is Associated with Reduced *BRCA1* Copy Number and Chromosome 17 Aneusomy

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Abstract

To explore the molecular mechanisms for the similarities between inherited and noninherited forms of breast cancer, we tested the hypothesis that inactivation of *BRCA1* by promoter hypermethylation is associated with reduced gene copy number and chromosome 17 aneusomy as observed in tumors from *BRCA1* mutation carriers. Using a combination of methylation-specific PCR analysis and fluorescence *in situ* hybridization, we observed varying degrees of promoter methylation in 39 of 131 (29.8%) primary tumors. Despite significant tumor heterogeneity, mean copy numbers of *BRCA1* and *CEP17* per cell were lower in methylated cases compared with unmethylated cases [1.78 versus 2.30 (P = 0.001) and 1.85 versus 2.29 (P = 0.005), respectively]. Methylation was more frequently observed in younger women (P = 0.05) with high-grade (P = 0.001), estrogen receptor–negative (P = 0.04), and progesterone receptor–negative (P = 0.01) tumors. Moreover, methylation was associated with reduced or absent *BRCA1* transcripts, which was reversible in the heavily *BRCA1*-methylated cell line UACC3199 following treatment with 5-aza-2′-deoxycytidine and trichostatin A. We identified five CpGs at positions −533, −355, −173, −21, and +44 as critical in the reexpression of *BRCA1*. We conclude that *BRCA1* methylation contributes to a subset of sporadic breast cancers with the resulting molecular and clinicopathologic phenotype similar to that of hereditary *BRCA1*-associated breast cancers. Our data support a model of carcinogenesis in which *BRCA1* promoter methylation may serve as a "first hit," much like an inherited germ line mutation, and promote tumor progression down a restricted set of molecular pathways. (Cancer Res 2005; 65(23): 10692-9)

Introduction

Whereas the majority of breast cancers occur sporadically, 5% to 10% of cases are caused by inherited mutations in breast cancer susceptibility genes *BRCA1* and *BRCA2*. *BRCA1* (OMIM 113705) is a classic tumor suppressor gene on 17q12-21; loss of the wild-type allele [loss of heterozygosity (LOH)] is required for tumorigenesis in germ line mutation carriers. Deletions in *BRCA1* locus are often accompanied by loss of chromosome 17 (1). *BRCA1* mutations are associated with cancer in women with a family history of breast or ovarian cancer or early onset of disease. *BRCA1*-associated breast cancers are more likely to occur at an early age (average of 44 years) and are characterized by poor histologic differentiation, aneuploidy, high S-phase fraction, and hormone receptor negativity (2). These aggressive pathologic features are also characteristic of breast cancers, which occur in young women without a family history, and suggest that alterations in BRCA1 or BRCA1-related pathway(s) might contribute to nonhereditary forms of breast cancer. By understanding the mechanisms of tumor initiation and progression, novel approaches for prevention and treatment of breast cancer can be developed.

Hypermethylation of the *BRCA1* promoter has been proposed as one mechanism for functionally inactivating BRCA1 and has been reported to occur in 7% to 31% of sporadic breast and ovarian cancers (3). *BRCA1*-methylated sporadic tumors have been described in young women and display pathologic features similar to those of *BRCA1*-mutated hereditary breast cancers (3–5). Interestingly, sporadic tumors with aberrant methylation of the *BRCA1* promoter can be clustered with tumors derived from women with inherited *BRCA1* germ line mutations because of similarities in their global gene expression profiles (5, 6).

Thus, an important role of functional inactivation of *BRCA1* by promoter methylation in sporadic breast cancer seems plausible but the mechanisms of tumor initiation and progression in these tumors are largely unknown. It has not been shown if the silencing of *BRCA1* in sporadic tumor cells is associated with the deletion of the *BRCA1* locus and/or the loss of chromosome 17, as is often the case in *BRCA1*-mutated cells. In this study, we have done an extensive analysis of *BRCA1* promoter methylation in relation to *BRCA1* gene copy number to gain a better understanding of the mechanisms involved in epigenetic regulation of the gene.

Materials and Methods

Cell lines. Four breast cancer cell lines, MCF-7, MDA-MB-231, HCC1937, and Hs578T, were obtained from American Type Culture Collection (Rockville, MD). UACC3199 cells were obtained from the University of Arizona Cancer Center (Tucson, AZ). MCF-7, MDA-MB-231, and Hs578T were grown in DMEM (Fisher Scientific, Hanover Park, IL). HCC1937 and UACC3199 were grown in RPMI 1640 (Invitrogen, Grand Island, NY). The media were supplemented with 10% FCS and 1% penicillin/streptomycin. For HCC1937 and Hs578T, 0.5 μg/mL insulin was also added. All cells were grown at 37°C in a humidified 5% CO_2 atmosphere. In addition, normal human lymphocytes from one healthy individual were used as reference cells (7).

Note: M. Wei and T.A. Grushko contributed equally to this work.

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Patient materials. The study was conducted under research protocols approved by the University of Chicago Institutional Review Board. Sporadic breast tumors were randomly selected from the pathology archives and tumor bank at the University of Chicago Hospitals as previously described (7). Of the 131 breast tumors analyzed in this study, 64 were paraffin-embedded specimens and 67 were fresh frozen specimens. Samples containing >50% tumor area were selected after microscopic examination of representative sections from each case. Frozen breast tissue specimens containing normal breast epithelia from three patients were used as controls.

DNA isolation. Genomic DNA was extracted using either QIAamp kit (Qiagen, Inc., Valencia, CA) for paraffin-embedded tissue or DNAzol isolation protocol (Invitrogen) for frozen tissue. The integrity of DNA extracted from paraffin-embedded tissue was confirmed by PCR with primers for exon 11A of the *BRCA1* gene (8, 9). Microdissection was done on 8-μm cryosections from 18 frozen specimens using Leica Laser Microdissection technology (Leica, Bensheim, Germany). From microdissected breast tumor cells, the DNA was extracted using the QIAamp kit (Qiagen).

DNA bisulfite modification. Sodium bisulfite conversion was done on 1 μg of tumor DNA per sample using previously described methods (10). Alkali-denatured DNA was incubated in 3 mol/L $NaHSO_3$ and 0.5 mmol/L hydroquinone for 16 hours at 54°C. Modified DNA was purified using the Wizard DNA Clean-up System (Promega, Madison, WI) and eluted into 50 μL of sterile water. DNA was precipitated with 0.5 mol/L ammonium acetate (pH 4.6), 1.5 μL of 20 mg/mL glycogen, and ethanol and then resuspended in Tris-EDTA.

***BRCA1* methylation.** Methylation analysis was done using a methylation-specific PCR–based approach as previously described (11). Primer sequences of *BRCA1* for the methylated reaction were 5′-TCGTGGTAACGGAAAAGCGC-3′ (sense) and 5′-AACGAACTCACGCCGCGCAA-3′ (antisense) and for the unmethylated reaction, 5′-TTGAGAGGTTGTTGTTTAGTGG-3′ (sense) and 5′-AACAAACTCACACCACACAA-3′ (antisense). The sense primer of the methylated reaction begins at 1,543 bp and the sense primer of the unmethylated reaction begins at 1,507 bp. The primers were predicted to give two PCR products with a 36-bp difference in size, each containing the transcription start site at 1,581 bp (GenBank *BRCA1* sequence U37574).

RNA isolation and reverse transcription-PCR. Total cellular RNA was isolated from microdissected frozen tumor cells using the RNAeasy Midi kit (Qiagen) and from breast cancer cells lines using Trizol reagents (Invitrogen). Reverse transcriptase reactions were done with 2 μg of DNase-treated RNA and 1 μL of oligo(dT)12-18 as the reverse transcription primer using the SuperScript One-Step reverse transcription-PCR (RT-PCR) System and Platinum Taq DNA Polymerase kit (Invitrogen). cDNA primers were designed to amplify exon 20 of the *BRCA1* gene: 5′-TGCTGAGTTTGTGTGTGAACGG-3′ (sense) and 5′-CTGTGCCAAGGGTGAATGATG-3′ (antisense). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a normalization control.

Fluorescence *in situ* hybridization. A BAC probe containing DNA sequences specific for the human *BRCA1* gene on 17q12-q21 (283G2, P1 library dmpc-HFF-1; ref. 12) was indirectly labeled by nick translation with biotin-conjugated dUTP (Boehringer Mannheim, Indianapolis, IN) and was detected with FITC-conjugated avidin as a green fluorescent signal (13, 14). A commercial *CEP17* probe is directly labeled with SpectrumOrange and contains α-satellite DNA for hybridization to the centromeric region of chromosome 17 (17p11.1-q11.1; Vysis/Abbott Labs, Downers Grove, IL).

Metaphase cell preparations from cell lines and from normal peripheral blood lymphocytes were done according to routine protocols (13). Imprint touch preparations of fresh frozen breast tumors and two-color fluorescence *in situ* hybridization (FISH) procedure were as described previously (14). In each sample, hybridization signals from 50 to 100 nuclei were scored. The absolute number of signals and the ratio of *BRCA1* signals to chromosome 17 centromere signals were recorded. Chromosome 17 aneusomy was estimated as previously described (7). The hybridization efficiency of the *BRCA1* probe was confirmed by performing *BRCA1/CEP17* FISH on normal lymphocytes as previously determined (7, 14, 15).

Demethylation of UACC3199 cell line. Cells were seeded at a density of 5×10^5 per 100-mm plate. The cells were allowed to attach for 48 hours. They were then exposed to 5-aza-2′-deoxycytidine (5-aza-dC) or trichostatin A (Sigma, St. Louis, MO) at 10, 50, or 100 ng/mL dose levels of each reagent or to the combination of 50 ng/mL 5-aza-dC and 50 ng/mL trichostatin A in triplicates. In parallel, untreated cells in triplicates were used as a control. After 48 hours of continuous exposure, the medium was changed. Cells were harvested and DNA was extracted after an additional 48 hours of incubation.

Sodium bisulfite genomic sequencing of the *BRCA1* promoter in UACC3199 cells after demethylation. The functional *BRCA1* promoter region of 658 bp, which includes 30 CpG dinucleotides, was sequenced for cytosine methylation using two rounds of PCR with nested primers specific to the bisulfite-modified sequence as previously determined (16). Purified PCR products were cloned into a TA vector according to the instructions of the manufacturer (TOPO TA Cloning kit for Sequencing, Invitrogen). Ten recombinants 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 estimated by determining the number of bisulfite-converted cases that differed from corresponding wild-type cases. For each dinucleotide, the proportion of methylated clones to the total number of clones analyzed ($n = 10$ in each experiment) was calculated.

Statistical analysis. Summary statistics were computed for patient demographic and disease characteristics, expressed on a continuous scale, and compared between *BRCA1*-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.

Mean numbers of *BRCA1* and *CEP17* signals per tumor for *BRCA1*-methylated and unmethylated cases were plotted, and for each tumor, the ratio of mean *BRCA1* signals to *CEP17* signals was computed. Mean copy numbers and mean ratios were compared between groups using the two-sample *t* test. For each tumor sample, proportions of cells exhibiting one, two, and three or more copies of *BRCA1* and *CEP17* signals were determined. Methylated and unmethylated tumor groups were compared for proportions of cells with aneusomy via *t* tests on arcsine transforms of these values, which are approximately normally distributed. For these tests and those described above, results of the *t* test and its nonparametric counterpart (Wilcoxon rank-sum test) were similar.

To evaluate the relationship between *BRCA1* methylation status and gene copy number while accounting for association with other patient/tumor characteristics, a linear regression model was fit, with *BRCA1* mean copy number as the response variable and covariates for methylation status and other patient/tumor characteristics associated with methylation status as the predictors. Similar adjusted comparisons were conducted for *CEP17* copy number and mean *BRCA1/CEP17* ratio.

Results

***BRCA1* promoter methylation and mRNA expression in normal human lymphocytes, normal breast epithelia, and breast cancer cell lines.** In optimizing our methylation-specific PCR assay, we confirmed that normal lymphocytes and normal breast epithelium were completely unmethylated at the *BRCA1* promoter. In agreement with prior publications (16–19), only *in vitro* methylated DNA and one of four breast cancer cell lines analyzed here, UACC3199, had aberrant *BRCA1* methylation (Fig. 1*A*).

Expression of *BRCA1* transcripts was observed in all unmethylated breast cancer cell lines and reference cells as determined by semiquantitative RT-PCR (Fig. 1*C*). As expected (16, 20), we observed no expression of *BRCA1* transcripts in the *BRCA1*-methylated UACC3199 cells (Fig. 1*C*).

***BRCA1* methylation in primary breast tumors.** Thirty-nine of 131 (29.8%) tumors were *BRCA1* methylated. Prevalence of *BRCA1*

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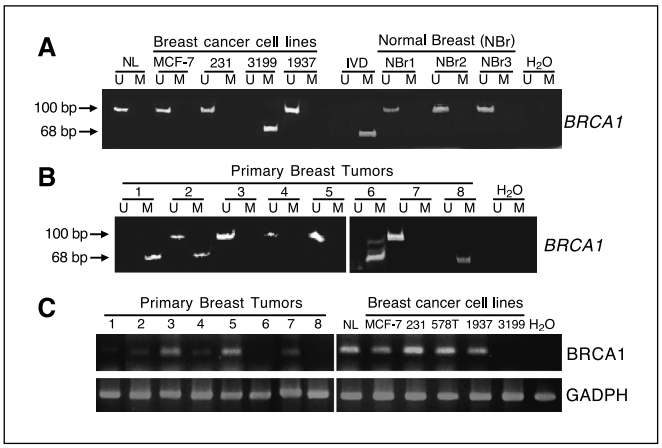

Figure 1. Methylation (*A* and *B*) and expression (*C*) analyses of *BRCA1* in breast cancer cell lines MCF-7, MDA-MB-231 (*231*), UACC3199 (*3199*), Hs578T (*578T*) and HCC1937 (*1937*), normal breast tissues (*NBr*), and primary breast tumors. *A,* optimization of the methylation-specific PCR assay in four breast cancer cell lines and three normal breast tissues. *B,* methylation-specific PCR analysis of *BRCA1* promoter methylation in eight microdissected primary breast tumors. *In vitro* methylated DNA (*IVD*) was used as a positive control for methylation; DNA from normal lymphocytes (*NL*) was used as a negative control for methylation. The 100-bp product corresponds to the unmethylated *BRCA1* product and the 68-bp product corresponds to the methylated *BRCA1* product. *Lanes M,* methylated products; *lanes U,* unmethylated products. UACC3199 cells and tumors 1, 6, and 8 show complete *BRCA1* methylation whereas tumor 2 provides an example of incomplete methylation of *BRCA1* alleles. *C,* patterns of *BRCA1* mRNA expression by RT-PCR in the same eight tumor samples as in (*B*) and in five breast cancer cell lines. Total RNA of normal lymphocytes was used as a positive control and H_2O was used as a negative control for *BRCA1* expression. *GAPDH* gene expression was used as a normalization control. All methylated *BRCA1* breast tumors (1, 2, 6, and 8) and methylated UACC3199 cells have significantly reduced or absent expression of *BRCA1*. With the exception of tumor 4, all other unmethylated tumors, cell lines, and normal lymphocytes highly express *BRCA1* transcripts.

methylation was similar in paraffin-embedded samples (32.8%) and fresh frozen samples (26.9%; $P = 0.57$). Among methylated tumors, we observed significant heterogeneity because *BRCA1* methylation was incomplete in the majority of cases. Both methylated and unmethylated products were present in 32 of 39 (82%) *BRCA1*-methylated tumors. Therefore, complete *BRCA1* promoter methylation (only methylated product present) was detected in only 7 of 39 or in 5.4% (7 of 131) of the primary breast carcinomas by conventional methylation-specific PCR assay.

***BRCA1* methylation and mRNA expression in microdissected tumors.** To determine if the unmethylated product in cases with incomplete *BRCA1* methylation is due to contamination by adjacent normal tissue or to incomplete methylation of one or both *BRCA1* alleles, methylation-specific PCR was done on tumor cells isolated by microdissection in 18 randomly selected fresh frozen cases (Table 1; tumors 1-8 from Table 1 are also depicted in Fig. 1*B*). Four of six *BRCA1*-methylated cases with additional unmethylated product showed only a methylated product after microdissection of tumor tissue. In two methylated cases, unmethylated band remained even after microdissection (tumors 2 and 11), suggesting that incomplete methylation of one or both *BRCA1* alleles is accountable for the methylation heterogeneity in these tumors (2 of 7, 30%). In all 18 cases, regardless of the DNA isolation method used, the methylation status of the *BRCA1* promoter remained unchanged.

Expression of *BRCA1* message, determined by semiquantitative RT-PCR, was observed in all *BRCA1*-unmethylated breast tumors (Table 1), with only one case (tumor 4) showing markedly reduced *BRCA1* transcripts (tumors 1-8 from Table 1 are also depicted in Fig. 1*C*). In contrast, no expression of the *BRCA1* transcripts was observed in four of five heavily *BRCA1*-methylated samples (tumors

Table 1. *BRCA1* promoter methylation, mRNA expression, and gene copy number in 18 microdissected breast tumors

Tumor	*BRCA1* status				*CEP17* status		
	Promoter methylation		mRNA expression	Mean *BRCA1* copies/cell	Proportion of cells (%) with one copy/cell	Mean *CEP17* copies/cell	Proportion of cells (%) with one copy/cell
	No microdissection	Microdissection					
8	M	M	−	1.15	84.71	1.24	76.47
9	M (U)	M	−	1.23	79.38	1.20	79.38
10	M (U)	M	−	1.60	43.16	1.97	5.26
6	M (U)	M	−	1.99	1.09	1.95	3.26
1	M (U)	M	+	2.01	2.08	1.99	2.05
11	M (U)	M (U)	+	1.97	3.09	1.96	6.19
2	M (U)	M (U)	+	2.00	3.09	1.99	3.09
12	U	U	+++	1.71	25.77	1.74	25.77
13	U	U	+++	1.99	3.09	1.97	3.09
14	U	U	+++	2.01	2.08	1.98	5.21
15	U	U	+++	2.03	4.12	2.05	3.09
16	U	U	+++	2.08	4.04	2.04	0.00
4	U	U	+	2.07	0.00	1.97	6.82
3	U	U	+++	2.08	4.04	1.97	3.03
7	U	U	++	2.10	2.02	2.08	1.01
17	U	U	+++	2.30	2.06	2.02	6.19
5	U	U	+++	3.14	0.0	3.17	0.00
18	U	U	+++	3.63	0.00	3.53	0.00

NOTE: M, methylated; U, unmethylated. −, no expression; +, markedly reduced expression; ++, reduced expression; +++, normal expression.

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6 and 8-10). Both tumors with incomplete methylation (tumors 2 and 11) and one specimen with complete methylation (tumor 1) had markedly reduced expression of *BRCA1*. Overall, a strong correlation between *BRCA1* methylation and mRNA expression was detected ($P < 0.001$).

Correlation of *BRCA1*/promoter methylation with clinicopathologic features of breast tumors. *BRCA1* methylation was more frequently observed in tumors from premenopausal or perimenopausal women (Table 2). Of the 58 cases from women <55 years old, 40% were methylated whereas only 22% of the 67 cases from women >55 years old were methylated ($P = 0.05$). *BRCA1*-methylated tumors were also more likely to be of higher grade, with 70.0% of the methylated cases classified as grade 3, whereas only 33.8% of the unmethylated cases fall in this category ($P = 0.001$).

Table 2. Clinicopathologic features of sporadic *BRCA1*-methylated and unmethylated breast tumors

Feature	Breast tumors ($N = 131$)		P*
	BRCA1-methylated ($n = 39$)	*BRCA1*-unmethylated ($n = 92$)	
Age at diagnosis, y	$n = 38$	$n = 87$	
≤55	23 (60.5) †	35 (40.2)	0.05
>55	15 (39.5)	52 (59.8)	
Race	$n = 26$	$n = 72$	
Caucasian	12 (46.2)	35 (48.6)	
African American	13 (50.0)	37 (51.4)	0.39
Hispanic	1 (3.8)	0 (0.0)	
Tumor size, cm	$n = 36$	$n = 86$	
Mean ± SD	4.6 ± 4.2	3.4 ± 2.1	0.11
Histologic type	$n = 38$	$n = 87$	
Ductal	31 (83.8)	79 (89.8)	
Lobular	5 (12.2)	5 (6.8)	0.22
Medullary	2 (7.3)	3 (3.4)	
Tumor grade	$n = 30$	$n = 74$	
1	3 (10.0)	8 (10.8)	
2	6 (20.0)	41 (55.4)	0.001
3	21 (70.0)	25 (33.8)	
Disease stage	$n = 29$	$n = 83$	
I	4 (13.8)	16 (19.2)	
II	13 (44.8)	52 (62.7)	
III	9 (31.0)	10 (12.1)	0.14
IV	3 (10.3)	5 (6.0)	
Nodes involved	$n = 36$	$n = 86$	
0	15 (41.7)	43 (50.0)	
1-3	6 (16.7)	22 (25.6)	0.16
4-9	12 (33.3)	13 (15.1)	
≥10	3 (8.3)	8 (9.3)	
Estrogen receptor status	$n = 37$	$n = 82$	
Negative	21 (56.8)	29 (35.4)	0.04
Positive	16 (43.2)	53 (64.6)	
Progesterone receptor status	$n = 23$	$n = 51$	
Negative	17 (73.9)	20 (39.2)	0.01
Positive	6 (26.1)	31 (60.8)	

*Test for difference in frequency distribution (features expressed in categories factors) or mean (features expressed on a continuous scale) between methylated and unmethylated tumors.
†Numbers in parentheses are percentages.

A strong correlation was also found with estrogen receptor negativity ($P = 0.04$) and progesterone receptor negativity ($P = 0.01$), with both being more likely a feature of *BRCA1*-methylated tumors. Correlation was found between the methylation status of *BRCA1* and tumor size, stage, histologic type, number of nodes involved, and patient's ethnicity, but did not reach statistical significance.

***BRCA1* copy number in BRCA1-deficient breast cancer cell lines.** We previously reported that the cell line HCC1937, derived from a *BRCA1* germ line mutation carrier, was polysomic for chromosome 17 (7). Here, we show that in these cells, the increased chromosome 17 copy number was accompanied by a concomitant increase in copy number of the mutated (17) *BRCA1* gene (Fig. 2*A*). We observed three copies in 33% of cells and four copies in 41% of cells for both *CEP17* and *BRCA1* signals (mean ratio, 1.05).

The *BRCA1*-methylated UACC3199 cells were mostly disomic for chromosome 17 with intact two copies of *BRCA1* (87.5%). However, the majority of cells showed a gain of *BRCA1* in the range of three to six copies per nucleus. The ratio of four copies of *BRCA1* to two copies of *CEP17* was most frequent and occurred in 51% of nuclei. The two extra copies of the *BRCA1* gene were translocated on two nonhomologous chromosomes (Fig. 2*B*).

***BRCA1* copy number in primary breast tumors in relation to promoter methylation and mRNA expression.** Imprint touch preparations for FISH were available from 50 of 67 frozen tissues [or 50 of 131 (38%); 37 unmethylated and 13 methylated] including the subset of 18 microdissected tumors. These cases were randomly selected but their clinical characteristics seemed to be representative of the entire cohort (data not shown). Figure 3*A* shows a plot of the mean number of *BRCA1* copies per cell versus the mean number of *CEP17* copies per cell, depicted for *BRCA1*-methylated and unmethylated tumors. The mean number of *BRCA1* copies was smaller in methylated cases as compared with unmethylated cases (1.78 versus 2.30, $P = 0.001$). Similarly, the *CEP17* mean copy number was smaller among methylated cases as compared with unmethylated cases (1.85 versus 2.29, $P = 0.005$). Because methylation was frequently associated with monosomy for chromosome 17 and loss of one copy of *BRCA1*, the overall *BRCA1*/*CEP17* ratio did not differ between the groups (mean of 0.97 in methylated cases versus 1.01 in unmethylated cases, $P = 0.24$).

Across all methylated and unmethylated cases, we also compared the proportion of tumor cells exhibiting abnormal copies of *BRCA1* and *CEP17*. The percentages of tumor cells with one, two, and three or more copies of *BRCA1* and *CEP17* were graphed by methylation status in Fig. 3*B*. The proportion of cells with reduction of *BRCA1* to one copy was significantly greater in methylated cases compared with unmethylated cases (24.3% versus 2.7%, $P = 0.003$). A similar pattern was seen with respect to the proportion monosomic for chromosome 17 cells (25.9% versus 3.2%, $P = 0.001$). In contrast, significantly higher proportions of cells with gain of *BRCA1* and *CEP17* were detected in unmethylated cases than in methylated tumors [*BRCA1*, 21% versus 2% ($P = 0.005$); *CEP17*, 22% versus 1.6% ($P = 0.01$)].

Results of *BRCA1/CEP17* FISH from 18 microdissected primary breast carcinomas are given in Table 1. The mean number of *BRCA1* copies per cell among methylated tumors was significantly smaller that that among unmethylated tumors (1.71 versus 2.29, $P = 0.02$) and correlated with expression of *BRCA1* transcripts. Three of four predominantly methylated tumors with no *BRCA1* expression (tumors 8-10) had the smallest number of *BRCA1* copies per cell, ranging from 1.15 to 1.60, and the highest proportion of

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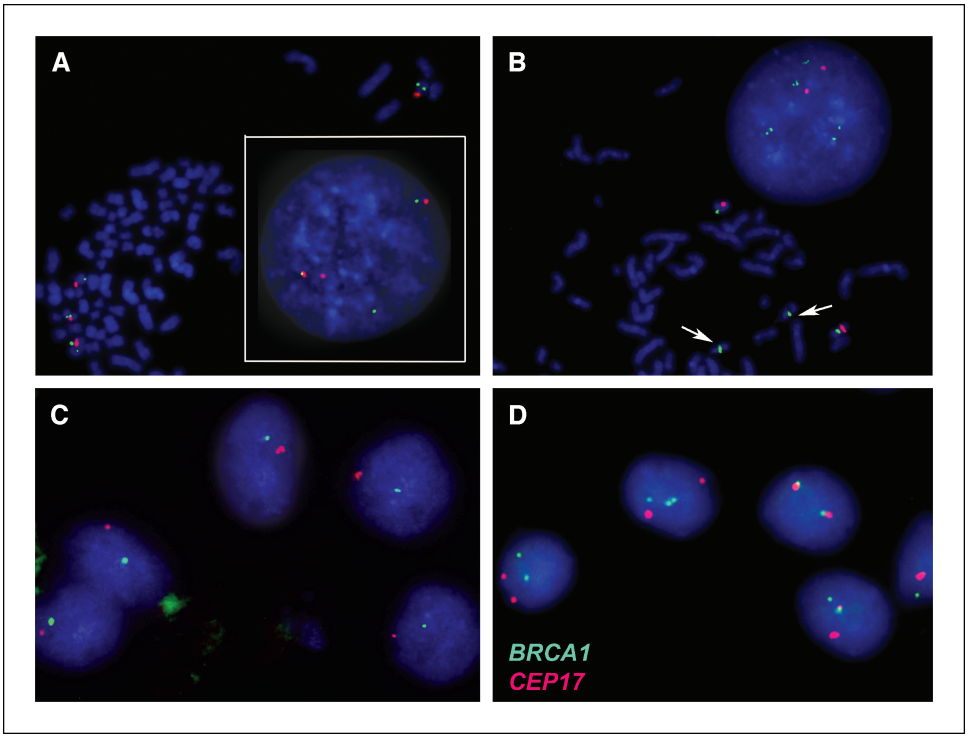

Figure 2. Representative photomicrographs of breast cancer cell lines HCC1937 (*A*) and UACC3199 (*B*) and *BRCA1*-methylated (*C*) and unmethylated (*D*) primary breast carcinomas after FISH. The *BRCA1* gene is localized by green fluorescent signals and the chromosome 17 centromere (*CEP17*) is localized by red fluorescent signals. The cells were counterstained with 4′,6-diamidino-2-phenilindole (blue). *A,* HCC1937 cells are mostly tetrasomic (metaphase) and trisomic (interphase) for chromosome 17 with concordant multiplication of *BRCA1*. *B,* UACC1399 cells in metaphase and interphase showing two presumably normal *BRCA1* signals on two chromosome 17 and two extra *BRCA1* signals duplicated and translocated to two nonhomologous chromosomes (*arrows*). *C,* nuclei from a typical example of a *BRCA1*-methylated tumor displaying monosomy for chromosome 17 with corresponding loss of one copy of the *BRCA1* gene are shown. *D,* tumor cells from an unmethylated case show two copies of *BRCA1* and two copies of *CEP17* signals per cell.

cells with *BRCA1* copy number loss, ranging between 43.16% and 84.71% (a representative photomicrograph of the *BRCA1*-methylated tumor 8 after FISH is shown in Fig. 2*C*; see also Table 1 and Fig. 1*B* and *C*). Conversely, the unmethylated tumors displayed the highest mean number of *BRCA1* copies per cell (range of the means, 2.01-3.63) and normal mRNA expression (a representative photomicrograph of the unmethylated tumor after FISH is shown in Fig. 2*D*). Gene copy numbers from the methylated cases, either with incomplete methylation of *BRCA1* promoter or reduced *BRCA1* transcripts (tumors 1, 2, and 11), were intermediate between those of tumors that were heavily methylated/undetectable transcripts and unmethylated/normal transcripts. In three unmethylated cancers (tumors 4, 7, and 12) and one methylated case (tumor 6), there was no correlation between methylation-specific PCR, RT-PCR, and FISH results, supporting the suggestion of other investigators that mechanism(s) other than aberrant methylation can be involved in LOH and diminished expression of *BRCA1* gene product (1, 21).

A similar pattern was seen with respect to chromosome 17 across all 18 cases in which chromosome 17 aneusomy was frequently associated with *BRCA1* copy number alterations. However, in methylated tumor 10 (Table 1), the proportion of cells with reduction of *BRCA1* to one copy (43.16%) was much higher than the proportion of cells monosomic for *CEP17* (5.26%). The *BRCA1/CEP17* ratio of 1:2 was recorded in 38% of tumor cells, suggesting that in this tumor the physical deletion of *BRCA1* was submicroscopic and did not involve the loss of whole chromosome 17. Such event has been observed in hereditary *BRCA1*-associated tumors (15).

Thus, there was good correlation between gene copy number, degree of *BRCA1* promoter methylation, and mRNA expression. In a multivariable model including estrogen receptor and progesterone receptor status, tumor grade, and patient age, methylation status remained a significant predictor of reduced *BRCA1* and *CEP17* copy number (P = 0.01). When patient and tumor characteristics were taken into account via inclusion in a statistical model predicting methylation status with the reduced *BRCA1* and *CEP17* copy number and other factors associated with methylation, a similar trend was observed.

Demethylation and reexpression of *BRCA1* after treatment of UACC3199 cells with 5-aza-2′-deoxycytidine and trichostatin A. Methylation-specific PCR analysis of DNA extracted before and after exposure to 5-aza-dC and/or trichostatin A showed restoration of the unmethylated state of *BRCA1* at all applied dose levels (Fig. 4*A*). The intensity of unmethylated band correlated with drug concentration. However, RT-PCR analysis revealed that only 50 and 100 ng/mL 5-aza-dC and 100 ng/mL trichostatin A or the combination of 50 ng/mL 5-aza-dC and 50 ng/mL trichostatin A was needed to induce the reexpression of *BRCA1* transcripts (Fig. 4*B*). We found that the combination of 5-aza-dC and trichostatin A led to a higher degree of *BRCA1* promoter demethylation and higher levels of mRNA reexpression than treatment with either inhibitor alone. Bisulfite DNA sequencing confirmed the partial demethylation of the *BRCA1* promoter by these agents (Fig. 4*C*). After combined 5-aza-dC/trichostatin A drug treatment, the demethylation event of different degrees was observed in 13 of 30 (43%) CpG sites. In five of these sites (or 5 of 30, 17%) demethylation was significant. The percent methylation at CpG dinucleotides, located at positions −533, −355, −173, −21, and +44 from the transcription start site, was 90%, 50%, 90%, 40%, and 100% before treatment and decreased to 20%, 0%, 40%, 0%, and 20% after treatment, respectively.

Discussion

The proportion of methylated cases detected in our study was 29.8%, which falls in the range of 7% to 31% methylated sporadic breast cancers previously reported in the literature (3, 19). We

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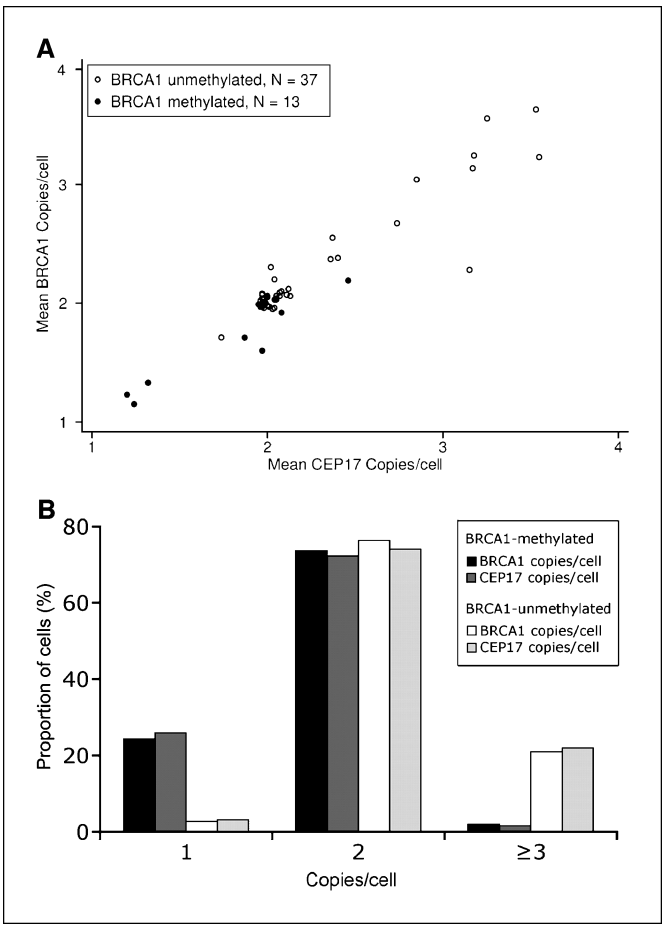

Figure 3. Distribution of the mean number of *BRCA1* copies relative to chromosome 17 centromere signals in *BRCA1*-methylated and *BRCA1*-unmethylated breast cancer tissues (*A*) and the proportion of cells with a given copy number of *BRCA1* and *CEP17* across the two groups of tumors (*B*). *A,* the *BRCA1*-methylated cases show smaller mean numbers of *BRCA1* ($P = 0.001$) and *CEP17* ($P = 0.005$) copies per cell than unmethylated cases. *B,* the percentage of cells with loss of *BRCA1* and *CEP17* to one copy is significantly greater in methylated cases compared with unmethylated cases (*BRCA1,* $P = 0.003$; *CEP17,* $P = 0.001$).

found that methylation was more common in high-grade, estrogen receptor–negative, and progesterone receptor–negative cancers from young women, a phenotype that is similar to that observed in hereditary *BRCA1*-associated breast cancers (1, 7). Similar to other reports (21, 22), we observed significant heterogeneity in methylation patterns. Over 80% of tumors with methylated DNA had varying amounts of unmethylated DNA. Nevertheless, the microdissection of 18 tumors allowed us to discriminate between methylation heterogeneity due to contamination with normal cells and methylation heterogeneity due to incomplete methylation of the CpG island and to examine the relationship between patterns of DNA methylation, mRNA expression, and gene copy number of *BRCA1* (Table 1). For the first time, we showed that tumors with methylation heterogeneity due to incomplete methylation of one or both alleles show levels of mRNA transcripts and gene copy numbers that were intermediate between unmethylated tumors and tumors with complete methylation of *BRCA1*. However, all tumors with any degree of DNA methylation had statistically significant reduction in *BRCA1* transcript levels and loss of copies of the gene when compared with unmethylated tumors.

We have previously shown that tumors from both *BRCA1*-mutated hereditary cancers and sporadic controls displayed chromosome 17 aneusomy and were often monosomic for chromosome 17 (7). In the present study, FISH analysis clearly showed significant differences in chromosome 17 copy number among methylated and unmethylated cases. The significant proportion of cells with chromosome 17 monosomy in the methylated tumors suggests that *BRCA1* methylation might have resulted in loss of chromosome 17. The initial reduction in expression of *BRCA1* could create subsequent genomic instability. It is likely that chromosome 17 aneusomy was specific and nonrandom because we had previously shown that *BRCA1*-methylated cells are more likely to have *MYC* gene copy number gain/amplification and chromosome 8 polysomy compared with unmethylated tumors (11). Thus, our previous and current data suggest that loss of *BRCA1* in some sporadic breast cancers through epigenetic mechanism contributes to the development of those tumors and seems to precede and possibly promote specific chromosomal changes, such as chromosome 17 aneusomy and *MYC* amplification (7, 11).

HCC1937, a breast cancer cell line from a *BRCA1* germ line mutation carrier, is polysomic for chromosome 17 and shows concomitant gain of copies of *BRCA1* gene and yet has only mutated *BRCA1* allele with the 5382insC mutation (17). This suggests that loss of the wild-type *BRCA1* allele due to deletion of a whole chromosome 17 was an early somatic genetic event in these cells, followed by gain of the remaining mutant homologue by nondisjunction (15). In addition, our methylation-specific PCR analysis, showing that the retained mutant allele of *BRCA1* remained unmethylated in HCC1937 cells, confirmed a previous observation that aberrant methylation of the CpG island rarely occurs when only a mutant *BRCA1* allele is present in the tumor (4). However, if two alleles are present and one of them has a germ line mutation, the biallelic inactivation of the gene may be accomplished by promoter methylation of the retained wild-type allele (4, 23). The *BRCA1*-methylated cell line UACC3199 was disomic for chromosome 17 and yet the cells had a mean *BRCA1* copy number of 4.09 and absent mRNA transcripts. Interestingly, a similar paradoxical copy number gain of a mutant *BRCA1* allele relative to chromosome 17 has been observed in *BRCA1*-associated hereditary breast cancers (15). Extrapolating from data on HCC1937 and UACC3199 cells, we suggest that those primary tumors, which were detected in our study as being *BRCA1* methylated, yet having two copies or gain of copies of *BRCA1* (tumors 1-2, 6, and 11 from Table 1), accomplished biallelic inactivation of *BRCA1* by promoter methylation of both alleles and without involvement of genetic deletions/LOH. Alternatively, the possibility of endoreduplication of one methylated allele in conjunction with loss of another allele cannot be ruled out at this time (24).

Esteller et al. (4, 18, 25) previously proposed the functional equivalency between the effect and significance of the epigenetic silencing of *BRCA1* in sporadic breast cancer and genetic suppression of the gene in *BRCA1* mutation carrier. The results of our methylation-specific PCR, RT-PCR, and FISH analyses indicate that *BRCA1* methylation contributes to a subset of sporadic breast cancers with the resulting phenotype similar to that of hereditary *BRCA1*-mutated breast cancers. These data support a model of breast carcinogenesis in which *BRCA1* promoter methylation may serve as a "first hit," much like an inherited germ line mutation, and the "second hit" results in

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reduced *BRCA1* copy number and/or chromosome 17 aneusomy. Our study supports the hypothesis that *BRCA1* promoter hypermethylation occurs early and, when complete, causes defects in chromosome structure, cell division, and viability. Thus, a BRCA1-deficient cell must acquire additional alterations that overcome these problems and force tumor progression down the same limited set of molecular pathways, which are characteristic of the progression of hereditary tumors (26, 27).

During the elaboration of our study, Staff et al. (28) reported FISH data on 60 primary tumors in which 45% were found to have a relative loss of *BRCA1* copy number that was highly correlated with reduced mRNA expression and DNA aneuploidy. Consistent with our data, they also reported a strong association between reduced *BRCA1* expression and progesterone receptor negativity. However, only 11% of their tumors were methylated, and the authors suggested alternative upstream mechanisms for down-regulating *BRCA1* as they did not find an association between *BRCA1* deletion and methylation. Other groups found that the rate of LOH at the *BRCA1* locus may be higher than the percentage of *BRCA1*-methylated tumors or tumors with low mRNA or protein expression (25). However, loss of BRCA1 expression without LOH and allelic loss has also been reported (29). In our study, we were able to detect the correlation between mean *BRCA1* and *CEP17* copy numbers and methylation status because we took a different approach by recognizing the significance of methylation heterogeneity and we did not use an arbitrary cutoff point to classify tumors with deletions. Rather, we used increases and decreases in mean gene copy number of *BRCA1* as being suggestive of chromosomal gains or losses, along with the calculation of the proportion of tumor cells with a given copy number of the gene and chromosome 17. Whereas Staff et al.'s (28) conclusion that haploinsufficiency at the *BRCA1* locus may cause a gene dosage effect is conceivable, it is also possible that the methylation heterogeneity that we observed could explain the differences between our study and that of Staff et al. (28). Of course, both studies had limitations because of the number of tumors available for correlation analysis. Nevertheless, our findings are consistent with a recent FISH study of *BRCA1* copy number in ovarian cancer (30) in which 25 of 47 cases (53%) were found to be "*BRCA1* deletion positive" (i.e., having a high proportion of tumor cells with *BRCA1/CEP17* copy number ratios of 1:2 and 1:1). Sixteen of these cases (34% of the total) actually had a very high proportion of cells with *BRCA1* deletions. Whereas the methylation status of the tumors has not been analyzed, the patients with *BRCA1* deletion–positive tumors were significantly younger and showed a trend toward poorer survival.

Our study has implications for clinical practice on the use of demethylating agents in cancer prevention or treatment of patients with estrogen receptor– and progesterone receptor–negative *BRCA1*-methylated sporadic breast cancers. Achieving *BRCA1* demethylation and reexpression after treating *BRCA1*-methylated cells with 5-aza-dC and trichostatin A, we confirmed a reversible mechanism of *BRCA1* silencing in tumor cells and a potential role for DNA methylation in the control of *BRCA1* expression (31). The combinatorial effect of 5-aza-dC and trichostatin A on *BRCA1* reactivation also confirmed the previously published observations on other cancer-related genes that the use of inhibitors of DNA methylation and histone deacetylation in combination may be an effective chemotherapy

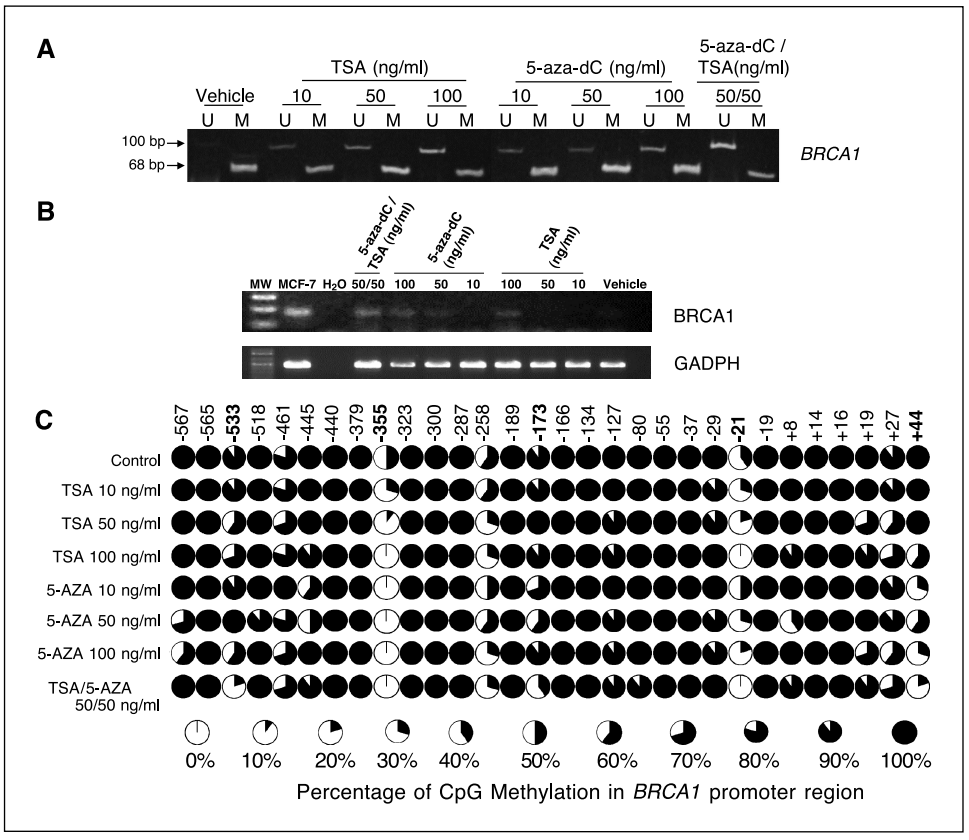

Figure 4. Effects of 5-aza-dC and trichostatin A (*TSA*) on *BRCA1* promoter CpG island methylation and gene expression in UACC3199 breast cancer cells. *A,* methylation-specific PCR analysis of *BRCA1* methylation after treatment. Untreated UACC3199 cells were used as a positive control for methylation and H_2O was used as a methylation-specific PCR negative control. *Lanes M,* methylated products; *lanes U,* unmethylated products. The combination of 50 ng/mL 5-aza-dC and 50 ng/mL trichostatin A led to higher levels of demethylation than either inhibitor alone. *B,* pattern of expression determined by RT-PCR of the *BRCA1* transcript after treatment. Total RNA from MCF-7 cells was used as a positive control. Total RNA of untreated UACC3199 cells and H_2O were used as negative controls for *BRCA1* expression. The levels of *BRCA1* mRNA were normalized to the level of *GAPDH* mRNA. The combination of 50 ng/mL 5-aza-dC and 50 ng/mL trichostatin A led to higher levels of *BRCA1* reexpression than either inhibitor alone. *C,* bisulfite-modified genomic DNA sequencing of *BRCA1* promoter in UACC3199 cells after demethylation treatment. Five CpG dinucleotides, at positions −533, −355, −173, −21, and +44 from the transcription start site, revealed the most significant demethylation after combined 5-aza-dC/trichostatin A drug treatment.

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for breast cancer (32). When we treated *BRCA1*-methylated cancer cells with 5-aza-dC and trichostatin A for only 48 hours (33), we saw a dramatic induction of *BRCA1* demethylation and reexpression levels despite the restricted number of CpGs that were affected. It is conceivable that those CpGs, at positions −533, −355, −173, −21, and +44 from the transcription start site, might be important in the transcriptional regulation of *BRCA1*. Indeed, a single CpG at position −355 was recently found to be one of four binding sites of *BRCA1* DNA for specific protein 1 (31) and is also located within 60 bp of one of the two binding sites of CTCCC binding factor (34). Specific protein 1 and CTCCC binding factor may play a role in maintaining a methylation-free state of the functional *BRCA1* promoter region, analyzed in our study, permitting transcription of the *BRCA1* gene. Disruption of the binding of specific protein 1 and CTCCC binding factor to DNA may facilitate aberrant DNA methylation of the *BRCA1* promoter, leading to genomic instability and development of sporadic breast cancer (34). In addition, methylation at positions −173 and −21 CpG sites may specifically inhibit binding of cAMP-responsive element binding protein and E2F transcription factors to *BRCA1* DNA, respectively (24, 35). Thus, the understanding of molecular mechanisms underlying tumor initiation and progression in cells lacking *BRCA1* is a critical step toward the development of more effective strategies for prevention, early detection, and treatment of aggressive breast cancer that disproportionately affects young women.

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