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PRECLINICAL STUDY

BRCA1 promoter methylation status does not predict response to tamoxifen in sporadic breast cancer patients

Jasmina Z. Cerne · Lin Zong · Jaroslav Jelinek · Susan G. Hilsenbeck · Tao Wang · Steffi Oesterreich · Sean E. McGuire

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Abstract The purpose of this study is to investigate whether *BRCA1* promoter methylation is associated with poorer outcome in sporadic breast cancer cases treated with tamoxifen. *BRCA1* promoter methylation was determined by bisulfite pyrosequencing in two groups of sporadic breast cancer patients, systemically untreated ($N = 497$) and treated with adjuvant tamoxifen ($N = 497$). Associations of *BRCA1* promoter methylation with clinopathological characteristics and the effect of *BRCA1* promoter methylation on time to first recurrence (TTR) and overall survival (OS) were examined. No significant differences were observed between *BRCA1* promoter methylation and clinopathological characteristics in untreated and tamoxifen-treated groups. Cut point analysis did not find any promising cut point for *BRCA1* promoter methylation that would differentially influence TTR and OS in untreated and tamoxifen-treated group. Using the median (2.53 %) and an arbitrary value of 10 % as a cut point for methylation, we still found no significant effect of *BRCA1* promoter methylation on TTR and OS in untreated and tamoxifen-treated group. Despite data suggesting that *BRCA1* levels impact estrogen receptor response to tamoxifen, our results indicate that *BRCA1* promoter methylation is not associated with poorer outcome in sporadic breast cancer cases treated with tamoxifen.

Keywords *BRCA1* promoter methylation · Breast cancer · Tamoxifen · Clinopathological characteristics · Time to first recurrence · Overall survival

Abbreviations

ER	Estrogen receptor
OS	Overall survival
TTR	Time to first recurrence

J. Z. Cerne · L. Zong · S. E. McGuire (✉)
Department of Molecular & Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
e-mail: semcguir@bcm.edu

J. Jelinek
Fels Institute for Cancer Research and Molecular Biology, Temple University, Philadelphia, PA, USA

S. G. Hilsenbeck · T. Wang
Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX, USA

S. Oesterreich
Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

S. E. McGuire
Department of Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Introduction

Breast cancer is the most common cancer among women in the industrialized world. Although germline mutations in the *BRCA1* gene are responsible for nearly half of the inherited breast cancer cases [1], there is very little evidence for *BRCA1* mutations in the sporadic form of the disease. Despite the lack of *BRCA1* somatic mutations, significantly reduced *BRCA1* expression has been observed in 30–40 % of sporadic breast cancer cases [2, 3]. Several mechanisms presumably cooperate to mediate *BRCA1* somatic inactivation with *BRCA1* promoter methylation being the most widely examined and present in 9–59 % of sporadic breast cancer cases [4–10].

BRCA1 encodes a multifunctional protein with roles in many important cellular functions, including DNA repair,

cell cycle control, protein ubiquitination, and transcriptional regulation [11]. Loss of these caretaker functions may explain why *BRCA1* inactivation predisposes to the development of cancer; however, it does not explain the strong predilection to specific tumor types, particularly breast and other hormone-responsive tumors. It has therefore been hypothesized that *BRCA1* may participate in sex steroid hormone activity. Indeed, prophylactic bilateral salpingo-oophorectomy has been shown to significantly reduce the rate of breast cancer in *BRCA1* mutation carriers by 47 % [12].

Paradoxically, the majority of breast cancers developed in *BRCA1* mutation carriers are estrogen receptor (ER) negative [13, 14]. Hosey and colleagues have suggested that *BRCA1* mutant tumors fail to express ER due to the loss of *BRCA1*-mediated transcriptional activation of ER and propose a model in which the observed loss of ER expression in *BRCA1* mutation carriers occurs only after the loss of the second *BRCA1* allele [15, 16]. Animal studies have supported this possibility by showing that ER is highly expressed in premalignant lesions and initiation stages of tumorigenesis, and that its expression gradually declines during mammary tumor progression in *BRCA1* mutant mice [17, 18]. Therefore, it is plausible that hormone deprivation could be preventive in *BRCA1* mutation carriers if initiated at early stages of tumor development. In agreement, the impact of prophylactic bilateral salpingo-oophorectomy on the subsequent risk of contralateral breast cancer was shown to be more effective if performed at a younger age [19].

Confirming a link between *BRCA1* levels and steroid hormone signaling in promoting mammary carcinogenesis, Lee and colleagues [20] demonstrated that mammary tumors in *BRCA1* mutant mice could be prevented by the administration of progesterone antagonist mifepristone. In an analogous attempt to prevent mammary cancer initiation in *BRCA1* mutant mice, Jones and colleagues found that tamoxifen paradoxically promoted the development of mammary tumors. In vitro experiments revealed that *BRCA1* knockdown in breast cancer cells induced the agonist activity of tamoxifen [21]. Interestingly, in a re-analysis of the National Surgical Adjuvant Breast and Bowel Project (NSABP-P1) by *BRCA* status, the authors found that tamoxifen use was associated with an increased incidence of breast cancer with a hazard ratio of 1.67 among *BRCA1* mutation carriers, whereas the incidence of breast cancer was decreased by 62 % in *BRCA2* mutation carriers and by 48 % in the study population as a whole [22]. In addition, Beiner and colleagues found an elevated risk for endometrial cancer among *BRCA1* mutation carriers who were treated with tamoxifen compared to non-carriers [23, 24]. Consistent with a possible enhanced agonistic role for tamoxifen in the presence of low levels of *BRCA1*, Wen and colleagues demonstrated that decreased *BRCA1* expression in breast cancer cells contributed to tamoxifen resistance due to altered ER-coregulator interactions; tamoxifen-bound ER-regulated gene promoters did not associate with corepressors, but instead inappropriately recruited coactivators [25]. Overall, these observations suggest that cellular levels of *BRCA1* not only play a role in breast cancer development but might also predict response to tamoxifen therapy.

The purpose of the current study was therefore to examine whether *BRCA1* promoter methylation, a mechanism of *BRCA1* down-regulation, is associated with poorer outcome in sporadic breast cancer cases treated with tamoxifen. To achieve this objective, we carried out a retrospective study to evaluate the association of *BRCA1* promoter methylation with time to first recurrence (TTR) and overall survival (OS) in two cohorts of sporadic breast cancer patients, systemically untreated and treated with adjuvant tamoxifen. We further aimed to investigate the associations of *BRCA1* promoter methylation with clinopathological characteristics.

Methods

Study population

Subjects presented in this study were diagnosed and treated between 1970 and 1999, and derived from two tumor banks (P01 and SPORE) with associated clinical annotations and long-term follow-up. These banks were constructed and maintained by the Smith Breast Center at Baylor College of Medicine, as previously described [26]. Briefly, the banks comprise 497 tumors from patients who did not receive adjuvant therapy after surgery (untreated) and 497 tumors from patients treated with tamoxifen (tamoxifen-treated). Additional selection criteria included: complete information about patient and tumor characteristics; early stage breast cancer; ER positivity by ligand-binding assay ($\geq$3 fml/mg protein), no neoadjuvant therapy; no adjuvant chemotherapy; and sufficient tumor material for DNA extraction. A total of 213 samples came from the P01 and 787 from the SPORE banks. OS data were equally reliably obtained from both banks. Follow-up of P01 cases was obtained directly from treating physicians and recurrence information is quite complete. Follow-up of SPORE cases was obtained from hospital tumor registries and recurrence is somewhat under-reported. There is no evidence that the under-reporting is associated with prognostic or predictive variables of interest.

The study protocol was approved by the Institutional Review Board of the Baylor College of Medicine with a waiver of consent.

Methylation analysis

The pattern of CpG methylation within the promoter region of *BRCA1* was quantified by bisulfite pyrosequencing as previously described [27]. The pyrosequencing assay assessed five adjacent CpG sites. These are located at −55, −37, −29, −21, and −19 relative to the *BRCA1* transcription start site +1 (Fig. 1).

Briefly, genomic DNA was extracted from frozen tumor tissues using the Puregene DNA Purification Kit (Qiagen) and subjected to bisulfite conversion using EZ DNA Methylation Gold Kit (Zymo Research). Bisulfite-modified DNA was amplified in a two-step PCR. First PCR reaction (20 µl) included bisulfite-treated genomic DNA, 1 unit of *Taq* DNA Polymerase, 67 mmol/L Tris–HCl (pH = 8), 16 mmol/L ammonium sulfate, 2 mmol/L $MgCl_2$, 0.125 mmol/L dNTPs, forward primer 5′-GGT GGT TAA TTT AGA GTT TAG AGA GA-3′ and reverse primer 5′-AAA ACT CCT AAC CTC ATA ACC AAC C-3′, each at 100 nmol/L. PCR conditions were as follows; initial denaturation at 95 °C for 5 min, followed by 38 cycles at 94 °C for 30 s, 62 °C for 45 s, 72 °C for 50 s, and a final extension at 72 °C for 10 min. The PCR product from the first PCR step was used as template to perform a second PCR step with forward primer 5′-TGA TTT AGT ATT TTG AGA GGT TGT TGT TTA -3′ and biotinylated reverse primer 5′-GGG ACA CCG CTG ATC GTT TAC AAT CCC AAT TTT AAT TTA TCT ATA ATT CCC-3′, which yielded a final 119-bp amplicon. PCR components and conditions for the second PCR reaction were as described before except that the annealing temperature was increased to 63 °C and 45 cycles were performed to completely exhaust the biotinylated primer. Biotin-labeled single-stranded templates were isolated and pyrosequencing reactions were run on PSQ HS 96 Pyrosequencing System (Biotage) with a specific pyrosequencing primer 5′-TTG AGA GGT TGT TGT TTA G-3′ and Pyro Gold CDT Reagents (Biotage).

Duplicate measurements were carried out for each sample. The pyrosequencing assay failed completely in two (0.2 %) samples due to poor DNA quality. For the CpG1, CpG2, CpG3, CpG4, and CpG5, the pyrosequencing assay failed in one of the two replicates in 30 (3.0 %), 40 (4.0 %), 67 (6.7 %), 111 (11.2 %), and 160 (16.1 %) samples, respectively. 95 % of samples had a difference between replicates of less than 15 % for all five CpG sites analyzed.

Statistical analysis

We found highly concordant methylation patterns between adjacent CpG positions (pairwise Spearman's correlation coefficients ranged from 0.22 to 0.55, $p < 0.001$). In view of the redundancy of the sites, we constructed a single measure of methylation using the maximum of the duplicates and averaging the five sites.

Descriptive and summary statistics were used to describe patient and tumor characteristics. Associations between clinopathological characteristics and adjuvant therapy (untreated vs. tamoxifen-treated) and associations between clinopathological characteristics and *BRCA1* promoter methylation (<median vs. >median) in untreated and

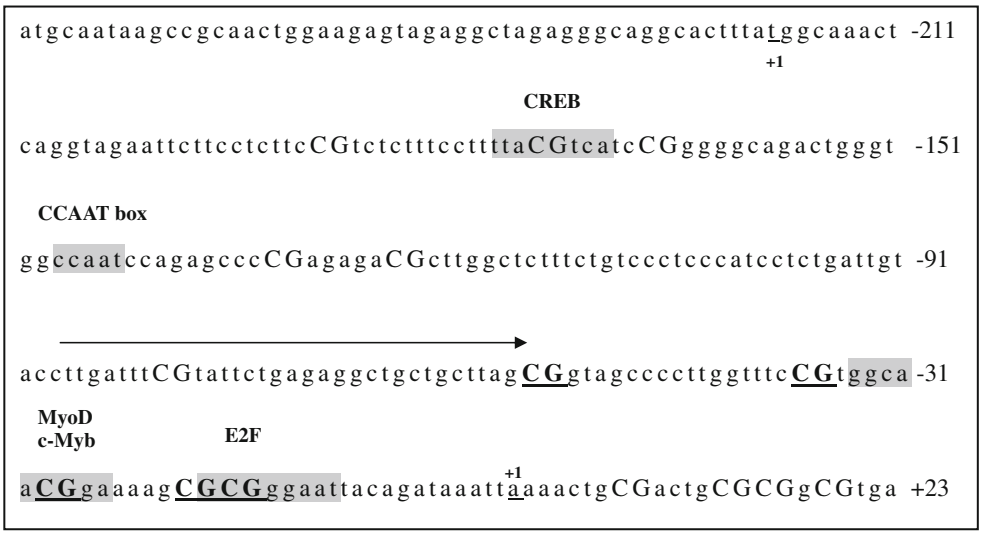

Fig. 1 Schematic representation of the *BRCA1* and *NBR2* bidirectional core promoter. Legend: CpG dinucleotides are in *upper case letters*. CpG dinucleotides analyzed in the present study are in *bold* and *underlined*, located at −55, −37, −29, −21 and −19. The *underlined* '*a*' indicates the *BRCA1* transcriptional start site (+1). The *underlined* '*t*' indicates the *NBR2* transcriptional start site (+1). *Gray boxes* represent CREB, MyoD, c-Myb and E2F transcription factor binding sites, which are potentially regulated by methylation at −173, −29, −29 and −21 to −19, respectively. The *arrow* indicates the position of the pyrosequencing primer used in the present study

tamoxifen-treated group were evaluated by χ^2 test and Spearman's rank correlation. Outcomes were TTR, defined as time from diagnosis to first recurrence or last follow-up whichever was first and censoring at death, and OS, defined as time from diagnosis to death or last follow-up. The effects of *BRCA1* promoter methylation on TTR and OS were summarized by the Kaplan–Meier method and compared by log-rank test. Survival analyses were performed for untreated and tamoxifen-treated group and for each bank separately, as well as in combination by stratifying on bank. Cut points for methylation in the analyses were the median (2.53 %) and an arbitrary value of 10 %. To find an optimal dichotomous cut point, the functional form of continuous methylation was evaluated by means of Martingale residuals. In addition, a data-driven cut point in methylation percentage for both endpoints was identified by the method of Hilsenbeck and Clark [28].

A p value <0.05 was considered to indicate statistical significance. Statistical analyses were done using SAS 9.2 (SAS institute Inc., Cary, NC) and R 2.14.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

A total of 994 sporadic breast cancer patients were included in the present study. The first group consisted of 497 systemically untreated patients who underwent breast cancer surgery or surgery plus local radiation (untreated group). The second group comprised 497 patients who, in addition to breast cancer surgery ± local radiation, received adjuvant tamoxifen treatment (tamoxifen-treated group). Median follow-up time for patients alive at last follow-up was 123 months (range, 0–253 months), for untreated patients was 124 months (range, 0–253 months), and for tamoxifen-treated patients was 122 months (range, 1–225 months).

Clinopathological characteristics for the untreated and tamoxifen-treated groups are summarized in Table 1. More cases in the untreated group were derived from the SPORE bank. Cases in the untreated group were slightly younger ($p < 0.001$), had smaller tumors ($p < 0.001$), fewer positive nodes ($p < 0.001$), and were more likely to be diploid ($p = 0.036$), as be expected for cases not thought to need additional therapy. No differences were observed between the two groups with respect to other characteristics, including histological type, PR status, and S-phase fraction ($p > 0.05$).

The overall distribution of *BRCA1* promoter methylation values for both groups is illustrated in Fig. 2. The first quartile, the median, and the third quartile were 1.72, 2.53, and 3.90 %, respectively, and 9.7 % of patients had *BRCA1* promoter methylation of ≥10 %. Clinopathological characteristics according to *BRCA1* promoter methylation (<median vs. >median) in untreated and tamoxifen-treated group are shown in Table 2. The small number of untreated cases derived from P01 bank tended to have lower methylation than SPORE cases ($p < 0.001$). No other differences were observed between *BRCA1* promoter methylation and clinopathological characteristics, including age, histological type, tumor size, nodal status, PR status, ploidy, and S-phase fraction in untreated and tamoxifen-treated group ($p > 0.05$).

Table 1 Clinopathological characteristics in untreated and tamoxifen-treated group

Characteristics	Untreated group $N = 497$ N (%)	Tamoxifen-treated group $N = 497$ N (%)	p Value
Bank			<0.001
P01	64 (13)	149 (30)	
SPORE	433 (87)	348 (70)	
Age (years)			<0.001
<50	76 (15)	33 (7)	
≥50	416 (85)	457 (93)	
Missing	5	7	
Histological type			0.656
IDC	421 (84)	416 (84)	
ILC	44 (9)	52 (10)	
Others	32 (6)	29 (6)	
Tumor size (cm)			<0.001
≤2	279 (56)	200 (40)	
$2 < x \leq 5$	198 (40)	271 (55)	
>5	20 (4)	26 (5)	
Nodal status			<0.001
Node negative	402 (81)	256 (52)	
1–3 positive nodes	68 (14)	167 (34)	
4–9 positive nodes	20 (4)	52 (10)	
≥10 positive nodes	7 (1)	22 (4)	
PR status			0.198
Negative: <5 fmol/mg	121 (24)	104 (21)	
Positive: ≥5 fmol/mg	376 (76)	393 (79)	
Ploidy			0.036
Diploid	249 (52)	212 (46)	
Aneuploid	226 (48)	253 (54)	
Missing	22	32	
S-phase fraction			0.862
Low: <6 %	213 (55)	221 (57)	
Medium: 6–10 %	90 (23)	85 (22)	
High: >10 %	82 (21)	85 (22)	
Missing	112	106	

IDC invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *PR* progesterone receptor

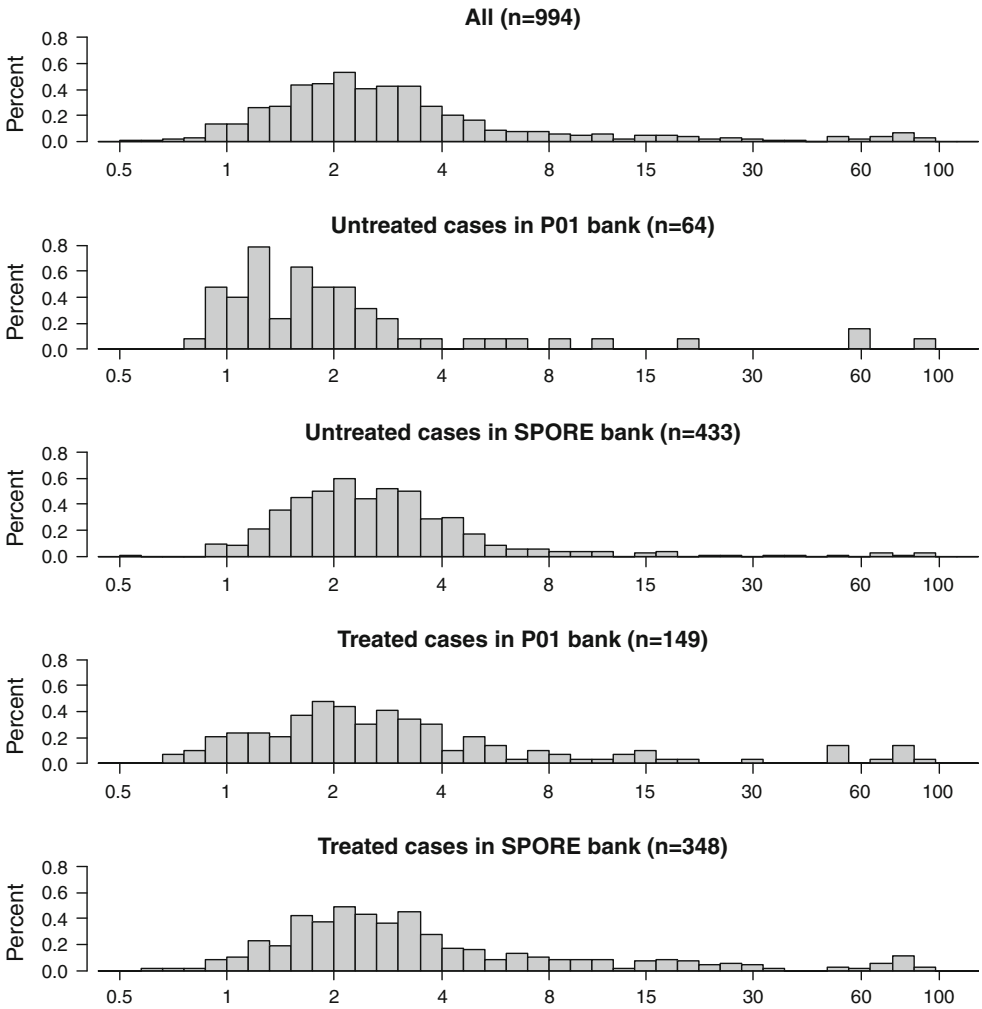

Fig. 2 Distribution of the *BRCA1* promoter methylation

Using the median (2.53 %) and an arbitrary value of 10 % as a cut point for methylation, we found no significant effect of *BRCA1* promoter methylation on TTR (Fig. 3—presented for each bank separately) and OS (data not shown) in untreated and tamoxifen-treated group. Similarly, cut point analysis, which included adjustment for multiple comparisons, did not find any promising cut point for *BRCA1* promoter methylation that would differentially influence TTR or OS in untreated or tamoxifen-treated group (Table 3). A retrospective power analysis indicated that, at the 5 % level of significance, the study had 80 % power to detected hazard ratios of at least 1.76 and 1.92 in TTR for the treated and untreated group, respectively; 1.49 and 1.53 in OS for the treated and untreated group, respectively.

Discussion

In the breast, tamoxifen acts as an ER antagonist and its widespread use has significantly improved OS in women with ER-positive breast cancer [29]. Despite the obvious benefits, 30–50 % of women develop drug resistance in which either they do not respond to tamoxifen at all (intrinsic resistance) or they do initially respond but eventually relapse (acquired resistance) [30]. In order to better predict therapeutic responses to tamoxifen treatment, further improvements in understanding the mechanisms underlying tamoxifen resistance are thus of great importance. Given the recent findings of potential implications of *BRCA1* function in tamoxifen resistance, we sought to evaluate the role of *BRCA1* promoter methylation in predicting tamoxifen sensitivity in sporadic breast cancer patients.

The expression of *BRCA1* gene is under complex regulation [31, 32]. *BRCA1*'s 5′ neighbor gene *NBR2* lies in a head-to-head orientation with the *BRCA1* gene, and both share the same bidirectional promoter (−218 to +1, relative to the *BRCA1* transcription start site; Fig. 1). This essential regulatory region is part of a large, 2.7-kb long, CpG island and spans 11 CpG sites, which have been shown to be unmethylated in normal human mammary epithelial cells [4–8]. On the other hand, methylation of the same region was

Table 2 Clinopathological characteristics according to *BRCA1* promoter methylation (<median vs. >median) in untreated and tamoxifen-treated group

Characteristics	Untreated group $N = 497$					Tamoxifen-treated group $N = 497$				
	BRCA1 promoter methylation				p Value	*BRCA1* promoter methylation				p Value
	<median		>median			<median		>median		
	N	% Row	*N*	% Row		*N*	% Row	*N*	% Row	
Bank					<0.001					0.128
P01	47	75	16	25		75	51	73	49	
SPORE	223	52	209	48		150	43	197	57	
Age (years)					0.691					0.684
<50	43	57	33	43		16	48	17	52	
≥50	224	54	190	46		204	45	251	55	
Missing	3		2			5		2		
Histological type					0.440					0.565
IDC	229	55	190	45		184	44	230	56	
ILC	21	48	23	52		27	52	25	48	
Others	20	63	12	38		14	48	15	52	
Tumor size (cm)					0.337					0.965
≤2	152	55	126	45		89	45	110	55	
$2 < x \leq 5$	104	53	93	47		124	46	146	54	
>5	14	70	6	30		12	46	14	54	
Nodal status					0.580					0.255
Node negative	214	53	187	47		109	43	146	57	
1–3 Positive nodes	41	60	27	40		86	51	81	49	
4–9 Positive nodes	10	53	9	47		22	43	29	57	
≥10 Positive nodes	5	71	2	29		8	36	14	64	
PR status					0.687					0.243
Negative: <5 fmol/mg	63	53	56	47		42	40	62	60	
Positive: ≥5 fmol/mg	207	55	169	45		183	47	208	53	
Ploidy					0.204					0.328
Diploid	126	51	122	49		100	47	112	53	
Aneuploid	128	57	98	43		107	43	144	57	
Missing	16		5			18		14		
S-phase fraction					0.441					0.714
Low: <6 %	111	52	102	48		97	44	123	56	
Medium: 6–10 %	53	60	36	40		38	45	47	55	
High: >10 %	47	57	35	43		33	39	51	61	
Missing	59		52			57		49		

IDC invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *PR* progesterone receptor

found to be a fairly frequent event in sporadic breast cancer cases with frequencies ranging from 9 to 59 % with most studies using 10 % of cells methylated as a cutoff for methylation if a quantitative method was applied [9, 10]. In our study, 9.7 % of patients had *BRCA1* promoter methylation of ≥10 %. Large discrepancies in the reported frequency of *BRCA1* promoter methylation across studies might be explained by potential infiltration with the unmethylated DNA from normal cells adjacent to the tumor, by the variable extent of *BRCA1* regulatory region covered, which might lead to erroneously including the methylation signature related to the neighboring *NBR2* gene and by different experimental methods employed. Most of the previous studies used methylation-specific PCR or restriction enzyme digestion as a method to detect *BRCA1* promoter methylation, which, unlike pyrosequencing, are known to be prone to false positive results. Assay failure is another potential consideration in our study. However, since

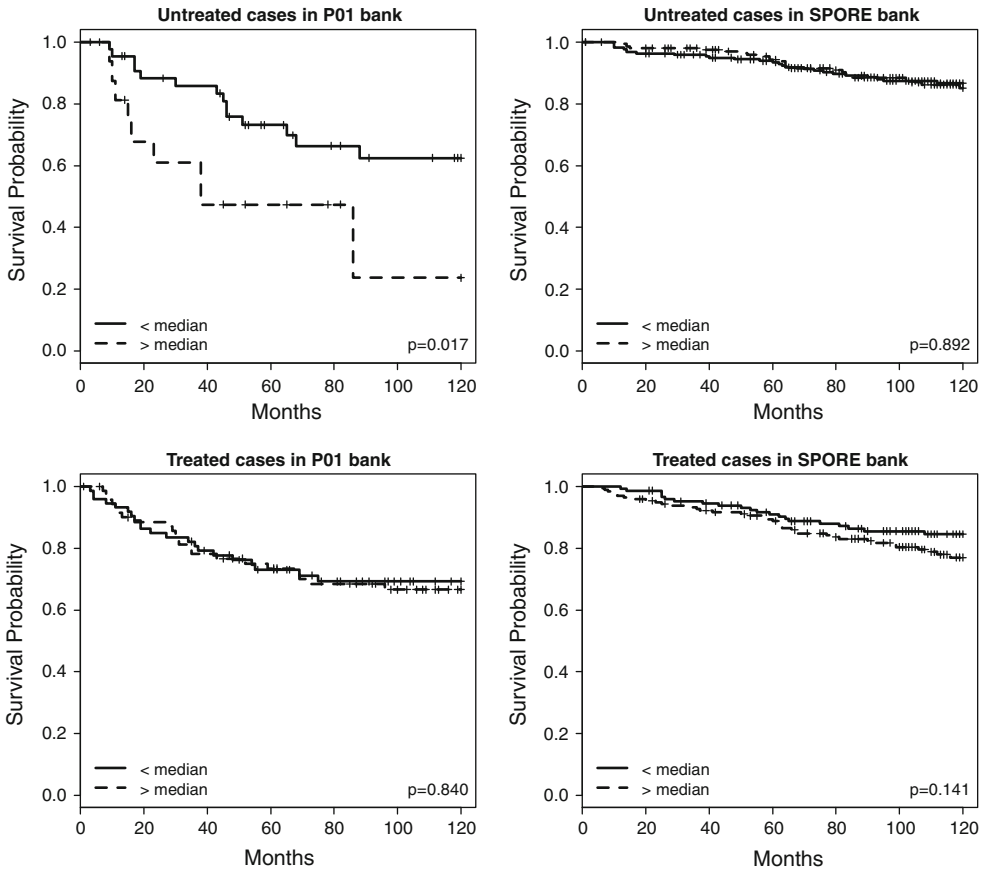

Fig. 3 Kaplan–Meier estimates of TTR with median (2.53 %) as a cut point for *BRCA1* promoter methylation in untreated and tamoxifen-treated group and for each bank separately

Table 3 Cut point analysis

	TTR				OS			
	Cut point[a]	<Cut point *N* (%)	>Cut point *N* (%)	*p* Value[b]	Cut point[a]	<Cut point *N* (%)	>Cut point *N* (%)	*p* Value[b]
Untreated group								
All	1.28	48 (9.7)	447 (90.3)	0.17	53.8	487 (98.4)	8 (1.6)	0.952
SPORE	1.28	28 (6.5)	404 (93.5)	0.184	5.21	388 (89.8)	44 (10.2)	0.588
P01	2.47	46 (73)	17 (27)	0.085	2.56	49 (77.8)	14 (22.2)	0.06
Tamoxifen-treated group								
All	0.858	8 (1.6)	487 (98.4)	0.006	0.727	3 (0.6)	492 (99.4)	<0.001
SPORE	2.18	123 (35.4)	224 (64.6)	0.304	25.8	324 (93.4)	23 (6.6)	0.346
P01	0.858	5 (3.4)	143 (96.6)	0.002	0.858	5 (3.4)	143 (96.6)	<0.001

[a] Cut point was selected by minimum *p* value method

[b] *p* Value was adjusted by empirical permutation method

95 % of the samples had the difference between the both replicates of less than 15 % for all five CpG sites analyzed and the higher percent of methylation from the two replicates was used in the analyses, the impact of the potential additional assay failure could not have been substantial.

The possibility of using epigenetic alteration as a surrogate marker to predict adjuvant tamoxifen efficacy was demonstrated by Fiegl and colleagues [33]. They showed that methylated *RASSF1A* in serum of breast cancer patients correlated with tamoxifen resistance, whereas loss of methylation portended a good response [33]. In order to explore if *BRCA1* promoter methylation may serve as a potential additional biomarker to identify patients most likely to respond to adjuvant tamoxifen therapy, a pyrosequencing assay was developed to examine the methylation pattern of the *BRCA1* promoter region directly upstream of the *BRCA1* transcriptional start site, which encompasses MyoD, c-myc, and E2F transcription factor binding sites

and was previously suggested to be a region of methylation initiation (Fig. 1) [34]. Since *BRCA1* promoter methylation has been consistently observed to contribute to the *BRCA1* gene silencing [3, 35, 36], we reasoned that, by reducing accessibility of transcription factors, the methylation of this particular region might have a considerable impact on repression of *BRCA1* gene expression [34, 37, 38]. Nevertheless, no significant effect of *BRCA1* promoter methylation on TTR and OS was found in either untreated or tamoxifen-treated patients. Owing to the minor differences between the P01 and SPORE bank in sampling and data collection and differences in the proportion of cases derived from each bank in the two treatment groups and somewhat lower methylation rate in untreated P01 cases, we performed stratified analyses and also analyzed each bank separately. By means of several methods, none of the approaches were able to find a promising cut point for *BRCA1* promoter methylation that would differentially influence TTR and OS in the untreated and tamoxifen-treated groups. Our attempts to define a clinically meaningful data-driven cut point were similarly unsuccessful. There were some statistically significant cut points identified with the cut point analysis ($p < 0.05$); however, these were mostly at methylation values of less than 1 %, and consequently, only 0.6–3.4 % of cases ended up being potentially good responders. Furthermore, their significance was not distributed more widely around the proposed value, which would provide a better indication that this might indeed be an area of interest. Although our study may be biased by the retrospective design and lack of data on corresponding *BRCA1* gene expression, we had a large community-based cohort of sporadic breast cancer patients and used a highly accurate method for detection and quantitation of DNA methylation. Retrospective power analysis indicated that we had excellent power to detect clinically meaningful difference.

In conclusion, our results suggest that *BRCA1* promoter methylation is not associated with poorer outcome in sporadic breast cancer cases treated with tamoxifen.

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Conflict of interest The authors declare that they have no conflict of interest.

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