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BRCA1 promoter methylation associated with poor survival in Chinese patients with sporadic breast cancer

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Transcriptional inactivation of breast cancer gene 1 (*BRCA1*) by DNA methylation is a frequent event in sporadic breast cancers. To investigate whether *BRCA1* methylation is associated with survival in Chinese patients with sporadic breast cancer, *BRCA1* methylation was determined using methylation specific PCR in 536 sporadic breast cancers. Survival curves for patients with methylated and unmethylated *BRCA1* were compared using the log-rank tests. Twenty-six percent (139/536) of patients exhibited *BRCA1* methylation in their tumors. The degree of *BRCA1* methylation was correlated with clinical stages of breast cancer, but was not significant. Patients with *BRCA1* methylated tumors had a significantly worse 5-year disease-free survival (DFS) and 5-year disease-specific survival (DSS) than did patients with unmethylated tumors (DFS: 73.2% *vs* 82.6%, $P = 0.045$; DSS 80.5% *vs* 87%, $P = 0.038$, two-sided). In conclusions, *BRCA1* methylation is a frequent event in breast cancer and is associated with poor clinical outcome in Chinese women with breast cancer. (*Cancer Sci* 2009; 100: 1663–1667)

Breast cancer gene 1 (*BRCA1*) encodes a multifunctional protein involved in DNA repair, cell cycle control, protein ubiquitinylation, and chromatin remodeling.[1,2] It is well known that germline mutations of *BRCA1* lead to familial breast cancer. The reported *BRCA1* mutation rate was up to 45% in familial breast cancer, but only 1% in sporadic breast cancer.[3] However, downregulation of *BRCA1* is a very frequent event in sporadic breast cancer and correlates with its progression.[4]

Most housekeeping genes and 40% of tissue-specific genes contain CpG islands around the transcription starting sites. Methylation of these CpG islands silences gene transcription epigenetically. Aberrant methylation of tumor suppressor genes including *BRCA1* is a frequent event that may play an important role in carcinogenesis.[5–8] It was reported that DNA methylation was the major cause of transcriptional silence of *BRCA1*, ranging from 13–40% in sporadic breast cancer.[9–11] Recently, Xu *et al.* reported that *BRCA1* methylation was correlated with the prognosis of Caucasian patients with breast cancers.[12] However, the role of *BRCA1* methylation is not well characterized in prognosis of sporadic breast cancers among other populations. In the present study, we determined the methylation status of *BRCA1* in 536 Chinese patients with sporadic breast cancer and investigated whether the *BRCA1* methylation was associated with clinical outcomes.

Materials and Methods

Study patients. A total of 611 patients with operable primary breast cancer (stage I–III) who had enough tumor DNA samples were selected from a pool of 857 consecutive breast cancer patients treated at the Breast Center, Peking University School of Oncology, from December 1994 to September 1999. A PCR product could not be obtained due to poor-quality DNA in 75 of 611 available DNA samples. Thus, 536 patients with sporadic primary breast cancer were analyzed in the present study. Pathological diagnosis was performed for all patients. The patient ages ranged from 25 to 86 years, with a median of 49 years. A total of 290 patients were premenopausal, and 246 patients were postmenopausal. The stage of the tumors was classified according to the tumor-node-metastasis classification of the International Union Against Cancer (UICC). Patients received a radical or modified radical mastectomy, and the axillary lymph nodes were routinely dissected to at least level I and II. The status of lymph node metastasis was determined based on the histological examination. The majority of patients received adjuvant treatment, including chemotherapy, endocrine therapy, or combined treatment, as summarized in Table 1.

The levels of ER and progesterone receptor (PR) were measured by the dextran-coated charcoal method. ER or PR was considered positive when the samples contained at least 10-fmol/mg protein. HER2 expression was determined by immunohistochemistry as described elsewhere.[13] BRCA1 expression was also determined by immunohistochemistry using a BRCA1 polyclone antibody (clone Ab-1423; dilution 1:100; Signalway, Pearland, TX, USA). BRCA1 immunostaining was considered positive when more than 10% of tumor cells showed positive nuclear staining. The follow-up data was available for all patients, with a median follow-up of 8 years (range, 0.4 to 11.6 years). During the follow-up period, 133 patients had developed distant metastases or local recurrences. Among them, 96 died of breast cancer. This study was approved by the Research and Ethical Committee of Peking University School of Oncology.

DNA extraction and bisulfite modification. Tumor DNA was extracted from the paraffin-embedded breast tumor specimens by a phenol-chloroform method. Briefly, three to four sections, 10 μm thick, were cut from each sample. After xylene deparaffination and washing with absolute ethanol, the sections were digested overnight with proteinase K (0.1 mg/mL) in 200 mL of DNA extraction buffer at 56°C.

Tumor DNA (1–2 μg) and controls were treated with sodium bisulfite and purified by the Wizards DNA Clean-Up System Kit (Promega, Madison, WI, USA). During the modification, the unmethylated cytosines of the genomic DNA were converted to uridines, but the methylated cytosines remained unchanged.[14]

Methylation-specific PCR (MSP). The methylation status of the *BRCA1* CpG islands (NC_000017) were determined by MSP as described.[15] The primers for detection of the unmethylated *BRCA1* CpG islands were 5′-ttggt ttttg tggta atgga aaagt gt-3′

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(sense) and 5′-caaaa aatct caaca aactc acacc a-3′ (antisense). The primers for detecting methylated *BRCA1* CpG islands were 5′-tcgtg gtaac ggaaa agcgc-3′ (sense) and 5′-aaatc tcaac gaact cacgc cg-3′ (antisense).[16] Approximately 60 ng of the bisulfite-modified DNA was used as a template for MSP. The PCR conditions were as follows: initial denaturation at 95°C for 15 min to activate HotStar Taq DNA polymerase followed by amplification at 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s for 38 cycles, and a final extension at 72°C for 10 min. PCR products were loaded onto 8% PAGE gel and visualized under UV illumination. The unmethylated and methylated PCR products were 86 bp and 75 bp, respectively. Genomic DNA of the human breast cancer cell line MCF7 was used as the unmethylated *BRCA1* negative control. The M.Sss I-modified genomic DNA of blood from healthy persons was used as the methylated *BRCA1* positive control.

Table 1. Association between methylation statuses of *BRCA1* CpG island and clinicopathological characteristics in 536 patients with breast cancer

Characteristics		*n*	*BRCA1* methylation (%)
Age	<50 years	273	26.0
	≥50 years	263	25.9
Tumor size	<2 cm	246	25.2
	≥2 cm	288	26.7
	(Unknown)	2	0
Histological type	Invasive ductal	397	25.7
	Medullary	61	26.2
	Invasive lobular	27	29.6
	Mucinous	20	35.0
	Others*	29	20.7
	(Unknown)	2	0
Clinical stage	I	232	22.8
	II	189	26.5
	III	112	32.1**
	(Unknown)	3	0
Lymph node status	Positive	209	29.2
	Negative	325	24.0
	(Unknown)	2	0
ER status	Positive	339	24.2
	Negative	182	30.2
	(Unknown)	15	13.3
PR status	Positive	248	22.6
	Negative	273	29.3
	(Unknown)	15	20.0
HER2 status	Positive	117	31.6
	Negative	396	24.5
	(Unknown)	23	21.7
Adjuvant therapy	C	225	24.4
	C + TAM	215	24.2
	TAM alone	52	26.9
	No treatment	17	47.1
	(Unknown)	27	37.0
Total		536	25.9

*Including five lipid-rich carcinomas, two Paget's disease, nine scirrhous adenocarcinomas; **stage I *versus* stage II *versus* stage III, χ^2 trend test, $P = 0.066$. BRCA1, breast cancer gene 1; C, chemotherapy; ER, estrogen receptor; HER2, neuroblastoma/glioblastoma derived oncogene homolog; M, methylated; PR, progesterone receptor; TAM, tamoxifen; U, unmethylated.

Clone sequencing. Fresh MSP products of *BRCA1* were cloned with the AT clone kit (Ao Ke Company, Beijing, China), and sequenced on the ABI Prism 3730 DNA Analyzer (Foster City, CA, USA).

Statistical analysis. The correlation between *BRCA1* methylation status, clinicopathologic characteristics, and adjuvant treatment was determined using Pearson's χ^2-test. DFS was defined as the time from the date of diagnosis to first recurrence (local or distant) or death from breast cancer without a recorded relapse. DSS was defined as the time from the date of diagnosis to death from breast cancer. Survival curves were assessed using the Kaplan–Meier method with log-rank tests. A Cox regression model was applied to determine whether a factor was an independent predictor of survival in multivariate analysis. All statistical tests were two-sided, and *P*-values less than 0.05 were considered statistically significant. The statistical analyses were performed using SPSS 15.0 software (SPSS, Chicago, IL, USA).

Results

Prevalence of *BRCA1* methylation in sporadic breast cancer. In this study of 611 tested patients, information on *BRCA1* methylation status in primary breast carcinoma tissues was obtained for 536 patients (87.7%). Twenty-six percent (139/536) exhibited *BRCA1* methylation in tumors by MSP (Fig. 1). To confirm the results of MSP, six representatives of MSP products were further analyzed by sequencing. The sequencing data showed that all cytosines in the CpG sites in the methylated samples remained as cytosines and those cytosines not in the CpG sites were converted to thymidines, indicating that all CpG sites were indeed methylated (Suppl. Fig.). The degree of *BRCA1* methylation was correlated with the clinical stages, but was not significant ($P = 0.066$) (Table 1). In addition, prevalence of *BRCA1* methylation in 95 basal-like related breast cancers (ER-, PR-, and HER2-concomitant triple negative) was slightly higher than that in 423 cancers without the concomitant negative (32.6% *vs* 24.8%, $P = 0.118$).

Breast cancer gene 1 (BRCA1) protein expression was analyzed by immunohistochemistry in 41 representative samples of breast tumors with or without *BRCA1* methylation in this cohort. The clinicopatholigical characteristics were not significantly different between the *BRCA1*-methylated and -unmethylated tumors in these 41 patients (data not shown). There was a trend association that *BRCA1*-methylated tumors exhibited a lower level (29%, 6/21)

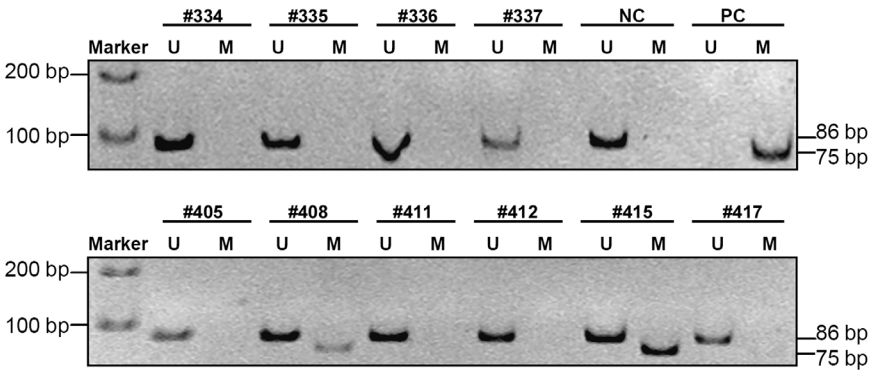

Fig. 1. Detection of methylated and unmethylated breast cancer gene 1 (*BRCA1*) by methylation-specific PCR. TE buffer was used as reagent control. Genomic DNA of human breast cancer cell line MCF7 was used as the unmethylated *BRCA1* negative control (NC). The M.Sss I-modified genomic DNA of blood from healthy persons was used as the methylated *BRCA1* positive control (PC). Methylation-specific PCR products (M, methylated, 75 bp; U, unmethylated, 86 bp) were run on an 8% PAGE gel.

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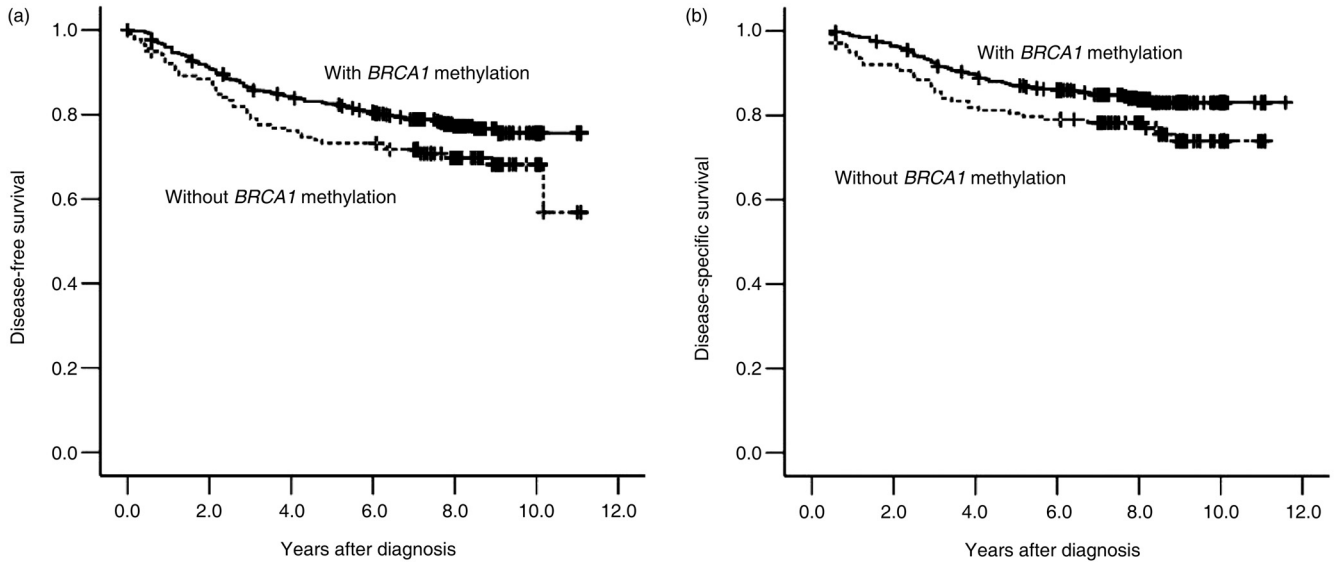

Fig. 2. Kaplan–Meier survival curves for disease-free survival (a) and disease-specific survival (b) for patients with breast cancer gene 1 (*BRCA1*)-methylated and -unmethylated sporadic breast cancer. Patients with *BRCA1*-methylated breast cancer had a shorter disease-free survival ($P = 0.045$, two-sided) and shorter disease-specific survival ($P = 0.038$, two-sided) in univariate analysis.

Table 2. Univariate and multivariate analysis of prognostic factors in Chinese patients with breast cancer*

Factor	DFS		DSS	
	HR (95% CI)	*P*-values**	HR (95% CI)	*P*-values
Univariate Analysis				
BRCA1 methylation	1.45 (1.01–2.09)	0.045	1.56 (1.02–2.37)	0.038
Clinical stage (I&II *vs* III)	5.67 (4.02–8.01)	<0.001	5.35 (3.58–7.99)	<0.001
Positive lymph node status	4.71 (3.24–6.86)	<0.001	5.55 (3.50–8.81)	<0.001
Tumor size (≥2 cm)	1.52 (1.07–2.16)	0.020	1.87 (1.22–2.86)	0.004
Positive HER2 status	1.46 (0.99–2.15)	0.055	2.07 (1.35–3.16)	0.001
Negative ER status	1.73 (1.22–2.44)	0.002	1.36 (0.90–2.04)	0.142
Multivariate analysis				
BRCA1 methylation	1.23 (0.84–1.80)	0.295	1.27 (0.81–1.99)	0.290
Clinical stage (I&II *vs* III)	3.30 (2.04–5.33)	<0.001	2.69 (1.57–4.64)	<0.001
Positive lymph node status	2.13 (1.26–3.58)	0.005	2.85 (1.53–5.32)	0.001
Tumor size (≥2 cm)	0.98 (0.67–1.45)	0.932	1.20 (0.75–1.94)	0.444
Positive HER2 status	1.42 (0.95–2.10)	0.086	2.06 (1.33–3.20)	0.001
Negative ER status	1.60 (1.12–2.29)	0.009	1.13 (0.71–1.80)	0.604

*Including treatment adjustment; **P-value, two-sided. BRCA1, breast cancer gene 1; CI, confidence interval; DFS, disease-free survival; DSS, disease-specific survival; ER, estrogen receptor; HER2, neuroblastoma/glioblastoma derived oncogene homolog; HR, hazard ratio.

of BRCA1 expression as compared with *BRCA1*-unmethylated tumors (55%, 11/20) ($P = 0.086$).

Association between *BRCA1* methylation and survival. *BRCA1* methylation was significantly associated with poorer DFS ($P = 0.045$, Fig. 2a) and DSS ($P = 0.038$, Fig. 2b). The 5-year DFS rate was 73.2% in patients with *BRCA1* methylation as compared with 82.6% in patients without *BRCA1* methylation; and the 5-year DSS rate was 80.5% in patients with *BRCA1* methylated tumors compared with 87% in patients with unmethylated tumors.

Using univariate analysis, the clinical stage, lymph node status, HER2 status, and tumor size were also significantly linked to both DFS and DSS. ER status was significantly associated with DFS but not with DSS, whereas PR status and age of diagnosis were not associated with survival. Thus, multivariate analysis was used further to evaluate whether *BRCA1* methylation was an independent factor of survival in this study. In multivariate analysis, clinical stage, lymph node involvement, and HER2 expression were significant independent factors, but *BRCA1* methylation was not (DFS: HR = 1.23, 95% CI: 0.84–1.80, $P = 0.295$; DSS: HR = 1.27, 95% CI: 0.81–1.99, $P = 0.290$; Table 2).

Discussion

Previous studies showed that the methylation rate for *BRCA1* varies from 12% to 40%.[9–11] In the present study, we investigated the methylation status of *BRCA1* in 536 Chinese patients with sporadic breast cancer. Twenty-six percent of the patients exhibited *BRCA1* methylation in their tumors. Our study extends previous research and is consistent with the observations that *BRCA1* methylation is a relatively frequent event in sporadic breast cancers and that methylation of *BRCA1* CpG islands might play an important role in breast carcinogenesis.

Several studies investigated the association between the *BRCA1* methylation and tumorigenesis.[16,17] *BRCA1* methylation can occur

in early stages of breast and ovarian carcinogenesis.[18] But few studies have been performed to investigate the prognostic role of *BRCA1* methylation in breast cancer. In the present study, we found that Chinese patients with *BRCA1* methylated tumors had a significantly poorer DFS and DSS than patients with *BRCA1* unmethylated tumors in univariate analysis (Table 2). Xu *et al*. also observed that *BRCA1* methylation was an independent risk factor for high mortality in 851 Caucasian women with sporadic breast cancer.[12] It was reported that the clinicopathological characteristics of breast cancers from Caucasian women and Asian women were different.[19,20] Whether these differences might result in different contribution of *BRCA1* methylation to patient survival could not be excluded. Anyway, in view of the positive observation in both populations, it is highly likely that *BRCA1* methylation correlates with poor prognosis in patients with breast cancer.

Breast cancer gene 1 (*BRCA1*) is a tumor suppressor gene; thus, mutations within this gene increase a person's susceptibility to breast cancer and ovarian carcinoma. In familial breast cancer, the mutation rate is as high as 45%.[21] However, the somatic mutation rate for *BRCA1* is only 1.23% in sporadic breast cancer.[3] The prevalence of somatic mutations of *BRCA1* in the present tested samples is under investigation. Aberrant methylation of *BRCA1* CpG islands may occur in a substantial number of sporadic breast cancers and may be associated with poor survival. It is well known that *BRCA1* methylation results in downregulation of *BRCA1* expression.[16,18] We also observed a low positive rate of BRCA1 protein in breast tumors with *BRCA1* methylation as compared with tumors without *BRCA1* unmethylation ($P = 0.086$). However, the exact underlying mechanism(s) of *BRCA1* dysfunction /methylation and its association with clinical outcomes in sporadic breast cancer is not clear.

Breast cancer gene 1 (*BRCA1*) is a crucial component for double-strand DNA break repair. Dysfunction of *BRCA1* might promote loss of *PTEN* expression, thus promoting cell transformation, proliferation, migration, angiogenesis, and genomic instability; inhibiting apoptosis; maintaining stem cell compartments; and finally leading to poor prognosis in breast carcinoma through the PTEN–PI3K pathway.[22] There are number of factors related to the prognosis of breast cancer;[23] whether *BRCA1* methylation promotes proliferation and metastasis of sporadic breast cancer should be studied further.

In conclusion, the present study shows that methylation of *BRCA1* CpG islands is associated with poor survival in patients with sporadic breast cancer, suggesting that inactivation of the *BRCA1* gene by CpG methylation may be involved in the progression of breast cancer and affect the clinical course and treatment of patients with this disease.

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Abbreviations

BRCA1	breast cancer gene 1
CI	confidence interval
DFS	disease-free survival
DSS	disease-specific survival
ER	estrogen receptor
HR	hazard ratio
HER2	neuroblastoma/glioblastoma derived oncogene homolog
MSP	methylation-specific polymerase chain reaction
PR	progesterone receptor
PTEN	phosphatase and tensin homolog

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

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Fig. S1. Sequencing chromatograms of the methylation-specific PCR products of breast cancer gene 1 (*BRCA1*) CpG islands in a representative sample by clone sequencing. The sequence of the methylated *BRCA1* CpG islands with bisulfite modification was listed above each corresponding chromatogram. Red Ts were converted from the unmethylated Cs. The highlighted Cs were the methylated CpG sites that were not converted. Underlined fragments were partial primer sequences.