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BRCA1 promoter methylation of normal breast epithelial cells as a possible precursor for *BRCA1*-methylated breast cancer

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Key words

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The breast cancer susceptibility gene 1 (*BRCA1*) and glutathione S-transferase P1 (*GSTP1*) promoters are reportedly often methylated in breast cancer tissues. Their methylation status in surrounding normal breast tissues has not been examined thoroughly although this may well be important for a better understanding of breast carcinogenesis. In this study, *BRCA1* and *GSTP1* promoter methylation was examined by methylation-specific PCR assay. Patients with *BRCA*1-methylated (*n* = 15) or *BRCA*1-unmethylated (*n* = 15) tumors and those with *GSTP1*-methylated (*n* = 9) or *GSTP1*-unmethylated (*n* = 11) tumors were included in the present study. Methylation status of manually micro-dissected normal epithelial cells from the formalin-fixed paraffin-embedded sections of normal breast tissues adjacent to and distant from the tumors was examined at multiple sites (*n* = 1–5). Of the 15 patients with *BRCA1*-methylated tumors, 9 harbored *BRCA1* promoter methylation in at least one site of the normal breast tissues. However, no *BRCA1* promoter methylation was observed at any site of the normal tissues of the 15 patients with *BRCA1*-unmethylated tumors. No *GSTP1* promoter methylation was observed in the normal tissues regardless of the methylation status of the tumors. The presence of *BRCA1* promoter methylation in the normal tissues was confirmed in the epithelial cells enriched with the magnetic-activated cell sorting method. Our findings suggest that a small proportion of normal breast epithelial cells with *BRCA1* promoter methylation can be precursor cells from which *BRCA1*-methylated breast tumors may originate. This does not apply to *GSTP1* promoter methylation.

The breast cancer susceptibility gene 1 (*BRCA1*) is a typical tumor suppressor gene, and its germline mutation is found in 40–50% of hereditary breast cancers.[1,2] Although the function of *BRCA1* is still not fully understood, it has been shown that it is implicated in the repair of double strand DNA breaks, various transcriptional pathways and regulation of cell cycle, ubiquitination and apoptosis.[1,3] While somatic mutation of *BRCA1* is a very rare event in sporadic breast cancers, promoter methylation of *BRCA1* has been observed in 5–31% of breast cancers,[1,4–10] and is reportedly strongly associated with a loss of *BRCA1* expression. Tumors with *BRCA1* promoter methylation are more likely to be estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, human epidermal growth factor receptor type 2 (HER2)-negative and to have a high histological grade.[4,11] Therefore, *BRCA1* promoter methylation is thought to play a more important role in the pathogenesis of ER-negative than of ER-positive breast cancer.

A few studies of *BRCA1* promoter methylation in normal breast tissues have identified it in 8.3–22% of these tissues.[5–7,12] However, these studies did not confirm the absence of tumor cells and benign proliferative lesions in the analyzed tissues. Contamination by tumor cells or some benign lesions harboring *BRCA1* promoter methylation[8,13] can lead to a false-positive result. Moreover, the geometrical relationship between the index tumor and *BRCA1*-methylated normal breast tissue has not yet been examined, even though it would be of interest to investigate whether the *BRCA1*-methylated sites in normal breast tissues are near to or far from the index tumor in order to further clarify the role of *BRCA1* promoter methylation in breast carcinogenesis, especially from the viewpoint of field cancerization.

The glutathione S-transferase P1 (*GSTP1*), which belongs to phase two metabolic enzymes, plays an important role in the detoxification of toxic substances, and its silencing by promoter methylation is suggested to be implicated in the pathogenesis of breast cancer.[14] *GSTP1* promoter methylation, unlike *BRCA1* promoter methylation, is thought to be implicated in the pathogenesis of ER-positive rather than ER-negative breast cancer.[15] It is reported that *GSTP1* promoter methylation is observed in 14–40% of breast cancers,[9,14,16] but relatively few studies have been reported on its methylation status in normal breast tissues, and their results are also conflicting.[7,14,17–19]

Therefore, in the study presented here, we investigated promoter methylation of *BRCA1* and *GSTP1* in tumor tissues as well as multiple sites of normal breast tissues. Special attention has been paid to the histological confirmation of the absence of tumor cells or benign proliferative lesions in the sections analyzed with the real-time methylation-specific polymerase chain reaction (MSP) assay as well as to geometrical aspects of methylated sites in the normal breast tissues in relation to the index tumor.

Materials and Methods

Patients. Female primary breast cancer patients who underwent mastectomy or breast conserving surgery (wide resection) between 2001 and 2012 at Osaka University Hospital were included in the present study. Patients treated with neoadjuvant chemotherapy and/or hormonal therapy were excluded. The surgical specimens from these patients were fixed in 10% buffered formalin and sliced at 5-mm intervals. Formalin-fixed paraffin embedded (FFPE) tissues were prepared from all these slices.

First, six paraffin sections were cut from the FFPE tumor tissues and subjected to the methylation assay for *BRCA1*. Of the total 147 tumors assayed, *BRCA1* promoter methylation was found in 15 of them. Of the 132 remaining tumors, 15 were selected as controls with optimal matching of age, tumor size and nodal status (Table 1). Similarly, 9 tumors with and 11 tumors without *GSTP1* methylation were selected from among 45 tumors. Eventually, 15 tumors with and 15 tumors without *BRCA1* promoter methylation, and 9 tumors with and 11 tumors without *GSTP1* promoter methylation were further analyzed.

Formalin-fixed paraffin embedded tissues from tumor and normal breast (adjacent to and distant from the tumor) of each patient were used for the following study. The first few sections (4 μm) obtained from each FFPE tissue were subjected to H&E staining for histological confirmation of the presence of tumor cells in FFPE tumor tissues as well as the absence of tumor cells or benign proliferative lesions in normal breast FFPE tissues, and also immunohistochemically stained for *BRCA1* and *GSTP1*. The next few sections (6–8 sections/tissue) with a thickness of 10 μm were H&E stained and tumor cells manually microdissected from the FFPE tumor tissues under a stereomicroscope as were normal epithelial cells from the FFPE normal breast tissues. The microdissected samples were then subjected to an assay to identify promoter methylation of *BRCA1* and

Table 1. Patient clinicopathological characteristics and their relationship with *BRCA1* or *GSTP1* promoter methylation

Clinicopathological factors	*BRCA*1		*GSTP1*	
	Methylated (n = 15)	Unmethylated (n = 15)	Methylated (n = 9)	Unmethylated (n = 11)
Age				
<50	7	8	5	7
≧50	8	7	4	4
T status				
1	9	7	5	5
2	6	8	4	6
Nodal status				
Positive	1	2	0	1
Negative	14	13	9	10
Histological grade				
1 + 2	7*	13	6	7
3	7	2	3	4
Unknown	1			
ER				
Positive	9	12	6	6
Negative	6	3	3	5
PR				
Positive	6	11	6	6
Negative	9	4	3	5
HER2				
Positive	0	2	3	0
Negative	15	13	6	11
Triple negative				
Yes	5	2	1	4
No	10	13	8	7
BRCA1 protein				
Positive	4**	11		
Negative	10	4		
Unknown	1			
GSTP1 protein				
Positive			1***	8
Negative			7	3
Unknown			1	

*P = 0.041, **P = 0.016, ***P = 0.015. ER, estrogen receptor; PR, progesterone receptor.

GSTP1. The present study was approved by the Institutional Review Board for Clinical Research, Osaka University Graduate School of Medicine.

DNA extraction and sodium bisulfite treatment. DNA was extracted from the FFPE tissue sections with the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. A maximum of 1 μg DNA was treated with sodium bisulfite with the aid of the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's protocol.

Assay for *BRCA1* and *GSTP1* promoter methylation. Methylation of the *BRCA1* and *GSTP1* promoter was assessed with real-time MSP. The methylation and unmethylation primer sets and probes for *BRCA1* and *GSTP1* were designed as previously reported.[10,15] Their sequence are shown in Supplementary Table S1 and conditions for MSP are shown in Supplementary Table S2. The standard curve was raised for each run to quantify the methylated or unmethylated *BRCA1* and *GSTP1* promoter using the standard oligonucleotides (Table S3). The completely methylated bisulfite-converted human DNA and the completely unmethylated bisulfite-converted human DNA (Human EpiTect Control DNA; Qiagen) were used as positive and negative controls, respectively, in each assay. The DNA samples that showed no amplification by both methylation and unmethylation primer sets were not evaluated because this indicates a poor quality of DNA used for the assay. Each sample was assayed in triplicate. When all of the triplicate assay results were above the detection limit, the sample was defined as positive. The representative results of real-time MSP are shown in Supplementary Figure S1. Finally, PCR products were also assessed by means of 2% agarose gel electrophoresis, staining with ethidium bromide and visualization under ultraviolet illumination. The methylation index (MI) was calculated as follows: MI (%) = 100 × methylated gene copy numbers/(methylated + unmethylated gene copy numbers).

Immunohistochemical assay. Immunohistochemistry was performed as previously reported for BRCA1 (Ab-1, monoclonal, 1:70; Calbiochem, San Diego, CA, USA) and with a slight modification for GSTP1 using anti-GSTP1 mouse monoclonal antibody (3F2) (Cell Signaling Technology, Danvers, MA, USA) as the primary antibody and Histofine Simple Stain MAX-PO (M) (Nichirei Biosciences, Tokyo, Japan) as the secondary antibody.[10,15] ER and PR was evaluated by immunohistochemistry (ER: clone 6F11; PR: clone 16; Ventana Japan and SRL) or by enzyme immunoassay (Abbott Laboratories, North Chicago, IL, USA) according to the manufacturer's instructions. The cut-off value was 10% for BRCA1, GSTP1, ER and PR. HER-2 was evaluated by HercepTest (Dako, Tokyo, Japan) or FISH as previously described.[20] Breast tumors with either 3+ immunostaining or a gene copy number ≥2.0 were considered HER-2 positive.

Magnetic-activated cell sorting. To confirm that the methylated DNA was derived from the normal epithelial cells, magnetic-activated cell sorting (MACS) using the EasySep Human EpCAM Positive Selection Cocktail, the EasySep Human MUC1 Positive Selection Cocktail and EasySep Magnetic Particles (Stem Cell Technologies, Vancouver, BC, Canada) was performed according to the manufacturer's protocol with some modification. In brief, six sections of FFPE normal tissues with a thickness of 50 μm were deparaffinized and rehydrated as described above. The sections were then minced with scissors and digested with Proteinase K (Invitrogen, Yokohama, Japan) for 2 h at 37°C. After passing through a 100-μm nylon mesh (BD Falcon, San Jose, CA, USA) the filtrate (cell suspension) was treated with anti-EpCAM, anti-MUC1 antibody and magnetic particles. The cell suspension was then placed in a magnet for 5 min and the supernatant poured off. This process was repeated twice. Finally, the enriched breast epithelial cells were assayed for promoter methylation of *BRCA1*. An internal control marker (bisulfite control [BSC]) was included to verify the conversion of cytosine to uracil by bisulfite treatment as well as DNA integrity.[21] The primers and probe sequence of BSC and conditions for PCR are shown in Supplementary Table S4.

Cytokeratin staining was used to confirm that the breast epithelial cells were actually enriched after MACS. In brief, the cells retrieved by MACS were attached to slides with Tissue Capture (Daido Sangyo, Saitama, Japan). After quenching of endogenous peroxidase with 3% H_2O_2 in methanol for 20 min, the slides were treated with trypsin (Nichirei Biosciences, Tokyo, Japan) at 37°C for 10 min, and non-specific binding was blocked with Block Ace (AbD Serotec, Raleigh, NC, USA) for 30 min. The slides were then incubated with anti-cytokeratin mouse mAb (AE1, AE3) (Nichirei Biosciences) for 1 h at room temperature. The subsequent process for visualization of the immunostaining was the same as described above.

Statistical analysis. The SPSS software package version 11.0.1J (SPSS, Chicago, IL, USA) was used for all statistical analyses. Differences in *BRCA1* MI were evaluated with Mann–Whitney's *U*-test. Associations between various parameters were assessed using the χ^2 test or Fisher's exact test.

Results

***BRCA1* promoter methylation in tumor and normal breast tissues.** *BRCA1* promoter methylation was analyzed in multiple sites, with an average 3 sites (range: 1–5 sites) per case, of the normal breast tissues surrounding the breast tumors with ($n = 15$) or without ($n = 15$) *BRCA1* promoter methylation. The distribution of specimens analyzed and their methylation ratio for 4 representative cases are shown in Figure 1. In 15 breast tumors with *BRCA1* promoter methylation, the median MI for *BRCA1* was 13.6%, ranging from 0.2% to 80.3%. Of these 15 patients, 9 were found to harbor *BRCA1* promoter methylation in at least one site of the normal breast tissues (Table 2). The median MI for *BRCA1* in the normal tissues was 2.1%, ranging from 0.4% to 11.9%, and was, thus, significantly ($P < 0.001$) lower than that in the aforementioned tumor tissues. The normal tissues with *BRCA1* promoter methylation were distributed widely, from immediately adjacent to the tumor to more than 6 cm away from the index tumor. The clinicopathological characteristics of the patients with *BRCA1*-methylated tumors are shown in Supplementary Table S5 according to the presence or absence of *BRCA1* promoter methylation in the normal tissues.

However, no *BRCA1* promoter methylation was observed in any sites of the normal breast tissues of the 15 patients with *BRCA1*-unmethylated tumors.

***GSTP1* promoter methylation in tumor and normal breast tissues.** Similarly, *GSTP1* promoter methylation was analyzed in multiple sites, with an average 3 sites (range: 2–5 sites) per case, of the normal breast tissues surrounding the breast tumors with ($n = 9$) or without ($n = 11$) *GSTP1* promoter methylation. No *GSTP1* methylation was detected in any sites

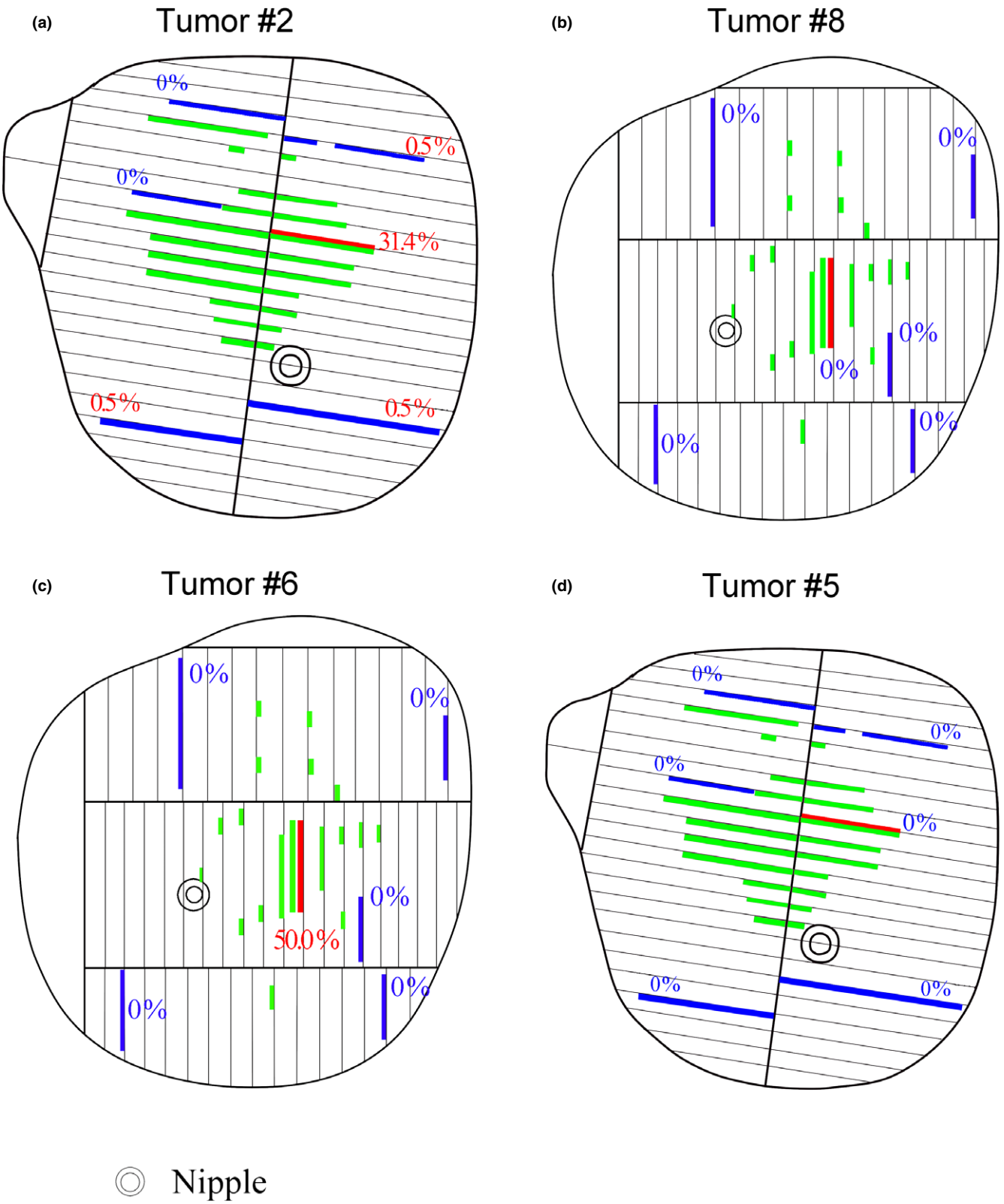

Fig. 1. Schematic presentation of representative results of methylation assay for *BRCA1* and *GSTP1*. Formalin-fixed surgical specimens (breast glands) were sliced at 5-mm intervals as indicated in each illustration by the multiple parallel lines, and paraffin blocks were prepared from the whole breast gland. In addition to tumor sites, multiple sites of the normal breast tissues were analyzed by promoter methylation assay for *BRCA1* (a, b) or *GSTP1* (c, d). The sites subjected to methylation assay are shown in bold lines; that is, (▬) tumor site and (▬) normal breast site. Distribution of tumor cells is indicated by (▬). The numbers represent the methylation index. Tumor numbers (#) correspond to those in Table 2.

of the normal tissues regardless of the *GSTP1* promoter methylation status of the tumor tissues (Table 3).

Immunohistochemistry of BRCA1 and GSTP1. The relationship between BRCA1 and GSTP1 promoter methylation with protein expression was investigated by means of IHC. *BRCA1* promoter methylation was found to be significantly associated with a loss of BRCA1 expression, as was GSTP1 methylation with a loss of GSTP1 expression (Table 1). Representative results of IHC of BRCA1 (Fig. 2a–d) and GSTP1 (Fig. 2e–g) are shown in relation to their promoter methylation status.

All the normal tissues assayed for BRCA1 promoter methylation were also subjected to the IHC for BRCA1, and BRCA1 expression was observed in the luminal epithelial cells in all of them regardless of methylation status. Similarly, GSTP1 expression in the epithelial cells was observed in all the normal tissues assayed for GSTP1.

***BRCA1* methylation assay for normal breast epithelial cells enriched by the magnetic-activated cell sorting.** To confirm that *BRCA1* is actually methylated in the epithelial cells of the normal breast tissues, the epithelial cells from 4 normal breast tissues from three different patients with *BRCA1*-methylated tumors were enriched as a result of MACS. The enriched cells were confirmed to be, in fact, the epithelial cells by immunohistochemical staining with anti-cytokeratin antibodies (Fig. 3a). The enriched epithelial cells were then subjected to the *BRCA1* promoter methylation assay, which confirmed the actual presence of *BRCA1* promoter methylation in these cells derived from all the four normal breast tissues (Fig. 3b).

Discussion

In the present study, we investigated *BRCA1* promoter methylation in multiple sites of normal breast tissues, and found that, of the 15 patients with *BRCA1*-methylated tumors, 9 showed *BRCA1* promoter methylation in the normal breast tissues, which were histologically confirmed not to contain any tumor cells or benign proliferative lesions. The methylated sites were distributed widely, from immediately adjacent to the tumor to more than 6 cm away from the index tumor, indicating that a certain proportion of histologically-normal breast epithelial cells harbor *BRCA1* promoter methylation and that such cells are not necessarily located adjacent to the tumor. Interestingly, no site in the normal breast tissues of the patients with *BRCA1*-unmethylated tumors was methylated.

The percentage of *BRCA1*-methylated alleles in the normal breast tissues was very low, with a median of 2.1% and a range from 0.4% to 11.9%. In addition, IHC of BRCA1 was almost

Table 2. *BRCA1* promoter methylation in breast tumors and normal breast tissues

Tumors			Distance from tumor margin						
			Adjacent	1–2 cm	2–3 cm	3–4 cm	4–5 cm	5–6 cm	>6 cm
BRCA1-methylated tumors	1	26.0	0.8	1.2		2.4	0.4		
	2	31.4	0			0.5	0.5	0.5	
	3	7.0			1.0				
	4	13.6	0						0
	5	19.7		0		0			
	6	8.4	5.9			2.5			0.9
	7	13.0	0			0			
	8	80.3	0		6.5	5.0			
	9	41.2	0			0			
	10	23.2	11.9	0					9.4
	11	1.5	1.7	0			2.4		3.5
	12	0.8	0	0					
	13	0.3	2.4	1.5					
	14	0.2	0	0					
	15	57.1	3.5	4.4					
BRCA1-unmethylated tumors	1	0			0	0		0	0
	2	0					0	0	0
	3	0	0		0			0	0
	4	0	0						0
	5	0		0	0				0
	6	0	0			0	0		
	7	0		0			0		
	8	0	0	0	0	0	0		
	9	0	0				0		
	10	0	0			0	0		
	11	0	0		0				
	12	0	0				0		
	13	0	0	0					
	14	0	0	0			0		
	15	0					0	0	

Multiple sites of the normal breast tissues were analyzed by methylation-specific PCR assay for *BRCA1* promoter. Distance from the tumor margin to each site was measured. The numbers in the Table represent the methylation index (MI) for *BRCA1* promoter. Black cells show the presence and "0"s the absence of *BRCA1* promoter methylation. Blank cells indicate that the assay for *BRCA1* promoter methylation was not performed.

Table 3. *GSTP1* promoter methylation in breast tumors and normal breast tissues

Tumors			Distance from tumor margin						
			Adjacent	1–2 cm	2–3 cm	3–4 cm	4–5 cm	5–6 cm	>6 cm
GSTP1-methylated tumors	1	3.4			0	0			0
	2	53.9	0			0	0		
	3	91.9	0			0			0
	4	23.4				0			0
	5	79.1		0			0		
	6	50.0	0	0	0	0	0		
	7	9.6	0				0		
	8	45.0	0				0		
	9	38.3	0			0	0		
GSTP1-unmethylated tumors	1	0	0				0		
	2	0	0				0		
	3	0	0	0				0	0
	4	0	0			0		0	
	5	0	0			0	0	0	
	6	0			0				
	7	0		0		0			0
	8	0	0		0	0			
	9	0	0			0			0
	10	0	0			0		0	
	11	0	0			0			

Multiple sites of the normal breast gland were analyzed using the methylation-specific PCR assay for *GSTP1* promoter. Distance from the tumor margin to each site was measured. The numbers in the table represent the methylation index (MI) for *GSTP1* promoter. Black cells show the presence and "0"s the absence of *GSTP1* promoter methylation. Blank cells indicate that the assay for *GSTP1* promoter methylation was not performed.

exclusively positive even in the epithelial cells within the normal breast tissues harboring *BRCA1* promoter methylation, indicating that only a very small percentage of epithelial cells are positive for *BRCA1* promoter methylation. Furthermore, to exclude the possibility that the positive results for *BRCA1* promoter methylation originated not from the breast epithelial cells but from the contaminated cells such as fibroblasts and lymphocytes within the microdissected areas, the epithelial cells were enriched by MACS to confirm that the enriched epithelial cells actually possessed the *BRCA1* methylation.

Taken together, these findings seem to indicate that *BRCA1* promoter methylation is harbored by a very small proportion of the histologically-normal epithelial cells which are widely distributed over the breast gland. This is essentially consistent with a previous report that *BRCA1* promoter methylation was found in 8.3% of normal breast tissues taken from the quadrant opposite to the tumor containing one.[7] Interestingly, *BRCA1* promoter methylation was also reportedly observed in 17–22% of fine needle aspirates or frozen tissues taken from asymptomatic women at high risk of breast cancer.[5,6,12] The fact that no methylation was observed in any sites of the normal breast tissues of the patients with *BRCA1*-unmethylated tumors seems to suggest that *BRCA1*-methylated tumors are unlikely to develop without a predisposing condition; that is, the presence of *BRCA1*-methylated epithelial cells.

We have previously shown that patients harboring *BRCA1* promoter methylation in peripheral blood mononuclear cells (PBMNC) have an increased risk for developing *BRCA1*-methylated breast cancer but not *BRCA1*-unmethyhlated breast cancer.[10] In that study, however, correlation of *BRCA1* promoter methylation between breast tumors and normal breast tissues was not investigated. Thus, in the present study, we have studied this issue and demonstrated that *BRCA1* promoter methylation can be seen in the normal breast tissues of the patients

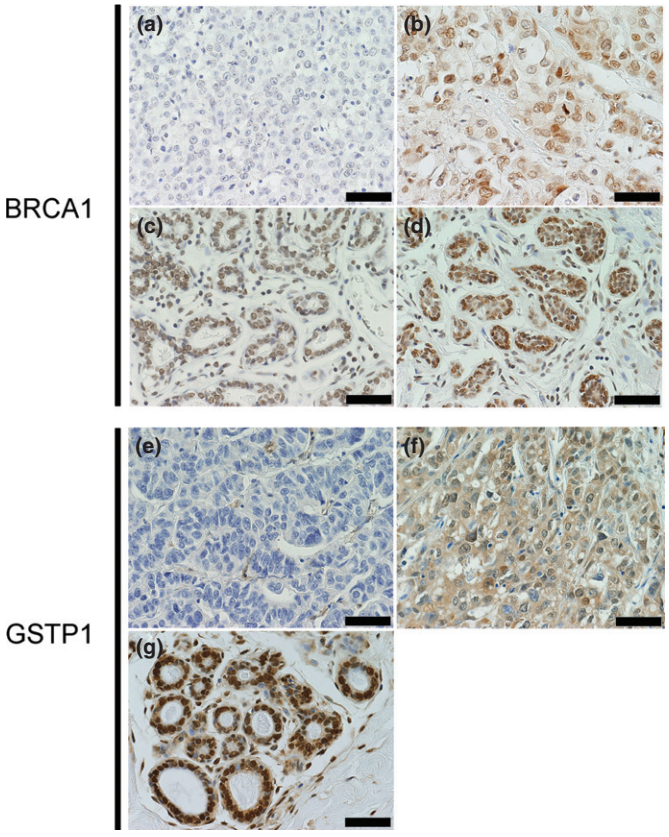

Fig. 2. Immunohistochemistry for BRCA1 and GSTP1. Immunostaining of BRCA1 in breast tumors with (a) and without (b) *BRCA1* methylation and in normal breast tissue with (c) and without (d) *BRCA1* methylation. Immunostaining of GSTP1 in breast tumors with (e) and without (d) *GSTP1* methylation and in normal breast tissue without *GSTP1* methylation (g). The scale bar indicates 100 μm.

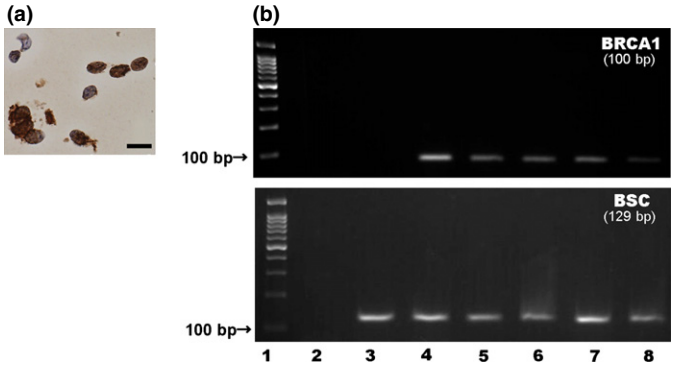

Fig. 3. *BRCA1* promoter assay for enriched epithelial cells from normal breast tissue. (a) Normal epithelial cells were enriched with the magnetic-activated cell sorting (MACS) method, and the enriched cells were immunostained using anti-cytokeratin antibody. All the cells stained positive, confirming that the enriched cells were epithelial cells. Scale bar: 10 μm. (b) The enriched epithelial cells prepared with the MACS method from the normal breast tissues of the three patients (#1, #2 and #3) with *BRCA1*-methylated breast tumors were subjected to *BRCA1* promoter methylation assay using the methylation set primers (upper panel [*BRCA1*]) as well as bisulfite control assay (lower panel [BSC]) which was done to confirm the conversion from cytosine to uracil by bisulfite treatment and DNA integrity. Lane 1, marker ladder; lane 2, negative control (water); lane 3, negative control (unmethylated control); lane 4, positive control (methylated control); lane 5, *patient #1-1; lane 6, *patient #1-2; lane 7, patient #2; lane 8, patient #3. *Samples were obtained from two different sites in patient #1.

with *BRCA1*-methylated tumors but not of those with *BRCA1*-unmethylated tumors. We have conducted additional study, although preliminary, to see whether or not *BRCA1* promoter methylation is correlated between PBMNC and normal breast tissues in breast cancer patients, and have been able to show a significant association (Table S6), suggesting that women with *BRCA1*-methylated PBMNC are at an increased risk for *BRCA1* promoter methylation in the normal breast tissues, which might lead to carcinogenesis of *BRCA1*-methylated tumors. Our preliminary results presented here only demonstrate the association of *BRCA1* promoter methylation between tumors and normal breast tissues. Direct evidence of progression from *BRCA1*-methylated normal breast epithelial cells to *BRCA1*-methylated breast cancer needs to be shown in future studies.

Unlike *BRCA1*, no site of the normal breast tissues analyzed for the *GSTP1* promoter methylation was found to carry methylation regardless of the *GSTP1* methylation status of the tumors, which is consistent with the findings of previous studies.[14,17,18] In contrast, it has been reported that *GSTP1* promoter methylation was observed in 16.7% and 17.0%, respectively, of the normal breast tissues of breast cancer patients.[7,19] However, these two studies did not confirm the absence of contamination by tumor cells or by benign proliferative lesions which can harbor methylation. Differences in assay conditions, including sensitivity for *GSTP1* promoter methylation detection, may explain such a discrepancy. In any case, our observation that *GSTP1* promoter methylation was not observed in any sites of the normal breast tissues irrespective of the *GSTP1* methylation status of the tumors seems to indicate that *GSTP1* promoter methylation occurs in a later phase in breast carcinogenesis than *BRCA1* promoter methylation.

In conclusion, we showed that a significant proportion of patients with *BRCA1*-methylated tumors harbored *BRCA1* promoter methylation in normal breast tissues but those with *BRCA1*-unmethylated tumors did not, and that no *GSTP1* methylation was observed in any of the normal tissues regardless of the *GSTP1* promoter methylation status of the tumors. This suggests a possibility that a small proportion of the epithelial cells with *BRCA1* promoter methylation can be precursor cells from which *BRCA1*-methylated breast tumors originate, but that this is not the case for *GSTP1* promoter methylation. Although our preliminary results presented here need to be validated by future studies, they may provide further insight into the different roles of promoter methylation of these genes in breast carcinogenesis.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Representative results of MSP assay for *BRCA1* and *GSTP1* promoter methylation.

Table S1. Sequences of primers and probe sets for *BRCA1* and *GSTP1*.

Table S2. Conditions for methylation-specific PCR for *BRCA1* and *GSTP1*.

Table S3. Sequences of standard oligonucleotides for methylation and unmethylation assays for *BRCA1* and *GSTP1* promoter region.

Table S4. Information on bisulfite control (BSC).

Table S5. Clinicopathological characteristics of patients with *BRCA1*-methylated breast tumors according to the *BRCA1* methylation status of the normal tissues.

Table S6. Correlation of *BRCA1* promoter methylation between normal breast tissues and peripheral blood mononuclear cells (PBMNC).