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PRECLINICAL STUDY

BRCA1 promoter methylation in peripheral blood cells is associated with increased risk of breast cancer with BRCA1 promoter methylation

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Abstract BRCA1 promoter methylation reportedly plays an important part in the pathogenesis of human breast cancer. In the present study, we investigated whether or not BRCA1 promoter methylation in peripheral blood cells (PBCs) can serve as a risk factor for developing breast cancer. The association of BRCA1 promoter methylation in PBCs with breast cancer risk was examined in a case–control study (200 breast cancer patients and 200 controls). BRCA1 promoter methylation in PBCs and breast tumors was determined with a methylation-specific quantitative PCR assay. BRCA1 promoter methylation in PBCs was seen in 43 (21.5%) of the breast cancer patients and in 27 (13.5%) of the controls. The odds ratio for breast cancer adjusted for other epidemiological risk factors was 1.73 (1.01–2.96) and was statistically significant ($P = 0.045$). When breast tumors were classified into those with and without BRCA1 promoter methylation, the odds ratio was 0.84 (0.43–1.64) ($P = 0.61$) for BRCA1 promoter methylation-negative and 17.78 (6.71–47.13) ($P < 0.001$) for BRCA1 promoter methylation-positive breast tumors. BRCA1 promoter methylation in PBCs is significantly associated with risk of breast cancer with BRCA1 promoter methylation. This seems to indicate that BRCA1 promoter methylation in PBCs may constitute a novel risk factor for breast cancer with BRCA1 promoter methylation.

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Introduction

BRCA1 is a well-established breast cancer susceptibility gene [1], and its germline mutations are found in 40–50% of hereditary breast cancers [2, 3]. It is a typical tumor suppressor gene, and loss of a wild allele has been reported in almost all breast tumors originating in BRCA1 germline mutation carriers [4–6]. Although its function is still not fully understood, it has been shown that BRCA1 is implicated in repair of double strand DNA breaks [7, 8], various transcriptional pathways [9], and regulation of the cell cycle [10, 11]. Moreover, recent studies have shown that most BRCA1 functions can be explained by its E3 ubiquitin ligase activity in cooperation with BARD1 [12, 13].

Somatic mutations of BRCA1 are very rare [14, 15], but its promoter methylation, which leads to silencing of the gene, is reportedly observed in 10–30% of sporadic breast tumors [16–18], suggesting that this gene is involved in the pathogenesis of a significant proportion of sporadic breast cancers. Interestingly, phenotypes of breast tumors with BRCA1 promoter methylation, which lack expression of the BRCA1 protein due to BRCA1 gene silencing [19, 20], are reported to be similar to those arising in patients with germline BRCA1 mutations in that they are more likely to be ER, PR [21–23], and HER2 negative tumors [24] as well as histologically high grade tumors [17]. These results seem to indicate that BRCA1 promoter methylation in sporadic breast tumors can be considered to occur relatively early in their pathogenesis. Such tumors thus appear to share similar phenotypes with hereditary breast tumors

with BRCA1 germline mutations, which, according to the two-hit theory, are thought to lose their BRCA1 function from the beginning of pathogenesis.

Snell et al. [25] recently studied BRCA1 promoter methylation in peripheral blood cells (PBCs) from seven familial breast cancer patients whose tumors were pathologically similar to BRCA1-mutated tumors but lacked BRCA1 and BRCA2 germline mutations, and were able to demonstrate BRCA1 promoter methylation in PBCs of three of them. They also reported that BRCA1 promoter methylation was observed in all breast tumors in these three patients, which suggests that BRCA1 promoter methylation in PBCs is associated with risk of developing breast cancer, especially breast cancer with BRCA1 promoter methylation. Moreover, Flanagan et al. [26] recently demonstrated that gene body hypermethylation of the ATM gene in PBCs is significantly associated with breast cancer risk [26], indicating a possible association of tumor suppressor gene methylation in PBCs with breast cancer risk.

These results taken together led us to hypothesize that BRCA1 promoter methylation in PBCs may indicate the propensity of a woman's normal breast tissue to have BRCA1 promoter methylation and that such would become susceptible to breast cancer pathogenesis. This would mean that BRCA1 promoter methylation in PBCs could constitute a risk factor for breast cancer. In the case–control study presented here, we, therefore, investigated whether BRCA1 promoter methylation in PBCs is associated with risk of breast cancer, especially breast cancer with BRCA1 promoter methylation.

Materials and methods

Patients

Cases were breast cancer patients who underwent breast conserving surgery or mastectomy at Osaka University Hospital between October 2000 and November 2008, and controls were women who received breast cancer screening by physical examination and mammography and/or ultrasonography at the screening institutes in Osaka and were found to be free of breast cancer during the period of June 2001 to September 2005. In order to minimize the influence of age, cases were composed of 50 patients in every 10 years-stratum (30–39, 40–49, 50–59, and 60–69) and controls were also composed of 50 patients in every 10 years-stratum (30–39, 40–49, 50–59, and 60–69) as shown in Table 1. Cases were selected in order of date of surgery, and controls were selected in order of date of breast cancer screening in each stratum. Peripheral blood was collected from breast cancer patients before surgery, and the patients who received neoadjuvant chemotherapy and/or hormonal therapy were excluded. Informed consent was obtained from each patient or control before blood collection.

DNA extraction and sodium bisulfite treatment

DNA was extracted from whole peripheral blood cells (PBCs) of 200 cases and 200 controls and from 162 breast tumor tissues as previously described [27–29] and 1 μg of the DNA was subjected to sodium bisulfite treatment using the EpiTect Bisulfite Kit (48) (QIAGEN, Inc. Valencia, CA) according to the manufacturer's protocol.

Fresh tumor tissues were obtainable from 162 tumors and kept at −80°C until use. They were not obtainable in the remaining 38 patients mostly due to their small size for sampling. The backgrounds of breast tumors with fresh tumors available were not significantly different from those with fresh tumor tissues unavailable in terms of age, tumor size, histological type, lymph node status, histological grade, and positivity of ER, PR, and HER2, except for tumor size, i.e., the former tumors included Tis ($n = 0$), T1 ($n = 105$), T2 ($n = 57$), and T3 ($n = 0$), and the latter tumors included Tis ($n = 3$), T1 ($n = 27$), T2 ($n = 7$), and T3 ($n = 1$)($P < 0.001$).

Analysis of BRCA1 promoter methylation

Methylation of the BRCA1 promoter was assessed with real-time methylation-specific polymerase chain reaction (MSP). The methylated primer set and probe for BRCA1 were used as previously reported [30]. The forward primer was 5′-TTT CGTGGTAACGGAAAAGCG-3′, the backward primer was 5′-CCGTCCAAAAAATCTCAACGAA-3′, and the probe was FAM-5′-CTCACGCCGCGCAATCGCAATT T-3′-DDQ1. The unmethylated primer set for BRCA1 was modified very slightly from the one previously reported [17] with forward primer 5′-TGGTTTTTGTGGTAATGGAA AAGTGTG-3′, backward primer 5′-CCCATCCAAAAAA TCTCAACAAA-3′, and probe FAM-5′-CTCACACCAC ACAATCACAATTTTAAT-3′-DDQ1. For a quantification of the methylated and unmethylated BRCA1 promoter, methylated standard oligoDNA, 5′-TTTCGTGGTAACGG AAAAGCGCGGGAATTATAGATAAATTAAAATTGCG ATTGCGCGGCGTGAGTTCGTTGAGATTTTTTGGACG G-3′ and unmethylated standard oligoDNA, 5′-TGGTTT TTGTGGTAATGGAAAAGTGTGGGAATTATAGATAA ATTAAAATTGTGATTGTGTGGTGTGAGTTTGTTGAG ATTTTTTGGATGGG-3′, were synthesized so as to include the aforementioned forward primer, backward primer, and probe sequences. EpiTect® Control DNA (human) (QIAGEN) was used as both the positive and negative control for methylated alleles.

For PCR amplification, 2 μl of bisulfite-modified DNA was added in a final volume of 20 μl PCR mix containing

Table 1 Demographics of breast cancer patients and controls included in this study and their association with BRCA1 promoter methylation in peripheral blood cells

	Breast cancer patients			Controls		
	Total 200	Me (−)[a] 157	Me (+)[b] 43	Total 200	Me (−)[a] 173	Me (+)[b] 27
Median age (range)	50 (30–69)	49 (30–69)	52 (30–69)	50 (30–69)	51 (30–69)	46 (31–66)
30–39 years	50	40	10	50	45	5
40–49 years	50	41	9	50	40	10
50–59 years	50	41	9	50	44	6
60–69 years	50	35	15	50	44	6
Menopausal status						
Premenopausal	111	88	23	104	90	14
Postmenopausal	89	69	20	96	83	13
Age at menarche						
≤12	71	56	15	71	58	13
13–14	104	82	22	105	93	12
15≤	25	19	6	24	22	2
Age at menopause						
<50	23	20	3	29	26	3
50≤	66	49	17	67	57	10
BMI						
<20	54	42	12	58	54	4
20–23	83	69	14	86	73	13
23<	63	46	17	56	46	10
Family history						
Yes	29	23	6	17	15	2
NO	171	134	37	183	158	25

BMI body mass index

[a] BRCA1 promoter methylation-negative in peripheral blood cells

[b] BRCA1 promoter methylation positive in peripheral blood cells

10 µl Fast Start Universal Probe Master (Rox; Roche Applied Science, Mannheim, Germany), 8 µl distilled water, probe, and primers. PCR amplifications were performed using the Light Cycler® 480 Real-Time PCR System (Roche Applied Science) under the following conditions: 1 cycle at 95°C for 10 min followed by 45 cycles at 95°C for 30 s, at 58°C for 30 s, and at 72°C for 30 s. The standard curve was raised for each run for quantification of the methylated BRCA1 promoter by using standard oligo DNA from 120 to 120,000 copies (Fig. 1). Each sample was analyzed in triplicate assays, and those which showed 120 or more copies in all samples were considered positive. Finally, PCR products were also confirmed by means of 2% agarose gel electrophoresis, staining with ethidium bromide and visualization under UV illumination. The percentage of methylation was calculated as M% = M/(M + UM). Finally, all data were corrected by quantification of the positive control.

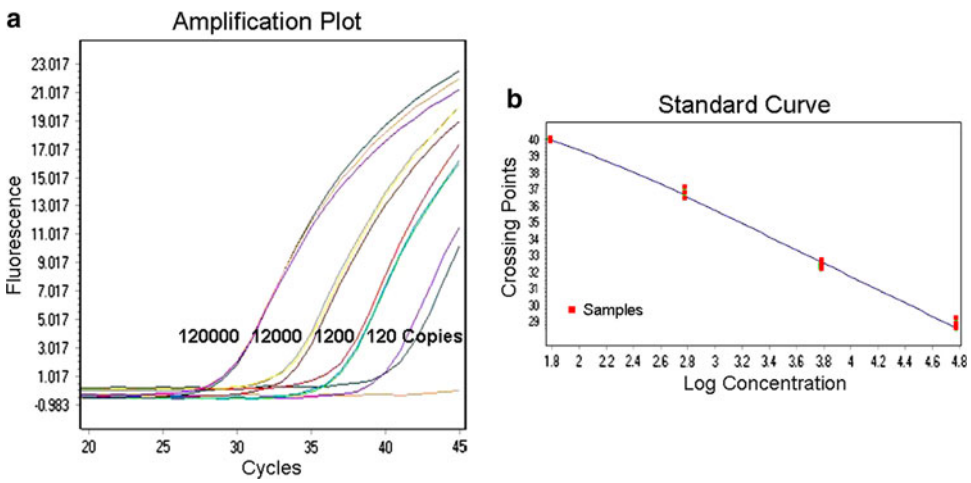

Fig. 1 MSP assay standard curve. **a** *Amplification plot* Samples containing five different copies (120000, 12000, 1200, 120, and 12) of standard oligo DNA were subjected to MSP assay. Cycle numbers are plotted against changes in normalized reporter signals. **b** *Standard curve plot* Log starting copy number was plotted against C_t. *Dots* represent data obtained from standard curve point samples

Detection sensitivity of MSP assay for methylated against unmethylated BRCA1 promoter

Human genome DNA completely methylated by Sss1 methylase treatment (EpiScope™Methylated HeLa Genomic DNA, Takara Bio, Shiga, Japan) was diluted against unmethylated human genome DNA obtained from the healthy controls at various ratios [methylated DNA/(methylated DNA + unmethylated DNA) = 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}]. These mixtures (total DNA content: 1 μg) were then treated with sodium bisulfite treatment and subjected to the MSP assay as described above.

Antibodies

The antibodies used were: BRCA1 (Ab-1, monoclonal, IgG isotype, 1:70; Calbiochem, San Diego, CA), estrogen receptor (ER) (polyclonal, IgG isotype, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), progesterone receptor (PR) (clone 636, monoclonal, IgG isotype, 1:800; Dako, Kyoto, Japan), and c-erbB2 (HER2) (polyclonal, IgG isotype, 1:100; Nichirei, Kyoto, Japan),

Immunohistochemical assay

Paraffin sections (3 μm) prepared from the formalin-fixed paraffin-embedded tumor specimens were immunohistochemically stained with the avidin–biotin-peroxidase method. The paraffin sections were deparaffinized and then rehydrated in graded alcohols. In brief, antigens for ER and PR were retrieved by heating the samples in a target retrieval solution (Dako) at 98°C for 40 min while antigen BRCA1 was retrieved with microwave oven treatment at 500 W for 15 min. Sections to be stained for HER2 were not pretreated. After quenching of endogenous peroxidase with 3% H_2O_2 in methanol for 20 min, non-specific binding was blocked by incubating the slides with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min, followed by incubation of the sections with the primary antibody at 4°C overnight. Next, the sections were treated with a biotin-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, after treatment with the avidin–biotin-peroxidase complex system (VECTASTAIN® Elite ABC kit, Vector Laboratories, Burlingame, CA), the sections were visualized with 3,3′-diaminobenzidine tetrahydrochloride (Merck, Darmstadt, Germany). The sections were then counter stained with hematoxylin.

Nuclear-stained tumor cells in three non-overlapping fields were identified with a 20× lens and counted. When tumor cells positive for nuclear BRCA1, ER, or PR staining were observed in more than 10% of all tumor cells, the tumor was considered positive for the corresponding protein. Staining of HER2 was scored with four grades (0, 1, 2, and 3) according to a previously described method [31], and tumors with scores of 2+ and 3+ were considered to be HER2 (+).

Statistics

BRCA1 promoter methylation status in PBCs of cases and controls was assessed and compared by using a logistic regression method to obtain the odds ratios (OR) and 95% confidence intervals (CI) after adjustment for other epidemiological risk factors such as age, family history, age at menarche, parity, menopausal status, and body mass index (BMI). These factors were categorized as (1) family history of breast cancer or ovarian cancer in a first-degree relative (yes or no); (2) age at menarche (12 years or younger, 13–14 years, and 15 years or older); (3) parity (first live birth at 25 years or younger, 26–29 years, 30 years or older, or nulliparity); (4) menopausal status (post-menopausal at less than 50 years, 50 years or older, premenopausal); (5) BMI (<20 kg/m^2, 20 to <23 kg/m^2, and 23.0 kg/m^2 or more). The association between BRCA1 promoter methylation status and clinicopathological characteristics of breast tumors was assessed with a chi-square test. A P value of <0.05 was considered significant. SPSS software (SPSS Inc., Chicago, IL) was used for all statistical analyses.

Results

BRCA1 promoter methylation in PBCs from breast cancer patients and controls

DNA samples extracted from PBCs from 200 breast cancer patients and 200 healthy controls were subjected to MSP assay for BRCA1 promoter methylation. Demographics of breast cancer patients and controls are shown in Table 1. BRCA1 promoter methylation in PBCs could be detected in 43 (21.5%) breast cancer patients and in 27 (13.5%) controls. BRCA1 promoter methylation was not significantly associated with age, body mass index, age at menarche, age at menopause, or family history of either breast cancer patients or controls. The average percentages of methylated against total BRCA1 genes were 1.82% (S.E. 0.49) for BRCA1 promoter methylation-positive breast cancer patients and 1.58% (S.E. 0.72) for BRCA1 promoter methylation-positive controls.

BRCA1 promoter methylation in breast tumors

DNA samples available from 162 tumor tissues were subjected to MSP assay for BRCA1 methylation and 31 (19.1%)

were found to have BRCA1 promoter methylation. BRCA1 promoter methylation-positive tumors were more likely to be ER-negative ($P = 0.068$) and PR-negative ($P = 0.006$) (Table 2). Furthermore, when breast tumors were classified into four intrinsic subtypes based on the immunohistopathological results of ER, PR, and HER2 [32], the BRCA1 promoter methylation-positive tumors were significantly more likely to be triple-negative tumors ($P = 0.025$) (Table 2). The average percentage of methylated against total BRCA1 genes was 19.0% (S.E. 5.12) for the BRCA1 promoter methylation-positive breast tumors.

The relationship between BRCA1 promoter methylation in tumor tissues and BRCA1 protein expression was also examined immunohistochemically in 50 representative breast tumors (Fig. 2). Of the 40 tumors without BRCA1 promoter methylation in tumor tissues, 33 were immunohistochemically positive for BRCA1, but only one of the ten tumors with BRCA1 promoter methylation in tumor tissues showed such positivity ($P < 0.001$).

Association between BRCA1 promoter methylation in PBCs and breast cancer risk

Association of BRCA1 promoter methylation with breast cancer risk was evaluated by means of a case–control study (Table 3), which demonstrated that the odds ratio for breast cancer adjusted for other epidemiological risk factors was 1.73 (1.01–2.96) and had statistical significance of $P = 0.045$.

The breast tumors were then classified into those with and without BRCA1 promoter methylation, and odds ratios for each class of breast tumors were calculated (Table 3). The odds ratios were 0.84 (0.43–1.64) ($P = 0.61$) for BRCA1 promoter methylation-negative and 17.78 (6.71–47.13) ($P = 0.001$) for BRCA1 promoter methylation-positive breast tumors, indicating a significant association of BRCA1 promoter methylation in PBCs with a risk of developing breast tumors with BRCA1 promoter methylation but not of tumors without such methylation.

No interference with MSP assay by circulating tumor cells

PBCs may be contaminated by circulating tumor cells with BRCA1 promoter methylation so that the MSP assay may detect BRCA1 promoter methylation of such tumor cells even though the normal PBCs do not show methylation. To rule out this possibility, we carried out a study to assess the detection sensitivity of the MSP assay using the method described in "Materials and methods" section (Fig. 3). The detection sensitivity of the MSP assay was found to be 10^{-3}, which was far higher than the reported ratios (10^{-6}–10^{-5}) of circulating tumor cells to PBCs [33]. It is thus unlikely that our MSP assay was interfered with by contamination from circulating tumor cells.

Table 2 Clinicopathological characteristics of breast tumors with or without BRCA1 promoter methylation in tumor tissues

	BRCA1 promoter methylation in breast tumors			
	Total 162	Positive 31	Negative 131	*P* value[a]
Menopausal status				
Premenopausal	90	17 (18.9%)	73 (81.1%)	0.929
Postmenopausal	72	14 (19.4%)	58 (80.6%)	
Tumor size				
T1	105	16 (15.2%)	89 (84.8%)	0.087
T2	57	15 (26.3%)	42 (73.7%)	
Lymph node metastasis				
Negative	120	24 (20%)	96 (80%)	0.636
Positive	42	7 (16.7%)	35 (83.3%)	
Histological type				
DCIS	11	2 (18.2%)	9 (81.8%)	0.825
IDC	138	26 (18.8%)	112 (81.2%)	
ILC	7	1 (14.3%)	6 (85.7%)	
Others	6	2 (33.3%)	4 (66.7%)	
Histological grade				
1	53	8 (15.1%)	45 (84.9%)	0.117
2	80	14 (17.5%)	66 (82.5%)	
3	21	7 (33.3%)	14 (66.7%)	
Unknown[b]	8	2 (25.0%)	6 (75%)	
Estrogen receptor				
Negative	33	10 (30.3%)	23 (69.7%)	0.068
Positive	129	21 (26.3%)	108 (83.7%)	
Progesterone receptor				
Negative	55	17 (30.9%)	38 (69.1%)	0.006
Positive	107	14 (13.1%)	93 (86.9%)	
HER2				
Negative	118	24 (20.3%)	94 (79.7%)	0.462
Positive	38	7 (18.4%)	31 (81.6%)	
Unknown[c]	6	0 (0%)	6 (100%)	
Luminal subtype				
Luminal A	104	17 (17.3%)	87 (83.7%)	0.025
Luminal B	26	4 (15.4%)	22 (84.6%)	
HER2	12	3 (25%)	9 (75%)	
Triple negative	14	7 (50%)	7 (50%)	
Unclassified[d]	6	0 (0%)	6 (100%)	

DCIS ductal carcinoma in situ, *IDC* intraductal carcinoma, *ILC* intralobular carcinoma, *HER2* human epidermal growth factor receptor 2, *Luminal A* ER(+) and/or PR(+) and HER2(−), *Luminal B* ER(+) and/or PR(+) and HER2(+), *HER2* ER(−) and PR(−) and HER2(+), *Triple negative* ER(−) and PR(−) and HER2(−)

[a] Chi-square test

[b,c] Unknown and [d] unclassified tumors were excluded from a chi-square test

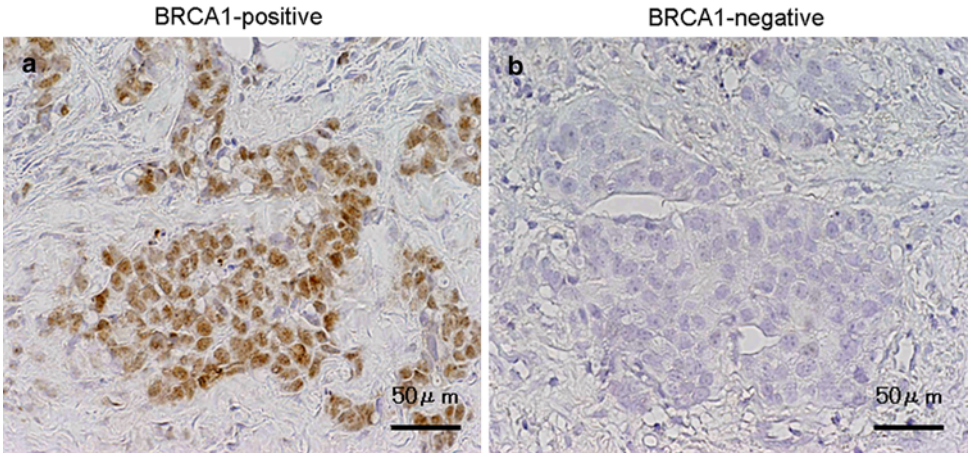

Fig. 2 Immunohistochemical staining of BRCA1. Representative immunohistochemical results for BRCA1-positive tumors (**a**) and BRCA1-negative tumors (**b**) (400×)

Table 3 Relation between BRCA1 promoter methylation in peripheral blood cells and breast cancer risk

	BRCA1 promoter methylation in PBCs			
	Positive	Negative	Adjusted OR (95% CI)	*P* value
Total breast cancers				
Controls	27	173	1	
Cases	43	157	1.73 (1.01–2.96)	0.045
Breast cancers without BRCA1 promoter methylation in tumor tissues				
Controls	27	173	1	
Cases	16	115	0.84 (0.43–1.64)	0.610
Breast cancers with BRCA1 promoter methylation in tumor tissues				
Controls	27	173	1	
Cases	21	10	17.78 (6.71–47.13)	< 0.001

PBCs peripheral blood cells, *OR* odds ratio, *CI* confidence interval

Discussion

We detected BRCA1 promoter methylation in PBCs in 21.5% of breast cancer patients and 13.5% of controls, while women with BRCA1 promoter methylation showed a significantly higher risk (odds ratio = 1.73) of breast cancer as compared with those without it. We next examined the association between the presence of BRCA1 promoter methylation in PBCs and risk of breast cancer with BRCA1 promoter methylation. We first analyzed BRCA1 promoter methylation in 162 breast tumors and found BRCA1 promoter methylation in 31 of them. These tumors were more likely to be ER- and PR-negative [21–23] and lacked BRCA1 protein expression [23, 34–36], which is consistent with the findings of previous studies. Of special interest is that we could establish a significant association (odds ratio = 17.78; $P < 0.001$) between BRCA1 promoter methylation in PBCs and risk of breast cancer with BRCA1 promoter methylation, but not with breast cancer without such methylation (odds ratio = 0.84, $P = 0.61$).

Our results are consistent with those reported by Snell et al. [25], who showed that BRCA1 promoter methylation in PBCs could be detected in three of seven familial breast cancer patients and that all breast tumors arising in such patients carried BRCA1 promoter methylation. However, Chen et al. [37] reported that BRCA1 promoter methylation could not be detected in PBCs of any of 41 familial breast cancer patients. The reason for this discrepancy is explained, at least in part, by the fact that Snell et al. [25] examined only those tumors which were pathologically similar to BRCA1-mutated tumors, whereas Chen et al. [37] did not select breast tumors in this manner.

It is well established that the aging process is associated with a global hypomethylation of the genome but an increase in methylation of specific gene promoters is also observed in conjunction with aging [38, 39]. Interestingly, a significant number of genes that are hypermethylated during aging are also hypermethylated during carcinogenesis [16, 40], indicating that hypermethylation of such genes in normal tissues may be associated with heightened cancer susceptibility. The association between DNA methylation and aging seems to imply that DNA methylation can be a somatic and acquired change which can accrue under the influence of various environmental factors later in life. However, no association of frequency of BRCA1 promoter methylation in PBCs with age was observed in our study nor in a study by Kontorovich et al.

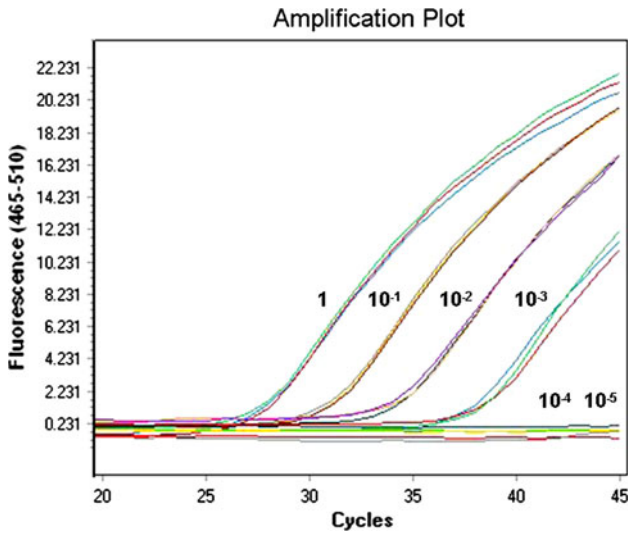

Fig. 3 Detection sensitivity of MSP assay for methylated against unmethylated BRCA1 promoter. Human genome DNA completely methylated by Sss1 methylase treatment was diluted against unmethylated human genome DNA obtained from healthy controls at various ratios (methylated DNA/(methylated DNA + unmethylated DNA) = 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}). These mixtures (total DNA content = 1 μg) were subjected to sodium bisulfite treatment and MSP assay and detection sensitivity of the assay was established as 10^{-3}. Amplification plots of the MSP assay for these mixtures are shown

[41]. These observations seem to suggest that BRCA1 promoter methylation may represent a germ line change (either trans-generational or single-generational) or a somatic change occurring in the early developmental stages and that it may not be influenced very much by environmental factors later in life. Since the presence of a heritable germline epimutation (MSH2 methylation) has been reported in a family with hereditary non-polyposis colon cancer [42], it is possible that BRCA1 promoter methylation is also a heritable germline epimutation. This possibility could not be investigated in our study, however, because DNA samples were not available from the family members of the breast cancer patients with BRCA1 promoter methylation in their PBCs. Lack of a significant association of BRCA1 promoter methylation in PBCs with a family history of breast cancer may indicate that BRCA1 promoter methylation, even if it is a heritable germline epimutation, is unlikely to be a major cause of hereditary breast cancer.

Our present study, though still preliminary, has suggested that analysis of BRCA1 promoter methylation in PBCs might be clinically useful in the identification of women at high risk for developing breast tumors with BRCA1 promoter methylation. It is well established that breast tumors with BRCA1 methylation are more likely to be triple-negative (basal-like) tumors [43, 44]. Since tamoxifen or raloxifene has been shown to be efficacious in the prevention of ER-positive tumors but not ER-negative tumors [45, 46], women with BRCA1 promoter methylation are expected to gain no benefits from these agents. Recently, it has been reported that poly-ADP ribose polymerase (PARP) inhibitors are very efficacious for triple-negative tumors [47, 48], suggesting a possibility that PARP inhibitors would be useful in the prevention of breast tumors with BRCA1 methylated tumors. Breast tumors are essentially heterogeneous, and recent development of gene expression profiling-based analysis has classified breast tumors into the several intrinsic subtypes [49–51]. Since it is speculated that causes of carcinogenesis might be different among these intrinsic subtype, it would be infeasible to identify a marker(s) associated with an increased risk for every type of breast tumors. More feasible approach would be detecting a marker(s) associated with a specific type of breast tumors. Such a tailored approach seems to be important for the development of chemoprevention of breast tumors in future.

Recently, it has been found that circulating tumor cells (CTCs) can occur in a significant proportion of breast cancer patients, even in those without metastases. Since we extracted DNA from whole PBCs, it is possible that the PBCs were contaminated by CTCs with BRCA1 promoter methylation and that the results of our MSP assay might be flawed. However, and as already mentioned under "Results" section, we analyzed the detection sensitivity of our MSP assay and found that it was around 10^{-3}, which is far higher than the reported ratios (10^{-6}–10^{-5}) of circulating tumor cells to PBCs. It is, therefore, very unlikely that the results of our MSP assay have been affected by CTCs, and can thus be considered to represent the actual BRCA1 promoter methylation status of PBCs.

In conclusion, we detected BRCA1 promoter methylation in PBCs in 21.5% of breast cancer patients and in 13.5% of controls, indicating that BRCA1 promoter methylation in PBCs is significantly associated with a risk of developing breast cancer with BRCA1 promoter methylation. These results suggest that BRCA1 promoter methylation may constitute a novel risk factor for breast cancer with BRCA1 promoter methylation.

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