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BRCA1 Promoter Methylation is Associated with Increased Mortality among Women with Breast Cancer

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Abstract

Promoter-CpG island hypermethylation is a common molecular defect in cancer cells. It has been proposed as an alternative mechanism to inactivate *BRCA1*in the breast where somatic mutations of *BRCA1* are rare. To better understand breast cancer etiology and progression, we explored the association between *BRCA1* promoter methylation status and prognostic factors as well as survival among women with breast cancer. We also examined whether dietary methyl content and functional polymorphisms of genes involved in one-carbon metabolism influenced the methylation pattern. Promoter methylation of *BRCA1* was assessed in 851 archived tumor tissues collected from a population-based study of women diagnosed with invasive or *in situ* breast cancer in 1996–1997, and who were followed for vital status through the end of 2002. About 59% of the tumors were methylated at the promoter of *BRCA1*. The *BRCA1* promoter methylation was more frequent in invasive cancers (p=0.02) and among premenopausal cases (p=0.05). *BRCA1* promoter methylation was associated with increased risk of breast cancer-specific mortality (age-adjusted HR 1.71; 95% CI: 1.05–2.78) and all-cause mortality (age-adjusted HR 1.49; 95% CI: 1.02–2.18). Among dietary methyl intakes in the year prior to the baseline interview examined, cases with lowest quintile of choline intake (<20%) had higher *BRCA1* methylation level in the tumor compared to the rest (66.1% vs. 57.7%, p=0.04). Functional polymorphisms in one-carbon metabolism were not correlated with *BRCA1* methylation status. Our study is the first epidemiological investigation on the prognostic value of *BRCA1* promoter methylation in a large population-based cohort of breast cancer patients.

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Our results indicate that *BRCA1* promoter methylation is an important factor to consider in predicting breast cancer survival.

Keywords

BRCA1; methylation; epigenetics; one-carbon; survival; breast cancer

INTRODUCTION

Breast cancer is the leading cause of cancer mortality among women 20 – 59 years of age and the second leading cause of cancer mortality among all women[1]. Breast cancer is a manifestation of abnormal genetic as well as epigenetic changes[2]. Promoter-CpG island hypermethylation, accompanied by global hypomethylation, are common molecular defects in cancer cells[3, 4]. Although the causal relationship is still being debated, evidence has shown that hypermethylation is associated with silencing of many crucial genes in the neoplastic process[5]. This phenomenon has also been reported in a large panel of genes in breast cancer[6].

Breast cancer gene 1(BRCA1), located on chromosome 17q21 (Figrue 1), encodes a multifunctional protein involved in DNA repair, control of cell-cycle checkpoints, protein ubiquitinylation and chromatin remodeling[7]. It was originally cloned as a gene responsible for familial breast cancer[8]. About 5–50% of familial breast cancers could be explained by inherited mutations of *BRCA1* in different populations[9]. However, somatic mutations of *BRCA1* are rare in sporadic breast cancers despite the high degree of loss of heretozygosity (LOH) at this locus[6, 10]. Therefore, other mechanisms for loss of function must exist. DNA methylation has been proposed as an alternative mechanism to inactivate *BRCA1*[11]. Results from various methods of detection revealed that 9–44% breast cancer samples harbored a hypermethylated promoter at *BRCA1*[11, 12].

BRCA1 status may potentially be used as a prognostic marker as studies have shown that breast cancers with *BRCA1* mutations are usually poorly differentiated, highly proliferative, ER-, PR-, and harbor *p53* mutations[13]. *BRCA1* mutated breast cancer is also associated with poor survival in some studies[14–18].

One-carbon metabolism may be involved in the DNA methylation process as it provides the universal methyl donor, S-adenosylmethionine (SAM). Folate, methionine and choline are the major sources of methyl groups in foods[19]. There is evidence that dietary methyl donors are capable of modulating methylation patterns in both animal models and humans[20–23]. Furthermore, functional polymorphisms in one-carbon metabolizing genes could in principle modify DNA methylation status[24–26].

We previously reported that intakes of B vitamin as well as common polymorphisms in one-carbon metabolizing genes were associated with breast cancer risk in the population-based Long Island Breast Cancer Study Project [27–29]. Herein, we investigated promoter methylation status of *BRCA1* in relation to clinical/pathological factors and breast cancer survival in the same population. The influence of dietary methyl intake as well as polymorphisms in one-carbon metabolizing genes on *BRCA1* promoter methylation was also examined.

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MATERIALS AND METHODS

Study population

We utilized the resources of the parent case-control as well as the follow-up study of the Long Island Breast Cancer Study Project, a population-based study. The study participants included women newly diagnosed with a first primary breast cancer who participated in the original case-control study[30] and were subsequently re-interviewed about five years later and followed for vital status[31]. Details of the study design have been described in detail previously[30–33].

Exposure data was obtained as part of the 1) case-control (baseline) interview; 2) follow-up interview; and 3) medical record abstraction. The questionnaires were administrated to assess the demographic characteristics, breast cancer-related factors, tumor characteristics and treatment information. The study protocol was approved by the Institutional Review Boards of the collaborating institutions.

Study outcome

The National Death Index was used to ascertain all-cause and breast cancer-specific mortality. Among the 1508 women diagnosed with breast cancer in 1996–1997, 198 (13.1%) deaths occurred by December 31, 2002. The mean follow up time was 5.6 years (range: 0.2–7.4). Based on International Classification of Diseases (ICD) codes 174.9 and C-50.9 listed as a primary or secondary code on the death certificate, 124 (62.6%) of these 198 deaths were due to breast cancer.

Tumor block retrieval and DNA extraction

Tumor tissue blocks were requested from all 35 participating hospitals of the parent study. In total, breast cancer tissue blocks were successfully retrieved for 975 case participants (67.2%). We compared the demographic and clinicopathological features between cases with or without tumor block available for methylation analysis in our study. Although most characteristics are similar between these two groups, some factors were different. Case women who had tumor samples available for methylation analysis tended to be older (mean age 59.6 vs. 57.9; $p=0.005$); to have an invasive tumor (87.8% vs. 80.1%; $p<0.001$); and to be post-menopausal (70.7% vs. 64.6; $p=0.01$).

The paraffin blocks from each case participant were used to generate 15x 5 micron and 10x 10 micron thick slides. Tumor tissues were isolated from 10 micron paraffin sections by microdissection. Tumor DNA was isolated by adding 30 ul of proteinase K-digestion buffer (50mM Tris, pH 8.1, 1 mM EDTA, 0.5% Tween 20, 10 ug/ml proteinase K) to the tube and incubating overnight at 37ºC. Proteinase K was inactivated incubating the samples at 95ºC for 10 min and centrifugation.

Analysis of *BRCA1* promoter methylation

BRCA1 promoter methylation was determined by methylation-specific PCR (MSP) with bisulfite-converted DNA (illustrated in Figure 1). Bisulfite modification of DNA to convert unmethylated cytosine residues to uracil was carried out using the CpGnome DNA Modification Kit (Chemicon International, Purchase, NY) following the protocol from the manufacturer. The sequences of the primers were: i) Methylated primer: forward-5'-GAG AGG TTG TTG TTT AGC GG-3'; backward-5'-CGC GCA ATC GCA ATT TTA AT-3'; ii) Unmethylated primers: forward-5'-TGG TAA TGG AAA AGT GTG GGA A-3'; backward-5'-CCC ATC CAA AAA ATC TCA ACA AA-3'. PCR was carried out in a total volume of 20 μl containing 0.5 U of AmpliTaq Gold II (Roche, Nutley, NJ, USA). The amplicon is 146bp in length. Each PCR reaction underwent initial denaturation at 95°C for 10 min, and 40 cycles of the following profile: 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C followed by a

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final 10-min extension at 72°C. The PCR products were then analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized by UV transillumination. DNA is considered methylated if PCR product is yielded using methylation-specific primers. Bisulfite-modified universal methylated DNA (Chemicon International, NY) and distilled water were used as positive and negative controls in the assay. The assay was successfully completed for 851 subjects; the main reason for failure of methylation assessment was insufficient DNA from tumor blocks.

Dietary assessment

As part of the baseline interview, participants were asked to complete a modified Block food frequency questionnaire (FFQ), which assessed intake of over 100 food items in the year prior to the interview [33]. The frequency and portion size data were translated to daily intakes of nutrients from both dietary and supplement sources using the National Cancer Institute's DietSys version 3 for folate, and a previously described protocol for choline, methionine and betaine [34]. Habitual use of multivitamin supplements was also obtained from the FFQ. Dietary intake values for one-carbon related micronutrients and compounds were calculated based on interview data assessed from this FFQ.

Blood sample collection and genotyping

Blood samples were collected from 73% of the cases at the time of the baseline interview by trained field staff [30] and DNA was isolated from blood specimens using the methods previously described[32]. Genotyping was conducted on 9 polymorphisms in the one-carbon metabolism pathway using methods described elsewhere [27, 28].

Statistical Analysis

Correlation of *BRCA1* promoter methylation status of the tumor tissue with patient demographic characteristics, other factors that may affect prognosis, and with known characteristics of the breast cancer diagnosis was examined using the chi-square statistic for categorical variables and by two-sample t-test for continuous variables. The Kaplan-Meier and the log-rank test were used to examine the crude association between *BRCA1* promoter methylation status and survival[35]. The Cox proportional hazard regression[35] was used to estimate the hazard ratio (HR) and 95% confidence interval (CI) for breast cancer-specific and all-cause mortality, with adjustments made for age at diagnosis (continuous). Potential confounding effect by other factors known to influence survival among breast cancer patients was evaluated by adjustment in the Cox model. These factors include age at diagnosis, cancer type (*in situ* vs. invasive), menopausal status (pre- vs. post-), race, family history of breast cancer and history of benign breast. One-carbon metabolism-related nutrient intakes in the year prior to the interview were categorized based on the distributions observed in cases with methylation data. Percentages of cases with methylated *BRCA1* promoter were compared within each quintile. Nutrients examined in the study include folate, methionine, choline, betaine and B vitamins (B_1, B_2, B_3, B_6, B_{12}). Whether the distribution of the one-carbon genotypes differed with respect to *BRCA1* methylation status was examined using the chi-square statistic. All statistical analyses were performed using SAS statistical software version 9.1(SAS Institute, Cary, NC).

RESULTS

BRCA1 methylation and clinicopathological characteristics of breast cancer

Promoter methylation status of *BRCA1* was assessed in breast tumor samples from a population-based sample of 851 women, including 104 *in situ* and 747 invasive cases (Table 1a). Overall 504/851 (59.2%) of tumors showed methylation at the promoter of *BRCA1*. Table

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1 summarizes the relationship between *BRCA1* methylation status and potential prognostic factors. *BRCA1* promoter methylation was more frequent in cancers that were classified as invasive (p=0.02) and among premenopausal women (p=0.05). *BRCA1* promoter methylation was not associated with age at diagnosis or family history of breast cancer.

As shown in Table 1b, hormone receptor status, as recorded on the medical record, was determined by immunohistochemistry and was available on a subset of the samples (625 out of 851). *BRCA1* promoter methylation status was not associated with ER or PR status in this subpopulation. For a smaller subset of women, we were able to obtain information on tumor size (n=321) and node involvement (n=327) from the medical record. *BRCA1* promoter methylation was more frequent in cancers with at least one node involved (p=0.003) and with tumor size greater than 2 cm (p=0.003).

BRCA1 methylation and survival

Among the 851 women with methylation data available, a total of 122 (14.3%) deaths were observed; 79 (64.8%) of these were due to breast cancer. As showing in Figure 2, *BRCA1* methylation was associated with breast cancer-specific mortality among the cohort of women in our analysis (p for log-rank test =0.03). Compared to cases with an unmethylated *BRCA1* promoter, those who had a methylated *BRCA1* promoter had 72% increased risk of dying from breast cancer at the end of follow up (age-adjusted HR: 1.72, 95% CI: 1.06–2.79). A similar result was observed for all-cause mortality with borderline significance (p for log-rank test =0.05); cases with methylated *BRCA1* promoters had 45% increased mortality risk when compared to those who had unmethylated *BRCA1* promoters (age-adjusted HR: 1.45, 95% CI: 0.99–2.11). The *BRCA1* methylation and survival association was of borderline significance after adjusting for age, cancer type (*in situ* vs. invasive), menopausal status (pre- vs. post-), race, family history of breast cancer and history of benign breast disease in a multivariate model (multi-variate adjusted HR for breast cancer-specific mortality: 1.67, 95% CI: 0.99–2.81; for all-cause mortality: 1.40, 95% CI: 0.94–2.08).

One-Carbon metabolism and *BRCA1* methylation

We explored whether dietary intake of one-carbon related nutrients was associated with *BRCA1* methylation status. As shown in Figure 3, none of the nutrient intakes was associated with *BRCA1* methylation except choline; very low choline intake (lowest quintile) was associated with higher methylation (66.1% vs. 57.7%, p=0.04) compared to the rest.

We did not observe any relationships between *BRCA1* promoter methylation and functional polymorphisms in the one-carbon metabolizing genes [(*MTHFR C677T* (rs1801133) and *A1298C* (rs1801131); *TYMS* 5'-UTR tandem repeat; *DHFR* 19bp deletion; *MTR A2756* (rs1805087); *MTRR A66G* (rs1801394); *BHMT G742A* (rs3733890); *RFC1 A80G* (rs1051266); and *cSHMT C1420T* (rs1979277)] (data not shown).

DISCUSSION

To reduce the disease burden of breast cancer, it is important to identify etiologic factors of the disease as well as factors that predict survival. We studied *BRCA1* promoter methylation because the importance of this gene has been well documented in breast carcinogenesis [36]. To the best of our knowledge, this is the first epidemiologic study on the prognostic value of *BRCA1* methylation. The cohort of women with breast cancer was drawn from a large population-based sample that encompassed a broad age range making the study results more generalizable than a series of cases from a single institution. Furthermore, the comprehensive lifestyle and dietary information as well as collection of biological samples allowed us to

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examine interactions among environmental, genetic, and epigenetic factors in relation to breast cancer mortality.

We found *BRCA1* promoter methylation in ~59% of tumors, a level higher than other published studies from other groups, which ranged from 9–44% [11, 12]. Several factors may account for these differences. First, the assay used for methylation measurement varied from study to study. Because contamination from adjacent tissue may occur during tissue dissection, unmethylated DNA from the normal cells might attenuate the methylation levels of the tumor tissue. In our study, tumor tissues were micro-dissected from paraffin-embedded sections so the contamination was minimized. Secondly, MSP detects differential methylation status by amplification of bisulfite-treated DNA with primers specific for methylated vs. unmethylated DNA[37]. CpG sites residing within the primer sets were used as a proxy for the methylation status of the region of interest. Although most published studies mentioned above used MSP, the primer sequences and target regions varied from study to study.

We found that *BRCA1* promoter methylation was more frequent in invasive than in *in situ* carcinomas. Since information on breast cancer subtype was not readily available for the other published studies, we could not compare the methylation-cancer type relationship observed here directly with other studies. In a small subset of our population for whom medical record data were available, we also found a higher prevalence of *BRCA1* promoter methylation in cases with at least one node involved and with tumor size greater than 2 cm. Taken together, these associations suggest that methylation occurs in sequence during tumor development and progression. More advanced tumor stage could have higher methylation level. Thus, it is possible that the variation in methylation prevalence reported across studies could also be due to the variation in the distributions of cases' stage at diagnosis across studies.

We found that *BRCA1* promoter methylation was more frequent in tumors from premenopausal women despite the fact that age of diagnosis was not associated with *BRCA1* methylation. Studies have indicated that estrogens stimulate the expression of *BRCA1* [38]. On the other hand, BRCA1 was shown to have an ability to inhibit the cellular response to estrogens by direct interaction with estrogen receptor[39, 40]. Nevertheless, we did not observe any association between *BRCA1* methylation and ER/PR status in our study.

BRCA1 mutation status as a potential prognostic marker had been explored previously. A recent review by Liebens et al [41] summarized differences in survival outcome in relation to *BRCA1* germline mutations. Evidence exists indicating that *BRCA1* mutation carriers had a worse survival [14–18]. Carriers of *BRCA1* mutations had functionally defective proteins, resulting in some loss of function. Promoter methylation represented an alternative mechanism for loss of function of *BRCA1*. Our study is the first to report that *BRCA1* promoter methylation influences breast cancer survival in an epidemiologic study. Decreased survival associated with methylated *BRCA1* corroborates the findings on *BRCA1* mutations. This finding may have clinical significance as it identifies a subgroup of patients with worse survival and could help in tailoring of breast cancer treatment based on epigenetic profiles.

With the exception of choline intake, we found no association between intake of nutrients involved with one-carbon metabolism and *BRCA1* methylation status. Although previous studies have shown that dietary methyl content and one-carbon gene polymorphisms were capable of modulating global DNA methyl content in animal models and humans[20–26], modulation of promoter methylation of specific genes has not been demonstrated. Choline is an essential nutrient required for one-carbon metabolism [42]. Evidence from animal studies has implied a causal relationship between choline-deficiency and carcinogenesis [43, 44]. The recent results from the Nurses' Health Study showed that increasing choline intake was associated with an elevated risk of colorectal adenoma [45]. Our results from the same population used in

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this study showed that higher choline intake was associated with decreased breast cancer risk [29]. Here, we observed that very low choline intake was associated with more frequent *BRCA1* promoter methylation. Global hypomethylation of the genome of the tumor cells are well-observed phenomenon[4] and it is linked with deficiency of methyl-content diet[46]. However, the relationship between methyl-content in diet and gene-specific methylation is not well-known. Since the epigenetic changes may be reversible and diet is a modifiable factor, further investigation into this relationship could aid in our search for breast cancer chemoprevention strategies.

One limitation of our study is that BRCA1 expression was not measured in the tumor tissues. Thus, we could not explore the functional mechanism of gene silencing through DNA methylation. Previous studies have generated conflicting results on *BRCA1* expression and the subcellular localization of this protein by using various antibodies for immunohistochemical staining [47–49]. This discrepancy was even more pronounced when paraffin-embedded tissues were used [50]. Nevertheless, the fact that our results corroborate those from *BRCA1* mutation studies suggests that promoter methylation indeed results in gene silencing or loss of gene function.

Another limitation of our study is that tumor DNA was not available for all case participants of the Long Island Breast Cancer Study Project, which is a population-based study. Although there were some differences between those with and without tumor DNA available for our analyses (described in the Methods section), the benefit of utilizing our population-based sample is that we are able to quantify the differences between the two groups. This valuable contrast aids in our interpretation of the generalizability of our study results to the general population. It is this type of information that is often unavailable from other study populations, such as those derived from a hospital-based case series. Nonetheless, caution should be taken when comparing our results across studies, or to the general population.

In summary, we examined *BRCA1* promoter methylation status and explored its relationship with clinicopathological factors and breast cancer survival. Our study, which is based on data drawn from a large population-based sample, is the first to report on the prognostic value of *BRCA1* promoter methylation status in breast cancer in an epidemiologic study. Our results indicate that *BRCA1* promoter methylation is an important factor to consider in predicting breast cancer survival.

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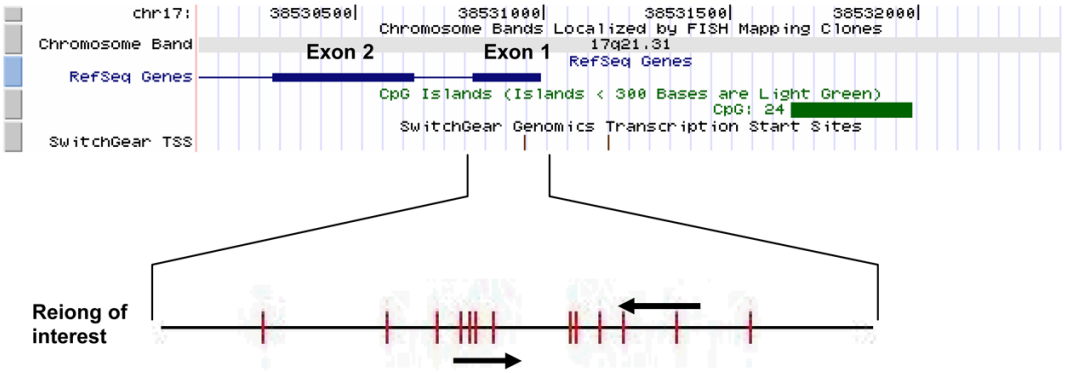

Figure 1.

Schematic illustration of the *BRCA1* promoter region for methylation analysis. The view of the genomic context was adapted from USCS Genome Browsers (http://genome.uscs.edu). *BRCA1* gene is in the reverse strand. Blue bar shows the exons of the RefSeq Genes and the green bar shows the predicted CpG island in the promoter region. Location of transcription start sites (TSS) was shown also shown. Region examined (Exon 1 and nearby region) was zoomed in to show the location of the primers for methylation-specific PCR and CpG sites (red bars) in the sequence.

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Fig.1a Association between BRCA1 promoter methylation and breas t cancer-specific mortality

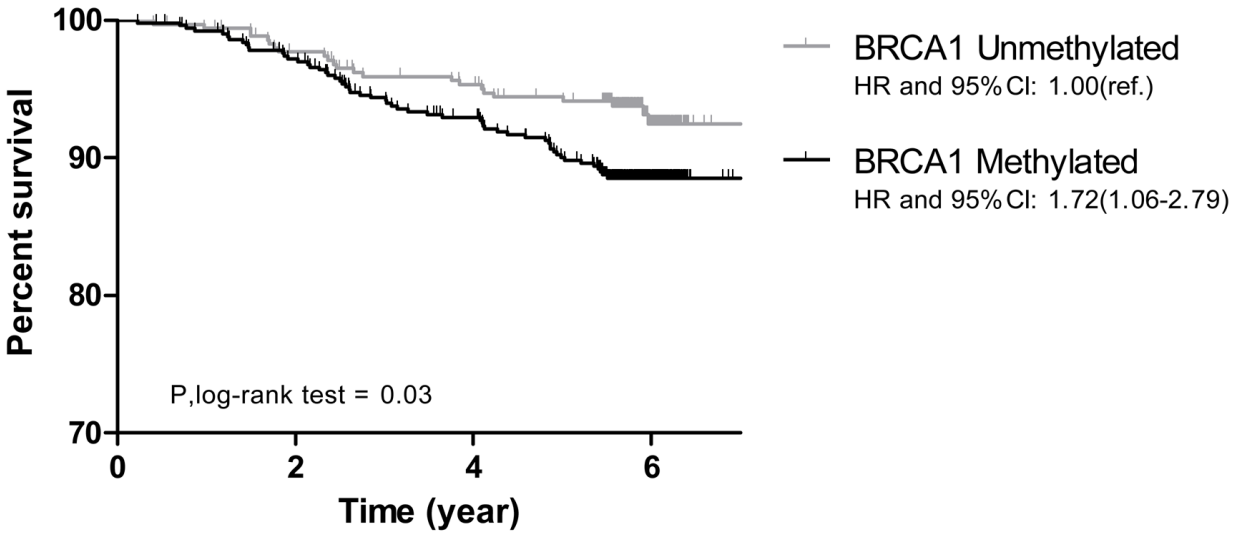

Fig.1b Association between BRCA1 promoter methylation and all-cause mortality

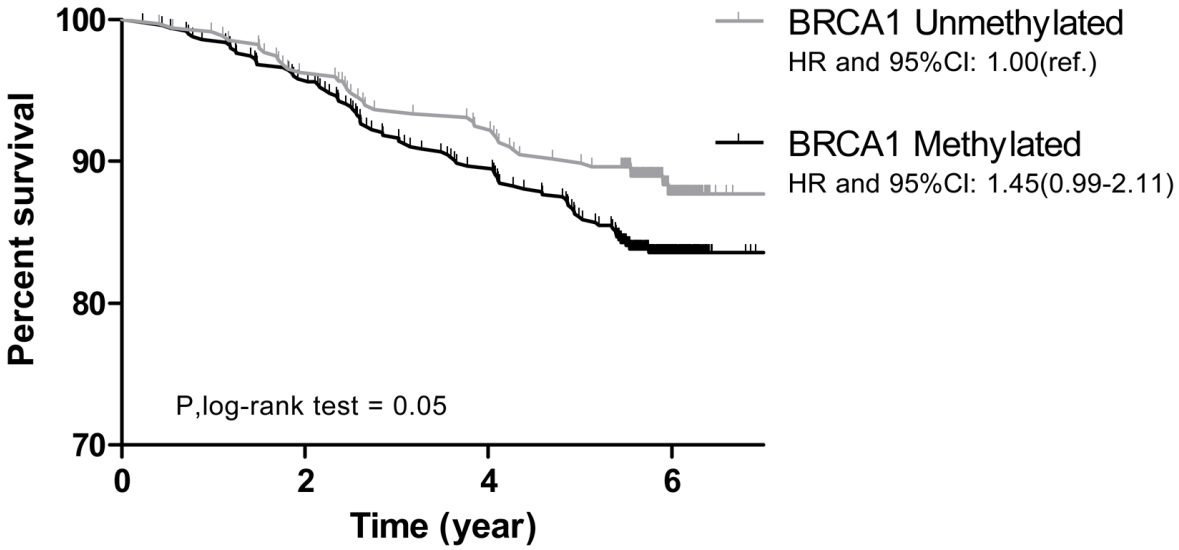

Figure 2.

Survival plot for breast cancer patients by *BRCA1* promoter methylation status in the tumor tissue - Kaplan–Meier analyses of survival among all 851 breast cancer cases. Vertical lines in the curve represent the death events.

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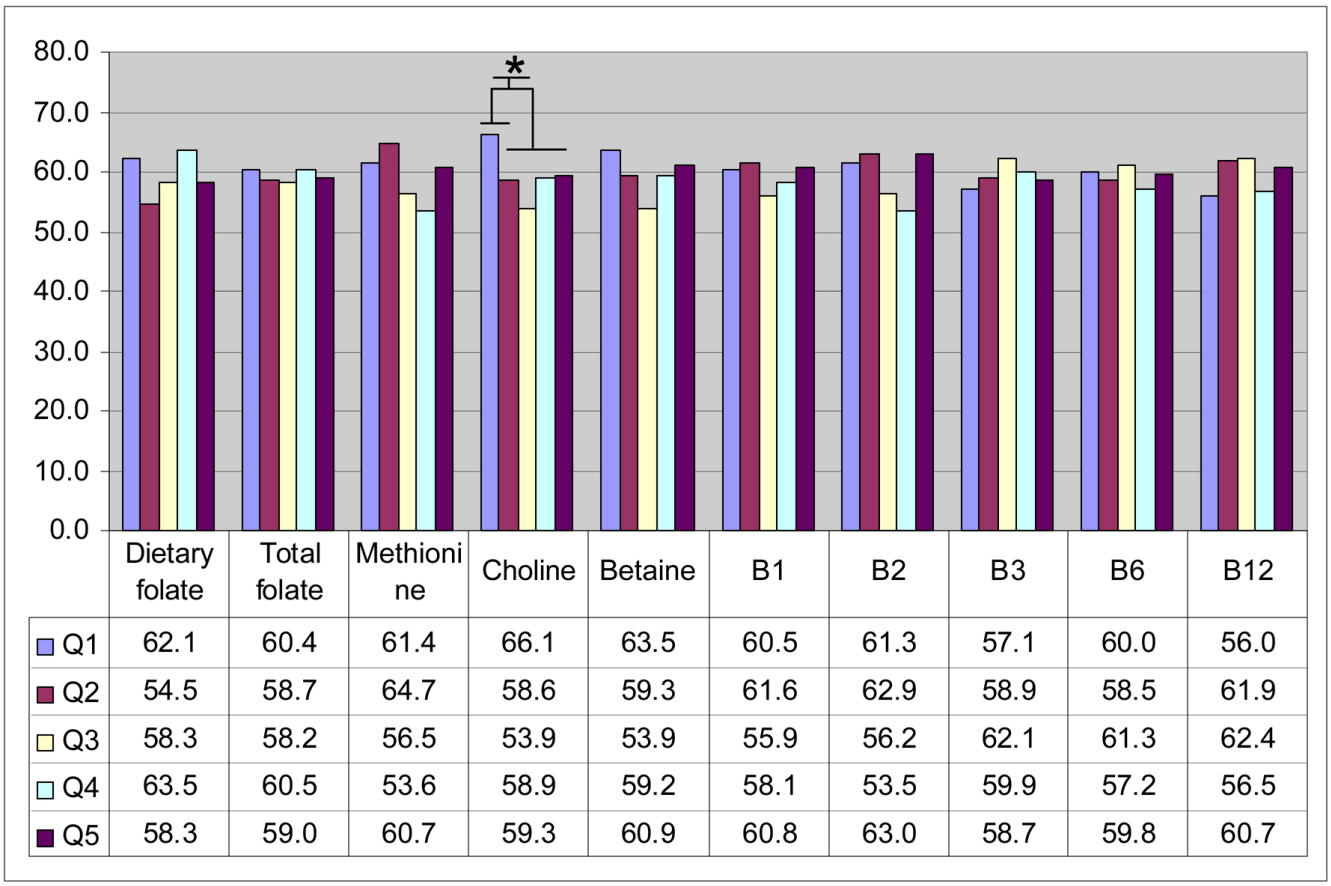

	Dietary folate	Total folate	Methionine	Choline	Betaine	B1	B2	B3	B6	B12
Q1	62.1	60.4	61.4	66.1	63.5	60.5	61.3	57.1	60.0	56.0
Q2	54.5	58.7	64.7	58.6	59.3	61.6	62.9	58.9	58.5	61.9
Q3	58.3	58.2	56.5	53.9	53.9	55.9	56.2	62.1	61.3	62.4
Q4	63.5	60.5	53.6	58.9	59.2	58.1	53.5	59.9	57.2	56.5
Q5	58.3	59.0	60.7	59.3	60.9	60.8	63.0	58.7	59.8	60.7

Figure 3.

Methyl-nutrient intake and *BRCA1* promoter methylation. Nutrient intakes in the year prior to the baseline interview were categorized into quintiles based on the distributions observed among cases with methylation data shown in the figure as Q1–Q5. Numbers shown are percentages of cases with methylated *BRCA1* promoter in tumor tissue within each quintile. (*choline intake: lowest quintile with the other four quintiles: 66.1% vs. 57.7%, p=0.04)

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Table 1

Table 1a. Association between *BRCA1* promoter methylation and potential predictors of breast cancer survival assessed at the baseline interview (N=851)[1]

Table 1b. Association between *BRCA1* promoter methylation and potential predictors of breast cancer survival assessed at the baseline interview

Feature	*BRCA1* methylated, n=504 (59.2)	*BRCA1* unmethylated, n=347 (40.8)	p-value
Age at diagnosis (y)			
≤60	259(59.7)	175(40.3)	0.78
>60	245(58.8)	172(41.2)	
mean age	59.0	60.4	0.12 (t-test)
Race	n=503	n=346	
White	463(58.5)	329(41.5)	0.21
Black	29(69.0)	13(31.0)	
Other	11(73.3)	4(26.7)	
Cancer type	n=504	n=347	
in situ	51(49.0)	53(51.0)	0.024
invasive	453(60.6)	294(39.4)	
Menopausal status	n=493	n=340	
pre-	157(64.3)	87(35.7)	0.051
post-	336(57.0)	253(43.0)	
Family history	n=488	n=333	
no	399(59.9)	267(40.1)	0.570
yes	89(57.4)	66(42.6)	
History of benign breast disease	n=503	n=347	
no	417(60.2)	276(39.8)	0.214
yes	86(54.8)	71(45.2)	
Supplement use[2]	n=498	n=341	
no	262(60.0)	175(40.0)	0.713
yes	236(58.7)	166(41.3)	
Active smoking	n=504	n=347	
never	229(60.6)	149(39.4)	0.192
current	88(53.0)	78(47.0)	
past/former	187(60.9)	120(39.1)	
Passive smoking	n=491	n=340	
never	95(58.6)	67(41.4)	0.397

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Feature	***BRCA1* methylated, n=504 (59.2)**	***BRCA1* unmethylated, n=347 (40.8)**	**p-value**
current	73(54.1)	62(45.9)	
past/former	323(60.5)	211(39.5)	
Folate intake (ug/day)	n=498	n=341	
mean (dietary)	264.9	258.7	0.52(t-test)
mean (total)	443.8	450.1	0.79(t-test)
Feature	*BRCA1* methylated, n=504 (59.2)	*BRCA1* unmethylated, n=347 (40.8)	p-value
ER status	n=372	n=253	
negative	89(59.7)	60(40.3)	0.952
positive	283(59.4)	193(40.6)	
PR status	n=372	n=253	
negative	135(59.2)	93(40.8)	0.905
positive	237(59.7)	160(40.3)	
Node	n=202	n=125	
0	142(57.3)	106(42.7)	0.003
1+	60(76.0)	19(24.0)	
Tumor size	n=199	n=122	
≤ 2cm	141(57.1)	106(42.9)	0.003
2cm-5cm	52(78.8)	14(21.2)	
>5cm	6(75.0)	2(25.0)	

[1] Numbers in the parenthesis are percentages of methylated or unmethylated case in each category.

[2] Supplement: multivitamin supplement use in the 5 years prior to the baseline interview.

* Fisher's Exact Test