RESEARCH ARTICLE

Clinicopathological Significance of BRCA1 Promoter Hypermethylation in Thai Breast Cancer Patients

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Abstract

Breast cancer susceptibility gene 1 (BRCA1), mapped on chromosome 17q21, is implicated in the mechanisms of cellular DNA repair. Inactivation of this gene is involved in the development of many human cancers, including breast cancer. This study aimed to investigate the prognostic value of BRCA1 promoter hypermethylation and expression in breast cancer cases. Sixty-one breast cancers were examined for BRCA1 hypermethylation by methylation-specific polymerase chain reaction (PCR), and 45 paired normal breast tissues were analyzed for altered BRCA1 mRNA levels by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Aberrant methylation status in BRCA1 was detected in 15 of 61 cases (24.6%), while reduced expression was found in 7 of 45 (15.6%). BRCA1 hypermethylation was statistically associated with tumor grade III (p=0.04), a high frequency of stage IIB (p=0.02), and triple-negative phenotype (OR= 3.64, 95%CI =1.1-12.3, p=0.03). Our findings indicated that BRCA1 promoter hypermethylation is a useful prognostic marker for breast cancer.

Keywords: Breast cancer - BRCA1 - DNA methylation - methylation-specific PCR - gene expression

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Introduction

Breast cancer susceptibility gene 1 (BRCA1) is mapped on chromosome 17q21; it encodes a multifunctional protein involved in DNA repair, cell-cycle check-point control, protein ubiquitinylation and chromatin remodeling (Miki et al., 1994; Ralhan et al., 2007). Inactivation of this gene has been implicated in the development of many human cancers, including breast cancer.

Several studies have reported that BRCA1 genetic abnormality may lead to carcinogenesis, e. g., BRCA1 mutations account for about 40-45% of hereditary breast cancer patients (Rosen, 2013). The loss of heterozygosity (LOH) at the BRCA1 locus was also more frequent among sporadic breast and ovarian cancers (Cropp et al., 1993; Saito et al., 1993; Ford et al., 1994).

In the emerging epigenetic field, hypermethylation of CpG islands in the gene promoter region is an important mechanism leading to changes in gene expression, without altering genetic code (Paluszczak et al., 2006). This study aimed to investigate the role of the BRCA1 hypermethylation epigenetic mechanism in Thai breast-cancer patients, to clarify the relevant prognostic biomarkers for breast cancer. We evaluated BRCA1 promoter hypermethylation, since, gene expression might play a role in the development of breast cancer, and the correlation of BRCA1 hypermethylation and BRCA1 mRNA expression with clinicopathological data from breast cancer-patients.

Materials and Methods

Tumor specimens

Sixty-one breast tumors and 45 available paired, normal breast tissues, were collected from the National Cancer Institute, Bangkok, Thailand, during the period 2007-2011. This study was approved by the Institutional Review Board (IRB) of the National Cancer Institute, Bangkok, Thailand. Invasive ductal breast carcinoma patients who had not undergone chemotherapy or radiotherapy were recruited into this study. Tissue samples were snap frozen in liquid nitrogen and kept at -80°C until used. Patients' clinicopathological data--age at diagnosis, tumor size, histological grade, axillary lymph-node status, number of lymph nodes, staging, triple-negative tumor (ER-, PR- and HER2-), immunohistochemistry staining of ER, PR and HER2-- were collected from patient files.

DNA extraction and sodium bisulfite modification

Sixty-one breast tumor tissues were isolated by proteinase K digestion and salting-out method (Miller et al., 1988). The DNA solution was kept at -20°C. Treatment of DNA with sodium bisulfite resulted in unmethylated cytosines being converted into uracil, while methylated

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cytosines remained unchanged. Bisulfite conversion used an EZ DNA Methylation Gold kit (Zymo Research, Orange, CA). One µg of DNA was treated with sodium bisulfite according to the manufacturer's protocol. The bisulfite-converted DNA was eluted in a total volume of 25 µl and stored at -20°C for later use.

RNA preparation and cDNA synthesis

Total RNA was extracted from 45 breast-tumor and their corresponding normal breast tissues using Trizol reagent, according to the instruction manual (Invitrogen, Carlsbad, CA, USA). mRNA was isolated by Oligotex mRNA purification kit (QIAGEN, Gmbh, Germany). Reverse transcription reactions were conducted according to the manufacturer's instructions, using the iScriptTM Select cDNA Synthesis Kit (Bio-Rad Laboratories, Inc, Hercules, CA) for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA, USA).

Methylation analysis by methylation-specific PCR

The aberrant methylation status of the BRCA1 promoter region in 61 breast tumors was analyzed by methylation specific-PCR on sodium bisulfite modified DNA. The primers for the methylated and unmethylated sequences were FM-BRCA1-5' GGT TAA TTT AGA GTT TCG AGA GAC G 3'; RM-BRCA1-5' TCA ACG AAC TCA CGC CGC GCA ATC G 3'; FU-BRCA1-5' GGT TAA TTT AGA GTT TTG AGA GAT G 3'; RU-BRCA1-5' TCA ACA AAC TCA CAC CAC ACA ATC A 3'. (Baldwin et al., 2000). The reactions were carried out in a total volume of 25 µl, containing 100 ng of bisulfite-treated DNA, 1X PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.4 µM of sense and antisense primers, 0.5X GC-rich solution and 1 unit of FastStart TaqDNA Polymerase (Roche Diagnostics, Mannheim, Germany). Reaction mixtures were hot-started at 95°C for 5 min. Amplification was performed in a Mastercycler gradient (Eppendorf) for 30 cycles (1 min at 95°C, 30 sec at 65°C (methylated sequence) and 62°C (unmethylated sequence) and 30 sec at 72°C, followed by a final extension of 5 min at 72°C. 25 microliters of PCR product were electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and photographed under UV light (Figure 1.). To confirm complete sodium bisulfite modification, the three differences in the PCR products of methylated and unmethylated bands were cut from the agarose gel, purified and sequenced. A representative chromagram of the sequencing results is shown in Figure 2.

BRCA1 expression analysis by quantitative real-time reverse transcription-PCR

Alterations in BRCA1 mRNA expression levels were analyzed by LightCycler Instrument (Roche Applied Science). The reaction mixture was 20 ng of template cDNA, 1x LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Germany), 4 mM MgCl2 and 0.5 µM forward and reverse primers in a final volume of 10 µl. The primer sequences were designed by Primer3 program, forward F-BRCA1 (5'- AAG ACA GAG CCC CAG AGT CA -3') and reverse R-BRCA1 (5'- CCC TGC TCA CAC TTT CTT CC -3'. β-globin housekeeping gene was used as an endogenous reference to obtain relative expression values (Figure 3). PCR was started at 95°C for 5 min (to activate the FastStartTaq), followed by 40-cycle amplification (95°C for 10 s, 62°C for 30 s, and 72°C for 30 s). After the PCR, each amplification reaction was checked using a dissociation curve. PCR product purity was checked by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light. Relative gene expression level was determined as previously described by Livak and Schmittgen, 2001. The cutoff values for gene expression were adopted from median expression levels. Tumor gene expression <1.5-fold was assigned as under-expression for BRCA1.

Statistical analysis

The association between BRCA1 hypermethylation, BRCA1 mRNA expression level, and clinicopathological characteristics--age at diagnosis, tumor size, histological grade, axillary lymph node-status, number of lymph nodes, staging, triple-negative breast tumor-- was examined statistically by chi-square test. P value < 0.05 was considered a significant correlation.

Results

Methylation-specific PCR was used to examine 61 invasive ductal breast carcinomas for aberrant methylation status of the BRCA1 gene. The results revealed BRCA1 hypermethylation in 15 of 61 (24.6%) tumor samples. A significant association was observed between BRCA1 hypermethylation and tumor grade III, a high frequency of stage IIB with P value 0.04 and 0.02, respectively, as well as triple-negative tumor (OR= 3.64, 95%CI =1.1-12.3, p=0.03) (Table I).

Complete of sodium bisulfite modification was confirmed. The three different PCR products from the methylated and unmethylated bands were cut from the agarose gel, purified, and sequenced. The representative chromagram shows that cytosine residue from the CpG

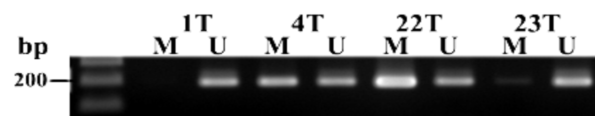

Figure 1. Representatives of BRCA1 Promoter Hypermethylation in Breast-tumor (T) Samples by Methylation-specific PCR. M = methylated sequence (182 bp), U = unmethylated sequence (182 bp); bp = basepair

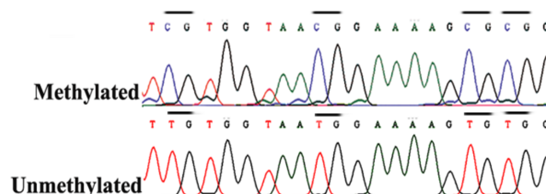

Figure 2. Representative Chromagram of PCR products by direct Sequencing. The CpG sites (bold bar); the methylated cytosines remained unchanged and the unmethylated cytosines were substituted by "T residue" in methylated and unmethylated samples, respectively

regions of the methylated PCR band remained unchanged. However, thymine-residue substitution was found in the unmethylated PCR band of the BRCA1 promoter region (Figure 2).

To assess the promoter hypermethylation status of BRCA1 effect on mRNA expression level, 45 breast tumors and their corresponding normal breast tissues were examined by quantitative real-time reverse transcription-PCR. Of 45 cases, 7 (15.6%) showed BRCA1 under-expression. A negative correlation between BRCA1 promoter hypermethylation and BRCA1 under-expression was found (Table I). No association was found between BRCA1 under-expression and the clinicopathological features of breast-cancer patients (Table II).

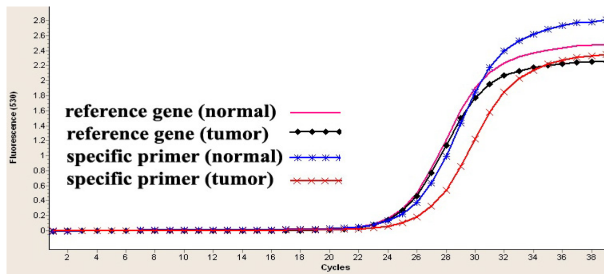

Figure 3. Representative Amplification Plots of BRCA1 Under-expression. SYBR Green I fluorescence signal versus cycle number of specific primer of BRCA1 under-expression and single copy gene of β-goblin was used as reference control in tumor and corresponding normal cDNA

Discussion

Several studies have reported BRCA1 promoter hypermethylation in breast cancer; however, its mechanism in the pathogenesis of breast cancer is not yet clearly understood. Our study found BRCA1 hypermethylation associated significantly with tumor grade III, with a high frequency of stage IIB. This suggests that BRCA1 methylation is involved in late-stage progression among breast-cancer patients. Likewise, the study by Wei et al., 2005 suggested a role for BRCA1 methylation in the

Table 1. Association between BRCA1 Methylation Status and Clinicopathological Data of 61 Breast Tumors and Reduced BRCAL Expression

Parameter	No.	BRCA1 methylation U n (%)	M n (%)	Odds ratio (95%CI)	P
Age				1.25 (0.39-4.01)	0.71
≤50	31	24(77)	7(23)		
>50	30	22(73)	8(27)		
Tumor size(cm)				1.00 (0.23-4.19)	0.97
≤2	12	9(75)	3(25)		
>2	49	37(76)	12(24)		
Histologic grade				-	0.04*
I	6	5(83)	1(17)		
II	30	26(87)	4(13)		
III	23	13(56)	10(44)		
Axillary lymph node status				1.17 (0.34-4.01)	0.80
Negative	22	17(77)	5(23)		
Postive	39	29(74)	10(26)		
Lymph Nodes (no.)				0.57 (0.16-2.09)	0.53
0-2 positive	37	27(73)	10(27)		
>2 positive	23	19(83)	4(17)		
Stage grouping				-	0.02*
I	5	5(100)	0(0)		
IIA	18	12(67)	6(33)		
IIB	10	4(40)	6(60)		
IIIA	13	12(92)	1(8)		
IIIB	15	13(87)	2(13)		
Immunohistochemical					
ER status				0.43 (0.08-2.22)	0.48
Negative/reduced expression (0,1+, 2+)	43	31(72)	12(28)		
Positive (3+)	14	12(86)	2(14)		
PgR status				0.20 (0.02-1.69)	0.15
Negative/reduced expression (0,1+, 2+)	44	31(71)	13(29)		
Positive (3+)	13	12(92)	1(8)		
HER2 status				0.59 (0.06-5.48)	
Negative/reduced expression (0,1+, 2+)	51	38(75)	13(25)		1.00
Positive (3+)	6	5(83)	1(17)		
Triple negative tumor					
ER,PR,HER2 positive	42	35(83)	7(17)	3.64 (1.1-12.3)	0.033
ER,PR, HER2 negative	19	11(58)	8(42)		
BRCA1-expression					
Positive	38	27(71)	11(29)	0.41 (0.04-3.81)	0.66
Reduced	7	6(86)	1(14)		

CI = confidence interval; M= methylated sequence; U= unmethylated sequence * statistically significant association

Table 2. Association between BRCA1 Under-Expression and Clinicopathological Data of 45 Breast Cancers and their Corresponding Normal Breast Tissues

Parameter	No.	BRCA1 under-expression BRCA1 - n (%)	BRCA1+ n (%)	Odds ratio,(95%CI)	P
Age				1.65,(0.32-8.39)	0.69
≤50	24	21(88)	3(12)		
>50	21	17(81)	4(19)		
Tumor size (cm)				0.57,(0.09-3.53)	0.61
≤2	9	7(78)	2(22)		
>2	36	31(86)	5(14)		
Histologic grade				-	0.59
I	5	4(80)	1(20)		
II	23	19(83)	4(17)		
III	15	14(93)	1(7)		
Axillary lymph node status				0.87,(0.17-4.45)	1.00
Negative	18	15(83)	3(17)		
Postive	27	23(85)	4(15)		
Lymph Nodes (no.)				0.26,(0.03-2.34)	0.39
0-2 positive	29	23(79)	6(21)		
>2 positive	16	15(94)	1(6)		
Stage grouping				-	0.25
I	5	3(60)	2(40)		
IIA	12	12(100)	0(0)		
IIB	8	6(75)	2(25)		
IIIA	10	9(90)	1(10)		
IIIB	10	8(80)	2(20)		
Immunohistochemical					
ER status				0.46,(0.05-4.39)	0.66
Negative/reduced expression (0,1+, 2+)	31	25(81)	6(19)		
Positive (3+)	10	9(90)	1(10)		
PgR status				1.87,(0.29-12.01)	0.61
Negative/reduced expression (0,1+, 2+)	33	28(85)	5(15)		
Positive (3+)	8	6(75)	2(25)		
HER2 status				0.81,(0.69-0.95)	1.00
Negative/reduced expression (0,1+, 2+)	37	30(81)	7(19)		
Positive (3+)	4	4(100)	0(0)		
Triple Negative tumor				1.12,(0.12-6.72)	0.90
ER,PR,HER2 positive	33	28(85)	5(15)		
ER,PR, HER2 negative	12	10(83)	2(17)		

CI = confidence interval; -= no under-expression; + = under-expression

aggressiveness of breast carcinomas, and that BRCA1 methylated tumors were found mainly in tumor grade III rather than grades I and II (Birgisdottir et al., 2006; Gacem et al., 2012).

The study also found that methylated BRCA1 correlated significantly with breast cancer subtype triple-negative (ER-, PR- and HER2-) tumor. This type of tumor is known to have a poor prognosis and to be more aggressive than hormone receptor-positive cancers (Camirand et al., 2013). The present study indicated that aberrant BRCA1 methylation status was associated with the pathogenesis of breast-cancer subtype. Similarly, several studies have shown that BRCA1 methylation correlates with triple-negative breast tumors (Galizia et al., 2010; Stefansson et al., 2011; Gacem et al., 2012).

In previous studies, the loss of gene expression in breast cancer was, often related to BRCA1 promoter hypermethylation. For example, several studies showed that BRCA1 promoter methylation is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens, and also with reduced protein levels in breast-cancer cell lines and sporadic breast carcinomas (Thompson et al., 1995; Sourvinos et al., 1998; Baldwin et al., 2000; Niwa et al., 2000; Rice et al., 2000; Matros et al., 2005; Mirza et al., 2007; Bal et al., 2012). The present study showed that 7 of 45 cases (15.6%) referred to BRCA1 under-expression, of which only 1 (14.0%) case with BRCA1 hypermethylation showed reduced gene expression, and another 6 (86.0%) cases demonstrated BRCA1 unmethylation. Indicating that methylation was not the sole mechanism accounting for reduced BRCA1 protein expression (Sharma et al., 2010). Therefore, multiple mechanisms effect the inactivation of BRCA1 function in breast cancer (Rice et al., 2000). Several studies have demonstrated that mutations, loss of heterozygosity, and deletions, can also suppress BRCA1 expression in invasive sporadic breast tumors (Birgisdottir et al., 2006). However, our findings demonstrated that BRCA1 under-expression did not correlate significantly with BRCA1 hypermethylation in breast-cancer patients. In consistent with our study, Pal et al., 2010 reported a negative correlation between methylation status and transcript expression levels for BRCA1 CpG sites in sporadic breast cancer, and that BRCA1 promoter methylation was not associated with loss of protein expression (Sharma et al., 2010).

In conclusion, our findings suggest that aberrant BRCA1 promoter hypermethylation is associated with tumor grade, late stage, and breast cancer subtype triple-negative tumor. This finding indicates that BRCA1 methylation is involved in the late-stage progression of breast-cancer and is a useful prognostic marker for breast-cancer development. .

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