BRCA1 Promoter Methylation and Clinicopathological Characteristics in Sporadic Breast Cancer Patients in Indonesia

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BRCA1 Promoter Methylation and Clinicopathological Characteristics in Sporadic Breast Cancer Patients in Indonesia

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Abstract

Objective: The aim of this study was to investigate the *BRCA1* promoter methylation and clinicopathological characteristics in sporadic breast cancer patients in Indonesia. **Methods:** In this cohort study, we selected 90 patients with stage I-III who had definitive surgery at our institution in 2011-2014. Demographic and clinical data regarding pathological stage, breast cancer treatment, outcome etc. were collected from the medical records. Twelve patients had incomplete information on follow up and 18 samples had insufficient tissues for the experiment. Sixty patients with adequate cancer tissues and complete follow up record were analyzed, only 56 patients were analyzed because 4 samples mRNA expression could not be detected. The Mann–Whitney U tests for non-normally distributed groups were used to compare the levels expression of *BRCA1* mRNA between methylated and non-methylated samples. Chi-square tests were used to compare methylation status, *BRCA1* mRNA expression and clinicopathological characteristics. P value < 0.05 was considered as statistically significant correlation. Data analysis was held by using the GraphPad PRISM 7 (GraphPad Software Inc., USA). **Results:** DNA and RNA were isolated from primary tumor tissues of 56 breast cancer patients. *BRCA1* promoter methylation was detected in 48 of 56 patients (85%). Level of *BRCA1* mRNA expression was associated with decreased methylation level in the *BRCA1* promoter regions suggesting the role of epigenetic silencing. However, there was no statistically significant association among methylation levels, *BRCA1* mRNA transcript level with clinicopathological factors. **Conclusion:** To our knowledge, this is the first study investigating methylation status and level of *BRCA1* mRNA transcripts among breast cancer patients in Indonesia. We found that the prevalence of *BRCA1* promoter methylation is higher than other studies from different populations. However, further investigation involving larger number of patients is required.

Keywords: Breast cancer- *BRCA1*- DNA methylation- *BRCA1* promoter methylation- gene expression

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Introduction

Breast and cervical cancers have the highest cancer prevalence rates that cause the most common cancer-related mortality among women in Indonesia. The incidence of breast cancer tends to increase and it is suggested that it largely contributes to the rise of newly diagnosed cancers among young patients (Youlden et al., 2014; Nindrea et al., 2017). Several studies have shown that the peak age of onset in Indonesia is 45–50 years of age, whereas it is 55–60 years in the West countries. The characteristic of breast cancer among Indonesian patients are more aggressive with lymph node involvement, larger tumor size, higher histological grade, higher mitotic index, higher c-erbB2 and p53 expression and higher MIB-1 proliferation index than West patients (Aryandono et al., 2006; Ng et al., 2010; Prajoko and Aryandono, 2014). Molecular and cellular heterogenesity combinated with sedentary lifestyle, high fat diet and exposure to environmental chemicals have been predicted as the risk factors of breast cancer (Leong et al., 2010; Bhoo-Pathy et al., 2013). Understanding tumor characteristics of breast cancer patients is important to select effective therapies and reduce treatment resistance (Alizadeh et al., 2015; Nindrea et al., 2018).

BRCA1 is a human tumor suppressor gene with play crucial role in repairing DNA damage and has been intensively be a subject of investigation since discovered 1990 (Wooster et al., 1994). *BRCA1*deficiency caused either by germ-line mutations or by down-regulation of gene expression, leads to tumor formation inappropriate target tissue. Decreased expression of the *BRCA1* gene has been contributes in both inherited and sporadic breast cancer, and the magnitude of the decreased is correlated with tumor progression. The lowest level of *BRCA1* protein commonly found in high-grade tumor and exhibit higher proliferation rates. There are several possible molecular mechanisms that could lead to permanent

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decreased of *BRCA1* levels in tumor cell including allelic loss of heterozygosity (LOH) and hypermethylation *BRCA1* gene promoter region (Mueller and Roskelley, 2003; Venkitaraman, 2002).

It was reported that DNA methylation was the major cause of transcriptional silence of *BRCA1*, ranging from 13–40% in sporadic breast cancer (Butcher and Rodenhiser, 2007). Several studies has been reported that *BRCA1* methylation was correlated with the prognosis of breast cancer in Caucasian patients (Baldwin et al., 2000; Cho et al., 2012). However, the role of *BRCA1* methylation is not well characterized in the prognosis of sporadic breast cancers among other populations. In the present study, we determined the methylation status of *BRCA1* in 90 Indonesian patients with sporadic breast cancer and investigated whether the *BRCA1* methylation was associated with clinical outcomes.

Materials and Methods

Study design and research sample

In this cohort study, the data were collected from Dr. M Djamil General Hospital Padang. We selected 90 patients with stage I-III who had definitive surgery in 2011-2014. Demographic and clinical data regarding pathological stages, breast cancer treatment, outcome etc. were collected from the medical records. Twelve patients had incomplete follow up data. Frozen tumor tissue samples were retrieved from the Biobank Faculty of Medicine Universitas Andalas. Eighteen samples had insufficient tissue materials for this experiment. Sixty patients with adequate cancer tissues and complete follow up data were further analyzed. Unfortunately, *BRCA1 mRNA* expression from 4 samples cannot be detected. Therefore, the final analysis included 56 patients (Figure 1).

Ethics statement

The study was approved by the ethical committee board of Faculty of Medicine Universitas Andalas, Padang City, Indonesia. Written informed consent was obtained from all patients.

DNA extraction and bisulfite modification

Genomic DNA was extracted from frozen breast cancer tissues using PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific, catalog number K182001) according to the manufacturer's instructions. Genomic DNA concentration and purity was quantified in duplicate using NanoDrop spectrophotometer. DNA samples were stored at -20°C.

Genomic DNA (200-500 ng) from each patient was converted using MethylCodeTM Bisulfite Conversion Kit (Invitrogen, catalog number MECOV-50) according to the manufacturer's manual instruction. The bisulfite-converted DNA was then stored at -20°C until further analysis.

Methylation-specific PCR

Methylation-Specific PCR was performed using primers specific to methylated DNA sequence in the promoter region of *BRCA1* (MF: 5'-TCGTGGTAACGGAAAAGCGC-3' and MR: 5'-AAATCTCAACGAACTCACGCCG-3', PCR product size: 75 bp). The unmethylated DNA sequence was amplified using primer specific to unmethylated-bisulfite-converted DNA sequence, which the C's (cytosines) in the template should be treated as T's (UF: 5'-TTGGTTTTTGTGGTAATGGAAAAGTGT-3' and UR: 5'-AAAAAATCTCAACAAACTCACACCA-3', PCR product size: 86 bp). The methylation and unmethylation-specific primers were adopted from Esteller et al., (2000) and Butcher and Rodenhiser, (2007). These primers annealed in the promoter region and flanked the transcription site of *BRCA1* (Figure 2).

MS-PCR reactions were performed in 25 µl total volume which contained 400 nM MF primer and 400 nM MR primer (synthesized by Integrated DNA Technologies, Singapore), 1x GoTaq Green Master mix (Promega, catalog number M7122), and 2 µl template of bisulfite-converted DNA. The cycling conditions were initial denaturation at 96°C for 4 minutes followed by 35 cycles of denaturation at 95°C for 50 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 30 seconds, and followed by final extension at 72°C for 5 minutes, using T100 thermocycler (Bio-Rad, USA). The reaction of unmethylated-specific PCR was also performed in different PCR tube with the same reaction except the primer. The PCR products were separated in 2% agarose gel and visualized after staining with GelRed (Biotium).

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen breast cancer tissues, using TRIzol Reagent with the PureLink® RNA Mini Kit (Ambion, Life Technologies, catalog number 15596-026 and 12183018A, respectively) according to the manufacturer's instructions. Total RNA concentration and purity were quantified in duplicate using NanoDrop spectrophotometer. RNA samples were stored at -80°C.

In total 1 µg RNA was used for cDNA conversion using iScript cDNA synthesis kit (Bio-Rad, catalog number 1708890). The total volume reaction 20 µl was incubated according to the manufacturer's instruction. The converted cDNA was stored at −20°C.

BRCA1 expression analysis by quantitative real-time reversetranscription-PCR

Quantitative reverse-transcription PCR primers were designed to span an exon-exon junction to reduce the risk of false positives from amplification of any contaminating genomic DNA (Figure 3). *BRCA1*-Forward (5'-CAGGAGTGGAAAGGTCATC-3') annealed in exon 13-exon 14 junctions and *BRCA1*-Reverse (5'-TCCCTCTAGATCTTGCCTT-3') annealed in exon 14-exon 15 junctions. GAPDH *mRNA* expression level also quantified as a housekeeping gene for normalization using GAPDH-Forward (5'-CATTGACCTCAACTACATGGTTT-3') and GAPDH-Reverse (5'-GAAGATGGTGATGGGATTTCC-3'). Both primers were designed using bioinformatics software Geneious 7.0.6.

Real-time PCR was performed in optical strip PCR tubes in duplicate which contained 500 nM *BRCA1*-Forward and 500 nM *BRCA1*-Reverse (synthesized by Integrated DNA

Technologies, Singapore), 1x Sso Evagreen Supermix (Bio-Rad, catalog number 172-5200), and 2 µl template (cDNA or *BRCA1* plasmid standard) in a final volume 20 µl. The cycling conditions were enzyme activation at 95°C for 30 seconds followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing/extension at 52.5°C for 5 seconds, and followed by melt curve from 65°C to 95°C, using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The *BRCA1* standard curve in linear regression was automatically generated by software CFX96 touch system by plotting the quantification cycle (Cq) against the log of copy number, so that the *BRCA1* copy number from samples will be known. Quantification cycle (Cq) or threshold cycle (CT) is the number of PCR cycles for which enough fluorescence was detected above background. Real-time PCR for GAPDH also conducted in a different plate with the same reactions above except the primer and the annealing/extension temperature (57.1°C).

Statistical analysis

The Mann–Whitney U test for non-normally distributed groups was used to compare the levels expression of *BRCA1 mRNA* between methylated and unmethylated samples. The association among methylation status, *BRCA1 mRNA* expression and clinicopathological characteristics was examined by chi-square test. P value < 0.05 was considered statistically significant correlation. Data analysis was carried out with the GraphPad PRISM 7 (GraphPad Software Inc., USA).

Results

The Methylation-Specific PCR was used to investigate methylation status of *BRCA1* promoter. It was successfully performed in 97% (58/60) samples to detect the methylation status of *BRCA1* promoter region. The typical results of methylated and unmethylated status were shown in Figure 4. *BRCA1* promoter methylation was

Table 1. Association between Methylation Status of *BRCA1* and Clinicopathological Characteristics Breast Cancer Patients

Characteristics		f	(%)
Age (years)	47,5 years	56	100
Tumor Size	8.3 cm	56	100
Clinical stage	Early BC (I-II)	22	39.3
	Advance (III-IV)	34	60.7
Histological grade	I	1	1.8
	II	51	91
	III	4	7.2
Lymph node status	N0	19	34
	N1	28	50
	N2	9	16
ER status	Negative	33	59
	Positive	23	41
PR status	Negative	39	69.6
	Positive	17	30.4
KI67	< 14%	28	50
	> 14%	28	50
HER2	HER2 (-)	35	62.5
	HER2 (+++)	21	37.5
TNBC	TNBC	15	26.8
Relapse status	No relapse	43	76.7
	Relapse	13	23.3
Current status	Die	49	87.5
	Survive	7	12.5

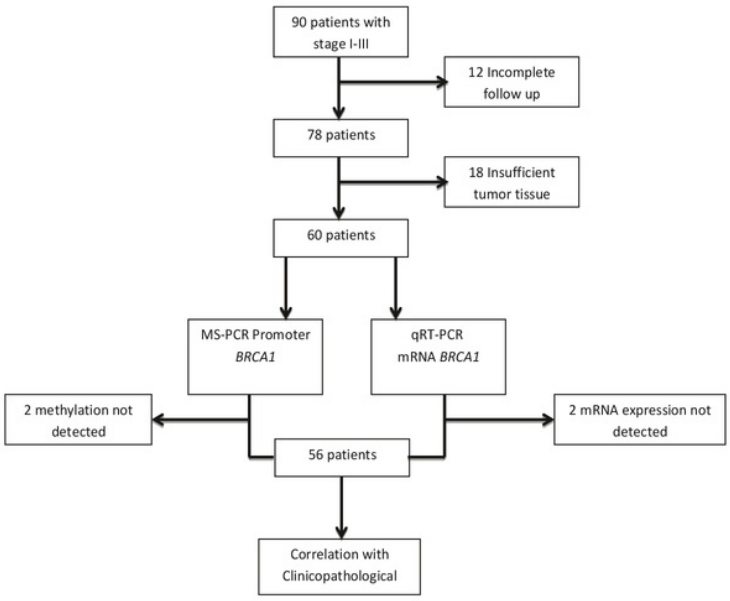

Figure 1. Number of Selected Specimens for Analysis

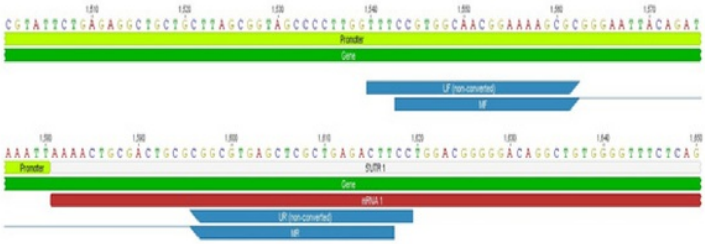

Figure 2. Location of Methylation-Specific Primers (MF-MR) and Unmethylation-Specific Primers (UF-UR) in *BRCA1* Sequence (NCBI Accession Number: NG_005905.2)

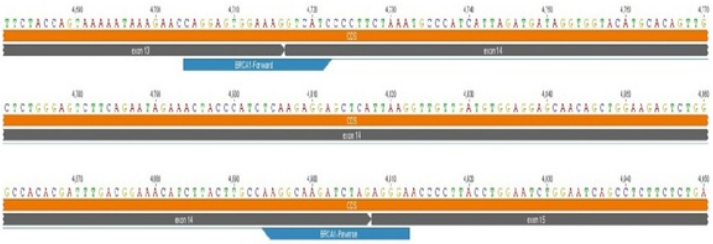

Figure 3. Location of *BRCA1* Forward and Reverse Primers in *BRCA1 mRNA* Sequence (NCBI Accession Number: NM_007294.3)

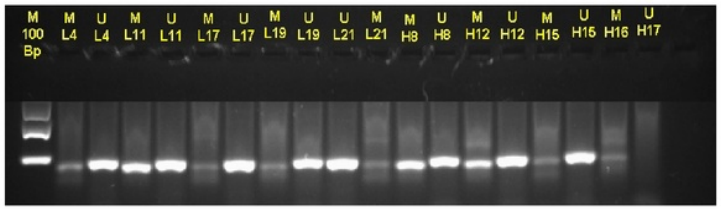

Figure 4. Electrophoresis of Amplification Products Specific Promoter Region *BRCA1* from Bisulfite-Treated DNA in Human Tumor Tissue. Each line consists amplification products from methylated (U), 75bp or unmethylated (M), 86bp primer

detected in 48 out of 60 (80%) sporadic breast tumors. The methylation status was not statistically significant associated with any clinicopathological parameters ($p > 0.05$) (Table 1).

Level of *BRCA1 mRNA* expression in tissue tumor specimen was successfully detected by using qRT-PCR. The median *mRNA* expression level of *BRCA1* relative to reference gene of GADPH were 2.27 (range 0.014-13.335). There was statistically significant association between methylation status promoter region and *mRNA* expression level of *BRCA1* (p-value 0.042), suggesting epigenetic silencing activities in the *BRCA1* promoter region. However there was no statistically significant correlation between levels of *mRNA BRCA1* and clinicopathological parameters including estrogen receptor (p=0.839), progesterone receptor (p=0.382), Ki67 (p=0.644), histological grade (p=0.329), stage (p=0.764), tumor size (p=0.148), and lymph node status (p=0.730) (Table 2).

There was an inverse relationship between *BRCA1* promoter methylation and *mRNA* transcript levels (p-value< 0,05) (Figure 5).

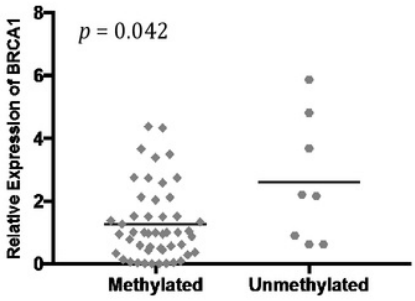

Figure 5. The Relationship between *BRCA1* Promoter Methylation and *mRNA* Transcript Levels. Lines note median expression value, p-value represents Mann-Whitney test.

Discussion

Cancer is resulted from the accumulation of altered genetic regulations which cause abnormal cell growth

Table 2. The Relationship between *BRCA1* mRNA Expression and Clinicopathological Factors

Clinicopathology	n	Mean ± SD mRNA BRCA1	p-value
Estrogen receptor			
Negative	33	3.43 ± 3.18	0.839
Positive	23	3.26 ± 3.11	
Progesterone receptor			
Negative	39	3.61 ± 3.17	0.382
Positive	17	2.81 ± 3.03	
KI67			
< 14%	28	3.56 ± 3.17	0.644
> 14%	28	3.17± 3.11	
HER2			
HER2 (-)	27	3.06 ± 2.92	0.840
HER2 (+)	6	3.05 ± 3.10	
HER2 (++)	2	4.54 ± 4.45	
HER2 (+++)	21	3.72 ± 3.44	
TNBC			
Luminal A	23	3.26 ± 3.10	0.977
HER2	18	3.47 ± 3.25	
TNBC	15	3.39 ± 3.20	
Histological Grade			
I	1	7.9	0.329
II	51	3.31 ± 3.18	
III	4	3.36 ± 3.12	
Stage			
Stage I	3	2.30 ± 3.12	0.764
Stage II	19	3.67 ± 3.36	
Stage III	34	3.28 ± 3.05	
Tumor size			
T1	3	2.30 ± 3.12	0.198
T2	11	4.79 ± 3.78	
T3	10	1.95 ± 1.88	
T4	32	3.42 ± 3.09	
Lymph node status			
N0	19	3.31 ± 3.28	0.730
N1	28	3.16 ± 2.95	
N2	9	4.12 ± 3.55	

and expansion. In addition to genetic mutations, epigenetic alteration also play an important role in breast carcingenesis involving changes in DNA methylation (global hypomethylation and locus-specific hypermethylation), altered histone tail modifications patterns and nucleosomal remodeling. Methylation of *BRCA1* promoter tumor suppressor gene has been known as one of the gene expression loss mechanisms, and has been identified in 10–30% of sporadic early-onset breast cancer with aggressive pathological features (Esteller et al., 2000; Wei et al., 2005; Matros et al., 2005; Turner et al., 2007).

We report for the first time from Indonesian population, promoter methylation of *BRCA1* gene is present in 48 of 60 (80%) patients with early-stage sporadic breast carcinomas. The promoter methylation status is significantly higher than previously reported from other population such as Taiwanese present in 56%, Thailand 24.6%, India 45% but present similar highest status with Vietnamese, 82,1% and France, 89,1% (Bosviel et al., 2012; Hsu et al., 2013; Hasan et al., 2013; Saelee et al., 2014; Truong et al., 2014). Our study supports previous premise that the promoter methylation status is depending on race/ethnicity and population, which also relates to tumor characterization (Wiencke, 2004). The promoter methylation of *BRCA1* gene relates to tumor grade III and is frequently found in stage IIB. The data of 536 sporadic breast cancer studied at Chinese population found 26% hypermethylation on *BRCA1* promoters has a poor prognosis effect (Chen et al., 2009).

In our study, expression of *mRNA* in tissue tumor specimen was successfully detected by using qRT-PCR. There was statistically significant association between methylation status promoter region and *mRNA* expression level of *BRCA1* (p-value 0.042), suggesting epigenetic silencing activities in the *BRCA1* promoter region. However there was no statistically significant correlation between levels of *mRNA BRCA1* and clinicopathological parameters including estrogen receptor, progesterone receptor and expression of KI 67.

Hypermethylation of the *BRCA1* gene promoter is present in 56% (78 of 139) of Taiwanese women with early-stage sporadic breast carcinomas, which is significantly higher than previously reported frequencies for this alteration in unselected sporadic breast tumors (Hsu et al., 2013). The result of meta-analysis study showed *BRCA1* methylation prevalence in Asians population (OR= 4.03, 95%CI 1.07–15.18, P= 0.04) was higher than in Caucasians populations (OR= 3.16, 95%CI 1.78–5.62, P< 0.001) and in Australians populations (OR= 3.27, 95% CI 1.37–7.84, P= 0.008) in breast cancers compared with non-cancer controls (Zhang and Long, 2015). The prevalence of *BRCA1* mutations in a cohort of Nigerian (predominantly Yoruba) breast cancer patients unselected for age of onset or family history. Surprisingly, 31/434 (7.1%) patients carried *BRCA1* mutations. This result show consistently lower frequencies of *BRCA1* mutations in non-founder populations, and especially low frequencies in African Americans (Fackenthal et al., 2011). Another study examined 1,628 population-based breast cancer cases and showed that mutations occurred in 2.9% of White cases and only 1.4% of Black cases (Malone et al., 2006). Our study supports the role of *BRCA1* methylation in the aggressiveness of breast cancer.

In our study, there was no any significant correlation between the hypermethylated *BRCA1* promoter and patient's age, tumor grades, and clinical stages which was similar with a study from Vietnamese breast cancer cohort (Truong et al., 2007). Decreased estrogen receptor expression is found in *BRCA1* promoter hypermethylated patients. More than 25% (15/56) patients with *BRCA1* promoter methylation were triple-negative breast cancers, even though a significant number of triple-negative breast cancer patients do not carry *BRCA1* mutations. Our study finding supported that hypermethylation at *BRCA1*

promoter region playing a role on etiological of triple-negative phenotype breast cancer (Miyoshi, 2008). Some study indicated that hypermethylation of promoter *BRCA1* gen was associated with the pathogenesis of breast-cancer subtype. Breast cancer with have hypermethylation on *BRCA1* promoter region are more likely to be of high grade or estrogen-receptor negative, and p53 positive (Johannsson et al., 1997; Hsu et al., 2013). It has been postulated that breast cancer with has hypermethylation on *BRCA1* promoter region is more aggressive. The benefits of epigenetic research, especially on gene promoter methylation are linked to the prevention and treatment of breast cancer.

The use of antioxidants as an antimethylation agent can reduce the occurrence of methylation in certain genes especially the proliferative gen and then can prevent the occurrence of breast cancer in the community. Likewise in the treatment of breast cancer, the use of demethylation drugs will be able to reduce the recurrence and mortality in cancer patients (Fleischauer et al., 2003).

This study is the first study involving Indonesian breast cancer. But there are are some limitations of this study including relatively small number of patient cohort, no survival or progression-free survival data comparing hypermethylated and non- methylated, and as focused in sporadic cancer. This study is lack of environmental-associated data including diet, smoking habit, Body Mass Index (BMI), obesity, menarche, hormonal contraception use, and physical activity levels. The genetic and environmental factors are postulated to be playing a role for breast cancer risk. Environmental factors may be associated with the methylation of the promoter regions within tumor suppressor and DNA repair genes *BRCA1*.

In conclusion, our study found that methylation rates in Indonesian breast cancer women is higher than in the literature, possibly due to advanced cancer stages. The presence of hypermethylation in the *BRCA1* promoter does not affect the type of breast cancer. This finding indicates that *BRCA1* methylation is involved in the late-stage progression of breast-cancer and can be used as one of the prognostic marker in breast cancer.

Conflict of interest

The authors declare no conflict of interest.

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