RESEARCH ARTICLE

BRCA1 Promoter Hypermethylation Signature for Early Detection of Breast Cancer in the Vietnamese Population

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Abstract

Breast cancer, a leading cause of death among women in most countries worldwide, is rapidly increasing in incidence in Vietnam. One of biomarkers is the disruption of the genetic material including epigenetic changes like DNA methylation. With the aim of finding hypermethylation at CpG islands of promoter of *BRCA1* gene, belonged to the tumor suppressor gene family, as the biomarker for breast cancer in Vietnamese population, sensitive methyl specific PCR (MSP) was carried out on 115 samples including 95 breast cancer specimens and 20 normal breast tissues with other diseases which were obtained from Ho Chi Minh City Medical Hospital, Vietnam. The result indicated that the frequency of *BRCA1* hypermethylation reached 82.1% in the cases ($p<0.001$). In addition, the DNA hypermethylation of this candidate gene increased the possibility to be breast cancer with high incidence via calculated odd ratios ($p<0.05$). In conclusion, hypermethylation of this candidate gene could be used as the promising biomarker application with Vietnamese breast cancer patients.

Keywords: *BRCA1* - methylation - bisulfite modification - breast cancer

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Introduction

In Vietnam, breast cancer is the most frequent malignancy leading to the death for female, constituting in certain has increased from a crude rate of 13.8 per 100,000 women in 2000 to 28.1 per 100,000 women in 2010 (Nguyen at al., 2013). The early detection is necessary to treat and improve the survival of breast cancer patients. In patient with breast cancer, gene amplification of HER2 which is a member of the epidermal growth factor receptor family, is found to be present in 15-30% newly diagnosed breast cancer cases and the mutations in the tumor suppressor gene p53 is presented in 18%-25% of primary breast carcinomas (Alsner at al., 2000; Kotoe at el., 2009). The overexpression of the p53 and HER-2/neu oncogenes are the two most common genetic abnormalities associated with breast cancer (Alsner at el., 2000). Besides, recently studies showed that the epigenetic alterations, especially in tumor suppressor genes, that initiate tumorgenesis could be used as the biomarkers for the prognosis and diagnosis of neoplasm in body (Dulaimi et al., 2004). So that, the objective of present study was to determine the hypermethylation occurred in breast cancer gene (*BRCA1* gene), one of tumor suppressor genes, applied in Vietnamese population.

Epigenetics changes, as DNA methylation is one of the most common changes of epigenetics events, covalent addition of the methyl group to DNA, which played an important role in driving tumorgenesis (Pongtheerat et al., 2011; Tanett et al., 2011; Fang et al., 2012; Fatemeh et al., 2012; Ramezani et al., 2012; Wei-Jia et al., 2012). The DNA methylation usually occurs in the CpG islands located in or near the promoter of over 70% of all gene (Tanett et al., 2011; Fatemeh et al., 2012; Wei-Jia et al., 2012). In breast cancer, hypermethylation occurs in *BRCA1* gene's promoter leaded to the cancer development and progression (Evron et al., 2001; Xu et al., 2009; Kulis at al., 2010; Ramezani et al., 2012). *BRCA1* is identified at 17q12-21. Its protein, breast cancer type 1 susceptibility protein is directly involved in preventing cells from growing and dividing in a controlled way and repairing damaged DNA. Recently, it is found that hypermethylation in promoter CpG islands of *BRCA1* leads to the loss of expression of *BRCA1*, especially, in breast cancer (Xu et al., 2009). According to the research of Hedenfalk et al., aberrant methylated of *BRCA1* promoter is responsible for functional inactivation of *BRCA1* and plays an important role in breast tumorigenesis (Hedenfalk et al., 2009).

In the present study, we determined quantitatively the hypermethylation at CpG islands of promoter belongs to *BRCA1*, in both Vietnamese breast cancer patients of all stages from premalignant to advanced metastatic breast

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tumor and healthy specimens by the MSP (Methylation specific PCR) method.

Materials and Methods

Sample collection

A total of 115 samples were composed of 95 breast cancer specimens that were enrolled in evaluating predictive factors including immunohistochemistry with two antibodies were HER2/neu and p53. HER2/neu status was originally scored 0 to 3+, and 0 tumors were regarded as HER2/neu negative. Moreover, the histologic grade and tumor grade of tumor specimens were also determined according to the American Joint Committee on Cancer 6th Edition Guidelines; 20 healthy specimens were obtained from women who underwent a biopsy of the mammary gland because of mammographic screening and for whom histology confirmed the present of only normal tissue. Those patients had the well information about the clinical and molecular characteristics of the tumors are given. Among these patients, the median of age was 48 years old (ranging from 25 to 88 years old). Moreover, the stages of tumors were large in stage I, II, III and IV which were accounted for 53 (55.8%), 27 (28.4%), 9 (9.5%) and 6 (6.3%) of 95 specimens. According to the tumor grade, grade 1, 2 and 3 were 5 (5.3%), 55 (57.9%), 35 (36.8%) of 95 samples, respectively. For HER and p53 staining, HER positive and p53 positive were detected in 59 (62.1%) and 17 (17.0%), respectively. The sum of sample with both HER and p53 negative was counted for 33 (28.7%) of 95 breast cancer specimens. Otherwise, the both HER and p53 positive was 82 (71.3%). All the samples were admitted to the Ho Chi Minh city Medical Hospital in Vietnam from 2010 to 2011. These tissues were obtained from the surgical specimens and then, embedded in the paraffin and stored at -20oC until the further used.

DNA bisulfite modification and Methylation assays

DNA extractions were performed by phenol chloroform method (Chomczynski et al., 1987). In addition, DNA modifications were carried out by the DNA modification kit (Epitect Kit, Qiagen). Approximate 2 µg genomic DNA of each sample is bisulfite-modified and purified, then, the final precipitate was eluted in a volume of 20 µl. MSP used specific primers to evaluate the situation of methylated and unmethylated status for a given gene. Primer sequences included methylated primers and unmethylated primers were given in Table 1. Besides, the *BRCA1* sequence, CpG sites and transcriptional factors were showed in Fig 1. The amplifications were done in a total volume of 15µl, containing 3µl bisulfite modified template DNA, 0.75 unit iTaq DNA polymerase (Biorad). MSP reaction was subjected to initial incubation at 95°C for 5 min, followed by 40 cycles at 95°C for 30s, 51°C for 30s, 72°C for 30s and 72°C for 6 min for final incubation. Each PCR product was directly loaded onto a 2.0% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

Table 1. Methylation and Unmethylation *BRCA1* Primer Sequences

Primer name	Primer Sequence (5' – 3')*
BRCA1 methylated (Forward)	TCGTGGTAACGGAAAAGCGC
BRCA1 methylated (Reverse)	AACGAACTCACGCCGCGCAA
BRCA1 unmethylated (Forward)	TTGTGGTAATGGAAAAGTGT
BRCA1 unmethylated (Reverse)	AACAAACTCACACCACACAA

Statistical analysis

The status of methylation of *BRCA1* gene were calculated. Differences in the presence of methylation were determined by a two sided Fisher test and Chi squared tests for variables. The relative risk (RR), associations between the methylated status of candidate gene and breast cancer, as well as any correlation between methylation status and other clinical parameters were examined using the Chi-square test. Moreover, the Odd ratio (OR) and 95% confidence intervals (CIs) were also calculated. Statistical analyses were performed by using Medcalc® Version 12.7.0.0. Statistical significance was assumed at two-side p value of $p<0.05$.

Results

Methylation analysis

For evaluation of promoter of *BRCA1* genes methylation status, the MSP method was carried out for 95 breast cancer specimens and 20 healthy DNA samples. According to the result, regarding to the tumor specimens, the frequency of methylation and unmethylation of *BRCA1* was 78 (82.1%), 17 (17.9%) of 95 ($p<0.001$), respectively. For the healthy specimens, there was no any considerably methylation occurred in *BRCA1* gene's promoter, counting for 0% ($p<0.001$) (Figure 2). Moreover, the MSP product sequencing was shown in Figure 3.

The correlation between the methylation frequencies of the *BRCA1* gene and clinicopathological parameters in breast cancer

Chi quare analysis revealed that there were no any significant differences between the methylation of promoter of *BRCA1* gene and clinicopathological parameters including age, stage, tumor grade which were shown in table 2 ($p>0.05$).

To evaluate the correlation between the DNA methylation and protein expression including HER/neu and p53, the immunochemical staining of HER, p53 was carried out. The results were shown in table 2.

Table 2. The Correlation between *BRCA1* Gene's Promoter Methylation and Clinicopathological Parameters

Characteristics		Age		Stage				Grade			HER/neu		p53		HER(-)p53(-)	
		≤ 48	> 48	I	II	III	IV	1	2	3	Positive	Negative	Positive	Negative	Yes	No
BRCA1	U	11	6	11	6	0	0	0	14	3	9	26	19	16	19	16
	M	38	40	42	21	9	6	5	41	32	30	50	62	19	14	66
	p value	0.35		0.27				0.07			0.31		0.02		0.0002	

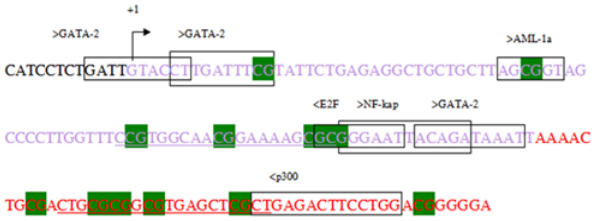

Figure 1. *BRCA1* Sequence, MSP Primer Sequences and Transcription Factor Binding Site. Promoter is black, 5'UTR is purple, exon 1a is red, CpG sites are green and underlined nucleotides are chosen for primer designed

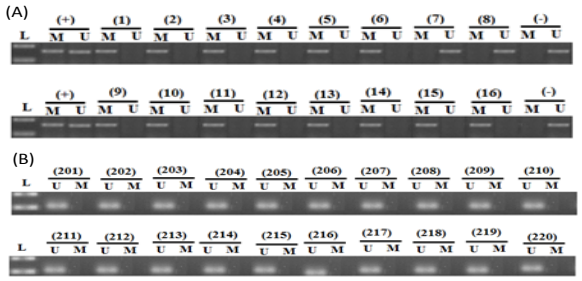

Figure 2. Methylation of *BRCA1* Gene Analysis on Some Clinical Samples by MSP. The MSP products are 68 bp. U: unmethylated; M: methylated; L: 50 kp Ladder; (+) positive control; (-) negative control; (A) tumor specimens; (B) non-cancer specimens

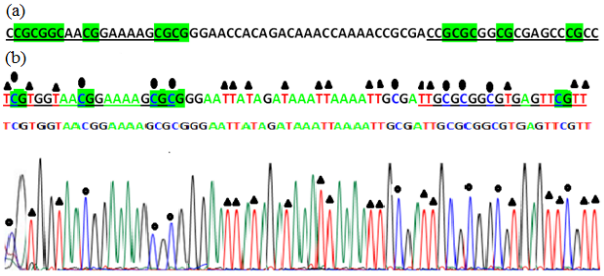

Figure 3. Sequencing Profile of Methylated of *BRCA1*. CpG sites were in green highlight; The cytosine did not depend on the CpG site were marked as the black triangle, the cytosine depend on the CpG site were marked as the black circle; (a) DNA sequence was without bisulfite modified; (b) DNA sequence was bisulfite modified; (c) The *BRCA1* sequencing by using the forward methylated primer

The significant correlations were found out between the methylation status of *BRCA1* gene's promoter with p53, HER(-)p53(-) characteristic ($p<0.05$). As for the HER/neu expression, there was no any signification was found out ($p=031$). Therefore, according to our study, the results of comparison of methylation frequency of candidate gene's promoter also showed the strong correlation between the methylation frequency and breast cancer ($p<0.05$). It meant that the mean concentration of methylated status of *BRCA1* gene's promoter in breast cancer patient was higher than in the normal healthy control groups, which was statistically significant ($p<0.05$). Therefore, it could be deduced that the methylation status in *BRCA1* gene's promoter was the significant feature in breast cancer tumorgenesis. In addition, to evaluate the applicability of *BRCA1* methylation in breast cancer patients as a biomarker for breast cancer, the Chi2 test between the relative risk, odd ratio and the disease was found significant at 95% confidence interval with the odd ratio and relative risk were 183.91 ($p<0.001$) and 33.64 ($p=0.01$), respectively, with the strong association.

Discussion

The find out the newly biomarker was the important aim for the prognosis and diagnosis of breast cancer, because the incident rate of breast cancer, especially in Vietnamese population, has considerably increased with a faster pace over past decades (Nguyen et al., 2013). Therefore, the purpose of present study was to investigate the methylation status of *BRCA1* gene's promoter in breast cancer tumor in Vietnamese population. The methylation of tumor suppressor gene, *BRCA1*, was early detectable, which provided evidences for early diagnosis of tumor (Manel et al., 2001; Ibanez at al., 2004). In this study, we successfully carried out the evaluation of methylation status of *BRCA1*, by sequencing, we observed total of 10 CpG sites which were totally methylated located at the region of forward and reverse methylated primers binding sites (Figure 3). Moreover, firstly, the signals of peaks in MSP product sequencing were unique, clear and specific. Secondly, making a comparison between DNA sequence without bisulfite modified with DNA sequence was bisulfite modified, a clearly results demonstrated that all the unmethylated nucleotides were totally conversed into uracil. Therefore, we concluded that the bisulfite modification was successfully carried out. The mean of *BRCA1* methylated promoter frequency, in our report, was 82.1% (78 of 95 tumor specimens). For the *BRCA1* promoter methylation, based on comparison to the worldwide researches, in our study the mean of frequency of hypermethylated *BRCA1* was rather higher than Tapia et al. (2008), Nicholas et al. (2013), William et al. (2013) showing the methylation frequencies as 51%, 56% and 29%, respectively. Concerning to non-cancer specimens or healthy specimens, the similar to those studies was considered that no any methylation frequency was found in *BRCA1* gene's promoter. In another word, the hypermethylation of *BRCA1* gene's promoter was the specific outcome of Vietnamese breast cancer patients.

Regarding to clinical parameters, in our report, there were no any significant differences between the hypermethylated *BRCA1* promoter and the patient ages, tumor grades and stages, according to the studies of Nicolas et al. (2013), Valgerdur et al. (2006). Concerning to HER/neu staining and *BRCA1* methylation, based on the results, it was clearly to consider that more than 75% of positive HER/neu cases were methylation, contract to negative HER/neu cases in which the methylated status, counting for more than 65% was also higher than unmethylated. Therefore, in addition to the statistical analysis ($p>0.05$), it could be clearly included that there was poor correlation between the HER/neu expression and *BRCA1* hypermethylation, according to Nicolas et al. (2013). In addition, the overexpression of mutant p53 had been indicated that to have a considerable relationship to the tumor rate, in our report, we conducted only the frequent methylation of *BRCA1* were associated to the

p53 stages (p=0.02). Especially, taken p53 and HER/neu staining together, the mean of hypermethylated *BRCA1* frequency was strongly associated with HER/neu(-)p53(-) stage (p=0.0002), meant that the quantitative of marker gene methylation were clearly associated as the useful biomarker for classify and diagnosis for breast tumor.

According to the odd ratio and risk ratio showed in table 2, it indicated that *BRCA1* methylated was highly correlated with breast cancer risk via the odd ratio with the significant statistic with OR=183.91 at 95%CI (p<0.001). It meant that in this model, the odds for a positive hypermethylation of *BRCA1* promoter in breast cancer was 183.91 times higher than in the case of cancer without methylation. In addition, the RR was 33.64 (p=0.01), meant that the hypermethylation of *BRCA1* gene's promoter was 33.64 times higher than unmethylation in breast tumor. Based on these results, it was tentatively inferred that the aberrant hypermethylation of *BRCA1* promoter as the role of driving the breast tumorgenesis. Consequently, the hypermethylation in *BRCA1* gene's promoter could be useful phenomenon applied in early detection of breast cancer in Vietnamese population.

In conclusion, in our study, we highlighted the mean methylation *BRCA1* gene's promoter frequency was 82.1%. This hypermethylation of candidate gene's promoter was the specific characteristic of Vietnamese breast cancer patients, especially, strongly associated with breast tumorgenesis. Furthermore, patients with the hypermethylation in *BRCA1* gene's promoter were higher than whom without methylation (OR=183.91 at 95%CI (p<0.001)). Based on these data, increased understanding of epigenetics changes as DNA hypermethylation in *BRCA1* may involve in pathogenesis of breast cancer and provided the basic method for early detection. Besides, these data could be inferred that the role of abnormal methylation at CpG islands of promoter belonged to *BRCA1* information as a potential biomarker for early diagnosis application in Vietnamese population. .

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