RESEARCH ARTICLE

Editorial Process: Submission:01/14/2018 Acceptance:05/03/2018

Methylation Profiles of *BRCA1, RASSF1A* and *GSTP1* in Vietnamese Women with Breast Cancer

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Abstract

Objective: This study investigated the DNA promoter methylation profiles of *BRCA1*, *RASSF1A* and *GSTP1* genes, both individually and in an integrative manner in order to clarify their correlation with clinicopathological parameters of breast cancer from Vietnamese patients, and establish new potential integrative methylation biomarkers for breast cancer detection. **Material and methods:** The methylation frequencies of *BRCA1*, *RASSF1A* and *GSTP1* were analyzed by methylation specific polymerase chain reaction (MSP) in 70 specimens of breast carcinomas and 79 pairs of tumor and matched adjacent normal tissues from breast cancer patients. **Results:** All the three analyzed genes showed a concordance concerning their promoter methylation in tumor and adjacent normal tissue. The methylation of *BRCA1*, *RASSF1A* and *GSTP1* was found in 58.23 %, 74.68 % and 59.49 % of tumor tissues and 51.90 %, 63.29 % and 35.44 % of corresponding adjacent tissues, respectively. When each gene was assessed individually, only the methylation of *GSTP1* was significantly associated with tumor tissues (p=0.003). However, the methylation frequency of at least one of the three genes and the methylation frequency of all the three genes both showed significant association with tumor (p=0.008 and p=0.04, respectively). The methylation of *BRCA1* was found to be significantly associated with tumor grade (p=0.01). **Conclusion:** This study emphasized that the panel of the three genes *BRCA1*, *RASSF1A* and *GSTP1* can be further developed as potential biomarkers in diagnosis and classification of breast cancer in Vietnamese women.

Keywords: Breast cancer 1 (*BRCA1*)- RAS-association domain family member 1 (*RASSF1A*)

Asian Pac J Cancer Prev, **19 (7)**, 1887-1893

Introduction

DNA methylation occuring at CpG dinucleotides that frequently locate in promoter regions is well known as an epigenetic regulation mechanism for transcriptionally silencing gene expression (Kagohara et al., 2017). Alteration of the DNA methylation pattern may inhibit tumor repressor genes that are involved in DNA repair, apoptosis, detoxification, thus promoting cell differentiation, proliferation, malignant transformation and tumorigenesis (Sharma et al., 2010; Baylin and Ohm, 2006). Aberrant DNA methylation is the earliest molecular alteration occurring during carcinogenesis and specific for the malignant state; therefore, since a long time, it has been considered as powerful potential biomarkers for cancer diagnosis (Teschendorff et al., 2016; Leygo et al., 2017; Hao et al., 2017). For instance, the DNA methylation of the *SEPTIN9, APC*, *GSTP1* and *RASSF1A* genes has been applied as biomarkers for clinical diagnosis of colorectal and prostate cancers, respectively (Nian et al., 2017; Cucchiara et al., 2017). Currently, DNA methylation profile in various types of cancers including lung, colon and breast has been extensively explored by genome wide analysis as well as by targeting a particular gene (He et al., 2016; Huang et al., 2014; Su et al., 2016).

Breast cancer is the most common type of cancer and leading cause of cancer death in women all over the world (Siegel et al., 2016). Among a large number of genes that have been identified as methylated genes in breast cancer, three critical tumor suppressor genes *BRCA1*, *RASSF1A* and *GSTP1* were extensively studied because their multifunctional roles in numerous cellular pathways. The *BRCA1* gene encodes a protein involved in DNA repair, cell cycle control and chromatin remodeling (Deng, 2006). The *RASSF1A* gene regulates cell proliferation, cellular integrity and cell death (Agathanggelou et al., 2005). The *GSTP1* gene encodes a detoxification enzyme involved in protecting cells from carcinogens (Laborde, 2010). Increasing number of meta analyses of the methylation status of those three genes has clarified a significant correlation of *BRCA1*, *RASSF1A* and *GSTP1* methylation with lymph node metastasis, triple-negative phenotype, high risk of relapse and a worse survival in

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patients with breast cancer (Zhang and Long, 2015; Jiang et al., 2012; Sheng et al., 2017). Currently, the methylation profiles of these genes are the most widely investigated as blood-based biomarkers for breast cancer (Tang et al., 2016). Indeed, assessing the DNA methylation profile of several genes in an integrative manner could greatly increase the sensitivity of cancer detection without affecting specificity. For instance, a 7-gene methylation panel predicts breast cancer progression with 93 % sensitivity and 100 % specificity while individual gene performances showed sensitivities of 63–79 % and specificities of 53–84 % (Li et al., 2015). Furthermore, DNA methylation having occurred at the primary tumor can progressively radiate to surrounding tissues (Teschendorff et al., 2016). A genome-wide analysis for breast tumor and adjacent tissues has clearly demonstrated that increased DNA methylation level in ductal carcinoma in situ is related with future development of invasive breast cancer and with cancer metastasis distance (Johnson et al., 2015; Fleischer et al., 2014). By examining *BRCA1* methylation status in normal tissues adjacent to and distant from tumor, Otani and et al., (2014) found that *BRCA1* methylation can be precursor for *BRCA1*-methylated breast tumors. Similarly, a significant difference of *RASSF1A* and *GSTP1* methylation in breast tumor as compared with normal adjacent tissues was respectively associated with early stage and advanced stage of breast cancer (Hesson et al., 2007; Fang et al., 2015). Therefore, the analysis of DNA methylation profiles in tumor and normal adjacent tissues will provide integrative data to understand malignant progression, metastasis and local recurrence (Casadio et al., 2013).

A high frequency of the methylation status of *BRCA1* (82.1 %) has been primarily described only in tumor but not to normal adjacent tissues collected from Vietnamese women suffering from breast cancer (Truong et al., 2014). In this study, by using the methylation specific polymerase chain reaction (MSP), we investigated the methylation status at the promoter of the three genes encoding *BRCA1*, *RASSF1A* and *GSTP1* in tumor and normal adjacent tissues from Vietnamese breast cancer. It has been remarked that *BRCA1* promoter methylation takes place almost exclusively in the sporadic setting and rarely occurs in patients with *BRCA1* mutations (Esteller et al., 2001; Dworkin et al., 2009). On the other hand, mutations of *BRCA1* in Vietnamese breast cancer patients are among the lowest reported worldwide (Ginsburg et al., 2011). Therefore, this study aims at evaluating the methylation profile of *BRCA1* in particular, as well as those of *RASSF1A* and *GSTP1* genes, both individually and in an integrative manner in order to establish new potential integrative methylation biomarkers for breast cancer detection. Furthermore, the comparison of the methylation profiles of these genes in breast tumor and in normal adjacent tissues will highlight the epigenetically concomitant changes of these genes in breast cancer.

Materials and Methods

Sample collection

Seventy specimens of breast carcinomas and 79 pairs of tumor and matched adjacent normal tissues were collected from breast cancer patients undergoing mastectomy at the Department of Pathology, National Cancer Hospital K, Hanoi, the largest cancer hospital in Vietnam, between 2014 and 2015. The corresponding adjacent tissue samples were selected 3-5 cm away from the site at which the primary tumor was obtained. Breast tumor and corresponding adjacent tissues were snap-frozen in liquid nitrogen immediately after resection and examination by pathologists, and stored at -80^{o}C until further used. The study was approved by the guidelines of the local ethical committee in Vietnam (106-YS.06-2015.07).

Genomic DNAs extraction and bisulfite modification

Genomic DNAs were extracted from freshly frozen tumor and normal adjacent tissues by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Five hundred ng of genomic DNAs were treated with sodium bisulfite by using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA). The efficiency of bisulfite conversion was evaluated using polymerase chain reaction (PCR) that amplifies the bisulfite-treated DNA with primer sets specific to unmethylated sequences of the *β-globin* gene (Lan et al., 2014).

Methylation specific PCR (MSP)

The methylation status of the investigated genes was evaluated by using Methylation Specific Polymerase chain reaction (MSP) with the primers that distinguish methylated (Me) from unmethylated (Un) DNA (Herman et al., 1996). The accuracy of primers specific to only modified targets has been validated as described previously (Lan et al., 2014). The nucleotide primers and MSP conditions for detecting the methylation status of *BRCA1*, *RASSF1A* and *GSTP1* were described previously (Lan et al., 2013, Lan et al., 2016). Bisulfite treated DNAs were subjected to single or nested PCR depending on the particular targeted genes. The MSP products were resolved by electrophoresis in 8% polyacrylamide gel, then stained with ethidium bromide and imaged with the UVP (USA). Genomic DNAs extracted from the PC3 cell line and from lymphocytes of healthy volunteers were treated with bisulfite and used as positive and negative controls for methylation of the targeted genes, respectively. Water with no DNA template was included in each PCR reaction as a control for contamination. All MSP reactions were performed in triplicate.

Statistical Analysis

Chi-square test and Fisher's exact test were used to determine the difference in methylation level of each gene, individually or in combination, between tumor and normal adjacent tissues, as well as their association with clinicopathological characteristics. The Kappa statistic was used to assess the concordance between the methylation status of the studied genes in tumor versus

normal adjacent tissue, as well as the methylation status of genes when assessed two by two in a given tissue type. For all statistical analyses, a p-value of ≤ 0.05 was considered as significant. All analyses were done by using the STATA program version 12 (https://www.stata.com/).

Results

Methylation status of the BRCA1, RASSF1A and GSTP1 in breast tumor and matched normal tissues

To confirm primers specificity to target genes, we first set up a specific MSP assay using native DNAs and primer sets specific to the methylated status of *BRCA1*, *RASSF1A* and *GSTP1*. No MSP products were amplified from untreated DNAs extracted from lymphocytes of healthy donors (Figure 1). Moreover, the MSP products specific to methylated alleles were amplified from bisulfite treated DNA extracted from PC3 cell line (Figure 1). These results confirmed the accuracy of the MSP primers specifically designed for the methylated targets; thus, false positive results were avoided. Subsequently, bisulfite treated DNAs extracted from the tumor and adjacent normal tissues were subjected to the MSP assays. The MSP products representative of methylated/unmethylated sequences of the three promoters *BRCA1*, *RASSF1A* and *GSTP1* were illustrated in Figure 1.

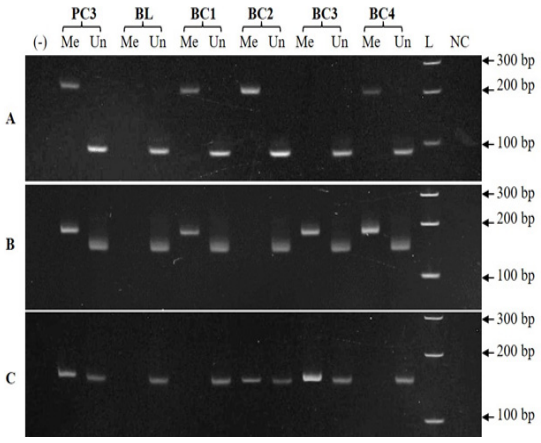

Figure 1. Representative Analysis of MSP Products Showing the Detection of Methylated Sequences (Me) and Unmethylated Ones (Un) of the *BRCA1* (A); *RASSF1A* (B) and *GSTP1* (C) Genes from Breast Cancer (BC) Samples. BL, lymphocytes of the healthy volunteers. PC3, cancer cell line. NC, Native DNA untreated by bisulfite and amplified with primer set specific to methylated targets. (-), Negative control without DNA templates. M, DNA ladder 100 bp. MSP product sizes of the methylated sequences of *BRCA1*, *RASSF1A* and *GSTP1* are 195 bp, 175 bp and 155 bp, and those corresponding to the unmethylated sequences are 77 bp, 135 bp and 149 bp, respectively.

The methylation frequencies of *BRCA1*, *RASSF1A* and *GSTP1* detected from 79 breast tumor samples were 58.23 %, 74.68 % and 59.49 %, respectively, while those detected from 79 matched normal adjacent tissue samples were 51.90 %, 63.29 % and 35.44 %, respectively (Table 1). Only the difference concerning *GSTP1* promoter methylation frequency between these two tissues was statistically significant (p=0.003).

The methylation frequency of at least one of the three target genes was 94.9 % in tumor samples, which is significantly higher compared with 79.8 % found in normal adjacent samples and thus significantly associated with breast cancer (OR=4.76, 95 % CI: 1.51-14.97, p=0.008) (Figure 2). Similarly, the methylation frequency of all the three genes was 31.7 % in breast tumors, which is significantly higher compared to 17.7 % in normal adjacent tissues (OR=2.15, 95 % CI: 1.01-4.58, p=0.04).

As assessed by the calculation of the Chi-square test, there is a high concordance between the methylated status of each gene in tumors and in normal adjacent tissues (p=0.0001; <0.0001; <0.0001, respectively) (Table 1). However, when genes were compared two by two for their methylation state in a given tissue type, only *BRCA1* showed a concordance concerning the methylation state with *RASSF1A* but not *GSTP1*, in normal adjacent tissues but not in tumor (Table 2). Notably, methylated *GSTP1* did not show concordance with any methylated genes in both tumor and normal adjacent tissues.

Association of the methylation status with clinicopathological parameters of breast cancer

Besides the 79 pairs of breast tumor and matched

Table 1. Methylation Frequencies of the Three Genes *BRCA1*, *RASSF1A* and *GSTP1* in Tumor (TU) and Normal Adjacent Tissues (AD). Methylation status was indicated as (+) for methylated and (-) for unmethylated. Numbers in parentheses represent frequencies.

	Number of methylated cases (%)		
	BRCA1	*RASSF1A*	*GSTP1*
TU (n=79)	46 (58.23)	59 (74.68)	47 (59.49)
AD (n=79)	41 (51.90)	50 (63.29)	28 (35.44)
p-value	0.424	0.122	0.003
Methylation status (n=79)			
TU+/AD+	32 (40.5)	48 (60.8)	26 (32.9)
TU+/AD-	14 (17.7)	11 (13.9)	21 (26.6)
TU-/AD+	9 (11.4)	2 (2.5)	2 (2.5)
TU-/AD-	24 (30.4)	18 (22.8)	30 (38.0)
Kappa	0.41	0.62	0.45
p-value	0.0001	<0.0001	<0.0001

Table 2. Concordance of the Methylation Status of *BRCA1*, *RASSF1A* and *GSTP1* Genes in Tumor and Normal Adjacent Tissues

	Kappa efficiency			
	In tumor tissue		In normal adjacent tissue	
Genes	*BRCA1*	*RASSF1A*	*BRCA1*	*RASSF1A*
RASSF1A	0.1064		0.3095	
	p= 0.0913		p=0.0024	
GSTP1	0.1119	-0.0436	0.1236	0.1541
	p=0.0783	p=0.7029	p= 0.1226	p=0.0548

Table 3. Association of the Methylation Status of the Three Genes *BRCA1*, *RASSF1A* and *GSTP1* with Clinicopathological Parameters Analyzed on 149 Breast Cancer Patients. IDC, Invasive Ductal Carcinoma. ILC, Invasive Lobular Carcinoma. Me, Un, methylation and unmethylation status, respectively. p-value is calculated by the Chi-square test, p-value* is calculated by the Fisher's test

Clinicopathological parameters	*BRCA1*			*RASSF1A*			*GSTP1*		
	Me	Un	p value	Me	Un	p value	Me	Un	p value
Age (n=149)									
<50 (n=62)	50	12	0.247	41	21	0.938	37	25	0.382
≥50 (n=87)	63	24		57	30		58	29	
Histological tumor type (n=149)									
IDC (n=120)	89	31	0.332	78	42	0.686	76	44	0.826
Others (n=29)	24	5		20	9		19	10	
Tumor grade (n=112)									
1 (n=11)	9	2		8	3	1*	9	2	
2 (n=86)	57	29	0.01*	57	29		51	35	0.180*
3 (n=15)	15	0		10	5		12	3	
Metastasis status (n=149)									
Yes (n=44)	32	12	0.566	29	15	0.982	32	12	0.140
No (n=105)	81	24		69	36		63	42	
ER status (n=38)									
Positive (n=22)	20	2	1*	12	10	0.309	17	5	0.080
Negative (n=16)	14	2		12	4		8	8	
PR status (n=38)									
Positive (n=20)	18	2	1*	14	6	0.503	14	6	0.564
Negative (n=18)	16	2		10	8		11	7	
Her2 status (n=38)									
Positive (n=29)	25	4	0.554*	19	10	0.67*	18	11	0.456*
Negative (n=9)	9	0		5	4		7	2	
Triple (n=38)									
ER+/PR+/HER2+ (n=11)	9	2	0.497*	8	3	0.627*	7	4	0.596*
ER+/PR+/HER2- (n=7)	7	0		4	3		6	1	
Others (n=37)	33	4	1*	24	13	0.368*	24	13	1*
ER-/PR-/HER2- (n=1)	1	0		0	1		1	0	

normal adjacent tissues, we included 70 additional tumor samples in order to study the association between the methylation status of *BRCA1*, *RASSF1A* and *GSTP1* with patients' clinicopathological features (Table 3). *BRCA1* methylation was significantly associated with tumor grade (p=0.01). However, no significant association between the methylated status of *RASSF1A* and *GSTP1* was observed with any clinocopathological parameter.

Discussion

Breast cancer-associated changes in promoter methylation of numerous genes have been validated by a genome wide analysis based on technological advances such as DNA microarrays or by the analysis of a particular gene based on PCR approaches (Van der Auwera et al., 2010; Teschendorff et al., 2016). The validation showed

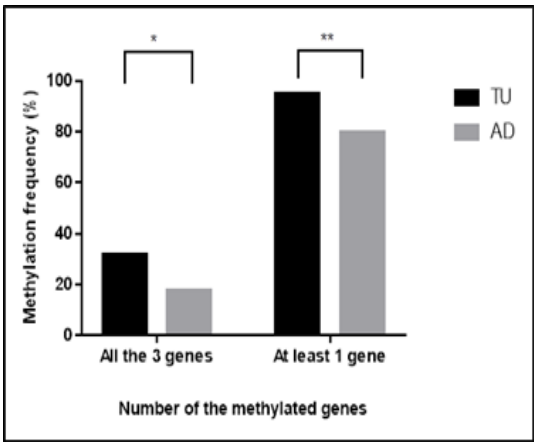

Figure 2. Methylation Frequency of *BRCA1*, *RASSF1A* and *GSTP1* in Tumors (TU-black bar) and Normal Adjacent Tissues (AD-grey bar).

that breast tumor tissue at different stages and the tissue adjacent to the tumor can be distinguished from each other based on the methylation frequency of a particular gene or gene panel (Lewis et al., 2005; Zhu et al., 2010, Johnson et al., 2015). Among the genes whose aberrant methylation is closely involved in carcinogenesis, the three genes *BRCA1*, *RASSF1A* and *GSTP1* have been previously shown to be the most frequently methylated in breast cancer. Their methylation in breast tumor has been found to be significantly elevated in comparison with normal adjacent tissues and usually associated with clinicopathological features (Yan et al., 2006; Lee, 2007, Zhang and Long, 2015). Therefore, their methylation status has been considered as a potential biomarker panel for diagnosis and prognosis of breast cancer (Cheuk et al., 2017; Geng and Wu, 2016).

In the present study, we investigated the methylation frequency of the three genes *BRCA1*, *RASSF1A* and *GSTP1* in Vietnamese women suffering from breast cancer using the MSP method (Herman et al., 1996). Frequent occurrence of methylation at the three promoters was found in both breast tumor and normal adjacent tissues; however, only *GSTP1* methylation frequency was significantly associated with tumor (p=0.003) (Table 1). Significant association between *GSTP1* methylation and breast tumor has been also described previously (Fang et al., 2015; Bhat et al., 2017). Concerning the association of *BRCA1* and *RASSF1A* methylation with breast tumor as shown in some previous studies (Cho et al., 2010; Grawenda and O'Neill, 2015), our results are in line with several other previous reports showing an absence of association. No difference in methylation of *BRCA1* and *RASSF1A* was found in breast ductal carcinoma in situ (DCIS) samples and paired normal adjacent samples (Honorio et al., 2003; Pang et al., 2014), neither in breast tumor relative to matched adjacent tissue (Jung et al., 2013, Yeo et al., 2005). Choosing the end-point MSP method as in our study, previous reports did not find any significant difference in *BRCA1* and *RASSF1A* methylation between the tumor and corresponding adjacent tissues (Cho et al., 2010, Hosny et al., 2016). Recently, comprehensive reviews have concluded that the difference in *BRCA1* and *RASSF1A* methylation was pretty marginal between tumor and adjacent tissues, supporting our finding (Zhang and Long, 2015; Geng and Wu, 2016). This insignificant difference in *BRCA1* and *RASSF1A* methylation frequencies from tumor to adjacent tissues in previous studies as in ours could be explained by the difficulty to get an adjacent tissue uncontaminated with malignant cells and the unclear determination of the geographic site of the adjacent tissue away from the tumor site (Yan et al., 2006; Otani et al., 2014). Alternatively, the wide variance in DNA methylation of a particular gene could be explained by epigenetic and cellular heterogeneity in breast cancer (Tian et al., 2016; Beca and Polyak, 2016).

Although when considered individually only *GSTP1* methylation is associated with tumor tissue, when the three genes were assessed together as a gene panel, we showed that the methylation of at least one of the three genes or all the three genes are both significantly associated with breast tumor (Figure 2). Additionally, the methylation of each gene was concordant in tumor and adjacent tissues, and especially, the methylation of *BRCA1* and *RASSF1A* was concordant in adjacent tissue. These results emphasized the need for application of different biomarkers including this three-gene panel in breast cancer diagnosis (Zardavas et al., 2015; Song et al., 2016; Choi et al., 2017). Moreover, it has been proposed that *BRCA1* promoter methylation takes place almost exclusively in the sporadic setting and rarely occurs in patients with *BRCA1* mutations (Esteller et al., 2001; Dworkin et al., 2009). The lowest frequency of *BRCA1* mutations (1 %) but the highest frequency of *BRCA1* methylation (82.1 %) worldwide so far were previously found in Vietnamese patients with breast cancer as in this study (75.8 %, 113/149 tumor samples) (Ginsburg et al., 2011; Truong et al., 2014; Zhang and Long, 2015). Therefore, it is reasonable to propose that the *BRCA1* methylation could serve as a prescreening test in our country where a hereditary nature is inappreciable.

Significant association between methylation frequency with clinicopathological parameters of breast cancer patients was shown for *BRCA1* but neither for *RASSF1A* nor *GSTP1* (Table 3). A comprehensive review has concluded that *RASSF1A* methylation is frequently elevated in primary tumor tissues and remains constant across all stages during breast cancer development (Geng and Wu, 2016). Moreover, a meta-analysis has also concluded that no significant association was identified between *GSTP1* promoter methylation and histological grade (Fang et al., 2015). These conclusions support to our finding showing no relation between *RASSF1A* and *GSTP1* methylation and clinicopathological parameters of breast cancer patients. In the other hand, although *BRCA1* methylation cannot be discriminated between tumor and adjacent tissue, it is significantly associated with breast tumor grade. Similar result was reported in Korean, Chinese and Thai patients with breast cancer (Jung et al., 2013; Chen et al., 2009; Saelee et al., 2014). Recently, a critical value of *BRCA1* methylation in prognosis has been confirmed and supports our finding concerning

the association of *BRCA1* methylation with histologic tumor grade (Guo et al., 2015; Li et al., 2015). However, we did not find any significant association of *BRCA1* methylation with hormone phenotypes, which has been more often occurred among breast cancer patients with negative ER, PR and HER2 expression (Jung et al., 2013; Sharma et al., 2014). It is noteworthy that *BRCA1* methylation was considerably dependent on the targeted CpG sites in triple negative breast cancer (Daniels et al., 2016); thus, the end-point MSP method used in this study, an assay extremely sensitive for detecting any DNA methylation at priming site only, could be unsuitable to find out the association and should be substituted by a quantitative method.

To summarize, this study has chosen the non quantitative MSP method for the analysis of DNA methylation, a method that has been widely used in numerous studies numerous studies given its simplicity, high sensitivity and low cost (Kristeen et al., 2009). given its simplicity, high sensitivity and low cost. The encouraging results obtained here now prompt us to quantitatively investigate the methylation level of the three promoters of *BRCA1*, *RASSF1A* and *GSTP1*, as well as the methylation spectrum at CpG sites in their promoter regions in breast cancer. Women in Vietnam are diagnosed with breast cancer at an early age with more aggressive tumors and an increasing incidence rate that exceeds that of the Western world (Trieu et al., 2015). Therefore, choosing suitable DNA methylation markers and optimizing detection techniques will considerably contribute to the effective breast cancer diagnosis in our country.

Conflict of interest

The authors Vu Lan Trang, Nguyen Thu Trang, Doan Thi Hong Van and Vo Thi Thuong Lan declare that they have no conflict of interest.

Acknowledgments

This study was financially supported by Grant 106-YS.06-2015.07 from the Ministry of Science and Technology, Viet Nam.

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