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ABERRANT METHYLATION OF CANCER-RELATED GENES IN VIETNAMESE BREAST CANCER PATIENTS: ASSOCIATIONS WITH CLINICOPATHOLOGICAL FEATURES

Background. Epigenetic alteration is one of the most common molecular changes identified in the progression of breast cancer (BC). **Aim.** To study the frequency and relation between methylation of *BRCA1, MLH1, MGMT, GSTP1, APC, RASSF1A, p16, WIF,* and *EGFR* and the clinicopathological features in Vietnamese BC patients. **Materials and Methods.** Methylation-specific polymerase chain reaction (MS-PCR) and SPSS 20.0 software were utilized in order to identify methylated frequency as well as evaluate its relationship with the patient's clinical features. **Results.** In 162 BC cases, the methylation rates of the selected genes were 53.7%, 22.8%, 38.9%, 34.6%, 29.0%, 46.3%, 20.4%, 18.5%, and 28.4% respectively. In 32 cases of benign breast diseases (BBD) – 12.5%, 15.6%, 6.3%, 3.1%, 12.5%, 21.9%, 3.1%, 15.6% and 3.1%. BC samples displayed higher *BRCA1, MGMT, GSTP1, APC, RASSF1A, WIF1*, and p16 methylation levels than BBD samples ($p < 0.001$). Hypermethylation of *BRCA1, GSTP1*, and *RASSF1A* was predominant in the invasive ductal carcinoma, while hypermethylation of *BRCA1, GSTP1, RASSF1A, WIF-1*, and p16 was found to significantly correlate with lymph node metastasis ($p < 0.05$). Hypermethylation of *BRCA1, MGMT*, and GSTP1 was more common in stage III ($p < 0.05$) than in stages I/II, whereas MLH1 methylation was predominant in stage I and *APC* methylation was less common in stage III ($p = 0.03$). In addition, methylation of *RASSF1A* and *EGFR* was more frequent in younger patients ($p < 0.01$) than in elder patients. **Conclusion.** These data suggest that a gene panel (*BRCA1/MGMT/GSTP1*) can be used to support the diagnosis and screening of Vietnamese patients' BC with a sensitivity of 70%, and a specificity of 85%.

Keywords: DNA methylation, breast cancer, clinicopathological features, panel, sensitivity/specificity.

Worldwide, breast cancer (BC) has become one of the most common types of solid cancer in women. Although BC used to be a common disease in the developed world, recently ~50% of new BC cases and related deaths have occurred in developing countries [1]. Understanding

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how epigenetic changes are related to BC clinicopathological features could lead to a better understanding of carcinogenesis and possibly to improvements in prevention, diagnosis, and treatment [2—4]. BC at an initial stage has a better prognosis and requires less extreme therapeutics with a high survival rate, up to more than 95%. However, the diagnosis after tumor metastasis significantly reduces the survival rate to 27% [5]. Molecular biomarkers serve as an revolutionary approach in BC diagnosis and prognosis.

Epigenetic alteration is one of the most common molecular changes identified in the progression of human cancer [6, 7]. The epigenetic mechanisms include aberrant DNA methylation, changes in the histone and chromatin structure by post-translational modification of histone proteins, and alterations in the expression of microRNA [8]. DNA methylation occurring at CpG dinucleotides, which are frequently located in the promoter regions, is well known as an epigenetic regulation mechanism for the transcriptionally silencing gene expression [9]. CpG islands that are normally unmethylated may become methylated in cancer cells, which can result in altered expression, including silencing of tumor suppressor or DNA repair genes [10].

Over 100 genes have been identified as aberrantly methylated in breast tumors or breast cancer cell lines [10]. These genes comprise functionally essential genes, such as tumor suppressor genes, DNA repair genes, genes linked with detoxification, and genes involved in signaling pathways and cell cycle control [11—13]. However, there is limited evidence regarding the extent to which alterations in methylation are associated with the clinicopathological features. In addition, conflicting results have been reported, which may be caused by varying populations and patient characteristics. In order to better understand the role of methylation in breast carcinogenesis, we evaluated the association of methylation of 9 genes significant in the regulation of cellular processes (BRCA1, MLH1, MGMT, GSTP1, APC, RASSF1A, p16, WIF1, and EGFR) in breast tumors with the clinicopathological features in a population-based study.

Materials and Methods

Sample collection. A total of 162 female patients with BC and 32 female patients with benign breast diseases (BBD) were recruited from the Vietnam National Cancer Hospital (Hanoi, Vietnam) between December 2020 and December 2021. Patients were diagnosed via pathological evidence. The patients gave their informed consent for using their samples for the research purposes. All the procedures were approved by the Vietnamese Ethics Committee (Circular No.458/2012/QĐ-BYT). The formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were cut into slices (5 μm thick) and stained with hematoxylin & eosin (H&E) using the Dako Coverstainer system (Agilent, US) for histopathological evaluation, following the manufacturer's protocol.

DNA extraction. Genomic DNAs were isolated from FFPE blocks of tumor and normal adjacent tissues with the QIAamp DNA Mini Kit (Qiagen, US).

Bisulfite modification. 300—500 ng of total DNA were treated with sodium bisulfite by utilizing the EpiTect Bisulfite Kit (Qiagen, US). The capability of DNA modification was analyzed by polymerase chain reaction (PCR) technique, which amplifies the bisulfite-treated DNA with primer sets specific to the housekeeping gene (β-globin).

Methylation-specific PCR (MS-PCR). The methylation patterns of 9 selected genes were determined through MS-PCR with specific primers for DNA methylated sequences (Me) and unmethylated sequences (Un).

Statistical analysis. The Chi-square test and Fisher's exact test were utilized to measure the

variation in methylation level concerning targeted gene, individual or else in combination, between BC and BBD tissues, along with their relation with the patient's clinicopathological parameters. For each comparison, $p \leq 0.05$ was considered significant. All analyses were done by using the SPSS software 20.0.

Results

Methylation status of BRCA1, MLH1, MGMT, GSTP1, APC, RASSF1A, WIF1, EGFR, and p16 in BC and BBD. The methylation frequencies of *BRCA1, MLH1, MGMT, GSTP1, APC, RASSF1A, WIF1, EGFR,* and *p16* detected in 162 BC samples were 53.7%, 22.8%, 38.9%, 34.6%, 29.0%, 46.3%, 20.4%, 18.5%, and 28.4% while those detected in 32 BBD samples were 12.5%, 15.6%, 6.3%, 3.1%, 12.5%, 21.9%, 3.1%, 15.6%, and 3.1% (Table 1).

Overall, BC samples displayed higher *BRCA1, MGMT, GSTP1, APC, RASSF1A, WIF1* and p16 methylation levels than BBC samples ($p < 0.001$ for 7 genes), whereas no differences were found for *MLH1* and *EGFR* methylation.

The methylation frequency of at least 1 of 9 target genes was 94.4% (153/162) in tumor samples, which is significantly higher compared to 59.4 % (19/32) found in BBD and thus significantly associated with BC ($p < 0.0001$). Except for *MLH1* and EGFR, the rate of methylation of at least 1 of 7 genes in BC and BBD was 148/162 (91.4%) *vs.* 14/32 (43.8%), respectively ($p < 0.05$). With the preliminary results when analyzing the methylation of 9 genes related to BC, we suggested choosing a panel of 2 or 3 genes (preferring genes with high methylation frequency in tumor tissue as well as distinguishing from BBD).

Subsequently, the sensitivity and specificity of the DNA methylation signatures in the BC samples were evaluated using the receiver operating characteristic (ROC) analysis to further assess the predictive accuracy of the DNA methylation signature. The sensitivity and specificity of each gene in distinguishing BC were calculated (Table 1). The AUC for selected genes ranged from 0.514 to 0.706. The sensitivity of each gene ranged from 18.5% to 53.7% and the specificity ranged from 78.1% to 96.9%. The areas under ROC curves (AUC) were also calculated when the combination of 2 or/and 3 genes was used. For 2 genes, in case of hypermethylation in at least one gene, the AUC for *BRCA1/MGMT, MGMT/GSTP1*, and *BRCA1/GSTP1* was 0.758, 0.740, and 0.737 (95% confidence interval (CI),

Table 1. **Diagnostic performance of methylated genes-candidates**

Gene	BC pos./total	BBD pos./total	Sensitivity (%)	Specificity (%)	AUC	95% CI	*p*
BRCA1	87/162	4/32	53.7	87.5	0.706	0.618—0.794	< 0.00001
MLH1	37/162	5/32	22.8	84.4	0.536	0.430—0.642	0.365
MGMT	63/162	2/32	38.9	93.7	0.663	0.574—0.752	0.0004
GSTP1	56/162	1/32	34.6	96.9	0.657	0.569—0.745	0.0004
APC	47/162	4/32	29.0	84.4	0.567	0.464—0.670	0.05
RASSF1A	75/162	7/32	46.3	78.1	0.622	0.522—0.722	0.01
WIF1	33/162	1/32	20.4	96.9	0.586	0.488—0.684	0.02
EGFR	30/162	5/32	18.5	84.4	0.514	0.406—0.623	0.697
p16	46/162	1/32	28.4	93.7	0.611	0.515—0.706	0.002

0.675—0.841, 0.658—0.822, 0.652—0.822), with a sensitivity of 64.2%, 57.4%, and 59.9% and a specificity of 87.5%, 90.6%, and 87.5%, respectively. When 3 candidate genes were used, the AUC for the selected genes (*BRCA1/MGMT/GSTP1*) was 0.80, with a sensitivity of 70% and a specificity of 85%, approximately.

Association of methylation status with clinicopathological parameters in breast tumors. *BRCA1, GSTP1,* and *RASSF1A* hypermethylation was predominant in the invasive ductal carcinoma ($p < 0.05$). Together, methylation of *BRCA1, GSTP1, RASSF1A, WIF1*, and *p16* was found to significantly correlate with lymph node metastasis ($p < 0.05$). In addition, the *RASSF1A* and *EGFR* methylation was more frequent in younger patients ($p < 0.01$) than in elder ones, while *MLH1* methylation was predominant at stage I of the disease ($p = 0.014$). *BRCA1, MGMT,* and *GSTP1* hypermethylation was more common at stage III ($p < 0.05$) than at stages I and II, whereas APC methylation was less common at stage III ($p = 0.03$) (Table 2).

Discussion

Promoter methylation of BC-related genes has been demonstrated by a specific molecular analysis based on DNA microarrays or else by the analysis of a particular gene based on the PCR approaches [14]. The aberrant DNA methylation has some analytical features that are appealing and potentially useful. It is clear that BC tissue at different stages and the tissue adjacent to the tumor and/or benign tissues can be differentiated from each other by the DNA methylation status of a particular gene or a gene panel [15, 16]. Among the genes whose aberrant methy-

Table 2. ***BRCA1, MLH1, MGMT, GSTP1, APC, RASSF1A, WIF1, EGFR,*** **and** ***P16*** **association with clinico-**

		BRCA1		MLH1		MGMT		GSTP1	
		Yes	*p*	Yes	*p*	Yes	*p*	Yes	*p*
	162	87		37		63		56	
Age			0.912		0.826		0.203		0.838
< 50.8	77	41		17		26		26	
> 50.8	85	46		20		37		30	
Histological subtypes			< 0.001		0.854		0.072		0.009
IDC*	113	82		30		56		52	
Others	29	5		7		7		4	
Tumor grade			< 0.001		0.072		< 0.001		< 0.001
1	4	1	0.255	3	0.014	3	0.133	0	0.296
2	79	41	0.856	16	0.255	24	0.065	19	0.001
3	56	41	0.008	14	0.384	32	< 0.001	32	< 0.001
Unclassified	23	4		4		4		5	
Lymph node metastasis			< 0.001		0.626		0.411		< 0.001
Yes	58	44		12		25		42	
No	104	43		25		38		14	
Tumor size			0.655		0.126		0.922		0.582
< 5 cm	121	67		31		47		44	
≥ 5 cm	20	10		2		8		6	
Unknown	21	10		4		8		6	

Notes: *Invasive ductal carcinoma; $\chi 2$ test; Fisher's exact test.

lation is closely involved in carcinogenesis, 9 genes (*BRCA1, MLH1, MGMT, GSTP1, APC, RASSF1A, WIF1, EGFR,* and *p16*) have been previously shown to be frequently methylated in BC. Their methylation in BC has been found to be significantly elevated in comparison with normal adjacent tissues and is considered a potential biomarker panel for BC diagnosis and prognosis [17—19].

In the present study, we investigated the methylation frequency of the genes *BRCA1, MLH1, MGMT, GSTP1, APC, RASSF1A, WIF1, EGFR,* and *p16* in Vietnamese women suffering from BC and BBD using the MS-PCR method. The frequent occurrence of methylation at 9 promoters was found in both BC and benign tissues; however, only *BRCA1, MGMT, GSTP1, APC, RASSF1A, WIF1,* and *p16* methylation frequency was significantly associated with tumor ($p < 0.05$) as has been shown in some previous studies [20—22]. Patients with localized BC have a 5-year survival rate of 98%. Nevertheless, in the case of diagnosis with metastasis, the survival percentage falls dramatically to approximately 20%. These reports point out the benefit of screening and early determination, and the vital significance of searching for novel biomarkers to go hand in hand with mammography results. Therefore, the methylation status has been considered as a potential biomarker panel for the BC diagnosis and prognosis.

In this study, we evaluated associations between methylation of 9 selected genes and clinicopathological features of BC cases. Several associations were identified and were gene-specific. For instance, hypermethylation of *BRCA1, GSTP1,* and *RASSF1A* tended to be more frequent in an invasive ductal carcinoma, while

pathological parameters

APC		RASSF1A		WIF1		EGFR		p16	
Yes	*p*	Yes	*p*	Yes	*p*	Yes	*p*	Yes	*p*
47		75		33		30		46	
	0.819		< 0.001		0.789		< 0.001		0.962
23		44		15		25		22	
24		31		18		5		24	
	0.474		0.026		0.332		0.740		0.310
37		67		29		24		40	
10		8		4		6		6	
	0.061		0.078		0.550		0.304		0.248
2	0.313	1	0.6215	0	0.577	1	0.519	2	0.587
28	0.065	39	0.610	18	0.693	1	0.374	21	0.203
9	0.028	26	0.838	12	0.971	7	0.292	20	0.317
8		9		3		7		3	
	0.252		0.252		0.035		0.340		0.044
20		36		17		13		22	
27		39		16		17		24	
	0.074		0.074		0.557		0.597		0.982
36		55		25		24		36	
10		9		3		5		6	
1		11		5		1		4	

associations between methylation of *BRCA1, MGMT, GSTP1, APC,* and *MLH1* and prognostic factors depended on the patient's age. Additionally, we found that methylation of the selected genes, such as *RASSF1A* and *EGFR* genes, was only associated with younger BC patients. We also observed that methylation of *BRCA1, GSTP1, RASSF1A, WIF-1* and *p16* was strongly associated with lymph node metastasis. The results of prior studies of methylation of selected genes in BC are contradictory [23]. The present study has reported that BRCA1 methylation is involved in BC late-stage progression. Likewise, prior reports suggested a role of *BRCA1* methylation in the aggressiveness of BC and that *BRCA1* methylated tumors were found mainly in grade III tumors rather than in grades I and II [24, 25]. A comprehensive review has concluded that *RASSF1A* methylation is frequently elevated in primary tumor tissues and remains constant across all stages during BC development [18]. Furthermore, a meta-analysis has additionally declared that no meaningful relation was confirmed between *GSTP1* hypermethylation and the histological grade [26]. These conclusions were inconsistent with our findings showing the relation between *RASSF1A* and *GSTP1* methylation and clinicopathological parameters of BC patients. This suggests that BC patients with *GSTP1* and/or *RASSF1A* promoter hypermethylation may have a biologically aggressive phenotype.

Additionally, *RASSF1A* and *EGFR* methylation was highly correlated with younger age of the patients ($p < 0.01$). Although associations between gene promoter methylation and age have been reported, they are not a universal finding, and results are even conflicting concerning breast tissues [27, 28]. Our study hypothesized that the age-associated pattern of *RASSF1A* and/or *EGFR* methylation suggests that aging is associated with the acquisition of low levels of methylation in more patients. Moreover, methylation of both *MLH1* and *APC* is related to the early stages compared with the late stages, suggesting that methylation of these selected genes is an early event that may predispose to methylation of other tumor suppressor genes [29]. This understanding suggests a potential of using the epigenetic markers, alone or in combination with others, either for predictable detection of BC or for clinical routine risk evaluation.

Our study has several limitations, in particular, the small sample size, while the difference in each molecular diagnosis might slightly affect the overall frequency of DNA hypermethylation. These challenges require further study in future research.

To sum up, aberrant DNA methylation has some signature features that may potentially be useful for early screening of solid cancer. Our data suggest that the gene panel (*BRCA1/MGMT/GSTP1*) could be used to support the diagnosis and screening of Vietnamese BC patients with a sensitivity of 70% and a specificity of 85%.

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АБЕРАНТНЕ МЕТИЛЮВАННЯ АСОЦІЙОВАНИХ З РАКОМ ГЕНІВ У ХВОРИХ НА РАК МОЛОЧНОЇ ЗАЛОЗИ З В'ЄТНАМУ: ЗВ'ЯЗОК З КЛІНІКО-ПАТОЛОГІЧНИМИ ОСОБЛИВОСТЯМИ

Вступ. Епігенетичні альтерації є одними з найпоширеніших молекулярних змін, які беруть участь у прогресуванні раку молочної залози (РМЗ). **Мета.** Дослідити частоту між метилюванням генів *BRCA1, MLH1, MGMT, GSTP1, APC, RASSF1A, p16, WIF1* та *EGFR*, а також його зв'язок з клініко-патологічними особливостями хворих на РМЗ з В'єтнаму. **Матеріали та методи.** Для визначення частоти метилювання використано метод метил-специфічної полімеразної ланцюгової реакції (MS-PCR). Оцінку зв'язку рівнів метилювання досліджуваних генів з клініко-патологічними особливостями пацієнтів проводили із застосуванням програмного забезпечення SPSS 20.0. **Результати.** У 162 зразках тканини РМЗ рівень метилювання досліджуваних генів становив 53,7%, 22,8%, 38,9%, 34,6%, 29,0%, 46,3%, 20,4%, 18,5% і 28,4%. У зразках доброякісних новоутворень молочної залози (ДНМЗ) у 32 пацієнток рівні метилювання зазначених генів складали 12,5%, 15,6%, 6,3%, 3,1%, 12,5%, 21,9%, 3,1%, 15,6% та 3,1%. Встановлено, що зразки РМЗ характеризувалися вищими рівнями метилювання *BRCA1, MGMT, GSTP1, APC, RASSF1A, WIF1* і p16, у порівнянні з ДНМЗ ($p < 0,001$). Встановлено, що гіперметилювання *BRCA1, GSTP1 і RASSF1A* є характерною ознакою інвазивної протокової карциноми молочної залози, тоді як гіперметилювання *BRCA1, GSTP1, RASSF1A, WIF1* і p16 асоціюється з наявністю метастазів у лімфатичні вузли ($p < 0,05$). Визначено, що гіперметилювання *BRCA1, MGMT і GSTP1* частіше зустрічалось у пацієнтів з III стадією РМЗ ($p < 0,05$), у порівнянні з хворими на I/II стадії. Метилювання *MLH1* частіше спостерігали у хворих з I стадією РМЗ, а також варто зазначити, що метилювання *APC* рідше виявлялось у пацієнток із III стадією РМЗ ($p = 0,03$). Крім того, у пацієнток молодшого віку частіше спостерігалось метилювання *RASSF1A* та *EGFR* ($p < 0,01$) порівняно з хворими на РМЗ старшого віку. **Висновок.** Отримані нами дані свідчать про можливість використання для діагностики та скринінгу РМЗ у в'єтнамських пацієнтів генної панелі (*BRCA1/MGMT/GSTP1*) із чутливістю 70,0% та специфічністю 85,0%.

Ключові слова: метилювання ДНК, рак молочної залози, клініко-патологічні ознаки, панель, чутливість/специфічність.