VNU Journal of Science: Natural Sciences and Technology, Vol. 32, No. 1S (2016) 207-212

Aberrant Promoter Methylation of *BRCA1* and *RASSF1A* in Tumor and Paired Adjacent Normal Tissues from Vietnamese Patients with Breast Cancer

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Received 02 June 2016
Revised 02 August 2016; Accepted 09 Septeber 2016

Abstract: DNA promoter methylation, a main way of epigenetic regulation, has been studied for detection, prognosis and treatment of breast cancer. In this study, methylation specific polymerase chain reaction (MSP) was used to analyze the promoter methylation of 2 tumor suppressor genes *BRCA1* and *RASSF1A* in tumor and paired adjacent normal tissues of 76 Vietnamese breast cancer patients. We found that tumor and paired adjacent normal tissues were frequently hypermethylated for the two tested genes. The *BRCA1* and *RASSF1A* were highly methylated in tumors (60.5% and 76.3%) and adjacent normal tissues (52.6% and 65.8%), respectively. Further more, there was a high agreement between *BRCA1* and *RASSF1A* methylation in tumor and adjacent tissues ($p=0.000050$ and $p<0.000001$). But the differences between methylation in tumor and adjacent tissues were not observed with these genes. On the other hand, there was a significant association between tumor grade and *BRCA1* methylation in tumor tissues ($p=0.035430$), but not with *RASSF1A*. Beside that, no significant association was observed between methylation status of the two genes and other clinicopathological factors of tumors (age, histological tumor type and metastasis status).

Keywords: Promoter methylation, *BRCA1*, *RASSF1A*, adjacent normal tissues, breast cancer.

1. Introduction

Breast cancer is the most common cancer and the first cause of cancer-death among females worldwide. In Vietnam, this is the most common cancer with 11,067 new cases and the leading cause of death in cancers with 4,671 deaths in 2012 [1]. Even though diagnosis by screening mammography is believed to be responsible for the significant decline in breast cancer mortality, the limitations of mammography are well recognized, especially for women with premenopausal breast cancer [2]. Thus, new approaches for breast cancer detection are clearly needed to improve diagnosis and prognosis.

Many studies have demonstrated that DNA methylation can contribute to the inactivation of tumour suppressor genes, which is a key event in tumorigenesis of a spectrum of human tumours. Nowadays, DNA methylation is

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widely accepted as a potential source of biomarkers for breast cancer detection, prognosis and treatment [3, 4]. Aberrant methylation is frequently found in breast tumors with more than 40 tumor suppressor genes shown to be inactivated by CpG promoter hypermethylation [5]. Among these genes, breast cancer susceptibility gene 1 (*BRCA1*) [3, 4, 6] and Ras association domain family 1A gene (*RASSF1A*) [4, 7] are frequently methylated. They are important tumor suppressor genes in breast cancer. The *BRCA1* gene encodes a multifunctional protein that is involved in DNA repair, cell cycle control and chromatin remodeling [3, 6]. The *RASSF1A* is involved in several growth regulating and apoptotic pathways; and regulates cell proliferation, cellular integrity and cell death [4, 14].

Recent studies have reported on the increases in aberrant DNA methylation in adjacent normal tissues of the both two genes [4, 7, 8]. Although data so far have been limited, information on the presence of *BRCA1* and *RASSF1A* methylation in the adjacent normal tissues to breast cancer may be an important predictor of breast cancer risk or help explain the high local recurrence rate with breast conserving surgery alone [4, 7].

In our previous works, we examined the methylation status of *BRCA1* and *RASSF1A* in ovarian and breast tumors in Vietnamese women [9, 10]. Until now, DNA methylation in adjacent normal tissues of breast cancer patients has not been reported in Vietnamese women yet. Therefore in the present study, we primarily investigated the methylation status of *BRCA1* and *RASSF1A* in breast tumors and paired adjacent normal tissues using the methylation specific polymerase chain reaction (MSP) assay. The specific aims were to: (1) determine aberrant methylation of *BRCA1* and *RASSF1A* genes in breast tumor and paired adjacent normal tissues; (2) compare the aberrant methylation between the breast tumour and paired adjacent normal tissues, and (3) assess if methylation status correlates with clinicopathological factors in the patients.

2. Materials and methods

Sample collection

Surgically resected specimens from breast carcinomas, matched adjacent normal tissues were collected from 76 breast cancer patients undergoing mastectomy at the Department of Pathology, National Cancer Hospital K, Hanoi, the largest cancer hospital in Vietnam between 2012 and 2013 after approval of the study by the local ethical committee in Vietnam. The corresponding adjacent normal tissue sample was selected 3-5 cm away from the site at which the primary tumor was sampled.

DNA preparation/sodium bisulfite conversion

Genomic DNAs were extracted by using the E.Z.N.A.® Tissue DNA Kit (Omega) and then treated with bisulfite using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions.

Methylation analysis

After sodium bisulfite conversion, genomic DNA was analyzed by the MSP assay as described by Herman et al. [11]. The primers and MSP conditions for detection of *BRCA1* and *RASSF1A* methylation were previously described [9, 10]. Then PCR products were resolved by electrophoresis in a 8% polyacrylamide gel, and the ethidium bromide-stained PCR products were imaged with the UVP (USA).

Statistical analysis

Statistical analyses were done with MedCalc version 13.0.6.0 (http://www.medcalc.org/). P-values were calculated using Fisher's exact test (2-sided). $P<0.05$ were considered statistically significant.

3. Results

3.1. Promoter methylation in tumor and paired adjacent normal tissues

The genomic DNAs extracted from tumor and paired adjacent normal tissues of 76 breast cancer patients were treated with bisulfite and subjected directly to the MSP.

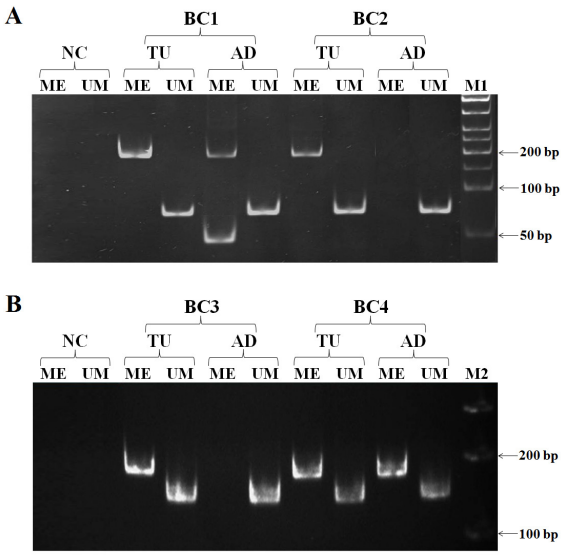

Fig. 1. Representative results of the methylation analysis of *BRCA1* (A) and *RASSF1A* (B) in tumor (TU) and paired adjacent normal (AD) tissues from the breast cancer patients (BC1-BC4). The PCR products in lanes ME and UM indicate the presence of methylated (195 bp with *BRCA1*, 170 bp with *RASSF1A*) and unmethylated (77 bp with *BRCA1*, 135 bp with *RASSF1A*) sequences. NC: Negative control without DNA templates. M1: 50-bp DNA ladder. M2: 100-bp DNA ladder.

Representative results of the MSP products for methylation status of *BRCA1* and *RASSF1A* were shown in Figures 1A and 1B, respectively. The MSP analysis revealed that tumor and paired adjacent normal tissues were frequently hypermethylated for two genes tested. In particular, *BRCA1* and *RASSF1A* were highly methylated in tumors (60.5% and 76.3%, respectively) and paired adjacent normal tissues (52.6% and 65.8%), respectively (Table 1).

Table 1. Promoter methylation of *BRCA1* and *RASSF1A* in tumor, paired adjacent normal tissue from breast cancer cases

Number of methylated cases (%)		
Source of DNA	*BRCA1*	*RASSF1A*
TU (n=76)	46 (60.5%)	58 (76.3%)
AD (n=76)	40 (52.6%)	50 (65.8%)
p value	0.4133	0.2104
Methylation status		
TU ME/AD ME	33 (82.5%)	48 (96.0%)
TU UM/AD ME	7 (17.5%)	2 (4.0%)
p value	0.000050	< 0.000001

TU: Tumor tissue, AD: Adjacent tissue, ME: Methylated, UM: Unmethylated

3.2. Comparison of aberrant methylation of BRCA1 and RASSF1A between breast tumor and paired adjacent normal samples

In order to determine concordance between promoter methylation in tumor and paired adjacent normal tissues, the pair-wise agreement was estimated for each gene. As shown in Table 1, hypermethylation of *BRCA1* and *RASSF1A* in tumor and paired adjacent normal tissues were highly concordant ($p=0.000050$ and $p<0.000001$, respectively, Fisher's exact test). But there was no significant difference in promoter methylation of *BRCA1* or *RASSF1A* between tumor and paired adjacent normal tissues ($p=0.4133$ and $p=0.2104$, respectively, Fisher's exact test) (Table 1).

3.3. Relationship between BRCA1 and RASSF1A promoter methylation in breast cancer tissues and clinicopathological factors

The age, histological tumor type, tumor grade and metastasis status of the 76 breast cancer patients, and promoter methylation of the *BRCA1* and *RASSF1A* were illustrated in Table 2. There was a significant association between tumor grade and *BRCA1* methylation in tumor tissues ($p=0.035430$), but not with *RASSF1A*. No significant association was

observed between methylation status of two tested genes and other clinicopathological factors of tumors (age, histological tumor type and metastasis status).

Table 2. Patient clinicopathological characteristics and their relationship with *BRCA1* or *RASSF1A* promoter methylation

Clinicopathological factors	*BRCA1*			*RASSF1A*		
	Methylated	Unmethylated	p value	Methylated	Unmethylated	p value
Age						
<50 (n=33)	22	11	0.356283	25	8	1.000000
≥50 (n=43)	24	19		33	10	
Histological tumor type						
IDC (n=64)	38	26	0.754124	50	14	0.462547
Others (n=12)	8	4		8	4	
Tumor grade						
1+2 (n=56)	30	26	0.035430	44	12	1.000000
3 (n=6)	6	0		5	1	
Metastasis status						
Yes (n=41)	22	19	0.803455	33	8	0.388912
No (n=27)	16	11		19	8	

IDC: Invasive Ductal Carcinoma.

4. Discussion and conclusion

DNA methylation of many tumor suppressor genes plays an important role in tumorigenesis. Promoter hypermethylation of the *BRCA1* and *RASSF1A* have been detected frequently in breast cancer in many studies [3, 4, 7]. In the present study, *BRCA1* hypermethylation was detected in 60.5% of the cases, which was relatively high and consistent with other previous reports (5.2% to 65.2%) [12, 13]. As the same way, our study revealed that the majority of breast cancer tumor tissues demonstrated hypermethylation (76.3%) in the *RASSF1A* promoter, consistent with the findings from other investigators (9% to 95%) [14, 7]. Differences in the frequency of hypermethylation among studies may be accounted for by several factors including methods, study cohort, adjacent normal tissues contaminated by cancer cells and population differences due to exposure to specific environmental factors.

In this study, the promoter methylation frequency of *BRCA1* was only significantly correlated with tumor grade. This result was consistent with previous reports in which the frequency of *BRCA1* methylation is higher in high grade [3, 6]. On the contrary, no significant association was observed between *RASSF1A* methylation status and the clinicopathological factors from the Vietnamese breast cancer patients. It suggests that this frequent and ubiquitous epigenetic alteration of *RASSF1A* promoter may potentially be a very early and critical event during breast cancer pathogenesis [7, 15].

So far, data on aberrant methylation in the adjacent normal tissues has been limited, especially in Vietnamese patients suffered on cancers. By extending the detection of promoter hypermethylation from tumor tissues to non-tumorous DNA, we found that promoter hypermethylation of *BRCA1* and *RASSF1A* was frequent in their paired adjacent normal tissues (52.6% and 65.8%, respectively), slightly lower than in their breast tumor tissues. *RASSF1A* methylation in paired adjacent normal tissues was consistent with other previous reports (7.5% to 92.5%) [15, 7]. In contrast to our results, some studies reported that a low level of

BRCA1 promoter methylation occurs in adjacent normal tissues (0% to 22.4%) [3, 8]. The main reasons causing the different frequency of methylation among studies may be the distance from selected adjacent tissues to the tumors, methods and study cohort.

Moreover, hypermethylation of BRCA1 and RASSF1A was positively correlated between tumor and adjacent normal breast tissues. This observation suggests that the pattern of methylation in adjacent normal breast tissue DNA may be an important predictor of breast cancer risk. Indeed, *BRCA1* and *RASSF1A* methylation in non-tumorous tissues has been considered as a sign of tumor progression [4, 7]. However, lack of methylation levels of these genes in normal breast tissue from controls and the relatively small sample size limit our conclusion. Therefore, further studies with normal breast tissue from controls, larger sample sizes and investigation of additional tumor suppressor genes are required in order to determine the relationship between DNA methylation in tumor and normal breast tissue. Despite of these limitations, however, the strength of this study is that this is the first one investigated promoter methylation of two specific genes in tumor and paired adjacent normal tissue from the same breast cancer patients in Vietnam.

In conclusion, our study showed that the promoter methylation of *BRCA1* and *RASSF1A* may be potential biomarkers for the determination of breast cancer risk in Vietnamese women.

Acknowledgments

This study was financially supported by grants KC.04.05/11-15 from the Ministry of Science and Technology, Vietnam.

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Hiện tượng methyl hóa bất thường vùng promoter gen *BRCA1* và *RASSF1A* của các mẫu u và liền kề ở bệnh nhân Việt Nam bị ung thư vú

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Tóm tắt: Methyl hóa DNA vùng promoter là biến đổi phổ biến của di truyền ngoại gen xảy ra ở ung thư. Hiện tượng này được nghiên cứu để phục vụ chẩn đoán, tiên lượng và điều trị ung thư vú. Methyl hóa promoter 2 gen ức chế khối u *BRCA1* và *RASSF1A* được phân tích bằng kỹ thuật MSP (PCR với cặp mồi đặc hiệu methyl) cho các mẫu u và liền kề của 76 bệnh nhân Việt Nam bị ung thư vú. Chúng tôi nhận thấy methyl hóa quá mức *BRCA1* xảy ra 60.5% với mẫu u và 52.6% với mẫu liền kề. Tương tự, methyl hóa quá mức *RASSF1A* xảy ra 76.3% với mẫu u và 65.8% với mẫu liền kề. Mối liên quan giữa tỉ lệ methyl hóa ở các mẫu u và liền kề với *BRCA1* và *RASSF1A* đều ở mức cao (p lần lượt là 0.000050 và <0.000001); tuy nhiên sự khác biệt không có ý nghĩa thống kê. Tỉ lệ methyl hóa *BRCA1* ở các mẫu u có mối liên quan với độ mô học (p=0.035430) nhưng mối liên quan với các đặc điểm mô bệnh học khác đều không có ý nghĩa thống kê.

Từ khóa: Methyl hóa DNA, *BRCA1*, *RASSF1A*, mô liền kề, ung thư vú.