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Original article

Clinical Utility of promoter methylation of the tumor suppressor genes DKK3, and RASSF1A in breast cancer patients

Marwa H. Saied [a,*], Aya Samy Rady [a], Galal Mustafa Abo El Naga [b], Ola A. Sharaki [a]

[a] Clinical Pathology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt
[b] Surgery Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt

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ABSTRACT

Background: DNA methylation is the commonest known epigenetic change that results in silencing of tumor suppressor genes. Promoter methylation of tumor suppressor genes has the potential for early detection of breast cancer.
Aim: Aim is to examine the potential usefulness of blood based methylation specific polymerase chain reaction (MSP) of methylated DKK3 and RASSF1A genes in early detection of breast cancer.
Method: Methylation status of DKK3 and RASSF1 was investigated in forty breast cancer patients, twenty fibroadenoma patients and twenty healthy ladies as control group using MSP.
Results: Methylation of DKK3 promoter was found in 22.5% of breast cancer patients, while DKK3 methylation was absent in both fibroadenoma patients and control group. Similarly, methylation of RASSF1 promoter was found in 17.5% of breast cancer patients and in none of fibroadenoma and control group.
Conclusion: Promoter methylation of DKK3 and RASSF1 was found in breast cancer patients while absent in control group suggesting that tumorspecific methylation of the two genes (DKK3 and RASSF1A) might be a valuable biomarker for the early detection of breast cancer.

1. Introduction

The World Health Organization has ranked breast cancer (BC) as the most common type of cancer among women. BC remains the most invasive cancer and the first leading cause of cancer related deaths among women worldwide [1].

Survival rates vary greatly worldwide with the lowest rates in developing countries. These low rates can be explained mainly by the lack of early detection programs as well as inadequate diagnosis and shortage of treatment facilities, resulting in a high proportion of women presenting with late-stage disease [2]. Diagnosis of BC in early stage has a 5-year survival rate up to 98% [3].

Mammography has become the gold standard tool for BC screening as it is sensitive enough to detect ductal carcinoma in situ (DCIS). However, it has many limitations to stand alone. The worst limitation of mammography is that clinically occult early-stage BC is often similar in appearance to a benign breast lesion, resulting in unnecessary subsequent biopsies [4,5].

Therefore, non-invasive sensitive tests are required to complement mammography or even stand alone as a primary screening and diagnostic tool for early detection of BC. The epigenetic markers fulfill these criteria such as methylated DNA and miRNAs since changes in their levels have been associated with tumor burden and malignant progression [6].

Determination of promoter methylation of tumor-suppressor genes in the DNA of blood is a rapidly growing research field in cancer detection. The principle of DNA methylation markers for early BC discovery is based on evidence that growing tumors release significant amounts of circulating DNA into bloodstream through cellular necrosis, apoptosis, or spontaneous release of DNA from primary and metastatic tumors [7].

DKK3 gene is a member of the human *Dickkopf* (*Dkk*) family (WNT signaling pathway inhibitors) located on short arm of chromosome 11 p15.3. The transcript length equals to 566 bps [8,9]. *DKK3* expression is frequently suppressed by its promoter methylation in BC [10].

Additionally, *RASSF1A* gene is a Ras association domain family member 1, which is located on short arm of chromosome 3 p21.3. Methylation of the *RASSF1A* promoter region is the most frequently described epigenetic event associated with the development of human cancer. It occurs early in the carcinogenesis of BC

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* Corresponding author.
E-mail address: marwahanafi@yahoo.co.uk (M.H. Saied).

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leading to silencing of *RASSF1A* gene with loss of its tumor suppressor function [11,12].

Therefore, in the present study, two tumor-suppressor genes (*DKK3* and *RASSF1A*) were investigated among Egyptian females with BC, and comparing the results to those of fibroadenoma patients (the most common benign breast disease) [13] and healthy controls using methylation specific polymerase chain reaction (MSP).

2. Subjects and methods

2.1. Patients

This study was carried out on 80 females; forty BC patients, twenty fibroadenoma patients and twenty matched age healthy ladies as control group. The study was approved by local ethics committee. All BC patients undergo full history taking, clinical examination, mammography and prognostic markers (progesterone receptors [PR], estrogen receptors [ER], and human epidermal growth factor receptor-2 [Her-2/neu]). The control group was free of family history of BC. Clinicopathological parameters were investigated in BC patients including: possible family history of BC, the histopathology of tumor, tumor stage, lymph nodes involvement and BC hormonal assay (ER, PR and Her-2/neu).

2.2. Determination of the methylation status of DKK3 and RASSF1A promoters using (MSP)

Firstly, DNA was extracted from blood using QIAGEN total DNA purification kit (QIAamp DNA Blood Mini Kit), QIAGEN, Germany. The extracted DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). The methylation status of the promoter of *DKK3* and *RASSF1A* genes was determined qualitatively by MSP [14]. Briefly, the bisulfite-converted DNA was amplified using primer sets specific for both unmethylated and methylated promoter sequences of *DKK3* and *RASSF1A* genes. 25 μl PCR mix containing 2.5 μl bisulfite-modified DNA, 1 μl forward primer, 1 μl reverse primer, 12.5 μl of Epitect MSP master mix and 8 μl RNase free water was prepared. The previous mix was subjected to 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, primer annealing at 63 °C for *DKK3* gene and at 55 °C for *RASSF1A* gene for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. MSP primer sequences and annealing temperature are listed in Table 1. PCR amplicons were electrophoresed on 2% agarose gels and visualized using transilluminator ultraviolet light. Universal methylated and unmethylated control (Epi Tect Control DNA Set, Qiagen, Germany) were included in each run. The sample is considered to have methylated or unmethylated gene allele depending on the primer pairs used. Consequently, sample is considered methylated when band appears only with methylated primers or unmethylated when band appears only with unmethylated primers) or hemimethylated if bands were appeared with both methylated and unmethylated primers; meaning that the patient has both alleles: methylated and unmethylated.

2.3. Statistical methods

Data analysis was performed using the software package SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA). ANOVA was used to analyze differences between groups. The chi-squared test is used to determine whether there is a significant difference between the expected frequencies and the observed frequencies in one or more categories. Results were considered significant if the p value was less than 0.05.

3. Results

The age of BC patients and healthy controls ranged from 28 to 61 years, and from 23 to 60 years respectively. However, fibroadenoma patients' age ranged from 21 to 54 years. The age of fibroadenoma patients was significantly low with $p < 0.001$, that in line with the fact that fibroadenoma is a disease of young ladies [13]. Comparing the menopausal status between the studied groups revealed that 60% of BC patients and controls were in pre-menopausal state, while the rest (40%) were in post-menopausal period with no significant difference between both groups. However, most of fibroadenoma group were in pre-menopausal state (90%) with significant difference versus BC and controls ($p < 0.001$).

3.1. Clinicopathological characteristics of BC

Regarding BC patients, about 37.5% of patients had positive family history of BC. Positive ER were encountered among 57.5% of patients, while positive PR were encountered among 30% of cases. On the other hand, Her2/neu receptors were negative in more than four fifths of cases (92.5%) (Table 2). Furthermore, metastasis were found in 15% of patients varing between bone and liver metastasis, while lymph nodes involvement were found in 40% of cases. Regarding the stage of the tumor, 72.5% of patients were stage II, 12.5% were stage III and about 15% of cases were stage IV. The histopathological findings revealed that the majority of BC (95%) had infiltrating ductal carcinoma, while 5% had infiltrating lobular carcinoma (Table 2).

3.2. Assessment of DKK3 and RASSF1A promoter methylation using MSP

Next, *DKK3* and *RASSF1A* promoter methylation were assessed in the three studied groups. *DKK3* and *RASSF1A* methylation were present only in BC patients [*DKK3* in 9 patients (22.5%), *RASSF1A* in 7 patients, (17.5%)]. Either *DKK3* or *RASSF1A* methylation was not detected in fibroadenoma or controls (Table 3).

Then, we studied potential associations between *DKK3* and *RASSF1A* promoter methylation and the clinicopathological parameters (Table 4). Only significant correlation between *DKK3* promoter methylation and distant metastasis was identified ($p = 0.041$). No significant correlation was detected between *RASSF1A* methylation and any of the studied parameters e.g. family history, tumor stage, distant metastasis (unpublished data).

Table 1
MSP primer sequences and annealing temperature for *DKK3* and *RASSF1*.

Gene	Primer sequences(5′ → 3′)	Annealing Temperature	Cycles	Product size
DKK3 (Unmethylated)	TTTGGTTGGTTAATGGTTGGGTTGTGGTTTTTTTG CCCTCACCCACCCCAACTAAACCAAATTACA	63 °C	40	278 bp
DKK3 (Methylated)	GTTGGTTAATGGTCGGGTTGCGGTTTTTTC ACCCACCCCGACTAAACCGAATTACG	63 °C	40	276 bp
RASSF1 (Unmethylated)	GGTTGTATTTGGTTGGAGTG CTACAAACCTTTACACACAACA	55 °C	40	180 bp
RASSF1 (Methylated)	GTTGGTATTCGTTGGGCGC GCACCACGTATACGTAACG	55 °C	40	160 bp

Table 2
Distribution of the studied BC cases according to different studied parameters.

Characteristic	No.	%
Family History	15	37.5
*ER positivity	23	57.5
+PR positivity	12	30.0
#HER2 positivity	3	7.5
Metastasis	6	15.0
Lymph node	16	40.0
Histopathology:		
• Ductal Carcinoma.	38	95.0
• Lobular Carcinoma.	2	5.0
Tumor stage:		
• Stage II	29	72.5
• Stage III	5	12.5
• Stage IV	6	15

*Estrogen Receptor, + progesterone Receptor, # human epidermal growth factor receptor-2.

3.3. Correlation between DKK3 and RASSA1 promoter methylation in BC patients

Methylation of *DKK3* was significantly associated with methylation of *RASSA1* (chi-square test-p = 0.0005). Both gene promoters were methylated in 6 patients (15%) and were unmethylated in 18 patients (45%). One patient was *DKK3* methylated-*RASSA1* unmethylated (2.4%) and one patient was *DKK3* unmethylated-*RASSA1* methylated (2.4%)

3.4. Determination which gene is better as blood-based BC test with qualitative MSP

Initially, the sensitivity and specificity of each gene was determined. Sensitivity of *DKK3* methylation alone in BC was 22.5% (9 out of 40 patients were methylated) with specificity 100% as all fibroadenoma patients and controls were methylation negative. Besides, *RASSF1A* methylation has sensitivity of 17.5% (7 out of 40 patients were methylated) with specificity 100% as the fibroadenoma and controls showed no methylation (Table 5). Combining both of *DKK3* and *RASSF1A* methylation revealed an improvement in the sensitivity of the panel up to 40% (16 out of 40 patients) for the detection of BC and a specificity of 100% (20 out of 20 were negative for methylation) in fibroadenoma and controls (Table 5).

Table 5
Sensitivity and specificity of blood-based detection of methylated *DKK3* and *RASSF1* genes in the three studied groups.

Gene	Sensitivity in Breast Cancer Methylation Positive%	Specificity in benign breast disease (fibroadenoma) Methylation negative%	Specificity in Healthy controls Methylation negative%
DKK3	9 of 40 22.5%	20 of 20 100%	20 of 20 100%
RASSF1	7 of 40 17.5%	20 of 20 100%	20 of 20 100%
*DKK3 &RASSF1**	16 of 40 40%	20 of 20 100%	20 of 20 100%

*Either gene methylated.

4. Discussion

In the present study we evaluated the performance of the promoter methylation of two tumor suppressor genes i.e. *DKK3* and *RASSF1A*. Each gene has different role in BC pathogenesis e.g. *DKK3* is *WNT* signaling pathway inhibitor [8,15–17], while *RASSF1A* regulates the apoptotic process. Therefore, promoter methylation followed by silencing of the previous genes would assist in the disease carcinogensis. Here, for the first time we used qualitative MSP

Table 3
Comparison between the three studied groups according to the MSP results of *DKK3* and *RASSF1* genes.

	Patients (n = 40)		Fibroadenoma (n = 20)		Control (n = 20)		X^2	^{MC}p
	No.	%	No.	%	No.	%		
Result of *DKK3*								
Unmethylated	19	47.5	10	50.0	11	55.0	9.921*	0.030*
Hemimethylated	12	30.0	10	50.0	9	45.0		
Methylated	9	22.5	0	0.0	0	0.0		
Result of *RASSF1*								
Unmethylated	28	70.0	20	100.0	20	100.0	11.366*	0.008*
Hemimethylated	5	12.5	0	0.0	0	0.0		
Methylated	7	17.5	0	0.0	0	0.0		

χ^2: Chi square test, MC: Monte Carlo test.
*: Statistically significant at $p \leq 0.05$.

Table 4
Association between the results of *DKK3* gene and different studied parameters in Breast cancer cases group.

Cases group	Result of DKK3						χ^2	^{MC}p
	Unmethylated (n = 19)		Hemimethylated (n = 12)		Methylated (n = 9)			
	No.	%	No.	%	No.	%		
Family History	6	31.6	4	33.3	5	55.6	1.625	0.488
Positive ER	11	57.9	7	58.3	5	55.6	0.019	1.000
Positive PR	5	26.3	5	41.7	2	22.2	1.157	0.669
Positive Her2	2	10.5	1	8.3	0	0.0	0.866	1.000
Lymph node	9	47.4	5	41.7	2	22.2	1.585	0.498
Metastasis	1	5.3	1	8.3	4	44.4	6.296*	0.041*

*Estrogen Receptor, +progesterone Receptor, # human epidermal growth factor receptor-
χ^2: Chi square test, MC: Monte Carlo test.
*: Statistically significant at $p \leq 0.05$.

for *DKK3* and *RASSF* promoter methylation assay in the blood of Egyptian BC patients. To see the potential usefulness of both genes in BC, we compared the results to those of both genes in most common benign breast disease (fibroadenoma) and in healthy controls. Also, blood was used as non-invasive sample to investigate the suitability of this technique in different stages of BC patients.

DKK3 promoter methylation was not found in either fibroadenoma patients or controls. However, nine out of forty BC patients (22.5%) showed methylation of *DKK3*. Correlating the clinicopathological parameters with *DKK3* promoter methylation revealed no association with any of the previous parameters except distant metastasis ($p = 0.041$). Previously, *DKK3* methylation showed significant correlation with poor overall patients [18]. That might indicate the useful prognostic role of *DKK3* methylation in predicting patient survival. Additionally, downregulation of *DKK3* expression was detected in highly aggressive basal BC subtype [19].

On the other hand, methylated *RASSF1A* was detected in 7 patients out of the forty BC patients (17.5%), without any methylation detection in fibroadenoma patients or controls. *RASSF1A* promoter methylation was previously detected in BC tissues and BC cell lines [20], in plasma DNA of BC patients [21], also in serum DNA of BC patients [22,23]. *RASSF1A* showed different sensitivity and specificity in different studies which is related most probably to number of the samples included. For instance, in a big cohort study of 261 BCE serum, 112 paired BC tissue and 237 of cancer free individuals showed high sensitivity of *RASSF1A* methylation (47%) with quite low specificity (74%), which may indicate that *RASSF1A* alone is not highly specific [15]. Opposite to *DKK3*, *RASSF1A* did not show significant correlation with distant metastasis. However, this does not exclude the association of *RASSF1A* methylation with tumor progression and metastasis [24]. Given the frequencies of *RASSF1A* methylation in cancer, *RASSF1A* was included in our study besides *DKK3* in a panel for detection of BC. Such addition has raised the sensitivity to be 40% comparing to sensitivity of applying each gene alone e.g. 22.5% using *DKK3* and 17.5% using *RASSF1A*.

The epigenetic changes in cancer are very heterogeneous [25], so using panels of more than one gene will improve the screening procedures. Therefore, promoter methylation of the *DKK3*/*RASSF1A* genes combination allowed significant discrimination of BC blood from benign breast disease and control groups with sensitivity higher than using each one alone. This panel represents a simple and non-invasive blood based test that can complement mammography in early detection of BC.

Conflict of interest

We have no conflict of interest to declare

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