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Original Research Article

Evaluation of Promoters Hypermethylation of APC and RASSF1A Genes for Early Detection of Breast Carcinoma

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Abstract: Breast cancer cells illustrate major disruption in their DNA methylation patterns as compared with the normal breast cells. Authors aimed to identify the epigenetic molecular markers for "APC and RASSF1A" genes, in serum samples depending on the fact that the DNA is released into the peripheral circulation when the necrotic and apoptotic cells detached from the tumor for breast cancer detection. Serum samples were collected from 93 breast cancer patients, 55 patients with benign breast lesions, and 30 healthy individuals for detection of methylated genes using relative quantitative methylation specific PCR. Overall significant differences in methylation levels of the promoters of APC and RASSF1A genes ($p<0.0001$, for both) were detected. They were significantly higher in the breast cancer patients (95.7% and 96.7%, respectively) than in the benign ones (10.9% and 52.7%, respectively); but were not detected in the healthy volunteers (0% at $p< 0.0001$). Both methylated genes' promoters showed no significant difference among the clinicopathological factors apart from the reported significance between APC gene with positive progesterone receptor (PgR) and HER-2neu. In conclusion, the quantitative detection of aberrant methylated promoters of the two genes "APC and RASSF1A" in the serum samples is a promising approach for diagnosis of breast cancer-patients.
Keywords: Promoter methylation; breast cancer; early diagnosis; APC; RASSF1A

INTRODUCTION

Breast cancer is a global public health issue characterized by its heterogeneity; having different characteristics and qualities in the clinical management, early detection of breast cancer is essential for the success of patients' favorable evaluation and the efficacy of subsequent therapies [1]. Although commonly used breast cancer detection methods depend on palpation and /or radiological images, still many tumors failed to be detected until the patient reached advanced stage. Consequently, developing more sensitive methods for cancer detection especially in women under age of 50 years who usually develop aggressive cancers is of great interest.

It has been reported that aberrant gene function results from CpG islands which affect both gene and epigenetic changes [2]. Epigenetics are changes in the phenotype or gene expression and they are due to other mechanisms other than changes in DNA sequence, these changes commonly occur in human cancer and continue in cell division and even may persist through several cohorts [3]. Thus, alteration in gene expression may results from hypermethylation of promoter regions rich in CpG islands [4].

Detection of methylated biomarkers may be a useful means for detection of cancer at its early stage [5]. An ideal diagnostic marker should be obtained with minimally invasive methods. Circulating markers separated from blood of cancer patients are usually a prospective source of tumor cells with genetically – altered DNA. And they can be detected by using commercial available kits, hence they can be considered as promising marker for detection of cancer [6].

Authors investigated the methylation status of the promoters for APC and RASSF1A genes to evaluate the presence of epigenetic alterations accompanied with breast cancer, and then correlate their early diagnostic efficacy with other clinicopathological factors.

MATERIALS AND METHODS

Sample collection

A group of 93 women (mean age: 47 years, range: 23–70 years) with breast cancer (including 41 non-invasive duct carcinoma [NIDC] and 52 invasive

duct carcinoma [IDC]) were registered in this study in the period between May 2013 and June 2014. All patients had no evidence of other cancers based on clinical and radiological evaluations; also, none of them received chemotherapy or radiotherapy prior to blood collection. Pathological staging and grading were assessed using the Tumor-Node-Metastases classification (TNM) [7] and the modified Scarff-Bloom-Richardson histologic grading system [8], respectively.

Using an age-matched approach with the already-mentioned breast cancer group, 55 patients with benign breast lesions (mean age: 40 years, range: 28 – 58 years), and 30 control healthy volunteers (mean age: 40 years, range: 25 – 60 years) were included in this study. The collected blood samples were provided from Ain Shams University Hospital, Cairo, Egypt. Blood samples were obtained after signing the informed consent from all participants, and collecting the clinical and pathological data from their medical reports met their approval. Whole blood was drawn from all the individuals before surgery in a plain tube with anticoagulant; after centrifugation at 10.000 rpm for 10 min at 4 °C, the samples were stored at -80 °C until been analyzed.

DNA Extraction and Purification:

Cell-free DNA in serum was extracted using the QIAamp DNA Blood Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol.

DNA Treatment and Bisulphite Conversion

Bisulphite conversion was performed based on the principle that bisulphite treatment of DNA converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues would remain unmodified. Thus, after bisulphite conversion, methylated and unmethylated DNA sequences would be distinguished by sequence-specific primers. Each DNA sample was treated with sodium bisulphite using the EpiTectPlus DNA bisulphite Kit (QIAgen, Hilden, Germany). DNA was converted and purified using the EpiTect Bisulphite Kit (QIAgen, Hilden, Germany), and all the converted DNA samples were assessed for their DNA purity and quantified on a Q-5000 Spectrophotometer (Quawell Technology, Inc., San Jose, USA), and then stored at – 20°C.

Methylation Specific PCR

Methylation-specific PCR (MSP) was performed using the EpiTect MSP Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol. Sense and antisense primer sequences for methylated "APC" gene's promoters are as follows: 5'-TATTGCGGAGTGCGGGTC-3'(sense), 5'-TCGACGAACTCCCGACGA-3' (antisense), which amplify a 98 bp product. Sense and antisense primer sequences for unmethylated "APC" gene's promoters are as follows: 5'-GTGTTTTATTGTGGAGTGTGGGTT-3' (sense), 5'-CCAATCAACAAACTCCCAACAA-3' (antisense), which amplify a 108 bp product.

Sense and antisense primer sequences for methylated gene's promoters "RASSF1A" are as follows: 5'-GGGTTTTGCGAGAGCGC-3' (sense), 5'-GCTAACAAACGCGAACCG-3' (antisense), which amplify a 260 bp product. Sense and antisense primer sequences for unmethylated gene's promoters "RASSF1A" are as follows: 5'-GGTTTTGTGAGAGTGTGTTTAG-3'(sense), 5'-CACTAACAAACACAAACCAAAC-3' (antisense), which amplify a 172 bp product. APC and RASSF1A genes' promoters regions have been previously described [9].

PCR was performed in a thermal cycler (Biometra,German) using specific primers in reactions containing: 2µl of bisulphite-treated DNA which was added to bring the used reaction volume up to 50µl, containing 1.25 mM dNTP, 16.6 mM (NH4) $2SO_4$, 67 mM Tris, pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 U RedTaq genomic DNA polymerase (Sigma-Aldrich, Inc., St. Louis, MO) and 25 pmol for each of the forward and reverse primers specific to the methylated and unmethylated DNA sequences. Both methylated and unmethylated primers were tested in separate reactions.

PCR conditions were as follows for both genes' promoters: activation at 95°C for 5 minutes; then 35 cycles for each gene as follows: denaturation at 94°C for 1 minute, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds, followed by final extension at 72°C for 10 minutes and hold at 4°C. The amplified PCR products were electrophoresed through a 2% agarose and visualized by ethidium bromide staining, and gel photos were captured using ultraviolet Gel Doc analyzer (G: Box F3, Court Suite, Frederick, USA) [10].

Relative Quantitative Gene Expression Analysis of the Promoters of APC and RASSF1A

To control the test efficiency and normalize it for sample to sample variation in DNA amount as well as to quantify the methylated and unmethylated levels of the investigated genes' promoters relatively to the expression of β-actin as a housekeeper in each sample, β-actin-specific primers were used as follows: 5\-GCGGGAAATCGTGCGTG-3\ (sense), 5\-CAGGGTACATGGTG GTGCC-3\ (antisense) with generation of a 309 base pair fragment.

PCR conditions were as follows: Activation at 95°C for 5 minutes; then 35 cycles as follows:

Denaturation at 94°C for 1 minute; annealing at 58°C for 30 seconds; and extension at 72°C for 30 seconds, followed by final extension at 72°C for 10 minutes and hold at 4°C. The signal intensities in agarose gel for each of the promoters of APC and RASSF1A in each sample were determined relative to that of β-actin in the same sample using Gel-pro [version 3.1], Media cybernetics, USA software, thus determining the relative amount of different samples. The fragments' sizes of the PCR products were estimated by comparison with DNA molecular weight marker (1 kb) (provided by Promega, GE Healthcare Bio-science, UK limited) (Figure 1).

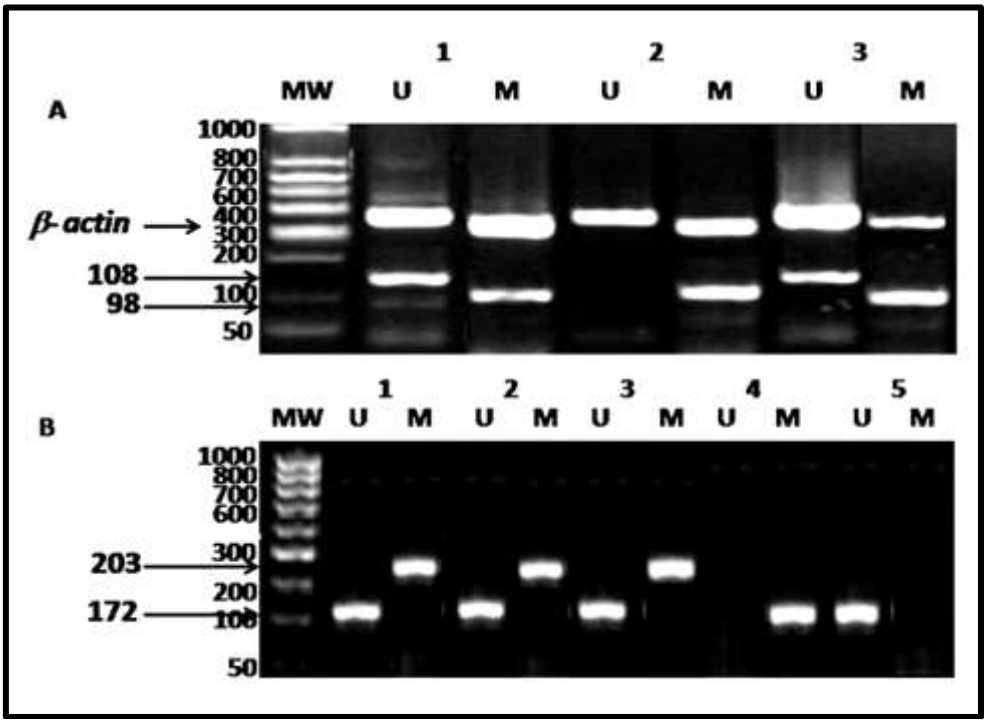

Fig-1: Detection of aberrant methylation of cancer-related genes APC (A) and RASSIF1A (B) genes in serum by agarose gel electrophoresis and ethidium bromide staining

Each sample was represented by two successive lanes, one for unmethylatied (U) band and the second for methylated (M) band. APC Positive bands for both unmethylated and methylated bands are shown at 108 and 98 bp., respectively. Lanes 1-2 are breast cancer samples; lanes 3 are benign breast samples. RASSIF1A Positive bands for both unmethylated and methylated bands are shown at 172 and 203 bp., respectively. Lanes 1-4 are breast cancer samples, lanes 5 are benign breast samples.

Statistical Analysis

The Kruskal-Wallis ANOVA test was used to identify the differences in methylation levels among the three groups (malignant tumors, benign lesions and control). The methylation thresholds of the two genes for cutoff values were established by receiver-operating-characteristic (ROC) analysis, where an optimal cutoff was chosen by maximizing the sensitivity [11].

In the present study, the promoter hypermethylation (positive vs. negative) using the selected cutoff values were defined. The sensitivity, specificity, and the area under the curves (AUC) of each methylation markers were calculated. We also explored the combinations of genes that can improve the performance of the relative Q-MSP test in differentiating between the three investigated groups. The sensitivity and the specificity for each gene's promoter alone or in combination for evaluating their diagnostic efficacy especially at the early-stage and the low-grade tumors were also examined. For all the statistical analyses, the SPSS system for the personal computer (version 11.0 for Windows; SPSS INC., Chicago, IL, USA) was used, with $p<0.05$ regarded as statistically significant. All aspects of the present study were approved by the ethics committee of Ain-shams University.

RESULTS

One-hundred seventy eight Egyptian individuals were enrolled in this study; they were categorized into 30 control healthy individuals, 55 patients with benign breast diseases (23 with breast cyst, 19 with duct ectasia and 13 with fibroadenoma)

and 93 patients with breast cancer (41 with non-invasive duct carcinoma [NIDC], and 52 with invasive duct carcinoma [IDC]). The clinicopathological data for the enrolled breast cancer patients are summarized in "Table 1".

Table 1: Clinicopathological characteristics of breast cancer patients (n = 93)

Characteristics	N (%)
Age	
< 45 years	42 (45.2%)
≥ 45 years	51 (54.8%)
Clinical Stage	
Early stage	68 (73.1%)
Late stage	25 (26.9%)
Histological Grade	
Low grade	74 (79%)
High grade	19 (20.4%)
Pathological Type	
NIDC	41 (44.1%)
IDC	52 (55.9%)
Lymph Node Involvement	
Negative	25 (26.9%)
Positive	68 (73.1%)
ER	
Negative	52 (55.9%)
Positive	41 (44.1%)
PgR	
Negative	41 (44.1%)
Positive	52 (55.9%)
Her2/neu	
Negative	38 (40.9%)
Positive	55 (59.1%)

Abbreviations: NIDC: Non-invasive ductal carcinoma; IDC: Invasive ductal carcinoma; ER: Estrogen receptor; PgR: Progesterone receptor; Her2/neu: Human epidermal growth factor receptor-2; N: Number of samples.

Methylation Pattern of APC and RASSF1A Genes' Promoters

We examined the methylation level of promoters for the two genes APC and RASSF1A in serum samples of age-matched controls, benign breast lesions and breast cancer patients. Breast cancer samples revealed the highest levels of methylation followed by benign breast lesions; whereas methylation was not detected in the control individuals. Overall significant differences in methylation levels were observed in both the investigated genes' promoters ($p< 0.001$ for all comparisons) (Table 2).

Tabel 2: DNA methylation levels in serum among the investigated groups

Investigated Genes' promoters	Mean ± SD			Significance level (*p*)			
	Control	Benign	Malignant	Overall	Control *vs* Benign	Control *vs* Malignant	Benign *Vs* Malignant
APC	0	4.7 ± 0.5	7 ± 0.8	<0.0001	<0.0001	<0.0001	<0.0001
RASSF1A	0	4 ± 0.3	8.6 ± 0.2	<0.0001	<0.0001	<0.0001	<0.0001

Assessment of frequency for Promoter Hypermethylation

To detect the consistency of the cutoff-based methylation patterns with the quantitative methylation values; a select cutoff values for promoter hypermethylation were selected based on ROC curve and these values were recognized as cutoff points as they can discriminate between malignant and non-malignant cases (combination of the control and the benign individuals). Accordingly cutoff value for APC and RASSF1A genes were 5.64 copies and 1.06 copies, respectively. Using these cutoff points, the frequency distribution among the three groups are shown in "Table 3".

Promoter hypermethylation frequencies were significantly detected in malignant group followed by benign cases while they were not detected in the control

ones (p< 0.001 for all comparisons) (Figure 2). We found no significant difference between methylation frequency of the two investigated genes' promoters and clinicopathological factors, except for a significant relation was reported between APC with PgR (p= 0.021) and HER-2/ neu (p= 0.04) that are reported in Table 3.

Table 3: Frequency of hypermethylation of *APC* and *RASSF1A* genes among the investigated groups and the clinicopathological factors

	APC gene N (%)		*RASSF1A* gene N (%)	
	< 5.64	≥ 5.64	< 1.06	≥ 1.06
Groups				
Control (n=30)	30 (100%)	0 (0%)	30 (100%)	0 (0%)
Benign (n=55)	49(89.1%)	6 (10.9%)	26 (47.3%)	29 (52.7%)
Malignant (n=93)	4 (4.3%)	89 (95.7%)	3 (3.2%)	90 (96.7%)
Clinicopathological Factors				
Histological Grades				
Low grade (n=74)	4 (5.4%)	70 (94.6%)	3 (4.1%)	71 (95.9%)
High grade (n=19)	0 (0%)	19 (100%)	0 (0%)	19 (100%)
Clinical Stages				
Early stage (n=68)	4 (5.9%)	64 (94.1%)	3 (4.1%)	65 (95.9%)
Late stage (n=25)	0 (0%)	25 (100%)	0 (0%)	25 (100%)
Lymph Node Involvement				
-ve (n=25)	0 (0%)	25 (100%)	0 (0%)	25 (100%)
+ve (n=68)	4 (5.9%)	64 (94.1%)	3 (4.4%)	65 (95.6%)
ER				
-ve (n=52)	4 (7.7%)	48 (92.3%)	3 (5.8%)	49 (94.2%)
+ve (n=41)	0 (0%)	41(100%)	0 (0%)	41(100%)
PgR				
-ve (n=41)	4 (4.8%)	37 (90.2%)	2 (4.9%)	39 (95.1%)
+ve (n=52)	0 (0%)	52 (100%)	1 (1.9%)	51 (98.1%)
	X^2=5.3, p=0.021			
HER-2/neu				
-ve (n=38)	4 (10.5%)	34 (89.5%)	2 (5.3%)	36 (94.7%)
+ve (n=55)	0 (0%)	55 (100%)	1 (1.8%)	54 (98.2%)
	X^2=6, p=0.04			

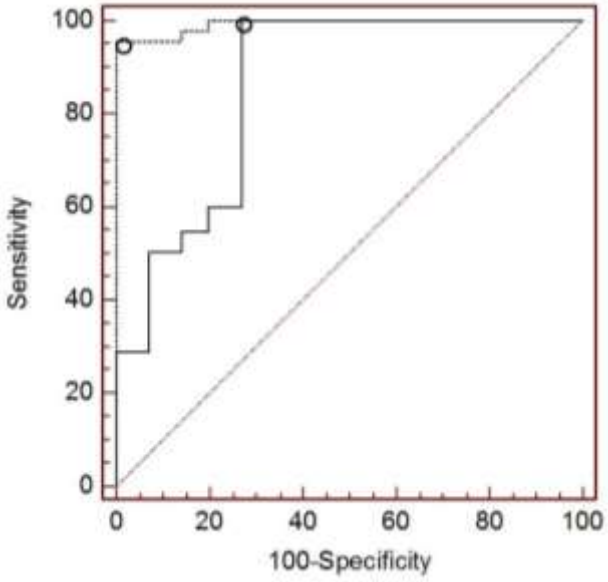

Fig-2: ROC curve analysis for APC and RASSIF1A genes to discriminate between malignant and non-malignant groups

Open circles denote best cutoff points of APC (dashed line) as 5.64 copies [sensitivity = 95.7% at absolute specificity, area under the curve (AUC) (SE) = 0.986 (0.015), 95% confidence limits range = 0.955 – 0.998, at P<0.0001], of RASSIF1A 1.06 copies [sensitivity = 96.7% at absolute specificity, area under the curve (AUC) (SE) = 0.86 (0.016), 95% confidence limits range = 0.801 – 0.906, at P<0.0001.

Diagnostic Efficacy of APC and RASSF1A Using Quantitative Methylation Specific PCR

The sensitivities and the specificities of the methylation markers either alone or in combinations were detected for early diagnosis of breast cancer, early-stage and low-grade tumors (Table 4). The sensitivities for both genes' promoters were nearly the same; whereas the specificity of APC's promoter was superior to RASSF1A's promoter for detection of breast cancer, early-stage and low-grade tumors. We also found that the combination of the two genes' promoters improves the specificity of RASSF1A.

Table 4: Estimated sensitivities, specificities for hypermethylation of *APC* and *RASSF1A* genes' promoters for early diagnosis of breast cancer, both for early-stage and low-grade

Genes' Promoters	Breast Cancer Diagnosis		Early Stages		Low Grades	
	Sensitivity%	Specificity%	Sensitivity%	Specificity%	Sensitivity%	Specificity%
APC	95.7	92.6	94.6	89.1	94.1	98.1
RASSF1A	96.8	65.9	95.6	47.3	95.4	47.3
APC + RASSF1A	95.7	92.9	94.6	89.1	94.6	89.1

DISCUSSION

Early detection of breast cancer is a major challenge for an effective treatment with a better outcome [12]. The major screening method for breast cancer detection is mammography however its limitations (example: false-positive results, false-negative results, over-diagnosis and over-treatment) are well-recognized [13]; thus there is an urgent need for applicable minimally invasive markers for screening and diagnostic purposes. Circulating cell free-DNA has been assessed in many cancers [14], thus they maybe promising minimally non-invasive diagnostic markers since they reported similar alterations to those detected from breast cancer tissues [15].

For conventional MSP, the PCR products were separated on gel, and the results were represented according to the methylation status resulting in lack of information about the recognition of the partial levels of methylation. To avoid these drawbacks, relative Q-MSP has been exploiting in the recent years since it is highly specific and sensitive over conventional MSP [16] as well as it has the ability to characterize precise cutoff point between malignant and non-malignant.

Relative MSP for APC and RASSF1A promoters were detected for one hundred seventy eight serum samples from breast cancer patients (n=93), patients with breast lesion (n=55), and 30 controls served as healthy individuals. Promoters of both genes reported significantly higher methylation levels in breast cancer patients followed by benign breast lesions; while they were not detected in the control individuals. These results agreed with our previous reports [17] indicating that assessment of these methylation levels in the circulating samples may be a powerful molecular marker for diagnosis of breast cancer. Moreover, the presence of methylated promoters of the APC and RASSF1A genes in the blood serum samples and its advantage to be used to differentiate between breast cancer and other breast lesions was further investigated using ROC analysis which revealed a significantly higher prevalence of the investigated methylated genes in breast cancer than in the benign lesions; whereas they were undetectable in the control individuals. The usefulness of using methylated genes in cancer diagnosis is established on the hypothesis that promoter methylation at specific sites of certain genes is confined to malignancy [18].

In the present study no significant relation reported between the investigated aberrant methylated genes' promoters and the clinicopathological factors, apart from the relation between PgR and HER-2/neu with hypermethylated promoter of the APC gene which reveals their importance for personalized therapy as well. Aberrant methylated genes' promoters during malignancy progression are potential prognostic markers [19]. The presence of aberrant methylated genes' promoters in all the grades and stages of breast cancer indicates the convenience of detection of this marker in all the types of cancer and not only in those of high grade or high stage. Moreover, association between methylated APC and RASSF1A genes and patients with IDC (Invasive Ductal Carcinoma) reported to be non-significant, indicating that it is important to perform long-term follow-up for aberrant promoter methylation in IDC patients which is in progress.

To explore further diagnostic utility of the genes APC and RASSF1A promoters' methylation as markers in breast cancer diagnosis; their sensitivities and specificities were assessed. We achieved reasonable high sensitivity and specificity in the breast cancer diagnosis patients especially those with early-stage and low-grade tumors using promoters of APC methylation

followed by promoters of RASSF1A; their combination reported the same sensitivities and specificities as those of APC gene alone. Further investigations are necessary for understanding methylation in detail and to accumulate more evidence as a biomarker for personalized medicine [20].

CONCLUSION

In conclusion, this study demonstrated the methylation status of the promoters of the two genes APC and RASSF1A among breast diseases' patients using relative Q-MSP method, thus providing a promising new diagnostic tool using the blood serum instead of surgical biopsies or mammography, for the early diagnosis and prediction of breast cancer especially in the patients who are at high risk. However, further multifaceted studies are needed to define the impact of these molecular markers for early diagnosis and disease monitoring. Also, further follow-up of different breast cancer subtypes is needed to understand the association of methylation of candidate genes in cancer development.

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ABBREVIATIONS

APC: the gene of adenomatous polyposis coli – AUC: area under the curve – CpG: cytosine preceding guanine – HER-2neu: human epidermal growth factor receptor-2 – IDC: invasive ductal carcinoma – NIDC: non-invasive ductal carcinoma – PgR positive progesterone receptor – QMSP: quantitative methylation specific PCR – RASSF1A: the gene of Ras association domain family protein 1A – ROC: receiver operating characteristic

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