Assessment of Methylation of RASSF1A Gene in Circulating Tumor DNA of Breast Cancer Patients by Methylation-Specific Polymerase Chain Reaction

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Abstract: Background: The aim of the present study was to evaluate the methylation status of RASSF1A tumor suppressor gene in circulating tumor DNA in sera of a group of Egyptian breast cancer patients by methylation-specific PCR. **Methods:** 50 subjects were included: 36 breast cancer patients and 14 healthy controls. Following breast examination, imaging studies and tissue biopsies (for patients group), CA 15-3 was assessed by electrochemiluminescence and RASSF1A gene methylation status was evaluated by methylation-specific PCR. **Results:** RASSF1A gene was methylated in 17/36 (47.2%) of patients and in none of the 14 control samples. Heterozygous pattern of RASSF1A gene methylation was detected in 2 patient samples. There was a significant association between tumor stage subgroups and each of CA 15-3 serum levels and RASSF1A gene methylation. **Conclusion:** RASSF1A gene methylation is a potential epigenetic marker for breast cancer. Further improvement of its screening efficiency might be reached by the use of more than one gene in combination. This gene is a likely candidate for tumor demethylation therapy.

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1. Introduction

In Egypt, breast cancer is the most frequent malignancy among women, constituting in certain governorate up to 38.6% and is more prominent among young premenopausal Egyptian women characterized by poor prognosis and low survival rate [(1)]. Worldwide, it is the most frequent malignancy among women [(2)], with more than 1.15 million new cases diagnosed each year [(3)], and is the second leading cause of cancer-related deaths [(4)].

Epigenetic changes, as DNA methylation and acetylation, are one of the most common molecular alterations in human neoplasia including breast cancer. DNA methylation occurs in cytosine ring of those cytosines preceding a guanosine (referred to as CpG dinucleotides) to form 5-methyl cytosine. CpG dinucleotides are found at increased frequency in the promoter region of many genes and are referred to as CpG islands (CpGIs). Methylation in the promoter region is frequently associated with "gene silencing" [(5)]. Many reports demonstrated epigenetic inactivation of tumor suppressor genes playing important roles in cancer development, including Ras-association domain family of genes. Epigenetic inactivation of RASSF1A (Ras-association domain family 1, isoform A) through methylation of its CpGI silences expression of the gene in many cancers including breast cancer [(6)].

The aim of the present study was to test for the presence of methylation of RASSF1A tumor suppressor gene in sera of a group of Egyptian breast cancer patients by methylation-specific PCR (MSP).

2. Material and Methods:

I-Study Design:

This study was approved by the Institutional Review Board of Ain Shams University Ethic committee. It is a cross-sectional study conducted on 36 female patients with breast cancer, with median age and interquartile range (IQR) of 51.5 (44.0-56.0) years, recruited from General Surgery and Oncology Departments at Ain Shams University Hospital, in addition to 14 age matched healthy women serving as a normal control group, with median age and IQR of 46.5 (37.7-52.5) years.

According to the primary tumor (T) of the ***Tumor-Node-Metastasis (TNM) staging system***, patients were subdivided into: *Tis, T1 and T2 subgroup* (n = 22) and *T3, T4 subgroup* (n = 14). Moreover, according to the ***histopathological types***, patients were subdivided into *ductal carcinoma subgroup* (n = 27) and *lobular carcinoma subgroup* (n = 9). Lastly, according to the ***RASSF1A gene methylation status***, patients were subdivided into: *methylated RASSF1A gene subgroup* (n= 17) and *unmethylated RASSF1A gene subgroup* (n= 19).

All individuals included in this study were subjected to full history taking with emphasis on family history of breast cancer and reproductive history, local breast examination, imaging studies (ultrasound for both groups followed by mammography and CT in patients group only), breast biopsy (for patients only), serum CA 15-3 assay by electrochemiluminescence and evaluation of RASSF1A gene methylation status by methylation-specific PCR.

II. Samples:

Five milliliters of venous blood were withdrawn under complete aseptic conditions, 24 hours before surgical intervention from patients, and from healthy controls. Samples were left to clot for 30 minutes in sterile dry vacutainers. Serum was separated by centrifugation and divided into 2 aliquots to be stored at -20 ºC until the assay of CA 15-3 and RASSF1A gene methylation.

III. Laboratory Analysis:

A- Assay of CA 15-3 by electrochemiluminescence: This was done on Cobas e 411 immunoassay autoanalyser (Roche Diagnostics, Indianapolis, USA) using the manufacturer's reagents. The antigen (sample) and the two provided antibodies, one biotinylated and the other monoclonal CA15-3-specific labeled with ruthenium complex, are allowed to react to form a sandwich complex. After addition of streptavidin-coated microparticles (solid phase), the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed by wash using system buffer. Application of a voltage to the electrode induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve generated by a 2-point calibration.

B- RASSF1A Gene Methylation Assay: Serum was used for detection tumor DNA instead of whole blood in which a huge amount of white blood cells DNA could be extracted alongside with tumor DNA. All reagents were supplied by Q1AGEN (Q1AGEN Incorporation, Stanford Valencia, USA).

1) DNA Extraction: This was performed using QIAamp DNA Blood Midi kit

2) Bisulfite Modification: using EpiTect Bisulfite kit, where incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues to uracil, leaving the methylated cytosines unchanged. Therefore, bisulfite treatment gives rise to different DNA sequences for methylated and unmethylated DNA. Bisulfite modification comprised the following steps: i) bisulfite-mediated conversion of unmethylated cytosines, by thermal cycling through 3 alternating denaturation and incubation cycles (denaturation always being at 99ºC for 5 minutes, while the 3 incubations being at 60 ºC for 25 minutes, 85 minutes and 175 minutes); ii) binding of the converted single-stranded DNA to the membrane of an EpiTect spin column; iii) washing; iv) desulfonation of membrane-bound DNA; v) washing of the membrane-bound DNA to remove desulfonation agent; and vi) elution of the pure, converted DNA from the spin column.

3) Amplification by PCR: Amplification of modified DNA was done using EpiTect MSP and primers. Two sets of primers were used: the first set for methylated RASSF1A gene (forward: 5′-GTTGGTATTCGTTGGGCGC-3′ and reverse: 5′-GCACCACGTATACGTAACG-3′) and the second for the unmethylated gene (forward: 5′-GGTTGTATTTGGTTGGAGTG-3′ and reverse: 5′-CTACAAACCT TTACACACAACA-3′). Amplification was repeated in 30 cycles according to the following protocol: 15 seconds at 94 ºC (denaturation), 30 seconds at 56 ºC (annealing), 30 seconds at 72 ºC (extension) and, lastly, 10 minutes at 72 ºC (final extension). In each run, a negative control tube containing water instead of DNA was included to exclude contamination. The amplified products were stored at -20˚C until time of detection step.

4) DNA Detection by Gel Electrophoresis: Amplified products were run on 2% agarose gel stained with ethidium bromide. A DNA molecular weight marker was also run in each gel to identify different separated bands. The amplified products were loaded on 2 wells for each studied subject, one for methylated and the other for unmethylated. Methylated samples gave bands at 160 bp and unmethylated ones at 180 bp. Heterozygous samples gave 2 bands (160 and 180 bp) at both methylated and unmethylated lanes as shown in figure (1).

IV- Statistical Analysis:

Statistical analysis was done through IBM SPSS statistics (Version 20.0, 2011, IBM Corporation, USA). Data were expressed descriptively as median and IQR for quantitative non-parametric values and as percent for qualitative data. Comparison between 2 independent non-parametric groups was done using the Mann Whitney U test while Chi square test was used for comparison of categorical data. $p < 0.05$ was considered significant and $p < 0.01$ was considered highly significant.

3. Results:

On comparing the demographic and laboratory data of patients and controls (Table 1), a significantly higher percentage of breast cancer cases

of 47.2% (17/36) had methylated RASSF1A gene than controls (0%) ($p < 0.01$). This also applies for CA15.3 which was significantly higher in patients (25.5 IU/mL) than controls (19 IU/mL) ($p < 0.05$). However, no significant difference was found between patients and control groups as regard age, menopausal age and menopausal status.

Statistical comparison between RASSF1A gene methylated and unmethylated patients' groups (Table 2) revealed no significant difference as regards age, family history of breast cancer, menopausal age, menopausal status and CA15.3 serum levels.

Table (3) shows different stage subgroups where the median serum CA15.3 levels (22.0 IU/mL) of Tis, T1 and T2 subgroup was significantly lower than that of T3, T4 subgroup (45.5 IU/mL) ($p < 0.05$). Also, RASSF1A gene was methylated in a significantly lower percentage of 31.8 % (7/22) in Tis, T1 and T2 subgroup than in T3, T4 subgroup which was 71.4 % (10/14) ($p < 0.05$).

The same comparison was performed among different breast cancer histological type subgroups (Table 4), where median CA15.3 serum levels were 27.0 IU/mL in lobular type and 23.0 IU/mL in ductal type with no significant difference. Also, no significant difference was found between lobular and ductal type subgroups as regards RASSF1A gene methylation [6/9 (66.6%) versus 11/27 (40.7%), respectively).

Table (1): Statistical Comparison of the Demographic and Laboratory Data Among Breast Cancer Patients and Healthy Controls Using Mann Whitney U Test and Chi square Test*

Parameter	Patients (n=36)	Controls (n=14)	Z/ X2*	Significance (*P*)
Age (years):				
Median	51.5	46.5	-1.33	> 0.05
IQR	44.0-56.0	37.7 - 52.5		
Menopausal age (years):				
Median	49.0	47.0	-0.326	> 0.05
IQR	46.0-52.0	43.7- 53.5		
Menopausal status:				
Menopaused	26 (72.2 %)	8 (57.1 %)	1.053*	> 0.05
Non-menopaused	10 (27.8 %)	6 (42.8 %)		
CA15.3 (IU/mL):				
Median	25.5	19	-2.337	**< 0.05**
IQR	20.0 - 39.0	13.7- 25.2		
RASSF1A methylation status				
Methylated	17 (47.2 %)	0 (0 %)	10.017*	**< 0.01**
Unmethylated	19 (52.8 %)	14 (100 %)		

IQR: Interquartile range.

Table (2): Statistical Comparison of the Demographic and Laboratory Data among RASSF1A Gene Methylated versus Unmethylated Patients' Subgroups

Parameter	RASSF1A gene methylated patients (n=17)	RASSF1A gene unmethylated patients (n=19)	Z\ X2*	Significance (*P*)
Age (years):				
Median	51.0	52.0	-0.286	> 0.05
IQR	44.0 – 55.0	44.0-57.0		
Family history of breast cancer:			0.007*	> 0.05
Positive	1 (5.9%)	1 (5.3%)		
Negative	16 (94.1 %)	18 (94.7 %)		
Menopausal age (years):				
Median	48.0	51.0	-0.801	> 0.05
IQR	46.0 -49.7	45.5-52.5		
Menopausal status:				
Menopaused	12 (70.5 %)	14 (73.7 %)	0.043*	> 0.05
Non-menopaused	5 (29.5 %)	5 (26.3 %)		
CA15.3 (IU/mL):				
Median	27.0	25.0	-0.46	> 0.05
IQR	18.0 -77.0	20-36.0		

IQR: Interquartile range.

Table (3): Statistical Comparison of CA15.3 Serum Levels and RASSF1A Gene Methylation Status Among Different Stage Subgroups of Breast Cancer Patients using Mann Whitney U Test and Chi Square test*

Parameter	T3, T4 subgroup (n=14)	Tis, T1 and T2 subgroup (n=22)	Z \X2*	*P*
CA15.3(IU/mL)				
Median	45.5	22.0	2.876	< 0.05
IQR	22.2-279.0	17.5-27.0		
RASSF1A methylation	10 (71.4 %)	7 (31.8%)	5.386*	< 0.05

IQR: Interquartile range.

Table (4): Statistical Comparison of CA15.3 Serum Levels and RASSF1A Gene Methylation Status among Different Histological Type Subgroups of Breast Cancer Patients using Mann Whitney U Test and Chi Square test*

Parameter	Lobular type (n=9)	Ductal type (n=27)	Z /X2*	*P*
CA15.3(IU/mL)				
Median	27.0	23.0	0.494	> 0.05
IQR	18.5-160.5	20.0-36.0		
RASSF1A methylation	6 (66.6 %)	11 (40.7 %)	7.34*	> 0.05

IQR: Interquartile range.

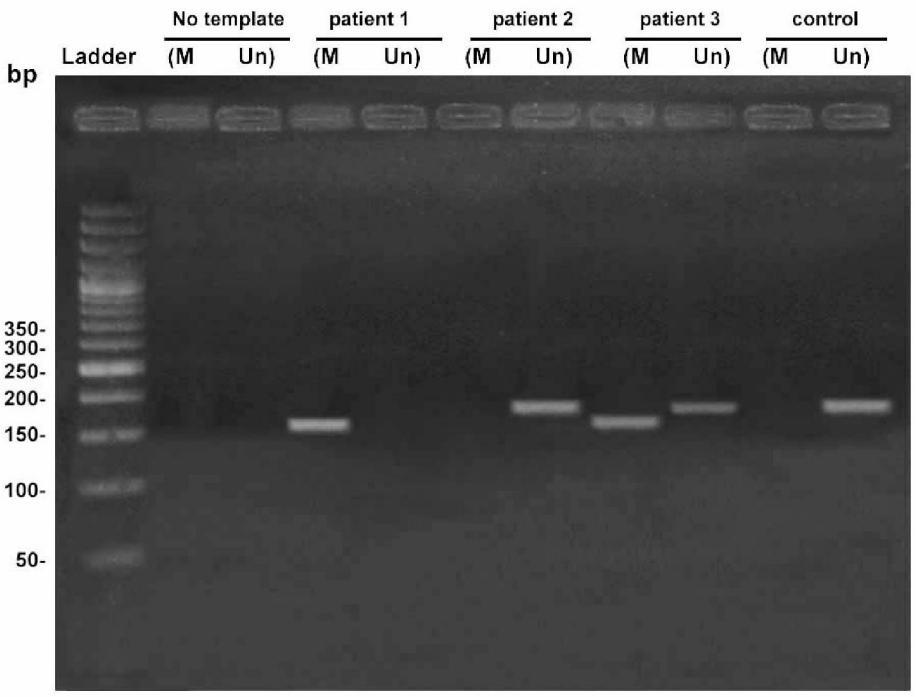

Figure (1): RASSF1A gene methylation pattern. Lanes viewed from left to right include a 50-bp ladder as a molecular weight marker, a water negative control, a methylated DNA band in patient 1, an unmethylated DNA band in patient 2, a heterozygous pattern (both methylated and unmethylated bands) in patient 3 and an unmethylated DNA band in a control sample.

4. Discussion:

Data of the present study revealed highly significant increase of RASSF1A methylation in patients serum versus controls, with methylation detected in 47.2% (17/36) and in none of the controls (100% specificity). These data are in accordance with the finding of ***Hoque and his colleagues*** [5] where RASSF1A methylation was detected in plasma of 43% (16/37) breast cancer patients and 13% (5/38) of controls. The methylation found in some of the controls might reflect chronic exposure of healthy subjects to still unidentified environmental carcinogenic factors, or could be a sign of an early starting neoplastic event, or both. ***Dulaimi et al.*** [7] revealed RASSF1A methylation in 56% (19/34) of breast cancer patients and none of controls. In

another study by ***Shukla et al.*** [8], RASSF1A was found to be methylated in 75% (15/20) of sera from breast cancer patients.

The low diagnostic sensitivity of assaying a single gene methylation is most probably related to the presence of normal DNA in serum which masks minute amounts of circulating tumor-related DNA. Therefore, detection of multiple genes increases the probability of detecting circulating tumor-related DNA [9]. In a study conducted by ***Dulaimi et al.*** [7] examining the promoter methylation status of three normally unmethylated genes; RASSF1A, adenomatous polyposis coli (APC), and death-associated protein kinase (DAP-kinase) in serum; hypermethylation of one or more genes was found in 76% (26/34) versus 56% (19/34) for RASSF1A only. The same was concluded in a study by ***Hoque and his colleagues*** [5] who determined aberrant methylation of four genes (APC, GSTP1, RASSF1A, and RARβ2) in plasma. Methylation of one of the four genes of interest was detected in 62% (29/47) versus 43% (16/37) for RASSF1A only. Furthermore, ***Jing et al.*** [10] investigated methylation profile of ten genes in serum of 50 breast cancer subjects and 50 healthy controls and found that methylation of at least one gene was found in 92% (46/50) versus 74% (37/50) for RASSF1A only.

Data of the present study showed no significant difference of age between RASSF1A gene methylated and unmethylated patients, in accordance to the studies of ***Burbee et al.*** [11], ***Shukla et al.*** [8] and ***Feng et al.*** [12]. In contrast, ***Euhus and his colleagues*** [13] reported that methylation increases in benign breast epithelium with increasing age and linearly between 32 and 55 years, and ***Hoque et al.*** [5] who found that RASSF1A gene methylated was associated with increasing age.

No statistically significant difference was found in the present study between RASSF1A gene methylated and unmethylated patients as regards presence of family history of breast cancer. This suggests the possibility of environmental factors having the upper hand for the methylation process, which was supported by many studies linking environmental risks to DNA methylation, as low folate [14], high alcohol intake [14, 15], smoking [15], menopausal status [5], early age of menarche and late age of menopause [16]. Some of these risk factors, namely menopausal status and menopausal age, were non-significantly different in the present study between those patients with methylated gene and those without, denoting the necessity of further exploration of these risks on a wider-scale study.

As regards, CA 15-3 serum levels, no statistically significant difference was found between RASSF1A gene methylated and unmethylated patients. This is in accordance with ***Jing et al.*** [10] who found that RASSF1A methylation was not associated with elevated serum CA 15-3 levels. This may be related to our study group which included breast cancer patients of different stages, with or without metastasis. CA15-3 is known to fall within normal reference limits in primary breast cancer, and is more observed to increase in cases of metastasis [17].

One prognostic aspect of CA15-3 and RASSF1A in breast cancer was evaluated in the current study through studying association of CA15-3 concentration and RASSF1A methylation status with tumor invasiveness. There was a significant association between stage subgroups and each of CA 15-3 serum levels and RASSF1A gene methylation status. Similarly, ***Safi et al.*** [18] and ***Gion et al.*** [19] concluded that CA 15-3 serum levels were associated with tumor burden, being significantly higher in late versus early stages. Also, it was reported that RASSF1A methylation was associated with advanced stage in a study carried out by ***Hoque et al.*** [5]. This may be referred to that the normally unmethylated RASSF1A gene inhibits cell migration and enhances cell adhesion [20]. Therefore, RASSF1A gene methylation and down-regulation result in disruption of cell adhesion. Furthermore, more methylation and loss of function, which inhibits cell growth and induces cell death, is a characteristic of aggressive malignant cells with high proliferative power [21]. Both increased malignant cell shedding and amplified proliferation contribute to tumor invasiveness and metastasis. Other prognostic aspects of RASSF1A were evaluated in different studies. ***Shukla and his colleagues*** [8] determined that RASSF1A methylation correlated with poor breast cancer prognosis and distant metastases. Furthermore, RASSF1A gene methylation proved to be associated with lower disease-free survival, increased chance of disease recurrence and was identified as an independent predictor of poor prognosis [22]. The same was revealed by a study conducted by ***Gobel et al.*** [23] on 428 breast cancer patients' plasma. They concluded that methylated RASSF1A was also an indicator for poor overall survival and distant disease free survival and, therefore, methylated RASSF1A appears to have promising potential as prognostic markers in clinical use. Because of their potential for reversal, epigenetic modifications are also appealing as targets for preventive care and therapeutics [24].

In the context of histological type, we found no statistically significant association between RASSF1A gene methylation and lobular or ductal types, which was in accordance with ***Burbee et al.*** [11] and ***Bae et al.*** [25].

It is noteworthy that we observed heterozygous pattern of RASSF1A gene methylation in 5.5% of patient samples (2/36) which gave 2 bands at both methylated and unmethylated lanes (160 and 180 bp, respectively). Most probably, the unmethylated band originated from normal DNA circulating in serum. The same pattern of RASSF1A gene methylation was observed by ***Maat et al.*** [26] in uveal melanoma tissue. They found a mixture of methylated and unmethylated DNA in 23% samples (9/39) and concluded that it was probably a result of contamination of the sample with normal tissue. Another explanation of heterozygous pattern by ***Peters and his colleagues*** [27] was due to the presence of undetected fibroblasts or tumor infiltrating lymphocytes. The probability of methylation of only one RASSF1A allele as a cause of our observed heterogenous pattern is unlikely. This is supported by the finding of many studies, in different tumors, that a "second hit" through promoter methylation of the remaining allele, following allelic loss, is indispensable for gene inactivation and tumor development[28]. ***Grote and his colleagues*** [28] demonstrated that allelic loss is found in 90% of small cell lung cancer and 80% of non-small cell lung cancer. However, promoter methylation is necessary before gene inactivation and tumor development. This is also supported by ***Maat et al.*** [26] who concluded that loss of one copy of chromosome 3 (monosomy 3) in approximately 50% of all uveal melanomas and hypermethylation of the other copy could promote progression of uveal melanoma tumor.

In conclusion, RASSF1A gene methylation was statistically higher in cases than controls. Therefore, it can be considered a potential epigenetic marker for breast cancer, preferably as a part of a panel of genes to improve the diagnostic performance. Also, RASSF1A gene methylation was significantly associated with stage subgroups, which gives it an additional prognostic benefit. Its role as a demethylation anti-cancer therapy awaits further investigation.

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