RASSF1A Gene Hypermethylation in Tissue and Serum Together with Tissue Protein Expression in Breast Cancer Patients

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Abstract: Background: Recently genetics and epigenetics alterations have been found to be characteristic of malignancy and hence can be used as targets for detection of neoplasms. RASSF1A genehypermethylation has been a subject of interest in recent researches on cancer breast patients. **Design and methods:** We investigated 30 breast cancer patients and 10 control subjects diagnosed with benign lesions of the breast for RASSF1A methylation status in paired tissue and serum samples using MSP and we evaluated RASSF1A protein expression in tissues by IHC .Results were studied in relation to known prognostic clinicopathplogical features in breast cancer. **Results:** We evaluated 30 breast cancer patients mean age (50.9±7.7) years and 10 control patients mean age (38.4±8.6 **years**). Frequency of RASSF1A methylation in tissues, serum were 73% and 63.3% respectively and RASSF1A protein expression showed frequency of 46.7%. There was an association between RASSF1A methylation in tissues, serum and loss of protein expression in tissues with invasive carcinoma, advanced stage breast cancer, L.N metastasis, ER/PR negativity and HER2 positivity.RASSF1A methylation in serum showed high degree of concordance with methylation in tissues (Kappa =0.851, P <0.001). **Conclusion:** RASSF1A hypermethylation in tissues and serum and its protein expression may be a valid, reliable and sensitive tool for detection and follow up of breast cancer patients.

1-Introduction:

Breast cancer is the most common cancer and the second most common cause of death from cancer in women. Every year more than one million women are diagnosed with breast cancer and approximately 400,000 die[1].Breast cancer is the most common malignancy among Egyptianwomen[2],.For successful treatment and outcome, early detection of breast cancer is a necessity. Despite the availability of mammography and prevalence of self-examination, there is still additional benefit to be gained from additional screening methodologies.

The genetic and epigenetic alterations that initiate and drive tumorgenesis can be used as targets for detection of neoplasms in body fluids[3],because they may precede clinically obvious cancer, can be detected at sensitive levels, may be specific for tumor cells, and can potentially provide information about the prognosis and treatment of the disease[4,5]. CpG islands located in promoter regions of genes are normally unmethylated. In cancer cells, aberrant hypermethylation of these promoter regions is associated with transcriptional silencing. Hypermethylation is therefore an alternative mechanism for inactivation of tumor suppressor genes[6,7].

Also It has been found that gene hypermethylation is a common and early alteration in many tumor types[8-10], including breast[11,12], hence it is considered as a promising target for detection strategies in clinical specimens[4,5].

RASSF1 encodes several isoforms, including *RASSF1A, RASSF1B*, and *RASSF1C*, which are derived from alternative mRNA splicing and promoter usage[13].

RAS association domain family protein 1A (*RASSF1A*) methylation status has been examined in different tumors[13,14,15] and breast cancer[3,14]. *RASSF1A* identified at 3p21.3 was suggested as the major target tumor suppressor on the basis of its frequent epigenetic silencing[13].It was reported previously that *RASSF1A* is epigenetically inactivated in 40 –72% of primary lung tumors by *de novo* methylation at the CpG island in the promoter[16,17,14]. Methylation-associated inactivation of *RASSF1A* was also observed in a considerable proportion of breast, ovarian, and nasopharyngeal cancer cell lines and primary tumors[14,17-20]. In small cell lung cancers, allelic deletion at 3p21.3 is associated with *RASSF1A* methylation, suggesting that both genetic and epigenetic steps are crucial for *RASSF1A* inactivation in some tumor types. The tumor suppressor function of RASSF1A has been suggested by observations that exogenous expression of RASSF1A decreases *in vitro*-colony formation, suppresses anchorage-independent growth, and dramatically reduces tumorigenicity*in*

vivo[16,17]. With these tumor suppression effects, the presence of a RAS association domain suggests that RASSF1 proteins may function as effector molecules in Ras or related growth inhibitory signaling pathways.

Aim of the Work:

In this study we aim to study the methylation status of RASSF1A gene in paired serum and tissue samples in cancer breast patients together with immunohistochemical analysis of RASSF1A protein. Results will be studied in relation to prognostic clinicopathological features in a trial to reveal RASSF1A gene role in prognosis.

2-Materials and Methods
2.1 Specimen Collection:

Thirty consecutive patients diagnosed with breast cancer who were admitted to Zagazig University hospitals, in the period from January 2011 to December 2011, were enrolled in this study. Patients ages ranged from 34 to 62 years. There were 5 cases of ductal CIS, 2 lobular CIS, 20 invasive ductal, and 3 invasive lobular carcinomas. Matched preoperative serum and tissue specimens were obtained from breast cancer patients and from control group that included 10 patients with benign breast lesions (7 fibroadenomas; 3 fibrocystic changes).

As regards tissue samples, Four μm thick sections from formaline-fixed, paraffine-embedded tissue blocks were stained with hematoxylin–eosin for morphological assessment. Tumors were evaluated for tumor grade using the Elston and Ellis grading system for invasive carcinoma, and the criteria of the European Breast Screening Group for DCIS, and tumor stage based on TNM, according to the 2003 WHO classification of breast tumors[20].

Ethical consideration: A written consent was taken from all of the participants after explaining details, benefits as well as risks to them.

2.2 Immunohistochemistry

Immunohistochemical staining was carried out using streptoavidin-biotin immunoperoxidase technique (Dako-cytomation, Glostrup, Denmark). Three μm thick sections, cut from formalin fixed paraffin embedded blocks, were deparaffinized in Xylene and rehydrated in graded alcohol. Sections were boiled in citrate buffer (pH 6.0) for 20 min for antigen retrieval and then washed in phosphate buffer saline (pH 7.3). Blocking of endogenous peroxidase activity by 3% H_2O_2 in methanol was attained. The slides were then incubated over night with the monoclonal antibodies: anti-RASSF1A (mouse monoclonal IgG, clone 3F3, code number AB23950), anti-ER(mouse monoclonal IgG, code number sc-56833, Santa Cruz Biotechnology, CA), anti-PR (rabbit polyclonal IgG, code number sc-539, Santa Cruz Biotechnology, CA) and anti-HER2 (mouse monoclonal IgG, code number sc-33684,

Santa Cruz Biotechnology, CA). Incubation with secondary antibody and product visualization was performed employing (DakoCytomation, Glostrup, Denmark) method with Diaminobenzidine (DAB) substrate chromogen. Slides were finally counterstained with Mayer's haematoxylin. The primary antibody was replaced by phosphate buffer solution (PBS) for negative controls.

RASSF1A protein expression appeared as yellowish brown staining in the cytoplasm of the cells. Positivestaining in more than 10% of tumor cells in the examined area was considered.we calculated a score (intensity x % area) for each tumor as follows: weak <100, moderate 100–200, and strong >200. Then a score equal or over 100 was considered positive expression, and bellow 100 considered as significant loss of expression[22].

2.3 DNA Extraction: DNA was extracted from fresh frozen tissue or from blood using a standard technique according to the manufacturer's instructions(QIAampDNAMinikit,QIAGEN GmbH, Hilden, Germany).

2.4 Methylation Analysis: Specimen DNA was modified with sodium bisulfite, converting all unmethylated, but not methylated, cytosine to uracil followed by amplification with primers specific for methylated versus unmethylatedDNA[23]*by* using a commercial kit (EpiTect Bisulfite,QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions in brief the procedure comprises a few simple steps: bisulfite-mediated conversion of unmethylatedcytosines; binding of the converted single-stranded DNA to the membrane of an EpiTect spin column; washing; desulfonation of membrane-bound DNA; washing of the membrane-bound DNA to remove desulfonation agent; and elution of the pure, converted DNA from the spin Column then kept at -20°C for further using.

2.5 Methylation-specific PCR analysis:

PCR was performed with methylation specific primers RASSF1A (U)
F(5ʹTGGTTTTTTTTAGTTTTTTTTTGTT-3ʹ)
R(5ʹACTACCATATAAAATTACACACA-3ʹ)
RASSF1A (M)
F(5ʹGGTTTTTTTTAGTTTTTTTTTCGTC-3ʹ)
R(5ʹ- CTACCGTATAAAATTACACGCG -3ʹ)
using 200 ng of the bisulfite-modified genomic DNA as templates and EpiTect MSP kit (QIAGEN GmbH, Hilden, Germany) kit ,the cycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 57 °C for 30 s, and 72°C for 45 s the PCR products (15 μl) were resolved on a 2% agarose gel.

2.6 Statistical analysis:

Data was analyzed using SPSS win statistical package version 17 (SPSS Inc., Chicago, IL). Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For not-normally distributed quantitative

data, comparison between two groups was done using Mann-Whitney test. Odds ratio (OR) with it 95% confidence interval (CI) were used for risk estimation. A p-value < 0.05 was considered significant.

3- Results:

In the current study we evaluated 30 breast cancer patients mean age (50.9±7.7)years and 10 patients diagnosed as benign breast lesions (7 fibroadenomas and 3 fibrocystic change) used as a control group. The mean age was 38.4±8.6 years.

All patients were subjected to clinical and histopathologicalevaluation .Both patients and control groups were evaluated for RASSF1A gene hypermethylation in paired tissue and serum samples, furthermore RASSF1 protein expression in tissues was evaluated by Immunohistochemistry.

Demographic and clinicopathologic data of breast cancer patients and their frequencies as regards RASSF1A methylation status in tissues and serum and RASSF1A protein expression are shown in table (1).

	RASSF1 in tissue		RASSF1 in serum		RASSF1 IHC score	
Frequency	M	U	M	U	<100	≤ 100
N (%)	31 (70%)	9 (30%)	19(63.3%)	11(36.7%)	16(53.4%)	14(46.6%)
Age: < 50 y n= 13 (43.3)	8 (61.5)	5 (38.5)	7 (53.8)	6 (46.2)	6 (46.2)	7 (53.8)
≤ 50 y n= 17 (56.7)	13 (76.5)	4 (23.5)	12 (70.6)	5 (29.4)	10 (58.8)	7 (41.2)
P- value	0.376		0.346		0.491	
Type:						
carcinoma in situ n=7 (23.3)	3(42.9)	4(57.1)	2(28.6)	5(71.4)	3(42.9)	4 (57.1)
Invasive carcinoma n=23 (76.7)	18(78.3)	5(21.7)	17(73.9)	6(26.1)	13(56.5)	10 (43.5)
P- value	0.153		0.068		0.675	
Low grade (I, II) n=14 (46.7)	9(64.3)	5(35.7)	8(57.1)	6(42.9)	5 (35.7)	9 (64.3)
High grade (III) n= 14 (46.7)	12(85.7)	2(14.3)	11(78.6)	3(21.4)	10 (71.4)	4 (28.6)
P- value	0.385		0.42		0.058	
Early stage (0& I) n=12 (40)	5(41.7)	7(58.3)	4(33.3)	8(66.7)	4 (33.3)	8 (66.7)
Advanced stage (II& III) n= 18 (60)	16(88.9)	2(11.1)	15(83.3)	3(16.7)	12 (66.7)	6 (33.3)
P- value	0.006		0.005		0.073	
ER						
-ve n=9 (30)	9(100)	0(0)	9(100)	0(0)	8 (88.9)	1 (11.1)
+ve n=21 (70)	12(57.1)	9(42.9)	10(47.6)	11(52.4)	8 (38.1)	13 (61.9)
P- value	0.019		0.006		0.017	
PR						
-ve n=13 (43.3)	13(100)	0(0)	13(100)	0(0)	10 (76.9)	3 (23.1)
+ve n=17 (56.7)	8(47.1)	9(52.9)	6(35.3)	11(64.7)	6 (35.3)	11 (64.7)
P- value	0.002		< 0.001		0.024	
HER2						
-ve n=15 (50)	7(46.7)	8(53.3)	7(46.7)	8(53.3)	7 (46.7)	8 (53.3)
+ve n=15 (50)	14(93.3)	1(6.7)	12(80)	3(20)	9 (60)	6 (40)
P- value	0.014		0.058		0.464	
Lymph node						
-ve n=20 (66.7)	12(60)	8(40)	10(50)	10(50)	8 (40)	12 (60)
+ve n=10 (33.3)	9(90)	1(10)	9 (90)	1(10)	8 (80)	2 (20)
P -Value	0.091		0.032		0.038	

3.1 *Comparison among different clinicopathological groups as regards RASSF1A:*

There was near significant difference (*P*=0.153) between in situ and invasive carcinoma when compared as regards RASSF1A methylation in tissues , similarly it showed near significant difference (*P*=0.068) when compared as regards

methylation status in serum, as there was association of RASSF1A methylation with invasive breast cancer. while there was no statistical difference when compared as regards RASSF1A protein expression in tissue.

Comparison between low grade and high grade tumors (cut off point was grades I& II versus

Grade III). patients showed none significant difference when compared as regards methylation status in tissues and serum, while it showed near significant difference when compared as regards RASSF1A protein expression (P=0.058) .with higher frequency of loss of protein expression in tissues of high grade patients.

We found that when comparing early stage to advanced stage patients (cut of point was stage 0 & I versus stage II & III) as regards RASSF1A methylation in tissues and serum , it showed significant difference (P=0.005, 0.006, respectively) between both groups with association of RASSF1A methylation and advanced tumor stage, while when compared as regards RASSF1A protein expression in tissues it showed near significant difference (p=0.073) with higher frequency of loss of protein expression in tissues with advanced stage patients.

Patients without lymph node metastasis were compared to patients with LN metastasis as regards RASSF1A methylation in tissue and serum and protein expression in tissues , near significant difference was found between the two groups (P=0.091) when compared in tissue . A statistical significant difference was found when compared in serum or protein expression in tissues (P= 0.032, 0.038 respectively), as there was an association between methylation in tissue and serum on one hand and lymph node metastasis on the other, moreover lymph node metastasis was associated with loss of protein expression.

3.2 Comparison according to hormone receptors and HER2 status as regards RASSF1A:

In our study all patients were evaluated according to their hormone receptor status, we found that there was a significant difference between ER-ve and ER+ve patients as regards RASSF1A methylation in tissue, serum and protein expression in tissue (P= 0.019 , 0.006, 0.017, respectively) as there was higher frequency of methylation in tissues and serum in ER-ve patients, moreover there was an association between ER negativity and loss of protein expression.

There was a significant difference between PR-ve and PR+ve patients as regards RASSF1A methylation in tissue, serum and protein expression in tissue (P=0.002 ,<0.001, 0.024 respectively), there was an association between methylation in tissue and serum ,also loss of protein expression and PR-ve patients.

HER2-ve patients showed statistically significant difference from HER2+ve patients as regards RASSF1A methylation in tissue (P=0.014) while it showed near significant difference as regards methylation in serum (p=0.058) with higher frequency of methylation in HER2 +vepatients , while there was no significant difference as regards RASSF1A protein expression in tissues. Moreover Triple negative patients(ER-ve,PR-ve,HER2-ve) showed methylation in both tissue and serum and loss of protein expression in all 4 cases.

3.3 Case-control comparison and risk estimate:

10 patients diagnosed as benign lesions of the breast, there was a highly significant statistical difference between patients group and control group when compared as regards age(P<0.001) with the older age incidence in cancer breast patients.

Comparison between Breast cancer patients and control group as regards RASSF1A methylation in tissue and serum ,showed highly statistical significant difference(P<0.001) with risk estimate (odd's ratio 2.1, 1.9 respectively) 95% confidence interval (1.3-3.4) and (1.3-2.9), while it showed significant difference when both groups where compared as regards RASSF1 protein expression by IHC (P=0.003) with risk estimate (odd's ratio: 1.7), (95% confidence interval 1.2-2.4).

3.4 Measurement of agreement for RASSF1A in tissue, serum and protein expression by IHC:

In the present study, we evaluated the concordance (measurement of agreement)between RASSF1A methylation in tissue and serum it showed a highly significant agreement (Kappa=0.851, p<0.001) with a sensitivity of serum testing 90.5% and a specificity 100%, while the positive predictive value of serum was 100% the negative predictive value was 81.1% in reference to RASSF1A methylation in tissue.

As for symmetric measures for both RASSF1A protein expression by immunohistochemistry compared to RASSF1A methylation in tissue showed significant measurement of agreement(kappa=0.521, p=0.004),while it showed non significant agreement between RASSF1A protein expression and methylation in the serum.

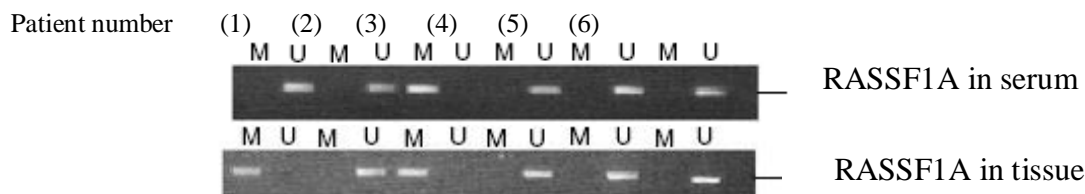Patient number   (1)   (2)   (3)   (4)   (5)   (6)

Fig.1. Representative samples of methylation specific PCR assays of RASSF1A in tissue and serum Methylated alleles (M) 269 bpunmethylated alleles (U) 271bp
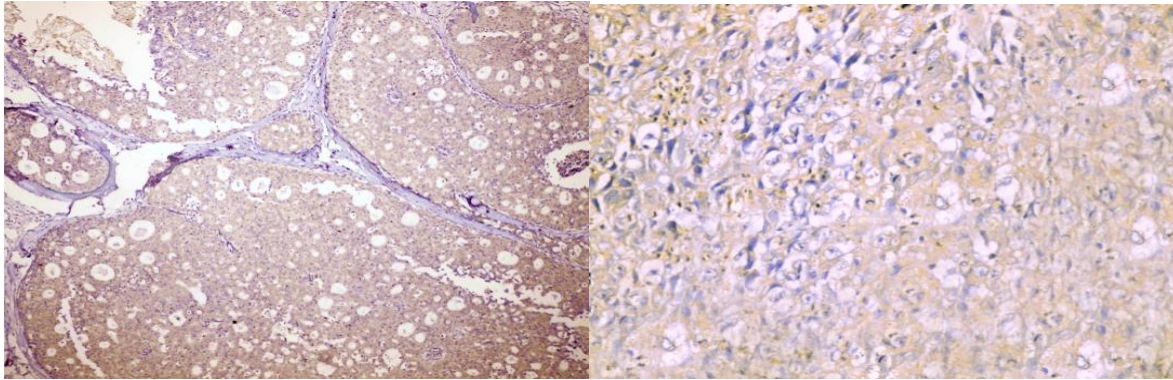
Fig. (2A): A case of ductal carcinoma in situ (UM) showing strong RASSF1A immunoreactivity (original magnification X 200)

Fig. (2B): A case of invasive duct carcinoma (M) showing moderate RASSF1A immunoreactivity (original magnification X 400)
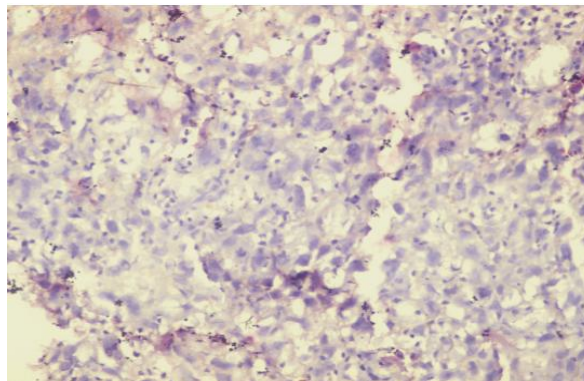

Fig. (2C): A case of invasive duct carcinoma (M) showing negative RASSF1A immunoreactivity (original magnification X 400)

4- Discussion:

Alteration in the methylation status of DNA are amongst the most frequent molecular changes associated with human cancers[24,4,25]. Aberrant promoter methylation has been described for several genes in various malignancies and the wide spectrum of genes involved suggest that specific tumors may have their own distinct methylation profile[25,26].

RASS1A gene has been a common factor in recent studies using a panel of genes to study hypermethylation in cancer breast patients[27-29].They tried to explore the role of RASSF1A and other genes epigenetics in the prognosis, early detection and differentiation between malignant and non malignant lesions.

Similarly, we conducted our study to explore the importance of RASSF1A gene methylation and protein expression in breast cancer patients and study the link with clinicopathological characteristics in an attempt to assess its role in prediction of prognosis.Moreover, we tried to assess the sensitivity of non invasive , accessible serum samples as a potential tool for follow up of patients.

In the current study, we investigated 30 breast cancer patients with mean age (50.9±7.7years) and 10 benign breast lesions(38.4±8.6years) ,as control group for RASSF1A methylation status in tissues ,and serum together with RASSF1A protein expression in tissues. We also studied clinicopathological features and hormone receptor status of cancer breast patients.

We found that there was no statistical significant difference between patients of different age groups (>,<50) as regards methylation status in tissue or serum or protein expression in tissues (*P*= 0.376,0.346,0.491 respectively). This is similar to previous studies[27,30]who didn't find any correlation between age and gene promoter methylation or protein expression. While it is different from another Tunisian study that found an association of age at diagnosis and methylation of RASSF1A gene (*P*=0.048) and they concluded that silencing of tumour suppressor gene by abnormal methylation is a prevalent event in tumors from younger patients[31]also other previous studies found association between age and methylation[32,33]. The discrepancy among studies may be explained by the fact that methylation profile of cancers is ethnicity specific[34,35].

Frequency of methylation of RASSF1A gene in tissues and serum was 70% and 63.3%respectively). In tissues it is lower than Karray –chouayekh*et al.*[31]who found that frequency of methylation in breast cancer patients is 87% and somehow it is comparable to another study by Park *et al.*[28) who found that frequency of

methylation in tissues was 76% , ours was higher than another study[22] who found that methylation frequency among breast cancer patients was 67%.As regards serum , hypermethylation frequency in another study was 65%[3]. Regarding RASSF1A protein expression, 53.4% of our cases showed weak or absent expression. This is lower than the incidence in the work of Li *et al.*[30] (72.2%). These differences may be attributed to different selection criteria and difference in sensitivity of MSP technique and anti-RASSF1A antibodies.

Comparison between in situ and invasive breast cancer as regards RASSF1A methylation in tissue, serum and protein expression revealed near significant difference between the groups with association of hypermethylation and invasive tumors. This is similar to a previous study[3] who found an association of hypermethylation of RASSF1A and invasive tumors .this can be explained by the fact that RASSF1A modulates multiple apoptotic cell cycle checkpoints pathways and hence its methylation may lead to progression of the disease[36,37].

As for protein expression in tissues, we found no significant difference between in situ and invasive carcinomas. However, Alvarez *et al.*[22] found a significant decrease in protein expression in cases of in situ carcinoma which is contradicting our study this can be explained by the difference in genetic behavior among ethnic populations.

There was no statistical significant difference between low and high grade tumors as regards RASSF1A methylation in tissues and serum this is similar to a recent study[31]. While there was near significant difference (P=0.058) when compared as regards protein expression in tissues with association between loss of expression and high tumor grade.

These findings are different from Alvarez *et al.*[22] who did not find any correlation between RASSF1A and protein expression this can be explained by difference in sample size and selection criteria.

In the present study we found that there was a statistical difference between patients diagnosed with early and advanced stages. this is similar to another study[31] who found an association between RASSF1A methylation and advanced tumor stage.(P=0.03).

On the other hand comparison between the same groups as regards protein expression in tissues showed near significant difference with association between loss of protein expression and advanced tumor stage, this is similar to a previous study[22]. This can be explained by the fact that promoter hyper methylation is a relevant molecular mechanism in inhibiting protein expression

Comparing patients with lymph node metastasis to patients without L.N metastasis as regards RASSF1A methylation in tissues showed

near significant difference, and there was a statistical significant difference when compared as regards RASSF1A methylation in serum or protein expression in tissues, as there was an association between L.N metastasis and methylation in tissues and serum also an association with loss of protein expression in tissues. This is similar to a study by Muller *et al.*[38]who found that L.N metastasis had a trend of high prevalence of methylation compared to the primary breast carcinoma which suggests that RASSF1A methylation may be a participant of key molecular pathways in tumor progression and aggressive tumor behavior.

In our study there was a significant association between RASSF1A methylation in tissue, serum and loss of protein expression and ER/PR negativity .This is similar to Sunami*et al.*[39]who found a strong correlation between double negative marker and hypermethylation. Similarly a recent study[36] found a strong correlation between ER/PR/HER2 triple negative and hypermethylation, this may have been explained by the possibility that RASSF1A methylation is associated with bad prognosis and poor clinical outcome, but the findings by previous studies[40,22,41,29,30] contradicted with our results as they found an association between ER/PR positivity and RASSF1A methylation , we recommend further studies in this context with larger number and more sensitive MSP techniques.

On the other hand a significant association between HER2 positivity and RASSF1A methylation in tissues and serum, but not with protein expression. Previous studies[31,40] found non significant correlation between methylation and Her2 status. The contradiction can be explained by the difference in distribution of grades and stages among patients.

In the present study all cases with RASSF1A methylation showed loss of protein expression in tissues, this is in agreement with Alvarez et al*et al.*[22] who found a highly significant association (P= 0.0063) between RASSF1A promoter hypermethylation and loss of protein expression, and they explained that promoter hypermethylation is a relevant molecular mechanism in inhibiting protein expression. furthermore, Li *et al.*[30] suggested that methylation may be responsible for alleles silencing. The silencing of gene expression may also be explained by gene deletion or point mutation, tumors having deletion of RASSF1 and presenting M and UM PCR products ,show a significant loss of protein expression[22].

In the current study we compared our breast cancer patients to a control group(n=10) diagnosed as fibroadenomaand fibrocystic disease, they were all negative for RASSF1A methylation and strongly expressing RASSF1A protein.
We found that there was a highly significant statistical difference between patients and controls

as regards age, with older age incidence in cancer breast patients. This is similar to a recent study by Cho *et al.*[29],this can be explained by the fact that cancer breast occurs in older age

Comparison between patients and control groups as regards RASSF1A methylation in tissue and serum showed highly significant difference with risk estimate (odd's ratio 2.1,1.9) respectively while it showed a significant difference as regards protein expression by IHC with risk estimate (odd's ratio1.7) .

This means that RASSF1A methylation and protein expression could be valuable tests in discrimination of malignant from non malignant breast lesions. This is consistent with a previous study[4] who stated that RASSF1A methylation could be used as a cancer molecular marker.

Also we are in agreement with several previous studies[42,43,44,38]who demonstrated that the acquisition of high level methylation at RASSF1A gene promoter and other studied genes is relevant for breast tumorgenesis, enabling their use as a specific breast cancer marker.

Aberrant promoter methylation needs to be used as a routine clinical test for breast cancer detection which obligates the use of more accessible samples,less painful and less intruding with female privacy.

In a trial to evaluate how serum samples can be trusted with suspecting, diagnosis and follow up of cancer patients , we studied the degree of concordance between RASSF1A methylation in tissues and serum ,we found that measurement of agreement showed high degree of concordance(Kappa=0.851, *P* <0.001)

Moreover we found that sensitivity of serum testing of RASSF1A was 90.5%, specificity 100% ,positive predictive value was 100% and negative predictive value was 81.1%. This is in context with Dulaimi*et al.*[3] who confirmed that hypermethylation can be detected by MSP in serum DNA and it can be considered as a screening method which may enhance early detection of breast cancer.

Moreover a recent study by Yamamoto *et al.*[45] evaluated paired serum and tissue samples from breast cancer patients for detection of hyper methylation in a panel of genes including RASSF1A and concluded that the use of more sensitive MSP technique is promising for enhancing the sensitivity for diagnosis of metastatic breast cancer and moreover this can be used as a potential tumor marker for early detection of cancer breast. They also evaluated RASSF1A gene methylation before and after surgery and they found that it turned to be negative after surgery which confirms that the origin of serum DNA is the tumor itself.

Conclusion:

RASSF1A gene hypermethylation in tissue and serum together with loss of RASSF1A protein expression are associated with clinicopathological features of bad prognosis in breast cancer patients. RASSF1A hypermethylation in serum shows high concordance with hypermethylation in tissue and shows reasonable sensitivity and specificity. In this context RASSF1A may be used in prediction , early diagnosis , follow up in breast cancer patients.

Recommendations:

More researches should be done on gene hypermethylation including larger number of patients, and different panels of genes should be tried to come up with a panel that can be used as routine investigation for diagnosis and follow up of breast cancer patients. Indeed, researches on more accessible body fluids specially serum and blood plasma should be addressed for better screening procedures. Moreover, more sensitive MSP techniques should be enhanced and developed for more accurate detection.

Conflict of interest: None

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5. References:
1- Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics,.CA Cancer J Clin.2002; 55: 74–108.
2- Kassim SK, Fawzy H, El Rassad MM, Khalil FK, and KhalifaA. Telomerase activity and tissue polypeptide specific antigen (TPS) in Egyptian breast cancer patients. ClinBiochem.2001; 34(6): 499-504.
3- Dulaimi E, Hillinck J, Ibanez de Caceres I, Al-Saleem T, Cairns P.Tumor suppressor gene promoter hypermethylation in serum of breast cancer patients. Clin Cancer Res.,2004; 10: 6189–6193.
4- Sidransky D. Emerging molecular markers of cancer.Nat Rev Cancer 2002; 2: 210–219.
5- Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer, 2003; 3: 253–66.
6- Baylin SB, Herman JG, Graff JR, Vertino PM, Issa J-PJ. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res., 1998; 72: 141–96.
7- Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet.,1999; 21: 163–7.
8- Belinsky SA, Nikula KJ, WAP**, et al.**Aberrant methylation of p16INK4a is an early event in lung cancer and a potential biomarker for early diagnosis. Proc NatlAcadSci USA 1998; 95: 11891–6.
9- EstellerM, Sparks A, Toyota M, *et al.*Analysis

of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res**.,** 2000; 60: 4366 –71.

10- Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer.Cancer Re**s.,** 2001; 61: 3225– 3229.

11- Lehmann U, Langer F, Feist H, Glockner S, Hasemeier B, Kreipe H. Quantitative assessment of promoter hypermethylation during breast cancer development. Am J Pathol., 2002; 160: 605–12.

12- Holst CR, Nuovo GJ, Esteller M, Chew K, Baylin SB, Herman JG, Tlsty TD/ Methylation of p16(INK4a) promoters occurs *in vivo* in histologically normal human mammary epithelia. Cancer Res., 2003; 63: 1596–601.

13- Lee MG, Kim HY, Byun DS, Lee SJ, Lee CH, Kim JI , Chang SG, Chi SG. Frequent epigenetic inactivation of RASSF1A in human bladder carcinoma.Cancer Res., 2001; 61: 6688–6692.

14- Agathanggelou Angelo, HonorioSoa, MacartneyDonia P, Martinez Alonso, Dallo Ashraf, Rader Janet, Fullwood Paul, Chauhan Anita *et al.,* Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours Oncogene 2001; 20: 1509-1518.

15- Byun Do-Sun, Lee Min-Goo, Chae Kwon-Seok, RyuByung- Gyu, and Chi Sung-Gil. Frequent Epigenetic Inactivation of *RASSF1A* by Aberrant Promoter Hypermethylation in Human Gastric Adenocarcinoma. Cancer Research, 2001; 61: 7034 –7038.

16- Dammann R, Li C, Yoon J-H, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet., 2000; 25: 315–9.

17- Burbee DG, Forgacs E, Zochbauer-Muller S, Shivakumar L, Fong K, Gao B, Randle D, Virmani A, Bader Y, Sekido Y, Latif F, Milchgrub S, Toyooka S, Gadzar AF, Lerman MI, Zabarovsky E, White M and Minna JD. The RASSF1A locus in the 3p21.3 homozygous deletion region: epigenetic inactivation in lung and breast cancer and suppression of the malignant phenotype. J Natl Cancer Inst (Bethesda) 2001; 93: 691–699.

18- Dammann R, Yang G, Pfeifer GP. Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res.,2001; 61: 3105-3109.

19- Lo KW, Kwong J, Hui ABY, Chan SYY, To KF, Chan ASC, Chow LSN, Teo PML, Johnson PJ, Huang DP. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma.Cancer Res., 2001; 61: 3877– 3881.

20- Vos MD, Ellis CA, Bell A, Birrer MJ, Clark GJ. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. J BiolChem 2000; 275: 35669–35672.

21- Tavassoéli FA, Devilee P. Pathology and Genetics Tumours of the Breast and Female Genital Organs [M] , IARC Press, Lyon, 2003, p. 10

22- Alvarez Carolina, Tapia Teresa, Cornejo Valeria, Fernandez Wanda, oz Alex Mun˜, Camus Mauricio, Alvarez Manuel, Devoto Luigi, and CarvalloPilar Silencing of Tumor Suppressor Genes RASSF1A, SLIT2, and WIF1 by Promoter Hypermethylation in Hereditary Breast Cancer. (2012) Wiley Periodicals, INC. Molecular Carcinogenesis. Published online in Wiley Online Library DOI 10.1002/mc.21881.

23- Herman J, Graff J, Myhanen S, Nelkin B, Baylin S. Methylation specific PCR: a noval PCR assay for methylation status of CpG islands. Proc NatlAcad Sci., 1996; 93: 9821-9826.

24- Rountree MR, Bachman KE, Herman JG, et al. DNA methylation, chromatin inheritance, and cancer. Oncogene 2001; 20: 3156–3165.

25- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet., 2002; 3: 415–428.

26- EstellerM.CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002; 21: 5427–5440.

27- Jeronimo Carmen, Monteiro Paula, Henrique Rui, Dinis-Ribeiro Ma´rio, Costa Isabel, Costa Vera L, Filipe Lu´ısa, Carvalho Andre´ L, Hoque Mohammad O, Pais Irene, Leal Conceic¸a˜o, Teixeira Manuel R, Sidransky David. Quantitative hypermethylation of a small panel of genes augments the diagnostic accuracy in fine-needle aspirate washings of breast lesions. Breast Cancer Res Treat .,2008; 109: 27–34.

28- Park So Yeon, HyeongJu, Lee HeeEun, Ryu Han Suk, Kim Sung-Won, Kim Jee Hyun, Kim In Ah. Jung Namhee, Cho Nam-Yun, Kang GyeongHoon. Promoter CpG island hypermethylation during breast cancer progression Virchows Arch 2011; 458: 73–84.

29- Cho Yoon Hee，Shen Jing，Gammon Marilie D，Zhang Yu-Jing，Wang Qiao，Gonzalez Karina，XuXinran， Bradshaw Patrick T， Teitelbaum Susan L， Garbowski Gail， HibshooshHanina，Neugut Alfred I，Chen Jia，Santella Regina M. Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012; 131: 197–205.

30- Li Y, Wei Q, Cao F, Cao X. Expression and promoter methylation of the RASSF1A gene in sporadic breast can- cers in Chinese women. Oncol Rep., 2008;19:1149–1153.

31- Karray-ChouayekhSondes, TrifaFatma, KhabirAbdelmajid, BoujelbaneNouredine, Sellami-BoudawaraTahia, DaoudJamel, FrikhaMounir, JlidiRachid, Gargouri Ali, Mokdad-Gargouri Raja Aberrant methylation of *RASSF1A* is associated with poor survival in Tunisian breast cancer patients. JCancer Res ClinOncol., 2010; 136: 203–210.

32- Issa JP, Ottaviano YL, CelanoP ,Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 1994; 7: 536–540.

33- Waki T, Tamura G, Sato M, Motoyama T. Age-related methylation of tumor suppressor and tumour-related genes: an analysis of autopsy samples. Oncogene 2003; 22: 4128–4133.

34- Bae YK, Brown A, Garrett E, Bornman D, Fackler MJ, Sukumar S*etal.*Hypermethylation in histologically distinct classes of breast cancer. Clin Cancer Res 2004; 10: 5998–6005.

35- Mehrotra J, Ganpat MM, Kanaan Y, Fackler MJ, McVeigh M, Lahti-Domenici J, Polyak K, Argani P, Naab T, Garrett E, Parmigiani G, Broome C, Sukumar S. Estrogen receptor/ progesterone receptor-negative breast cancers of young African- American women have a higher frequency of methylation of multiple genes than those of Caucasian women. Clin Cancer Res.,2004; 10: 2052–2057.

36- Gobel Georg, Auer Doris,GauggInge,SchneitterAlois,Lesche Ralf, Muller-Holzner Elisabeth, Marth Christian, Daxenbichler Gunter. Prognostic significance of methylated RASSF1A and PITX2 genes in blood- and bone marrow plasma of breast cancer patient.Breast Cancer Res Treat., 2011; 130: 109–117.

37- Donninger H, Vos MD, Clark GJ. The RASSF1A tumor suppressor. J Cell Sci., 2007; 120: 3163–3172.

38- Muller HM, Widschwendter A, Fiegl H, Ivarsson L, Goebel G, Perkmann E, Marth C, Widschwendter M. DNA methylation in serum of breast cancer patients: an independent prog- nostic marker. Cancer Res., 2003; 63: 7641–7645.

39- Sunami E, Shinozaki M, Sim M, Nguyen SL, Vu A, Giuliano AE, Hoon DSB. Estrogen receptor and HER2/neu status affect epigenetic differences of tumor-related genes in primary breast tumors. Breast Cancer Res 2008; 10: R46.

40- FengWeiwei, Orlandi Rosaria, Zhao Naiqing, Carcangiu Maria Luisa, Tagliabue Elda, XuJia, Robert C BastJr, Yu Yinhua Tumor suppressor genes are frequently methylated in lymph node metastases of breast cancers. BMC Cancer 2010; 10: 378.

41- AuweraIlse Van der, Bovie Catherine, Svensson Cecilia, Trinh Xuan B, LimameRidha, Dam Peter van, Laere Steven J van, Marck Eric A van, Dirix Luc Y, Vermeulen Peter B Quantitative methylation profiling in tumor and matched morphologically normal tissues from breast cancer patients. BMC Cancer 2010; 10:97.

42- Evron E, Dooley WC, Umbricht CB, Rosenthal D, Sacchi N, Gabrielson E, Soito AB, Hung DT, Ljung B, Davidson NE, Sukumar S.Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet 2001; 357:1335–1336.

43- Fackler MJ, McVeigh M, MehrotraJ ,Blum MA, Lange J, Lapides A, Garrett E, Argani P, Sukumar S.Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. Cancer Res 2004; 64(13):4442–4452.

44- Hoque MO, Feng Q, Toure P, Dem A, Critchlow CW, Hawes SE, Wood T, Jeronimo C, Rosenbaum E, Stern J, Yu M, Trink B Kiviat NB, Sidransky D. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. J ClinOncol2006; 24:4262–4269.

45- Yamamoto Noriaki, Nakayama Takahiro,Kajita Masahiro,Miyake Tomohiro,Iwamoto T, Kim SJ, Sakai A, Ishihara H, Tamaki Y, Noguchi S. Detection of aberrant promoter methylation of GSTP1, RASSF1A, and RARb2 in serum DNA of patients with breast cancer by a newly established one-step methylation-specific PCR assay. Breast Cancer Res Treat 2012; 132:165–173.