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THE CLINICAL SIGNIFICANCE OF RASSF1A AND CDH1 HYPERMETHYLATION IN BREAST CANCER PATIENTS

Biochemistry

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ABSTRACT

Introduction: Aberrant DNA methylation patterns in serum DNA might be used as a biomarker for the early diagnosis and management of cancer patients. The aim of present study was to evaluate DNA methylation of *RASSF1A* and *CDH1* in circulating cell free DNA (cfDNA) from serum and paired tissue DNA samples of breast cancer patients.

Material and methods: Methylation-specific PCR was used to assess the methylation status of the two genes in serum and paired tissue sample DNA of 50 breast cancer patients. Biochemical parameters were assessed using an electrochemiluminescence analyzer.

Results: Significant correlation found between methylation status of *RASSF1A* and *CDH1* in serum and paired tissue samples of patients. Among clinicopathological findings, *CDH1* methylation showed significant association with advance staging and tumor and methylation of *RASSF1A* exhibited significant association with progesterone receptor and estrogen receptor status in both serum and paired tissue. Vitamins levels were significantly high in cases compared to control group. High folic acid levels were significantly associated with the *RASSF1A* methylation.

Conclusions: These findings suggest that methylation of cfDNA may be important in the early detection of breast cancer.

KEYWORDS

Methylation, RASSF1A, CDH1, Methylation-specific PCR, Vitamin B12, Folic acid

Introduction

Breast cancer is the most commonly diagnosed and leading cause of cancer death in females worldwide. The global burden of breast cancer exceeds all other cancers and incidence rates of breast cancer are increasing, accounting for 23% (1.38 million) of total new cancer cases in 2008 [1]. Detection of breast cancer at an early stage has improved the prognosis and survival rate up to 98% [2] and requires a less severe treatment, however diagnosis following tumor metastasis has decreased the survival rate by up to 27% [3]. Screening mammography sensitivity varies from about 68-93% [4]. There remains a requirement for a minimally invasive, cost-effective procedure that can be used along with mammography to improve screening sensitivity.

Epigenetics is defined as a stable change in gene expression potential that takes place throughout development and cell proliferation, with no associated changes in gene sequences. Gene silencing through hypermethylation is a well-known, common event in carcinogenesis, and is thought to be contributing factor for the overall genetic instability and gives selective growth advantage to the tumor [5]. Hypermethylation is known to be a frequent and early alteration in several tumor types, including breast, and has emerged as a promising target for the detection and diagnosis of various diseases [6]. Previous studies on DNA hypermethylation in breast cancer have identified a number of vital genes as targets for epigenetic events, including cell signaling intermediates (*RASSF1A)*, adhesion molecule (*E-cadherin)*, DNA repair *(BRCA1*), receptors such as the estrogen receptor, and retinoic acid receptor-beta *(RAR-beta*), cytokines such as *HIN-1*, cell cycle regulators (*Cyclin D2)* and many more [7].

One promising approach to the detection of breast cancer is via study of the circulating DNA from cancer cells [8]. A number of previous studies have revealed that cell-free DNA, present in the plasma or serum of cancer patients possess the same methylation patterns as that of primary source tumor DNA. Based on the previous studies, cfDNA may be used as a valuable source of genetic material for the molecular analysis in cancer and pre-cancer patients [9].

RASSF1A promoter hypermethylation is probably the most frequently studied epigenetic event in human cancers including breast cancer, and it was detected even in the serum of breast cancer patients. *RASSF1A* methylation frequencies range from 10-95% in breast cancer. Analysis of the methylation pattern of *RASSF1A* can be used as screening technique for the early detection of cancer [10].

E-cadherin is an important molecule in cell-cell adhesion in epithelial tissues. In addition to its role in normal cell function, it is important in malignancy, particularly in tumor development and progression. Thus, loss of E-cadherin function due to hypermethylation associates with increased invasiveness and metastasis of the tumors, and it has been proposed as a marker of poor prognosis [11].

The role of nutrients in affecting gene expression through their interaction with polymorphism and variation of DNA methylation has received considerable attention recently. B vitamin coenzymes such as folate, vitamin B6, and vitamin B12 are important in maintaining DNA integrity and stability, and inadequacy of these B vitamins may promote carcinogenesis. In a recent study, the influence of one-carbon metabolism on promoter methylation in breast-cancer related genes in a relatively large epidemiologic study was demonstrated [12]. As it is known that epigenetic modifications are reversible and occur early in tumor development, dietary intervention may possibly enhance cancer prevention. However, data relating to circulating levels of folate and vitamin B12 association with promoter hypermethylation of tumor suppressor gene in breast cancer patients are very limited.

The present study evaluated the promoter methylation status of *RASSF1A* and *CDH1* in serum samples, paired tumor tissue of breast cancer patients and its association with noncancerous controls patients with benign breast disease and normal healthy individuals using MS-PCR and their correlation with clinicopathological features, serum vitamin B12 and folic acid levels.

Materials and methods

Study population. The present study was performed on 50 samples of histopathologically confirmed, newly diagnosed cases of North Indian patients with breast cancer, including 25 age-matched non-cancerous benign breast disease cases and 25 healthy controls, collected from the Department of Surgery, Lok Nayak Jayprakash Hospital, New Delhi between October 2011 and October 2012. Blood samples (5ml) were obtained at the time of diagnosis before any treatment and serum samples were separated and stored at -80˚C until further processing. The present study was approved by the Institutional Ethics Committee of Maulana Azad Medical College, New Delhi. Written informed consent was obtained from each patient, including all non-cancerous

benign cases and healthy controls. Patients with a history of any other malignancy or metastasized cancer from any other organs were excluded from the present study. Demographic data of patients and controls are shown in (Table 1).

DNA extraction from serum samples and tissue samples and bisulfite modification. DNA was extracted from the paraffin-embedded tissues of breast cancer patients and non-cancerous benign breast disease patients using a tissue DNA extraction kit (Geneaid Biotech Ltd, New Taipei City Taiwan) according to the manufacturer's protocol. cfDNA was isolated from serum samples using the FitAmp™ Plasma/Serum DNA Isolation Kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer's instructions. DNA concentrations were measured and a total of 1 µg of DNA was used for bisulfite modification. Aberrant DNA methylation in CpG islands was determined by chemical modification of cfDNA and tissue DNA with sodium bisulfite treatment. Sodium bisufite conversion of cfDNA and tissue DNA was performed by using the BisulFlash™ DNA Modification Kit (Epigentek) according to the manufacturer's instructions.

Methylation specific-polymerase chain reaction (MS-PCR). Methylation analysis of RASSF1A and CDH1 genes were analyzed by Methylation Specific Polymerase Chain Reaction (MS-PCR). The primer sequences of RASSF1A and CDH1 genes were reported previously [13,14]. PCRs were run in a volume of 25 µl, containing 2 µl bisulfite-modified DNA, 12 µl of 2x Hot Start PCR Master mix (Fermentas International Inc., Ontario, Canada), 0.25 µl sense primer (25 pM), 0.25 µl antisense primer (25 pM), and 12.5 µl H2O. The PCR profile was 95˚C for 10 min, 40 cycles at 95˚C for 45 sec, primer annealing at 52 to 56˚C for 45 sec, 72˚C for 45 sec, and a final extension step at 72˚C for 10 min. The amplified PCR products were electrophoresed on 2% agarose gels and further evaluated under ultraviolet light gives 169 bp for both methylation and unmethylation of RASSF1A gene and 116 bp for methylation and 97 bp for unmethylation of CDH1 gene (Figure 1).

Determination of serum levels of vitamin B12 and folic acid levels. Serum levels of vitamin B12 and folic acid levels were quantified by using fully automated electrochemiluminescence analyzer (ELECSYS 2010, Hitachi High-Technologies Corp., Tokyo, Japan).

Statistical analysis. Statistical analysis was performed using SPSS ver. 16 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism ver 7.0 (GraphPad Software Inc., La Jolla, CA, USA). Categorical data were analyzed by chi square or Fisher's exact test. Correlation between methylation statuses of different genes was analyzed using Spearman correlation coefficient. The Mann-Whitney U test was used to assess differences between nonparametric distributed variables. Kruskal-Wallis test was used to assess differences between nonparametric distributed variables for more than two groups, and followed by *post-hoc* tests if indicated. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The clinicopathological characteristics of breast cancer patients, patients with non-cancerous benign lesions, and healthy volunteers are presented in (Table1). All patients had histopathologically confirmed invasive ductal carcinoma (IDC). Among these cases, 35 (70%) were ≤45 years, and 15 (30%) were >45 years. The mean age of the patients was 49.22 years (range 30-75 years). Menopausal conditions demonstrated that 17 (34%) samples were in premenopausal status and 33 (66%) samples were in postmenopausal status. Out of total cases, 32 (64%) samples were in early stages (I and II) and 18 (36%) samples were in advanced stages (III & IV). Tumor grading revealed that 5 (10%), 24 (48%) and 21 (42%) patients were in well, moderately and poorly differentiated groups, respectively. Lymph node status demonstrated that 30 (60%) cases were lymph node positive. Hormone receptor status revealed that 22 (44%) samples were positive for estrogen receptor (ER), 20 (40%) samples were positive for progesterone receptor (PR) and 32 (64%) were HER2/neu positive. The mean age of the healthy controls was 38.48 years (range 17-70 years).

Gene promoter hypermethylation in cfDNA and paired tissue samples of breast cancer patients. The frequency of promoter hypermethylation in serum DNA of *RASSF1A* and *CDH1* were 64 and 48%, respectively and as compared with the paired tissue samples, 70% of cases were methylated for *RASSF1A* followed by *CDH1* (50%). By contrast, in benign breast diseases patients the frequency of *RASSF1A* and *CDH1* was found to be 20 and 4%, respectively. None of the 25 healthy control serum samples contained hypermethylated DNA sequences (Table 2). A significant positive correlation between promoter methylation of genes in paired tissue DNA and cfDNA was identified (Table 3). Sensitivity and specificity of *RASSF1A* and *CDH1* were observed in all three study groups (breast cancer cases, benign breast diseases and healthy controls). In addition, the combination of different markers was also examined to maximize specificity and sensitivity (Tables 4). Among all the three genes, *RASSF1A* has better sensitivity (64%) and *CDH1* methylation revealed increased specificity of 96 and 100% in benign breast lesions and healthy controls, respectively. Demographic and clinicopathological features of breast cancer patients and their frequencies as in tissues and serum are shown in (Table 5). There was a significant association observed between methylation status of the *CDH1* gene with the tumor size and TMN staging ($P<0.05$). On the other hand, *RASSF1A* methylation showed a statistically significant association with the PR and ER status of breast cancer patients in both serum and paired tissue samples (Table 5).

Comparison of serum levels of vitamin B12 and folic acid among the cases and control groups. A statistically significant values of Vitamin B12 level was observed between cases and control groups (benign cases and healthy controls) with the median value of 430 pg/ml, 274 and 300 pg/ml respectively similarly in case of folic acid levels, with median value of 9.5 ng/ml level in cases was significantly higher ($P=0.02$) than in the control group The breast cancer patients had significantly high levels of both serum Vitamin B12 and folic acid levels than controls (benign and healthy controls) (Table 6). *Post hoc* test was performed for multiple comparisons (Dunn's Multiple Comparisons Test) was done for the both parameters (Table 7). *Post hoc* test was performed for multiple comparisons (Dunn's Multiple Comparisons Test) was done for the both parameters. Multiple comparisons test revealed the significant difference in the serum levels of vitamin B12 in cases and controls but not in the case of folic acid levels.

Association of methylation status of RASSF1A and CDH1 gene with the serum levels of vitamin B12 and folic acid of breast cancer patients. Statistically significant association was observed in the present study between methylation frequency of *RASSF1A* and serum levels of folic acid ($P=0.01$). We observed a statistically significant inverse association between methylation status of *CDH1* and serum vitamin B12 levels i.e. high vitamin B12 levels in methylation negative patients (Table 8).

Discussion

The present study investigated the diagnostic utility of promoter hypermethylation of the tumor suppressor genes (*RASSF1A* and *CDH1*) in the serum of breast cancer patients, and correlated it with the corresponding cancerous breast tissue. Cancer-specific methylation changes in plasma and serum could be used in developing blood-based tests, which may be used for the risk assessment, timely diagnosis and monitoring of various cancers. Frequency of methylation of *RASSF1A* gene in serum and tissue of breast cancer patients was 64 and 70%, respectively. The frequency of *RASSF1A* methylation was 20% in the serum of patients of benign breast diseases and 0% in the serum of healthy controls. Gawish et al. [15] have reported similar frequency of *RASSF1A* methylation in serum and tissue samples of breast cancer patients. A case-control study by Brooks et al. [16] reported different results, and stated that increased methylation frequency in benign breast diseases patients and healthy controls (22.9 and 17.2% respectively) was similar to the cases (22%).

The present study found no statistically significant difference between patients of reproductive age group and post-menopausal patients with regard to methylation status in serum and tissue, this is similar to the results of other studies [17]. However, a previous study [18] reported different results, and found an association between age at diagnosis and methylation of *RASSF1A* ($P=0.048$), concluding that silencing of the tumor suppressor gene by abnormal methylation is a phenomenon that is prevalent in the tumors of younger patients. In present study, no significant association was observed between *RASSF1A* and any clinicopathlogical parameters.. The *RASSF1A* promoter was significantly methylated at higher levels both in ER-positive and PR-positive breast cancer patients in both serum and paired tissue samples was found [19].

CDH1 is characterized as a potent suppressor of invasion and metastasis, and plays a major role in malignant cell transformation, especially in tumor development and progression.

We detected *CDH1* methylation in 24/50 (48%) of breast cancer patients in serum and 25/50 (50%) in paired tissue samples. Our results were similar to a previous study conducted [20] on western population. Differences among our result with other studies may be due to sample size or different study population. A 4% (1/25) methylation frequency found in benign breast diseases and sera from healthy controls did not show any methylation for *CDH1* (0/25). Our findings were in agreement with the results of previous studies in respect of healthy controls [21]. It has been reported previously that *CDH1* promoter methylation increases with breast tumor progression. Therefore, *CDH1* promoter methylation is an important event associated with the progression of breast cancer [22]. Consistent with these reports, we found statistically significant association between the methylation status with the tumor size and clinical staging of tumor. Large sized tumor and clinical stage III showed higher *CDH1* methylation frequency compared to small sized tumor. Our findings are similar to the previously published study [23].

Some components in food and dietary nutrients particularly micronutrients involved in folate-mediated one-carbon metabolism [24,25]. In one-carbon metabolism, S-adenosylmethionine (SAM) is a universal methyl donor and other components are folate, methionine and several B vitamins (B2, B6 and B12) that are essential co-factors in this pathway. It has been observed in both animal models and humans that dietary methyl donors are capable of modulating methylation patterns [26-29].

The present study explored association of methylation status of tumor suppressor genes (*RASSF1A* and *CDH1*) in the serum of breast cancer patients. We investigated the serum levels of vitamin B12 and folic acid. In addition, we investigated whether there were any differences in the serum levels of vitamin B12 and folic acid among cases and controls (benign breast diseases and healthy controls).

The current study identified a statistically significant association between the methylation status of *CDH1* gene with the serum levels of vitamin B12 i.e., high vitamin B12 levels in methylation negative sample as compared with the methylation positive samples. Our findings are in accordance with the results of study performed by Xu et al. [12], inverse associations were observed for *CDH1* with vitamin B12 i.e., higher intake is associated with lower likelihood for a case to possess a methylated promoter for the *CDH1* gene.

We found that serum folic acid levels were significantly associated with increasing methylation levels of *RASSF1A*. Our findings support the results of a study performed by Vineis et al. [30], although their findings concerned lung cancer samples. These results suggest that intake of certain B vitamins may affect the supply of key cofactors required for preserving the proper functions of one-carbon metabolism in the cell, consequently influencing methylation reactions. Our results provide preliminary results that dietary nutrients can affect breast cancer epigenetics. Further studies are required on larger population to establish association between methylation status and serum vitamin B12 and folic acid levels in breast cancer.

In conclusion, the present study demonstrated the diagnostic utility of serum and tissue DNA methylation in breast cancer patients. In this report we demonstrated the possibility of finding the presence of alterations in serum DNA. We found that frequent promoter methylation of *CDH1* and *RASSF1A* may play a key role in early diagnosis of breast cancer. Moreover, we also observed that promoter methylation of *CDH1* may be associated with increased tumor size and advanced tumor stage of breast cancer patients. In addition, serum levels of vitamin B12 and folic acid with methylation status of tumor suppressor genes may increase the possibility of breast cancer diagnosis. However, a large pooled study is required to confirm these findings.

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Table 1: Demographic data of breast cancer patients and healthy Controls

Variables	Breast cancer patients (%)	Controls (%)
	50(100)	50(100)
Age group		
Age>45	15(30)	32(64)
Age <45	35(70)	18(36)
Mean + SD (Y)	49.22+12.37	38.48+13.89
Menopause status (cases)		
Pre	33(66)	
Post	17(34)	
Tumor size		
T1+ T2	22(44)	
T3	22(44)	
T4	6(12)	
Tumor Stage		
Early(I & II)	32(64)	
Advanced (III & IV)	18(36)	
Tumor Grading		
Well differentiated	5(10)	
Moderately diffrentiated	24(48)	
Poorly differentiated	21(42)	
Lymph nodes		
Positive	30(60)	
Negative	20(40)	
Estrogen Receptor (ER)		
Positive	22(44)	
Negative	28(56)	
Progesterone Receptor (PR)		
Positive	20(40)	
Negative	30(60)	
HER2/Neu Status		
Positive	32(64)	
Negative	18 (36)	

Table 2: Frequency Promoter hypermethylation of different genes in serum of breast cancer patient and control group

Genes	Methylation in serum DNA sample N=50 (%)	Methylation in cancerous tissue sample N=50(%)	Controls		p value
			Benign breast diseases (25) Methylation positive (%)	Healthy controls(25) Methylation positive (%)	
RASSF1A	32 (64%)	35 (70%)	5 (20%)	0	< 0.0001*
CDH1	24 (48%)	25 (50 %)	1 (4%)	0	< 0.0001*

* Statistically significant value

Table 3: Correlation of the methylation status in both tissue and serum DNA of the breast cancer patients

	***RASSF1A* tissue DNA**			
	N	**unmethylated**	**methylated**	**Correlation[a]**
RASSF1A serum cfDNA	50			**0.7819**
unmethylated		14	4	
methylated		1	31	
	***CDH1* tissue DNA**			
***CDH1* serum cfDNA**	50			**0.9608**
unmethylated		25	1	
methylated		0	24	

Table 4: Sensitivity and specificity of aberrant methylated gene and on combination of genes

	Sensitivity in breast cancer N=50		Specificity in benign breast diseases N=25		Specificity in healthy controls N=25		PPV	NPV
GENES	**Methylation positive**	**%**	**Methylation negative**	**%**	**Methylation negative**	**%**		
RASSF1A	32	64	20	80	25	100		
CDH1	24	48	24	96	25	100		
RASSF1Aa CDH1	38	76	45			90	88	78

*PPV: Positive Predictive value; NPV: Negative Predictive value
[a] Either gene methylated

Table 5: Clinicopathological characteristics of breast cancer patients with aberrant methylation in serum and paired tissue samples

Patients enrolled	RASSFIA methylation		CDH1 methylation	
	Serum n (%)	Tissue n (%)	Serum n (%)	Tissue n (%)
Menopausal status				
Pre-menopausal (17)	8 (47)	10 (59)	5 (29)	5 (29)
Post-menopausal (33)	24 (73)	25(76)	19 (57)	20 (61)
p value	0.11	0.10	0.07	0.07
Tumor size				
T1+ T2 (22)	12 (55)	12(55)	6 (27)	7 (32)
T3 (22)	15 (68)	17 (77)	12(55)	12(55)
T4 (6)	5 (83)	6 (100)	6 (100)	6 (100)
p value	0.38	0.06	**0.002***	**0.004***
TMN staging				
Early disease (32) Stage I & II	18 (56)	19 (59)	11(34)	12 (38)
Advance diseases Stage III&IV (18)	14 (78)	16 (89)	13(72)	13 (72)
p value	0.21	**0.05**	**0.01***	**0.03***
Grading				
G1 (5)	2 (40)	4(80)	1(20)	1(20)
G2 (24)	15(63)	16(67)	12(50)	13(54)
G3 (21)	15(71)	15 (71)	11(52)	11(52)
p value	0.47	0.91	0.50	0.50
Lymph node status				
Positive (30)	16(53)	18(60)	15(50)	16(53)
Negative (20)	16 (80)	16(80)	9(45)	9(45)
p value	0.07	0.2	0.9	0.77
ER				
Positive (22)	18(81)	19(86)	10(45)	10(45)
Negative (28)	11(40)	11(40)	14(50)	15(54)
p value	**0.02***	**0.001***	0.78	0.77
PR				
Positive (20)	16(80)	18(90)	12(60)	12(60)
Negative (30)	14(47)	15(50)	20(55)	22(63)
P value	**0.03***	**0.005***	0.76	0.36
Her2/Neu				
Positive (32)	22(69)	23(72)	21(66)	23(72)
Negative (18)	9(50)	9(50)	7(39)	8(44)
p value	0.23	0.14	0.08	0.07

* Statistically significant value

Table 6: Comparison of serum B12 levels and folic acid levels in breast cancer patients & controls

		Breast cancer pts (50)	Controls		*p* value
			Benign breast diseases (25)	Healthy controls(25)	
Serum B12 levels (pg/mL)	Median	430	274	300	0.003*
	Range	16-4947	154-2000	180-1386	
Serum folic acid levels (ng/mL)	Median	9.5	8.0	8.0	0.02*
	Range	3-40	4-22	4-14	

* Statistically significant value

Table 7: Dunn's multiple comparisons test

Comparisons	Serum Vitamin B12 Levels		Serum Folic Acid Levels	
	Mean Rank Difference	p value	Mean Rank Difference	P value
Breast Cancer Pts Vs. Benign Cases	21.140	P < 0.01	15.530	P > 0.05
Breast Cancer Pts Vs. Healthy Controls	17.820	P < 0.05	15.230	P > 0.05
Benign Cases Vs. Healthy Controls	-3.320	P > 0.05	-0.3000	P > 0.05

Table 8: Association of genes methylation status with the serum levels of vitamin B12 and folic acid levels

		RASSF1A		*CDH1*	
		Methylation positive samples	Methylation negative samples	Methylation positive samples	Methylation negative samples
Serum B_{12} levels (pg/mL)	Median	430	528.5	316.50	420
	Range	166 -1158	174-4947	166 -1575	174-4947
	P value	0.24		0.03*	
Serum folic acid levels (ng/mL)	Median	15	7.5	13.50	8.5
	Range	4 - 40	3 - 32	3 - 32	4 - 40
	P value	0.01*		0.52	

* Statistically significant value

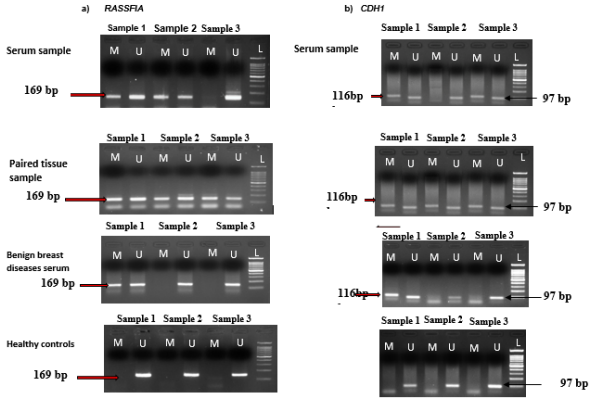

FIG 1: *Representative gel electrophoresis pictures demonstrating aberrant methylation in RASSF1A (a) and CDH1 (b) for serum DNA of breast cancer patients (top row). The corresponding tissue DNA methylation (middle row). There were no hypermethylation in the healthy control serum sample (bottom row). RASSF1A methylation (product size: 169 bp for both methylated and unmethylated DNA) and CDH 1 methylation (product size: 116 bp for methylated and 97bp for unmethylated DNA).*
L is the100 bp DNA ladder

Lanes M and U, amplified products with primers recognizing methylated and unmethylated sequences, respectively

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