Original Article
Middle East Journal of Cancer; January 2016; 7(1): 9-20

Assessment of Hypermethylation of RASSF1A and Protocadherin-10 Tumor Suppressor Genes in Breast Cancer Females: A Six-Year Disease-Free Survival Case-Control Study

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Abstract

Background: This study intends to determine the diagnostic and prognostic roles of hypermethylation of serum RASSF1A and protocadherin-10 promoters in females with breast cancer.

Methods: This study enrolled 80 breast cancer patients and 80 apparently normal healthy controls. The promoter methylation status of serum RASSF1A and PCDH10 genes was investigated using methylation specific PCR.

Results: We detected no hypermethylation of the two genes in serum DNA of normal healthy controls (100% specificity). Of the 80 patients, 50 (62.5% sensitivity) displayed hypermethylated RASSF1A, whereas 34 (42.5% sensitivity) showed hypermethylated PCDH10 and 64 (80% sensitivity) were hypermethylated in at least one of these two genes. A significant association existed between hypermethylated RASSF1A and axillary lymph node involvement. There was a significant association between hypermethylated PCDH10 and axillary lymph node involvement, tumor size and pathological grade. Hypermethylated RASSF1A and PCDH10 combination was significantly associated with axillary lymph node involvement and Her-2 expression. Patients with methylated RASSF1A or PCDH10 had significantly shorter survival rates compared to those with unmethylated RASSF1A or PCDH10.

Conclusion: Methylated RASSF1A is superior to methylated PCDH10 for diagnosis of breast cancer patients. Addition of methylated PCDH10 to methylated RASSF1A significantly improves the diagnostic accuracy of RASSF1A. The present study suggests that hypermethylated RASSF1A and PCDH10 may be independent prognostic indicators for disease-free survival in breast cancer patients.

Keywords: Breast cancer, Hypermethylation, RASSF1A, PCDH10

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Middle East Journal of Cancer

Received: May 6, 2015; Accepted: October 7, 2015

Introduction

Breast cancer is one of the most common cancers worldwide. Egypt is no exception, with increasing incidence rates. Generally, human cancer represents a heterogeneous group of diseases driven by progressive genetic and epigenetic abnormalities. Previous studies of breast cancer have identified numerous genetic changes in tumor suppressor genes and oncogenes along with various chromosomal abnormalities. On the other hand, epigenetics describe heritable changes in gene activities that occur without changes in the DNA sequence itself. These molecular events involve hyper- and hypo-methylation of DNA and altered patterns of histone modification with resultant remodeling of the chromatin structure. Epigenetic changes in neoplastic cells result in altered expression of many cancer-associated genes compared with normal cells without transformation.[1]

DNA hypermethylation refers to a relative increase of methylation at normally undermethylated CpG islands. In the context of epigenetic dysregulation in cancer, hypermethylation of CpG island-containing promoters and concomitant inhibition of gene expression is the most frequently and consistently observed epigenetic abnormality in cancer cells. This hypermethylation is regarded as a crucial event in cancer progression.[2] In breast cancer alone, over 100 hypermethylated and transcriptionally silenced genes have been found.[3]

Hypermethylation is therefore an alternative mechanism for inactivation of tumor suppressor genes.[4] Because gene hypermethylation has been found to be a common and early alteration in many tumor types,[5] including breast,[6] it has emerged as a promising target for detection strategies in clinical specimens.[7] Several tumor suppressor and other cancer genes have been found to be hypermethylated in normal breast cells such as RAS-association domain family 1 isoform A (RASSF1A) and protocadherin-10 (PCDH10).[8]

RAS-association domain family 1 isoform A is a tumor suppressor gene that codes a protein which is a member of a new group of RAS effectors. Particular investigation of RASSF1A has revealed its involvement in regulation of the cell cycle, apoptosis and microtubule stability.[9] Inactivation of the RASSF1A gene is a frequent phenomenon in many types of tumors including those of the breast. This inactivation can be caused by genetic events, such as loss of heterozygosity at the 3p21.3 region where this gene is located or rarely by point mutations. Epigenetic inactivation by DNA hypermethylation, however, has been found in a substantial percentage of various primary tumors.[10] This epigenetic alteration in the RASSF1A gene was observed in 62%-81% of primary breast cancers methylated in both primary and metastatic tissues.[11] Göbel et al.[12] reported that

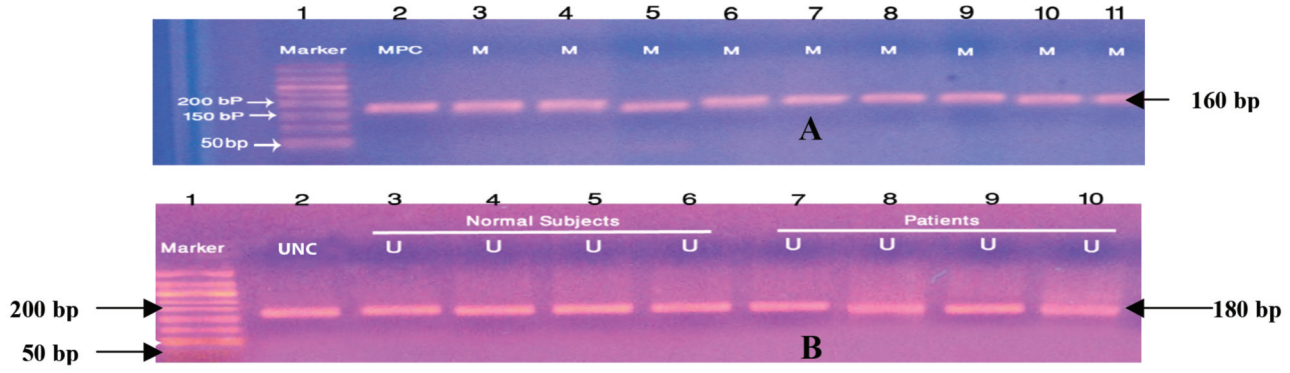

Figure 1. Electrophoresis of methylation-specific PCR (MS-PCR) products for the methylated (M) and unmethylated (U) RASSF1A gene at 160 and 180 bp, respectively. (A) Positive methylated bands (M) of RASSF1A in serum DNA of breast cancer patients where lane 1 is the molecular weight marker (50 bp DNA ladder), lane 2 is the methylated positive control (MPC) and lanes 3-11 are methylated RASSF1A (M). (B) Unmethylated (U) bands of RASSF1A among patients and normal healthy controls. Lane 2 is the unmethylated negative control (UNC), lanes 3-6 are unmethylated RASSF1A (U) in normal healthy controls and lanes 7-10 are unmethylated RASSF1A (U) in patients.

methylated RASSF1A in peripheral blood plasma appeared to have promising potential as a prognostic marker in breast cancer patients.

Protocadherin-10 has been implicated as a tumor suppressor gene.[13] This gene is down-regulated or lost in multiple human cancer types.[14] Promoter hypermethylation and chromatin remodeling have emerged as the main mechanism for the down regulation or loss of PCDH10 in cancers.[15] Re-expression of PCDHIO can reduce tumor formation and tumor invasiveness both *in vivo* and *in vitro*.[16]

Detection of tumor suppressor gene hypermethylation in serum or plasma, as more readily accessible bodily fluids, does not require the presence of a specialist for obtaining of the sample. DNA is known to be released into serum and plasma. In cancer patients, tumor DNA is enriched.[17] Importantly, tumor cell-specific DNA alterations in serum are not limited to patients with metastatic cancer but have also been found in sera from patients with early or organ-confined tumors.[18]

The aim of this study was to determine the diagnostic accuracy of methylated RASSF1A and PCDH10 genes to predict breast cancer and to investigate whether these two genes have a prognostic role as predictors of clinical outcome in females with breast cancer.

Subjects and Methods

One hundred and sixty females were enrolled in this study. Participants were divided into two groups: group I included 80 patients who were 30-70 years of age and had recently diagnosed clinical stages II and III invasive ductal carcinoma of the breast.[19] These patients had undergone no surgical intervention or received chemotherapy. Patients were recruited from the Experimental and Clinical

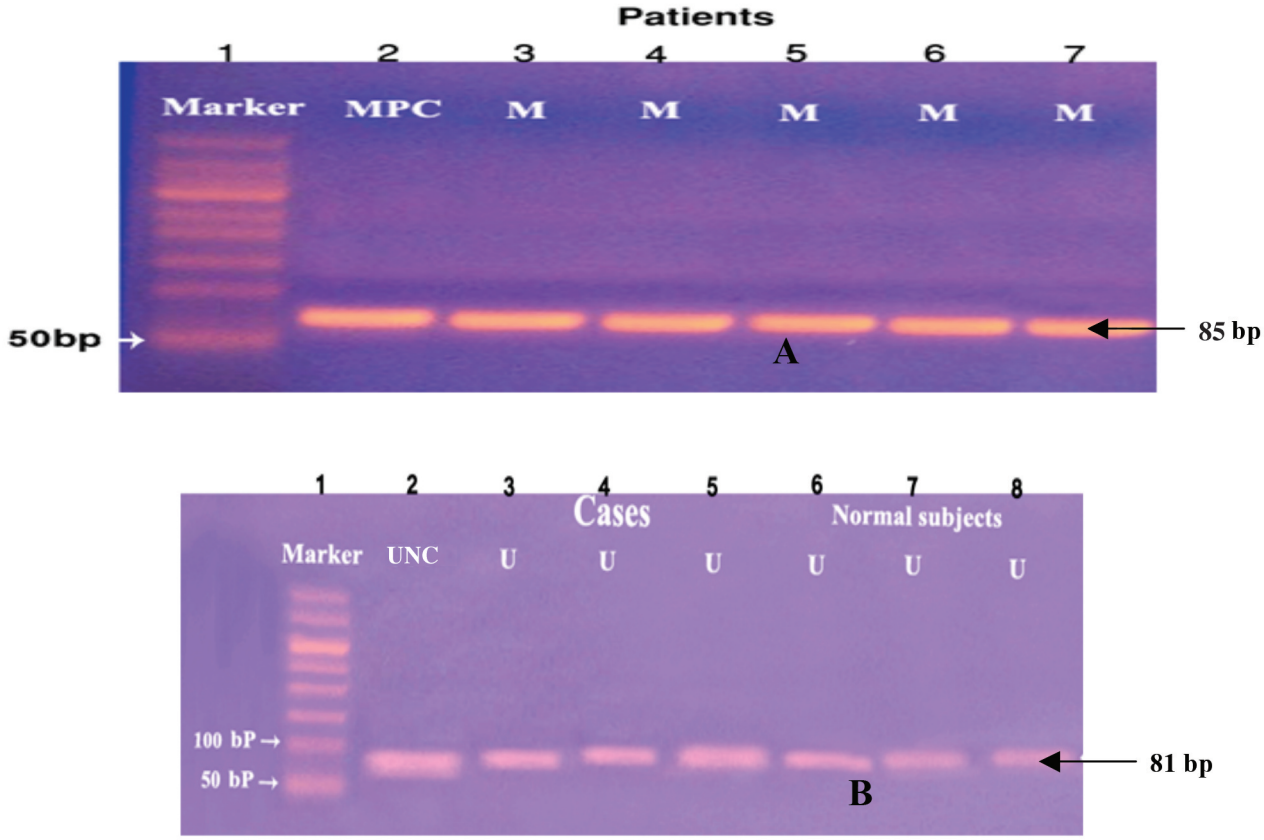

Figure 2. Electrophoresis of methylation-specific (MS)-PCR products for the methylated (M) and unmethylated (U) PCDH10 gene at 85 and 81 bp, respectively. (A) Positive methylated bands (M) of PCDH10 in serum DNA of breast cancer patients, where lane 1 is the molecular weight marker (50 bp DNA ladder), lane 2 is the methylated positive control (MPC), and lanes 3-7 show bands of methylated (M) PCDH10. (B) Unmethylated (U) bands of PCDH10 among patients and normal healthy controls. Lane 2 is the unmethylated negative control (UNC), lanes 3-5 represent unmethylated (U) bands in cases, and lanes 6-8 represent unmethylated bands (U) in normal healthy controls.

Table 1. Clinicopathological data of breast cancer patients (n=80).

Tumor pathological data		No. (%)
Age (years)	<50	33 (41.25)
	≥50	47 (58.75)
Menopausal status	Premenopausal	37 (46.25)
	Postmenopausal	43 (53.75)
Tumor Size (cm)	2 - 5	39 (48.70)
	>5	41 (51.30)
Pathological grade	II	69 (86.25)
	III	11 (13.75)
Clinical stage	II	40 (50.00)
	III	40 (50.00)
Vascular invasion	Negative	17 (21.25)
	Positive	63 (78.75)
Her2/neu expression	Negative	68 (85.00)
	Positive	12 (15.00)
Estrogen receptor status (ER)	Negative	4 (5.00)
	Positive	76 (95.00)
Progesterone receptor status (PR)	Negative	9 (11.25)
	Positive	71 (88.75)
Axillary lymph node involvement	Negative	16 (20.00)
	Positive	64 (80.00)

Surgery and Cancer Management and Research Departments at the Medical Research Institute, University of Alexandria. Group II included 80 normal healthy controls matched for age (31-68 years), menstrual and socioeconomic status with the first group. Controls were selected from female workers at the Medical Research Institute who underwent routine check-ups that included mammography and were negative for breast cancer.

After obtaining approval from the Ethical Committee of the Medical Research Institute (Alexandria University, Egypt), signed informed consents were received from all subjects who agreed to participate in this study. A full history was recorded and each patient underwent a thorough clinical examination, routine laboratory investigations, mammography of both breasts, radiological investigations that included chest X-ray, ultrasonography of the abdomen and liver, computed tomography (CT) scan of the chest and abdomen, and bone scan when needed. Fine needle aspiration cytology (FNAC) of the breast mass was performed to establish the pathological diagnosis in the cancer patients.

Clinicopathological data were obtained from patients' pathology reports. The collected data included tumor size, tumor pathological grade, axillary lymph node involvement, vascular invasion, status of estrogen receptor (ER) and progesterone receptor (PR), and Her2 expression (Table 1). Each breast cancer patient's clinical stage was determined by the oncologist according to the tumor-node-metastasis (TNM) staging system.[20]

All 80 breast cancer patients underwent modified radical mastectomies, then received six cycles of adjuvant combined chemotherapy comprised of 5-fluorouracil, adriamycin and cyclophosphamide (FAC).[21] The patients were

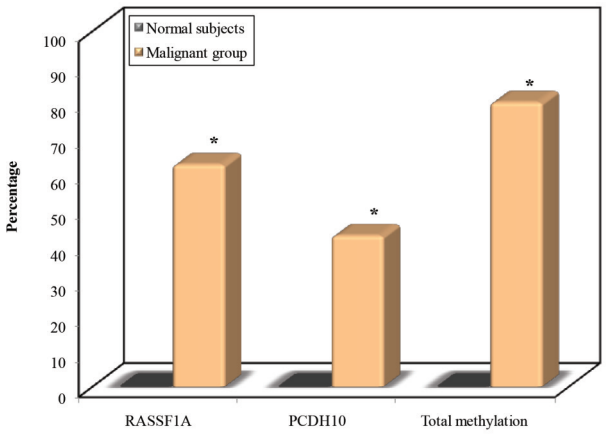

Figure 3. Frequency of aberrant methylation genes (% positive) in breast cancer patients and normal healthy controls.

Table 2. The area under the curve, sensitivity, specificity, PPV and NPV for hypermethylated RASSF1A, PCDH10 and RASSF1A/PCDH10 combination.

Gene	Area under the curve	*P*-value	Sensitivity (%)	Specificity (%)	PPV	NPV
RASSF1A	0.813*	<0.001	62.50	100.0	100.0	57.14
PCDH10	0.713*	<0.001	42.50	100.0	100.0	46.51
RASSF1A/PCDH10 combination	0.900*	<0.001	80.0	100.0	100.0	71.43

PPV: Positive predictive value; NPV: Negative predictive value; *: Significance was considered at $P<0.05$.

re-evaluated after three and six cycles of chemotherapy to estimate clinical response. Patients were followed up for 72 months for assessment of disease-free survival (DFS) based on detection of metastasis or recurrence.

Sample collection and DNA extraction

We obtained one blood sample from patients before surgery and from controls. Blood samples were centrifuged at 3500 rpm for 10 min at room temperature. Serum samples were separated and stored at -80°C until DNA extraction. Free serum DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Sodium bisulfite modification of extracted DNA

The extracted DNA was modified according to the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Bisulfite treatment is used to ascertain the methylation status of individual cytosines in DNA. Ideally, bisulfite treatment deaminates unmethylated cytosines to uracils, and leaves 5-methylcytosines unchanged. This allows their differentiation by methylation specific polymerase chain reaction (MSP).

Methylation-specific PCR (MSP)

The bisulfite modified DNA was used as a template for MSP using primers specific for either the methylated or the modified unmethylated sequences. The sequences of PCR primers (two sets of primers) used to distinguish methylated and unmethylated RASSF1A and PCDH10 genes, annealing temperatures, and the expected sizes of PCR products were described previously.[22, 23] The RASSF1A and PCDH10 specific primers were ordered from Qiagen company (Qiagen, Hilden, Germany). PCR was carried out according to the EpiTect PCR Control DNA Set (Qiagen, Hilden, Germany) with some modifications. PCR reactions were carried out in a total volume of 50 µl that contained 25 µl of Taq PCR Master Mix (Qiagen, Hilden, Germany), 2.5 µl of the forward primer (0.1-0.5 µM), 2.5 µl of the reverse primer (0.1-0.5 µM) and 20 µl of template modified DNA (<1 µg/reaction). PCR conditions were as follows: 35 cycles of denaturation at 94°C for 1 min, annealing at 50-68°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. For each PCR reaction, in order to check the specificity of the primers and for monitoring the bisulfite conversion efficiency, a

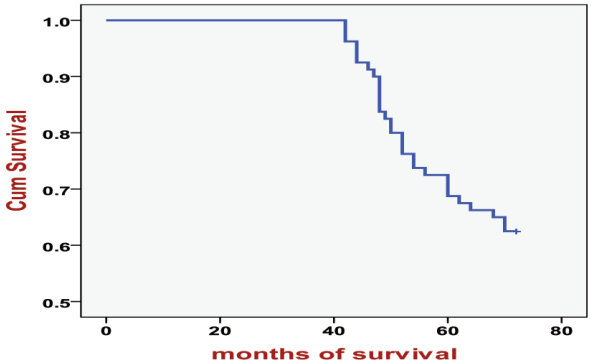

Figure 4. Kaplan-Meier curve for overall disease-free survival (DFS) of breast cancer patients.

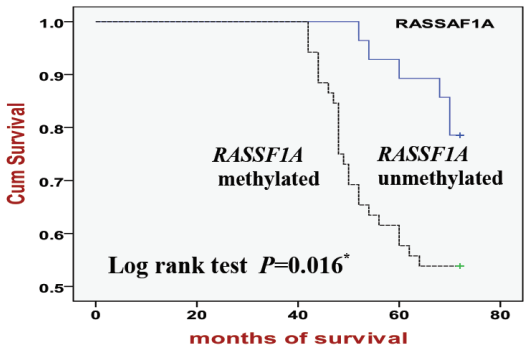

Figure 5. Kaplan-Meier curve for disease-free survival (DFS) of breast cancer patients in relation to RASSF1A methylation status.

Table 3. Association of methylation status of the RASSF1A and PCDH10 genes in breast cancer patients.

Gene	Breast cancer patients				Total	
	Methylated		Unmethylated			
	No.	%	No.	%	No.	%
RASSF1A	50	62.5	30	37.5	80	100.0
PCDH10	34	42.5	46	57.5	80	100.0
Chi-square (P)			6.416* (0.011)			

P: *P*-value for the chi-square test; *: Significance was considered at $P<0.05$.

methylated positive control DNA (bisulfite converted) and unmethylated negative control DNA (bisulfite converted) were used (EpiTect PCR Control DNA, Qiagen, Germany). The amplified products were run on 2% agarose gel with a 50 bp DNA ladder, stained with ethidium bromide, and visualized under UV light (Figures 1, 2).

Statistical analysis

Statistical analyses were performed using Predictive Analytics software (PASW Statistics 18). Qualitative data were described using numbers and percentages. Associations between variables were tested with the chi-square test. When more than 20% of the cells had an expected count less than 5, correction for the chi-square was conducted using Fisher's exact test. The area under the receiver operating characteristic curve (ROC) has denoted the diagnostic performance of the test. An area more than 50% gives acceptable performance and an area about 100% is the best performance. Disease-free survival was calculated from the date of diagnosis of the primary tumor to last follow-up or date of death. Disease-free survival curves were calculated with the Kaplan-Meier method. Results were considered statistically significant if the *P*-value was <0.05.

Results

Frequency of hypermethylated RASSF1A and PCDH10 genes in breast cancer patients and normal healthy controls

Although we observed promoter hypermethylation of both genes in breast cancer patients, there was no methylation of RASSF1A and PCDH10 observed in serum DNA of normal healthy controls. Of 80 breast cancer patients, 50 (62.5%) displayed promoter hypermethylation in the RASSF1A gene ($P=0.00$), 34 (42.5%) had hypermethylation of the PCDH10 gene ($P=0.00$), and 64 (80%) had evidence of hypermethylation in at least one of the two genes ($P=0.00$; Figure 3).

Diagnostic accuracy of methylated RASSF1A and PCDH10 genes in breast cancer patients

The area under the curve (diagnostic accuracy) for methylated RASSF1A was 81.3%, whereas for methylated PCDH10 it was 71.3%. Methylated RASSF1A had a sensitivity of 62.50% and a specificity of 100%. PCDH10 had a sensitivity of 42.5% and 100% specificity. The area under the curve for the combination of the two genes was 90% with sensitivity of 80% and specificity of 100% (Table 2).

Methylation status association of RASSF1A and PCDH10 genes in breast cancer patients

The analysis of methylation distribution demonstrated a significant association between methylation of the RASSF1A gene and methylation of the PCDH10 gene (chi-square: 6.416*, $P=0.011$; Table 3).

Association of promoter hypermethylation of RASSF1A and PCDH10 with breast cancer clinicopathological data

We studied the clinical significance of promoter hypermethylation of RASSF1A and PCDH10 genes by investigating the relationship between methylation of these genes and clinicopathological data of breast cancer patients. There was a significant association between hypermethylated RASSF1A and axillary lymph node involvement ($P=0.008$), while hypermethylated PCDH10 was significantly associated with tumor

Table 4. Association between RASSF1A and PCDH10 gene methylation and clinicopathological characteristics of breast cancer patients.

Clinicopathological characteristics	No. (%)	Methylated promoter region				Total methylation	
		RASSF1A		PCDH10		No. (%)	
		No. (%)	*P*-value	No. (%)	*P*-value		*P*-value
Total	80 (100)	50 (62.5)		34 (42.5)		64 (80)	
Age (years)							
<50	33 (41.2)	20 (60.6)	0.769	14 (42.4)	0.991	26 (78.8)	0.820
≥50	47 (58.8)	30 (63.8)		20 (42.6)		38 (80.9)	
Tumor size (Cm)							
2.5 -≤5	39 (48.7)	25 (64)	0.773	24 (61.5)	0.019*	34 (87.2)	0.117
>5	41 (51.3)	25 (61)		10 (24.4)		30 (73.2)	
Clinical stage							
II	40 (50.0)	26 (65)	0.107	21 (52.5)	0.070	35 (87.5)	0.094
III	40 (50.0)	24 (60)		13 (32.5)		29 (72.5)	
Pathological grade							
II	69 (86.2)	43 (62.3)	FEp = 1.000	33 (47.8)	FEp =0.020*	57 (82.6)	FEp = 0.217
III	11 (13.8)	7 (63.6)		1 (9.1)		7 (63.6)	
Vascular invasion							
- ve	17 (21.2)	11 (64.7)	0.832	9 (52.9)	0.326	14 (82.4)	FEp = 1.000
+ ve	63 (78.8)	39 (61.9)		25 (39.7)		50 (79.4)	
Estrogen receptor (ER) status			FEp = 0.628				
- ve	4 (5.0)	2 (50.0)		2 (50.0)	FEp = 1.000	2 (50.0)	FEp = 0.579
+ ve	76 (95.0)	48 (63.2)		32 (42.1)		62 (81.6)	
Progesterone receptor (PR) status							
- ve	9 (11.2)	4 (44.4)		6 (66.7)	FEp = 0.159	8 (88.9)	FEp = 0.679
+ ve	71 (88.8)	46 (64.8)	FEp = 0.159	28 (39.4)		56 (78.9)	
Her2/neu expession							
- ve	68 (85.0)	40 (58.8)	FEp = 0.052	32 (47.1)	FEp = 0.062	57 (83.8)	0.042*
+ ve	12 (15.0)	10 (83.3)		2 (16.7)		7 (58.3)	
Lymph node involvement							
- ve	16 (20.0)	5 (31.3)	0.008*	3 (18.8)	FEp = 0.047*	7 (43.8)	<0.001*
+ ve	64 (80.0)	45 (70.3)		31 (48.4)		57 (89.1)	
Menopausal status							
Premenopausal	37 (46.2)	22 (59.5)	0.563	17 (45.9)	0.563	30 (81.1)	0.823
Postmenopausal	43 (53.8)	28 (65.1)		17 (39.5)		34 (79.1)	

FEp: *P*-value for Fisher's exact test. All other values are the *P*-values for the Chi-square test; –ve: negative; +ve: positive; *: Statistically significant at $P < 0.05$.

size (P=0.019), tumor pathological grade (P=0.020), and axillary lymph node involvement (P=0.047). A significant association existed between hypermethylated RASSF1A and/or PCDH10 with axillary lymph node involvement (P=0.001) and Her2 expression (P=0.042; Table 4).

Disease-free survival (DFS) analysis of breast cancer patients

Testing of hyermethylated RASSF1A and PCDH10 as predictors of disease-free survival (DFS) was performed using Kaplan-Meier analysis with log-rank statistics. After a follow up period of 72 months, 30 out of 80 patients had recurrence or metastasis. The cumulative overall DFS was 62.5% (Figure 4, Table 5). Disease-free survival was higher in patients with unmethylated RASSF1A (78.6%) and PCDH10 (75.6%) with a *P*-value of 0.016 compared to patients who had hypermethylated RASSF1A (53.8%) and PCDH10 (45.7%) with a *P*-value of 0.003; (Figures 5, 6; Table 5). The combination of RASSF1A and/or PCDH10 had overall DFS of 73.3% for unmethylated and 60% for methylated genes, which was not significant (P=0.219, Figure 7; Table 5).

Table 5. Disease-free survival (DFS) among breast cancer patients according to RASSF1A and PCDH10 methylation status.

Gene	Methylation status	DFS (months) Mean±SD	Recurrent/ metastatic cases No. (%)	Non- recurrent/ metastatic cases No. (%)	*P*-value
RASSF1A	Unmethylated (n=28)	69.9± 0.99	6 (21.4)	22 (78.6)	0.016*
	Methylated (n= 52)	61.85±1.63	24 (46.2)	28 (53.8)	
PCDH10	Unmethylated (n= 45)	68.24±1.22	11 (24.4)	34 (75.6)	0.003*
	Methylated (n=35)	60.09±1.99	19 (54.3)	16 (45.7)	
RASSF1A and/or PCDH10	Unmethylated (n= 15)	70.67±0.79	4 (26.7)	11 (73.3)	0.219
	Methylated (n=65)	63.29±1.41	26 (40)	39 (60)	

P: Value for log-rank test. *: Statistically significant at $P<0.05$.

Cox proportional hazards analysis of disease-free survival (DFS) in relation to hypermethylated RASSF1A, PCDH10 and breast cancer clinico-pathological data

We conducted Cox proportional hazards analysis for the breast cancer patients in order to control for potential confounding effects of menopausal status, pathological grade, clinical stage, axillary lymph node involvement, status of ER and PR, Her2 expression, tumor size, vascular invasion, and to calculate the hazard ratios (HRs). Table 6 shows that methylated RASSF1A and PCDH10 represent a significant hazard for metastasis and poor prognosis after adjustment for the other prognostic factors. Patients with methylated PCDH10 had approximately a six-fold higher risk (hazard ratio: 5.642; CI: 2.313-13.763) for metastasis compared with patients with unmethylated PCDH10. Patients with methylated RASSF1A had approximately 4 times greater risk (hazard ratio: 3.52; CI: 1.653-7.516) for metastasis. The combination of RASSF1A and/or PCDH10 revealed no significant risk for metastasis (hazard ratio: 2.95; CI: 0.85- 6.219).

Discussion

In the present study, we identified RASSF1A as a target for methylation and silencing in 62.5% of breast cancer patients which suggested that inactivation of this gene was a frequent event in the process of mammary tumorigenesis. In the normal healthy controls RASSF1A was unmethylated. Among independent studies the

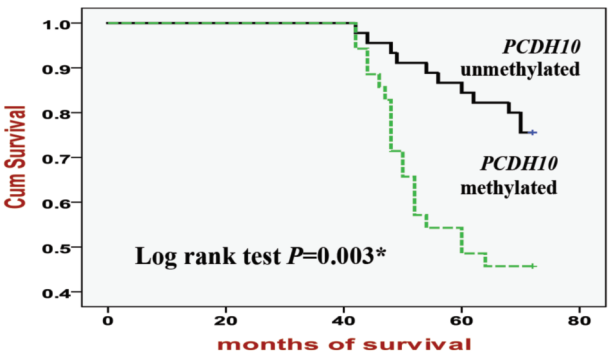

Figure 6. Kaplan-Meier curve for disease-free survival (DFS) of breast cancer patients in relation to PCDH10 methylation status.

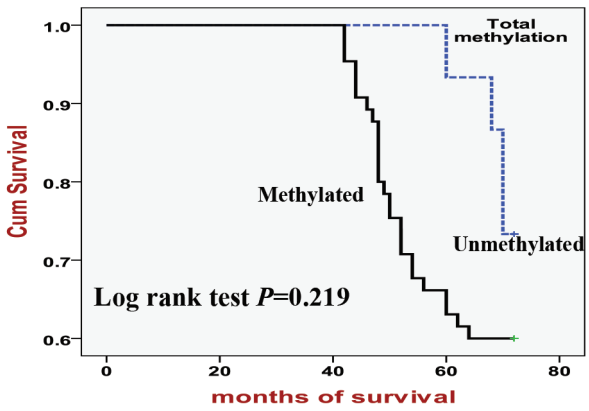

Figure 7. Kaplan-Meier curve for disease-free survival (DFS) of breast cancer patients in relation to combined methylation status of the RASSF1A and/or PCDH10 genes.

Table 6. COX regression model fit to hypermethylated RASSAF1A and PCDH10 plus clinicopathological data in breast cancer patients versus normal healthy controls.

Variable	Regression coefficient (b)	Standard error (SE) (b)	*P*-value	e^b hazard ratio *	95% CI for hazard ratio	
					Lower	Upper
PCDH10	1.730	0.455	0.000	5.642	2.313	13.763
RASSF1A	1.260	0.386	0.001	3.525	1.653	7.516
Total methylation	1.082	0.381	0.064	2.950	0.850	6.219
Menopausal status	-0.134	0.394	0.734	0.875	0.404	1.894
Family history	0.405	0.419	0.333	1.500	0.660	3.407
Grade	0.390	0.614	0.525	1.477	0.444	4.919
Axillary lymph node status	-0.610	0.624	0.328	0.543	0.160	1.845
Estrogen receptor (ER)	0.864	0.861	0.316	2.373	0.439	12.836
Progesterone receptor (PR)	0.255	0.680	0.708	1.291	0.340	4.898
Her2	0.740	0.808	0.360	2.096	0.430	10.219
Tumor size (Cm)	-0.702	0.468	0.133	0.496	0.198	1.240
Vascular invasion	-0.613	0.577	0.288	0.542	0.175	1.679

* Risk of metastasis according to treatment assignment and prognostic variables; CI: Confidence intervals-

incidence of methylated RASSF1A in breast cancer patients was approximately 60%.[24] The current study results agreed with this high incidence of RASSF1A methylation. In particular, Dammann et al.[25] detected RASSF1A promoter methylation in 62% of primary mammary carcinomas. Honorio et al.[26] reported that the RASSF1A promoter was methylated in 65% of invasive breast carcinomas and in 42% of corresponding ductal carcinoma in situ. Sharma et al.[27] observed ASSF1A promoter hypermethylation in 63% of breast cancer patients.

In line with results obtained from other authors,[28-30] there was no RASSF1A methylation detected in serum DNA of normal healthy controls. This result reinforced the notion that methylated RASSF1A might be considered a specific marker to differentiate breast cancer patients from healthy subjects.

In the present study we detected aberrant methylation of the PCDH10 gene in 42.5% of breast cancer patients, but not in the serum from normal healthy controls. These results supported previous findings[31, 32] where the authors reported that the incidence of aberrant methylation of PCDH10 in primary cancers was considered to be a major determinant of the sensitivity of the gene in biopsies or blood specimens. In this regard, methylation of PCDH10 might be considered a good candidate for breast cancer diagnosis.

We did not observe aberrant methylation in at least one of the RASSF1A and PCDH10 genes in the normal healthy controls. However it was detected in 80% of breast cancer patients, while the frequency of promoter methylation of each of the two genes examined varied from 42.5% for PCDH10 to 62.5% for RASSF1A. These results suggested that the simultaneous methylation of multiple genes might be important for carcinogenesis. How these specific epigenetic alterations affect the tumor behavior remains to be fully understood. In numerous types of cancers, abnormal methylation and subsequent silencing of genes play important roles in tumor growth, cell cycle regulation, apoptosis, DNA repair, and metastatic potential.[31] Thus the methylation of these genes may cause RASSF1A and PCDH10 repression. Such reduced expression may be an additional mechanism that contributes to malignant progression by facilitating cell aberrant growth, which results from loss of the growth inhibitory activity of PCDH10 and RASSF1A during breast carcinogenesis.

In this study, the diagnostic accuracy for each individual tumor suppressor gene and for the combination of the two genes were significantly

greater than 50% (P<0.05). We found that methylated RASSF1A (accuracy: 81.3%) was a better diagnostic marker than methylated PCDH10 (accuracy: 71.3%). The sensitivity and specificity of methylated RASSF1A was 62.5% and its specificity was 100%, whereas the sensitivity of PCDH10 was 42.5% and its specificity was 100% for detecting breast cancer. The accuracy for the two-gene combination (90%) was greater when compared with the accuracy for the RASSF1A gene (81.3%). The two-genes combination had a sensitivity of 80% and a specificity of 100%. These findings suggested that the addition of PCDH10 to RASSF1A significantly improved the test performance compared with RASSF1A alone.

We observed a significant association between methylation at the RASSF1A and PCDH10 promoter regions (chi-square: 6.416*, P=0.011). Coincident methylation of a number of other tumor suppressor genes has also been reported previously in breast cancer. This association meant that the epigenetic inactivation of tumor suppressor genes did not occur randomly and has suggested the existence of specific molecular associations between hypermethylation of RASSF1A and PCDH10 genes in breast tumors. Possibly the promoters of these tumor suppressor genes are susceptible for CpG methylation that leads to transcriptional silencing of these genes and establishment of breast cancer.[33, 34]

There was a nonsignificant difference in the methylation status of RASSF1A and PCDH10 genes between younger and older breast cancer patients, which has been reported by other studies.[35] This meant that no relationship existed between the accumulation of promoter methylation in tumor suppressor genes and aging. Of note, that there was a nonsignificant difference in methylation status of RASSF1A, and PCDH10 genes between pre- and post-menopausal women which suggested that no link existed between methylation of these genes and menopausal status.

Tumor size and number of axillary lymph node involvement are the two most important prognostic determinants for breast cancer. In the present study, RASSF1A promoter hypermethylation has been observed in 70.3% of patients who were axillary lymph node positive and significantly associated with axillary lymph node involvement. PCDH10 promoter hypermethylation was observed in 48.4% of axillary lymph node positive cases and a significant association existed with lymph node involvement. In addition, methylated PCDH10 was significantly associated with tumor size and pathological grade. The combination of methylated RASSF1A and/or PCDH10 genes was significantly associated with lymph node involvement and Her-2 expression. Our findings supported previous data regarding the association of methylation of these genes with breast cancer progression and metastasis. Increased tumor size and nodal metastasis have been reported as two criteria for cancer progression.[22] Therefore we can use these epigenetic markers as predictors for tumor prognosis. The association between hypermethylated RASSF1A and PCDH10 with breast cancer clinicopathological data has been further confirmed by the finding of a significant correlation between the hypermethylated status of these two genes and breast cancer DFS.

We investigated the relationship between methylated RASSF1A, PCDH10 and RASSF1A and/ or PCDH10 and breast cancer prognosis by evaluating DFS. After 72 months of follow-up, 30 out of 80 patients (37.5%) had either recurrence or metastasis. Disease-free survival analysis showed a significant association between methylated RASSF1A and poor prognosis in breast cancer patients. Patients with hypermethylated RASSF1A had shorter DFS compared to those without. Our results agreed with the results reported by Kioulafa et al.[24] and Göbel et al.[12] who found significantly shorter DFS in patients with methylated RASSF1A. It was probable that RASSF1A gene silencing due to promoter methylation deactivated its tumor suppressor role which could possibly contribute to a shorter survival in breast cancer patients.

Our results also demonstrated that PCDH10 methylation in serum DNA of breast cancer patients provided important prognostic information, since patients with PCDH10 promoter methylation had shorter DFS than those

without. This result was validated in other studies. Yu et al.[31] reported that PCDH10 methylation was significantly associated with shortened survival in stage I-III gastric cancer patients. Therefore, PCDH10 methylation could be regarded as a valuable new prognostic factor for breast cancer patients. On the other hand, this was not the case in the combination of RASSFIA and/or PCDH10 as the overall DFS was 73.3% and 60% in the unmethylated and methylated patients respectively, which was nonsignificant. Therefore, the combination of methylated RASSF1A and/or PCDH10 genes could not be regarded as a prognostic marker for breast cancer patients.

We used Cox proportional hazards analysis to evaluate the risk of metastasis according to hypermethylated RASSF1A and PCDH10. After adjustment for the most important breast cancer prognostic factors, our results revealed that patients with hypermethylated PCDH10 were more likely to have metastatic disease compared to patients with hypermethylated RASSF1A. Our results confirmed the studies of Ko et al.[36] and Heitzer et al.[37]

From this study, we anticipate that the detection of hypermethylated RASSF1A or PCDH10 in serum DNA of breast cancer patients can provide the clinician with additional information regarding the patient's risk of relapse or recurrence. Accordingly, patients with unmethylated RASSF1A or PCDH10 may indeed be at low risk. However, patients with detectable methylated RASSF1A or PCDH10 may be at higher risk of relapse or recurrence.

Methylated RASSF1A is superior to methylated PCDH10 in the diagnosis of breast cancer patients. The addition of methylated PCDH10 to methylated RASSF1A significantly improves the diagnostic accuracy of RASSF1A alone. Methylated RASSF1A or methylated PCDH10 can be used as a prognostic marker for predicting the clinical outcome of breast cancer patients. We have reported that addition of methylated PCDH10 to methylated RASSF1A significantly did not improve the prognostic accuracy of RASSF1A alone.

Conflict of Interest

No conflict of interest is declared.

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