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RASSF1A Promoter Methylation Levels Positively Correlate with Estrogen Receptor Expression in Breast Cancer Patients[1,2]

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Abstract

The aim of this study was to investigate the relationship between the promoter methylation in five cancer-associated genes and clinicopathologic features for identification of molecular markers of tumor metastatic potential and hormone therapy response efficiency in breast cancer. The methylation levels in paraffin-embedded tumor tissues, plasma, and blood cells from 151 sporadic breast cancer patients and blood samples of 50 controls were evaluated by quantitative multiplex methylation-specific polymerase chain reaction. DNA methylation of *RAS-association domain family member 1* (*RASSF1A*), *estrogen receptor 1* (*ESR1*), *cadherin 1*, *type 1*, *E-cadherin* (*CDH1*), *TIMP metallopeptidase inhibitor 3* (*TIMP3*) and *spleen tyrosine kinase* (*SYK*) genes was detected in the tumors of 124, 19, 15, 15, and 6 patients with mean levels of 48.45%, 3.81%, 2.36%, 27.55%, and 10.81%, respectively. Plasma samples exhibited methylation in the same genes in 25, 10, 15, 17, and 3 patients with levels of 22.54%, 17.20%, 22.87%, 31.93%, and 27.42%, respectively. Cumulative methylation results confirmed different spectra in tumor and plasma samples. Simultaneous methylation in tumors and plasma were shown in less than 17% of patients. *RASSF1A* methylation levels in tumor samples statistically differ according to tumor size (P = .029), estrogen receptor (ER) and progesterone receptor (PR) status (P = .000 and P = .004), and immunohistochemical subtype (P = .000). Moreover, the positive correlation was found between *RASSF1A* methylation levels and percentage of cancer cells expressing ER and PR. The direct relationship between *RASSF1A* promoter methylation and expression of ER could aid the prognosis of hormonal therapy response.

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Introduction

Breast cancer is the most common cancer in women worldwide. According to data published by the International Agency for Research on Cancer, in 2008, 1,383,000 breast cancer patients were newly diagnosed and 458,000 breast cancer–related deaths occurred [1]. More than 25% of breast cancer patients develop metastatic disease that is mostly incurable and for which there are only palliative therapeutic options [2]. Clinicopathologic characteristics such as tumor size, lymph node (LN) status, invasion of vessels, and hormone receptor status play important roles in metastasis risk [3]. However, the results of a recent multicenter study found differences in clinicopathologic features between patients with and without primary metastases, and for

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[2]This article refers to supplementary material, which is designated by Table W1 and is available online at www.transonc.com.

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metastasis risk, the lobular histology and luminal B positivity in T1 primary metastatic breast cancer were determined [2].

Similar to other cancer types, breast tumorigenesis is characterized by the progressive accumulation of genetic and epigenetic changes in many genes that regulate cell proliferation and differentiation. Therefore, molecular characterization of tumor tissues allows determination of novel cancer markers including those predicting metastatic potential and therapy response.

Epigenetic abnormalities in neoplastic cells, such as hypermethylation and hypomethylation of DNA, altered patterns of histone modification, and remodeled chromatin structure, result in the modified expression of many essential genes. A well-categorized epigenetic change is hypermethylation of tumor-suppressor promoters that led to inappropriate transcription silencing of these genes [4]. The tumor suppressor gene *RAS-association domain family member 1* (*RASSF1A*) encodes a member of the group of RAS effectors that regulates cell proliferation, apoptosis, and microtubule stability. Hypermethylation of *RASSF1A* was found in a substantial percentage of various primary tumors [5]. Epigenetic inhibition of *RASSF1A* is considered to be an early cancer biomarker; however, this phenomenon is extended from primary to metastatic tumors during tumor progression [6]. Moreover, in invasive breast cancers, significantly higher *RASSF1A* methylation levels were shown compared with *in situ* carcinomas [7]. These results indicate the possible association of *RASSF1A* silencing with metastasis. Other studies reported higher frequencies of methylation in *RASSF1A* alone or in combination with *HIN-1* in estrogen receptor (ER)–positive cases compared with ER-negative cases [8,9]. Moreover, a recent *in vitro* study revealed that RASSF1A inhibits ERα expression and function [product of *estrogen receptor 1* (*ESR1*) gene]; thereby, it plays a key role in suppressing transformation of mammary epithelial cells and ERα-positive breast cancer initiation [10]. In addition to the potential *RASSF1A*-mediated epigenetic regulation of *ESR1*, mild or moderate DNA methylation of the *ESR1* promoter alone was observed in breast tumorigenesis, indicating the possible influence of epigenetic processes on hormonal therapy response [11,12]. In tumorigenesis, there are numerous changes in the cadherin-catenin adhesion complexes, including the cell adhesion protein E-cadherin encoded by *cadherin 1*, *type 1*, *E-cadherin* (*CDH1*). In primary breast cancer, the heterogeneous loss of E-cadherin expression corresponding with variable patterns of promoter methylation was observed in the early stages before cell invasion [13]. *CDH1* hypermethylation with loss of protein expression was found in both ductal and lobular breast carcinomas; however, no significant correlation was observed between E-cadherin expression and the *CDH1* promoter methylation profile [14]. The tissue inhibitors of metalloproteinase (TIMPs) prevent degradation of the extracellular matrix by the metalloproteinases. TIMP metallopeptidase inhibitor 3 (TIMP3) is a matrix-bound protein regulating matrix composition that affects tumor growth, angiogenesis, invasion, and metastasis. *TIMP3* promoter methylation was observed in 21% to 27% of breast cancer patients and in invasive ductal carcinomas that were associated with high tumor grading and LN metastasis [15,16]. The spleen tyrosine kinase (SYK) is an intracellular receptor protein kinase involved in cell proliferation, differentiation, and phagocytosis and plays a suppressive function in breast cancer progression and metastasis [17]. The frequencies of *SYK* promoter hypermethylation at different stages of breast cancer indicate its occurrence shortly before the development of the invasion phenotype [18]. The objective of the present study was to determine the association of the promoter methylation profiles of five genes related to invasion and metastasis with breast cancer clinicopathologic features to identify useful molecular markers indicating the metastatic potential of tumors and patient response to hormonal therapy.

Materials and Methods

Patients

A total of 151 paraffin-embedded tumor tissue samples and matched 151 peripheral blood samples from nonfamilial breast cancer patients and blood samples of 50 healthy controls were obtained from the Department of Pathology and Department of Senology at hospitals in Bratislava, Slovakia. This study was approved by Ethics Committee of the University Hospital in Bratislava, and written informed consent was obtained from all patients and controls. Relevant clinical and pathologic data were retrieved from the patients' clinical records, and tumors were characterized according to the primary tumor, regional lymph nodes, distant metastasis (TNM) classification. The age of patients ranged from 23 to 91 years (mean, 61.2 ± 10.8 years) at the time of breast cancer diagnosis. Typing was performed according to the current World Health Organization (WHO) classification for breast neoplasms (Table 1). No preoperative radiotherapy or chemotherapy had been performed in any of the cases. Controls included 25 individuals of <50 years and 25 individuals of >50 years who had no signs and symptoms of cancer or other serious diseases.

DNA Extraction and Sodium Bisulfite Modification

Blood samples of patients and controls were collected in EDTA-treated tubes and centrifuged at 1000*g* for 10 minutes at room temperature within 2 hours of venepuncture. Then, supernatants were collected and centrifuged at 1000*g* for 10 minutes at room temperature to prevent cellular DNA contamination. Plasma samples were stored at −70°C until further processing. Cell-free DNA from plasma samples was isolated using a QIAamp DSP Virus Kit (Qiagen, Hilden, Germany), DNA from paraffin-embedded tumor tissues was isolated by the MagneSil Genomic, Fixed Tissue System (Promega, Madison, WI), and genomic DNA from peripheral blood was obtained using a FlexiGene DNA Kit (Qiagen) according to the manual instructions. DNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany). Tumor DNA (2 μg), cell-free DNA (2 μg), and genomic DNA (1 μg) were modified by sodium bisulfite treatment according to the protocols of the EpiTect Bisulfite Kit (Qiagen) and CpGenome DNA Modification Kit (Chemicon, Billerica, MA), respectively. DNA was stored at −18°C until use.

Quantitative Multiplex Methylation-Specific Polymerase Chain Reaction Analysis

For quantitative evaluation of promoter methylation, the two-color modification of quantitative multiplex methylation-specific polymerase chain reaction (QM-MSP) technology was used [19]. QM-MSP was performed in two sequential polymerase chain reaction (PCR) reactions. In the first step, co-amplification of three and two gene loci (*RASSF1A*, *CDH1*, *SYK* and *ESR1*, *TIMP3*) was performed using three and two pairs of methylation-independent external primers, respectively. Multiplex PCRs were performed in 30-μl volumes containing 30 to 60 ng of modified DNA, 15 μl of 2× QIAGEN Multiplex PCR Master Mix (Qiagen), and aliquots of six/four primers at a final concentration of 0.2 μM. PCR conditions were 95°C for 15 minutes, 35 cycles at 94°C for 30 seconds, 62/56°C for 60 seconds, hybridization at 72°C for 90 seconds, and final extension at 72°C for 10 minutes.

Table 1. *RASSF1A* Methylation Levels in Different Clinical and Histopathologic Categories in Breast Cancer Patients.

	N	*RASSF1A* Methylation in Tumor Samples			*RASSF1A* Methylation in Plasma Samples		
		Mean	Median	*P* Value	Mean	Median	*P* Value
	151						
Age				.773			.669
≤50	17	37.9	44.4		1.8	0	
>50	134	40.0	40.5		4.0	0	
Histologic type				.864			.660
DIC	131	40.1	40.6		3.8	0	
LIC	10	40.1	49.1		5.2	0	
Others	10	35.1	32.5		1.2	0	
Tumor size				**.029**			.643
≤20	90	41.9	42.6		4.1	0	
>20 ≤ 50	47	32.1	31.5		3.9	0	
>50	4	64.7	64.2		0	0	
Histologic grading				.668			.102
1	36	40.6	43.2		2.5	0	
2	75	41.3	42.3		5.1	0	
3	38	36.3	35.8		2.4	0	
LN status				.718			.297
0	86	42.2	43.2		3.7	0	
1	36	36.5	36.8		2.2	0	
2	11	36.1	40.4		13.4	0	
3	6	43.1	49.6		0	0	
TNM staging				.066			.271
I	80	43.4	43.2		4.3	0	
II	50	31.2	28.8		1.5	0	
III	8	46.7	56.9		15.8	0	
IV	1	65.9	65.9		0	0	
ER status				**.000**			.266
Negative	21	18.0	3.7		2.1	0	
Positive	129	43.6	44.4		4.0	0	
PR status				**.004**			.863
Negative	33	27.4	22.4		5.1	0	
Positive	117	43.5	44.4		3.4	0	
HER2 expression				.069			.419
Negative	128	38.2	39.3		3.3	0	
Positive	22	50.8	43.8		6.7	0	
IHC subtypes				**.000**			.252
ER+/PR+ HER2−	114	42.2	44.2		3.3	0	
ER+/PR+ HER2+	16	51.0	46.2		9.2	0	
ER− PR− HER2+	7	43.1	42.3		0	0	
ER− PR− HER2−	13	5.8	0		3.4	0	

DIC indicates ductal invasive carcinomas; LIC, lobular invasive carcinomas; Others, tubular, micropapillar invasive, cribriform invasive, or mucinous breast carcinomas; LN status, lymph node status; ER status, estrogen receptor status; PR status, progesterone receptor status; IHC subtypes, immunohistochemical subtypes.

$P < .05$ was regarded as statistically significant (in **bold**). LN status was categorized according to the number of cancer cell–positive nodes as 0, 1, 2, and 3 with none, 1 to 3, 4 to 10, and >10 of positive LNs, respectively. ER or PR status was considered as positive in cases with ≥1% of positively responding cells. HER2 expression was regarded as positive, if the intensity of IHC reaction was 3+ in 30% of tumor cells or with fluorescence *in situ* hybridization proven *HER2* gene amplification in cases with ambiguous IHC positive at 2+ intensity reaction. According to ER, PR, and HER2 expression, four IHC subtypes were recognized, luminal A and B (ER+ and/or PR+ HER− and ER+ and/or PR+ HER2+), HER2 overexpression positive (ER− PR− HER2+), and triple negative (ER− PR− HER2−). Tumor sizes are shown in millimeters.

In the second step (quantitative real-time PCR), 1 µl of the first reaction PCR product was used at a dilution of up to $1:10^2$ in a duplex reaction with both pairs of primers and specific TaqMan probes for methylated and unmethylated DNA substrates for each gene. PCRs were performed in 15-µl volumes containing 7.5 µl of Maxima Probe qPCR Master Mix (2×; Fermentas, Amherst, NY), methylation- and unmethylation-specific primers for *RASSF1A*, *ESR1*, *CDH1*, *TIMP3*, or *SYK* gene at a final concentration of 0.3 µM, and methylation- and unmethylation-specific TaqMan probes at concentrations ranging from 0.1 to 0.27 µM. The reaction conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles at 95°C for 15 seconds and 60°C for 30 to 90 seconds with final extension at 72°C for 30 seconds. For quantitative PCR, a CFX96TM Real-Time PCR System (Bio-Rad, Hercules, CA) was used. Standard curve establishment and evaluation of quantitative analysis of DNA methylation were performed as previously described [20]. The relative amount of methylation (%) was calculated in each sample according to the formula $[M/(U + M)] \times 100$. Concentrations of methylated (M) and unmethylated (U) portions were determined from simultaneously amplified standard curves for each gene. Methylation levels up to 0.5% were considered to be the background of this sensitive quantitative method. The cumulative methylation index (CMI) was calculated as the sum of percentage methylation for all evaluated genes. For all five genes, CMI of 500 was the maximum value of methylation. Primers and TaqMan probes are summarized in the supplementary material (Table W1) [21].

Statistical Analysis

For statistical analyses, SPSS statistics 15.0 was applied, with $P < .05$ regarded as statistically significant. Normally distributed data were tested by Pearson correlations, Student's t tests, or analysis of variance with Bonferroni or Tamhane tests for multiple comparisons, depending on homogeneity of variance. For non-normally distributed data, Spearman correlations, nonparametric Mann-Whitney U or Kruskal-Wallis H tests were used. Normality of distribution was assessed by

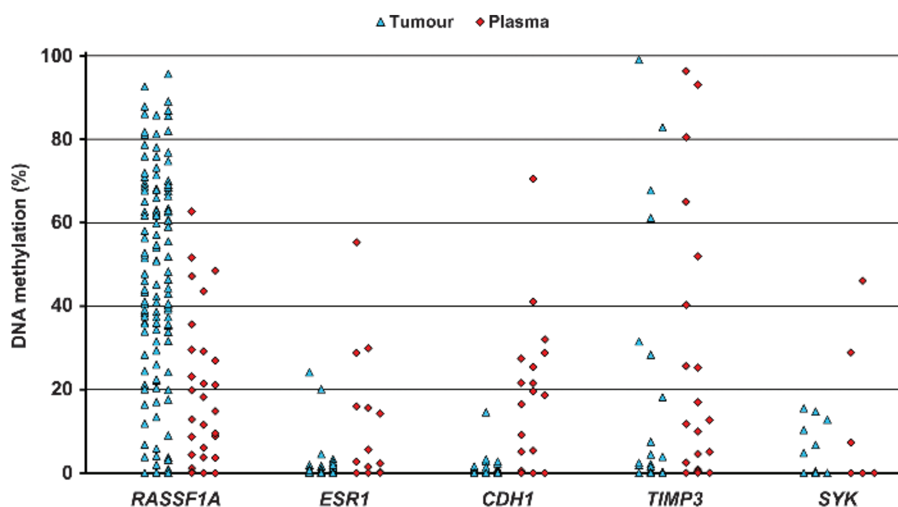

Figure 1. Methylation levels of five genes evaluated in tumor and plasma samples of breast cancer patients.

Kolmogorov-Smirnoff tests. All tests were two-tailed. Categorical data were tested by Chi square.

Results

DNA Methylation in Tumor and Plasma Samples

Quantitative analyses of DNA methylation were performed in paraffin-embedded tumor tissues, blood cells, and plasma samples from 151 breast cancer patients. Of these, 129 patients (85.4%) demonstrated different levels of methylation in at least one of the evaluated promoters in their tumors: in 124, 19, 15, 15, and 6 patients, mean methylation levels of 48.45%, 3.81%, 2.36%, 27.55%, and 10.81% were found in *RASSF1A*, *ESR1*, *CDH1*, *TIMP3*, and *SYK*, respectively. In plasma samples, *RASSF1A* methylation was observed at a markedly lower frequency of 22.54% in 25 patients. *ESR1*, *CDH1*, *TIMP3*, and *SYK* were methylated in 10, 15, 17, and 3 patients at 17.20%, 22.87%, 31.93%, and 27.42%, respectively. DNA methylation levels of five evaluated genes in tumor and plasma samples are graphically depicted in Figure 1. Simultaneously methylated promoters in both tumor and plasma samples were found in 25, 1, 2, 4, and 1 patients in *RASSF1A*, *ESR1*, *CDH1*, *TIMP3*, and *SYK* genes, respectively (Table 2). Low levels of *TIMP3* methylation (0.53–5.15%) were detected in the genomic DNA of four breast cancer patients. Of 50 healthy controls, methylation levels of >0.5% in the *CDH1* promoter was observed in the genomic DNA of one person alone. However, in the control plasma samples, rare methylation events in *CDH1* were found in one person (1.67%), *TIMP3* in two (16.52% and 20.40%), and *ESR1* in two (7.12% and 16.72%). The number of methylated genes was determined in individual patients. Of 129 patients with any methylation in tumor tissues, 91, 30, and 7 samples were methylated in one, two, and three evaluated genes, respectively. One patient manifested promoter methylation in all five genes; however, the CMI was only 77.14. Of 49 patients with methylation in plasma, 37, 7, and 5 samples were methylated in one, two, and three genes, respectively (Figure 2). The cumulative methylation levels for the five evaluated genes were significantly higher in the tumors and plasma than in the genomic DNA of the same patients. In the methylation-positive tumor and plasma samples, the mean CMIs were 50.52 and 32.96, respectively, compared with no methylation in genomic DNA except for four patients with *TIMP3* methylation. However, a similar range of cumulative methylation in tumor and plasma DNA (0.74–156.57 and 0.51–151.62) was observed. In the majority of tumors, the substantial portion of CMI was represented by *RASSF1A* methylation when compared with more frequent methylation of other genes in plasma samples.

RASSF1A *Methylation Levels and Clinicopathologic Categories*

Statistical analysis of the correlation between methylation levels and clinicopathologic features of 151 breast cancer patients was performed for the highly and frequently methylated *RASSF1A* gene. The evaluated categories were age, histologic type, tumor size, histologic grading, LN status, TNM staging, ER status, progesterone receptor (PR) status, human epidermal growth factor receptor 2 (HER2) expression, and immunohistochemical (IHC) subtypes. In plasma samples, *RASSF1A* methylation ranged from 0% to 15.8%, with no significant differences between the subgroups of each clinicopathologic category. The tumor samples of these patients exhibited visibly higher levels of *RASSF1A*

Table 2. Frequencies of Breast Cancer Patients with DNA Methylation in Tumor and Plasma Samples.

Evaluated Genes	Promoter Methylation in Tumor, *N* (%)	Promoter Methylation in Plasma, *N* (%)	Promoter Methylation in Both Tumor and Plasma, *N* (%)
RASSF1A	124 (82.1)	25 (16.6)	25 (16.6)
ESR1	19 (12.8)	10 (6.7)	1 (0.7)
CDH1	15 (9.9)	15 (10)	2 (1.3)
TIMP3	15 (9.9)	17 (11.5)	4 (2.7)
SYK	6 (4)	3 (2)	1 (0.7)

methylation, and statistically significant differences were observed between patients with various tumor size (P = .029), ER-negative and ER-positive status (P = .000), PR-negative and PR-positive status (P = .004), and different IHC subtypes (P = .000; Table 1 and Figure 3). Moreover, there was a positive correlation between *RASSF1A* methylation levels and the percentage of cancer cells with ER (r = 0.251, P = .002) or PR expression (r = 0.200, P = .014).

Discussion

Quantification of DNA methylation levels in cancer-associated genes contributes to the more complex molecular characterization of tumors required for the development of new diagnostic and therapeutic strategies for cancer patients. High methodical diversity of DNA methylation status evaluation was found in the current literature; therefore, we compared our results with these studies using quantitative methods based on real-time technology. In tumor samples, we found *RASSF1A* methylation in 82.1% of evaluated breast cancer patients, with a mean level of 48.45% in methylated cases. In other studies, analogous frequencies (68% and 82.5%) but lower means of methylation levels (18.5 ± 4.7% and more than 10%) were found in the majority of patients [21,22]. Similar to our results, previous studies recorded low incidences of *ESR1* methylation in breast cancers and weak correlation with low ERα expression levels, indicating a sporadic role of DNA methylation in ER silencing [11,23]. In the present study, 9.9% of patients exhibited *CDH1* promoter methylation levels of up to 14.56% in tumor tissues, similar to other groups of patients where low levels of *CDH1* methylation frequencies from 5.8% to 22.5% were observed [20,22,24]. Variable *TIMP3* methylation levels of 3% to 42% were identified in the samples of nine patients [25], in accordance with our findings; however, to our knowledge, quantitative evaluation of *SYK* methylation has not yet been performed. Comparison of studies in European, American, and Saudi Arabian females revealed that the highest methylation levels were in *RASSF1A* similar to our study, confirming the important role of *RASSF1A* epigenetic silencing in breast cancer regardless of ethnicity [21,23,24].

Circulating cell-free DNA from plasma, serum, or other body fluids seems to be an appropriate biologic material for qualitative or quantitative testing of tumor-specific molecular alterations including DNA methylation. The serum of patients with invasive ductal carcinomas showed significantly higher *RASSF1A* methylation frequencies compared with control persons [26], as in our study. We found significantly lower frequencies of *RASSF1A* methylation in plasma samples (16.6%) than in tumors (82.1%); however, all 25 patients with positive findings in plasma had simultaneous methylation in their tumor samples. For *ESR1*, *CDH1*, *TIMP3*, and *SYK*, rare incidences were observed in both tumor and plasma samples. *ESR1* was evaluated in the serum of healthy controls and disease-free breast cancer and metastatic breast

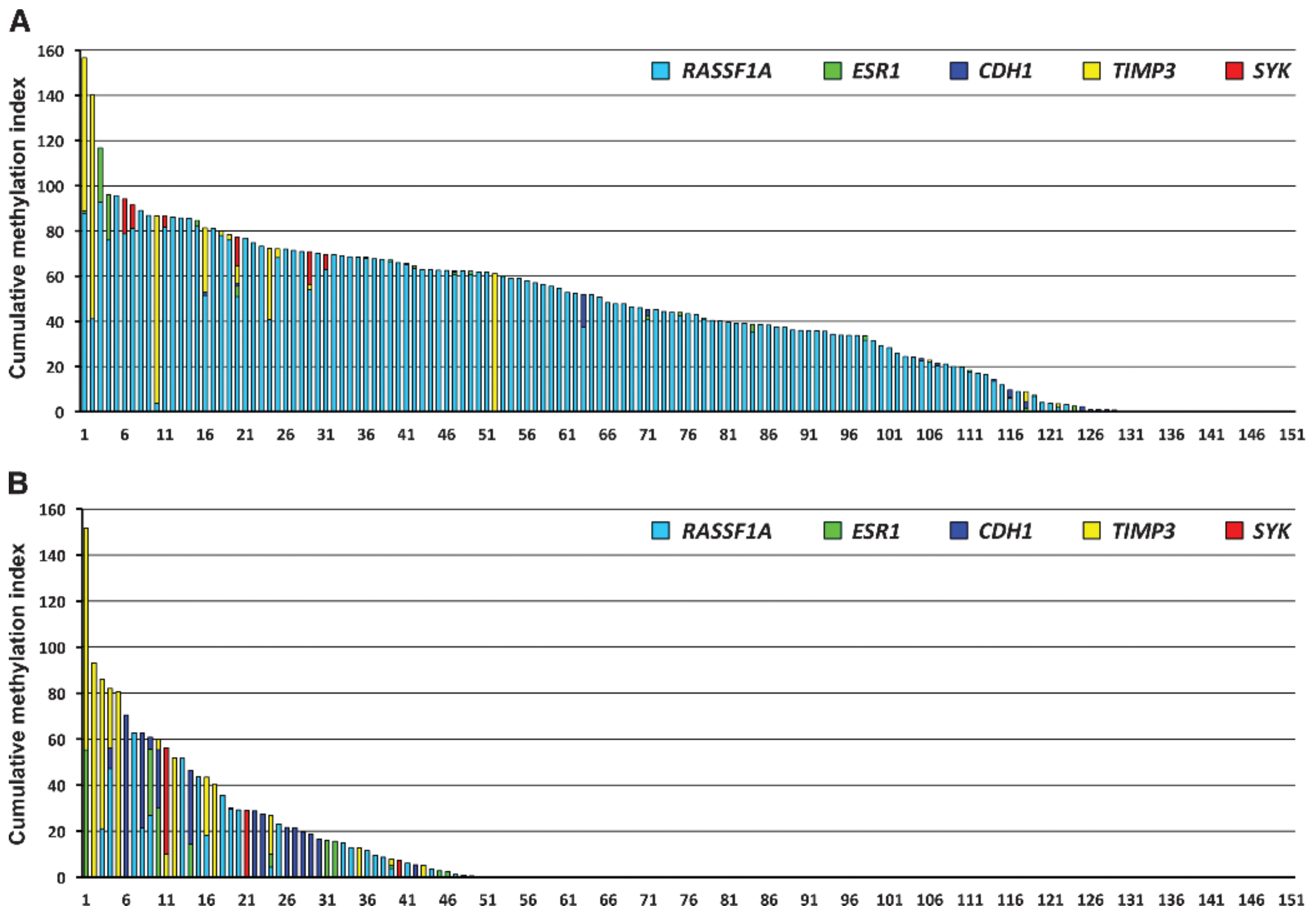

Figure 2. Cumulative DNA methylation levels in breast cancer patients. The results from tumor tissues of 129 patients (A) and plasma samples of 49 patients (B) are shown. The CMI is the sum of percentage methylation for five evaluated genes.

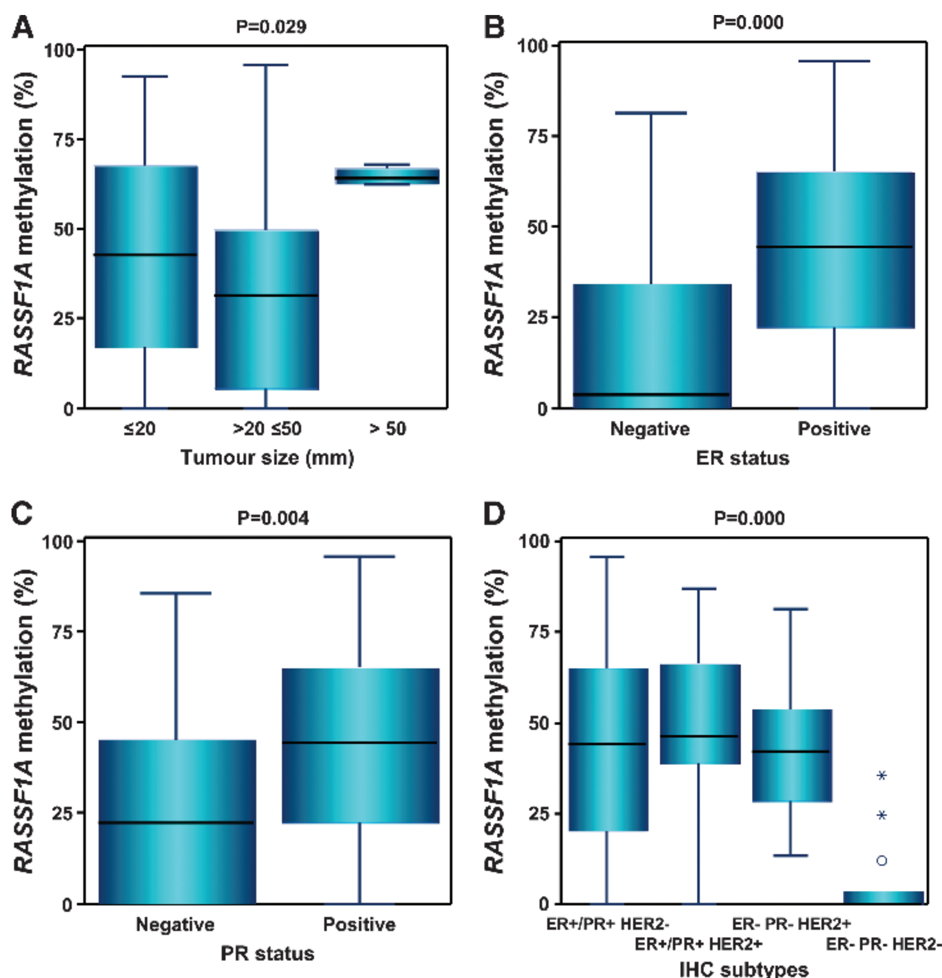

Figure 3. Distribution of *RASSF1A* methylation levels in four clinical and histopathologic categories of breast cancer patients. Box plots show the significant differences in the subgroups of patients with different tumor sizes (A), negative and positive estrogen or progesterone status (B and C), and different IHC subtypes (D). The length of the boxes is the interquartile range (IQR) that represents values between the 75th and 25th percentiles. Values more than three IQRs from the end of a box are labeled as extreme (*). Values more than 1.5 IQRs but less than 3 IQRs from the end of the box are labeled as outliers (O). The median is depicted by a horizontal line.

cancer patients and revealed no differences in the low levels of *ESR1* methylation between these three groups [27]. Our results oppose the above-mentioned hypothesis describing the possible influence of *ESR1* epigenetic silencing alone in the strategy of breast cancer therapy. Many researchers have focused on the identification of useful sets of methylated genes to improve diagnosis, prognosis, or therapeutic strategy; therefore, CMI appears to be a useful parameter. The high incidence of *RASSF1A* methylation in CMI shows the value of this silenced gene in tumor development in our patients. However, in 49 plasma samples, we found a different spectrum in CMI, with visibly higher occurrences of *TIMP3* and *CDH1* methylation. These results indicate that cell-free DNA could be derived from a degraded cell subpopulation, which is active in invasive and metastatic processes, for example, circulating tumor cells, rather than from products of apoptosis and necrosis in heterogeneous tumor masses [28]; therefore, these DNA samples could be used for metastatic potential testing. However, after the critical evaluation of methodical diversity, variability of results, and limited diagnostic sensitivity and specificity of cell-free DNA alterations in many published studies, we agree that clinical utilization of such DNA requires further studies to assess sample collection, processing, analysis, and measurement of results [29].

In our previous study, no relationship between tumor size and *RASSF1A* methylation levels was observed [20], but in the presented group of patients we found significantly higher levels in four cases with breast tumors larger than 50 mm, three of which were at an advanced stage of disease. The accumulation of DNA methylation changes could be associated with aggressive phenotype rather than larger size of tumor, because small cancers can also invade and metastasize as a result of higher numbers of molecular changes compared with early-stage cancer. Most importantly, we found a relationship between *RASSF1A* methylation levels and expression of hormonal receptors. Previous studies showed higher frequency of *RASSF1A* methylation in breast cancers with ER+ and PR+ status than in ER− and PR− cases [24,30]; however, we observed a positive correlation between *RASSF1A* methylation levels in tumor tissues and number of cancer cells with positive expression in both ER and PR. Analyses of *RASSF1A* methylation in four different IHC subtypes showed very low levels in ER− PR− HER2− but not in ER− PR− HER2+. Moreover, in cancers with HER2 overexpression, higher but statistically insignificant differences in methylation levels were observed compared with HER2-negative cancers. These results indicate the possible influence of HER2 on DNA methylation processes.

In normal human breast epithelium, ERα expression is fairly consistent over time, and women with ER overexpression in the normal breast may have increased estrogen sensitivity that is associated with higher breast cancer risk [31]. In previous case-control studies, the incidence of breast epithelial cells expressing ER was higher in breast cancer cases than benign breast disease controls [32]; however, Woolcott and colleagues did not confirm this strong association [33]. ER expression in luminal breast cancers varied from 1% to 100% of positively stained cells, and even patients with 1% of ER-expressing tumor cells experience some clinical benefit from endocrine therapies [34]. Therefore, in addition to ER levels, ER dynamics could play an important role in tumor behavior including therapy response. In a recent study, a mouse model using patient-derived ER+ tumor xenografts was developed for the evaluation of intratumoral hormone and receptor action. The researchers reported that analysis of the ER transcriptome in selected tumors showed notable differences in the ER mechanism of action and downstream-activated signaling networks, in addition to identifying a small set of common estrogen-regulated genes. Mapping of conserved and tumor-unique ER programs can contribute to the development of more personalized therapeutic strategies [35]. Both estrogen signaling and epigenetic modifications, in particular DNA methylation, are involved in the regulation of gene expression in breast cancers. Putnik and colleagues investigated the potential regulatory cross talk between these two pathways in human MCF-7 breast cancer cells. They identified approximately 140 genes that were influenced by both 17β-estradiol and a demethylating agent 5-aza-20-deoxycytidine; however, they did not show a direct molecular interplay of estrogen mediators and epigenetic signaling at the promoters of regulated genes [36]. Furthermore, in a recent study of the *RASSF1A* tumor-suppressive function in MCF-7 cells, reconstitution of RASSF1A expression decreased ERα levels, followed by reduced expression of Id1 and the E2-responsive genes *BCL-2* and *C-MYC*, up-regulation of p21Cip1/Waf1 induction of cell-cycle arrest and senescence, and inhibition of signaling pathways involved in breast epithelial cell transformation. These findings indicate a central role of RASSF1A in suppressing transformation of human breast epithelial cells in part through ERα inhibition [10]. This hypothesis is supported by the present study, because we observed a positive correlation between *RASSF1A* methylation levels and percentage of ER- or PR-positively stained cells in contrary to hormone receptor–negative cases with low levels of methylation. In another study of heterogeneity of matched breast primary tumors and metastases, 3 of 10 evaluated patients exhibited no *RASSF1A* methylation and a hormone-negative phenotype in both types of tissues [37]. Previous work carried out *in vitro* is convincing; however, detailed studies of cancer-associated changes in the ER mechanism of action in human tumors will enable association of the "ER-dependent pathway patterns" with the effectiveness of hormonal therapy and may help to develop new therapeutic molecules.

To summarize, in the present study, we observed high frequencies of *RASSF1A* methylation and positive correlation of *RASSF1A* methylation levels with ER and PR expression in breast cancer patients. Therefore, we speculate that the levels of *RASSF1A* methylation in ER+ breast cancer patients could be helpful in determining prognosis and hormonal therapy response. Clinical utility of cell-free DNA isolated from plasma for cancer-associated molecular characteristics testing, namely, DNA methylation, is unsatisfactory because of its low detection sensitivity. Furthermore, the specific methylation spectra in plasma samples related to invasive and metastatic processes need to be further evaluated in larger study cohorts.

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Table W1. Primers and TaqMan Probes.

Primer Name	Oligonucleotide Sequence (5′–3′)	Product Size (bp)	Reference
RASSF1A F	TTTAGTTTGGATTTTGGGGG	139	Present study
RASSF1A R	CAACTCAATAAACTCAAACTCCC		Present study
RASSF1A Met F	GCGTTGAAGTCGGGGTTC	85	[21]
RASSF1A Met R	CCCGATTAAACCCGTACTTCG		Present study
RASSF1A Met R Probe	FAM–ACAAACGCGAACCGAACGAAACCA–BHQ-1		[21]
RASSF1A Umet F	GGTGTTGAAGTTGGGGTTTG	86	[21]
RASSF1A Umet R	CCCAATTAAACCCATACTTCACT		Present study
RASSF1A Umet R Probe	HEX–CTAACAAACACAAACCAAACAAAACCA–BHQ-1		[21]
CDH1 F	GGAATTGTAAAGTATTTGTGAGTTTG	169	Present study
CDH1 R	AAATACCTACAACAACAACAACAAC		Present study
CDH1 Met F	AGTTCGTTTTAGTTCGGTTCG	98	Present study
CDH1 Met R	GCCGAAAAACTACGACTCCA		Present study
CDH1 Met F Probe	FAM–TTCGTTCGGCGTTTTCGGTTAGT–BHQ-1		Present study
CDH1 Umet F	AGTTTGTTTTAGTTTGGTTTGATT	98	Present study
CDH1 Umet R	ACCAAAAAACTACAACTCCAAAA		Present study
CDH1 Umet F Probe	HEX–TTTGTTTGGTGTTTTTGGTTAGTTATG–BHQ-1		Present study
ESR1 F	GGGAGATTAGTATTTAAAGTTGGAG	205	Present study
ESR1 R	CCACCTAAAAAAAAAACACAACC		Present study
ESR1 Met F	CGGAGGGCGTTCGTTTT	115	Present study
ESR1 Met R	GCATATCCCGCCGACACG		Present study
ESR1 Met F Probe	FAM–TTCGTCGGGTCGTTCGGTTTTATCG–BHQ-1		Present study
ESR1 Umet F	GGTGGAGGGTGTTTGTTTT	118	Present study
ESR1 Umet R	CACATATCCCACCAACACAC		Present study
ESR1 Umet F Probe	HEX–TTTGTTGGGTTGTTTGGTTTTATTGGA–BHQ-1		Present study
TIMP3 F	GGAGGTTAAGGTTGTTT	175	Present study
TIMP3 R	CAAACTCCAACTACCCAAAAAC		Present study
TIMP3 Met F	AGGTTAAGGTTGTTTCGTACGGTTC	122	Present study
TIMP3 Met R	CGCTACTACCGCCGCTACC		Present study
TIMP3 Met F Probe	FAM–CGGGCGAGCGAGTTCGGGT–BHQ-1		Present study
TIMP3 Umet F	TAAGGTTGTTTTGTATGGTTTG	126	Present study
TIMP3 Umet R	ATCATTACCACTACTACCACCACTAC		Present study
TIMP3 Umet F Probe	HEX–TGGGTGAGTGAGTTTGGGTTGTAGT–BHQ-1		Present study
SYK F	GGAAGTTGTTTAAAATGAGGAAGA	180	Present study
SYK R	TAACCTCCTCTCCTTACCAAA		Present study
SYK Met F	CGGCGGTTGGAGAGC	82	Present study
SYK Met R	GCGACCACACCTACCTACG		Present study
SYK Met F Probe	FAM–TTCGCGTTGCGTTCGTTTTCGT–BHQ-1		Present study
SYK Umet F	GGTGGTGGTTGGAGAGTG	87	Present study
SYK Umet R	AACACAACCACACCTACCTACA		Present study
SYK Umet F Probe	HEX–TGGTTTTGTGTTGTGTTTGTTTTTGTTT–BHQ-1		Present study