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Advances in Brief

DNA Methylation in Serum of Breast Cancer Patients: An Independent Prognostic Marker

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Abstract

Changes in the status of DNA methylation are one of the most common molecular alterations in human neoplasia. Because it is possible to detect these epigenetic alterations in the bloodstream of patients, we investigated whether aberrant DNA methylation in patient pretherapeutic sera is of prognostic significance in breast cancer.

Using MethyLight, a high-throughput DNA methylation assay, we analyzed 39 genes in a gene evaluation set, consisting of 10 sera from metastasized patients, 26 patients with primary breast cancer, and 10 control patients. To determine the prognostic value of genes identified within the gene evaluation set, we finally analyzed pretreatment sera of 24 patients having had no adjuvant treatment (training set) to determine their prognostic value. An independent test set consisting of 62 patients was then used to test the validity of genes and combinations of genes, which in the training set were found to be good prognostic markers.

In the gene evaluation set we identified five genes (*ESR1*, *APC*, *HSD17B4*, *HIC1*, and *RASSF1A*). In the training set, patients with methylated serum DNA for *RASSF1A* and/or *APC* had the worst prognosis ($P < 0.001$). This finding was confirmed by analyzing serum samples from the independent test set ($P = 0.007$). When analyzing all 86 of the investigated patients, multivariate analysis showed methylated *RASSF1A* and/or *APC* serum DNA to be independently associated with poor outcome, with a relative risk for death of 5.7.

DNA methylation of particular genes in pretherapeutic sera of breast cancer patients, especially of *RASSF1A/APC*, is more powerful than standard prognostic parameters.

Introduction

Involvement of axillary lymph nodes and tumor size are the most important prognostic factors in breast cancer (1–4). Although the presence or absence of metastatic involvement in the axillary lymph nodes is the most powerful prognostic factor available for patients with primary breast cancer, it is only an indirect measure reflecting the tendency of the tumors to spread. In approximately one-third of women with breast cancer and negative lymph nodes, the disease recurs, whereas about one-third of patients with positive lymph nodes are free of recurrence 10 years after locoregional therapy (2, 3). These data highlight the need for more sensitive and specific prognostic indicators, ideally reflecting the presence or absence of tumor-specific alterations in the bloodstream that may eventually even after years lead to metastasis. It is now widely accepted that adjuvant systemic therapy substantially improves disease-free and overall survival in both pre- and postmenopausal women up to the age of 70 years with lymph node-negative or lymph node-positive breast cancer (2, 3). It is also generally accepted that patients with poor prognostic features benefit the most from adjuvant therapy, whereas some patients with good prognostic features may be overtreated (1, 4, 5). Moreover, many other factors have been investigated for their potential to predict disease outcome, but in general they have only limited predictive value (4). Interesting prognostic parameters including gene expression profiles (6, 7), cell cycle regulating proteins (8), and occult cytokeratin-positive metastatic cells in the bone marrow (9) have been added to the list of prognostic factors recently, but their prognostic relevance needs to be additionally evaluated.

Changes in the status of DNA methylation, known as epigenetic alterations, are one of the most common molecular alterations in human neoplasia (10), including breast cancer (11). Cytosine methylation occurs after DNA synthesis by enzymatic transfer of a methyl group from the methyl donor *S*-adenosylmethionine to the carbon-5 position of cytosine. Cytosines are methylated in the human genome mostly when located 5′ to a guanosine. Regions with a high G:C content are so-called CpG islands. It has been increasingly recognized over the past 4–5 years that the CpG islands of many genes, which are mostly unmethylated in normal tissue, are methylated to various degrees in human cancers, thus representing tumor-specific alterations (10, 12). The presence of abnormally high DNA concentrations in the serum of patients with various malignant diseases was described several years ago (13). The discovery that cell-free DNA can be shed into the bloodstream has generated great interest. Numerous studies have demonstrated tumor-specific alterations in DNA recovered from plasma or serum of patients with various malignancies, a finding that has potential for molecular diagnosis and prognosis. The nucleic acid markers described in plasma and serum include oncogene mutations, microsatellite alterations, gene rearrangements, and epigenetic alterations, such as aberrant promoter hypermethylation (13). During recent years some studies have reported cell-free DNA in serum/plasma of breast cancer patients at diagnosis (14–17) and in some cases persistence after primary therapy (17, 18). Nevertheless an increasing number of studies have reported the presence of methylated DNA in serum/plasma of patients with various types of malignancies, including breast cancer, and the absence of methylated DNA in normal control patients (14, 17, 19–22). Thus far, only few studies have addressed the prognostic value of these epigenetic alterations in the bloodstream of patients (23, 24).

This study evaluated the prognostic potential of DNA methylation-based markers in pretherapeutic sera of breast cancer patients. We decided to investigate these markers in women who had not undergone any form of adjuvant systemic treatment, because this group of patients has the greatest potential for testing the relevance of a prognostic factor (5). Of 39 markers tested, preoperative serum showing *APC* and/or *RASSF1A* methylation was an independent prognostic marker for overall survival in breast cancer.

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Materials and Methods

Patients. The gene evaluation set consisted of patients with recurrent disease ($n = 10$; sera obtained at diagnosis of metastasis in the bone, lung, brain, or liver) and pretherapeutic sera of recently diagnosed primary breast cancer patients ($n = 26$; age range, 36.1–83.9 years; mean, 59.3 years; 2, 18, and 6 patients had pT1, pT2, and pT3 cancers, respectively; 15, 10, and 1 patients had lymph node-negative, -positive, and unknown disease, respectively) and normal controls ($n = 10$; age range, 20.5–71.5 years; mean, 44.6 years; all underwent a core biopsy and were confirmed to have benign disease of the breast).

To assess prognostic significance we used pretherapeutic sera in independent training ($n = 24$) and test ($n = 62$) sets consisting of patients who did not receive any adjuvant treatment after surgery.

Systemic adjuvant therapy was either not necessary, or the patients were not eligible or refused any additional treatment. The primary surgical procedure included breast-conserving lumpectomy or modified radical mastectomy and axillary lymph node dissection. Median age of the study population was 60 years (range, 28–86 years). After a median follow-up of 3.7 years (range, 1 month to 12.2 years) 17 of the 86 patients (20%) had died. Distribution of aberrant serum DNA methylation of the 86 patients, and association with clinical and histopathological characteristics are shown in Table 1.

Serum Samples and DNA Isolation. Patient blood samples were drawn before therapeutic intervention. The blood was centrifuged at $2000 \times g$ for 10 min at room temperature, and 1 ml aliquots of serum samples were stored at −30°C.

Genomic DNA from serum samples was isolated using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol with some modifications for multiple loading of the DNA extraction columns to gain a sufficient amount of DNA. Thus, 4×200 μl of a serum sample were each mixed with 200 μl of working solution (binding buffer supplemented with polyA carrier RNA) and 50 μl proteinase K (18 mg/ml), and incubated for 10 min at 72°C. After adding 100 μl isopropanol the solution was mixed, loaded onto the extraction column, and centrifuged for 1 min at $8,000 \times g$. The flow-through was pipetted back into the same column reservoir and centrifuged a second time. This procedure was repeated four times for each serum sample. After these "pooling steps" the DNA isolation was processed as described in the manufacturer's protocol. For DNA elution 55 μl of AE buffer (Qiagen, Hilden, Germany) were added, incubated for 20 min at 45°C, and centrifuged for 3 min at $12,000 \times g$. For both, normal sera and cancer sera analysis the same amount of serum for DNA extraction was used.

Table 1 *Characteristics of training and test sets*

Characteristics	Training set ($n = 24$) %	Test set ($n = 62$) %	P^a
Size of tumor[b]			0.024
T1	62.5	79	
T2	37.5	13	
T3 + T4	0	7	
Histologic type			n.s.
Invasive ductal	67	63	
Invasive lobular	8	13	
Others	25	24	
Tumor grade[c]			n.s.
1	46	44	
2	33	39	
3	17	10	
Lymph node metastases			n.s.
No	75	65	
Yes	12.5	11	
Unknown	12.5	24	
Menopausal status			n.s.
Premenopausal	33	16	
Postmenopausal	67	84	
Estrogen-receptor status			n.s.
Positive	54	40	
Negative	42	45	
Progesterone-receptor status			n.s.
Positive	58	45	
Negative	38	40	
Hormone-receptor status[d]			n.s.
Positive	63	50	
Negative	33	36	

[a] P for the comparison of numbers of patients were calculated by means of the χ^2 test. n.s., not significant; median age: training set (54.2 years; 37.6–83.2), test set (65.7 years; 28.2–86.2), $P = 0.052$; follow-up: training set (8.0 years; 1 month to 12.2 years), test set (3.1 years; 1 month to 11 years), $P < 0.001$.
[b] Tumor size was unknown in 1 case.
[c] Tumor grade was unknown in 6 cases.
[d] Hormone-receptor status was unknown in 10 cases.

Analysis of DNA Methylation. Sodium bisulfite conversion of genomic DNA was performed as described previously (25). Sodium bisulfite-treated genomic DNA was analyzed by the MethyLight, a fluorescence-based, real-time PCR assay, as described previously (25, 26). Briefly, two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest and a reference set, β-actin (*ACTB*), to normalize for input DNA. Serum samples of patients with recurrent disease revealed the highest amount of β-actin, whereas no difference between β-actin values from serum samples of patients with primary breast cancer and sera of normal controls was observed. Specificity of the reactions for methylated DNA was confirmed separately using *Sss*I (New England Biolabs) -treated human WBC DNA (heavily methylated). The percentage of fully methylated molecules at a specific locus was calculated by dividing the *GENE*:*ACTB* ratio of a sample by the *GENE*:*ACTB* ratio of *Sss*I-treated WBC DNA and multiplying by 100. The abbreviation PMR indicates this measurement. For each MethyLight reaction 10 μl of bisulfite-treated genomic DNA was used.

A gene was deemed methylated if the percentage of fully methylated reference value was >0. Primer and probes specific for methylated DNA and used for MethyLight reactions are listed in Supplemental Data.

Statistical Analysis. We used Pearson's χ^2 or, in the case of low frequencies per cell, Fisher's exact method to test associations between categorically clinicopathological features. The Mann-Whitney U test was used to assess differences between nonparametric distributed variables. Overall survival was calculated from the date of diagnosis of the primary tumor to the date of death or last follow-up. Overall survival curves were calculated with the Kaplan-Meier method. Univariate analysis of overall survival according to clinicopathological factors [histological type, tumor stage, nodal status, grading, menopausal status, hormone receptor status (estrogen and/or progesterone receptor positivity), and estrogen and progesterone receptor status] and gene methylation were performed using a two-sided log-rank test.

Multivariate Cox proportional hazards analysis was used to estimate the prognostic effect of methylated genes.

A $P < 0.05$ was considered a statistically significant difference. All of the statistical analyses were performed using SPSS Software 10.0.

Results

Determination of Appropriate Genes in a Gene Evaluation Set. We initially investigated 39 genes in the sera of 10 patients with metastasized breast cancer for the presence of aberrant methylation. The 33 genes positive in the sera of the metastasized patients were additionally evaluated in an independent sample set of pretherapeutic sera of 26 patients with primary breast cancer and 10 healthy controls. An overview of the frequency of methylation in the investigated serum samples is given in Table 2. The most appropriate genes for our additional analyses were determined to be those that met one of the following criteria: (*a*) unmethylated in serum samples from healthy controls and >10% methylated in serum samples from primary breast cancer patients; or (*b*) ≤10% methylated in serum samples from healthy controls and >20% methylated in serum samples from primary breast cancer patients. A total of five genes, namely *ESR1*, *APC*, *HSD17B4*, *HIC1*, and *RASSF1A*, met at least one of these criteria (Table 2).

Prognostic Significance in Training and Test Set. Pretreatment serum samples from patients included in the training set were used to evaluate the prognostic value of the methylation status of these five genes. In this training set we identified *ESR1*, *APC*, or *RASSF1A* methylation in primary breast cancer patient sera to be markers of poor prognosis, whereas *HSD17B4* reached only borderline significance, and aberrant methylation of *HIC1* showed no significant results (Table 3). Furthermore, various combinations of the investigated

genes were analyzed. Patients were classified as methylation-positive if at least one of the genes included in the combination showed aberrant methylation. Patients with methylated serum DNA for *RASSF1A* and/or *APC* had the worst prognosis ($P < 0.001$), even worse than when each gene was analyzed individually (Table 3).

The highly significant prognostic value for *APC* and/or *RASSF1A* methylation in serum samples from breast cancer patients was confirmed by analyzing the test set ($P = 0.007$, log rank test). *ESR1* and *APC* methylation as single genes or the combinations *ESR1/RASSF1A* and *ESR1/APC* no longer had prognostic significance (Table 3).

Combined analysis of the training and test sets ($n = 86$) showed correlation between *ESR1* and *RASSF1A* ($P = 0.005$), and between *ESR1* and *APC* ($P = 0.031$), whereas no correlation was observed between *RASSF1A* and *APC*. In patients with advanced tumors *RASSF1A* and *ESR1* methylation, and in patients with progesterone receptor-negative tumors *APC* methylation was more prevalent in pretherapeutic sera, whereas no additional associations were seen between clinicopathological features and DNA methylation of *APC*, *ESR1*, or *RASSF1A* (Table 4). *RASSF1A* methylation in pretherapeutic sera was more prevalent in older than in younger patients, whereas age had no effect on DNA methylation of *ESR1* or *APC*.

Univariate analysis of all 86 of the investigated patients (training set plus test set) revealed prognostic significance for tumor size, lymph node metastases, and methylation status of *APC*, *RASSF1A*, and the combination of *RASSF1A/APC* (Table 5; Fig. 1). Because *ESR1* methylation correlates with *APC* as well as with *RASSF1A* methylation, we did not test the triple combination in the univariate or the multivariate analyses of all 86 of the patients.

Multivariate Analysis. The Cox multiple regression analysis included tumor size, lymph node metastases, age, and methylation status of the investigated genes. Beside lymph node status, methylated *RASSF1A* and/or *APC* serum DNA was strongly associated with poor outcome, with a relative risk for death of 5.7 (Table 6).

Table 2 *Frequency of methylated serum DNA in the gene evaluation set*

Gene	Healthy controls ($n = 10$)	Primary breast cancer ($n = 26$)	Recurrent breast cancer ($n = 10$)
	% positive		
ESR1	0	27	70
APC	0	23	80
HSD17B4	0	12	30
CDH13	0	8	40
ESR2	0	4	20
MGMT	0	4	10
SYK	0	4	10
HIC1	10	39	90
RASSF1A	10	23	80
GSTP1	10	12	60
MYOD1	20	27	80
CDH1	20	20	90
PTGS2	30	39	100
PGR	30	46	80
CALCA	40	50	60
HLAG	60	69	100
BLT1	60	85	100
ARHI	100	100	100
MLLT7	100	100	100
TFF1	100	100	100
SOCS2	0	0	40
SOCS1	0	0	30
TERT	0	0	30
DAPK1	0	0	30
TIMP3	0	0	20
BRCA1	0	0	20
GSTM3	0	0	20
MT3	0	0	20
TWIST	0	0	10
MLH1	0	0	10
CYP1B1	0	0	10
TITF1	0	0	10
FGF18	0	0	10
CDKN2A	n.d.[a]	n.d.	0
HSPA2	n.d.	n.d.	0
PPP1R13B	n.d.	n.d.	0
TP53BP2	n.d.	n.d.	0
REV3L	n.d.	n.d.	0
IGFB2	n.d.	n.d.	0

[a] n.d., not done.

Table 3 *Univariate analysis of methylation status in training and test sets*

Genes	Training set ($n = 24$) P[a]	Test set ($n = 62$) P
ESR1	0.018	0.555
APC	0.002	0.307
HSD17B4	0.056	
HIC1	0.796	
RASSF1A	0.042	0.014
RASSF1A/APC	<0.001	0.007
ESR1/APC	0.001	0.951
ESR1/RASSF1A	0.032	0.138

[a] P for each variable were calculated by means of the log rank test.

Table 4 *Frequency of methylated genes according to clinicopathological features*

DNA methylation of *RASSF1A* for 1 case was missing. χ^2 pearson: tumor size, *ESR1* ($P = 0.005$); tumor size, *RASSF1A* ($P = 0.049$); progesterone-receptor, *APC* ($P = 0.036$). Median age, *RASSF1A* methylated (79.0 yrs.; 49.6–86.2), *RASSF1A* unmethylated (59.4 yrs.; 28.2–82.3), $P = 0.009$.

Characteristics	No. of patients	*ESR1*	*APC*	*RASSF1A*	*RASSF1A* and/or *APC*
		% positive			
Size of tumor[a]					
T1	64	14	11	9	19
T2	17	12	12	19	31
T3 + T4	4	75	25	50	50
Histologic type					
Invasive ductal	55	18	15	11	22
Invasive lobular	10	20	0	30	30
Others	21	10	10	10	20
Tumor grade[b]					
1	38	11	11	13	21
2	32	19	16	16	31
3	10	30	10	11	11
Lymph node metastases					
No	58	12	9	9	18
Yes	10	20	30	20	40
Unknown	18	28	11	22	28
Menopausal status					
Premenopausal	18	28	11	11	22
Postmenopausal	68	13	12	13	22
Estrogen-receptor					
Positive	38	16	11	16	21
Negative	38	16	16	11	27
Progesterone-receptor					
Positive	42	14	5	14	18
Negative	34	18	24	12	33
Hormone-receptor status[c]					
Positive	46	15	9	15	20
Negative	30	17	20	10	31

[a] Tumor size was unknown in 1 case.
[b] Tumor grade was unknown in 6 cases.
[c] Hormone-receptor status was unknown in 10 cases.

Discussion

Prognosis in patients with newly diagnosed breast cancer is determined primarily by the presence or absence of metastases in draining axillary lymph nodes (1–4). Nevertheless, the life-threatening event in breast cancer is not lymph node metastasis *per se*, but hematogenous metastases, which mainly affect bone, liver, lung, and brain. Therefore, we aimed to develop a prognostic test that is sensitive for hematogenous metastases and could be performed in pretherapeutic serum of patients.

In recent years several studies have reported cell-free tumor-specific DNA in serum/plasma of breast cancer patients at diagnosis (14–17). Aberrant methylation of serum/plasma DNA of patients with

Table 5 *Results of univariate analysis*

Variable	No. of patients who died/total no.	Relative risk of death (95% confidence interval)	*P*
Size of tumor			0.018
T1	10/64		
T2	5/17	2.2 (0.6–7.8)	
T3 + T4	2/4	5.4 (0.7–42.9)	
Histologic type			0.296
Invasive ductal	13/55		
Invasive lobular	1/10	0.4 (0–3.1)	
Others	3/21	0.5 (0.1–2.1)	
Tumor grade			0.310
1	6/38		
2	9/32	2.1 (0.7–6.7)	
3	2/10	1.3 (0.2–7.9)	
Lymph node metastases			0.005
No	7/58		
Yes	5/10	7.3 (1.7–31.7)	
Unknown	5/18	2.8 (0.8–10.3)	
Menopausal status			0.062
Premenopausal	1/18		
Postmenopausal	16/68	5.2 (0.6–42.4)	
Estrogen-receptor			0.369
Positive	10/38	1.9 (0.6–5.9)	
Negative	6/38		
Progesterone-receptor			0.766
Positive	9/42	1.1 (0.3–3.2)	
Negative	7/34		
Hormone-receptor status			0.799
Positive	10/46	1.1 (0.4–3.5)	
Negative	6/30		
ESR1 methylation			0.370
Unmethylated	13/72		
Methylated	4/14	1.8 (0.5–6.7)	
APC methylation			0.001
Unmethylated	12/76		
Methylated	5/10	5.3 (1.3–21.3)	
RASSF1A methylation			0.001
Unmethylated	11/74		
Methylated	6/11	6.9 (1.8–26.5)	
RASSF1/APC methylation			<0.001
Unmethylated	7/66		
Methylated	10/19	9.5 (2.9–31.4)	

various types of malignancies, including breast cancer, has been described (14, 17, 19–22).

In light of these observations, we examined the methylation status of 39 genes, which, on the one hand, are known to be frequently methylated in breast cancer and other malignancies (10, 11) and, on the other hand, were reported to be abnormally regulated in tumors of patients with poor prognostic breast cancer (6, 7). Because levels of circulating DNA in metastasized patients are known to be higher (18) and because the loss of genetic heterogeneity of disseminated tumor cells with the emergence of clinically evident metastasis was reported recently (27), we firstly investigated these 39 genes in 10 sera of metastasized patients to determine the overall prevalence of methylation changes in breast cancer. As a next step we analyzed the 33 genes that were positive in the metastasized patients, in the pretreatment sera of 26 patients with primary breast cancer, and in 10 benign controls to identify the most important genes for additional analysis. Eventually we came up with five genes (*ESR1*, *APC*, *HSD17B4*, *HIC1*, and *RASSF1A*), which were primarily analyzed in a group of 24 patients (training set). To confirm the significance of this result we tested these genes in an independent set of 62 patients (test set). To apply the strictest criteria for testing the potential of a prognostic factor (5), we investigated these markers in women who had not undergone adjuvant systemic treatment. DNA methylation of *APC* and *RASSF1A* in pretherapeutic sera, both frequently methylated and abnormally regulated in human primary breast cancers (28, 29), turned out to be a strong independent prognostic parameter. These genes are involved in pathways counteracting metastasis: mediation of intercellular adhesion, stabilization of the cytoskeleton, regulation of the cell cycle, and apoptosis (30, 31). Methylated DNA in the pretherapeutic serum of patients coding for these two genes reflects poor prognosis. The source of the tumor-specific DNA and its definite role in metastasis remains elusive (13): circulating tumor-specific altered

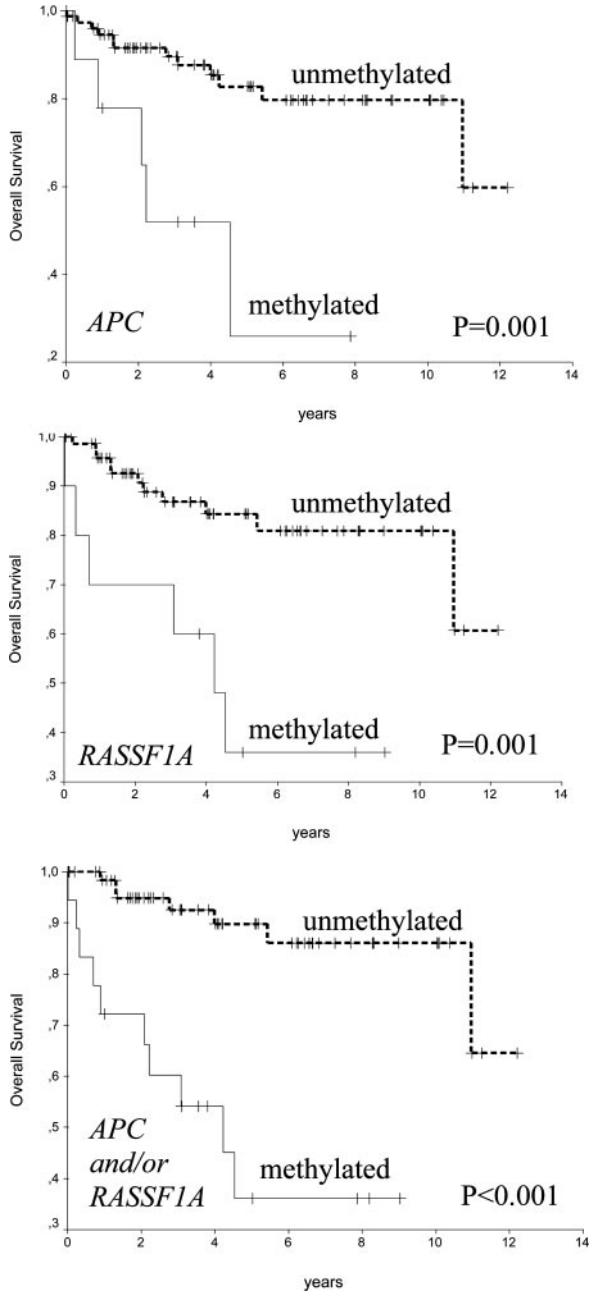

Fig. 1. Kaplan-Meier analysis of the probability of overall survival among all 86 patients.

Table 6 *Multivariate analysis*

Variable	Relative risk of death (95% confidence interval)	*P*
Size of tumor		0.19
T2 (*vs.* T1)	2.7 (0.8–9.3)	
T3 + T4 (*vs.* T1)	2.9 (0.4–20.5)	
Lymph node metastases		0.039
Yes (*vs.* no lymph node metastases)	3.9 (1.1–13.9)	
Unknown (*vs.* no lymph node metastases)	5.2 (1.2–22.4)	
Age	1.0 (1.0–1.1)	0.06
RASSF1A and/or *APC* methylated (*vs.* unmethylated)	5.7 (1.9–16.9)	0.002

genetic information may serve as a surrogate marker for circulating tumor cells that ultimately cause distant metastases. An alternative, but equally attractive, hypothesis is that circulating altered DNA *per se* may cause *de novo* development of tumor cells in organs known to harbor breast cancer metastases. This so-called "Hypothesis of Genometastasis" suggests that malignant transformation might develop as a result of transfection of susceptible cells in distant target organs with dominant oncogenes that circulate in the plasma and are derived from the primary tumor (32). Interestingly, irrespective of the source of DNA in the serum, it is noteworthy that some genes provide prognostic information when methylated in patient sera, whereas genes like *HIC1*, which is methylated in ~40% and 90% of primary and metastasized breast cancer patients, respectively, but in only 10% of healthy individuals, are not at all a prognostic parameter.

Irrespective of the mechanistic role of methylated DNA with regards to metastasis in breast cancer patients, these epigenetic changes in serum have several advantages as indicators of poor prognosis as compared with currently used or studied prognostic parameters: DNA in serum is stable and can be analyzed by a high-throughput method like MethyLight. Compared with bone marrow aspiration, a simple blood draw (which can be repeated any time throughout the follow-up period) is sufficient. The more screening mammographies are performed, the more small cancers are treated, and after histopathological examination no tumor material will remain to perform RNA- and/or protein-based assays for risk evaluation.

This article demonstrates a useful and easy approach for risk assessment of breast cancer patients.

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