CXCL12 and *ADAM23* hypermethylation are associated with advanced breast cancers

IVANA FRIDRICHOVA, BOZENA SMOLKOVA, VIERA KAJABOVA, IVETA ZMETAKOVA, TOMAS KRIVULCIK, MICHAL MEGO, ZUZANA CIERNA, MARIAN KARABA, JURAJ BENCA, DANIEL PINDAK, MARTIN BOHAC, VANDA REPISKA, and LUDOVIT DANIHEL

BRATISLAVA, SLOVAK REPUBLIC

More than 25% of the patients with breast cancer (BC) develop metastatic disease. In the present study, we investigated the relationship between DNA methylation levels in genes regulating cell growth, invasiveness, and metastasis and advanced BCs and evaluated the clinical utility of methylation profiles for detecting metastatic potential. Pyrosequencing was used to quantify methylation levels in 11 cancer-associated genes in primary tumors (PTs), lymph node metastases (LNMs), plasma (PL), and blood cells from 206 patients with invasive BC. Protein expression was evaluated using immunohistochemistry. PTs showed hypermethylation of A isoform of the RAS-association domain family 1 (*RASSF1A*), adenomatous polyposis coli (*APC*), chemokine C-X-C motif ligand 12 (*CXCL12*), and disintegrin and metalloprotease domain 23 (*ADAM23*) (means 38.98%, 24.84%, 12.04%, and 10.01%, respectively). Positive correlations were identified between methylations in PTs and LNMs, but not between PL and PTs. The cumulative methylation of PTs and LNMs manifested similar spectrums of methylated genes that indicate the maintaining of aberrant methylation during breast tumorigenesis. Significantly increased methylation levels in *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* were found in estrogen receptor (ER) positive BCs in comparison with ER negative cases. Regarding these results, the evaluation of DNA methylation could be more informative in testing of patients with ER positive BC. The risk for LNMs development and higher proliferation of cancer cells measured through Ki-67 expression was increased by hypermethylation of *CXCL12* and *ADAM23*, respectively. Therefore, the quantification of *CXCL12* and *ADAM23* methylation could be useful for the prediction of advanced stage of BC. (Translational Research 2015;165:717–730)

Abbreviations: ADAM23 = disintegrin and metalloprotease domain 23; APC = adenomatous polyposis coli; BC = breast cancer; BRMS1 = breast cancer metastasis suppressor 1; CDH1 = cadherin 1, type 1; cfDNA = cell-free DNA; CMI = cumulative methylation index; CXCL12 = chemokine C-X-C motif ligand 12; DIC = ductal invasive carcinoma; ER = estrogen receptor; ERα = estrogen receptor alpha; HER2 = human epidermal growth factor receptor 2; IHC = immunohistochemical; IRS = immunoreactive score; Ki-67 = index of proliferative activity; LIC = lobular invasive carcinoma; LN = lymph node; LNM = lymph node metastasis; PBCs = periph-

From the Department of Genetics, Cancer Research Institute of SAS, Bratislava, Slovak Republic; Faculty of Medicine, Second Department of Oncology, Comenius University, National Cancer Institute, Bratislava, Slovak Republic; Faculty of Medicine, Institute of Pathological Anatomy, Comenius University, University Hospital, Bratislava, Slovak Republic; Department of Surgical Oncology, National Cancer Institute, Bratislava, Slovak Republic; Department of Plastic, Aesthetic and Reconstructive Surgery, University Hospital, Bratislava, Slovak Republic; Faculty of Medicine, Institute of Medical Biology, Genetics and Clinical Genetics, Comenius University, University Hospital, Bratislava, Slovak Republic; Pathological-Anatomical Workplace, Health Care Surveillance Authority, Bratislava, Slovak Republic.

Submitted for publication October 13, 2014; revision submitted December 17, 2014; accepted for publication December 29, 2014.

Reprint requests: Ivana Fridrichova, Department of Genetics, Cancer Research Institute of SAS, Vlarska 7, 833 91 Bratislava, Slovak Republic; e-mail: ivanafrid@gmail.com.

1931-5244/$ - see front matter

http://dx.doi.org/10.1016/j.trsl.2014.12.006

eral blood cells; PL = plasma; PPs = percentage of positive cells; PR = progesterone receptor; PR-B = B isoform of the progesterone receptor; PT = primary tumor; RASSF1A = A isoform of the RAS-association domain family 1; SD = standard deviation; SI = staining intensity; SOCS1 = suppressor of cytokine signaling 1; SYK = spleen tyrosine kinase; TIMP3 = tissue inhibitor of the metalloproteinases 3; TNM = TNM classification

AT A GLANCE COMMENTARY

Fridrichova I, et al.

Background

The aberrant methylation profiles in tumor tissues lead to the deregulation of transcription activities in many genes that strongly influence the cancer cell behavior including the invasivity and metastases forming. We investigated the relationship between DNA methylation levels in genes regulating cell growth, invasiveness, and metastasis and advanced breast cancers (BCs) and evaluated the clinical utility of the methylation profiles for detecting metastatic potential.

Translational Significance

The results of our study indicate that the methylation profiles of *RASSF1A*, *APC*, *CXCL12*, or *ADAM23* could be more informative in testing of patients with estrogen receptor positive BC, and the hypermethylation of *CXCL12* and *ADAM23* genes could be useful for the prediction of advanced stage of BC.

INTRODUCTION

Breast cancer (BC) is the most common malignancy in women and it represents 28.8% of all the female cancers diagnosed in 40 European states in the year 2012.[1] One of the primary causes of the high mortality is that more than 25% of patients with BC develop metastatic disease, and approximately 6% of the patients are diagnosed with metastatic disease at the time of the initial diagnosis.[2]

Metastasis development is a complex process that is defined by distinct steps involving the local invasion of cancer cells, their intravasation into adjacent vessels, transit through the circulatory system and evasion of the host immune system, extravasation into the parenchyma of distant organs, and the colonization and formation of micrometastases, followed by the proliferation and progression of macrometastases.[3]

The invasive behavior of cancer cells is associated with the presence of tumor DNA fragments in the peripheral blood of patients with cancer. In healthy human plasma (PL), the cell-free DNA (cfDNA) is derived from apoptotic cells with a primarily hematopoietic origin.[4,5] In patients with cancer, the apoptotic and necrotic cancer cells are the primary source of the tumor cfDNA.[6] Many studies have used cfDNA for the qualitative or quantitative evaluation of cancer-specific alterations, including changes in methylation profiles; however, the clinical utility of cfDNA has been critically re-evaluated because of the high methodical diversity and limited diagnostic sensitivity and specificity.[7]

Cancer cells intravasate into both blood and lymphatic vessels, but the hematogenous circulation is considered to be the major route for metastatic dissemination. To date, it is not clear, whether tumor cells actually metastasize from the lymph nodes (LNs) to the secondary organs, or whether the presence of tumor cells in the LNs only reflects their intrinsic invasiveness.[8] Regardless the molecular characters of lymph node metastasis (LNM) provide useful information for the development of more effective therapy.

Human cancer represents a heterogeneous group of diseases driven by progressive genetic and epigenetic alterations including the hyper- and hypomethylation of DNA and changed histone modifications that result in remodeling of the chromatin structure.[9] The aberrant methylation profiles in genes, which are responsible for specific processes in tumorigenesis, could be used as prognostic or predictive markers. Moreover, dynamic methylation changes during tumorigenesis modulate the presence of variable expression profiles in cancer cells that lead to different behaviors including sensitivity to therapy.

In our study, we were focused on the DNA methylation changes in genes that regulate cell growth and act in the inhibition of invasivity and metastasis processes. All evaluated genes have the cytosine-phosphate-guanine (CpG) islands in their promoter sequences; therefore, their expression could be epigenetically regulated. The multifunctional A isoform of the RAS-association domain family 1 (*RASSF1A*) and adenomatous polyposis coli (*APC*) genes belong to the group of highly methylated genes found in many types of cancers, including BC,[10] and their methylation levels increase from the preinvasive and early stage of

BC through tumorigenesis.[11,12] The tumor suppressor gene *RASSF1A* is functionally involved in apoptotic signaling, mitotic progression, and microtubule stabilization[10,13]; therefore, its inactivation deregulates these events. The APC is an antagonist of the Wnt signaling pathway and is involved in the transcriptional activation, apoptosis, adhesion, and migration of cells. In BC, inhibition of *APC* gene expression and increasing levels of β-catenin were observed,[14] predominantly through *APC* promoter methylation, which has been identified in 40% of patients with BC.[12] Variable frequencies of promoter methylation in BC were also observed in the *SOCS1* (suppressor of cytokine signaling) and *SYK* (spleen tyrosine kinase) genes.[15-18] The SOCS1 is a negative regulator of cytokine signaling that promotes the regulation of proliferation, differentiation, apoptosis, and immune surveillance in normal mammary epithelium, and epigenetic inactivation of the *SOCS1* gene in BC can cause the increase of epithelial proliferation and cell survival in response to cytokines and growth factors.[15] SYK is an intracellular receptor protein kinase involved in cell proliferation, differentiation, and phagocytosis. Its suppressive function in tumorigenesis and metastasis formation was documented in many clinical studies, where the correlation between reduced SYK expression and an increased risk for metastasis was found.[19]

Invasion, the first critical step in the metastatic process, requires changes in cell-cell adhesion and adhesion to the extracellular matrix. In tumors, many changes in the cadherin-catenin adhesion complexes were observed, including the cell adhesion protein, E-cadherin, encoded by the *CDH1* (cadherin 1, type 1) gene, which suppresses tumor cell invasion and metastasis. In primary BC, the heterogeneous loss of E-cadherin expression in correspondence with the heterogeneous pattern of promoter methylation was observed early, before cell invasion, and also in the non-neoplastic breast tissues of patients with BC.[20] The transmembrane glycoproteins from the disintegrin and metalloprotease (ADAM) family participate in invasion processes via the negative modulation of cell proliferation, adhesion, and migration through integrin regulation.[21] Epigenetic inactivation of the *ADAM23* gene through promoter hypermethylation and a decrease in the messenger RNA and relevant protein expression is correlated with more advanced BC.[22] The other important event in the invasivity of cancer cells is the failure of specific chemokines. Chemokine C-X-C motif ligand 12 (CXCL12) is constitutively expressed in various organs and tissues and together with the signal receptor CXCR4 regulates adhesion, proliferation, and the physiological migration of cells. In previous studies, a strong association between *CXCL12* hypermethylation and a histologically advanced disease, the presence of metastases, and death in patients with BC was found.[23,24] The tissue inhibitors of metalloproteinases (TIMPs) prevent the degradation of the extracellular matrix. TIMP3 is a matrix-binding protein that regulates the matrix composition and affects tumor growth, angiogenesis, invasion, and metastasis. Methylation of the *TIMP3* promoter was frequently observed in many human tumors including BC[25] and in ductal invasive carcinomas, it may be associated with high tumor grading and LNM.[26] Furthermore, the low expression levels of breast cancer metastasis suppressor 1 (BRMS1) have been correlated with the metastatic potential of human BC cell lines in nude mice.[27] Hypermethylation of the *BRMS1* promoter was frequently found in primary tumors (PTs) and matched LNMs of patients with BC.[28]

For more than 2 decades, the expression of estrogen receptor alpha (ERα), encoded by the *ESR1* gene, has been used to predict the benefit of endocrine therapy in patients with BC. ERα is a transcription factor that regulates the transcription of its target genes and mediates estrogen functions during the proliferation and differentiation of reproductive organs. Deregulation of estrogen signaling is associated with the initiation and progression of BC.[29] The longer B isoform of the progesterone receptor (PR-B), encoded by the *PGR B* gene, is also a transcription factor that regulates distinct target genes through complex interactions between PR-B and other regulatory factors; the understanding of these interactions could be critical for BC antiprogestin treatment.[30] In patients with BC, the inactivation of ERα and PR through methylation of both *ESR1* and *PGR* promoters was found[31,32]; however, the mean methylation levels were not higher than 2.2%.[33,34]

Many research teams have investigated the molecular mechanisms of invasivity and metastasis for the identification of more precise predictive markers and new therapeutic targets that aim to delay or fully halt the dissemination of the cancer. In tissues, epigenetic changes often arise before structural changes; therefore, the methylation profiles of specific genes could be useful for the identification of new biomarkers. The aim of the present study was to investigate the relationship between the DNA methylation levels of selected genes that are responsible for cell growth, invasivity and the regulation of metastasis, and the advancing degrees of sporadic BC to evaluate the clinical utility of the methylation profiles for monitoring metastatic potential.

Table I. Clinical characteristics and histopathologic features of patients with breast cancer

Clinicohistopathologic variables (N = 206)	N	(%)
Age (y)		
≤ 50	48	23.3
> 50	158	76.7
Tumor histology		
DIC	178	86.4
LIC	24	11.7
Others	4	1.9
Tumor size (mm)		
≤20	136	67.0
>20≤50	59	29.1
>50	8	3.9
Histologic grading		
1	22	10.9
2	105	52.0
3	75	37.1
LN status		
0	120	58.8
1–3	48	23.5
4–10	17	8.3
>10	19	9.3
Distant metastasis		
Negative	201	98.5
Positive	3	1.5
TNM classification		
I	98	47.8
II	66	32.2
III	38	18.5
IV	3	1.5
ER status		
Negative	27	14.8
Positive	155	85.2
PR status		
Negative	50	27.6
Positive	131	72.4
HER2 expression		
Negative	174	84.5
Positive	32	15.5
IHC subtypes		
ER+/PR+ HER2−	133	73.1
ER+/PR+ HER2+	25	13.7
ER−PR− HER2+	5	2.7
ER−PR− HER2−	19	10.4
Ki-67 proliferative index		
≤14% of cells	94	45.9
>14% of cells	111	54.1
Tumor multifocality		
Negative	183	89.3
Positive	22	10.7

Abbreviations: DIC, ductal invasive carcinoma; *ER*, estrogen receptor; *IHC*, immunohistochemical; *LIC*, lobular invasive carcinoma; *LN*, lymph node; *Others*, tubular or mucinous breast carcinoma; *PR*, progesterone receptor.
LN status was categorized according to the number of metastatic LNs. 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.

PATIENTS AND METHODS

Patient samples. In the present study, paraffin-embedded PT and LNM samples and the corresponding peripheral blood cells (PBCs) and PL samples from 206 nonfamilial patients with BC, PBC and PL samples from 56 healthy women, and normal breast tissues from the mammoplasties of 9 healthy women were collected between March 2012 and March 2014 at the National Cancer Institute in Bratislava. This research was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). The informed consent was obtained from patients and controls and the study has approved the author's institutional review board. The relevant clinical and histopathologic data were retrieved from the patients' clinical records, and tumors were characterized according to the PT, regional LNs, and distant metastasis (TNM) classification. At the time of the initial BC diagnosis the age of the women ranged from 28 to 83 years with a mean of 59.30 ± 11.05. Typing was performed according to the current World Health Organization classification for breast neoplasms (Table I). No preoperative radiotherapy or chemotherapy had been performed in any of the cases. The control individuals for the blood sample collection were aged between 29 to 85 years (mean 56.54 ± 12.21). The age of the healthy breast tissue donors ranged from 31 to 56 years. These individuals showed no signs or symptoms of cancer or other serious diseases.

DNA extraction and sodium bisulfite modification. The PL from the blood samples was processed and stored as previously described.[35] The cfDNA from the PL was isolated using a QIAamp DSP Virus Kit (Qiagen, Hilden, Germany), and the PBC DNA was obtained using a FlexiGene DNA Kit (Qiagen). For the DNA isolation from the paraffin-embedded PTs (>80% cancer cells), LNMs, and healthy breast tissue samples, the representative samples were selected by a pathologist, 3-mm-diameter cores from the blocked region were cut and the DNA was isolated using the MagneSil Genomic, Fixed Tissue System (Promega, Madison, Wisconsin). All the kits were used according to the manufacturer's instructions. The DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo

According to ER, PR, and HER2 expression, 4 IHC subtypes were recognized, luminal A and B (any ER+ or PR+ HER− and any ER+ or PR+ HER2+), HER2 overexpression positive (ER− PR− HER2+), and triple negative (ER− PR− HER2−). Ki-67 proliferative index was considered as low and high according to the number of stained cancer cell with cutoff 14%.

Fisher Scientific, Bremen, Germany). The DNA samples were modified via a sodium bisulfite treatment procedure, where the unmethylated cytosines were converted into uracils and the 5-methylcytosines remain unaltered. For the PBC DNA (1 μg), the CpGenome DNA Modification Kit (Chemicon, Billerica, Massachusetts) was used. For the cfDNA (2 μg) and paraffin-embedded PTs, LNMs, and the healthy breast DNA (2 μg), the EpiTect Bisulfite Kit (Qiagen) was used. The aliquots of the modified DNAs were stored at -18°C until use.

Methylation analyses. The quantitative DNA methylation analyses of the bisulfite-modified DNA samples were performed using pyrosequencing in 11 genes (*APC*, *ADAM23*, *CXCL12*, *ESR1*, *PGR B*, *CDH1*, *RASSF1A*, *SYK*, *TIMP3*, *BRMS1*, and *SOCS1*). Between 5 and 8 CpG sites located in the CpG islands of the promoter region flanking the transcription start site were evaluated in each gene. The primer sequences and the polymerase chain reaction conditions used were previously reported.[35] The pyrosequencing analyses were performed using a PyroMark Q24 System (Qiagen), and the results were evaluated using the PyroMark Q24 2.0.6. software (Qiagen). The methylation data are presented as the percentage of the average methylation in all the CpG sites in each gene. The analyzed sample was considered to be hypermethylated when the average methylation for the individual gene was higher than the cutoff value for the identical gene, which was defined as the mean methylation level determined in the normal blood cells, PL, or healthy mammary glands plus 2 standard deviations.[36,37] The cumulative methylation index (CMI) was calculated as the sum of the methylation percentage for all the 11 evaluated genes.

Immunohistochemistry. The protein expressions in the healthy and malignant breast tissues and the LNM were detected via semiquantitative immunohistochemical (IHC) analyses of tissue microarrays using specific antibodies against the 11 corresponding proteins. The primary antibody specification and the laboratory procedures used were previously described.[35] The results are expressed as an immunoreactive score (IRS), also known as the German IRS, which evaluates both the percentage of positive cells and the staining intensity of the nuclei or the cytoplasm.[38] The staining intensity and percentage of positive cells were multiplied, giving 4 IRS categories: negative, weak, moderate, and high protein expression.

Statistical analyses. The normality assumption hypothesis was tested using the Kolmogorov-Smirnoff test. Statistical comparisons between the groups were carried out in accordance with the normality test using Student's t test or analysis of variance with Bonferroni's or Tamhane's tests for multiple comparisons. The nonparametric Mann-Whitney U or Kruskal-Wallis H tests were used for non-normally distributed data. The associations between the categorical variables were analyzed using a chi-squared test in contingency tables. For variables with normal distributions, the Pearson's coefficient was calculated, and for the non-normally distributed variables, Spearman's correlation coefficient was calculated. The association of the hypermethylation in the evaluated genes with the clinicohistopathologic features was evaluated using an analysis of covariance, with age as the covariate. The presence of age-related methylation in the peripheral blood was tested using the same approach. A logistic regression adjusted for age was used to determine the effect of the independent categorical variables on the advancing degree of BC, which was measured by the presence of LNM, the Ki-67 proliferation rate, and the tumor size. This determination included the computation of the risk estimate. A P value <0.05 was considered to indicate statistical significance. The statistical computations were performed using the SPSS 17.0 software programme (SPSS Inc, Chicago, Illinois).

RESULTS

DNA methylation in different types of samples in patients with BC. In the present study, the quantitative analyses of the DNA methylation levels were performed in the paraffin-embedded PT and LNM tissues, the PBCs, and the PL samples from 206 patients with invasive BC. The highest methylation levels were found in the PT samples in the *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* genes, with means of 38.98%, 24.84%, 12.04%, and 10.01%, respectively. In the same genes, similar methylation levels were observed in the LNM samples (Table II), with a positive correlation between the PT and LNM samples ($r = 0.426$, $P = 0.001$; $r = 0.755$, $P < 0.001$; $r = 0.291$, $P = 0.028$; and $r = 0.423$, $P = 0.001$), but no correlations were found between the PL and PT samples in any of analyzed genes. On the contrary, significant differences between the 3 types of patient samples, with increasing values from PBC to PL to PT, were observed in most of the evaluated genes (Table II). DNA methylation levels that exceeded the cutoff values (Table III) were found in 156, 144, 104, and 52 of the PT samples and 37, 33, 30, and 13 of the LNM samples for the *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* genes, respectively. Moreover, a hypermethylation value in at least 1 of the 4 aforementioned genes was observed in the PT samples from 190 of the 206 patients with BC (92.2%). However, only 3, 12, 7, and 5 patients

Table II. The mean DNA methylation levels and the significant differences between different types of samples in 206 patients with breast cancer

	DNA methylation levels				Significant differences					
	Mean ± SD (in %)				*P* value					
Genes	PBCs	PL	PT	LNM	PBC vs PL	PBC vs PT	PBC vs LNM	PL vs PT	PL vs LNM	PT vs LNM
APC	2.12 ± 1.04	4.60 ± 6.14	24.84 ± 22.28	22.14 ± 22.40	<0.001	<0.001	<0.001	<0.001	<0.001	0.208
ADAM23	2.64 ± 1.42	3.57 ± 3.89	10.01 ± 11.88	9.26 ± 11.50	<0.001	<0.001	<0.001	<0.001	<0.001	0.895
CXCL12	2.83 ± 1.46	3.60 ± 4.37	12.04 ± 11.96	11.30 ± 10.49	0.015	<0.001	<0.001	<0.001	<0.001	0.918
ESR1	4.40 ± 1.31	4.55 ± 3.30	8.92 ± 3.77	8.09 ± 4.37	0.054	<0.001	<0.001	<0.001	<0.001	0.016
PGR B	5.04 ± 1.62	3.89 ± 3.28	7.20 ± 6.22	7.21 ± 4.34	<0.001	<0.001	0.001	<0.001	<0.001	0.453
CDH1	9.69 ± 1.93	7.82 ± 5.08	7.47 ± 2.96	10.20 ± 5.19	<0.001	<0.001	0.866	0.407	<0.001	<0.001
RASSF1A	1.09 ± 0.39	3.48 ± 3.70	38.98 ± 24.90	32.39 ± 27.11	<0.001	<0.001	<0.001	<0.001	<0.001	0.076
SYK	1.65 ± 0.70	2.62 ± 2.27	5.79 ± 3.90	5.40 ± 2.99	<0.001	<0.001	<0.001	<0.001	<0.001	0.465
TIMP3	2.80 ± 1.68	3.15 ± 3.41	5.67 ± 6.54	7.40 ± 8.93	0.057	<0.001	<0.001	<0.001	0.018	0.161
BRMS1	1.75 ± 1.06	2.55 ± 2.19	3.15 ± 1.34	3.29 ± 2.04	<0.001	<0.001	<0.001	<0.001	0.001	0.957
SOCS1	1.44 ± 0.78	2.22 ± 2.24	4.85 ± 3.00	4.61 ± 3.10	0.002	<0.001	<0.001	<0.001	<0.001	0.362

Abbreviations: LNM, lymph node metastasis; *PBC*, peripheral blood cells; *PL*, plasma; *PT*, primary tumor.

Table III. The DNA methylation levels in the peripheral blood (N = 56) and breast tissue (N = 9) samples of healthy women, and the cutoff values for evaluation of the hypermethylation status in breast cancer

	DNA methylation levels in the controls mean ± standard deviation (in %)			Hypermethylation cutoff values (in %)*		
Genes	Peripheral blood cells	Plasma	Mammary glands	Peripheral blood cells	Plasma	Mammary glands
APC	1.30 ± 0.57	3.84 ± 4.59	5.33 ± 1.23	2.44	13.02	7.79
ADAM23	2.14 ± 0.40	3.04 ± 3.50	4.89 ± 2.32	2.94	10.04	9.53
CXCL12	2.34 ± 0.51	4.05 ± 4.68	4.33 ± 1.73	3.36	13.41	7.79
ESR1	3.32 ± 0.90	4.88 ± 4.20	10.11 ± 2.71	5.12	13.28	15.53
PGR B	4.45 ± 1.24	4.31 ± 5.55	5.11 ± 2.42	6.93	15.41	9.95
CDH1	9.39 ± 1.68	7.71 ± 3.64	7.56 ± 2.96	12.75	14.99	13.48
RASSF1A	1.00 ± 0.27	3.48 ± 5.68	5.33 ± 3.04	1.54	14.84	11.41
SYK	1.18 ± 0.39	2.29 ± 2.23	5.22 ± 1.39	1.96	6.75	8.00
TIMP3	2.91 ± 1.34	3.61 ± 4.35	5.13 ± 0.99	5.59	12.31	7.11
BRMS1	1.30 ± 0.66	2.65 ± 1.43	3.67 ± 2.29	2.62	5.51	8.25
SOCS1	1.04 ± 0.38	2.18 ± 1.89	4.67 ± 2.18	1.80	5.96	9.03

*The hypermethylation cutoff values were counted as the sum of the mean methylation levels plus 2 standard deviations in the identical gene from the control DNA samples.

showed hypermethylation in the *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* genes, respectively, in the PL samples. Simultaneous hypermethylation in the PT and PL samples were only rarely observed in 1, 9, 4, and 4 patients for the *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* genes, respectively. The DNA methylation levels in the PBCs, PL, PTs, and LNMs of the patients for the *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* genes are depicted in Fig 1.

To determine level of DNA methylation in normal blood cells and breast tissues, the PBCs from 56 healthy women and 9 normal mammary glands were analyzed. On comparison of the PBC methylation levels in the patients and controls, significant differences were found in 8 genes (*APC*, *ADAM23*, *CXCL12*, *ESR1*, *PGR B*, *SYK*, *BRMS1*, and *SOCS1* with *P* values <0.001, 0.001, 0.005, 0.001, 0.002, <0.001, 0.001, and <0.001, respectively); however, none of the genes had mean methylation levels that exceeded the cutoff value for hypermethylation in the PBCs (Tables II and III).

In the PL of the patients and controls, only the *ADAM23* gene showed a significant difference in the mean methylation ($P = 0.016$), and the mean methylation level was lower than the cutoff value for PL (Tables II and III). On comparison of the normal mammary glands with the PT and LNM tissues, the *APC*, *CXCL12*, and *RASSF1A* genes showed statistically significant differences in their mean methylation levels between the normal and cancerous tissues ($P = 0.001$, $P = 0.002$, and $P = 0.001$, respectively) and between the normal tissues and the LNM samples ($P = 0.012$, $P = 0.008$, and $P = 0.000$, respectively).

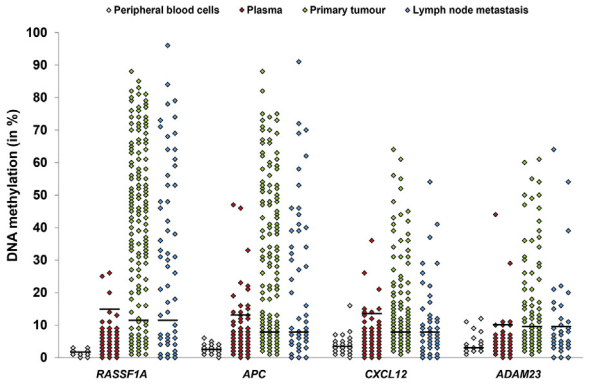

Fig 1. The DNA methylation levels of 4 hypermethylated genes in different types of samples from 206 patients with breast cancer. The hypermethylation cutoff values are denoted by black lines.

We also tested the potential effect of age on the level of methylation. There was a significant association between age and increasing methylation levels in the PBCs of the controls (*ADAM23*, *CXCL12*, *PGR B*, *CDH1*, and *TIMP3*) and the patients (*ESR1*, *PGR B*, *CDH1*, and *TIMP3*) but not in the normal and cancerous breast tissues.

In 57 patients who had available LN samples, the CMIs of the 11 genes increased from the mean value 35.00 ± 7.25 in the PBCs to 43.16 ± 12.23 and 140.7 ± 56.71 in the PL and PT samples, respectively. In the LNM samples, a CMI value of 119.04 ± 46.55 was found. The cumulative methylation results of the PT and LNM samples manifested a very similar spectrum of methylated genes, with the highest levels at 313 and 268, respectively. In both the PT and LNM samples, the *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* genes were highly methylated in the CMI profiles, which was contrary to the results observed in the PBCs. The cfDNA from the PL manifested as a combined profile of methylated genes that was observed in the PBCs and PTs of the patients (Fig 2).

DNA methylation and protein expression. IHC analyses of the expression of the 11 proteins were performed in the tissue microarray sections from the patients and controls to investigate the effect of the identified methylation profiles on both gene silencing and the subsequent protein expression. No associations were observed between the methylation levels and the related protein expression in both the PT and LNM samples, with the exception of the *PGR B* gene in the LNM samples ($P = 0.034$) (Fig 3). However, the *PGR B* methylation levels were lower than the hypermethylation cutoff value that was determined in the normal mammary glands. In the PT samples, an increasing trend in the mean methylation levels, from 17.25% to 24.21% and from 9.44% to 14.29%, was observed in the *APC* and *CXCL12* genes, respectively, in association with decreasing protein expression (from high to negative); however, neither were significant (Fig 3). An example of the differential expression of the ADAM23 protein in relation to the methylation levels of *ADAM23* gene in the PT samples is depicted in Fig 4.

DNA methylation and clinicohistopathologic features. In the 206 patients with BC, the association between the clinical features and the histopathologic characteristics of the PT and DNA methylation levels in both the PT and LNM tissues was investigated for 4 genes with mean DNA methylation levels that exceeded the cutoff values (Table IV). In the *RASSF1A* gene, significant differences in the methylation levels were identified in the estrogen receptor (ER) status and triple-negative BC phenotype in the PT samples and in the human epidermal growth factor receptor 2 (HER2) overexpression, the LNM categories and the PT size in the LNM tissues. Significant differences in the *APC* methylation levels were observed in the IHC subtypes and the ER, TNM, and Ki-67 categories in the PT samples and according to the histologic type in the LNM tissues. The *CXCL12* methylation levels differed significantly according to the ER and PR status, as well as the LNM status, in the LNM samples. Finally, the methylation levels of the *ADAM23* gene in the PT samples were significantly different in the different categories depending on the ER and HER2 status and the Ki-67 proliferative index. In the LNM tissues, a significant difference was observed in the *ADAM23* methylation levels in groups of patients with different PT histologic grades and HER2 and PR status. The mean methylation levels in the various clinicopathologic categories and the corresponding *P* values are summarized in Table IV.

A logistic regression adjusted for age was used to determine the effect of the independent categorical variables on the advancing degree of BC, as measured by the presence of LNM, the Ki-67 proliferation rate, and the PT size. Several variables in the PT contributed to the risk of LNM development, such as a higher T stage, high proliferation, ER positivity, and hypermethylation of the *CXCL12* gene. The presence of hypermethylation increased the risk for LNM by 2.1-fold. Histologic grade 3 and ER negativity increased the risk of having BC with a high Ki-67 proliferation ratio. Moreover, hypermethylation in the *ADAM23* and *PGR B* genes indicate a 5.2- and 2.7-fold greater risk for high tumor cell proliferation, respectively. On the basis of the results of the tested models, an increased tumor size (>20 mm) is associated with ER negativity, HER2 overexpression, and lobular histology but not with hypermethylation in the analyzed genes. The results of

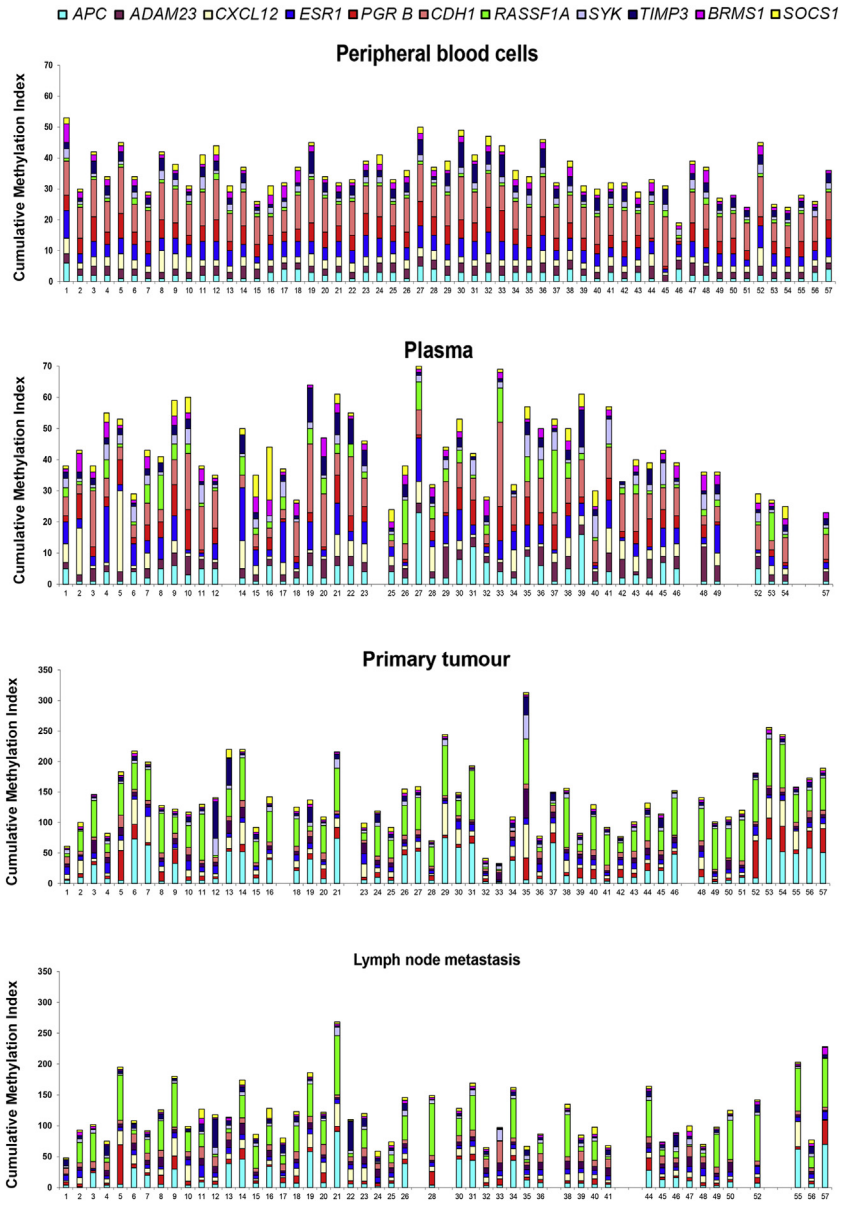

Fig 2. The cumulative DNA methylation levels in different types of samples from the patients with breast cancer with lymph node metastasis. The patient order in each of the 4 graphs is the same, to simplify the evaluation of each individual case. Only the cumulative methylation results that were counted from all 11 genes (*APC*, *ADAM23*, *CXCL12*, *ESR1*, *PGR B*, *CDH1*, *RASSF1A*, *SYK*, *TIMP3*, *BRMS1*, and *SOCS1*) are depicted in the graphs. Patients with incomplete results were excluded from the analyses (*empty columns*).

the logistic regression analyses are summarized in Table V.

DISCUSSION

Metastatic cancer causes serious medical problems, because this disease state is mostly incurable and there are only palliative therapeutic options available. For this reason, the prediction of the metastatic potential of a tumor is necessary in newly diagnosed patients for proper therapy management and subsequent medical care.

In the present study, increased methylation levels in 10 evaluated genes (*APC*, *ADAM23*, *CXCL12*, *ESR1*, *PGR B*, *RASSF1A*, *SYK*, *TIMP3*, *BRMS1*, and *SOCS1*) were observed in the PT samples when compared with the PBCs of patients with BC. In our analyses, the physiological DNA methylation levels in the relevant samples (PBCs, PL, and mammary glands) from healthy women were considered in the evaluation of the methylation results. Then, the "pathologic" DNA methylation levels were determined to be greater than the cutoff that

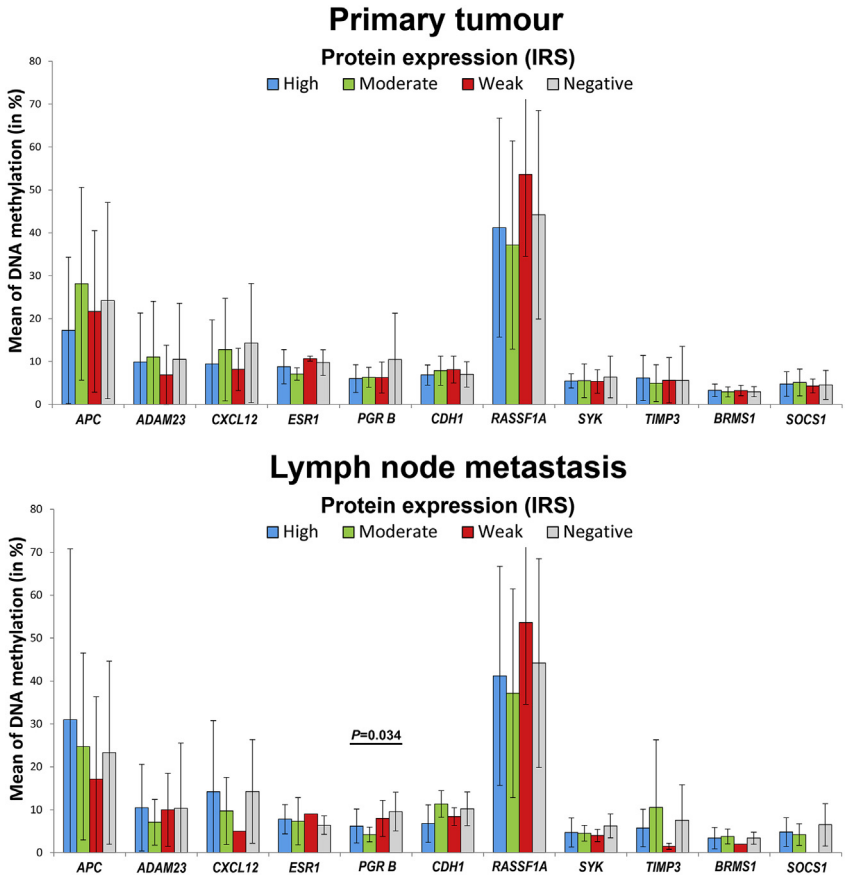

Fig 3. The DNA methylation levels for 4 categories of protein expression in 206 primary tumors and 57 lymph node metastases. For the semiquantitative evaluation of the protein expression, the immunoreactive score (IRS) was used, which involves the staining intensity (SI) and the percentage of positive cells (PP). The SI was subdivided into 4 categories (0 = negative, 1 = weakly positive, 2 = moderately positive, and 3 = strongly positive). The PP was graded as follows: 0 = negative, 1 = up to 10% positive cells, 2 = from 11% to 50% positive cells, 3 = from 51% to 80% positive cells, and 4 = more than 80% positive cells. The SI and PP were multiplied, producing 4 IRS categories: negative (0), weak (1–3), moderate (4–6), and high (8–12) protein expression.[38] The *P* value is for the analysis of variance; this association did not persist following Bonferroni correction.

was represented by the mean methylation level in the relevant healthy samples plus 2 standard deviations. These significantly increased methylation levels in the cancer-associated genes could influence the gene expression and the overall stability of the protein complexes and lead to the subsequent downregulation of cell proliferation and suppression of tumorigenesis. Using the more precise criterion of a cutoff value, the hypermethylation status was identified in the *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* genes in both the PT and LNM samples (with the exception of *ADAM23*, which was close to the cutoff in the LNM samples), but no hypermethylation was observed in the PBC and PL samples from the patients with BC. In our group of patients, *RASSF1A* and *APC* hypermethylation was identified in 75.5% and 69.9% of the patients, respectively. In *RASSF1A* gene, we previously found the frequency from 71.9% to 82.6%[35,39,40] and similar results were observed in other studies, with the frequencies of *RASSF1A* methylation at 82.5% and 68% and of *APC* at 52.5%.[41,42] When compared with our results, the frequencies of the methylated BC samples from other studies were different for the *CXCL12* gene (25.2% vs 14.5% and 52.4%, respectively),[23,24] and for *ADAM23* (50.4% vs 69.2%),[22] but these authors only used qualitative methods for their methylation analyses.

Regardless of the fact that PL is a readily available material for molecular analyses and many studies use cfDNA for the qualitative and quantitative evaluation of cancer-specific alterations, such as DNA strand integrity, mutations, changes in microsatellites, and methylation,[7] our present results did not show any correlations between the PL and PT samples, similarly as in our

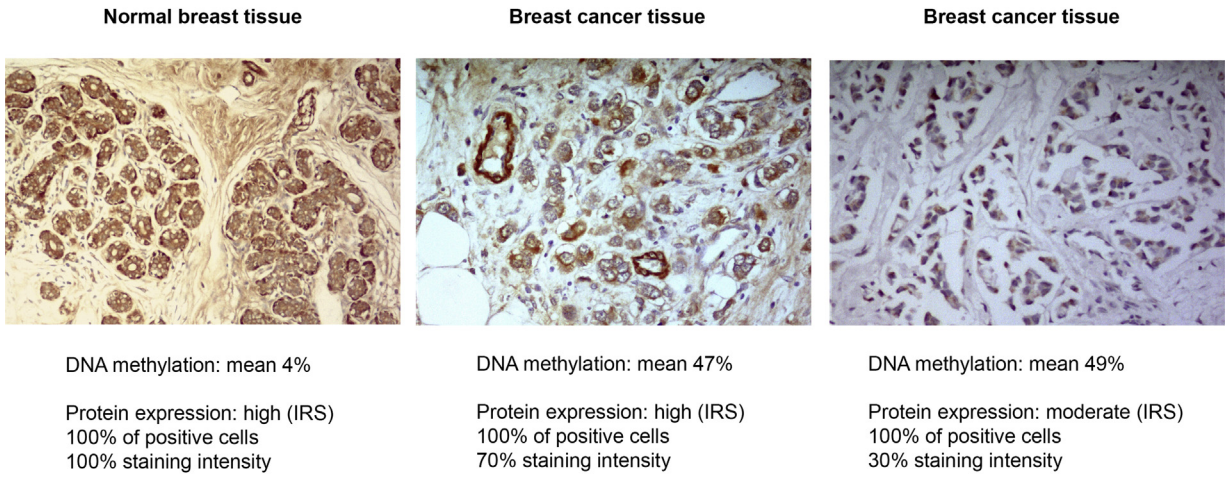

Fig 4. The differential expression patterns of the ADAM23 protein in normal breast tissue and 2 breast primary tumors that had similar methylation levels in the *ADAM23* gene. *IRS*, immunoreactive score.

previous study.[35] Moreover, the different methylation profiles were found between PL and PT samples that we observed previously in other group of patients with BC.[40] In another study, a relatively consistent variation in the methylation patterns of the PL samples among the controls and the BC patients with and without LNM was observed. However, the modest differences between the cases and controls were not suitable for clinical utility.[43] Additionally, the cancer-specific cfDNA concentration is normally very low, and there is a high portion of genomic DNA that originates from the physiological degradation processes in the PL samples, which was documented in our study via the cumulative methylation profiles in the PL; therefore, the cancer specificity of the methylation analyses could be substantially underestimated.

Several studies have shown that during breast tumor progression there is a change in the ER, PR, and HER2 status. The receptor is normally conversed from ER+/PR+ to ER−/PR− but less frequently vice versa; however, the HER2 conversion occurred equally in both directions between the PTs and the relapsed lesions or the non-bone distant BC metastases.[44,45] The altered hormonal receptors and HER2 status in metastatic BC could lead to inappropriate therapy if it is managed according to the IHC results determined via the PT. In our study, we hypothesized that the ER and PR expressions could be epigenetically regulated; therefore, we introduced the *ESR1* and *PGR B* genes in the methylation analyses and found the means to be 8.92% and 7.2% in the PTs, respectively. However, no association was observed between the increasing methylation levels in the *ESR1* and *PGR B* genes and the decreasing relevant protein expression in the PT samples. In the LNM samples, significant differences were observed in the *PGR B* methylation levels between all the evaluated protein groups; however, the mean methylation level of *PGR B* was lower than the hypermethylation cutoff. Very low *ESR1* and *PGR B* methylation levels were shown in previous studies, and the means varied from 0% to 2.2% and 2% in sporadic BC, respectively.[33,34,37,46] Therefore, we do not presume that DNA methylation plays a crucial role in the gene silencing of these hormonal receptors in association with breast tumorigenesis. However, some of the epigenetic events in estrogen signaling cannot be excluded because in ER-targeted gene expression, the complex corecruitment of coactivators and corepressors with ER was observed, including DNA methyltransferases, histone acetyltransferases, histone deacetylases, histone methyltransferases, and polycomb proteins.[47] PR also interacts with other regulatory factors, which frequently lead to post-translational modifications that can dramatically change its function in normal and malignant breast tissues.[30] The roles of ER and PR in the regulation of DNA methylation processes support the results of a previously published report, where different methylation profiles (93 hypermethylated and 55 hypomethylated genes) in ER+/PR+ and ER−/PR− BC were observed.[48] In our study, the significantly lower methylation levels identified in the *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* genes were observed in the patients with ER− or ER−PR−HER2− BC. These findings indicate a hormonal regulation of the DNA methylation process, which is visible in the hypermethylated genes and in the functional relationship between the individual genes and the hormone receptors, as was previously observed in an in vitro study showing that *RASSF1A* regulated ER expression in an estrogen-independent manner.[49] This

Table IV. The mean DNA methylation levels (in %) in the PTs and LNM samples in different categories, based on the clinical features and histopathologic characteristics of the PTs

	PTs (N = 206)				LNM (N = 57)			
Genes	Clinicopathologic category		DNA methyl. (mean)	*P** value	Clinicopathologic category		DNA methyl. (mean)	*P** value
RASSF1A	ER status	Negative	22.70	<0.001	HER2	Negative	27.12	0.004
		Positive	41.88			Positive	45.88	
	IHC subtype	ER+/PR+ HER2−	41.17	0.003†	LNM status	1–3	20.37	0.018
		ER+/PR+ HER2+	43.60			4–10	38.42	
		ER−PR− HER2+	33.20			>10	46.39	
		ER−PR− HER2−	19.68		PT size	≤20 mm	25.66	0.021
						>20≤50 mm	39.83	
						>50 mm	36.50	
APC	ER status	Negative	10.93	0.005	Histologic type	DIC	18.25	0.017
		Positive	27.85			LIC	42.89	
	IHC subtype	ER+/PR+ HER2−	27.41	0.007†				
		ER+/PR+ HER2+	27.48					
		ER−PR− HER2+	23.40					
		ER−PR− HER2−	8.53					
	TNM	I and II	23.13	0.022				
		III and IV	31.38					
	Ki-67	≤14% of cells	21.44	0.018				
		>14% of cells	27.43					
CXCL12	ER status	Negative	11.27	0.022	LNM status	1–3	9.04	0.004
		Positive	12.39			4–10	20.00	
	PR status	Negative	14.88	0.004		>10	8.89	
		Positive	11.22					
ADAM23	ER status	Negative	6.37	0.001	Histologic grade	1 and 2	6.71	<0.001
		Positive	11.25			3	12.64	
	HER2	Negative	8.76	0.001	HER2	Negative	7.02	
		Positive	16.81			Positive	15.00	<0.001
	Ki-67	≤14% of cells	7.19	0.008	PR status	Negative	15.00	<0.001
		>14% of cells	12.24			Positive	7.81	

Abbreviations: DIC, ductal invasive carcinoma; *ER*, estrogen receptor; *HER2*, HER2 overexpression; *IHC*, immunohistochemical; *Ki-67*, Ki-67 proliferation index; *LIC*, lobular invasive carcinoma; *LNM*, lymph node metastasis (classified according to the number of metastatic lymph nodes); *PR*, progesterone receptor; *PT*, primary tumor; *TNM*, TNM classification.
*Analysis of covariance adjusted for age.
†$P < 0.01$ for the triple-negative phenotype vs the other IHC categories after adjusting for multiple comparisons.

association was supported by our finding of positive correlation between *RASSF1A* methylation levels and the percentage of cancer cells expressing ER and PR in BC samples.[40]

During the progression of BC, many combinations of genetic and epigenetic changes occur, resulting in mutations or altered gene expression. The functions of the subsequent protein products could be influenced not only by changes in the protein structure but also by abnormal interactions between partners in active protein complexes. Concerning the regulation complexity, we expected to find link between the DNA methylation levels and inhibition of the protein expression of *RASSF1A*, *APC*, *CXCL12*, and *ADAM23*. On the basis of our results, we deduced that the detected cancer-specific DNA methylation profiles contribute to destabilization of the regulation processes in breast tumorigenesis, which could have prognostic value.

Previous studies have shown that the DNA methylation of several genes, including *RASSF1A* and *APC*, accumulate during tumorigenesis; this was identified in preinvasive breast lesions and significantly increased from hyperplasia to ductal carcinoma in situ and invasive ductal carcinoma. The results also indicate that methylation in the evaluated genes is an early epigenetic event in BC.[11,37,50] The metastatic potential of cancerous cells that are distributed throughout lymphatic vessels could be evaluated by comparing the molecular characteristics in LNMs and PTs. In recent studies, the simultaneous presence of *RASSF1A* and *APC* methylation from PTs to LNMs was detected.[36,51] In 57 patients, we observed very similar DNA methylation levels in all 4 hypermethylated genes (*RASSF1A*, *APC*, *CXCL12*, and *ADAM23*) in both the LNM and PT tissues. Taking into account the tumor heterogeneity, we hypothesize that there are

Table V. The risk estimation of the selected risk factors for advancing tumorigenesis in breast cancer (logistic regression adjusted for age)

Risk factor	Variables	Risk value	*P* value	Odds ratio	95% Confidence interval
Lymph nodes metastases*	ER status	≥1% of positive cells	0.010	4.41	1.42–13.72
	ADAM23 DNA methylation	>9.53%	0.184	0.56	0.24–1.31
	CXCL12 DNA methylation	>7.79%	0.039	2.10	1.04–4.24
	Ki-67	>14% of cells	0.016	2.63	1.20–5.74
	T stage	T2 and higher	<0.001	4.43	2.11–9.31
	ESR1 DNA methylation	>15.53	0.111	4.61	0.71–30.05
Ki-67 proliferation rate†	Histologic grade	3	0.003	3.15	1.48–6.68
	ER status	≥1% of positive cells	0.001	0.10	0.03–0.38
	ADAM23 DNA methylation	>9.53%	<0.001	5.23	2.27–12.05
	PGR B DNA methylation	>9.95%	0.050	2.69	1.00–7.24
PT size‡	Histologic grade	3	0.059	3.97	0.95–16.62
	ER status	≥1% of positive cells	0.006	0.26	0.10–0.68
	HER2	Overexpression	0.021	2.85	1.17–6.95
	Histologic type	LIC	0.002	5.08	1.79–14.41

Abbreviations: ER, estrogen receptor; *HER2,* HER2 overexpression; *Ki-67,* Ki-67 proliferation index; *LIC,* lobular invasive cancer; *PT,* primary tumor; *TNM,* TNM classification.
*−2 Log likelihood = 194.84; R^2(Cox and Snell) = 0.17; R^2(Nagelkerke) = 0.23.
†−2 Log likelihood = 181.22; R^2(Cox and Snell) = 0.24; R^2(Nagelkerke) = 0.32.
‡−2 Log likelihood = 190.42; R^2(Cox and Snell) = 0.16; R^2(Nagelkerke) = 0.22.

partially degraded cell populations present in the circulation, originating from detached cancer cells that are not able to survive and form metastases but have different methylation profiles; their fragments could be represented by a portion of cfDNA in the PL. The surviving cancer cells could represent more aggressive populations that are resistant to apoptosis and are predisposed to metastasis. Therefore, this hypothesis could explain the discrepancies between the methylation profiles in the PL and PT samples and in concordance with the PT and LNM tissues in our study.

To evaluate the association of the clinicohistopathologic characteristics of the patients with BC in regards to the methylation levels in the 4 hypermethylated genes, we found significantly higher methylation levels in the ER-positive PT samples, which were also observed in *RASSF1A* and other frequently methylated genes.[36,37] These results indicate that the methylation levels of *RASSF1A*, *APC*, *CXCL12*, and *ADAM23* in ER-positive tumors can be more easily distinguished from the physiological methylation "background;" therefore, they could be more informative in testing of patients with ER-positive BC.

Recent epigenetic studies have been focused not only on the general investigation of cancer-specific changes but also on the methylation profiles of genes that are active in partial processes of tumor progression, such as the adhesion between cancer cells and with other cells and the extracellular matrix. The transmembrane glycoprotein ADAM23 negatively regulates integrins through the binding of the ADAM23 disintegrin domain with integrins, which prevents their activation.[21] Epigenetic silencing of the *ADAM23* gene allows integrin activation that could stimulate cancer cell migration. Moreover, activated integrins promote interactions between circulating tumor cells and thrombocytes to protect cancer cells against degradation in the vasculature.[52] Furthermore, another important group of substances that influence cancer cell invasivity are the chemokines, namely CXCL12. In cancer tissues, both *CXCL12*, which is secreted by the myoepithelial cells and myofibroblasts of the tumor stroma, and its receptor *CXCR4* are expressed in BC cells and are epigenetically regulated during the modulation of the metastatic potential in BC; *CXCL12* methylation and *CXCR4* overexpression without methylation were found in patients with the histologically advanced disease and was associated with a poor chance of survival.[23,53,54] Alternatively, CXCL12 was significantly overexpressed in the organs where BC frequently forms metastases.[55]

In our study, the increasing methylation levels that manifested in any of the hypermethylated genes were associated with a higher disease stage, increased PT size, increased Ki-67 proliferation, or an increased number of LNMs, which reflects the accumulation of epigenetic changes during tumor progression. However, on evaluation of the risk for advancing BC, we found that the risk for the presence of LNM is associated with *CXCL12* hypermethylation, and the risk for increased Ki-67 proliferation is influenced by *ADAM23* hypermethylation. These data confirm that epigenetic inhibition of the *CXCL12* and *ADAM23* genes results in the destruction of cell-cell and cell-extracellular matrix

adhesion and can stimulate the detachment and mobility of cancer cells in advanced BC.

CONCLUSIONS

Our results documented that *RASSF1A* and *APC* hypermethylation, which frequently develops in early BC, is maintained during breast tumorigenesis and indicates a key role for epigenetic inhibition of the *RASSF1A* and *APC* tumor suppressor genes in BC. On the contrary, *CXCL12* and *ADAM23* hypermethylation are associated with LNMs development and higher proliferation of cancer cells, respectively, that could be useful for the prediction of advanced stage of BC. The differences or coincidences in the cancer-specific methylation profiles between the cfDNA from the PL, PT, and LNM tissues could contribute not only to clarifying the investigated gene functions during tumorigenesis but also to determining the sample type, which reflects the molecular characteristics of the BC disease in real time, for more tailored management and therapy. To increase the clinical utility of these epigenetic markers, it is critical to obtain the available biological material in a noninvasive manner, as our expectations regarding the PL samples were not fulfilled. However, the growing research on circulating tumor cells could bring a promising solution to this problem.

ACKNOWLEDGMENTS

Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

This study was supported by the Slovak Research and Development Agency (APVV) grant No. APVV-0076-10; the Research and Development Operational Programme (ERDF), grant No. 26240220058; the Scientific Grant Agency (VEGA), grants No. 2/0120/13, No. 2/0169/14, and No. 1/0724/11; and the RFL2010 programme funded by the Slovak Cancer Research Foundation.

The authors would like to thank Gabriela Gasajova, Denisa Manasova, Ludovit Gaspar, Emilia Klincova, and Peter Rozsa for their excellent technical assistance and Jan Luha and Ladislava Wsolova for their assistance in the statistical analyses. We would also like to acknowledge our collaborators from the Department of Pathology: Gabriela Sieberova, Jan Macuch, Michal Majercik, and Peter Jani.

All authors declare that they have read the *Translational Research* journal's authorship agreement. The manuscript has been reviewed and approved by all named authors. NPG Language Editing supported the preparation of this manuscript by a native English speaker editing.

REFERENCES

1. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 2013;49:1374–403.
2. Barinoff J, Hils R, Bender A, et al. Clinicopathological differences between breast cancer in patients with primary metastatic disease and those without: a multicentre study. Eur J Cancer 2012;49:305–11.
3. Klein CA. Cancer. The metastasis cascade. Science 2008;321:1785–7.
4. Suzuki N, Kamataki A, Yamaki J, Homma Y. Characterization of circulating DNA in healthy human plasma. Clin Chim Acta 2008;387:55–8.
5. Ziegler A, Zangemeister-Wittke U, Stahel RA. Circulating DNA: a new diagnostic gold mine? Cancer Treat Rev 2002;28:255–71.
6. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001;61:1659–65.
7. Jung K, Fleischhacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker—a critical appraisal of the literature. Clin Chim Acta 2010;411:21–2.
8. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer 2009;9:239–52.
9. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
10. Dammann R, Schagdarsurengin U, Seidel C. The tumor suppressor RASSF1A in human carcinogenesis: an update. Histol Histopathol 2005;20:645–63.
11. Pasquali L, Bedeir A, Ringquist S, Styche A, Bhargava R, Trucco G. Quantification of CpG island methylation in progressive breast lesions from normal to invasive carcinoma. Cancer Lett 2007;257:136–44.
12. Van der Auwera I, Van Laere SJ, Van den Bosch SM, et al. Aberrant methylation of the adenomatous polyposis coli (APC) gene promoter is associated with the inflammatory breast cancer phenotype. Br J Cancer 2008;99:1735–42.
13. Dallol A, Agathanggelou A, Fenton SL, et al. RASSF1A interacts with microtubule-associated proteins and modulates microtubule dynamics. Cancer Res 2004;64:4112–6.
14. Jönsson M, Borg A, Nilbert M, Andersson T. Involvement of adenomatous polyposis coli (APC)/beta-catenin signalling in human breast cancer. Eur J Cancer 2000;36:242–8.
15. Sutherland KD, Lindeman GJ, Choong DY, et al. Differential hypermethylation of SOCS genes in ovarian and breast carcinomas. Oncogene 2004;23:7726–33.
16. Rottapel R, Ilangumaran S, Neale C, et al. The tumor suppressor activity of SOCS-1. Oncogene 2002;21:4351–62.
17. Yuan Y, Mendez R, Sahin A, Dai JL. Hypermethylation leads to silencing of the SYK gene in human breast cancer. Cancer Res 2001;61:5558–61.
18. Widschwendter M, Siegmund KD, Müller HM, et al. Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. Cancer Res 2004;64:3807–13.
19. Coopman PJ, Mueller SC. The Syk tyrosine kinase: a new negative regulator in tumor growth and progression. Cancer Lett 2006;241:159–73.
20. Zou D, Yoon HS, Perez D, Weeks RJ, Guilford P, Humar B. Epigenetic silencing in non-neoplastic epithelia identifies E-cadherin (CDH1) as a target for chemoprevention of lobular neoplasia. J Pathol 2009;218:265–72.

21. Verbisck NV, Costa ET, Costa FF, et al. ADAM23 negatively modulates alpha(v)beta(3) integrin activation during metastasis. Cancer Res 2009;69:5546–52.
22. Costa FF, Verbisck NV, Salim AC, et al. Epigenetic silencing of the adhesion molecule ADAM23 is highly frequent in breast tumors. Oncogene 2004;23:1481–8.
23. Ramos EA, Camargo AA, Braun K, et al. Simultaneous CXCL12 and ESR1 CpG island hypermethylation correlates with poor prognosis in sporadic breast cancer. BMC Cancer 2010;10:23.
24. Zhou W, Jiang Z, Liu N, et al. Down-regulation of CXCL12 mRNA expression by promoter hypermethylation and its association with metastatic progression in human breast carcinomas. J Cancer Res Clin Oncol 2009;135:91–102.
25. Bachman KE, Herman JG, Corn PG, et al. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. Cancer Res 1999;59:798–802.
26. Lui EL, Loo WT, Zhu L, Cheung MN, Chow LW. DNA hypermethylation of TIMP3 gene in invasive breast ductal carcinoma. Biomed Pharmacother 2005;59(Suppl 2):363–5.
27. Seraj MJ, Samant RS, Verderame MF, Welch DR. Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. Cancer Res 2000;60: 2764–9.
28. Metge BJ, Frost AR, King JA, et al. Epigenetic silencing contributes to the loss of BRMS1 expression in breast cancer. Clin Exp Metastasis 2008;25:753–63.
29. Keeton EK, Brown M. Coregulator expression and breast cancer: improving the predictive power of estrogen receptor alpha. Clin Cancer Res 2003;9:1229–30.
30. Hagan CR, Lange CA. Molecular determinants of context-dependent progesterone receptor action in breast cancer. BMC Med 2014;12:32.
31. Parrella P, Poeta ML, Gallo AP, et al. Nonrandom distribution of aberrant promoter methylation of cancer-related genes in sporadic breast tumors. Clin Cancer Res 2004;10:5349–54.
32. Lapidus RG, Ferguson AT, Ottaviano YL, et al. Methylation of estrogen and progesterone receptor gene 5′ CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin Cancer Res 1996;2:805–10.
33. Suijkerbuijk KP, Fackler MJ, Sukumar S, et al. Methylation is less abundant in BRCA1-associated compared with sporadic breast cancer. Ann Oncol 2008;19:1870–4.
34. Lee JS, Fackler MJ, Lee JH, et al. Basal-like breast cancer displays distinct patterns of promoter methylation. Cancer Biol Ther 2010;9:1017–24.
35. Zmetakova I, Danihel L, Smolkova B, et al. Evaluation of protein expression and DNA methylation profiles detected by pyrosequencing in invasive breast cancer. Neoplasma 2013;60: 635–46.
36. Feng W, Orlandi R, Zhao N, et al. Tumor suppressor genes are frequently methylated in lymph node metastases of breast cancers. BMC Cancer 2010;10:378.
37. Muggerud AA, Rønneberg JA, Wärnberg F, et al. Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma in situ and early invasive breast cancer. Breast Cancer Res 2010;12:R3.
38. Remmele W, Schicketanz KM. Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer. Computer-assisted image analysis (QIC score) vs. subjective grading (IRS). Pathol Res Pract 1993;189:862–6.
39. Sebova K, Zmetakova I, Bella V, et al. RASSF1A and CDH1 hypermethylation as potential epimarkers in breast cancer. Cancer Biomark 2011–2012;10:13–26.
40. Kajabova V, Smolkova B, Zmetakova I, et al. RASSF1A promoter methylation levels positively correlate with estrogen receptor expression in breast cancer patients. Transl Oncol 2013;6: 297–304.
41. Cho YH, Yazici H, Wu HC, et al. Aberrant promoter hypermethylation and genomic hypomethylation in tumor, adjacent normal tissues and blood from breast cancer patients. Anticancer Res 2010;30:2489–96.
42. Buhmeida A, Merdad A, Al-Maghrabi J, et al. RASSF1A methylation is predictive of poor prognosis in female breast cancer in a background of overall low methylation frequency. Anticancer Res 2011;31:2975–81.
43. Sturgeon SR, Balasubramanian R, Schairer C, Muss HB, Ziegler RG, Arcaro KF. Detection of promoter methylation of tumor suppressor genes in serum DNA of breast cancer cases and benign breast disease controls. Epigenetics 2012;7:1258–67.
44. Hoefnagel LD, van de Vijver MJ, van Slooten HJ, et al. Receptor conversion in distant breast cancer metastases. Breast Cancer Res 2010;12:R75.
45. Lindström LS, Karlsson E, Wilking UM, et al. Clinically used breast cancer markers such as estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 are unstable throughout tumor progression. J Clin Oncol 2012;30: 2601–8.
46. Feng W, Shen L, Wen S, et al. Correlation between CpG methylation profiles and hormone receptor status in breast cancers. Breast Cancer Res 2007;9:R57.
47. Hervouet E, Cartron PF, Jouvenot M, Delage-Mourroux R. Epigenetic regulation of estrogen signaling in breast cancer. Epigenetics 2013;8:237–45.
48. Li L, Lee KM, Han W, et al. Estrogen and progesterone receptor status affect genome-wide DNA methylation profile in breast cancer. Hum Mol Genet 2010;19:4273–7.
49. Thaler S, Schmidt M, Schad A, Sleeman JP. RASSF1A inhibits estrogen receptor alpha expression and estrogen-independent signalling: implications for breast cancer development. Oncogene 2012;31:4912–22.
50. Park SY, Kwon HJ, Lee HE, et al. Promoter CpG island hypermethylation during breast cancer progression. Virchows Arch 2011; 458:73–84.
51. Barekati Z, Radpour R, Lu Q, et al. Methylation signature of lymph node metastases in breast cancer patients. BMC Cancer 2012;12:244.
52. Felding-Habermann B, O'Toole TE, Smith JW, et al. Integrin activation controls metastasis in human breast cancer. Proc Natl Acad Sci U S A 2001;98:1853–8.
53. Allinen M, Beroukhim R, Cai L, et al. Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 2004;6:17–32.
54. Ramos EA, Grochoski M, Braun-Prado K, et al. Epigenetic changes of CXCR4 and its ligand CXCL12 as prognostic factors for sporadic breast cancer. PLoS One 2011;6:e29461.
55. Müller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature 2001;410:50–6.