Evaluation of protein expression and DNA methylation profiles detected by pyrosequencing in invasive breast cancer

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Breast carcinoma is the most common cancer with high mortality caused by metastatic disease. New molecular biomarkers predicting the tumour's metastatic potential would therefore improve metastasis prevention and personalised care. The aim of the study was to investigate the relationship between DNA methylation levels in invasivity and metastasising associated genes with aberrant protein expression and also to evaluate whether a similar DNA methylation level is present in the tumour and circulating cell-free DNA for utilising plasma DNA methylation as prognostic biomarker. By using pyrosequencing, we analysed DNA methylation levels of 11 genes, namely *APC, ADAM23, CXCL12, ESR1, PGR B, CDH1, RASSF1A, SYK, TIMP3, BRMS1* and *SOCS1* in tumour, plasma and peripheral blood cells from 34 patients with primary breast cancer, as well as plasma and peripheral blood cells from 50 healthy controls. Simultaneously, the expression of related proteins in paraffin-embedded tumour samples was evaluated by immunohistochemistry. Statistical analysis was performed by SPSS statistics 15.0 software. Tumour DNA hypermethylation was found in most commonly methylated *RASSF1A* (71.9%), *APC* (55.9%), *ADAM23* (38%) and *CXCL12* (34.4%) genes with methylation levels up to 86, 86, 53 and 64%, respectively. In tumours, significantly higher methylation levels were found in nine genes, compared with the patients´ peripheral blood cell DNA. Furthermore, in patients methylation levels in peripheral blood cell DNA were significantly higher than in controls in *CXCL12, ESR1* and *TIMP3* genes, but the values did not exceed 15%. On the other hand, no correlations were observed in patients between DNA methylation in tumours and cell-free plasma DNA. Moreover, in patients and controls nearly identical values of cumulative DNA methylation (43.6% ± 20.1 vs. 43.7% ± 15.0) were observed in plasma samples. A variable spectrum from high to none expressions presented in tumour tissues in all of the proteins evaluated, however in *APC* and *CXCL12* genes a visible decreasing trend of mean DNA methylation level with increasing expression of the corresponding protein was observed. The DNA methylation profiles manifested in our group of breast carcinomas are cancer specific, but they are not the only cause that affects the silencing of evaluated genes and the decrease of relevant protein products. The clinical utility of DNA methylation testing in peripheral blood cell DNA for cancer diagnosis and therapy need to be further investigated.

Key words: breast cancer, protein expression, DNA methylation, invasivity and metastasising associated genes, pyrosequencing analysis

Breast cancer (BC) is associated with a high mortality rate in women. Despite the fact that more targeted therapies have improved survival of patients with advanced stages; these tumours frequently relapse due to drug resistance mechanisms.

Thus a detailed study of DNA, RNA and protein alterations in mechanisms regulating the metastatic processes would improve metastasis prevention and personalised care [1]. It is generally accepted that in addition to genetic alterations, also

abnormal epigenetic events are responsible for the development and progression of cancer. DNA methylation along with histone modifications and miRNA regulation generates the epigenetic control mechanism of germline and tissue specific gene expression. The results of many studies have shown that aberrant DNA methylation initiates carcinogenesis and promotes cancer progression by activating oncogenes, suppressing tumour suppressor genes and inducing chromosome instability. DNA methylation as an early event in breast carcinogenesis has been frequently studied in tumour samples, breast fluids and blood derivatives with the aim to find reliable, non-invasive cancer biomarkers. Numbers of genes have been reported as being methylated in breast and other cancers, with focus on potential clinical application of this information in cancer diagnosis, prognosis, and therapeutics [2]. DNA methylation of *RASSF1A*, *RARbeta2* and *APC* genes in benign tissue was used for cancer risk prediction. The analysed genes had a higher methylation frequency in unaffected women at high risk for BC compared with those at low or intermediate risk [3]. Recent research has indicated that simultaneous assessment of multiple genes involved in different biological processes is more reasonable compared to single marker analysis. Several studies published panels of genes that in combination exhibited adequate sensitivity and specificity compared to single-marker analysis. Esteller and colleagues reported a sensitivity of 73% using a panel of four genes (*GSTP1*, *BRCA1*, *CDH1* and *P16*) based on 45 BC tumours [4]. Pyrosequencing analyses of DNA methylation in promoters of four genes (*RASSF1A*, *GSTP1*, *RARβ* and *CDH1*) was investigated in a spectrum of neoplastic breast lesions in breast tissue samples. DNA methylation increased from normal tissue to hyperplasia and a significantly higher increase was found in invasive and *in situ* tumours for *RASSF1A* gene [5]. High sensitivity (84%) and specificity (89%) for detection of BC was observed on using a panel of four genes (*CCND2*, *HIN1*, *RASSF1* and *TWIST*) that was tested in 9 normal mammoplasty and 19 tumour specimens [6].

Several studies showed that disrupted protein expressions were associated with deregulation of DNA methylation of various genes in breast, lung, endometrial and other cancers [7-10].

As the potential of circulating cell-free DNA (cfDNA) was broadly recognised as a biomarker for early detection, diagnosis, prevention and treatment of cancer, huge effort has been concentrated on analysis of DNA methylation in serum/plasma samples [11-16]. Although numerous biomarker candidates have been reported, only few methylation based biomarkers, including *SEPT9*, *VIM*, *SHOX2* and *MGMT,* have been adopted into clinical use for early detection of colorectal, lung and brain cancers [17]. Moreover, some systemic epigenetic changes were detected in peripheral blood cells in BC patients [18, 19]. A recent article by Li and collaborators provided a comprehensive literature review of blood-based DNA methylation. The authors concluded that DNA methylation in

peripheral blood of patients had a great potential to become useful as informative biomarkers of cancer risk and prognosis, yet large systematic and unbiased prospective studies considering biological plausibility and data analysis issues will be needed [20].

In our study, we evaluated a DNA methylation panel of eleven target genes responsible for self-sufficiency in growth signals (*APC*, *ESR1*, *PGR B, RASSF1A*, *SYK* and *SOCS1*) or for inhibition of cell invasion and metastasis formation (*ADAM23*, *CXCL12*, *CDH1*, *TIMP3* and *BRMS1*). Selection of the genes was based on their previous association with advanced breast cancer [21-26]. The DNA methylation status of the selected genes was analysed by pyrosequencing, which offers a quantitative sequence-based analysis of multiple CpG sites and provides a comprehensive picture of the distribution of DNA methylation throughout the promoter regions of the genes studied. The aim of the study was to investigate the relationship between DNA methylation of promoter sequences and protein expression and to evaluate whether in any of the selected genes or their combinations similar DNA methylation levels could be observed simultaneously in tumours and circulating cfDNA; thus DNA methylation in plasma samples could be utilised as cancer prognostic biomarker.

Patients and methods

Patient samples. In the current study paraffin-embedded tumour tissue samples and corresponding peripheral blood cells and plasma samples from 34 newly diagnosed non-familial BC patients and 50 control blood and plasma samples were obtained from four hospitals in Bratislava and Malacky, Slovakia. This study was approved by the ethical committee and all patients and controls signed a written informed consent permitting the use of their samples for research. The associated clinico-pathological data were retrieved from the patients' clinical records and they are summarized in Table 1. At the time of BC diagnosis, the age of the patients ranged from 41 to 90 years. In the study group 27 (79.4%) ductal, 4 (11.8%) lobular and 3 (8.8%) mucinous invasive breast carcinomas were included. Tumours were graded and staged according to the current WHO classification for breast neoplasms. The patients were diagnosed with stages I to IV with frequencies of 13 (38.2%), 14 (41.2%), 5 (14.7%) and 2 (5.9%). HER2 positivity was present in 5 (14.7%) and hormone receptor negativity in 13 (38.2%) patients. No neoadjuvant therapy or radiotherapy had been performed in any of the cases. The age of the controls ranged from 20 to 78 years. These persons had no signs and symptoms of cancer or other serious diseases.

DNA extraction and sodium bisulfite modification. Blood samples were obtained in EDTA-treated tubes and centrifuged at 1000 g for 10 min at room temperature within 2 h of venepuncture. To avoid cellular DNA contamination, supernatants were carefully collected and centrifuged again at 1000 g for 10 min at room temperature. Plasma samples

were kept at −70°C until further processing. DNA from paraffin-embedded tumour tissues was isolated by the MagneSil Genomic, Fixed Tissue System (Promega, Madison, WI), cfDNA from plasma samples was isolated using a QIAamp DSP Virus Kit (Qiagen, Hilden, Germany), and peripheral blood cell DNA was obtained using a FlexiGene DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For measurements of DNA concentration, a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany) was used. For the DNA modification procedure we performed sodium bisulfite treatment of peripheral blood cells DNA (1 μg), cfDNA (2 μg) and tumour DNA (2 μg) using an established protocol of the CpGenome DNA Modification Kit (Chemicon, Billerica, MA) and EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), respectively. The principle of sodium bisulfite modification is based on the conversion of unmethylated cytosines to uracils in CpG dinucleotides, while 5-methylcytosines remain unaltered. The aliquots of modified DNAs were stored at −18°C until use.

Pyrosequencing methylation analysis. Quantitative DNA methylation analysis of bisulfite-treated DNA was performed by pyrosequencing in 11 genes. For each gene, we selected the CpG islands in the promoter area flanking the transcription start site at 5'UTR. Five to eight CpG sites were studied for each particular CpG island. Oligonucleotides for PCR amplification and pyrosequencing were designed using PyroMark Assay Design software 2.0 (Qiagen, Hilden, Germany) and sequences are summarized in Table 2. For genes *RASSF1A* and *CDH1,* the pyrosequencing assays, primer sequences and PCR conditions were adopted from PyroMark Assay Database (http://techsupport.pyrosequencing.com). We performed validation of all our designed pyrosequencing assays according to the manufacturer's recommendations. PCR reactions were done using the PyroMark PCR Kit (Qiagen, Hilden, Germany) according to the manual instructions, with several modifications. A total of 25 μl reactions contained 2 μl or 4 μl sodium bisulfite-treated DNA for peripheral blood cells or cfDNA and paraffin-embedded tumour tissues, respectively. Final concentrations of the primers were 0.24 μM for genes *RASSF1A*, 0.32 μM for *APC, CXCL12, ESR1, PGR B, CDH1, SYK, BRMS1* and *SOCS1* genes and 0.4 μM for *ADAM23* and *TIMP3* genes. The annealing conditions were modified for gene *ADAM23* to 52°C for 30 s and for *TIMP3* gene to 54°C for 30 s. Pyrosequencing was carried out using a PyroMark Q24 system and the PyroGold Reagent Kit (Qiagen, Hilden, Germany). The results of the analyses were evaluated using the PyroMark Q24 2.0.6. software (Qiagen, Hilden, Germany). Methylation data are presented as the percentage of average methylation in all CpG sites observed and were calculated for each sample and each gene. Analyzed samples were defined as hypermethylated if average methylation was more than 2 standard deviations from the identical gene mean value determined in peripheral blood DNA of controls. The cumulative methylation index (CMI) was calculated as

Table 1. Patients and tumour characteristics

Clinico-pathological variables (N = 34)	N (%)
Age (yrs)	
≤ 50	8 (23.5)
> 50	26 (76.5)
Tumour histology	
DIC	27 (79.4)
LIC	4 (11.8)
MUC	3 (8.8)
Tumour size (mm)	
≤ 20	17 (50)
> 20 ≤ 50	14 (41.2)
> 50	3 (8.8)
Histological grading	
1	8 (23.5)
2	9 (26.5)
3	15 (44.1)
nd	2 (5.9)
TNM staging	
I.	13 (38.2)
II.	14 (41.2)
III.	5 (14.7)
IV.	2 (5.9)
ER/PR status	
Negative	13 (38.2)
Positive	21 (61.8)
HER2 expression	
Negative	27 (79.4)
Positive	5 (14.7)
nd	2 (5.9)

DIC, ductal invasive carcinomas; LIC, lobular invasive carcinomas; MUC, mucinous breast carcinomas; ER status, oestrogen receptor status; PR status, progesterone receptor status; nd, non-detected data.

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 tumour cells or with fluorescence in situ hybridization proven HER2 gene amplification in cases with ambiguous IHC positive at 2+ intensity reaction.

the sum of % M for all genes evaluated. For 11 genes the maximum value of methylation was CMI 1100.

Immunohistochemistry. Protein expression in malignant breast tissues was detected with specific antibodies against 11 corresponding proteins. Tissue samples were fixed in 4% formaldehyde and routinely processed in paraffin. Sections were matched to their corresponding wax blocks (the donor blocks) and 3 mm diameter cores of the tumour and control tissue were removed from these donor blocks with the multipurpose sampling tool Harris Uni-Core (Sigma -Aldrich, Steinheim, Germany) and inserted into the recipient master block. The recipient block was cut into 4μm thick sections and the sections were transferred to coated slides. The slides were deparaffinised, rehydrated and immersed in phosphate buffered saline buffer (10 mM, pH 7.2). Primary antibodies

Table 2. List of PCR and pyrosequencing primers

Gene	RefSeqID	Orientation	5' - 3' Sequence	PCR product size		Number of CpG analyzed
			Amplification primers		Pyrosequencing primers	
APC	NT_034772	Forward	GGGAAGAGGAGAGAGAAGTAGTTG	131bp	AGAGAAGTAGTTGTGTAAT	5
		Reverse	Biotin-AACTACACCAATACAACCACATATC			
ADAM23	NT_005403	Forward	Biotin-GCGTCGTTTTAGTATTTTTAGGTT	89bp	ACTACTCCCTCCCCC	8
		Reverse	TCCCCAACCACTACTCCCT			
BRMS1	NT_026437	Forward	TATTTTTTTGAGTTGGGGGTGG	125bp	TTTGAGTTGGGGGTGGG	8
		Reverse	Biotin-ACCTACAAAAAAAACCCTAATAATTCA			
CXCL12	NT_033985	Forward	TAGTGGGGTTTTGTTATAGGGATA	121bp	GGGTTTTGTTATAGGGATAAT	7
		Reverse	Biotin-ACCTTTAACCTTCTCAAACTC			
ESR1A1	NT_025741	Forward	GGGATGGTTTTATTGTATTAGATTTAAGG	115bp	GTTTTATTGTATTAGATTTAAGGG	5
		Reverse	Biotin-ACTTACTACTATCCAAATACACC			
PGRB	NT_033899	Forward	AGGGATTGAGAGTTTTATAGTATGT	169bp	GTTTGATGTTAGAGAAAAAGT	7
		Reverse	Biotin-CCAAAAAAATTCTCCAACTTCTATCC			
SOCS1	NT_010393	Forward	AGGGTTTAGAAGAGAGGGAAATA	83bp	GAGAGGGAAATAGGG	7
		Reverse	Biotin-CCCAACTCCACTTTTAATTTCTC			
SYK	NT_008470	Forward	GTTAAGGAAGTTGTTTAAAATGAGG	83bp	GAAGTTGTTTAAAATGAGGAA	7
		Reverse	Biotin-TCCTCCTCGCTCTCCAAC			
TIMP3	NT_011520	Forward	GGAGGTTAAGGTTGTTT	175bp	GTAAGGTAATTTTGGAGAGG	5
		Reverse	Biotin-CAAACTCCAACTACCCAAAAAC			
CDH1*	NT_010498	Forward	ATTTTAGTAATTTTAGGTTAGAGGGTTA	106bp	ACCACAACCAATCAACAAC	7
		Reverse	Biotin-ACCACAACCAATCAACAAC			
RASSF1A*	NT_022517	Forward	AGTTTGGATTTTGGGGGAGG	136bp	GGGTTAGTTTTGTGGTTT	7
		Reverse	Biotin-CAACTCAATAAACTCAAACTCCCC			

*primer sequences and PCR conditions were adopted from PyroMark Assay Database

against ADAM 23 (Abcam, Cambridge, UK) BRMS1 (Abcam, Cambridge, UK), SOCS1 (Abcam, Cambridge, UK), Syk (Abcam, Cambridge, UK), TIMP3 (Abcam, Cambridge, UK), APC (Abcam, Cambridge, UK), RASSF1 (Abcam, Cambridge, UK), Cdh1 Ab-1 (Thermo Scientific, Fremont, USA), CXCL 12 (Life Span BioSciencies), oestrogen and progesterone receptors (Dako, Glostrup, Denmark) were used for tumour staining. The slides were incubated with the primary antibody according to the manufacturer's instructions. For visualisation, we used the EnVision system with horseradish peroxidase and diaminobenzidine chromogen solution (DAB, Dako, Glostrup, Denmark). The slides were counterstained with haematoxylin. As positive control, we used normal tissue that was included on the tissue microarrays. As negative control, breast tissue was used, omitting the primary antibody from the staining protocol. The result of the immunohistochemical analyses was expressed by an ImmunoReactive Score (IRS), so-called German IRS, evaluating both the percentage of positive cells (PP) and the staining intensity (SI) of the nuclei or cytoplasm [27]. Shortly, SI was subdivided into four categories (0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive). PP was graded as follows: 0 = negative, 1 = up to 10 percentage positive cells, 2 = from 11 to 50 percentage positive cells, 3 = from 51 to 80 percentage positive cells, and 4 = more than 80 percentage positive cells. SI and PP were then combined by multiplication giving four IRS categories: negative, weak, moderate and high protein expression.

Statistical analysis. Statistical software SPSS statistics 15.0 was used for statistical analyses of the data. Normality of distribution was tested by the Kolmogorov-Smirnoff test. If normally distributed, sample means were tested by Student t-test or analysis of variance (ANOVA) with Bonferroni's or Tamhane's corrections depending on homogeneity of variance. Nonparametric Mann-Whitney U or Kruskal-Wallis H test were used for non-normally distributed data. Pearson's or Spearman's correlations were used according to the normality of data. All tests were two-tailed performed at the significance level $P < 0.05$. Categorical data were tested by Chi square. Adjustment for age was performed using analysis of covariance (ANCOVA).

Results

Bisulfite pyrosequencing was used to investigate DNA methylation profiles in formalin fixed paraffin-embedded tumour tissues, plasma samples and blood cells. The results of DNA methylation levels in 11 genes were compared in 34 BC patients and 50 healthy controls. We observed hypermethyla-

tion of four evaluated genes in tumours. The most commonly hypermethylated gene was *RASSF1A* in 71.9% of patients with methylation level up to 86%. *APC*, *ADAM23* and *CXCL12* genes were hypermethylated in 55.9%, 38% and 34.4% of cases with respective maximal methylation values of 86, 53 and 64%. DNA methylation levels were significantly higher in peripheral blood cells of patients compared to controls in *CXCL12*, *ESR1* and *TIMP3* genes (Table 3), although levels of DNA methylation did not exceed 15% in any of the genes studied. Moreover, significant differences between methylation levels in tumours and peripheral blood cell DNA of patients were observed in *APC*, *ADAM23*, *CXCL12*, *ESR1, CDH1, RASSF1A, SYK, BRMS1* and *SOCS1* genes.

None or only mild DNA methylation levels up to 23% were found in most of patients´ plasma samples, however in rare cases DNA methylation levels 47, 44, 30 and 50% in respective *APC*, *ADAM23, SYK* and *TIMP3* genes were manifested. Though there were no significant differences between DNA methylation in plasma samples between cases and controls, with exception of *CDH1* gene ($P = 0.045$), this result is nevertheless controversial for the higher mean in control plasma and higher maximal methylation level in patient plasma (Table 4). DNA methylation in plasma samples of BC patients was significantly lower than in tumour samples for the majority of the genes studied, with exception of *CDH1*, *TIMP3* and *BRMS1;* there were thus no correlations between DNA methylation in tumours and plasma samples from patients in any of the genes studied (Fig. 1).

The CMI means in tumour tissues and plasma samples were 137.5 ± 60.3 and 43.6 ± 20.1, respectively, compared with CMI mean 39.1 ± 8.1 in patients' peripheral blood cell DNA. In patients, the cumulative DNA methylation in tumours was significantly higher than in plasma samples ($P < 0.001$). Comparison of CMIs in patients' tumour and cfDNA are presented in Fig. 2. On the other hand, the CMI mean in the healthy control group of women was 43.7 ± 15.0 and 33.2

Table 3. Comparison of DNA methylation (in %) between breast cancer patients and controls in peripheral blood cells

Genes	Controls N=50		Patients N=34		P value*
	Mean ± SD	Range	Mean ± SD	Range	
APC	1.28 ± 0.57	(1 - 4)	1.68 ± 1.04	(1 - 6)	0.082
ADAM23	2.18 ± 0.39	(2 - 3)	2.29 ± 0.58	(1 - 3)	0.511
CXCL12	2.28 ± 0.50	(1 - 3)	2.79 ± 0.84	(2 - 5)	0.044
ESR1	3.22 ± 0.86	(2 - 6)	4.09 ± 1.44	(2 - 9)	0.026
PGRB	4.32 ± 1.13	(1- 7)	5.41 ± 2.03	(3- 13)	0.056
CDH1	9.02 ± 1.60	(4 - 12)	9.64 ± 2.10	(4 - 15)	0.698
RASSF1A	1.04 ± 0.28	(0 - 2)	1.00 ± 0.00	(1 - 1)	0.475
SYK	1.06 ± 0.24	(1 - 2)	1.15 ± 0.44	(1 - 3)	0.638
TIMP3	2.50 ± 0.81	(1 - 5)	3.65 ± 2.55	(1 - 14)	0.036
BRMS1	1.40 ± 0.70	(1 - 3)	1.56 ± 0.93	(1 - 6)	0.726
SOCS1	0.96 ± 0.28	(0 - 2)	1.09 ± 0.38	(1- 3)	0.271

*P value for the difference between controls and patients adjusted for age by the analysis of covariance (ANCOVA).

Table 4. Comparison of DNA methylation (in %) between breast cancer patients and controls in plasma samples

Genes	Controls N=50		Patients N=34		P value*
	Mean ± SD	Range	Mean ± SD	Range	
APC	2.53 ± 1.56	(0 - 6)	4.41 ± 7.81	(1 - 47)	0.060
ADAM23	2.69 ± 1.50	(1 - 9)	3.82 ± 7.33	(1 - 44)	0.073
CXCL12	2.68 ± 1.92	(1 - 11)	2.85 ± 2.84	(1 - 15)	0.879
ESR1	5.24 ± 4.33	(1 - 23)	4.18 ± 4.07	(1 - 18)	0.338
PGRB	4.69 ± 5.85	(1- 32)	2.97 ± 2.15	(1- 8)	0.206
CDH1	7.73 ± 3.60	(1 - 15)	6.53 ± 5.32	(1 - 23)	0.045
RASSF1A	4.02 ± 6.62	(1 - 36)	2.85 ± 3.13	(1 - 18)	0.404
SYK	2.14 ± 2.28	(0 - 12)	3.00 ± 5.65	(0 - 31)	0.488
TIMP3	3.92 ± 4.54	(0 - 31)	3.97 ± 8.43	(0 - 50)	0.697
BRMS1	2.56 ± 1.57	(0 - 8)	3.12 ± 2.36	(0 - 10)	0.516
SOCS1	2.08 ± 1.91	(1 - 11)	1.76 ± 0.90	(1 - 4)	0.358

*P value for the difference between controls and patients adjusted for age by the analysis of covariance (ANCOVA).
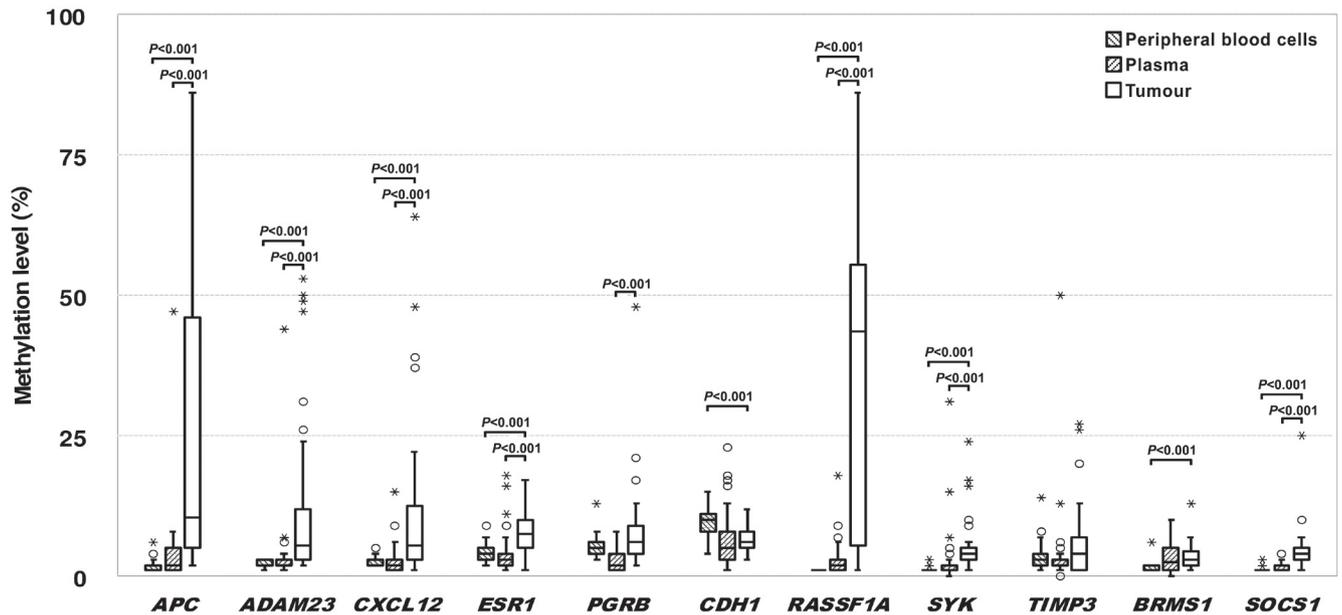
Figure 1. DNA methylation levels (%) for 11 analysed genes in peripheral blood cells, plasma and paraffin-embedded tumour samples of breast cancer patients
P values represent differences between peripheral blood cells and tumour samples or between plasma and tumour samples. The length of the boxes is the interquartile range (IQR) that represents values between the 75th and 25th percentiles. Values more than three IQR's from the end of a box are labelled as extreme (*). Values more than 1.5 IQR's but less than 3 IQR's from the end of the box are labelled as outliers (O). The median is depicted by horizontal line.
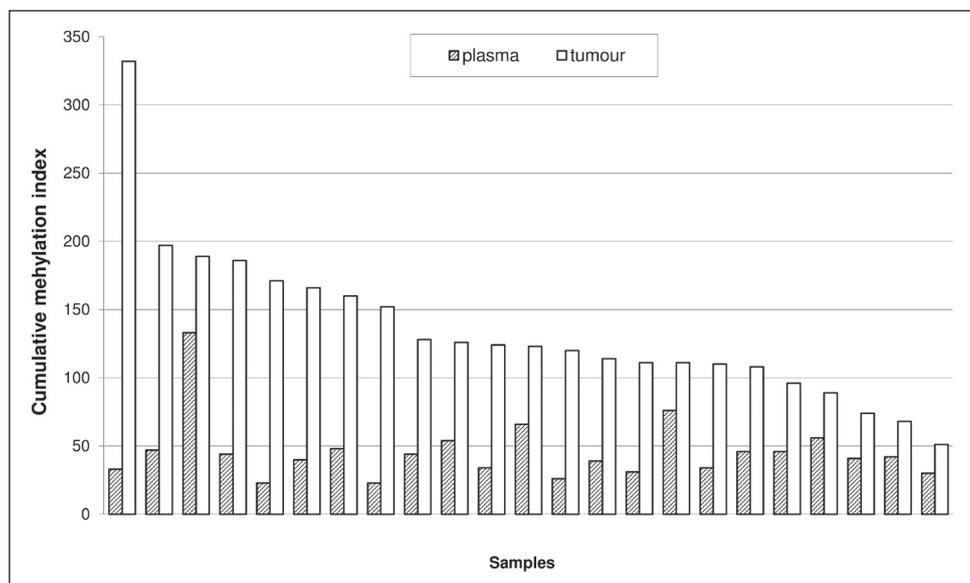

Figure 2. Cumulative methylation index for paraffin-embedded tumour and plasma samples in breast cancer patients
Cumulative methylation index is the sum of %M for eleven evaluated genes.

± 4.1 in plasma and peripheral blood cell DNA, respectively. The value of cumulative methylation in peripheral blood cell DNA of patients and controls was significantly different (39.1 ± 8.1 vs. 33.2 ± 4.1; P < 0.006), in contrast to nearly identical

cumulative methylation in plasma DNA of patients and controls (43.6 ± 20.1 vs. 43.7 ± 15.0).

Immunohistochemical staining was used to determine relevant protein expression. Variable expression profiles were
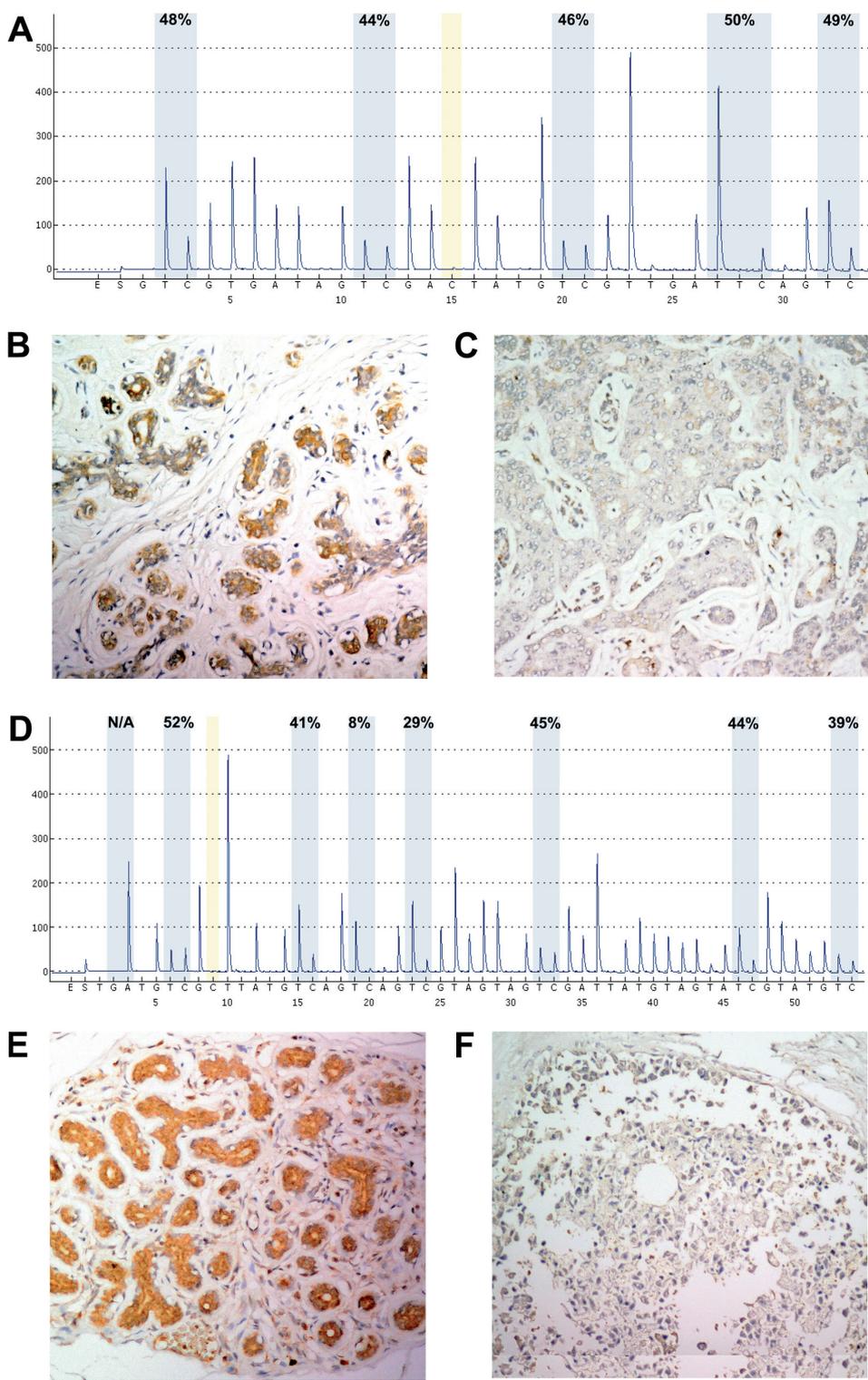
Figure 3. Association between promoter methylation and inhibition of relevant protein expression
A: Pyrosequencing result of the *APC* gene promoter region with average DNA methylation in the tumour of 47%
B: Normal breast tissue. Immunohistochemical staining with antibody against APC Magnification 350 x
C: Breast carcinoma. Immunohistochemical staining with antibody against APC Magnification 350 x
D: Pyrosequencing result of the *CXCL12* gene promoter region with average DNA methylation in the tumour of 39%
E: Normal breast tissue. Immunohistochemical staining with antibody against CXCL 12 Magnification 350 x
F: Breast carcinoma. Immunohistochemical staining with antibody against CXCL 12 Magnification 350 x

identified for the proteins analysed, ranging from negative to high expression. The highest frequency of negative protein expression was found for TIMP3 protein (70%) and positive protein expression for ESR1 (60.6%). In the highly methylated *APC* gene a visible decreasing trend of mean methylation levels from 68% to 19% was observed in association with an increasing protein expression. Similar results were found in *CXCL12*, where the mean methylation level 24.17% was reduced to 4.33%; however in both genes without significant difference. The examples of pyrosequencing results in patients with average methylation level 47% in *APC* and 39% in *CXCL12* gene and with low protein expressions in both proteins are presented in Figure 3.

Discussion

DNA methylation analysis in promoters of 11 genes, namely, *APC, ADAM23, CXCL12, ESR1, PGR B, CDH1, RASSF1A, SYK, TIMP3, BRMS1* and *SOCS1,* as well as immunohistochemical analysis of related proteins in paraffin-embedded tumour tissues were realised with the purpose to investigate the relationship between DNA methylation and protein expression and to find a useful plasma-based marker for BC. In our study, tumour samples showed a wide range of promoter DNA methylation as well as protein expression. The most frequent methylation was found in *RASSF1A* gene promoter that was hypermethylated in 71.9% of the BC patients assessed, with the mean level 50.3%. Comparable results were described for BC cases in our previous study, i.e.82.1% methylation frequency with the mean level 48.45% in *RASSF1A* gene using QM-MSP [28]. Other authors showed similar frequencies of 68% and 82.5% or notably lower means of *RASSF1A* methylation levels, i.e. 18.5±4.7% [6, 29]. The important role of *RASSF1A* epigenetic silencing in BC was presented by very high methylation levels in *RASSF1A* gene in many studies regardless ethnicity of the population studied [6, 30]. In another study, the association of *RASSF1A* methylation with time to first recurrence and overall survival was observed. These data suggest that *RASSF1A* methylation could be a potential prognostic biomarker [31]. Costa and co-workers showed higher methylation frequency of *ADAM23* promoter in primary tumours of BC patients compared to our results (69.2% vs. 38.2%) [25]. Other authors reported in BC different *CXCL12* methylation frequencies, i.e. 14.5% and 52.4% [23, 32], compared to our study where the frequency was 34.4%. Patients with *CXCL12* methylation had a shorter overall survival and disease-free survival. These findings suggest that the DNA methylation status of *CXCL12* gene could be used as a prognostic biomarker in BC [33, 34]. Our results showed that *APC* belonged also to the most frequently methylated genes. The frequency of hypermethylation was 55.9%, with the mean level 40.3%. Other studies reported lower or similar frequencies of *APC* gene methylation, ranging from 28 to 53% [35, 36], which correlated with reduced APC protein levels [37]. In our study, the results of quantification of *APC* gene methylation with immunohisto-

chemical evaluation of protein expression indicate that DNA methylation of *APC* gene could initiate inhibition of relevant protein expression. We also observed a decreasing trend of methylation levels in association with increasing protein expression for *CXCL12* gene. A strong relationship between *CXCL12* methylation and protein expression was verified by other authors [32, 38]. Moreover, a low CXCL 12 protein level was associated with poor prognosis [33, 39]. In other genes studied, we did not find a clear association between DNA methylation levels and relevant protein expressions, evaluated by the semi-quantitative IRS method. Our results suggest that the DNA methylation profiles observed in our group of breast carcinomas are not the only cause that affects the silencing of the genes evaluated and the decrease of relevant protein products. Other mechanisms regulating gene expression may be involved, such as post-transcriptional modifications of histones and chromatin rearrangement, as well as aberrant miRNA regulation. All of these modifications participate in compression or relaxation processes of chromatin structures that contribute to gene expression regulation [40]. In a recently published review, Hervouet and co-authors provided strong evidence that expression of oestrogen target genes was tightly regulated by multiple and highly dynamic mechanisms using classical co-recruitment of oestrogen receptors and co-activators/co-repressors or by epigenetic manner, including receptor complexes with histone acetyltransferases, histone deacetylases, histone methyltransferases, DNA methyltransferases and Polycomb proteins [41]. Another example of the complexity of gene expression regulation was presented in a cell line study where the regulation of *RASSF1A* gene was affected by simultaneous DNA and protein methylation. The investigators demonstrated that simultaneous targeting of DNA and E2F1 transcription factor methylation was an effective epigenetic treatment reactivating *RASSF1A* expression and inducing apoptosis in breast cancer cells [42]. Other authors showed the influence of miRNAs on epigenetic switches in DNA methylation and histone modification of *RASSF1A* gene in BC [20]. The above mentioned results indicate that in addition to epigenetic silencing of gene expression through DNA methylation of promoter sequences there are other mechanisms that affect gene and the following protein expression. Thus we did not observe a clear association between DNA methylation and relevant protein expression, despite the findings of a wide range of promoter DNA methylation levels and/or variable protein expression in the samples evaluated.

To determine whether DNA methylation patterns identified in tumours can be found in peripheral blood, we analysed DNA methylation levels of cfDNA and peripheral blood cells DNA of BC patients and controls. Peripheral blood is a very attractive medium for the development of molecular biomarkers for cancer detection, prognostic assessment and treatment response assays as it is obtained through a simple, relatively non-invasive procedure. A list of studies reported aberrant plasma/serum DNA methylation of a single gene or a panel of genes in BC patients, using different quantitative or

qualitative assays [11-15]. Variations in results from different studies could be attributed to differences in clinical status and methodological approaches used for analysis. The widely used semi-quantitative methods, such as MSP, bear a significant risk of false-positive or false-negative results, especially when the DNA quality and/or quantity is low, which is often the case in clinical settings. In comparison to the other quantitative methods as COBRA (combined bisulfite restriction analysis), SIRPH (SNuPE ion pair-reverse phase high-performance liquid chromatography) or Q-MSP (quantitative methylation specific PCR), pyrosequencing has been shown to be the most reliable method that fulfils also the basic requirements of clinical settings, such as robustness, cost efficiency and easy use [43, 44]. As a general observation from DNA methylation studies, the *RASSF1A* gene appeared to be a promising biomarker of BC in plasma/serum. However, several authors questioned the serious clinical utility of circulating DNA as a biomarker of cancer for moderate concordance in terms of the presence of methylation of genes between tumour tissues and plasma/serum of cancer patients and overall relatively low methylation frequencies in serum compared to matched tumour samples [reviewed in 45, 46]. Similarly to our results, modest differences of cfDNA methylation between BC cases and controls were reported [16, 47]. Korshunova and colleagues found a high background of overall methylation in serum of cancer-free individuals and nearly identical DNA methylation levels in four BC associated genomic regions for BC cases and controls [48]. Massively parallel bisulphite pyrosequencing revealed the complex nature of the cytosine-methylation landscape of both serum and tissue DNA and no special type of methylation pattern was found in a statistically meaningful way exclusively in cancerous or normal tissues, including serum [47]. Circulating tumour cells as a source of cancer-related DNA methylation were reported by Matuschek and colleagues. They observed a correlation between DNA methylation in *APC* and *GSTP1* genes in serum with the presence of circulating blood tumour cells, which are associated with a more aggressive and advanced BC disease [49]. Our current results are in concordance with our previous study where we found different spectra of methylated genes in plasma compared to tumour samples, using the RT-based quantitative method [28]. Estimations of the concentration of tumour-derived DNA in serum, based on detection of tumour-specific mutations, demonstrated that the amount of this DNA was less than 0.2% [50]. As discussed in Korshunova and co-authors, the expected methylation signals from such minute amounts are so close to the background level that robust detection of tumour-shed DNA would seem to be problematic, especially in the case of an epigenetically complex background [47]. For this reason, the use of PCR-based methods that are able to detect no less than a twofold difference will remain challenging. This could be the main obstacle in finding tumour specific differences in sera/plasma and the main reason of the lack of sensitivity of the epigenetic biomarkers studied [45]. The implication of these findings for further blood-based epigenetic research is the need for identification of markers with the largest possible epigenetic differences between diseased and normal serum.

Recently, several articles were published discussing the role of aberrant global and gene specific DNA methylation in peripheral blood cells in breast and other cancers [reviewed in 51, 20]. The results showed that some systemic epigenetic changes would be detected in peripheral blood DNA in breast, pancreatic, bladder and colorectal cancers [18, 52-55]. In our study, significantly higher levels of promoter DNA methylation of three genes were found in peripheral blood cells of BC patients compared to controls. It has been suggested that cancer specific genetic variants or immunologic processes related to inflammation in cancer development may lead to methylation changes in leukocyte subpopulations that are manifested in peripheral blood [56, 57]. Greater insight into the origin and nature of DNA methylation changes in peripheral blood as well as information about DNA methylation pattern in heterogeneous leukocyte subpopulations are needed to determine if DNA methylation in peripheral blood cells could serve as an informative biomarker in cancer diagnosis and treatment [20].

Conclusions

Despite the finding of no clear association between DNA methylation and protein expression, we can conclude that the quantitative analyses of tumour DNA methylation in any of *RASSF1A, ADAM23, CXCL12* and *APC* genes could have prognostic potential. DNA methylation in plasma samples of BC patients is currently not suitable for translation into clinical use due to the technological challenges and insufficient knowledge of origin and nature of cfDNA methylation. However, the significantly higher level of promoter DNA methylation in peripheral blood cells of BC patients compared to controls in our study support the idea that the analyses of DNA methylation in peripheral leukocytes of cancer patients could have a potential to become an informative biomarker of cancer risk and prognosis.

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