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DNA Hypermethylation Detected in Invasive Breast Cancer

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DNA methylation as an early event in breast carcinogenesis has been frequently studied in tumor samples. The aim of this study was to compare the relationship between DNA methylation levels of genes associated with invasivity and metastasising and aberrant protein expression. A total 55 healthy controls and 185 patients with primary breast cancer, as well as plasma and peripheral blood cells has been analysed by using pyrosequencing method. The expression of 11 proteins in paraffin-embedded biopsy was evaluated by immunohistochemistry. Genes containing CpG islands in promotor region were suggested for screening because they could be epigenetically regulated with high probability. DNA methylation of APC, ADAM23, CXCL12, ESR1, PGR B, CDH1, RASSF1A, SYK, TIMP3, BRMS1 and SOCS1 genes has been detected. DNA hypermethylation of tumor suppressor genes is tumor-specific and could be used for recognition of tumor cells. We observed higher methylation status for 4 genes (RASSF1A, APC, CXCL12 and ADAM23) from 11 genes evaluated in tumors. The highest promoter methylation level was 88%, detected in RASSF1A

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and APC genes. Variable expression profiles were identified in analyzed genes ranging from negative expression to high expression. We can conclude that the quantitative analyses of tumor DNA methylation in any of RASSF1A, ADAM23, CXCL12 and APC genes could have prognostic potential. *Supported by the grant APVV-0076-10 - the Slovak Research and Development Agency and Research and Development Operational Programme (ERDF)-26240220058*

Introduction

Breast cancer is one of the major health problem that affect 1676633 human worldwide. With this number, breast cancer is the leading cancer diagnosed in the women and is second only to lung cancer in terms of total fatalities [1]. Survival of breast cancer patients have increased tremendously over the last few decades. However, this applies only if the breast cancer is diagnosed at an early stage and is limited to the primary organ. Once breast cancer metastasizes to other organs, the therapeutic options are very limited and the success rate of managing such patients in clinics is dismal. Breast cancer is driven by progressive genetic changes and epigenetic abnormalities [2]. It includes changed patterns of histone modification, with resultant remodeling of structure of chromatin and hypo- and hypermethylation of DNA. These changes cause deregulation of the transcription activity of many genes. Epigenetic regulation is critical for normal growth and development and provides a layer of transcriptional control. Therefore, the evaluation of the cancer-specific methylation pattern changes in the promoter regions has an important potential to improve an early detection of cancer and prediction of therapy and prognostic response. The aim of this study was to compare the relationship between DNA methylation levels of 11 genes participated on growth signal transferring (*APC, ESR1, PGR B, RASSF1A, SYK* and *SOCS1*) and metastasis formation (*ADAM23, CXCL12, CDH1, TIMP3* and *BRMS1*). Since the selected genes contain CpG islands in promotor area they are epigenetically regulated with a high probability. Moreover, in breast carcinomas the different levels of methylation of the genes concerned were observed, suggesting different reduction rates of protein products synthesis.

Methods

A total of 185 paraffin-embedded tumor tissue samples from non-familiar patients with primary breast cancer and 55 healthy controls as well as plasma and peripheral blood cells have been analysed by using highly quantitative method of pyrosequencing. Tumors obtained from Slovak patients were staged and graded according to the current WHO classification for breast neoplasms. DNA from paraffin-embedded tumor tissues was isolated by the MagneSil

Genomic, Fixed Tissue System (Promega, Madison, WI), from peripheral blood cell by using a FlexiGene DNA kit (Qiagen, Hilden, Germany) and from plasma samples by using a QIAamp DSP Virus Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA methylation in 11 genes (*APC, ESR1, PGR B, RASSF1A, SYK, SOCS1, ADAM23, CXCL12, CDH1, TIMP3, BRMS1*) containing CpG islands in promoter region was evaluated after the sodium bisulfite-treatment of isolated DNA [3, 4, 5, 6]. CpG islands in the promoter area flanking the transcription start site at 5'UTR were selected for each gene. Five to eight CpG sites were studied for each particular CpG island. In *ESR1*gene the methylation status for two regions in the promoter A and in the first exon was evaluated, completely 14 CpG sites.

Pyrosequencing assays primer sequences and PCR conditions were taken from [7]. For *ESR1* gene was designed additional set of the oligonucleotides for PCR amplification and pyrosequencing of the promoter A using PyroMark Assay Design software 2.0 (Qiagen, Hilden, Germany). PCR primers for *ESR1* promoter A region 2: F primer - GTTGGAGGTTAGGGAGTTTAGG, R primer - biotin- TCTAACCCCCACCCTACCCC. Sequence for pyrosequencing primer is GGGAGTTTAGGAGTTGG. PCR product size is 120bp and it is analysed other 9 CpG sites.

A total 1 μg of peripheral blood cells DNA was bisulfite converted using the CpGenome DNA Modification Kit (Chemicon, 150 Billerica, MA). Cell free DNAs from plasma (2 μg) and tumor DNA (2 μg) were modified according to the protocol of EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). PCR reactions were provided using the PyroMark PCR Kit (Qiagen, Hilden, Germany) according to the manual instructions with several modifications [7]. PCR conditions for *ESR1* promoter A region 2 were modified for final PCR primer concentrations to 0.28 μM. The annealing temperature was modified to 58°C for 30 seconds (Table 1). 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. Protein expression in malignant breast tissues was detected with specific antibodies against 11 corresponding proteins. Statistical analysis was performed by SPSS statistics 15.0 software.

Table 1. *The Composition and Profile of PCR reaction for A2 ESR1 Gene Promoter Amplification*

PCR components	**Concentration for 25 µl reaction**	**Volume for 25 µl reaction**	
Pyromark 2x concentrated	1x concentrated	12.5 µl	
Coralload 10x concentrated	1x concentrated	2.5 µl	
Primer F	0.28 µmol/l	0.7 µl	
Primer R	0.28 µmol/l	0.7 µl	
H_20		6.6 µl	4.6 µl
DNA		2 µl	4 µl
PCR program	**Temperature**	**Time**	**Number of cycles**
First denaturation	95°C	15 min	1x
Denaturation	94°C	30 sec	45 x
Annealing	58°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	10 min.	1x

PCR reactions contained 2 µl sodium bisulfite-treated DNA for peripheral blood cells and 4 µl sodium bisulfite-treated DNA for cfDNA and paraffin-embedded tumour tissues.

Results and Discussion

DNA hypermethylation of tumor suppressor genes is tumor-specific process and could be used for tumor cells recognition. Bisulfite pyrosequencing was used to investigate DNA methylation profiles in formalin fixed paraffin-embedded tissues, blood cells and plasma samples in 185 patients with primary breast cancer.

Clinical and histopathological characteristics of patients are summarized in table 2.

Table 2. *Clinico - Histopathological Characteristics of Patients*

	N = 185	**Frequency (%)**
Age		
≤ 50	40	21.6
> 50	145	78.4
Histological type		
ductal invasive carcinoma	155	83.8
lobular invasive carcinoma	24	13.0
OTHER	6	3.2
Tumor size		
≤ 20 mm	120	65.93
> 20 mm ≤ 50 mm	55	30.22
> 50 mm	7	3.85
Histological grading		
1	25	13.7
2	88	48.4
3	69	37.9
Lymph node status (LN)		
negative	103	59.9
positive	69	40.1
Tumor node metastases staging (TNM)		
I	89	48.4
II	62	33.7
III	30	16.3
IV	3	1.6
Estrogen receptor status (ER)		
negative	29	18
positive	132	82
Progesterone receptor status (PR)		
negative	47	29.4
positive	113	70.6

We observed higher methylation status in 4 genes (*RASSF1A*, *APC*, *CXCL12* and *ADAM23*) from 11 genes evaluated in tumors. Average DNA methylation levels in tumor tissue were as follows: 38.57% ± 25.76 for *RASSF1A* gene, 23.73% ± 22.38 for *APC* gene, 9.36% ± 10.58 for the gene *ADAM23* and 11.83% ± 12.01 for the gene *CXCL12*.The highest promoter methylation level was 88% detected in both *RASSF1A* and *APC* genes (Table 3).

Table 3. *Levels of DNA Methylation in Breast Cancer Patients and Controls*

	Controls			Patients			Patients		
Genes	**DNA from peripheral blood cells**			**DNA from peripheral blood cells**			**DNA from tumors**		
	N	**Mean (%) ± SD**	**Range (%)**	**N**	**Mean (%) ± SD**	**Range (%)**	**N**	**Mean (%) ± SD**	**Range (%)**
APC	55	1.31 ± 0.57	1 - 4	184	2.11 ± 1.03	0 - 6	185	23.73 ± 22.38	1 - 88
ADAM23	55	2.13 ± 0.39	1 - 3	185	2.44 ± 1.00	1 - 12	185	9.36 ± 10.58	1 - 60
CXCL12	55	2.35 ± 0.51	1 - 3	185	2.85 ± 1.39	1 - 16	185	11.83 ± 12.01	1 - 64
ESR1	55	3.33 ± 0.90	2 - 6	184	4.36 ± 1.31	1 - 9	184	8.89 ± 4.00	1 - 31
PGRB	55	4.45 ± 1.25	1 - 7	181	5.19 ± 1.67	0 - 13	182	7.26 ± 6.51	1 - 48
CDH1	55	9.36 ± 1.68	4 - 13	188	9.80 ± 1.70	4 - 15	179	7.49 ± 2.96	1 - 20
RASSF1A	55	1.00 ± 0.27	0 - 2	183	1.06 ± 0.30	0 - 3	182	38.57 ± 25.76	1 - 88
SYK	55	1.16 ± 0.37	1 - 2	183	1.55 ± 0.64	1 - 4	184	6.02 ± 4.32	1 - 39
TIMP3	55	2.93 ± 1.35	1 - 9	183	3.04 ± 1.95	0 - 14	175	6.14 ± 7.38	0 - 60
BRMS1	55	1.31 ± 0.66	0 - 3	182	1.68 ± 1.04	0 - 7	183	3.20 ± 1.59	0 - 13
SOCS1	55	1.04 ± 0.38	0 - 2	183	1.45 ± 0.79	0 - 4	184	5.02 ± 3.45	0 - 25

One third of all breast cancer patient display ER negative phenotype and to date it is still not clear if chromosomal alteration or mutations might be responsible for this phenotype. Moreover, changes in the ER tumor expression status were observed, when the ER positive tumors became ER negative during the patient treatment. Hypermethylation in the promoter region of the gene *ESR1* represents one of the possible mechanisms for loss of *ESR1* gene expression since methylation represents a reversible and dynamic process. The expressions of ER and PR receptors need to be monitored to identify patients that will positively respond to hormonal antiestogen treatment [8, 9].

In our results, the values of *ESR1* gene methylation in paraffin embedded tumor tissue were relatively low and ranged from 1-31 % among the individual patients (Figure 1) with average value 8.89 % ± 4.00. Average value of methylation measured in patients peripheral blood lymphocytes is 4.36 % ± 1.31 (range of 1-9 %) which is just slightly higher when compared to healthy controls 3.33 ± 0.90 % (range 2-6 %). In similar study reported by other authors the aberrant DNA methylation was not detected in gene *ESR1* [4, 9]. In contrast to the authors who have demonstrated significant levels of DNA methylation in gene *ESR1* [11, 12, 13], we have analyzed the higher number of CpG dinucleotides in the *ESR1* gene and we used pyrosequencing as more sensitive quantitative method for analyzing methylation of specific CpG dinucleotides. The average values of methylation in studied tumor samples of the 131 ER positive and 29 ER negative patients were 8.76 % ± 4.065 (range 1-31 %) and 9 % ± 3.625 (range 2-16 %), respectively. These methylation levels are nearly identical, and they are most likely not responsible for partial or complete inhibition of gene expression. In literature, complete absence of *ADAM23* tumor suppressor gene expression was observed for breast cancer patients with the DNA methylation levels in range of 40-60 % [14], and for

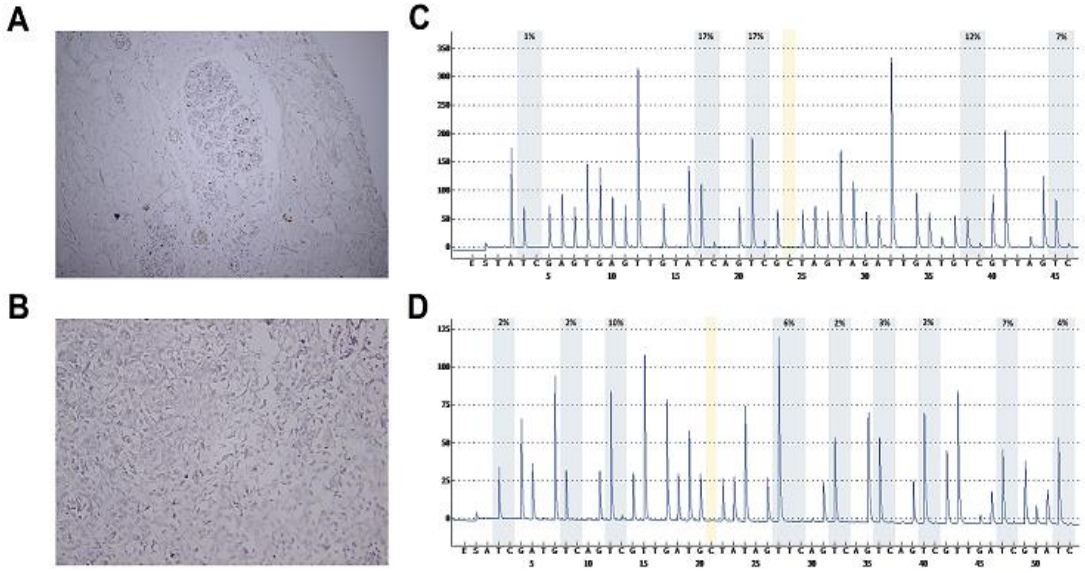
colorectal cancer patients in range of 70-90 % [15]. Based on these findings we can conclude that low level of *ESR1* gene methylation we identified in analyzed tumor samples is not responsible for the lack of ER expression. The relationship between *ESR1* promoter methylation and ER expression was statistically not significant with P = 0.464. ER expression in the tumor ranged from 0-100 %. It is currently estimated that the immunohistochemical determination of estrogen receptor expression is determined incorrect (false positive or false negative) in up to 20 % of cases a worldwide. The subjective evaluation of positive stained cells and the staining intensity of the signal, and also definition of thresholds for ER positive and negative tumors could be the reasons of this mistake. Tumors were considered to be positive tumors in case of at least 1 % of the tumor tissue cells shown immunoreactivity.

Some studies indicate that also other mechanisms regulating *ESR1* gene expression may be involved, such as aberrant miRNA regulation and post-transcriptional modifications of histones [16]. MicroRNAs are small noncoding RNAs that suppress post-transcriptional gene expression by pairing with the 3 'UTR of target mRNAs. Several studies have demonstrated a correlation between microRNA-212/222 and ER expression. Over-expression of microRNA-212/222 in ER positive breast tumors showed a suppression of expression of ER and other proteins [17]. Also reduction of the histone deacetylase HDAC1 level in advanced stage cancer associated with negative expression of ER has been described in regard to the status of estrogen receptors of breast carcinomas [18].

The data obtained for the progesterone receptor PR were as follows: average values of methylation in tumor tissue 7.26 ± 6.51% (range 1-48%), in peripheral blood lymphocytes of patients 5.19% ± 1.67 (range 0-13 %) and in healthy controls, 4.45% ± 1.25 (range 1-7%). Although the significant correlation between the level of methylation and expression of progesterone receptor was found in tumor of the patients (P = 0.020), the mean DNA methylation values in tumor and peripheral blood cell in *PGR B* are very similar and therefore the low DNA methylation of *PGR B* gene could not initiate inhibition of relevant protein expression. It was demonstrated that expression of the two promoters in *PGR B* gene is induced by the ER in function of transcription factor [19]. It suggests that in ER negative patients, the progesterone receptor should not be expressed, independently on methylation status.

In our study the variable expression profiles in all analyzed genes ranging from negative expression to high expression were found, but no relationship with biological significance between DNA methylation and protein expression was demonstrated.

Figure 1. *Expression of ER receptors in normal breast tissue and invasive ductal carcinoma of the ER negative patient with low/normal DNA methylation.*
A: Normal breast tissue with focal expression of ER. Immunohistochemical staining with antibody against ER. Magnification 300 x.
B: Invasive ductal carcinoma of the breast. Negative immunohistochemical staining with antibody against ER. Magnification 300 x.
C: Pyrosequencing result of the ESR1 gene promoter A region 1 with average DNA methylation in the tumor of 11%.
D: Pyrosequencing result of the ESR1 gene promoter A region 2 with average DNA methylation in the tumor of 4%.



Conclusion

Breast cancer is a heterogeneous disease for which alterations in DNA methylation patterns have been shown to be of biological and clinical importance. We can conclude that the quantitative analyses of tumor DNA methylation in any of *RASSF1A, ADAM23, CXCL12* and *APC* genes could have prognostic potential.

Surprisingly, the determination of methylation in genes *ESR1* and *PGR B* encoded hormonal receptors have not a relevant for prognosis or development of the disease.

Identification of early epigenetic changes in breast cancer might give valuable markers for early detection and contribute to the understanding of how these changes affect the progression of the disease and prognosis for the patient.

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