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Hypermethylation of the GSTP1 promoter region in breast cancer is associated with prognostic clinicopathological parameters

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Breast cancer is one of the most common cancer affecting women and the recent research is focused on identifying new genetic and epigenetic prognostic and predictive factors. Glutathione S-transferase P1 (GSTP1) is a biotransformation enzyme expressed in normal breast epithelial cells which can be epigenetically inactivated in breast cancer. We have shown, that application of nested two-stage methylation-specific PCR (MSP) is a suitable method for analysis of epigenetically silenced GSTP1 in formalin-fixed paraffin-embedded (FFPE) tissues from breast cancer patients. Of 45 breast tumors, 11 (24, 4%) were found to have methylated GSTP 1 promoter region. We were able to demonstrate the correlation between the hypermethylation of the GSTP1 promoter region and histological grade of the tumor ($p < 0.01$), Nottingham Prognostic Index ($p < 0.01$) and lymph node metastasis ($p < 0.05$). Our results indicate that the GSTP1 hypermethylation is putative prognostic factor in breast cancer but a confirmation of its prognostic value is desirable in larger studies.

Key words: breast cancer, prognostic factors, hypermethylation, GSTP1, methylation-specific PCR

Breast cancer is one of the most common cancers affecting women with increasing incidence. In Slovakia, nearly 2000 women are diagnosed for breast cancer each year [1]. Based on morphological taxonomy and application of antibodies to formalin fixed paraffin-embedded (FFPE) tumor sections, several prognostic parameters have been identified for routine prognostic use like tumor size, histological grade, regional lymph node status, human epidermal growth factor receptor 2 (HER2) gene amplification, expression of estrogen receptors (ER) and progesterone receptors (PR) [2–4]. Since the completion of the human genome sequencing, analysis of new genetic and epigenetic prognostic factors has become possible. This led to the identification of a variety of genes that are involved in breast cancer pathogenesis and progression [5–7].

Epigenetic changes are produced by two different mechanisms – methylation of CpG islands maintaned by methyltransferases and acetylation and/or methylation of histones. In breast cancer, methylation changes like global hypomethylation and hypermethylation of promoter regions in several genes has been identified and related to oncogene activation and tumor suppressor gene silencing, respectively [8, 9]. The hypomethylation is believed to occur in the late stage of the disease, while the hypermethylation is assumed to be an early event of carcinogenesis and a possible biomarker for cancer detection, prognosis and potential chemoprevention and therapeutic target [10]. Genes involved in different cellular processes like cell cycle regulation, or with known function like steroid receptors, cell adhesion molecules or biotransformation enzymes can be inactivated by hypermethylation of their promoter regions [7].

Glutathione S-transferase P1 (GSTP1) is a biotransformation enzyme involved in the detoxification, and it is expressed in normal breast epithelial cells and prostate epithelium [11, 12]. GSTP1 is epigenetically inactivated in prostate cancer and is one of the most specific methylation biomarkes in this tumor [13]. In addition to detoxifying function of the GSTP1 gene product, it has been shown, that GSTP1 has a negative influence on JNK1 signaling [14] suggesting its function as putative tumor suppressor gene. It is known, that loss of the GSTP1 protein expression occurs in approximately 53% of breast cancers as detected by immunohistochemistry [11]. The hypermethylation of the promoter region of the GSTP1 gene has been suggested as one of the possible mechanisms leading to its down regulation [15].

In our study, we established a nested two stage methylation-specific PCR (MSP) on bisulfite modified DNA isolated from formaline-fixed paraffin-embedded (FFPE) breast cancer tissue and from control samples to examine hypermethylation

Table 1. Clinicopathological parameters of tested samples

Variable	n (%)
Age (mean / range)	60.5 (39-79)
Histological type	
Ductal	39 (81.3)
Lobular	5 (10.4)
Other	4 (8.3)
Histological grade	
Grade 1	9 (18.8)
Grade 2	18 (37.5)
Grade 3	21 (43.8)
Tumor size	
≤ 2cm	23 (47.9)
> 2cm	25 (52.1)
Lymph node status	
LN negative	24 (50.0)
LN positive	22 (45.8)
Unknown LN status	2 (4.2)
NPI	
NPI 1	15 (31.3)
NPI 2	20 (41.7)
NPI 3	11 (22.9)
Unknown	2 (4.2)
ER status	
ER-negative	12 (25.0)
ER-positive	36 (75.0)
PR status	
PR-negative	17 (35.4)
PR-positive	31 (64.6)
HER2 status	
HER2- negative	43 (89.6)
HER2- positive	5 (10.4)

LN – lymph node; NPI – Nottingham prognostic index; ER – estrogen receptor; PR – progesterone receptor

of the promoter region of the GSTP1 gene. The nested PCR in combination with MSP is a very sensitive method, which has not been used on clinical breast cancer samples from FFPE yet. The breast cancer clinical samples were tested by this approach with the goal to investigate epigenetic differences in GSTP1 promoter region and to correlate the methylation status of GSTP1 to known and accepted prognostic parameters in breast cancer.

Patients and methods

Patients and tumor characterization. Paraffin-embedded tissue sections were obtained from biopsy specimens of 48 breast cancer patients with histological and immunohistochemical characterized breast cancers that were presented to the department of pathology. The study was approved by Institutional Review Board. The parameters of the histopathological examinations including tumor size, histological grade, regional lymph node status, estrogen receptor (ER) status, progesterone receptor (PR) status and HER-2 status as shown in table 1. The mean age of the patients was 60.5 (range 39-79 years).

Patients after neodjuvant therapy, cases with in situ medullary carcinomas were excluded, because it was not possible perform the evaluation of the histological grading. Histological assessments were performed on 4-5 μm thick H&E- stained sections of formalin-fixed, paraffin-embedded tumours. Typing was evaluated according to the criteria outlined in the World Health Organization Classification of Tumours [16] and histological grading was performed according to the method of Elston and Ellis [17]. Histological staging was evaluated in line with TNM classification (2002). For study purposes, the size of the tumor (2 cm) was used for group selection. The presence or absence of metastases in lymph nodes was the criterion for axillary lymph node status. The Nottingham prognostic index was calculated using formula: NPI = 0,2x tumor size (cm) + LN status (1-3) + tumor grade (1–3) [18]. Final score was used for stratification of patients in three groups: group1 - NPI ≤ 3, 4; group 2 –NPI 3,5-5,4; group 3 - NPI > 5,4.

The anti-ER (clone ER1D5, Immunotech) and anti-PR (clone 1A6, Immunotech) antibodies were used for the detection of ER and PR, respectively. The ER and PR status was interpreted semiquantitatively as positive when more than 10% of tumour cells showed positive nuclear staining. HER2 immunohistochemical analysis was performed using the HercepTest kit (DakoCytomation, Glostrup). The results were interpreted as follows: negative – equal to absent or faint (partial) membrane staining; positive - equal to more than 10% of invasive cancer cells. In one case a chromogene in-situ hybridization (CISH) was performed for the exact determination of HER2 status. Both CISH and immunohistochemical examination controls were included in each stain.

DNA preparation. Genomic DNA was isolated from paraffin sections after deparaffinization in xylen and rehydratation through a series of descending concentrations of alcohol. DNA was isolated using the Wizard® Genomic DNA purification kit (Promega, USA) according to the manufacturer protocol. Briefly, 50 μl of Nuclei Lysis Solution was added to the tube containing the resuspended cells from the deparaffinized tumor tissue section and the samples were incubated at 56°C overnight. The proteins were precipitated using 50 μl of Protein Precipitation Solution. After centrifugation, the supernatant was transferred to a clean microcentrifuge tube containing 150 μl of isopropanol, DNA was precipitated by centrifugation, washed in 70% ethanol and resolved in TE buffer. The concentration of DNA was determined at 260 nm by spectrophotometry.

Bisulfite treatment. Sodium bisulfite conversion of unmethylated cytosine residues to uracil in samples of genomic DNA obtained from breast cancer tumor tissue was performed using the CpGenome™ DNA Modification Kit (Millipore, USA) according to the manufacturer's protocol with our modifications concerning DNA concentration, because of highly degraded DNA isolated from FFPE tissues, where we sequenced the methylation specific PCR products, to confirm the DNA bifulfite modification [9]. Briefly, 5-8 μg of genomic DNA resolved in 100 μl of H_2O were

denaturated with 7.0 µl of 3 M NaOH and incubated overnight. DNA was then bound to a 5 µl micro-particulate carrier in the presence of 750 µl of Reagent II responsible for desulfonation. Then, the modified DNA was desalted by repeated washing with 70% ethanol and subsequent centrifugation. The DNA was finally eluted from the carrier by heating in 25 µl TE buffer, divided into 5 µl aliquots and stored at -20°C.

Methylation specific polymerase chain reaction (MSP). The methylation status of the promoter region of the GSTP1 gene was determined by MSP. We used a nested two-step approach (20) with our own modifications. The PCR reaction was performed with 1.0 µl of bisulfite modified DNA template in 25 µl of reaction mixture containing 2.5 mmol/L $MgCl_2$, 10 pmol/L of each forward and reverse primer, 0.5 mmol/L of each of the four dNTPs, 2.5 µl of 10x PCR Buffer (ABgene®, United Kingdom) and 1 unit of Thermostart Taq polymerase (ABgene®, United Kingdom). The primer sequences and PCR product size for this nested MSP approach are shown in the Table 2. Briefly, the first step PCR was carried out with primer sets GSTP1Fext and GSTP1Rext designed to bind to both, methylated and unmethylated DNA. The PCR products from the first step were diluted 1:500 and subjected to the

Table 2. Primer sequences for nested MSP.

Primer	Sequence	Size (bp)
GSTP1Fext	GGGATTTTAGGGYGTTTTTTTG	
GSTP1Rext	ACCTCCRAACCTTATAAAAATAATCCC	159
GSTP1MF	TTCGGGGTGTAGCGGTCGTC	
GSTP1MR	GCCCCAATACTAAATCACGACG	91
GSTP1UF	GATGTTTGGGGTGT-AGTGGTTGTT	
GSTP1UR	CCACCCCAATACTAAATCACAACA	97

second PCR with primer sets GSTP1MF and GSTP1MR, and GSTP1UF and GSTP1UR, binding to methylated DNA and unmethylated DNA, respectively. The PCR reaction was performed in duplicate and was subjected to hot start at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72 °C for 30 seconds, and the final step of 4 minutes at 72°C. The second step PCR was performed by the identical PCR program with the exception of annealing temperature, which was 60°C. For each set of bisulfite modification and PCR, a positive and negative control for methylated and unmethylated DNA, CpGenome Universal Methylated DNA (Millipore, USA) and CpGenOme Unmethylated DNA (Millipore, USA) were used, respectively. Water with no DNA template was included as a control for possible contamination.

Twelve µl of each PCR reaction were loaded onto 1.75% agarose gel, stained with ethidium bromide and visualized using UV illuminator. The 50 bp ladder (Fermentas, Germany) was used as molecular weight standard.

Statistical evaluation The chi-square test for trend and Fisher's exact test were applied to determine whether the observed variable frequencies markedly differ from the frequencies that were expected by methylation changes in GSTP1. A $p < 0.05$ was considered statistically significant. All calculations were performed using the Excel 2000 (Microsoft Corporation®).

Results

Initially, 48 patients were included in the study group with histological diagnosis of invasive breast carcinoma. Three patients were excluded from the MSP analysis because of highly degraded DNA isolated from the FFPE tissue section. Using the nested MSP, we examined the methylation status of promoter region of the GSTP1 gene in FFPE sections from 45 patients with breast cancer. In the first round PCR, the primers amplify both methylated and unmethylated DNA. In the second round PCR, the primers specific for unmethylated DNA and methylated DNA binding to converted or methylated cytosines, respectively, are used. Figure 1 shows representative results of the MSP on breast cancer tissue samples. The presence of visible PCR product in the lanes 4m and 5m of the tumor DNA indicates the presence of methylated alleles of GSTP1 promoter region. The absence of a PCR product in the lanes 1m, 2m indicates that these clinical samples have unmethylated alleles only. The PCR products in lanes 1u, 2u, 3u, 4u, 5u of the tumor DNA most likely represents presence of the normal cells with unmethylated GSTP1 or partially methylated tumor cells. The positive control, CpGenome Universal Methylated DNA yielded a PCR product with the primers specific for methylated DNA (lane 6m), because this control sample contains methylated alleles only. Likewise, the CpGenome Universal Unmethylated DNA contains unmethylated alleles only and a PCR product is visible by use of primers specifically binding to unmethylated DNA (lane 7u). Of all tested patients, 11 (24.4%) samples showed GSTP1 promoter region methylation. By application of GSTP1 primer set binding to unmethylated DNA we were able to detect the presence of unmethylated DNA in each tested sample.

The evaluation of relationship between the GSTP1 promoter region hypermethylation and the selected clinicopathologic parameters summarized in the table 2 showed a significant association between GSTP1 promoter region hypermethylation and tumor grade ($p = 0.0016$) with approximately 50% samples in grade 3 being positive for GSTP1 methylation, and lymph node metastases pN ($p = 0,0142$). Both, ER and PR status showed significant positive correlation with methylation changes in GSTP1 $p= 0.0225$ and $p=0.0306$. When we applied NPI into statistical analysis, the significant association between the presence of methylation in GSTP1 and higher NPI ($p = 0.0017$) was calculated. Analyzing the relationship between age of disease onset and GSTP1 hypermethylation, we found significant correlation neither in general group, nor by stratification of subjects by age decades. No significant association was found between GSTP1 and tumor type ($p=0.0912$), tumor size ($p=0.2984$) and HER2 expression ($p=0.9999$).

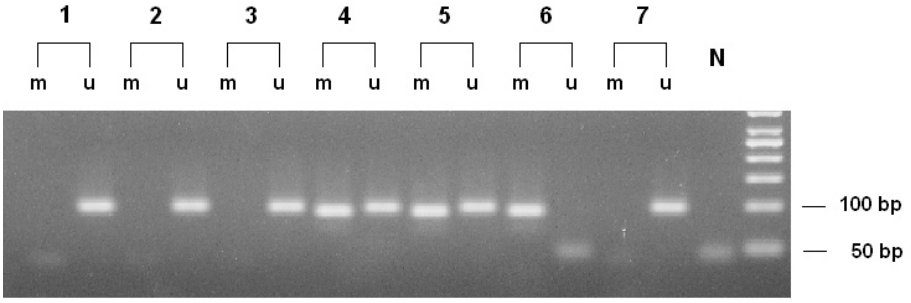

Figure 1. Representative agarose gel electrophoresis of methylation-specific PCR for GSTP1 in bisulfite modified DNA isolated from FFPE breast cancer tissue and in bisulfite modified control DNA samples.

The samples 1 to 5 correspond to DNA from patients with breast cancer, sample 6 is CpGenome Universal Methylated DNA, and sample 7 is CpGenome Universal Unmethylated DNA. In lane N (negative control), only water was used instead of DNA. The lanes m and u are PCR products yielded from methylated and unmethylated DNA, respectively. Primer set GSTP1MF and GSTP1MR was used for the detection of methylated DNA, primer set GSTP1UF and GSTP1UR was used for detection of unmethylated DNA yielding 91 and 97 bp PCR product, respectively. As molecular weight standard, 50 bp ladder (Fermenats, Germany) was used.

Table 3. Degree of association between clinicopathological parameters and GSTP1 methylation in tumors

Clinicopathological parameter	n	GSTP1 Methylation		P value
		Present	Absent	
Histological type				
Ductal	37	11	26	
Lobular	4	0	4	
Other	4	0	4	p = 0.0912[#]
Histological grade				
Grade 1	8	0	8	
Grade 2	16	1	15	
Grade 3	21	10	11	p = 0.0016[#]
Tumor size				
≤ 2cm	24	4	20	
> 2cm	21	7	14	p = 0.2984[†]
Lymph node status				
LN negative	23	2	21	
LN positive	21	9	12	p = 0.0142[†]
NPI				
NPI 1	14	0	14	
NPI 2	19	5	15	
NPI 3	11	6	5	p = 0.0017[#]
ER status				
ER-negative	12	0	12	
ER-positive	33	11	22	p = 0.0225[†]
PR status				
PR-negative	18	1	17	
PR-positive	27	10	17	p = 0.0306[†]
HER2 status				
HER2- negative	5	1	4	
HER2- positive	40	10	30	p = 0.9999[†]

LN – lymph node; NPI – Nottingham prognostic index; ER – estrogen receptor; PR – progesterone receptor

[#]P value obtained from Chi-square test for trend, [†]P value obtained from Fisher´s exact test

Discussion

Aberrant promoter methylation has been reported for different genes and is thought to become a serious marker for cancer detection, prognosis and therapy [8]. Promoter hypermethylation of GTSP1 is very specific for prostate cancer and is suggested as a marker for early disease detection [12, 13]. In breast cancer, numerous epigenetic molecular biomarkers have been discovered and considered as target for chemoprevention and therapy (9, 10). Using different MSP approaches, several studies indicated the impact of the GTSP1 expression and/or hypermethylation in breast cancer tissues on prognosis and response to chemotherapy [22, 25–27].

In our study, we established a nested MSP for the detection of methylation status of the GSTP1 promoter region. The methylated GSTP1 alleles were present in 24,4% (11 from 45 tested) of breast cancer sections cases. That is within the range of 13 to 30 % reported by others using qualitative approaches [23–25, 30, 31] but bellow the 50% of a quantitative MSP approach (32). Currently, at least three different approaches are used for testing of GSTP1 methylation in clinical samples and more modification of these methods exist. MSP is highly sensitive and can identify 1 methylated allele in 1000 unmethylated alleles [23], while the nested MSP has a sensitivity 1 methylated allele in 50 000 [29, 30]. Quantitative MSP shows the highest sensitivity, and can detect GSTP1 methylation in a histological as normal appearing tissue also [13, 28], which allows also quantification and comparison of the methylation levels in samples of different patients after setting an appropriate threshold value. The sensitivity of the detection methods can be influenced by the sample preparation such as use of snap frozen (32) or FFPE sections [23–25, 30]. When the DNA is isolated from FFPE, it is recommended to use sections with more than 70% target cells after deparaffinization [32], which is a good way for standardization of the first steps for methylation testing.

As previously reported GSTP1 is frequently hypermethylated in breast cancer [15] and prostate cancer [12], but infrequently in other cancer tissues (15) and is therefore considered as suitable biomarker [22, 31]. It has been shown by two groups [25, 26], that methylation of GSTP1 correlates with lymph node metastasis. Our data are consistent with these reports. Furthermore, we demonstrated a significant correlation of GSTP 1 hypermethylation with tumor grade (p=0.0016) as well as with NPI (p=0.0017). The previous reports were not able to show a correlation to histological grade, which may be due to different constitution of patient's groups concerning grading. In our group, grade 1 was represented by 18.8%, grade 2 by 37.5% and grade 3 by 43.8%. In the above mentioned reports [25, 26], grade 1 represents 12 and 20%, grade 2 78% and 66%, and grade 3 15% and 14%, respectively. When compared with patients groups from the study dealing with the prognostic relevance of histological grade (34), where from 372 patients 15.3% patients were with grade 1, 40.9% patients with grade 2 and 43.8% patients with grade 3, is our constitution of patients group more representative. NPI is widely used method of integration independently significant factors [18] and can be suitable as a basis of analysis of new prognostic factors. We were able to demonstrate a significant correlation of the methylation of GSTP1 with NPI, which supports the data obtained by Arai and coworkers [26], where a significant correlation between relapse-free survival rates and methylation status of GSTP1 ($p < 0.01$) was shown. These findings indicate, that the hypermethylation of GSTP1 promoter region is a putative prognostic factor in breast cancer, however, we suggest, that this correlation have to be confirmed on a larger patients group.

Our statistical analysis showed significant correlation between GSTP1 methylation and ER and PR status. Sunami and coworkers [22] reported about a significantly higher GSTP1 methylation in ER-positive tumor group and showed that in both LN metastasis positive and negative groups, GSTP1 hypermethylation is more common in ER-positive tumors. Recently, it was demonstrated that GTSP1 hypermethylation is an early event in breast carcinogenesis and occurs in ductal hyperplasia also [30]. Furthermore, the differences in methylation status of other genes involved in breast carcinogenesis RASF1A and CCND between ER-positive and ER-negative groups can be recognized in early stages of cancer [22, 30] and it is known that methylation and ER status change with tumor progression [36], suggesting that ER expression may influence epigenetic changes in early stages of the disease.

It has been shown that GSTP1 methylation could be a putative biomarker for disease detection and putative new prognostic factor [22–26], and the association with resistance to docetaxel and paclitaxel and GSTP1 methylation was reported [27] also. Recently, the efforts of developing a sensitive screening test for diagnostic accuracy in fine needle aspirate washing of breast lesion were reported [32], where a panel of 23 genes was analyzed. The GSTP1 was one of 10 genes showing significant differences between breast carcinoma, fibroadenoma and normal breast samples. When six genes were examined for methylation status, the GSTP1 methylation was demonstrated in a nipple aspirate fluids [23] in 7 out of 22 (31%), and in 6 out for 7 matched probes of aspirate/tissue samples, demonstrating also a suitability of GSTP1 for early disease detection. However, more examinations are needed to proof, if patient with GSTP1 hypermethylation in early stage of breast cancer have a worse prognosis taking into consideration the ER and PR status, until GSTP1 could be considered as both – a marker of an early stage of the disease and a prognostic marker.

Even though the GSTP1 hypermethylation is reported to occur with different frequencies in breast cancer tissue, it is considered to be a putative prognostic and predictive parameter and a biomarker of the disease. However, only when the results of multiple studies are confirmed in larger group of patients together with other genes involved in epigenetically regulation of breast cancer, detection of promoter hypermethylation can find application in clinical practice.

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