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ORIGINAL ARTICLE

GSTP1 promoter hypermethylation is an early event in breast carcinogenesis

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Abstract Promoter hypermethylation in precursor lesions of the breast cancer may be biomarkers of cancer risk and targets for cancer chemoprevention. Pi-class glutathione-*S*-transferases (GSTP1) is inactivated by promoter hypermethylation in invasive breast cancers. However, little is known about epigenetic silencing of GSTP1 gene by promoter hypermethylation in precursor lesions. To determine the significance of GSTP1 promoter hypermethylation in breast carcinogenesis, methylation status of GSTP1 gene was studied by nested methylation-specific polymerase chain reaction, and GSTP1 expression was studied by immunohistochemistry in invasive ductal carcinoma (IDC), ductal carcinoma in situ (DCIS), usual ductal hyperplasia (UDH), and normal breast tissue. GSTP1 promoter hypermethylation was detected in 4/24 (16.7%) of UDH, 18/49 (36.7%) of DCIS, and 14/36 (38.9%) of IDC. No hypermethylation was detected in normal breast tissues. GSTP1 promoter hypermethylation was found to be progressively elevated during breast carcinogenesis ($p<0.01$). GSTP1 promoter hypermethylation was associated with loss of GSTP1 expression ($p<0.01$ for UDH, $p<0.001$ for DCIS and IDC). Our results suggest that GSTP1 promoter hypermethylation is an early event in breast carcinogenesis and appears to functionally silence GSTP1 expression. GSTP1 promoter hypermethylation in the precursor lesions of breast cancer may be used as a target for cancer chemoprevention.

Keywords Breast · Carcinogenesis · Methylation · GSTP1

Introduction

The currently favored working hypothesis of breast cancer evolves in a linear progression through sequential stages of hyperplastic benign breast lesions with or without cellular atypia, carcinoma in situ, and ultimately, invasive cancer [2, 7]. Molecular studies have shown that numerous genetic alterations found in invasive cancer are also found with increased frequency during progression in this proposed continuum [5, 16, 25]. Inactivation of tumor suppressor genes by promoter hypermethylation has been considered as a potentially important mechanism involved in the development of breast cancer. It is generally assumed that breast cancer develops in a stepwise manner from normal breast epithelium towards cancer by the accumulation of epigenetic alterations [24].

Glutathione-*S*-transferases (GST) are an enzyme family that can detoxify reactive chemical species by catalyzing their conjugation to reduced glutathione [6]. Among the isoenzymes, the role of pi-class GST (GSTP1) is involved in the metabolism, detoxification, and elimination of potentially genotoxic foreign compounds and, thus, acts to protect cells from DNA damage and cancer initiation [13]. Previous studies have shown that the CpG-rich promoter regions are methylated in several types of human cancers [4, 23, 26]. GSTP1 promoter hypermethylation is also associated with loss of GSTP1 expression, as measured by immunohistochemistry [4, 26]. Previous studies have

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already investigated the methylation status of GSTP1 in invasive breast cancer [1, 8, 11, 19, 21]. Promoter hypermethylation of GSTP1 is associated with a poor prognosis of breast cancer [1]. However, little is known about epigenetic silencing of GSTP1 gene by promoter hypermethylation in the precursors of breast cancer. Consequently, the significance of alterations in GSTP1 promoter hypermethylation status during multistage carcinogenesis of the breast is not fully understood. Cancer chemoprevention can be defined as the prevention of cancer or treatment of identifiable precursors [22]. If GSTP1 promoter hypermethylation occurs in precursor lesions, then this opens new avenues for cancer chemoprevention based on the inhibition or reversal of epigenetic alterations before the onset of invasive breast cancer. Moreover, the detection of promoter hypermethylation in precursor lesions at risk for progression to invasive cancer paves the way for the use of DNA methylation markers in risk assessment.

To elucidate the role of GSTP1 in breast carcinogenesis, we have investigated the promoter methylation status of GSTP1 in normal breast tissues, usual ductal hyperplasia (UDH), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC). We have also studied the expression of GSTP1 protein by immunohistochemistry and compared its expression with GSTP1 methylation status.

Materials and method

Tissues

A total of 124 breast specimens, representing 15 normal breast tissues, 24 UDH, 49 DCIS, and 36 IDC, not otherwise specified, were selected from the files of the Department of Pathology, Chonnam National University Hospital, Gwangju, South Korea. We only selected stage I IDC to rule out complex genetic alterations relating to metastasis.

Nested methylation-specific PCR

For DNA extraction, one 5-μm tissue section was deparaffinized with xylene. On a mirror-imaged hematoxylin–eosin stained slide, the region of interest was manually scraped off to ensure a specific cell population of more than 80% in the preparation. The scraped tissue extracted in 27 μl TNES (10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% SDS) containing 3 μl proteinase K for 4 h at 52°C. The tissue extract was heat inactivated at 99°C for 10 min and clarified by centrifugation at 13,000 rpm for 30 s. Supernatant (13.5 μl) was used directly as a source of DNA for sodium bisulfite treatment. DNA was heated 10 min to 99°C, quick chilled, and incubated with 1.5 μl of 2 mol/l NaOH for 30 min at 42°C. Freshly prepared 3.6 mol/l sodium bisulfite containing 1 mmol/l hydroquinone was mixed with DNA, then overlaid with oil, and incubated at 55°C for 5 h in the dark. The sample was desalted using ion exchange columns (Amersham, Piscataway, NJ, USA). DNA was precipitated with 200-μl absolute alcohol, washed with 70% ethanol, air dried, and resuspended in water (20 μl). Samples were stored at −80°C until use.

To facilitate methylation-specific PCR (MSP) analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, the methylation status of GSTP1 was analyzed by a nested, two-step PCR approach, as described previously, with slight modifications [18]. Step 1 of the nested MSP was performed to amplify a 303-bp fragment of the GSTP1 gene with primer sets flanking the CpG-rich promoter region. Hence, these primers did not discriminate between methylated and unmethylated nucleotides after bisulfite treatment. The primer sequences for step 1 were 5′-ATT TGG GAA AGA GGG AAA GGT-3′ as the forward and 5′-ACT AAA AAC TCT AAA CCC CAT C-3′ as the reverse. Reactions were carried out using the following conditions: 95°C for 5 min; then 36 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 40 s; and a final extension step at 72°C for 5 min. A negative control for the assay (water only) and unmethylated (human sperm DNA) and methylated controls (MDA-MB-231 cells) were included in step 1 reactions.

PCR products from step 1 reactions were diluted 1:5 in water and subjected to the second step of MSP, which incorporated one set of primers (labeled as unmethylated or methylated) that were designed to recognize bisulfite-induced modifications of unmethylated cytosines. The sense and antisense primers for the methylated GSTP1 sequence were 5′-TTC GGG GTG TAG CGG TCG TC-3′ and 5′-GCC CCA ATA CTA AAT CAC GAC G-3′, respectively. The sense and antisense primers for the unmethylated sequence were 5′-GAT GTT TGG GGT GTA GTG GTT GTT-3′and 5′-CCA CCC CAA TAC TAA ATC ACA ACA-3′, respectively. The PCR amplification protocol for step 2 was as follows: 95°C for 5 min, then denature at 95°C for 30 s, anneal at 60°C for 30 s, extension at 72°C for 40 s for 30 cycles followed by a 5-min final extension. Primer sequences used in the step 2 amplification were previously described [8].

The PCR products were resolved by electrophoresis in a 2% agarose gel, and the ethidium bromide-stained PCR products were imaged with the Eagle Eye II Video System (Stratagene, La Jolla, CA, USA). Any tumor sample that reliably yielded a PCR product in the methylated reaction visible by ethidium bromide staining was considered positive for promoter hypermethylation.

Immunohistochemistry

Immunohistochemical analysis of GSTP1 protein expression in matching tissue sections was performed by applying the avidin–biotin–peroxidase complex method. Sections were deparaffinized and then were placed in a microwave oven with a 2.1% citric acid buffer solution (pH 6.0) for 10 min to retrieve the antigens. After microwave processing, the sections were incubated overnight at 4°C with primary anti-GSTP1 antibody (1:100 dilution, clone GSTpi, Novocastra, Burlingame, CA, USA). The streptavidin–horseradish peroxidase (Research Genetics, Huntsville, AL, USA) detection system was then applied, followed by 30 min of incubation at room temperature. After washing, the tissue sections were developed with a peroxidase substrate solution (3,3-diaminobenzidine tetrahydrochloride). The sections were counterstained with hematoxylin, dehydrated, and mounted. For negative controls, sections were treated similarly with the exception of the primary antibody.

GSTP1 expression was considered to be positive when >10% of cells exhibited cytoplasmic or nuclear staining [12].

Statistical analysis

The χ^2 test was used for group comparison. For the statistical analysis, a two-sided value of $p<0.05$ was considered to indicate statistical significance. Data were analyzed using the SPSS software 11.5 for windows.

Results

Frequency of GSTP1 promoter hypermethylation

GSTP1 promoter hypermethylation was detected in 16.7% (4 of 24) of UDH, 36.7% (18 of 49) of DCIS, and 38.9% (14 of 36) of IDC samples. No hypermethylation was detected in normal breast tissues. Representative results of MSP assay are shown in Fig. 1. The frequency of GSTP1 promoter hypermethylation was found to be progressively increased along the continuum from normal breast tissue to IDC ($p<0.01$). DCIS and IDC showed statistically higher frequency of GSTP1 promoter methylation compared to normal breast tissues ($p<0.01$ and $p<0.01$, respectively). Although not statistically significant, the incidence of GSTP1 promoter hypermethylation was higher in high nuclear grade DCIS: 18.2% (2 of 11) for grade 1, 35.3% (6 of 17) for grade 2, and 47.6% (10 of 21) for grade 3.

We also determined the association of risk factors, such as age, tumor grade, and estrogen receptor status with GSTP1 promoter hypermethylation in IDC. None of the risk factors showed significant association with promoter hypermethylation of GSTP1 (data not shown).

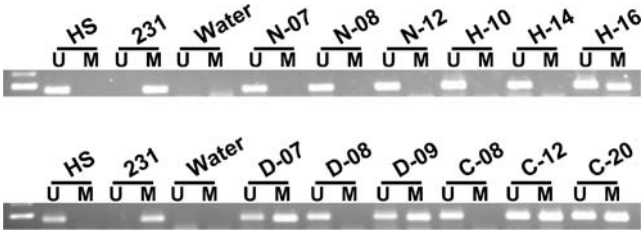

Fig. 1 Representative results of nested methylation-specific PCR analysis for GSTP1 in breast carcinogenesis. *Lanes U and M* correspond to unmethylated and methylated DNA, respectively. DNA from human sperm (*HS*) and MDA-MB-231 (*231*) breast cancer cell line served as negative and positive controls for methylated genes, respectively. Water served as negative control for both unmethylated and methylated genes. *N* normal breast case; *H* usual ductal hyperplasia case; *D* ductal carcinoma in situ case; *C* invasive ductal carcinoma case

Immunohistochemical analysis of GSTP1

To determine whether the GSTP1 promoter hypermethylation was correlated with the loss of GSTP1 expression, immunohistochemical staining using an anti-GSTP1 antibody was carried out on all samples that were subjected to methylation analysis in this study. Representative results of GSTP1 immunohistochemistry are shown in Fig. 2. In all of the 15 normal breast tissues, mammary epithelium including myoepithelial cells showed both nuclear and cytoplasmic immunoreactivity for GSTP1. GSTP1 was also detected in non-epithelial cells (e.g., stromal cells and inflammatory cells). Loss of GSTP1 expression (negative) was observed in 8.3% of UDH, 49.0% of DCIS, and 41.7% of IDC. Strong staining was found in non-neoplastic breast tissues of all samples with UDH, DCIS, and IDC. When the GSTP1 expression was compared against the methylation status of GSTP1, GSTP1 expression was more frequently lost in breast tissues with methylation ($p<0.01$ for UDH, $p<0.001$ for DCIS and IDC; Table 1).

Discussion

GSTP1 is known to play a role in detoxification of potential carcinogens [13]. Breast epithelial cells with lack the expression of GSTP1 is supposed to suffer from DNA damage more easily upon exposure to carcinogens. Loss of GSTP1 expression is observed in approximately two thirds of breast cancers, which indicates its potential role in breast carcinogenesis [1]. GSTP1 promoter hypermethylation appears likely to be responsible for absence of GSTP1 expression [4, 26]. Previous studies have described the significance of GSTP1 promoter hypermethylation in invasive breast cancer [1, 8, 11, 19, 21]. To determine when, in the breast carcinogenesis, promoter hypermethylation of the GSTP1 gene begins to play a role, we analyzed the

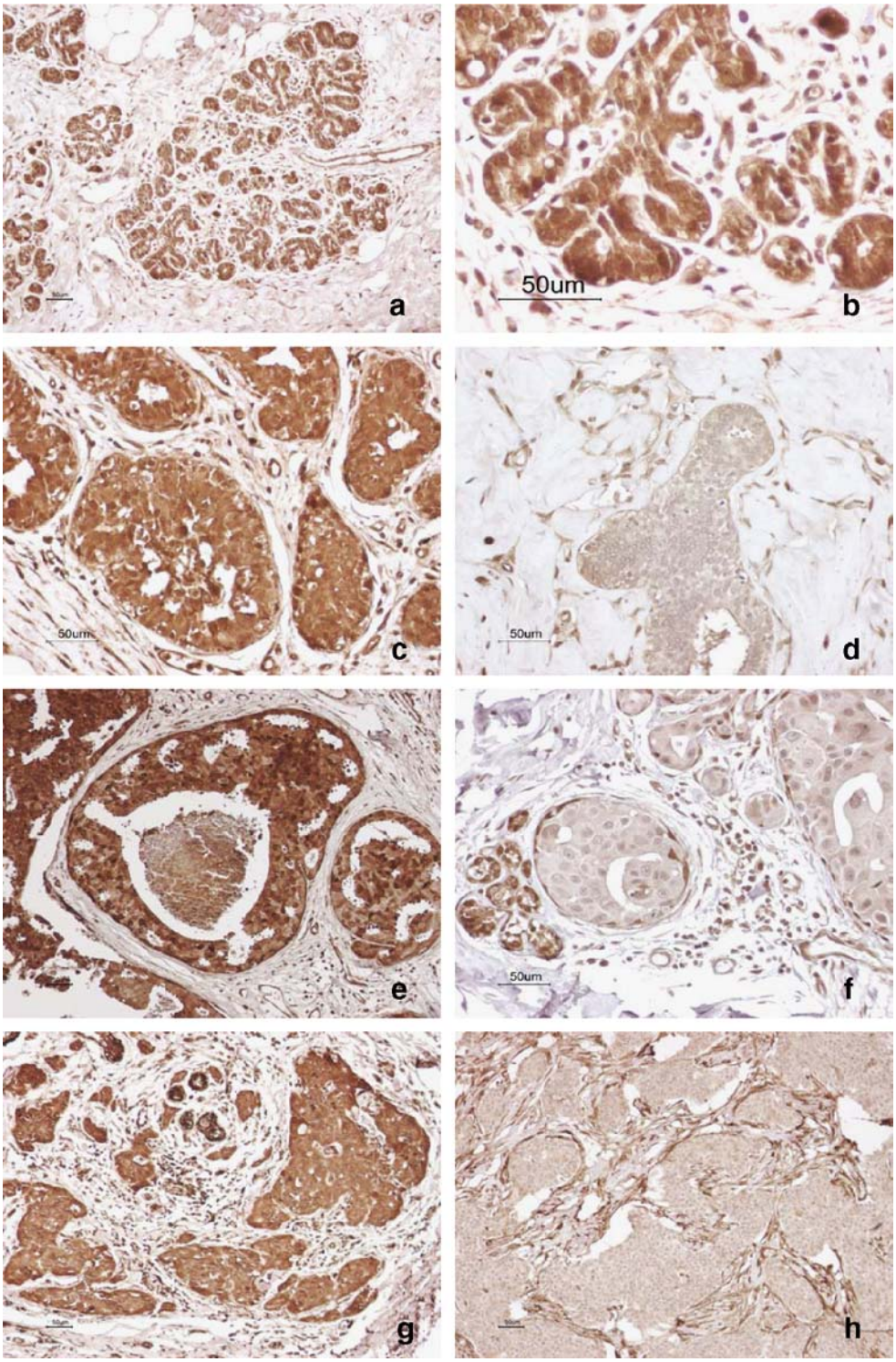

Fig. 2 Positive and negative immunohistochemical staining of GSTP1 in normal breast tissue, UDH, DCIS, and IDC. Normal breast tissues show strong, diffuse nuclear and cytoplasmic staining representing a positive expression of GSTP1 (**a** and **b**). UDH (**c**), DCIS (**e**), and IDC (**g**) show strong positivity in unmethylated cases. In methylated UDH (**d**), DCIS (**f**), and IDC (**h**), loss of GSTP1 expression is observed. Adjacent non-neoplastic breast tissues show strong GSTP1 expression

promoter hypermethylation of GSTP1 in invasive breast cancer as well as its precursor lesions. We found out that GSTP1 promoter hypermethylation is an early event in breast carcinogenesis.

The frequency of GSTP1 promoter hypermethylation in IDC (38.9%) was found to be higher than reported previously. The frequency given in other studies of breast cancer varied between 13 and 30% [1, 8, 11, 19, 21]. The higher detection rate in our study might be due to the nested MSP. A PCR-based approach called MSP can detect 1 copy of methylated DNA in 1,000 unmethylated copies of genomic DNA [10]. We used nested MSP. This method is very sensitive and can detect 1 methylated allele in >50,000 unmethylated alleles [18].

Table 1 GSTP1 expression in usual ductal hyperplasia, ductal carcinoma in situ, and invasive ductal carcinoma according to GSTP1 promoter hypermethylation

	Usual ductal hyperplasia			Ductal carcinoma in situ			Invasive ductal carcinoma		
	GSTP1 negative	GSTP1 positive	*p* value	GSTP1 negative	GSTP1 positive	*p* value	GSTP1 negative	GSTP1 positive	*p* value
GSTP1 unmethylated	0 (0%)	20 (100%)	<0.01	9 (29.0%)	22 (71.0%)	<0.001	4 (18.2%)	18 (81.8%)	<0.001
GSTP1 methylated	2 (50%)	2 (50%)		15 (83.3%)	3 (16.7%)		11 (78.6%)	3 (21.4%)	

Previous studies reported that GSTP1 promoter hypermethylation is associated with loss of GSTP1 protein expression, as measured by immunohistochemistry [1, 4, 26]. In this study, we have demonstrated a significant correlation between the promoter hypermethylation of GSTP1 and the data obtained from immunohistochemical analyses. Promoter hypermethylation of GSTP1 resulted in loss of GSTP1 protein expression in samples with methylation. These data provide evidence that GSTP1 promoter hypermethylation is a major mechanism involved in GSTP1 gene inactivation, resulting in impaired GSTP1 function during breast cancer development. While GSTP1 promoter hypermethylation status correlated well with GSTP1 expression, 21 cases of IDC and its precursors gave discordant data. The inconsistency between GSTP1 promoter hypermethylation and protein expression has been reported previously [1, 4, 8, 26]. Twenty-one cases can be divided into two groups: methylated and positive group (8 cases) and unmethylated and negative group (13 cases). The finding of GSTP1 hypermethylation in the fraction of IDC and its precursors that retained GSTP1 expression could be due to incomplete or low methylation. We used nested MSP. This is a very sensitive method, but it cannot distinguish high from low levels of promoter methylation. Using quantitative methylation analysis, Ogino et al. [17] found that tumors that show low levels of methylation in the gene promoters do not silence protein expression. GSTP1 unmethylated and negative group might be caused by mechanisms other than hypermethylation of GSTP1 promoter region. We concluded that GSTP1 is inactivated not only by promoter hypermethylation but also by other factors or mechanisms.

The potential reversibility of epigenetic states offers exciting opportunities for novel cancer drugs that can reactivate epigenetically silenced tumor suppressor genes. If epigenetic changes occur in precursor lesions of cancer, these changes may be targets for chemoprevention. GSTP1 detoxify carcinogens by conjugating them with glutathione. It is suggested that GSTP1 plays an important role in the prevention of the development of cancer upon exposure to carcinogens [13]. GSTP1 is a good candidate for cancer chemoprevention. To date, almost all of the studies on GSTP1 promoter hypermethylation have been analyzed in invasive breast cancer. To our knowledge, there are no data available about GSTP1 promoter hypermethylation in precursor lesions of breast cancer. In this study, promoter region of GSTP1 gene was methylated in 36.7% of DCIS and in 16.7% of UDH. However, GSTP1 promoter hypermethylation was not detected in normal breast tissues. Although not statistically significant, the incidence of GSTP1 promoter hypermethylation was higher in high nuclear grade DCIS. Loss of GSTP1 expression was observed in 49.0% of DCIS and in 17.7% of UDH. The data of GSTP1 immunohistochemistry in DCIS are consistent with previous report [3]. Promoter hypermethylation and loss of GSTP1 expression in DCIS and UDH provide opportunities for breast cancer prevention strategies including restoration of GSTP1 function via treatment with inhibition of promoter methylation.

Consistent with our results, previous studies reported the detection of promoter hypermethylation for other genes in tissue samples from precursor lesions of breast cancer [9, 14, 15]. The histologic appearance of precursor lesions is similar regardless of whether they progress or stabilize, suggesting that there may be morphologically silent molecular differences that result in progression, regression, or stabilization of these precursor lesions [2]. Promoter hypermethylation in precursor lesions can be used as a cancer risk marker. Promoter hypermethylation of p16, RUNX3, and HPP1 in Barrett's esophagus or low-grade dysplasia was independently associated with an increased risk of progression of Barrett's esophagus to high-grade dysplasia or esophageal adenocarcinoma [20]. It is presently unknown whether precursor lesions with GSTP1 promoter hypermethylation harbor an increased risk of malignant transformation. It would be interest to test in retrospective studies whether precursor lesions with and without promoter hypermethylation of GSTP1 may differ for the risk of developing invasive breast cancer.

In conclusion, we have shown that GSTP1 promoter hypermethylation occurred almost exclusively in IDC and its precursor lesions. GSTP1 promoter hypermethylation was found to be progressively elevated along the continuum from normal breast tissue to invasive breast cancer. GSTP1 promoter hypermethylation was associated with loss of

GSTP1 expression. Our results suggest that GSTP1 promoter hypermethylation is an early event in breast carcinogenesis and appears to functionally silence GSTP1 expression. GSTP1 promoter hypermethylation in the precursor lesions of breast cancer may be used as a target for cancer chemoprevention and a cancer risk marker.

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