

# Inactivation of Glutathione S-Transferase *P1* Gene by Promoter Hypermethylation in Human Neoplasia<sup>1</sup>

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## Abstract

Glutathione S-transferases (*GSTs*) are a family of isoenzymes that play an important role in protecting cells from cytotoxic and carcinogenic agents. The  $\pi$ -class *GST* has been associated with preneoplastic and neoplastic changes. Recently, it has been reported that regulatory sequences near the *GSTP1* gene, which encodes the human  $\pi$ -class *GST*, are commonly hypermethylated in prostatic carcinomas. In the present study, we studied more than 300 primary human tumors originating in other organs for aberrant methylation of *GSTP1* using methylation-specific PCR. *GSTP1* hypermethylation was most frequent in breast and renal carcinoma, showing aberrant methylation in 30 and 20% of the cases, respectively. Other tumor types showed promoter methylation only rarely or not at all. Hypermethylation of *GSTP1* was associated with loss of expression demonstrated by immunohistochemistry. Our results suggest that aberrant methylation of *GSTP1* may contribute to the carcinogenic process in breast and renal carcinomas.

## Introduction

The *GSTs*<sup>3</sup> are a family of enzymes implicated in the detoxification of a wide range of xenobiotics and chemotherapeutic agents (1, 2). *GSTs* catalyze the conjugation of glutathione with electrophilic compounds including carcinogens and exogenous drugs (1), resulting in less toxic and more readily excreted metabolites. There are four distinct classes ( $\alpha$ ,  $\mu$ ,  $\pi$ , and  $\theta$ ) of isozymes in the *GST* superfamily, each encoded by a different gene at different loci and with peculiar structural and functional characteristics (2). Consistent with its role in defending normal cells against electrophilic carcinogens, inherited homozygosity for null *GSTM1* and *GSTT1* alleles and a genetic variant in *GSTP1* may confer an increased risk of cancer (3–5). Indeed, *GSTP1* null mice show an increased risk of skin tumorigenesis induced by carcinogens (6).

The  $\pi$  class *GST* (*GSTP1*) is of particular interest to the study of cancer biology. *GSTP1* is expressed in normal tissues at varying levels in different cell types, and abnormal *GSTP1* activity and expression have been reported in a wide range of tumors including those of the breast and kidney (7–10). *GSTP1* is encoded by the *GSTP1* gene located in chromosome 11 (11). The 5' region of *GSTP1* contains a CpG island, and in cancer cells, the hypermethylation of the CG-rich area in the promoter region of tumor suppressor genes correlates with its loss of transcription, as demonstrated for many tumor suppressor genes. Recently, hypermethylation of regulatory sequences at *GSTP1*

associated with the loss of *GSTP1* expression has been found in the vast majority of human prostate carcinomas (12). However, it is not known if the epigenetic silencing of *GSTP1* occurs in other human tumor types.

To study the relevance of the promoter hypermethylation of the *GSTP1* gene in human neoplasia, other than prostate cancer, we examined more than 300 primary tumors for *GSTP1* aberrant methylation using MSP. The tumors included were breast, endometrial, ovarian, renal, bladder, colon, pancreatic, lung, head and neck carcinoma, melanomas, leukemias, lymphomas, gliomas, and meningiomas. Among the tumor types studied, only breast and renal carcinomas were common targets of *GSTP1* methylation-associated inactivation. Our results suggest an important role for the epigenetic silencing of the *GSTP1* gene in breast and renal carcinogenesis and may provide new clues for treatment strategies in these tumors.

## Materials and Methods

**MSP.** DNA methylation patterns in the CpG island of *GSTP1* gene were determined by chemical modification of unmethylated, but not the methylated, cytosines to uracil, and subsequent PCR using primers specific for either methylated or the modified unmethylated DNA (13). Primer sequences for *GSTP1* were for the unmethylated reaction 5'-GAT GTT TGG GGT GTA GTG GTT GTT-3' (upper primer) and 5'-CCA CCC CAA TAC TAA ATC ACA ACA-3' (lower primer) and for the methylated reaction 5'-TTC GGG GTG TAG CGG TCG TC -3' (upper primer) and 5'-GCC CCA ATA CTA AAT CAC GAC G-3' (lower primer). The annealing temperature was 59°C. Placental DNA treated *in vitro* with *SssI* methyltransferase was used as positive control for methylated alleles of *GSTP1*, and DNA from normal lymphocytes was used as negative control for methylated alleles of *GSTP1*.

Briefly, 1  $\mu$ g of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol, and resuspended in water. Controls without DNA were performed for each set of PCR. Ten  $\mu$ l of each PCR reaction were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

**Immunohistochemical Staining for *GSTP1*.** Formalin-fixed paraffin-embedded breast carcinoma sections were stained with anti-*GSTP1* polyclonal antibody (1:100 dilution; Immunotech), using an immunoperoxidase method (Vectastain ABC kit; Vector Laboratories), with 3,3'-diaminobenzidine as the peroxidase substrate on Bio-Tech Mate 1000 automated stainer (Ventana-BioTek Solutions, Inc., Tucson, AZ). Expression was determined by a single author (E. G.), who did not have knowledge of the molecular analysis of those samples.

## Results

***GSTP1* Promoter Methylation.** DNA obtained from 339 primary human tumors of different cell types was subjected to *GSTP1* promoter methylation study using MSP. The region chosen for *GSTP1* spans the area of greatest CpG density immediately 5' to the transcription start site, in an area studied previously for methylation changes (12). Normal lymphocytes, breast, kidney, lung, and liver were found completely unmethylated at the *GSTP1* promoter (Fig. 1). The prostate carcinomas showed a high rate of methylated tumors

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<sup>3</sup> The abbreviations used are: *GST*, glutathione S-transferase; *GSTP1*, *GST P1*; MSP, methylation-specific PCR.

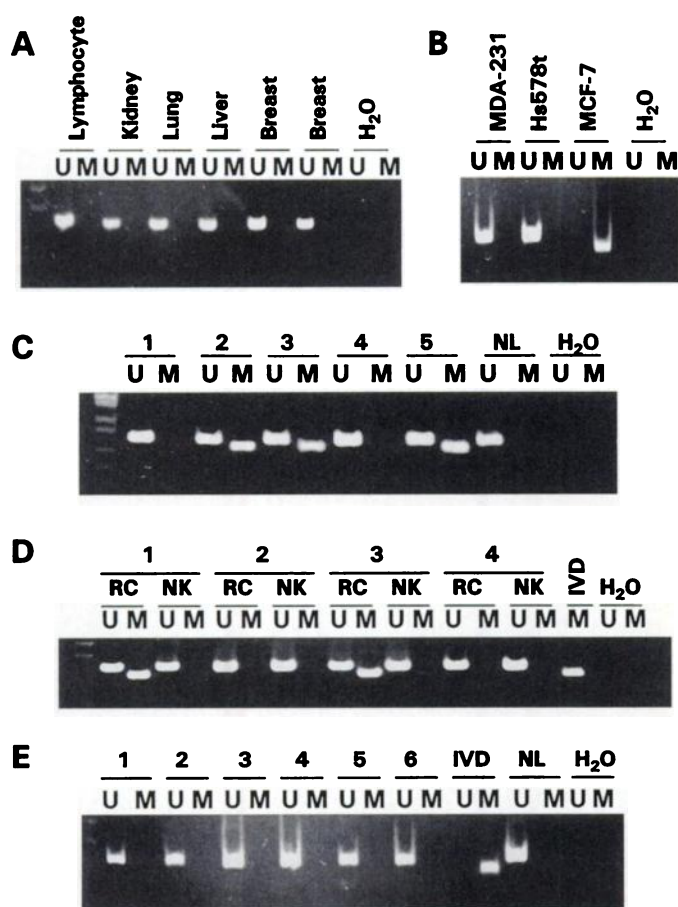


Fig. 1. Methylation-specific PCR of *GSTP1*. Left, molecular weight markers (PBR322/Msp digest). The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated genes of *GSTP1*; the presence of product in those lanes marked M indicates the presence of methylated genes. *H<sub>2</sub>O*, water control for PCR reaction. A, MSP of *GSTP1* in normal tissues. B, MSP of *GSTP1* in breast cancer cell lines. Corresponding lanes are: MDA-MB-231, Hs578t, and MCF-7. C, MSP of *GSTP1* in primary breast carcinomas (1–5). Normal lymphocyte (NL) DNA served as negative control. D, MSP of *GSTP1* in primary renal carcinomas. Primary tumors (RC) and adjacent normal kidney (NK) from patients (1–4) are shown. *In vitro* methylated DNA (IVD) was used as positive control for methylation. E, MSP of *GSTP1* in other primary human malignancies. Lanes 1 and 2, endometrial carcinoma; Lanes 3 and 4, ovarian carcinoma; Lanes 5 and 6, acute myelogenous leukemias; Lane IVD, *in vitro* methylated DNA as positive control for methylation; Lane NL, normal lymphocytes as negative control for methylation.

(five of six), and the prostate carcinoma cell line LNCaP was methylated as described previously (12).

The promoter of *GSTP1* was found unmethylated in all of the primary cases of endometrial ( $n = 20$ ), ovarian ( $n = 10$ ), bladder ( $n = 12$ ), pancreatic ( $n = 18$ ), and head and neck ( $n = 11$ ) carcinomas, as well as in melanomas ( $n = 12$ ), meningiomas ( $n = 18$ ), and leukemias ( $n = 10$ ; Fig. 1). A small percentage of abnormal *GSTP1* methylation was found in lung (2 of 21) and colon (1 of 23) carcinomas and non-Hodgkin's lymphomas (1 of 47). We also examined carcinoma cell lines derived from the same tissues and found that among 29 such cell lines, only 2 of lung origin were methylated at *GSTP1*. Table 1 summarizes the distribution of *GSTP1* promoter hypermethylation in all of the primary carcinomas and cancer cell lines studied.

In contrast to these tumor types, 24 of 77 (31%) breast and 7 of 41 (17%) renal primary carcinomas were hypermethylated at the *GSTP1* promoter (Fig. 1). Again, cell lines of these tumor types matched the incidence determined in primary tumors, with 8 of 18 (44%) breast and 3 of 11 (27%) renal cancer cell lines having aberrant methylation

at the *GSTP1* CpG island (Fig. 1). Abnormal methylation of the *GSTP1* promoter region in the breast carcinomas was not associated with any significant difference with the age of onset, histological type, cellular grade, tumoral size, nodal metastasis, DNA ploidy, or estrogen receptor status. However, the presence of methylated alleles showed a positive correlation with those tumors positive for the progesterone receptor ( $P = 0.05$ ). Among the renal carcinomas studied, 6 of 34 (18%) clear cell carcinomas and 1 of 7 (14%) collecting ductal tumors demonstrated evidence of methylation. No correlation was found between this epigenetic alteration and the age of onset, cellular grade, or clinical stage of the renal tumors. This change was slightly more frequent in the renal tumors in women (4 of 14 in women versus 2 of 20 in men), although this difference was not statistically significant. Paired normal kidney tissue from each patient was completely unmethylated, suggesting that this alteration arose during tumor progression and is not present in normal tissue (Fig. 1).

**Immunohistochemistry GST $\pi$ .** Paraffin sections from 47 breast and 6 renal carcinomas were studied immunohistochemically for GST $\pi$  expression using a standard peroxidase-antiperoxidase technique. Examples of the results obtained are shown in Fig. 2. All of the breast carcinomas studied showed expression of GST $\pi$  in adjacent normal mammary epithelium and nonepithelial cells (e.g., fibroblasts and inflammatory cells). Of the 47 breast carcinomas, 18 (38%) were classified as negative for GST $\pi$  expression, and 29 (62%) expressed GST $\pi$ . Of the 18 tumors that lacked GST $\pi$  expression, 14 (78%) displayed *GSTP1* promoter hypermethylation ( $P < 0.0001$  by Fisher's exact test), whereas none of the 29 tumors that expressed GST $\pi$  was methylated at the *GSTP1* locus. Among the six renal carcinomas in which GST $\pi$  expression was studied, three had complete loss of GST $\pi$  expression in tumor cells, all of which were hypermethylated at the *GSTP1* promoter region. Three other tumors retained GST $\pi$  expression and were unmethylated at the *GSTP1* locus. In both groups, GST $\pi$  expression was present in the adjacent normal renal tissue.

**Discussion**

Our data demonstrate the pattern of distribution of the aberrant promoter methylation-associated inactivation of the *GSTP1* gene in human primary tumors. Previously, the hypermethylation of CpG dinucleotide sequences located in the CpG island of *GSTP1* was reported in ~90% of human prostatic carcinomas (12). Our results suggest that outside of prostate carcinoma, silencing of *GSTP1* by aberrant methylation is restricted primarily to breast and renal carcinoma, at least within the tumor types examined in this study.

Nearly one-third of the primary human breast carcinomas studied showed *GSTP1* promoter hypermethylation. Previous studies have reported lack of GST $\pi$  expression in a similar percentage of primary

Table 1. Hypermethylation of *GSTP1* promoter in primary tumors and cell lines

	Primary tumors	Cell lines
Breast cancer	24/77 (31%)	8/18 (44%)
Renal cancer	8/35 (20%)	3/11 (27%)
Lung cancer	2/21 (9%)	2/9 (27%)
Colon cancer	1/23 (4%)	0/5
Endometrial carcinoma	0/20	
Ovarian cancer	0/10	0/5
Glioma	1/19	0/4
Meningioma	0/18	
Melanoma	0/12	
Leukemias	0/10	0/4
Lymphomas	1/47 (2%)	
Head and Neck carcinoma	0/11	0/1
Bladder carcinoma	0/12	
Pancreatic carcinoma	0/18	0/1
Prostate carcinoma	5/6 (83%)	

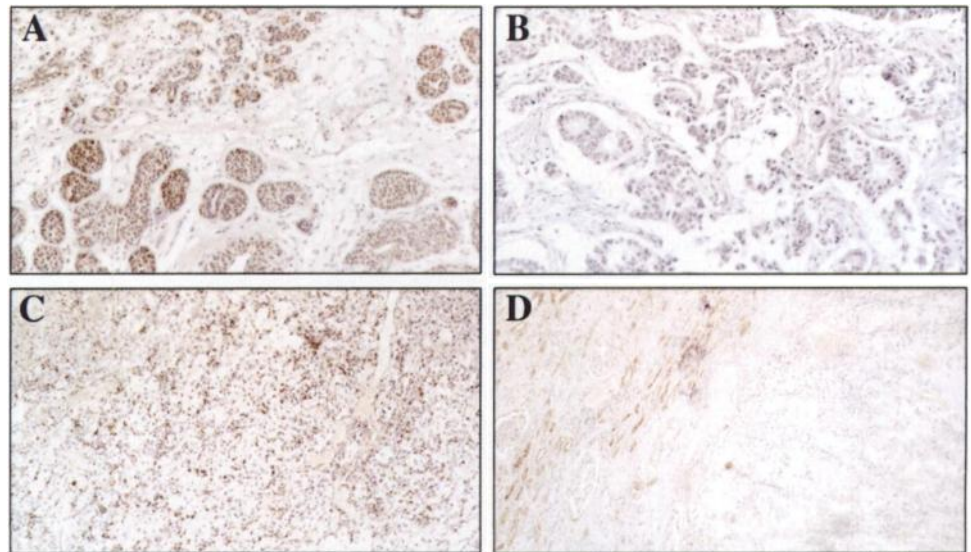


Fig. 2. Immunohistochemistry of GST $\pi$  in primary breast and renal carcinomas. A is a breast carcinoma unmethylated at *GSTP1* showing expression of the protein in all cells, whereas the breast carcinoma in B, which has *GSTP1* promoter hypermethylation, does not express the protein. C, a renal carcinoma unmethylated at *GSTP1*, which expresses the protein. D: right half, a renal carcinoma with aberrant methylation at *GSTP1* and lack of GST $\pi$  protein; left half, the normal kidney, which retains the expression of the protein.

breast carcinomas (8, 10), whereas the normal mammary epithelium always expresses GST $\pi$  (8). In our samples, the presence of the epigenetic alteration in *GSTP1* was associated with loss of expression of GST $\pi$  protein determined by immunohistochemistry, whereas all of the tumors that expressed GST $\pi$  were unmethylated at the *GSTP1* promoter. A minor percentage of tumors (8%) showed loss of expression without hypermethylation, and posttranscriptional differences have been described in human breast cancer cell lines (14). However, the overall strong association between hypermethylation and loss of expression suggests a causative role for aberrant methylation of the *GSTP1* promoter and the silencing of the gene in the majority of breast cancers lacking GST $\pi$  expression.

The involvement of GST $\pi$  in breast cancer is further supported by studies in breast cancer cell lines. A positive selection system designed to identify new candidate tumor suppressor genes by mRNA subtractive hybridization, comparing normal and tumor-derived human mammary epithelial cells, identified *GSTP1* as a gene whose expression was reduced in the breast cancer cells (15). The GST $\pi$  protein was also down-regulated in a number of mammary tumor-derived cell lines but strongly expressed in normal and immortalized mammary epithelial cells grown in culture (15). We confirmed that the *GSTP1* nonexpressing cell line MCF-7 (15) was fully methylated at the *GSTP1* promoter in our study, whereas the expressing cell lines, such as Hs578T and MDA-MB-231 (16), were completely unmethylated at this locus. A recent study has also shown that the use of the demethylating agent 5-aza-2'-deoxycytidine can induce the expression of *GSTP1* RNA in MCF7 cells (16).

How might loss of GST $\pi$  expression by promoter hypermethylation be involved in the development of breast carcinoma? Estrogen carcinogenesis has been mainly focused on the mitogenic effects generated by receptor-mediated processes. Evidence also suggests the effects of these compounds on genotoxic damage (17). The estrogens 17 $\beta$ -estradiol and estrone are metabolized via two major pathways: 16 $\alpha$ -hydroxylation and formation of catechol estrogens, the 2-hydroxy and 4-hydroxy derivatives. Oxidation of catechol estrogens to catechol estrogen quinones results in electrophilic intermediates that are conjugated with glutathione by the GSTs. If these inactivating processes are incomplete, the electrophilic intermediates react covalently with DNA, forming apurinic stable adducts. The resultant apurinic sites may generate mutations. Further supporting the involvement of this pathway in breast cancer is a recent report that genetic alterations in the catechol-*O*-methyltransferase gene, which encodes

for the enzyme involved in the first step of inactivation of the catechol estrogens, is associated with breast cancer risk (18). Epigenetic silencing of *GSTP1* by promoter methylation might similarly facilitate the carcinogenic action of estrogens as endogenous tumor initiators.

It is noteworthy that methylation of the *GSTP1* promoter was not observed in tumors originating in two other female tissues, ovary and uterus. Estrogens are clearly implicated as risk factors for the development of endometrial cancer (19). However, the role for estrogen in this process is primarily mitogenic (growth stimulatory) rather than mutagenic (20). Thus, the lack of *GSTP1* inactivation in endometrial carcinoma is consistent with the selective role of estrogen as the substrate for the generation of endogenous carcinogens. The risk for ovarian carcinoma, on the other hand, does not seem to be associated with estrogen administration. In fact, oral contraceptives appear to be protective against ovarian carcinoma (19).

The other tumor type where aberrant methylation of the *GSTP1* promoter was found fairly frequently was renal carcinoma. Our immunohistochemical study demonstrated that tumors harboring this epigenetic alteration did not express the GST $\pi$  protein, whereas the unmethylated did. Former studies of GST $\pi$  expression in renal neoplasia had reported a similar rate of 25% tumors losing GST $\pi$  expression (7). Estrogens may also be involved in this tumor type as well. Estrogen-induced carcinogenesis in animal models, and in particular the induction in Syrian golden hamsters of kidney tumors, suggest a causal role for this pathway in some renal tumor formation (21).

Inactivation of *GSTP1* might also affect the sensitivity of tumor cells to chemotherapeutic agents. It will be interesting to know the role that the hypermethylation-associated inactivation of *GSTP1* plays in antineoplastic drug sensitivity. Several studies suggest that GST $\pi$  contributes to the resistance of tumor cells to a number of antineoplastic agents, including alkylating agents and anthracyclines (22). Transfection of a cDNA encoding GST $\pi$  into drug-sensitive cells resulted in increased resistance to doxorubicin (23), whereas the reduction of GST $\pi$  levels using an antisense approach resulted in increased sensitivity (24). Furthermore, the use of GST class-selective inhibitors and the depletion of reduced glutathione by buthionine sulfoximine sensitizes cancer cells to a variety of antineoplastic drugs (25). Because alkylating agents and anthracyclines are commonly used in breast carcinoma chemotherapeutic regimens, the response of tumors may be affected by *GSTP1* promoter hypermethylation and subsequent loss of expression. However, studies of GST $\pi$  expression



in breast cancer have not uniformly demonstrated a difference in prognosis according to *GST $\pi$*  expression levels (8–10). The noted improvement in prognosis in women with node-negative breast cancer who lacked *GST $\pi$*  expression cannot be attributed to such an effect, because none of these women received adjuvant chemotherapy (9). The inability to detect a difference in node-positive breast cancer cases was attributed to variation in stage and treatments among these patients. A careful analysis of a larger group of similarly treated breast cancer patients may resolve this issue.

In summary, all of the data collected in the present study suggest that *GSTP1* promoter hypermethylation is the major factor underlying loss of *GST $\pi$*  expression in breast and renal carcinomas. Such inactivation of *GSTP1* may expose these cells to the action of a wide range of electrophilic carcinogens, including estrogen metabolites.

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