Cytidine methylation of regulatory sequences near the \(\pi\)-class glutathione \(S\)-transferase gene accompanies human prostatic carcinogenesis

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ABSTRACT Hypermethylation of regulatory sequences at the locus of the \(\pi\)-class glutathione \(S\)-transferase gene GSTPI was detected in 20 of 20 human prostatic carcinoma tissue specimens studied but not in normal tissues or prostatic tissues exhibiting benign hyperplasia. In addition, a striking decrease in GSTPI expression was found to accompany human prostatic carcinogenesis. Immunohistochemical staining with anti-GSTPI antibodies failed to detect the enzyme in 88 of 91 prostatic carcinomas analyzed. In vitro, GSTPI expression was limited to human prostatic cancer cells lines containing GSTPI alleles with hypomethylated promoter sequences; a human prostatic cell line containing only hypermethylated GSTPI promoter sequences did not express GSTPI mRNA or polypeptides. Methylation of cytidine nucleotides in GSTPI regulatory sequences constitutes the most common genomic alteration yet described for human prostate cancer.

Human cancer cells typically contain somatically altered genomes, characterized by mutation, amplification, or deletion of critical genes (1). In addition, the DNA template from human cancer cells often displays somatic changes in DNA methylation (1–8). However, the precise role of abnormal DNA methylation in human tumorigenesis has not been established (2–8). For human prostate cancer, the most commonly diagnosed cancer in men in the United States (9), we have found uniform somatic changes in DNA methylation at the locus of the glutathione \(S\)-transferase gene GSTPI. Glutathione \(S\)-transferases (GSTs; EC 2.5.1.18) catalyze intracellular detoxification reactions, including the inactivation of electrophilic carcinogens, by conjugating chemically reactive electrophiles to glutathione (10–12). Human GSTs, encoded by several different genes at different loci, have been classified into four families, which have been referred to as \(\alpha\), \(\mu\), \(\pi\), and \(\theta\) (13). We report here that most human prostate cancers fail to express the \(\pi\)-class GST, and that regulatory sequences near the GSTPI gene, which encodes the human \(\pi\)-class GST, appear to be commonly hypermethylated during prostatic carcinogenesis.

MATERIALS AND METHODS

Immunohistochemical Staining for GSTPI. Formalin-fixed paraffin-embedded prostatic tissue sections were stained with anti-GSTPI antisera (1:100 dilution; Oncor) using an immunoperoxidase method (Vectastain ABC Kit; Vector Laboratories), with either 3,3'-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) as the peroxidase substrate. As a control for the specificity of staining, tissue sections prepared from normal human liver and normal human kidney were also stained with the anti-GSTPI antisera. Each of the tissues exhibited the characteristic staining pattern previously described (14).

Immunoblot Analysis for GSTPI Polypeptides. Saline-washed cultured prostatic carcinoma cells (15–19) were collected by centrifugation at 14,000 \(\times\) \(g\) for 10 min at room temperature and then lysed in a protein extraction buffer [2% SDS/10% (vol/vol) glycerol/10 mM dithiothreitol in 62 mM Tris\(\cdot\)HCl, pH 7.8] by heating to 95\(^\circ\)C for 10 min. The DNA content of each cell extract was estimated by using a diphenylamine assay (20). Equivalent extracts from each of the cultured cell lines were subjected to immunoblot analysis for GSTPI and DNA topoisomerase I polypeptides, using specific antisera (rabbit anti-GSTPI from Oncor; human anti-topoisomerase I provided by W. C. Earnshaw, Johns Hopkins University) as previously described (21).

Northern Blot Analysis for GSTPI mRNA. Total RNAs were extracted from cultured cells by the method of Chomczynski and Sacchi (22) and then quantitated by using an orcinol assay (23). Purified RNAs (20 \(\mu\)g) were electrophoresed on 1.5% agarose gels in the presence of 2.2 M formaldehyde, transferred to Zeta-Probe (Bio-Rad) filters, and then assessed for GSTPI and TOP1 mRNA levels by hybridization with specific \(32\)P-labeled cDNA probes (prepared using the Random Primers DNA Labeling System, Gibco/BRL). Following hybridization at 50\(^\circ\)C for 16 hr (in 50% formamide/7% SDS/0.5% p-1,3-dioxane milk/heat-denatured salmon sperm DNA at 200 \(\mu\)g/ml/300 mM NaCl/2 mM EDTA/20 mM sodium phosphate, pH 7.4), blots were washed with 0.3\(\times\) SSC (1\(\times\) SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0) and 0.3% SDS for 60 min at 65\(^\circ\)C before being exposed to X-Omat AR Kodak film at \(-70^\circ\)C. Plasmid pGST-\(\pi\)-1 containing the entire GSTPI coding sequence (24) was obtained from the American Type Culture Collection; plasmid pHToPl-D1 containing 704 bp of human TOP1 cDNA (25) was provided by L. F. Liu (Robert Wood Johnson–University of Medicine and Dentistry of New Jersey).

Southern Blot Analysis for GSTPI Promoter Methylation. DNA was isolated from human prostatic cancer lines and from a variety of normal and neoplastic human tissues as previously described (26, 27). Normal tissue specimens, including seminal vesicle, esophagus, kidney, liver, lung, and spleen, were recovered at autopsy. Normal prostate tissue specimens were obtained from autopsies of men of different ages (18 to 69 years) who did not suffer with prostatic diseases. Neoplastic prostate tissues, along with matched adjacent normal prostate or matched normal seminal vesicle

Abbreviations: GST, glutathione \(S\)-transferase; BPH, benign prostatic hyperplasia.

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tissues, were dissected from resection specimens obtained from men treated for localized prostatic carcinoma by radical prostatectomy. For analysis of \( \text{GSTP1} \) promoter methylation, purified DNAs were digested first with \( \text{HindIII} \) and \( \text{EcoRI} \) and then extensively with the \( m^3 \text{C} \)-sensitive restriction endonuclease \( \text{BssHII} \). Digested DNAs were then electrophoresed on agarose gels, transferred to \( \text{Zeta-} \)Probe (Bio-Rad) membranes, and then hybridized with \( ^{32} \text{P} \)-labeled \( \text{GSTP1 cDNA} \) as previously described (26).

**RESULTS AND DISCUSSION**

Many human cancers exhibit increased \( \text{GSTP1} \) expression relative to their tissues of origin (28). Surprisingly, we found that most prostatic cancers contained decreased levels of detectable \( \text{GSTP1} \) polypeptides relative to normal prostatic tissue. To assess \( \text{GSTP1} \) expression in normal and neoplastic prostatic cells in vivo, we subjected tissue specimens to immunohistochemical staining using anti-\( \text{GSTP1} \) antibodies (Fig. 1). Normal prostatic tissues exhibited intense staining for \( \text{GSTP1} \) in almost all basal epithelial cells (Fig. 1A). Some normal glandular epithelial cells also appeared to contain immunoreactive \( \text{GSTP1} \), while most contained no detectable enzyme (Fig. 1B). Lobules with \( \text{GSTP1} \)-positive or with \( \text{GSTP1} \)-negative glandular epithelial cells did not differ in their histologic appearance or anatomic distribution. Otherwise, \( \text{GSTP1} \)-positive epithelial cells were detected in all normal epithelial structures within prostatic tissue, including the verumontanum, the urethra, prostatic ducts, ejaculatory ducts, and the prostatic urethra (not shown). Positive immunohistochemical staining for \( \text{GSTP1} \) was also seen in foci of transitional cell metaplasia, squamous cell metaplasia, and hyperplasia associated with benign prostatic hypertrophy (not shown). Despite the presence of abundant \( \text{GSTP1} \) in most normal prostatic epithelial cells as well as in cells making up benign proliferative prostatic lesions, 88 of 91 specimens of prostatic carcinoma contained no detectable \( \text{GSTP1} \) (Fig. 1D–F). Prostatic intraepithelial neoplasia (PIN) has been proposed to represent a premalignant lesion (29). Of interest, in some PIN lesions, many epithelial cells were also devoid of \( \text{GSTP1} \) (Fig. 1C).

To better understand \( \text{GSTP1} \) regulation in human prostate cancer, five established human prostatic carcinoma cell lines (15–19) were assessed for \( \text{GSTP1} \) expression by immunoblot analysis for \( \text{GSTP1} \) (Fig. 2A) and by Northern blot analysis for \( \text{GSTP1} \) mRNA (Fig. 2B). In accordance with the lack of \( \text{GSTP1} \) expression by human prostatic cancers in vivo, immunoblot analysis failed to detect \( \text{GSTP1} \) polypeptides in a protein extract prepared from \( \text{LNCaP} \) cells (Fig. 2A, lane 1), and Northern blot analysis failed to detect \( \text{GSTP1} \) mRNA in RNA recovered from the \( \text{LNCaP} \) cell line (Fig. 2B, lane 1). In contrast, Du-145 cells, PC-3 cells, PPC cells, and Tsu-Pt1 cells contained abundant \( \text{GSTP1} \) polypeptides and \( \text{GSTP1} \) mRNA (Fig. 2A and B, lanes 2–5).

Sequence analysis of the 5' regulatory region upstream of the \( \text{GSTP1} \) gene has revealed a TATAA box, two potential SP1 binding sites, and a consensus AP-1 site (refs. 30 and 31; Fig. 3A). Promoter studies have established that a 72-nt sequence located 5' of the transcriptional start site (from nucleotide −80 to nucleotide −8) exhibits basal promoter activity when ligated to a promoterless chloramphenicol acetyltransferase (CAT) reporter gene in transient transfection assays (32, 33). The 400 nt lying immediately 5' of the transcriptional start site contain nearly 72% C and G nucleotides, with 41 CpG dinucleotides. Methylation of CpG dinucleotides in regulatory regions of mammalian genes has been frequently associated with diminished transcriptional activity (34–38). To determine whether differences in \( \text{GSTP1} \) promoter methylation correlate with the differences in \( \text{GSTP1} \) expression detected in the human prostatic cancer cell lines, DNA from each of the cell lines was digested with the \( m^3 \text{C} \)-sensitive restriction enzyme \( \text{BssHII} \) and then subjected to Southern blot analysis using a \( \text{GSTP1} \) probe (Fig. 3). \( \text{GSTP1} \) promoter DNA from \( \text{LNCaP} \) cells failed to cut with

![Fig. 1. π-class GST expression in normal and neoplastic human prostatic tissues. Tissue sections were assessed for \( \text{GSTP1} \) expression by immunohistochemical staining with anti-\( \text{GSTP1} \) antiserum. (A) Normal prostatic epithelium with intensely staining basal prostatic epithelial cells. (×70.) (B) Normal prostatic tissue exhibiting lobular heterogeneity in \( \text{GSTP1} \) expression. (×40.) (C) Diminished \( \text{GSTP1} \) expression in prostatic intraepithelial neoplasia (PIN). (×70.) (D–F) Prostatic cancer tissues with no detectable \( \text{GSTP1} \) expression in the neoplastic cells. (D, ×40; E, ×70; F, ×40.)](image-url)
While LNCaP cells, which did not express GSTP1 mRNA or polypeptides (Fig. 2 A and B, lanes 1), contained only hypermethylated GSTPI promoter sequences (Fig. 3B, lane 6), Tsu-Pr1 cells, which expressed abundant GSTP1 mRNA and polypeptides (Fig. 2 A and B, lanes 5), appeared to contain only hypomethylated GSTPI promoter sequences (Fig. 3B, lane 10). In addition, hypomethylated GSTP1 promoter sequences were detected in Du-145 cells, PC-3 cells, PPC cells, and Tsu-Pr1 cells (Fig. 3B, lanes 7–9). Each of these cells also expressed abundant GSTP1 mRNA and polypeptides (Fig. 2 A and B, lanes 2–4), suggesting a strong correlation between GSTPI transcriptional activity and the presence of hypomethylated GSTPI promoter sequences. As has been proposed for some C+G-rich 5' regulatory sequences in other genes, the GSTP1 promoter sequences may constitute a methylation-sensitive "CpG island" (41).

Clearly, the data presented thus far have demonstrated that the great majority of human prostatic carcinoma cells fail to express GSTP1 polypeptides in vivo. In addition, our studies have revealed that the lack of GSTP1 expression by LNCaP cells in vitro appears to be accompanied by hypermethylation of regulatory sequences in all GSTP1 alleles. These data suggest that a possible mechanism for the failure of human prostate cancer cells to express GSTP1 polypeptides in vivo could be transcriptional inactivation of the GSTPI gene as a result of methylation of CpG island sequences in the transcriptional regulatory region. Alternatively, the absence of GSTP1 expression in human prostate cancer cells could also result from other genomic alterations at the GSTP1 locus, from a lack of transcriptional activating factors, from the presence of transcriptional repressors, or by a posttranscriptional mechanism. Furthermore, even though LNCaP cells exhibit evidence of methylation-inactivation of GSTP1 expression, DNA methylation patterns have been found to change as mammalian cells are propagated in vitro (42). Thus, to determine whether lack of GSTP1 expression by human prostate cancer cells in vivo might be accompanied by hypermethylation of GSTP1 regulatory sequences, we undertook an analysis of GSTPI promoter methylation, using DNA isolated from a variety of normal tissues as well as from prostatic carcinomas.

GSTP1 promoter hypermethylation has not, to the best of our knowledge, been previously described (43). To ascertain whether GSTP1 promoter hypermethylation occurs during normal physiologic cellular differentiation, DNA isolated from normal human tissues with different GSTP1 expression patterns was subjected to Southern blot analysis following digestion with the mC-sensitive restriction enzyme BssHII (Fig. 4). No methylated GSTP1 promoter sequences were detected in DNA isolated from normal prostate, seminal
The GSTPI promoter methylation status in vivo in normal adult tissues. Shown are Southern blot analyses using a GSTPI cDNA probe of DNA isolated from normal human tissues. (A) DNA from normal tissues digested first with EcoRI and HindIII and then with (+) or without (−) BssHII. (B) DNA, prepared from normal prostatic tissues recovered at autopsy from men of different ages who did not suffer from prostatic diseases, digested with EcoRI, HindIII, and BssHII. Arrow denotes migration position of GSTPI restriction fragment uncut by BssHII.

To assess whether hypermethylation of GSTPI promoter sequences occurs during aging in normal prostatic cells, DNA prepared from normal prostatic tissue specimens recovered from men of different ages was also analyzed (Fig. 4B). Again, there was no evidence of GSTPI promoter hypermethylation in any of the normal prostatic tissues.

To discover whether GSTPI promoter hypermethylation occurs during prostatic carcinogenesis in vivo, Southern blot analyses were performed on BssHII-digested DNA isolated from human prostatic carcinoma specimens (Fig. 5). Each of the prostate cancers studied displayed evidence of hypermethylation of promoter sequences in GSTPI alleles relative to matched control DNA prepared from normal tissues (Fig. 5A and B). Prostatic cancer tissue contains an admixture of normal stromal cellular elements and carcinoma cells. Whether differences in the abundance of hypermethylated GSTPI regulatory sequences among the different cancers reflect different abundances of normal and neoplastic cells in the cancer specimens, or whether some of the carcinoma cells contain both hypermethylated and hypomethylated GSTPI promoter sequences, has not been determined. Nevertheless, each of the prostate cancers contained GSTPI alleles with hypermethylated regulatory sequences. Furthermore, for prostatic epithelial cells, hypermethylation of GSTPI promoter sequences appeared specific to the process of malignant transformation. For five cases of prostate cancer removed by radical prostatectomy, DNA was selectively prepared from different areas of the same resection speci-mens containing normal prostate, BPH tissue, or prostatic cancer (Fig. 5C). When Southern blots containing BssHII-digested DNA samples were hybridized with a GSTPI probe, apparent hypermethylation of GSTPI promoter sequences was detected in each of the cancer specimens, but none of the BPH specimens (Fig. 5C). In one of the cases, DNA prepared from grossly normal prostatic tissue also appeared to contain GSTPI alleles with some methylated promoter sequences (Fig. 5C, lane 10). Whether this resulted from infiltration of grossly normal prostatic tissue by microscopic carcinoma containing methylated GSTPI promoters, from GSTPI promoter hypermethylation in prostatic intraepithelial neoplasia (PIN) lesions in the grossly normal prostatic tissue, or from GSTPI promoter hypermethylation in histologically normal prostatic epithelial cells has not been established. However, hypermethylation of GSTPI promoter DNA nonetheless appears specific to the process of neoplastic transformation in the prostate: normal prostatic tissues from men without prostate cancer did not contain detectable methylation of GSTPI regulatory sequences (Fig. 4B).

All of the data collected in this study suggest that decreased GSTPI expression and GSTPI promoter hypermethylation occur commonly during the malignant transformation of...
prostatic epithelial cells. Regional methylation changes, often manifest as hypermethylation of CpG island sequences, have been proposed to play an important role in carcinogenesis (2–8). The prevalent finding of hypermethylated 5′ GSTP1 regulatory sequences in human prostatic cancers, but not normal tissues, supports this general proposal. Unfortunately, the mechanism by which de novo regional hypermethylation of specific autosomal alleles occurs in cancer cells has not been well established. Abnormal DNA methylation accompanying neoplastic transformation has been suggested to result from an enhanced DNA methylation capacity in the neoplastic cells. Both increased DNA methytransferase activity (44) and elevated DNA-MT mRNA levels (45) have been detected in neoplastic cells. Of interest, DNA-MT mRNA increases were observed early in the process of colonic carcinogenesis, even in some histologically normal colonic mucosa specimens (45). Whether an increased DNA methylation capacity in human prostatic results in GSTP1 promoter hypermethylation remains to be established.

GSTs have been proposed to play a critical role in defending normal cells against electrophilic carcinogens (10–12). Consistent with this role, inherited homozygosity for null GSTM1 alleles may confer an increased risk of cigarette smoke-related lung cancer (46). In addition, overexpression of c-met GST in cultured cells has been shown to reduce toxicity after benzo[a]pyrene exposure (49). An intriguing implication of the apparent methylation-inactivation of GSTP1 expression associated with prostatic carcinogenesis is the hypothesis that some normal prostatic epithelial cells might acquire an increased vulnerability to electrophilic carcinogens by virtue of containing abnormally methylated GSTP1 promoters. Abundant epidemiologic evidence supports a significant role for environmental factors both in prostatic carcinogenesis and in prostate cancer progression to a life-threatening disease (50). Normal prostatic tissue appears to contain epithelial cells with diminished GSTP1 expression but hypomethylated GSTP1 regulatory sequences. Perhaps, rare cells with hypermethylated GSTP1 promoter sequences arise among these GSTP1-negative epithelial cells and undergo clonal expansion as a consequence of carcinogen exposure. Were this hypothesis true, a possible prostate cancer prevention strategy might be the therapeutic augmentation of GST activity by using GST inducers. Recent studies have identified several GST inducers which may be useful for such an approach (51–53). We hope that further studies of GSTP1 regulation in normal and neoplastic prostatic epithelial cells will help engender several new diagnostic and treatment strategies for prostate cancer.

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