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GSTP1 CpG Island Hypermethylation Is Responsible for the Absence of GSTP1 Expression in Human Prostate Cancer Cells

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GSTP1 CpG island hypermethylation is the most common somatic genome alteration described for human prostate cancer (PCA); lack of GSTP1 expression is characteristic of human PCA cells *in vivo*. We report here that loss of *GSTP1* function may have been selected during the pathogenesis of human PCA. Using a variety of techniques to detect *GSTP1* CpG island DNA hypermethylation in PCA DNA, we found only hypermethylated *GSTP1* alleles in each PCA cell in all but two PCA cases studied. In these two cases, CpG island hypermethylation was present at only one of two *GSTP1* alleles in PCA DNA. In one of the cases, DNA hypermethylation at one *GSTP1* allele and deletion of the other *GSTP1* allele were evident. In the other case, an unmethylated *GSTP1* allele was detected, accompanied by abundant GSTP1 expression. *GSTP1* CpG island DNA hypermethylation was responsible for lack of *GSTP1* expression by LNCaP PCA cells: treatment of the cells with 5-azacytidine (5-aza-C), an inhibitor of DNA methyltransferases, reversed the *GSTP1* promoter DNA hypermethylation, activated *GSTP1* transcription, and restored *GSTP1* expression. *GSTP1* promoter activity, assessed via transfection of *GSTP1* promoter-*CAT* reporter constructs in LNCaP cells, was inhibited by *Sss*I-catalyzed CpG dinucleotide methylation. Remarkably, although selection for loss of *GSTP1* function may be inferred for human PCA, *GSTP1* did not act like a tumor suppressor gene, as LNCaP cells expressing GSTP1, either after 5-aza-C treatment or as a consequence of transfection with *GSTP1* cDNA, grew well *in vitro* and *in vivo*. Perhaps, *GSTP1* inactivation may render prostatic cells susceptible to additional genome alterations, caused by electrophilic or oxidant carcinogens, that provide a selective growth advantage. *(Am J Pathol 2001, 159:1815–1826)*

Somatic genome lesions, including mutations, translocations, amplifications, and deletions, are characteristic of cancer cell DNA.[1–4] Often, these lesions target critical genes involved in cell transformation or in the maintenance of the neoplastic phenotype. At other times, these genome lesions do not seem to target such cancer genes. Somatic changes in deoxycytidine methylation are also frequently found in human cancer cell DNA.[5,6] Many of these DNA methylation changes seem to target critical genes associated with cancer pathogenesis. Other somatic changes in DNA methylation found in cancer cells may not involve critical genes. Ideally, if a cancer cell DNA alteration has targeted a critical gene for cancer development, the DNA lesion has likely provided a selective cell growth or survival advantage at some point during cancer initiation or malignant progression. To infer such selection *in vivo* for a somatic DNA change found in human cancer cells, the DNA alteration must change the function of a specific gene or its product and must be selectively present in a specific cell population (eg, cancer cells *versus* normal cells or metastatic cancer cells *versus* primary site cancer cells).

In a previous study,[7] we reported the detection of somatic changes in deoxycytidine methylation affecting a CpG island encompassing the 5′-regulatory region of the human π-class glutathione *S*-transferase (GST) gene, *GSTP1*, in human prostatic carcinomas (PCAs). The specific DNA methylation change, a somatic increase in CpG dinucleotide methylation at a *Bss*HII endonuclease recognition site in the transcriptional promoter near *GSTP1*, was present in DNA isolated from 20 of 20 PCA specimens. Furthermore, the presence of this DNA alteration correlated with a lack of GSTP1 polypeptide expression in PCA cells *in vivo* and *in vitro*, raising the possibility that the DNA methylation change might be associated with gene inactivation. These findings of *GSTP1* CpG island DNA methylation and lack of GSTP1 expression in human

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PCA have now been reported in several subsequent studies from several different laboratories.[8–18] Somatic alterations in CpG dinucleotide methylation, especially alterations targeting CpG dinucleotides clustered into CpG islands at the regulatory region of genes, usually result in changes in gene expression, but not in changes in gene product function.[5,6] To infer selection *in vivo* for *GSTP1* CpG island DNA hypermethylation and loss of *GSTP1* function in PCA, *GSTP1* CpG island DNA hypermethylation must be associated with gene inactivation and must be selectively present in PCA cells *versus* normal cells. Furthermore, PCA cells must contain only inactivated *GSTP1* genes. *GSTP1* is an autosomal gene located at chromosome 11q13.[19–21] To permit selection during prostatic carcinogenesis, prostatic cells must either contain CpG dinucleotide changes affecting both *GSTP1* alleles or DNA hypermethylation affecting one *GSTP1* allele in association with another gene-inactivating lesion affecting the other *GSTP1* allele.

We present here evidence that *GSTP1* genes are inactivated in prostatic cells during the pathogenesis of human PCA as a consequence of CpG island DNA hypermethylation, and that cells with inactivated *GSTP1* genes may have been selected during human prostatic carcinogenesis. PCA cells in most PCA cases stereotypically fail to express GSTP1 polypeptides. Using a variety of analytic approaches to detect *GSTP1* CpG island hypermethylation in PCA cell DNA, we found that all PCA cells in all but one PCA case contained only hypermethylated *GSTP1* CpG islands *in vivo*. In this one PCA case, in which each of the PCA cells carried an unmethylated *GSTP1* CpG island allele, all of the cells expressed high levels of GSTP1 polypeptides. In addition, studies of *GSTP1* promoter function in LNCaP PCA cells *in vitro* further supported the notion that CpG island DNA hypermethylation was responsible for *GSTP1* transcriptional inactivation. Finally, although PCA cells with *GSTP1* CpG island hypermethylation and loss of GSTP1 expression seemed to have been selected during human prostatic carcinogenesis, restoration of GSTP1 expression in fully transformed LNCaP PCA cells, either via 5-aza-C treatment or by transfection with *GSTP1* cDNA, failed to reduce LNCaP PCA growth *in vitro* or tumorigenicity *in vivo*, suggesting that *GSTP1* does not likely function as a tumor suppressor gene in the pathogenesis of PCA.

Materials and Methods

Isolation of Genomic DNA from Normal and Neoplastic Human Cells and Tissues

Genomic DNA was isolated from LNCaP PCA cells,[22] and from PCA tissues, along with normal prostate tissues and normal seminal vesicle tissues, obtained at radical prostatectomy or pelvic lymph node dissection, as previously described.[7,23] The collection of such tissues was conducted as part of a clinical research protocol approved by the Joint Committee on Clinical Investigation at the Johns Hopkins Medical Institutions. Genomic DNA was also isolated from normal and neoplastic tissues, obtained at surgery for carcinomas of the kidney, endometrium, uterine cervix, bladder, and ureter.[24–26] DNA quantity was estimated using a diphenylamine assay.[27]

Immunohistochemical Detection of GSTP1, Prostate-Specific Antigen, and Keratin Polypeptides in Human Tissue Sections

Formalin-fixed, paraffin-embedded tissues, were cut into 5-μm sections and stained with anti-GSTP1 antibodies (1:3000 dilution; DAKO, Carpinteria, CA), anti-prostate-specific antigen antibodies (1:25 dilution, DAKO), and anti-prostate-specific acid phosphatase antibodies (1:20,000 dilution, DAKO), using an immunoperoxidase method (ChemMate Universal Detection System; Ventana Medical Systems, Tucson, AZ) with diaminobenzidine as a peroxidase substrate.[7,28] Immunostained tissue sections were counterstained with hematoxylin.

Southern Blot Analyses for GSTP1 CpG Island Hypermethylation and for Other Somatic Genome Alterations

Southern blot analysis of DNA from LNCaP PCA cells, and from normal tissues and PCA tissues, was accomplished as described previously.[7,23] To detect *GSTP1* CpG island hypermethylation, purified DNAs were digested first with *Eco*RI and *Hin*dIII, and then with *Bss*HII, an enzyme that will not cut its recognition sequence, GCGCGC, if it contains $^{5\text{-m}}$C. To detect somatic loss of polymorphic alleles at different chromosomal loci, including 8p, 16q, and 17p, purified DNAs were digested with relevant restriction endonucleases recognizing cutting sites present on only one of two alleles at the various loci. Digested DNAs were electrophoresed on agarose gels, transferred to Zeta-Probe membranes (Bio-Rad, Richmond, CA), hybridized with ^{32}P-labeled *GSTP1* cDNA[21] or ^{32}P-labeled genomic probe DNA (probes KS-2, CI-8319, MSR, KSR, and K26 for 8p, HPO-4 for 16q, and YNZ-22 for 17p[23]), and visualized by autoradiography. Autoradiographs were then subjected to quantitative densitometry using a Scanmaster scanner (Howtek).

A CpG Dinucleotide Methylation-Sensitive Endonuclease/Polymerase Chain Reaction (PCR) Assay for the Simultaneous Discrimination of Maternal and Paternal GSTP1 Alleles and Detection of GSTP1 CpG Island Hypermethylation

Purified DNAs were digested extensively with *Hpa*II, with *Msp*I, or left undigested, and then subjected to PCR amplification using primers encompassing a polymorphic $[ATAAA]_n$ repeat sequence and two *Hpa*II/*Msp*I sites in the 5′ region of *GSTP1* (GenBank positions −535 to −509, 5′-AGCCTGGGCCACAGCGTGAGACTACGT-3′, and −246 to −266, 5′-GGAGTAAACAGACAGCAGGAA-

GAGGAC-3′) using reaction conditions described previously.[13] As a control, the DNAs were also subjected to PCR amplification with primers encompassing the polymorphic $[ATAAA]_n$ repeat sequence but not the two *Hpa*II/*Msp*I sites (GenBank positions −535 to −509, 5′-AGCCTGGGCCACAGCGTGAGACTACGT-3′, and −364 to −337, 5′-TCCCGGAGCTTGCACACCCGCTTCACA-3′). PCR products were visualized, after end-labeling the downstream primer with [γ-^{32}P]ATP using T4 polynucleotide kinase, by electrophoresis on 6% polyacrylamide DNA sequencing gels containing 8 mol/L urea run at 60 W for 2.5 hours, gel mounting, and drying on filter paper (Whatman), and exposure to X-OMAT film (Eastman-Kodak, Rochester, NY).

A Bisulfite Genomic-Sequencing Approach for the Detection of Somatic GSTP1 CpG Island DNA Hypermethylation

To map CpG dinucleotide changes throughout the *GSTP1* CpG island, bisulfite genomic sequencing, which permits discrimination of 5-^{m}C from C,[29] was undertaken. Purified DNAs (200 ng) were digested with *Eco*RI, admixed with salmon sperm DNA (2.5 μg), and then treated with sodium bisulfite as described previously.[30] Bisulfite-treated DNA was then subjected to two rounds of PCR to amplify *GSTP1* CpG island alleles, using primers that recognize antisense strand *GSTP1* sequences after conversion of C to T (first PCR reaction primers: GenBank positions −636 to −613, 5′-AC$^{A}/_{G}$CAACCTATAATTC-CACCTACTC-3′, and +117 to +94, 5′-GT$^{T}/_{C}$GGGAGTT-GGGGTTTGATGTTG-3′; second PCR reaction primers: GenBank positions −535 to −512, 5′-AACCTAAACCA-CAAC$^{A}/_{G}$TAAAACAT-3′, and +89 to +66, 5′-TTGGTTT-TATGTTGGGAGTTTTGA-3′). The first PCR reaction contained 100 ng bisulfite-treated DNA, 1 μmol/L primers, 250 μmol/L deoxyribonucleotide triphosphates, and 2.5 Units Platinum *Taq* polymerase (Life Technologies, Inc., Rockville, MD) in OptiPrime buffer no. 7 (Stratagene, La Jolla, CA). The reaction mixture was heated to 94°C for 2 minutes, then subjected to PCR with incubation at 94°C for 1 minute, 58°C for 2 minutes, and 72°C for 3 minutes for five cycles, followed by incubation at 94°C for 30 seconds, 63°C for 2 minutes, and 72°C for 1.5 minutes for 25 cycles before a final extension at 72°C for 6 minutes. The second nested PCR reaction mixture, which contained 15 ng of DNA, 1 μmol/L of primers, 250 μmol/L of deoxyribonucleotide triphosphates, and 2.5 U of *Taq* polymerase in OptiPrime buffer no. 8 (Stratagene), was heated to 94°C for 2 minutes, then subjected to PCR with incubation at 94°C for 1 minute, 57°C for 2 minutes, and 72°C for 3 minutes for five cycles, followed by incubation at 94°C for 30 seconds, 62°C for 2 minutes, and 72°C for 1.5 minutes for 25 cycles before a final extension at 72°C for 6 minutes. To permit DNA sequencing of individual *GSTP1* CpG island alleles, PCR products were first purified by separation on 1% agarose gels (Life Technologies), isolated from the agarose (using a QIAquick gel extraction kit; Qiagen, Valencia, CA), and recovered by ethanol precipitation, and then cloned by ligation into pCR 2.1pTOPO cloning vectors (using a TOPO kit; Invitrogen, Carlsbad, CA) followed by introduction into TOP 10 One-Shot competent bacteria. Plasmid DNAs isolated from independent drug-resistant bacterial clones (a minimum of 10 clones for each PCR reaction product) were subjected to DNA sequence analysis using a cycle-sequencing approach with M13-sequencing primers dye-labeled terminators (Abi Prism Dye Terminator Cycle Sequencing Ready Reaction kit; Perkin Elmer, Emeryville, CA), and an ABI automated sequencer.

Propagation of LNCaP Human PCA Cells in Vitro *and* in Vivo*, Assessment of Effects of GSTP1 CpG Island Methylation on GSTP1 Regulation in LNCaP Human PCA Cells, and Isolation of LNCaP Variants Expressing GSTP1 Polypeptides*

LNCaP PCA cells, which contain hypermethylated *GSTP1* CpG island alleles and fail to express GSTP1,[7] and PC-3 PCA cells, which contain unmethylated *GSTP1* CpG island alleles and express abundant GSTP1,[7,31] were propagated *in vitro* in RPMI 1640 (Mediatech) supplemented with 10% fetal calf serum (Life Technologies). *GSTP1* transcription by isolated nuclei from LNCaP and from PC-3 was assessed via nuclear run-on transcription assay accomplished as previously described,[32] using *GSTP1* genomic DNA, *hAR* cDNA and *TOP1* cDNA as hybridization targets for radiolabeled nuclear RNA. To reverse *GSTP1* CpG island DNA hypermethylation in LNCaP PCA cells, the cells were treated with 5 μmol/L 5-aza-C in complete growth medium. *GSTP1* expression was monitored via Northern blot analysis, using radiolabeled *GSTP1* cDNA probes (with *TOP1* and *H4* cDNA probes as controls), and immunoblot analysis, using anti-GSTP1 antibodies (with anti-lamin B antibodies as controls), in a manner previously described.[7] The LNCaP-5azaC subline, isolated by treatment of LNCaP cells with 5-aza-C for more than 30 generations, was maintained by propagation *in vitro* in growth medium containing 5-aza-C.

To ascertain the effect of CpG island DNA hypermethylation on *GSTP1* promoter function in LNCaP PCA cells, *GSTP1* transcriptional regulatory sequences (GenBank positions −408 to +36) were isolated, treated with *Sss*I (New England BioLabs, Beverly, MA), a bacterial CpG methylase, or left untreated, and then ligated to a linearized pCAT-Basic vector (Promega, Madison, WI), without propagation in bacteria, before transfection into LNCaP PCA cells using Lipofectamine Plus reagent (Life Technologies). *GSTP1* promoter activity in LNCaP PCA cells was also evaluated using a series of unmethylated *GSTP1* promoter/*CAT* reporter constructs as previously described for MCF-7 breast cancer cells.[33] CAT reporter expression was assessed 48 hours after transfection using an enzyme activity assay (Flash Cat nonradioactive assay kit, Stratagene). The plasmids pCAT-Control (Promega) and pCMV-*β-gal* (Stratagene) served as controls for transient transfection analyses.

LNCaP-GSTP1 subclones were generated via transfection of pCMV-*GSTP1neo*, prepared by ligating *GSTP1* cDNA[21] into pCMV-*neo*, selection of G418 (Life Technologies)-resistant subclones, and verification of GSTP1 expression by immunoblot analysis using anti-GSTP1 antibodies. Growth rates of LNCaP cells, LNCaP-5-aza-C cells, and LNCaP-GSTP1 subclones were determined by estimation of cell number throughout time during propagation *in vitro* in complete growth medium (in the absence of 5-aza-C or G418). Tumorigenicity for LNCaP cells and each of the LNCaP variants was assessed by inoculation of 10^6 cells in 0.1 ml of saline solution admixed with 75% Matrigel into the subcutaneous region of the flanks of athymic mice.[34] Tumor size was determined by caliper measurement. At 8 weeks after inoculation, tumors were excised and subjected to immunohistochemical staining with anti-GSTP1 antibodies as described above.

Results

Southern Blot Analyses Reveal that Most PCA Cells Contain Only Hypermethylated GSTP1 CpG Island Sequences in Vivo

Most PCA tissues are composed of admixtures of normal and neoplastic cells. Normal cells, including fibroblasts, vascular endothelial cells, and inflammatory cells, may comprise up to 30 to 50% or more of the cells in different prostate tumor specimens. Not surprisingly, analyses of DNA isolated from such tumors for the presence of somatic genome alterations are frequently confounded by the presence of normal cell DNA among the tumor DNA in the various samples. In our initial study, we used Southern blot analysis to assess *GSTP1* CpG island hypermethylation in DNA from 20 matched normal tissue and PCA specimens.[7] Hypermethylated *GSTP1* CpG island sequences were detected as *GSTP1* sequences that failed to cut with the $^{5\text{-m}}$C-sensitive restriction endonuclease *Bss*HII, an enzyme that cuts at the sequence GCGCGC in DNA only when the sequence does not contain $^{5\text{-m}}$CpG. Using this approach, we found a varied abundance of abnormal hypermethylated *GSTP1* promoter alleles amid normal unmethylated *GSTP1* promoter alleles in the PCA DNA samples.[7] To determine whether the normal unmethylated *GSTP1* promoter sequences in the PCA DNA specimens were present in PCA cells or were present only in normal cells located in the tumor specimens, we compared the abundance of unmethylated and methylated *GSTP1* alleles against the abundance of retained and lost polymorphic sequences on chromosomes 8p, 16q, and 17p for each matched normal tissue and PCA DNA specimen (Figure 1). In the majority of cases studied (eight of nine), an equivalent level of retained polymorphic DNA sequences at chromosomal loci exhibiting allelic loss and retained unmethylated *GSTP1* alleles were present in PCA DNA specimens (Figure 1B). These retained normal alleles were likely contributed by normal cells admixed with tumor cells in the PCA specimens. For one case (case no. 96), a significantly greater level of retained unmethylated *GSTP1* alleles than retained polymorphic DNA sequences at an allelic loss locus was evident in the PCA DNA specimen (Figure 1B). The simplest explanation for the discrepancy in the level of retained normal alleles present in this case was that some or all of the PCA cells contained unmethylated *GSTP1* promoter alleles or that

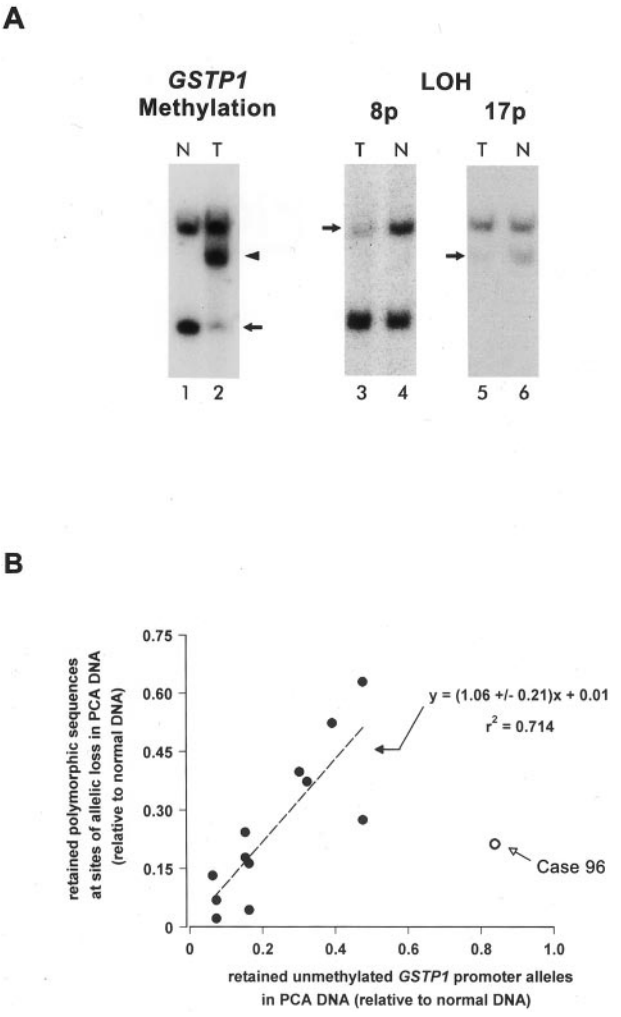


Figure 1. Equivalence of *GSTP1* CpG island hypermethylation and chromosome deletions in DNA from prostate cancer (PCA) cases. Southern blot analysis (see Materials and Methods) was used to determine the abundance of normal unmethylated *GSTP1* alleles[7] and of retained polymorphic sequences at sites of allelic loss on chromosomes 8p, 16q, and 17p[23] for DNA from PCA (T; **lanes 2**, **3**, and **5**) and from matched normal tissues (N; **lanes 1**, **4**, and **6**). **A:** Representative Southern blots for one PCA case are displayed. To discriminate *GSTP1* CpG island hypermethylation (**lanes 1** and **2**), DNAs were digested first with *Eco*RI and *Hin*dIII, and then with *Bss*HII, an enzyme that will not cut its recognition sequence, GCGCGC, if it contains $^{5\text{-m}}$C. An **arrow** denotes the position of normal unmethylated *GSTP1* alleles; the position of hypermethylated *GSTP1* alleles is indicated by an **arrowhead**. Loss of polymorphic alleles (LOH) at chromosomal loci on 8p (**lanes 3** and **4**) and 17p (**lanes 5** and **6**) were discriminated by digestion with relevant restriction endonucleases recognizing sites present on only one of two alleles at each locus. **Arrows** denote normal retained polymorphic sequences at sites of allelic loss. **B:** The quantities of retained unmethylated *GSTP1* alleles for nine PCA cases were plotted as a function of the quantities of retained polymorphic DNA sequences at chromosomal loci exhibiting allelic loss. PCA DNA from case 96 exhibited a significantly greater level of retained unmethylated *GSTP1* alleles than retained polymorphic DNA sequences at an allelic loss locus.

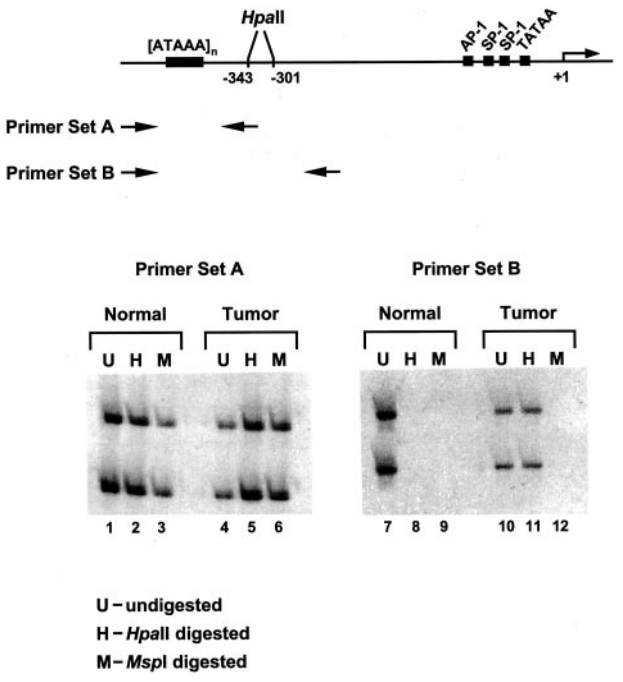


Figure 2. Discrimination of DNA hypermethylation at maternal and paternal *GSTP1* alleles using a PCR strategy. DNA from matched normal (normal) and neoplastic (tumor) prostate tissues was left untreated (U; **lanes 1**, **4**, **7**, and **10**), or was treated with *Hpa*II (H; **lanes 2**, **5**, **8**, and **11**), which cuts CCGG but not $C^{5\text{-}m}CGG$, or treated with *Msp*I (M; **lanes 3**, **6**, **9**, and **12**), which cuts CCGG and $C^{5\text{-}m}CGG$, before being subjected to PCR amplification using oligonucleotide primers targeting a polymorphic $[ATAAA]_n$ repeat sequence near the *GSTP1* regulatory region. For primer set B, the amplification of polymorphic *GSTP1* promoter sequences after *Hpa*II digestion, but not after *Msp*I digestion, indicated the presence of CpG dinucleotide methylation at the *Hpa*II/*Msp*I sites in the DNA analyzed.

some or all of the PCA cells contained less extensively methylated *GSTP1* promoter alleles. To evaluate this possibility, strategies for assessing allele-specific *GSTP1* hypermethylation and for determining the extent of hypermethylation throughout the *GSTP1* CpG island region were used.

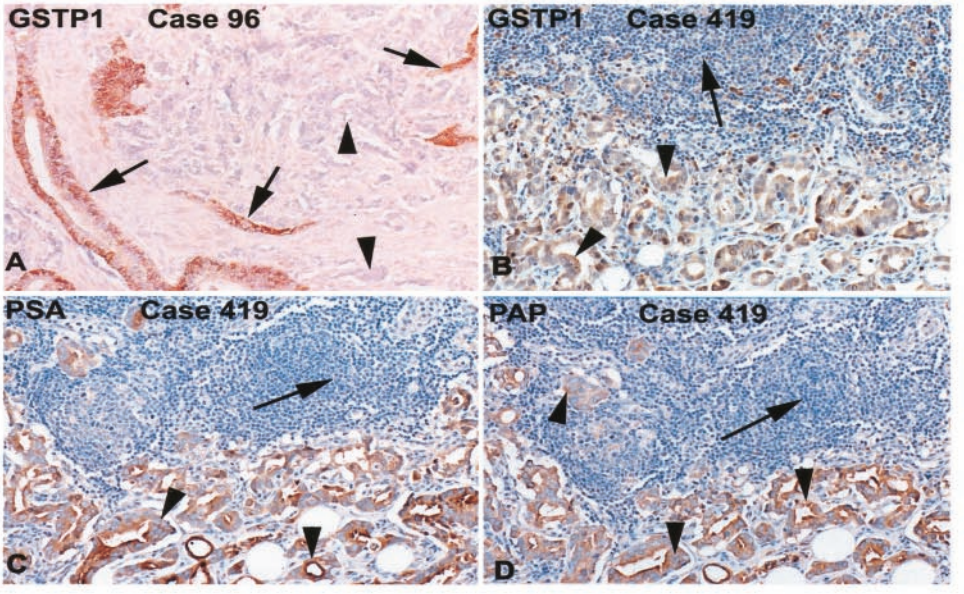
Somatic GSTP1 CpG Island DNA Hypermethylation Changes Affect Both Maternal and Paternal GSTP1 Alleles in Most PCA Cases

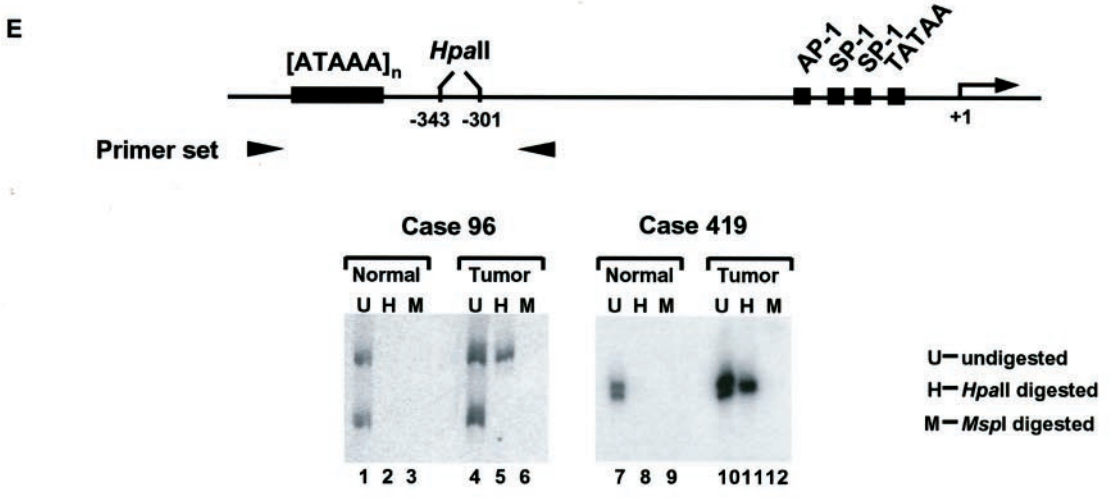
GSTP1 CpG island hypermethylation might contribute to the neoplastic transformation of PCA cells or might appear in PCA cells as a consequence of the process of prostatic carcinogenesis. To infer selection of inactivating *GSTP1* promoter hypermethylation during the pathogenesis of prostate cancer, *GSTP1* DNA hypermethylation must affect both *GSTP1* alleles in prostatic cells, or if present at one *GSTP1* allele, must be accompanied by other somatic genome lesions affecting the other *GSTP1* allele. To determine whether *GSTP1* promoter DNA hypermethylation was present at one or both *GSTP1* alleles, a PCR strategy was used to distinguish DNA hypermethylation at maternal and paternal *GSTP1* alleles (Figure 2). After treatment of DNA from matched normal and neoplastic prostate tissues with the restriction endonuclease *Hpa*II, which cuts at the sequence CCGG but not at the sequence $C^{5\text{-}m}CGG$, or with *Msp*I, which cuts both sequences CCGG and $C^{5\text{-}me}CGG$, the digested DNA specimens were subjected to PCR amplification using oligonucleotide primers targeting a polymorphic $[ATAAA]_n$ repeat sequence near two *Hpa*II/*Msp*I sites at the *GSTP1* regulatory region (Figure 2). The amplification of polymorphic *GSTP1* promoter sequences after *Hpa*II digestion, but not after *Msp*I digestion, indicated the presence of CpG dinucleotide methylation at the *Hpa*II/*Msp*I sites in the DNA analyzed. Using this approach, *GSTP1* CpG island DNA hypermethylation was detected in the majority of PCA DNA specimens (40 of 42 or 95%) and not in normal prostate DNA specimens (Table 1). Furthermore, no *GSTP1* CpG island DNA hypermethylation was detected in any of the *GSTP1* alleles present in either normal or neoplastic tissues from kidney, bladder, ureter, uterus, or uterine cervix (Table 1). Of informative PCA cases containing DNA heterozygous for polymorphic *GSTP1* $[ATAAA]_n$ repeat sequences, 28 of 33 (85%) exhibited

Table 1. Detection of *GSTP1* CpG Island Hypermethylation in Cancer DNA Using an Assay Capable of Discriminating CpG Hypermethylation Affecting Maternal and Paternal *GSTP1* Alleles13

Cancer organ site*	Number of cancer cases with *GSTP1* CpG island hypermethylation†
Prostate‡	40/42
Noninformative (homozygous for *GSTP1* $[ATAAA]_n$ repeats)	11/11
Informative (heterozygous for *GSTP1* $[ATAAA]_n$ repeats)	29/31 (27 cases with 2 hypermethylated *GSTP1* alleles, 2 cases with 1 hypermethylated *GSTP1* allele, and 2 cases with 0 hypermethylated *GSTP1* alleles)
Kidney	1/10
Endometrium	0/10
Uterine cervix	0/10
Bladder/ureter	0/5

*For each case, DNA was isolated from cancer tissues, and from normal tissues, as described in the Materials and Methods.
†None of the DNA isolated from normal tissues displayed any *GSTP1* CpG island DNA hypermethylation.
‡Control normal DNA for prostate cancer cases included DNA from normal prostate tissue adjacent to cancer, DNA from seminal vesicles without cancer involvement, and DNA from white blood cells.


GSTP1 Case 96
A
GSTP1 Case 419
B
PSA Case 419
C
PAP Case 419
D


E
[ATAAA]n
HpaII
AP-1
SP-1
SP-1
TATAA
-343 -301
+1
Primer set
Case 96
Case 419
Normal
Tumor
U H M
1 2 3 4 5 6 7 8 9 101112
U—undigested
H—HpaII digested
M—MspI digested


F
[ATAAA]n
-547
AP-1
SP-1
SP-1
TATAA
+1
+83
WBC
Case 96
Case 419
0%
1-20%
21-40%
41-60%
61-80%
81-100%

DNA hypermethylation affecting both *GSTP1* alleles, 1 of 33 (3%) exhibited allelic loss, 2 of 33 (6%) exhibited DNA hypermethylation affecting one of two *GSTP1* alleles (cases no. 96 and no. 419, see Figure 3), and 2 of 33 (6%) failed to exhibit DNA hypermethylation at either *GSTP1* allele.

Bisulfite Genomic Sequencing Analyses Reveal that DNA from One PCA Case, Containing PCA Cells that Express High Levels of GSTP1 Polypeptides, Displays CpG Island Hypermethylation Affecting One GSTP1 Allele but Not the Other

For the four cases that did not appear to contain somatic *GSTP1* CpG island DNA hypermethylation at both maternal and paternal *GSTP1* alleles using the allele-specific *GSTP1* PCR DNA methylation assay described, the failure to detect CpG island hypermethylation could have been a result of a true absence of somatic *GSTP1* CpG island hypermethylation in PCA cells. Alternatively, *GSTP1* CpG island hypermethylation may have been present in PCA cell DNA, but not at the specific CpG dinucleotides sampled in the assay used (an assay false-negative). To resolve this issue, genomic DNA from each of these four cases was subjected to analysis using a bisulfite genomic-sequencing approach capable of ascertaining the extent of CpG island DNA hypermethylation at maternal *versus* paternal *GSTP1* alleles. One of the prostate cancer cases (case no. 96) that showed *GSTP1* hypermethylation affecting only one of two *GSTP1* alleles in PCA DNA by the $^{5\text{-m}}$CpG-sensitive restriction endonuclease/PCR assay (Figure 3E and Table 1) also showed less *GSTP1* promoter methylation, relative to loss of polymorphic DNA sequences at an allelic loss locus, by Southern blot analysis (Figure 1B). When DNA from this PCA case was subjected to bisulfite genomic-sequencing analysis (Figure 3F), *GSTP1* hypermethylation was evident at both *GSTP1* alleles, although the extent of CpG dinucleotide methylation throughout each *GSTP1* CpG island allele was different, with the most dense area of CpG dinucleotide methylation clustered near the known *cis* promoter regulatory elements.[19,20,33,35–41] Immunohistochemical-staining analysis of PCA tissues from this case revealed an absence of GSTP1 expression in all PCA cells, consistent with inactivation of both *GSTP1* alleles (Figure 3A). Similarly, DNA from both of the PCA cases that appeared not to contain *GSTP1* hypermethylation at either *GSTP1* allele when assessed using the allele-specific *GSTP1* PCR DNA methylation assay did contain *GSTP1* DNA hypermethylation affecting both *GSTP1* alleles when assessed using bisulfite genomic sequencing (not shown). Neither of these cases expressed immunoreactive GSTP1 in PCA cells when PCA tissues were stained with anti-GSTP1 antibodies (not shown). The remaining PCA case that showed *GSTP1* hypermethylation at only one of two *GSTP1* CpG island alleles (case no. 419, see Figure 3E) when assessed using the allele-specific *GSTP1* PCR DNA methylation assay appeared also to contain *GSTP1* DNA hypermethylation at only one of two *GSTP1* CpG island alleles when assessed using bisulfite genomic sequencing (Figure 3F). Immunohistochemical staining of PCA tissues from this PCA case revealed abundant GSTP1 expression (Figure 3B), as well as expression of prostate-specific antigen (Figure 3C) and prostate-specific acid phosphatase (Figure 3D) consistent with uninhibited transcription of the unmethylated *GSTP1* promoter alleles present in PCA cells in this PCA case. Of interest, the PC-3 and DU145 PCA cell lines also contain both unmethylated and hypermethylated *GSTP1* CpG island alleles, and each cell line also exhibits high-level *GSTP1* mRNA and GSTP1 polypeptide expression.[7] Also, although GSTP1-expressing PCA cells are extremely rare in PCAs at the time of initial presentation, GSTP1-expressing PCA cells have been detected in locally recurrent or persistent PCAs after radiation therapy in as many as 62% cases,[42] suggesting that reactivation of *GSTP1* expression may well occur under certain circumstances *in vivo* as well as *in vitro*. For case no. 419, whether the expressed *GSTP1* allele carries a somatic mutation that affects GSTP1 function has not been determined.

GSTP1 CpG Island Hypermethylation Prevents GSTP1 Expression in LNCaP PCA Cells

We previously reported that LNCaP PCA cells contain only hypermethylated *GSTP1* CpG island alleles and fail to express either *GSTP1* mRNA or GSTP1 polypeptides.[7] To determine whether diminished *GSTP1* transcription might be responsible for the lack of *GSTP1* mRNA expression in LNCaP cells, nuclear run-on transcription analysis was undertaken. Significantly reduced *GSTP1* transcription in LNCaP PCA cells was evident in comparison with PC-3 PCA cells (Figure 4A), known to contain

Figure 3. Analysis of GSTP1 expression and of *GSTP1* CpG island methylation for prostate cancer (PCA) case 96 and case 419. Both case 96 and case 419 showed *GSTP1* hypermethylation affecting only one of two *GSTP1* alleles in PCA DNA by the $^{5\text{-m}}$CpG-sensitive restriction endonuclease/PCR assay (see Figure 2 and Table 1). Immunohistochemical staining with anti-GSTP1 antibodies revealed an absence of GSTP1 expression in PCA cells (**arrowheads**) *versus* normal cells (**arrows**) in case 96 (**A**), but an abundance of GSTP1 expression in PCA cells (**arrowheads**) in case 419 (**B**). PCA cells in case 419 nonetheless appeared to express prostate-specific antigen (**C**) and prostate-specific acid phosphatase (**D**) as evidenced by immunohistochemical staining with appropriate antibodies. **E:** DNA from case 96 and from case 419 was subjected to analysis using the $^{5\text{-m}}$C-sensitive restriction endonuclease-PCR assay described for Figure 2. DNA from matched normal (normal) and neoplastic (tumor) prostate tissues was left untreated (U; **lanes 1**, **4**, **7**, and **10**), or was treated with *Hpa*II (H; **lanes 2**, **5**, **8**, and **11**), which cuts CCGG but not C$^{5\text{-m}}$CGG, or treated with *Msp*I (M; **lanes 3**, **6**, **9**, and **12**), which cuts CCGG and C$^{5\text{-m}}$CGG, before being subjected to PCR amplification using oligonucleotide primers targeting a polymorphic $[ATAAA]_n$ repeat sequence near the *GSTP1* regulatory region. DNA from both of the PCA cases was also subjected to bisulfite genomic sequencing analysis (**F**), using an assay capable of distinguishing CpG dinucleotide methylation patterns at both maternal and paternal *GSTP1* alleles (see Materials and Methods). For each case, a minimum of eight PCR clones was sequenced; the fraction of PCR clones with $^{5\text{-m}}$C at each CpG site is indicated for each polymorphic $[ATAAA]_n$ repeat allele using the gray scale provided. For case 96, although the extent of CpG dinucleotide methylation throughout each *GSTP1* CpG island allele was different, both *GSTP1* alleles displayed CpG dinucleotide hypermethylation, particularly near known *cis* regulatory elements. For case 419, *GSTP1* DNA hypermethylation appeared to be present on only one of two *GSTP1* CpG island alleles.

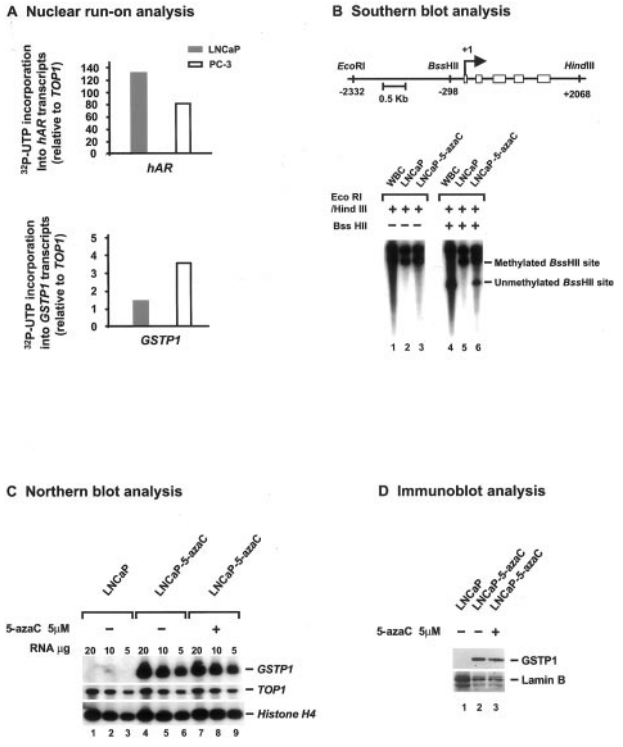


Figure 4. Contribution of *GSTP1* CpG island hypermethylation to lack of GSTP1 expression by LNCaP prostate cancer (PCA) cells. **A:** Nuclear run-on transcription analyses of *GSTP1*, *hAR*, and *TOP1*, using nuclei from LNCaP PCA cells, which fail to express *GSTP1* mRNA, and PC-3 PCA cells, which express high levels *GSTP1* mRNA, were undertaken. The amount of ^{32}P-UTP-labeling of *GSTP1* and *hAR* transcripts, relative to ^{32}P-UTP-labeling of *TOP1* transcripts, is displayed. **B–D:** LNCaP PCA cells propagated *in vitro* were treated with the DNA methyltransferase inhibitor 5-aza-C. By Southern blot analysis (**B**), 5-aza-C treatment resulted in the appearance of unmethylated *GSTP1* CpG island alleles in LNCaP DNA, as evidenced by the appearance of unmethylated *Bss*HII recognition sites in the *GSTP1* promoter region. By Northern blot analysis (**C**) and by immunoblot analyses (**D**), 5-aza-C treatment triggered a restoration of *GSTP1* expression in LNCaP PCA cells, detected whether or not 5-aza-C was present in the growth medium.

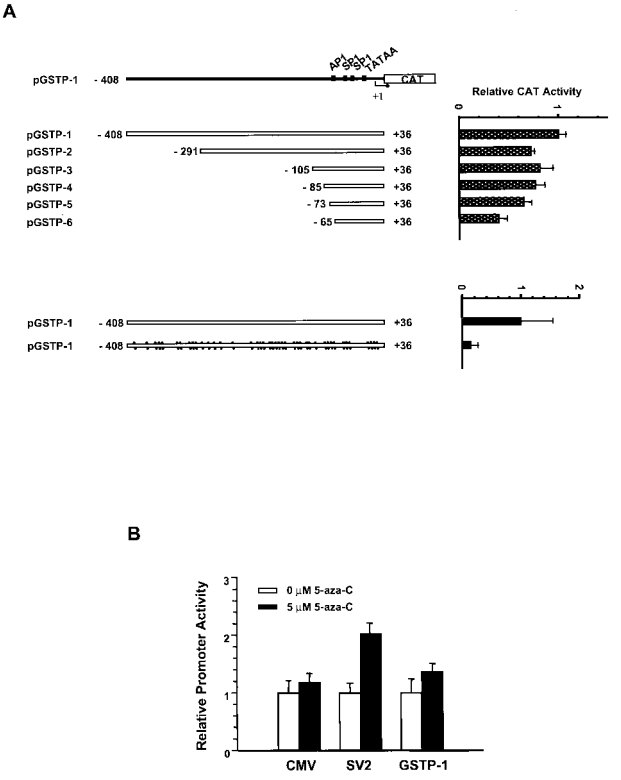


Figure 5. Effects of CpG island DNA hypermethylation on *GSTP1* promoter function in LNCaP prostate cancer (PCA) cells. **A:** Unmethylated *GSTP1* promoter/*CAT* reporter constructs were used for *GSTP1* promoter mapping,[33] revealing transcriptional enhancing sequences at −408 to −291 and at −73 to −65 5′ of the transcription start site after transfection into LNCaP PCA cells. When methylated *GSTP1* promoter sequences (**black dots**), prepared by treatment with *Sss*I methylase, were ligated to *CAT* reporter sequences and transfected into LNCaP PCA cells, a reduction in CAT reporter activity, in comparison to unmethylated *GSTP1* promoter/*CAT* reporter-transfected LNCaP cells, was evident. **B:** The *trans*-activation effects of 5-aza-C exposure (**black bars**) on the activity of unmethylated *CMV*, *SV2*, and *GSTP1* promoters in LNCaP PCA cells were assessed. 5-Aza-C treatment of unmethylated *GSTP1* promoter/*CAT* reporter-transfected LNCaP cells triggered only minimal increases in *GSTP1* promoter activity.

unmethylated *GSTP1* CpG island alleles and to express high levels of *GSTP1* mRNA and GSTP1 polypeptides.[7] Treatment with inhibitors of DNA methyltransferases has been reported to result in reversal of *GSTP1* CpG island hypermethylation and restoration of *GSTP1* expression in MCF-7 breast cancer cells[43] and in Hep3B liver cancer cells.[30] To ascertain whether the *GSTP1* CpG island hypermethylation might contribute to the reduced *GSTP1* transcription in LNCaP PCA cells, we subjected LNCaP PCA cells propagated *in vitro* to treatment with the DNA methyltransferase inhibitor 5-aza-C. Exposure of LNCaP PCA cells to 5-aza-C resulted in a reversal of *GSTP1* DNA hypermethylation evident by Southern blot analysis (Figure 4B) and a restoration of *GSTP1* mRNA and GSTP1 polypeptide expression seen using Northern blot and immunoblot analyses, respectively (Figure 4, C and D). Increased *GSTP1* expression by 5-aza-C-treated LNCaP cells did not seem to be merely the result of 5-aza-C induction of *GSTP1* transcription. LNCaP cells containing unmethylated *GSTP1* promoter alleles after 5-aza-C treatment expressed similar amounts *GSTP1* mRNA and GSTP1 polypeptides in the presence or absence of 5-aza-C (Figure 4, C and D).

To directly determine the effect of CpG island DNA hypermethylation on *GSTP1* promoter function, we conducted transient expression assays using hypermethylated and unmethylated *GSTP1* promoter/*CAT* reporter DNA constructs, prepared by ligating *Sss*I CpG-methylase-treated and untreated *GSTP1* promoter sequences to unmethylated *CAT* reporter sequences, transfected into LNCaP cells (Figure 5). In initial experiments using unmethylated *GSTP1* promoter/*CAT* reporter constructs transfected into LNCaP cells, transcriptional enhancing sequences were evident at −408 to −291 and at −73 to −65 5′ of the transcription start site (Figure 5A). The region −73 to −65 has also been found to augment *GSTP1* promoter function in human MCF-7 breast cancer (BCA) cells in previous studies.[33,35,38,39] No evidence for a *cis*-acting transcriptional silencer, as has been reported at −105 to −86 5′ of the transcription start site for MCF-7 cells,[39] was seen (Figure 5A). However, when hypermethylated *GSTP1* promoter/*CAT* reporter constructs were transfected into LNCaP cells, a reduction in CAT reporter activity, in comparison to unmethylated *GSTP1* promoter/*CAT* reporter-transfected LNCaP cells, was found (Figure

Table 2. Forced GSTP1 Expression in LNCaP Cells Fails to Reduce Proliferation *in Vitro* or Tumorigenicity *in Vivo*

Cell line	GSTP1 expression*	Doubling time *in vitro* (days)	Tumorigenicity *in vivo* (fraction of mice with tumors at 8 weeks)†
LNCaP	−	1.11 ± 0.07	9/15
LNCaP-5-aza-C	+	Not determined	8/10
LNCaP-*neo*	−	0.09 ± 0.14	15/15
LNCaP-*GSTP1*-1	+	1.04 ± 0.04	15/15
LNCaP-*GSTP1*-3	+	0.88 ± 0.04	15/15
LNCaP-*GSTP1*-5	+	1.06 ± 0.10	10/15

*GSTP1 expression assessed by immunoblot analysis with anti-GSTP1 antibodies.
†Cells (10^6) admixed with Matrigel were inoculated subcutaneously into athymic mice. At 8 weeks after inoculation, animals were sacrificed and the appearance of tumors >4 mm^3 was scored.

5A), consistent with an inhibitory effect of *GSTP1* CpG island hypermethylation on *GSTP1* transcription in PCA cells. Of note, although 5-aza-C treatment of unmethylated *SV2* promoter/*CAT* reporter-transfected LNCaP cells resulted in a substantial induction of *CAT* reporter expression, 5-aza-C treatment of unmethylated *GSTP1* promoter/*CAT* reporter-transfected LNCaP cells triggered only minimal increases in *GSTP1* promoter activity (Figure 5B), confirming that 5-aza-C treatment of LNCaP cells was unlikely to have elevated *GSTP1* mRNA and GSTP1 polypeptide expression (Figure 4) via *GSTP1* promoter *trans*-activation.

Restoration of GSTP1 Expression in LNCaP Cells Fails to Abrogate LNCaP Proliferation in Vitro *or Tumorigenicity* in Vivo

Somatic *GSTP1* inactivation seems to be selected during human prostatic carcinogenesis. Adler and colleagues[44] have reported that π-class GSTs inhibit Jun N-terminal kinase (JNK) activity. If expression of GSTP1 in PCA cells inhibited PCA growth by interfering with growth-promoting signal transduction pathways, loss of *GSTP1* function might provide a selective growth advantage for PCA cells. To determine whether restoration of GSTP1 expression affected PCA growth, GSTP1 expression was restored in LNCaP cells, either by 5-aza-C treatment or by transfection with pCMV-*GSTP1*. When the proliferation of LNCaP cells, LNCaP-5-aza-C cells, LNCaP-*neo* cells, and three independent LNCaP-*GSTP1* subclones, in tissue culture flasks *in vitro* was assessed, no consistent inhibition of cell growth was evident (Table 2). In addition, when each of the cell lines was admixed with Matrigel and injected subcutaneously into immunodeficient mice, no consistent differences in tumorigenicity was seen (Table 2).

Discussion

Hypermethylation CpG island sequences encompassing the transcriptional promoter of *GSTP1* has been reported to be the most common somatic genome alteration in human PCA.[7–12] Furthermore, loss of *GSTP1* function seems to occur very early in prostatic carcinogenesis, as loss of GSTP1 expression and *GSTP1* CpG island DNA hypermethylation have been detected in the majority of prostatic intraepithelial neoplasia lesions.[13] The data presented in this study, which focused on localized PCA removed at prostatectomy, revealed that somatic *GSTP1* defects, whether CpG island hypermethylation or gene deletions, were present in all of the PCA cases studied. For the PCA cases in which PCA cells failed to express GSTP1 *in vivo*, defective *GSTP1* alleles, and only defective *GSTP1* alleles, were present in all of the cancer cells. For LNCaP PCA cells propagated *in vitro*, which contained only defective *GSTP1* alleles and also failed to express GSTP1, reversal of abnormal *GSTP1* CpG island DNA hypermethylation resulted in restoration of GSTP1 expression. The *GSTP1* CpG island DNA hypermethylation also likely prevented GSTP1 expression by PCA cells *in vivo*. In the single case studied in which PCA cells expressed abundant GSTP1 polypeptides, although one of the *GSTP1* alleles carried CpG island DNA hypermethylation, the other allele was free of any somatic *GSTP1* defects. To be subject to selection in cancer cells, somatic genome alterations, including CpG island DNA hypermethylation, must be maintained through cell division and must affect gene and/or gene product function. CpG dinucleotide methylation patterns can be maintained through mitosis by the action of DNA methyltransferases at the site of DNA replication.[45–47] Taken together, all of the data collected for this manuscript strongly suggest that selection for *GSTP1* inactivation during the pathogenesis of human PCA can be inferred for most PCA cases.

The mechanisms by which critical genes, such as *GSTP1*, acquire somatic CpG island DNA hypermethylation during cancer pathogenesis have not been established. Nonetheless, abnormal actions of DNA methyltransferases likely play some sort of role. Forced expression of DNA methyltransferases in immortalized mammalian cells has been shown to result both in *de novo* hypermethylation and in transformation *in vitro*.[48–50] Transformation by *c-fos* seems to require DNA methyltransferase expression.[51] Mice carrying defective *Apc* alleles and disrupted *Dnmt1* alleles exhibit fewer intestinal polyps.[52] Often, silenced genes manifest a repressed chromatin conformation along with carrying increased CpG island hypermethylation. In fact, recent data have suggested that DNA methyltransferases and $^{5\text{-m}}$C-binding proteins may interact directly with chromatin remod-

eling enzymes, such as histone deacetylases, to repress gene expression.[53–62] In contrast, transcriptionally active genes seem relatively resistant to *de novo* CpG island DNA methylation.[63,64] Whether a possible coordination of DNA methyltransferase activity and transcriptional inactivity may lead to specific gene silencing during the development of human cancers has not been determined. Nonetheless, an inducible gene such as *GSTP1* might be especially vulnerable to inactivation, while in a nonexpressed state, via this type of mechanism. Genes encoding GSTs are characteristically expressed at very low levels in many tissues until induced, via an increase in transcriptional promoter activity, on exposure to oxidants and electrophiles.[65–67] Perhaps, in the absence of inducer exposure, low level *GSTP1* transcription might render the *GSTP1* CpG island vulnerable to *de novo* DNA hypermethylation.

How might the phenotype of lack of GSTP1 expression be subject to selection during prostatic carcinogenesis? In one selection model, *GSTP1* might act like a tumor suppressor gene, which when inactivated leads to tumor growth. Favoring this type of model, Adler and colleagues[44] have reported that π-class GSTs can interfere with *N*-terminal c-Jun kinase signaling. Against this model, our studies of LNCaP PCA cell growth and tumorigenicity discerned no role for *GSTP1* expression in abrogation of LNCaP PCA cell proliferation *in vitro* or *in vivo*. In another selection model, *GSTP1* might act like a caretaker gene, which when inactivated leads to additional somatic genome alterations that promote tumor growth.[4] GSTP1, like other GSTs, can catalyze the detoxification of oxidants and electrophiles that threaten genome damage.[66] As an example, mice carrying disrupted *Gstp* alleles display enhanced skin tumorigenesis on exposure to 7,12-dimethylbenz anthracene.[68] In addition, recent data indicate that GSTP1 may provide prostate cells protection against DNA adduct formation associated with ingestion of dietary heterocyclic aromatic amine carcinogens, such as 2-amino-1-methyl-6-phenylimidazo[4,5-β]pyridine (PhIP), present in many foods in the stereotypical North American diet, particularly well-done or charred meats.[69] However, in these studies, when LNCaP cells were genetically modified to express GSTP1, the resultant cells appeared protected not only against DNA adduct formation on exposure to *N*-OH-PhIP, an activated PhIP metabolite, but also against *N*-OH-PhIP cytotoxicity.[69] Loss of *GSTP1* function thus rendered LNCaP cells vulnerable to both genome damaging and cell killing effects of *N*-OH-PhIP. For lack of GSTP1 expression to be selected in the face of PhIP exposure, PhIP-mediated genome damage must target another gene involved in prostate cell growth regulation. In this way, loss of *GSTP1* caretaker function might indirectly lead to selection during prostatic carcinogenesis. The data presented in this article permit only the inference that selection for *GSTP1* inactivation during the pathogenesis of human PCA has likely occurred. To prove selection, model studies demonstrating a selective growth or survival advantage for loss of *GSTP1* function in prostate cells will be required.

In our study, using a combination of assays, *GSTP1* CpG island hypermethylation was detected in DNA from every prostate cancer case surveyed. As such, sensitive and specific detection of *GSTP1* CpG island hypermethylation might offer an opportunity for molecular detection, diagnosis, and staging of human PCA. Thus far, two basic PCR strategies have emerged. The first features the use of $^{5\text{-m}}$CpG-sensitive restriction endonucleases before PCR amplification of *GSTP1* CpG island sequences. One version of this PCR strategy seems capable of detecting PCA DNA in 91% of PCA cases at a limiting sensitivity of 2 pg. This assay has been reported to detect as little as 2 ng PCA DNA when the PCA DNA is admixed with 1 μg of white blood cell DNA.[9] The second PCR strategy for detecting hypermethylated *GSTP1* CpG island sequences involves the use of the bisulfite reaction followed by PCR, which results in the conversion of C, but not of $^{5\text{-m}}$C, to T. Primers specific for converted target sequences derived from $^{5\text{-m}}$CpG-containing *versus* CpG-containing *GSTP1* alleles are then used to selectively amplify products from hypermethylated *versus* unmethylated *GSTP1* CpG islands (methylation-specific PCR or MSP).[30,70,71] In a recent report, a version of this PCR strategy, able to discriminate as few as 200 LNCaP PCA cells, detected PCA DNA in 94% of PCA tissues, 72% of plasma or serum specimens, 50% of ejaculates, and 36% of urine specimens from men with known PCA.[8] As more data become available regarding consensus *GSTP1* CpG island DNA methylation patterns characteristic of PCA, both of these PCR strategies can be refined to discriminate a greater fraction of PCA cases, perhaps permitting *GSTP1* CpG island DNA hypermethylation to serve as a potentially useful molecular biomarker for PCA detection, diagnosis, and staging.

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