Haplotypes, Loss of Heterozygosity, and Expression Levels of Glycine *N*-Methyltransferase in Prostate Cancer

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Abstract **Purpose:** Glycine *N*-methyltransferase (GNMT) affects genetic stability by regulating DNA methylation and interacting with environmental carcinogens. In a previous study, we showed that *GNMT* acts as a susceptibility gene for hepatocellular carcinoma. Here, we report on our efforts to characterize the haplotypes, loss of heterozygosity (LOH), and expression levels of the *GNMT* in prostate cancer.

Experimental Design: Peripheral blood mononuclear cell DNA collected from 326 prostate cancer patients and 327 age-matched controls was used to determine *GNMT* haplotypes. Luciferase reporter constructs were used to compare the promoter activity of different *GNMT* haplotypes. *GNMT* LOH rates in tumorous specimens were investigated via a comparison with peripheral blood mononuclear cell genotypes. Immunohistochemical staining was used to analyze GNMT expression in tissue specimens collected from 5 normal individuals, 33 benign prostatic hyperplasia patients, and 45 prostate cancer patients.

Results: Three major *GNMT* haplotypes were identified in 92% of the participants: A, 16GAs/DEL/C (58%); B, 10GAs/INS/C (19.9%); and C, 10GAs/INS/T (14.5%). Haplotype C carriers had significantly lower risk for prostate cancer compared with individuals with haplotype A (odds ratio, 0.68; 95% confidence interval, 0.48-0.95). Results from a phenotypic analysis showed that haplotype C exhibited the highest promoter activity ($P < 0.05$, ANOVA test). In addition, 36.4% (8 of 22) of the prostatic tumor tissues had LOH of the *GNMT* gene. Immunohistochemical staining results showed abundant GNMT expression in normal prostatic and benign prostatic hyperplasia tissues, whereas it was diminished in 82.2% (37 of 45) of the prostate cancer tissues.

Conclusions: Our findings suggest that *GNMT* is a tumor susceptibility gene for prostate cancer.

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Prostate cancer is the third most common cancer for men throughout the globe and the most common cancer among men in North America, Europe, and some parts of Africa (1). Asian populations have much lower incidence rates, especially among Chinese (2). Well-documented risk factors associated with prostate cancer include age, race, and family history of prostate cancer. Secondary risk factors that have been implicated are diet, androgens, occupational chemicals, smoking, inflammation, and obesity (2).

Changes in DNA methylation are found during the initial and progressive stages of many human tumors. Alterations appearing as either hypomethylation or hypermethylation can lead to chromosomal instability and transcriptional gene silencing (3), both of which have been identified in prostate cancer cases (4). Results from animal experiments show that diets deficient in methyl group donors (e.g., choline and methionine) or methyl group metabolism coenzymes (e.g., folate and vitamin B12) result in disturbances in intracellular S-adenosylmethionine levels, trigger DNA hypomethylation, and promote cancers of the liver, prostate, and other organs (5).

Glycine *N*-methyltransferase (GNMT; EC2.1.1.20), a protein with multiple functions, affects genetic stability by regulating the ratio of S-adenosylmethionine to S-adenosylhomocysteine, binding to folate (6, 7), and interacting with environmental carcinogens, such as benzo(*a*)pyrene (8). Previously, we identified *GNMT* as a tumor susceptibility gene for human hepatocellular carcinoma (HCC; refs. 9, 10). We mapped the human *GNMT* gene to the 6p12 chromosomal region and characterized its polymorphism (10, 11). Genotypic analyses of several human *GNMT* gene polymorphisms using a special

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Received 6/26/06; revised 10/23/06; accepted 12/12/06.

Grant support: Veterans General Hospital, Tsou Foundation, and National Yang-Ming University Joint Research Program grant VTY92-P5-32 and Mackay Memorial Hospital and National Yang-Ming University Joint Research Program grants MMHYM93-N010-016 and MMHYM94-016.

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doi:10.1158/1078-0432.CCR-06-1551

formula, we reported that 36% to 47% of the HCC tissues have loss of heterozygosity (LOH) of *GNMT* gene (11). Because GNMT only expresses in the liver, pancreas, prostate, and kidney (10), we hypothesized that *GNMT* is also a tumor susceptibility gene for prostate cancer.

In this report, we designed a case-control study to analyze associations between different *GNMT* haplotypes and prostate cancer. Results from a phenotypic analysis showed that one *GNMT* haplotype exerting a protective effect against prostate cancer had the highest level of promoter activity. We also investigated the *GNMT* LOH rate in tumorous specimens by comparing their genotypes with those found in peripheral blood mononuclear cells (PBMC). Finally, immunohistochemical staining was used to analyze GNMT gene expression level; the results indicate that *GNMT* was down-regulated in most of the prostatic tumor specimens.

Materials and Methods

Participants and specimens. Between 2000 and 2005, 326 prostate cancer patients were recruited from postsurgery follow-up clinics at three Taiwanese hospitals: Taipei Veterans General Hospital (Taipei, Taiwan), Taipei Mackay Memorial Hospital (Taipei, Taiwan), and Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan). Pathologic examinations of specimens obtained from transrectal ultrasound-guided biopsy have been done on all the patients and the diagnosis was confirmed. The demographic and clinical data, including disease stage, Gleason scores, and prostate-specific antigen (PSA) levels, were collected through reviewing medical records of the patients. The disease stages of the patients were determined using the definition proposed by the American Joint Committee on Cancer tumor-nodes-metastasis system (2002). All the patients were Chinese descendants and none of them were aborigines. For the case-control study, 327 normal adult males matched by age and ethnic background to the patients were recruited from Taipei Municipal Hospital and Kaohsiung Yuan's General Hospital. All the controls had received physical examination, including digital rectal examination and serobiochemical tests, to rule out malignancy. Men with abnormal PSA level (>4.0 ng/mL) were excluded from the control group. This study has been reviewed and approved by the institutional review boards of all the participating hospitals. Informed consent was obtained from patients who participated in this study.

For LOH and immunohistochemical staining studies, paraffin-embedded tissue blocks that contained sufficient amounts of surgical specimens from 45 prostate cancer patients were available for the analysis. These patients had received either transurethral resections of prostate or radical retropubic prostatectomies. For comparison, paraffin-embedded tissue blocks from 30 patients with benign prostatic hyperplasia (BPH) and a tissue array (US Biomax, Inc., Rockville, MD) containing five normal prostate tissues (spotted in triplicates), three BPH, and three prostate cancer tissues (spotted in duplicates) were also used.

Cell lines and culture. Two human prostate cancer cell lines, PC-3 (12) and LNCaP (13), one human HCC cell line, HA22T/VGH (14), and one human embryonic kidney cell line, HEK293A (15), were used in this study. The PC-3 cells were maintained in Ham's F-12K medium (Life Technologies, Rockville, MD) supplemented with 7% heat-inactivated fetal bovine serum (HyClone, Logan, UT). The LNCaP cells were cultured in RPMI 1640 (HyClone) supplemented with 10% heat-inactivated fetal bovine serum. Both HA22T/VGH and HEK293A cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) with 10% heat-inactivated fetal bovine serum. All the culture media mentioned above contained penicillin (100 IU/mL), streptomycin (100 mg/mL), fungizone (2.5 mg/mL), and L-glutamine (2 mmol/L). The cell cultures were maintained in a humidified incubator with 5% CO_2.

Determination of the genetic polymorphisms of GNMT using GeneScan, Taqman, and denaturing high-performance liquid chromatography assays. Genomic DNA was obtained from the PBMCs using a conventional phenol/chloroform extraction method followed by ethanol precipitation. All DNA samples were stored at −20°C. As shown in Fig. 1A, three polymorphisms of *GNMT*, including the short tandem repeat polymorphism 1 (STRP1), 4-bp insertion/deletion polymorphisms (INS/DEL), and single-nucleotide polymorphism 1 (SNP1), were analyzed in this study. Automated fragment analysis (GeneScan) was used to detect instances of STRP1 and INS/DEL. Details of the methods and primers used have been described previously (11). In brief, PCR was done using a GeneAmp PCR 9700 thermocycler (Applied Biosystems, Foster City, CA). The sizes of the PCR products were elucidated by using an ABI 377 PRISM sequencer (Applied Biosystems) with a GeneScan 400 size standard and Genotyper analytic software (Applied Biosystems). For quality control purposes, DNA samples from Huh 7 and Hep 3B cell lines whose genotypes have been determined previously were included in each set of experiments (11).

The SNP1 was determined using a Taqman 5′-nuclease assay, which has been described previously (11). In addition, about one third (112) of samples were randomly selected for denaturing high-performance liquid chromatography assay (16). Denaturing high-performance liquid chromatography was carried out using an automated high-performance liquid chromatography instrument equipped with a DNA separation column (Transgenomic, San Jose, CA). PCR was done in a 25 μL reaction volume containing 25 ng of genomic DNA, 0.3 μmol/L of each primer, 200 μmol/L deoxynucleotide triphosphates, 2.5 μL of 10× PCR buffer containing $MgCl_2$ (Applied Biosystems), and 0.625 unit of AmpliTaq Gold polymerase (Applied Biosystems). The primers used for denaturing high-performance liquid chromatography were identical to those used in the Taqman assay. The PCR consisted of 35 cycles at 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s in a GeneAmp PCR 9700 thermocycler followed by denaturation at 95°C for 10 min and gradually cooling to 35°C using a 1-h ramping time. Initially, the PCR products were examined directly for the presence of any heteroduplex by injecting 5 μL of each sample at a column temperature of 65.5°C. Therefore, the C/T genotype of SNP1 can be distinguished from C/C or T/T genotype. To further differentiate between C/C and T/T genotypes, 2.5 μL of PCR products were mixed with 2.5 μL of PCR products from a positive control with known homozygous genotype (e.g., T/T) followed by denaturation and gradual renaturation. For a homozygous C/C sample, it will display double peaks on a chromatogram, and for a T/T sample, it will display a single peak. The results of the 112 samples on the denaturing high-performance liquid chromatography assay were 100% concordant with that obtained using the Taqman assay.

Microdissection and DNA extraction. The tumorous portions of prostate cancer tissues on a pathologic slide, which had been stained with H&E, were identified and marked by a pathologist. Subsequently, two other slides containing adjacent sections with a thickness of 10 μm were deparaffinized and used for microdissection. To prevent cross-contamination, cases were dissected with different needles. The dissected tissues were further digested with proteinase K at 55°C for 2 days. DNA extraction protocols have been described previously (17, 18).

LOH analysis. Forty-five DNA specimens from the microdissected tumorous tissues and matched PBMCs were used for LOH analysis. The intensity of each fluorescent peak of the STRP1 and INS/DEL alleles from the GeneScan data was used to calculate the LOH index using the following formula: LOH index = $(T1/T2)/(P1/P2)$, where T1 and T2 are the allelic intensities of the tumor samples and P1 and P2 are the allelic intensities of the PBMC samples. LOH was defined as a LOH index value <0.6 or ≥ 1.7, meaning that the reduction of a single tumor tissue allele by at least 40% when compared with a corresponding allele in the PBMC sample (19). Individual PCR was repeated for all loci with LOH for confirmation. Examples of LOH of STRP1 and INS/DEL were illustrated in Fig. 1B and C, respectively.

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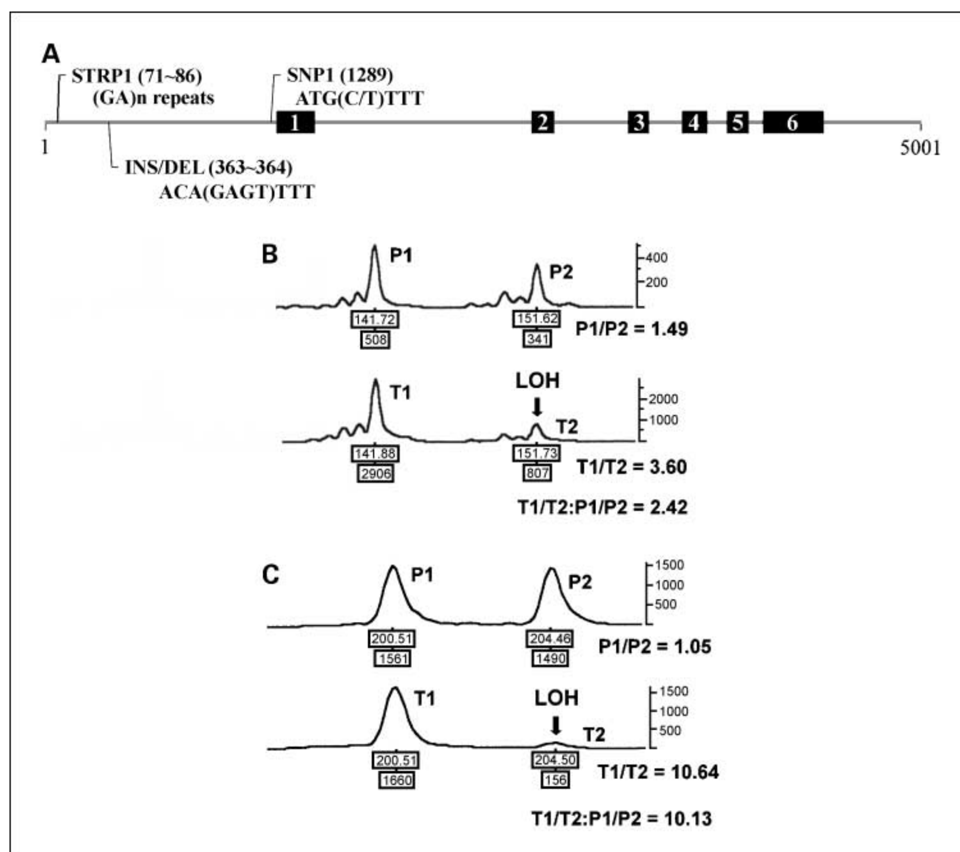

Fig. 1. *A,* the human *GNMT* gene that contains six exons is located at chromosome 6p12. The following three genetic polymorphisms of *GNMT* gene, localized in the upstream promoter region, were genotyped in this study: STRP1, 4-bp INS/DEL, and SNP1. *B,* example of LOH of STRP1 of *GNMT. C,* example of LOH in the INS/DEL genotype of *GNMT*. LOH index = (T1/T2)/(P1/P2), where T1 and T2 are the allelic intensities of the tumor samples and P1 and P2 are the allelic intensities of the PBMC samples. LOH was defined as LOH index value <0.6 or ≥1.7.

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Immunohistochemical staining. In total, 5 normal prostate tissues, 33 BPH, and 45 prostate cancer specimens were subjected to immunohistochemical staining using a GNMT monoclonal antibody 14-1 at 1:25 dilution (9). A ready-to-use biotinylated secondary antibody was applied to bind the primary antibody. A streptavidin-peroxidase conjugate was also applied to tissue slides (HistoST5050 detection kit, Zymed Laboratories, San Francisco, CA). The presence of peroxidase was revealed by the addition of 3,3′-diaminobenzidine tetrahydrochloride solution as described previously (20).

Construction of a plasmid for phenotypic analysis. Detailed descriptions of the construction of pGNMT-1.8k-16GAs/DEL/C and pGNMT-1.8k-10GAs/INS/C are given in ref. 11. pGNMT-1.8k-10GAs/INS/C was used as a template in a PCR to generate the pGNMT-1.8k-10GAs/INS/T plasmid. Two separate PCRs were done using the following two primer pairs, respectively: PS4565 (5′-GGGGTACCAGCATCTTGGC-CAGGCTG)/PA6358 (5′-CCGCACTTAAAACATAAGCACTGC) and PS6335 (5′-GCAGTGCTTATGTTTTAAGTGCGG)/PA6391 (5′-GCGA-GATCTCCTGCGCCGCGCCTGGCT). DNA products from both PCRs were mixed and used as the template for another PCR with PS4565 and PA6391 primers. The PCR was done in 50 μL reaction mixtures containing 200 μmol/L each of the four deoxynucleotide triphosphates, 0.2 μmol/L of each primer, 1.5 mmol/L $MgCl_2$, and 2.5 units of AmpliTaq DNA polymerase. Thirty-five amplification cycles were done under the following conditions: 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min. PCR products were digested with *Kpn*I and *Bgl*II and cloned to pGL3-Basic. The resultant plasmid (pGNMT-1.8k-10GAs/INS/T) was confirmed using DNA sequencing.

Luciferase and β-galactosidase assays. The cells were plated in six-well culture dishes at a density of 2×10^5 per well and maintained at 37°C in 5% CO_2 overnight. Transfection of PC-3 and LNCaP cells was done using LipofectAMINE (Invitrogen, Carlsbad, CA). Calcium phosphate coprecipitation method was used for transfection of HA22T/VGH and HEK293A cells. The cells were cotransfected with 4 μg of *GNMT* promoter containing luciferase plasmid and 1 μg of pCMVβ plasmid DNAs. The cultured medium was changed 18 h after transfection, and the cells were maintained for another 48 h before they were washed twice with PBS and lysed with 130 μL of Reporter Lysis Buffer (Promega, Madison, WI). Protein concentrations of the lysates were measured using the Bradford method (Bio-Rad, Hercules, CA). Luciferase and the β-galactosidase activity were measured using the Luciferase Assay and β-Galactosidase Enzyme Assay systems (Promega), respectively. Relative luciferase activity was normalized by the β-galactosidase activity.

Statistical analysis. Pearson χ^2 or Fisher's exact tests were used to assess the difference of the allelic frequencies of the *GNMT* gene between the case and control groups and to compare the tumor-node-metastasis (TNM) stages between groups with or without LOH and GNMT expression. Student's *t* test was used to compare the mean age of diagnosis, mean Gleason score, and mean pretreatment PSA levels between patients with or without LOH of *GNMT*. One-way ANOVAs were used to assess mean luciferase activity for three *GNMT* haplotypes. The GENECOUNTING software (version 2.0), which implements an estimation-maximization algorithm, was used to estimate the frequencies of haplotypes of these three loci and calculating linkage disequilibrium (r^2 or D') between any two loci (21, 22). Odds ratios (OR) and 95% confidence intervals were estimated for the associations between different haplotypes and prostate cancer by using logistic regression. Statistical analyses were done using the Statistical Package for the Social Sciences software package (version 11.0), and P value of <0.05 was considered significant.

Results

The clinicopathologic characteristics of the patients with prostate cancer. In total, 326 patients with prostate cancer were recruited for this case-control study. The mean age of the

prostate cancer cases was 73.0 ± 7.3 years. The mean pretreatment PSA level (±SD) of the patients was 256.5 ± 971.8 ng/mL (n = 299; range, 0.11-10,651; median, 34.0 ng/mL). The distribution of TNM stages I to IV of the patients was 8.1% (25 of 309), 23.9% (74 of 309), 19.4% (60 of 309), and 48.5% (150 of 309), respectively. In terms of Gleason score, 24.8% (75 of 303) of the patients were 2 to 5, 51.2% (155 of 303) were 6 to 7, and 24.1% (73 of 303) were 8 to 10.

Comparison of GNMT allele frequencies between prostate cancer patients and controls. To study the association between *GNMT* genetic polymorphisms and susceptibility to prostate cancer, we established a case-control study with 326 cases and 327 controls. As shown in Table 1, among the study subjects, we observed 11 different STRP1 allelic types using automated sequencer with GeneScan software assay. The numbers of GA repeats ranged from 9 to 20, with the exception of 14 GA repeats. The most common genotype of STRP1 was 16 GA repeats. No statistically significant differences were noted for the STRP1 and INS/DEL allelic distributions between the case and control groups, but a significant difference was noted for SNP1 C allele frequencies between the two groups (88.7% versus 84.9%, respectively; P = 0.04). Individuals that carry the T allele were at significantly lower risk of developing prostate cancer than those carrying the C allele (OR, 0.72; 95% confidence interval, 0.52-0.99).

Comparison of the rates of different GNMT haplotypes in a case-control study. Subsequently, we compared the distributions of different *GNMT* haplotypes in the case-control study. Results from a pairwise linkage disequilibrium analysis revealed strong linkage disequilibrium between any two loci of the three polymorphic sites ($D' > 0.94$; $P < 0.00001$). As shown in Table 1, >90% of our study populations carried the following three major *GNMT* haplotypes: A, 16GAs/DEL/C (60.0% and 58.0% for the cases and controls, respectively); B, 10GAs/INS/C (21.5% and 19.9% for the cases and controls, respectively); and C, 10GAs/INS/T (10.2% and 14.5% for the cases and controls, respectively). In addition, all the 16, 17, and 18 GA repeat alleles cosegregated with the DEL allele and that the SNP1 T allele cosegregated with the INS allele. The 10 GA repeat alleles only occurred in haplotypes containing the INS allele. Results from a comparison of haplotype frequencies between the case and control groups showed a significantly higher frequency of haplotype C in the control group (10.2% versus 14.5%; OR, 0.68; 95% confidence interval, 0.48-0.95).

Phenotypic analysis of different GNMT haplotypes. To study the phenotypes (promoter activity) of different *GNMT* haplotypes, three luciferase reporter gene plasmids were constructed. Two prostate cancer cell lines (PC-3 and LNCaP), a HCC cell line (HA22T/VGH), and a human embryonic kidney

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Table 1. *GNMT* genetic polymorphic site allele and haplotype frequencies among patients with prostate cancer and matched controls

***GNMT* alleles/ haplotypes**				**Prostate cancer cases (N = 326), n (%)**	**Controls (N = 327), n (%)**	**OR (95% CI)***
Alleles						
STRP1				n = 636	n = 646	
9 GA repeat				5 (0.8)	0	—
10 GA repeat				203 (31.4)	227 (35.1)	0.87 (0.69-1.09)
11 GA repeat				0	2 (0.3)	—
12 GA repeat				4 (0.2)	4 (0.6)	—
13 GA repeat				3 (0.5)	0	—
14 GA repeat				0	0	—
15 GA repeat				2 (0.3)	1 (0.2)	—
16 GA repeat				385 (60.5)	377 (58.4)	1.09 (0.88-1.37)
17 GA repeat				13 (2.0)	11 (1.7)	—
18 GA repeat				16 (2.5)	17 (2.6)	—
19 GA repeat				2 (0.3)	2 (0.3)	—
20 GA repeat				3 (0.5)	5 (0.8)	—
INS/DEL				n = 636	n = 654	
DEL				431 (67.8)	426 (65.1)	1.00 (Reference)
INS				205 (32.2)	228 (34.9)	0.89 (0.71-1.12)
SNP1				n = 652	n = 654	
C				578 (88.7)	555 (84.9)	1.00 (Reference)
T				74 (11.3)	99 (15.1)	0.72 (0.52-0.99)
Haplotypes †						
	STRP1	INS/DEL	SNP1	n = 640 (%)	n = 654 (%)	
A	16GAs	DEL	C	384 (60.0)	379 (58.0)	1.00 (Reference)
B	10GAs	INS	C	138 (21.5)	130 (19.9)	1.05 (0.79-1.38)
C	10GAs	INS	T	65 (10.2)	95 (14.5)	0.68 (0.48-0.95)
D	18GAs	DEL	C	15 (2.3)	17 (2.6)	0.87 (0.43-1.77)
E	17GAs	DEL	C	12 (1.9)	11 (1.7)	1.08 (0.47-2.47)
Others ‡				26 (4.1)	22 (3.4)	1.17 (0.65-2.09)

Abbreviation: 95% CI, 95% confidence interval.
*STRP1 ORs were compared with all others alleles; listed allele frequencies ≥5%.
† Only haplotypes with estimated frequencies ≥1% are listed.
‡ Haplotypes with estimated frequencies <1% are pooled into the "others" category.

cell line (HEK293A) were used for the transfection experiments. The results showed that, in all the cell lines tested, the plasmid containing haplotype C had the highest level of promoter activity and the plasmid containing haplotype A had the lowest promoter activity (Fig. 2). The differences of the promoter activities of these three plasmids were statistically significant (ANOVA tests, $P = 0.001$ for PC-3; $P = 0.007$ for LNCaP; $P = 0.001$ for HA22T/VGH; and $P = 0.033$ for HEK293A).

LOH rate at GNMT locus in prostate cancer patients. Of the 45 prostate cancer patients, 23 (51.1%) and 21 (46.7%) had heterozygous genotypes of the STRP1 and INS/DEL alleles in their PBMCs, respectively. Subsequently, we analyzed the STRP1 and INS/DEL alleles in the tumorous tissues and found that 27.3% (6 of 22) of them had LOH of STRP1 (data missing from one case) and 20.0% (4 of 20) of them had LOH of INS/DEL (data missing from one case). In total, 8 of 22 (36.4%) patients' tumorous specimens had LOH of either STRP1 or INS/DEL allele of *GNMT* gene (Table 2). We further compared the clinical features and pretreatment PSA levels between groups of patients with or without LOH of *GNMT* in their tumorous tissues. The results showed that the mean age of the diagnosis of patients with LOH of *GNMT* was 5 years younger than that of the patients without LOH of *GNMT*, although the difference was not statistically significant (68.3 ± 7.8 versus 73.7 ± 5.3; $P = 0.065$). In addition, patients whose tumor specimens did not have LOH of *GNMT* had lower rate of TNM stages III and IV than patients whose tumor specimens had LOH [76.9% (10 of 13) versus 87.5% (7 of 8)]. In terms of the mean Gleason scores and mean pretreatment PSA levels, there was no significant difference between the two groups of patients.

GNMT expression was down-regulated in the tumorous tissues from prostate cancer patients. Immunohistochemical staining with anti-GNMT monoclonal antibody 14-1 was used to analyze GNMT expression levels in the following three groups of tissues: 5 normal prostate tissues, 33 BPH, and 45 prostate cancer. The results showed that all five normal prostate tissues had abundant levels of GNMT in the cytoplasm of the cells. Both the peripheral and transitional zones of the normal prostate had positive staining (Fig. 3A-D). In addition, all 33 BPH cases had high expression levels of GNMT in the nuclei and cytoplasm of the cells (Fig. 3E and F). In contrast, only 8 of 45 (17.7%) prostate cancer tissues and 11 of 38 (28.9%) tumor-adjacent tissues had weak to moderate level of GNMT staining. GNMT was primarily expressed in the cytoplasm of the cells in the prostate cancer and tumor-adjacent tissues (Fig. 3G, H, J). The differences of the positive rates of GNMT expression between prostate cancer and normal/BPH patients prostate tissues were statistically significant ($P < 0.0001$; Table 3). We also observed a significant trend of positive GNMT expression in normal/BPH, tumor-adjacent, and tumorous tissue samples ($P_{\text{trend}} < 0.0001$). Moreover, prostate cancer patients with TNM stages I and II had significantly higher rate of positive GNMT expression compared with the group of patients with negative GNMT expression [50.0% (4 of 8) versus 11.8% (4 of 34); $P = 0.03$, Fisher's exact test]. In other words, 50% (4 of 8) of patients with positive GNMT staining were in the stages III and IV, whereas 88.2% (30 of 34) of patients with negative GNMT staining were in the stages III and IV. Results from an analysis of 38 paired tumor (T) and adjacent-tissue (N) specimens taken from the same patients showed that 15.8% (6 of 38) had the T+/N+ staining pattern, 13.2% (5 of 38) had the T−/N+ staining pattern, 71.0% (27 of 38) had the T−/N− pattern, and 0% had the T+/N− pattern (Table 3).

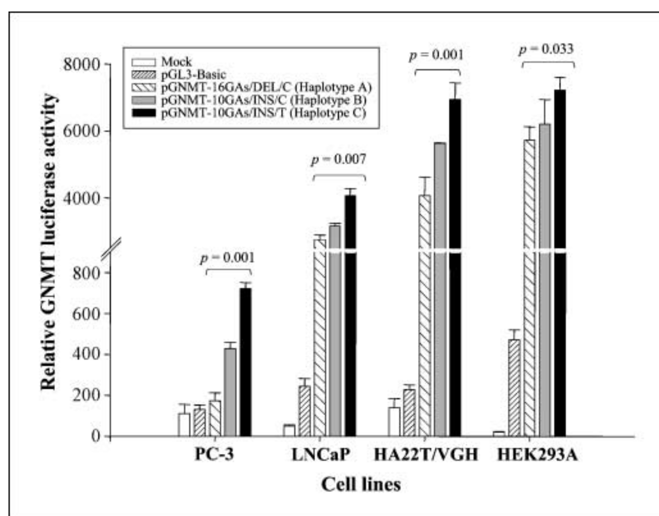

Fig. 2. Phenotypic analysis of three GNMT haplotypes, A, B, and C, in four human cell lines, including two prostate cancer cell lines (PC-3 and LNCaP). The relative GNMT promoter activity was normalized by using the β-galactosidase. Columns, mean; bars, SD.

Discussion

Results from this case-control study of the association between *GNMT* polymorphism and prostate cancer showed that there were three major *GNMT* haplotypes in our populations and haplotype C conferred protection from the development of prostate cancer (OR, 0.68; 95% confidence interval, 0.48-0.95). In addition, phenotypic analysis of three haplotypes indicated that haplotype C had the highest promoter activity and that haplotype B had significantly higher activity than the haplotype A ($P < 0.05$, ANOVA test). The results of the promoter activities were consistent in all four cell lines that we tested. It is also consistent with our previous finding that a *GNMT* promoter containing the 10GAs/INS allele expressed significantly greater activity than that containing the 16GAs/DEL allele (11). The difference between haplotypes B and C is due to the T allele of SNP1, which exerts a strong disequilibrium and is linked with the INS allele and 10 GA repeats. Results from a sequence analysis show that the *GNMT* promoter with the SNP1 T allele produces a glucocorticoid-responsive element TG*T*TTT (23) that may contribute to the higher level of haplotype C promoter activity.

GNMT is an abundant cytosolic enzyme that catalyzes the methylation of glycine into sarcosine and plays a key role in the one-carbon metabolism pathway (6). Beagle et al. (24) recently reported dual associations between the SNP1 *GNMT* and both methylenetetrahydrofolate reductase 677-C/T SNP and levels of homocysteine in the plasma of a group of young women. Specifically, they found that, after a 2-week restriction of folate intake in a group of women, individuals with the *GNMT* SNP1 T/T genotype had significantly higher plasma levels of homocysteine than individuals with the *GNMT* SNP1 C/T or

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Table 2. Comparison of the clinical features and pretreatment PSA levels between two groups of prostate cancer patients whose tumor specimens had LOH of GNMT or not

Patients	LOH of *GNMT* markers*		Age of diagnosis †	TNM stage †	Gleason score †	Pretreatment PSA level (ng/mL) †
	STRP1	INS/DEL				
Patients without LOH of *GNMT*						
P002	–	–	72	II	7 (4 + 3)	5.9
P092	–	–	76	II	5 (3 + 2)	69.0
P117	–	–	76	II	5 (2 + 3)	5.3
P034	–	–	70	III	6 (4 + 2)	38.1
P085	–	–	76	III	NA	26.2
P099	–	–	73	III	7 (4 + 3)	23.0
P168	–	–	67	III	7 (4 + 3)	23.4
P112 ‡	F	NI	72	III	7 (3 + 4)	15.0
P048	–	–	70	IV	7 (4 + 3)	120.0
P024	–	–	79	IV	7 (4 + 3)	101.0
P036	–	–	65	IV	8 (5 + 3)	43.9
P094	–	–	68	IV	9 (5 + 4)	230.0
P134	–	–	83	IV	7 (4 + 3)	154.0
P018	–	F	80	IV	7 (4 + 3)	666.0
P177	–	NI	77	NA	9 (5 + 4)	48.0
Mean ± SD			73.7 ± 5.3		7.0 ± 1.2	111.0 ± 172.0
Patients with LOH of *GNMT*						
P004	+	-	71	III	7 (4 + 3)	21.0
P032	+	-	55	III	7 (4 + 3)	9.1
P050	+	-	69	III	9 (5 + 4)	65.7
P015	+	-	81	IV	7 (4 + 3)	2,150.0
P041	-	+	66	II	5 (3 + 2)	64.0
P025	-	+	75	III	7 (4 + 3)	29.7
P051	+	+	63	IV	6 (2 + 4)	10,567.0
P109	+	+	66	IV	7 (3 + 4)	
Mean ± SD			68.3 ± 7.8		6.9 ± 1.1	1,614.0 ± 3,692.7
LOH frequency (%)	6/22 (27.3)	4/20 (20.0)				
Total (%)	8/22 (36.4)					

Abbreviations: NA, not available; F, failed amplification in tumor tissue sample; NI, noninformative (homozygous genotype in the PBMC DNA).
*+, LOH detected; –, LOH not detected.
† No statistically significant differences in mean age of diagnosis ($P = 0.065$), TNM stage ($P = 0.800$), mean Gleason score ($P = 0.818$), or mean pretreatment PSA level ($P = 0.288$) in prostate cancer patients with or without LOH of GNMT.
‡ Case no.112 was excluded from the analysis of mean age of diagnosis, TNM stage, mean Gleason score, and mean pretreatment PSA level.

C/C genotype. These results suggest that individuals carrying the *GNMT* SNP1 T/T genotype may express greater GNMT enzyme activity leading to increased plasma concentrations of homocysteine. Hultdin et al. (25) previously reported that an increase in plasma homocysteine is associated with reduced prostate cancer risk. We therefore suggest that the *GNMT* SNP1 T allele may confer protection from prostate cancer development.

In the present study, we observed *GNMT* gene LOH in 36.4% (8 of 22) of prostate cancer tissue samples. Because the tumorous tissue samples used in this part of the experiment were obtained via microdissection, the true LOH rate might be higher. Previously, we compared the fluorescent signals of each allele of *GNMT* genotypes in the tumorous and nontumorous liver tissues from the same patients with HCC and found that the rates of LOH for STRP1and INS/DEL were 41% (7 of 17) and 36% (4 of 11), respectively (11). To avoid misclassification due to genetic alterations in the nontumorous tissues, so-called field effect, we used paired PBMC and tumor DNA samples from the same patients to analyze the LOH of *GNMT*. In terms of the pattern of the LOH, we found that six of six LOH of STRP1 were from 16 GA repeats toward 10 GA repeat alleles and three of four LOH of INS/DEL were toward DEL allele. In contrast, the directions of the LOH of STRP1 in HCC patients were more complicated: among seven HCC cases with LOH, one was from 10 GA repeats toward 16 GA repeats, one was from 10 GA repeats toward 17 GA repeats, two were from 16 GA repeats toward 10 GA repeats, two were from 16 GA repeats toward 18 GA repeats, and one was from 20 GA repeats toward 16 GA repeats.[10]

When we compared the clinical features of patients whose tumor specimens had LOH of *GNMT* or not, several interesting findings were noted. The mean age of diagnosis of cases with LOH was 5 years younger than that of patients who did not have LOH of *GNMT* (Table 2). Moreover, the former had higher percentage of stages III and IV patients than the latter (87.5% versus 76.9%; Table 2). These findings imply that LOH of *GNMT* may be associated with early prostate cancer development and more malignant disease progression. In terms of other prostate cancer clinical characteristics, such as Gleason score and pretreatment PSA level, no significant difference was noted between prostate cancer patients whose tumor specimens had LOH of *GNMT* or not. During the past few years, our knowledge of genetic alterations in prostate cancer has significantly increased. For example, several

[10] Y.M. Chen, personal communication.

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chromosomal loci possibly harboring predisposing or somatically mutated genes have been suggested, but only a few genes have been found to be aberrated in a significant proportion of prostate cancer. These include *GSTP1*, *PTEN*, *TP53*, and *androgen receptor* (see ref. 26 for review). The present study is the first to show the relatively high rate of LOH of *GNMT* gene in prostate cancer.

Immunohistochemical staining results show that all of the tissue samples from the normal prostate and BPH participants expressed higher GNMT levels than tumor-adjacent or tumorous tissue samples. According to a paired sample analysis, 71% of the prostate cancer patients lost GNMT expression in tumor-adjacent and tumorous tissue. Specifically, we did not observe a single prostate cancer case of concurrent positive GNMT staining in tumorous tissue and negative staining in tumor-adjacent tissue—evidence in support of the idea that GNMT expression in prostate cancer is down-regulated. In addition to the cytoplasmic staining pattern, GNMT was expressed in the nuclei of all 33 BPH tissue samples that were examined. In contrast, none of the eight prostate cancer tissue samples showing GNMT expression had a nuclear staining pattern. GNMT, which is primarily expressed in cell cytoplasm (6, 9, 20), is a polycyclic aromatic hydrocarbon-binding protein (27). Previously, we and other groups showed that if the cells were treated with benzo(*a*)pyrene, GNMT will be translocated into the nuclei (8, 28, 29). Because cigarette smoking has been identified as a possible prostate cancer risk factor and GNMT inhibits benzo(*a*)pyrene-7,8-diol-9,10-epoxide-DNA adduct formation (8), the GNMT expression in the prostate may exert a protective effect for individuals exposed to benzo(*a*)pyrene and its down-regulation may be an early sign for prostate cancer carcinogenesis.

Epigenetic mechanisms, such as DNA methylation and histone modification, play important roles in normal developmental processes, gene imprinting, and human carcinogenesis. Methylation is catalyzed by DNA methyltransferases and can be reversed by demethylases or by treatment with demethylating drugs, such as 5-azacytidine. Aberrant DNA methylation, considered a hallmark of carcinogenesis, has been recognized in cancer cells for more than 20 years (30). The role of DNA methylation in the malignant transformation of prostate tissue has been thoroughly studied, from its contribution to early tumor development stages to advanced androgen independence stages. The most significant research findings involve the discovery of targets, such as GSTP1, Ras association domain family 1A, and retinoic acid receptor β2, which are rendered inactive via promoter hypermethylation during disease initiation and progression (see ref. 31 for a detailed review).

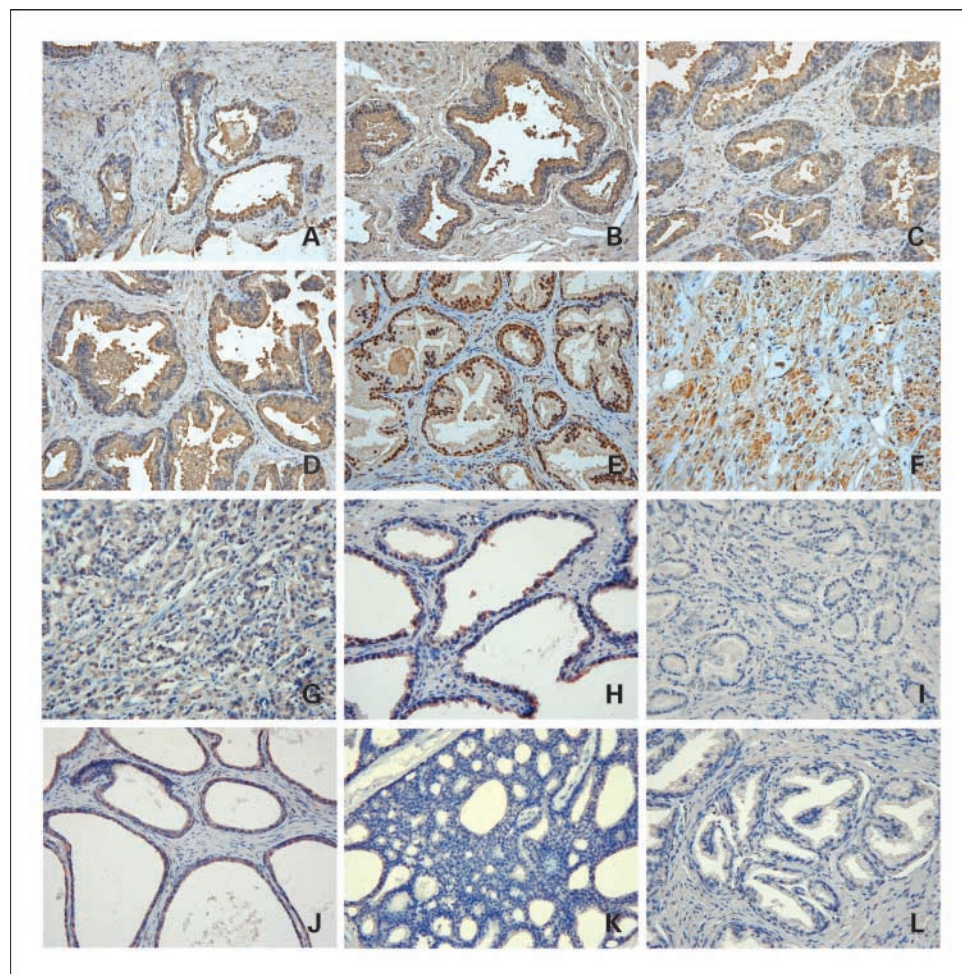

Fig. 3. Immunohistochemical staining of GNMT expression in different human prostate tissues. *A* to *D,* normal prostate tissues. Magnification, ×200. *A* and *B,* positive staining in the peripheral zone of normal prostate tissues. *C* and *D,* positive staining in transitional zone of normal prostate tissues. *E* and *F,* BPH tissues. *E,* positive cytoplasmic and nuclei staining. *F,* positive staining in the fibromuscular stromal component of BPH. *G* to *L,* paired tumor and tumor-adjacent tissues from three prostate cancer patients. *G, I,* and *K,* tumorous tissues. *H, J,* and *L,* tumor-adjacent tissues. Magnification, ×200. *G* and *H,* patient case no. 99, an example of the T+/N+ staining pattern. *I* and *J,* patient case no. 112, an example of the T−/N+ staining pattern. *K* and *L,* patient case no. 36, an example of the T−/N− staining pattern.

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Table 3. Results from the immunohistochemical staining of GNMT in human prostatic tissue

Tissue compartment	n	GNMT		P*
		Positive staining†	Negative staining	
Normal prostate tissue (%)	5	5/5 (100.0)†	0	
Peripheral zone (%)	2	2/2 (100.0)	0	
Transitional zone (%)	4	4/4 (100.0)	0	
BPH (%)	33	33/33 (100.0)	0	
Tumor-adjacent tissue (%)	38	11/38 (28.9)	27/38 (71.1)	<0.0001
Tumorous tissue (%)	45	8/45 (17.8)	37/45 (82.2)	<0.0001

Tissue pairs with different GNMT staining§

T+/N+ (%)	T+/N− (%)	T−/N+ (%)	T−/N− (%)	Total pairs
6 (15.8)	0 (0)	5 (13.2)	27 (71.0)	38

*Normal prostate tissue and BPH tissue were pooled together for comparison with tumor-adjacent and tumor tissues.
† P_{trend} < 0.0001.
‡ Two peripheral zones and four transitional zones from five normal individuals.
§ T+/N+, positive GNMT staining in both tumorous and tumor-adjacent tissue; T+/N−, positive GNMT staining in tumorous tissue and negative GNMT staining in tumor-adjacent tissue.

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Limited sample size is one concern of this study. Because the allelic frequencies of *GNMT* genotypes may differ among different ethnic groups (11, 24), future studies with larger sample size in other populations are recommended. GNMT is believed to play a role in monitoring the S-adenosylmethionine/S-adenosylhomocysteine ratio (7). Because S-adenosylmethionine is the sole methyl group donor for DNA methyltransferases, GNMT may be involved in DNA methylation regulation. According to Rowling et al. (32), the induction of GNMT enzyme activity by all-*trans*-retinoic acid in rats results in DNA hypomethylation in rat hepatocytes. Thus, the down-regulation of GNMT expression in prostate tissue may result in aberrant DNA methylation and carcinogenesis. Further studies are needed to elucidate the mechanism underlying the transcriptional control of GNMT gene expression and its relationship to DNA methylation in the prostate.

Acknowledgments

We thank the personnel at the National Yang-Ming University Genome Research Center Genotyping Lab (Taipei, Taiwan) and the medical staffs of the hospitals who participated in this study; Drs. Huang-Kuang Chang, Jong-Ming Hsu, Wun-Rong Lin, Wei-Kung Tsai, and Wen-Chou Lin (Department of Urology, Mackay Memorial Hospital) for their help with collecting clinical specimens and questionnaires; Drs. Ting-Yao Chen (Department of Pathology, Taipei Medical University, Taipei, Taiwan) and Anna Fen-Yau Li (Department of Pathology and Laboratory Medicine, Taipei Veterans General Hospital) for their helpful feedback and ideas; and Drs. Shao-Yuan Chuang and Ching-Heng Lin (Institute of Public Health, National Yang-Ming University) for their help with statistical analyses.

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