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Genotypic and Phenotypic Characterization of a Putative Tumor Susceptibility Gene, *GNMT*, in Liver Cancer[1]

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ABSTRACT

Glycine *N*-methyltransferase (GNMT), a multifunctional protein involved in the maintenance of the genetic stability, is often down-regulated in hepatocellular carcinoma (HCC). Using genotypic characterization of *GNMT* in hepatoma cell lines and in a Taiwanese population with a high incidence of liver cancer we have investigated the role of this gene in the progression of liver cancer. Six novel polymorphisms, including two short tandem repeats, one 4-nucleotide insertion/deletion polymorphism, and three single nucleotide polymorphisms, in *GNMT* were identified in this study. The rates of loss of heterozygosity at the *GNMT* locus in pairs of normal and tumor tissue from the HCC patients were approximately 36–47%. In addition, the observed heterozygosity of *GNMT* decreases in tumor adjacent liver DNA from HCC patients compared with that observed in blood DNA from normal individuals and HCC patients. This may result from the early event of loss of heterozygosity within the *GNMT* gene in the liver tissues of HCC patients. However, in this study, we did not observe the association of polymorphic *GNMT* alleles as inherited risk factors for HCC. We also elucidated the functional impact of genetic markers in the *GNMT* promoter by performing luciferase reporter gene and gel mobility shift assays. The results indicate that two polymorphisms, short tandem repeat 1 and insertion/deletion polymorphism, in the promoter region could cause allelic specific effects on the transcriptional activity of *GNMT*. The risk genotypes of *GNMT*, which presumably have a lower expression level, as estimated from *in vitro* functional studies, are over-represented in tumor-adjacent tissues from HCC patients. In summary, our results suggest that *GNMT* alteration may be an early event in HCC development and that *GNMT* could be a new tumor susceptibility gene for HCC.

INTRODUCTION

GNMT[3] is a protein with multiple functions. It has the potential to influence the genetic susceptibility through two of these functions (1). First, GNMT is involved in cellular one-carbon metabolism, and it can regulate the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine (2). In liver, GNMT is also a major folate binding protein (3). Thus, GNMT may induce changes in tissue folate status resulting in chromosome breakage or abnormal DNA methylation (4). Second, GNMT is an enzyme participating in detoxification. In addition, GNMT may have a protective effect against the exposure to carcinogens by decreasing DNA adduct formation.[4]

The expression of GNMT is highly responsive to environmental factors such as dietary intake (5, 6), and the expression of *GNMT* mRNA is tissue-specific, most abundant in liver, pancreas, and prostate (7, 8). *GNMT* mRNA has been shown recently to be down-regulated in HCC as well as in hepatitis C virus (HCV)-induced and alcoholic cirrhotic livers (9, 10). Here we investigate the role of *GNMT* in liver cancer predisposition by genotypic and phenotypic characterization in liver cancer cell lines and in a Taiwanese population with a high incidence of liver cancer. We have identified six novel polymorphisms in the *GNMT* gene, and determined the allelic and genotypic distribution of *GNMT* among two groups, normal individuals and patients with HCC. We additionally developed quantitative methods for assessing allelic loss at *GNMT* and determined the LOH rate of *GNMT* in HCC normal-tumor adjacent tissue pairs. Our functional characterization suggests that two polymorphisms in the promoter region could result in allelic-specific effects on the transcriptional level of *GNMT*.

MATERIALS AND METHODS

Human Subjects and Study Population. DNA samples used for the initial detection of sequence variations in the *GNMT* gene were derived from seven cell lines, Hep G_2, Hep 3B, Huh 6, Huh 7, Sk-Hep-1, PLC/PRF/5, HA22T/VGH, and 16 unrelated Taiwanese individuals. Blood samples from two groups of subjects, normal individuals ($n = 274$) and patients with HCC ($n = 71$), were collected. Tumorous and nontumorous liver tissues were collected from 42 HCC patients obtained. Risk factors associated with HCC were recorded by chart review. The subjects providing normal-tumor pairs of HCCs were mostly under grade 2, 2–3, or 3; 75% were hepatitis B surface antigen-positive and 25% were HCV (enzyme immuno assays) positive. The human subjects used in this study were collected in Taiwan, and approved by the Institutional Review Boards at the Taipei Veterans General Hospital (approval number: 90-02-01A).

Cell Lines and Culture. Five human HCC cell lines, HuH 7 (11), HA22T/VGH (13), Hep 3B, SK-Hep-1, and PLC/PRF/5 (13–15); and two hepatoblastoma cell lines, HuH 6 (12) and Hep G_2 (15, 16), used in this study, were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), penicillin (100 IU/ml), streptomycin (100 mg/ml), fungizone, (2.5 mg/ml), and L-glutamine (2 mM) in a humidified incubator with 5% CO_2.

Primers for Sequencing the *GNMT* Gene. The sequence of primer pairs to amplify the locus, and size of products for sequencing the *GNMT* gene are GM1 forward: AAAGGAAAGGGAGAAAAATGAATC and GM1 reverse: TGGGCAACAGAGCAAGACT (promoter region, 488 bp), GM2 forward: AAATGAAGAGGATGAAGTAAAGTT and GM2 reverse: CCCAGCGAAGGAAGGCATCAGC (promoter ~ 5′UTR region, 547 bp), GM3 forward: GCACCGGCTGACTA TACCTACACA and GM3 reverse: TCTCCGATATACAGCTGCCACACG (5′UTR ~ Exon 1 region, 564 bp), GM4 forward: CGCGCTCACCTGCTATTG and GM4 reverse: AGGGACGCT-

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[3] The abbreviations used are: GNMT, glycine *N*-methyltransferase; HCC, human hepatocellular carcinoma; UTR, untranslated region; STRP, short tandem repeat polymorphism; INS/DEL, insertion/deletion; dNTP, deoxynucleotide triphosphate; SNP, single nucleotide polymorphism; TAMRA, 6-carboxytetramethylrhodamine; EMSA, electrophoretic mobility shift assay; LOH, loss of heterozygosity; CI, confidence interval; T:N ratio, allele ratio of tumorous DNA:allele ratio of nontumorous DNA; HNF, hepatocyte nuclear factor; PBMC, peripheral blood mononuclear cell.

[4] Yi-Ming Arthur Chen, personal communication.

CACTTTTTCTG (Exon 1 region, 584 bp), GM5 forward: CTGCG AGTGCCCCGTGAGG and GM5 reverse: CTTGCAGTGAGCCGAGATG (Intron 1 region, 494 bp), GM6 forward: GATGAAGTCGTGCTCTGTCG and GM6 reverse: AGTCCGTTCCTCTGCCTCCTCT (Intron 1 region, 536 bp), GM7 forward: CGCGCCCGGCTTTGTCCT and GM7 reverse: ACCTGCATACCCCACTTGTCG (Exon2 region, 507 bp), GM8 forward: TGGCCGTACTCAGGGTGGACT and GM8 reverse: TCAGGAAGAGAAAGAGGAATCAGG (Intron 2 region, 557 bp), and GM9 forward: CTTCTCCCTTGATCCCTCTTTTCT and GM9 reverse: GCCGGTGCTCACTCTGGTC (Exon 3 region, 484 bp).

GeneScan Analysis. Fragment analysis was used for the detection of STRPs (STRP1 and STRP2) and INS/DEL polymorphism. Primers for the STRP1 marker are Forward STRP1F: 6-FAM-CAAGTTGGAAAGGAAGGAGGAGAG, and reverse STRP1R: GCGAGCCAGCCAGCAGAAAGA. Primers for STRP2 are STRP2F: HEX-ACAGGCCCGGGTTGGTTA, and STRP2R: CTTGCAGTGAGCCCGAGATGGA. Primers for Insertion are INSF: NED-GCACAAACAAAGCAAGAAAG, and INSR: ATGCCCGC CATTAATAAC. The 10-μl PCR mixture contained 1× Gold Buffer (Applied Biosystems), approximately 0.8–1 μl 25 mM $MgCl_2$, 1 μl 800 μM dNTPs, 1 μl 5 μM concentrations of each primer pair, 0.5 μl 0.25 unit Amplitaq Gold (Applied Biosystems), and 1 μl of the 10 ng extracted DNA. The thermal profile was 95°C for 10 min, followed by 9 cycles consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by 19 cycles consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A 30-min final extension step was included at the end to ensure maximum nontemplate-A addition and, thus, eliminate split leaks. One μl of diluted PCR product was mixed with 9 μl of loading buffer (formamide: Rox 500 size standard, 1:39). The mixture was denatured at 95°C for 5 min, chilled on ice, and loaded on an ABI PRISM 3100 automatic sequencer. The data were analyzed using the GeneScan and Genotyper software (Applied Biosystems).

TaqMan Genotyping. The TaqMan-Allelic Discrimination method was used for the detection of SNPs including SNP1, SNP2, and SNP3. All of the assays were conducted in 96-well PCR plates. Each PCR plate included 8 wells of no template control, 8 allele 1 template-containing controls, and 8 allele 2 template-containing controls. The amplification of region containing SNP was performed with either an allele 1 or allele 2 specific fluorogenic probe in combination with common nonfluorogenic primers. The primers for detecting SNP1, SNP2, and SNP3 in the GNMT gene were GSNP1-F: GCGCGCTCACCTGCTATT and GSNP1-R: GGAGCGGGTCCGGTACAC for SNP1, GSNP2-F: CGCGTGTGGCAGCTGTATAT and GSNP2-R: CCACCCG TTCCAGGATTG for SNP2, and GSNP3-F: CCTTGTGGTGACAGGAAACAGAT and GSNP3-R: AACCCTCTTCCACCAGCAT for SNP3. The allelic-specific flurogenic probes were GSNP1-Ael-1: VIC-TCCGCACTTAAAGCATAAGCACTGCT-TAMRA for C allele and GSNP1-Ael-2: 6-FAM-CGCTCCGCACTTAAAACATAAGCACT-TAMRA for T allele of SNP1 (antisense), GSNP2-Ael-1: VIC-AGTACAGGCTGAGACAGAC CCCGATCTAMRA for allele T and GSNP2-Ael-2: 6-FAM-TACAGGCTGAGCC AGACCCCGAT-TAMRA for allele G of SNP2 (antisense), GSNP3-Ael-1: VIC-CAGAGTCCGTTCCTCTGCCTCCTCT-TAMRA for allele G and GSNP3-Ael-2: 6-FAM-CAGAGTCCGTTCTTCTGCCTCCTCTC-TAMRA for allele A of SNP3 (antisense). Each PCR reaction mixture contained 2.5 μl 10× Buffer A, 3.5 μl 25 mM $MgCl_2$, 2 μl 200 μM dNTPs, 3 μl 2.5 μM primers, 1 μl 5 μM Probe 1, 1 μl Probe 2, 0.125 μl 5 units/μl TaqGold, 9.375 μl water, and 2.5 μl 10 ng DNA. The thermal profile was 95°C for 5 min followed by 40 cycles consisting of 95°C for 15 s and 64°C for 1 min. After PCR was completed, plates were brought to room temperature, read in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), and results analyzed using the Allelic Discrimination software.

EMSA. For EMSA studies, nuclear extracts of Hep G_2 cells were prepared according to Dignam *et al.* (17). The protein concentration in the nuclear extracts was determined. Nuclear extract (10.92 μg) per gel-shifting lane was used to detect binding of HNF-3 and HNF-4, transcription factors using EMSA kits (Geneka Biotechnology, Montreal, Quebec, Canada). The following double-stranded DNA probes were used: GS1-W, GS1-I, HNF-3, mutant HNF-3 mutant, HNF-4, and mutant HNF-4. The HNF-3 and HNF-4 oligos used here recognize all of the HNF-3 and HNF-4 variants. The sequences of GS1-W and GS1-I are shown in Fig. 4, and the sequences of other probes are HNF-3 probe: F: GCCCATTGTTTGTTTTAAGCC, R: CGGGTAACAAA CAAAATTCGG; HNF-3 mutant probe: F: GCCCATTGGGCCATTTAAGCC, R: CGGGTAACCCGGTAAATTCGG; HNF-4 probe F: GGAA AGGTCCAAAGGGCG CCTTG, R: CCTTTCCAGGTTTCCCGCGGAAC; Oct probe F: CCTCTTGGATTTGCATATGGGCTG, R: GGAGAACCTAAACGTATACCCGAC and Oct mutant probe F: CCTCTTGGATGATTATATGGGCTG, R: GGAGAACCTAGTAATATATACCCGAC. Binding reactions were performed according to the manufacturer's protocol. For binding reactions, nuclear extracts were incubated at room temperature with double-stranded DNA probes, prepared by annealing complementary oligonucleotides and labeled with [γ-^{32}P]dATP. The binding reaction mixtures were run on a 5% polyacrylamide gel in 0.25 × Tris-borate-EDTA at 12.5 V/cm for 2 h.

Construction of *GNMT* Promoter-Luciferase Plasmids. Plasmid pBS-6.5k (8), which contains the promoter region of the GNMT gene, was used as the template in the PCR. A 1.8-kb DNA fragment, which contains the 5′ upstream region of GNMT, was amplified. The PCR conditions were as recommended by the manufacturer (Perkin-Elmer, Norwalk, CT), except that the $MgCl_2$ was 1.5 mM, and the primers were 200 nM. Thirty-five cycles of amplification were performed in a DNA thermal cycler (Perkin-Elmer) using their Gold Amplitaq Taq DNA polymerase. Each PCR cycle used a primer-annealing step at 60° for 1 min and an extension step at 72° for 2 min. The following primers were used: PS4565 (5′-GGGGTACCAGCATCTT GGCCAGGCTG), and PA6391 (5′-GCGAGATCTCCTGCGCCGCGCCTGGCT). Immediately after amplification, SDS and EDTA were added to the PCR to 0.1% and 5 mM, respectively, and DNA was precipitated with 2.5 M ammonium acetate and 70% ethanol. After digestion with *Kpn*I and *Bgl*II, the DNA fragment was isolated by elution from agarose gel electrophoresis. The fragment was ligated to a vector, pGL3-basic (Promega), that had been digested previously with *Kpn*I and *Bgl*II. The resultant plasmid, designated as pGNMT-1.8k-16GA, contains 16 GA repeats in its GNMT promoter region. The pGNMT-1.8k-16GA was additionally used as a template in PCR to generate another plasmid, pGNMT-1.8k-10GA, containing 10 GA repeats in the promoter region. Different PCRs were performed with the following two pairs of primers separately: PS5010 (5′-ACAGAGCGAGACTGTGTCTC)/PA5148 (5′-TCTCTCTCTCTCTCTCTC TCTGC) and PS5141 (5′-GAGAGAGAGAGAGAGAGAAGC)/PA5671 (5′-CAGAGCAAGACTCCGTCTCA). DNA products from both PCRs were mixed and used as the template in the third PCR with the PS5010 and PA5671 primers. The PCR reactions were performed in 50-μl reaction mixtures containing 200 μM of each of the four dNTPs, 0.2 μM of each of the primers, 1.5 mM $MgCl_2$, and 2.5 units AmpliTaq DNA polymerase (Perkin-Elmer). Thirty-five amplification cycles were performed at the following conditions: 94° for 30 s, 60° for 30 s, and 72° for 1 min. The PCR products were additionally digested with Tth111I and replaced the Tth111I-Tth111I region of pGNMT-1.8k16GA to generate the pGNMT-1.8k10GA. Nucleotide sequences of the constructs were confirmed by automated DNA sequencing. To generate GAGT inserts, the primers PS5010(5′-ACAGAGCGAGACTGTGTCTC)/PA5421(5′-ACTCGTAACAG GGCCTTTGAGCCC) and the primers PS5411(5′-GCCCTGTTACAGAGTTTTT GTGAG) PA5671(5′-CAGAGCAAGACTCCGTCTCA) were used to amplify the fragments in the first round of PCR. The PCR products were then mixed and used as template for the second PCR. With primers PS5010/PA5671, PCR was performed in 50-μl reaction mixtures containing 200 μM of each of the four dNTPs, 0.2 μM of each of the primers, 1.5 mM $MgCl_2$, and 2.5 units AmpliTaq DNA polymerase (Perkin-Elmer). The amplification cycles were at 94° for 5 min × 1 cycle; denaturation at 94° for 30 s, annealing at 60° for 30 s, an extension at 72° for 1 min, × 35 cycles, with a final extension at 72° for 10 min × 1 cycle. The final products were digested with Tth111I. The 1.8 kb Tth111I-Tth111I region of pGNMT was replaced by the Tth111I-Tth111I fragment generated from pGNMT with 4 bp GAGT insertion.

Luciferase and β-Galactosidase Assay. Cells were plated in six-well culture dishes at a density of 2×10^5 cells/well and maintained at 37° with 5% CO_2 overnight. The transfection was performed using the calcium phosphate coprecipitation method. Duplicated wells were transfected with 4-μg pGNMT-1.8k10GA or pGNMT-1.8k16GA plasmid DNAs, which had been mixed with 2-μg pCMVβ previously. The plasmid pCMVβ was used to monitor the transfection efficiency. Plasmid DNAs from pGL3-contral and pGL3-basic were also used as the background and positive control, respectively. After transfection for 18 h, the cultured medium was changed, and the cells were maintained for another 48 h. Then, the cells were washed with PBS twice and lysed with 70-μl Reporter Lysis Buffer (Promega). The protein concentration was measured using the Bradford method (Bio-Rad). The luciferase and the

β-galactosidase activity were measured using the Luciferase Assay System (Promega), and the β-galactosidase Enzyme Assay System (Promega), respectively.

Statistical Analysis. Expected genotype frequencies were calculated from the allele frequencies under the assumption of Hardy-Weinberg equilibrium. Allele frequencies and genotypic frequencies were calculated, and the differences between paired groups were determined using a χ^2 test. A two-tailed P of 0.05 was interpreted as indicating a statistically significant difference. All of the statistical analyses were done with SAS software, version 8 (SAS Institute).

RESULTS

Identification of Novel Polymorphisms in the *GNMT* Gene. To develop genetic markers for *GNMT*, *GNMT* was resequenced from multiple independent sources: 5 HCC cell lines, 2 hepatoblastoma cell lines, and blood from 16 unrelated Taiwanese individuals. Regions resequenced included partial coding regions, 5′UTR, and promoter regions. Samples were sequenced in both the forward and reverse orientations. No sequence differences were observed in the coding regions of *GNMT* (GenBank accession no. AF101475.1), but we observed three common SNPs (Fig. 1*A*), SNP1, SNP2, and SNP3, at nucleotide positions 1289, 1586, and 2666 in the 5′UTR, intron 1, and intron 2 of *GNMT*, respectively. We also observed two STRPs, STRP1 and STRP2, starting at nucleotide positions 71 and 2117 in the promoter region and intron 2, respectively. An additional 4 nucleotide (GAGT) INS/DEL polymorphism (Fig. 1*B*) was identified between nucleotide positions 363 and 364 in the promoter region, at a location only 120 bp away from the transcription initiation site. A summary of the novel inherited polymorphisms in *GNMT* identified in this paper is shown in Table 1.

Development of High Throughput Assays for Genotyping. GeneScan assays were developed to allow fragment analysis of the STRP1, STRP2, and INS/DEL polymorphisms (Fig. 2*A*), and allelic discrimination assays were developed for detecting SNP1, SNP2, and SNP3 (Fig. 2*B*). The allele sizes shown in Fig. 2*A* were obtained using an ABI Prism 3100 Genetic Analyzer. The genotypes at the *GNMT* locus are summarized in Table 2. In this study, seven, three, and two alleles were identified at STRP1, STRP2, and INS/DEL locus, respectively (Table 3). The alleles were named based on the sizes of fragments determined using the ABI Prism 3100 platform. The numbers of GA repeats in the 139, 144, 150, 152, 154, 156, and 158 alleles for STRP1 ranges among 10, 13 16, 17, 18, 19, and 20. The numbers of T contained in the 120, 128, and 135 alleles for STRP2 were 13, 19, and 25. In allelic discrimination assays, the control templates of di-allelic SNPs for TaqMan genotyping are the DNA samples with known genotypes from resequencing *GNMT*.

Allelic and Genotypic Distribution of the *GNMT* Gene in Taiwanese Population. Allelic and genotypic distribution of *GNMT* in DNA specimens extracted from PBMCs from two subject groups, normal individuals and patients with HCC, as well as DNA from liver tissues

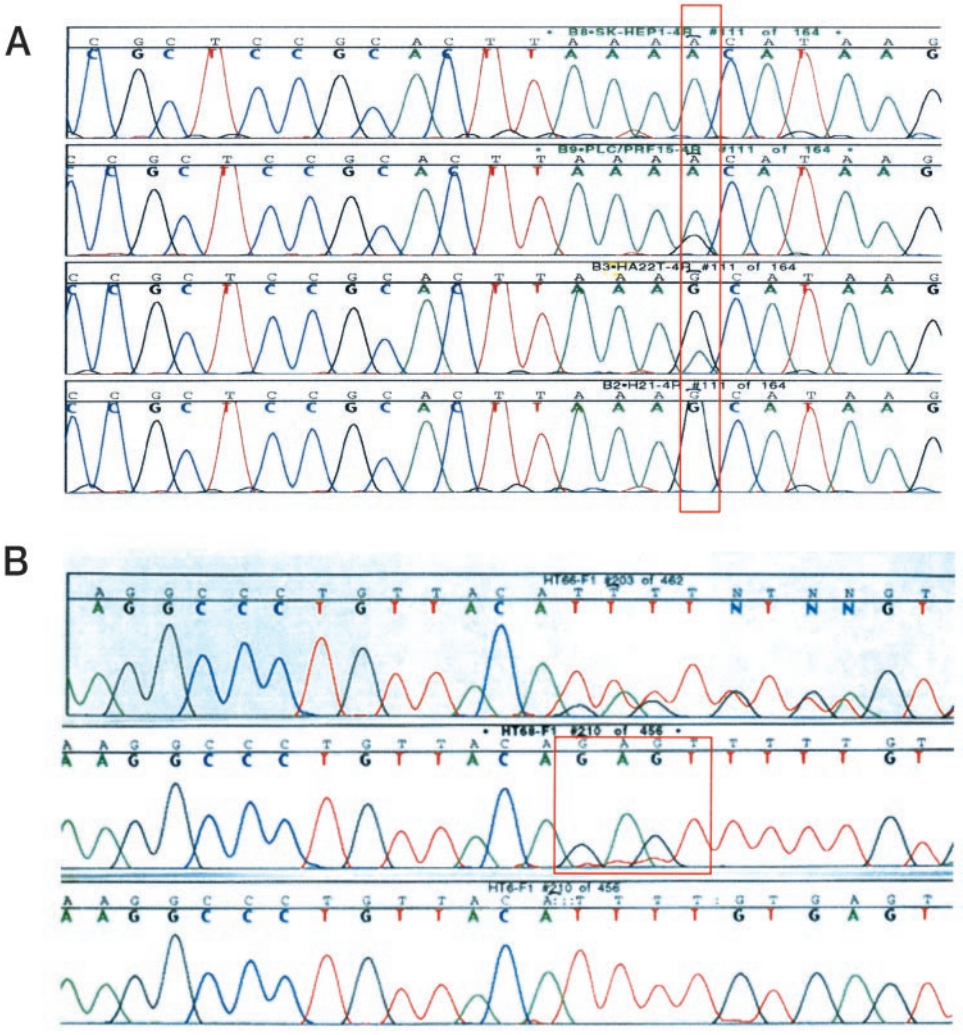

Fig. 1. Identification of novel polymorphisms in the *GNMT* gene. *A*, an example of SNP identification by resequencing the *GNMT* gene. The reverse sequences of *GNMT* containing the SNP1 are shown in *A*. A homozygous genotype A/A in Sk-Hep1, G/G in a patent with hepatoma (H21), and a heterozygous A/G in PLC/PRF-15 and HAT22. *B*, identification of an INS/DEL polymorphism. The forward sequences containing the INS/DEL polymorphism are shown in *B*. A homozygous GAGT insertion in a tumorous liver DNA HT68 (*middle*), homozygous deletion in a tumorous liver DNA HT6 (*bottom*), and a heterozygous genotype in tumorous liver DNA HT66 (*top*).

Table 1 *Summary of novel polymorphisms in GNMT*

Marker	Polymorphic type	Location[a]		Sequence
STRP1	Dinucleotide repeats	71~86	Promoter	(GA)n, N = 10, **16**, 17, 18, 20
INS/DEL	4-nucleotide INS/DEL	363~364[b]	Promoter	TTACA (GAGT) TTTTG
SNP1	SNP	1289	5′ UTR	TTATG(**C**/T) TTTAA
SNP2	SNP	1586	Intron 1–5′ end	GTCTG (**T**/G) CTCAG
STRP2	Single Nucleotide Repeats	2117~2135	Intron 1	TTTTTCTC (T) n, N = 13, **19**, 25
SNP3	SNP	2666	Intron 1–3′ end	GCAGA (**G**/A) GAACG

[a] Location represents the nucleotide position in the sequence from GenBank accession no. AF 101475.1, and this DNA sequence represents the major allele in Taiwanese population are shown in bold.

[b] The 4-bp insertion does not exist in AF 101475.1, and it is inserted between nucleotides 363~364.

from HCC patients were determined (Tables 3 and 4). The distribution of the *GNMT* genotypes in the Taiwanese control population was found to be in Hardy-Weinberg equilibrium based on the results of χ^2 tests. In blood DNA, the allelic and genotypic distribution of *GNMT* is similar between normal individuals and HCC patient group. However, the allelic distribution of STRP1 ($P = 0.0164$) and SNP1 ($P = 0.0196$), as well as genotypic distribution of STRP1 ($P = 0.0109$), INS/DEL ($P = 0.0403$), SNP1 ($P = 0.0157$), and SNP2 ($P = 0.0320$) is significantly different between blood DNA and liver DNA from the HCC patient group (χ^2 test, $P < 0.05$; Table 4). The observed heterozygosity of *GNMT* decreases in liver DNA from HCC patients compared with that observed in blood DNA from normal or HCC patients (Table 4).

Development of Quantitative Methods for Assessing Allelic Loss at *GNMT*. Quantitative methods for assessing allelic loss at the *GNMT* locus were established to standardize LOH assessment for the novel *GNMT* genetic markers. The relative density ratio of two alleles (allele ratio) in each blood DNA sample with heterozygous genotypes at STRP1, INS/DEL, and STRP2 was calculated, 1.4004 ± 0.1774, 1.0512 ± 0.0929, and 1.1727 ± 0.8305, respectively (mean ± SD). The allele ratio varied widely in STRP2 assays; therefore, only STRP1 and INS/DEL assays were selected for the additional development of LOH assessment standards. One-hundred eleven INS and 115 STRP1 values of allele ratio were obtained, and the CIs of the normal distribution of allele ratio of STRP1 and INS/DEL were determined.

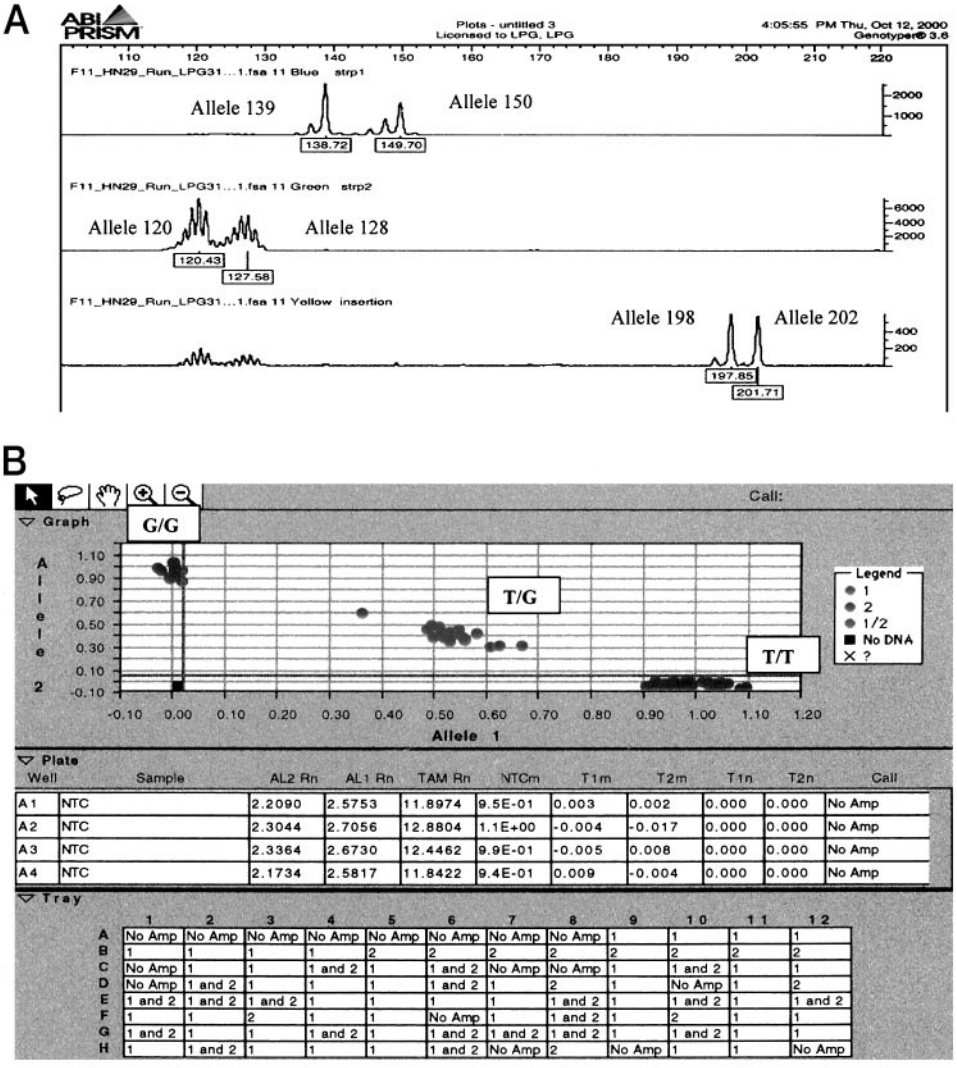

Fig. 2. Developing genotyping assays for novel *GNMT* genetic markers. *A*, GeneScan analysis of STRP1, STRP2, and INS/DEL. Electropherotograms of *GNMT* genotyping in ABI3100. An example of a heterozygous genotype of three markers, STRP1 (*top*, 139/150 alleles), STRP2 (*middle*, 120/128 alleles), and INS/DEL (*bottom*, 198/202 alleles) are shown in *A*. *B*, an example of the TaqMan-Allelic Discrimination method was used for the detection of SNPs (SNP2 in *B*).

Table 2 *List of genotypes at GNMT in liver cancer cell lines*

Cell lines	Cancer type	STRP1	INS/DEL	SNP1	SNP2	STRP2	SNP3
HA22T/VGH	HCC	139/139	202/202	C/T	G/G	120/135	G/A
Huh 7	HCC	150/150	198/198	C/C	T/T	128/128	G/G
Hep 3B	HCC	139/139	202/202	T/T	G/G	120/120	A/A
Sk-Hep-1	HCC	139/139	202/202	T/T	G/G	120/120	A/A
PLC/PRF/5	HCC	139/139	202/202	T/T	G/G	120/120	G/A
Huh 6	Hepatoblastoma	139/150	198/202	C/C	T/G	120/128	G/A
Hep G2	Hepatoblastoma	139/152	198/202	C/C	T/G	128/135	G/G

The allele ratios located within the 99% interval were selected for calculating pair ratio (allele ratio 1/allele ratio 2). The pair ratio from genotypes obtained from normal blood DNA was used to calculate an expected distribution for the observed T:N ratio. LOH was assigned if the observed ratio was outside the 99% (CI) obtained for the empirically generated distribution.

Assessment of the LOH in Tumor and Nontumor DNA. To study whether LOH at the *GNMT* gene was present or not, the genotypes of *GNMT* in 42 pairs of tumor and nontumor from HCC patients were determined (Table 5). The allele ratio in each DNA sample was calculated and compared with their allele ratio in corresponding tumorous and normal tissues (T:N ratio). Samples were scored as positive for LOH if the calculated value of T:N ratio was not within the 99% CI of normal distribution for STRP1 and INS/DEL, 0.66–1.38 and 0.74–1.26, respectively (Fig. 3). Eleven of 42 HCC pairs for INS/DEL and 17 of 41 HCC pairs for STRP1 are informative. In tumors and corresponding nontumor liver tissues, we detected 36% (4 of 11) of LOH for INS/DEL and 41% (7 of 17) for the STRP1 (Table 5). There were no significant differences observed in patients with and without LOH with respect to tumor size, gender, HBV/HCV infection, and other demographic status based on the available information.

Gel Mobility Shift Assay for Different Motifs Containing Insertion or Deletion Genotype. Computational analysis predicted that several transcription factors could potentially bind to the motif surrounding the INS/DEL polymorphism. To determine whether the INS/DEL polymorphism could influence the binding affinity of transcription factors, gel shift experiments were performed with Hep G_2 nuclear extracts and with either GS1-D (allele 198) or GS1-I (allele 202 contains GAGT 4-nucleotide insertion) probe (Fig. 4*A*). A specific complex was detected with GS1-I but not GS1-D, which does not contain GAGT (*Lane 2* and *Lane 1* in Fig. 4*B*, respectively). Because the consensus sequences of the HNF-3, HNF-4, and Oct binding sites closely resembled the sequences found in this region, we additionally performed competition assays for those transcription factors (Fig. 4, *B* and *C*). A double-strand DNA probe containing the consensus HNF-3 binding site can compete with GS1-I (*Lane 6* in Fig. 3*B; Lane 4* in Fig. 4*C*) but not GS1-D and Oct (*Lanes 2* and *13* in Fig. 4*C*, respectively). Similarly, a weaker reduction of the complex was also observed with HNF-4 probe (*Lane 3* in Fig. 4*B; Lane 7* in Fig. 4*C*). As shown in Fig. 3*C*, the specificity of HNF-3 and HNF-4 binding was additionally confirmed by competition assays with mutant oligonucleotides. Neither mutant HNF-3 nor mutant HNF-4 probes (HNF3∗ and HNF4∗), *Lanes 5* and *8*, respectively, in Fig. 4*C*, compete as well as the GS1-D probe for forming a complex. These results indicate that the HNF-3 (maybe also HNF-4) transcription factor can bind to the 202 allele at INS/DEL and that allele 198 abolishes its binding site near the region where the INS/DEL polymorphism is surrounded.

Phenotypic Analysis of the Promoter Constructs with Different STRP1 Genotypes. In the Taiwanese population, STRP1 $(GA)_{10}$ -INS and STRP1 $(GA)_{16}$ -DEL represent the two major haplotypes in the promoter region of *GNMT*, the 139(STRP1)-202(INS/DEL) and 150(STRP1)-198(INS/DEL) allele. To elucidate the impact of each genotype on the activity of the *GNMT* promoter, we cloned 10, 14, 15, 16, and 20 GA repeats upstream of the luciferase reporter gene construct (Fig. 5). The *GNMT* promoter constructs were transfected into Hep G_2. The absolute luciferase activity of the *GNMT* promoter, which contains 10 GA repeats (allele 139) and INS (allele 202), was set to 100%, and all of the other constructs were compared accordingly. When the *GNMT* promoter, containing 16 repeats (allele 150) and DEL (allele 198), was transfected into Hep G_2, its transcriptional activity was reduced to 67%, relative to the promoter containing 10 GA repeats. In addition, our results also indicate that the transcriptional activity of the *GNMT* promoter is inversely affected by the number of GA repeats at STRP1.

Table 3 *Allelic distribution of GNMT*

Allele	Normal PBMC	HCC PBMC	Odds ratio	95% CI	HCC nontumor tissue	Odds ratio	95% CI
STRP1[a,b]							
139	174/544 (0.320)	47/142 (0.331)	1.09	0.73–1.63	23/82 (0.280)	0.88	0.52–1.49
144	2/544 (0.004)	–	–	–	–	–	–
150	340/544 (0.625)	84/142 (0.592)	1.00	Referent	51/82 (0.622)	1.00	Referent
152	21/544 (0.039)	10/142 (0.070)	1.93	0.87–4.25	2/82 (0.024)	0.63	0.14–2.79
154	5/544 (0.009)	1/142 (0.007)	0.81	0.09–0.85	5/82 (0.061)	6.67	1.86–23.84
156	1/544 (0.002)	–	–	–	–	–	–
158	1/544 (0.002)	–	–	–	1/82 (0.012)	6.67	0.41–108.26
STRP2[c]							
120	29/154 (0.188)	28/142 (0.197)	1.08	0.60–1.94	12/84 (0.143)	0.73	0.35–1.53
128	107/154 (0.695)	96/142 (0.676)	1.00	Referent	61/84 (0.726)	1.00	Referent
135	18/154 (0.117)	18/142 (0.126)	1.11	0.55–2.26	11/84 (0.131)	1.07	0.48–2.42
INS/DEL[d]							
198	372/548 (0.679)	95/142 (0.669)	1.00	Referent	62/84 (0.738)	1.00	Referent
202	176/548 (0.321)	47/142 (0.331)	1.05	0.71–1.55	22/84 (0.262)	0.75	0.45–1.26
SNP1[e]							
C	133/156 (0.853)	118/138 (0.855)	1.00	Referent	80/84 (0.952)	1.00	Referent
T	23/156 (0.147)	20/138 (0.145)	0.98	0.51–1.87	4/84 (0.048)	0.29	0.10–0.87
SNP2							
T	104/146 (0.712)	20/26 (0.769)	1.00	Referent	59/82 (0.720)	1.00	Referent
G	42/146 (0.288)	6/26 (0.231)	0.74	0.28–1.98	23/82 (0.280)	0.97	0.53–1.76
SNP3							
G	110/134 (0.821)	23/26 (0.885)	1.00	Referent	64/76 (0.842)	1.00	Referent
A	24/134 (0.179)	5/26 (0.192)	1.00	0.34–2.89	12/76 (0.158)	0.86	0.40–1.83

[a] The numbers of GA repeat contained in the 139, 144, 150, 152, 154, 156, and 158 alleles were 10, 13, 16, 17, 18, 19, and 20.
[b] Normal PBMC *versus* HCC nontumor tissue (χ^2 test, $P = 0.0164$).
[c] The numbers of T contained in the 120, 128, and 135 alleles were 13, 19, and 25.
[d] The allele 202 contained 4-nucleotide (GAGT) insertion.
[e] Normal PBMC *versus* HCC nontumor tissue (χ^2 test, $P = 0.0196$).

Table 4 *Genotypic distribution of GNMT*

For 198/202: 0.262(HCC PBMC)—0.467 (normal PBMC) = −0.205, 198/198: 0.595(HCC nontumor tissue)—0.445(normal PBMC) = 0.150, 202/202: 0.143(HCC nontumor tissue) −0.088(normal PBMC) = 0.055; therefore, 0.150/0.205 (~73%) of 198/202 in blood tends to be 198/198 in liver from HCC patients.

Genotype	Expected	Normal PBMC	HCC PBMC	HCC nontumor tissue
STRP1[a]				
139/139	0.102	24/272 (0.088)	4/71 (0.056)	6/41 (0.146)
139/150	0.400	119/272 (0.438)	39/71 (0.549)	9/41 (0.220)
150/150	0.391	102/272 (0.375)	21/71 (0.296)	18/41 (0.439)
150/>=152	0.066	14/272 (0.052)	2/71 (0.028)	6/41 (0.146)
139/>=152	0.031	7/272 (0.025)	–	2/41 (0.048)
152/>=152	0.003	4/272 (0.015)	4/71 (0.056)	–
Others	0.007	2/272 (0.007)	–	–
STRP2				
120/120	0.036	–	1/71 (0.014)	1/42 (0.024)
120/128	0.262	25/77 (0.325)	24/71 (0.338)	6/42 (0.143)
128/128	0.476	34/77 (0.442)	29/71 (0.408)	25/42 (0.595)
128/135	0.166	14/77 (0.182)	13/71 (0.183)	5/42 (0.119)
120/135	0.046	4/77 (0.052)	3/71 (0.042)	4/42 (0.095)
135/135	0.014	–	1/71 (0.014)	1/42 (0.024)
INS[b]				
198/198	0.461	122/274 (0.445)	29/71 (0.408)	25/42 (0.595)
198/202	0.436	128/274 (0.467)	37/71 (0.52)	11/42 (0.262)
202/202	0.103	24/274 (0.088)	5/71 (0.070)	6/42 (0.143)

[a] Normal PBMC *versus* HCC nontumor tissue (χ^2 test, $P = 0.0109$).
[b] Normal PBMC *versus* HCC nontumor tissue (χ^2 test, $P = 0.0403$).

Table 5 *Summary of LOH at the GNMT locus in HCC pairs*

HCC	Marker	Allele 1	Allele 2	T:N Ratio	LOH Assessment
LN5	INS/DEL[a]	198	202		
LT5	INS/DEL	198	202	0.74[b]	LOH
HN3	INS/DEL	198	202		
HT3	INS/DEL	198	202	1.83	LOH
HN38	INS/DEL	198	202		
HT38	INS/DEL	198	202	0.59	LOH
HN57	INS/DEL	198	202		
HT57	INS/DEL	198	202	0.66	LOH
LN5	STRP1[c]	139	150		
LT5	STRP1	139	150	0.50	LOH
LN6	STRP1	150	154		
LT6	STRP1	150	154	0.44	LOH
LN9	STRP1	139	152		
LT9	STRP1	139	152	0.63	LOH
HN3	STRP1	139	150		
HT3	STRP1	139	150	0.58	LOH
HN11	STRP1	150	154		
HT11	STRP1	150	154	0.66[b]	LOH
HN38	STRP1	139	150		
HT38	STRP1	139	150	1.41	LOH
HN45	STRP1	150	158		
HT45	STRP1	150	158	1.54	LOH

[a] The normal range of T:N ratio for INS/DEL is 0.74~1.26, and LOH rate for INS/DEL is 36% (4/11).
[b] The T:N ratio is of borderline statistical significance at the 99% CI.
[c] The normal range of T:N ratio for STRP1 is 0.66~1.38, and LOH rate for STRP1 is 41% (7/17).

DISCUSSION

In this study, we have identified six novel polymorphisms (Fig. 1; Table 1) and developed several genotyping assays for high-throughput platforms (Fig. 2). The accuracy of those assays has been additionally validated based on the linkage analysis of the CEPH families (data not shown). The best interval of the INS/DEL marker is D6S426-D6S271 in the ABI reference map and D6S1019-D6S1280 in the WEBER reference genetic map.[5] When placed in these locations, no double recombination events or map expansion was observed. This genetic localization is consistent with the previous result of cytogenetic localization to chromosome 6p12 (Ref. 8).

[5] Internet address: http://lpg.nci.nih.gov/html-chlc/ChlcMaps.html.

The observed heterozygosity of *GNMT* is decreased in tumor-adjacent liver DNA from HCC patients compared with that observed in blood DNA from normal individuals and HCC patients (Table 4). This may result from the early event of LOH within the *GNMT* gene in the liver of HCC patients or the subpopulation structure in the DNA resources used here. We have genotyped DNA samples with nine unlinked and highly polymorphic genetic markers (Applied Biosystems AmpFESTER Profiler Plus). On the basis of the distribution of those genetic markers, we rule out the latter possibility (data not shown). LOH of the *GNMT* markers was also observed in tumor and nontumor liver tissues from the sample patients, and the LOH rates were between 36 and 41% (Table 5). However, the hypothesis of high LOH of *GNMT* in the early stage of HCC development remains to be tested by genotyping *GNMT* in the blood and liver DNA from the same individuals on a large scale. If the hypothesis regarding high LOH of *GNMT* in the early stage of HCC development is true, the LOH rate might be underestimated: some cases that scored negative or noninformative could be because of the early alterations in the nontumor liver tissue that was used as reference for LOH assessment (18, 19). Therefore, the ratio of LOH in HCC pairs could be much higher if we were to use normal blood DNA instead of nontumor liver DNA as reference (18).

HBV and/or HCV infection could be one of the triggers that induce LOH of the *GNMT* gene in the liver tissues (20, 21). It has been shown that chronic viral infection or environmental carcinogens can induce the destruction of hepatocytes (22, 23). Subsequently, the high rate of

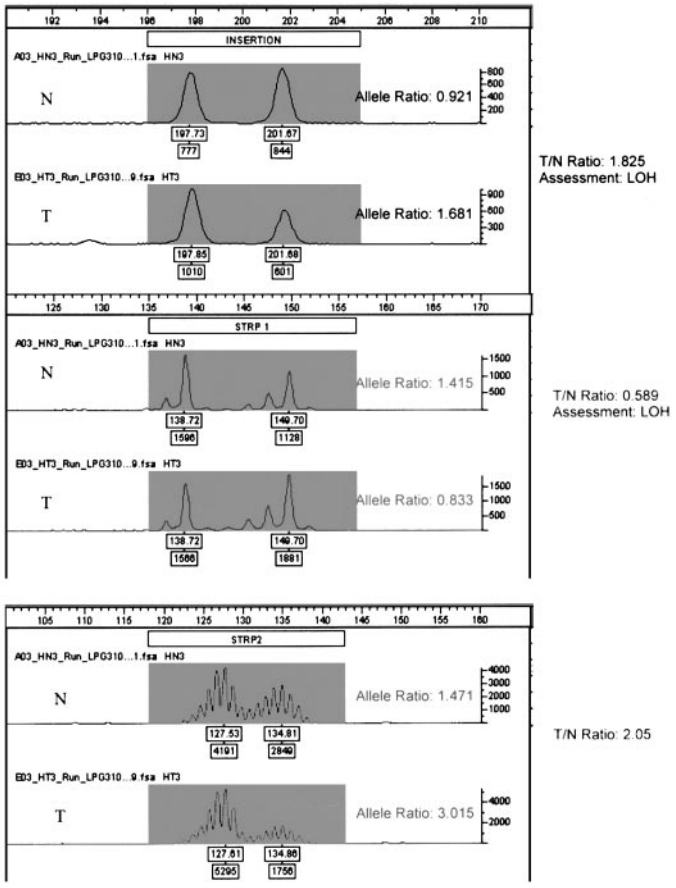

Fig. 3. LOH at the *GNMT* locus. An example of LOH in tumor (HT3) and nontumor (HN3) DNA from a patient with HCC. The chromatography of the INS/DEL polymorphisms is shown on the *top panels*, the STRP1 are shown on the *middle panels*, and the STRP2 are shown on the *bottom panels*. The calculated allele ratio (pick height in Allele1/pick height in Allele2) and T:N ratio. The LOH assessment for INS/DEL and STRP1 is determined by the standard developed in this report.

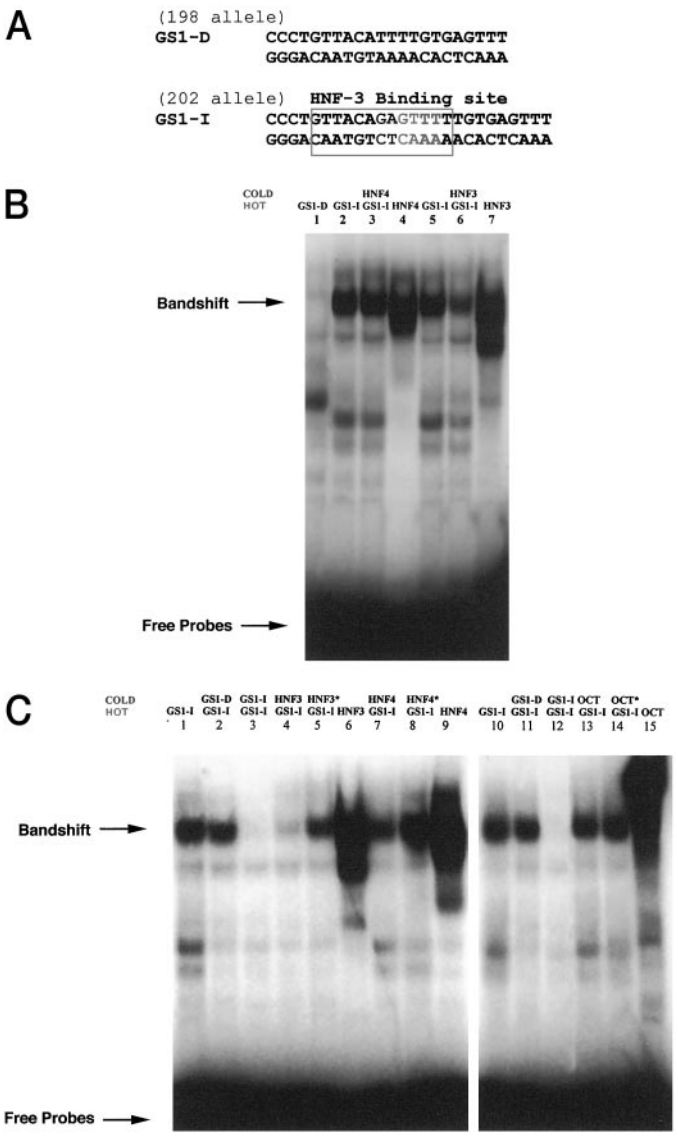

Fig. 4. Gel mobility shift assay for the motif containing INS/DEL polymorphism. *A*, the sequences of double-stranded DNA corresponding to the containing allele (202, probe: GS1-I) or deletion allele (198, probe: GS1-D) at the INS/DEL locus. *B*, differential gel shift pattern of ^{32}P-labeled GS1-I (*Lane 1*) and GS1-D (*Lane 2*), HNF-3 (*Lane 7*), and HNF-4 (*Lane 4*) probes. The same set of extracts was treated with 150-fold excess of unlabeled HNF-3 (*Lane 6*) and HNF-4 (*Lane 3*) probes as a competitor before the addition of [γ-^{32}P]ATP-labeled GS1-I probe. The ^{32}P-labeled probes are shown as *HOT* and unlabeled probes are shown as *COLD*. *C*, gel shift assays using hot GS1-I (*Lanes 1–5, 7, 8, 10–14*) and HNF-3 (*Lane 6*), HNF-4 (*Lane 9*), and Oct (*Lane 15*) probes in HepG_2 nuclear extracts. An excess ($\times$150) of cold GS1-D, GS1-I, HNF-3, HNF-4, and Oct probes were used as a competitor in *Lanes 2/11, 3/12, 4, 7,* and *13*, respectively. An excess ($\times$150) of cold mutant HNF-3$*$, HNF-4$*$, and Oct$*$ probes were used as a competitor in *Lanes 5, 8,* and *14*, respectively.

liver regeneration in virus-induced cirrhosis liver may increase the opportunity of LOH at the *GNMT* locus. Furthermore, early alterations within the *GNMT* gene in the nontumor liver tissues imply a critical role in liver cancer development. For example, LOH at *M6P/IGF2R*, a tumor suppressor gene, has also been shown to be an early event in liver carcinogenesis (24). The allelic loss patterns of *M6P/IGF2R* in liver cirrhosis were identical to those in the corresponding HCC. The authors suggest that HCC could develop from one of the cells in which *M6P/IGF2R* encoding had been lost. It is possible that the high LOH rate of *GNMT* in liver tissues resulted from the similar mechanisms inducing the LOH of *M6P/IGF2R* in liver.

To address the functional significance of INS/DEL we first performed a gel mobility assay using probes containing either the insertion or the deletion allele. Our study suggests that the 198 allele may abolish an HNF-3 recognition site (Fig. 4). Therefore, the 198 and 202 alleles potentially could have different effects on the transcriptional level of the *GNMT* gene that might be HNF-3-dependent. In addition, our results show that the luciferase activity of the Luc construct of the *GNMT* promoter with 10 GA repeats plus insertion (202 allele) had even higher activity than a construct with 10 GA repeats only (198 allele; Fig. 5). This provides additional evidence that allele 198 and allele 202 at INS/DEL may have allelic-specific effects on the transcriptional level of *GNMT*. However, direct evidence of HNF-3 binding to this region will need to be tested by supershift assay with monoclonal antibodies against transcription factor HNF-3. HNF-3 belongs to a large family of forkhead transcription factors (25). HNF-3 is liver-enriched, and involved in the differentiation of hepatocytes and the maintenance of liver-specific functions. Expression of the HNF-3 members is differentially regulated by nutritional and hormonal factors (25). Therefore, the functional effect of the INS/DEL polymorphism could be differentially dependent on the nutritional and hormonal status of liver tissues. Furthermore, we used a reporter gene system to demonstrate that the number of GA repeats influences the transcriptional efficiency of the *GNMT* promoter and differentially modulates *GNMT* expression among different human hepatoma cell lines. In Hep G_2 cells, promoters with the shorter repeat, $(GA)_{10}$, showed higher expression levels than those containing promoters with the longer repeats, $(GA)_{14}$, $(GA)_{15}$, $(GA)_{16}$, and $(GA)_{20}$ (Fig. 5). It has been reported that repetitive dinucleotide sequences may stimulate the activity of RNA polymerase II, and a variety of nuclear proteins have been found to bind to repetitive elements (Ref. 26). Presumably, the polymorphic repetitive sequences could have allelic-dependent effects on gene transcription. Several STRP sequences in the regulatory region of promoters have also been shown to confer different transcriptional efficiencies (27, 28).

In addition to the above functional polymorphisms in promoter region, SNP1 in 5′UTR, and intronic SNP2, SNP3, and STRP2 may also have a functional impact on *GNMT*. An increasing volume of evidence indicates that the polymorphisms in noncoding regions of genes, including the 5′ and 3′UTRs, and introns could influence gene

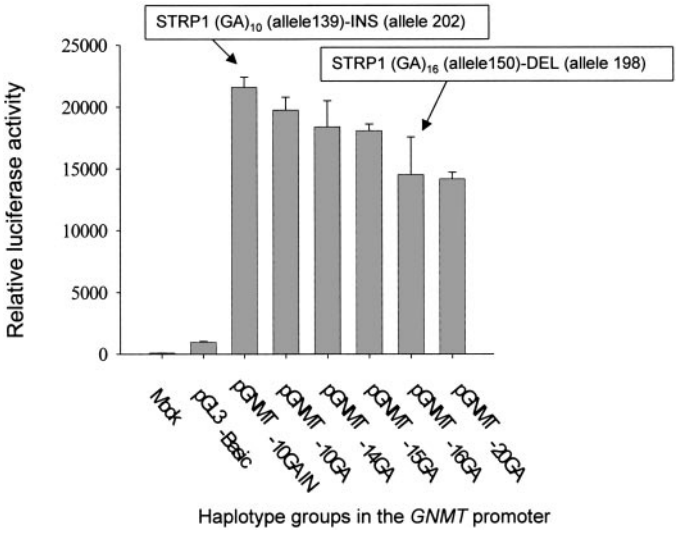

Fig. 5. Effects of the STRP1 and INS/DEL motifs on the promoter activity of the *GNMT* gene. Hep G_2 cells transfected with the recombinant gene carrying $(GA)_{10}$, $(GA)_{14}$, $(GA)_{15}$, $(GA)_{16}$, or $(GA)_{20}$ without the 4-bp GAGT insertion, or $(GA)_{10}$ with 4 bp insertion at the IND/DEL motif. In the Taiwanese population, STRP1 $(GA)_{10}$ (allele 139)-INS (allele 202) and STRP1 $(GA)_{16}$ (allele150)-DEL (allele 198) represent the two major haplotypes in the promoter region of *GNMT;* bars, $\pm$*SD*.

transcription and have relevance for complex traits and diseases (29–32). The functional relevance of those polymorphisms remains to be additionally characterized and determined. Intriguingly, the frequency of the C allele of SNP1 increased dramatically in tumor-adjacent liver DNA from HCC patients as compared with the blood DNA from the non-HCC group ($P = 0.0196$ in a χ^2 test; Table 3). This could be because of the functional deficiency caused by the C allele in SNP1 or result from the linkage between the C allele and an undetected functional variation near by. We will additionally address this question by resequencing *GNMT* more extensively and designing a functional assay for SNP1 in the future studies.

On the basis of the phenotypic results, we selected homozygous 198/198 at the INS/DEL locus and genotypes containing long repeats (repeat number N> = 16) at both STRP1 alleles as risk genotypes. Presumably, these risk genotypes have a lower GNMT expression level as compared with nonrisk genotypes. Intriguingly, we observed that the risk genotypes are over-represented in tumor-adjacent liver DNA from HCC patients. For example, 73% (198 of 202) in blood DNA from non-HCC group tends to be 198 of 198 in tumor-adjacent liver DNA from HCC patients (Table 4). This suggests that the inactivation of GNMT may be important in the initiation or early progression of tumorigenesis, and increasing risk genotypes could result from the high LOH rate of *GNMT* in liver from HCC patients. Therefore, investigating the early alteration of the *GNMT* genetic markers in blood DNA and liver DNA could be used as a method to screen individuals with a high risk of developing HCC early in the disease process (33). The major functions of GNMT are related to the maintenance of genetic stability in cells; thus, genetic alteration of *GNMT* could act as a mutator phenotype that drives the carcinogenic process (34). It might be possible to prevent liver cancer or delay its development through a better understanding the role of GNMT in HCC development.

In conclusion, we have developed new genetic markers at the *GNMT* locus and observed that risk genotypes of *GNMT* as estimated from *in vitro* functional studies are increased in tumor-adjacent tissues from HCC patients. Our results suggest that *GNMT* alteration may be an early event in HCC development and could represent a new tumor susceptibility gene for liver cancer.

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