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CHARACTERIZATION OF GLYCINE-N-METHYLTRANSFERASE-GENE EXPRESSION IN HUMAN HEPATOCELLULAR CARCINOMA

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Messenger RNA differential display was used to study liver-gene expression in paired tumor and non-tumor tissues from hepatocellular carcinoma (HCC) patients. mRNA differential display and Northern-blot analyses showed that a 0.8-kb cDNA fragment was diminished or absent from the tumorous tissues of 7 HCC patients. The cDNA fragment was sequenced and found to have 98.7% nucleotide sequence homology with human glycine-N-methyltransferase cDNA (GNMT). In addition, there was no detectable level of GNMT expression in 4 human HCC cell lines, SK-Hep1, Hep 3B, HuH-7 and HA22T, examined by Northern-blot assay. A full-length *GNMT* cDNA clone-9-1-2 was obtained by screening a Taiwanese liver cDNA library. In comparison with the *GNMT* cDNA sequence reported elsewhere, clone 9-1-2 had 4 nucleotide differences resulting in 1 amino-acid change. Immunohistochemical staining with rabbit anti-recombinant GNMT serum showed that GNMT protein almost completely disappeared in liver-cancer cells, while it was abundant in the non-tumorous liver cells. Down-regulation of *GNMT* gene expression may be involved in the pathogenesis of liver cancer. *Int. J. Cancer* 75:787–793, 1998.
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Hepatocellular carcinoma (HCC) is the leading cause of cancer deaths in sub-Saharan Africa and in Asia, including Taiwan and the southern provinces of mainland China (Li and Shiang, 1980; Lin *et al.*, 1977). It has been estimated that 250,000 to 1 million new HCC cases are found each year in the world (Simonetti *et al.*, 1991). Infection with the hepatitis-B virus, ingestion of aflatoxin-B1-contaminated foods, alcoholic cirrhosis and other factors associated with chronic inflammatory and hepatic regenerative changes are important risk factors for HCC (Beasley *et al.*, 1981; Wogan 1992; Harris, 1990). Mutations of various proto-oncogenes and tumor-suppressor genes have been reported in hepatocarcinogenesis (Pascale *et al.*, 1993); epigenetic mechanisms are also involved. Abnormal DNA methylation is involved in carcinogenesis, but the mechanism underlying this effect is still elusive (Lapeyre *et al.*, 1981; Kautiainen and Jones, 1986).

In this study, mRNA differential display (Liang and Pardee, 1992) was used to identify genes expressed differentially in tumor and non-tumor tissues from HCC patients. A cDNA fragment which has 98.7% sequence homology with human glycine-N-methyltransferase (GNMT) cDNA (Ogawa *et al.*, 1993) was found to be diminished or absent in tumor tissues from 7 HCC patients. GNMT (EC 2.1.1.20) is an enzyme regulating the ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) by catalyzing the synthesis of sarcosine from glycine and SAM (Kerr, 1972).

MATERIAL AND METHODS

Tumors

Human HCC tumors and their corresponding non-tumorous liver tissues were obtained, with their informed consent from patients at Taipei Veterans General Hospital. All specimens were frozen immediately after surgical resection and stored in liquid nitrogen before extraction of RNA and DNA materials. All cancerous and non-cancerous tissue specimens were confirmed by pathologic examination.

Cell lines and culture

Four human HCC cell lines, HuH 7 (Nakabayashi *et al.*, 1982), HA22T/VGH (Chang *et al.*, 1983), Hep 3B, and SK-Hep-1 (Aden *et al.*, 1979; Fogh and Trempe, 1976; Fogh *et al.*, 1977); and 2 hepatoblastoma cell lines, HuH 6 (Nakabayashi *et al.*, 1982) and Hep G2 (Aden *et al.*, 1979; Javitt, 1990) were used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), penicillin (100 IU per ml), streptomycin (100 µg per ml), fungizone, (2.5 mg per ml) and L-glutamine (2 mM) in a humidified incubator with 5% CO_2.

RNA extraction and mRNA differential display

Total RNA from tissues or cell lines were purified using the Ultraspec RNA extraction kit (Bio Tecx, Houston, TX). The extracted RNA had been treated with RNase-free DNase I before it was used for mRNA differential display (RNAmap kit, GenHunter, Brookline, MA). Detailed procedures for mRNA differential display have been described previously (Liang and Pardee, 1992; Liang *et al.*, 1992). The primers used in the assay included 4 T_{12}MN primers and 20 arbitrary oligonucleotide primers (AP-1 to AP-20) (GenHunter). The α-[^{35}S] dATP was obtained from NEN (Boston, MA).

Band recovery, amplification and characterization of the cDNA fragment

The cDNA fragments showing differential expression were cut from the dried gel and eluted by boiling the gel in 100 µl H_2O for 15 min. The cDNA was then recovered by ethanol precipitation in the presence of 0.3 M sodium acetate (pH 5.2) and glycogen (1 mg/ml). The precipitated cDNA was re-dissolved in 10 µl of H_2O and 4 µl of it was re-amplified in a reaction volume of 40 µl by PCR with the same primers (T_{12}MC and AP-16). The cDNA fragment was then subcloned to a pGEM-T vector (Promega, Madison, WI) and its nucleotide sequence was determined by a dideoxynucleotide sequencing method (Toneguzzo *et al.*, 1988) with T7 and Sp6 primers (Promega). The sequencing data were analyzed using the BLAST software program (Stephen *et al.*, 1990).

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Northern-blot and Southern-blot analyses

For Northern-blot analysis, 20 μg of RNA of each specimen was electrophoresed in a 1.2% agarose-formaldehyde gel and transferred to a nitrocellulose (NC) membrane (Stratagene, La Jolla, CA). For Southern-blot analysis, 10 μg of genomic DNA of each specimen was electrophoresed in a 0.8% agarose gel and transferred to a NC membrane. The 0.8-kb cDNA fragment was used as the probe and labeled with α-[^{32}P] dCTP (Amersham, Aylesbury, UK) using the Redi-Prime kit (Amersham). The hybridization conditions for Northern-blot and Southern-blot assays were as follows: 5 × SSPE, 5× Denhardt's, 50% formamide, salmon sperm DNA (100 μg per ml), 0.1% SDS and radiolabeled probe (1×10^6 cpm per ml) at 42°C for 24 hr. The membrane was washed twice with 2× SSC and 0.1% SDS at room temperature for 15 min, then with 0.1% SSC and 0.1% SDS at 50°C for 60 min. The washed membrane was autoradiographed with Kodak XAR-5 film at −80°C.

GNMT cDNA screening

A Taiwanese liver-cDNA library constructed using the phage ZAP-II vector system was obtained from Dr. S.-F. Tsai (Institute of Genetics, National Yang-Ming University, Taipei, Taiwan). The library contained 1.1×10^6 clones, the titer being approximately 1×10^9 pfu per ml. In the primary screening, 1×10^6 pfu of phages were plated out on 10 15-cm plates at high density (1×10^5 pfu/plate), and the replica filters were screened with a ^{32}P-labeled 0.8-kb cDNA fragment. All positive plaques were picked up, titrated and plated at 100 to 1,000 pfu per plate for secondary screening and final recovery. The positive clones were converted into phagemid using a helper phage in a non-suppressive bacterial host system (ExAssist Interference-Resistant Helper Phage with XLOLR strain, Stratagene).

Nucleotide sequence analysis of the GNMT cDNA clone

The phagemid (pBluescript-GNMT-9-1-2), obtained from the cDNA library screening, was sequenced using a dideoxynucleotide sequencing method (Toneguzzo *et al.*, 1988) with 12 different primers which included T3, T7 primers (Stratagene), as well as 5 GNMT-sense (S) and 5 GNMT-anti-sense (A) primers. The GNMT primers were designed according to the GNMT cDNA sequence reported by Ogawa *et al.* (1993), as follows: S1, 5′-TGTGGCAGCTGTATATCGGA-3′ (89–108); S2, 5′-GAGGGCTTCAGTGTGACGAG-3′ (232–251); S4, 5′-TGTGCACCCCCAGGGAAGAA-3′ (559–578); S5, 5′-CTACCCACACTGTCTGGCAT-3′ (732–751); S6, 5′-CCTCTGCCCAGGCACTGCTA-3′ (907–926); A1, 5′-TGCTCTAGAGGCTGGCCCTG-3′ (983–964); A2, 5′-GGGTTTGTAAGGCTTGAAGT-3′ (828–809); A3, 5′-CCTGGGGGTGCACAGCCTGT-3′ (572–553); A4, 5′-CTTGTCGAAGGCGGGCTCGT-3′ (330–311); and A5, 5′-AAGCAGCCATGCCTTGTACT-3′ (150–131).

Confirmation of the Taiwanese GNMT cDNA sequence

To confirm the differences of the nucleotide sequences between GNMT cDNA clone 9-1-2 and the reported sequence from Ogawa *et al.* (1993), RNA was extracted from normal liver tissues from 3 patients. The cDNA was prepared and used as templates for PCR with primers S7 and A1. Detailed procedures for the cDNA preparation have been described (Chen *et al.*, 1997). The nucleotide sequence of primer S7 was 5′-TGGCCAGGATGGGTGTCCTG-3′ (690-709). PCR conditions were as recommended by the manufacturer except that $MgCl_2$ was 2 mM and the primers were 0.5 μM (Innis and Gelfand, 1990). The PCR products were analyzed by electrophoresis on 2% agarose gels, then subject to nucleotide sequence analyses as described above.

Immunohistochemistry

Normal and tumor tissues from 3 HCC patients were used for immunohistochemical study with rabbit anti-GNMT antibodies. The tissue blocks fixed in paraffin were sliced into 6-μm-thick sections, de-paraffinized, and immersed in 3% hydrogen peroxide for 5 min to abolish the endogenous peroxidase reaction. Then, the sections were stained with rabbit pre-immunized and anti-GNMT sera at 1/100, 1/200, 1/500 and 1/1,000 dilutions at room temperature for 10 min. After washing in PBS, these slides were incubated with biotinylated antibody and peroxidase-labeled streptavidin (DAKO, Carpinteria, CA) for 10 min at room temperature. These slides were further incubated with 3,3′-diaminobenzidine tetrahydrochloride solution for color reaction.

RESULTS

mRNA differential display, Northern-blot and Southern-blot analyses

In order to run the assay efficiently, among 80 combinations of primer pairs (4 T_{12}MN primers *vs.* 20 AP primers), 12 pairs able to amplify more bands in the mRNA differential display of one set of tumor and non-tumor liver tissues were chosen (Table I). Then, these 12 primer pairs were used to amplify cDNA from 7 sets of tumor and non-tumor liver tissues from HCC patients in mRNA differential display. Among them, 4 primer pairs were found to amplify genes expressed differentially in at least 4 of 7 sets of the tumor and non-tumor tissues in the mRNA differential display (Table I). As shown in Figure 1, in comparison with results in the non-tumor tissues, a cDNA fragment was found absent or diminished in the tumor tissues from 6 HCC patients (lanes 1, 3, 5, 7, 11 and 13) in the mRNA differential display with primers T_{12}MC and AP-16. For patient HCC-9, the cDNA fragment was found diminished in the non-tumorous and in the tumorous specimens (lanes 9 and 10).

The results of mRNA differential display were reproducible, and the cDNA band identified in the non-tumorous tissue of HCC-7 in the dried gel was cut, recovered and re-amplified using PCR with the same primers. The resultant 0.8-kb cDNA fragment, designated 7N1, was sub-cloned to a pGEM-T vector and the nucleotide sequence was determined. The 7N1 DNA insert had 818 base pairs and shared 98.7% nucleotide sequence homology with a cDNA of the human *GNMT* gene (Ogawa *et al.*, 1993; data not shown).

The 7N1 DNA fragment was labeled with α-[^{32}P] dCTP and used as a probe in the Northern-blot assay. As shown in Figure 2, a 1.4-kb mRNA of the putative *GNMT* gene was detected in the non-tumorous liver tissue specimens of all of the HCC patients (the even-number lanes), while it was absent from the tumorous specimens of 5 HCC patients (lanes 3, 5, 7, 11 and 15). For patients HCC-1 and HCC-20, expression levels of the *GNMT* gene in the tumor tissues (lanes 1 and 13) were much lower than that in the non-tumorous specimens (lanes 2 and 14).

Expression of the *GNMT* gene in the human HCC and hepatoblastoma cell lines was also studied by Northern-blot analysis. There was no detectable level of GNMT mRNA in 4 HCC cell lines, HA22T/VGH, SK-Hep1, Hep 3B and HuH-7 (Fig. 3, lanes 1, 2, 3 and 6). The levels of GNMT mRNA in 2 hepatoblastoma cell lines, Hep G2 and HuH-6 (lanes 4 and 5) were significantly lower than that in the non-tumorous liver tissue from an HCC patient (lane 7).

TABLE I – THE PRIMER PAIRS USED FOR mRNA DIFFERENTIAL DISPLAY

T_{12}MN	Arbitrary primers (AP-1 to AP-20)	
	Amplify more bands	Have found genes expressed differentially in at least 4 of 7 sets of tumorous and non-tumorous tissues
T_{12}MA	AP-1, -11 and -18	AP-18
T_{12}MT	AP-1, -15 and -18	AP-15
T_{12}MG	AP-1, -11 and -18	AP-18
T_{12}MC	AP-1, -5 and -16	AP-16

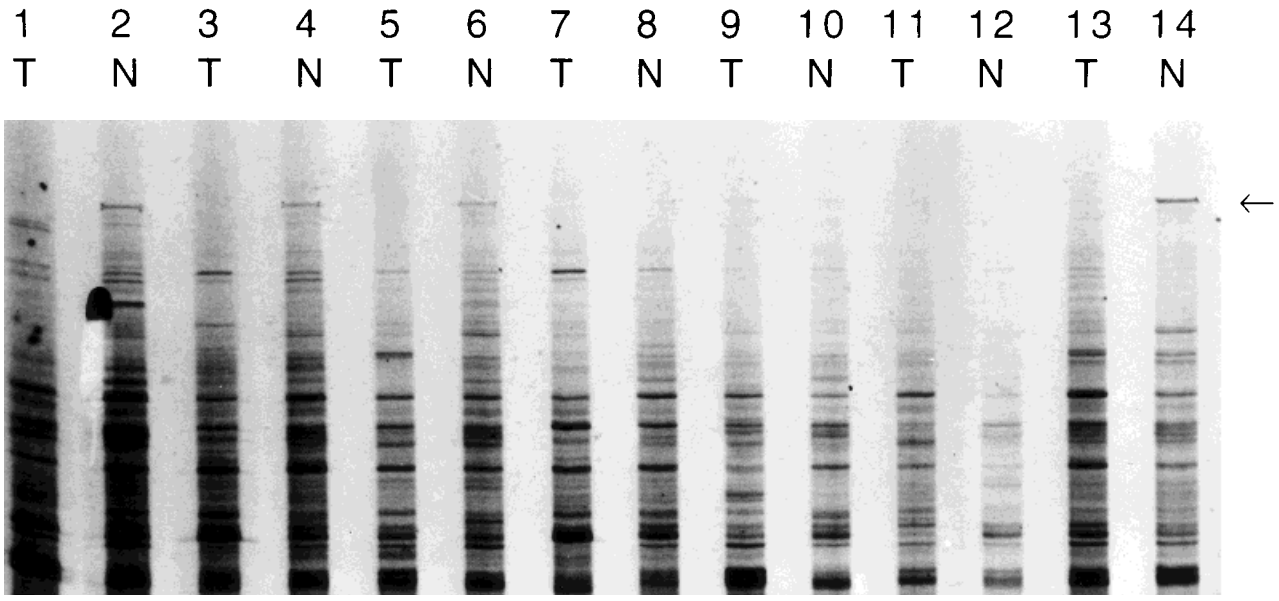

FIGURE 1 – Results of mRNA differential display. Total RNA of human tumorous (T) and non-tumorous (N) liver tissues from 7 HCC patients were extracted and their gene expression was studied by using differential display with $T_{12}MC$ and AP-16 primers. Lanes 1 and 2, HCC-1; lanes 3 and 4, HCC-5; lanes 5 and 6, HCC-7; lanes 7 and 8, HCC-8; lanes 9 and 10, HCC-9; lanes 11 and 12, HCC-14; lanes 13 and 14, HCC-20. Arrow indicates cDNA fragment-7N1 identified for further study.

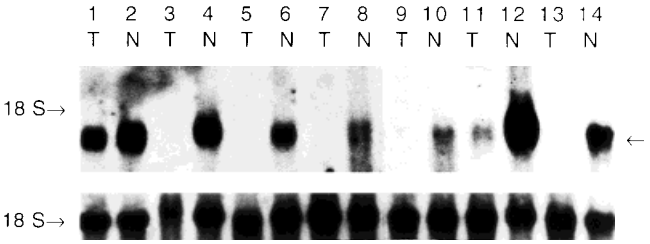

FIGURE 2 – Northern-blot analysis of *GNMT* gene expression in tumorous (T) and non-tumorous (N) liver tissues from HCC patients. *Upper panel:* 20 µg RNA from 7 HCC patients, HCC-1 (lanes 1 and 2), HCC-5 (lanes 3 and 4), HCC-7 (lanes 5 and 6), HCC-8 (lanes 7 and 8), HCC-14 (lanes 9 and 10), HCC-20 (lanes 11 and 12), and HCC-24 (lanes 13 and 14), were blotted onto the nitrocellulose membrane and hybridized with the GNMT-7N1 cDNA probe. *Lower panel:* The 18S rRNA from each specimen was probed as a control. Arrow indicates position of *GNMT* mRNA.

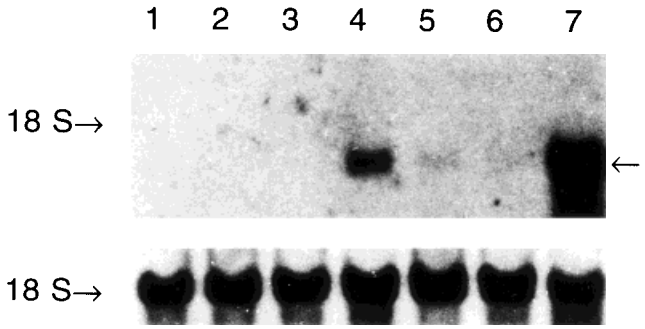

FIGURE 3 – Northern-blot analysis of *GNMT* expression in different human HCC and hepatoblastoma cell lines. *Upper panel:* 20 µg RNA from 4 HCC cell lines, HA22T/VGH (lane 1), SK-Hep 1 (lane 2), Hep 3B (lane 3), and HuH-7 (lane 6), 2 hepatoblastoma cell lines, Hep G2 (lane 4) and HuH-6 (lane 5), and non-tumorous liver tissue from patient HCC-24 (lane 7) were blotted onto the nitrocellulose membrane and hybridized with the GNMT-7N1 cDNA probe. *Lower panel:* 18S rRNA from each specimen was also probed. Arrow indicates position of *GNMT* mRNA.

Furthermore, Southern blot hybridization with [^{32}P]-labeled-7N1 DNA probe demonstrated that the GNMT gene was present in the Hep 3B cells (Fig. 4, lanes 1 and 3).

Isolation and sequencing of a full-length human GNMT cDNA clone

The 7N1 cDNA fragment was used as a probe to isolate a full-length GNMT cDNA in a Taiwanese liver-cDNA library. After screening of 1×10^6 phages, 9 positive clones were obtained, and 1 clone, 9-1-2, which had the largest size of insert, was selected for sequence analysis. As shown in Figure 5, clone 9-1-2 had 1096 nucleotides. The first ATG codon begins an open reading frame that extends to the termination codon located downstream at the 295th triplets. At the 3′ end, a poly(A) sequence was observed. In comparison with the *GNMT* cDNA sequence reported by Ogawa *et al.* (1993), the clone 9-1-2 had 4 nucleotide differences in the coding region which resulted in 1 amino-acid change: amino-acid residue 24 changed from glutamic acid to aspartic acid (Fig. 5). To elucidate the difference, additional *GNMT* cDNA was obtained from the non-tumorous liver tissues of 3 HCC patients by using RT-PCR. Sequencing results showed that these 3 cases had identical sequences of the coding region of clone 9-1-2.

Immunohistochemical studies

The 1.2-kb full-length *GNMT* cDNA fragment was sub-cloned to an expression vector and a GST-GNMT recombinant fusion protein was induced in *Escherichia coli.* The recombinant GST-GNMT protein was purified and used as antigen to raise rabbit anti-sera (data not shown). The GNMT expression in the HCC tissues from 3 patients was investigated by immunohistochemical analysis with the rabbit anti-serum. As shown in Figure 6*a*, GNMT was

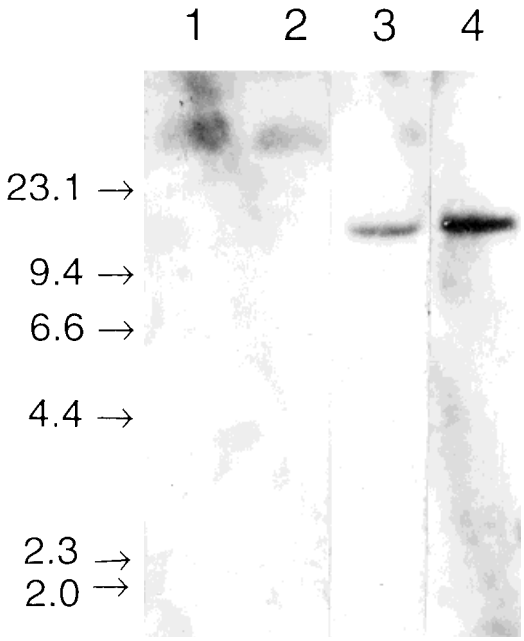

FIGURE 4 – Southern-blot analysis of the *GNMT* gene in Hep 3B cells. Genomic DNA (10 µg) from Hep 3B cells (lanes 1 and 3) and normal peripheral-blood mononuclear cells (lanes 2 and 4) was treated (lanes 3 and 4) or not treated (lanes 1 and 2) with HindIII restriction enzyme for Southern-blot assay. Size markers are shown in the left margin.

expressed abundantly in the cytosols of most of the normal liver cells, while it was not expressed in the cancer cells from the same patient (Fig. 6*b*,*c*). Similar distinctive results were also found in the other 2 HCC patients. As shown in Figure 6*d*, GNMT was expressed in the normal liver cells surrounded by tumor nodules which had no GNMT staining. There were no positive results when the pre-immunized rabbit serum or a rabbit anti-GST anti-serum was used in the study (data not shown).

DISCUSSION

According to the study designed by Liang *et al.* (1992), it is feasible to display all the mRNA present in a cell using mRNA differential display with 80 different primer pairs. However, when the method was used to study the gene expression of human cancers, many cDNA fragments amplified in the tumor tissues may originate from normal cells present in the tissues, which may affect the results of the analyses. To solve this problem, we decided to use 7 sets of tumor and non-tumor tissues for the assay and displayed the results simultaneously. In addition, 12 of 80 primer pairs were selected, based on the relative numbers of cDNA bands that they can amplify, for differential display (Table I). Through this study design, 4 cDNA fragments which including GNMT showed distinctive patterns in more than 4 of 7 sets of specimens were identified. In fact, all of the tumor tissues from 7 HCC patients had diminished or undetectable levels of *GNMT* gene expression in mRNA differential display assays.

The results of the Northern-blot assays were consistent with those of mRNA differential display. Due to scarcity of tissues, HCC-9 was not available for Northern-blot analysis. Furthermore, the gene expression of *GNMT* was found to disappear almost completely in 4 HCC cell lines tested. Interestingly, the level of *GNMT* gene expression also decreased in 2 hepatoblastoma cell lines-Hep G2 and HuH 6. This implied that the significance of down-regulation of *GNMT* gene expression in the pathogenesis of HCC and of hepatoblastoma may be different. The relative levels of *GNMT* gene expression may be used as a marker for distinguishing these 2 types of cancer.

In comparison with the coding region of the *GNMT* cDNA clone reported by Ogawa *et al.* (1993), the *GNMT* cDNA clone-9-1-2 we obtained has 4 nucleotide differences resulting in 1 amino-acid change (amino-acid residue 24, glutamic acid to aspartic acid). The differences have been confirmed by sequencing the PCR-amplified *GNMT* cDNA fragments from 3 Taiwanese' normal liver tissues. It is worthy to note that in rabbits, pigs and rats whose *GNMT* cDNA has been studied, the predicted amino-acid residue 24 of their GNMT proteins were all aspartic acid (Ogawa *et al.*, 1993). Further studies therefore are needed to determine whether the difference we found was due to an error in the cited study or to other mechanisms.

In this study, a rabbit anti-GNMT anti-serum was used for immunohistochemical analysis. The anti-serum was generated by immunized rabbits with a recombinant GST-GNMT protein produced in *E. coli.* In addition, a protein with similar size of GNMT (about 32 kDa) in normal liver cell lysates was reactive with the rabbit anti-serum in a Western-blot assay (data not shown). Therefore, the rabbit anti-GNMT anti-serum can specifically react to the GNMT present in the normal liver cells. Immunohistochemical staining with the rabbit anti-GNMT antibody showed that GNMT was almost undetectable in the hepatoma cells, while it was abundant in liver cytosol, especially in the periportal region (Yeo and Wagner, 1994). For case J95-3707, the normal liver cells surrounded by tumor nodules can be considered as an internal control (Fig. 6*d*). More pathological studies need to be performed to determine the usefulness of the anti-GNMT antibody in differentiating benign and malignant hepatic lesions. At present, monoclonal antibodies against GNMT are in preparation for further immunohistochemical and biochemical studies.

It has been shown that GNMT activity was significantly decreased in rat hepatoma (Heady and Kerr, 1975). Moreover, in a rat hepatoma model induced using N-2-fluorenylacetamide, GNMT enzyme activity decreased gradually and became undetectable in the liver tumor 8 months after treatment (Tsukada *et al.*, 1985). This indicates that down-regulation of *GNMT* gene expression is present in naturally occurring and in carcinogen-induced liver cancers.

The GNMT protein has been shown to be an important factor for regulation of the SAM/SAH ratio (Kerr, 1972). Through the enzyme, glycine receives a methyl group from SAM and becomes sarcosine, which can be oxidized back to glycine by sarcosine dehydrogenase. This reaction will generate energy and release one carbon unit from SAM. It has also been reported that the activity of GNMT in rats fluctuates and correlates with the level of methionine in the diet (Ogawa and Fujioka, 1982*b*). Results from various laboratories indicate that lipotropic compounds, such as SAM and its precursors-methionine, choline and betaine, prevent development of liver tumors induced by various carcinogens in a rat or mouse model (Farber and Ichinose, 1958; Shivapurkar *et al.*, 1986; Pascale *et al.*, 1992). The mechanism by which SAM administration prevents the development of pre-neoplastic and neoplastic tissues is still not clear. Since GNMT tightly controls the level of SAM in the liver cells, its enzyme activity may be activated by SAM and involved in the chemopreventive pathway of liver cancer (Pascale *et al.*, 1995).

It is important to note that GNMT exhibits multiple functions. It is a folate-binding protein (Cook and Wagner, 1984). Protein-sequence analysis of rat GNMT demonstrated that it contains a potential nucleotide-binding domain (Ogawa *et al.*, 1987). In addition, it shares amino-acid-sequence homology with liver

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 GGCACGAGGG ATG GTG GAC AGC GTG TAC CGG ACC CGC TCC CTG GGG GTG GCG GCC     55
            Met Val Asp Ser Val Tyr Arg Thr Arg Ser Leu Gly Val Ala Ala
             1               5                  *  10                  15

GAA GGG CTC CCG GAC CAG TAC GCG GAC GGG GAG GCG GCG CGC GTG TGG CAG CTG    109
Glu Gly Leu Pro Asp Gln Tyr Ala Asp Gly Glu Ala Ala Arg Val Trp Gln Leu
                  20              ***  25                  30

TAT ATC GGA GAC ACC CGC AGC CGC ACC GCC GAG TAC AAG GCA TGG CTG CTT GGG    163
Tyr Ile Gly Asp Thr Arg Ser Arg Thr Ala Glu Tyr Lys Ala Trp Leu Leu Gly
     35                  40                  45                  50

CTG CTG CGC CAG CAC GGC TGC CAG CGG GTG CTC GAC GTA GCC TGT GGC ACT GGG    217
Leu Leu Arg Gln His Gly Cys Gln Arg Val Leu Asp Val Ala Cys Gly Thr Gly
              55                  60                  65

GTG GAC TCC ATT ATG CTG GTG GAA GAG GGC TTC AGT GTG ACG AGT GTG GAT GCC    271
Val Asp Ser Ile Met Leu Val Glu Glu Gly Phe Ser Val Thr Ser Val Asp Ala
 70                  75                  80                  85

AGT GAC AAG ATG CTG AAG TAT GCA CTT AAG GAG CGC TGG AAC CGG CGG CAC GAG    325
Ser Asp Lys Met Leu Lys Tyr Ala Leu Lys Glu Arg Trp Asn Arg Arg His Glu
          90                  95                 100                 105

CCC GCC TTC GAC AAG TGG GTC ATC GAA GAA GCC AAC TGG ATG ACT CTG GAC AAA    379
Pro Ala Phe Asp Lys Trp Val Ile Glu Glu Ala Asn Trp Met Thr Leu Asp Lys
                 110                 115                 120

GAT GTG CCC CAG TCA GCA GAG GGT GGC TTT GAT GCT GTC ATC TGC CTT GGA AAC    433
Asp Val Pro Gln Ser Ala Glu Gly Gly Phe Asp Ala Val Ile Cys Leu Gly Asn
     125                 130                 135                 140

AGT TTC GCT CAC TTG CCA GAC TGC AAA GGG GAC CAG AGT GAG CAC CGG CTG GCG    487
Ser Phe Ala His Leu Pro Asp Cys Lys Gly Asp Gln Ser Glu His Arg Leu Ala
              145                 150                 155

CTG AAA AAC ATT GCG AGC ATG GTG CGG GCA GGG GGC CTA CTG GTC ATT GAT CAT    541
Leu Lys Asn Ile Ala Ser Met Val Arg Ala Gly Gly Leu Leu Val Ile Asp His
160                 165                 170                 175

CGC AAC TAC GAC CAC ATC CTC AGT ACA GGC TGT GCA CCC CCA GGG AAG AAC ATC    595
Arg Asn Tyr Asp His Ile Leu Ser Thr Gly Cys Ala Pro Pro Gly Lys Asn Ile
          180                 185                 190                 195

TAC TAT AAG AGT GAC TTG ACC AAG GAC GTC ACA ACA TCA GTG CTG ATA GTG AAC    649
Tyr Tyr Lys Ser Asp Leu Thr Lys Asp Val Thr Thr Ser Val Leu Ile Val Asn
                 200                 205                 210

AAC AAG GCC CAC ATG GTG ACC CTG GAC TAT ACG GTG CAG GTG CCG GGG GCT GGC    703
Asn Lys Ala His Met Val Thr Leu Asp Tyr Thr Val Gln Val Pro Gly Ala Gly
     215                 220                 225                 230
                                                 *
CAG GAT GGC TCT CCT GGC TTG AGT AAG TTC CGG CTC TCC TAC TAC CCA CAC TGT    757
Gln Asp Gly Ser Pro Gly Leu Ser Lys Phe Arg Leu Ser Tyr Tyr Pro His Cys
              235                 240                 245

CTG GCA TCC TTC ACG GAG CTG CTC CAA GCA GCC TTC GGA GGT AAG TGC CAG CAC    811
Leu Ala Ser Phe Thr Glu Leu Leu Gln Ala Ala Phe Gly Gly Lys Cys Gln His
250                 255                 260                 265
                                       *   *
AGC GTC CTG GGC GAC TTC AAG CCT TAC AAG CCA GGC CAA ACC TAC ATT CCC TGC    865
Ser Val Leu Gly Asp Phe Lys Pro Tyr Lys Pro Gly Gln Thr Tyr Ile Pro Cys
          270                 275                 280                 285

TAC TTC ATC CAC GTG CTC AAG AGG ACA GAC TGA GTG TGG CCT CAG CTC CCA CAA    919
Tyr Phe Ile His Val Leu Lys Arg Thr Asp
                 290                 295

GCC TCT GCC CAG GCA CTG CTA GGC TCT GTC TGG AAG ATG GGG ACC AGC AGC CCC    973
ACA CCA GGG CCA GCC TCT AGA GCA GAC TAC AGC TGG GGT GCA GGG ATG TGG GTT   1027
CCA CAG ACG GAA GGG TAA ACA ATA TAG TCT TTT TCA GTT CCT GCA AAA AAA AAA   1081
AAA AAA AAA AAA AAA A                                                   1097
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FIGURE 5 – The nucleotide and predicted amino-acid sequences of a full-length human *GNMT* cDNA clone-9-1-2. *Nucleotide and amino-acid residues differing from those reported by Ogawa *et al.* (1993).

4S-benzo[a]pyrene-binding protein which interacts with the 5′-flanking regions of the cytochrome p4501A1 gene in a rat model (Raha *et al.*, 1994, 1995; Ogawa *et al.*, 1997). Therefore, when *GNMT*-gene expression is suppressed, not only will the epigenetic modification (methylation) of many cellular genes be altered, but the hepatic detoxication pathway may also be affected.

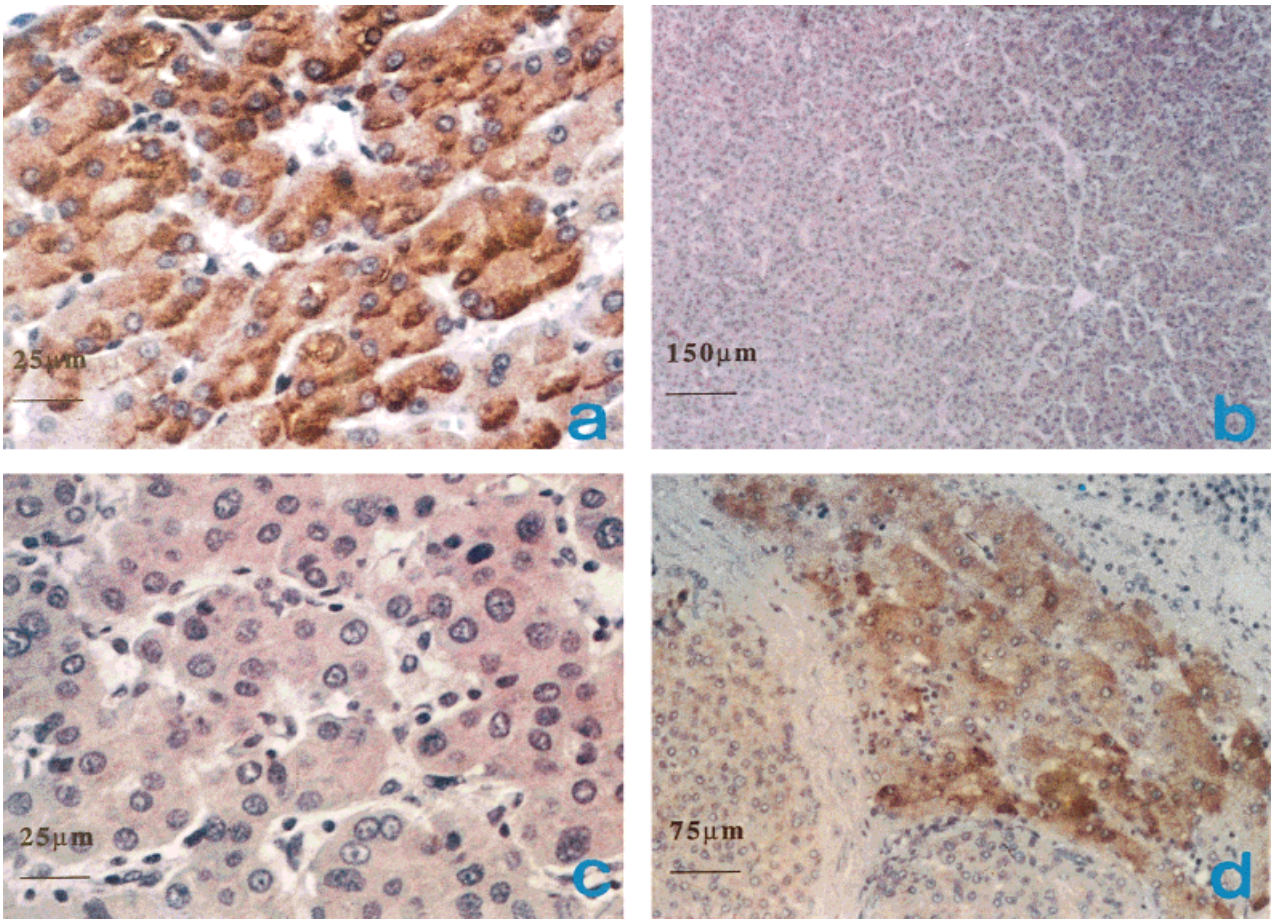

FIGURE 6 – Immunohistochemical analysis of the GNMT expression in the paraffin-fixed tissue sections from 2 HCC patients. The sections were exposed to rabbit anti-GNMT anti-serum at 1/500 dilution and visualized by a labeled streptavidine-biotin method. Non-tumorous *(a)* and tumorous tissues *(b, c)* from an HCC patient, J96-1415; *(d)* tumorous tissue from another HCC patient, J95-3707.

In the rat model, the activity of GNMT is stringently restricted to liver, kidney and pancreas cells (Kerr, 1972). One may suspect that the down-regulation of *GNMT* in HCC is a result of genomic abnormality. However, Southern-blot analysis demonstrated that Hep-3B cells, whose *GNMT*-gene expression was totally suppressed, contained the *GNMT* gene (Fig. 4). Further studies on the genomic DNA level are needed to elucidate the mechanism of down-regulation of *GNMT*-gene expression. Finally, the potential application of GNMT in clinical medicine deserves special attention.

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REFERENCES

ADEN, D.P., FOGEL, H., PLOTKIN, S., DAMJANOV, I. and KNOWLES, B.B., Controlled synthesis of HBsAg in a differentiated human liver-carcinoma-derived cell line. *Nature (Lond.),* **282,** 615–616 (1979).

BEASLEY, R.P., HWANG, L.Y., LIN, C.C. and CHIEN, C.S., Hepatocellular carcinoma and hepatitis-B virus. A prospective study of 22,707 men in Taiwan. *Lancet,* **II,** 1129–1133 (1981).

CHANG, C., LIN, Y., O-LEE, T., CHOU, C., LEE, T., LIU, T., P'ENG, F., CHEN, T. and HU, C., Induction of plasma protein secretion in a newly established human hepatoma cell line. *Mol. cell. Biol.,* **3,** 1133–1137 (1983).

CHEN, Y.-J., SHIH, L.-S., CHANG, J.-G., WHANG-PENG, J. and CHEN, Y.-M.A., Frequent detection of aberrant RNA transcripts of the *CDKN2* gene in human gastric adenocarcinoma. *Int. J. Cancer,* **71,** 350–354 (1997).

COOK, R.J. and WAGNER, C., Glycine N-methyltransferase is a folate binding protein of rat liver cytosol. *Proc. nat. Acad. Sci. (Wash.),* **81,** 3631–3634 (1984).

FARBER, E. and ICHINOSE, H., The prevention of ethionine-induced carcinoma in the liver in rats by methionine. *Cancer Res.,* **18,** 1209–1213 (1958).

FOGH, J. and TREMPE, G., New human tumor cell line. *In:* J. Fogh (ed.), *Human tumor cell in vitro,* pp. 115–119, Plenum, New York (1976).

FOGH, J., WRIGHT, W.C. and LOVELESS, J.D., Absence of HeLa-cell contamination in 169 cell lines derived from human tumors. *J. nat. Cancer Inst.,* **41,** 209–214 (1977).

HARRIS, C.C., Hepatocellular carcinogenesis: recent advances and speculation. *Cancer Cells,* **2,** 146–148 (1990).

HEADY, J.E. and KERR, S.J., Alteration of glycine-N-methyltransferase activity in fetal, adult and tumor tissues. *Cancer Res.,* **35,** 640–643 (1975).

INNIS, M.A. and GELFAND, D.H., Optimization of PCRs. *In:* M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (eds.), *PCR protocols,* pp. 3–12, Academic Press, San Diego (1990).

JAVITT, N.B., Hep-G2 cells as a resource for metabolic studies: lipoprotein, cholesterol, and bile acids. *FASEB J.,* **4,** 161–168 (1990).

KAUTIAINEN, T.L. and JONES, P.A., DNA methyltransferase levels in tumorigenic and non-tumorigenic cells in culture. *J. biol. Chem.,* **261,** 1594–1598 (1986).

KERR, S.J., Competing methyltransferase system. *J. biol. Chem.,* **247,** 4248–4252 (1972).

LAPEYRE, J.-N., WALKER, M.S. and BECKER, F.F., DNA methylation and methylase levels in and malignant mouse hepatic tissues. *Carcinogenesis,* **2,** 873–878 (1981).

LI, F.P. and SHIANG, E.L., Cancer mortality in China. *J. nat. Cancer Inst.,* **65,** 217–221 (1980).

LIANG, P., AVERBOUKH, L., KEYOMARSI, K., SAGER, R. and PARDEE, A.B., Differential display and cloning of messenger RNAs from human breast cancer *versus* mammary epithelial cells. *Cancer Res.,* **52,** 6966–6968 (1992).

LIANG, P. and PARDEE, A.B., Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science,* **257,** 967–971 (1992).

LIN, T.M., CHANG, L.C. and CHEN, R.P., A statistical analysis of mortality of malignant neoplasms in Taiwan. *J. Formosan med. Ass.,* **76,** 656–668 (1977).

NAKABAYASHI, H., TAKETA, K., MIYANO, K., YAMANE, T. and SATO, J., Growth of human hepatoma cell lines with differentiated functions in chemical medium. *Cancer Res.,* **42,** 3858–3862 (1982).

OGAWA, H. and FUJIOKA, M., Purification and properties of glycine N-methyltransferase. *J. biol. Chem.,* **257,** 3447–3452 (1982*a*).

OGAWA, H. and FUJIOKA, M., Induction of rat-liver glycine N-methyltransferase by high methionine diet. *Biochem. biophys. Res. Commun.,* **108,** 227–232 (1982*b*).

OGAWA, H., GOMI, T. and FUJIOKA, M., Mammalian glycine N-methyltransferase. Comparative kinetic and structural properties of the enzymes from human, rat, rabbit and pig livers. *Comp. Biochem. Physiol.,* **106B,** 601–611 (1993).

OGAWA, H., KONISHI, K., TAKATA, Y., NAKASHIMA, H. and FUJIOKA, M., Rat glycine methyltransferase. Complete amino-acid sequence deduced from a cDNA clone and characterization of the genomic DNA. *Europ. J. Biochem.,* **168,** 141–151 (1987).

OGAWA, H., GOMI, T., IMAMURA, T., KOBAYASHI, M. and HUH, N-H., Rat liver 4S-benzo[a]pyrene-binding protein is distinct from glycine N-methyltransferase. *Biochem. Biophys. Res. Commun.,* **233,** 300–304 (1997).

PASCALE, R.M., MARRAS, V., SIMILE, M.M., DAINO, L., PINNA, G., BENNATI, S., CARTA, M., SEDDAIU, M.A., MASSARELLI, G. and FEO, F., Chemoprevention of rat-liver carcinogenesis by S-adenosyl-L-methionine: a long-term study. *Cancer Res.,* **52,** 4979–4986 (1992).

PASCALE, R.M., SIMILE, M.M. and FEO, F., Genomic abnormalities in hepatocarcinogenesis. Implication for a chemopreventive strategy. *Anticancer Res.,* **13,** 1341–1356 (1993).

PASCALE, R.M., SIMILE, M.M., DE MIGLIO, M.R., NUFRIS, A., DAINO, L., PINNA, G., SEDDAIU, M.A., RAO, P.M., RAJALAKSHMI, S., SARMA, D.S.R. and FEO, F., Chemoprevention by S-adenosyl-L-methionine of rat-liver carcinogenesis initiated by 1,2-demethylhydrazine and promoted by orotic acid. *Carcinogenesis,* **16,** 427–430 (1995).

RAHA, A., JOYCE, T., GUSKY, S. and BRESNICK, E., Glycine N-methyltransferase is a mediator of cytochrome p4501A1 gene expression. *Arch. Biochem. Biophys.,* **322,** 395–404 (1995).

RAHA, A., WAGNER, C., MACDONALD, R.G. and BRESNICK, E., Rat-liver cytosolic 4 S polycyclic aromatic-hydrocarbon-binding protein is glycine N-methyltransferase. *J. biol. Chem.,* **269,** 5750–5756 (1994).

SHIVAPURKAR, N., HOOVER, K.L. and POIRIER, L.A., Effect of methionine and choline on liver-tumor promotion by phenobarbital and DDT in diethylnitrosamine-initiated rats. *Carcinogenesis,* **7,** 547–550 (1986).

SIMONETTI, R.G., CAMMA, C., FIORELLO, F., POLITI, F., D'AMICO, G. and PAGLIARO, L., Hepatocellular carcinoma: a worldwide problem and the major risk factors. *Digest. Dis. Sci.,* **36,** 962–972 (1991).

STEPHEN, F.A., GISH, W., MILLER, W., MYERS, E.W. and LIPMAN, D.J., Basic local alignment search tool. *J. mol. Biol.,* **215,** 403–410 (1990).

TONEGUZZO, F., GLYNN, S., LEVI, E., MJOLSNESS, S. and HAYDAY, H., Use of a chemically modified T7 DNA polymerase for manual and automated sequencing of supercoiled DNA. *Biotechniques,* **6,** 460–469 (1988).

TSUKADA, K., ABE, T., KUWAHATA, T. and MITSUI, K., Metabolism of S-adenosylmethionine in rat hepatocytes: transfer of methyl group from S-adenosylmethionine by methyltransferase reactions. *Life Sci.,* **37,** 665–672 (1985).

WOGAN, W.N., Aflatoxins as risk factors for hepatocellular carcinoma in human. *Cancer Res.,* **52,** 2114s–2118s (1992).

YEO, E.-J. and WAGNER, G., Tissue distribution of glycine N-methyltransferase, a major folate-binding protein of liver. *Proc. nat. Acad. Sci. (Wash.),* **91,** 210–214 (1994).