Epigenetic Silencing of *GNMT* Gene in Pancreatic Adenocarcinoma

ANCA BOTEZATU[1], CORALIA BLEOTU[2], ANCA NASTASE[3], GABRIELA ANTON[1], NICOLAE BACALBASA[4], DAN DUDA[5], SIMONA OLIMPIA DIMA[3] and IRINEL POPESCU[3]

[1]*Viral Genetic Engineering Laboratory, Romanian Academy "Stefan S. Nicolau" Virology Institute, Bucharest, Romania;*
[2]*Antiviral Therapy Engineering Laboratory, Romanian Academy "Stefan S. Nicolau" Virology Institute, Bucharest, Romania;*
[3]*Center of General Surgery and Liver Transplantation "Dan Setlacec", Fundeni Clinical Institute, Bucharest, Romania;*
[4]*"Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania;*
[5]*Edwin L. Steele Laboratory for Tumor Biology, Charlestown, MA, U.S.A.*

Abstract. *Background/Aim: Although pancreatic ductal adenocarcinoma (PDAC) remains a major challenge for therapy, biomarkers for early detection are lacking. Epigenetic silencing of tumor suppressor genes is a majorcontributor to neoplastic transformation. The aim of this study was to identify new factors involved in PDAC progression. The GNMT gene possesses CpG islands in the promoter region and is important in methyl-group metabolism and in maintaining a normal methylation status of the genome. Materials and Methods: To test the hypothesis whether GNMT is epigenetically regulated in PDAC, we evaluated the GNMT gene expression and promoter methylation status in 30 paired samples of PDAC and normal pancreatic tissue. Results: We found significantly higher methylation frequencies ($p<0.001$) in PDACs (2.82-100%; median, 36.05%) than in controls (0.28-14.02%; median, 4.39%). The GNMT gene expression was decreased in PDACs compared to normal pancreatic tissues in 26/30 cases (86.67%). Furthermore, we showed that treatment with 5-aza-2-deoxycytidine (5-aza-dC) increased GNMT mRNA expression and decreased viability in PDAC cells. Conclusion: Collectively, these data indicate that GNMT is aberrantly methylated in PDAC representing, thus, a potential major mechanism for gene silencing. Methylation of GNMT gene is directly correlated with disease stage and with tumor grade indicating that these epigenetic effects may be important regulators of PDAC progression.*

Correspondence to: Anca Botezatu, Viral Genetic Engineering Laboratory, Romanian Academy "Stefan S. Nicolau"Virology Institute, Bucharest 285 Mihai Bravu Ave., Bucharest, Romania. Tel/Fax: +40 213242590, e-mail: gnanka30@yahoo.com and Simona Olimpia Dima, Center of General Surgery and Liver Transplantation "Dan Setlacec", Fundeni Clinical Institute, Bucharest, 258 Fundeni Ave.,Bucharest, Romania. Tel: +40 212750700/212750500, Fax: +40 213180444, e-mail: simonaolimpiadima@yahoo.com

Key Words: Epigenetic alteration, promoter methylation, pancreatic cancer.

Pancreatic ductal adenocarcinoma (PDAC) is a malignant neoplasm characterized by a very poor prognosis with a median survival of less than 6 months. The non-specific symptoms of PDAC, as well as the high metastatic potential caused by lymphatic dissemination, make diagnosis difficult in the early stages of the disease. Less than 10% of patients are diagnosed with pancreatic cancer at early-stages (1).

The factors involved in early PDAC and the molecular mechanisms leading to the development of PDAC remain unknown. The detection of pancreatic cancer at early stages, the prediction of the potential resectability or response to therapy are the current major challenges in improving the clinical outcome of PDAC. Molecular biomarkers are important tools for early diagnosis and prognosis, not only for therapeutic strategy but also for the identification of novel and efficient therapeutic agents. Clinical research in the field of cancer biomarkers is essential in understanding the aggressive nature and the heterogeneity of the disease, as well as the resistance to conventional therapeutic agents (2). Therefore, predictive markers of responsiveness to adjuvant therapy would allow for selection of patients for appropriate treatment (3).

So far, few studies have identified cancer biomarkers with clinical significance. A biomarker must be easily quantified in order to minimize the invasiveness of the therapeutic interventions (2).

Changes in the pattern of DNA methylation is a feature of initial and progressive stages of many human tumors. Hypomethylation or hypermethylation are both leading to chromosomal instability, silencing of gene transcription and, subsequently, to malignant transformation. Research in the epigenetic field has demonstrated the involvement of an intensive reprogramming of epigenetic machinery, such as DNA methylation, histone modifications and microRNA expression. The reversed features of epigenetic abnormalities have opened new opportunities for epigenetic therapy.

DNA hypermethylation is a known epigenetic modification during oncogenesis, which leads to gene silencing. Some tumor suppressor genes presenting aberrant CpG island hypermethylation have been found in PDAC. The first tumor suppressor gene reported to be specifically methylated in PDAC was *p16* (4). Recent studies showed that several hypermethylated genes are associated with PDAC (*hMLH1, E-cadherin (CDH1), ppENK, CDKN1C, SPARC, TFPI-2, GATA4,5, BNIP3, TSLC1, HHIP, MUC2,* reprimo *(RPRM), CXCR4* and *SOCS1*) (5). Sato *et al*. noted that the gene *CDKN1C/p57KIP2* presented a decreased expression in intraductal papillary mucinous neoplasm (6). Partial methylation of the pathway of tissue factor inhibitor 2 (*TFPI-2*) – a Kunitz-type serine proteinase inhibitor – has been identified as a putative tumor-suppressor gene. Aberrant methylation of *TFPI-2* was reported in PDAC xenografts and in primary PDACs. Re-expression of the *TFPI-2* gene led to proliferation, migration and invasion of cancer cells (7). At the same time, a number of genes, such as claudin 4 (*CLDN4*), lipocalin 2 (*LCN2*), 14-3-3sigma/stratifin (*SFN*), trefoil factor 2 (*TFF2*), *S100A4*, mesothelin (*MSLN*), *PSCA*, *S100P* and maspin (*SERPINB5*) are over-expressed in PDAC cells due to promoter hypomethylation (8).

Folate is essential for the synthesis of nucleotides. Low levels of folate correlate with alterations in cell replication, DNA excision and repair and DNA methylation pattern. Those alterations lead to an increased risk for developing cancer (9). One of the most important pieces in the metabolism of folate is methylenetetrahydrofolate reductase (MTHFR), an enzyme that catalyses the reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyl THF, the methyl donor for methionine synthesis from homocysteine (10). Glycine *N*-methyltransferase (GNMT) is another key regulatory protein in folate metabolism, methionine availability and transmethylation reactions. Low levels of GNMT may lead to aberrations in homocysteine metabolism. The abnormal levels of homocysteine have consequences in the development of numerous pathologies like cognitive disorders (11), cardiovascular disease (12), neural tube and other birth defects, as well as pregnancy complications (13). DNA methylation depends on methyl-group donor S-adenosylmethionine (SAM), which is reduced to S-adenosylhomocysteine (SAH). The SAM:SAH ratio is regulated by an interplay between the two key enzymes of folate metabolism. MTHFR is inhibited by high levels of SAM, whereas low levels of SAM lead to an increase in 5-methyl-THF production, which inhibits GNMT and methyl-groups for transmethylation reactions (*i.e.*, DNA methylation) (14-17).

Certain inherited variations in the *GNMT* gene have been associated with an increased risk of liver and prostate cancers (18, 19). On the other hand, *GNMT* gene mutations impair glycine N-methyltransferase functions, such as processing potential cancer-causing substances in the liver and helping to regulate other genes, including those responsible for controlling cell growth.

In a previous study of gene expression profiles in human cancers, we discovered a decreased *GNMT* gene expression in pancreatic adenocarcinomas (20). Herein, we aimed to comparatively evaluate the *GNMT* promoter methylation profiles in PDAC samples, in adjacent normal tissues and in PDAC cell lines.

Materials and Methods

Specimens. The present study included 30 patients with PDA who underwent pancreatic resection at the Center of Digestive Disease and Liver Transplantation of Fundeni Clinical Institute, Bucharest, Romania.

All patients provided written, informed consent for the use of their samples and the study was approved by the Ethics Committee of Fundeni Clinical Institute Bucharest, Romania, in accordance with ethical standards included in the current revision of the Helsinki Declaration. The clinical characteristics of patients are presented included in Table I.

Tissue samples were collected at the time of surgery from all PDA and CP patients.

Cell lines. PANC-1, MiaPaCa-2, and BxPC-3 cells were obtained from the American Type Culture Collection (ATCC, cat no. CRL-1420, CRL-1687, address) and cultured according to the ATCC recommendations. The PCL-35and PK-9 cell line were a gift from Dr. Akira Horii (Tohoku University School of Medicine, Sendai, Japan). Cell lines were cultured in appropriate medium (DMEM F-12), (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum at 37˚C with 5% CO_2.

DNA isolation. DNA isolation from tissues and cell lines was performed with the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer's recommendations. Isolated DNAs were subsequently stored at –20˚C. The concentration and purity of each DNA sample was evaluated with a NanoDrop spectrophotometer (NanoDropTechnologies, Montchanin, DE, USA).

Bisulfite treatment. Bisulfite treatment for unmethylated C residues conversion was performed with the EpiTect Bisulfite kit (Qiagen, Valencia, CA, USA). Seven hundred ng of each isolated DNA in a volume of 20 μl was bisulfite-treated along with positive (CpGenome Universal Methylated DNA) and negative (CpGenome Universal Unmethylated DNA) controls (Millipore, Billerica, MA, USA).

Table I. *Clinical characteristics of patients*.

Clinical characteristics	
Age, years (mean±SD)	61±8.16
Male, n (%)	16 (53.33)
Women, n (%)	14 (46.67)
Tumor grade, n	
G1	19
G1-G2	3
G2	5
G2-G3	2
G3	1
Tumor dimension (cm), n	
<1.4	5
1.5-2.9	9
3-4.5	14
>4.6	2
Disease stage, n	
IA	4
IIA	4
IB	7
IIB	12
IV	3

Methylation-specific PCR (MS-PCR). MS-PCR was performed in a final volume of 25 μl using Platinum Taq DNA Polymerase (1U), enzyme buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.3 μM of each specific primers and 50 ng target DNA.

Primers were selected using MethPrimer (*GNMT*-MF 5'-TTAAAAGGATTTAGTTTAGGATTGC-3', *GNMT*-MR 5'-AACATAAACACTACTAACAACCGAC-3'; *GNMT*-UF 5'-AAAAGGATTTAGTTTAGGATTGTGA-3', *GNMT*-UR 5'-AAACATAAACACTACTAACAACCAAC-3'.

The PCR conditions were as follows: 95˚C for 5 min, 35cycles of 95˚C/30 s, 56˚C/30 s, 72˚C/30 s and a final elongation of 72˚C for 7 min. The methylated/unmethylated status of the target gene was estimated in 2% agarose gel electrophoresis.

Direct quantitative methylation-specific PCR (Q-MSP) of genomic DNA. This method was used for evaluating the degree of sample methylation. According to Applied Biosystem Guide (Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System, Foster City, CA, USA), standard curves were designed using serial dilutions (10 pg, 100 pg, 1 ng and 10 ng) of positive (DNA fully methylated) and negative controls (DNA fully unmethylated). The quantity of unmethylated and methylated DNA for each sample was extrapolated using the standard curves. Direct Q-MSP was performed in 50 μl final volume comprising 50 ng/μltarget DNA, 25 μl FastStart Universal SYBR Green Master (ROX; Roche Molecular Biochemicals, Mannheim, Germany) and 0.3 μM primers. PCR parameters we used were as follows: 95˚C/10 min (1 cycle), 40 cycles at 95˚C/15 s and 60 s/specific annealing temperature. qPCR experiments were performed in duplicate and mean values were used for calculations. To control the quality of DNA samples, the *ACTB* gene was used as reference (21). Methylation percentage was calculated according to method described by Fackler *et al*. (% M=100([ng methylated gene A/(ng methylated gene A+ unmethylated gene A)]) (21).

RNA extraction and integrity evaluation. Total RNA was isolated from both pathological and control tissue samples using 1 ml Tri Reagent (Sigma, St. Louis, MO, USA) for each 50 to 100 mg of tissue, according to the manufacturer's instructions, followed by further purification using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). The quantity and quality of the total RNA were assessed by spectrophotometry (Nano Drop 1000; Thermo Scientific, Arlington, TX, USA) and by lab-on-a-chip Agilent 2100 technology (Agilent Technology, Santa Clara, CA, USA). Samples with an RNA integrity number (RIN) greater than 7 were used for gene expression.

cDNA synthesis and Real-time qPCR. A 2-step relative quantification was performed with the 7300 Real-Time PCR (ABI, Foster City, CA, USA). Firstly, cDNA was obtained from 2 μg of total RNA by means of the High Capacity cDNA Reverse Transcription Kit (ABI) in a total volume of 20 μl. cDNA samples were diluted to 2 ng/μl and qPCR amplification was performed in duplicate for each sample in a total volume of 25 μl with FastStart Universal SYBR Green Master (Roche Molecular Biochemicals) under the following conditions: 95˚C for 10 min, 95˚C for 15 s and 1 min at 60˚C for 40 cycles. We determined fold changes in the pathologic tissues compared with paired control tissue, while the level of each mRNA was normalized to the reference gene *GAPDH*. The primers' sequences were selected at exon-exon junction (to avoid genomic DNA amplification) and according to NCBI (National Centre of Biotechnology Information) with the Primer3-Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). We employed, for the housekeeping gene *GAPDH* (NM_001256799.1) - Forward: 5'-ACC ACA GTC CAT GCC ATC AC -3', Reverse: 5' -TCC ACC ACC CTG TTTTTG CTG TA- 3' and for the target gene *GNMT* (NM_018960.4)- Forward: 5'-CCTGTGGCACTGGGGT GGACT-3', Reverse: 5'-TTGTCGAAGGCGGGCTCGTG-3'. We analyzed the data with the SDS 1.4 software (ABI, Foster City, CA, USA) using the comparative Ct method [2^(-delta delta Ct)].

Demethylation treatment of cell lines. To analyze the restoration of GNMT expression, five PDAC cell lines (PANC-1, BxPC-3, PCL-35, PK-9 and MIAPaCa-2) were seeded at a density of 1×10^5, cultured for 24 hours and treated with 5-aza-2-deoxycytidine (5-aza-dC) (Sigma) at concentrations of 5 μM and 10 μM for 3 days. The medium containing the drug was replaced every 24 h. The cells were harvested after 24 and 72 h of treatment.

Detection of apoptosis. In order to establish the effect of 5 azaC on cell apotosis, we used the Annexin V-FITC Apoptosis Detection Kit I (BD Bioscience Pharmingen, San Diego, CA, USA, USA) according to manufacturer's protocol. Briefly, 1×10^5 cells (MiaPaCa-2, PANC-1, BxPC-3, PK-9, PCI-35) were seeded in a 24-well plate and treated with 5 μM or 10 μM 5azaC for 24, 48 and 72 h. Total cells were re-suspended in 100 μl of binding buffer (10 mM of HEPES/NaOH, pH: 7.4, 140 mM of NaCl and 2.5 mM of $CaCl_2$) and stained with 5 μl Annexin V-FITC and 5μl propidium iodide for 10 min in the dark. At least 10,000 events from each sample were acquired using a Beckman Coulter flow cytometer. The percentage of treatment-affected cells was determined by subtracting the percentage of apoptotic/necrotic cells in the untreated population from the percentage of apoptotic cells in the treated population.

Statistical analysis. To examine differences in gene expression, the Student's *t*-test (when values were normally distributed) or the non-parametric Mann–Whitney test (when the distribution normality test

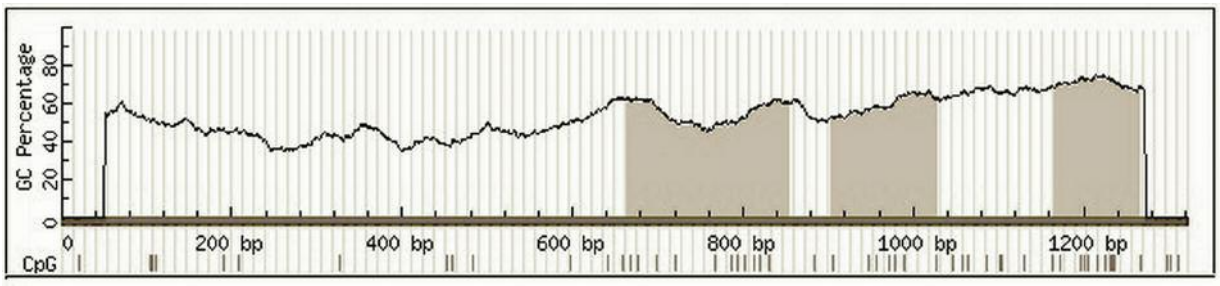

Figure 1. *The density of CpG islands of GNMT promoter prediction using the MethPrimer software.*

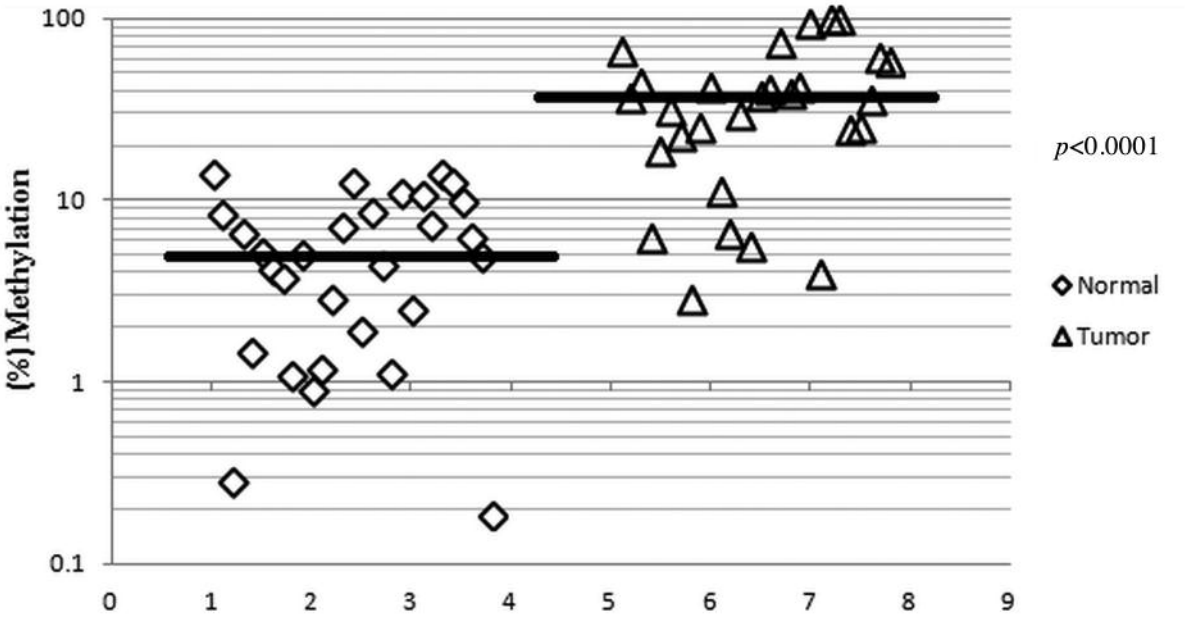

Figure 2. *Scattered plots in logarithmic scale of GNMTpromoter methylation percentage in PDAC versus adjacent pancreatic tissues. *p<0.05; the p-value was calculated using the two-sided non-parametric Mann-Whitney test. 0 values cannot be plotted on a logarithmic scale.*

failed) were used. For subgroup analyses and the comparison of methylation status, the Kruskal–Wallis test followed by the Dunn's Multiple Comparison post hoc test was used. A p-value <0.05 was considered statistically significant. All calculations were performed using the GraphPad Prism, version 5.02, for Windows (GraphPad Software, San Diego, CA, USA).

Results

GNMT is frequently hypermethylated in PDACs. The bioinformatics analysis using the MethPrimer software (for CpG islands prediction and primer design; (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) of the *GNMT* gene promoter sequence (AF101475.1) showed the presence of three CpG islands (Island 1, 191 bp (664-854); Island 2, 124 bp (904-1027); Island 3, 101 bp (1165-1265) (Figure 1).

GNMT methylation was observed in 23/30 (76.367%) of PDAC cases. All cases were above the established threshold of 11% according to the methylation level found in normal tissue samples. Significantly higher methylation frequencies (p<0.001) were found in PDAC samples (2.82-100%; median, 36.05%) than in adjacent pancreatic tissues (0.28-14.02%; median, 4.39%) (Figure 2).

Data regarding *GNMT* gene hypermethylation in control *vs.* PDAC tissues were analyzed using the Fisher's exact test. Specificity (percentage of healthy patients identified as not having the condition) and sensitivity (prediction of the capacity to identify all patients from the tumor group presenting the disease) were calculated. We found specificity=0.8, sensitivity=0.9; odds ratio (OR)=21.357; relative risk (RR)=3.714; p<0.0001, confidence interval (CI)=95%.

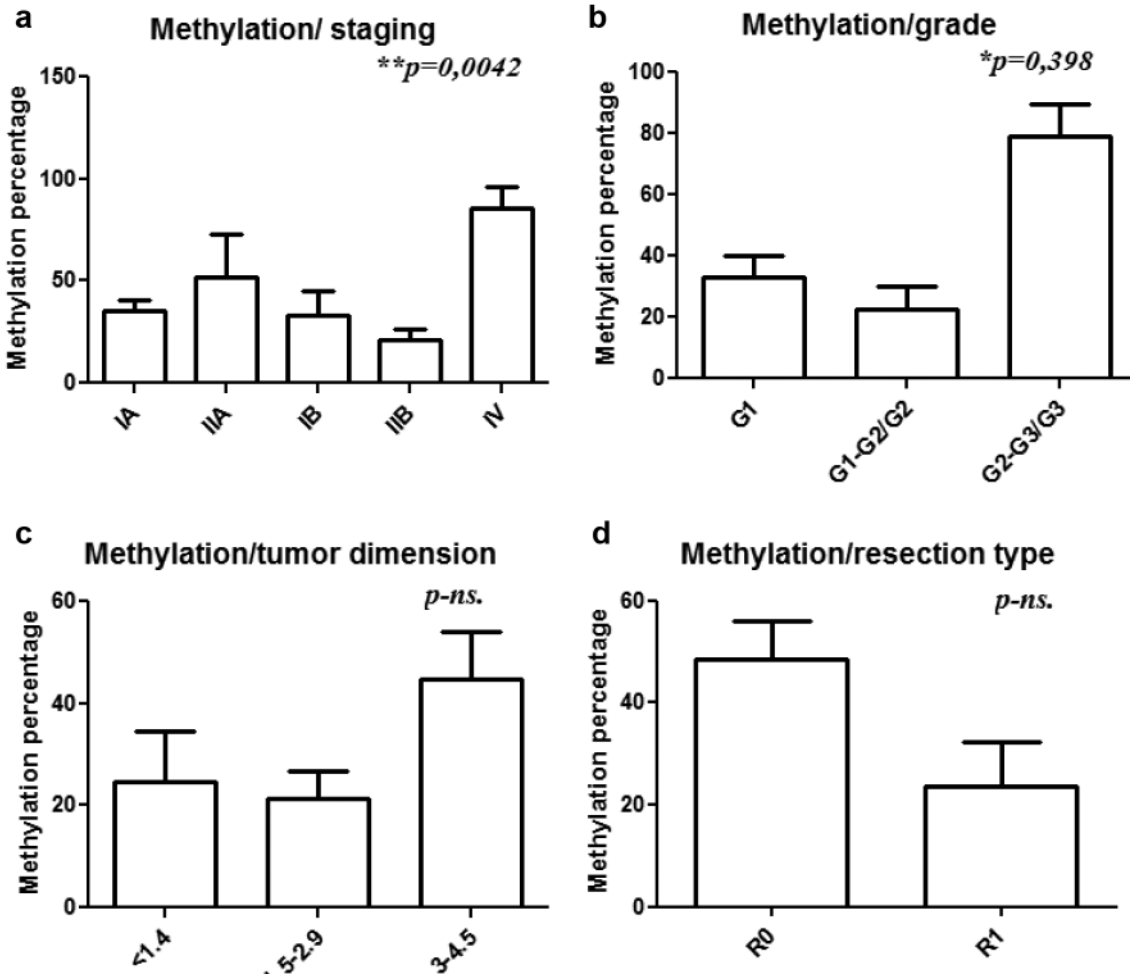

Figure 3. *Box plot graphs representing the correlation between methylation percentage, stage (a), tumor grade (b), dimension (c) and resection type (d). Significance was assessed by the non-parametric Kruskal-Wallis test.*

Correlation between GNMT methylation and clinicopathologic features in PDAC. The analysis of data regarding the GNMT promoter methylation level and the characteristics of the tumors (grade, stage, dimension) showed a significant correlation between tumor grade and methylation percentage (p=0.0398) and between tumor stage and methylation percentage (p=0.0042) (Figure 3).

GNMT mRNA expression is decreased in PDAC. The GNMT gene expression was decreased in PDAC tissues compared to controls. Only in 4 cases (13.33%) GNMT expression levels were similar with paired pancreatic tissues, while the other 26 cases (86.67%) presented a decreased expression. The median fold change expression of the gene was –2.42 (range between, –0.02, –4.55). The Fisher's exact test, applied to analyze *GNMT* gene expression differences between control/tumor, revealed specificity=0.9, sensitivity= 1; OR=359.22; RR=7.5; p<0.0001, CI=95%. The analysis of correlations between the *GNMT* expression level and tumor characteristics (grade, stage, dimension, type of resection) showed a significant association between tumor grade and *GNMT* expression level (p=0.0265), as well as between the type of resection and *GNMT* expression level (p=0.0187) (Figure 4).

PDAC cell apoptosis and necrosis is increased after inhibition of DNA methyltransferase activity. Cell proliferation is also suppressed in a concentration-dependent manner, while apoptosis is increased. In order to demonstrate that a decreased *GNMT* gene expression in PDAC is determined by CpG island methylation, lower 5-aza-dC concentrations were used for the treatment of PDAC cell lines. We observed a slow increase of apoptotic and necrotic cells for 10 μM 5-aza-dC after 72 h (day 3-D3)

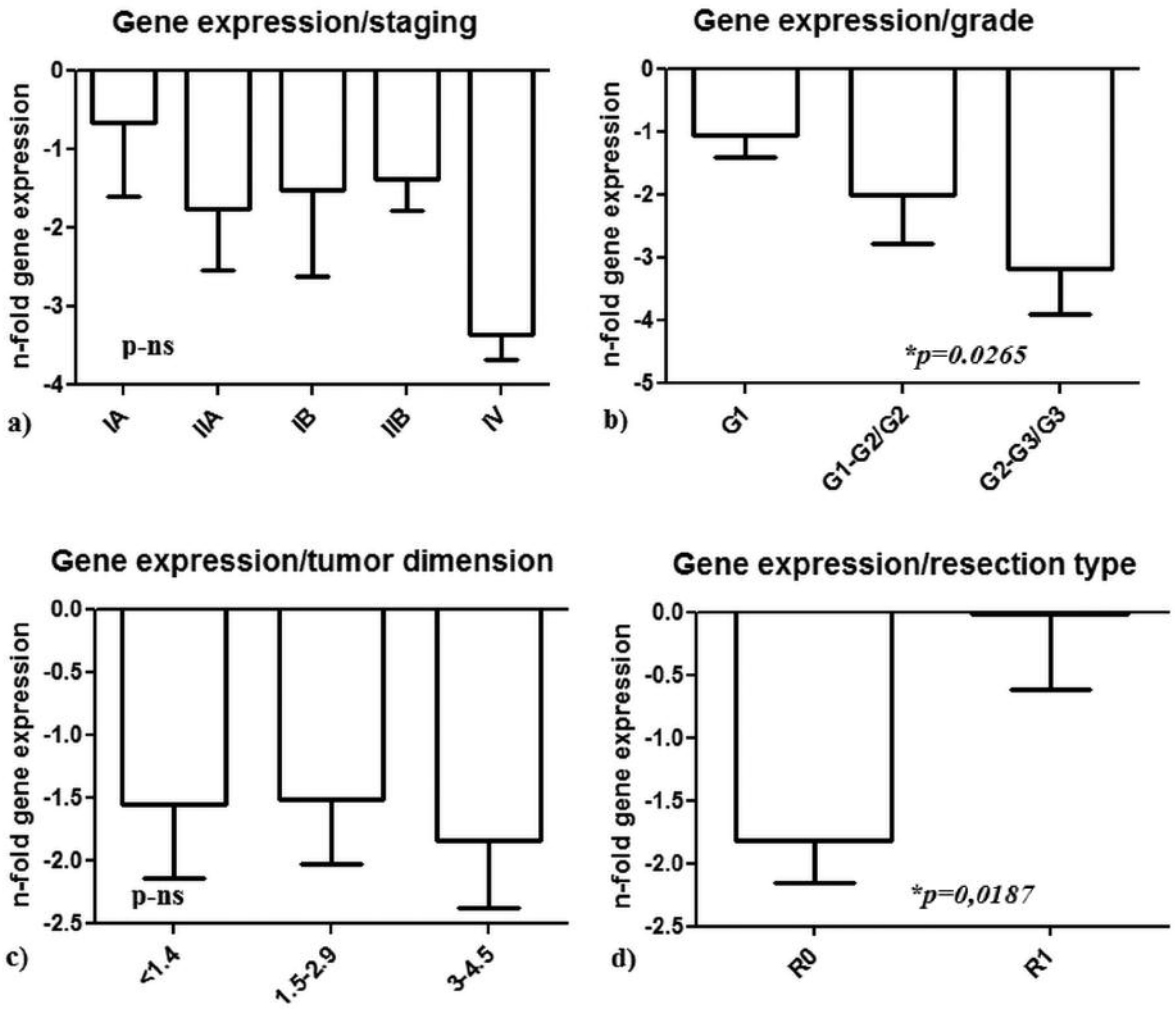

Figure 4. *Box plot graphs representing the correlation between gene expression, stage (a), tumor grade (b), dimension (c) and resection type (d). Significance was assessed by the non-parametric Kruskal-Wallis test.*

(Table II). The highest percentage of apoptotic cells was found in PK-9 cell line treated with 10 μM 5-aza-dC/D3 (16.6%) and in MiaPaCa-2 (11.3%) for the same conditions. The highest percentage of necrotic cells was found in MiaPaCa-2 cell line treated with 10 μM 5-aza-dC/D3 (4.7%) (Table II).

Inhibition of DNA methyltransferase activity leads to preferential accumulation of PDAC cells in the S phase of the cell cycle. Flow cytometry analysis of PDAC cell lines exposed to 5-aza-dC illustrated the dose-time effect dependency of the drug. Exposure of cells to 5-aza-dC affected progression of cells through the S phase. The higher the dose, the higher the accumulation of S-phase cells, especially for PCI-35 and MiaPaca-2 cell lines. On the other hand, cells accumulated preferentially in the G_2/M phase with increasing drug concentration and time exposure. The higher the drug concentration and the longer the exposure, the fewer were the cells in G_0/G_1.

Methylation status and mRNA expression of GNMT in pancreatic cell lines. *GNMT* mRNA expression evaluated in all human pancreatic carcinoma cell lines used in this study, as well as in normal pancreatic tissues, showed a significant down-regulation in all PDAC cell lines *versus* normal pancreatic tissues (BxPC-3 (–0.25-fold), MiaPaca-2 (–1.4-fold), PCI-35 (–1.2-fold), PK-9 (–0.3-fold) and PANC-1 (–2.3-fold)). On the other hand, analysis of the methylation status of *GNMT* promoter revealed gene hypermethylation in all 5 PDAC cell lines (Figure 5). Following 5-aza-dC treatment, *GNMT* promoter demethylation was observed for all PDAC cell lines in a manner dependent of the time of

Table II. *Apoptosis - Annexin V-FITC results*.

	24 h(D1)			48 h(D2)			72 h(D3)		
	Control	5 μM	10 μM	Control	5 μM	10 μM	Control	5 μM	10 μM
PANC-1									
Dead cells	0.8	0.5	1	0.6	0.3	0.9	0.7	0.9	1.5
Late apoptosis	0.1	0.1	0.1	0.2	0.2	0.4	0.3	0.1	0.3
Viable cells	98.9	98.9	98.4	98.8	98	98	98.9	98.9	98
Early apoptosis	0.2	0.5	0.5	0.4	1.5	0.7	0.2	0.1	0.1
PCI-35									
Dead cells	0.9	0.9	0.6	0.4	1.4	1.3	0.7	1.9	1.5
Late apoptosis	0.2	0.3	0.2	0.6	0.6	0.7	0.8	0.2	0.9
Viable cells	98.2	97.7	97.5	97.6	96.8	94	98.2	97.6	94.8
Early apoptosis	0.6	1	1.6	1.5	1.3	4	0.3	0.3	2.8
BxPC-3									
Dead cells	0.1	0.3	1	0.4	0.4	1.2	0.4	1.3	1.8
Late apoptosis	0.1	0.4	0.5	0.3	0.4	0.5	0.1	0.4	0.5
Viable cells	99.3	98.5	96.1	99	97.9	96.9	99.4	98	96.4
Early apoptosis	0.5	0.9	2.4	0.3	1.2	1.3	0.1	0.4	1.4
MiaPaCa-2									
Dead cells	0.6	0.9	1.1	0.8	2.8	3.9	0.8	2.8	4.7
Late apoptosis	0.3	1.5	1.2	0.4	3.2	4.4	0.3	3.5	8.8
Viable cells	98.1	95.1	94.6	98.2	92.3	90.7	98.1	91.9	84
Early apoptosis	1	2.5	3.1	0.6	1.7	1	0.8	1.8	2.5
PK-9									
Dead cells	1	1.3	0.8	1.4	1	2.2	0.3	0.6	1.1
Late apoptosis	0.8	1.2	0.7	1.6	2.7	2.4	2.6	2.3	6.3
Viable cells	97.2	94.1	92.2	96	92.8	91.7	95.2	93.3	85.3
Early apoptosis	1	3.5	6.4	1	3.5	3.7	1.9	3.8	7.28

exposure and the drug concentration. Using Q-MSP, a significant de-methylation was detected in PCL-35 cells, whereas a slight de-methylation was detected in the BxPC-3 cell line, thus indicating that the methylation of *GNMT* promoter was partially reversed. For all cell lines, except PK-9, the highest de-methylation percentage were noticed after 72 h of exposure with 10 μM 5-aza-dC. In the PK9 cell line, an important de-methylation was found at 48 h of exposure with 10μM 5-aza-dC. Regarding the efficiency of 5-aza-dC, the optimum drug concentration was 10 μM (Table III), except for PK-9 cells, which required treatment with a dose of 5 μM of demethylating agent.

GNMT mRNA expression in PDAC cell lines after treatment by 5-aza-dC. Using Q-RT-PCR, 5-aza-dC-treated PDAC cell lines were evaluated for their mRNA expression. The drug restored the expression of *GNMT* in all cell lines according to drug concentration and exposure time. The level of *GNMT* mRNA expression increased 64-and 9-fold following treatment with 5 μM 5-aza-dC for 3 days in the PANC-1 and BxPC-3 cell lines, respectively (Figure 5).

Interestingly, the expression of *GNMT* mRNA in the PCI-35 cells significantly increased after treatment with 10 μM 5-aza-dC for 3 days (1,197-fold) (data not shown). In contrast, the *GNMT* gene expression was found moderately increased after treatment with 10 μM 5-aza-dC for 3 days in MiaPaca-2 and PK-9 cells (2- and 1.5-fold, respectively) (Figure 5). In MiaPaca-2 cells, the *GNMT* mRNA expression was higher for the 5 μM concentration after 24 h of exposure (7.8-fold), while in PK-9 cells it increased after 48 h of exposure (32-fold). Treatment with 5 μM 5-aza-dC/ 48h also had a moderate effect on the increase of *GNMT* mRNA expression in the PANC and BxPC-3 cell lines (5- and 4-fold, respectively) (Figure 5). In all treated cell lines, the methyltransferase inhibitor restored the expression of *GNMT* indicating the role of DNA methylation in down-regulation of *GNMT* expression in PDAC.

Discussion

Many genes are commonly silenced epigenetically in PDAC. Among them are *BNIP3* (coding BH3 domain protein with pro-apoptotic function) (22) *CSMD2, SLC32A1, TMEM204*

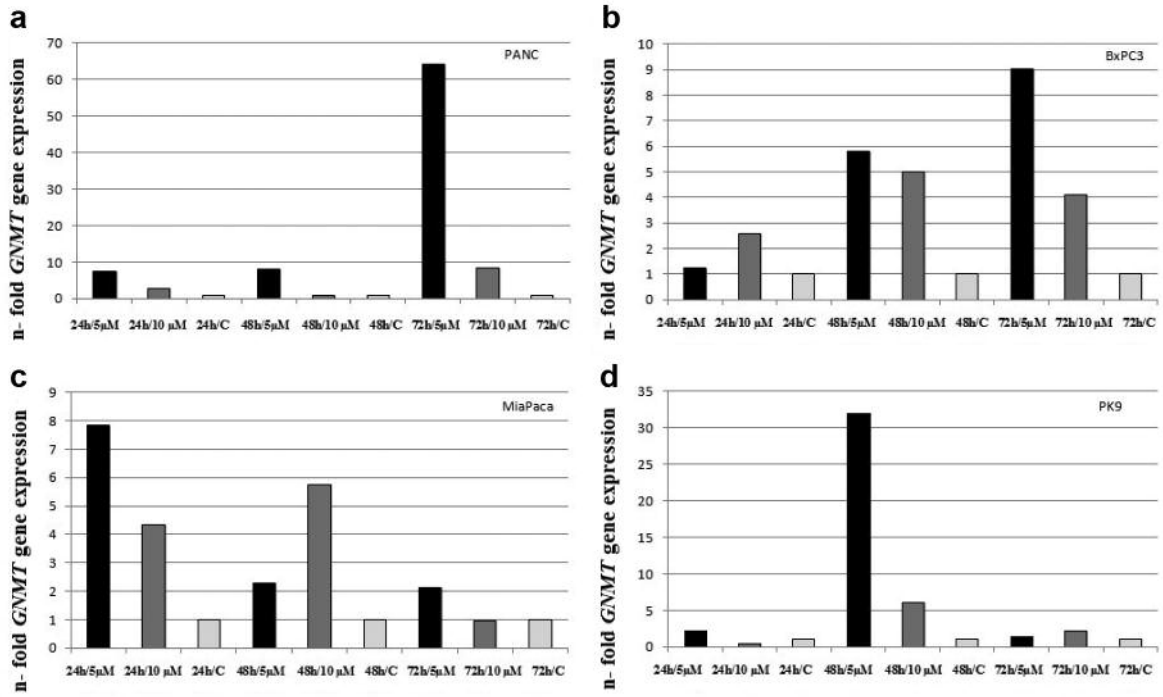

Figure 5. *Graphic representation of n-fold GNMT gene expression in four cell lines PANC-1 (a), BxPC-3 (b), MiaPaca-2 (c) and PK-9 (d) treated with 5-aza-dC (5 and 10 μM).*

and *TRH* (23, 24). Recently, several novel and important epigenetically silenced factors have been also identified (hsa-miR-9-1, *ZNF415, CNTNAP2* and *ELOVL4*) (5, 24-27). Ueki and colleagues found that the promoter of pre-proenkephalin (*ppENK*) gene is hypermethylated in most PDACs (28). SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin/BM40) is a factor involved in many processes like cell migration, proliferation, matrix cell adhesion. The gene coding for this protein was found aberrantly methylated in PDAC (29, 30). Recently, *GNMT* was identified as a tumor suppressor gene, which is subjected to mutations in liver and prostate cancer (31, 32). SAM levels depend on the glycine-N-methyltransferase, a one-carbon group methyltransferase, which catalyzes the conversion of SAM to SAH, making *GNMT* a key regulator of the SAM:SAH ratio (33). These data point the importance of *GNMT* gene in the regulation of methyl group metabolism, maintaining the normal genome methylation pattern.

In the present study, hypermethylation of *GNMT* was found in all 5 human PDAC cell lines tested and in 23/30 (76.367%) human PDAC samples but not in any of the corresponding non-neoplastic adjacent pancreatic tissues.

The methylation of *GNMT* promoter was found to be tumor-specific and its effect consisted in the down-regulation of gene expression. The results are consistent with the involvement of epigenetic alterations in pancreatic

Table III. *Methylation percentage according to 5-aza-dC treatment.*

	%M		
	Minimum	Maximum	Median
PANC-1			
Control	90.68	95.00	94.70
5 μM	33.51	93.67	58.65
10 μM	9.75	83.36	53.80
PCI-35			
Control	47.82	70.65	70.53
5 μM	9.51	50.98	14.80
10 μM	1.69	34.54	3.04
BxPC-3			
Control	99.17	99.65	99.42
5 μM	55.96	93.46	65.93
10 μM	34.05	67.63	62.37
MiaPaca-2			
Control	62.07	80.22	73.54
5 μM	38.26	60.81	42.49
10 μM	32.45	44.80	34.21
PK-9			
Control	96.37	99.85	99.36
5 μM	49.05	97.86	56.04
10 μM	17.75	84.13	67.89

carcinogenesis. *GNMT* is a good candidate for epigenetic modifications in PDAC. Furthermore, we showed that treatment with 5-aza-dC increased *GNMT* mRNA expression in PDAC cell lines. Exposure of cells to 5-aza-dC affected the cycling cells in the S phase and correlated with the de-methylation process. Collectively, these data indicate that *GNMT* is aberrantly methylated in PDAC and may represent a major mechanism for gene silencing. To our knowledge, this is the first study to report the methylation status of *GNMT* in PDAC. The data reported by Huidubro *et al*. on *GNMT* methylation in hepatocellular carcinoma support our results. *GNMT* gene hypermethylation could contribute to gene repression and its restoration in cell lines displaying hypermethylation leads to a reduced tumor growth *in vitro* (34). We showed that methylation of the *GNMT* gene is directly correlated with disease stage and with tumor grade. This indicates that these epigenetic effects may be important regulators of PDAC progression and may serve as prognostic biomarkers for PDAC.

Conflicts of Interest

The Authors declare no conflict of interest.

Acknowledgements

The Authors would like to thank for valuable comments hosting this research to Teh Bin Tean Senior Principal Investigator, Cancer Science Institute of Singapore, NUS Professor, Duke-NUS Graduate Medical School, Principal Investigator, National Cancer Centre (Singapore).

The study was supported by POSDRU 89/1.5/S/60746 and PN-II-PT-PCCA90/2012 research grants.

References

1 Takayama R, Nakagawa H, Sawaki, Mizuno N, Kawai H, Tajika M, Yatabe Y, Matsuo K, Uehara R, Ono K, Nakamura Y and Yamao K: Serum tumor antigen REG4 as a diagnostic biomarker in pancreatic ductal adenocarcinoma. J Gastroenterol *45*: 52-59, 2010.

2 Dima SO, Tanase C, Albulescu R, Botezatu A and Popescu I: Novel Biomarkers in Pancreatic Cancer.In:Pancreatic Cancer - Clinical Management. Sanjay K. Srivastava.InTech, pp. 31-54, 2012.

3 Duffy MJ: Role of tumor markers in patients with solid cancers: A critical review. Eur J Intern Med *18*: 175-184, 2007.

4 Schutte M, Hruban RH, Geradts J, Maynard R, Hilgers W, Rabindran SK, Moskaluk CA, Hahn SA, Schwarte-Waldhoff I, Schmiegel W, Baylin SB, Kern SE and Herman JG: Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res *57*: 3126-3130, 1997.

5 Omura N, Li CP, Li A Hong SM, Walter K, Jimeno A, Hidalgo M and Goggins M: Genome-wide profiling of methylated promoters in pancreatic adenocarcinoma. Cancer Biol Ther *7*: 1146-1156, 2008.

6 Sato N, Matsubayashi H, Abe T, Fukushima N and Goggings M: Epigenetic down-regulation of CDKN1C/p57KIP2 in pancreatic ductal neoplasms identified by gene expression profiling. Clin Cancer Res *11*: 4681-4688, 2005.

7 Sato N, Parker AR, Fukushima N, Miyagi Y, Iacobuzio-Donahue CA, Eshleman JR and Goggins M: Epigenetic inactivation of TFPI-2 as a common mechanism associated with growth and invasion of pancreatic ductal adenocarcinoma. Oncogene *24*: 850-858, 2005.

8 Sato N, Maitra A, Fukushima N, Van Heek NT, Matsubayashi H, Iacobuzio-Donahue CA, Rosty C and Goggins M: Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. Cancer Res *63*: 4158-4166, 2003.

9 Fowler BM, Giuliano AR, Piyathilake C, Nour M and Hatch K: Hypomethylation in cervical tissue: is there a correlation with folate status? Cancer EpidemiolBiomarkers Prev *7*: 901-906, 1998.

10 Bailey LB and Gregory JF III: Polymorphisms of methylenetetrahydrofolate reductase and other enzymes: metabolic significance, risks and impact on folate requirement. J Nutr *129*: 919-922, 1999.

11 Miller JW: Folate, cognition, and depression in the era of folic acid fortification. J Food Sci *69*: SNQ61-SNQ67, 2004.

12 Caudill MA: The role of folate in reducing chronic and developmental disease risk: an overview. J Food Sci *69*: SNQ55-SNQ60, 2004.

13 Vollset SE, Refsum H, Irgens LM,Emblem BM, Tverdal A, Gjessing HK,Monsen AL and Ueland PM: Plasma total homocysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine study. Am J Clin Nutr *71*: 962-968, 2000.

14 Yeo EJ, Briggs WT and Wagner C. Inhibition of glycine N-methyltransferase by 5-methyltetrahydrofolate pentaglutamate. J Biol Chem *274*: 37559-37564, 1999.

15 Kerr SJ and Heady JE: Modulation of tRNA methyltransferase activity by competing enzyme systems. Adv Enzyme Regul *12*: 103-117, 1974.

16 Takata Y, Huang Y, Komoto J, Yamada T, Konishi K, Ogawa HGomi T, Fujioka M and Takusagawa F: Catalytic mechanism of glycine N-methyltransferase. Biochemistry *42*: 8394-8402, 2003.

17 Beagle B, Yang TL, Hung J,Cogger EA, Moriarty DJ and Caudill MA: The glycine N-methyltransferase (GNMT) 1289 C->T variant influences plasma total homocysteine concentrations in young women after restricting folate intake. J Nutr *135*: 2780-2785, 2005.

18 Huang YC, Chen M, Shyr YM,Su CH, Chen CK, Li AF, Ho DM and Chen YM: Glycine N-methyltransferase is a favorable prognostic marker for human cholangiocarcinoma. J Gastroenterol Hepatol *23*: 1384-1389, 2008.

19 Huang YC, Lee CM, Chen M,Chung MY, Chang YH, Huang WJ, Ho DM, Pan CC, Wu TT, Yang S, Lin MW, Hsieh JT and Chen YM: Haplotypes, loss of heterozygosity, and expression levels of glycine N-methyltransferase in prostate cancer. Clin Cancer Res *13*: 1412-1420, 2007.

20 Badea L, Herlea V, Dima SO,Dumitrascu T and Popescu I: Combined gene expression analysis of whole-tissue and microdissected pancreatic ductal adenocarcinoma identifies genes specifically overexpressed in tumor epithelia. Hepatogastroenterology *55*: 2016-2027, 2008.

21 Fackler MJ, McVeigh M, Mehrotra J,Blum MA, Lange J, Lapides A,Garrett E, Argani P and Sukumar S: Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. Cancer Res *64*: 4442-4452, 2004.

22 Okami J, Simeone DM and Logsdon CD: Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. Cancer Res *64*: 5338-5346, 2004.

23 Shimizu H, Horii A, Sunamura M, Motoi F, Egawa S, Unno M and Fukushige S: Identification of epigenetically silenced genes in human pancreatic cancer by a novel method "microarray coupled with methyl-CpG targeted transcriptional activation" (MeTA-array). Biochem Biophys Res Commun *411*: 162-167, 2011.

24 Grady WM, Parkin RK, Mitchell PS, Lee JH, Kim YH, Tsuchiya KDWashington MK, Paraskeva C, Willson JK, Kaz AM, Kroh EM, Allen A, Fritz BR, Markowitz SD and Tewari M: Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. Oncogene 27: 3880-3888, 2008.

25 Lehmann U, Hasemeier B, Christgen M, Muller M, Romermann D, Langer F andKreipe H: Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol *214*: 17-24, 2008.

26 Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K and Croce CM: MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci USA *104*: 15805-15810, 2007.

27 Morris KV, Chan SW, Jacobsen SE and Looney DJ: Small interfering RNA-induced transcriptional gene silencing in human cells. Science *305*: 1289-1292, 2004.

28 Ueki T, Toyota M, Skinner H, Walter KM, Yeo CJ, Issa JP, Hruban RH and Goggins M: Identification and characterization of differentially methylated CpG islands in pancreatic carcinoma. Cancer Res *61*: 8540-8546, 2001.

29 Sato N, Fukushima N, Maehara N, Matsubayashi H, Koopmann J, Su GH, Hruban RH and Goggins M: SPARC/osteonectin is a frequent target for aberrant methylation in pancreatic adenocarcinoma and a mediator of tumor-stromal interactions. Oncogene *22*: 5021-5030, 2003.

30 Guo Y, Yuan F, Deng H, Wang HF, Jin XL and Xiao JC: Paranuclear dot-like immunostaining for CD99: a unique staining pattern for diagnosing solid-pseudopapillary neoplasm of the pancreas. Am J Surg Pathol *35*: 799-806, 2011.

31 Tseng TL, Shih YP, Huang YC, Wang CK, Chen PH, Chang JG,Yeh KT, Chen YM and Buetow KH: Genotypic and phenotypic characterization of a putative tumor susceptibility gene, GNMT, in liver cancer. Cancer Res *63*: 647-654, 2003.

32 Song YH, Shiota M, Kuroiwa K, Naito S and Oda Y: The important role of glycine N-methyltransferase in the carcinogenesis and progression of prostate cancer. Mod Pathol *24*: 1272-1280, 2011.

33 Wang YC, Tang FY, Chen SY, Chen YM and Chiang EP: Glycine-N methyltransferase expression in HepG2 cells is involved in methyl group homeostasis by regulating transmethylation kinetics and DNA methylation. J Nutr *141*: 777-782, 2011.

34 Huidobro C, Torano EG, Fernandez AF, Urdinguio RG, Rodríguez RM, Ferrero CMartínez-Camblor P, Boix L, Bruix J, García-Rodríguez JL, Varela-Rey M, Mato JM, Martínez-Chantar ML and Fraga MF: A DNA methylation signature associated with the epigenetic repression of glycine N-methyltransferase in human hepatocellular carcinoma. J Mol Med (Berl) *91*: 939-950, 2013.

Received October 16, 2014
Revised November 2, 2014
Accepted November 5, 2014