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Alterations in S-adenosylhomocysteine metabolism decrease O^6-methylguanine DNA methyltransferase gene expression without affecting promoter methylation

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ABSTRACT

The DNA repair enzyme O^6-methylguanine DNA methyltransferase (MGMT) protects cells against the cytotoxic effects of alkylating agents. Therefore, modulation of MGMT expression in tumors is a possible strategy for improving the efficiency of cancer therapy. MGMT expression and activity is lost frequently in association with DNA hypermethylation of the *MGMT* promoter region. Since DNA and mRNA methylation are controlled by intracellular S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) levels, we hypothesized a role for AdoMet/AdoHcy ratio in the regulation of *MGMT* promoter methylation and mRNA expression.

Our initial studies showed that AdoMet/AdoHcy ratios vary over a wide range (7.0–50) in different glioblastoma and hepatoma cell lines. The studied cell lines exhibit distinct *MGMT* promoter methylation patterns: *MGMT* promoter was completely unmethylated in LN-18 and Tu 132 cells, hypermethylated in LN-229, U87-MG, and Tu 113 cells, and partially methylated in HepG2 cells. Furthermore, *MGMT* promoter methylation patterns and global DNA methylation are not related to intracellular AdoMet/AdoHcy ratio under control conditions. To lower AdoMet/AdoHcy ratio to values <1 we used AdoHcy hydrolase inhibitor adenosine-2′,3′-dialdehyde (30 μM) and found that neither short-term (24 h) nor long-term changes (7 weeks) in AdoMet/AdoHcy ratio altered global or *MGMT* promoter methylation. However, experimentally elevated AdoHcy levels significantly decreased MGMT mRNA levels by >50% in all MGMT-expressing cell lines, which is most likely the result of impaired mRNA methylation. Thus, the present study suggests elevation of AdoHcy levels by AdoHcy hydrolase inhibition as a novel pharmacological approach to modulate MGMT expression and to increase the responsiveness to alkylating agents.

1. Introduction

Alkylating agents are among the most widely used chemotherapeutic drugs in the treatment of human cancer. Several alkylation sites in DNA have been described as being targets of these compounds. The most frequent site is the O^6 position of guanine. However, the toxicity of alkylating agents is reduced in the presence of O^6-methylguanine DNA

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Abbreviations: MGMT, O^6-methylguanine DNA methyltransferase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; DNMT, DNA methyltransferase; NCS, newborn calf serum; FCS, fetal calf serum; Adox, adenosine-2′,3′-dialdehyde; HPLC, high performance liquid chromatography; EC, energy charge; RT-PCR, reverse transcription-polymerase chain reaction.

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methyltransferase (MGMT). MGMT exerts its protective effect by transferring small alkyl adducts, such as methyl groups, from the O^6 atom of guanine in DNA to an internal cysteine residue, with concomitant inactivation of the methyltransferase activity [1]. Thus, MGMT activity is a major mechanism of resistance to alkylating drugs [2] and the modulation of MGMT expression in tumors and normal tissue is currently being investigated as a possible strategy for improving cancer therapy [3].

MGMT is expressed in all normal human cells, however, MGMT levels vary widely between different species and tissues [4,5]. In humans, liver contains the highest level of MGMT activity [4]. In certain human cancer, MGMT function is lost frequently and this event may be an important step in human tumorigenesis [6,7]. For example, 30% of gliomas [8,9] and 39% of hepatocellular carcinomas [10] lack MGMT. Cells deficient in the MGMT protein are not able to reverse the formation of adducts at the O^6 position. Therefore, MGMT-deficient cells are sensitive to chemotherapeutic alkylating agents [11], whereas cells with high MGMT activity are resistant to alkylating agents.

Loss of expression is rarely due to deletion, mutation, or rearrangement of the *MGMT* gene, but epigenetic changes, specifically promoter-region methylation, have been associated with the silencing of the gene. Methylation of CpG sequences in both promoter and neighboring regions of the *MGMT* gene results in an inactive condensed chromatin state of the gene [12,13]. Therefore, CpG island hypermethylation of *MGMT* causes reduced MGMT expression and activity and is a good predictive marker for chemotherapy with alkylating agents [7,14,15]. For instance, Hegi et al. [14] have recently shown that epigenetic silencing of *MGMT* is associated with longer survival in patients with glioblastoma who receive alkylating agents.

Abnormal patterns of DNA methylation are hallmarks of most malignancies: global DNA hypomethylation is accompanied by region-specific hypermethylation [16,17]. However, to date it is not clear why the promoter of *MGMT* and other tumor suppressor genes becomes hypermethylated during tumorigenesis [18]. The process of DNA methylation is mediated by at least three DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) that catalyze the transfer of a methyl group from *S*-adenosylmethionine (AdoMet, methyl donor) to cytosine bases in DNA [19]. After transfer of the methyl group, AdoMet is converted to *S*-adenosylhomocysteine (AdoHcy), which is a potent product inhibitor of most, if not all, AdoMet-dependent methyltransferases [20]. In recent years, the ratio of AdoMet and AdoHcy, also termed methylation potential, has been used frequently as an indicator of cellular methylation capacity [21]. Increased AdoMet concentration stimulates DNMT reactions and inhibits intracellular demethylase activity, which results in DNA hypermethylation [22,23], whereas, elevation of intracellular AdoHcy concentration leads to inhibition of DNMTs, DNA hypomethylation, and decreases overall mRNA methylation [24–26]. Since AdoHcy levels are controlled by AdoHcy hydrolase, AdoHcy hydrolase activity might also contribute to the regulation of DNA and mRNA methylation [27].

Based on these previous results, we wanted to analyze whether the AdoMet/AdoHcy ratio and AdoHcy hydrolase activity under control conditions are related to *MGMT* methylation patterns and whether an experimentally altered AdoMet/AdoHcy ratio might influence *MGMT* promoter hypermethylation and global DNA methylation in glioblastoma and hepatoma cell lines. Since a decreased AdoMet/AdoHcy ratio leads to impaired mRNA methylation [25], we hypothesized that inhibition of AdoHcy hydrolase decreases MGMT mRNA expression. Pharmacological inhibition of MGMT expression (caused by *MGMT* promoter hypermethylation or reduced mRNA methylation) could thus be used as a novel therapeutic approach to increase the responsiveness to temozolomide and other alkylating agents.

2. Materials and methods

2.1. Materials

The human malignant glioma cell lines LN-18, LN-229, U87-MG, Tu 113, Tu 132 were kindly provided by PD Dr. Naumann (Hertie Institute for Clinical Brain Research, University of Tübingen, Germany). The human hepatoblastoma cell line HepG2 [28,29] was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig, Germany). RPMI 1640, newborn calf serum (NCS), 0.05% trypsin:0.02% EDTA, Dulbecco's phosphate buffered saline (PBS), and penicillin (100 U/ml)/streptomycin (100 μg/ml) were provided by Invitrogen (Karlsruhe, Germany). DMEM was obtained from Cambrex (Vervier, Belgium) and fetal calf serum (FCS) from Biochrom (Berlin, Germany). AdoMet, AdoHcy, adenosine, N^6-methyladenosine, nucleoside phosphorylase, adenosine-2′,3′-dialdehyde (Adox), and ammonium phosphate were obtained from Sigma (Taufkirchen, Germany). Adenosine deaminase and xanthine oxidase were purchased from Roche (Mannheim, Germany), AMP, ADP, and ATP from Boehringer (Mannheim, Germany), heptanesulfonic acid sodium salt from Fluka (Buchs SG, Switzerland). HPLC grade methanol and acetonitrile were from Merck (Darmstadt, Germany).

2.2. Cell culture

Glioma cell lines were cultured in DMEM supplemented with 10% "heat-inactivated" (58 °C, 1 h) FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HepG2 cells were cultured in RPMI 1640 containing glutamine supplemented with 10% "heat-inactivated" NCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines were grown in a humidified atmosphere containing 5% CO_2 at 37 °C and subcultured at a split ratio of 1:4 once or twice a week using trypsin/EDTA. In order to change the AdoMet/AdoHcy ratio, cells were rinsed with PBS and incubated with AdoHcy hydrolase inhibitor Adox (30 μM) diluted in medium supplemented with calf serum.

2.3. Metabolite measurement

AdoMet, AdoHcy, adenosine, AMP, ADP, and ATP concentration in different cell lines was measured in perchloric acid cell extracts. Culture medium was removed, cells were rinsed with PBS, immediately lysed in pre-cooled 0.6 M perchloric acid in

Table 1 – The primer sequences used in this study

Gene	Primer sequences (F, forward; R, reverse)	Product size (bp)
MGMT [13]	F: CGA CTT GGT ACT TGG AAA AAT G R: CGC ATC CGA TGC AGT GTT AC	702
18S rRNA [35]	F: CGG CTA CCA CAT CCA AGG AA R: GCT GGA ATT ACC GCG GCT	187
MGMT unmethylated [7]	F: TTT GTG TTT TGA TGT TTG TAG GTT TTT GT R: AAC TCC ACA CTC TTC CAA AAA CAA AAC A	94
MGMT methylated [7]	F: TTT CGA CGT TCG TAG GTT TTC GC R: GCA CTC TTC CGA AAA CGA AAC G	81

culture dishes and harvested by scraping. The protein precipitate was removed by centrifugation (10 min, 20,000 × *g*) and the supernatant was adjusted to pH 5.5–6.0 by adding 2 M K_2CO_3/1 M KH_2PO_4. All samples and standards were supplemented with 1 μM N^6-methyladenosine as internal standard. The precipitated potassium perchlorate was discarded after centrifugation at 20,000 × *g* and the supernatant was applied onto solid-phase extraction column (BondElut, ICT, Germany). Elution of the compounds was performed with 0.1 M HCl and the eluate was analyzed by HPLC as described previously [30,31]. Absorbance at 254 nm was monitored, and peaks were identified with authentic standards. To determine adenine nucleotide concentration perchloric acid cell extracts were adjusted to pH 8.0 by adding 2 M K_2CO_3/1 M KH_2PO_4 and samples were analyzed by HPLC without prior extraction [31].

Since results were expressed as nanomoles per 1×10^7 cells, cell number was determined using a hemocytometer. To determine the cell number, culture medium was removed and cells were rinsed with PBS, harvested by incubation with trypsin/EDTA and diluted in 15 ml medium supplemented with calf serum. An aliquot of 100 μl was then withdrawn for determination of cell number. The remaining 14.9 ml were centrifuged (200 × *g*, 10 min) and processed as described under ''*Preparation of total protein and protein quantification*''.

2.4. Preparation of total protein and protein quantification

The pelleted cells were washed with PBS and homogenized in potassium phosphate buffer (50 mM, pH 7.0) by sonication. The homogenate was centrifuged at 20,000 × *g* for 15 min and supernatant was processed for photometrical determination of enzyme activity as described below. The protein concentration was determined according to the method of Bradford using bovine serum albumin as the standard.

2.5. Assay of AdoHcy hydrolase activity

AdoHcy hydrolase activity was assayed in the hydrolytic direction. Briefly, enzymatic activity in crude cell extracts was measured photometrically in a total volume of 500 μl in 50 mM potassium phosphate (pH 7.0) at room temperature. The reaction mixture contained total cell lysate, adenosine deaminase (1 U), nucleoside phosphorylase (0.09 U), and xanthine oxidase (0.08 U). Therefore, adenosine generated by AdoHcy hydrolysis is processed to uric acid, which can be measured photometrically at 292 nm. The reaction was started by the addition of 50 μM AdoHcy [32].

2.6. DNA isolation and bisulfite treatment

Genomic DNA was isolated using DNeasy Tissue Kit (Qiagen, Hilden, Germany). DNA methylation pattern in the CpG island of the *MGMT* gene was determined by chemical modification of unmethylated, but not methylated, cytosines to uracil using EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In the subsequent PCR primers specific for either methylated or the modified unmethylated DNA were used (Table 1). Lymphocyte DNA treated in vitro with *Sss*I methyltransferase (New England Biolabs, Frankfurt am Main, Germany) was used as a positive control for methylated alleles of *MGMT*, and DNA from normal human lymphocytes was used as negative control for methylated alleles of *MGMT*.

2.7. RNA isolation and reverse transcription

Total RNA was isolated with Tri-Reagent/Trizol as described previously [33]. Total cellular RNA was reverse transcribed by incubating a 20 μl reaction mixture composed of 300 ng of RNA as template, 1× NH_4-reaction buffer for PANScript Polymerase (PAN, Aidenbach, Germany), 1 mM dNTPs (Eppendorf, Hamburg, Germany), 0.25 μg random hexamers (Promega, Mannheim, Germany), 15 U RNase inhibitor (Eppendorf, Hamburg, Germany), and 12.5 U AMV reverse transcriptase (PeqLab, Erlangen, Germany) at 20 °C for 10 min and 42 °C for 1 h. The reaction was then stopped by incubation at 95 °C for 5 min.

2.8. PCR analysis for MGMT promoter methylation and MGMT expression

For LightCycler reaction a master mix of the following reaction components was prepared to the indicated end-concentration: 12.6 μl H_2O, 2.4 μl $MgCl_2$ (4 mM), 0.5 μl forward primer (0.5 μM), 0.5 μl reverse primer (0.5 μM), and 2.0 μl Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). 2 μl cDNA (for expression analysis) or 2 μl sodium bisulfite-treated DNA (20–60 ng, for methylation-specific PCR) were added to 18 μl LightCycler master mix as

PCR template. The primer sequences used in this study are given in Table 1. The following LightCycler experimental run protocol was used: denaturation program (95 °C for 10 min), amplification and quantification program repeated 45 times (95 °C for 10 s, "touch down PCR" 68–58 °C (MGMT expression 62–52 °C, *MGMT* promoter methylation with primers specific for the methylated allele 65–55 °C) with a step size of 0.5 °C/cycle for 10 s, 72 °C for 16 s (MGMT expression 20 s) with a single fluorescence measurement), melting curve program (65–95 °C with a heating rate of 0.1 °C/s and a continuous fluorescence measurement) and a finally cooling step to 37 °C. PCR products of promoter methylation analysis were analyzed by agarose gel electrophoresis. Relative MGMT mRNA expression was calculated according to a mathematical model established by Pfaffl [34]. As an example for a non-regulated reference gene we chose 18S rRNA [35], because several studies have shown that 18S rRNA is more reliable than glyceraldehyde-3-phosphate dehydrogenase and beta-actin as an internal control gene for quantitative comparison of mRNA expression [36,37]. "Fit point method" was performed in the LightCycler software 3.5.3 (Roche Diagnostics, Mannheim, Germany).

2.9. Determination of global DNA methylation

Methylation pattern in global DNA was determined using cytosine extension assay as described previously [25,38]. This assay provides sensitive and quantitative measurement across a wide spectrum of DNA methylation densities: 10% decrease in methylation density results in a significant increase in [^{3}H]dCTP incorporation [38]. Briefly, genomic DNA (2 μg) was digested with 20 U *HpaII* (Fermentas, St. Leon-Rot, Germany) for 15 h. A second DNA aliquot was incubated without enzyme and served as background control. For cytosine extension assay 0.5 μg DNA were incubated with 1× NH_4-reaction buffer for PANScript Polymerase, 1 mM $MgCl_2$, 0.5 U PANScript DNA Polymerase (PAN, Aidenbach, Germany), and 0.1 μl [^{3}H]dCTP (40–60 Ci/mmol, NEN Life Science Products, Boston, USA) in a total volume of 25 μl for 1 h at 56 °C. Duplicate 10 μl aliquots from each reaction were filtered through Whatman DE-81 ion exchange filters and filters were washed three times with 3 ml 0.5 M sodium phosphate buffer (pH 7.0). Radioactivity incorporated in DNA and adsorbed on the filters was determined by liquid scintillation counting.

2.10. Calculation and statistics

The energy charge (EC) of the adenylate system was calculated according to Atkinson and Walton [39], defined as

$$\frac{[ATP] + 0.5[ADP]}{[AMP] + [ADP] + [ATP]}$$

Data were analyzed by Student's *t*-test if variability is the same in each group, because *t*-test assumes that standard deviations of two datasets are equal. If variances of two datasets are significantly different, we used Welch's alternate *t*-test, because this test does not assume equal variances (InStat). A difference between groups was considered to be significant when *p*-value was <0.05.

Table 2 – Endogenous adenine nucleoside and nucleotide levels in the different glioma cell lines

Cell line	Experimental condition	*n*	AdoMet (nmol/10^7 cells)	AdoHcy (nmol/10^7 cells)	AdoMet/AdoHcy	Adenosine (nmol/10^7 cells)	AMP (nmol/10^7 cells)	ADP (nmol/10^7 cells)	ATP (nmol/10^7 cells)	EC
LN-229	Control	6	1.08 ± 0.13	0.030 ± 0.006	40.87 ± 5.23	0.026 ± 0.005	2.52 ± 0.49	16.90 ± 3.14	77.20 ± 10.30	0.809 ± 0.006
	Adox	6	2.89 ± 0.28*	5.82 ± 0.58*	0.50 ± 0.01*	0.008 ± 0.005*	6.35 ± 1.74	18.58 ± 1.34	51.48 ± 4.38	0.799 ± 0.013*
LN-18	Control	10	1.98 ± 0.14	0.089 ± 0.013	24.96 ± 2.86	0.064 ± 0.015	1.34 ± 0.32	9.80 ± 0.76	82.24 ± 7.27	0.932 ± 0.004
	Adox	6	3.01 ± 0.06*	3.65 ± 0.12*	0.83 ± 0.03*	0.016 ± 0.001*	1.02 ± 0.02	5.88 ± 0.14*	65.98 ± 1.08	0.946 ± 0.002*
U87-MG	Control	7	2.69 ± 0.27	0.179 ± 0.025	15.45 ± 1.51	0.035 ± 0.005	2.53 ± 0.34	15.21 ± 0.93	124.32 ± 4.52	0.929 ± 0.003
	Adox	5	3.90 ± 0.19*	8.43 ± 0.94*	0.48 ± 0.05*	0.090 ± 0.035	3.73 ± 0.68	16.22 ± 1.10	116.38 ± 6.07	0.912 ± 0.010*
Tu 113	Control	5	2.39 ± 0.17	0.227 ± 0.048	13.01 ± 3.24	0.169 ± 0.031	4.07 ± 0.85	14.48 ± 2.96	109.29 ± 10.63	0.915 ± 0.007
	Adox	3	2.64 ± 0.06	5.58 ± 0.18*	0.47 ± 0.01*	0.063 ± 0.003*	2.42 ± 0.06	8.14 ± 0.28	96.21 ± 2.53	0.939 ± 0.003*
Tu 132	Control	9	3.74 ± 0.13	0.602 ± 0.073	6.97 ± 0.82	0.288 ± 0.081	9.07 ± 0.99	22.89 ± 1.08	237.09 ± 17.42	0.901 ± 0.017
	Adox	5	3.60 ± 0.27	5.56 ± 0.32*	0.71 ± 0.01*	0.093 ± 0.040	1.44 ± 0.99*	17.12 ± 2.04*	184.18 ± 9.55	0.951 ± 0.004

*$p < 0.05$ versus control.

3. Results

3.1. Comparison of AdoMet/AdoHcy metabolism in different glioblastoma cell lines

Based on the hypothesis that AdoMet/AdoHcy ratio might be involved in the regulation of *MGMT* promoter methylation and mRNA expression, we first compared the AdoMet and AdoHcy tissue content to calculate the AdoMet/AdoHcy ratio under physiological conditions in different glioma cell lines. HPLC analysis showed that the intracellular tissue content of AdoMet, which is a methyl group donor for most biological methylation reactions, differed moderately among the five glioma cell lines (Table 2). Lowest AdoMet levels were observed in LN-229 cells (1.08 nmol/10^7 cells), highest in Tu 132 cells (3.74 nmol/10^7 cells). In contrast, the mean content of AdoHcy, an accurate inverse indicator of DNA methylation [24,26], strongly differed among the different cell lines. For instance, Tu 132 cells exhibited 20-fold higher AdoHcy levels than LN-229 cells. The great differences in intracellular AdoHcy concentrations lead to various AdoMet/AdoHcy ratios in different glioma cell lines. LN-229 cells exhibit the highest AdoMet/AdoHcy ratio (40.9), Tu 132 cells the lowest AdoMet/AdoHcy ratio (7.0). LN-18, U87-MG, and Tu 113 exhibit an AdoMet/AdoHcy ratio of 25.0, 15.5, and 13.0, respectively. As shown previously, in HepG2 cell line, the intracellular concentration of AdoMet and AdoHcy is 1.76 ± 0.1 nmol/10^7 cells and 0.034 ± 0.002 nmol/10^7 cells, respectively, resulting in an AdoMet/AdoHcy ratio of 53.4 ± 3.3 [25].

3.2. Changes in methylation potential by inhibition of AdoHcy hydrolase

Alterations in AdoMet/AdoHcy ratio were achieved by AdoHcy hydrolase inhibitor Adox, which appears to be selective for AdoHcy hydrolase at low concentrations [40], although it lowers ribonucleotide reductase activity at high concentrations (630 μM) [41]. As shown in Table 2, administration of Adox 30 μM resulted in enhanced accumulation of AdoHcy between 3.7 nmol/10^7 cells (LN-18 cell line) and 8.4 nmol/10^7 cells (U87-MG cells). Therefore, the AdoMet/AdoHcy ratio decreases to values <1 in all investigated cell lines (Table 2). Since AdoHcy hydrolysis is blocked, adenosine levels decrease in LN-229, LN-18, Tu 113, and Tu 132 cells. In HepG2 cells, inhibition of AdoHcy hydrolase also leads to an enhanced accumulation of intracellular AdoHcy to 2.25 ± 0.16 nmol/10^7 cells, resulting in an AdoMet/AdoHcy ratio of 1.4 [25].

3.3. Enzymatic activity of AdoHcy hydrolase in glioblastoma cell lines

Since intracellular AdoHcy levels are controlled by AdoHcy hydrolase, AdoHcy hydrolase activity might also play an important role in the regulation of genomic and promoter DNA methylation. As shown in Fig. 1, AdoHcy hydrolase activity differs among the different glioblastoma cells. Interestingly, all glioma cell lines showed relative low enzymatic activity compared to hepatoma cell line HepG2. However, there is no apparent relationship between AdoHcy levels and AdoHcy hydrolase activity in crude cell extracts.

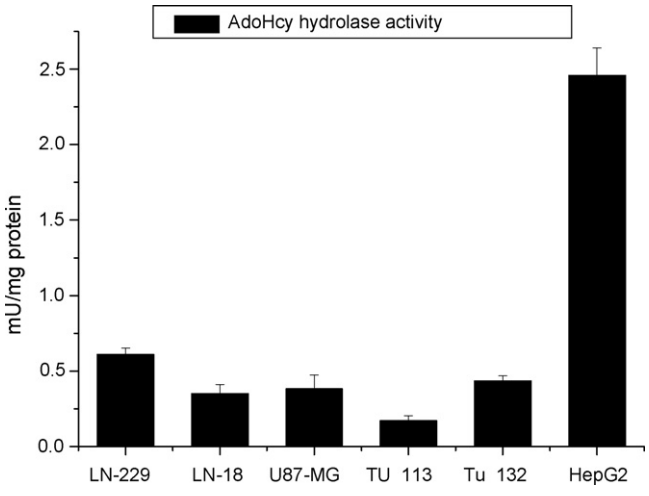

Fig. 1 – AdoHcy hydrolase activity in different glioma cell lines and HepG2 cells. Enzyme activity was determined photometrically in total cell lysates. Data represent averages ± S.E.M. for *n* = 6–12 experiments.

3.4. Influence of AdoMet/AdoHcy metabolism on global DNA methylation

All glioma and hepatoma cell lines showed a similar extent of [^{3}H]dCTP incorporation into DNA after treatment with the methyl-sensitive restriction enzyme *HpaII* under control conditions. Thus, the number of unmethylated cytosine residues does not depend on AdoMet/AdoHcy ratio under physiological conditions. Furthermore, inhibition of AdoHcy hydrolase for 24 h did not significantly change the extent of genomic DNA methylation in LN-229, LN-18, U87-MG, Tu 113,

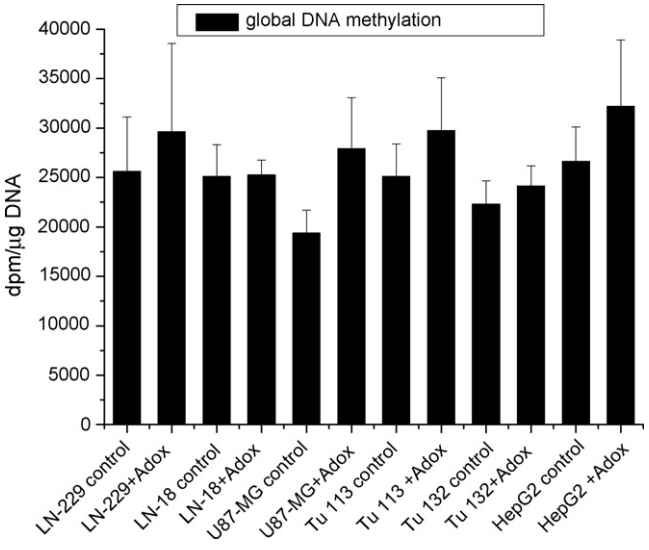

Fig. 2 – Comparison of the global DNA methylation in untreated and Adox-treated cells. All glioma cell lines were incubated with Adox 30 μM for 24 h, whereas, HepG2 cells were cultured in the presence of Adox for 7 weeks. The degree of global DNA methylation was determined using cytosine extension assay as described in Section 2.

and Tu 132 cells (Fig. 2). Based on these findings and on our previous studies showing that administration of Adox for 24 h did not change global DNA methylation in HepG2 cells [25], we speculated that long-term changes in AdoMet/AdoHcy ratio might lead to inhibition of DNA methylation. However, even inhibition of AdoHcy hydrolase by Adox for 7 weeks did not significantly change in vitro methyl incorporation into DNA compared to untreated control in HepG2 cells.

3.5. *MGMT promoter hypermethylation in different glioma and hepatoma cell lines*

DNA obtained from all cell lines was subjected to *MGMT* promoter methylation study using methylation-specific PCR. The region chosen for *MGMT* spans the area of greatest CpG density immediately 5′ to the transcription start site, in an area previously found to be hypermethylated in cell lines where MGMT expression is silenced [42,43]. To determine the correctness of our method, untreated DNA from human lymphocytes and lymphocyte DNA in vitro treated with *Sss*I methyltransferase were used as negative control and positive control for methylated alleles of *MGMT*. As expected, *MGMT* promoter in normal lymphocytes was completely unmethylated, whereas, *Sss*I treatment resulted in a completely methylated *MGMT* promoter (Fig. 3). Measuring *MGMT* promoter methylation in different glioma and hepatoma cell lines revealed a hypermethylated *MGMT* promoter in LN-229, U87-MG, and Tu 113 cell lines, whereas the promoter was completely unmethylated in LN-18 and Tu 132 cells. In HepG2 cells, unlike all glioma cell lines that were either completely methylated or completely unmethylated in this region of *MGMT* CpG island, amplification of the *MGMT* promoter was observed in the unmethylated and in the methylated reaction (Fig. 3), suggesting that the *MGMT* promoter in HepG2 cells is partially hypermethylated. Elevation in AdoHcy levels, which is a potent inhibitor of DNMTs, did not result in *MGMT* promoter hypomethylation (Fig. 3). Notably, even treatment of HepG2 cells with Adox for 7 weeks did not result in pharmacological reversal of *MGMT* promoter hypermethylation.

3.6. *Expression of MGMT in the different cell lines*

Since *MGMT* promoter methylation status is an indicator of the transcriptional activity of the gene in glioma cells, we hypothesized that MGMT mRNA is detectable in LN-18, Tu 132, and HepG2 cells. In fact, reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed presence of MGMT transcripts in these three cell lines, but absence of MGMT mRNA in LN-229, U87-MG, and Tu 113 cells. Although the above findings clearly show that Adox does not change *MGMT* promoter methylation, we explored the possibility that Adox reduces MGMT mRNA expression, since previous studies have clearly shown that inhibitors of AdoHcy hydrolase interfere with mRNA methylation and alter mRNA expression [44,25]. *Real-time* RT-PCR analysis showed that inhibition of AdoHcy hydrolase by Adox significantly decreases MGMT mRNA levels by approximately 50% in HepG2, LN-18, and Tu 132 cells (Fig. 4). A decrease in MGMT mRNA expression was also observed when these cell lines were incubated with adenosine plus homocysteine or adenosine plus pentostatin, which are also known to decrease AdoMet/AdoHcy ratio by elevating AdoHcy levels [44,25].

3.7. *Energy metabolism in different glioma cells*

To determine whether changes in energy metabolism are involved in the regulation of MGMT mRNA expression, we next measured AMP, ADP, and ATP concentrations in control and Adox-treated cells. Under control conditions, all investigated cell lines exhibit an EC between 0.8 and 1.0 (Table 2). Inhibition of AdoHcy hydrolase only slightly increased or decreased EC depending on the cell line examined.

4. Discussion

MGMT is a major determinant of susceptibility to methylating carcinogens and the tumor resistance to anticancer methylating and chloroethylating drugs [45]. Therefore, the modulation of MGMT expression in tumors and normal tissue is currently being investigated as a possible strategy for improving cancer therapy [3]. MGMT expression and activity is lost frequently in association with DNA hypermethylation of the *MGMT* promoter region in several human tumors including colon and lung cancers, gliomas, lymphomas, and hepatocellular carcinomas [5,6,46,47,8]. The mechanisms responsible for eliciting promoter hypermethylation are not well understood [18]. Since the ratio of AdoMet and AdoHcy has been used frequently as an indicator for cellular methylation status, we pursued if the AdoMet/AdoHcy ratio and AdoHcy hydrolase activity are related to *MGMT* methylation patterns in glioblastoma and hepatoma cell lines. Furthermore, we examined if alterations in AdoMet/AdoHcy ratio might influence *MGMT* promoter methylation, MGMT expression and thus, sensitivity to alkylating agents.

Determination of AdoMet/AdoHcy ratio in different glioma cell lines is consistent with earlier studies showing that the AdoMet/AdoHcy ratio greatly varies among different cell lines [31] and that values between 5 and 80 are consistent with physiological cell function [48–51]. Although previous studies showed that AdoHcy hydrolase controls intracellular AdoHcy levels [52,27] in our study intracellular AdoHcy levels did not correlate with AdoHcy hydrolase activity in the different glioblastoma cell lines. However, adenosine acts as a substrate and an inhibitor of AdoHcy hydrolase [32], suggesting that high AdoHcy levels, for instance in Tu 113 and Tu 132 cells, might be the result of high intracellular adenosine levels (Table 2). Furthermore, we are aware of the fact that the utilization of a cell culture model does not necessarily reflect physiological conditions.

Since the AdoMet/AdoHcy ratios vary over a wide range, these glioblastoma cell lines are a valuable tool to study the impact of differences in AdoMet/AdoHcy ratio on global DNA and *MGMT* promoter methylation under control conditions. In addition, we studied DNA methylation patterns in HepG2 cells, because this cell line exhibits an AdoMet/AdoHcy ratio >50 [31,53,25]. Despite the great differences in AdoMet/AdoHcy ratios in vitro methyl incorporation into DNA was similar in all glioblastoma and hepatoma cell lines showing that genomic

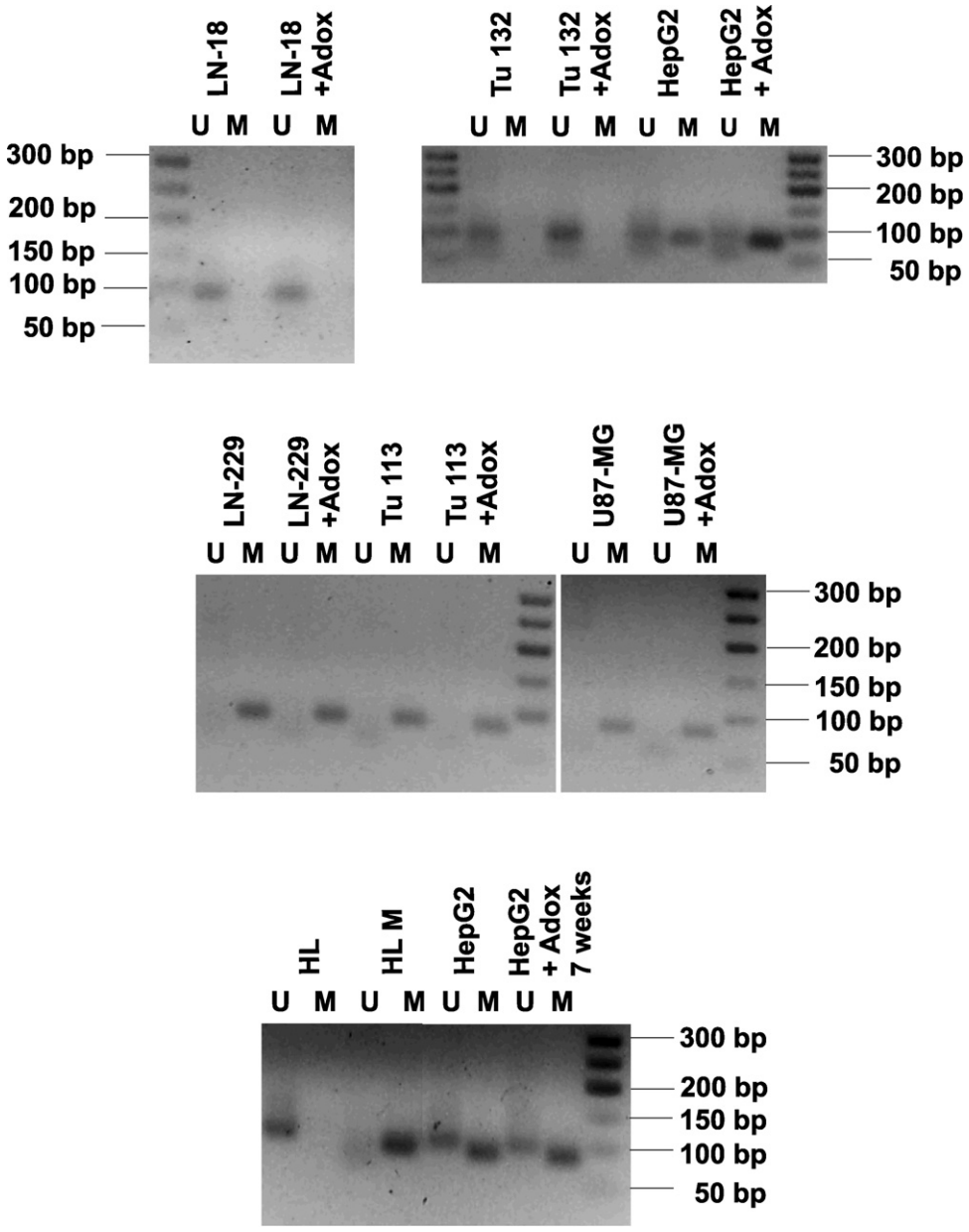

Fig. 3 – Influence of AdoHcy hydrolase inhibition on *MGMT* promoter methylation in the different cell lines. Bisulfite-treated DNA was used for PCR amplification using primer sets designed for methylated and unmethylated *MGMT* promoter. The PCR products were analyzed by agarose gel electrophoresis. The presence of a visible PCR product in lane U indicates the presence of unmethylated genes of *MGMT*; the presence of product in lane M indicates the presence of methylated genes. Lengths of the different PCR products are: 94 bp (unmethylated promoter) and 81 bp (methylated promoter). DNA from human lymphocytes (HL) served as a negative control for *MGMT* methylation; lymphocyte DNA methylated in vitro with *SssI* methyltransferase (HL M) served as a positive control for *MGMT* promoter methylation.

DNA is methylated to the same extent in all cell lines under physiological conditions. In addition, *MGMT* promoter methylation does not seem to depend on intracellular AdoMet/AdoHcy ratio. For example, LN-18 cells exhibit a completely unmethylated *MGMT* promoter although LN-18 cells show low AdoHcy levels, suggesting that AdoHcy does not inhibit DNMTs. In contrast, *MGMT* promoter is hypermethylated in LN-229, U87-MG, and Tu 113 cell lines although the AdoMet/AdoHcy ratio greatly varies among these cell lines. Intriguingly, *MGMT* promoter was partially methylated in HepG2 cells, although Zhang et al. showed that this cell line exhibits a completely unmethylated promoter [10]. In view of this finding and the fact that other studies showed that CpG islands of genes that are nonessential in culture (e.g. the erythropoietin gene in HepG2 cells) become methylated after multiple rounds of passaging [54,55], it seems likely that the *MGMT* promoter methylation observed in HepG2 cells in the present study is caused by de novo methylation. Taken together, these data

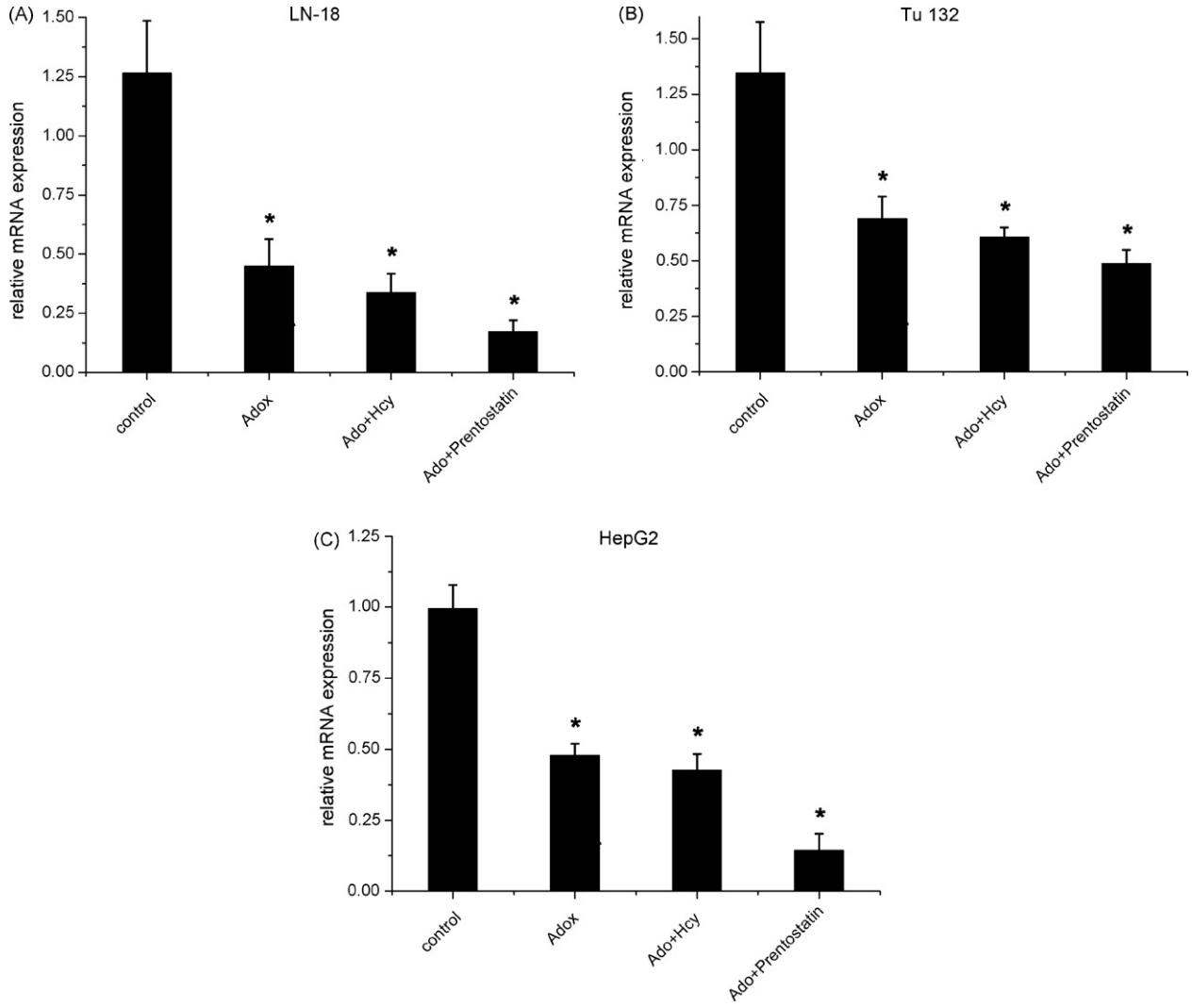

Fig. 4 – Relative amounts of MGMT mRNA levels in LN-18 (A), Tu 132 (B), and HepG2 cells (C). The different cell lines were cultured for 24 h in the presence of Adox, adenosine plus homocysteine (Ado + Hcy, each 1 mM), or adenosine (Ado, 1 mM) plus pentostatin (1 μM). The relative expression level was determined by *real-time*-RT-PCR and calculated according to Pfaffl [34]. The expression of 18S rRNA served as internal standard. Data are mean ± S.E.M. for 3–9 determinations. *$p < 0.05$ versus control.

clearly show that AdoMet/AdoHcy ratio is not causally related to *MGMT* promoter hypermethylation and global DNA methylation under cell culture conditions.

Based on these results, we hypothesized that an experimentally altered AdoMet/AdoHcy ratio might influence *MGMT* promoter methylation, global DNA methylation, and MGMT expression. A pharmacological approach (AdoHcy hydrolase inhibition), allowed us to decrease AdoMet/AdoHcy ratio in glioblastoma cell lines. We chose Adox as AdoHcy hydrolase inhibitor, because it appears to be selective for AdoHcy hydrolase at low concentrations [25,40]. Consistent with our previous studies in HepG2 cells [44], Adox increases AdoHcy levels and decreases AdoMet/AdoHcy ratio to values <1 without affecting EC in the glioblastoma cell lines. Conceptually, increases in intracellular AdoHcy levels result in impaired transmethylation activity because AdoHcy is a potent inhibitor of most, if not all, AdoMet-dependent methyltransferases [56,48,57]. However, our data show that global DNA methylation and *MGMT* promoter methylation are not influenced by short-term alterations (24 h) in AdoMet/AdoHcy ratio in the different glioma and hepatoma cell lines although the AdoMet/AdoHcy ratio is reduced about >90%.

Similar results showed the induction of acute hyperhomocysteinemia in healthy male volunteers: Acute elevation of total homocysteine is not sufficient to induce DNA hypomethylation in lymphocytes [58]. This observation is not surprising since DNA methylation occurs concurrently with replication during S-phase of cell cycle [59] and indicates that epigenetic mechanisms follow a slow time course. Therefore, we next investigated if long-term (7 weeks) alterations in AdoMet/AdoHcy ratio change the global DNA methylation or the *MGMT* promoter methylation. We chose HepG2 cells as an experimental model, because our previous studies have shown that these cells are viable in the presence of Adox

for several weeks (unpublished data). As shown in Figs. 2 and 3 also long-term inhibition of AdoHcy hydrolase did not change DNA methylation pattern, suggesting that AdoHcy levels are not sufficient to inhibit DNMTs to a significant extent in our cell culture model. This is a surprising finding considering that AdoHcy is a potent inhibitor of DNMTs and intracellular AdoHcy levels have recently been shown to be an accurate predictor of genomic DNA hypomethylation [24,26]. One explanation for the lack of correlation between intracellular AdoHcy levels and DNA methylation is that lowering AdoMet/AdoHcy reduces the rate of DNA methylation by DNMT, but not, over the time course of our experiments, the overall extent of DNA methylation. Another explanation is that a possible compensatory up-regulation of DNMTs might have offset the inhibitory effect of AdoHcy. In this regard, combined methyl deficiency has been shown to up-regulate DNMT activity in rat liver [60–63]. Moreover, recent studies showed that leukocyte DNA is hypermethylated in three patients with AdoHcy hydrolase deficiency, despite enormously increased AdoHcy levels [64–66] and DNA hypomethylation due to elevated AdoHcy levels seems to be tissue-specific [67]. Taken together, these studies and the results from our present study strongly suggest, that regulation of DNA methylation is a complex phenomenon and neither short-term nor long-term alterations in AdoHcy levels inevitably lead to changes in DNA methylation. A very recent study analyzing the relationship between the pattern of *MGMT* promoter methylation and MGMT protein expression in colorectal cancer showed that extensive promoter methylation (including the Mp and Eh region) is required for the resultant loss/reduction of MGMT expression [68]. In light of this study it would be very interesting to investigate the effect of AdoMet/AdoHcy ratio on the specific patterns of *MGMT* promoter methylation in further studies.

Gene expression analysis showed that MGMT mRNA was only detectable in LN-18, Tu 132, and HepG2, which showed an unmethylated (LN-18, Tu 132) or partially hypermethylated (HepG2) *MGMT* promoter. Consistent with previous studies, methylation of discrete regions of the *MGMT* promoter in LN-229, Tu 113, and U87-MG cells was associated with the silencing of the gene in these cell lines [69,42,43]. Based on previous studies showing that increased AdoHcy levels are associated with reduced mRNA methylation and differential mRNA expression in HepG2 cells [44,25], we hypothesized that an experimentally altered AdoMet/AdoHcy ratio might influence MGMT expression although *MGMT* promoter methylation is not affected. In fact, inhibition of AdoHcy hydrolase by Adox resulted in decreased MGMT mRNA levels. Interestingly, co-incubation with adenosine plus homocysteine or adenosine plus pentostatin also decreased MGMT expression in all MGMT-expressing cell lines. This result suggests that impaired MGMT mRNA levels are caused by elevated AdoHcy levels, because the simultaneous addition of adenosine and homocysteine or adenosine and pentostatin elevates AdoHcy levels to a similar extent like the AdoHcy hydrolase inhibitor Adox [44,25,70,71]. Since elevated AdoHcy levels result in uncapped mRNA, lowered MGMT mRNA levels are probably due to decreased stability of the respective mRNA [72,73], because uncapped mRNAs are degraded by 5′–3′-exoribonuclease before translation takes place [72,74]. This hypothesis is supported by studies in *Xenopus laevis* showing that inhibitors of AdoHcy hydrolase interfere with mRNA methylation and elongation of poly(A)$^+$ RNA [75,76].

In summary, the present study demonstrates that the AdoMet/AdoHcy ratio and AdoHcy hydrolase activity are not related to *MGMT* methylation patterns under control conditions in different glioma and hepatoma cell lines. Moreover, neither short-term nor long-term inhibition of AdoHcy hydrolase, resulting in alterations in AdoMet/AdoHcy levels, influenced global DNA methylation or *MGMT* promoter methylation. However, changes in AdoMet/AdoHcy ratio achieved by inhibition of AdoHcy hydrolase or induction of AdoHcy synthesis by administration of adenosine plus homocysteine significantly decreased MGMT mRNA levels in all MGMT-expressing glioma and hepatoma cell lines. Based on previous studies showing that MGMT mRNA levels in human tumor cells correlate with MGMT activity [77] and the observation that glioblastoma patients with low MGMT activity survived better after therapy with alkylating agents than patients expressing high MGMT levels in the tumor [3], we suggest that inhibition of MGMT expression by alterations in AdoMet/AdoHcy ratio could be used as a novel pharmacological strategy to improve the responsiveness to temozolomide and other alkylating agents.

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REFERENCES

[1] Demple B, Jacobsson A, Olsson M, Robins P, Lindahl T. Repair of alkylated DNA in *Escherichia coli*. Physical properties of O^6-methylguanine-DNA methyltransferase. J Biol Chem 1982;257:13776–80.
[2] Kaina B, Fritz G, Mitra S, Coquerelle T. Transfection and expression of human O^6-methylguanine-DNA methyltransferase (MGMT) cDNA in Chinese hamster cells: the role of MGMT in protection against the genotoxic effects of alkylating agents. Carcinogenesis 1991;12:1857–67.
[3] Kaina B, Christmann M, Naumann S, Roos WP. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. DNA Repair (Amst) 2007;6:1079–99.
[4] Gerson SL, Trey JE, Miller K, Berger NA. Comparison of O^6-alkylguanine-DNA alkyltransferase activity based on cellular DNA content in human, rat and mouse tissues. Carcinogenesis 1986;7:745–9.
[5] Citron M, Decker R, Chen S, Schneider S, Graver M, Kleynerman L, et al. O^6-Methylguanine-DNA methyltransferase in human normal and tumor tissue from brain, lung, and ovary. Cancer Res 1991;51:4131–4.
[6] Citron M, Graver M, Schoenhaus M, Chen S, Decker R, Kleynerman L, et al. Detection of messenger RNA from O^6-methylguanine-DNA methyltransferase gene MGMT in

human normal and tumor tissues. J Natl Cancer Inst 1992;84:337–40.
[7] Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 2000;343:1350–4.
[8] Silber JR, Mueller BA, Ewers TG, Berger MS. Comparison of O^6-methylguanine-DNA methyltransferase activity in brain tumors and adjacent normal brain. Cancer Res 1993;53:3416–20.
[9] Silber JR, Bobola MS, Ghatan S, Blank A, Kolstoe DD, Berger MS. O^6-Methylguanine-DNA methyltransferase activity in adult gliomas: relation to patient and tumor characteristics. Cancer Res 1998;58:1068–73.
[10] Zhang YJ, Chen Y, Ahsan H, Lunn RM, Lee PH, Chen CJ, et al. Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and p53 mutation in hepatocellular carcinoma. Int J Cancer 2003;103:440–4.
[11] Glassner BJ, Weeda G, Allan JM, Broekhof JLM, Carls NHE, Donker I, et al. DNA repair methyltransferase (Mgmt) knockout mice are sensitive to the lethal effects of chemotherapeutic alkylating agents. Mutagenesis 1999;14:339–47.
[12] Bhakat KK, Mitra S. CpG methylation-dependent repression of the human O^6-methylguanine-DNA methyltransferase gene linked to chromatin structure alteration. Carcinogenesis 2003;24:1337–45.
[13] Danam RP, Howell SR, Brent TP, Harris LC. Epigenetic regulation of O^6-methylguanine-DNA methyltransferase gene expression by histone acetylation and methyl-CpG binding proteins. Mol Cancer Ther 2005;4:61–9.
[14] Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005;352: 997–1003.
[15] Paz MF, Yaya-Tur R, Rojas-Marcos I, Reynes G, Pollan M, Aguirre-Cruz L, et al. CpG Island hypermethylation of the DNA repair enzyme methyltransferase predicts response to temozolomide in primary gliomas. Clin Cancer Res 2004;10:4933–8.
[16] Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 1998;72:141–96.
[17] Rountree MR, Bachman KE, Herman JG, Baylin SB. DNA methylation, chromatin inheritance, and cancer. Oncogene 2001;20:3156–65.
[18] Clark SJ, Melki J. DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene 2002;21:5380–7.
[19] Bestor TH. The DNA methyltransferases of mammals. Hum Mol Genet 2000;9:2395–402.
[20] Chiang PK. Biological effects of inhibitors of S-adenosylhomocysteine hydrolase. Pharmacol Ther 1998;77:115–34.
[21] Cantoni GL, Richards HH, Chiang PK. Inhibitors of S-adenosylhomocysteine hydrolase and their role in the regulation of biological methylation. In: Usdin E, Borchardt RT, Creveling CR, editors. Transmethylation. New York: Elsevier/North-Holland; 1979. p. 155–64.
[22] Detich N, Hamm S, Just G, Knox JD, Szyf M. The methyl donor S-adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of S-adenosylmethionine. J Biol Chem 2003;278:20812–20.
[23] Pascale RM, Simile MM, De Miglio MR, Feo F. Chemoprevention of hepatocarcinogenesis: S-adenosyl-L-methionine. Alcohol 2002;27:193–8.
[24] Caudill MA, Wang JC, Melnyk S, Pogribny IP, Jernigan S, Collins MD, et al. Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine beta-synthase heterozygous mice. J Nutr 2001;131:2811–8.
[25] Hermes M, Osswald H, Mattar J, Kloor D. Influence of an altered methylation potential on mRNA methylation and gene expression in HepG2 cells. Exp Cell Res 2004;294:325–34.
[26] Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. J Biol Chem 2000;275:29318–23.
[27] Hoffman DR, Cornatzer WE, Duerre JA. Relationship between tissue levels of S-adenosylmethionine, S-adenosylhomocysteine, and transmethylation reactions. Can J Biochem 1979;57:56–65.
[28] Aden D, Fogel A, Plotkin S, Damjanov I, Knowles B. Controlled synthesis of HbsAg in a differentiated human liver carcinoma-derived cell line. Nature 1979;282:615–6.
[29] Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 1980;209:497–9.
[30] Delabar U, Kloor D, Luippold G, Muehlbauer B. Simultaneous determination of adenosine, S-adenosylhomocysteine and S-adenosylmethionine in biological samples using solid-phase extraction and high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 1999;724:231–8.
[31] Hermes M, Hippel von S, Osswald H, Kloor D. S-Adenosylhomocysteine metabolism in different cell lines: effect of hypoxia and cell density. Cell Physiol Biochem 2005;15:233–44.
[32] Kloor D, Kurz J, Fuchs S, Faust B, Osswald H. S-Adenosylhomocysteine-hydrolase from bovine kidney: enzymatic and binding properties. Kidney Blood Press Res 1996;19:100–8.
[33] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162:156–9.
[34] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
[35] Broackes-Carter FC, Mouchel N, Gill D, Hyde S, Bassett J, Harris A. Temporal regulation of CFTR expression during ovine lung development: implications for CF gene therapy. Hum Mol Genet 2002;11:125–31.
[36] Tsuji N, Kamagata C, Furuya M, Kobayashi D, Yagihashi A, Morita T, et al. Selection of an internal control gene for quantitation of mRNA in colonic tissues. Anticancer Res 2002;22:4173–8.
[37] Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O. Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem 2001;295:17–21.
[38] Pogribny I, Yi P, James SJ. A sensitive new method for rapid detection of abnormal methylation patterns in global DNA and within CpG islands. Biochem Biophys Res Commun 1999;262:624–8.
[39] Atkinson DE, Walton GM. Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. J Biol Chem 1967;242:3239–41.
[40] Ramakrishnan V, Borchardt R. Adenosine dialdehyde and neplanocin A: potent inhibitors of S-adenosylhomocysteine hydrolase in neuroblastoma N2a cells. Neurochem Int 1987;10:423–31.

[41] Cory JG, Mansell MM. Studies on mammalian ribonucleotide reductase inhibition by pyridoxal phosphate and the dialdehyde derivates of adenosine, adenosine 5′-monophosphate, and adenosine 5′-triphosphate. Cancer Res 1975;35:390–6.
[42] Qian XC, Brent TP. Methylation hot spots in the 5′ flanking region denote silencing of the O^6-methylguanine-DNA methyltransferase gene. Cancer Res 1997;57:3672–7.
[43] Watts GS, Pieper RO, Costello JF, Peng YM, Dalton WS, Futscher BW. Methylation of discrete regions of the O^6-methylguanine DNA methyltransferase (MGMT) CpG island is associated with heterochromatinization of the MGMT transcription start site and silencing of the gene. Mol Cell Biol 1997;17:5612–9.
[44] Hermes M, Osswald H, Kloor D. Role of S-adenosylhomocysteine hydrolase in adenosine-induced apoptosis in HepG2 cells. Exp Cell Res 2007;313:264–83.
[45] Pegg AE. Mammalian O^6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. Cancer Res 1990;50:6119–29.
[46] Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 1999;59:793–7.
[47] Esteller M, Gaidano G, Goodman SN, Zagonel V, Capello D, Botto B, et al. Hypermethylation of the DNA repair gene O^6-methylguanine DNA methyltransferase and survival of patients with diffuse large B-Cell lymphoma. J Natl Cancer Inst 2002;94:26–32.
[48] Cantoni G, Chiang P. The role of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase in the control of biological methylations. In: Cavalli D, Gaull G, Zappia V, editors. Natural sulfur compounds: novel biochemical and structural aspects. New York: Plenum Press; 1980. p. 67–80.
[49] Jhaveri MS, Wagner C, Trepel JB. Impact of extracellular folate levels on global gene expression. Mol Pharmacol 2001;60:1288–95.
[50] Kloor D, Delabar U, Muehlbauer B, Luippold G, Osswald H. Tissue levels of S-adenosylhomocysteine in the rat kidney: effects of ischemia and homocysteine. Biochem Pharmacol 2002;63:809–15.
[51] Pilz RB, Van den Berghe G, Boss GR. Adenosine dialdehyde and nitrous oxide induce HL-60 differentiation. Blood 1987;70:1161–4.
[52] Eloranta TO. Tissue distribution of S-adenosylmethionine and S-adenosylhomocysteine in the rat. Effect of age, sex and methionine administration on the metabolism of S-adenosylmethionine, S-adenosylhomocysteine and polyamines. Biochem J 1977;166:521–9.
[53] Hermes M, Osswald H, Kloor D. Adenosine metabolism and its effect on methylation potential in cultured cells: methodological considerations. Cell Mol Biol (Noisy-le-grand) 2006;52(Suppl.):OL874–81.
[54] Antequera F, Boyes J, Bird A. High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 1990;62:503–14.
[55] Wenger RH, Kvietikova I, Rolfs A, Camenisch G, Gassmann M. Oxygen-regulated erythropoietin gene expression is dependent on a CpG methylation-free hypoxia-inducible factor-1 DNA-binding site. Eur J Biochem 1998;253:771–7.
[56] Cantoni GL, Scarano E. The formation of S-adenosylhomocysteine in enzymatic transmethylation reactions. J Am Chem Soc 1954;76:4744.
[57] Clarke S, Banfield K. S-Adenosylmethionine-dependent methyltransferases. In: Carmel R, Jacobsen D, editors. Homocysteine in health and disease. Cambridge University Press; 2001. p. 63–78.
[58] Fux R, Kloor D, Hermes M, Roeck T, Proksch B, Grenz A, et al. Effect of acute hyperhomocysteinemia on methylation potential of erythrocytes and on DNA methylation of lymphocytes in healthy male volunteers. Am J Physiol 2005;289:F786–92.
[59] Araujo FD, Knox JD, Szyf M, Price GB, Zannis-Hadjopoulos M. Concurrent replication and methylation at mammalian origins of replication. Mol Cell Biol 1998;18:3475–82.
[60] Pogribny IP, Poirier LA, James SJ. Differential sensitivity to loss of cytosine methyl groups within the hepatic p53 gene of folate/methyl deficient rats. Carcinogenesis 1995;16:2863–7.
[61] Pogribny IP, Miller BJ, James SJ. Alterations in hepatic p53 gene methylation patterns during tumor progression with folate/methyl deficiency in the rat. Cancer Lett 1997;115:31–8.
[62] Wainfan E, Dizik M, Stender M, Christman JK. Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. Cancer Res 1989;49:4094–7.
[63] Wainfan E, Poirier LA. Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. Cancer Res 1992;52:2071s–7.
[64] Baric I, Cuk M, Fumic K, Vugrek O, Allen RH, Glenn B, et al. S-Adenosylhomocysteine hydrolase deficiency: a second patient, the younger brother of the index patient, and outcomes during therapy. J Inherit Metab Dis 2005;28:885–902.
[65] Baric I, Fumic K, Glenn B, Cuk M, Schulze A, Finkelstein JD, et al. S-Adenosylhomocysteine hydrolase deficiency in a human: a genetic disorder of methionine metabolism. Proc Natl Acad Sci USA 2004;101:4234–9.
[66] Buist NR, Glenn B, Vugrek O, Wagner C, Stabler S, Allen RH, et al. S-Adenosylhomocysteine hydrolase deficiency in a 26-year-old man. J Inherit Metab Dis 2006;29:538–45.
[67] Choumenkovitch SF, Selhub J, Bagley PJ, Maeda N, Nadeau MR, Smith DE, et al. In the cystathionine beta-synthase knockout mouse, elevations in total plasma homocysteine increase tissue S-adenosylhomocysteine, but responses of S-adenosylmethionine and DNA methylation are tissue specific. J Nutr 2002;132:2157–60.
[68] Nagasaka T, Goel A, Notohara K, Takahata T, Sasamoto H, Uchida T, et al. Methylation pattern of the O(6)-methylguanine-DNA methyltransferase gene in colon during progressive colorectal tumorigenesis. Int J Cancer 2008.
[69] Costello JF, Futscher BW, Tano K, Graunke DM, Pieper RO. Graded methylation in the promoter and body of the O^6-methylguanine DNA methyltransferase (MGMT) gene correlates with MGMT expression in human glioma cells. J Biol Chem 1994;269:17228–37.
[70] Hershfield MS, Kredich NM, Koller CA, Mitchell BS, Kurtzberg J, Kinney TR, et al. S-Adenosylhomocysteine catabolism and basis for acquired resistance during treatment of T-cell acute lymphoblastic leukemia with 2′-deoxycoformycin alone and in combination with 9-beta-D-arabinofuranosyladenine. Cancer Res 1983;43:3451–8.
[71] Hershfield MS, Kurtzberg J, Harden E, Moore JO, Whang-Peng J, Haynes BF. Conversion of a stem cell leukemia from a T-lymphoid to a myeloid phenotype induced by the adenosine deaminase inhibitor 2′-deoxycoformycin. Proc Natl Acad Sci USA 1984;81:253–7.
[72] Furuichi Y, LaFiandra A, Shatkin AJ. 5′-Terminal structure and mRNA stability. Nature 1977;266:235–9.
[73] Furuichi Y. "Pretranscriptional capping" in the biosynthesis of cytoplasmic polyhedrosis virus mRNA. Proc Natl Acad Sci USA 1978;75:1086–90.

[74] Lockard RE, Lane C. Requirement for 7-methylguanosine in translation of globin mRNA in vivo. Nucleic Acids Res 1978;5:3237–47.
[75] Radomski N, Kaufmann C, Dreyer C. Nuclear accumulation of S-adenosylhomocysteine hydrolase in transcriptionally active cells during development of *Xenopus laevis*. Mol Biol Cell 1999;10:4283–98.
[76] Radomski N, Barreto G, Kaufmann C, Yokoska J, Mizumoto K, Dreyer C. Interaction of S-adenosylhomocysteine hydrolase of *Xenopus laevis* with mRNA (guanine-7-) methyltransferase: implication on its nuclear compartmentalisation and on cap methylation of hnRNA. Biochim Biophys Acta 2002;1590:93–102.
[77] Pieper RO, Futscher BW, Dong Q, Ellis TM, Erickson LC. Comparison of O-6-methylguanine DNA methyltransferase (MGMT) mRNA levels in Mer+ and Mer− human tumor cell lines containing the MGMT gene by the polymerase chain reaction technique. Cancer Commun 1990;2:13–20.