

Alterations of methionine metabolism in hepatocarcinogenesis: the emergent role of glycine N-methyltransferase in liver injury

Maria M. Simile^a, Gavinella Latte^a, Claudio F. Feo^b, Francesco Feo^a, Diego F. Calvisi^a, Rosa M. Pascale^a

University of Sassari, Sassari, Italy

Abstract

The methionine and folate cycles play a fundamental role in cell physiology and their alteration is involved in liver injury and hepatocarcinogenesis. Glycine N-methyltransferase is implicated in methyl group supply, DNA methylation, and nucleotide biosynthesis. It regulates the cellular S-adenosylmethionine/S-adenosylhomocysteine ratio and S-adenosylmethionine-dependent methyl transfer reactions. Glycine N-methyltransferase is absent in fast-growing hepatocellular carcinomas and present at a low level in slower growing HCC ones. The mechanism of tumor suppression by glycine N-methyltransferase is not completely known. Glycine N-methyltransferase inhibits hepatocellular carcinoma growth through interaction with Dep domain-containing mechanistic target of rapamycin (mTor)-interacting protein, a binding protein overexpressed in hepatocellular carcinoma. The interaction of the phosphatase and tensin homolog inhibitor, phosphatidylinositol 3,4,5-trisphosphate-dependent rac exchanger, with glycine N-methyltransferase enhances proteasomal degradation of this exchanger by the E3 ubiquitin ligase HectH. Glycine N-methyltransferase also regulates genes related to detoxification and antioxidation pathways. It supports pyrimidine and purine syntheses and minimizes uracil incorporation into DNA as consequence of folate depletion. However, recent evidence indicates that glycine N-methyltransferase targeted into nucleus still exerts strong anti-proliferative effects independent of its catalytic activity, while its restriction to cytoplasm prevents these effects. Our current knowledge suggest that glycine N-methyltransferase plays a fundamental, even if not yet completely known, role in cellular physiology and highlights the need to further investigate this role in normal and cancer cells.

Keywords Hepatocellular carcinoma, Methionine cycle, Folate cycle, tumor suppression, S-adenosylmethionine

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Introduction

Interest in the role of methionine, its active derivative S-adenosylmethionine (SAM), and the methionine metabolic cycle, in hepatic injury and hepatocarcinogenesis, was stimulated by the discovery of a decrease in SAM levels

Departments of ^aClinical, Surgical and Experimental Medicine, Division of Experimental Pathology and Oncology (Maria M. Simile, Gavinella Latte, Francesco Feo, Diego F. Calvisi, Rosa M. Pascale);

^bClinical, Surgical and Experimental Medicine, Division of Surgery (Claudio F. Feo), University of Sassari, Sassari, Italy.

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Correspondence to: Rosa M. Pascale, Department of Clinical, Surgical and Experimental Medicine, Division of Experimental Pathology and Oncology, University of Sassari, Sassari, Italy; via P. Manzella 4, 07100 Sassari, Italy, e-mail: patsper@uniss.it

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during liver injury, provoked by ethanol and hepatotoxins, and in hepatocellular carcinoma (HCC), induced by different carcinogens, in animals fed diets containing adequate amounts of methyl donors [1-3]. Reconstitution of the liver's SAM pool by administration of exogenous SAM, prevented liver damage and HCC [3-6]. These pioneering findings were followed by several studies of the mechanisms involved in the antitumor effect of SAM, which directed their attention to the main metabolic cycles implicated in SAM production: the methionine and folate cycles. This led to the discovery of the leading role of glycine N-methyltransferase (GNMT) in liver injury and hepatocarcinogenesis.

The methionine and folate metabolic cycles

SAM, synthesized from methionine and ATP by methionine adenosyltransferases [7], is the methyl donor for many reactions producing methylated compounds and S-adenosylhomocysteine (SAH), a potent inhibitor of

most methyltransferases [8] (Fig. 1). SAH is converted to homocysteine and adenosine via a reversible reaction catalyzed by SAH hydroxylase (SAHH). However, the equilibrium of SAHH favors the formation of homocysteine and adenosine. The latter must be transported outside of the cells or metabolized, to avoid homocysteine accumulation. Homocysteine is an amino acid not used for protein synthesis, but is an intermediate in methionine metabolism, irreversibly transformed to cystathionine by cystathionine β -synthetase (CBS), and then to GSH through the transsulfuration pathway, or methylated to form methionine, thus completing the “methionine cycle” (Fig. 1). Homocysteine remethylation may occur during betaine (tri-methylglycine) conversion to di-methylglycine in a reaction catalyzed by betaine-homocysteine methyltransferase (BHMT). Di-methylglycine may be transformed to glycine by a dimethylglycine dehydrogenase (DMGDH) [N,N-dimethylglycine:(acceptor) oxidoreductase (demethylating)] (Fig. 1). Betaine is formed through choline oxidation, catalyzed by choline oxidase, and followed by oxidation of betaine aldehyde by a specific dehydrogenase [9]. In animals, choline may be acquired from the diet and through the transmethylation pathway [10] in which phosphatidylethanolamine is methylated

to phosphatidylcholine by phosphatidylethanolamine N-methyltransferase (PEMT) [10] in the presence of SAM (Fig. 1). This pathway is particularly active during choline deficiency [11].

Another source of methionine is the so called “folates cycle” [12] in which 5-tetrahydrofolate (THF) is oxidized to 5,10-methylenetetrahydrofolate (MeTHF), in a reaction catalyzed by sarcosine dehydrogenase (SARDH). MeTHF is transformed to 5-methyltetrahydrofolate (MTHF) by a specific reductase (MeTHFR). Homocysteine plus MTHF leads to methionine and THF in a reaction catalyzed by a 5-methyltetrahydrofolate homocysteine transferase (MHMT) that requires cobalamin as cofactor (Fig. 1).

Glycine methyltransferase (GNMT) plays a fundamental role in methyl group supply, DNA methylation, and nucleotide biosynthesis. GNMT catalyzes the SAM-dependent methylation of glycine to form sarcosine [13] (Fig. 1). SARDH catalyzes glycine resynthesis associated with the formation of MeTHF from THF. GNMT expression regulates the cellular folate status: it binds MTHF and is inhibited by it and increases cellular MTHF retention thus allowing MTHF-dependent remethylation [14,15]. GNMT and PEMT are among the largest

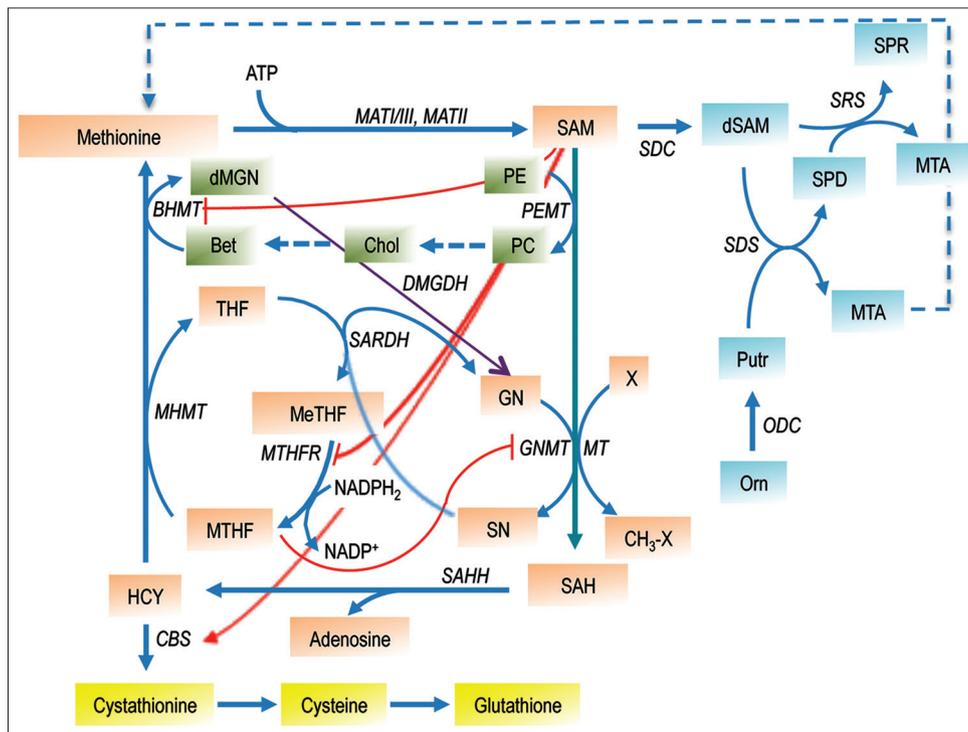


Figure 1 Figure 1 Methionine and folates cycles

The dotted line refers to the “salvage pathway” for methionine biosynthesis. The purple arrow indicates the conversion of dimethyl glycine to sarcosine, the red arrows indicate the inhibition of BHMT and MTHFR and the activation of CBS by SAM, and the inhibition of GNMT by MTHF. Bet, betaine; Chol, choline; dMGN, di-methylglycine; dSAM, decarboxylated S-adenosylmethionine; GN, glycine; HCY, homocysteine; MeTHF, 5,10-methylenetetrahydrofolate; MTA, 5-methylthioadenosine; MTHF, 5-methyltetrahydrofolate; Orn, ornithine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Putr, putrescine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SN, sarcosine; SPD, spermidine; SPR, spermine; THF, tetrahydrofolate; BHMT, betaine homocysteine methyltransferase; CBS, cystathionine beta-synthetase; MATI/III, methionine adenosyltransferase I/III; MATII, methionine adenosyltransferase II; MHMT, methyltetrahydrofolate homocysteine methyltransferase; MT, methyltransferases; MTHFR, 5-methyltetrahydrofolate reductase; ODC, ornithine decarboxylase; PEMT, phosphoethanolamine methyltransferase; SAHH, S-adenosylhomocysteine hydroxylase; SARDH, sarcosine dehydrogenase; SDC, SAM decarboxylase; SDS, spermidine synthetase; SRS, spermine synthetase

consumers of methyl groups derived from SAM and thus play a major role in homocysteine generation and regulation of methyl group metabolism [16].

Numerous regulatory mechanisms contribute to maintaining an optimal metabolism of folate, methyl groups and homocysteine. The liver is the principal organ in which the excess of methionine is degraded. In it different enzymes, including MATI/III, CBS, BHMT and GNMT, maintain homocysteine at adequate level. Furthermore, the so-called SAM “long-range” interactions [17], which affect enzymes at distance in the metabolic network, induces CBS activation (Fig. 1); this enhances the transsulfuration pathway and inhibits BHMT and MeTHFR (Fig. 1), thus decreasing homocysteine re-methylation to methionine [17]. Indeed, MATI/III Michaelis constant (K_m) is so high that the elevated methionine liver levels produce large amounts of SAM. High SAM levels inhibit MTHFR while stimulating CBS activity [17,18] (Fig. 1). Therefore, a methionine excess, by producing a SAM excess, induces homocysteine degradation through the transsulfuration pathway, whereas low methionine and SAM levels activate homocysteine re-methylation. Furthermore, SAM allosterically inhibits MeTHFR, thus decreasing the production of 5-methyl-TNF for the subsequent remethylation of homocysteine [19-21] (Fig. 1). SAM also inhibits homocysteine remethylation by inhibiting the expression of BHMT. These SAM effects are associated with the activation of CBS and consequent homocysteine consumption by transsulfuration pathway (Fig. 1).

GNMT binds DMGDH and SARDH (Fig. 1) [21] and is allosterically inhibited by 5-methyl-THF [14,22]. In addition to the allosteric regulation, phosphorylation of GNMT represents an additional post-translational control of this protein [12].

The central role of the methionine and folate cycles in cell metabolism also depends on the multiple interactions of these cycles with cell metabolism. Folate cycle is involved in dTMP synthesis, thus allowing DNA production and synthesis of purine and nucleic acids synthesis [22,23]. Furthermore, SAM decarboxylation by a specific decarboxylase is a fundamental step of polyamine biosynthesis. 5-methylthioadenosine (MTA), an end-product of this biosynthesis, inhibits SAM-decarboxylase [2]. This control step is regulated by the activity of MTA phosphorylase, which catalyzes the transformation of MTA into 5-methylthioribose and adenine, allowing the methionine resynthesis in the “salvage pathway” (Fig. 1).

The SAM/SAH ratio plays a key role in SAM-dependent methyltransferase reactions. MATI and MATIII isozymes, respectively the tetramer and dimer of the subunit $\alpha 1$ isozymes, are encoded by the liver-specific *MAT1A* gene [24] (Fig. 1). The widely distributed MATII isoform (the $\alpha 2$ -subunit) is codified by the *MAT2A* gene, whose expression, prevalent in fetal liver, is gradually replaced by *MAT1A* in adult liver [24]. The K_m for methionine of MATI and MATIII are 23 μM –1 mM and 215 μM –7 mM, respectively. Therefore, SAM, at physiological liver level (0.1-0.2 $\mu\text{mol/g}$ of liver, about 60 μM) slowly inhibits MATI and stimulates MATIII activity. In contrast, MATII, with a lower K_m for methionine (4–10 μM), is inhibited by its reaction product [24]. A third gene, *MAT2B*, encodes the MATs

β -subunit, devoid of catalytic activity, which regulates MATII by lowering its K_m for methionine and inhibition constant (K_i) for SAM [25]. Therefore, the β -subunit association with the α -subunit renders MATII more susceptible to inhibition by SAM [25].

GNMT is a major regulator of the cellular SAM/SAH ratio and SAM-dependent methyl transfer reactions. Low SAM levels favor homocysteine remethylation, whereas high SAM levels activate CBS. Furthermore, GNMT provides an alternative route for the conversion of SAM excess to SAH and the preservation of the SAM/SAH ratio. GNMT is abundant in liver and its product sarcosine, which has no known physiological role, is converted back to glycine by sarcosine dehydrogenase [14,21,22]. GNMT is a major regulator of the cellular SAM/SAH ratio and SAM-dependent methyl transfer reactions. SAM-dependent methyltransferases are generally strongly inhibited by the product SAH, and the cellular SAM/SAH ratio plays a key role in methyl-transfer reactions [14]. Unlike most SAM-dependent methyltransferases, GNMT has a relatively high K_m value for SAM and is weakly inhibited by SAH: the K_i values of GNMTs for SAH are 35-80 μM , much higher than those for other methyltransferases [14,21]. Therefore, at physiological levels of SAM (0.1-0.2 $\mu\text{mol/g}$ of liver) and SAH (0.02-0.06 μmol /of liver), GNMT exhibits appreciable activity. Therefore, the fluctuations in GNMT activity could alter the SAM/SAH ratio, thus influencing the activity of methyltransferases.

Further, GNMT, as a major hepatic folate binding protein, binds to and may be inhibited by MTHF [14-17,22]. Therefore, when SAM levels increase, MeTHFR inhibition leads to a decrease in free MTHF and dissociation of the complex GNMT-MTHF [14,22]. The consequent rise in free GNMT prevents the SAM level from rising excessively. Conversely, when SAM concentration tends to decrease, the amount of free GNMT falls, MeTHFR inhibition is released and more MTHF is available. Thus, GNMT increases the cells' folate content and the remethylation of MTHFR-dependent homocysteine. For this reason, the GNMT pathway may be considered a “salvage pathway”.

Alterations of the methionine cycle in liver cancer

Decreases in SAM liver content characterize liver injuries that lead to inflammation, necrosis, cirrhosis and eventually cancer, such as alcoholic steatohepatitis [26,27], and D-galactosamine [28], acetaminophen [29] and CCl_4 [30,31] toxicity. In cirrhotic liver, the oxidation of cysteine residue at the ATP binding site is involved in MATI/III downregulation, decrease in MAT1A: MAT2A ratio (MAT1A/MAT2A switch), and SAM and GSH contents [32,33]. This favors hepatic oxidative stress and fibrosis followed by the development of HCC. These alterations could be partly attributable to the absence of prohibitin (PHB)-1. This, together with PHB-2, forms multimeric ring complexes in the mitochondrial inner membrane and plays a role in mitochondrial maturation [34,35]. SAM contributes to PHB1 stability and a drop in SAM is

associated with PHB1 deficiency [36]. This deficiency leads to increases in the production of reactive oxygen species, impairs the formation of mitochondrial respiratory supercomplexes, disorganizes the mitochondrial cristae morphology, and increases the sensitivity to stimuli-elicited apoptosis [37].

The strong involvement of the MAT1A/MAT2A switch and the fall in SAM levels during hepatocarcinogenesis is further demonstrated by the observation that the Mat1A-KO mouse model, which features a chronic SAM deficiency that is not compensated for by MAT2A induction, is characterized by hepatomegaly at 3 months of age, macrovesicular steatosis involving 25-50% of hepatocytes, mononuclear cell infiltration in periportal areas, at 8 months, and HCC development at 18 months [38].

A sharp decrease in SAM content and SAM/SAH ratio has been documented in liver of rats fed an adequate diet, during hepatocarcinogenesis induced by different carcinogens, and is still present in dysplastic nodules (DN) and HCC several weeks after carcinogen administration is discontinued [3-5]. Decreases in SAM content and SAM/SAH ratio also occur in human HCC and, to a lower extent, in the cirrhotic liver surrounding a tumor [39]. SAM administration reconstitutes the GSH pool, has beneficial effects against liver fibrosis in both rats and humans [24,31] and prevents HCC development in rats and mice [3-6,39].

The genesis of the MAT1A/MAT2A switch is complex. A mechanism deals with changes of promoter methylation of the *MAT1A* and *MAT2A* genes in HCC [40-43]. Methylation of CCGG sequences of *MAT1A* promoter, associated with a decrease in MAT1A, occurs in cirrhotic liver of CCl₄-treated rats and in HepG2 and Huh7 liver cancer cell lines [40]. MAT1A expression is reactivated in the human hepatoblastoma cell line HepG2 treated with the demethylating agent 5-aza-2'-deoxycytidine or the histone deacetylase inhibitor trichostatin, suggesting a role for DNA hypermethylation and histone deacetylation in MAT1A silencing [40]. In HuH7 cells, *MAT1A* downregulation is also associated with CCGG methylation at +10 and +80 of the coding region [41]. Conversely, *MAT2A* upregulation in human HCC is associated with CCGG hypomethylation of the gene promoter [42,43].

The highest *MAT1A* promoter hypermethylation and *MAT2A* promoter hypomethylation were found in human HCC with poorer prognosis (HCCP), based on survival time after partial liver resection, whereas, in human HCC with a better prognosis (HCCB) and small changes in the MAT1A: MAT2A ratio, CpG methylation and histone H4 acetylation occurred [43]. Interestingly, the Mat1A/Mat2A switch and low SAM levels associated with CpG hypermethylation and histone H4 deacetylation in *Mat1A* promoter, and prevalent CpG hypomethylation and histone H4 acetylation in *Mat2A* promoter has also been found in fast growing HCC of Fisher 344 (F344) rats, whereas these alterations were almost absent in slowly growing HCC of brown Norway (BN) rats [43].

Post-transcriptional mechanisms are also involved in MAT1A: MAT2A switch. Among the mRNA-binding proteins (RBPs), AUF1, increases mRNA decay, whereas HuR binds to AU-rich elements promoting mRNA stabilization [43-45].

A Mat1A mRNA decrease in fetal rat liver is associated with increased interactions with AUF1, whereas a Mat2A mRNA increase is associated with its interaction with HuR [43,46]. A sharp increase in AUF1 and HuR occurs in F344 and human HCC associated with a consistent increase in MAT1A-AUF1 and MAT2A-HuR ribonucleoproteins in both HCC types [43].

The importance of these findings also lies in the fact that rats F344 and BN are, respectively, genetically susceptible and resistant to the development of liver tumors. Numerous loci responsible for the resistance to the development of dysplastic nodules and carcinomas whose effect predominates in resistant rats have been identified [reviewed in 47]. These observations indicate that the alterations of promoter methylation of *Mat1A* and *Mat2A* genes and histone deacetylation, responsible for the Mat1A: Mat2A switch, are genetically determined.

Recent data indicate that miRNAs may also contribute to the MAT1A: MAT2A switch. Knockdown of miR-664, miR-485-3p, and miR-495 individually in Hep3B and HepG2 cells was found to induce MAT1A expression, and Hep3B cells tumorigenesis in nude mice was decreased by stable overexpression of these miRNAs and increased by their knockdown [48].

GNMT in liver injury

GNMT was first described by Blumenstein and Williams in guinea pig liver [13]. *GNMT* gene expression is greatest in mammalian liver, followed by the acinar tissue of pancreas and kidney [49]. The presence of GNMT protein and enzymatic activity has been demonstrated in liver [49]. This protein is also expressed in fetal and adult rabbit liver and in rat liver, pancreas and prostate [50,51].

GNMT mRNA is downregulated in cirrhotic liver and in HCC induced by hepatitis C virus infection and alcohol abuse [52,53]. Recent findings showed that GNMT deficiency in mice induces steatohepatitis, fibrosis, cirrhosis, and HCC. A lack of GNMT triggers natural killer cell activation in *Gnmt*-KO mice and depletion of TRAIL-producing natural killer cells significantly attenuates acute liver injury and inflammation in these animals [54]. In contrast, 1,2,3,4,6-penta-O-galloyl- β -D-glucopyranoside, a GNMT inducer, has potent anti-HCC effects both *in vitro* and *in vivo* [55]. Interestingly, GNMT is completely absent in fast-growing HCC and present at a low level in slower growing HCC, which suggests that it has an impact on tumor progression [50].

Epigenetic silencing of the *GNMT* gene, due to promoter methylation was found in pancreatic adenocarcinoma [56]. Hypermethylation of *GNMT* gene also occurs in some HCC cell lines and in 20% of primary HCCs [57]. Furthermore, GNMT binds different carcinogens including polyaromatic hydrocarbons and aflatoxins and prevents the DNA adduct formation and cytotoxicity induced by these carcinogens [58,59]. Studies conducted in Taiwanese men, suggested that GNMT is a tumor susceptibility gene for

HCC [60] and prostate cancer [61]. However, in another study [62], GNMT short tandem repeat polymorphism (STRP1), and single nucleotide polymorphism rs10948059 were not associated with an increase in the risk of prostate cancer in Taiwanese men of European descent. An androgen response element within the first exon of the GNMT gene, which binds androgen receptor *in vitro* and *in vivo*, was identified in prostate cancer, which highlights androgen as an important feature of GNMT regulation [63,64].

Role of GNMT in hepatocarcinogenesis

A study of *Gnmt*-KO mice by Luka *et al* [65] made a crucial contribution to our recognition of the role of GNMT in hepatocarcinogenesis. In that study, the absence of liver *Gnmt* resulted in increases of up to a 7-fold and 35-fold in free methionine and SAM, respectively, while SAH level decreased 3-fold. Further studies using a *Gnmt*-KO mouse model with knockout of the 1-5 exons in *Gnmt* gene also resulted in HCC development [66,67]. These mice showed hypermethioninemia and elevated levels of serum aminotransferase and hepatic SAM. About 60% of these mice exhibited increased glycogen storage in the liver. HCC developed between 14-24 months of age, with a prevalence of female mice [67]. Wnt signaling pathway, global DNA hypomethylation and aberrant expression of DNA methyltransferases 1 and 3b were found even at early stages of HCC development [67]. *Gadd45a*, *Pak1*, *Mapk3* and *Dusp3* genes of the MAPK pathway were activated in *Gnmt*-KO mice, especially females [67].

Elevated serum aminotransferase, methionine, and SAM levels, associated with the development of liver steatosis and fibrosis were found by Martínez-Chantar *et al* [68] in a male *Gnmt*-KO model in which GNMT exon 1 was disrupted. HCCs developed at the age of 8 months [69]. Activation of Ras and Jak/Stat pathways and suppression of the Ras inhibitors, Ras-association domain family/tumor suppressor (RASSF) 1 and 4, and of the Jak/Stat inhibitors, SOCS 1-3 and cytokine-inducible SH2-protein, occurred in these mice [68]. Hypermethylation of *Rassf1* and *Socs2* promoters and binding of trimethylated lysine 27 of histone 3 to these two genes occurred in HCC from *Gnmt*-KO mice [68] indicating that GNMT loss induced aberrant methylation of DNA and histones resulting in epigenetic modulation of critical carcinogenic pathways. The reasons for the discrepancies between the two *Gnmt*-KO models are not clear and require additional investigation.

Some interesting relationships also occur between GNMT and LKB1/AMPK signaling [69]. Hepatoma cells from *Gnmt*-KO mice lacking exon 1 exhibit, like human HCC, increased activity of *Lkb1* and *Ras*. Ras-mediated *Lkb1* hyper-activation promotes Ampk activation and proliferation of GNMT-KO hepatoma cells, and requires Erk2 and p90rsk activity and Rasgrp3 expression. Noticeably, reduced levels of GNMT, phosphorylation of AMPK α at Thr172, and increased levels of RAS, LKB1 and RASGRP3 were found, in HCC samples from patients with short survival.

Mechanisms of the inhibition of hepatocarcinogenesis by GNMT

GNMT impacts on HCC growth through different mechanisms (Fig. 2). It inhibits HCC growth through interaction with Dep domain-containing mtor-interacting protein (DEPTOR), an mTOR binding protein overexpressed in HCC, especially in patients with poor prognosis [70]. GNMT overexpression causes a decrease in Mechanistic target of rapamycin (mTORC1) signaling, delays G2/M cell cycle progression, and inhibits HuH-7 cell proliferation [70]. DEPTOR over-expression activates AKT via the inhibition of the negative feedback loop from S6K to PI3K [71]. Knockdown of DEPTOR expression in HuH-7 cells causes activation of the mTORC1 targets S6K and 4E-BP, but suppresses AKT and reduces cell survival [70].

Recent evidence [72] revealed a new mechanism of the interaction between GNMT and AKT signaling. It was observed that the interaction of the phosphatase and tensin homolog (PTEN) inhibitor phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 2 (PREX2) with GNMT enhances PREX2 proteasomal degradation by the E3 ubiquitin ligase HectH9 (Fig. 2). GNMT or HectH9 depletion resulted in AKT activation in a PREX2 dependent manner and enhanced cell proliferation. Elevated Prex2 protein expression and

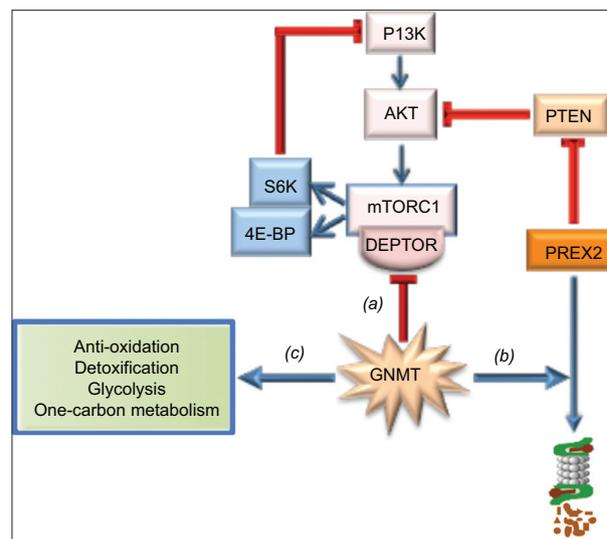


Figure 2 Mechanisms of inhibition of HCC growth by GNMT. (a) The inhibition of DEPTOR, with consequent activation of mTORC1 targets SK6 and 4E-BP, allows the feedback negative effect of SK6 on the PI3K/AKT axis. (b) GNMT impedes the proteasomal degradation of PREX2 by the E3 ubiquitin ligase HectH9, thus allowing the inhibition, by PREX2, of the AKT inhibitor PTEN. (c) The maintenance of adequate levels of proteins involved in the anti-oxidation/detoxification response, glycolytic energy metabolism and one-carbon metabolism pathways may impede oxidative stress and liver damage that should favor HCC development

AKT, murine thymoma viral oncogene homolog; DEPTOR, dep domain-containing mtor-interacting protein; GNMT, glycine N-methyltransferase; mTORC1, mechanistic target of rapamycin complex 1; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog

AKT activation occur in *GNMT*-KO mice liver. Interestingly, upregulation of PREX2 protein expression in human HCC is associated with poorer survival. No changes in PREX2 mRNA level were found, suggesting a post-translational alteration of PREX2 expression [72].

GNMT was also found to maintain normal levels of proteins related to detoxification and antioxidation pathways (Fig. 2). Proteins involved in the anti-oxidation/detoxification response, glycolytic energy metabolism and one-carbon metabolism pathways were found to be downregulated significantly and peroxidation was increased in *Gnmt*-KO mice with respect to wild mice [73]. In contrast, the survival of HuH-7 cells overexpressing GNMT, treated with H₂O₂, has been found to be significantly higher than that of controls [73].

The strong involvement of GNMT in cellular folate metabolism [14,22,23] may partly account for the clinical behavior of GNMT deficiency. Hepatic folate increases in *Gnmt*-TG mice whereas it decreases in *Gnmt*-KO mice [22]. GNMT overexpression, in addition to the increase in folate concentration, induces folate-dependent homocysteine methylation [23], which contrasts with a reduction in MHMT (Fig. 3) expression in *Gnmt*-KO mice, indicating that the GNMT deletion impairs methylfolate-dependent metabolism [22]. MeTHFR catalyzes the conversion of 5,10-methyltetrahydrofolate to 5-methyltetrahydrofolate (Fig. 3). The latter inhibits GNMT activity (Fig. 1) [74]. SAM accumulation in *Gnmt*-KO mice results in Methfr

down-regulation with a consequent decrease in the supply of methyl groups from folate metabolism [18,19]. Also, formiminotransferase cyclodeaminase (FTCD; Fig. 3) – which links histidine catabolism to folate metabolism and catalyzes the folate-dependent degradation of N-formiminoglutamic acid to form 5,10-methylenetetrahydrofolate, glutamate, and ammonia – is downregulated in the absence of GNMT activity [75]. These findings are in keeping with the presence in *Gnmt*-KO mice of a phenotype similar to that of patients with congenital GNMT deficiencies [75,76].

Studies of GNMT in human HCC have shown that *GNMT* gene alteration, including insertion/deletion polymorphism in the promoter region, is an early event in HCC development and that *GNMT* could be considered a new tumor susceptibility gene for HCC [61,77]. In respect to these findings, it is interesting the observation that some genes involved in methionine metabolism, such as *BHMT* and *GNMT*, are connected to the genetic susceptibility to liver cancer [77]. Chemically induced hepatocarcinogenesis in genetically susceptible F344 rats is characterized by the development of numerous DN that progress to HCCs, whereas the same treatments induce in the genetically resistant BN rats only a few slow-proliferating DN and HCCs [77]. A gene expression signature characterized by highest expression of the oncosuppressors *Csmd1*, *Dmbt1*, *Dusp1*, and *Gnmt*, in DN, and *Bhmt*, *Dmbt1*, *Dusp1*, *Gadd45g*, *Gnmt*, *Napsa*, *Pp2ca*, and *Ptpn13*, in HCCs, was identified in resistant rats. Greater aggressiveness linked to higher cell cycle

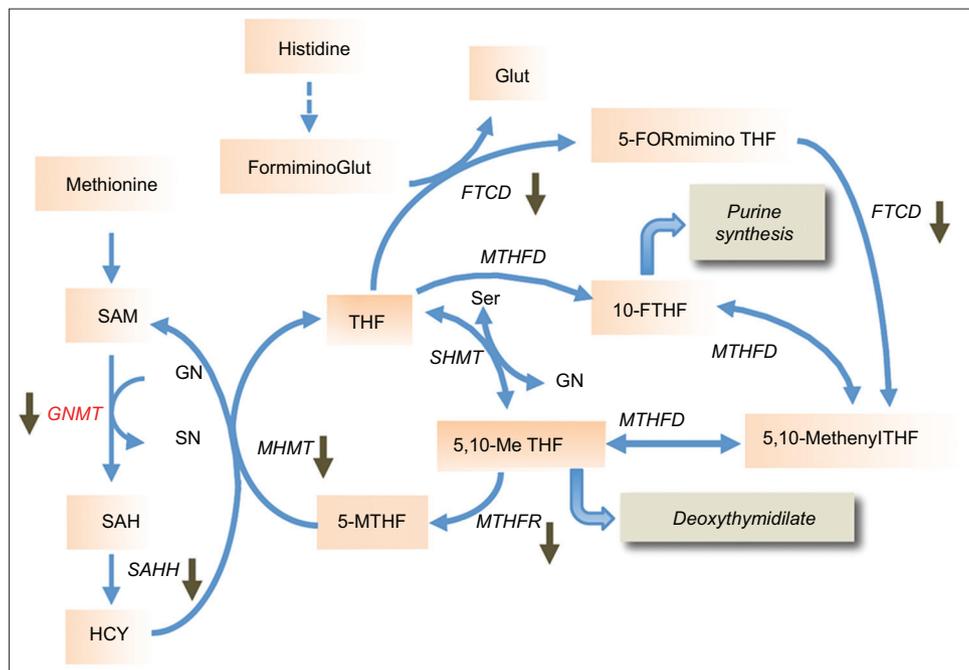


Figure 3 Effect of *gnmt* deficiency on the folate metabolism

The black arrows indicate the decrease in enzyme activities linked to that of GNMT activity

Formiminoglut, formiminoglutamate; 5-formimino-thf, 5-formimino-tetrahydrofolate; 10-FTHF, 10-formyltetrahydrofolate; Glut, glutamate; GN, glycine; HCY, homocysteine; 5,10-methenylTHE, 5,10-methenyletrahydrofolate; 5,10-MeTHE, 5,10-methylenetetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SN, sarcosine; Ser, serine; THF, tetrahydrofolate; FTCD, formiminotransferase cyclodeaminase; GNMT, glycine methyltransferase; MHMT, methyltetrahydrofolate homocysteine methyltransferase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, 5-methyltetrahydrofolate reductase SAHH, S-adenosylhomocysteine hydroxylase; SHMT, serine hydroxymethyl transferase

and signal transduction pathways activation was documented in human HCCP with respect to HCCB [77-79]. Integrated gene expression data revealed the highest expression of proliferation-related *CTGF*, *c-MYC*, and *PCNA*, and the lowest expression of *BHMT*, *DMBT1*, *DUSP1*, *GADD45g*, and *GNMT*, in more aggressive rat and human HCC [77]. These findings confirm that genes involved in the methionine cycle, such as *BHMT* and *GNMT*, contribute to determining the prognosis of HCC.

The mechanism by which GNMT can participate in tumor prevention/suppression in humans is not completely clear. It has been shown [80] that GNMT supports methylene-folate dependent pyrimidine synthesis and formyl-folate dependent purine syntheses (Fig. 3). It also minimizes uracil incorporation into DNA as a consequence of folate depletion and translocates into nuclei during prolonged folate depletion. According to these findings, loss of GNMT impairs nucleotide biosynthesis, whereas GNMT overexpression enhances nucleotide biosynthesis and improves DNA integrity by reducing uracil misincorporation in DNA both *in vitro* and *in vivo* [80].

Intriguingly, recent observations [81] indicate that GNMT has a secondary function, as a regulator of cellular proliferation, independent of its catalytic activity. According to this study, GNMT is strongly downregulated in human cancers and is undetectable in cancer cell lines while the transient expression of the protein in cancer cells induces apoptosis and the activation of ERK1/2 as an early pro-survival response. The anti-proliferative effect of GNMT is partially reversed by the pancaspase inhibitor zVAD-fmk. High GNMT levels were found in regenerating liver and in NIH3T3 mouse fibroblasts, where they did not produce cytotoxic effects. Notably, GNMT, a predominantly cytoplasmic protein, translocates into nuclei upon transfection of cancer cells [81]. The induction of apoptosis was associated with the GNMT nuclear localization but was independent of its catalytic activity or folate binding. GNMT targeted to nuclei still exerted strong anti-proliferative effects while its restriction to cytoplasm prevented these effects. Thus, according to these findings, the oncosuppressive action of GNMT would be independent of its catalytic activity.

Concluding remarks

Increasing evidence indicates that GNMT plays an essential regulatory role in liver cells. Its regulation of methionine and folate cycles is fundamental. GNMT preserves DNA integrity by promoting MeTHF-dependent pyrimidine synthesis and 10-formylTHF-dependent purine synthesis, it thus contributes to reducing uracil misincorporation in DNA and regulates genes related to detoxification and anti-oxidation pathways. Furthermore, GNMT may reduce the proliferative activity of HCC by controlling the Pi3K/AKT pathway through interaction with DEPTOR, an activator of AKT signaling, and enhancing the degradation of the PTEN inhibitor PREX2. In addition, WNT and MAPK signaling and *Cyclin D1* and *c-Myc* genes are activated in *GNMT*-KO mice. However, the mechanisms through which GNMT exerts an inhibitory effect on these and

others signaling pathways contributing to HCC growth are poorly known and need further investigation. Further studies should be devoted to the mechanisms of the oncosuppressor effect of the nuclear GNMT localization. Finally, the discovery of insertion/deletion polymorphisms in the promoter region of GNMT and the association of low GNMT expression with genetic susceptibility to HCC suggest that GNMT is implicated in the genetic susceptibility to this tumor. This is an attractive possibility that needs further experimental support. Overall, current information suggests that GNMT plays a basic, even if not yet completely known, role in the cellular physiology and highlights the need to investigate this role further in normal and cancer cells.

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