Regulation of Folate-Mediated One-Carbon Metabolism by Glycine N-Methyltransferase (GNMT) and Methyleneetetrahydrofolate Reductase (MTHFR)

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Summary: Folate-mediated one-carbon metabolism is an important therapeutic target of human diseases. We extensively investigated how gene-nutrient interactions may modulate human cancer risk in 2 major folate metabolic genes, MTHFR and GNMT. The biochemical impacts of MTHFR and GNMT on methyl group supply, global DNA methylation, nucleotide biosynthesis, DNA damage, and partitioning of the folate dependent 1-carbon group were carefully studied. The distinct model systems used included: EB virus-transformed lymphoblasts expressing human MTHFR polymorphic genotypes; liver-derived GNMT-null cell lines with and without GNMT overexpression; and HepG2 cells with stabilized inhibition of MTHFR using shRNA, GNMT wildtype, heterozygous (GNMThet) and knockout (GNMTnul) mice. We discovered that the MTHFR TT genotype significantly reduces folate-dependent remethylation under folate restriction, but it assists purine synthesis when folate is adequate. The advantage of de novo purine synthesis found in the MTHFR TT genotype may account for the protective effect of MTHFR in human hematological malignancies. GNMT affects transmethylation kinetics and S-adenosylmethionine (adoMet) synthesis, and facilitates the conservation of methyl groups by limiting homocysteine remethylation fluxes. Restoring GNMT assists methylfolate-dependent reactions and ameliorates the consequences of folate depletion. GNMT expression in vivo improves folate retention and bioavailability in the liver. Loss of GNMT impairs nucleotide biosynthesis. Over-expression of GNMT enhances nucleotide biosynthesis and improves DNA integrity by reducing uracil misincorporation in DNA both in vitro and in vivo. The systematic series of studies gives new insights into the underlying mechanisms by which MTHFR and GNMT may participate in human tumor prevention.

Background: Different folate cofactors carry one-carbon (1C) units that are essential for DNA synthesis and biological methylation in mammalian cells. The regulation of the key enzymes in the methionine cycle and cellular methylation reactions is specific to gene, tissue or cell type, and the stage of transformation (1). Perturbations in folate, vitamin B6, and homocysteine pathways have been associated with cancer (2) and many human pathological conditions (3–5); and folate-mediated 1C-metabolism and vitamin B6 dependent pathways are important therapeutic targets of numerous human diseases (6, 7). In the present study, we extensively investigated how gene-nutrient interactions may modulate human cancer risk in 2 major folate metabolic genes: MTHFR and GNMT. Loss of GNMT Is Associated with Human Hepatocellular Carcinoma: GNMT (EC 2.1.1.20) converts adoMet to S-adenosylhomocysteine (adoHcy) while generating sarcosine from glycine. GNMT serves as an alternative pathway to regulate the methyl donor availability and utilization (8). Missense mutations in GNMT lead to hepatic methionine and adoMet accumulation, hepatomegaly and elevated transaminase (9). GNMT is commonly diminished in human hepatocellular carcinoma (HCC) and hepatoma cell lines (10) thus defective GNMT has been suggested to be an early marker in human HCC development.

GNMT Regulates transmethylation Kinetics: GNMT knockout mice exhibited elevated hepatic adoMet levels and reduced MTHFR and S-adenosylhomocysteine hydrolase (SAHH) expression in the liver (11). We further demonstrated that GNMT expression enhances homocysteine transsulfuration and remethylation fluxes when methionine is in excess, and GNMT assists homocysteine clearance when needed (8). Importantly, GNMT overexpression does not exacerbate the methyl group deficiency when methionine supply is limited. In addition, GNMT-expressing cells (GNMT+) appeared to have elevated adoMet synthase (MAT) activity that could contribute to the adoMet supply in order to compensate for the accelerated transmethylation reactions in these cells. We suggest that GNMT+ cells can maintain adoMet homeostasis with the assistance of increased MAT activity. These experiments suggested that GNMT also protects cells in methionine depletion (8). GNMT Determines Folate Status and Folate-Dependent Remethylation: GNMT binds to 5-methyl-tetrahydrofolate (THF) and is inhibited by it (12). We discovered that GNMT+ cells are less sensitive to folate depletion, and that GNMT expression specifically improved folate status in folate restriction (13). Compared to wildtype mice that normally express GNMT at weaning, GNMT−/− that express human GNMT at birth had significantly elevated hepatic folate before weaning (13). Deletion of GNMT directly led to loss of hepatic folate in a dose-dependent manner. In addition, the hepatic methyl-folate-dependent enzyme MTR was reduced in GNMT−/−. The methylfolate-dependent homocysteine remethylation fluxes can be drastically increased when we restored GNMT function in GNMT−/− cells. We have reported concerns about perturbed methylation reactions in humans on methotrexate (MTX) therapy as this commonly used folate analogue (14) might also inhibit MAT gene, protein, and enzyme activity (15). We discovered that in addition to the improved folate status, restoring GNMT expression in HepG2 cells also significantly reduces MTX cytotoxicity (13). Taken together, GNMT expression increases cellular methyl-folate retention, and the retained folates are effectively used for 5-methyl-THF dependent remethylation.

GNMT is Essential for Genome Stability: GNMT is a susceptibility gene and a potential tumor suppressor for HCC (10). Deletion of GNMT increases the susceptibility
to liver cancer in mice (11, 16). GNMT might exert protective effects via benzo(a)pyrene detoxification and reduce DNA adduct formation in hepatocytes (17). Transient expression of GNMT induces apoptosis in cancer cells but not normal cells (18). Previous studies emphasize the antitumor activity of GNMT on carcinogen removal, detoxification, or prevention of abnormal cell proliferation. We proposed additional functions of GNMT on nucleotide biosynthesis and DNA stability. Our kinetic studies demonstrated that GNMT is critical for 10-formylTHF-dependent purine syntheses and it also assists methylene-THF-dependent purine syntheses particularly in folate depletion (19). The de novo thymidylate biosynthetic pathway in mammalian cells comprising multiple folate metabolic enzymes translocates to the nucleus for DNA replication and repair (20). Prolonged folate deficiency leads to chromosome damages including uracil misincorporation that can promote cancer risk. Restoring GNMT not only helps retain folate cofactors and improve nucleotide biosynthesis but, more importantly, it also protects cells from DNA damage during folate depletion (19). Our in vivo studies showed that GNMT deletion (GNMT<sup>−/−</sup>) resulted in significant uracil accumulation, regardless of dietary folate supply. Ample folate supplementation was effective in reducing uracil in mice with partial GNMT (GNMT<sup>−/+</sup>) to the same level as that of normal mice with an adequate folate supply; however, supplementation of 10 times the RDA folate failed to restore DNA integrity in GNMT<sup>−/−</sup> (19). Prior to this study, the tumor-suppressor role of GNMT was mainly linked to detoxification and anti-proliferation cell death role (21). Here we report a new role of GNMT in DNA protection that can account for human cancer prevention. The above series of studies showed the complex role of GNMT in maintaining optimal transmethylation, methylfolate dependent reactions, nucleotide biosynthesis and DNA integrity.

**Genetic Variations of MTHFR Modulate Cancer Risk**

MTHFR catalyzes the irreversible conversion of 5,10-methylene-THF to 5-methyl-THF, and it is inhibited by adoMet (21). This enzyme plays an important role in cellular folate coenzyme cycling and the partitioning of 1C units. The association between polymorphic variants of MTHFR and human cancer has been studied extensively. Polymorphisms in the MTHFR gene were identified as independent prognostic markers for progression-free survival in patients with early-stage B-chronic lymphocytic leukemia (22). A protective effect of the MTHFR T allele against human cancer has been reported in acute lymphocytic leukemia (23) and malignant lymphoma (24). However, MTHFR polymorphisms do not significantly contribute to an inherited genetic susceptibility to non-Hodgkin lymphoma or chronic lymphocytic leukemia (25). The protective effect of MTHFR was proposed to be closely associated with the folate status of these patients<sup>4</sup> and it is plausible that differences in folate status between study populations may in part account for the different findings on hematological malignancies. These studies indicated that MTHFR alters tumorigenesis in a tissue-specific manner, and this genetic predisposition is closely related to environmental factors including folate status.

**Cell Models of MTHFR Genetic Variations**

Homozygous mutation of cytosine to thymidine at nucleotide 677 (677C→T) of the MTHFR gene causes an alanine-to-valine substitution in this position in exon 4, resulting in a thermolabile enzyme with approximately 35% residual activity in vitro, and weaker binding with coenzyme riboflavin (26). MTHFR-deficient fibroblasts can synthesize normal or high serum from formate (27) suggesting that MTHFR not only regulates the availability of reduced folate coenzymes but also changes the partitioning of 1C moieties. Excessive L-methionine inhibits thymidine synthesis in Raji cells (28), presumably due to a shift of 1C unit utilization. Thymidine synthesis activity is highly dependent on methyleneTHF availability (27, 29); therefore MTHFR may modulate thymidine synthesis. It has been proposed that genetic variations of MTHFR may influence thymidine synthesis and improve the quality of DNA synthesis, which may confer the protective effect against the development of leukemia, but direct evidence was needed. A study using primed, constant infusions of isotopic tracers in healthy women found that total remethylation flux in monocytes was not affected by dietary folate restriction (30). The 677C→T polymorphism, or their combination. No significant impacts of MTHFR TT on nucleotide synthetic pathways were observed in these subjects (30). Kinetic studies on feasible in vitro models under more rigorously controlled folate conditions might help elucidate how MTHFR modulates utilization of 1C units between folate-dependent nucleotide synthesis and homocysteine remethylation. Stable isotopic kinetic studies enabled us to estimate enzymatic pools of cells (28). When comparing 1C metabolic kinetics between Epstein–Barr virus-transformed lymphoblasts expressing MTHFR 677C and MTHFR 677T, we discovered that MTHFR TT had stronger impacts on purine synthesis (34). TT cells can better utilize folate coenzymes for purine synthesis when folate is abundant, suggesting that increased cellular methyleneTHF would permit the use of more 1C units for nucleotide biosynthesis. On the other hand, the MTHFR TT genotype significantly reduced folate-dependent remethylation under folate restriction, reflecting limited methyl folates under folate restriction (34). The increased purine synthesis in adequate folate suggested an increased formedylate folate pool in MTHFR TT when folate supply is abundant. Moreover, the advantage of 1C partitioning into purine synthesis only exists when the folate supply is abundant. When folate was restricted, reduced MTHFR could no longer shift the partitioning toward formylTHF, and no such increase in purine synthesis flux was observed (34). The role of MTHFR in purine synthesis found in the MTHFR TT genotype may account for the protective effect of MTHFR in hematological malignancies. On the other hand, the mean percentage reduction in adoMet in TT was greater than that in CC cells in response to folate restriction, suggesting that TT was more sensitive to folate restriction with respect to adoMet homeostasis in these lymphoblasts (34). Mild folate restriction appeared to have stronger impacts on methionine and adoMet syntheses in the TT lymphoblasts. Under folate restriction the decreased MTHFR in TT lymphoblasts may direct 1C unit transfer toward purine synthesis at the expense of adoMet synthesis. These transformed cells are potential models for studying the consequences of human genetic variations.

**MTHFR C677T Polymorphism Modulates Cancer Risk in a Site-Specific Manner**

Previously, antiestrogen inhibition of MTHFR was found to decrease tumor cell survival in cancer cells derived from the colon, lung, breast, prostate and neuroblastoma. The compromised MTHFR activity was also associated with increased quantities of an apoptosis marker in cancer cells; hence MTHFR inhibition was proposed as a novel anticancer approach. MTHFR tends to reduce the risk of colorectal cancer, hepatocellular carcinoma (35), cervical cancer and certain leukemias and lymphomas but seems to increase the risk of cancer of the breast, endometrium, esophagus, stomach, pancreas and bladder (36). The modulation of MTHFR polymorphisms on tumorigenesis seems to occur in a site-specific manner, and it is closely related to folate status.

Human HCT116 colon and MDA-MB-435 breast cancer cells expressing mutant 677TT human MTHFR cDNA had accelerated cellular growth and elevated thymidine synthase activity (37), whereas we found no difference in thymidylate synthesis between TT and CC in the transformed lymphoblasts, suggesting tissue-specific impacts of MTHFR 677T on nucleotide synthesis. These findings partially explain the variability of association between MTHFR and occurrence of various types of cancer in humans. In HCT116 cells, the MTHFR 677T mutation exhibited significantly increased genomic DNA methylation in adequate or high folate but it exhibited reduced genomic DNA methylation in folate restriction. In contrast, the MTHFR 677T mutation in MDA-MB-435 cells had significantly decreased DNA methylation in adequate or high folate but had no effect in low folate. Data from these models give insights into how MTHFR C677T polymorphism may modulate the risk of colorectal and
breast cancers in a site-specific manner (38). In conclusion, the systematic series of studies gives new insights into the underlying mechanisms by which MTHFR and GNMT may participate in human tumor prevention.

Acknowledgments

Studied mentioned in this review were supported by the Ministry of Science and Technology, Taiwan, Republic of China NSC102-2320-B-005-006-MY3, NSC100-2628-B-005-002-MY4, TVGH-NCHU 997608, NSC-103-2911-I-005-301, and the Ministry of Education, Taiwan, R.O.C. under the ATU plan.

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