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The one-carbon metabolism pathway highlights therapeutic targets for gastrointestinal cancer (Review)

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Abstract. After the initial use of anti-folates for treatment of malignancies, folate metabolism has emerged as a rational diagnostic and therapeutic target in gastrointestinal cancer. The one-carbon metabolic pathway, which comprises three critical reactions (i.e., folate and methionine cycles), underlies this effect in conjunction with the trans-sulfuration pathway. Understanding of the one-carbon metabolism pathway has served to unravel the link between the causes and effects of cancer phenotypes leading to several seminal discoveries such as that of diadenosine tri-phosphate hydrolase, microRNAs, 5-FU and, more recently, trifluridine. In the folate cycle, glycine and serine fuel the mitochondrial enzymes SHMT2, MTHFD2 and ALDH1L2, which play critical roles in the cancer survival and proliferation presumably through purine production. In the methionine cycle, S-adenocyl methionine serves hydrocarbons and polyamines that are critical for the epigenetic controls. The trans-sulfuration pathway is a critical component in the synthesis of glutathione, which is involved in the production of reactive oxygen species in cancer stem cells. Therefore, characterization of one-carbon metabolism is indispensable to the development of precision medicine in the context of cancer diagnostics and therapeutics. In the present study, we review the historical issues associated with one-carbon metabolism and highlight the recent advances in cancer research.

Contents

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1. The multi-faceted one-carbon metabolic pathway

According to central dogma, information flow from the genome is dictated by the transcription of coding genes to mRNA, followed by translation to proteins. Multi-faceted omics information yields high-volume data associated with the whole-genome sequence, epigenome, methylome, transcriptome, proteome and metabolome, all of which have been linked to disease-specific cell phenotypes (1). The metabolome comprises of physiologically active substances such as nutrients (e.g., glucose), lipids, amino acids (e.g., serine and glycine) and nucleic acids. Importantly, in tumor cells, the processes of cell growth and proliferation requires construction of building blocks for new cellular components from substances associated with a redox status (Fig. 1) (2). One-carbon (C1) metabolism encompasses a complex metabolic network based on the chemical reaction of folate compounds (3). The folate cycle couples with the methionine cycle to form a bi-cyclic metabolic pathway that circulates carbon units as part of a process referred to as the C1 metabolism (3). These two cycles also link with the trans-sulfuration pathway, which plays a critical role in the regulation of the redox state by producing glutathione (3). C1 metabolism is critical for the maintenance of genomic stability through nucleotide metabolism as well as for the epigenetic control of DNA and histones, altered expression of which is a characteristic attribute of tumor cells. Ultimately, these findings should unravel new opportunities for translational approaches, drug discovery and studies of cancer pathogenesis. The study and control of C1 metabolism is the foundation for precision medicine in the context of disease prevention, identification of biomarkers, diagnosis, and treatment of various diseases, including cancer (3-5). High expression of C1 metabolic enzymes such as SHMT2, MTHFD2 and ALDH1L2 was shown to be independently associated with RFS. These findings suggest that mitochondrial folate metabolic enzymes could serve as potential therapeutic

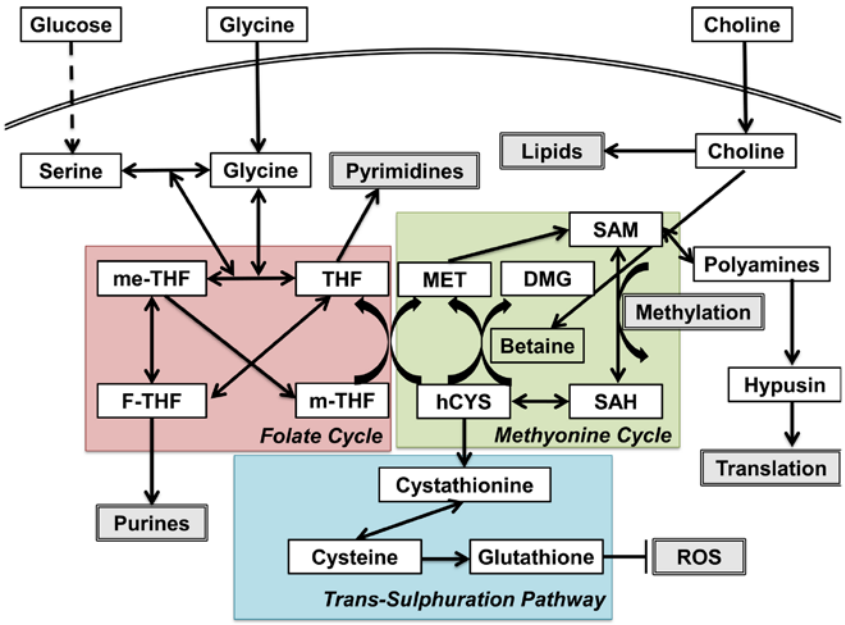

Figure 1. Multi-faceted functions of one-carbon metabolism. Three mitochondrial enzymes, SHMT2, MTHFD2 and ALDH1L2, play critical roles in cancer survival and proliferation presumably though purine production, and are thus suggested as potential diagnostic and therapeutic targets in gastrointestinal cancer cells. THF, tetrahydrofolic acid; me-THF, N5N10-methylene-tetrahydrofolic acid; m-THF, N5-methyl-tetrahydrofolic acid; F-THF, N10-formyl-tetrahydrofolic acid; MET, methionine; SAM, S-adenosylmethionine; SAH, S-adenosyhomocysteine; hCYS, homocysteine; DMG, dimethylglycine; ROS, reactive oxygen species.

targets for treatment of colorectal cancer (6). The genomic analysis of clinical samples is an entry point for developments in Precision Medicine. Here we highlight recent developments in C1 metabolism research.

2. Therapeutic targets in C1 metabolism

Naturally, researchers have considered folate metabolism as a plausible target for disease control. Antagonism of folate metabolism has been the principal plank of chemotherapeutic concept for more than 60 years. Farber and colleagues (7) noted that folic acid could stimulate proliferation of acute lymphoblastic leukemia (ALL) cells and wondered whether the intermediates of chemical synthesis could antagonize cell proliferation. They conducted a pioneering study in which they used aminopterin, one of the above-mentioned intermediates, to induce clinical remission in patients with ALL (8). Thereafter, multiple pathways downstream of C1 metabolism were identified and targeted by various cytotoxic chemotherapeutic agents. For example, methotrexate (MTX), an anti-folate agent that targets dihydrofolate reductase, is used to treat various cancer and is an effective therapy for rheumatoid arthritis (RA), despite its associated toxicity (9). The first documented use of 5-fluorouracil (5-FU) was reported by *Spears et al* (10); it was later approved for the treatment of colorectal cancer. 5-FU is an analogue of the DNA base, uracil, and is a potent thymidine synthase inhibitor that blocks methylation of dUMP to dTMP and disrupts the folate cycle (11). Similarly, gemcitabine, another nucleotide metabolism inhibitor in the C1 metabolic pathway, is used to treat pancreatic cancer (12). A previous study of gemcitabine-resistant pancreatic cancer cells indicated that microRNA-1246, which belongs to a class of non-coding RNAs, is involved in the modulation of chemotherapy resistance and cancer stem cell properties, which suggests a critical role of nucleotide metabolism in cancer cell metabolism (12). The conceptual basis of 5-FU has been used to develop a thymidine analog, trifluorothymidine (TFT), as discussed below.

Recently, C1 metabolic enzymes were shown to be novel therapeutic targets for cancer. Pandey *et al* (13) showed that inhibition of SHMT1 with targeted siRNAs reduced tumor size in a mouse xenograft model. Pickman *et al* (14) demonstrated inhibition of acute myeloid leukemia cells by MTHFD2 knockdown-induced suppression of TCA *in vivo*. Small compounds for inhibition of SHMT1 or MTHFD2 have already been identified (15-18). These compounds may undergo further development as novel drugs for cancer therapy in the foreseeable future.

Regarding nucleotide medicine, microRNAs have been shown to exert various effects on cells, such as epigenetic reprogramming via modulation of the methylation pathway (19,20). Later studies indicated that specific microRNAs, such as microRNA-302, could induce reprogramming in cancer cells, thus, identifying these as candidate moieties for treatment of refractory cancer cells from a nucleotide medicine perspective (21-23). Furthermore, microRNA-369 was shown to modulate the activity of a splicing factor of pyruvate kinase (PK), which induces metabolic reprogramming (24). Taken together, nucleotide metabolism plays a critical role in C1 metabolism and allows the generation of useful tools for mechanistic studies and therapeutic tools with which to target cancer cells.

Control of methylation events might be plausible, given the significance of epigenetic events with regard to the malignant phenotype of cancer (25,26). Previous research has shown that a temporarily distinct subpopulation of slow-cycling melanoma

cells in which the H3K4 demethylase JARID1B (KDM5B/PLU-1/RBP2-H1) play a role is required for continuous tumor growth (27). These slow-cycling cells, which exhibit slow DNA replication and are likely resistant to chemotherapeutic reagents (e.g., genotoxic agents) and radiation, may be instrumental in tumor relapse and metastasis. In solid cancers, KDM family members are implicated in carcinogenesis, and knockdown of associated genes has been shown to inhibit tumorigenicity and elicit cellular senescence (28,29). Several reagents, such as dimethyl sulfoxide (DMFO), have been developed to target methylation donors, ornithine decarboxylation (ODC), and polyamine metabolism and have been evaluated in clinical trials (30).

3. Application of nucleotide analogues in C1 metabolism

Nucleoside analogues, including deoxyadenosine analogues, adenosine analogues (31), deoxycytidine analogues, guanosine and deoxyguanosine analogues, thymidine and deoxythymidine analogues, and deoxyuridine analogues, can be used to target hepatitis B or C virus (HBV and HCV), herpes simplex virus (HSV) and human immunodeficiency virus (HIV). The uracil analogue, 5-FU, contains a fluorine atom in place of hydrogen at the C-5 position (32). 5-FU is the cornerstone of treatment for various malignancies, including colon, gastric and pancreatic cancers. Current strategy for cancer treatment usually includes a combination of cytotoxic drugs and more targeted drugs that affect, for example, signal transduction pathways. Furthermore, the efficacy of the combination drug tegafur/gimeracil/oteracil (TS-1 in Japan) in patients with advanced gastric cancer has been reported in an adjuvant setting (33). Gimeracil has been reported to inhibit tegafur degradation, thus, increasing the effect of tegafur. More recently, the thymidine analog TFT has been shown to be a potent inhibitor of DNA replication. Originally, the effects of TFT were evaluated in tumors transplanted into mice in the 1960s (34). However, the short half-life of TFT, which limits its clinical use as a chemotherapeutic agent, is yet to be overcome. TFT is an antiviral drug that interferes with DNA replication. This agent is thought to overcome signaling pathways involved in resistance to 5-FU derivatives (S-1) in several model settings. 5-chloro-6-(2-iminopyrrolidin-1-yl) methyl-2,4(1H,3H)-pyrimidinedione hydrochloride (TPI) is a potent inhibitor of thymidine phosphorylase, the enzyme that degrades FTD, and thereby potentiates the efficacy of TF *in vivo*. A TFT:TPI molecular ratio of 2:1 was used in TAS-102. Evaluation of this combination demonstrated that the cytotoxicity of TFT is enhanced by TPI. Furthermore, TPI also possesses antiangiogenic properties; specifically, this agent inhibits thymidine phosphorylase (TP). Evaluation of these drugs in combination with other cytotoxic agents for treatment of various cancers has also yielded consistent results. The combinatorial use of these agents with other targeted agents synergistically downregulates signal transduction pathways responsible for tumor growth, progression and metastasis. In patients with refractory colorectal cancer, TAS-102 was associated with a significant improvement in overall survival relative to the placebo in both phase II and phase III trials (35,36). Further studies to assess the efficacy of S-1 or TAS-102 in a neoadjuvant setting are underway (37-39). The above-described results clearly demonstrate that in the future, these agents will alter the effectiveness of anti-metabolite agents used for cancer chemotherapy.

4. Polyamines in C1 metabolism

The methionine cycle produces S-adenocyl methionine (SAM), which acts as a methyl donor in methylation reactions (40). SAM is involved in the methylation of histones, DNA and RNA, as well as of lysine and arginine in general proteins. SAM is coupled with ornithine metabolic pathway. In a study of PK, which catalyzes the last step of glycolysis, PKM2 knock-down in the allele contributed to the generation of SAM in mice (24), which suggests an important role of PKM2 in the modulation of cancer phenotypes via SAM-mediated control of methylation. PKM2, which results from alternative splicing of the PK gene, was preferentially expressed in tumors relative to PKM1, which is expressed in differentiated cells. PK contributes to the production and transportation of pyruvate in the mitochondria and is thus, associated with folate production in C1 metabolism. This gateway function of PK is altered in colorectal cancer, wherein the translocation of PKM2 protein into the nucleus via TGF-β stimulation has been observed in metastatic cancer cells (41); notably, pyruvate dehydrogenase is also affected in cancer cells (42).

SAM production is associated with polyamine metabolism in which ornithine decarboxylation (ODC) functions as a restricting step in the metabolic flow (43). Studies of an ODC enzyme revealed the characteristic cancer stem cell properties of fluorescent cancer cells harboring a GFP-ODC enzyme fusion cassette (44-46). These GFP-ODC labeled cancer cells exhibited the most aggressive tumorigenicity in immunodeficient mice, were resistant to chemotherapy and radiation therapy and exhibited reduced production of reactive oxygen species (ROS). A trans-omics mathematical analysis that linked metabolome data with transcriptome data revealed novel functions of the ornithine metabolic pathway in cancer stem cells (47). Given that ornithine is located upstream of polyamine metabolism, the polyamine flow might play a role in the maintenance of cancer stemness. Thus, C1 metabolism helps to control treatment-refractory cancer stem cells.

5. Diadenosine phosphate hydrolases in C1 metabolism

Although genetic alterations are not the sole pathogenetic mechanism of carcinogenesis, these factors undoubtedly play a significant role in disease initiation and progression (48-50). Studies of hereditary diseases that are known to predispose to cancer have indicated the involvement of ectopically activated oncogenes and the inhibition of tumor suppressor genes (51). In the 1990s, numerous studies suggested that in cancer patients, commonly deleted genomic regions might contain tumor suppressor genes (52); accordingly, introduction of these missing genes to cancer cells might inactivate tumor cell proliferation and cell cycle progression and thus suppress tumorigenicity (53). Positional cloning approaches to the identification of critical genes in the common fragile sites on chromosome 3p14 led to the identification of the fragile histidine triad (*FHIT*) gene, which encodes an enzyme with dinucleotide hydrolase activity (diadenosine tri-phosphate

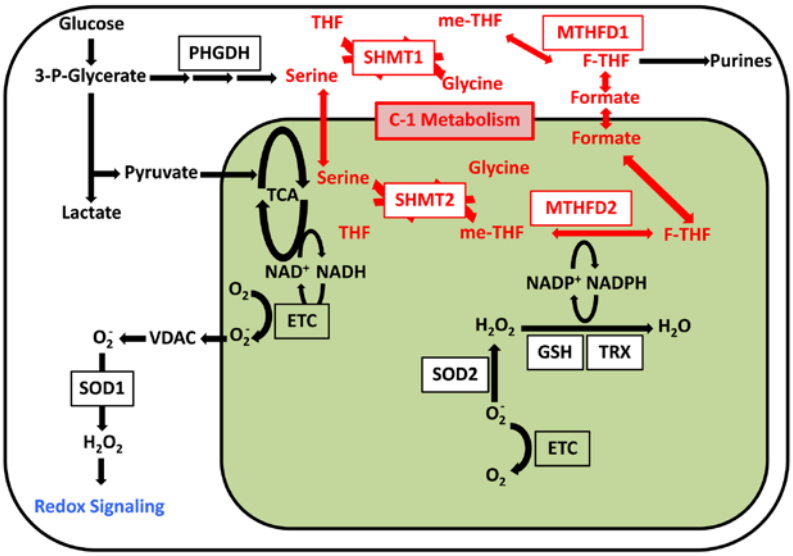

Figure 2. One-carbon metabolism in mitochondria. One-carbon metabolism comprises three critical reactions: the folate and methionine cycles and the trans-sulfuration pathway. In the folate cycle, glycine and serine fuel mitochondrial enzymes via purine production. In the methionine cycle, S-adenocyl methionine (SAM) serves both hydrocarbons and polyamines. The trans-sulfuration pathway is critical for the synthesis of glutathione, which is involved in the production of reactive oxygen species. Acting in unison, these molecules promote the survival and maintenance of gastrointestinal cancer cells. NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; ETC, electron transport chain; GSH, glutathione; PHGDH, 3-phosphoglycerate dehydrogenase; SOD1, superoxide dismutase 1; SOD1, superoxide dismutase 2; TRX, thioredoxin; VDAC, voltage dependent anion channel 1.

hydrolase) and a role in purine metabolism (54). A subsequent biochemical study indicated the importance of His96 as a catalyst for the hydrolysis of phosphoanhydrides such as Ap3A (55). More than 50% of human tumors exhibit focal deletion of this gene (56). Experiments in mice have indicated a deficiency in *FHIT*-induced genomic instability and spontaneous tumor formation, both of which were suppressed by the introduction of *FHIT* (3,57).

Studies of the *FHIT* loci genomic structure identified LINE-1, a human transposable element that is presumably involved in genomic deletion breakpoints associated with cancer (58,59). Since aphidicholine, an inhibitor of DNA polymerase α and δ, is known to affect the fragility of the above-mentioned common fragile sites (56), fragility in cancer cells might involve processes such as replication, recombination and DNA repair. Indeed, studies of gene function have indicated the involvement of Fhit protein in checkpoint system activation in response to genomic damage (60). In cancer, alterations to this checkpoint response have been linked to the activation of an Akt-survivin pathway-mediated cell survival mechanism (61). The mechanism by which the above-mentioned phenomenon occurs in tumors remains to be elucidated; however, DNA repair presumably requires the repair enzymes to appropriately incorporate nucleotide bases into DNA (3). Therefore, this historically important discovery of *FHIT* from the most active common fragile sites in the human genome indicates the homology of the encoded protein with dinucleotide hydrolase (62) and suggests that C1 metabolism leads to nucleotide metabolism in cancer cells.

6. ROS in C1 metabolism

Mitochondrial quality is known to influence cellular differentiation. For example, certain mutations in mitochondrial DNA (mtDNA) affect cellular reprogramming. Reprogramming induction in fibroblasts harboring mtDNA mutations revealed drastically reduced reprogramming efficiency of these cells relative to that of wild-type fibroblast cells (63). Reduced reprogramming efficiency has also been observed in human cells that harbor large mtDNA deletions (64), as well as in clonal human fibroblast cells with very high frequency of mt-tRNA point mutations. In addition, mtDNA has been suggested to affect reprogramming efficiency (57,58). However, the induced pluripotent stem cell lines showed different pathological mtDNA point mutations (20,25,63-66). In these cells, no significant difference in reprogramming efficiency was observed between the normal and mutated lines. Many studies have associated heteroplasmic mtDNA mutations with specific segregation patterns during reprogramming. This phenomenon was not only observed in the induced pluripotent stem cells, but also in mouse germ cells and during epiblast differentiation in monkey embryos (11,67).

Furthermore, tDNA mutation was found to induce ROS. ROS signaling determines cell fate. For example, mitochondrial ROS was shown to induce differentiation of hematopoietic stem cells (HSCs) (9,30). Therefore, ROS was thought to mediate signaling and thus affect cell differentiation. Induced pluripotent stem cells with mtDNA mutations retain high levels of ROS (63), although this phenotype can be rescued via treatment with antioxidants such as n-acetyl-l-cysteine (NAC). Altered ROS signaling is thought to induce the mtDNA mutation phenotype in stem cells (63). Therefore, the mitochondria is an organelle involved in signal transduction (Fig. 2).

7. Roles in cancer stem cell control

ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\cdot$), are highly chemically reactive species derived from molecular oxygen (68,69). ROS are generated

in the mitochondria (69). ROS can also be produced by various oxidases (e.g., NADPH oxidases and peroxidases) in different cellular compartments or organelles, such as the cell membranes, peroxisomes and the endoplasmic reticulum (70). Furthermore, chemotherapy, radioactivity, and even smoking can increase cellular ROS levels (66,71,72). A low level of ROS promotes cell proliferation and growth and increases cell survival (73). In contrast, a high level of ROS can cause cellular toxicity and trigger apoptosis (74,75). Cellular antioxidant systems can scavenge ROS and prevent irreversible cellular oxidative damage (76). It is important for cells to balance ROS generation and antioxidant activity, and redox regulation of cellular processes is essential for growth and development. ROS levels are increased in many cancer cells, and this is in part due to the higher metabolism rate (65,77). Aberrant ROS levels can elicit cancer cell apoptosis and necrosis (78). Cancer cells have a high antioxidant capacity to counteract and scavenge ROS. Because this high antioxidant capacity enhances cell survival and impairs cellular responses to anticancer therapy (79), induction of ROS-mediated damage in cancer cells with use of appropriate pharmacological agents that either promote ROS generation beyond the cellular antioxidative capacity or disable the cellular antioxidant system, has been considered as a radical therapeutic strategy for preferential targeting of cancer cells (79). Recently, cancer stem cells (CSCs) have gained attention as a subpopulation of cancer cells with stem cell-like properties and characteristics; these cells have been identified in the context of various cancers, including leukemia (80), breast (64) and pancreatic cancer (81). CSCs have the capacity to self-renew and differentiate and are thought to be responsible for cancer recurrence after chemotherapy or radiotherapy because of their ability to survive treatment and quickly generate new tumors (82,83). Characterization of CSCs have led to a perspective in which cancer therapeutic strategies should target not only normal cancer cells, but also CSCs. Given the importance of redox balance in cancer cells, conventional therapies (chemotherapy or radiotherapy) that target the redox balance could kill most cancer cells (67,79,84). However, the unique redox balance in CSCs and the underlying mechanisms that protect CSCs from ROS-mediated cell killing have not been fully elucidated (63,85,86).

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