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**Correlation between Amino Acid Metabolism and Self-renewal of Cancer Stem Cells: Perspectives in Cancer Therapy**

Amino Acid Metabolism and CSCs

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## **Abstract**

Cancer stem cells (CSCs) possess self-renewal and differentiation potential, which may be related to recurrence, metastasis, and radiochemotherapy resistance during tumor treatment. Understanding the mechanisms *via* which CSCs maintain self-renewal may reveal new therapeutic targets for attenuating CSC resistance and extending patient life-span. Recent studies have shown that amino acid metabolism plays an important role in maintaining the self-renewal of CSCs and is involved in regulating their tumorigenicity characteristics. This review summarizes the relationship between CSCs and amino acid metabolism, and discusses the possible mechanisms by which amino acid metabolism regulates CSC characteristics particularly self-renewal, survival and stemness. The ultimate goal is to identify new targets and research directions for elimination of CSCs.

**Key Words:** Amino acid metabolism; Cancer stem cell (CSC); Self-renewal; Resistance

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**Core Tip:** Amino acid metabolism plays an important role in maintaining the stemness of CSCs and is involved in regulating their self-renewal and differentiation potential. This review summarizes the relationship between CSCs and amino acid metabolism and discusses possible mechanisms *via* which amino acid metabolism regulates the self-renewal and differentiation potential of CSCs. The ultimate goal is to identify new targets and research directions for elimination of CSCs.

## **INTRODUCTION**

The concept of cancer stem cells (CSCs) has emerged in recent years. CSCs are a population of self-renewing cell types identified in many types of liquid and solid tumors, and persist predominantly in a low pH, low O<sub>2</sub>, and nutrient-deficient tumor

microenvironment (TME)<sup>[1, 2]</sup>. CSCs possess the capability to initiate cancer development, recurrence and metastasis<sup>[1-4]</sup>, and play important roles in radio-, chemo- and immunotherapy resistance<sup>[5, 6]</sup>. The TME is a dynamic milieu comprising of cancer cells and stromal cells<sup>[7-9]</sup> and provides specific conditions favorable to tumor growth such as low pH, hypoxia, ischemia, and limited nutrients<sup>[7]</sup>. TME regulates the morphology of cancer cells, induces tumor cell activation and CSC production, mediates immunosuppression, and determines tumor response to treatment<sup>[2, 7, 10-12]</sup>. The CSC niche is a part of the TME, in which perivascular, invasive, and hypoxic niches are involved in the generation and maintenance of CSCs<sup>[13, 14]</sup>. CSCs also rebuild the microenvironment by transdifferentiation into vascular endothelial cells, fibroblasts, and pericytes<sup>[7, 14]</sup>. CSCs obtain nutrients from TME to support their proliferation<sup>[15]</sup>. Owing to the increased interest in CSCs, the role of metabolism in the regulation of CSC biology is now being extensively investigated.

Amino acids are indispensable nutrients for the body and play important roles in TME<sup>[16]</sup>. The human body contains twenty amino acids, which are divided as essential and nonessential. Among these, eight essential amino acids are obtained from food, as they cannot be synthesized by the body or their rate of synthesis cannot meet the body requirements<sup>[17]</sup>. Although nonessential amino acids can be synthesized by the body, they are equally or more important than essential amino acids for cancer progression<sup>[18]</sup>. Arginine and histidine are semi-essential amino acids as their organic synthesis is not sufficient for metabolic requirements, and hence they have to be obtained from the environment<sup>[19]</sup>. However, some researchers consider cysteine and tyrosine as semi-essential amino acids as well because they can be converted from methionine and phenylalanine *in vivo*; thus, conversion and food intake can complement each other<sup>[20, 21]</sup>. In this review we have described these four amino acids as nonessential amino acids.

In addition to being the building blocks of proteins, amino acids participate in many biosynthetic pathways as intermediate metabolites<sup>[22]</sup>. Previously, researchers have studied the relationship between tumors and amino acid metabolism. In tumor cells,

nonessential amino acids may act as essential amino acids to meet the requirement of abnormal proliferation<sup>[23]</sup>. For example, glutamine is considered to be a “conditional” essential amino acid<sup>[24]</sup>, and therefore, it has been proposed that amino acid metabolism-related enzymes may be used to disrupt amino acid metabolism in targeted therapy<sup>[25]</sup>. Whether CSCs also harbor similar therapeutic targets warrants detailed investigation, but the exact relationship between CSCs and amino acid metabolism is not completely elucidated. This review tries to summarize the relationship between CSC self-renewal and other characteristics and amino acid metabolism to provide new targets for cancer therapy.

## **1. TUMORIGENICITY CHARACTERISTICS OF CSCS AND ESSENTIAL AMINO ACID METABOLISM**

In recent years, researchers have focused on the differences in amino acid metabolism between CSCs and tumor cells, in which essential amino acids play a major role. Several studies have investigated the relationship between methionine and tryptophan metabolism and CSCs; however, studies on phenylalanine metabolism are lacking, and those on the metabolism of the other five essential amino acids are limited. In this review, we have attempted to summarize the role of metabolism of these amino acids in CSC self-renewal.

### ***1.1 Methionine Metabolism***

The methionine cycle maintains the balance of methionine levels *in vivo*. Homocysteine, an intermediate of the methionine cycle, regenerates methionine and [tetrahydrofolate](#) (THF) with one-carbon THF (1C-THF) catalyzed by methyltransferase (MTase), while methionine reenters the methionine cycle. THF acts as a carrier in the transfer and utilization of 1C unit, which is crucial for biosynthesis of nucleic acids, DNA stability, and gene expression<sup>[26, 27]</sup>. As conversion of homocysteine to methionine is folic acid-dependent, content of folic acid affects the tumorsphere-forming ability, nucleotide biosynthesis, and DNA methylation in colon cancer cells and glioblastoma

cells<sup>[27-29]</sup>. Cancer cells consume higher amount of methionine than normal cells in some malignant tumors<sup>[30, 31]</sup>; hence, methionine and its derivatives may be labeled with radionuclides in clinics for identification of malignant recurrent glioma, meningioma, as well as prostate cancer and multiple myeloma<sup>[28, 32-34]</sup>. The methionine cycle is enhanced in CSCs of various cancers, such as lung, breast cancer, osteosarcoma, and brain tumor, owing to their disordered proliferation and higher rate of DNA biosynthesis<sup>[28, 35, 36]</sup>. As the concentration of methionine increases, the glioblastoma tumorsphere formation ability that supports the self-renewal capacity of CSCs increases; while methionine deprivation not only promotes embryonic stem cell (ESC) differentiation but also weakens clonal formation and tumorigenicity of lung and breast cancer tumorsphere cells, which can be rescued by the re-addition of methionine<sup>[28, 36]</sup>. It was further found that betaine, synthesized from choline, provides methyl group to homocysteine under the action of betaine homocysteine methyltransferases; this in turn leads to the recycling of methionine<sup>[29, 37, 38]</sup>. Stem cell reprogramming requires methionine metabolism and the choline/betaine axis to jointly regulate intracellular homocysteine, abnormality in which causes oxidative stress, mitochondrial toxicity, and inflammation<sup>[29]</sup>.

S-adenosine methionine (SAM) is a crucial intermediate of the methionine cycle, which acts as a direct donor of the methyl group and is involved in genome methylation *in vivo*<sup>[28, 39, 40]</sup>. In gastric CSCs, higher methylation of miR-7-5p promoter region reduces its intracellular expression, while in methionine-deficient medium, miR-7-5p expression is up-regulated and inhibits the formation of gastric CSCs by targeting Notch and Hedgehog signaling pathways<sup>[41]</sup>. Nuclear reprogramming is usually accompanied by an increase in DNA methylation level in ESCs<sup>[29]</sup>. Methionine adenosyltransferase (MAT) catalyzes the production of SAM and has two isozymes, MAT $\alpha$ 1 and MAT $\alpha$ 2<sup>[42]</sup>. MAT $\alpha$ 1 is mainly expressed in hepatocytes, while MAT $\alpha$ 2 is present in extrahepatic tissue<sup>[42, 43]</sup>. MAT $\alpha$ 2, which maintains the epigenome of CSCs, is a key enzyme involved in the SAM generation in lung, breast, and liver CSCs<sup>[36, 39, 44]</sup>. The inhibition of tumorsphere formation and genome methylation by MAT $\alpha$ 2 inhibitors FIDAS-5 and cycloleucine demonstrates that methionine circulation is necessary to

maintain CSC self-renewal and tumorigenicity<sup>[36, 39]</sup>. Another study found that sirtuin 1 (SIRT1), a NAD<sup>+</sup>- dependent protein deacetylase, regulated methionine metabolism and histone methylation by regulating MYC-mediated MAT expression in mouse ESCs (mESC)<sup>[45, 46]</sup>. Nicotinamide N-methyltransferase (NNMT) catalyzes the transfer of methyl groups from SAM to nicotinamide and is overexpressed in a variety of cancer cells. NNMT promotes cancer cell invasion, migration, and proliferation by inhibiting the methylation potential of cancer cells<sup>[47, 48]</sup>. Overexpression of NNMT in glioblastoma mesenchymal stem-like cells promotes hypomethylation of mesenchymal subtype genes by down-regulating DNA methyltransferase 1 (DNMT1) and DNMT3A<sup>[49]</sup>. Ras, Stat3, and NF-κB signaling pathways upregulate NNMT in cancer cells, which may be related to the epithelial-to-mesenchymal transition (EMT)<sup>[47]</sup>. Several other enzymes that catalyze SAM, such as DNMT1/3L, AMD1, SRM, and MTAP, are downregulated in colon CSCs. The reduction of DNMT1/3L, which catalyzes the transfer of methyl groups from SAM to DNA, leads to the accumulation of SAM in CSCs and thus affects DNA methylation<sup>[50]</sup>.

Overall, maintenance of CSC phenotype mainly requires methionine cycle and folic acid cycle, as they either directly supply CSCs with nutrients or participate in genome methylation as methyl donors. Therefore, reducing the exogenous intake of methionine and folic acid or blocking the methionine cycle may be new therapeutic directions, which are worth investigating<sup>[26, 51, 52]</sup>.

### ***1.2 Tryptophan Metabolism***

Tryptophan is a source of the 1C unit and high consumption causes changes in TME. Tryptophan 2,3-dioxygenase (TDO2), a rate-limiting enzyme in tryptophan metabolism, was overexpressed in esophageal CSCs and may promote their production by inducing Oct4 and CD44 expression and activating EGFR pathway, which stimulates EMT and invasion of esophageal CSCs<sup>[53, 54]</sup>. TDO2 is involved in the formation of tumorspheres of esophageal CSCs and TDO2 suppression reduces the size and number of spheres<sup>[53]</sup>. Indoleamine-2,3-dioxygenase-1 (IDO1), one of two IDO isozymes, is another rate-

limiting enzyme that catalyzes the production of kynurenine in tryptophan metabolism. Similar to TDO2, the expression of IDO1 is increased in breast and prostate CSCs as well as mesothelioma stem cells<sup>[55]</sup>. The increased IDO1 promotes immune escape by depleting tryptophan in TME and inducing the binding of tryptophan catabolites to aryl hydrocarbon receptor (AhR) resulting in regulatory T cell activation; this can be reversed by IDO1 inhibitors such as LW106<sup>[55-57]</sup>. Additionally, IDO also regulates tumor-related immune responses through molecular stress response pathways, mTOR kinase, and NF- $\kappa$ B pathway<sup>[56, 58, 59]</sup>. IDO1 and kynurenine pathway metabolites may promote colon cancer cell proliferation and cancer-therapy resistance by altering the PI3K/Akt and  $\beta$ -catenin pathways, which are known to be beneficial for self-renewal of colon CSCs<sup>[60-63]</sup>. <sup>1</sup>2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) is a tryptophan metabolite. ITE reduces the expression of Oct4 in CSCs by activating the AhR transcriptional pathway, thereby inducing CSC differentiation and ultimately reducing CSC tumorigenicity<sup>[53]</sup>. Tryptophan deprivation in TME decreases endogenous ITE level and increases Oct4 expression in CSCs, which subsequently maintains the stemness of CSCs<sup>[53, 64]</sup>. Recent findings on ITE synthesis and stimulation of the AhR transcriptional pathway have provided crucial targets for the treatment of CSCs<sup>[64, 65]</sup>. Tryptophan derivative, melatonin, may inhibit the proliferation and tumorigenicity of glioma stem cells by inhibiting the zeste homologue 2 and Notch pathways that are important for the survival of glioma stem-like cells<sup>[66, 67]</sup>. In conclusion, tryptophan metabolic enzymes or metabolites, rather than tryptophan itself, are more essential for CSC self-renewal and survival<sup>[53, 56]</sup> and provide new directions for eliminating CSCs.

### ***1.3 Threonine, Lysine, Leucine, Isoleucine, and Valine Metabolism***

Threonine is involved in the synthesis of nucleotides and is an important nutrient for mESCs<sup>[68]</sup>. Threonine content was significantly increased during tumorsphere formation of the colon cancer HCT116 cell line, but it was not explored in-depth<sup>[69]</sup>. Glycine and acetyl-CoA, produced by threonine dehydrogenase (TDH)-mediated threonine

metabolism, are involved in various biosynthetic pathways. Additionally, glycine produces 1C-THF, which results in SAM synthesis *via* the methionine cycle. SAM ultimately regulates epigenetic modifications in ESCs, which play a significant role in their stemness maintenance and self-renewal<sup>[68, 70]</sup>. TDH is highly expressed in mESCs, and inhibition of TDH or depletion of threonine in the growth medium reduces trimethylation of histone H3 Lysine 4 (H3K4me3) and ESC growth<sup>[68, 71]</sup>.

Studies on the relationship between lysine metabolism and CSCs are limited. Only a few studies have suggested that lysine metabolism in CD110+ colorectal CSCs not only reduces the production of reactive oxygen species (ROS), which suppress the proliferation of cancer cells, but also maintains the self-renewal of CSCs by activating the Wnt signaling pathway<sup>[72, 73]</sup>. Instead, researchers have extensively investigated the role of epigenetic modification of histone lysine residues in CSCs<sup>[74, 75]</sup>, which may be of greater relevance.

Studies on the relationship between leucine or isoleucine metabolism and CSCs are also limited. Several studies have focused on the leucine-rich repeat of G-protein coupled receptor 5, a gastrointestinal CSC biomarker<sup>[71, 76-78]</sup>. A few studies have found that leucine and isoleucine inhibit the stemness and self-renewal of EpCAM+ hepatocellular carcinoma stem cells by activating the mammalian target of rapamycin pathway complex 1 (mTORC1), in addition to enhanced chemotherapy sensitivity<sup>[72, 79]</sup>. But a recent study suggested that the reduction of leucine caused apoptosis of CD13+ CSCs in hepatocellular carcinoma, but the specific mechanism is not yet clear<sup>[80]</sup>.

Valine is reported to be elevated in canine mammary CSCs<sup>[72]</sup> and a decrease in valine can cause apoptosis of CD13+ CSCs in hepatocellular carcinoma by unknown mechanisms<sup>[80]</sup>. 3-Hydroxyisobutyryl-CoA hydrolase (HIBCH), which catalyzes 3-hydroxyisobutyryl-CoA to 3-hydroxyisobutyrate, is a key enzyme in valine metabolism and is highly expressed in a colorectal cancer, prostate cancer, and brain tumor. Elevated HIBCH promotes the initiation and progression of colorectal cancer by increasing the proliferation of tumor cells and the resistance to bevacizumab, while reducing cancer cell autophagy<sup>[81, 82]</sup>. In brain tissue with breast cancer metastasis,

HIBCH expression was significantly increased in the areas of reactive gliosis associated with metastatic cells, tumor margins, and hemorrhagic areas, which may provide metabolic substrates<sup>[82]</sup>. Although studies on metabolism of these five amino acids in CSCs is limited, we confirm their involvement in maintaining self-renewal, survival, and drug resistance of CSCs.

## **2. TUMORIGENICITY CHARACTERISTICS OF CSCS AND NONESSENTIAL AMINO ACID METABOLISM**

Humans do not have a dietary requirement for nonessential amino acids; however, they have crucial roles to play in CSC survival. To date, only one study illustrates the role of histidine metabolism in the central nervous system of *Drosophila*<sup>[83]</sup>. This section will further enumerate the roles of other nonessential amino acid metabolism in CSC biology.

### ***2.1 Glycine/Serine Metabolism***

Serine and glycine are commonly obtained *via* a branch of glycolysis and subsequent biosynthetic pathways. They can be interconverted by serine hydroxymethyl transferase (SHMT1/2), and participate in the folic acid cycle by providing a carbon unit<sup>[84]</sup>. Hence, in this review, we have jointly discussed the relationship between serine and glycine metabolism and CSCs. In colon CSCs, canine mammary CSCs, and neuroblastoma stem-like cells, the level of glycine is significantly higher than that in normal cancer cells<sup>[69, 72, 85-87]</sup>. If levels of glycine in colon CSC spheres are reduced, EMT suppression and induction of CSC apoptosis will occur<sup>[69]</sup>. Glycine decarboxylase (GLDC) is highly expressed in several cancers, including lung, ovarian, cervical, prostate, lymphoma, and breast except gastric cancer, catalyzes the conversion of glycine to 1C-THF, and participates in the methionine cycle<sup>[36, 84, 88-90]</sup>. The silent GLDC in gastric cancer may be due to hypermethylation of CpG islands in the promoter region of GLDC, which causes invasion and migration of gastric cancer cells<sup>[90, 91]</sup>. GLDC is also related to bone metastases from breast cancer and may increase the aggressiveness of malignant tumors

by aiding their metabolic adaptation to hypoxia<sup>[89, 92]</sup>. Overexpressed GLDC in non-small cell lung CSCs alters glycolysis, promotes cellular transformation and synthesis of pyrimidines for cell proliferation that eventually promotes tumorigenesis<sup>[88, 90]</sup>. GLDC knockout suppresses colony formation and CD166 on the surface markers of lung CSCs and reduces tumorigenicity<sup>[36, 88]</sup>. Glycine metabolism *via* glycine and glycine decarboxylase is a requirement to drive CSCs and promote tumorigenesis<sup>[72, 88]</sup>. Recently, it was found that a new splice variant of GLDC (GLDCV1) is overexpressed in non-small cell lung CSCs; its tumorigenic ability is similar to that of GLDC and can be exerted by activating MAPK/ERK signaling pathway and regulating cyclin<sup>[93]</sup>. The binding of c-Myc to GLDC promoter also results in GLDC overexpression in ESCs, which is critical for maintaining their stemness by adjusting H3K4me3 Levels; however, whether this is related to c-Myc in CSCs is not yet clear<sup>[94-96]</sup>. In glioblastoma multiforme, GLDC knockdown results in conversion of excess glycine into toxic aminoacetone and methylglyoxal by glycine C-acetyltransferase (GCAT), leading to highly expressing SHMT2 cell growth arrest<sup>[97]</sup>. Importantly, GCAT silencing and preemptive knockdown of SHMT2 can suppress the toxicity due to GLDC knockdown<sup>[97]</sup>. Thus, excess glycine is probably toxic to CSCs, and inducing excessive accumulation of glycine in CSC cytoplasm may be a new treatment strategy for glioblastoma<sup>[98]</sup>.

Serine is involved in nucleotide and one-carbon unit biosynthesis. It plays an important role in tumor cell proliferation and is found at a high level in colorectal CSCs, ovarian clear cell adenocarcinoma, cervical squamous cell carcinoma, and neuroblastoma stem-like cells<sup>[69, 87, 99]</sup>. In ovarian clear cell adenocarcinoma and cervical squamous cell carcinoma stem-like cells, the high levels of serine are accompanied by elevated levels of aspartate, glutamate, and glutamine, all of which are involved in the tricarboxylic acid cycle (TCA cycle)<sup>[99]</sup>. In neuroblastoma stem-like cells, upregulated activating transcription factor 4 can activate genes of the glycine/serine pathway to promote formation of tumorspheres<sup>[87, 100]</sup>. Moreover, in melanoma stem-like cells, up-regulated phosphoenolpyruvate carboxykinase, an enzyme in gluconeogenesis,

promotes tumorigenesis by promoting glycolysis and serine/glycine pathway<sup>[101]</sup>. Phosphoglycerate dehydrogenase (PHGDH) is the first key enzyme in the glycolytic serine biosynthetic pathway and is overexpressed in breast cancer<sup>[102-104]</sup>. The serine synthesized by PHGDH is converted to glycine by SHMT and then forms glutathione (GSH) to maintain intracellular redox balance<sup>[105, 106]</sup>. PHGDH was found to be preferentially expressed in hypoxia-induced breast CSCs and preserved the breast CSC stemness by maintaining the balance of redox reactions and shunting a portion of glucose-derived 3-phosphoglycerate<sup>[103, 104]</sup>. The shunt from glucose metabolism to serine metabolism produces NADPH, which can maintain the reduced state of GSH and forms an antioxidant barrier in breast CSCs<sup>[104, 107]</sup>. Additionally, high intra-tumoral co-expression of PHGDH and Oct4 in NT2/D1 (embryonal carcinoma stem-like cells) is beneficial for the survival of CSCs<sup>[103]</sup>. PHGDH can interact with kinesin family member 15, which is overexpressed in liver cancer cells and liver CSCs, and increase its stability to promote the liver CSC phenotype<sup>[108]</sup>. Because CSCs are more dependent on mitochondrial metabolic pathways than glycolytic pathways, mitochondrial inhibitors can limit their growth<sup>[107, 109, 110]</sup>. However, increased intracellular PHGDH expression was observed after the use of mitochondrial inhibitors, indicating that PHGDH may play a protective role against mitochondrial inhibitors in CSCs. Additionally, increasing the intake of exogenous serine or synthesis of intracellular serine also countervails the damage to CSCs caused by mitochondrial inhibitors<sup>[107]</sup>. PHGDH deficiency suppresses tumorsphere formation and reduces expression of stem factors (Oct4, Sox2, Nanog, Bmi-1) in breast CSCs, embryonal carcinoma, and brain tumor stem-like cells, and also impairs metastasis from breast to lung and increases chemotherapy sensitivity<sup>[103, 104, 106]</sup>. Mechanistically, the inhibition of PHGDH not only results in redox imbalance but also promotes the differentiation of CSCs through the degradation of Oct4 and the differential ubiquitination of  $\beta$ 3-tubulin; it also promotes p-AMPK mediated-Beclin-1 dependent autophagy in a p-mTOR-independent manner. These findings suggest that PHGDH is necessary for maintaining CSC stemness and self-renewal and may be a new metabolic target for eradication of CSCs<sup>[103, 104, 108]</sup>. Other enzymes, including SHMT1/2,

[phosphoserine phosphatase](#), [phosphoserine aminotransferase](#), and GCAT, required for glycine/serine metabolism, are up-regulated in non-small cell lung CSCs with different amplitudes and promote tumorigenesis by up-regulating glycine/serine metabolism<sup>[88]</sup>.

## 2.2 Glutamate and Glutamine Metabolism

Glutamate and glutamine, often upregulated in CSCs, have an amine group (-NH<sub>2</sub>) difference and glutamine is converted to glutamate *via* deamination by glutaminase (GLS), which constitutes the first step of glutaminolysis<sup>[111]</sup>. Glutaminolysis, a series of reactions in which glutamine is degraded to produce metabolic components and energy, may either replace or complement glucose dependence of cancer cells and CSCs<sup>[111-113]</sup>. Glutamine and glutamate are structurally similar and their roles *in vivo* are interrelated<sup>[114]</sup>. Glutamine is used in the biosynthesis of nucleotides, lipids, and amino acids; glutamate forms  $\alpha$ -ketoglutarate ( $\alpha$ -KG) catalyzed by glutamate dehydrogenase (GDH or GLUD), thereby producing ATP for cellular activities. Interestingly, the biological functions of both these amino acids may be specific to the cancer types<sup>[115-118]</sup>.

Glutamine acts as a “conditionally” essential amino acid in multiple CSCs because the biosynthesis of three major nutrients and nucleic acids requires glutamine to provide the source of carbon and amino nitrogen<sup>[99, 111, 112, 117, 119]</sup>. The glutamine transporter ASCT2 (also known as SLC1A5), encoded by *SLC1A5*, is highly expressed in various CSCs and is associated with tumor progression and poor prognosis<sup>[120, 121]</sup>. CD9-mediated ASCT2 plasma membrane localization increases glutamine uptake and provides energy for CSC growth in pancreatic ductal adenocarcinoma<sup>[122]</sup>. The up-regulation of MYC-regulated ASCT2 and GLS1 in colorectal CSCs increases glutamine metabolism and metformin resistance<sup>[118, 123-125]</sup>. MYC in CSCs is regulated by the tumor suppressor TP73/p73, and loss of TP73/p73 reduces the expression of MYC and GLS, thereby inhibiting ASCT2 and reducing glutamine-uptake and glutamine metabolism<sup>[126, 127]</sup>. ASCT2 also activates the downstream mTORC1 signaling pathway to promote the growth of prostate cancer cells or melanoma cells by increasing glutamine uptake<sup>[128, 129]</sup>. Of the other SLC1A family members, the upregulation of

SLC1A3 (also called glutamate aspartate transporter, GLAST) in CD133+ thyroid CSCs depends on the activation of the NF- $\kappa$ B pathway; SLC1A3 expression in glioblastoma stem-like cells depends on the activation of the STAT3 pathway triggered by glutamate, whereas SLC1A6 that acts as a glutamate exporter is down-regulated in EMT<sup>[130-132]</sup>. The higher glutamine and glutamate levels in ovarian clear cell adenocarcinoma and cervical squamous cell carcinoma stem-like cells are related to TCA cycle; in glioblastoma stem-like cells with high GLS expression, GLS inhibition attenuates the influx of glutamine metabolites into the TCA cycle<sup>[99, 111]</sup>. Exogenous glutamine *via* GLS induces tumorsphere formation and <sup>5</sup>expression of ALDH, a stem cell marker of head and neck squamous cell carcinoma, which can be prevented by glutamine deprivation and GLS inhibitors<sup>[112]</sup>. Glutamine not only promotes the expression of CSC markers and self-renewal potential of pancreatic CSCs, but also increases radiotherapy resistance by maintaining ROS stability<sup>[118, 133]</sup>. Glutamine also promotes clonogenic formation and stemness marker expression in non-small cell lung CSCs and hepatocellular carcinoma CSCs *via* the maintenance of redox balance and activation of the Wnt/ $\beta$ -catenin signaling pathway<sup>[119, 134]</sup>. Additionally, the AMPK-mTOR pathway is involved in the regulation of glutamine metabolism on the metformin sensitivity of colorectal CSCs; in absence of glutamine, the activation of AMPK and inhibition of mTOR will increase the sensitivity of metformin-resistant SW620 colorectal CSCs to metformin; however, as metformin-sensitive HT29 CSCs have an activated AMPK pathway, inhibition of glutamine metabolism will enhance the inhibitory effect of metformin<sup>[118, 125]</sup>. Moreover, mTOR inhibition in ovarian clear cell adenocarcinoma stem-like cells in absence of glutamine confirms that glutamine regulates CSCs through the mTOR pathway<sup>[135]</sup>. The ammonia molecule released by glutaminolysis also neutralizes the excess acid produced by the Warburg effect in epithelial CSCs, in which stemness and EMT are uncoupled<sup>[136]</sup>.  $\alpha$ -KG, another metabolite produced during glutaminolysis in ESCs, regulates the demethylation of DNA/histone to maintain pluripotency<sup>[137]</sup>. If the overexpressed GDH1 is suppressed in CSCs, the level of  $\alpha$ -KG will be reduced, which

not only reduces the production of ATP but also produces a large amount of ROS to damage CSCs<sup>[132]</sup>.

GLS has two isoenzyme forms, GLS1 and GLS2; GLS1 is a tumor promoter in many cancers, while GLS2 appears to be a tumor suppressor<sup>[123, 134, 138]</sup>. Recently, studies have found that GLS1 induced by distal-less homeobox-2 promotes the progression of transcription factor Snail-mediated EMT by negatively regulating p53 in colon and breast cancer. However, GLS2 inhibits Snail to prevent EMT in hepatocellular carcinoma independent of glutaminase activity; during breast cancer EMT, GLS2 and glutamine utilization are reduced, which can be rescued by the suppression of transcription factor, FOXC2<sup>[117, 123, 139, 140]</sup>. In intrahepatic cholangiocarcinoma and lung cancer, the expression of GLS1 is **negatively correlated with the expression of E-cadherin** but **positively correlated with** that of **vimentin**, and cells with low E-cadherin/high vimentin are more sensitive to GLS1 inhibitors<sup>[138, 141]</sup>. Additionally, aspartate aminotransferase (GOT1) is upregulated and system L-type amino acid transporter 1 is down-regulated in ovarian clear cell adenocarcinoma stem-like cells<sup>[135, 142]</sup>. Glutamine depletion and ASCT2/SLC1A3/GLS/GDH/GOT1 inhibition increase CSC apoptosis and sensitivity to therapy, all of which are new ways for CSC therapy<sup>[111, 119, 128, 133, 134]</sup>. For instance, GLS inhibitors CB839 and compound 968 suppress cloning ability of high GLS-expressing glioblastoma stem-like cells and reduce expression of stemness marker CD133. CB839 also selectively leads to cell cycle arrest without inducing apoptosis<sup>[111]</sup>. Other GLS inhibitors, such as BPTES and Zaprinast, effectively sensitizes pancreatic CSCs to radiotherapy and induces apoptosis through intracellular ROS accumulation<sup>[133]</sup>. SLC1A3 knockdown reduces intracellular glutamate levels and inhibits the self-renewal activity and tumorigenicity of CD133 + thyroid CSCs; SLC1A3 inhibitor UCPH-101 induces apoptosis of glioblastoma stem-like cells<sup>[130, 131]</sup>.

### 2.3 Cysteine Metabolism

Cysteine is a special amino acid that can be obtained not only from cystine conversion but also *via* homocysteine transsulfuration<sup>[31, 143]</sup>. The cystathionine produced from

homocysteine by cystathionine  $\beta$ -synthase (CBS) is further converted to cysteine by cystathionine  $\gamma$ -lyase (CGL). Cysteine then produces GSH so as to maintain the redox balance<sup>[143]</sup>. Increased homocysteine to cysteine metabolism is observed in tamoxifen-resistant breast cancer, and both CBS and CGL are significantly upregulated in CD133+ colon CSCs<sup>[50, 144]</sup>. The cystine-glutamate antiporter xCT (SLC7A11) on the cell membrane, which is stabilized by CD44/CD44 variant (CD44v) and overexpressed in breast CSCs, is associated with cystine intake as well as cysteine and GSH production<sup>[145-149]</sup>. CD133 in liver CSCs, CD44v in lung CSCs, and CD44v8-10 in esophageal squamous cell carcinoma and urothelial cancer stem-like cells upregulate or stabilize xCT against intercellular ROS, and overexpressed CD44v in lung CSCs is not related to stem-like properties<sup>[150-153]</sup>. Inhibition of xCT leads to changes in redox levels of breast CSCs, decreased survival rate, and reduced self-renewal<sup>[146, 147]</sup>. Sulfasalazine (SSZ), an inhibitor of xCT, selectively inhibits CD44+/CD44v+ CSCs, such as those in gastrointestinal tumors, metastatic bladder cancer, esophageal squamous cell carcinoma, and glioma, decreases intracellular GSH levels, and increases ROS levels<sup>[145, 152, 154-156]</sup>. The same SSZ effect also occurs in CD133+ liver CSCs<sup>[150, 157]</sup>. In CD44v<sup>high</sup> head and neck squamous cell carcinoma cells, the cytotoxicity of SSZ depends on ASCT2-dependent glutamine uptake and GDH-mediated production of  $\alpha$ -KG; GDH depletion and ASCT2 inhibition not only significantly attenuate SSZ-induced intracellular ROS accumulation but also weaken the inhibitory effect of SSZ on cell survival<sup>[120]</sup>. A phase I study on combined drug therapy in advanced non-small cell lung cancer (UMIN000017854) proposed that SSZ 1.5 g/day can be safely used in combination with standard-dose cisplatin and pemetrexed, but its side effects include intestinal toxicity and limited absorption<sup>[158]</sup>. Another phase 1 study in patients with refractory cisplatin CD44v+ gastric cancer (UMIN000015595) showed that a combination of 6 g dose of SSZ and cisplatin is feasible, but side effects and disappearance of the inhibitory effect of SSZ on xCT after oral administration were reported<sup>[159]</sup>. In addition, vaccines against xCT antigens induce xCT antibody production that mediates antibody-dependent cell cytotoxicity, resulting in redox

imbalance, inhibition of breast CSC phenotype and self-renewal, increased chemosensitivity, delay in primary tumor growth, and impaired pulmonary metastasis<sup>[146, 147, 160, 161]</sup>. Therefore, targeted inhibition and immunotargeting therapy of xCT promotes CSC apoptosis, which may provide new methods for adjuvant anti-cancer therapy<sup>[147, 150, 154, 155, 160]</sup>. Glutamate cysteine ligase (GCL) catalyzes condensation of cysteine, produced by above mentioned pathways, and glutamate to form  $\gamma$ -glutamyl-cysteine, which reacts with glycine to form GSH by the action of glutathione synthetase<sup>[162]</sup>. <sup>2</sup> GCL is composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM); upregulated GCLC, regulated by nuclear factor erythroid-derived 2-like 2, in breast CSCs can mediate the production of GSH to upregulate the expression of FoxO3a and Bmi-1, which are essential for maintaining stemness, whereas GCLM is induced in a HIF-1 dependent manner during chemotherapy or hypoxia<sup>[162, 163]</sup>. In short, GSH is the key to elucidating the role of cysteine metabolism in CSCs.

#### ***2.4 Aspartate and Asparagine Metabolism***

Aspartate and asparagine differ in cellular functions owing to their structural differences. In tumor cells, asparagine is involved in the synthesis of proteins and is a nitrogen source for the synthesis of purines and pyrimidines<sup>[164]</sup>. Recently, studies have focused on the regulatory role of asparagine in cancers. Asparagine regulates the cellular adaptation to glutamine depletion and inhibits glutamine depletion-mediated apoptosis; in case of sufficient availability of other amino acids, asparagine depletion also causes apoptosis<sup>[165]</sup>. Asparagine synthetase, which synthesizes asparagine from glutamine, is associated with tumorigenesis in lung cancer and poor prognosis in glioma and neuroblastoma as well as plays a crucial role in glutamine-dependent survival<sup>[165, 166]</sup>. Asparagine can also be used as an exchange factor for cellular uptake of amino acids, such as serine, arginine, and histidine, thereby activating mTORC1 and regulating amino acid metabolism<sup>[167]</sup>. Aspartate is also involved in the synthesis of nucleotides<sup>[168]</sup>. Aspartate and asparagine are upregulated in osteosarcoma stem-like cells, and GOT1, an enzyme that converts aspartate to oxaloacetate, is upregulated in

ovarian clear cell adenocarcinoma stem-like cells<sup>[35, 135, 169]</sup>. The upregulated aspartate in ovarian clear cell adenocarcinoma and cervical squamous cell carcinoma stem-like cells may be involved in TCA cycle reactions<sup>[99]</sup>. Although research on aspartate and asparagine in CSCs is limited, their established functions in cancer cells provides a basis for further research on CSCs.

### ***2.5 Alanine, Proline, Arginine, and Tyrosine Metabolism***

A recent study revealed that alanine and proline levels are increased in canine mammary CSCs, which may be related to maintenance of stemness<sup>[72]</sup>. Over-expression of glutamic pyruvate transaminase 2, which catalyzes the reaction between alanine and  $\alpha$ -KG to form pyruvate and glutamate, reduces the level of  $\alpha$ -KG in cells, thereby leading to proline hydroxylase 2 activity inhibition and HIF-1 $\alpha$  stabilization. HIF-1 $\alpha$  in turn activates the sonic hedgehog signaling pathway and promotes breast cancer tumorigenesis and CSC growth<sup>[170, 171]</sup>. Arginine and proline metabolism are upregulated in osteosarcoma stem-like cells. The level of arginine and ornithine, converted from arginine *via* arginase, increases significantly and participates in cell proliferation and urea cycle<sup>[35]</sup>. Addition of proline to Dulbecco's minimum essential media allows ESC to maintain pluripotency. Moreover, proline also induces ESC transformation to mesenchymal-like state and genome-wide reprogramming involving H3K9 and H3K36 methylation<sup>[172, 173]</sup>. TP73/p73 regulates proline metabolism in CSCs; loss of TP73/p73 reduces proline synthesis by inhibiting pyrroline-5-carboxylate reductase 1, which catalyzes proline formation from pyrroline-5-carboxylate<sup>[126]</sup>. In addition, proline metabolism also plays an important role in the self-renewal of human breast CSCs *via* proline dehydrogenase (PRODH), and inhibiting PRODH damages spheroidal growth and metastasis<sup>[72]</sup>. CD13+ CSCs are habituated to tyrosine metabolism in hepatocellular carcinoma; acetyl-CoA, produced by tyrosine metabolism, not only enters the TCA cycle to provide energy to CD13+ CSCs, but also promotes the transcription factor Foxd3 acetylation to maintain the CD13+ CSC self-renewal<sup>[80]</sup>. Due to the lack of phenylalanine hydroxylase in CD13+ CSCs, deprivation of phenylalanine

has no effect on cell survival<sup>[80]</sup>. Thus, the metabolism of arginine, alanine, proline, and tyrosine seem to be necessary in CSCs of specific cancer species.

### **3. RELATIONSHIP BETWEEN AMINO ACID METABOLISM AND TUMOR MICROENVIRONMENT**

In addition to regulating CSCs, amino acid metabolism is also interconnected with TME or CSC niches as TME plays an important role in maintaining the self-renewal of CSCs<sup>[119, 174]</sup>. As microenvironments of different cancer types exhibit variable conditions (glucose concentration and oxygen tension), CSCs display diverse metabolic phenotypes to adapt to these microenvironments<sup>[113, 175-177]</sup>. For instance, in the absence of glucose, glutamine compensates for the shortage of glucose<sup>[12, 113]</sup>. However, in the absence of glutamine, extracellular asparagine becomes critical because intracellular asparagine is redirected to glutamine synthesis to avoid apoptosis<sup>[165, 178, 179]</sup>. Glutamine metabolism is also affected in TME as interleukin-4 (IL-4), secreted by immune cells, increases ASCT2 expression in breast cancer cells<sup>[180, 181]</sup>. Growth factor IL-3 in TME, through binding to IL-3R $\alpha$ , up-regulates ASCT2 expression and promotes glutamine uptake *via* the JAK/STAT pathway<sup>[180, 182]</sup>. Hypoxic microenvironment causes the accumulation of lactate, which affects ASCT2 and GLS1 expression by activating c-Myc<sup>[180, 183]</sup>. Glutamine-dependent ovarian cancer cells form a glutamine loop with cancer-associated fibroblasts (CAFs) within TME; tumor cells convert glutamine to glutamate, which is regenerated into glutamine by CAFs to supply to tumor cells<sup>[12, 184]</sup>. CAFs also secrete cysteine and GSH, which are absorbed by ovarian cancer cells to induce resistance to platinum-based chemotherapy. However, drug resistance induced by TME is destroyed by effector T cells, which suppress xCT expression of CAFs through the JAK/STAT1 pathway<sup>[180, 185]</sup>.

Cancer cell metabolism produces an acidic, hypoxic, and malnourished TME, which is detrimental to the antitumor immune response<sup>[186]</sup>. The main amino acids in the tumor immune microenvironment are tryptophan and arginine, whose increased catabolism is a common marker of TME<sup>[187]</sup>. Cells that decompose tryptophan and

arginine, such as myeloid-derived suppressor cells and tumor-associated dendritic cells, induce regulatory T cells and suppress effector T cells to suppress antitumor immunity and promote tumorigenesis<sup>[187, 188]</sup>. Interestingly, tryptophan has a significant effect on T cell survival and function<sup>[189]</sup>. Tumor cells that overexpress IDO show reduced extracellular tryptophan, which affects the effector function of T cells<sup>[189]</sup>. Whereas kynurenine, an immunosuppressive product of tryptophan metabolism, induces CD4+ T cells to differentiate into regulatory T cells by activating AhR, which weakens the ability of the immune system to recognize and kill cancer cells<sup>[178, 190]</sup>. However, it does not seem to be a contradiction, as the expression of IDO, extracellularly consumed tryptophan, and synthesized kynurenine all synergistically inhibit T cell proliferation and activation<sup>[188]</sup>. The increased IDO1 in breast and prostate CSCs also activates regulatory T cells through the kynurenine pathway to promote immune escape<sup>[55]</sup>. The presence of arginine promotes the effector function and survival of T cells, which indicates that the lack of arginine in TME leads to T cell dysfunction<sup>[12, 184, 188]</sup>. Citrulline and ornithine, downstream metabolites of arginine, also affect T cell activation<sup>[178]</sup>. Glutamine is not only used for cancer cell metabolism, but also provides nitrogen and carbon sources for active T cells in TME<sup>[178, 191]</sup>. Glutamine metabolism is essential to B cell proliferation and differentiation into plasma cells, and macrophage antigen presentation and phagocytosis<sup>[188, 189]</sup>. Amino acids such as serine and alanine are also critical to the tumor immune microenvironment. Serine provides purines for T cell proliferation, but has no effect on T cell function. Conversely, alanine affects T cell effector function and proinflammatory cytokine secretion by promoting T cell protein synthesis and initial activation<sup>[189, 192]</sup>. In addition to tumor metabolism and immune microenvironment, amino acid metabolism also has an influence on the structural microenvironment. Collagen, the main component of the extracellular matrix in TME, is degraded to proline by the action of metalloproteinases and collagenases. The extracellular proline is an energy source for tumor cells and may be resynthesized into collagen to promote the extracellular matrix remodeling, which is responsible for cancer cell reprogramming<sup>[174, 193, 194]</sup>. High-density extracellular collagen matrix also shifts the

metabolism of metastatic breast cancer 4T1 cell line from glucose to glutamine<sup>[195]</sup>. All the above evidence shows that amino acid metabolism, cancer cells or CSCs, and TME form a complex regulatory network that can be efficiently applied in clinical research.

## **CONCLUSION**

The concept of CSCs, a class of cells with potential for self-renewal and differentiation, was proposed owing to the emergence of recurrence, metastasis, and drug or radiotherapy resistance in tumors, and renders tumor treatment challenging<sup>[196]</sup>. Currently, researchers are focusing on issues related to the metabolism within CSCs and attempting to identify new research directions and therapeutic targets to eliminate CSC population. In TME, researchers have indicated the involvement of amino acids as a nutrition and energy source, apart from glycolysis, by demonstrating abnormal mitochondrial function in tumor cells<sup>[197, 198]</sup>. Amino acids are not only involved in protein synthesis, but also participate in important biosynthetic pathways as intermediate metabolites. As amino acids are important nutritional components in TME, an increasing number of studies are focusing on the role of amino acids in self-renewal and other biological characteristics of CSCs. This review focuses on the role of amino acid metabolism in CSC biology, particularly self-renewal and their mechanism of action.

The role of 20 amino acids in CSCs is summarized in Table 1. The metabolism of certain amino acid plays an important role in the self-renewal of CSCs, such as methionine, tryptophan, glycine, serine, and glutamine. The effects of amino acids depletion in TME or inhibition of key enzymes on the self-renewal and survival of CSCs further illustrate the influence of amino acid metabolism on the characteristics of CSCs and provide potential targets for cancer therapy. An increasing number of clinical trials focus on targeting key proteins in amino acid metabolism pathways in CSCs. For example, xCT, a cystine-glutamate antiporter, plays an important role in CSC self-renewal; clinical studies involving its inhibitor, SSZ, in advanced non-small cell lung cancer (UMIN000017854) and refractory cisplatin CD44v+ gastric cancer

(UMIN000015595) suggest the potential feasibility of targeting amino acid metabolism transporters for tumor therapy<sup>[158, 159]</sup>. In two clinical cases of CD44v9-positive urogenital cancer, SSZ was also used as a new adjuvant treatment approach<sup>[199]</sup>. Further, parthenolide and piperlongumine targeting aberrant glutathione metabolism in leukemia stem cells<sup>[200]</sup>; pegcrisantaspase depleting plasma glutamine and asparagine in relapsed/refractory acute myeloid leukemia<sup>[201]</sup>; and L-asparaginase exhausting asparagine in acute lymphoblastic leukaemia<sup>[202]</sup> highlight the extensive prospect of targeting amino acid metabolism in cancer therapy. Additionally, key metabolic enzymes also act as potential targets for CSC-based cancer therapy; they are listed in Table 2 and Figure 1.

In conclusion, the role of amino acid metabolism is varied in different cancer types and metabolism of amino acids are interlinked, which adds to the complexity of TME. Based on these reports, we expect the future research on amino acid metabolism to be based on cancer types, amino acid interrelations, and TME. Only through this research path, can we propose better solutions for CSC clinical therapy and ultimately prolong patient life-expectancy.

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