HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2021 March 25.

Published in final edited form as:

Oncogene. 2020 October ; 39(44): 6747–6756. doi:10.1038/s41388-020-01480-z.

MULTIFACETED ROLE OF BRANCHED-CHAIN AMINO ACID METABOLISM IN CANCER

Hui Peng[1], **Yingfei Wang**[1,2,*], **Weibo Luo**[1,3,*]

[1]Department of Pathology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390, United States

[2]Department of Neurology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390, United States

[3]Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390, United States

Abstract

Metabolic reprogramming fulfils increased nutrient demands and regulates numerous oncogenic processes in tumors, leading to tumor malignancy. Branched-chain amino acids (BCAAs, i.e., valine, leucine, and isoleucine) function as nitrogen donors to generate macromolecules such as nucleotides and are indispensable for human cancer cell growth. The cell-autonomous and non-autonomous roles of altered BCAA metabolism have been implicated in cancer progression and the key proteins in the BCAA metabolic pathway serve as possible prognostic and diagnostic biomarkers in human cancers. Here we summarize how BCAA metabolic reprogramming is regulated in cancer cells and how it influences cancer progression.

Keywords

BCAA; BCAT; BCKDH; metabolic reprogramming; cancer progression

BCAA metabolism---An emerging oncogenic metabolic pathway in cancer

Metabolism is the collection of life-sustaining biological activities, fueling organisms with energy to drive cellular processes and providing the new cells with building blocks. Distinct to normal non-proliferating cells, the malignant properties of cancer cells, including rapid proliferation and aggressive invasion into normal tissues, require altered metabolism to meet increased nutritional and biosynthetic demands [1, 2]. A comprehensive understanding of how metabolic reprogramming engages in cancer development is beneficial for the identification of prognostic biomarkers and therapeutic targets leading to the development of better diagnosis and treatment.

*Correspondence: Weibo Luo, Weibo.Luo@UTSouthwestern.edu ; Yingfei Wang, Yingfei.Wang@UTSouthwestern.edu.

Conflict of interest

The authors declare no potential conflicts of interest.

As one of the most fundamental bricks of cell structure, amino acid is largely needed to support the synthesis of proteins for cell proliferation. Some amino acids can be synthesized intracellularly while others lack their *de novo* biosynthetic pathways inside the cell and have to be derived from the diet, therefore termed nonessential amino acids and essential amino acids, respectively. Branched-chain amino acids (BCAAs), i.e., valine, leucine, and isoleucine, belong to a group of essential amino acids. In addition to incorporation into proteins directly, BCAAs can be also broken down to produce a number of metabolites such as glutamate during the degradation process, thereby associating with other metabolic pathways that are critical for tumorigenesis. The significance and essentiality of BCAA metabolic reprogramming are recently highlighted in many types of human cancers [3-5], including glioblastoma [6, 7], pancreatic ductal adenocarcinoma (PDAC) [8-10], leukemia [11-13], non-small cell lung cancer (NSCLC) [14], breast cancer [15], ovarian cancer [16], clear cell renal cell carcinoma (ccRCC) [17], bone sarcomas [18], endometrial cancer [19], and hepatocellular carcinoma (HCC) [20]. Various models have been proposed to elucidate the role of altered BCAA metabolism in tumor progression, stem cell maintenance, and drug resistance, indicating that targeting BCAA metabolism is an appealing therapeutic approach for the treatment of human cancers. Here we summarize regulation of BCAA metabolic reprogramming in cancer cells and the mechanisms of altered BCAA metabolism-mediated cancer progression.

The BCAA metabolic network

BCAAs are imported into the cell by L-type amino acid transporters (LATs) [21-23] and SLC25A44 is responsible for BCAA transport into the mitochondria [24]. Intracellular BCAAs are then converted to branched-chain α-keto acids (BCKAs) including α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV), and α-ketoisovalerate (KIV) by branched-chain amino acid transaminases (BCATs), which meanwhile transfer the amino group from BCAAs to α-ketoglutarate (α-KG) to produce glutamate (Fig. 1). BCAT has two isoforms BCAT1 and BCAT2 encoded by their own genes and located in the cytosol and the mitochondrion, respectively. BCAT2 is ubiquitously expressed while the expression of BCAT1 is restricted to certain organs, such as the brain [25]. The BCAT-catalyzed transamination reaction is reversible, which enables the production of BCAAs by reamination of BCKAs that may be derived from other tissues [12]. Monocarboxylate transporter 1 was identified to mediate BCKA secretion into the extracellular space in glioblastoma [26]. By doing so, the balance between BCAAs and BCKAs is finely maintained in the cell. Apart from controlling BCAA and BCKA levels, BCAT is also critical for homeostasis of intracellular α-KG and glutamate levels [13]. α-KG is a key intermediate in the tricarboxylic acid (TCA) cycle and also an important co-substrate for a group of α-KG-dependent dioxygenases that are involved in hypoxia response, metabolism, and epigenetics [27, 28]. Glutamate contributes to a number of metabolic fates in proliferating cancer cells, including protein synthesis and synthesis of nucleotides and other nonessential amino acids [29, 30]. Therefore, BCAAs are the important nitrogen donors in cancer cells.

Immediately after BCAA catabolism, BCKAs undergo irreversible decarboxylation catalyzed by the branched-chain α-keto acid dehydrogenase (BCKDH) complex in the

mitochondria (Fig. 1). The catalytic activity of the BCKDH complex is negatively determined by its phosphorylation status, which is controlled by a pair of enzymes, branched-chain keto acid dehydrogenase kinase (BCKDK) and Mg^{2+}/Mn^{2+}-dependent 1K protein phosphatase (PPM1K) (Fig. 1). BCKAs are eventually metabolized into acetyl-coenzyme A (acetyl-CoA) and succinyl-CoA that can fuel into the TCA cycle for energy production. A recent report showed that about 1-3% of TCA intermediates are derived from BCKA oxidation in PDAC [10]. But this finding was not supported by other studies, which reveal no integration of the carbon flux of BCAAs into the intermediates in the TCA cycle in cancer cells [8, 11, 14].

The regulation of BCAA metabolism in cancer

Reprogramming of BCAA metabolism is determined by altered expression and activity of BCAA transporters and metabolic enzymes involved in the BCAA metabolic pathway. It has been reported that BCAT is regulated by oncogenes and tumor suppressors in cancer cells, leading to tumorigenesis (Table 1). The promoter region of *BCAT1* harbors the binding sites of several transcriptional regulators, including c-Myc [31-33], hypoxia-inducible factor (HIF) [6], and SMAD5 [10]. Both the mRNA and protein levels of BCAT1 have been demonstrated to be upregulated by HIF-1 in human glioblastoma cell lines and primary glioblastoma spheres under hypoxic conditions [6]. Although both HIF-1α and HIF-2α can directly bind to the hypoxia response element at the first intron of *BCAT1* gene, only HIF-1α is functional in activating BCAT1 transcription [6]. Similarly, the BCAA transporter LAT1 is also induced by HIF-1 and HIF-2 in human glioblastoma under hypoxia, while HIF-2 is responsible for LAT1 expression in ccRCC cells [6, 34]. BCAT2 expression is not affected by hypoxia or HIF in glioblastoma [6]. The metabolic tracing experiment showed that hypoxia increases nitrogen transfer from BCAAs to glutamate, which is abolished by knockout of HIF-1/2α, indicating that HIF is a key regulator of BCAA metabolic reprogramming in human glioblastoma cells in response to hypoxia [6]. Musashi2 (MSI2), which is abnormally activated in many human malignancies such as glioma and breast cancer, was suggested to bind to MSI binding elements located in the 3’ untranslated region of BCAT1 in human chronic myeloid leukemia cell line and positively regulates BCAT1 transcription [12]. Upon activation of transforming growth factor-β, SMAD5 is translocated into the nucleus and binds to the *BCAT1* promoter to induce its expression in cancer-associated fibroblasts from PDAC tumors [10]. The transcription factor sterol regulatory element-binding protein 1 (SREBP1) was shown to activate BCAT2 transcription in pancreatic cancer cells [35]. In addition, upregulation of BCAT2 expression is also controlled by mutant *KRAS* oncogene at post-translational levels. The tyrosine kinase SYK is downregulated in *KRAS*-mutant PDAC leading to reduced phosphorylation of tyrosine 228 on BCAT2 protein, thereby reducing the ubiquitin E3 ligase TRIM21-mediated ubiquitination and subsequent protein degradation of BCAT2 [8].

Several nuclear receptors were shown to control the expression of genes involved in BCAA metabolism. Peroxisome proliferator-activated receptor γ (PPAR-γ) coactivator 1α (PGC-1α) is able to induce BCAT2 and BCKDHA expression but not BCKDK expression in transgenic mice possibly through multiple nuclear receptors [36]. Transcription factors such as PPAR-γ and Krüppel-like factor (KLF) 4 were also shown by bioinformatics analysis to

be enriched in *Bcat2* and/or *Bckdha* genes in PGC-1α transgenic mice [36]. The glucocorticoid receptor-KLF15 axis co-activates BCAT2 expression in rat muscle cells by binding to its promoter [37]. Peroxisome proliferator-activated receptor-α (PPAR-α) is suggested to activate the BCKDH complex by downregulating BCKDK in the rat liver [38]. On the other hand, BCAA-fed mice showed higher expression levels of PPAR-α and PPAR-γ in muscle, liver, and white adipose tissue [39, 40], indicating a feedback mechanism between nuclear receptor and BCAA metabolism.

The transcription of BCAT1 is inhibited by DNA methylation at its promoter in isocitrate dehydrogenase (IDH) mutant anaplastic astocytoma and glioblastoma, which is correlated with BCAT1 downregulation [7]. Methylation of *BCAT1* promoter is reduced in HCC leading to increased BCAT1 expression [41]. Histone modifiers G9a and SUV39H1 catalyze di- and tri-methylation of lysine 9 of histone H3 (H3K9) at the promoter of *BCAT1* gene leading to downregulation of BCAT1 in lung cancer cells [42]. Likewise, EZH2, the catalytic subunit of the polycomb repressive complex 2 that causes H3K27 methylation, suppresses the expression of BCAT1 in leukemia [11]. The transaminase activity of BCAT1 and BCAT2 is also inhibited by the oncometabolite *R*-2-hydroxyglutarate (2-HG), which is produced by mutant IDH1/2 in glioma and competes with α-KG for binding [43]. Collectively, the expression levels of BCAT and BCKDH are finely controlled by oncogenic factors and tumor suppressors thus driving BCAA metabolic reprogramming (Table 1).

The mechanisms of altered BCAA metabolism-mediated cancer progression

Recent studies reveal the cell autonomous and non-autonomous roles of altered BCAA metabolism in cancer progression (Table 2). BCAA metabolic reprogramming produces the intermediates that rewire other metabolic pathways and also alter mitochondrial functions and gene expression, thereby promoting cancer cell proliferation.

BCAA catabolism and mTOR signaling pathway in cancer

Several studies implicated that low BCAA catabolism causes cancer progression [18, 20]. Elevated BCAA levels in plasma and tumor tissues, often accompanied by the reduction of BCAA catabolism, have been observed in many types of human cancers (Table 2), including HCC [20], breast cancer [15], leukemia [11, 12], early PDAC [44], and ccRCC [17]. BCAT1 catalyzes reamination of BCKAs possibly from blood circulation to enable these cancer cells to accumulate BCAAs [12]. It has been reported that leucine binds to its sensor Sestrin2 to activate the mechanistic target of rapamycin complex 1 (mTORC1) [45]. mTORC1 triggers a cascade of signaling pathway through phosphorylating its downstream effectors, including eukaryotic translation initiation factor 4E binding protein 1, p70S6 kinase, and SREBP, to regulate autophagy and the synthesis of lipids, nucleotides and proteins [46]. The aberrant activation of the mTOR signaling pathway resulting from genetic alterations or altered levels of the upstream signal has been implicated in tumor progression and becomes a target for the treatment of cancer [46, 47]. Indeed, increased BCAA levels in the aforementioned types of cancer cells have been suggested to promote tumor progression via the activation of mTORC1 (Fig. 1).

BCAA metabolism and α-KG-dependent gene expression in cancer

As an amino acceptor, α-KG is deprived by high BCAT1 levels in a broad range of different types of cancer cells, such as acute myeloid leukemia (AML), glioma, and breast cancer [13]. Raffel S *et al.* showed that increased BCAA degradation by BCAT1 overexpression is required for proliferation, survival, and stemness maintenance of leukemia stem cells from *in vitro* cell culture and *in vivo* tumors in AML patients via the restriction on α-KG levels [13].

The mechanism of α-KG-mediated tumor malignancy engages a group of dioxygenases, which use α-KG as a co-substrate and decarboxylate it into succinate in the reaction. α-KG-dependent dioxygenases require the involvement of oxygen, Fe(II), and ascorbate in addition to the obligatory co-substrate α-KG, meaning that the abundance of α-KG has a direct impact on the enzyme activity [13, 48]. This group of enzymes play important roles in hypoxia signaling and shaping epigenetic landscapes, which are frequently dysregulated in cancer cells [27, 48, 49]. As an example of α-KG-dependent dioxygenases, EGLN prolyl hydroxylases catalyze hydroxylation on proline residues of HIF-α, which is necessary for subsequent proteasomal destruction via the von Hippel-Lindau/cullin-2/Elongin-B/C complex under normoxia [50, 51]. When O_2 availability markedly drops (hypoxia), a hallmark of many solid tumors, HIF-α protein is stabilized and dimerizes with HIF-1β, thereby stimulating the downstream targets to promote cancer progression [52-54]. Reduced α-KG levels caused by increased BCAT1 activity in AML were shown to attenuate the EGLN prolyl hydroxylase activity, hence causing HIF activation to induce HIF target gene expression in order to help cancer cells survive even under normoxic conditions (Fig. 1) [13].

Similarly, diminished intracellular α-KG pools are likely to reduce the activity of another α-KG-dependent dioxygenase, the ten-eleven translocation 2 (TET2) DNA demethylase as well, which leads to DNA hypermethylation in AML with high BCAT1 activity (Fig. 1) [13]. This DNA hypermethylation phenotype is similarly observed in IDH mutant AML, where the oncometabolite 2-HG generated by mutant IDH competitively binds to and inactivates TET2 [13, 55, 56].

BCAA catabolism and glutamate in cancer

In addition to altering α-KG levels thus impacting oxygen sensing and DNA methylation pattern, some cancer cells favor BCAA degradation as it provides precursors such as glutamate for the biosynthesis of fundamental building blocks to sustain cancer cell proliferation [7, 8, 14].

Glutamate is a co-product in the first step of BCAA catabolism when converting BCAA to BCKA. BCAT1 expression is necessary for the release of glutamate in glioblastoma to sustain cell growth [7]. BCAA-derived glutamate also supports the amino acid pools for protein synthesis and the nucleotide pools for DNA synthesis (Fig. 1). The isotope tracing studies in PDAC organoids and cell lines provide direct evidence that BCAAs are the nitrogen donors for the synthesis of nucleotides and nonessential amino acids aspartate, serine, and alanine via glutamate as the intermediate [8, 35]. Furthermore, analyzing the

incorporation of dietary-derived isotope-labeled BCAA into mouse tissues also revealed that BCAA uptake is increased in mouse lung tumors and then used for the synthesis of proteins and nucleotides [14].

BCAA catabolism and reactive oxygen species (ROS) in cancer

Elevated BCAA levels have been shown to increase generation of mitochondrial ROS via PI3K/Akt-mTORC1 activation in peripheral blood mononuclear cells [57]. BCAA levels are also increased in plasma and tumors from breast cancer patients but expression of BCAT1 suppresses mitochondrial ROS in human breast cancer cells [15]. A recent study showed that BCAT1 mediates resistance of a tyrosine kinase inhibitor gefitinib in epidermal growth factor receptor-mutant lung cancer cells via the production of glutathione (GSH) to attenuate ROS accumulation [42]. Whether or not the increased levels of GSH stem from glutamate in the course of BCAA catabolism remains to be determined.

The BCKDH-BCKDK-PPM1K complex and cancer progression

The BCKDH complex plays a crucial role in cancers through its metabolic and non-metabolic activities (Fig. 1). Knockdown of the E1α subunit of the BCKDH complex (BCKDHA) substantially inhibits PDAC cell proliferation *in vitro* and in mice [9]. Although BCKDHA knockdown has no significant impact on the levels of TCA cycle intermediates or oxygen consumption rate, but significantly impedes fatty acid synthesis in PDAC cells [9]. Whether or not BCAAs are used as the carbon source for the biosynthesis of fatty acids in PDAC remains to be investigated.

A recent study showed that BCAT1 is predominantly expressed in cancer-associated fibroblasts but BCAT2 and BCKDH complex are primarily expressed in PDAC cells [10]. BCAT1 is responsible for BCKA production in cancer-associated fibroblasts, which is excreted and uptaken by PDAC cells within the tumor microenvironment [10]. BCKA can be reaminated to BCAA by BCAT2 in PDAC cells to support the *de novo* protein synthesis and cell prolifieration [10]. Meanwhile, BCKA can be also oxidized by the BCKDH complex to generate biomacromolecules in PDAC cells [10]. This cell non-autonomous pathway is very critical for PDAC growth under conditions of BCAA deprivation (Fig. 2).

BCKDK is upregulated in colorectal tumors and its expression promotes colorectal tumor growth and metastasis in mice [58, 59]. Xue P *et al.* showed that BCKDK directly phosphorylates MEK1 at serine 221 in colorectal cancer cells *in vitro* leading to activation of MAPK signaling [58]. MAPK has a broad function in cancer cell proliferation and thus the BCKDK-MEK1 axis may contribute to colorectal cancer development. BCKDK itself can be phosphorylated at tyrosine residue 246 by Src, which enhances protein stability and kinase activity of BCKDK [59]. Interestingly, phosphorylated BCKDK promotes epithelial-mesenchymal transition (EMT) by regulating EMT genes, leading to metastasis of colorectal cancer [59]. These studies implicate that BCKDK may be a possible target to treat colorectal cancer.

Another study reported a critical role of the phosphatase PPM1K in hematopoiesis and leukemogenesis. PPM1K deletion causes accumulation of the cellular BCAA levels but

decreases glycolysis and quiescence of hematopoietic stem cells by reducing the ubiquitin E3 ligase CDC20-mediated ubiquitination and degradation of MEIS1 and p21 [60].

BCAA metabolism and cancer immunity

Emerging evidences reveal that BCAA metabolic reprogramming could potentially affect cancer immunity. BCKAs excreted from glioblastoma cells are taken up by tumor-associated macrophages and then reaminated back to BCAAs in the *in vitro* model (Fig. 2). Increased exposure to BCKAs reduces the phagocytic activity of macrophages, indicating a possible role of tumor-excreted BCKAs in cancer immune suppression [26]. The import of BCAAs into $Foxp3^+$ regulatory T (Treg) cells through an amino acid transporter SLC3A2 helps maintain the proliferative status of Treg cells to suppress the immune response [61]. BCAT1 has also been shown to be an immunosuppressive enzyme that downregulates glycolysis in T cells [62]. Together, BCAA metabolic reprogramming plays a critical role in immune suppression, which boosts cancer progression.

Dietary BCAA supplementation and cancer progression

As the most straightforward strategy to manipulate BCAA metabolism *in vivo*, oral BCAA supplementation has been studied in the past decade for its effect on liver cancer in animal models and patients. Dietary BCAA supplementation can ameliorate fibrosis, suppress tumor growth (or prevent HCC), and increase the survival of mouse and rat in models of liver cirrhosis [63-65]. Over the years, clinical studies have suggested that oral BCAA supplementation during radiotherapy or drug treatment including Sorafenib and Levocarnitine can improve biochemical and amino acid profiles of HCC patients [66-68]. Dietary BCAA uptake has been shown to further prevent HCC recurrence after radiofrequency ablation and supportive therapies and preserve liver function [69-72]. Long-term BCAA supplementation can also prevent HCC and prolong survival of patients with cirrhosis [73-75]. However, recent studies reported that dietary BCAA levels seem to have a positive correlation with the development and growth of tumors such as liver cancer [20] and PDAC [8] in mouse. Nonetheless, it still requires a comprehensive investigation whether controlling dietary intake of BCAAs has a therapeutic benefit for certain types of human cancers.

Concluding Remarks and Future Perspectives

Here, we summarize the recent research progresses about altered BCAA metabolism-mediated tumorigenesis via diverse mechanisms (Fig. 1). Reprogramming of BCAA metabolism alters the levels of important metabolites, including BCAAs, α-KG, glutamate, and ROS, which are used to generate nutrients and building blocks, motivate the signaling pathways, shape the epigenetic landscapes, and improve the capacity of drug resistance, ultimately leading to cancer cell survival and rapid expansion.

It appears that the role of BCAA metabolic reprogramming in cancer progression highly depends on the tissue-of-origin, genetic mutations, and tumor microenvironment. We also need to bear in mind the complexity of the metabolic network in the cell, which may own the compensatory pathways when BCAA metabolism is inhibited. For example, the decrease

of BCAA-derived glutamate can be compensated by the increase of glutaminase activity, which generates glutamate from glutamine [43]. Thereafter, a systematic analysis should be applied to investigate BCAA metabolic reprogramming and its associated metabolic pathways in cancer cells in order to develop more effective strategies to treat cancer. In addition, many outstanding questions should be also investigated in the future (Box 1) to fully understand the role of BCAA metabolism in cancer progression. Nevertheless, given the great significance of BCAA metabolic reprogramming in cancer progression, the metabolic enzymes engaged in BCAA metabolism could be potential therapeutic targets for the treatment of human cancers.

Acknowledgement

Work in authors' laboratories was supported by grants from NIH (R01CA222393), CPRIT (RP190358), Mary Kay Foundation (08-19), and Welch Foundation (I-1903-20190330) to W.L.; and NIH (R01AG066166, R35GM124693) and UTSW startup funds to Y.W.. W.L. is a CPRIT Scholar in Cancer Research.

References

1. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. Science Advances 2016; 2: e1600200. [PubMed: 27386546]
2. Faubert B, Solmonson A, DeBerardinis RJ. Metabolic reprogramming and cancer progression. Science 2020; 368: eaaw5473. [PubMed: 32273439]
3. Sivanand S, Vander Heiden MG. Emerging roles for branched-chain amino acid metabolism in cancer. Cancer Cell 2020; 37: 147–156. [PubMed: 32049045]
4. Holeček M Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. Nutrition & Metabolism 2018; 15: 33. [PubMed: 29755574]
5. Ananieva EA, Wilkinson AC. Branched-chain amino acid metabolism in cancer. Current opinion in clinical nutrition and metabolic care 2018; 21: 64–70. [PubMed: 29211698]
6. Zhang B, Chen Y, Shi X, Zhou M, Bao L, Hatanpaa KJ et al. Regulation of branched-chain amino acid metabolism by hypoxia-inducible factor in glioblastoma. Cellular and Molecular Life Sciences 2020. doi: 10.1007/s00018-020-03483-1.
7. Tönjes M, Barbus S, Park YJ, Wang W, Schlotter M, Lindroth AM et al. BCAT1 promotes cell proliferation through amino acid catabolism in gliomas carrying wild-type IDH1. Nature Medicine 2013; 19: 901–908.
8. Li JT, Yin M, Wang D, Wang J, Lei MZ, Zhang Y et al. BCAT2-mediated BCAA catabolism is critical for development of pancreatic ductal adenocarcinoma. Nature cell biology 2020; 22: 167–174. [PubMed: 32029896]
9. Lee JH, Cho YR, Kim JH, Kim J, Nam HY, Kim SW et al. Branched-chain amino acids sustain pancreatic cancer growth by regulating lipid metabolism. Experimental & molecular medicine 2019; 51: 1–11.
10. Zhu Z, Achreja A, Meurs N, Animasahun O, Owen S, Mittal A et al. Tumour-reprogrammed stromal BCAT1 fuels branched-chain ketoacid dependency in stromal-rich PDAC tumours. Nature Metabolism 2020; 2: 775–792.
11. Gu Z, Liu Y, Cai F, Patrick M, Zmajkovic J, Cao H et al. Loss of EZH2 reprograms BCAA metabolism to drive leukemic transformation. Cancer discovery 2019; 9: 1228–1247. [PubMed: 31189531]
12. Hattori A, Tsunoda M, Konuma T, Kobayashi M, Nagy T, Glushka J et al. Cancer progression by reprogrammed BCAA metabolism in myeloid leukaemia. Nature 2017; 545: 500–504. [PubMed: 28514443]
13. Raffel S, Falcone M, Kneisel N, Hansson J, Wang W, Lutz C et al. BCAT1 restricts alphaKG levels in AML stem cells leading to IDHmut-like DNA hypermethylation. Nature 2017; 551: 384–388. [PubMed: 29144447]

14. Mayers JR, Torrence ME, Danai LV, Papagiannakopoulos T, Davidson SM, Bauer MR et al. Tissue of origin dictates branched-chain amino acid metabolism in mutant Kras-driven cancers. Science 2016; 353: 1161–1165. [PubMed: 27609895]
15. Zhang L, Han J. Branched-chain amino acid transaminase 1 (BCAT1) promotes the growth of breast cancer cells through improving mTOR-mediated mitochondrial biogenesis and function. Biochemical and biophysical research communications 2017; 486: 224–231. [PubMed: 28235484]
16. Wang Z-Q, Faddaoui A, Bachvarova M, Plante M, Gregoire J, Renaud M-C et al. BCAT1 expression associates with ovarian cancer progression: possible implications in altered disease metabolism. Oncotarget 2015; 6: 31522–31543. [PubMed: 26372729]
17. Qu YY, Zhao R, Zhang HL, Zhou Q, Xu FJ, Zhang X et al. Inactivation of the AMPK-GATA3-ECHS1 pathway induces fatty acid synthesis that promotes clear cell renal cell carcinoma growth. Cancer research 2020; 80: 319–333. [PubMed: 31690668]
18. Martin SB, Reiche WS, Fifelski NA, Schultz AJ, Stanford SJ, Martin AA et al. Leucine and branched chain amino acid metabolism contribute to the growth of bone sarcomas by regulating AMPK and mTORC1 signaling. Biochemical Journal 2020; 477:1579–1599. [PubMed: 32297642]
19. Wang P, Wu S, Zeng X, Zhang Y, Zhou Y, Su L et al. BCAT1 promotes proliferation of endometrial cancer cells through reprogrammed BCAA metabolism. International journal of clinical and experimental pathology 2018; 11: 5536–5546. [PubMed: 31949641]
20. Ericksen RE, Lim SL, McDonnell E, Shuen WH, Vadiveloo M, White PJ et al. Loss of BCAA catabolism during carcinogenesis enhances mTORC1 activity and promotes Tumor development and progression. Cell metabolism 2019; 29: 1151–1165.e1156. [PubMed: 30661928]
21. Kim DK, Kim IJ, Hwang S, Kook JH, Lee M-C, Shin BA et al. System L-amino acid transporters are differently expressed in rat astrocyte and C6 glioma cells. Neurosci Res 2004; 50: 437–446. [PubMed: 15567481]
22. Shennan DB, Thomson J, Gow IF, Travers MT, Barber MC. L-leucine transport in human breast cancer cells (MCF-7 and MDA-MB-231): kinetics, regulation by estrogen and molecular identity of the transporter. Biochimica et biophysica acta 2004; 1664: 206–216. [PubMed: 15328053]
23. Baracos VE, Mackenzie ML. Investigations of branched-chain amino acids and their metabolites in animal models of cancer. J Nutr 2006; 136: 237s–242s. [PubMed: 16365090]
24. Yoneshiro T, Wang Q, Tajima K, Matsushita M, Maki H, Igarashi K et al. BCAA catabolism in brown fat controls energy homeostasis through SLC25A44. Nature 2019; 572: 614–619. [PubMed: 31435015]
25. Hall TR, Wallin R, Reinhart GD, Hutson SM. Branched chain aminotransferase isoenzymes. Purification and characterization of the rat brain isoenzyme. The Journal of biological chemistry 1993; 268: 3092–3098. [PubMed: 8381418]
26. Silva LS, Poschet G, Nonnenmacher Y, Becker HM, Sapcariu S, Gaupel AC et al. Branched-chain ketoacids secreted by glioblastoma cells via MCT1 modulate macrophage phenotype. EMBO reports 2017; 18: 2172–2185. [PubMed: 29066459]
27. Abla H, Sollazzo M, Gasparre G, Iommarini L, Porcelli AM. The multifaceted contribution of α-ketoglutarate to tumor progression: an opportunity to exploit? Seminars in Cell & Developmental Biology 2020; 98: 26–33. [PubMed: 31175937]
28. Zdzisińska B, Żurek A, Kandefer-Szerszeń M. Alpha-ketoglutarate as a molecule with pleiotropic activity: well-known and novel possibilities of therapeutic use. Archivum Immunologiae et Therapiae Experimentalis 2017; 65: 21–36. [PubMed: 27326424]
29. Cluntun AA, Lukey MJ, Cerione RA, Locasale JW. Glutamine metabolism in cancer: understanding the heterogeneity. Trends in cancer 2017; 3: 169–180. [PubMed: 28393116]
30. Biswas D, Duffley L, Pulinilkunnil T. Role of branched-chain amino acid-catabolizing enzymes in intertissue signaling, metabolic remodeling, and energy homeostasis. FASEB journal 2019; 33: 8711–8731. [PubMed: 31084571]
31. Zhou W, Feng X, Ren C, Jiang X, Liu W, Huang W et al. Over-expression of BCAT1, a c-Myc target gene, induces cell proliferation, migration and invasion in nasopharyngeal carcinoma. Molecular cancer 2013; 12: 53. [PubMed: 23758864]

32. Zheng YH, Hu WJ, Chen BC, Grahn TH, Zhao YR, Bao HL et al. BCAT1, a key prognostic predictor of hepatocellular carcinoma, promotes cell proliferation and induces chemoresistance to cisplatin. Liver international 2016; 36: 1836–1847. [PubMed: 27246112]
33. Xu M, Liu Q, Jia Y, Tu K, Yao Y, Liu Q et al. BCAT1 promotes tumor cell migration and invasion in hepatocellular carcinoma. Oncology letters 2016; 12: 2648–2656. [PubMed: 27698837]
34. Elorza A, Soro-Arnáiz I, Meléndez-Rodríguez F, Rodríguez-Vaello V, Marsboom G, de Cárcer G et al. HIF2α acts as an mTORC1 activator through the amino acid carrier SLC7A5. Molecular cell 2012; 48: 681–691. [PubMed: 23103253]
35. Dey P, Baddour J, Muller F, Wu CC, Wang H, Liao W-T et al. Genomic deletion of malic enzyme 2 confers collateral lethality in pancreatic cancer. Nature 2017; 542: 119–123. [PubMed: 28099419]
36. Hatazawa Y, Tadaishi M, Nagaike Y, Morita A, Ogawa Y, Ezaki O et al. PGC-1α-mediated branched-chain amino acid metabolism in the skeletal muscle. PloS one 2014; 9: e91006. [PubMed: 24638054]
37. Shimizu N, Yoshikawa N, Ito N, Maruyama T, Suzuki Y, Takeda S-i et al. Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. Cell metabolism 2011; 13: 170–182. [PubMed: 21284984]
38. Shimomura Y, Murakami T, Nakai N, Nagasaki M, Harris RA. Exercise promotes BCAA catabolism: effects of BCAA supplementation on skeletal muscle during exercise. The Journal of Nutrition 2004; 134: 1583S–1587S. [PubMed: 15173434]
39. Terakura D, Shimizu M, Iwasa J, Baba A, Kochi T, Ohno T et al. Preventive effects of branched-chain amino acid supplementation on the spontaneous development of hepatic preneoplastic lesions in C57BL/KsJ-db/db obese mice. Carcinogenesis 2012; 33: 2499–2506. [PubMed: 23027617]
40. Arakawa M, Masaki T, Nishimura J, Seike M, Yoshimatsu H. The effects of branched-chain amino acid granules on the accumulation of tissue triglycerides and uncoupling proteins in diet-induced obese mice. Endocrine journal 2011; 58: 161–170. [PubMed: 21372430]
41. Zou H, Liao M, Xu W, Yao R, Liao W. Data mining of the expression and regulatory role of BCAT1 in hepatocellular carcinoma. Oncology letters 2019; 18: 5879–5888. [PubMed: 31788061]
42. Wang Y, Zhang J, Ren S, Sun D, Huang H-Y, Wang H et al. Branched-chain amino acid metabolic reprogramming orchestrates drug resistance to EGFR tyrosine kinase Inhibitors. Cell reports 2019; 28: 512–525.e516. [PubMed: 31291585]
43. McBrayer SK, Mayers JR, DiNatale GJ, Shi DD, Khanal J, Chakraborty AA et al. Transaminase inhibition by 2-hydroxyglutarate impairs glutamate biosynthesis and redox homeostasis in glioma. Cell 2018; 175: 101–116.e125. [PubMed: 30220459]
44. Mayers JR, Wu C, Clish CB, Kraft P, Torrence ME, Fiske BP et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. Nat Med 2014; 20: 1193–1198. [PubMed: 25261994]
45. Wolfson RL, Chantranupong L, Saxton RA, Shen K, Scaria SM, Cantor JR et al. Sestrin2 is a leucine sensor for the mTORC1 pathway. Science 2016; 351: 43–48. [PubMed: 26449471]
46. Tian T, Li X, Zhang J. mTOR signaling in cancer and mTOR inhibitors in solid tumor targeting therapy. International journal of molecular sciences 2019; 20: 755.
47. Mossmann D, Park S, Hall MN. mTOR signalling and cellular metabolism are mutual determinants in cancer. Nature reviews Cancer 2018; 18: 744–757. [PubMed: 30425336]
48. Kaelin William G Jr., McKnight Steven L. Influence of metabolism on epigenetics and disease. Cell 2013; 153: 56–69. [PubMed: 23540690]
49. Loenarz C, Schofield CJ. Expanding chemical biology of 2-oxoglutarate oxygenases. Nature chemical biology 2008; 4: 152–156. [PubMed: 18277970]
50. Epstein ACR, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR et al. C. elegans EGL-9 and Mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001; 107: 43–54. [PubMed: 11595184]
51. Ivan M, Kaelin WG Jr. The EGLN-HIF O_2-sensing system: multiple inputs and feedbacks. Molecular cell 2017; 66: 772–779. [PubMed: 28622522]
52. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 2010; 29: 625–634. [PubMed: 19946328]

53. Luo W, Wang Y. Hypoxia mediates tumor malignancy and therapy resistance. Advances in experimental medicine and biology 2019; 1136: 1–18. [PubMed: 31201713]
54. Luo W, Wang Y. Epigenetic regulators: multifunctional proteins modulating hypoxia-inducible factor-α protein stability and activity. Cellular and Molecular Life Sciences 2018; 75: 1043–1056. [PubMed: 29032501]
55. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim S-H et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. Cancer Cell 2011; 19: 17–30. [PubMed: 21251613]
56. Chowdhury R, Yeoh KK, Tian YM, Hillringhaus L, Bagg EA, Rose NR et al. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. EMBO reports 2011; 12: 463–469. [PubMed: 21460794]
57. Zhenyukh O, Civantos E, Ruiz-Ortega M, Sánchez MS, Vázquez C, Peiró C et al. High concentration of branched-chain amino acids promotes oxidative stress, inflammation and migration of human peripheral blood mononuclear cells via mTORC1 activation. Free Radical Biology and Medicine 2017; 104: 165–177. [PubMed: 28089725]
58. Xue P, Zeng F, Duan Q, Xiao J, Liu L, Yuan P et al. BCKDK of BCAA catabolism cross-talking with the MAPK pathway promotes tumorigenesis of colorectal cancer. EBioMedicine 2017; 20: 50–60. [PubMed: 28501528]
59. Tian Q, Yuan P, Quan C, Li M, Xiao J, Zhang L et al. Phosphorylation of BCKDK of BCAA catabolism at Y246 by Src promotes metastasis of colorectal cancer. Oncogene 2020; 39: 3980–3996. [PubMed: 32238881]
60. Liu X, Zhang F, Zhang Y, Li X, Chen C, Zhou M et al. PPM1K Regulates hematopoiesis and leukemogenesis through CDC20-mediated Ubiquitination of MEIS1 and p21. Cell reports 2018; 23: 1461–1475. [PubMed: 29719258]
61. Ikeda K, Kinoshita M, Kayama H, Nagamori S, Kongpracha P, Umemoto E et al. Slc3a2 mediates branched-chain amino-acid-dependent maintenance of regulatory T cells. Cell reports 2017; 21: 1824–1838. [PubMed: 29141216]
62. Ananieva EA, Patel CH, Drake CH, Powell JD, Hutson SM. Cytosolic branched chain aminotransferase (BCATc) regulates mTORC1 signaling and glycolytic metabolism in CD4+ T cells. The Journal of biological chemistry 2014; 289: 18793–18804. [PubMed: 24847056]
63. Takegoshi K, Honda M, Okada H, Takabatake R, Matsuzawa-Nagata N, Campbell JS et al. Branched-chain amino acids prevent hepatic fibrosis and development of hepatocellular carcinoma in a non-alcoholic steatohepatitis mouse model. Oncotarget 2017; 8: 18191–18205. [PubMed: 28212548]
64. Iwasa M, Kobayashi Y, Mifuji-Moroka R, Hara N, Miyachi H, Sugimoto R et al. Branched-chain amino acid supplementation reduces oxidative stress and prolongs survival in rats with advanced liver cirrhosis. PloS one 2013; 8: e70309. [PubMed: 23936183]
65. Cha JH, Bae SH, Kim HL, Park NR, Choi ES, Jung ES et al. Branched-chain amino acids ameliorate fibrosis and suppress tumor growth in a rat model of hepatocellular carcinoma with liver cirrhosis. PloS one 2013; 8: e77899. [PubMed: 24223741]
66. Lee IJ, Seong J, Bae JI, You SH, Rhee Y, Lee JH. Effect of oral supplementation with branched-chain amino acid (BCAA) during radiotherapy in patients with hepatocellular carcinoma: a double-blind randomized study. Cancer research and treatment 2011; 43: 24–31. [PubMed: 21509160]
67. Imanaka K, Ohkawa K, Tatsumi T, Katayama K, Inoue A, Imai Y et al. Impact of branched-chain amino acid supplementation on survival in patients with advanced hepatocellular carcinoma treated with sorafenib: a multicenter retrospective cohort study. Hepatology research 2016; 46: 1002–1010. [PubMed: 26690886]
68. Iwasa M, Sugimoto R, Ishihara T, Sekoguchi-Fujikawa N, Yoshikawa K, Mifuji-Moroka R et al. Usefulness of levocarnitine and/or branched-chain amino acids during invasive treatment for hepatocellular carcinoma. Journal of nutritional science and vitaminology 2015; 61: 433–440. [PubMed: 26875483]

69. Takami T, Yamasaki T, Saeki I, Matsumoto T, Suehiro Y, Sakaida I. Supportive therapies for prevention of hepatocellular carcinoma recurrence and preservation of liver function. World journal of gastroenterology 2016; 22: 7252–7263. [PubMed: 27621572]

70. Kuroda H, Ushio A, Miyamoto Y, Sawara K, Oikawa K, Kasai K et al. Effects of branched-chain amino acid-enriched nutrient for patients with hepatocellular carcinoma following radiofrequency ablation: a one-year prospective trial. Journal of gastroenterology and hepatology 2010; 25: 1550–1555. [PubMed: 20796154]

71. Nojiri S, Fujiwara K, Shinkai N, Iio E, Joh T. Effects of branched-chain amino acid supplementation after radiofrequency ablation for hepatocellular carcinoma: A randomized trial. Nutrition (Burbank, Los Angeles County, Calif) 2017; 33: 20–27.

72. Morihara D, Iwata K, Hanano T, Kunimoto H, Kuno S, Fukunaga A et al. Late-evening snack with branched-chain amino acids improves liver function after radiofrequency ablation for hepatocellular carcinoma. Hepatology research 2012; 42: 658–667. [PubMed: 22380706]

73. Kawaguchi T, Shiraishi K, Ito T, Suzuki K, Koreeda C, Ohtake T et al. Branched-chain amino acids prevent hepatocarcinogenesis and prolong survival of patients with cirrhosis. Clinical gastroenterology and hepatology 2014; 12: 1012–1018.e1011. [PubMed: 24036055]

74. Tada T, Kumada T, Toyoda H, Kiriyama S, Tanikawa M, Hisanaga Y et al. Oral supplementation with branched-chain amino acid granules prevents hepatocarcinogenesis in patients with hepatitis C-related cirrhosis: a propensity score analysis. Hepatology research 2014; 44: 288–295. [PubMed: 23607436]

75. Hayaishi S, Chung H, Kudo M, Ishikawa E, Takita M, Ueda T et al. Oral branched-chain amino acid granules reduce the incidence of hepatocellular carcinoma and improve event-free survival in patients with liver cirrhosis. Digestive diseases (Basel, Switzerland) 2011; 29: 326–332.

Box 1:

Outstanding questions

1. Why is BCAA contribution to the TCA cycle very low in cultured cancer cells and adipocytes? Is this due to technical limitations in current metabolic detection methods or because BCAA contributes to branched chain fatty acids instead? Does BCAA contribute to the TCA cycle in tumor cells *in vivo*?
2. What controls the utilization of BCAAs, the maintenance of the BCAA pool, and the biosynthesis of macromolecules from BCAA metabolism in human tumors? What is the mechanism that determines the fate of BCAAs in human tumors?
3. How are the transamination and reamination activities of BCAT controlled in cancer cells? How is the homeostasis of BCAAs and BCKAs maintained by BCAT in cancer cells?
4. To what extent do cancer cells use the BCKAs in the cell or from circulation to synthesize BCAAs?
5. Does BCAA metabolic reprogramming cooperate with other mechanisms to promote tumor growth? Are there any other metabolic pathways that can compensate to sustain cell survival when BCAA metabolism is inhibited in cancer cells?
6. Can the BCAA-controlled diet be used to treat cancer?

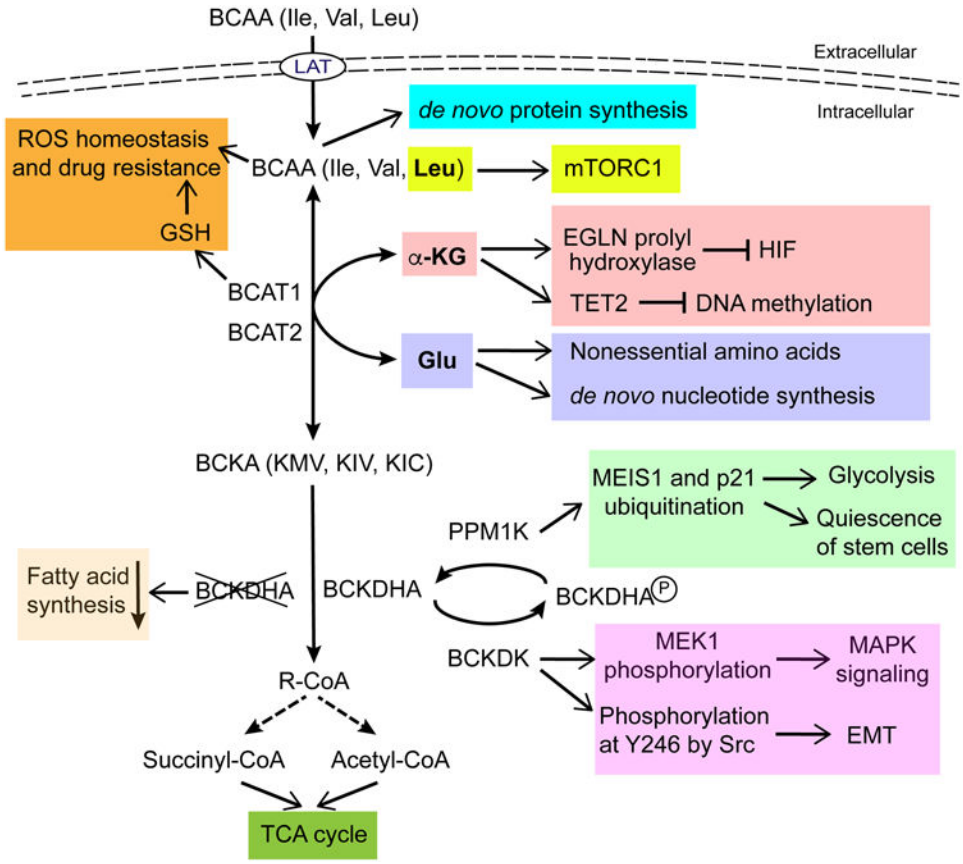

Figure 1. Role of BCAA metabolism in cancer progression.

BCAAs (i.e, Val, valine; Ile, isoleucine; Leu, leucine) are transported by LATs into the cell and reversibly metabolized by branched-chain amino acid transaminases (BCATs), followed by irreversible decarboxylation of branched-chain α-keto acids (BCKAs, i.e, KMV, α-keto-β-methylvalerate; KIV, α-ketoisovalerate; KIC, α-ketoisocaproate) by the branched-chain α-keto acid dehydrogenase (BCKDH) complex. The activity of the BCKDH complex is determined by its phosphorylation status modulated by a pair of enzymes, branched-chain keto acid dehydrogenase kinase (BCKDK) and Mg^{2+}/Mn^{2+}-dependent 1K protein phosphatase (PPM1K). Along the BCAA metabolic pathway, BCAAs (especially Leu), α-ketoglutarate (α-KG), glutamate (Glu), BCKDK, BCKDH, and PPM1K have been demonstrated to play significant roles in cancer progression via various mechanisms, which are highlighted in the colored boxes. The end products of BCAA catabolism, acetyl-coenzyme A (acetyl-CoA) and succinyl-CoA, were shown to contribute to 1-3% of the intermediates of the tricarboxylic acid (TCA) cycle, but their roles in cancers remain to be investigated. EMT, epithelial-mesenchymal transition; mTORC1, mechanistic target of rapamycin complex 1; TET, ten-eleven translocation; GSH, glutathione; ROS, reactive oxygen species.

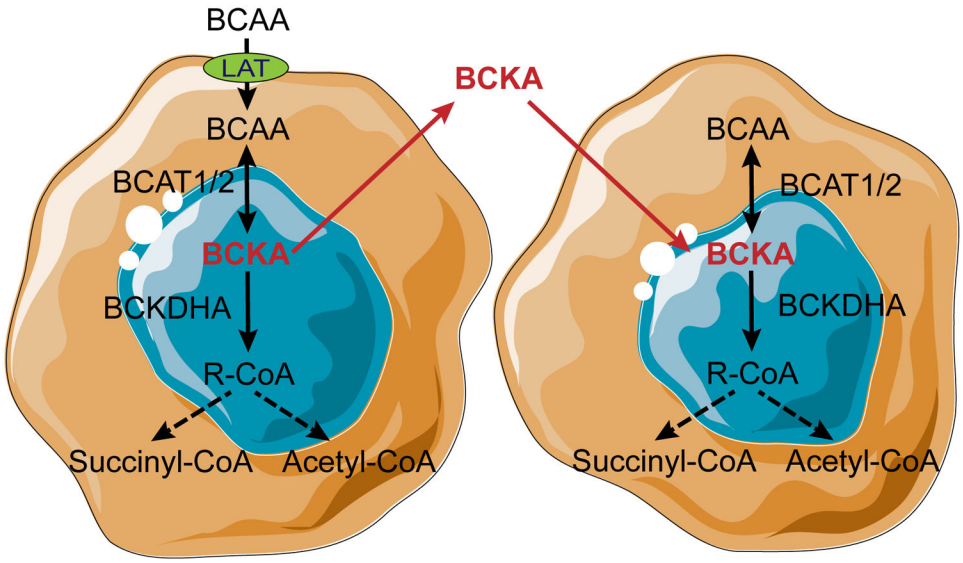

Figure 2. The intercommunication of BCAA metabolism in the tumor microenvironment.

Within the tumor microenvironment, BCKA produced by a cell is excreted and utilized by the adjacent cell. For example, cancer-associated fibroblasts excrete BCKA generated from BCAA deamination, which can be uptaken by the neighboring PDAC cells, where BCKA is either converted back to BCAA by BCAT2 to support the *de novo* protein synthesis or undergoes oxidation. In another case, glioblastoma cells can secrete BCKA into the microenvironment, and then tumor-associated macrophages uptake BCKA and reaminate it back to BCAA.

Table 1.

Regulation of proteins in the BCAA metabolic pathway

Regulator	Target	Expression fate	Mechanism	Reference
HIF-1/2	LAT1 (SLC7A5)	Upregulation	Direct binding to the promoter	Zhang et al. 2020 Elorza et al. 2012
c-Myc	BCAT1	Upregulation	Direct binding to the promoter	Zhou et al. 2013, Zheng et al. 2016, Xu et al. 2016
HIF-1	BCAT1	Upregulation	Direct binding to the promoter	Zhang et al. 2020
MSI2	BCAT1	Upregulation	Direct binding to 3' untranslated region	Hattori et al. 2017
SMAD5	BCAT1	Upregulation	Direct binding to the promoter	Zhu et al. 2020
KRAS	BCAT2	Upregulation	Blocking SYK-induced Y228 phosphorylation to inhibit ubiquitination and protein degradation of BCAT2	Li et al. 2020
PGC-1α	BCAT2	Upregulation	Possibly through multiple nuclear receptors	Hatazawa et al. 2014
PGC-1α	BCKDH	Upregulation	Possibly through multiple nuclear receptors	Hatazawa et al. 2014
SREBP1	BCAT2	Upregulation	Direct binding to the promoter	Dey et al. 2017
Mutant IDH	BCAT1	Downregulation	Promoter methylation	Tonjes et al. 2013
G9a	BCAT1	Downregulation	H3K9 methylation	Wang et al. 2019
SUV39H1	BCAT1	Downregulation	H3K9 methylation	Wang et al. 2019
EZH2	BCAT1	Downregulation	H3K27 methylation	Gu et al. 2019
R-2-HG	BCAT1	Inactivation	Direct inhibition of enzyme activity	McBrayer et al. 2018
R-2-HG	BCAT2	Inactivation	Direct inhibition of enzyme activity	McBrayer et al. 2018

Table 2.

Alterations of BCAAs and proteins involved in BCAA metabolism in human cancers and their effects on tumor progression

Cancer type	BCAA levels	Protein levels	Effect on cancer progression	Reference
PDAC	N.D.	BCAT1 ↓, BCAT2 ↑ in tumors; BCAT1 ↑, BCAT2 ↓ in stroma	Tumor growth	Zhu et al. 2020
	↑ in pancreas	BCAT2 ↑	Tumor growth	Li et al. 2020
	↑	SLC7A5, BCAT2 ↑	Tumor growth	Lee et al. 2019
	↓	BCAT2 ↑; BCAT1, BCKDH ↓	No effect of BCAT	Mayers et al. 2016
	↑ in plasma	N.D.	N.D.	Mayers et al. 2014
Leukaemia	↑	BCAT1 ↑	Tumor growth	Gu et al. 2019
	N.D.	BCAT1 ↑	Tumor growth	Raffel et al. 2017
	↑	BCAT1 ↑	Tumor growth	Hattori et al. 2017
	↑	BCAT1, PPM1K, BCKDHA, DBT BCKDH1B ↑ in HSPCs; BCKDK ↓ in MNCs.	Tumor growth	Liu et al. 2018
HCC	↑	BCAT1, BCAT2, BCKDH ↓	Tumor growth	Ericksen et al. 2019
	N.D.	BCAT1 ↑	Tumor growth	Zheng et al. 2016
ccRCC	↑	N.D.	Tumor growth	Qu et al. 2020
Bone sarcomas	N.D.	BCAT1 ↑ in Osteosarcoma; BCAT2 ↑ in Chondrosarcoma	N.D.	Martin et al. 2020
Endometrial cancer	N.D.	BCAT1 ↑	N.D.	Wang et al. 2018
Breast cancer	↑	BCAT1, BCAT2, PP2CM, BCKDH ↑	N.D.	Zhang and Han. 2017
NSCLC	↑	SLC7A5, BCAT1, BCAT2, p-BCKDH ↑	Tumor growth	Mayers et al. 2016
Ovarian cancer	N.D.	BCAT1 ↑	Tumor growth	Wang et al. 2015
Glioblastoma	N.D.	BCAT1 ↑	Tumor growth	Tonjes et al. 2013

↑, increase. ↓, decrease. N.D., not determined. p, phosphorylation. HSPCs, hematopoietic stem/progenitor cells. MNCs, mononuclear cells.