Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) *2022 23(9):760-769*
www.jzus.zju.edu.cn; www.springer.com/journal/11585
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https://doi.org/10.1631/jzus.B2100985

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BCAT1 promotes lung adenocarcinoma progression through enhanced mitochondrial function and NF-κB pathway activation

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Lung cancer is one of the most prevalent and malignant cancers, among which lung adenocarcinoma (LUAD) accounts for the majority and remains a major cause of cancer-related mortality worldwide (Cui et al., 2019). Despite the growing intensity of research on the pathobiology and progression of lung cancer and the fact that many genes have been identified as potential drivers and targets for therapy (Luo et al., 2019; Zhang et al., 2019), the treatment and prognosis of lung cancer patients have hardly improved. Therefore, this study aimed to investigate the precise mechanism of lung cancer development and explore efficient diagnostic and therapeutic methods for clinical treatment.

Branched-chain aminotransferase 1 (BCAT1) plays an important role in the metabolism of branched-chain amino acids (BCAAs). BCAAs, including leucine, isoleucine, and valine, are essential nutrients for protein synthesis and cell growth, also providing energy for cancer cell proliferation (Ananieva and Wilkinson, 2018). BCAT1 is upregulated in many types of cancer and has been proposed as a prognostic cancer cell marker (Zhang and Han, 2017; Xu et al., 2018). Besides, BCAT1 upregulation was reported to promote cancer cell proliferation, cell cycle progression, differentiation, invasion, and metastasis (Oktyabri et al., 2016; Zhu et al., 2017). BCAT1 and its metabolites are involved in the metabolism of cancer cells through different mechanisms, such as mitochondrial biogenesis and fatty acid metabolism. For example, glutamate generated from the metabolism of BCAAs is an important source of α-ketoglutarate, which is an intermediate product of the tricarboxylic acid (TCA) cycle and provides energy for cell growth (Fu et al., 2019). BCAT1 also promotes the growth of breast cancer cells by improving mammalian target of rapamycin (mTOR)-mediated mitochondrial biogenesis and function, while BCAT1 suppression results in the downregulation of genes implicated in lipid production and protein synthesis, and eventually inhibits tumor growth (Ananieva and Wilkinson, 2018). Taken together, BCAT1 plays an essential role in cancer development, and therefore, clarifying the associated functions and mechanisms during LUAD development is necessary and may guide improved treatments for LUAD patients.

Firstly, we investigated the protein expression levels of BCAT1 in tumors and surrounding normal lung tissues from LUAD patients. Tissue microarrays containing 86 pairs of tissue specimen were examined by immunohistochemical (IHC) staining. The BCAT1 expression rates were scored as 0, 1–2, and 3–4 for negative, weakly positive, and positive staining, respectively. As shown in Fig. 1a, the frequency of positive staining for BCAT1 in tumor tissues was significantly higher (69 of 86 (80.2%)) than that in normal

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Received Nov. 28, 2021; Revision accepted May 15, 2022;
Crosschecked Aug. 10, 2022

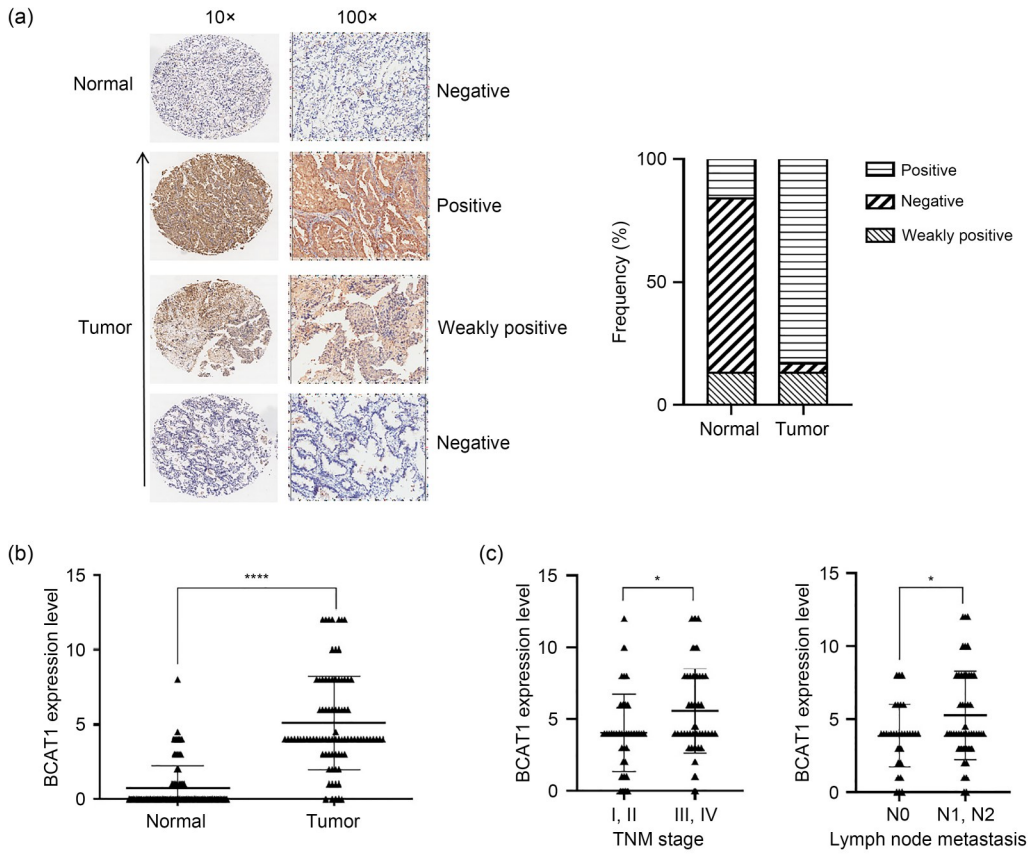

Fig. 1 Overexpression of branched-chain aminotransferase 1 (BCAT1) in lung adenocarcinoma (LUAD) tumor tissues. (a) Immunohistochemical (IHC) staining of BCAT1 in LUAD tumor and normal lung tissues. The histogram shows the frequency of positive, weakly positive, and negative staining. (b) Protein expression levels of BCAT1 in LUAD tumor and normal lung tissues. (c) Tumor node metastasis (TNM) stage and local lymph node metastasis frequency of LUAD patients with different BCAT1 expression levels. * $P<0.05$; **** $P<0.0001$.

tissues (14 of 86 (16.3%)), while there were 5 (5.8%) and 61 (70.9%) cases with negative BCAT1 staining in tumor and normal tissues, respectively. According to the "staining intensity score" and "staining positive rate score" based on the IHC results, the protein expression level of BCAT1 was significantly higher in LUAD tumor tissues compared to normal lung tissues (Fig. 1b, $P<0.0001$). Furthermore, the statistical analysis of LUAD specimens indicated that the expression level of BCAT1 was positively associated with local lymphatic metastasis ($P=0.0304$) and the tumor node metastasis (TNM) stage ($P=0.0189$), but not with the sex, age, tumor size, distant metastasis, or overall survival (Fig. 1c).

Next, we studied the functions of BCAT1 in tumor development of LUAD cells in vitro and in vivo. We detected the expression levels of BCAT1 in normal and LUAD cell lines, among which A549 presented a moderate expression level of BCAT1 and was used for the functional study of BCAT1 in LUAD development (Fig. S1). We constructed a BCAT1 knockdown LUAD cell line (BCAT1-sh1) and found that BCAT1 knockdown significantly decreased the proliferation rate and colony numbers of LUAD cells (Figs. 2a–2c). Besides, BCAT1 knockdown decreased the wound closure ability of LUAD cells and reduced the migratory and invasive cell numbers, which was consistent with the upregulation of E-cadherin and downregulation of vimentin (Figs. 2d–2f). Then, the proliferation, migration, and invasive abilities of another BCAT1 knockdown LUAD cell line (BCAT1-sh2) and control LUAD cells were examined, and the results showed that cells transfected with BCAT1-sh2 had similar characteristics to BCAT1-sh1-transfected cells when compared to control LUAD cells (Fig. S2). Therefore, we constructed a BCAT1-overexpressing LUAD cell line (BCAT1-OE) and found that the cell proliferation rate and colony formation ability of cells with BCAT1

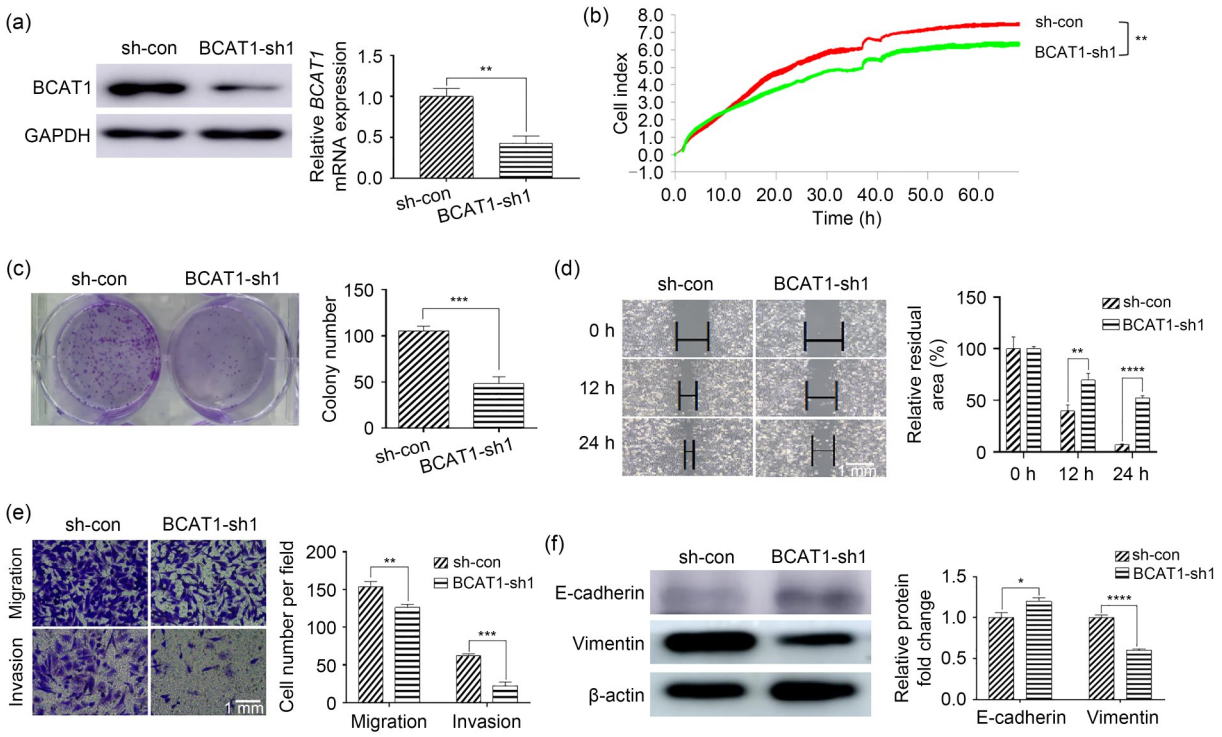

Fig. 2 Effects of branched-chain aminotransferase 1 (BCAT1) knockdown on cell proliferation, colony formation, migration, and invasion. (a) Western blot and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of BCAT1 in BCAT1 knockdown and control cells. (b–f) Cell proliferation rate (b), colony formation (c), wound healing (d), migration and invasion assays (e), and western blot analysis of E-cadherin and vimentin (f) in BCAT1 knockdown and control cells. BCAT1-sh1 and sh-con: BCAT1 knockdown and control LUAD cell lines, respectively. Data are expressed as mean±standard deviation (SD), n=3. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

overexpression were both considerably higher than those of control cells (Lv-con) (Figs. 3a–3c). BCAT1 overexpression also accelerated the speed of wound closure, and enhanced the migration and invasion abilities of LUAD cells (Figs. 3d and 3e). Besides, the expression level of E-cadherin was decreased while that of vimentin was increased in BCAT1-overexpressing cells (Fig. 3f).

Subsequently, BCAT1 overexpressing and control cells were implanted subcutaneously into nude mice. Five weeks later, the mice were sacrificed and tumors were collected. As shown in Figs. 3g and 3h, the volume and weight of tumor xenografts from the BCAT1 overexpressing group were higher than those from the control group. Additionally, IHC analysis revealed that BCAT1 overexpression was maintained in the tumor xenografts (Fig. 3i).

Since BCAT1 is an important enzyme in the metabolism of BCAAs, we examined the effects of BCAA supplementation on BCAT1-overexpressing LUAD cells (Fig. 4a). It was found that the cell proliferation ability was enhanced with increasing BCAA concentration. Then, we examined the sensitivity of BCAT1-overexpressing and control cells to BCAA supplementation.

As compared to the control cells, the cell proliferation ability of BCAT1-overexpressing cells was increased with leucine and isoleucine supplementation, while there was no difference in the cell proliferation ability under valine supplementation (Fig. 4b). To better understand the impact of BCAT1 overexpression on BCAA metabolism in LUAD cells, we determined the messenger RNA (mRNA) levels of several important genes associated with BCAA metabolism. As demonstrated in Figs. 4c and 4d, the transcription levels of genes promoting BCAA metabolism, including branched-chain ketoacid dehydrogenase E1α (*BCKDHA*), branched-chain ketoacid dehydrogenase E1β (*BCKDHB*), and enoyl-coenzyme A hydratase short chain 1 (*ECHS1*), were increased, whereas the level of branched-chain ketoacid dehydrogenase kinase (*BCKDK*), which is a BCAA metabolism inhibitor,

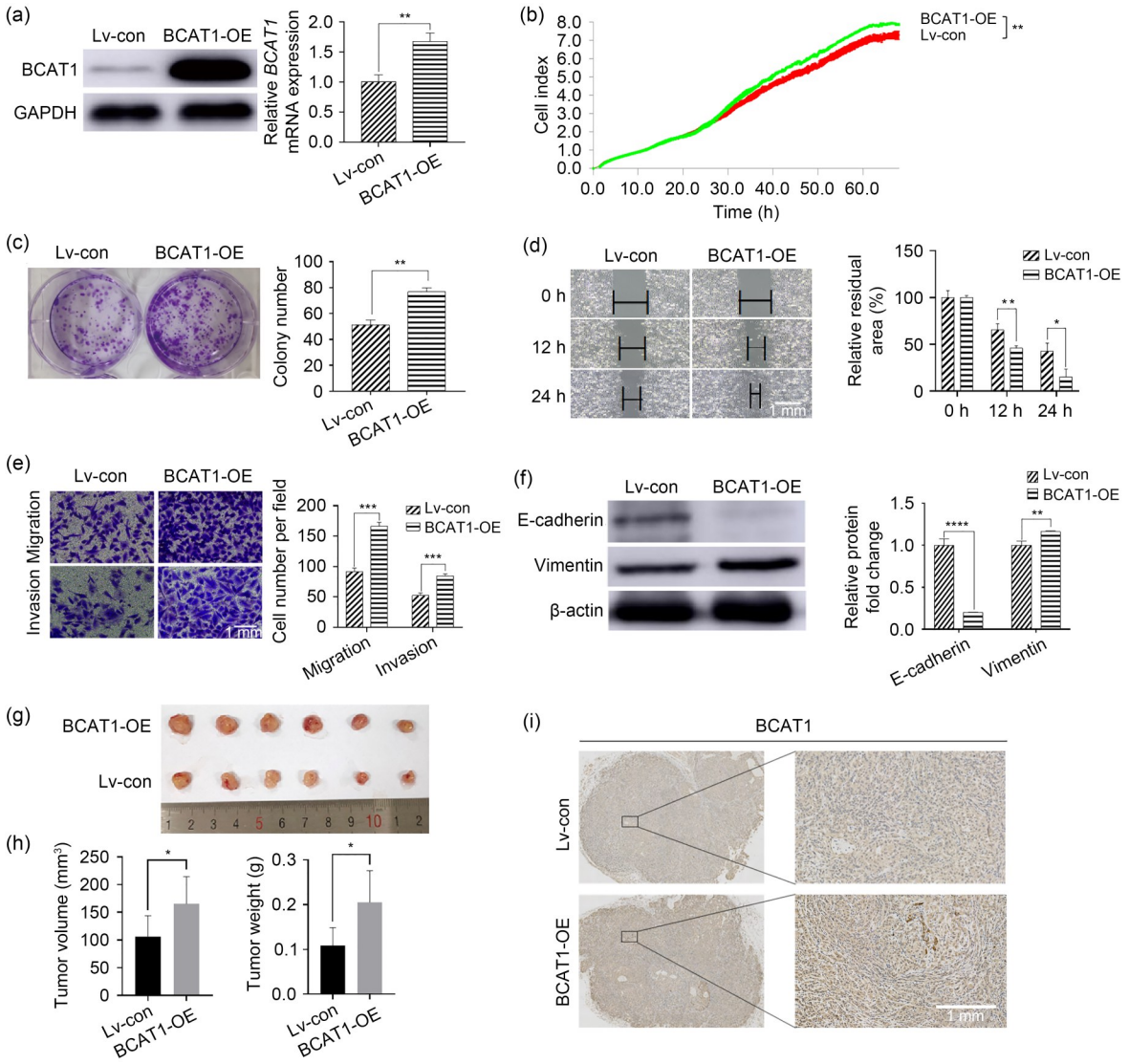

Fig. 3 Effects of branched-chain aminotransferase 1 (BCAT1) overexpression on cell proliferation, metastasis, and tumor growth. (a) Western blot and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of BCAT1 in BCAT1-overexpressing and control cells. (b–f) Cell proliferation rate (b), colony formation (c), wound healing (d), migration and invasion assays (e), and western blot analysis of E-cadherin and vimentin (f) in BCAT1-overexpressing and control cells. (g, h) Tumor xenografts were collected and measured for size and weight. (i) Immunohistochemical (IHC) staining of BCAT1 in tumor xenografts. BCAT1-OE and Lv-con: BCAT1-overexpressing and control LUAD cell lines, respectively. Data are expressed as mean±standard deviation (SD), n=3. * P<0.05; ** P<0.01; *** P<0.001; **** P< 0.0001.

was downregulated in BCAT1-overexpressing cells. Since there is evidence that enhanced BCAA metabolism is closely associated with mitochondrial respiration and reactive oxygen species (ROS) production (Ko et al., 2020), we determined the oxygen consumption by measuring the cellular oxygen consumption rate (OCR), which was increased in BCAT1 overexpressing cells, while BCAT1 inhibition decreased the OCR (Figs. 4e and S3a). The results for the expression levels of regulators of mitochondrial synthesis, including peroxisome proliferator-activated receptor-γ coactivator 1-α (*PGC1α*), nuclear respiratory factor 1 (*NRF-1*), and mitochondrial transcription factor A (*TFAM*) by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), showed that BCAT1 overexpression enhanced the mRNA levels of these regulators (Fig. 4f). Next, we determined the cellular ROS level and found that BCAT1 overexpression decreased

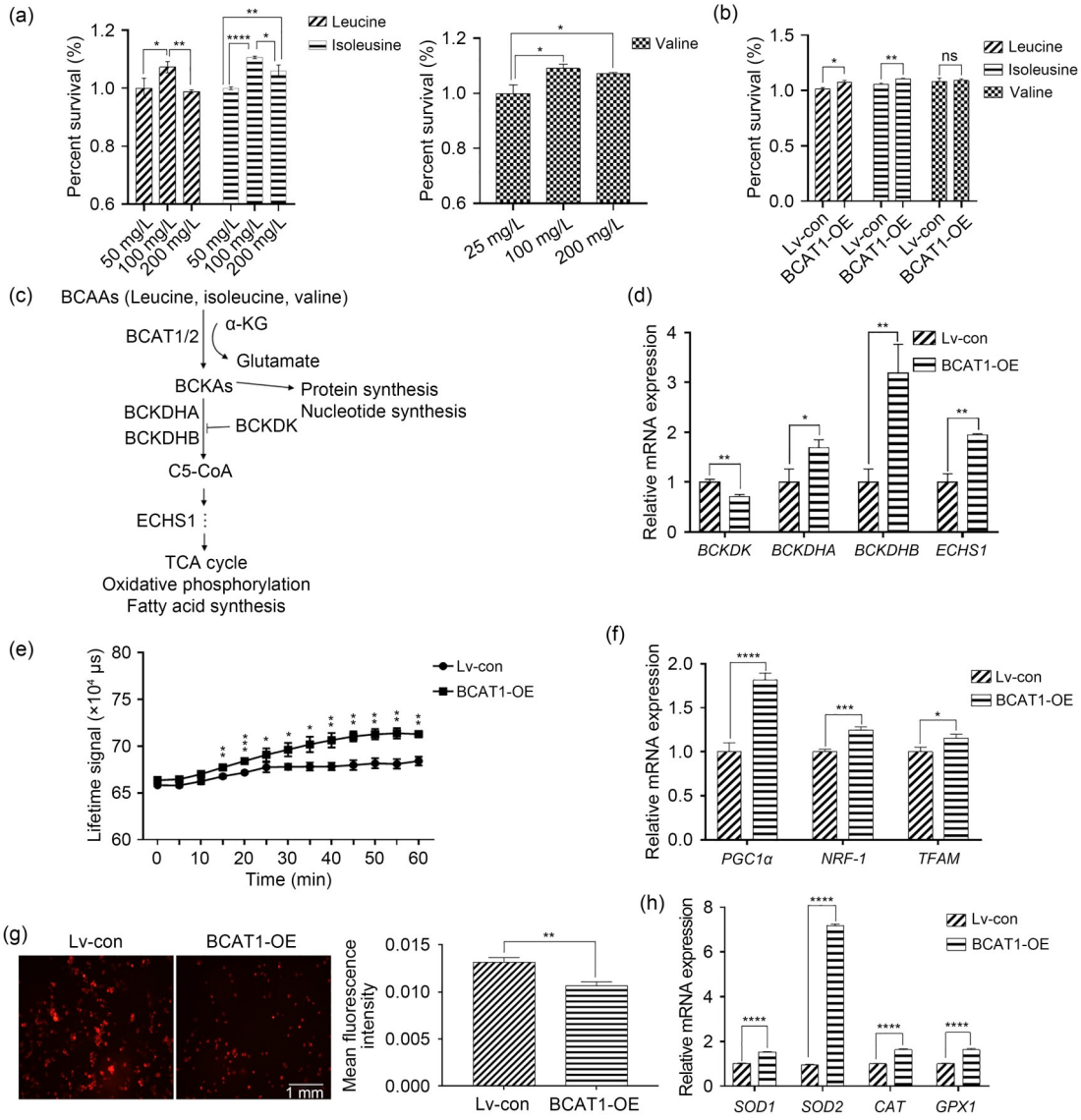

Fig. 4 Effects of branched-chain aminotransferase 1 (BCAT1) overexpression on branched-chain amino acid (BCAA) metabolism, mitochondrial function, and reactive oxygen species (ROS) content. (a) Cell proliferation ability detection of BCAT1-overexpressing cells with different BCAA supplementation; (b) Cell proliferation abilities of BCAT1-overexpressing and control cells with 100 mg/L BCAA supplementation; (c) Diagram of BCAA metabolic pathway; (d) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses of *BCKDK*, *BCKDHA*, *BCKDHB*, and *ECHS1*; (e) Oxygen consumption rate (OCR) measurement; (f) qRT-PCR analyses of *PGC1α*, *NRF-1*, and *TFAM*; (g) ROS content detection; (h) qRT-PCR analyses of *SOD1*, *SOD2*, *CAT*, and *GPX1* in BCAT1-overexpressing and control cells. *BCKDK*: branched-chain ketoacid dehydrogenase kinase; *BCKDHA*: branched-chain ketoacid dehydrogenase E1α; *BCKDHB*: branched-chain ketoacid dehydrogenase E1β; *ECHS1*: enoyl-coenzyme A hydratase short chain 1; *PGC1α*: peroxisome proliferator-activated receptor-γ coactivator 1-α; *NRF-1*: nuclear respiratory factor 1; *TFAM*: mitochondrial transcription factor A; *SOD1*: superoxide dismutase 1; *CAT*: catalase; *GPX1*: glutathione peroxidase 1; BCAT1-OE and Lv-con: BCAT1 overexpressing and control LUAD cell lines, respectively; α-KG: α-ketoglutarate; BCKAs: branched-chain keto acids; C5-CoA: C5-coenzyme A; TCA: tricarboxylic acid. Data are expressed as mean±standard deviation (SD), n=3. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001; ^{ns} not significant.

the ROS content, while BCAT1 knockdown increased the ROS content (Figs. 4g and S3b). Besides, the mRNA levels of antioxidants including superoxide dismutase 1 (*SOD1*), *SOD2*, catalase (*CAT*), and glutathione peroxidase 1 (*GPX1*) increased in BCAT1 overexpressing cells (Fig. 4h), which further confirmed that BCAT1 overexpression decreased oxidative stress in LUAD cells.

In order to further study the mechanism by which BCAT1 promotes LUAD development, RNA sequencing was performed in BCAT1-overexpressing and control LUAD cells. The global transcriptome maps revealed a total number of 17 503 genes. About 440 differentially expressed genes (DEGs) were identified, among which 139 genes were downregulated and 301 genes were upregulated in BCAT1-overexpressing cells (Fig. S4a). Gene Ontology (GO) analysis was performed and the molecular function analysis showed that the DEGs were mostly characterized by functions such as binding and catalytic activity (Fig. 5a). Besides, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification and enrichment analysis indicated that the DEGs were mainly enriched in advanced glycation end-product (AGE)-receptor for AGE (RAGE) signaling pathway, metabolic processes, cell adhesion, migration, and so on (Figs. 5b and S4b). These results demonstrated that signal transduction and metabolism were two important aspects that were mostly affected by BCAT1 in LUAD cells. Among the DEGs, nuclear factor-κB (NF-κB) inhibitor β (*NFKBIB*) was significantly downregulated in BCAT1-overexpressing cells, which possibly indicated the activation of NF-κB signaling pathway. To verify this hypothesis, we first detected the transcription level of *NFKBIB* by qRT-PCR, and found that it was decreased in BCAT1-overexpressing cells and increased in BCAT1 knockdown cells, as compared to control cells (Fig. 5c). Subsequently, the expression levels of proteins involved in the NF-κB signaling pathway were investigated (Fig. 5d). BCAT1 overexpression resulted in higher expression levels of inhibitor of κB kinase α/β (IKKα/β) and phosphorylated p65 (p-p65), and lower expression level of NF-κB inhibitor α (IKBα). We also detected the expression levels of IKKα/β and p-p65 in BCAT1 knockdown cells and found that both of them were decreased (Fig. S4c). Moreover, we treated BCAT1-overexpressing and control cells with ammonium pyrrolidinedithiocarbamate (PDTC), which is an inhibitor of NF-κB pathway activation. As shown in Fig. 5e, PDTC treatment inhibited the proliferation ability of both BCAT1-overexpressing and control cells, and the inhibitive effect was positively related to the concentration of PDTC. Besides, compared to the control cells, BCAT1-overexpressing cells were much more sensitive to PDTC treatment. We also examined the effects of PDTC on the migration and invasion abilities of BCAT1-overexpressing cells and found that PDTC treatment significantly decreased the numbers of migratory and invasive cells (Fig. 5f).

Our findings were partially consistent with the findings of a previous study, in which BCAT1 was upregulated in tumor tissues of lung cancer patients, and BCAT1 overexpression promoted lung cancer development (Lin et al., 2020). This suggested that BCAT1 protein could serve as a promising biomarker for lung cancer diagnosis including LUAD, and that BCAT1 is a carcinogenic factor during LUAD development. In addition, a complementary DNA (cDNA) microarray containing cDNA extracted from fifteen pairs of tissue samples was analyzed by qRT-PCR, and the transcription level of *BCAT1* showed no difference between the tumor and normal lung tissues of LUAD patients, which was consistent with the mRNA expression values in the Gene Expression Profiling Interactive Analysis (GEPIA) database, but different from that in the Cancer Genome Atlas (TCGA) database (Fig. S5). Therefore, the mRNA level of *BCAT1* was not stable in clinical tissue samples, and transcriptional and post-transcriptional regulations were probably important to maintaining high protein expression levels of BCAT1 in LUAD patients.

Enhanced BCAA metabolism impairs the tumor cell lifespan and tumor formation through enhanced mitochondrial biogenesis and function (Zhang and Han, 2017). We found that mitochondrial synthesis and function were enhanced in BCAT1-overexpressing LUAD cells. Moreover, transcriptomic analysis showed that the DEGs induced by BCAT1-overexpression were mainly clustered in metabolism and signal transduction. Among the metabolism-associated DEGs, most were primarily involved in fatty acid synthesis and differentiation. KEGG pathway enrichment revealed that these DEGs were also enriched in the AGE-RAGE signaling pathway. AGEs are a kind of metabolite produced from proteins and lipids, which induce signaling transduction through RAGE receptors in several diseases, including cancer and metabolic diseases (Rai et al., 2019). These findings indicate that BCAT1 overexpression promotes LUAD development through metabolism reprogramming and signal transduction.

NF-κB is a nuclear transcription factor that participates in apoptosis, tumorigenesis, inflammation, and metabolic processes. NF-κB pathway activation

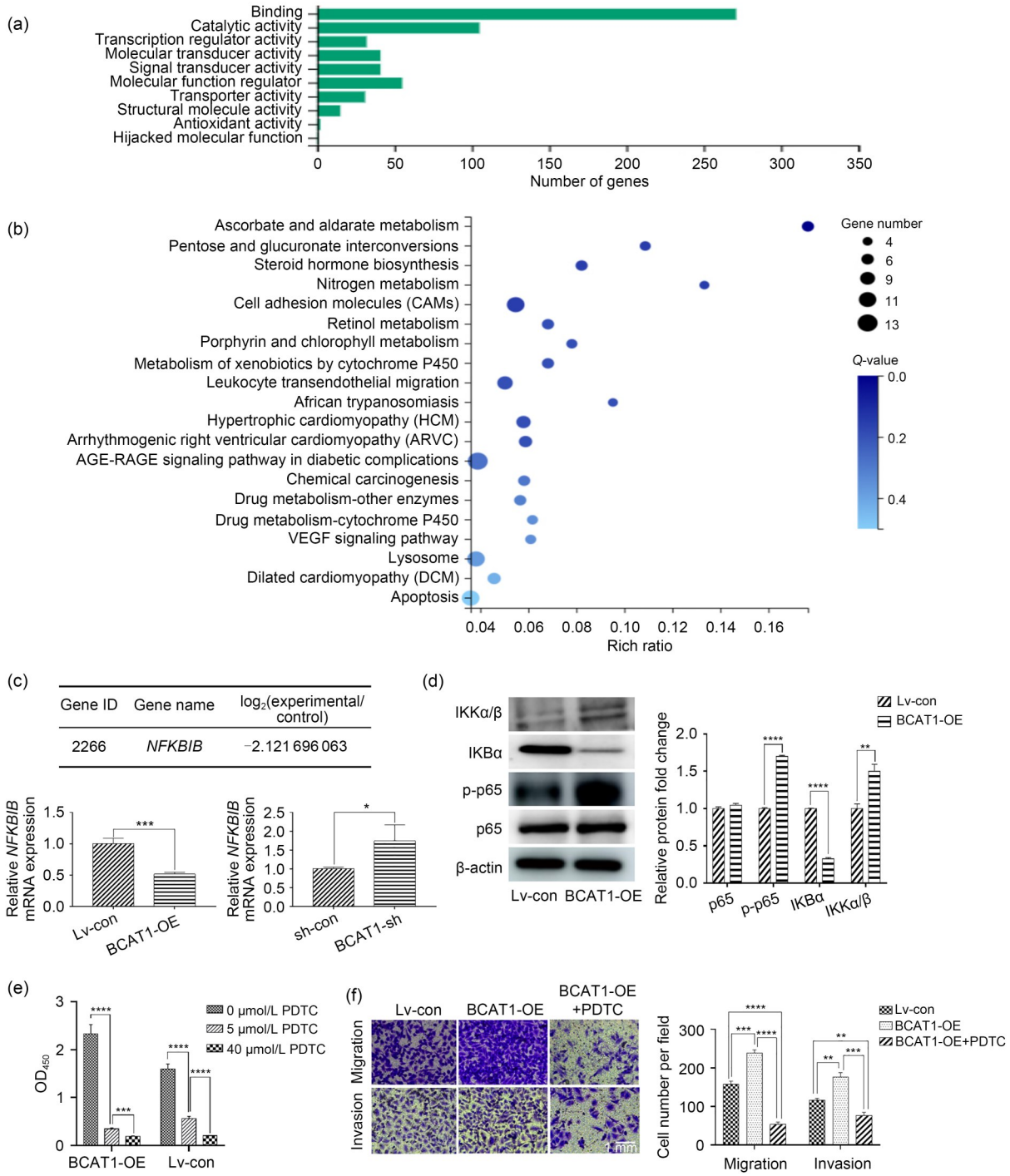

Fig. 5 Transcriptome analyses of NF-κB pathway activation in branched-chain aminotransferase 1 (BCAT1)-overexpressing cells. (a, b) Molecular function analyses based on Gene Ontology (GO) functional classification (a) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (b) of differentially expressed genes (DEGs); (c) The fold change of *NFKBIB* messenger RNA (mRNA) level from the RNA-sequencing result and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *NFKBIB*; (d) Western blot analyses of p65, p-p65, IKBα, and IKKα/β; (e, f) Cell proliferation, and detection of migration and invasion abilities of lung adenocarcinoma (LUAD) cells after pyrrolidinedithiocarbamate (PDTC) treatment (5 μmol/L). *NFKBIB*: nuclear factor-κB inhibitor β; p-p65: phosphorylated p65; IKBα: nuclear factor-κB inhibitor α; IKKα/β: inhibitor of κB kinase α/β; OD_{450}: optical density at 450 nm. BCAT1-OE and Lv-con: BCAT1 overexpressing and control LUAD cell lines, respectively. Data are expressed as mean±standard deviation (SD), n=3. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

occurs in several types of cancer, including lung cancer (Shamekhi et al., 2020). ROS are byproducts of metabolism, which have a double-edged role in NF-κB signaling. Usually, high levels of ROS lead to cell apoptosis, while low levels of ROS function as a signal transduction factor (Zhang et al., 2016; Zhou et al., 2021). Low levels of ROS and high expression levels of antioxidants have been usually reported to activate the NF-κB pathway, which further impairs ROS generation and antioxidant expression (Morgan and Liu, 2011). Herein, we found that the BCAT1-overexpressing cells showed decreased ROS content and increased expression levels of antioxidants, which probably led to NF-κB pathway activation. ROS function through various mechanisms in different cells and activate the NF-κB pathway through IKB degradation and the entrance of NF-κB protein into the nucleus. ROS also activate the NF-κB pathway together with inflammation factors in LUAD cells (Loukili et al., 2010). This study provides evidence for the participation of ROS and NF-κB pathway activation in the tumorigenesis of LUAD cells with BCAT1 overexpression.

Based on the accumulating evidence, in which NF-κB pathway activation promotes cancer development, inhibitors of the NF-κB pathway have been considered promising drugs for cancer treatment (Chen et al., 2019; Tyagi and Patro, 2019). In this study, we found that treatment with the NF-κB pathway inhibitor PDTC could significantly decrease the proliferation and invasive abilities of LUAD cells, while BCAT1-overexpressing LUAD cells seemed to be more sensitive to PDTC treatment. These findings indicate that BCAT1 overexpression-induced NF-κB pathway activation plays a pivotal role in promoting LUAD progression, and NF-κB pathway inhibition can prevent LUAD development in vitro. Furthermore, NF-κB pathway inhibitors may be potentially useful for the precise treatment of LUAD patients with BCAT1 overexpression in tumor tissues.

In summary, this study revealed that BCAT1 protein was upregulated in the tumor tissues of LUAD patients. BCAT1 overexpression promoted LUAD development through enhanced BCAA metabolism, mitochondrial biosynthesis, and respiration, and reduced ROS content, which finally led to the downregulation of *NFKBIB* and NF-κB pathway activation (Fig. 6). These findings suggest that BCAT1 is a promising biomarker for LUAD diagnosis, and NF-κB pathway inhibitors may have a therapeutic potential in LUAD treatment.

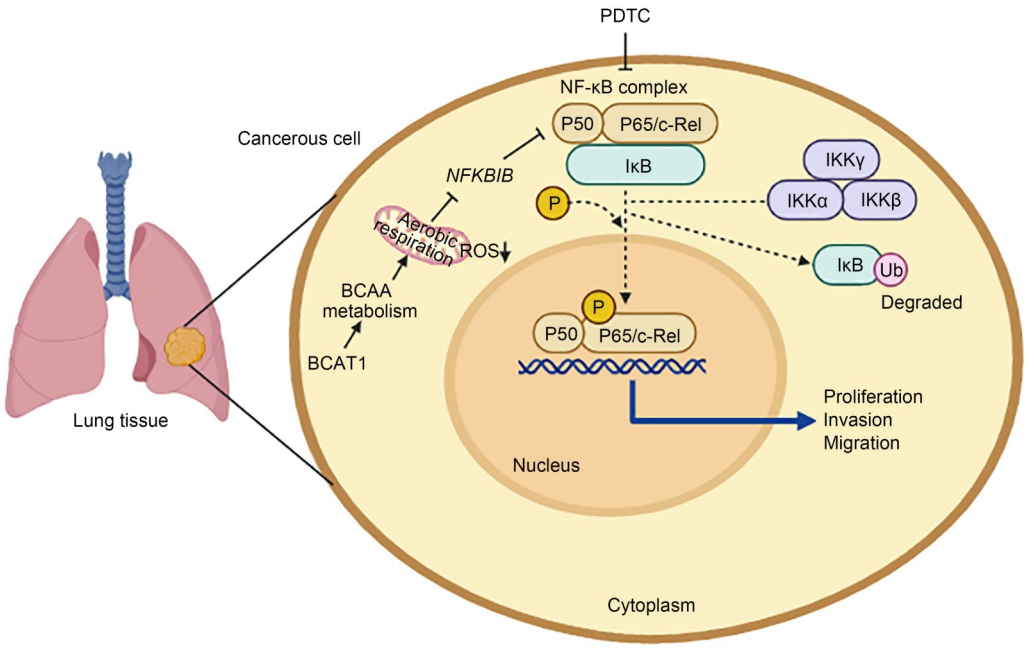

Fig. 6 A schematic model illustrating the role of branched-chain aminotransferase 1 (BCAT1) in lung adenocarcinoma (LUAD) development. PDTC: ammonium pyrrolidinedithiocarbamate; BCAA: branched-chain amino acid; ROS: reactive oxygen species; NF-κB: nuclear factor-κB; *NFKBIB*: NF-κB inhibitor β; IκB: NF-κB inhibitor; IKKα/β/γ: inhibitor of κB kinase α/β/γ.

Material and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Availability of data

The RNA sequencing data were submitted to BioSample database of NCBI (BioProject ID: PRJNA717911).

Acknowledgments

This work was supported by the National Science and Technology Major Project of China (No. 2018ZX10302205), the Major Project of Science and Technology in Henan Province (No. 161100311400), the Natural Science Foundation of Henan Province (Nos. 182300410361 and 222300420306), the Project of Basic Research Fund of Henan Institute of Medical and Pharmacological Sciences (No. 2022BP0103), and the Key Scientific and Technological Project of Henan Province (Nos. 212102310124 and 222102310083). We appreciate the continuous support of Henan Key Laboratory for Pharmacology of Liver Diseases where most of the experiments were performed.

Author contributions

Qianwei ZHAO, Jintao ZHANG, and Jianying ZHANG designed the study. Mengdan YU, Jinxia LI, and Zhibiao ZHANG performed the experiments and analyzed data. Fang XU, Yixian LIU, Liping DAI, and Bingxia ZHANG collected materials and helped in manuscript preparing. Qianwei ZHAO, Mengdan YU, and Jinxia LI wrote the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Mengdan YU, Qianwei ZHAO, Jinxia LI, Fang XU, Zhibiao ZHANG, Yixian LIU, Liping DAI, Bingxia ZHANG, Jianying ZHANG, and Jintao ZHANG declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. All institutional and national guidelines for the care and use of laboratory animals were followed.

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Supplementary information

Material and methods; Figs. S1–S5