BCAT1 Promotes Lung Adenocarcinoma Progression Through Enhanced Mitochondrial Respiration and NF-κB Pathway Activation

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Abstract

BCAT1 is up-regulated and acts as an oncogenic factor in many types of cancers, but its role in lung adenocarcinoma (LUAD) development is not clearly understood. Here we found BCAT1 protein level was up-regulated in tumor tissues, which was positively associated with TNM stage and local lymph node metastasis of LUAD patients. BCAT1 knockdown inhibited cell growth and mobility while BCAT1 overexpression promoted LUAD development both *in vitro* and *in vivo*. BCAAs metabolism and mitochondrial respiration were enhanced in BCAT1 overexpression cells, which were more sensitive to Leucine and Isoleucine supplements, compared to control cells. Moreover, RNA sequencing analysis suggested that differentially expressed genes (DEGs) in BCAT1 overexpression LUAD cells were enriched in metabolism, signal transduction, and immune response processes, and BCAT1 overexpression decreased NFKBIB mRNA level that induced NF-*κ*B pathway activation in LUAD cells. As an inhibitor of NF-*κ*B pathway, ammonium pyrrolidinedithiocarbamate (PDTC) treatment predominately counteracted the effect of NF-*κ*B pathway activation and inhibited LUAD cells proliferation and migration, especially cells with BCAT1 overexpression. Taken together, our findings point a key role for BCAT1 in promoting LUAD development through metabolic reprogramming and NF-*κ*B pathway activation, which provides promising molecular biomarker and therapeutic targets for LUAD diagnosis and treatment.

Introduction

Lung cancer is one of the most common and malignant cancers in the world, and non-small cell lung cancer (NSCLC) takes around 85% of all the cases[1]. NSCLC mainly includes LUAD and squamous cell carcinoma (SCC)[2]. Among NSCLC cases, LUAD accounts for about 60% and remains a major cause of cancer-related mortality worldwide[3,4]. In recent years, increasing researches on the pathobiology and progression of lung cancer have been performed and many genes have been identified as potential drivers and targets for therapy[5–9]. However, the cure and prognosis of lung cancer patients are hardly improved[10,11]. Therefore, it is necessary to reveal the precise mechanism of lung cancer development and develop more efficient diagnostic and therapeutic methods for clinical lung cancer patients.

Among the Branched-chain aminotransferases (BCAT) enzymes, BCAT1 is an enzyme that plays an important role in branched-chain amino acids (BCAAs) metabolism process. BCAAs including leucine, isoleucine and valine are essential nutrients for protein synthesis and cell growth, which can be consumed to provide energy for cancer cells growth and proliferation[12,13]. Increasing evidence showed that BCAT1 is a vital and well researched enzyme which is closely associated with cancers development. It was found that BCAT1 was up-regulated in many kinds of cancers, which has been proposed as a prognostic cancer cell marker[14–16]. Besides, researches showed that BCAT1 up-regulation promoted cancer cell proliferation, cell cycle progression, differentiation, apoptosis, invasion and metastasis in numerous types of cancers, such as gastric cancer, colorectal cancer, prostate cancer, breast cancer, ovarian cancer, hepatocellular cancer, and so on[17–20]. As an important enzyme, BCAT1 and its metabolite can interact with metabolism process in cancer cells through different ways, such as mitochondrial

biogenesis, fatty acid metabolism and so on. For example, glutamate generated from BCAAs metabolism is an important source of *α*-ketoglutarate, which is an intermediate product of TCA cycle and provides energy for cell growth and proliferation[21]. It is also found that BCAT1 promotes the growth of breast cancer cells through improving mTOR-mediated mitochondrial biogenesis and function, while BCAT1 suppression results in downregulation of numerous genes implicated in lipid production and protein synthesis, and finally inhibits tumor growth[12,16]. Taken together, BCAT1 plays an important role in cancer development, and making it clear of its functional mechanism during LUAD development possibly promotes the clinical treatment of LUAD patients.

In this study, we found BCAT1 was overexpressed in LUAD cancer tissues, compared to the normal lung tissues of LUAD patients. The *in vitro* and *in vivo* studies showed that BCAT1 overexpression promoted LUAD cells proliferation, migration and invasion. Besides, we found that BCAT1 overexpression promoted BCAAs metabolism and mitochondrial respiration of LUAD cells, and transcriptomic study indicated that NF-*κ*B signaling pathway was activated in BCAT1 overexpression LUAD cells. Moreover, treatment of LUAD cells with PDTC, which is an inhibitor of NF-*κ*B pathway, prominently inhibited cell proliferation and invasion abilities. In conclusion, our findings offer insights into the role of BCAT1 in NF-*κ*B pathway activation during LUAD development, which might provide new methods for clinical diagnose and treatment of LUAD patients.

Results

BCAT1 was up-regulated in tumor tissues of LUAD patients

To evaluate the significance of BCAT1 during LUAD development, we investigated the expression levels of BCAT1 in tumor and para-carcinoma normal lung tissues from clinical LUAD patients. TMAs containing 86 pairs of tissue specimens were examined by IHC staining using human BCAT1 antibody. The positive rates were scored as 0, 1–2, and 3–4 for negative, weak positive, and positive staining, respectively. As shown in Fig. 1A, the frequency of positive staining of BCAT1 in tumor tissues was significantly higher (69 of 86 [80%]) than that in normal tissues (14 of 86 [16%]), while there existed 5 (0.5%) and 61 cases (71%) in tumor and normal tissues where BCAT1 were negative staining, respectively. According to the "staining intensity score" and the "staining positive rate score" from IHC results, the expression level of BCAT1 was significantly higher in LUAD tumor tissues compared to normal lung tissues (Fig. 1B, $p < 0.0001$). Furthermore, statistical analysis of LUAD specimens indicated that BCAT1 expression level was positively associated with local lymphatic metastasis ($p = 0.0304$) and TNM stage ($p = 0.0189$), but not with the gender, age, tumor size, distant metastasis, and overall survival (Fig. 1C). Taken together, these results indicated that BCAT1 could be a promising biomarker for clinical LUAD diagnosis, and its up-regulation possibly contributed to LUAD initiation and progression.

BCAT1 knockdown inhibited proliferation, migration, and invasion of LUAD cells in vitro

Next, we detected the expression levels of BCAT1 in immortalized normal lung cell line (BEAS-2B) and LUAD cell lines including NCI-H1975, H1299, A549 and HCC827 by western blot, and results showed that BEAS-2B and H1299 showed high expression level of BCAT1 while NCI-H1975 and HCC827 showed low expression level of BCAT1. Besides, A549 performed moderate expression level of BCAT1, which was chosen for functional study of BCAT1 in LUAD development (Figure S1). Then lentiviral shRNA vector (BCAT1-sh) targeting BCAT1 were constructed and transfected into A549 cells. Western blot and qRT-PCR analysis in Fig. 2A showed that expression level of BCAT1 were knocked down by more than 60% with BCAT1-sh, compared to the cells transfected with empty vector (sh-con). Then cell proliferation abilities of BCAT1 knockdown and sh-con LUAD cells were detected by xCELLigence Real-Time Cell Analyzer (RTCA)-MP system. As shown in Fig. 2B, BCAT1 knockdown significantly decreased proliferation rate of LUAD cells *in vitro*. Besides, the colony formation assay in Fig. 2C showed that colony numbers were decreased in BCAT1 knockdown LUAD cells. Furthermore, wound healing, migration and matrigel invasion assays were performed in BCAT1 knockdown and control LUAD cells, and results in Fig. 2D-F showed that the wound closure ability was decreased and the migratory and invasive cell numbers were markedly reduced, which was consistent with the results that E-cadherin was up-regulated while Vimentin was down-regulated in BCAT1 knockdown cells. Collectively, these results demonstrated that BCAT1 suppression could inhibit tumorigenesis of LUAD cells *in vitro*.

BCAT1 overexpression promoted tumorigenesis of LUAD cells both in vitro and in vivo

Then we constructed the A549 cell line that stably overexpressing BCAT1 with lentivirus method. The expression levels of exogenous BCAT1 were demonstrated by western blot and qRT-PCR analysis in Fig. 3A. Then these two groups of cells were subjected to cell growth and colony formation assays (Fig. 3B and 3C), and results showed that the cell proliferation rate and colony formation ability of BCAT1-transfected cells were both much higher than those of control cells (Lv-con). Besides, the function of BCAT1 on motility and invasiveness of LUAD cells were investigated. The wound healing assay showed that overexpression of BCAT1 enhanced LUAD cells migration at the edge of scratched regions and significantly accelerated the speed of wound closure (Fig. 3D). Migration and matrigel invasion assays showed that the number of BCAT1-transfected cells migrating through the filter membrane remarkably increased compared to that of Lv-con cells (Fig. 3E). Besides, it was found that the expression level of E-cadherin was decreased while Vimentin was increased in BCAT1 overexpression cells (Fig. 3F). These results suggested that overexpression of BCAT1 enhanced the mobility and invasion capability of LUAD cells *in vitro*. Furthermore, to study the influence of BCAT1 on LUAD development *in vivo*, we established the xenograft mice model and A549 cells with BCAT1 overexpression and control cells were implanted subcutaneously into nude mice, respectively. Five weeks later, mice were sacrificed and the tumors were

collected for analysis. Results in Fig. 4A-C showed that the volumes and weights of LUAD tumors from BCAT1 overexpression group were larger and higher than those of control group. Additionally, IHC analysis was performed with the LUAD tumors and it showed that BCAT1 overexpression was maintained in the xenografts (Fig. 4D). In conclusion, these results suggested that BCAT1 overexpression promoted LUAD tumor formation and development both *in vitro* and *in vivo*.

BCAT1 overexpression enhanced BCAAs consumption and mitochondrial respiration of LUAD cells

Since BCAT1 is an important enzyme during BCAAs (Leucine, Isoleucine, Valine) metabolism process, we examined the effects of BCAAs supplementation on BCAT1 overexpression LUAD cells (Fig. 5A). It showed that the cell proliferation ability was enhanced with increasing BCAAs concentration. Moreover, we examined the sensitivity of BCAT1 overexpression and control cells to BCAAs supplementation, respectively. Results showed that compared to control cells, the cell proliferation abilities of BCAT1 overexpression cells were higher with Leucine and Isoleucine supplementation, while there was no difference in cell proliferation abilities between BCAT1 overexpression and control cells with Valine supplementation (Fig. 5B). BCAAs metabolism mainly provided metabolic intermediates for TCA cycle and mitochondrial oxidative phosphorylation that contributed to energy generation or biosynthesis (Fig. 5C), then we detected the mRNA expression level of several important genes associated with BCAAs metabolism in BCAT1 overexpression and control LUAD cells. Results in Fig. 5D showed that the transcription levels of genes promoting BCAAs metabolism including BCKDHA, BCKDHB, and ECHS1 increased and BCAAs metabolism inhibitor BCKDK was down-regulated in BCAT1 overexpression cells. It indicated that BCAAs metabolism possibly contributed to energy production in BCAT1 overexpression cells. Then we measured the effect of BCAT1 overexpression on oxygen consumption of LUAD cells. The mitochondrial respiration was assessed by measurement of OCR, which was increased in BCAT1 overexpression cells while BCAT1 inhibition decreased OCR (Fig. 5E and F). Taken together, these results proved that BCAT1 overexpression enhanced BCAAs metabolism and mitochondrial respiration to promote LUAD development.

RNA-seq and bioinformatics analysis of DEGs in BCAT1 overexpressed LUAD cells

To further study the mechanism of BCAT1 in promoting LUAD development, RNA-seq was performed in BCAT1 overexpressed and control LUAD cells, respectively. The global transcriptome maps showed that a total number of 17503 genes were detected both in BCAT1 overexpressed and control LUAD cells. About 440 DEGs were identified, among which 139 genes were down-regulated and 301 genes were up-regulated in BCAT1 overexpressed LUAD cells (Fig. 6A). Then GO analysis was performed with these DEGs based on biological process, cellular component, and molecular function (Fig. 6B). Biological process analysis indicated that DEGs were enriched in cellular process, biological regulation, response to

stimulus, multicellular organismal process, signaling, developmental process, metabolic process, and localization. Cellular component analysis showed that DEGs were mainly located in cell, organelle, membrane, and membrane part. Besides, molecular function analysis showed that DEGs were mostly characterized by functions including binding and catalytic activity. To better understand the cellular processes regulated by BCAT1 overexpression in LUAD cells, KEGG pathway classification analysis was carried out, and results in Fig. 6C showed that DEGs were primarily clustered in six categories, among which DEGs were mostly associated with signal transduction, cancer development, metabolism and immune system. Consistently, we performed KEGG functional enrichment analysis in Fig. 6D, and it indicated that DEGs were mainly enriched in metabolic processes, AGE-RAGE signaling pathway, cell adhesion and migration, and so on.

NF-κB signaling pathway was activated in BCAT1 overexpression LUAD cells

Among the DEGs, RNA-seq results showed that NFKBIB was significantly downregulated in BCAT1 overexpressed LUAD cells. Since NFKBIB combines with NF-κB in the cytoplasm and prevents its transportation into nucleus, inhibiting NF-κB signaling pathway activation. Downregulation of NFKBIB possibly indicated activation of NF-κB signaling pathway in BCAT1 overexpressed LUAD cells. To verify this hypothesis, we first detected the transcription level of NFKBIB by qRT-PCR, and found that it was decreased in BCAT1 overexpressed cells while increased in BCAT1 knockdown cells, as compared with control cells (Fig. 7A). Then the expression levels of proteins involved in NF-κB signaling pathway were investigated, as shown in Fig. 7B, BCAT1 overexpression resulted in higher expression levels of IKKα/β, phosphorylated p65 (p-p65) and lower expression level of IKBα. These findings suggested that BCAT1 overexpression promoted NF-κB signaling pathway activation in LUAD cells.

PDTC treatment inhibited cell proliferation, migration and invasion of BCAT1 overexpression LUAD cells in vitro

To verify the finding that BCAT1 overexpression activated NF-κB pathway to promote LUAD development, then we treated BCAT1 overexpression and control LUAD cells with PDTC, which is an inhibitor of NF-κB pathway activation. Results in Fig. 7C showed that PDTC treatment inhibited the proliferation ability of both BCAT1 overexpression and control LUAD cells, and the inhibiting effect was positively related to the concentration of PDTC. Besides, compared with control LUAD cells, BCAT1 overexpression LUAD cells were much more sensitive to PDTC treatment, which further indicated activation of NF-κB pathway in BCAT1 overexpression LUAD cells. Moreover, we detected the effect of PDTC on migration and invasion abilities of BCAT1 overexpressed LUAD cells. Results in Fig. 7D showed that PDTC treatment significantly decreased the number of migratory and invasive cells. In conclusion, these results suggested that BCAT1 overexpression could promoted LUAD development through NF-κB pathway activation, which could be

counteracted by the treatment of NF-κB pathway inhibitor. This finding provided promising therapeutic method for clinical LUAD cure.

Discussion

To study the relationship between BCAT1 and LUAD development, here we firstly detected the expression level of BCAT1 in tumor and normal lung tissues from LUAD patients. It showed that BCAT1 was significantly overexpressed in tumor tissues, and its expression level was associated with TNM stage and local lymphatic metastasis of clinical LUAD patients, but not with distant metastasis and overall survival. These findings were consistent with an earlier research that BCAT1 was up-regulated in tumor tissues of lung cancer patients and BCAT1 overexpression promoted NSCLC development[22]. Besides, here we found BCAT1 was overexpressed in several LUAD cell lines. These findings suggested that BCAT1 could be taken as a promising biomarker for lung cancer diagnosis including LUAD, and further indicated BCAT1 as a carcinogenic factor during LUAD development.

The function and mechanism study of BCAT1 in LUAD development showed that BCAT1 overexpression promoted tumorigenesis of LUAD cells both *in vitro* and *in vivo*. BCAT1 is an enzyme catalyzing the metabolism of BCAAs. It has been proved that BCAT1 overexpression promoted cancers development through regulating metabolic progresses, such as oxidative phosphorylation, fatty acid and amino acid metabolism[23–25]. Consistently, here we found that BCAT1 overexpression enhanced BCAAs metabolism and mitochondrial respiration in LUAD cells. Moreover, transcriptomic analysis of BCAT1 overexpression and Lv-con A549 cells showed that the DEGs induced by BCAT1 overexpression were mainly clustered into two main categories including metabolism and signal transduction. Among the metabolism associated DEGs, genes were primarily involved in fatty acid synthesis and differentiation. These results suggested that BCAT1 overexpression enhanced BCAAs metabolism, and lead to enhancement of mitochondrial respiration and fatty acid synthesis to promote LUAD cells growth. Besides metabolic reprogramming, KEGG pathway enrichment showed that DEGs were also enriched in AGE-RAGE signaling pathway. AGEs were a kind of metabolites produced from proteins and lipids that induced signaling transduction through RAGE receptors in several kinds of diseases including cancer, metabolic diseases, and so on[26,27]. BCAT1 and enhanced BCAAs consumption were reported to be involved in several signaling pathways associated with cancer development, such as MYC, mTOR, β-cactenin, NF-κB and so on[15,16,28]. Taken together, these results indicated that BCAT1 overexpression promoted metabolism reprogramming of LUAD cells, and lead to signal transduction processes to promote LUAD development.

NF-κB is a nuclear transcription factor which regulates expression of a large number of genes that are critical for the regulation of apoptosis, tumorigenesis, inflammation, and metabolic processes[29]. Increasing researches showed that NF-κB pathway activation was found in the tumor tissues of many types of cancers including lung cancer[30,31]. Besides, it was found that NF-κB pathway activation promoted cancer development through enhancing cell proliferation, invasion, and angiogenesis, inhibiting cell apoptosis, inducing metabolism reprogramming, and regulating inflammation and immune response,

and so on[32–34]. Therefore, more and more inhibitors of NF-κB pathway were being exploited and NF-κB pathway inhibition was taken as a promising method for cancer treatment[35–38]. In this study, we found that NF-κB pathway was activated in BCAT1 overexpression LUAD cells, and NF-κB pathway inhibitor of PDTC treatment could significantly decreased proliferation and invasion abilities of LUAD cells while BCAT1 overexpression LUAD cells seemed to be more sensitive to PDTC treatment. These findings indicated that NF-κB pathway activation was a pivotal reason to promote LUAD progression induced by BCAT1 overexpression, and proved that NF-κB pathway inhibition could prevent LUAD development *in vitro*. Furthermore, it provided possibility for application of NF-κB pathway inhibitors for precise treatment of LUAD patients with BCAT1 overexpression in tumor tissues.

In conclusion, this study revealed that BCAT1 was up-regulated in tumor tissues of LUAD patients. BCAT1 overexpression promoted LUAD development both *in vitro* and *in vivo* through enhancing BCAAs consumption and mitochondrial respiration, and finally activated NF-κB pathway, which could be counteracted by PDTC treatment (Fig. 8). These findings suggested BCAT1 as a promising biomarker for LUAD diagnosis, and provided NF-κB pathway inhibitors as potential therapeutic methods for LUAD treatment.

Material And Methods

Cell culture

The immortalized normal lung cell line (BEAS-2B) and LUAD cell lines including NCI-H1975, H1299, A549 and HCC827 were cultured in RPMI-1640 or DMEM with 10% FBS and incubated at 37°C in a humidified incubator with 5% CO_2. BEAS-2B was purchased from the Chinese Academy of Sciences Typical Culture Preservation Committee Cell Bank. A549 was purchased from Genetic Testing Biotechnology Corporation. HCC827 was gifted by Proteomics and Signal Transduction Center, Zhengzhou University. H1299 and NCI-H1975 were gifted by Henan Institute of Medical and Pharmaceutical Sciences, Zhengzhou University.

Reagents

Antibodies used included BCAT1 (Sangon Biotech, China), phospho-NF-κB p65 (Ser536) (CST, USA), IKKα/β (Wanleibio, China), IKBα (Wanleibio, China), p65 (Wanleibio, China), GAPDH (Sangon Biotech, China), β-Actin (proteintech, China), E-cadherin (proteintech, China), Vimentin (proteintech, China), Antibody cocktail to aerobic respiration (CST, USA). PDTC, L-Valine, L-Isoleucine, and L-Leucine (MCE, USA).

Immunohistochemistry (IHC) and Evaluation

The tissue microarrays (TMAs) consisting of 86 pairs of human LUAD and normal lung tissues were purchased from Outdo Biotech (China). This study was conducted with the informed consent obtained from all subjects. All experiments were performed in accordance with relevant guidelines and regulations of Declaration of Helsinki and has been approved by the Research Ethics Committee of Zhengzhou University. Anti-BCAT1 antibody was used for IHC detection of BCAT1 expression according to the manufacturer's suggested protocols. In brief, paraffin sections were dewaxed by heat treatment in EDTA buffer, pH = 9. After blocking for 30 min with 3% bovine serum albumin (BSA), tissue sections were incubated with anti-BCAT1 antibody diluted 1:400 overnight at 4 °C. Then they were incubated with the secondary antibody (HRP labeled) at room temperature for 50 min. The staining intensity of tissues was scored as 0 (negative), 1 (1+), 2 (2+), 3 (3+). For statistical evaluation, tissues were scored as 0 (non-staining); 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% (positive staining). BCAT1 protein expression was evaluated as negative (0), weak positive (1, 2), positive (3, 4) according to the staining positive rate score. The total histological score was defined as the product of "staining intensity score" and "staining positive rate score", and 0-5 indicated BCAT1 low expression while 6-12 indicated BCAT1 high expression.

Lentivirus transduction and Stable cell line construction

BCAT1 overexpression, shRNA and control lentiviral particles were purchased from Genechem and Hanbio Biotechnology (China). The shRNA sequences targeting BCAT1 were as follows: 5′-GGATCAAGAATGGGTCCCATATTCA-3′, The shRNA control sequence was: 5′-TTCTCCGAACGTGTCACGTAA-3′. A549 cells were seeded in 6-well plates and infected with BCAT1 overexpression, shRNA or control lentivirus respectively, followed by incubation overnight with 5 μg/mL polybrene (Sigma-Aldrich, USA). Stable BCAT1 knockdown and overexpression cells were selected with 2 μg/mL puromycin.

Protein extraction and Western blot

Cells were lysed in RIPA buffer (Solarbio, China) for 30 min at 4°C and then centrifuged at 12000 rpm for 15 min. The supernatant was collected and total protein was quantified using the BCA kit (Solarbio, China). Total protein was boiled and separated by 10% SDS–PAGE gel, and then transferred to PVDF membrane (Merck Millipore, USA). Membranes were blocked in 5% skim milk powder in TBS-T (TBS plus 0.5% Tween-20) at room temperature for 2 hours, and then blots were incubated with primary antibody at 4°C overnight. The membrane was probed with secondary antibody, and signals were detected with ECL Substrate (Beyotime Biotech, China) and Amersham Imager 600 System (General Electric Company, USA). Protein expression was quantified using Image J software, with GAPDH or *β*-Actin as an internal control.

RNA Extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells with TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instruction and cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa Clontech, China). qRT-PCR was performed using the ChamQ^{TM} Universal SYBR$^{®}$ qPCR Master Mix (Vazyme Biotech, China) and QuantStudio 5 Real-Time PCR System (Thermo Scientific, USA). The target mRNA expression level was quantified using the $2^{-\Delta\Delta Ct}$ method with normalized to the endogenous gene ACTB. The following primer sequences were used for qRT-PCR: BCAT1 forward, 5′-TGGAGAATGGTCCTAAGCTG-3′, reverse, 5′-GCACAATTGTCCAGTCGCT C-3′; NFKBIB forward, 5′-GCCCTGACCGTACTCCCGAC-3′, reverse, 5′-GGCCTCC ACTGCCAAATGAA-3′; BCKDK forward, 5′-CTTCTGAAAAGTGCTCGGTAC-3′, reverse, 5′-CTTCTGGAAGGCACGGATATAT-3′; BCKDHA forward, 5′-GAGGCAGG TGTGCTGATGTATCG-3′, reverse, 5′-AGTGGAGAGGAGATAGTGACGAAGTG-3′; BCKDHB forward, 5′-GGTGGCTCATTTTACTTTCCAG-3′, reverse, 5′-AGGATCTTT GGCCAATGAGTTA-3′; ECHS1 forward, 5′-GTGATATCATCTATGCCGGTGA-3′, reverse, 5′-GTCTCAACAGGACAAATCTTGC-3′; GAPDH forward, 5′-CTCAAGGG CATCCTGGGCTA-3′, reverse, 5′-CGTCAAAGGTGGAGGAGTGG-3′; ACTB forward, 5′-GGCATCCACGAAACTACCTT-3′, reverse, 5′-CTCCTGCTTGCTGATCCACA -3′. Primers used for qRT-PCR were synthesized by Shenggong, China.

RNA sequencing (RNA-seq) and Informatics analysis

RNA-seq was performed with BCAT1 overexpression and control A549 cells (each with three replicates) using BGISEQ-500 sequencing system. In brief, RNA sample was extracted and the quality was assessed, followed by generation of cDNA libraries. Gene expression levels for each sample were quantified using the FPKM method (fragments per kilobase of transcript per million mapped reads) and processed for analysis. DEGs were screened by fold change ≥ 1.5 and the adjusted p value ≤ 0.01. Gene Ontology (GO) annotation proteome was performed from the UniProt-GOA database (http://www.ebi.ac.uk/GOA). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was based on database (http://www.genome.jp/kegg/).

Cell proliferation assay

The cell proliferation ability was measured by using the xCELLigence Real-Time Cell Analyzer (RTCA)-MP system (Acea Biosciences, USA), which provided a continuous and quantitative measurement of the cell index to monitor cellular growth status. First, 50 μL of culture medium was added in each well of E-Plate 12 (Roche Applied Science, USA) to detect the baseline. Then cells were suspended in 100 μL medium and seeded in E-Plate 12 with a density of 5×10^4 cells/mL. After 30 min, the E-Plate 12 was inserted into RTCA-MP device and incubated at 37°C with 5% CO_2. Cell index was measured every 5 min to reflect the cellular proliferation ability.

CCK-8 assay

Cells in the logarithmic growth phase were seeded in a 96-well plate (3000 cells/well), and the total volume of cell suspension per well was 100 μL. After incubation for 24 h and 48 h, 10 μL CCK-8 reagent was added into each well, and then cells were incubated for 1-4 h at 37°C in the dark. The absorbance (A) value at 450 nm wavelength was detected with a microplate reader (Thermo, USA).

Colony formation assay

Cells were seeded into 6-well plates (1000 cells/well). Then cells were cultured at 37°C with 5% CO_2 for approximately one week. After that, clones were washed with PBS and then fixed with 4% paraformaldehyde, followed by staining with 1% crystal violet for 30 min. The colonies were examined and counted with a microscope.

Wound-healing assay

Cells were seeded with a density of 10^6 cells/well in 6-well plates, when the cell density reached 95%, monolayers were scratched with a 10 μL pipette tip to create scratch wounds. The suspended cells were washed away with PBS, and then cultured in RPMI-1640 medium with 2% FBS. The distance between scratches was measured at 0 h, 12 h, 24 h, and 48 h, and the relative residual area (%) was calculated using the following equation: (current gap distance/original gap distance) x 100%.

Cell migration and invasion assays

For migration assay, 5×10^4 cells were suspended in serum-free medium and seeded in the top chambers, then the lower chambers were filled with 600 μL medium containing 10% FBS. After incubation for 8~12 h, the migratory cells were fixed with methanol and stained with a 0.1% crystal violet solution. Then cells were counted with a microscope (magnification, x200; Olympus BX53, Japan). For invasion assay, the difference was that Matrigel (BD Biosciences, USA) was diluted in serum-free medium (1:6) and added to the upper chamber before cells were seeded.

Xenograft mice model

Animal study was performed in accordance with the guidelines of the National Act on the Use of Laboratory Animals (P. R. China), approved by the Animal Ethics Committee of Zhengzhou University. This study was reported in accordance with ARRIVE guidelines. BALB/c nude mice (female, aged 4 weeks) were upraised in SPF conditions in Henan Key Laboratory for Pharmacology of liver diseases, with a 12 h light/dark cycle and free access to food and water. BCAT1 overexpression and control A549 cells ($5x10^6$ in 100 μL PBS) were subcutaneously injected into the flanks of mice, respectively. Five weeks

after injection, mice were sacrificed by cervical dislocation and the tumors were collected for analysis. Tumor volume was evaluated with the formula: tumor volume = (width^2 x length)/2.

Cellular oxygen consumption rate (OCR) detection

Briefly, cells (8 x 10^4) were seeded into a 96-well plate and then cultured at 37°C with 5% CO_2 for overnight. Next, the BBoxiProbeTM R01 (BestBio, China) and oxygen blocking fluid were added into the 96-well plate chambers in sequence at 37℃, and the fluorescence intensity at the excitation wavelength of 468 nm was detected by the CLARIOstar Plus (BMG, Germany).

Statistical analysis

All statistical analyses were performed using SPSS 21.0 and visualized with GraphPad Prism 6.0. Data are shown as mean ± standard error of measurement (SEM). A p value ≤ 0.05 was considered as statistically significant.

Declarations

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Author contributions

Q.Z., J.Z., and J.Z. designed the study. J.L., M.Y., and Z.Z. performed the experiments and analyzed data. F.X., Y.L., L.D., and B.Z. collected materials and helped in manuscript preparing. Q.Z., M.Y. and J.L. wrote the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Additional information

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

Ethical statement

This study was conducted with the informed consent obtained from all subjects. All experiments were performed in accordance with relevant guidelines and regulations of Declaration of Helsinki and has been approved by the Research Ethics Committee of Zhengzhou University.

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Figures

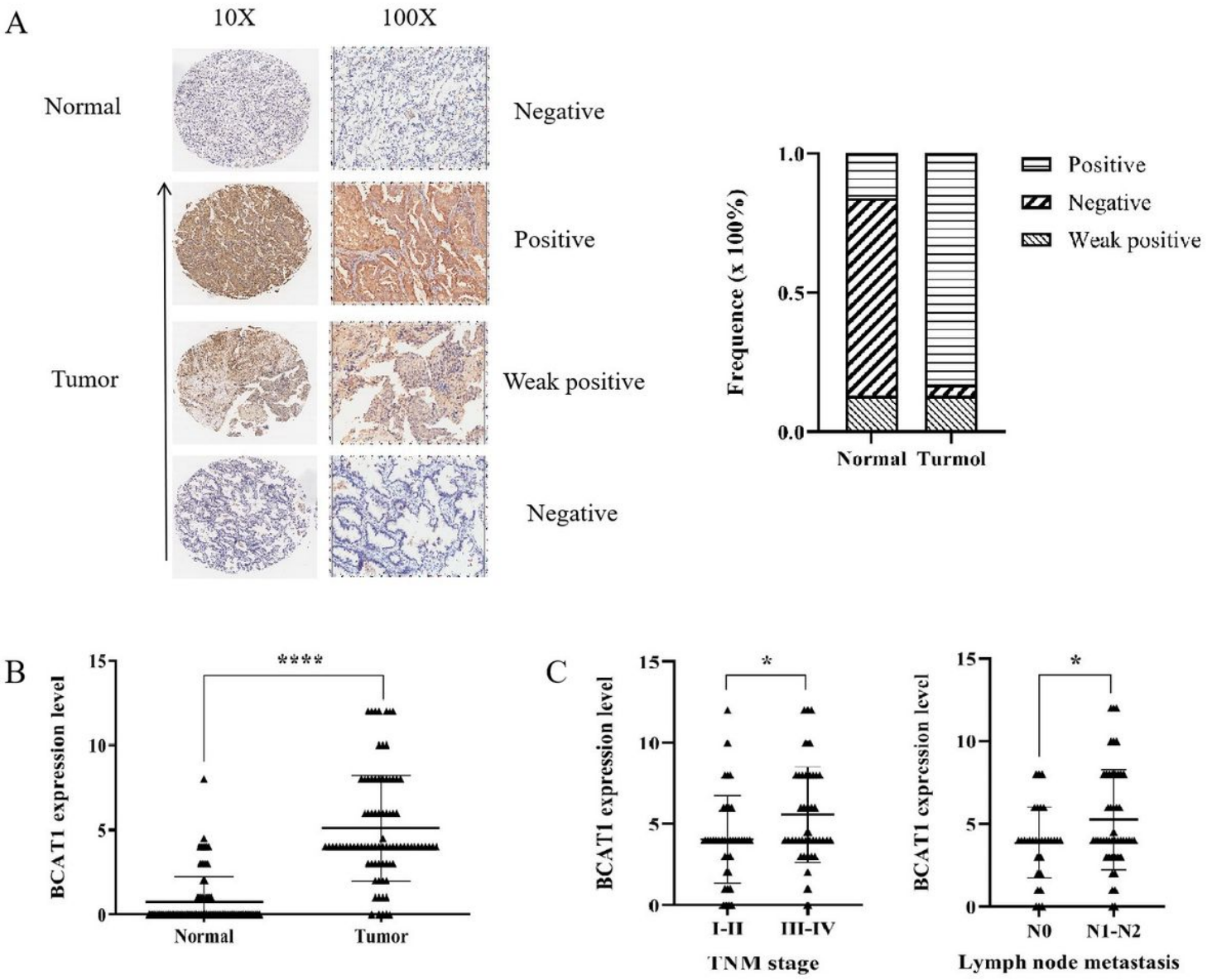

Figure 1

BCAT1 expression is increased in LUAD tumor tissues. (A) IHC staining of BCAT1 in LUAD tumor and normal lung tissues. Histogram showed the frequency of positive, weak positive and negative staining. (B) Expression levels of BCAT1 in LUAD tumor and normal lung tissues. (C) TNM stage and local lymph node metastasis frequency of LUAD patients with different BCAT1 expression levels.

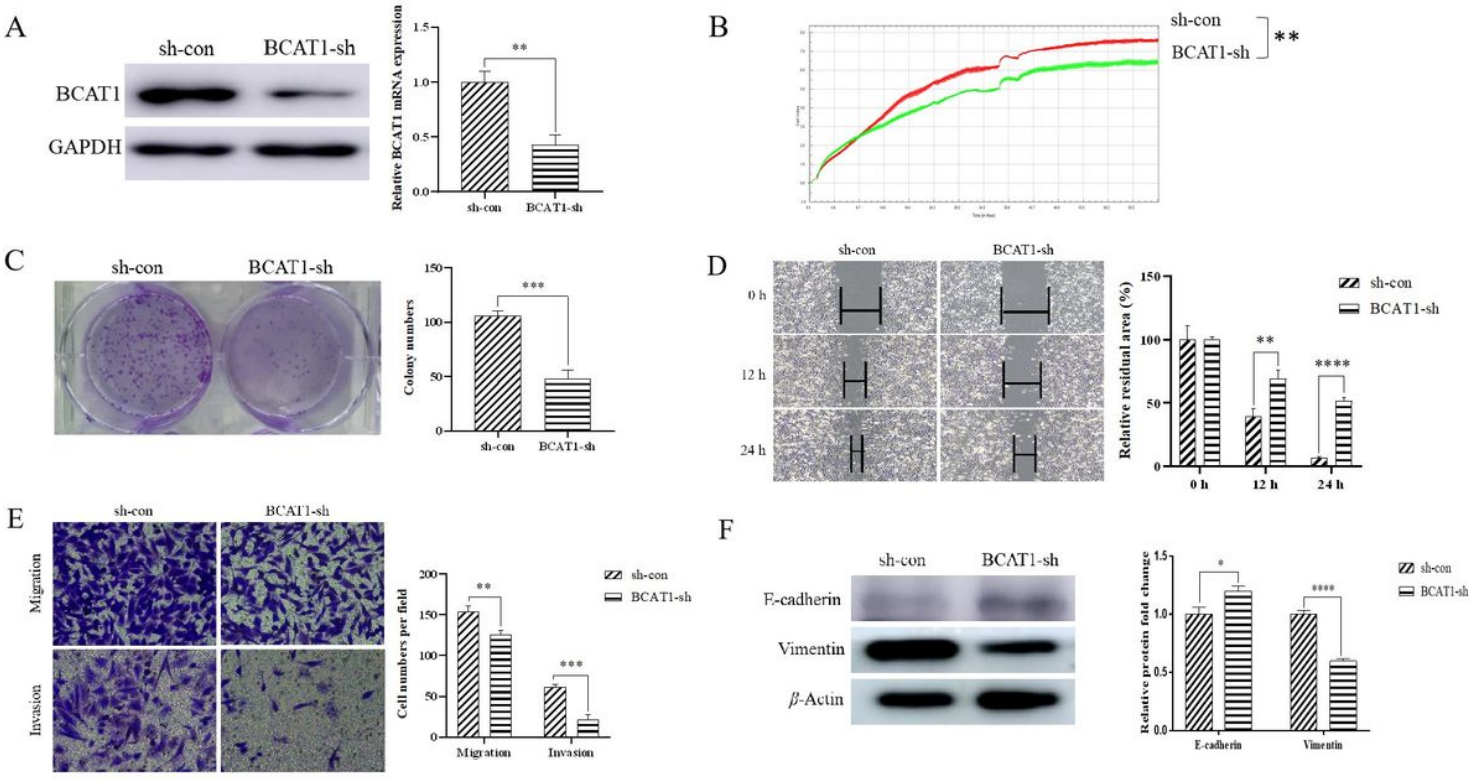

Figure 2

BCAT1 knockdown inhibits cell proliferation, colony formation, migration and invasion. (A) Western blot and qRT-PCR analysis of BCAT1 in A549 cells transfected with sh-con and shRNAs. (B) Cell proliferation rate detection by using the real-time cell electronic sensing system. (C-E) Colony formation, wound healing, migration and invasion assays of A549 cells transfected with sh-con and shRNAs. (F) Western blot analysis of E-cadherin and Vimentin in BCAT1 knockdown and control A549 cells.

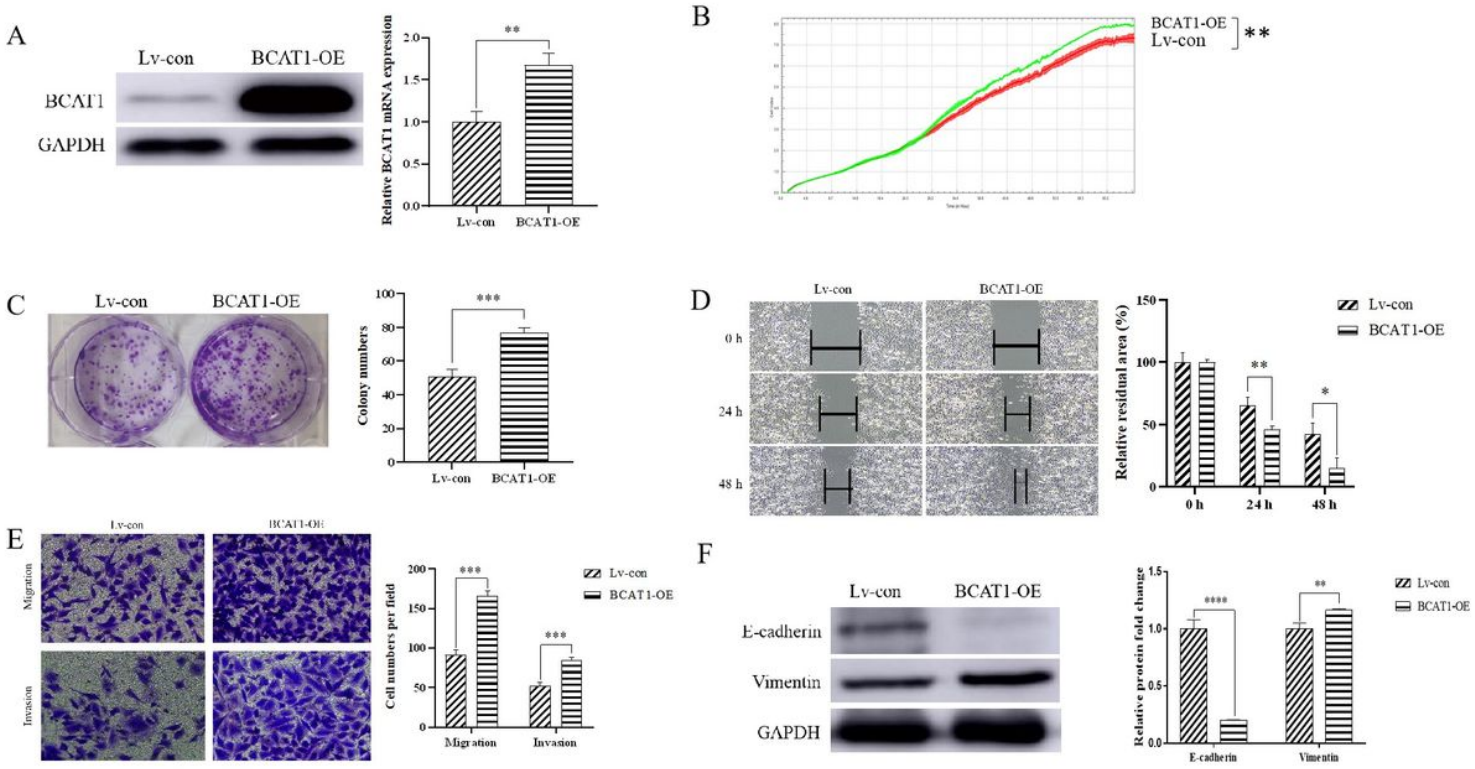

Figure 3

BCAT1 overexpression promotes cell proliferation, colony formation, migration and invasion. (A) Western blot and qRT-PCR analysis of BCAT1 in Lv-con and BCAT1 overexpression A549 cells. (B) Cell proliferation rate detection by using the real-time cell electronic sensing system. (C-E) Colony formation, wound healing, migration and invasion assays of Lv-con and BCAT1 overexpression A549 cells. (F) Western blot analysis of E-cadherin and Vimentin in BCAT1 overexpression and control A549 cells.

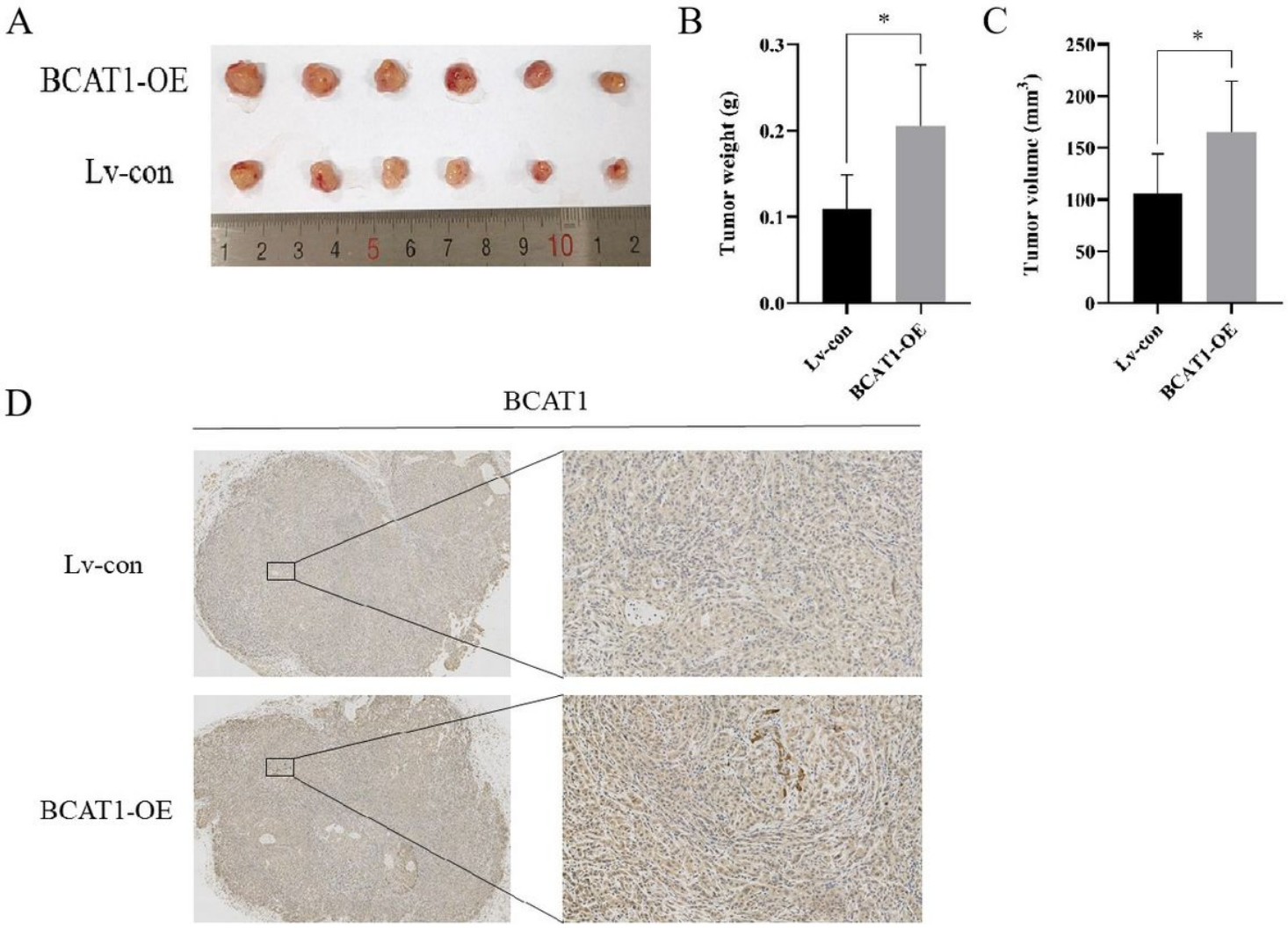

Figure 4

BCAT1 overexpression promotes LUAD tumor formation and growth in vivo. (A) Tumors were established in xenograft mice model after injection with BCAT1 overexpression and control LAUD cells, respectively. (B) and (C) Tumor weight and volume in xenograft mice model. (D) IHC staining of BCAT1 in LUAD tumors from xenograft mice model.

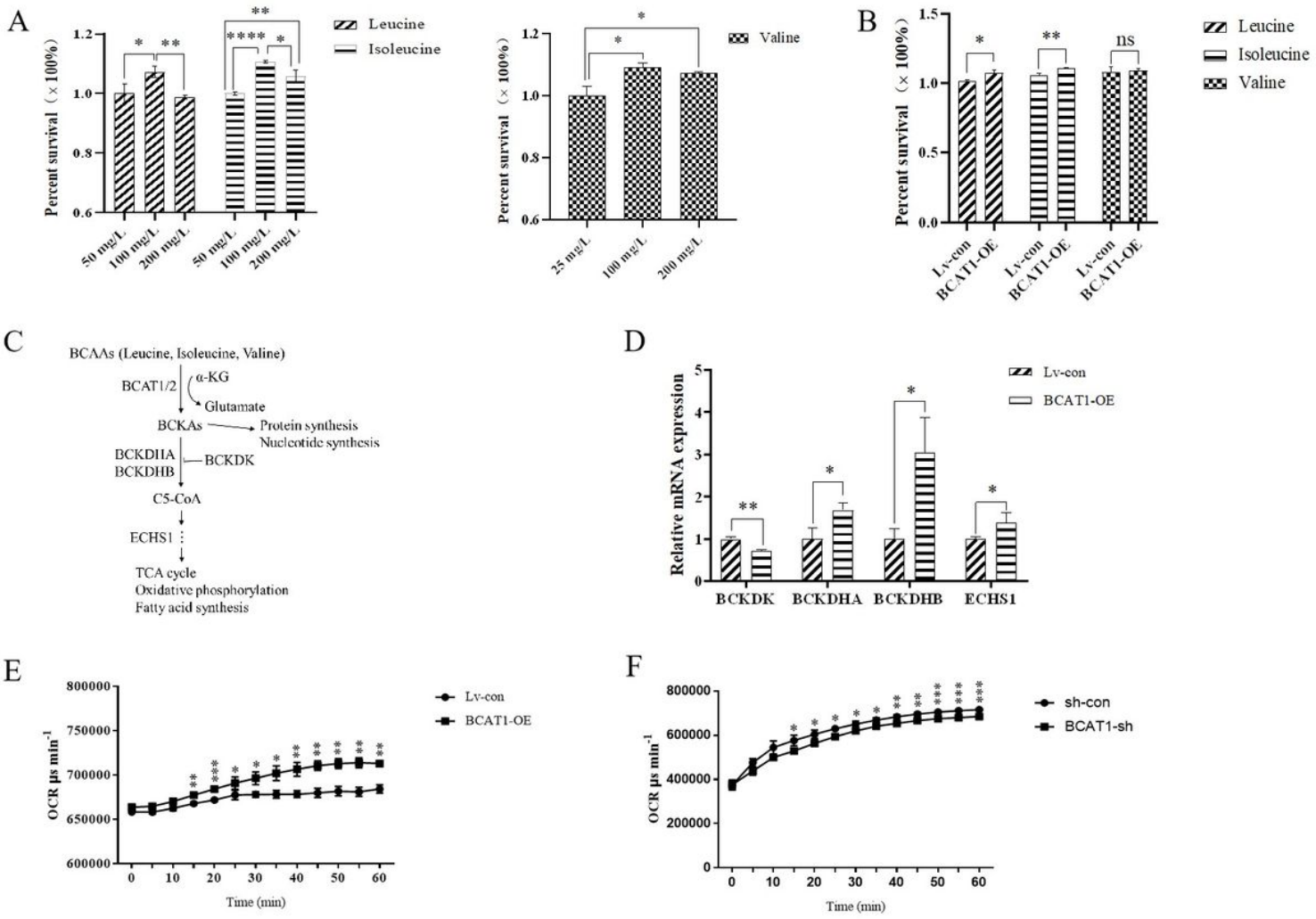

Figure 5

BCAT1 overexpression enhanced BCAAs metabolism and oxygen consumption of LUAD cells. (A) Cell proliferation ability detection of BCAT1 overexpression cells with different kinds and concentrations of BCAAs. (B) Cell proliferation ability detection of BCAT1 overexpression and control cells with different kinds of BCAAs (100 mg/L). (C) Diagram of BCAAs metabolic pathway. (D) qRT-PCR analysis of BCKDK, BCKDHA, BCKDHB, and ECHS1 in BCAT1 overexpression and control A549 cells. (E) and (F) OCR measurement of BCAT1 overexpression, knockdown, and control A549 cells.

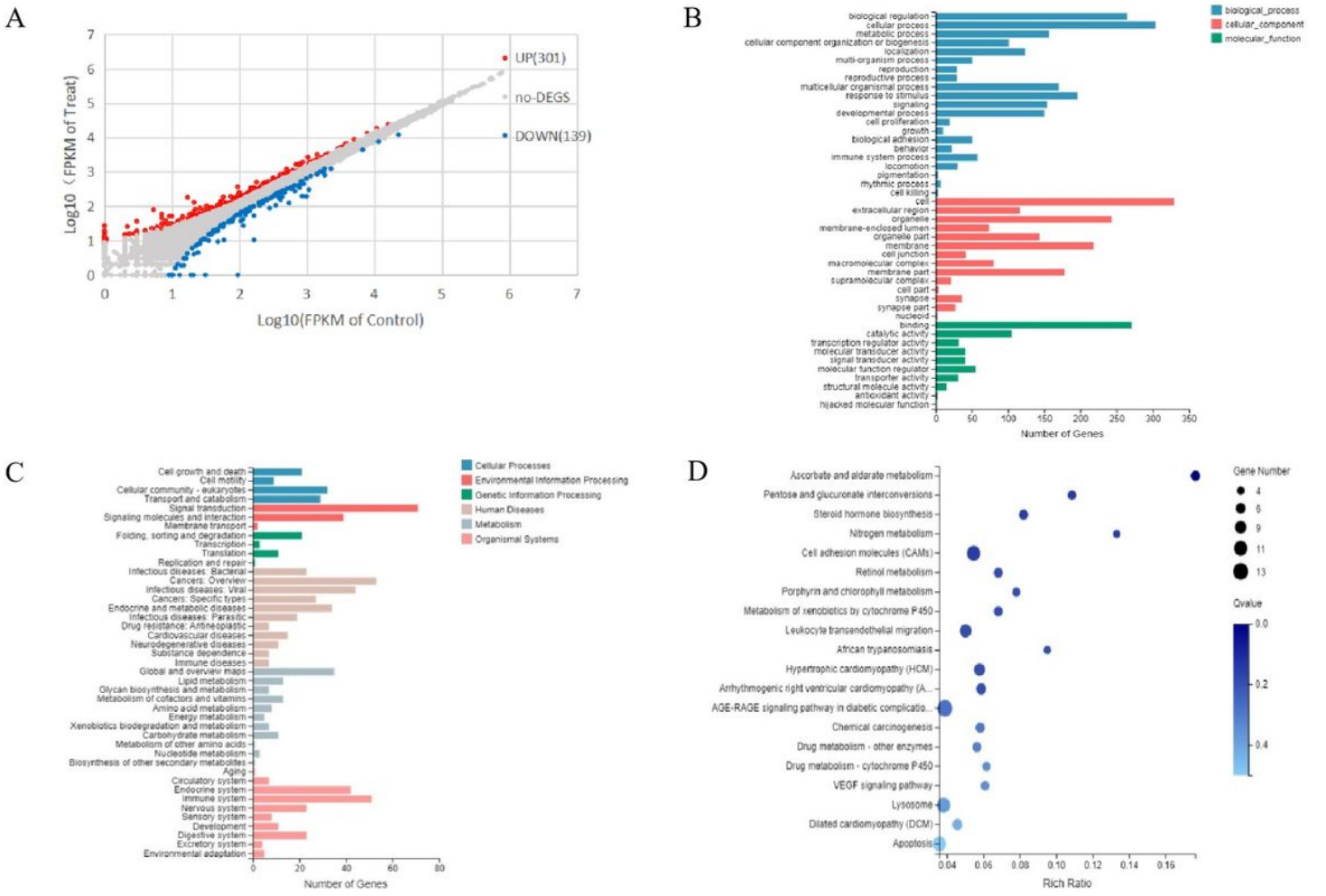

Figure 6

Transcriptome analysis of DEGs induced by BCAT1 overexpression in A549 cells. (A) Scatter plot of global gene expression pattern in Lv-con and BCAT1 overexpression A549 cells. (B) GO functional classification analysis including biological process, cellular component, and molecular function of DEGs. (C) KEGG pathway classification analysis of DEGs based on KEGG database. (D) KEGG functional enrichment analysis of DEGs.

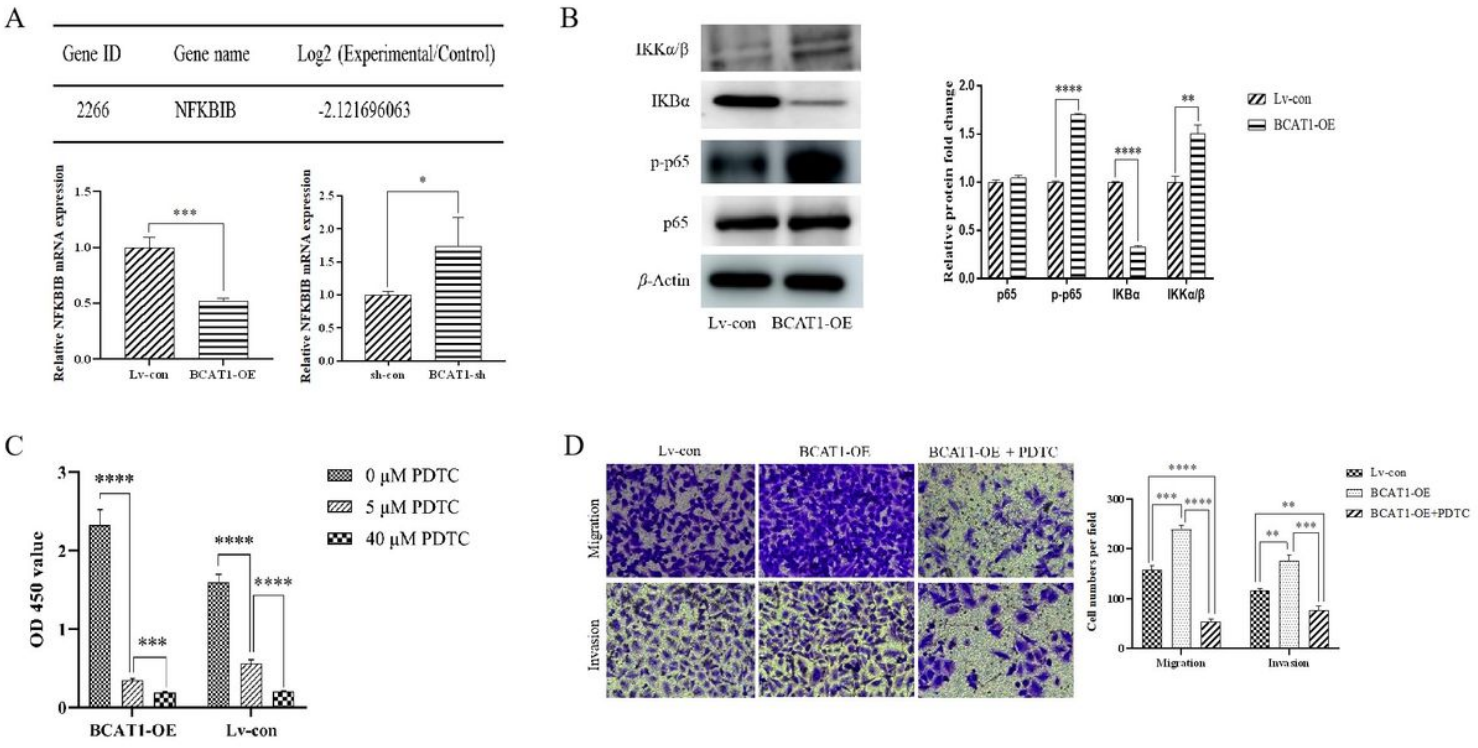

Figure 7

BCAT1 overexpression activates NF-κB pathway. (A) The fold change of NFKBIB mRNA level from RNA sequencing result, and qRT-PCR analysis of NFKBIB in BCAT1 overexpression, knockdown and control A549 cells. (B) Western blot analysis of p65, p-p65, IKBα, and IKKα/β in BCAT1 overexpression and control A549 cells. (C-D) Cell proliferation, migration, and invasion abilities detection of LUAD cells after PDTC treatment.

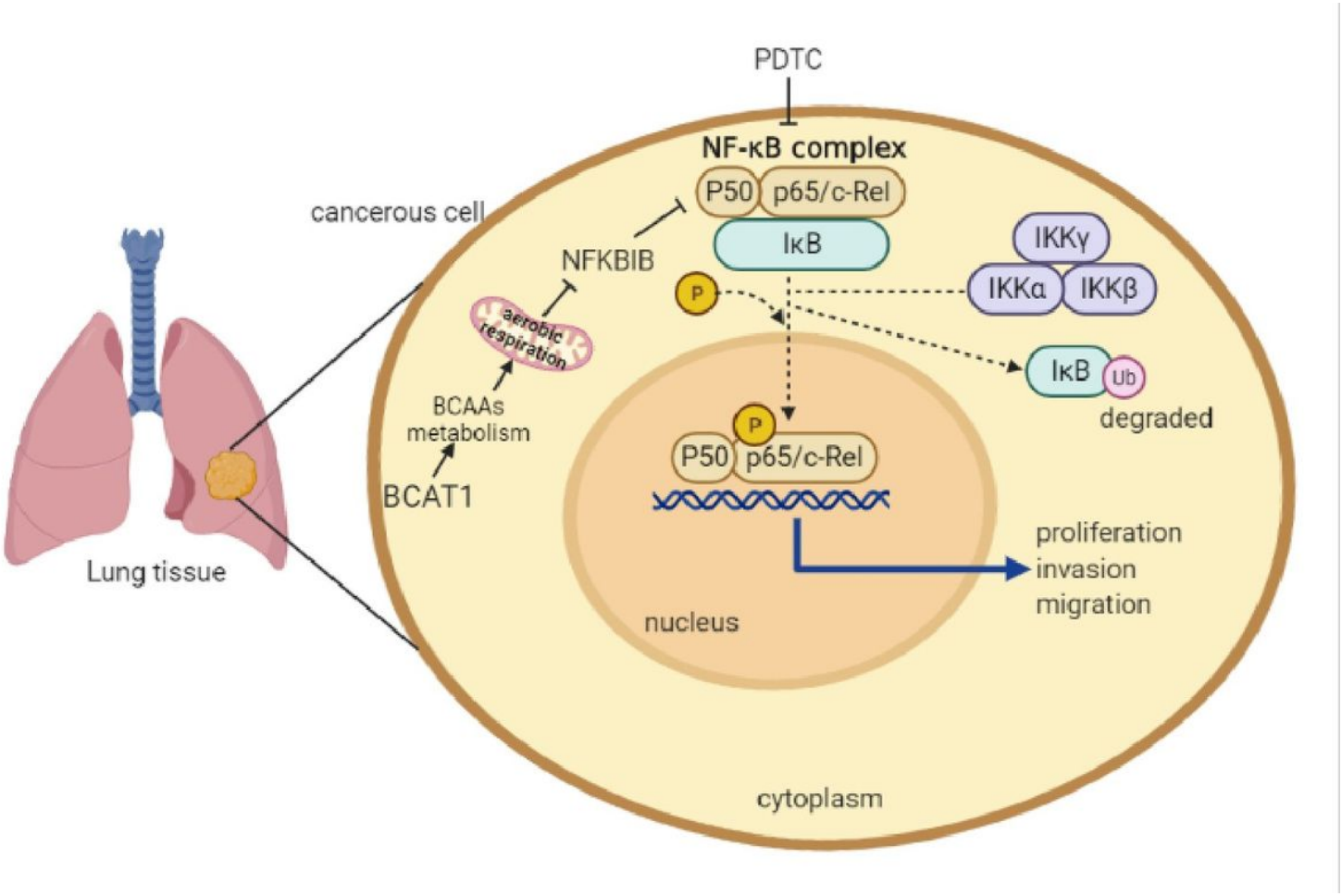

Figure 8

Schematic model of BCAT1 overexpression promotes LUAD development through activating NF-κB pathway, which could be prevented by PDTC treatment.

Supplementary Files

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- SupplementaryInformation.pdf