BCKDK Promotes Epithelial Ovarian Cancer Proliferation and Migration by Activating the MEK/ERK Signaling Pathway

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Research

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Abstract

Background: Ovarian cancer is the most fatal gynecologic cancer, and epithelial ovarian cancer (EOC) is the most common type. The branched-chain α -keto acid dehydrogenase kinase (BCKDK) plays an important role in many serious human diseases, including cancers. Its function in promoting cell proliferation and migration has been reported in various cancers. However, the biological role of BCKDK and its molecular mechanisms underlying EOC initiation and progression are unclear.

Methods: First, the expression level of BCKDK in EOC cell lines or tissues was determined using tissue microarray (TMA)-based immunohistochemistry or western blotting. Then, growth curve analysis, anchorage-independent cell transformation assays, wound healing assays, cell migration assays, and tumor xenografts were used to test whether BCKDK could promote cell transformation or metastasis. Finally, the signaling pathways involved in this process were investigated by western blotting or immunoprecipitation.

Results: We found that the expression of BCKDK was upregulated in EOC tissues and that high expression of BCKDK was correlated with an advanced pathological grade in patients. The ectopic overexpression of BCKDK promoted the proliferation and migration of EOC cells, and the knockdown of BCKDK with shRNAs inhibited the proliferation and migration of EOC *ex vivo* and *in vivo*. Moreover, BCKDK promoted EOC proliferation and migration by activating MEK.

Conclusions: Our results demonstrate that BCKDK promotes EOC proliferation and migration by activating the MEK/ERK signaling pathway. Targeting the BCKDK-MEK axis may provide a new therapeutic strategy for treating patients with EOC.

Background

Among women over 40 years old, ovarian cancer (OC) is the second common gynecological tumor after breast cancer, while it is the deadliest one ^{1,2}. Because it is difficult to diagnose in early stage of OC. OC is mainly divided into three types: epithelial, germ cell, and sex-cord stromal. Epithelial ovarian cancer (EOC) is the most common type, and approximately 95% of OC patients are diagnosed as EOC ^{2,3}. Because of the asymptomatic nature of EOC, about 75% of EOC patients are first diagnosed at an advanced stage.

At present, ovarian cancer screening mainly relies on colposcopy combined with OC biomarker screening, such as serum cancer antigen 125 (CA125) or human epididymis protein. However, an investigation of a randomized controlled trial involving more than 200,000 women showed that the screening group did not significantly reduce the mortality rate compared with the unscreening group ⁴. Therefore, the fatality rate of OC, including EOC, has not decreased significantly in the past 30 years ^{1,5}. On the other hand, although studies showed that the use of PARP1 inhibitors (Niraparib, Olaparib and Rucaparib) significantly improved the progression-free survival of patients with OC ⁶⁻⁹. However, the phenomenon of inherent or acquired resistance is existed in OC patients, and not all patients continue to be effective on PARP

inhibitor therapy⁹⁻¹¹. Therefore, new alternative or complementary targeted drugs are still need to develop urgently.

Branched-chain α -keto acid dehydrogenase kinase (BCKDK) located in the mitochondrial matrix, belonging to pyruvate dehydrogenase kinases (PDKs) family¹², which promoted the proliferation and metastasis of various tumors and was considered to be a strong therapeutic target for preventing tumors development¹³⁻¹⁸. Dysfunction of BCKDK was closely related to various human diseases, especially maple syrup urine disease. Bravo-Alonso and Oyarzabal found the excessive function of BCKDK resulted in maple syrup urine disease¹⁹⁻²¹. As diabetics had increased susceptibility to ovarian cancer, always divided into late stages when the first diagnosis and had a poor prognosis²². Over-expression of BCKDK resulted in branched-chain amino acid (BCAA) increase, elevated plasma levels of BCAA were associated with a greater than 2-fold increased risk of future pancreatic cancer diagnosis²³. Leu promoted adipose tissue protein synthesis through mTOR pathway²⁴, and then adipocytes promoted ovarian cancer metastasis and provided energy for rapid tumor growth²⁵. BCKDK promoted colorectal cancer and hepatocellular carcinoma metastasis and proliferation via the ERK signaling pathway²⁶⁻²⁸. Furthermore, BCKDK was highly expressed in DOX-induced ovarian cancer drug-resistant cell lines, and its expression level was twice as high as that of sensitive one²⁹. Inhibition of BCKDK increased the sensitivity of ovarian cancer cells to paclitaxel³⁰. We can't help but wonder if BCKDK could promote ovarian cancer proliferation and metastasis? Which pathways worked in this process?

In this study, We showed that the BCKDK had a higher expression in EOC patients versus normal tissues. The high expression of BCKDK was correlated with the advanced pathological grade for patients. Overexpression of BCKDK increased the clone formation and migration ability of SKOV3 and OVCAR3 cells *ex vivo*. Knockdown of BCKDK inhibited EOC tumor progression *ex vivo* and *in vivo*. And we identified BCKDK as an upstream kinase of MEK, which up-regulated MEK/ERK signaling by interacting with MEK. The above results suggest that BCKDK may promote EOC proliferation and migration through enhancing the MEK/ERK signaling pathway.

Methods

Plasmids, shRNA, antibodies, and other reagents

The plasmid of pCMV-C-Flag (catalog: D2632) was purchased from Beyotime Biotechnology (Shanghai, China). The plasmid of pDONR223-BCKDK (catalog: 23794) was purchased from Addgene (Cambridge, MA, USA). The plasmids of pCMV-BCKDK-Flag and pLKO.1-shBCKDK were constructed by our laboratories. 5 sequences were designed to knock down BCKDK, and the sequences are: 1. 5'-CCGGGATCTGATCATCAGGATCTCACTCGAGTGAGATCCTGATGATCAGATCTTTTTG-3'; 2. 5'-CCGGTCAGGACCCATGCACGGCTTTCTCGAGAAAGCCGTGCATGGGTCCTGATTTTTG-3'; 3. 5'-CCGGCGTCCGCTACTTCTTGGACAACCTCGAGTTGTCCAAGAAGTAGCGGACGTTTTTG-3'; 4. 5'-CGGACGCTGACTTCGAGGCTTGGACTCGAGTCCAAGCCTCGAAGTCAGCGTTTTTTG-3'; 5. 5'-

CCGGCCAGCACCAAGTTCCGTCATTCCTCGAGGAATGACGGAACTGGTGCTGGTTTTTG-3'. A mock shRNA with a sequence lacking significant homology to the human genome database was used as the mock shRNA. The sequence was: 5'-CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG-3'. The sense and anti-sense oligonucleotides were synthesized, annealed and cloned into the pLKO.1-TRC cloning vector at the *EcoRI* and *AgeI* sites as described by the manufacturer³¹.

Anti-p-MEK1/2 (ser221) (catalog: 2338), anti-t-MEK (catalog: 8727), anti-phospho-p44/42MAPK (Erk1/2) (T202/Y204) (D13.14.4E) (catalog: 4370), and anti-t-ERK (catalog: 4695) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-mouse BCKDK antibody (catalog: sc-374425) and anti- β -actin antibody (catalog: sc-130656) were purchased from Santa Cruz Technology, Inc (Santa Cruz, CA, USA). HRP-conjugated anti-rabbit (catalog: E030120) antibody and HRP-conjugated anti-mouse antibody (catalog: E030110) were purchased from EarthOx Life Sciences (San Francisco, CA, USA). Anti-Flag antibody (catalog: F1804, catalog: F7425) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The DAB Detection Kit (Polymer) (catalog: PV-6000-D) was purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd (Beijing, China). G418 (catalog: A1720) and puromycin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used for setting up stable cell lines. Simple-Fect (catalog: Profect-01) was purchased from Signaling Dawn Biotech (Wuhan, Hubei, China) for transfection.

Cell Culture

The human epithelial ovarian cancer cell lines (HO8910, HO8910-PM, SW626, SKOV3 and OVCAR3) and the normal cell lines (IOSE80 and HEK293T) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). IOSE80 and HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) and 2 mmol/L glutamine (Gibco, Gaithersburg, MD, USA). HO8910 and HO8910-PM cells were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS and 2 mmol/L glutamine. SKOV3 cells were cultured in McCoy's 5A (Gibco) supplemented with 10% FBS and 2 mmol/L glutamine. OVCAR3 were cultured in RPMI-1640 (Gibco) supplemented with 20% FBS and 2 mmol/L glutamine. All cells were grown in 5% CO₂ with saturated humidity at 37°C.

Western blot

Cells ($0.8-2 \times 10^6$) were cultured in 10 cm diameter dishes to 70–80% confluence, and then starved without serum for 24h. Then the cells were treated with 40 ng/mL epidermal growth factor (EGF) (R&D catalog: 236-EG-200) for 15min. EGF is a well-known tumor promotion agent used to study malignant cell transformation in animal and cell models of cancer³². After this, cells were washed twice in PBS before being lysed in RIPA buffer (Coolaber, China). Then, samples were sonicated in 15 seconds intervals three times, and insoluble debris was removed by centrifugation at 13000 rpm for 15 min. Protein content was determined by BCA method (Coolaber, China). 30–120 μ g of protein was separated by 10% SDS-PAGE and visualized by chemiluminescence (BIO-RAD, USA) in triplicate.

Growth curve analysis

2×10^5 cells were plated in each dish and counted at different times in triplicate, using a hemacytometer to generate a growth curve.

Anchorage-independent cell transformation assay

Different cell lines (8×10^3 /well) were seeded in 6-well plates. The cells were then cultured in 1 mL of agar (Sigma-Aldrich Corp.) containing 10% FBS, 0.33% BME (Eagle basal medium, Sigma-Aldrich Corp.), 25 μ g/mL gentamicin and 2 mM L-glutamine, with an additional 3 mL of 2 mM L-glutamine, 0.5% BME agar containing 10% FBS and 25 μ g/mL gentamicin being below. Then the cells were maintained in a 37°C, 5% CO₂ incubator for 7–14 days and the colonies were observed and assessed by microscopy in triplicate assays.

Tumor xenografts

Female athymic Balb/c nude mice (4–6-week-old) were purchased from Chongqing Tengxin Beer Experimental Animal Sales CO, LTD (Chongqing, China). Mice were kept in specific pathogen-free conditions according to the National Guidelines for Animal Usage in Research (set by the Chinese government) at the Chongqing Population and Family Planning Science and Technology Research Institute. Mice were divided and randomized into three groups. Each of the different cell lines (3×10^6 in 200 μ L PBS) was injected subcutaneously into the right flank. The tumor volumes were measured every three days and were calculated with the formula: $V = 0.52 (\text{length} \times \text{width} \times \text{height})$. Forty days after the injection, the mice were sacrificed with dislocation of cervical vertebra after injecting pentobarbital sodium (50 mg/kg, i.p.), 5 min. The tumor tissues were prepared with paraffin sections after fixation with formalin, and then stained with hematoxylin and eosin (H&E).

Patients samples and immunohistochemistry (IHC)

The tissue microarrays (TMA) of EOC (catalog: FOV 1006) were purchased from Xi'an Tabos Bio Co., Ltd. This study was approved by the ethical committee of Yichang Second People's Hospital. Samples were obtained with informed consent. Detailed information on patients was shown in Fig. 1d. The TMA was stained followed the standard protocol for IHC staining. High pressure repair was conducted with Tris/EDTA buffer (pH 10.0) for 120 °C, 5 min. The sections were incubated with primary antibody against BCKDK (1:50). The DAB Detection Kit (Polymer) was used as the secondary detection system. Positive staining of brownish-yellow particles was located in the cytoplasm. The immuno-scores were calculated following the Remmele scoring method³³, and the scores greater than 2 were used as positive group, the others were used as negative group. And the slides were independently examined by two pathologists.

Wound healing assay

The wound healing assays were applied to determine the migration ability of cells. 2×10^5 cells were cultured in a six-well plate until 80–90% confluence and then carefully scratched with a 10 μ L pipette tip.

After washing three times with 1×PBS to remove detached cells, images in 10 different wound fields were captured at respective time points (0 h and 48 h) to evaluate the migration of cells in triplicate assays.

Transwell assay

Chambers (catalog: 3422, 8µm pore, Corning, NY, USA) were used to investigate migration ability of cells. 1×10^5 cells suspended in 150 µL serum-free medium were seeded onto the upper chamber of 24-well plates, and 700 µL of medium with 10% FBS was added to the lower chamber. 48 h later, the medium was removed from the upper chamber. The non-migrating cells on the upper side of the chamber were removed thoroughly with a clean cotton swab. Then cells on the bottom side of the membrane were fixed with 4% paraformaldehyde for 30 min, then stained with 0.1% of crystal violet (Sangon Biotech) for 15 min. Finally, the stained cells were counted by microscopy. Results represent the average number of cells in three fields per membrane in triplicate inserts.

Immunoprecipitation

HO8910-PM were seeded in 10cm dishes for 24h. Then, cells were harvested in IP buffer (150mM NaCl, 50mM tris-HCl pH 7.4, 1% NP40, 1mM DTT and 1mM EDTA). 2mg proteins were subjected to immunoprecipitation following the manufacturer's instructions. ([Http://www.scbt.com/protocols.html?protocol=immunoprecipitation](http://www.scbt.com/protocols.html?protocol=immunoprecipitation)). The mouse source antibody was used for IP and the rabbit source antibody was used for western blotting.

Statistical analysis

All quantitative data in the present study were performed at least in triplicate. The results are expressed as the mean $\pm$ standard deviation. A two-tailed ANOVA or Student's t-test was used to evaluate the data. Correlation data were determined by using Pearson correlation coefficients. And $P < 0.05$ was considered significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

BCKDK is highly expressed in EOC and is associated with pathological grading of EOC patients

BCKDK expression level was detected in 1 normal ovarian epithelial cell line and 5 EOC cell lines (Fig. 1a). The results showed that the BCKDK level of normal IOSE80 cells was the lowest. BCKDK was poorly expressed in SKOV3 and OVCAR3 cells, moderately expressed in HO8910 cells, and highly expressed in HO8910-PM and SW626 cells. Then, the expression level of BCKDK was also detected in EOC tissue and corresponding tumor adjacent tissue samples. The results demonstrated that expression level of BCKDK was higher in EOC tissue than corresponding adjacent tissue (Fig. 1b, 1c), and is associated with pathological grading of patients(Fig. 1e). And detailed information of patients is shown in Fig. 1d.

BCKDK promotes EOC cell proliferation

To test whether BCKDK can promote cell proliferation, BCKDK was overexpressed in SKOV3 and OVCAR3 cells which poorly expressed BCKDK. SKOV3 and OVCAR3 stable cell lines were generated with transfecting the pCMV-c-Flag or pCMV-BCKDK-Flag plasmid into cells, and the growth curves of SKOV3-Mock or SKOV3-BCKDK cells were compared. The results demonstrated that SKOV3-Mock cells grew slower than SKOV3-BCKDK cells (Fig. 2a, inner session indicating BCKDK overexpression). Then, the anchorage-independent growth of SKOV3-Mock or SKOV3-BCKDK was also compared, and the result showed that the number of colonies in SKOV3-Mock cell cultures was less than that in SKOV3-BCKDK cell cultures (Fig. 2c *left panel*). The corresponding statistical significance is indicated in the right panel of Fig. 2c. Similar results were observed in the cultures of OVCAR3-Mock or OVCAR3-BCKDK stable cells (Fig. 2b, 2d). These results indicated that BCKDK promoted EOC cell proliferation.

BCKDK promotes EOC cell migration

Since BCKDK is closely associated to tumor migration in colorectal cancer²⁷, we wondered whether BCKDK also regulated EOC migration. To test this hypothesis, wound healing assay and transwell assay were used to detect the effects of BCKDK on the migration of SKOV3 or OVCAR3 cells. The wound healing assay results were shown in Fig. 3a and 3b, which demonstrated that SKOV3/ OVCAR3-Mock cells had weaker wound healing ability than SKOV3/ OVCAR3-BCKDK cells. Furthermore, transwell assays results indicated that fewer cells migrated from upper chamber in SKOV3/ OVCAR3-Mock cells than in SKOV3/ OVCAR3-BCKDK cells. over-expression of BCKDK accelerate the migration of SKOV3 and OVCAR3 cells (Fig. 3c, 3d). Therefore, results above suggested that over-expression of BCKDK accelerated the migration capability of EOC cells.

Knockdown of BCKDK attenuates EOC tumor properties

To further verify the above hypothesis, BCKDK was knocked down in HO8910-PM EOC cells to generate the stable shMock cell lines and the stable shBCKDK cell lines (HO8910-PM-shMock, HO8910-PM-shBCKDK). The result in Fig. 4a inner session of *left panel* showed that BCKDK was knocked down by shRNA sequence for lines 2 and 4. And growth curves of HO8910-PM-shMock, or shBCKDK cell lines were tested. The results indicated that HO8910-PM-shMock cells grew dramatically faster than HO8910-PM-shBCKDK cells (Fig. 4a). Then, the anchorage-independent growth of the HO8910-PM-shMock or shBCKDK cell lines was analyzed, and the results demonstrated that the number of colonies in HO8910-PM-shMock cell cultures was much more than in HO8910-PM-shBCKDK cell cultures (Fig. 4b). And wound healing assay and transwell assay of the HO8910-PM-shMock or -shBCKDK cell lines were also analyzed, and the results suggested that knockdown of BCKDK attenuated the migration of HO8910-PM cells (Fig. 4c, 4d). Therefore, these above results indicated that knockdown of BCKDK in EOC cells inhibited tumorigenesis and migration *ex vivo*. Furthermore, tumor xenograft assays were also performed in female athymic Balb/c nude mice. We injected HO8910-PM-shMock, or -shBCKDK cells (3×10^6) subcutaneously into the right flank, with tumor size assessed over 40 days. Tumors in HO8910-PM-shBCKDK-inoculated mice grew to a smaller size compared to those in HO8910-PM-shMock-inoculated mice (Fig. 5a, 5b). And the tumor growth curve was shown in Fig. 5c. The final weight of tumor was shown in Fig. 5d. The tumor tissues dissected from these xenografts in the study were stained with

hematoxylin & eosin (H&E) to verify these tissues belong to tumor tissues (Fig. 5e). These data indicated that inhibiting BCKDK expression in EOC cells significantly weaken their tumorigenic properties *ex vivo* and *in vivo*, and further verified that BCKDK promotes EOC cell proliferation and migration.

BCKDK promotes tumor properties through up-regulating the MEK-ERK signaling pathway

After we confirmed that BCKDK promoted cell proliferation and migration *ex vivo* and *in vivo*. We wondered which signaling pathway worked in this process. BCKDK promotes carcinogenesis has been reported in colorectal cancer and hepatocellular carcinoma, and MAPK signaling pathway was worked in this process^{26, 28}. Therefore, p-MEK1/2 (ser221) and p-ERK1/2 (T202/Y204) levels were detected in OVCAR3 or SKOV3 stable cell lines, and the results demonstrated that the level of p-MEK1/2 (ser221) and p-ERK1/2 (T202/Y204) were elevated when BCKDK was overexpressed (Fig. 6a, 6b). These results suggested that BCKDK promoted EOC through up-regulating MEK-ERK activity. Then, the level of BCKDK, p-MEK1/2 (ser221) and p-ERK1/2 (T202/Y204) were also detected in the HO8910-PM-shBCKDK cell lines. Both p-MEK1/2 (ser221) and p-ERK1/2 (T202/Y204) were dramatically decreased when BCKDK was knocked down in HO8910-PM cell lines (Fig. 6c). Furthermore, the expression level of BCKDK, p-MEK1/2 (Ser221), and p-ERK1/2(T202/Y204) were tested in dissected tumor tissues. The results confirmed that the level of BCKDK, p-MEK1/2 (Ser221), and p-ERK1/2(T202/Y204) were lower in the tumor tissue of HO8910-PM-shBCKDK mice than in the tumor tissue of HO8910-PM-shMock mice (Fig. 6d). These data confirmed that BCKDK promoted tumor properties through up-regulating the MEK-ERK signaling pathway.

BCKDK interacts with MEK

As we confirmed that BCKDK directly interacted with MEK in colorectal cancer cells in before research²⁶. We wondered whether BCKDK also interacted with MEK in EOC cells? Then, BCKDK was immunoprecipitated from HO8910-PM cells, and was detected with a MEK antibody by Western blotting. The results demonstrated that BCKDK could co-immunoprecipitate with MEK in HO8910-PM cells (Fig. 6e).

Taken together, our study indicates that BCKDK promotes EOC proliferation and migration by activating the MEK/ERK signaling pathway.

Discussion

OC is the deadliest gynecological tumor. The five-year survival rate of OC patients is as low as 15–45%³⁴. While, the survival rate of OC patients in FIGO's stage I can as high as 90% or above. To improve the diagnosis of ovarian cancer, a variety of serum markers have been developed and used in the diagnosis of ovarian cancer patients. In the 1980s, CA125 was reported as a tumor marker for ovarian tumors³⁵. However, the sensitivity and specificity of these markers are not as high as expected^{36, 37}. Therefore, there is still urgently need to develop specific serum markers that can be screened without internal inspections. As there are no external manifestations before the advanced stage of OC patients, and the

fatality rate, including EOC, has not decreased significantly in the past 30 years^{5, 38}. The new alternative or complementary targeted drugs also need to be researched and developed.

Previous studies demonstrated that the amino acid profile could be an effective diagnostic tool in various cancer patients^{39–42}, and some amino acids are associated with OC^{43–46}, for example, leu⁴⁷. The catabolism of BCAA is closely related to the development and progression of various tumors. Inhibition of BCAA catabolism can promote tumor growth and development^{48, 49}. Inhibiting the expression of BCAA catabolic enzymes can lead to the accumulation of BCAA in tumors, while the liver regeneration tissues were not accumulated⁴⁸. Current research focuses on BCAT1 or BCAT2, which works in the first step of BCAA catabolism, while there are relatively few studies on BCKDK, which is a key negative regulatory enzyme in BCAA catabolism^{50–53}. Despite this, studies have shown that the overexpression of BCKDK promotes the growth and metastasis of various tumors^{26–28}. In this study, we determined that BCKDK promoted the proliferation and metastasis of EOC, and BCKDK was expressed at higher levels in EOC tissues than in adjacent normal tissues (Fig. 1b) and is correlated with advanced pathological grade for patients (Fig. 1e). This suggests that BCKDK could be another potential biomarker for the treatment of EOC. Inhibitors targeting BCKDK will be examined in future research.

Current research of BCAA catabolism worked in tumors focused on BCAT. As the BCAT reaction is reversible and near equilibrium, its direction should respond to changes in concentrations of BCAA and BCKAs, and availability of the donors and acceptors of nitrogen, to some extent, the conclusion was opposite in different researches. For example, some studies confirmed that the high expression of BCAT promoted the transfer of the BCAA amino group to α -ketoglutarate (α -KG) to form glutamate and the corresponding branched-chain keto acids (BCKAs). The BCAA catabolism was increasing, then the BCA-CoA entering into tricarboxylic acid cycle that provided energy for tumor cells proliferation and growth^{53, 54}. The other studies verified that the catabolism of BCAA in tumor cells was decreasing, and the high expression of BCAT promoted the conversion of BCKAs to BCAA and α -KG, and then providing essential nutrients and energy for cancer growth^{48–49, 55}. Our research supports the second. The overexpression of BCKDK inhibits the conversion of BCKAs to BCA-CoA, which leads to the accumulation of BCKAs. Furthermore, the accumulation of BCKAs inhibits BCAA catabolism. Therefore, BCKAs are converted into BCAAs again through amination with the BCAT enzyme. In addition, there are studies showing that BCKDK and PPM1K make up a ChREBP-regulated node that integrates BCAA and lipid metabolism and promotes BCAAs as a material for fat synthesis for fat cells, which provide energy for tumor growth⁵⁶. It has been proven that leu was increased in OC⁴⁷. Other studies also found that the overexpression of BCAT promoted OC proliferation^{51–53}. Therefore, our research gave a further understand of BCAA catabolism worked in the ovarian cancer. While, how BCKDK coordinated with BCAT to balance the BCAA metabolism? And whether they could directly regulate each other was still unclear.

Furthermore, to confirm the function of BCKDK in EOC, BCKDK was overexpressed in SKOV3 and OVCAR3 cells which poorly expressed BCKDK. BCKDK gain significantly promoted the proliferation and migration *ex vivo*, whereas knocked down the expression of BCKDK in HO8910-PM EOC cells reduced the

proliferation and migration *ex vivo* and inhibited the tumor growth *in vivo*. Hence, these data supported the tumor-promoting function of BCKDK in EOC. These results were also consistent with previous findings demonstrating that BCKDK is a key regulator of cell proliferation and metastasis in colorectal cancer and hepatocellular carcinoma ^{26–28}. Moreover, BCKDK promotes EOC proliferation and migration by activating the MEK/ERK signaling pathway. In agreement with our previous study, our previous study demonstrated that BCKDK promoted colorectal cancer proliferation by targeting the MEK1 ²⁶. Another previous study also verified that BCKDK promoted hepatocellular cancer proliferation by MEK/ERK signaling pathway ²⁸. To our knowledge, this study was the first to report the ectopic expression of BCKDK in EOC, and uncovered the mechanism that BCKDK regulates EOC proliferation and migration by MEK/ERK signaling pathway.

Other studies also showed that BCKDK was related to lipid metabolism, which was upregulated by APN ⁵⁵. In addition, BCKDK promoted tumor growth and metastasis by interacting with SRC or mTOR in colon cancer or hepatic carcinoma (Fig. 7) ^{27,28}. Therefore, in addition to the MEK-ERK pathway, whether the APN, SRC, or mTOR signaling pathways are also involved in this process still needs further examination. In addition, the drug resistance of ovarian cancer is a thorny issue, the mitochondria are closely related to apoptosis and autophagy-induced drug resistance ^{58–60}. As BCKDK is located in the mitochondria, and related to drug resistance in ovarian cancer ^{29–30}. What is the relevant mechanism? Many questions need to be addressed in the future. Due to the follow-up data was missing, overall survival rates and progression-free survival rates were not analyzed in this study, future studies need to collect more clinical information.

Conclusions

These findings indicated that the expression of BCKDK was upregulated in EOC tissues and that high expression of BCKDK was correlated with advanced pathological grade in patients. BCKDK promoted EOC proliferation and migration by activating MEK. Targeting the BCKDK-MEK axis may provide a new therapeutic strategy for treating patients with EOC.

Abbreviations

OC: Ovarian cancer; EOC: epithelial ovarian cancer; BCKDK: branched-chain α -keto acid dehydrogenase kinase; CA125: cancer antigen 125; PDKs: pyruvate dehydrogenase kinases; BCAA: branched-chain amino acid; ERK: extracellular signal-regulated kinase; EGF: epidermal growth factor; TMA: tissue microarray; BME: Eagle basal medium; H&E: hematoxylin and eosin; α -KG: α -ketoglutarate; BCKAs: branched-chain keto acids.

Declarations

Ethics approval and consent to participate

The study involving human tissues got approved by the Ethics Committee of Yichang Second People's Hospital. The study involving animals got approved by the Ethics Committee of the Chongqing Population and Family Planning Science and Technology Research Institute. (Ethic approval number: 2019D001). The study followed the tenets of the Declaration of Helsinki, and informed consent was obtained from each participant.

Consent for publication

All authors involved in the authorship are consent for publication in the current form.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Dongyang Yu, Yi Liu, and Yafei Tian were involved in the acquisition of experimental data. Huashun Li and Juanjuan Xiao performed the tissue microarray analysis and analyzed the clinical data. Li Mu and Yijian Zhu housed and maintained the mice. Lianbing Li and Linbo Chen provided advice on the manuscript. Feng Zhu, Qihong Duan and Peipei Xue designed the study, analyzed the data and drafted the manuscript.

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Figures

Figure 1

Figure 1

BCKDK overexpression is associated with advanced pathological grade in EOC patients. (a) Expression of BCKDK in 6 different ovarian cell lines. (b) Immunohistochemical examination for the expression of BCKDK in 70 cases of human epithelial ovarian cancer tissues and 30 of adjacent tissues. Pictures from 1 representative case are shown in the upper panel, and the 2 scale bars from up to down in each group correspond to 100 and 40 μ m respectively. (c) Statistics of the IHC examination results are shown. The

IHC score of BCKDK is higher in EOC tissues than adjacent tissues. (d) The clinical characteristics of 70 patients with ovarian cancer. (e) The correlation between BCKDK expression and clinicopathologic features, Correlation data were determined by using Pearson correlation coefficients. Error bars represent the mean $\pm$ SD values. (***, $P < 0.001$).

Figure 2

Figure 2

BCKDK promotes EOC cell proliferation. (a) Growth curves of vector control cells (SKOV3-Mock) and BCKDK-overexpressing cells (SKOV3-BCKDK). Insert shows verification of the cell lines identified by Western blot. Data are represented as mean $\pm$ standard deviation from triplicate experiments. The asterisk indicates a significant increase in cell number in SKOV3-BCKDK cells compared with SKOV3-Mock cells (*, $P < 0.05$). (b) Growth curves of vector control cells (OVCAR3-Mock) and BCKDK-overexpressing cells (OVCAR3-BCKDK). Insert shows verification of the cell lines identified by Western blot. Data are represented as mean $\pm$ standard deviation from triplicate experiments. The asterisk indicates a significant increase in cell number in OVCAR3-BCKDK cells compared with OVCAR3-Mock cells (**, $P < 0.01$). (c) BCKDK can transform SKOV3 cells ex vivo as illustrated by growth of BCKDK transformed cells in soft agar. Photomicrograph of representative colony formation in soft agar of vector control cells (SKOV3-Mock) compared with BCKDK-overexpression cells (SKOV3-BCKDK) is shown (***, $P < 0.001$). (d) BCKDK can enhance the transformation of OVCAR3 cells ex vivo as illustrated by growth of BCKDK transformed cells in soft agar. Photomicrograph of representative colony formation in soft agar of vector control cells (OVCAR3-Mock) compared with BCKDK-overexpressing cells (OVCAR3-BCKDK) is shown (***, $P < 0.001$).

Figure 3

Figure 3

BCKDK promotes EOC cell migration. (a) Scratch wound assay demonstrating that SKOV3-Mock migrate faster than SKOV3-BCKDK cells. The dotted lines show the area where the scratch wound was created. The scratch wound assay was performed in triplicate experiments. (b) Scratch wound assay demonstrating that OVCAR3-Mock migrate faster than OVCAR3-BCKDK cells. The dotted lines show the area where the scratch wound was created. The scratch wound assay was performed in triplicate experiments. (c) Transwell assay. SKOV3-BCKDK have greater migration capacity than SKOV3-Mock cells. Representative images from transwell assays of SKOV3-Mock cells and SKOV3-BCKDK cells are shown. (d) Transwell assay. OVCAR3-BCKDK have greater migration capacity than OVCAR3-Mock cells. Representative images from transwell migration assays of OVCAR3-Mock cells and OVCAR3-BCKDK cells are shown.

Figure 4

Figure 4

Knockdown of BCKDK attenuates EOC tumor properties ex vivo. (a) Growth curves of HO8910-PM-shMock, HO8910-PM-shBCKDK2, and HO8910-PM-shBCKDK4 cells. Insert shows verification of the knockdown cell lines identification by Western blot. Data are represented as mean $\pm$ standard deviation from triplicate experiments. The asterisks indicate a significant increase compared with shMock cells (*, $P < 0.05$). (b) Knockdown of BCKDK reduces tumorigenic properties of HO8910-PM cells ex vivo.

Representative photomicrograph of colony formation in soft agar of vector control cells (shMock) compared with BCKDK-knockdown cells (shBCKDK2 or shBCKDK4) is shown. Data are represented as mean $\pm$ standard deviation from triplicate experiments (**, $P < 0.01$; ***, $P < 0.001$). (c) Scratch wound assay demonstrating that HO8910-PM-shMock migrate faster than HO8910-PM-shBCKDK2, 4 cells. The dotted lines show the area where the scratch wound was created. The scratch wound assay was performed in triplicate experiments. (d) Transwell assay. HO8910-PM-shMock have greater migration capacity than HO8910-PM-shBCKDK2, 4 cells. Representative images from transwell assays of HO8910-PM-shMock cells and HO8910-PM-shBCKDK2, 4 cells are shown.

Figure 5

Figure 5

Knockdown of BCKDK reduces tumorigenic properties of HO8910-PM cells in vivo. (a) Mice and (b) tumors dissected from each group are shown. (c) Final average tumor growth curve and (d) tumor weight of mice injected with HO8910-PM-shMock or HO8910-PM-shBCKDK cells is shown. Data are shown as means $\pm$ standard deviation of measurements. The asterisk indicates a significant decrease in tumor size of HO8910-PM-shBCKDK-injected mice compared with HO8910-PM-shMock-injected mice (*,

P<0.05). (e) Immunohistochemistry analysis was performed in the tumor tissues of HO8910-PM-shMock-injected mice or HO8910-PM-shBCKDK-injected mice.

Figure 6

Figure 6

BCKDK promotes tumorigenesis through up-regulating MEK-ERK signaling pathway. (a, b) The level of phosphorylation of MEKs and ERKs were increased in SKOV3/OVCAR3-BCKDK cells after EGF treatment for 15 min. (c) The level of phosphorylation of MEKs and ERKs were decreased in HO8910-PM-shBCKDK cells after EGF treatment for 15 min. (d) The level of phosphorylation of MEKs and ERKs were decreased in the tumor tissues from HO8910-PM-shBCKDK-injected mice compared to HO8910-PM-shMock-injected mice. (e) BCKDK binds with MEK in HO8910-PM cells. Endogenous BCKDK was immunoprecipitated from HO8910-PM cells and then probed with anti-MEK antibody. All Western blot data are representatives of results from triplicate experiments.

Figure 7

Figure 7

The signaling pathway of BCKDK in cancers 26-28.

Supplementary Files

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