EBioMedicine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

# BCKDK of BCAA Catabolism Cross-talking With the MAPK Pathway Promotes Tumorigenesis of Colorectal Cancer

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ARTICLE INFO

*Article history:*
Received 8 January 2017
Received in revised form 1 May 2017
Accepted 1 May 2017
Available online xxxx

*Keywords:*
Branched-chain amino acids catabolism
Colorectal cancer
BCKDK
Tumorigenesis
MEK
Phenyl butyrate

ABSTRACT

Branched-chain amino acids catabolism plays an important role in human cancers. Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, and the new global incidence is over 1.2 million cases. The branched-chain α-keto acid dehydrogenase kinase (BCKDK) is a rate-limiting enzyme in branched-chain amino acids catabolism, which plays an important role in many serious human diseases. Here we investigated that abnormal branched-chain amino acids catabolism in colorectal cancer is a result of the disease process, with no role in disease initiation; BCKDK is widely expressed in colorectal cancer patients, and those patients that express higher levels of BCKDK have shorter survival times than those with lower levels; BCKDK promotes cell transformation or colorectal cancer *ex vivo* or *in vivo*. Mechanistically, BCKDK promotes colorectal cancer by enhancing the MAPK signaling pathway through direct MEK phosphorylation, rather than by branched-chain amino acids catabolism. And the process above could be inhibited by a BCKDK inhibitor, phenyl butyrate.



## 1. Introduction

Tumor metabolism is closely related to tumorigenesis and tumor progression (Burrage et al., 2014; Gill et al., 2016; Nelson et al., 2013; Pike et al., 2011; Sonnet et al., 2016; Watanabe et al., 1984). Gill et al. (2016) presented that the increased rate of glycolysis can support cancer cells rapid proliferation through fulfilling cells biosynthetic demands. Pike et al. (2011) showed fatty acid oxidation promotes cancer cells growth and survival through providing bioenergetic fuel for cells. Nelson et al. (2013) demonstrated a primary metabolite of cholesterol, 27-hydroxycholesterol (27HC), promotes tumor cells growth and metastasis through signal transduction. The above studies show that the metabolism effects tumor growth in 3 ways: providing biosynthetic materials, providing energy, or through signal transduction. Branched-chain amino acids (BCAA) catabolism is abnormal in many human diseases (Burrage et al., 2014; Sonnet et al., 2016; Watanabe et al., 1984). Branched-chain amino transferase (BCAT) is the first enzyme in BCAA catabolism which has a higher activity in tumors and has been shown to be a useful marker for grading and genetic characterization in glioma, colorectal cancer (CRC) and medulloblastoma (Conway et al., 2016; de Bont et al., 2008; Mitchell et al., 2014). However, healthy human prostate tissue was found to have elevated BCAT levels relative to malignant tissue (Billingsley et al., 2014). Therefore, the role of BCAA catabolism in tumors need to be further understood.

BCKDK is a key negative regulation enzyme in BCAA catabolism that inhibits the dehydrogenase activity of the branched-chain α-keto acid dehydrogenase (BCKDH) complex by dephosphorylating the E1 component of the complex (Chuang et al., 2002). BCKDK plays an important role in many serious human diseases, including Huntington's disease (Mochel et al., 2007), Maple syrup urine disease (Beaudet, 2012;

*Abbreviations:* BCAA, branched-chain amino acids; BCKDK, branched-chain α-keto acid dehydrogenase kinase; CRC, colorectal cancer; BCAT, branched- chain amino transferase; 27HC, 27-hydroxycholesterol; BCKA, branched- chain α-keto acid; BCKDHA, branched-chain α-keto acid dehydrogenase E1; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PB, phenylbutyrate.

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http://dx.doi.org/10.1016/j.ebiom.2017.05.001

Sonnet et al., 2016), human autism with Epilepsy (Novarino et al., 2012), and Obesity (Burrage et al., 2014). However, the relationship between BCKDK and cancer is unknown.

CRC is the third most commonly diagnosed cancer in males and the second in females, and the new global incidence is over 1.2 million cases (Torre et al., 2015). Therefore, the mechanism of CRC tumorigenesis need to be further elucidated. Watanabe et al. (1984) reported that BCAA levels in the tumor tissue of colon cancers were generally higher than those in the respective nontumorous tissue. On the other hand, Wubetu et al. (2014) showed BCAA suppressed insulin-initiated proliferation of human Colon tumor cells through induction of autophagy. Yoshikawa et al. (2006) demonstrated BCAT1 over-expression is a sensitive marker of metastasis in CRC. Therefore, the role of BCAA catabolism in CRC is controversial. BCAA is a biosynthetic material, however, whether BCAA is a potential carcinogen is unclear.

In this study, we identify BCKDK as an upstream kinase of MEK. We show that the BCKDK positively regulates MEK/ERK signaling by directly phosphorylating MEK at Ser221. BCKDK has a higher expression in the colorectal tissues of CRC patients *versus* normal tissues. The higher that BCKDK is expressed, the shorter survival time is in CRC patients. Over-expression of BCKDK increases the clone formation of JB6 Cl41 and WiDr cells *ex vivo*. Knockdown of BCKDK inhibits colorectal tumor progression *ex vivo* and *in vivo*. The above suggests BCAA catabolism can crosstalk with MAPK signaling pathway, whereby BCKDK plays a crucial role in human CRC tumorigenesis through phosphorylating MEK at ser221.

## 2. Materials and Methods

### 2.1. Plasmids, shRNA, Antibodies, and Other Reagents

The plasmids of pBABEpuro-HA-MEK1 (catalog: 53195) and pDONR223-BCKDK (catalog: 23,794) were purchased from Addgene (Cambridge, MA, USA). The plasmid of pCMV-C-Flag (catalog: D2632) was purchased from Beyotime Biotechnology (Shanghai, China). The plasmid of pCMV-Myc (catalog: 631604) was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). The plasmid of pET23a-His (catalog: 69745-3) was purchased from Novagen (Darmstadt, Hessen, Germany). The plasmids of pCMV-BCKDK-Flag, pCMV-Myc-MEK1, pET23a-MEK1 (residues 62-393)-His, and pLKO.1-shBCKDK were constructed by our laboratories. The sense template sequence for BCKDK amplification was 5′-CGGAATTCCGATGATCCTGG.

CGTCGGTGCTGAGG-3′ (*Eco*RI end underlined) and the antisense template sequence was 5′-CCGCTCGAGGATCCGGAAGCTTTCCTCCCGGCC-3′ (*Xho*I end underlined). The amplified BCKDK and pCMV-c-Flag vector were digested with *Eco*RI and *Xho*I, ligation, and colony screening for pCMV-BCKDK-Flag. The sense template sequence for MEK1 amplification was 5′-CCGCTCGAGCGCCCAAGAAG AAGCCGACG-3′ (*Xho*I end underlined) and the antisense template sequence was 5′-ATAAGAATGCGGCCGCTTAAGCCAAATGGTGGAGCCAG-3′ (*Not*I end underlined). The amplified MEK1 was cloned into pCMV-Myc vector at *Xho*I and *Not*I sites. The sense template sequence for MEK1 (residues 62-393) amplification was 5′-CGGGATCCCGGAAGCGCCT TGAGGCCTTTCTGAC-3′ (*Bam*HI end underlined) and the antisense template sequence was 5′-CCGCTCGAGAGCCAAATGGTGGAGCCAGATC-3′ (*Xho*I end underlined). The amplified MEK1 (residues 62-393) was cloned into pet23a-his vector at *Bam*HI and *Xho*I sites. 5 sequences were designed to knock down BCKDK, and the sequences are: 1. 5′-CCGGCGTCCGCTACTTCTTGGACAACTCGAGTTGTCCAAGAAGTAGCGGAC-GTTTTTG-3′; 2. 5′-CCGGCCAGCACCAGTTCCGTCATTCCTCGAGGAATGA CGGAACTGGTGCTGGTTTTTG-3′; 3. 5′-CCGGGATCTGATCATCAGGATCTCA CTCGAGTGAGATCCTGATGATCAGATCTTTTTG-3′; 4. 5′-CCGGTCAGGACCC ATGCACGGCTTTCTCGAGAAAGCCGTGCATGGGTCCTGATTTTTG-3′; 5. 5′-C CGGACGCTGACTTCGAGGCTTGGACTCGAGTCCAAGCCTCGAAGTCAGCGT-TTTTTG-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′-CCGG

CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-TTTTTG-3′. The sense and anti-sense oligonucleotides were synthesized, annealed and cloned into the pLKO.1-TRC cloning vector at the *Eco*RI and *Age*I sites as described by the manufacturer (Moffat et al., 2006).

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). Anti-p-MEK1/2 (ser221) (catalog: 2338), phospho-p44/42MAPK (Erk1/2) (T202/Y204) (D13.14.4E) (catalog: 4370), t-MEK (catalog: 8727), and t-ERK (catalog: 4695) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Flag antibody (catalog: F1804, catalog: F7425) was purchased from Sigma-Aldrich (St. Louis, MO, USA). HRP-conjugated anti-mouse antibody (catalog: E030110) and HRP-conjugated anti-rabbit (catalog: E030120) antibody were purchased from EarthOx Life Sciences (San Francisco, CA, USA). Simple-Fect (catalog: Profect-01) was purchased from Signaling Dawn Biotech (Wuhan, Hubei, China) for transfection. G418 (catalog: A1720) and puromycin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used for setting up stable cell lines.

### 2.2. Cell Culture

The human colon cancer cell lines (DLD1, HCT8, SW480, WiDr, HCT116, and HCT15) and the normal cell lines (HIEC-6, JB6 C141, and HEK293T) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA).

### 2.3. Western Blot

Cells ($0.8–2 \times 10^6$) were cultured in 10 cm diameter dishes to 70–80% confluence, and then starved no serum for either 12 h (JB6 C141 cell lines) or 24 h (colon cancer cell lines). The cells were then treated with 20 ng/mL epidermal growth factor (EGF) (R&D catalog: 236-EG-200) for 15 min. EGF is a well-known tumor promotion agent used to study malignant cell transformation in animal and cell models of cancer (Hunter and Karin, 1992; Sachsenmaier et al., 1994; Sassone-Corsi et al., 1999; Scafidi et al., 2014). After this, cells were washed once in PBS before being lysed in RIPA buffer (1 × PBS, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mmol/L Na3VO4, 1 mmol/L aprotinin and 1 mmol/L phenylmethylsulfonyl fluoride). In addition, HCT116 cells were treated with 0–3200 μM BCKDK inhibitor for 48 h, with the later 24 h together with no serum. Then, samples were sonicated in 15 second intervals three times, and insoluble debris was removed by centrifugation at 13000 rpm for 15 min. Protein content was determined by Bradford method (Bradford, 1976). 30–120 μg of protein was separated by 10% SDS-PAGE and visualized by chemiluminescence (BIO-RAD, USA) in triplicate.

### 2.4. Growth Curve Analysis and MTS Assays

$2 \times 10^5$ cells were plated in each dish and counted at different times in triplicate, using a hemacytometer to generate a growth curve. For inhibition assays, $1 \times 10^3$ HCT116 cells were seeded in 96-well plates for 24 h, and then cells were fed with fresh medium containing different concentrations of BCKDK inhibitor, phenyl butyrate (PB), for 48 h. The cytotoxicity of inhibitors was measured with MTS assay kit (Promega catalog: G3580) according to the manufacturer's instructions in triplicate, with the results expressed as the percentage of inhibition.

### 2.5. Bacterial Expression and Purification of the MEK1-His

The pET23a-mek1 (residues 62-393)-his plasmid was expressed in *E. coli* BL21 bacteria (Novagen; Darmstadt, Hessen, Germany). Bacteria were grown at 37 °C overnight. Then, bacteria were harvested by

Please cite this article as: Xue, P., et al., BCKDK of BCAA Catabolism Cross-talking With the MAPK Pathway Promotes Tumorigenesis of Colorectal Cancer, EBioMedicine (2017), http://dx.doi.org/10.1016/j.ebiom.2017.05.001