Title page

Cordycepin inhibits cancer cell proliferation and angiogenesis through a DEK interaction via ERK signaling in cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) is a malignant tumor that arises from the epithelial cells of the bile duct and is notorious for its poor prognosis. The clinical outcome remains disappointing and thus more effective therapeutic options are urgently required. Cordycepin, a traditional Chinese medicine, provides multiple pharmacological strategies in anti-tumors, but its mechanisms have not been fully elucidated. In this study, we reported that cordycepin inhibited the viability and proliferation capacity of CCA cells in a time and dose dependent manner, determined by MTT and colony formation assay. Flow cytometry and hochest dye showed that Cordycepin induced cancer cell apoptosis via ERK1/2 deactivation. Moreover, cordycepin significantly reduced the angiogenetic capabilities of CCA in vitro as examined by tube formation assay. We also discovered that cordycepin inhibited DEK expression by using western blot assay. DEK serve as an oncogenic protein that is overexpressed in various gastrointestinal tumors. DEK silencing inhibited CCA cell viability and angiogenesis, but not apoptosis induction determined by western blot and flow cytometry. Furthermore, cordycepin significantly inhibited tumor growth and angiogenic capacities in a xenograft model by downregulating the expression of DEK, p-ERK1/2 CD31 and vWF. Taken together, we demonstrated that cordycepin inhibited CCA cell proliferation and angiogenesis with a DEK interaction via downregulation in ERK signaling. These data indicate that cordycepin may serve as a novel agent for CCA clinical treatment and prognosis improvement.
Significance statement

Cordycepin provides multiple strategies in anti-tumors, but its mechanisms are not fully elucidated, especially on CCA. We reported that cordycepin inhibited the viability of CCA cells and induced apoptosis via ERK1/2 deactivation and DEK inhibition, reduced the angiogenetic capabilities of CCA both in vivo and in vitro.

1. Introduction

Cholangiocarcinoma (CCA) is a malignant tumor that originated from the epithelial cells of the bile duct and accounts for 15% of primary liver cancers (Banales et al., 2016). As surgery is the only potential curative operation for CCA patients, the clinical outcome remains disappointing and the survival time is less than 2 years (Blechacz and Gores, 2008). In the last two decades, with the further development of efficient therapeutic strategies, surgical treatment of this disease has become optional. However, CCA is insensitive to radiochemotherapy and there are serious side effects with combined treatment, thus clinical outcome needs to be improved. Considering the rather unspecific presentation of CCA patients, and the aggressiveness of CCA, more specific molecular agents and drugs with low toxicity are urgently required.

Cordycepin, a traditional Chinese medicine extracted from *Cordyceps militaris* (Fig 1A), has been reported to exert significant anti-tumor activities in a wide spectrum of cancers. Cordycepin exerts anti-proliferative, anti-metastatic, and induces apoptosis in cancer cells in vitro (Jeong et al., 2011; Lee et al., 2010). More importantly, cordycepin was reported to have a potential role in RNA/DNA biosynthesis inhibition, anti-
angiogenesis, and immunomodulation (Lu et al., 2014; Nakamura et al., 2015). Though there are many studies on cordycepin inhibition of cancer, little is known about its molecular mechanisms in CCA.

DEK is an oncogene protein that is overexpressed in aberrant proliferating and malignant cells (Riveiro-Falkenbach and Soengas, 2010). Since its first description, DEK has been demonstrated playing roles in DNA repair, replication, and transcription (Kappes et al., 2011; Soekarman et al., 1992). Recent studies showed a pro-oncogenic role of DEK in promoting cell proliferation, survival, and transformation (Adams et al., 2015; Privette Vinnedge et al., 2015; Wise-Draper et al., 2009). DEK functions are well established in cellular processes but the regulation of DEK is not fully understood. E2 factor (E2F), nuclear transcription factor Y (NF-Y), Yin Yang 1 (YY-1), and estrogen receptor α are thought to directly modulate DEK gene transcription (Muller et al., 2001; Privette Vinnedge et al., 2012). DEK was also characterized as a critical regulator of G1/S transition in mammalian cells and was reported play a role in angiogenesis (Zhang et al., 2016). Since DEK is frequently upregulated in various human malignancies and exhibits oncogenic activities, therapeutic strategies that target DEK may form a new approach to cancer treatment.

There are few published reports of the use of cordycepin in CCA treatment, thus we decided to investigate whether cordycepin suppresses CCA progression and to elucidate its molecular mechanisms. MTT and colony formation assays were performed to investigate the impact of cordycepin treatment on cell proliferation, and tube formation assays were conducted to examine its roles in angiogenesis.
In this study, we provide a mechanistic insight into the inhibition of CCA progression and angiogenesis by cordycepin and revealed an interaction between cordycepin and DEK. Our data indicate that cordycepin may serve as a novel therapeutic target for CCA.

2. Materials and methods

2.1. Ethics statement

This research complied with the Helsinki Declaration and was approved by the Human Ethics Committee and the Research Ethics Committee of Yanbian University Medical College. Patients were informed that the resected specimens were stored by the hospital and potentially used for scientific research, and their privacy could be maintained.

2.2. Cell culture and Cordycepin treatment

Human normal biliary epithelial cell line HIBEpic and human CCA cell line QBC939 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The RBE CCA cell line was purchased from Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). CCA cell lines HuCCT1, CCKS1 and TFK1 were provided by the Department of Pathology of Kanazawa University (Kanazawa, Japan). HIBEpic, QBC939, RBE, HuCCT1 and TFK1 were grown in RPMI-1640 culture medium and CCKS1 was cultured in Dulbecco’s modified eagle media: nutrient Mixture F-12, containing 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin-streptomycin. Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC and grown in endothelial cell
growth medium. Cells were maintained in a 37°C incubator supplied with 5% CO₂.

For the cordycepin treatment, cordycepin was dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 400mM and further diluted to different concentrations with culture medium. For Erk1/2 inhibitor GDC-0994 treatment, HuCCT1 cells were treated with 1nM and 5nM GDC-0994 for 48h.

**2.3. Reagents and antibodies**

Cell culture medium (RPMI-1640, DMEM-F12) were purchased from CORNNING (USA). FBS was purchased from BI. For conditional media (CM), cancer cells were seeded after 24h, then treated with or without Cordycepin for another 48h and the supernatant was collected for Tube formation assay. (Described in the tube formation assay section). For basal media (BM) used for HUVECs can met the basic needs for endothelial cell growth and without cancer cell pre-incubation. The purpose of using different media was to avoid the impact on HUVECs caused by the media. Cordycepin was purchased from Sigma Chemical Co. (St Louis, MO, USA). Erk1/2 inhibitor was purchased from ApexBio (USA). The primary antibodies against Cleaved caspase 9, Cleaved caspase 3, Cleaved PARP, p-Erk1/2 and Erk1/2 were purchased from Cell Signaling Technology (Danvers, MA, USA). VEGF, VEGFR-2 and DEK antibodies were purchased from ProteinTech (USA). CD31 and β-actin was purchased from Abcam (USA). vWF was purchased from Santa Cruz (USA).

**2.4. MTT assay**

Cells were seeded 5000 cells / well in 96-well plates and cultured. The cells were then treated with cordycepin at various doses. After 24h, 48h and 72h, cells were added MTT solution for 4h in the incubator. Then, adding DMSO dissolved MTT and detecting at a full-wavelength spectrophotometer (Tecan, Switzerland).

2.5. Colony formation assay

Cells were seeded at a density of 2000 cells on 6-well plates and cultured. Then cells were treated with cordycepin respectively. Two weeks later, when colonies were visible, cells then fixed in formaldehyde 4%, stained by Gimsa 0.5% and counted manually.

2.6. Hoechst33342 staining

Cells were seeded in 6-well plates and cultured until 70-80% confluence. Then cordycepin treated after 48h, the cells were fixed in 4% formaldehyde for, stained for 30min at room temperature with Hoechst33342 and photographed under microscope (Leica SP5II).

2.7. Flow cytometry assay

CCA cells treated with cordycepin after 48h, harvested cells, washed with cold PBS, and re-suspended in binding buffer. The cells were stained with Annexin-V and/or PI for 15min respectively, and performed using BD Accuri C6 Software (BD Biosciences, San Jose, CA, USA).
2.8. Western blot analyses

Cells collected and lysed with T-PER buffer (Invitrogen, USA) supplemented with protease inhibitors. BCA protein assay (Beyotime Biotechnology, China) was used to measure protein concentration. Proteins were separated using 8-10% SDS-PAGE electrophoresis and transferred to PVDF membranes. The membranes were blocked and probed with appropriate antibodies. Dilutions of Cleaved caspase 9, Cleaved caspase 3, Cleaved PARP, p-Erk1/2, Erk1/2, VEGF, VEGFR-2 and DEK antibodies were 1:1000, β-actin was 1:3000. Plus HRP labeled secondary antibody was used for incubation and then ECL color luminescence (Beyotime Biotechnology, China) was used for detection. β-actin was used as a loading control.

2.9. Tube formation assay

Each well of prechilled 96-well plates were bedded with a layer of Matrigel and were polymerized at 37°C for 1h. HUVECs were then resuspended in FBS-free medium with cancer cell culture supernatant. HUVECs were seeded on the polymerized Matrigel. Then incubated for 2h. The wells were photographed on an inverted phase contrast microscope, the tube formation ability was evaluated by counting the number of tubes in three random fields.

2.10. siRNA Transfection

DEK siRNA 4 (5’-TGTCCTCATTTAAAGAAGA-3’) and control siRNA were purchased from RBIOBIO (Guangzhou, China). Cells were transfected with 100nM
siRNA using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions.

2.11. Immunohistochemical staining

A total 10 Human CCA patient specimens, collected from 2005 to 2013, were provided by the Affiliated Hospital of Kanazawa University in Japan. Human specimens and Xenograft tumor tissues fixed with 4% paraformaldehyde-PBS, embedded in paraffin and sectioned into 4μm thin slices. After antigen retrieval with citric acid (pH 6.0), endogenous peroxidase activity was blocked with 1% hydrogen peroxide. Primary antibody p-Erk1/2 antibody (1:200) and DEK antibody (1:250), CD31 antibody (1:100), and vWF (1:200) were applied. CD31 antibody and vWF were used to track the newly formed micro vessels. Three optional field were chosen randomly, and the number of vessels were counted. After incubated with secondary antibodies against goat IgG, sections were visualized with diaminobenzidine (DAB) and counterstained with hematoxylin. The staining was imaged by microscopy.

2.12. RT-PCR

The total RNA was extracted using Kit (QIAGEN Germany). cDNA was reversely transcript from 500 ng of total RNA using. The cDNA was amplified with the following primers:

5’-AAGGGTGGAATTAAGTACAAGCACA-3’ (forward),
5’-ATCCACGATTGCCTGCACA-3’ (reverse) as for DEK;
5'-GAGTCAACGGATTTGGTCGT-3' (forward),
5'-TTGATTTTGGAGGGATCTC-3' (reverse) as for GAPDH.

The real time PCR was carried out with using Kit (TaKaRa Japan). GAPDH was used as an internal control for each sample.

2.13. Xenograft model

This study was approved by the ethics committee of the Yanbian University Medical College. 4-week-old male nude mice were obtained from Hua Fu Kang bioscience company Beijin, China, and HuCCT1 (5×10⁶ cells/mouse) in 100 μl of FBS-Free medium were subcutaneously injected. Tumor nodules were examined every day and were evaluated using the following formula: tumor volume = (Width²×Length)/2. 7 days after tumor cell inoculation, 10 mice with different tumor volume were divided randomly into two groups (5 animals per group). The treatment group received cordycepin treatment via i.p. injection every day (50mg/kg), and the control group received the same volume of PBS. The bodyweight and tumor volume of each mouse was assessed every 2 days using caliper measurements. On day 45, the mice were sacrificed, and the livers and tumors were excised and fixed for immunohistochemistry staining and H&E staining. This experiment was accomplished in Changchun shengyu cell biotechnology co. LTD, China.

2.14. Statistics

Statistical analysis was conducted by SPSS 17.0 software (SPSS Inc. USA) and
GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA). Measurements were indicated as mean ± SD and evaluated using Student’s t-test and one-way ANOVA. A P-values < 0.05 was used as a measure of statistically significance.

3. Results

3.1 Cordycepin inhibits cancer cell growth and Erk1/2 phosphorylation in CCA.

Cordycepin is reported to exert significant anti-tumor activities (Chaicharoenaudomrung et al., 2018; Yang et al., 2017a; Zhou et al., 2017). To investigate whether cordycepin influences the progression of CCA, HuCCT1 and CCKS1 cells were treated with cordycepin in a time- and dose-dependent manner. Different doses of Cordycepin used for cancer cell treatment was based on the different IC50 value of the cell, as shown in figure 1B. The IC50 value of HuCCT1 was 100 μM and CCKS1 was 150μM. Cell viability was determined by MTT assay. Cordycepin significantly reduced cell viability in both HuCCT1 and CCKS1 cells (Fig1B). The colony formation ability of HuCCT1 and CCKS1 were both reduced in a dose-dependent manner after cordycepin treatment for two weeks (Fig1C). These results demonstrated that cordycepin inhibits CCA progression by inhibiting CCA cell proliferation. As mounting data has demonstrated that cordycepin provides multiple pharmacological strategies for apoptosis induction in various kinds of cancers, we examined whether cordycepin exerts apoptosis induction in both CCA cell lines. We found that after 48h of treatment with cordycepin in both HuCCT1 and CCKS1 cell lines, Hoechst 33342 staining revealed a significant increase in cell apoptosis (Fig.1D).
Quantitative measurement of Annexin-V and propidium iodide positivity was performed by flow cytometry. Both cell lines showed a significant increase in apoptosis after 48h of cordycepin treatment (Fig.1E). We then analyzed the expression of apoptosis markers by western blot assay. Cordycepin treatment resulted in a significant increase in cleaved caspase-9, cleaved caspase-3 and cleaved PARP (Fig.1F). Previous reports showed that the ERK pathway is involved in tumor initiation and progression, and a reduction in ERK activation resulted in CCA growth inhibition (Chen et al., 2018; Yokoi et al., 2018). Thus, western blot assays were performed to evaluate ERK expression. Protein expression was evaluated following treatment of both cell lines with various doses of cordycepin for 24 h. In both cancer cell lines, p-ERK1/2 levels were significantly decreased (Fig.1G). These results demonstrated that cordycepin attenuates CCA cell proliferation by apoptosis induction and ERK1/2 inhibition.

3.2 Cordycepin inhibits the tube formation capacities of HUVECs.

Earlier studies indicated that cordycepin has an anti-angiogenetic role in cancer suppression (Lu et al., 2014). Given that angiogenesis is essential for cancer progression and metastasis, we employed tube formation assays in HUVECs to determine whether cordycepin influences CCA angiogenesis. First, to abolish the influence of cell viability of HUVECs in tube formation assays, and to further elucidate how cordycepin inhibits CCA angiogenesis, MTT assays were performed. HUVECs were incubated in DMEM, HUVEC basal medium (BM), RPMI-1640/DMEM-F12 cancer cell culture BM, RPMI-1640/DMEM-F12 plus 100/150 μM cordycepin, condition medium (CM) from both
cancer cell lines, and the CM from both cancer cell lines after 48h cordycepin treatment. MTT assays revealed that only incubation with CM obtained from both cancer cell lines after 48h cordycepin treatment significantly reduced the viability of HUVECs. We speculated that cordycepin may inhibit CCA angiogenesis, and that the CM obtained from cancer cell lines after cordycepin treatment may contain some apoptotic factors that further influence HUVEC cell viability (Fig.2A). Next, we incubated HUVECs with the CM obtained from both cancer cell lines after 48h of cordycepin treatment. We found that the tube formation ability of HUVECs was attenuated in the cordycepin-treated group in both cell lines (Fig.2B). We then assessed the expression of VEGF, and VEGFR-2 by western blotting. Cordycepin treatment lowered VEGF, and VEGFR-2 expression, which further confirmed the tube formation results (Fig.2C). Our results indicated that cordycepin slowed CCA progression by angiogenesis inhibition.

3.3 Cordycepin downregulated DEK overexpression in CCA cell lines.

DEK is reported as an oncogene in many types of cancers, like acute myeloid leukemia, bladder cancer, hepatocellular cancer, and breast cancer (Liu et al., 2017). Recent reports showed that DEK interact with LCMR1 to enhance cancer cell growth and suppress apoptosis (Xu et al., 2017). A previous study also showed that DEK has critical roles in angiogenesis and PI3K/AKT/mTOR pathway modulation (Yang et al., 2017b). Considering these findings, and compared with our previous studies of cordycepin, we hypothesized that cordycepin may influence DEK expression. Evaluation of DEK expression in both cancer cell lines was undertaken by western blotting. We found an
inverse relationship between cordycepin and DEK expression, as shown in Figure 3A; DEK demonstrated significantly lower expression after cordycepin treatment. DEK expression in various cancer types was also confirmed. Data obtained from the UALCAN database showed that DEK is overexpressed in CCA (Fig.3B). To further confirm our results, IHC staining was undertaken. Compared with peri-tumor tissue, DEK showed strongly positive staining in CCA tumor tissue (Fig.3C). We then analyzed $DEK$ mRNA expression levels by RT-PCR in HIBEpic cells, a normal epithelial cell line of the bile duct, and five CCA cell lines, HuCCT1, CCKS1, QBC939, TFK1, and RBE. As shown in Figure 3D, the expression levels of DEK in five cancer cell lines were much higher than in HIBEpic cells. Together, these data implied that DEK is overexpressed in CCA cell lines and cordycepin suppression of DEK expression may contribute to its roles in CCA inhibition.

**3.4 DEK silencing inhibits CCA growth.**

To further confirmed the oncogenic roles of DEK in CCA and the correlation between cordycepin and DEK, we next silenced $DEK$ expression using siRNA. Western blotting analysis enabled selection of siRNA sequence 4 for further investigation (Fig.4A). MTT assay showed a significant decrease in cell viability in HuCCT1 cells after $DEK$ silencing (Fig.4B), and colony formation assays also revealed a reduction in colony formation capacity (Fig.4C). To gain a mechanistic understanding of the potential role of DEK in modulating tumorigenesis, flow cytometry and Hoechst 33342 staining were used to investigate the roles of DEK in apoptosis. As shown in Figure 4D and Figure
4E, no significant changes were observed in apoptosis induction, and western blotting also confirmed no changes in apoptosis markers (Fig. 4F). Herein, we indicate that DEK increased tumor cell growth by enhancing its proliferation rate and DEK knockdown is irrelevant to apoptosis induction.

3.5 DEK silencing attenuates the tube formation capacities of HUVECs and inhibited Erk1/2 signaling.

We next analyzed the role of DEK in angiogenesis, and tube formation assays revealed that DEK silencing significantly inhibited the ability of HUVECs to form tubes (Fig. 5A). Intriguingly VEGF and VEGFR-2 demonstrated no significant changes in expression (Fig. 5B). Therefore, further investigation is required. MTT assays showed that, compared with the other four groups, HUVECs incubated with CM obtained from DEK-silenced HuCCT1 cells underwent viability attenuation (Fig. 5C). Previous studies demonstrated that DEK has critical roles in tumorigenesis via ERK1/2 modulation, hence western blot assays were used to analyze p-ERK1/2 expression after DEK silencing. Compared with the control group, DEK silencing inhibited the phosphorylation of ERK1/2 (Fig. 5D). We next applied GCD-0994, an ERK1/2 inhibitor to further confirm the role of DEK in ERK1/2 activation. Western blot assay showed that there were no significant changes in DEK expression (Fig. 8E), suggesting that DEK exerts oncogenic functions via ERK1/2 modulation.

3.6 Cordycepin inhibits CCA progression by targeting DEK via ERK1/2 signaling
in a xenograft model.

To further confirm the anti-tumor effects of cordycepin in vivo, $5 \times 10^6$ HuCCT1 cells were subcutaneously transplanted into nude mice (n=5 biologically independent mice per group). Seven days after cancer cell injection, cordycepin at a dose of 50mg/kg was administered every 24 h for 45 days by intraperitoneal injection (i.p.), and then the mice were killed and the transplanted tumors were excised (Fig.6A). There were no significant changes in body weight (Fig.6B), and tumor volumes underwent a significant reduction compared with the control group (Fig.6C). We then evaluated the subcutaneously transplanted tumors at the end of experiment; after cordycepin treatment, tumor weight and tumor volume were both reduced (Fig.6D and E). It was also obvious that there were far fewer microvessels on the tumor surface compared with the control group and indicated that angiogenesis was inhibited by cordycepin (Fig.6F).

Hematoxylin and eosin (H&E) staining of the portal tract was undertaken to evaluate the toxicity of cordycepin and revealed no significant changes (Fig.6G), indicating that little damage was done by cordycepin at a dose of 50mg/kg. Next, we applied IHC and H&E staining of the xenograft tumor tissue. IHC staining showed a significant inhibition of p-ERK1/2 and DEK expression compared with the control group (Fig.6H). The IHC staining of two angiogenic markers CD31 and vWF showed that the newly formed endothelial cells and micro vessels were significantly reduced after cordycepin treatment (Fig.6I). Thus, our data indicated that cordycepin inhibited CCA progression by targeting angiogenic capacities and DEK via ERK1/2 signaling in vivo.
4. Discussion

Multiple studies have shown that cordycepin exerts anti-tumor effects in hepatocellular carcinoma, colorectal cancer, and gastric cancer (He et al., 2010; Nasser et al., 2017; Zeng et al., 2017). Researches have indicated that cordycepin suppresses tumor growth by apoptosis induction and angiogenesis inhibition. However, in CCA, the effects of cordycepin were not fully elucidated. Therefore, we performed MTT and colony formation assays to investigate the impact of cordycepin treatment on cell proliferation. MTT assays revealed attenuated cell viability and colony formation ability in both CCA cell lines. Further investigations utilizing Hoechst dye and flow cytometry revealed apoptosis induction in both cell lines following cordycepin treatment. Parallel with western blot results, the apoptosis markers, cleaved caspase-3, cleaved caspase-9 and cleaved PARP were upregulated, which further confirmed the apoptosis induction effects of cordycepin. Previous studies have suggested that cordycepin induces apoptotic effects by downregulating ERK signaling (Wang et al., 2017), and ERK1/2 activation promotes CCA cell proliferation, chemoresistance, and progression (Ewald et al., 2014; Yokoi et al., 2018). Thus, analysis of p-ERK1/2 expression after cordycepin treatment was performed. Compared with the control group, cordycepin treatment significantly inhibited ERK1/2 phosphorylation, suggesting that cordycepin inhibits CCA cell proliferation dependent upon ERK1/2 downregulation.

Angiogenesis, another aspect that promotes cancer progression was also investigated in this study. Given that angiogenesis provides various benefits and comfort microenvironment for tumor growth (Okkenhaug et al., 2016; Xiang et al., 2017; Yang
et al., 2018), CCA progression also take the advantages (Kangsamaksin et al., 2017; Xu et al., 2018). Indeed, tube formation assay which performed by HUVECs in vitro, revealed an association between angiogenesis and cordycepin anti-tumor functions. In our data, cordycepin inhibited HUVECs tube formation ability in vitro, and angiogenesis associated protein, VEGF and VEGFR-2 were also downregulated. We therefore believe cordycepin may play an anti-tumor role by targeting angiogenesis in CCA progression.

Cordycepin have been demonstrated targeting various oncogenes to achieve anti-tumor effects (Cui et al., 2018; Liang et al., 2017; Zeng et al., 2017). DEK acts as an oncogene, is overexpressed in various cancer types, including CCA. DEK was proved to enhance tumorigenesis and tumor associated angiogenesis. In this study, notably, we found DEK expression was downregulated by cordycepin treatment. To further elucidate the roles of cordycepin and DEK interplay, we silenced DEK using siRNA and examined its roles in tumor growth and angiogenesis. According to our studies, DEK silencing significantly reduced cell viability and colony formation capacities. However, DEK silencing was found irrelevant with apoptosis induction in CCA. In contrast, DEK silencing was found to promote apoptosis in cervical cancer and lung cancer (Feng et al., 2017; Liu et al., 2012), we speculated that, Cordycepin modulated cell death may mainly due to Erk1/2 deactivation but not DEK silencing. DEK silencing revealed no changes in cell apoptosis induction may due to cancer specificity, and further studies are required.

Finally, DEK silencing was also associated with tube formation in HUVECs and
downregulation of ERK1/2 signaling. These data revealed that cordycepin functions correspond with DEK silencing and that DEK may act as a potential target of cordycepin. We next generated a xenograft model to further confirm our findings. Cordycepin treatment significantly downregulated the relative volume of subcutaneously transplanted tumors, and IHC staining of tumor tissue revealed that p-ERK1/2 and DEK were inhibited. In conclusion, our study provides mechanistic insights into the inhibition of CCA progression and angiogenesis by cordycepin and revealed an interaction with DEK (Fig.7).

In sum, our study provides mechanistic insights into the inhibition of CCA progression and angiogenesis by cordycepin and revealed an interaction with DEK. Our data indicate that cordycepin may serve as a novel therapeutic target for CCA.

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5. References:


Footnotes

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Figure Legends

Figure 1. Cordycepin inhibits CCA cell growth and Erk1/2 phosphorylation.

A. The structure of Cordycepin. B. MTT assay of HuCCT1 and CCKS1 treated with cordycepin at 24h, 48h, 72h time point in a dose dependent manner, cordycepin significantly inhibited the viability of both cancer cell lines (**P<0.01 vs 24h 0μM group; ###P<0.01 vs 48h 0μM group; ^^P<0.01 vs 72h 0μM group, n=6). C. Colony formation assay of HuCCT1 and CCKS1 treated with Cordycepin in a dose dependent manner. Cordycepin significantly inhibited the colony formation ability of both cancer cell lines (*P<0.05, **P<0.01 vs 0 μM group, n=3). D. HuCCT1 and CCKS1 treated with cordycepin for 48h, Hochest33342 dye showed that the apoptotic body were significantly increased (200×, n=3). E. Flow cytometry showed an increase ratio of apoptosis cells after cordycepin treatment of both cell lines with a PI and Annexin V double staining (**P<0.01 vs 0μM group, n=3). F. Western bolt assay showed a significant increase of Cl-caspase 9, Cl-caspase 3, and Cl-PARP expressions of HuCCT1 and CCKS1 after cordycepin treatment. G. Western blot assay showed that after Cordycepin treatment, p-Erk1/2 expression was significantly downregulated.

Figure 2. Cordycepin inhibits tube formation capacities of HUVECs

A. MTT assay of HUVECs. HUVECs treated with different condition medium (CM) for 48h, compared with other groups, BM: basal medium. CM obtained from both cancer cell lines which treated with Cr: cordycepin for 48h significantly inhibited HUVECs viability. (**P<0.01 vs 0μM group; ###P<0.01 vs cordycepin 100/150μM
group, n=6). B. HUVECs treated with CM of HuCCT1 and CCKS1. Tube formation assay showed that, CM obtained from cancer cells treated with cordycepin significantly inhibited the tube formation ability of HUVECs (**\( P \leq 0.01 \) vs 0μM group, 40×, n=3). C. Western blot assay showed that after cordycepin treatment of both cells, expression of VEGF and VEGFR-2 were down regulated.

Figure 3. Cordycepin downregulated DEK overexpression in CCA.

A. Western blot assay showed that DEK expression in HuCCT1 and CCKS1 were downregulated after cordycepin treatment. B. UALCAN data base based on TCGA data showed that DEK is overexpressed in CCA. C. IHC staining of DEK. DEK showed a strongly positive staining in CCA tissue. D. RT-PCR assay showed that DEK is overexpressed in five different CCA cell lines.

Figure 4. Silencing DEK attenuates CCA cell growth.

A. Western blot assay to demonstrate the knock down significance of si DEK. B. MTT assay showed that the cell viability of HuCCT1 were downregulated by si DEK 4 (**\( P \leq 0.001 \) vs si Control group, n=6). C. Colony formation assay of HuCCT1. The ability of colony formation of HuCCT1 were inhibited by si DEK 4 (**\( P \leq 0.01 \) vs si Control group, n=3). D. Hochest33342 dye showed no significant changes in apoptosis induction in HuCCT1 after silencing DEK. (n=3) E. Flow cytometry showed no significant changes in apoptosis induction after silencing DEK. (n=3) F. Western bolt
assay showed no significant changes in expression of Cl-caspase 9, Cl-caspase 3, and Cl-PARP.

**Figure 5. DEK silencing attenuates tube formation capacities of HUVECs and inhibited Erk1/2 signaling**

A. Tube formation assay of HUVECs. The ability of tube formation were inhibited after HUVECs were treated with the culture supernatant obtained from DEK silencing cancer cell (\**(\*\*P<0.01 vs si Control group, 40×, n=3)\). B. Western blot assay showed no significant changes in VEGF and VEGFR-2 expression. C. MTT assay of HUVECs. HUVECs treated with different culture supernatant for 48h, compared with other groups, culture supernatant obtained from DEK silencing HuCCT1 significantly inhibited HUVECs viability. (\**(\*\*P<0.01 vs 0μM group, n=3)\) D. Western blot assay showed that the expression of p-Erk1/2 was downregulated by silencing DEK. E. Western blot assay showed no significant changes in DEK expression after GCD-0994 treatment.

**Figure 6. Cordycepin inhibits CCA growth via DEK and p-Erk1/2 inhibition in vivo.**

A. Representative images of nude mice xenografted with HuCCT1 cells and treated with or without cordycepin 50mg/kg. B. Changes of body weight after 45 days of cordycepin treatment. No significant changes were found. C. Changes of tumor volume after 45 days of cordycepin treatment. Cordycepin treatment significantly inhibited the
tumor growth of HuCCT1 in a xenograft model (*$P<0.05$, **$P<0.01$ vs Control group). D. Comparison of tumor weight and cordycepin efficacy. E. Comparison of tumor volume and cordycepin efficacy. F. Pictures of xenograft tumor after cordycepin treatment. Compared with control group, the tumor volume was much smaller after cordycepin treatment and few micro blood vessels were found. G. H&E staining of the portal tract to evaluate the toxicity of cordycepin. No significant changes were found.

H. IHC and H&E staining of the xenograft tumor tissue. IHC staining showed a significant inhibition of p-Erk1/2 and DEK expression. I. The IHC staining of two angiogenic markers CD31 and vWF showed that the newly formed endothelial cells and micro vessels (marked by red arrowhead) were significantly reduced after cordycepin treatment.

Figure 7. Schematic representation of the molecular mechanism of Cordycepin.

Schematic representation of the molecular mechanism of Cordycepin targeting DEK, inhibits cancer cells proliferation by apoptosis induction and angiogenesis inhibition.
Figure 2

A

HUVEC

HUVEC

Cell Viability rate (%)

B

HuCCT1 CM + HUVECs

HuCCT1

Number of tubules

Cordycepin (µM)

Cordycepin (µM)

CCKS1 CM + HUVECs

CCKS1

Number of tubules

Cordycepin (µM)

Cordycepin (µM)

C

HuCCT1

CCKS1

Cordycepin (µM)

Cordycepin (µM)

VEGF

VEGF

β-actin

β-actin

VEGFR2

VEGFR2

** #

**

**
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>HuCCT1</th>
<th>CCKS1</th>
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<td>Cordycepin (µM)</td>
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</tr>
<tr>
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<td>100</td>
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<td>DEK</td>
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<td></td>
</tr>
<tr>
<td>β-actin</td>
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B

TCGA

Transcript per million

Normal    Tumor

P = 7.07E-14

n=9        n=38

C

Pri-tumor

HE

DEK

Tumor

DEK

×200

×200

×400

D

<table>
<thead>
<tr>
<th></th>
<th>HIBE36c</th>
<th>HuCCT1</th>
<th>CCKS1</th>
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Figure 4

A. **HuCCT1**

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<th>si-control</th>
<th>si-DEK 1</th>
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<th>si-DEK 3</th>
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</tbody>
</table>

B. **Cell viability rate (%)**

- Control
- si-control
- si-DEK 4

C. **HuCCT1**

- Control
- si control
- si DEK 4

D. **HuCCT1**

- Control
- si control
- si DEK 4

E. **HuCCT1**

- Control
- si control
- si DEK 4

F. **HuCCT1**

- Cl-caspase 9
- Cl-caspase 3
- Cl-PARP
- β-actin

G. **Apoptosis rate (%)**

- Control
- si control
- si DEK 4

**n.s.** (not significant)
Figure 5

A. HuCCT1 CM + HUVECs

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<thead>
<tr>
<th></th>
<th>Control</th>
<th>si Control</th>
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<tbody>
<tr>
<td>Number of tubes</td>
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B. HuCCT1

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C. HUVEC

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<td>HuVEC BM</td>
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<tr>
<td>HuCCT1 BM</td>
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<td>HuCCT1 Control CM</td>
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<tr>
<td>HuCCT1 si Control CM</td>
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<td>HuCCT1 si DEK 4 CM</td>
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D. HuCCT1

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<th>Protein</th>
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E. HuCCT1

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</table>
Figure 6

A

Control

Cordycepin (50mg/kg)

Control

Cordycepin (50mg/kg)

B

Body weight (g) vs. Days

Control

50mg/kg

C

Tumor Volume (mm³) vs. Days

Control

50mg/kg

D

Tumor Weight (g)

Control

50mg/kg

E

Tumor Volume (mm³)

Control

50mg/kg

F

Control

50mg/kg

G

Control

50mg/kg

200x

400x
Figure 7

Cordyceps Militaris → Cordycepin

Cancer cell

DEK

ErK

Apoptosis

Angiogenesis

VEGF