Original Article

Cordycepin Suppresses The Malignant Phenotypes of Colon Cancer Cells through The GSK3ß/ß-catenin/cyclin D1 Signaling Pathway

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Abstract

Objective: Cordycepin, also known as 3′-deoxyadenosine, is the main bioactive ingredient of *Cordyceps militaris* and possesses various pharmacological effects. This study was performed to investigate the role of cordycepin in regulating the biological behaviors of colon cancer cells and the potential mechanism behind it.

Materials and Methods: In this experimental study, after treatment of colon cancer cells with different concentrations of cordycepin, inhibition of proliferation was detected by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Colon cancer cell migration and invasion abilities were analyzed by wound healing and Transwell assays. Flow cytometry was performed to detect cell apoptosis. A lung metastasis model in nude mice was utilized to examine the effect of cordycepin on the metastasis of colon cancer cells *in vivo*. Western blot was used to quantify GSK3β, β-catenin and cyclin D1 expression levels.

Results: Cordycepin inhibited colon cancer cell proliferation, migration and invasion, induced apoptosis *in vitro*, and inhibited lung metastasis of colon cancer cells *in vivo*. GSK-3β inhibitor (CHIR99021) treatment abolished the effects of cordycepin on cell viability, migration, invasion and apoptosis. Additionally, cordycepin promoted the expressions of GSK3β, and inhibited β-catenin and cyclin D1 in colon cancer cells, while co-treatment with CHIR99021 reversed the above effects.

Conclusion: Cordycepin suppresses the malignant phenotypes of colon cancer through the GSK3β/β-catenin/cyclin D1 signaling pathway.

Keywords: Colon Cancer, Cordycepin, Cyclin D1, GSK3β, β-Catenin

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Introduction

Colon cancer is one of the most common malignancies (1). Surgical resection, chemotherapy, and molecular targeted therapy are the main treatment strategies for colon cancer (2). Nonetheless, approximately 50% of colon cancer patients develop chemoresistance (3). Therefore, the search for possible therapeutic agents is important to improve the prognosis of colon cancer patients.

Natural products represent a rich source for the discovery and development of anti-cancer drugs (4-6). Cordycepin, also known as 3'-deoxyadenosine, is a kind of nucleoside analog, which is extracted from *Cordyceps militaris* (7, 8). Cordycepin has various pharmacological effects, including anti-tumor, anti-bacterial, and anti-aging activity, immunomodulation, scavenging of free radicals and anti-ischemia/reperfusion injury activity (8-10). Reportedly, cordycepin can inhibit the growth of colon cancer cells, suggesting that cordycepin is promising in the treatment of colon cancer (11). However, the detailed mechanism by which cordycepin exerts its tumor-suppressive functions is not clear.

Kinases play a critical role in cellular signal transduction, and many of them are associated with tumorigenesis and cancer progression (12). Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase, which has two isoforms, GSK3α and GSK3β (13). GSK3β has been found to regulate the survival and proliferation of colon cancer cells (14). Interestingly, GSK3β is reported to be involved in the phosphorylation of β-catenin, thereby activating the E3 ubiquitin ligase subunit β-Trcp and inducing the proteasome degradation of β-catenin (15). In addition, elevated levels of nuclear β-catenin is considered to be a hallmark of aggressive colon cancer, which activates Wnt-related targets including c-myc, cyclin D1, MMP2 and MMP9, thereby promoting cell proliferation, invasion and migratory potential (16-19). The present study aimed to investigate the role and mechanism of GSK3β/β-catenin/cyclin D1 pathway in colon cancer.

Materials and Methods

Cell lines and cell culture

In this experimental study, Cordycepin (3′-deoxyadenosine, CAS: 73-03-0) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Human immortalized colon mucosa cell line FHC, and human colon cancer cell lines (HT-29 and LoVo) were provided by the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and the cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Waltham, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA), 0.1 mg/mL streptomycin (Gibco, Waltham, MA, USA) and 100 U/mL penicillin (Gibco, Waltham, MA, USA) at 37˚C in a humidified environment with 5% CO_2. The medium was changed every 1-2 days, and when the confluence reached 80%, 0.25% trypsin was used for trypsinization, and the cells were passaged.

3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The cells were trypsinized, resuspended and then inoculated in 96-well plates at 5×10^3 cells/well, with 3 replicate wells for each group of cells. HT-29 and LoVo cells were treated with different concentrations of cordycepin (20, 40 and 80 μM) for 24 hours, respectively. Cells with no treatment served as the control group. After adding 10 μL of MTT (Sigma-Aldrich, St. Louis, MO, USA) per well, the cells were further incubated for 2 hours. Then dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added into the wells, and the crystals were dissolved, and the absorbance values were measured by a spectrophotometer at 490 nm.

Wound healing experiment

HT-29 and LoVo cells were inoculated into 6-well culture plates (5×10^5 cells/well) and cultured. When the confluency of the cells reached 90%, a scratch was made in the middle of the monolayer cells with a sterile 200 μL pipette tip. Then, the cells were gently washed 3 times with phosphate buffered saline (PBS), the width of the scratch was detected, and then the cells were cultured with serum-free medium. After 24 hours, the scratch was detected under the microscope again. Scratch healing rate (%)=(0 hours scratch width−24 hours scratch width)/0 h scratch width×100%.

Transwell assay

Cell invasion assays were performed with Transwell chambers (Corning, NY, USA). Matrigel (1:10; BD Biosciences, Franklin Lakes, NJ, USA) was used to cover the filter of Transwell chambers. The density of HT-29 cells was modulated to 1×10^6 cells/mL with serum-free medium, and 100 μL of cell suspension and 600 μL of the complete medium were supplemented to the upper compartment and lower compartment, respectively. After 2 days, the cells on the upper surface of the filter were removed, and the cells on the below surface of the filter were fixed with 4% paraformaldehyde, and subsequently stained with 0.1% crystal violet. The number of cells that passed through the filter were counted in five random fields of view, and the average was calculated to indicate the invasion ability of the cells.

Flow cytometry

The cells were trypsinized with EDTA-free trypsin, and the cells were collected by centrifugation. According to the manufacturer's instructions for Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen Biotech Co., Ltd., Shanghai, China), the colon cells were washed twice with PBS and resuspended with 1× binding buffer and accordingly incubated with 5 μL of Annexin V-FITC staining solution and 10 μL of propidium iodide (PI) staining solution for 15 min at ambient temperature in the dark. After the cells were washed by PBS, a flow cytometer (BD Biosciences, San Jose, CA, USA) was used to quantify apoptosis within 1 hours.

Lung metastasis assay

All animal experiments were approved by the animal Ethical Committee of Zhejiang Greentown Cardiovascular Hospital (2017A044). 12 nude mice (6-week-old, male) weighing 12-15 g, purchased from the Experimental Animal Center of Zhejiang University, were randomly grouped into 2 groups (n=6 in each group). The mice in each group were injected with HT-29 cells (1×10^7 cells per mouse) via the caudal vein, and then treated with or without cordycepin (20 mg/kg). After 2 weeks, the mice were euthanized, and the fresh lung tissues were harvested for histopathology analysis. The number of metastatic nodules in the lung of each mouse was counted with naked eyes. Next, formalin-fixed and paraffin-embedded lung tissues were prepared, and 4 μm of thick sections were stained with hematoxylin and eosin and then observed under the microscope (NikonEclipseE600; Nikon, Thornwood, NY, USA).

Western blot

The colon cancer cells were lysed in RIPA lysis solution (Beyotime Biotechnology, Shanghai, China), and the BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) was utilized to quantify the protein concentration. After adding protein loading buffer and boiling to denaturation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The protein samples were transferred onto the polyvinylidene fluoride

(PVDF) membranes, and then were blocked at ambient temperature for 1 hours using 5% skimmed milk. Then the membranes were incubated with the following primary antibodies: anti-GSK3β (1:2000, ab32391, Abcam, Shanghai, China), anti-β-catenin (1:2000, ab16051, Abcam, Shanghai, China) and anti-cyclin D1 (1:2000, ab16663, Abcam, Shanghai, China) overnight at 4˚C. The following day, the secondary antibody, Goat Anti-Rabbit IgG H&L (1:5000, ab6721, Abcam, Shanghai, China), was added, and the membrane was incubated for 1 hour at 37˚C. After the membranes were washed by tris buffered saline tween (TBST), the ECL chemiluminescence kit (Beyotime Biotechnology, Shanghai, China) was used for luminescence development, and an Odyssey imaging system was utilized to analyze the grayscale value of each band.

Statistical analysis

Statistical product and service solutions (SPSS) software (version 23.0, SPSS, Chicago, IL, USA) was adopted to process the data represented as "mean ± standard deviation". To make the comparison between two groups, a One-Sample Kolmogorov-Smirnov test was used to detect the normality of the data. If the data were normally distributed, an independent sample t test was utilized; paired sample Wilcoxon signed rank test was adopted to compare the data with skewed distribution. One-way ANOVA test was performed to make the comparison among three or more groups. If there was a significant difference, Newman-Keuls analysis was performed to make the comparison between 2 groups. The differences were considered statistically significant at P<0.05.

Results

Cordycepin suppresses the malignant phenotypes of colon cancer cells

The molecular structure of cordycepin is shown in Figure 1A. To probe the biological effect of cordycepin on the phenotypes of FHC and colon cancer cells, the proliferation of HT-29, LoVo and FHC cells was detected by the MTT method after treatment with different concentrations of cordycepin (20 μM, 40 μM and 80 μM). As shown, the viability of colon cancer cells was decreased by cordycepin treatment in a dose-dependent manner (P<0.05, Fig.1B); notably, only high doses of cordycepin (80 μM) could significantly suppress the viability of FHC cells (Fig.S1A, See Supplementary Online Information at www.celljournal.org), which suggested that cordycepin selectively kills cancer cells. The results of the scratch healing assay and Transwell assay showed that cordycepin treatment reduced the migration and invasion of HT-29 and LoVo cells compared with the control group (P<0.01, Fig.1C, D). In addition, flow cytometry revealed that cordycepin treatment promoted the apoptosis of HT-29 and LoVo cells compared with the control group (P<0.05, Fig.1E). Additionally, a lung metastasis model in nude mice was used to evaluate the metastasis of HT-29 cells *in vivo*, and it showed that cordycepin treatment inhibited the pulmonary metastasis of HT-29 cells *in vivo* (Fig.S1B, See Supplementary Online Information at www.celljournal.org).

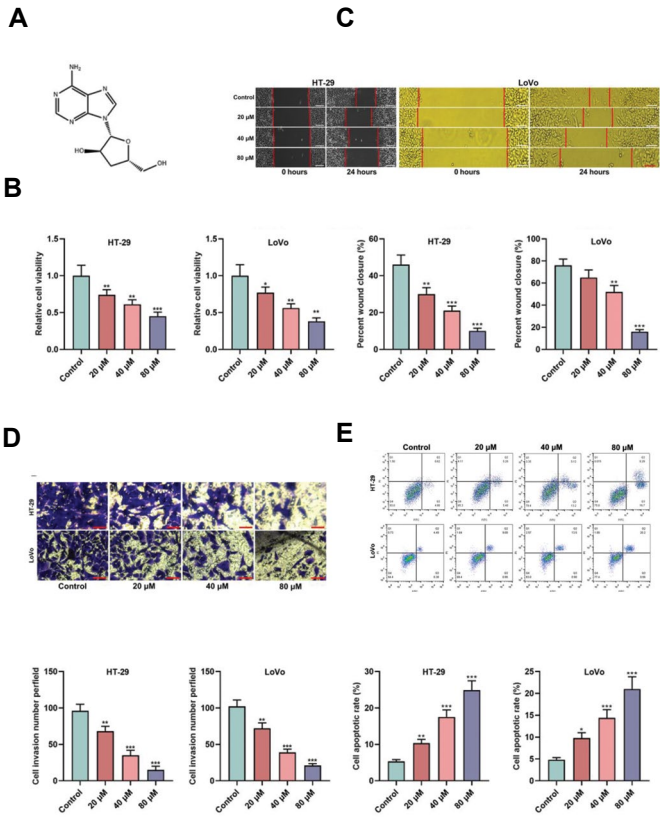

Fig.1: Effects of cordycepin on the biological behaviors of colon cancer cells**. A.** The molecular structure of cordycepin. **B.** Cell viability of HT-29 and LoVo cells was detected by MTT assay after treatment with different concentrations of cordycepin (control, 20 μM, 40 μM and 80 μM). **C.** Cell migration (scale bars: 50 μm) of HT-29 and LoVo cells was detected by scratch healing assay after treatment with different concentrations of cordycepin (control, 20 μM, 40 μM and 80 μM). **D.** Cell invasion (scale bars: 50 μm) of HT-29 and LoVo cells was detected by Transwell assay after treatment with different concentrations of cordycepin (control, 20 μM, 40 μM and 80 μM). **E.** Cell apoptosis of HT-29 and LoVo cells was detected by flow cytometry after treatment with different concentrations of cordycepin (control, 20 μM, 40 μM and 80 μM). All experiments were repeated at least 3 times and were performed in triplicates, and data were presented as mean ± SD. *; P<0.05, **; P<0.01, and ***; P<0.001.

Effects of cordycepin and GSK-3β inhibitor (CHIR99021) on GSK3β protein expression

To investigate the mechanism by which cordycepin suppresses the malignancy of colon cancer cells, Western blot was used to detect the expressions of GSK3β protein in colon cancer cells. Cordycepin treatment was found to promote GSK3β protein expression in HT-29 and LoVo cells in a dose-

dependent manner (P<0.001, Fig.2A). After the co-treatment with CHIR99021, the promoting effect of cordycpin on GSK-3β protein expression was reversed (P<0.001, Fig.2B).

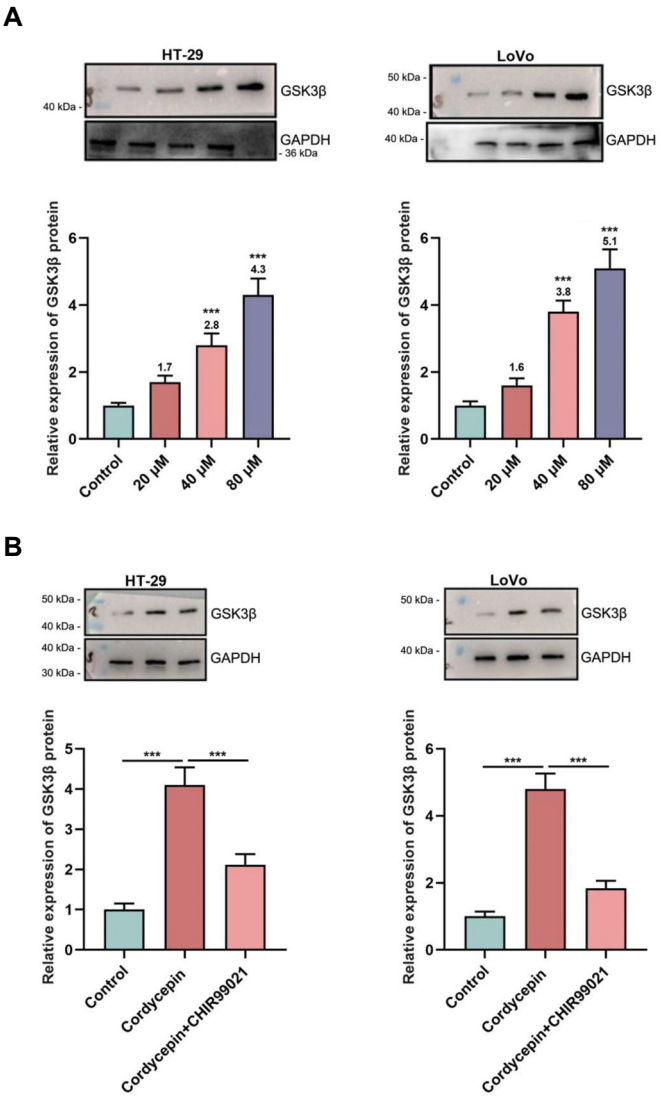

Fig.2: Effects of cordycepin and CHIR99021 on GSK3β expression**. A.** GSK3β protein expression in HT-29 and LoVo cells was detected by Western blot after treatment with different concentrations of cordycepin (control, 20 μM, 40 μM and 80 μM). **B.** GSK3β protein expression in HT-29 and LoVo cells was detected by Western blot after co-treatment with 80 μM cordycepin and 10 μM CHIR99021. All experiments were repeated at least 3 times and were performed in triplicates, and data were presented as mean ± SD. ***; P<0.001.

Effects of cordycepin and CHIR99021 on the biological behaviors of colon cancer cells

To investigate that whether the tumor-suppressive effects of cordycepin on colon cancer cells are dependent on GSK3β, after the HT-29 and LoVo cells were treated with cordycepin and CHIR99021, MTT assay, scratch healing assay, Transwell assay and flow cytometry were used to detect the viability, migration, invasion and apoptosis of the colon cells, repectively. It was found that, cordycepin treatment inhibited the viability, migration and invasion, and promoted apoptosis of HT-29 and LoVo cells; remarkably, co-treatment with CHIR99021 reversed the above effects of cordycepin (P<0.001, Fig.3A-D).

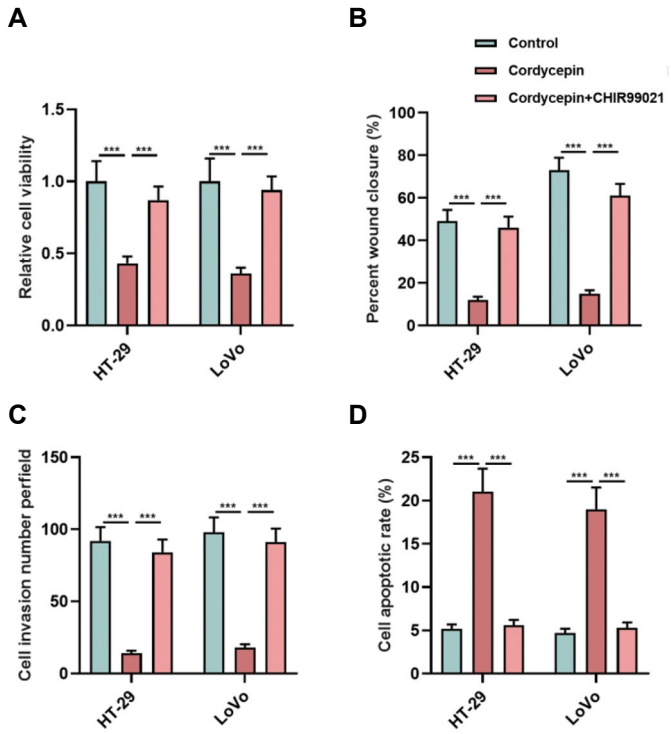

Fig.3: Effects of cordycepin and CHIR99021 on the viability, migration and apoptosis of colon cancer cells. **A.** Viability of HT-29 and LoVo cells was detected by MTT assay after treatment with 80 μM cordycepin and 10 μM CHIR99021 for 24 hours. **B.** Migration of HT-29 and LoVo cells was detected by scratch healing assay after treatment with 80 μM cordycepin and 10 μM CHIR99021 for 24 hours. **C.** Invasion of HT-29 and LoVo cells was detected by Transwell assay after treatment with 80 μM cordycepin and 10 μM CHIR99021 for 24 hours. **D.** Apoptosis of HT-29 and LoVo cells was detected by flow cytometry after treatment with 80 μM cordycepin and 10 μM CHIR99021 for 24 hours. All experiments were repeated at least 3 times and were performed in triplicates, and data were presented as mean ± SD. ***; P<0.001.

Effects of cordycepin and CHIR99021 on cyclin D1 and β-catenin protein expressions

Next, we investigated the regulatory effects of cordycepin and GSK3β on cyclin D1 and β-catenin, the expression levels of cyclin D1 and β-catenin proteins were detected by Western blot. The results showed that the expression levels of cyclin D1 and β-catenin protein were reduced upon cordycepin treatment; however, co-treatment with CHIR99021 inhibited the effects of cordycepin on cyclin D1 and β-catenin protein expression (P<0.001, Fig.4A, B). These data suggested that cordycepin could regulate the expression level of cyclin D1 and β-catenin in colon cancer cells via activating GSK3β.

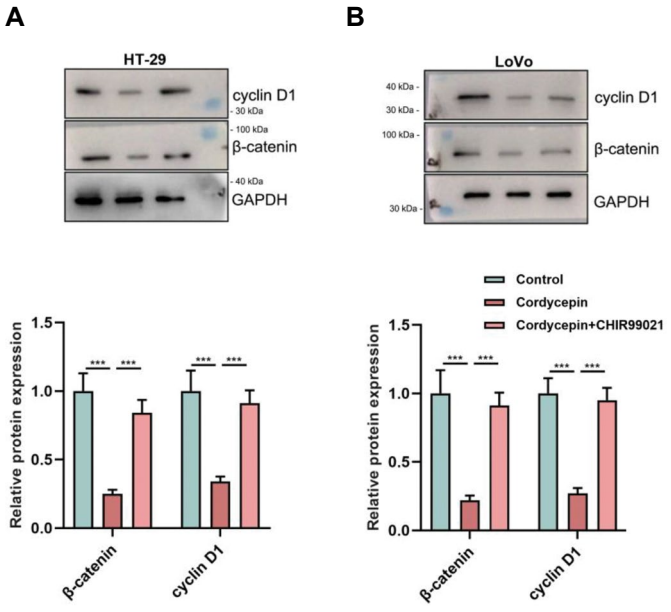

Fig.4: Effects of cordycepin and CHIR99021 on cyclin D1 and β-catenin protein expressions. **A, B.** Cyclin D1 and β-catenin protein expressions of HT-29 and LoVo cells were detected by Western blot after co-treatment with 80 μM cordycepin and 10 μM CHIR99021. All experiments were repeated at least 3 times and were performed in triplicates, and data were presented as mean ± SD. ***; P<0.001.

Discussion

Colon cancer patients with distant metastasis or recurrence after surgery have poor prognosis (20). In recent years, several studies have implied that cordycepin can induce apoptosis of cancer cells and promote DNA damage to inhibit the proliferation and metastasis of cancer cells, and it may also increase the chemosensitivity of cancer cells (21, 22). For example, cordycepin inhibits the proliferation of tongue cancer cells in a dose-dependent manner (23). Cordycepin can down-regulate the expression of C-X-C chemokine receptor type 4 in hepatocellular carcinoma cells in a dose-dependent manner, significantly inhibiting the migration and invasion of hepatocellular carcinoma cells (24). Another study shows that cordycepin can regulate the expressions of cyclin-dependent kinase 1 and cyclin B1, leading to cell cycle arrest of esophageal cancer cells (25). In addition, multiple previous studies report that cordycepin has the potential to block the progression of colon cancer (26-28). Specifically, cordycepin can inhibit the migration and invasion of HCT116 cells by regulating the expression of Prostaglandin E2 receptor 4 and the transduction of AMPK-CREB signaling pathway (27). Additionally, cordycepin can induce the activation of JNK1, leading to cell cycle arrest of HCT116 cells (28). Consistently, in the present study, cordycepin was found to significantly inhibit the proliferation, migration and invasion of colon cancer cells and promote their apoptosis.

GSK3β is a serine/threonine kinase partaking in modulating cell proliferation, DNA repair, cell cycle progression, signal transduction and metabolic pathways (29). Importantly, the dysfunction/dysregulation of GSK-3β is involved in tumorigenesis, and it may exert cancer-promoting function or tumor-suppressive function in different cancers (30-32). GSK3β promotes epithelial-mesenchymal transition (EMT) of triple-negative breast cancer cells, and GSK3β inhibitors selectively kill cancer cells with mesenchymal properties and are considered as potential therapeutic targets for triple-negative breast cancer (30). Resveratrol can inhibit the EMT of colon cancer through the AKT/GSK-3β/Snail signaling pathway (31). The occurrence, progression and drug resistance of pancreatic ductal carcinoma are thought to be related to the expression of GSK3β, and the inhibition of GSK3β induces apoptosis and slows the growth of tumors and metastases (32). β-catenin is a crucial component in Wnt signaling pathway; the abnormal activation of the Wnt/β-catenin signaling pathway facilitates the accumulation of β-catenin in the nucleus and the transcription of many oncogenes such as c-Myc and Cyclin D1, thereby contributing to the occurrence and development of a variety of cancers including colon cancer (33, 34). Importantly, GSK3β can phosphorylate β-catenin, leading to the ubiquitination and proteasomal-dependent degradation of β-catenin (15, 35). The dysregulation of the GSK3β/β-catenin pathway is involved in the regulation of the malignant biological behaviors of cancer cells (36-38). For instance, upregulated gene 4 can promote the proliferation of osteosarcoma cells through the GSK3β/β-catenin/cyclin D1 pathway (37). PIK3CD induces malignant phenotypes of colorectal cancer by activating AKT/GSK-3β/β-catenin signaling (38). Herein, we found that cordycepin could increase GSK3β expression and inhibit the expression levels of β-catenin and cyclin D1, which partly explains the mechanism of cordycepin's tumor-suppressive effects in colon cancer.

Conclusion

This study not only confirms the tumor-suppressive role of cordycepin in colon cancer, but also reports that the biological function of cordycepin on colon cancer cells is mediated by GSK3β/β-catenin/cyclin D1 pathway. It's worth noting that, even though cordycepin/*Cordyceps militaris* is widely used in traditional Chinese medicine, clinical trials are still needed to further validate its safety and efficacy in treating colon cancer.

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Authors' Contributions

Y.Z.; Conceived and designed the experiments. Y.Z.,

J.X., X.S., D.S.; Performed the experiments. Y.Z., J.X.; Analyzed the data. J.X.; Wrote the manuscript. All authors read and approved the final manuscript.

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