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Original Article

Cordycepin inhibits human ovarian cancer by inducing autophagy and apoptosis through Dickkopf-related protein 1/β-catenin signaling

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Abstract: Cordycepin, the major active component from *Cordyceps militaris*, has been reported to significantly inhibit some types of cancer; however, its effects on ovarian cancer are still not well understood. In this study, we treated human ovarian cancer cells with different doses of cordycepin and found that it dose-dependently reduced ovarian cancer cell viability, based on Cell counting kit-8 reagent. Immunoblotting showed that cordycepin increased Dickkopf-related protein 1 (Dkk1) levels and inhibited β-catenin signaling. Atg7 knockdown in ovarian cancer cells significantly inhibited cordycepin-induced apoptosis, whereas β-catenin overexpression abolished the effects of cordycepin on cell death and proliferation. Furthermore, we found that Dkk1 overexpression by transfection downregulated the expression of c-Myc and cyclin D1. siRNA-mediated Dkk1 silencing downregulated the expression of Atg8, beclin, and LC3 and promoted β-catenin translocation from the cytoplasm into the nucleus. These results suggest that cordycepin inhibits ovarian cancer cell growth, possibly through coordinated autophagy and Dkk1/β-catenin signaling. Taken together, our findings provide new insights into the treatment of ovarian cancer using cordycepin.

Keywords: Apoptosis, Dickkopf-related protein 1, β-catenin, autophagy, cordycepin

Introduction

Cordycepin (3'-deoxyadenosine) is the major bioactive component of *Cordyceps militaris* and has been reported to inhibit cell proliferation [1-3], induce apoptosis [4-7], inhibit platelet aggregation, regulate steroidogenesis, and reduce inflammation [8]. Moreover, cordycepin possesses anti-tumor activities [9]. However, studies on cordycepin inducing autophagy-mediated apoptosis through the regulation of Dkk1/β-catenin signaling pathways are not clearly unknown.

Autophagy is a process of self-degradation of cellular components [10], and can be considered as 'recycling' process cell undergo in order to overcome the effects of nutrient deprivation on the physiological cellular metabolism and to improve survival [11]; however, it may also cause cell death [12]. Consequently, autophagy plays a crucial role in cancer by significantly inhibiting tumor growth [13, 14]. Microtubule-associated protein 1A/1B light chain 3 (LC3) is a soluble protein that is encoded by the MAP1LC3B gene in humans [15]. During autophagy, autophagosomes engulf cytoplasmic components. Concomitantly, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form an LC3-phosphatidylethanolamine conjugate (LC3-II) [15, 16]. Therefore, the ratio of LC3-II to LC3-I represents the autophagic

rate [17, 18]. Beclin and Atg7 are known autophagy-related proteins [19].

Dickkopf-related protein 1 (Dkk1), a negative regulator of Wnt signaling, is a target of the β-catenin/TCF pathway and plays an important role in vertebrate head formation [20, 21]. Wnt ligands bind to the seven-pass-transmembrane receptor, Frizzled, and the single-pass low-density lipoprotein coreceptor-related proteins 5 and 6 (LRP5/6) [22]. Dkk1 forms a ternary complex with Dkk1 and LRP5/6, and induces the rapid endocytosis and removal of LRP5/6 from the cell surface [23]. Our data reveal a novel intranuclear link between β-catenin signaling and its antagonist, Dkk1.

Wnt/β-catenin signaling has been shown to play a crucial role in autophagy [24]. However, the role of Dkk1 in regulating autophagy remains to be elucidated. Autophagic and apoptotic programmed cell death, occurring via different mechanisms, are both implicated in cancer [25]. The inhibition of the Wnt/β-catenin signaling pathway suppresses survival signals and induces apoptosis, whereas autophagy is induced through stress-activated signaling pathways [24, 26]. In this study, we found that cordycepin triggers autophagic flux by suppressing the β-catenin signaling pathway. In addition, cordycepin promoted caspase-3 cleavage by upregulating Dkk1 expression in ovarian cancer cells. Taken together, our data suggest that cordycepin inhibits ovarian cancer cell growth, possibly through coordinated autophagy and Dkk1/β-catenin signaling.

Materials and methods

Reagents and antibodies

Fetal bovine serum (FBS), Antibiotic-Antimycotic (100X), and phosphate-buffered saline (PBS) were procured from Gibco™ (Waltham, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and Cordycepin (3'-deoxyadenosine from *C. militaris*) were purchased from Sigma-Aldrich™ (St. Louis, MO, USA). Annexin-V-FLUOS staining kit was purchased from two different sources, namely Roche Diagnostics GmbH (Mannheim, Germany) and Sigma-Aldrich™. The transfection reagent, Hilymax, and Cell counting kit-8 (CCK-8) were purchased from Dojindo (Dojindo, Japan). Antibodies against PARP1, caspase-8, -9, -3, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against Dkk1, beclin, β-catenin, Atg7, LC3, PCNA, cyclin D1, Bcl-2, and c-Myc were procured from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell lines and cytotoxicity

SKOV-3 and OVCAR-3 ovarian cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured in DMEM supplemented with 10% (v/v) FBS and 1% (w/v) penicillin-streptomycin in a humidified incubator with 5% (v/v) CO_2 at 37°C. The cells were allowed to adhere and grow for 24 h prior to exposure to cordycepin. In brief, SKOV-3 cells and OVCAR-3 were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 h of incubation, the cells were treated with various concentrations of cordycepin for 24 h. The optimal dose (without cytotoxic effects) was determined using the CCK-8 assay (Dojindo). The absorbance was measured at 450 nm using a Sunrise microplate absorbance reader (Tecan, Männedorf, Switzerland), relative to the untreated control in triplicate experiments.

Apoptosis assay

To detect the effect of cordycepin on apoptosis, we used the Muse Annexin V & Dead Cell reagent (Millipore) following the user's guide and the manufacturer's instructions. SKOV-3 and OVCAR3 cells were treated with cordycepin for 48 h, harvested by trypsinization and washed twice in PBS. The harvested cells were centrifuged at 1500 rpm for 5 min and the cell pellet was suspended in fresh growth media. Next, 100 μL of cells in suspension were transferred to a new tube, and then added the 100 μL of the Muse Annexin V and Dead Cell Reagent to each tubes. For staining, mixed cells were incubated at room temperature for 45 minutes to 1 hour. Measurements were conducted by using a Muse Cell Analyzer (Millipore, Billerica, MA, USA). The statistics were shown the percentages of the cells represented by alive, apoptosis and dead population.

Microarray analysis

Transcriptional profiling of the cordycepin-treated ovarian cancer cells was carried out using a

human twin 44K cDNA chip. Total RNA was extracted from vehicle- or cordycepin (100 μg/mL)-treated SKOV-3 and OVCAR-3 ovarian cancer cells. This was followed by the synthesis of cDNA probes using 50 mg RNA in the presence of aminoallyl-dUTP by reverse transcription. The cDNA was coupled with the Cy3 (vehicle) or Cy5 dye (cordycepin-treated). Genes were considered to be differentially expressed after a significance analysis of the microarray, when the global M and log2 (R/G) values exceeded |1.0| (two-fold) with $P < 0.05$. The Student's *t*-test was applied to assess the statistical significance of the differential expression of genes after cordycepin treatment. To analyze the biological significance of the changes, the array data were categorized into specific gene groups.

Gene ontology-based network analysis

To study the biological functions of the regulated genes through their interaction network, we used Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com) to examine the biological functions of the differentially regulated genes and proteins according to ontology-related interaction networks, including apoptosis signaling. Network generation was optimized based on the obtained expression profiles when possible and was aimed at producing highly connected networks.

Immunoblotting

Total cell lysates were prepared after homogenization in 2 mL of Tris-HCl (20 mM), containing a protease inhibitor cocktail (Roche), and left on ice for 30 min before centrifuging (10 min, 12,000 rpm, 4°C). The protein content of the supernatant was quantified using the BCA method. Denatured proteins (30 μg) were resolved using 12% SDS-polyacrylamide gel electrophoresis and transferred onto a 0.2-μm nitrocellulose membrane in a transfer buffer and kept for 2 h. The membrane was blocked for 1 h using 5% (w/v) skimmed milk in Tris-buffered saline with Tween-20 (TTBS), followed by incubation with the indicated appropriately diluted primary antibodies at room temperature for 2 h or at 4°C overnight. After washing the membrane three times (5 min each) with TTBS, it was incubated with horseradish peroxidase-conjugated goat anti-mouse or rabbit anti-goat IgG (1:2000 dilution) in TTBS containing 5% (w/v) skimmed milk at room temperature for 1 h. The membrane was rinsed three times (5 min each) with 0.1% (v/v) TTBS. An enhanced chemiluminescence system (Thermo Scientific) was used to visualize the bands on a ChemiDoc MP system (Bio-Rad, Hercules, CA, USA). Densitometric measurement of the bands was performed using the ImageJ software. Protein levels were quantitatively analyzed after normalizing with the level of β-actin as the internal control.

Wound healing assay

The Dkk1 plasmid, pCS2-hDkk1-FLAG, was purchased from Addgene (plasmid number, 154-94). To specifically silence Dkk1 and Atg7 expression, several siRNAs (Invitrogen) were tested for their capacity to reduce Dkk1 and Atg7 mRNA and protein levels in RNC transfections, and the most potent siRNAs were used for all subsequent experiments. A nonspecific siRNA was used as a negative control siRNA. The plated cells were transfected after 24 h with 100 mol/L siRNA using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) overnight. The wound healing assay was conducted on control siRNA-transfected and siDkk- or overDkk1-transfected cells after seeding them onto a 24-well plate (2×10^4 cells/well). After scraping the cell monolayer with a pipette tip to create a wound, the cells were treated with cordycepin (100 μg/mL) for 48 h. The plates were imaged using the TissueFAXS system (TissueGnostics, Vienna, Austria). Wound closure was analyzed by measuring the healed area and the proportion of the migrated cells using the HistoQuest software (TissueGnostics).

Immunofluorescence microscopy

The cells were fixed with 4% formamide for 15 min at room temperature for 24 h after an adherent culture was established. Thereafter, the cell membranes were permeabilized by treating with PBS containing 0.25% Triton X-100 for 10 min, blocked using TBST containing 1% BSA (Sigma-Aldrich) for 30 min, and incubated with the β-catenin primary antibody (Millipore, USA) for 1 h. The cells were then incubated with Alexa Fluor 488-conjugated anti-mouse secondary antibody (Cell Signaling Technology) for 1 h in the dark. After treating the cells with 4, 6-diamidino-2-phenylindole, fluorescence images were obtained by using a confocal microscope (Nikon, Japan).

Statistical analyses

GraphPad Prism (GraphPad, San Diego, CA, USA) was used for the statistical analyses. Intergroup differences were analyzed using the Student's *t*-test. The IC_{50} values were determined by nonlinear curve fitting using five data points and expressed as the mean ± SD.

Results

Cordycepin dose-dependently suppresses ovarian cancer cell growth

Cordycepin dose-dependently inhibits the cell viability of ovarian cancer cells. We assessed the effect of cordycepin on the viability of ovarian cancer cells, SKOV-3 and OVCAR-3. We treated the cells with different concentrations of cordycepin and found that it inhibits the viability of SKOV-3 cells in a dose-dependent manner (20-100 μg/mL), based on the results of CCK-8 (**Figure 1A**). To observe the cell death of cordycepin-treated SKOV-3 cells, the morphologies of human ovarian cancer cells were compared to those of untreated control cells by using light microscopy. The morphology of SKOV-3 cells changed drastically after 60 μg/mL cordycepin treatment for 48 h (**Figure 1B**). The ovarian cancer cells began to detach from the surface of the culture plate and appeared buoyant. Moreover, the cells appeared to be shrunken, resulting in reduced cell volume. These morphological changes preceded apoptosis. On the other hand, 40 μg/mL cordycepin induced less drastic changes at 48 h (**Figure 1B**). Similar results were obtained for OVCAR-3 cells (**Figure 1A** and **1B**). These data suggest that cordycepin inhibits ovarian cancer cell growth in a dose-dependent manner.

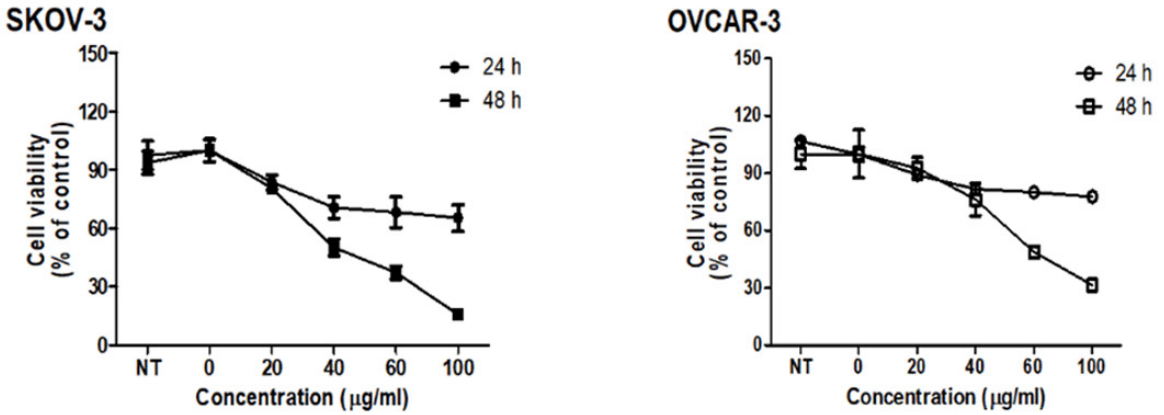
Cordycepin induces apoptosis in ovarian cancer cells

To evaluate the anti-cancer effect of cordycepin resulted in apoptosis in human ovarian cancer cells, we analyzed Annexin V expression on SKOV-3 and OVCAR3 cells by flow cytometry. After cordycepin treatment for 48 h, we measured apoptotic cells by Muse Annexin V and Dead Cell kit. As shown in the representative plots in **Figure 3A**, cordycepin potently induced apoptosis as detected by increased Annexin V expression in both SKOV-3 and OVCAR3 ovarian cancer cell lines. The addition, total fractions of apoptosis (early and late apoptosis) were increased by cordycepin treatment in dose-dependent manner. The apoptotic fractions of SKOV-3 and OVCAR-3 cells were increased from 3.2% and 2.4% in control group to 18.2% and 8.1% in cordycepin (100 μg/mL)-treated group, respectively (**Figure 1C**).

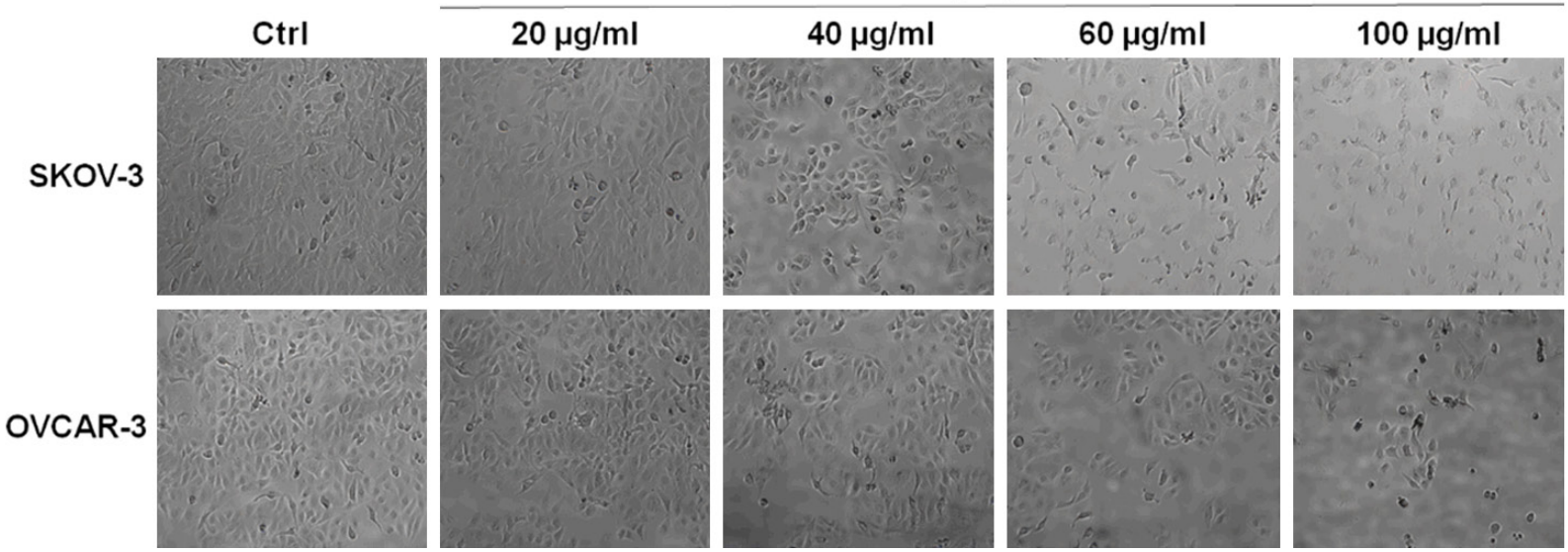
Cordycepin induces apoptotic gene expression in ovarian cancer cells

To investigate the genes involved in the cancer cell growth inhibition effect of cordycepin, microarray analyses of cordycepin (60 μg/mL)-treated SKOV-3 and OVCAR-3 ovarian cancer cells were conducted. Among the 63,785 genes assayed, 28,858 genes were expressed in the cordycepin-treated cells. Among 18,553 genes, cordycepin treatment upregulated and downregulated 784 and 680 genes, respectively, in comparison to the levels observed in the untreated control, at 48 h. Genes that were increased or decreased by more than 2-fold were categorized as being significant in data mining. Biologically relevant features were constructed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tools (http://david.abcc.ncifcrf.gov/). Lists of 2-fold upregulated and downregulated genes in cordycepin-treated SKOV-3 ovarian cancer cells were uploaded to DAVID for gene ontology analysis (**Figure 2A**). Upregulated genes included those involved in cell adhesion, inflammatory response, immune response, cell-cell signaling, autophagy, chemotaxis, cytokine-mediated signaling pathway, chemokine-mediated signaling pathway and positive-regulation of tumor necrosis-factor production. Downregulated genes included those involved in oxidation-reduction process, cell adhesion, cell surface receptor signaling pathway, regulation of cell proliferation and gene expression, chloride transmembrane transport, response to toxic substance, negative regulation of Wnt signaling pathway, and positive regulation of protein kinase B signaling. The genes upregulated by cordycepin treatment were compared with potential autophagotic genes by identifying candidate genes using the GeneCards database (http://www.genecards.org/) (**Figure 2B**). The intersection obtained by hierarchical clustering is presented along with the gene lists in **Figure 2C**. The signaling network of cordycepin-responsive autophagotic genes is shown in **Figure 2D**.

A


SKOV-3
OVCAR-3
24 h
48 h
Cell viability (% of control)
150
120
90
60
30
0
NT
0
20
40
60
100
Concentration (μg/ml)

B


Cordycepin 48 h
Ctrl
20 μg/ml
40 μg/ml
60 μg/ml
100 μg/ml
SKOV-3
OVCAR-3

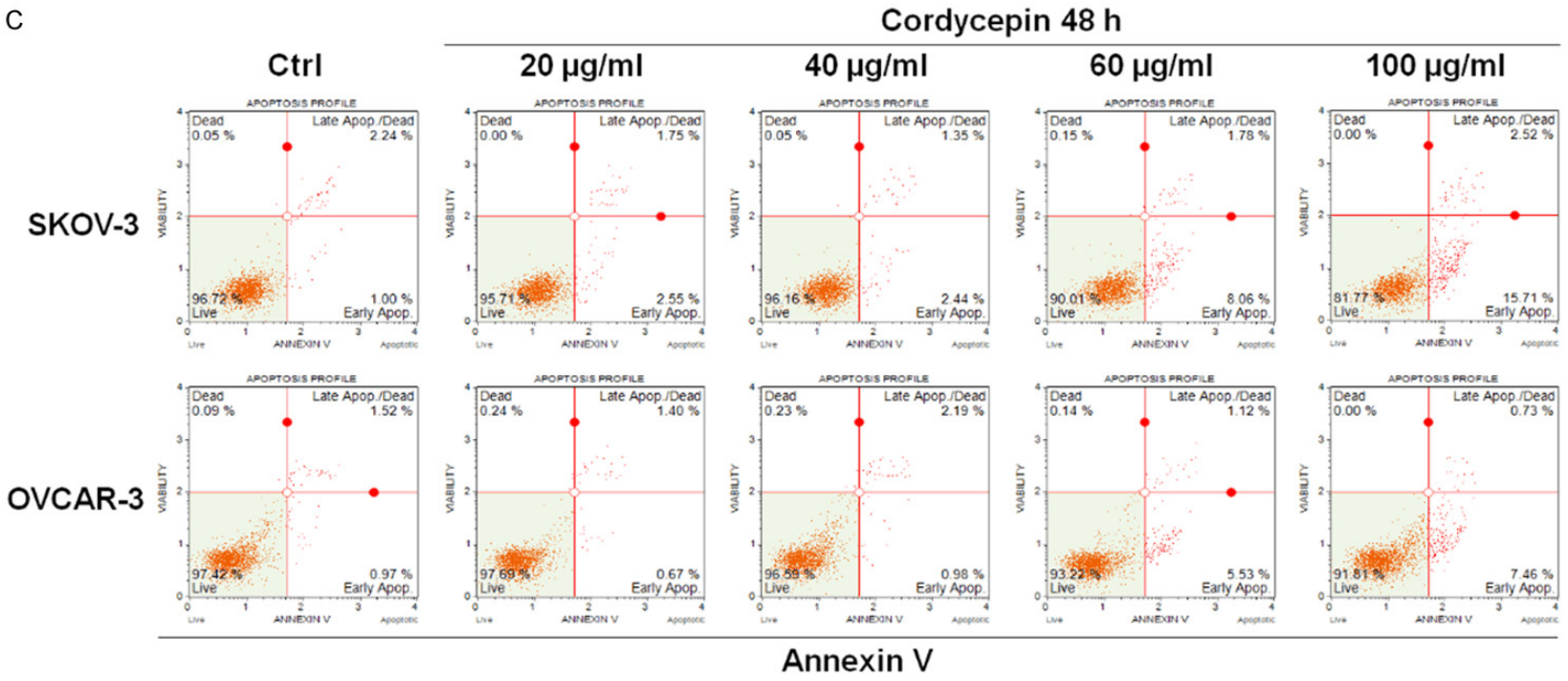
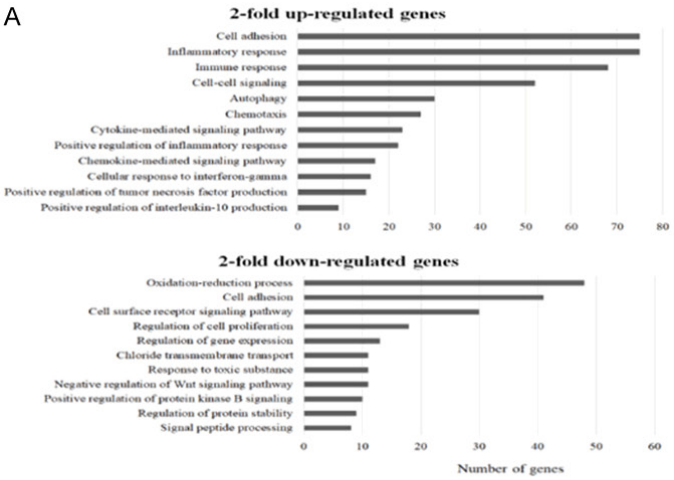


Figure 1. Cordycepin dose-dependently inhibits cell viability and induces apoptosis of ovarian cancer cells. A. Cell viability of SKOV-3 and OVCAR-3 cells were measured using the cell counting kit-8 reagent (dojindo) after treatment with indicated concentrations of cordycepin (0, 20, 40, 60, 100 ug/ml) for 24 and 48 h. B. The cells were exposed to various concentrations of cordycepin (0, 20, 40, 60, 100 ug/ml) for 48 h. C. Flow cytometry analysis was performed in SKOV-3 and OVCAR-3 cell lines after treatment with indicated concentrations of cordycepin (0, 20, 40, 60, 100 ug/ml) for 48 h. Data were normalized to control and represent the mean ± SEM for three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

A



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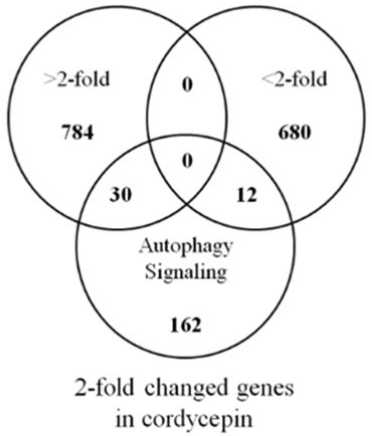

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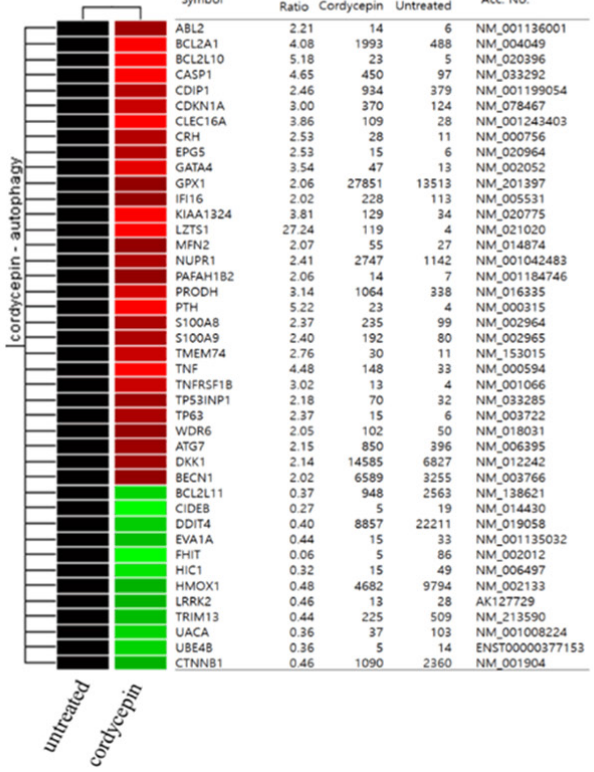


Gene Symbol	Normalized Ratio	Signal Intensity Cordycepin	Signal Intensity Untreated	RefSeq. Acc. No.
ABL2	2.21	14	6	NM_001136001
BCL2A1	4.08	1993	488	NM_004049
BCL2L10	5.18	23	5	NM_020396
CASP1	4.65	450	97	NM_033292
CDIP1	2.46	934	379	NM_001199054
CDKN1A	3.00	370	124	NM_078467
CLEC16A	3.86	109	28	NM_001243403
CRH	2.53	28	11	NM_000756
EPG5	2.53	15	6	NM_020964
GATA4	3.54	47	13	NM_002052
GPX1	2.06	27851	13513	NM_201397
IFI16	2.02	228	113	NM_005531
KIAA1324	3.81	129	34	NM_020775
LZTS1	27.24	119	4	NM_021020
MFN2	2.07	55	27	NM_014874
NUPR1	2.41	2747	1142	NM_001042483
PAFAH1B2	2.06	14	7	NM_001184746
PRODH	3.14	1064	338	NM_016335
PTH	5.22	23	4	NM_000315
S100A8	2.37	235	99	NM_002964
S100A9	2.40	192	80	NM_002965
TMEM74	2.76	30	11	NM_153015
TNF	4.48	148	33	NM_000594
TNFRSF1B	3.02	13	4	NM_001066
TP53INP1	2.18	70	32	NM_033285
TP63	2.37	15	6	NM_003722
WDR6	2.05	102	50	NM_018031
ATG7	2.15	850	396	NM_006395
DKK1	2.14	14585	6827	NM_012242
BECN1	2.02	6589	3255	NM_003766
BCL2L11	0.37	948	2563	NM_138621
CIDEB	0.27	5	19	NM_014430
DDIT4	0.40	8857	22211	NM_019058
EVA1A	0.44	15	33	NM_001135032
FHIT	0.06	5	86	NM_002012
HIC1	0.32	15	49	NM_006497
HMOX1	0.48	4682	9794	NM_002133
LRRK2	0.46	13	28	AK127729
TRIM13	0.44	225	509	NM_213590
UACA	0.36	37	103	NM_001008224
UBE4B	0.36	5	14	ENST00000377153
CTNNB1	0.46	1090	2360	NM_001904

D

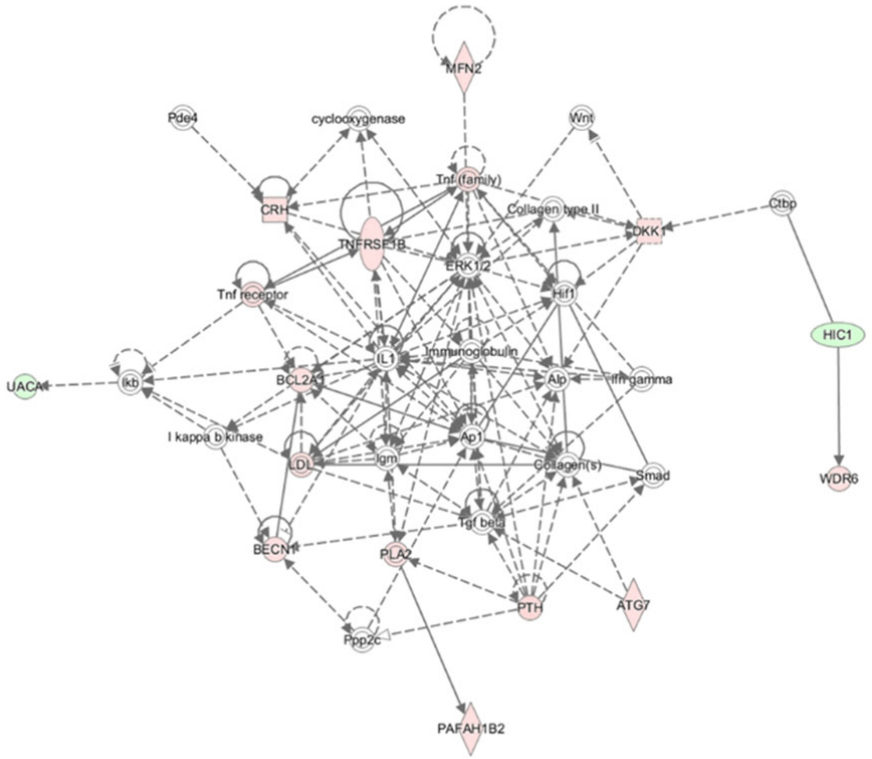
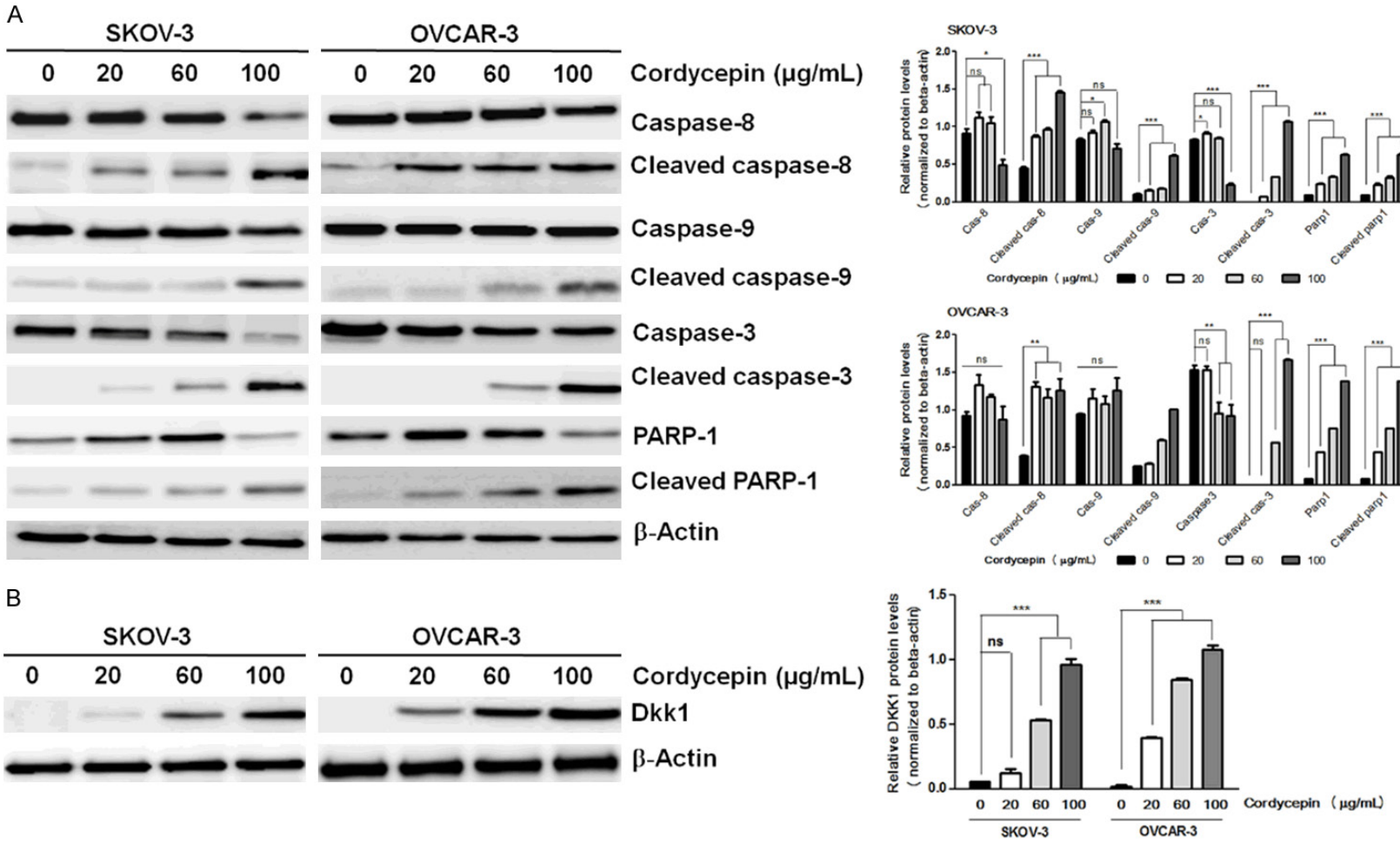


Figure 2. Gene expression analysis and signal network of apoptotic genes changed by cordycepin treatment. A. Results of microarray analysis of gene expression in response to 60 μg/mL of cordycepin for 48 h. Red and green represent more than 2-fold upregulated and downregulated genes, respectively. B. Venn diagram showing gene expression profiles based on microarray results in SKOV3 cells treated with cordycepin. The overlapped gene were differentially changed (up- and down-regulated) more than 2-fold, associated with autophagy. C. Lists of autophagy-related genes that were altered more than 2-fold in response to cordycepin. D. Signal network of the autophagotic genes generated by using a Qiagen IPA. Microarray experiments were performed in triplicate.



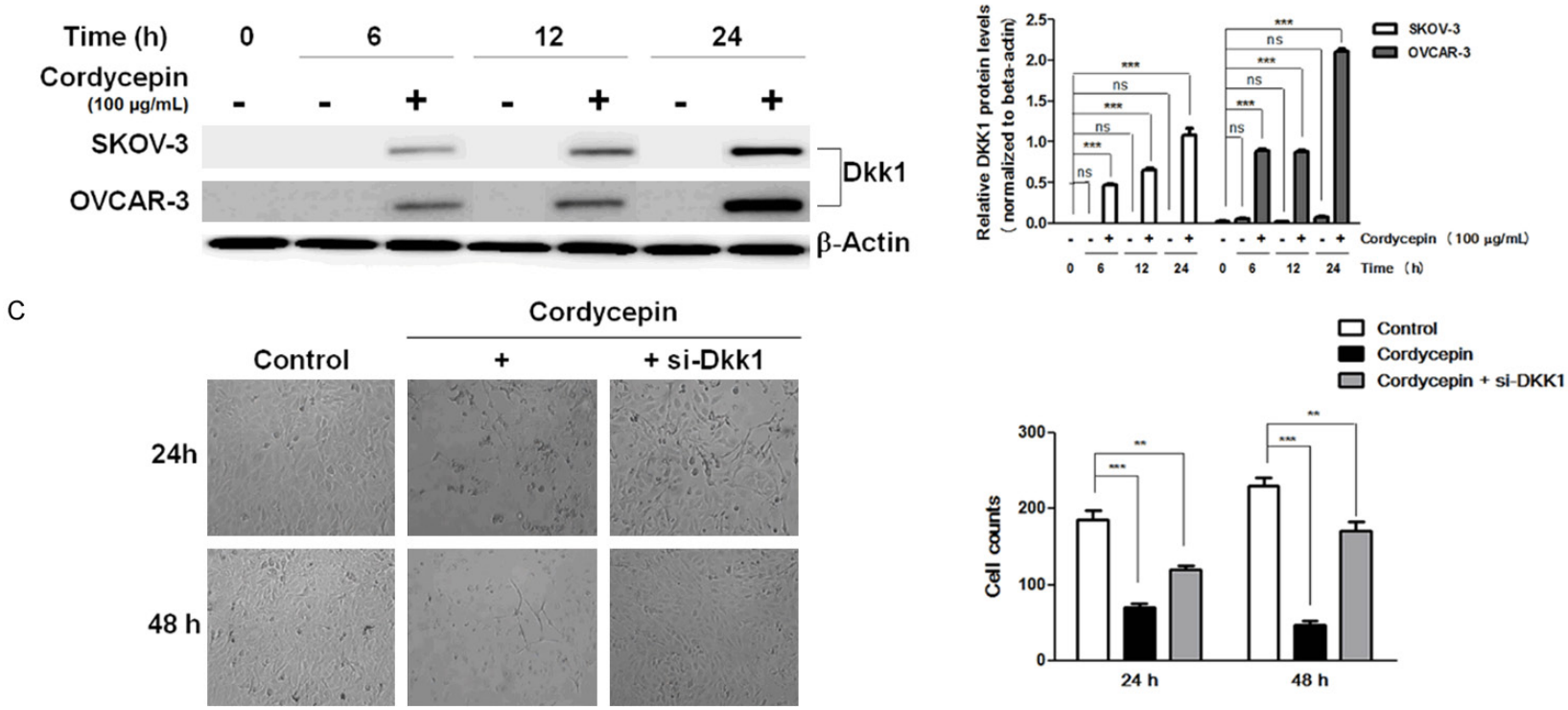


Figure 3. Cordycepin induces apoptosis through upregulation of Dkk1. A. The indicated cell lines were exposed to 0, 20, 60 and 100 μg/mL cordycepin for 12 h, and whole-cell protein lysates were harvested and analyzed for western blot for cleaved caspase-8, -9, and -3 and cleaved PARP-1. B. SKOV-3 and OVCAR-3 cells were exposed to 0, 20, 60 and 100 μg/mL cordycepin and western blot were performed for Dkk1 in a dose-dependent manner. And the indicated cell lines were exposed to 100 μg/ml cordycepin for 0, 6, and 24 h and western blot were performed for Dkk1. C. siRNA-mediated knockdown of Dkk1 efficiently protected against cordycepin-induced apoptosis. Data were normalized to controls and represent the mean ± SEM for three independent experiments (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Dkk1 is involved in cordycepin-induced apoptosis

To study the mechanism by which cordycepin inhibits cell proliferation and induces cell apoptosis, SKOV-3 and OVCAR-3 ovarian cancer cells treated with different doses of cordycepin (0, 20, 60, and 100 μg/mL) were used for protein expression analysis. Bax pro-apoptotic members were analyzed as target proteins by immunoblotting. The results showed that the protein expression levels of cleaved caspase-3, -8, -9, and -PARP-1 increased significantly after treatment with cordycepin (**Figure 3A**). Together, these results imply that cordycepin induced cell apoptosis through caspase dependent pathways. Dkk1 has been shown to be a key positive regulator of intrinsic apoptosis by inhibiting the activation of caspases in human ovarian cancer cells. Therefore, we evaluated whether cordycepin influences the levels of Dkk1 in ovarian cancer cells. SKOV-3 and OVCAR-3 cells were treated with different doses of cordycepin for 6 h interval. We found that cordycepin dose- and time-dependently increased the levels of Dkk1 (**Figure 3B**). Moreover, we silenced Dkk1 using a specific siRNA in SKOV-3 cells to validate its function in cordycepin-induced apoptosis. As shown in **Figure 3C**, compared with SKOV-3-lacZ cells, SKOV-3-siDkk1 cells significantly protected against cordycepin-induced apoptosis. Taken together, Dkk1 expression contributes to cordycepin-induced apoptosis in human ovarian cancer cells.

Cordycepin increases autophagy-related proteins in ovarian cancer cells by downregulation of β-catenin levels

We evaluated the molecular mechanisms underlying the inhibitory effects of cordycepin on ovarian cancer cells and studied the role of growth-regulatory factors in ovarian cancer. To investigate whether cordycepin could modulate autophagy in SKOV-3 cells, the expression of autophagy-related proteins was determined after treatment with cordycepin. Following treatment with different doses of cordycepin (0, 20, 60, and 100 μg/mL) for 24 h, SKOV-3 cells exhibited a significant increase of the ratio of LC3-II to LC3-I protein and a significant decrease of β-catenin in a dose-dependent manner compared to the non-treated control cells (**Figure 4A**). In addition, autophagy-related proteins, Atg7 and beclin was increased (**Figure 4A**). To elucidate the potential role of β-catenin in the regulation of autopahgy signaling, formation of the ratio of LC3-II to LC3-I in SKOV-3 cells was confirmed using immunofluorescence analysis. As a result, cordycepin also triggered a punctate immunostaining pattern for LC3, which is a marker protein of autophagosomes (**Figure 4B**). Using transmission electron microscopy (TEM), we visualized the autophagic morphology of cordycepin-treated SKOV-3 cells (**Figure 4C**). The untreated control cells showed normal organelles without autophagic bodies. In contrast, cordycepin treatment induced the formation of phagophores as well as lysosomes in the cells.

Upregulation of Dkk1 by cordycepin induces autophagy through abolishing the translocation of β-catenin to nucleus

We evaluated the inhibitory effects of cordycepin on the role of transcription factor for cell survival of β-catenin. In the previous results, cordycepin dose- and time-dependently increased Dkk1 (**Figure 3B**) and downregulated nuclear β-catenin protein levels (**Figure 4A**). Therefore, we confirmed the effects of Dkk1 on β-catenin by silencing Dkk1 with siRNA and overexpressing β-catenin. First, the inhibitory effect of cordycepin on nuclear translocation of β-catenin was detected through immunoblotting. As a result, in SKOV-3 cells treated with different doses of cordycepin (0, 20, 60, and 100 μg/mL), cordycepin inhibited β-catenin translocation from the cytoplasm into the nucleus (**Figure 5A**). Second, to elucidate the potential role of Dkk1 in the regulation of β-catenin signaling, Dkk1 siRNA was transfected into SKOV-3 cells. Dkk1 siRNA was transfected into SKOV-3 cells. As shown in **Figure 5B**, siRNA inhibition of Dkk1 upregulated nuclear β-catenin protein levels after cordycepin treatment. β-catenin nuclear translocation inhibition under cordycepin was measured by confocal microscopy. These results show that cordycepin effectively attenuates nuclear β-catenin translocation by modulating the Dkk1 signaling pathway. The overexpression of β-catenin, verified by immunoblotting, diminished the effects of cordycepin on autophagy-associated proteins, Atg7 and beclin, and the LC3-II to LC3-I ratio in SKOV-3 cells (**Figure 5C**).

Downregulation of Dkk1 decreased the effects of cordycepin on autophagy

Thus, we verified that β-catenin overexpression abolishes the effects of cordycepin on autoph-

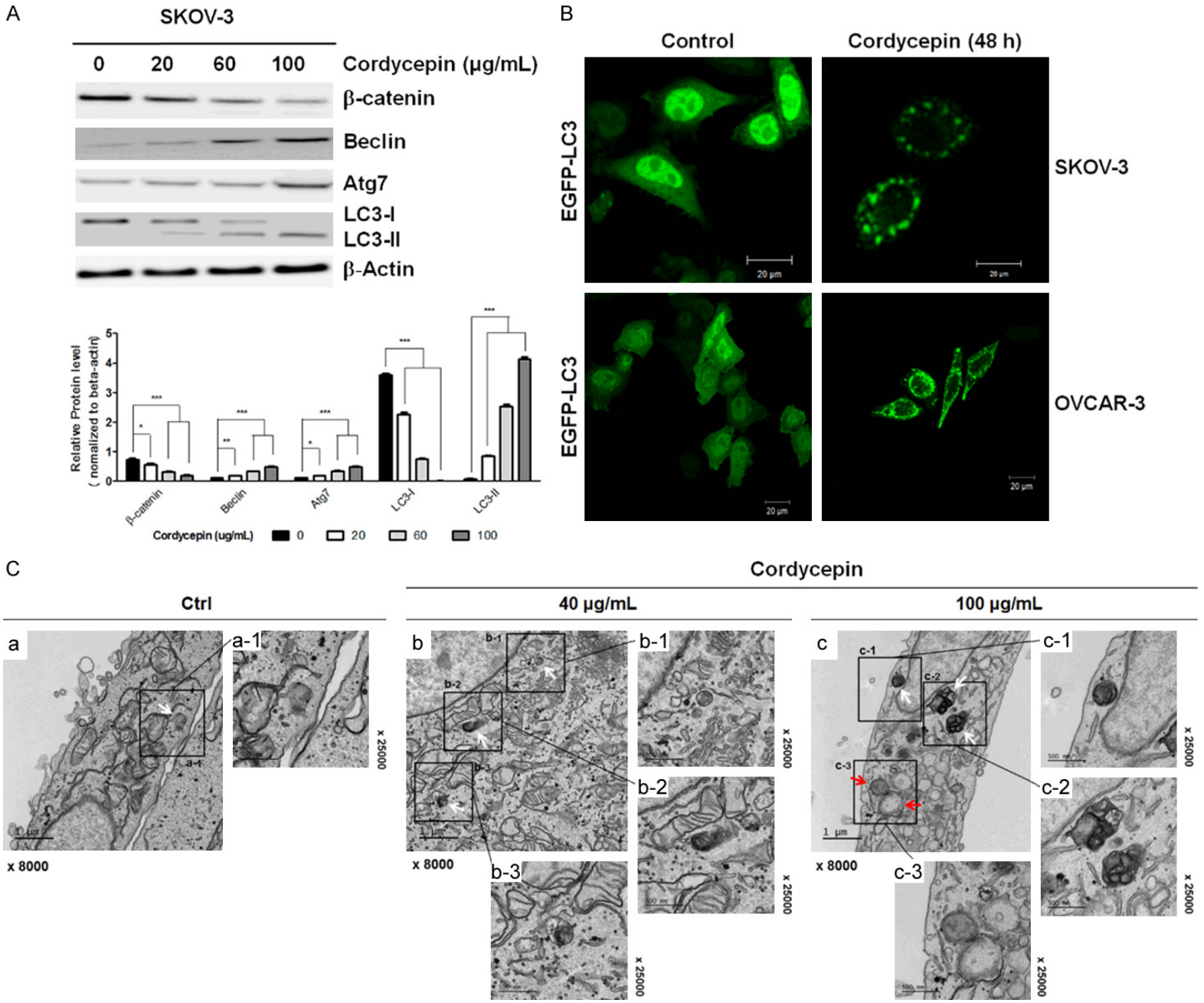

A
SKOV-3
0
20
60
100
Cordycepin (μg/mL)
β-catenin
Beclin
Atg7
LC3-I
LC3-II
β-Actin
Relative Protein level
(nomalized to beta-actin)
β-catenin
Beclin
Atg7
LC3-I
LC3-II
Cordycepin (ug/mL) 0 20 60 100
B
Control
Cordycepin (48 h)
EGFP-LC3
SKOV-3
EGFP-LC3
OVCAR-3
20 μm
C
Cordycepin
Ctrl
40 μg/mL
100 μg/mL
a
a-1
b
b-1
b-2
b-3
c
c-1
c-2
c-3
x 8000
x 25000
1 μm

Figure 4. Cordycepin-mediated Dkk1 upregulation increases autophagy by down-regulating β-catenin in ovarian cancer cells. A. SKOV-3 cells were exposed to 0, 20, and 100 μg/ml cordycepin and western blot were performed for β-catenin, beclin, Atg7, and LC3. B. Confirmation of autophagosome formation induced by cordycepin by LC3 antibody staining. SKOV-3 cells and OVCAR-3 were cultured for 1 h, stained, and analyzed. C. Morphological ultrastructural appearance of autophagy by transmission electron microscopy (TEM) in cordycepin-treated ovarian cancer cells. Untreated SKOV-3, and SKOV-3 cells were incubated with cordycepin at 40 μg/mL and 100 μg/mL for 48 h. The induction of autophagic cell death by cordycepin was analyzed by TEM. Morphologic findings characteristic of autophagy are enlarged to highlight the autophagic bodies indicated by the arrows. White arrows indicate phagophores/autophagosomes and red arrows indicate lysosomes/autolysosomes. Representative images are shown. Data represent the mean ± SEM for three independent experiments each $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

agy. Furthermore, we evaluated the effects of cordycepin on Dkk1 in SKOV-3 cells after silencing Dkk1 with siRNA, which was verified by immunoblotting (**Figure 5D**). Consequently, the effects of cordycepin on autophagy-associated proteins, Atg7 and beclin, and the LC3-II to LC3-I ratio were reduced in SKOV-3 cells. Thus, we verified that Dkk1 silencing abolishes the effects of cordycepin on autophagy. Furthermore, cordycepin (100 μg/mL) induced Dkk1 overexpression, which was confirmed at the transcriptional level (**Figure 5E**), while si-Dkk1 increased the migration of SKOV-3 cells in the wound healing assay (**Figure 5F**).

Cordycepin causes autophagy-triggered apoptosis in ovarian cancer cells

Since cordycepin induces apoptosis as well as autophagy in human ovarian cancer cells, we further explored the relationship between cordycepin-induced apoptosis and autophagy. An siRNA was used to inhibit Atg7 expression in ovarian cancer cells, and then apoptosis-related proteins were quantified by western blot analysis. These findings demonstrated that Atg7 silencing significantly abrogated cordycepin-induced cleavage of caspase-3 and PARP1 (**Figure 6A**). Moreover, autophagy inhibitors, such as LY294002 and chloroquine (CQ), which block the upstream and downstream steps of autophagy, respectively, were used to examine the impact of autophagy on apoptosis. We inhibited autophagy in SKOV-3 cells using LY294002 and CQ and analyzed the effects on the level of LC3-II and cordycepin-induced cell death. LY294002 is an inhibitor of PI3K, which inhibits autophagosome accumulation and inhibits the conversion of LC3-I to LC3-II. However, CQ, a lysosome inhibitor, could induce the aggregation of autophagosomes and increase LC3-II levels by blocking the fusion of autophagosomes and lysosomes. Western blot analysis indicated that cordycepin-induced autophagy was successfully inhibited by LY294002 and CQ (**Figure 6B**). To further understand the biological role of autophagy in cordycepin-induced cell death, we examined the changes of cordycepin-induced apoptosis. The results demonstrated that cordycepin in combination with LY294002 and CQ treatment reduced the cleavage of caspase 3 (**Figure 6B**), compared with cordycepin treatment alone, whereas cells treated with LY294002 and CQ showed anti-apoptosis-inducing effects on SKOV-3 cells. These results suggest that the inhibition of autophagy attenuates cordycepin-induced apoptosis, indicating that autophagy plays a crucial role in cordycepin-induced cell death in SKOV-3 cells.

Discussion

Although cordycepin-induced cell death has been reported, the molecular mechanisms underlying the relationship between cordycepin-induced apoptosis and autophagy have not been elucidated in ovarian cancer. Here, we focused on understanding the fundamental mechanisms of cordycepin-induced apoptosis and examined the relationship between apoptosis and autophagy. Cordycepin induces apoptosis in human ovarian cancer cells through extrinsic and intrinsic signaling pathways primarily regulated by Bcl-2 pro-apoptotic proteins [27]. Additionally, cordycepin induces the mitochondrial translocation of Bax in a dose-dependent manner, causing the release of cytochrome c and the activation of caspases [28]. In the present study, we found that cordycepin reduces the viability of human ovarian cancer cells in a dose- and time-dependent manner, verifying its apoptotic potential. In addition, flow cytometry analysis revealed that approximately 40% of SKOV-3 and OVCAR-3 cells exhibit early- and late-phase apoptosis after exposure to cordycepin (100 μg/mL) for 48 h (**Figure 1C**). Moreover, the cleaved forms of

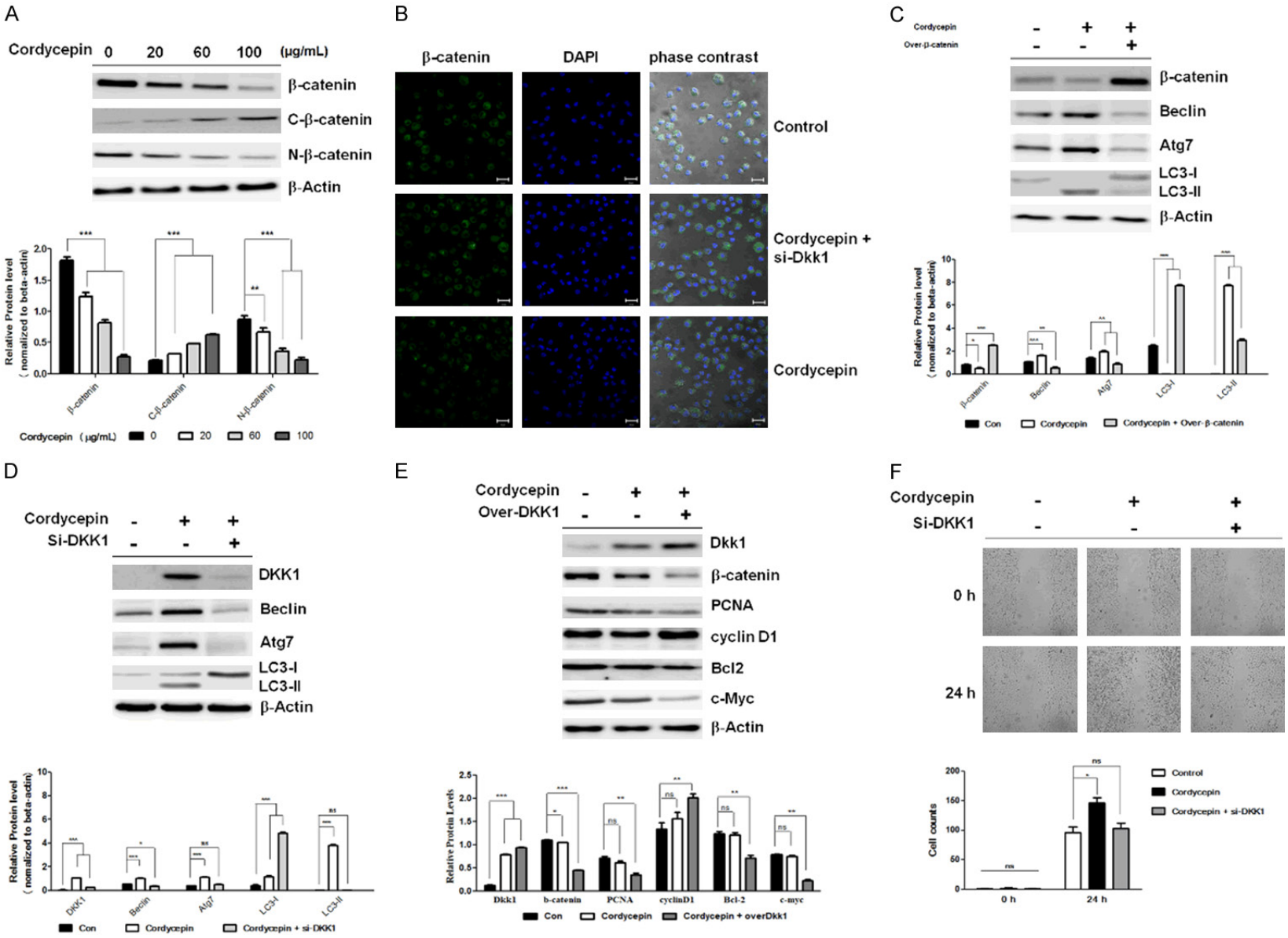


Figure 5. Overexpression of β-catenin and downregulation of Dkk1 abolishes the effects of cordycepin on autophagy. A. The indicated cell lines were exposed to 0, 20, and 100 μg/mL cordycepin and western blot analysis was performed for measuring translocation of β-catenin from cytosol to nucleus. B. β-catenin localization

was examined by immunofluorescence analysis after 24 h of Dkk1 siRNA transfection or cordycepin treatment. C. SKOV-3 cells were overexpressed with β-catenin or treated with cordycepin. And then, western blotting was performed for β-catenin, beclin, Atg7, and LC3. D. Western blotting for Dkk1, beclin, Atg7, and LC3 in Dkk1-silenced or cordycepin treated SKOV-3 cells. E. To assess the effect of Dkk1 downregulation on β-catenin expression, western blotting was performed for Dkk1, β-catenin, PCNA, cyclin D1 and c-Myc. F. The effect of si-Dkk1 on the migration of SKOV-3 cells was analyzed using the wound healing assay. Data represent the mean ± SEM for three independent experiments each $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

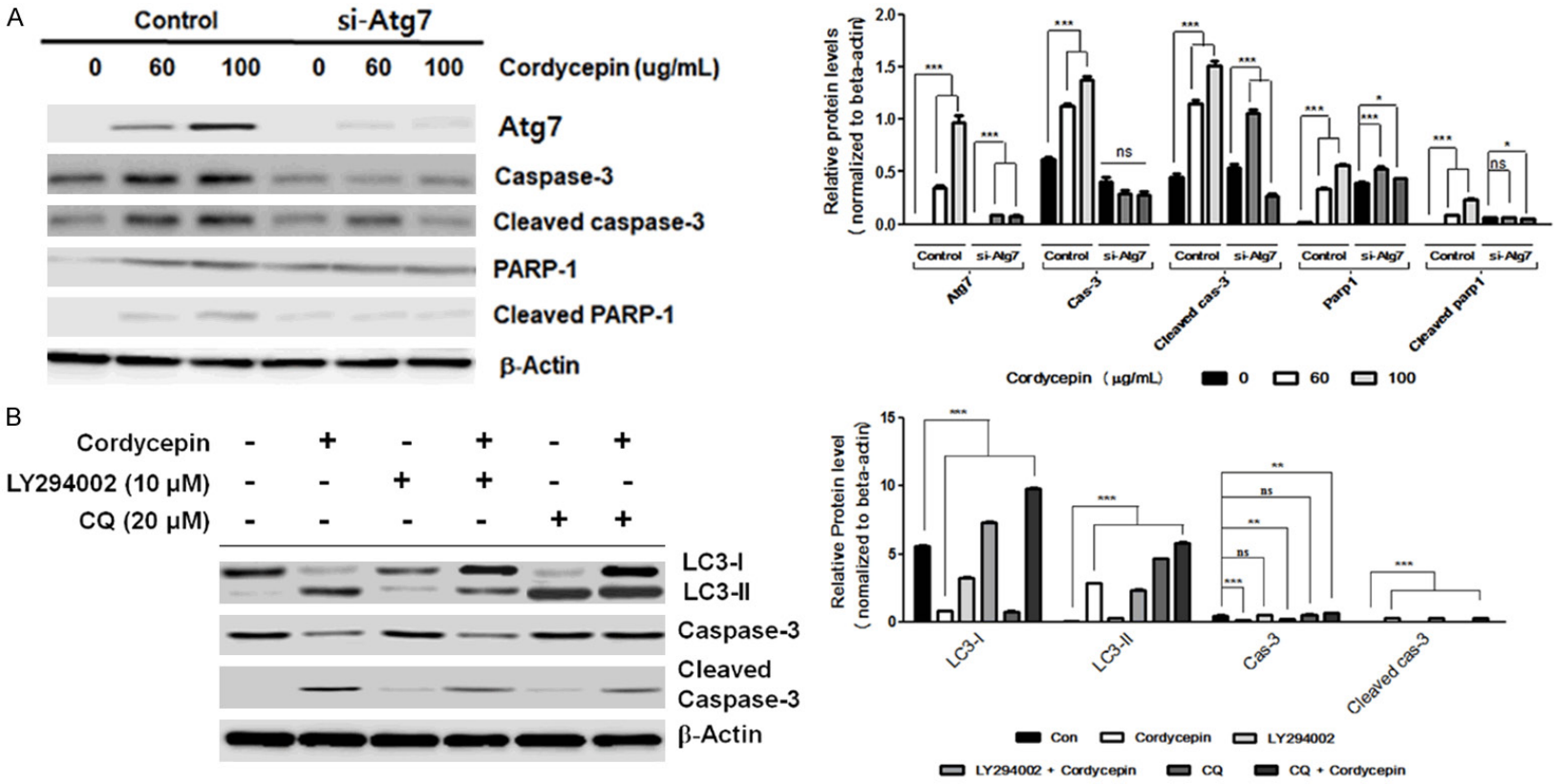


Figure 6. Cordycepin induces pro-apoptotic autophagy in SKOV-3 cells. A. SKOV-3 cells conducted with Atg7 siRNA transfection and exposed to 0, 60, and 100 μg/mL cordycepin for 12 h was analyzed by western blot for Atg7, caspase-3, and PARP. B. Cordycepin promotes apoptosis through activating autophagy in SKOV-3 cells. SKOV-3 cells were pretreated with 10 μM LY294002 or 20 μM CQ for 1 h, and then exposed to 100 μg/mL cordycepin for another 12 h. Data represent the mean ± SEM for three independent experiments each $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

caspase-9, caspase-3, and PARP1 increased in a dose- and time-dependent manner (**Figure 3A**), suggesting that cordycepin induces extrinsic as well as intrinsic apoptosis in human ovarian cancer cells. Dkk1 has been previously shown to positively regulate apoptosis by inhibiting the activation of caspases in human cancer cells [29]. Thus, we assessed the mechanism of cordycepin-induced apoptosis, and found that cordycepin increases Dkk1 expression (**Figure 3B**). However, Dkk1 silencing protected SKOV-3 cells from cordycepin-induced apoptosis (**Figure 3C**). These results suggest that the expression of Dkk1 contributes to cordycepin-induced apoptosis in human ovarian cancer cells. Dkk1, a negative regulator of Wnt signaling, is a target of the β-catenin/TCF pathway [30]. Nuclear β-catenin activates c-Myc, cyclin D1, and c-jun to promote cell proliferation, and activates Bcl-2 to inhibit apoptosis [31]. In this study, we found that cordycepin dose- and time-dependently increases the levels of Dkk1 (**Figure 3B**), autophagy-related proteins, Atg7, and beclin (**Figure 4A**). It also increased the LC3-II to LC3-I ratio in SKOV-3 cells in a dose-dependent manner (**Figure 4A**) and decreased β-catenin levels as compared to the non-treated control cells in a dose-dependent manner. We found that cordycepin also triggered a punctate immunostaining pattern for LC3, which is a marker protein of autophagosomes (**Figure 4B**). Moreover, siRNA inhibition of Dkk1 upregulated nuclear β-catenin protein levels after cordycepin treatment. The inhibition of the nuclear translocation β-catenin by cordycepin was detected by immunoblotting and confocal microscopy (**Figure 5A**, **5B**). These results show that cordycepin effectively attenuates nuclear β-catenin translocation by upregulating the Dkk1 signaling pathway.

Autophagy is induced in β-catenin-deficient cancer cells via Bax activation [24]. In addition, through the immunofluorescence analysis, β-catenin overexpression diminished the dose-dependent effects of cordycepin on the autophagy-associated proteins, Atg7 and beclin, and the LC3-II to LC3-I ratio (**Figure 5C**). However, β-catenin expression was significantly attenuated by Dkk1, and the autophagic effects of cordycepin were abolished by Dkk1 knockdown (**Figure 5D**, **5E**). These findings suggest that cordycepin induces autophagy through the downregulation of β-catenin and the upregulation of Dkk1 expression. The relationship between autophagy and apoptosis or other forms of cell death is complicated and poorly understood [32]. Under stress, cells exhibit autophagy as an adaptive mechanism to avoid stress-induced apoptosis [33]. Cordycepin induces autophagy-mediated c-FLIPL degradation, which leads to apoptosis in human non-small cell lung cancer cells [34]. However, the molecular mechanisms of autophagy-triggered apoptosis are not well understood. β-catenin silencing induces autophagy and apoptosis in MM cells [24]. We found that inhibiting autophagy by silencing Atg7 expression significantly abrogated cordycepin-induced cleavage of caspase-3 and PARP1 (**Figure 6A**), indicating that cordycepin induces autophagy-triggered apoptosis in ovarian cancer cells. To clarify this, we inhibited cordycepin-induced autophagy pharmacologically by using LY294002 and CQ in SKOV-3 cells (**Figure 6B**). We found that cordycepin-induced cell death significantly decreased by additional treatment with LY294002 and CQ, indicating that cordycepin-induced autophagy might play a cytotoxic role in SKOV-3 cells. To further confirm the cytoprotective role of autophagy induced by cordycepin in SKOV-3 cells, we detected apoptosis in SKOV-3 cells when cells were treated with cordycepin and autophagy inhibitors. Remarkably, treatment with LY294002 and CQ attenuated cordycepin-induced apoptosis, as evidenced by decreased caspase-3 cleavage. These results demonstrate that cordycepin-induced autophagy plays a cytotoxic role in SKOV-3 cells. Blocking autophagy could attenuate the efficacy of cordycepin in SKOV-3 cells and could be a promising new therapeutic strategy for ovarian cancer.

In summary, our results demonstrate that cordycepin triggers Dkk1-mediated autophagy by suppressing the β-catenin signaling pathway. In addition, cordycepin promotes the cleavage of caspase-3 by inducing autophagy in ovarian cancer cells. Our findings describe the molecular mechanisms of apoptosis induced by cordycepin, which may provide a theoretical basis for the application of cordycepin derivatives in cancer treatment.

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Disclosure of conflict of interest

None.

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