Cordycepin induces apoptotic cell death and inhibits cell migration in renal cell carcinoma via regulation of microRNA-21 and PTEN phosphatase

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ABSTRACT

Cordycepin is an active component extracted from Traditional Chinese medical herb Cordyceps militaris. Many reports demonstrated that cordycepin harbors antitumor activity in a broad spectrum of cancer types. In this study the actions and the underneath molecular mechanisms of cordycepin were investigated in renal cell carcinoma Caki-1 cell line. Results showed that cordycepin induced apoptotic cell death and inhibited cell migration in Caki-1 cells. Quantitative real-time PCR results and western blot analyses indicated cordycepin dose-dependently decreased microRNA-21 expression and Akt phosphorylation levels in Caki-1 cells, but increased PTEN phosphatase levels. Block of cordycepin-induced microRNA-21 decrease or PTEN increase in Caki-1 cells by transfection of microRNA-21 mimic or PTEN siRNA significantly attenuated cordycepin-induced cell death and inhibition of cell migration. Taken together, findings in present study suggested that cordycepin induced apoptotic cell death in renal cell carcinoma through regulation of microRNA-21 and PTEN phosphatase. Furthermore, present study also firstly illustrated that cordycepin inhibited cell migration of renal cell carcinoma, which also involved microRNA-21 and PTEN phosphatase.

Renal cell carcinoma (RCC) is the most lethal urological cancer, accounting for 87% of all renal malignancies and 2–3% of all cancers (4). In the past two decades, the global incidence of RCC has increased by 2% per year (4). Worldwide, the age-standardized incidence and mortality of RCC were 6 per 100,000 and 2.1 per 100,000 respectively (43). Despite advance in diagnostic techniques, up to 20–30% of patients with RCC present with metastasis (4). The prognosis of RCC has historically been poor, with 5-year survival rate of 8% among patients with metastatic RCC (9). Although recent progress about understanding of the pathogenesis of RCC has led to novel immune-based and targeted treatments for this chemoresistant cancer, no one drug obtains overall response rate of 100% and durable complete responses (4, 9). Development of neo-adjuvant therapy is still in a demand at present.

Cordycepin is a main active composition extracted from Cordyceps militaris and has been reported to harbor anti-tumor activity in a broad spectrum of cancer types, including lung cancer (50), ovarian cancer (46), leukemia (30), breast cancer (47), and so on (33). Two recent reports firstly demonstrated that cordycepin could induce apoptosis in renal cell carcinoma through Erk-JNK signaling pathway (20, 48) and indicated the potential application of cordycepin in treating renal cell carcinoma. However, the actions and the underneath molecular mechanisms of cordycepin in renal cell carcinoma are not definitely clear and warrant extensive and further investigations. Recently, a comprehensive molecular analysis

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of clear cell carcinoma indicated that decreased methylation of microRNA-21 gene promoter correlated with increased expression of microRNA-21 in cancer cells and was associated with worse survival (42). In addition, phosphatase and tensin homolog (PTEN) was a target of microRNA-21 and could be down-regulated by microRNA-21 (22, 42). PTEN is a tumor suppressor in renal cell carcinoma and inhibits tumor cell proliferation and migration in renal cell carcinoma (2, 5, 39). Nonetheless, PTEN gene is often mutated or down-regulated in renal cell carcinoma (1, 11, 17, 26, 39). So, besides the effects of cordycepin on renal cell carcinoma, the role of microRNA-21 and PTEN phosphatase in cordycepin actions was also investigated in present study.

MATERIALS AND METHODS

Cells and materials. Caki-1 renal cell carcinoma cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES buffer, and 100 μg/mL gentamicin in an incubator with 5% CO₂ at 37°C. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary antibody against poly (ADP-ribose) polymerase (PARP) (cat: 9542), PTEN (cat: 9552) and phospho-Akt (cat: 4060) were obtained from Cell Signaling Technology (Denver, Colorado, USA). β-actin primary antibody was bought from Millipore (cat: MAB1501, Darmstadt, Germany). MicroRNA-21 mimic and primers for real-time quantitative PCR assays was purchased from Guangzhou RiboBio Company (Guangzhou, Guangdong, China). Small interfering RNA specific to PTEN were as follows: sense, 5'-CUG CUA GCC UCU GGA UUU GTT-3'. Antisense, 5'-CUG CUA GCC UCU GGA UUU GTT-3'. Sequences of small interfering RNA (siRNA) specific to PTEN were as follows: sense, 5'-CAA AUC CAG AGG CUA GCA GTT-3'; anti-sense, 5'-CAT CUG CUA GCC UCU GGA UUU GTT-3'. Cell transfection was performed according to the manufacturer’s recommendations. Twenty-four hours

CCK-8 assay. CCK-8 assay was carried out to determine the effect of cordycepin on cell viability. Caki-1 cells were seeded into 96-wells plate (1 × 10⁴ cells/100 μL/well) the day before experiment. And on the next day cells were treated by cordycepin at the concentration of 0, 10, 20, 30, 40, and 50 μg/mL for 48 h. Then, 10 μL CCK-8 reagent was added into each well and incubated for 3 h at 37°C. Finally, the absorbance at 450 nm (OD₀₅₀) was measured using a plate reader (PerkinElmer, Waltham, USA). The cell survival (%) was calculated as (ODcontrol − ODdrug) / ODcontrol × 100%.

Transwell migration assays. Assays were performed using 24-well plate Transwell chambers (8-μm polycarbonate membrane; Costar, Corning Inc.). Caki-1 cells were harvested and adjusted to 5 × 10⁵ cells/mL in cell culture medium containing 0.02% FBS. 100 μL of cells were added to the upper chamber and incubated at 37°C in 5% CO₂. After 24 or 48 h of incubation, non-migrated cells on the upper side of the chamber were removed using a cotton swab. The migrated cells on the lower side of the chamber was fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. For each experiment, the numbers of cells were counted at ×200 magnification in five separate fields by light microscopy.

Caspase-3 activity assay. To evaluate caspase-3 activity, cells were lysed in lysis buffer (1% NP-40, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10% glycerol) containing protease inhibitors after their treatment with cordycepin. Then, 20 μL lyses were added to 100 μL reaction buffer (1% NP-40, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing a caspase substrate [Asp-Glu-Val-Asp-chromophore-p-nitroanilide (DVAD-pNA)] and incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured using a spectrophotometer (PerkinElmer).

MicroRNA-21 real-time quantitative PCR assay. Total RNA was extracted using Trizol reagent (Life Technologies). First-strand cDNA synthesis kit (RiboBio, Guangzhou, China) and microRNA-21 real-time quantitative PCR assay kit (RiboBio) were used to determine microRNA-21 transcripts in triplicate for each sample. U6 small nucleolar RNA was set as an internal control. Relative expression level of microRNA-21 was calculated using the comparative CT method (2                       −ΔΔCt).

Cell transfection. Sequences of small interfering RNA (siRNA) specific to PTEN were as follows: sense, 5'-CAA AUC CAG AGG CUA GCA GTT-3'; antisense, 5'-CUG CUA GCC UCU GGA UUU GTT-3'. Cell transfection was performed according to the manufacturer’s recommendations. Twenty-four hours
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P < 0.05 was considered as statistically significant. All the statistical analyses in this study were performed using SPSS software (SPSS version 17.0; Chicago, IL, USA).

RESULTS

Effects of cordycepin on renal cell carcinoma Caki-1 cells

In order to investigate the effect of cordycepin on renal cell carcinoma, Caki-1 cells were treated with gradually-increased dose of cordycepin as indicated in Fig. 1 for 48 h. Results showed that the viability of Caki-1 cells was gradually decreased by cordycepin treatment with the increase of cordycepin concentration (Fig. 1A). Biochemical assays demonstrated that cordycepin increased caspase-3 activities in Caki-1 cells in a concentration-dependent manner (Fig. 1B). These results indicated that cordycepin induced cell apoptosis in Caki-1 cells, which was further confirmed by cell culture experiments with caspase inhibitor and western blots analyses. Pretreatment of Caki-1 cells with 50 μM z-VAD-fmk, a pan-caspase inhibitor, significantly alleviated the cell death induced by cordycepin (Fig. 1C). Western blots analyses also showed that cordycepin induced cleavages of PARP and caspase-3, characteristics of apoptosis, in Caki-1 cells; however, the cleavages of PARP and
Regulation of microRNA-21 and PTEN by cordycepin in Caki-1 cells

Fig. 3 showed the effects of cordycepin on the levels of microRNA-21 and PTEN in Caki-1 cells. Western blots analyses showed that cordycepin gradually increased PTEN phosphatase expression in a concentration-dependent manner in Caki-1 cells. And phosphorylation of the substrate of PTEN phosphatase, Akt, was correspondingly decreased by cordycepin in a concentration-dependent manner (Fig. 3A–C). Quantitative real-time PCR results demonstrated caspase-3 induced by cordycepin was completely inhibited by z-VAD-fmk pretreatment (Fig. 1D).

In addition to the effects of cordycepin on cell death, the effects on cell migration were also investigated by Transwell migration assays. As indicated in Fig. 2A and 2B, 10 μg/mL cordycepin treatment significantly suppressed cell migration of Caki-1 cells at both 24 h and 48 h incubation.
that cordycepin dose-dependently down-regulated microRNA-21 levels in Caki-1 cells (Fig. 3D).

**Effects of microRNA-21 mimic and siRNA specific to PTEN on the actions of cordycepin in Caki-1 cells**

As mentioned above, cordycepin could regulate the expression levels of microRNA-21 and PTEN phosphatase in Caki-1 cells. So, in the following experiments microRNA-21 mimic and siRNA specific to PTEN (siRNA PTEN) were employed to transfect Caki-1 cells and the effects of cordycepin on the cell death and migration were then studied. Results showed that microRNA-21 mimic and siRNA PTEN transfections both greatly reduced cordycepin-increased PTEN phosphatase expression levels in Caki-1 cells (Fig. 4A, B). Apoptotic cell death induced by cordycepin was also significantly attenuated by microRNA-21 mimic and siRNA PTEN transfections (Fig. 5A). Furthermore, cordycepin-induced cell migration inhibition in Caki-1 cells was also significantly reversed by microRNA-21 mimic and siRNA PTEN transfections (Fig. 5B, C).

**DISCUSSION**

Because of chemoresistance (22), many complementary and alternative medicine are investigated in preventing and treating renal cell carcinoma (21, 28, 29, 34). Traditional Chinese medicine is one of them for complementary and alternative therapy. Cordycepin, an active compound extracted from one Traditional Chinese medical herb, *Cordyceps militaris*, has been studied to treat many cancers including lung cancer (50), ovarian cancer (46), leukemia (30), breast cancer (47), brain cancer (19), colon cancer (27), liver cancer (25), and so on (33). Recently, some efforts have been done to investigate the potential therapeutic effects of cordycepin in renal cell carcinoma (20, 48). Researchers found that cordycepin could induce cell apoptosis in renal cell carcinoma via ERK-JNK signaling pathways (20, 48).

Consistent with these reports, results from present study confirmed the apoptotic effects of cordycepin in Caki-1 cells, one of renal cell carcinoma cell lines. Furthermore, findings from this study indicated that cordycepin also suppressed the cell migration of renal cell carcinoma. The underneath molecular mechanisms of cordycepin actions on renal cell carcinoma were also investigated in present study.

MicroRNAs are evolutionarily conservative, endogenous non-coding small RNAs with length of 19–25 nucleotides (3, 40). Through complementarily binding target mRNA microRNAs cause degradation of target mRNA or stop the translation process leading to decreased gene expression (36). Previous studies have demonstrated that microRNAs play an important role in many pathophysiological processes including cancer development and progression (10, 36, 37). MicroRNAs may target tumor suppressors to promote cancer formation or target oncogenes to prevent cancer development (tumor suppressor miRs) (12, 24, 31). MicroRNA-21 is oncogenic and has been shown to be involved in many tumors development including breast, gastric, colon, lung, pancreatic and ovarian cancers (23, 41). Through increasing proliferation and decreasing apoptosis of tumor cells microRNA-21 increases cancer incidence (6). In renal cell carcinoma microRNA-21 is up-regulated and is associated with higher tumor stage and grade. RCC patients with positive microRNA-21 exhibit shorter disease-free and overall survival rates (13). Therefore, microRNA-21 is proposed as a diagnostic and prognostic marker in renal cell carcinoma (15, 44, 45). So, in this study influence of cordy-
PTEN/PI3K/Akt signaling pathway is an important pathway regulated by microRNA-21 (7). In that pathway, PI3K catalyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) followed by recruitment of Akt and phosphoinositide dependent kinase (PDK) to allow PDK to phosphate and activate Akt (8, 18). Akt activation promotes cell proliferation and inhibits cell apoptosis through multiple downstream target proteins (7). On the contrary, in that pathway, PTEN catalyzes PIP3 into PIP2, negatively regulates that pathway and consequently inhibits cell proliferation and promotes cell apoptosis (7). It has been shown that in renal cell carcinoma, PTEN was commonly mutated or down-regulated (1, 11, 17, 26, 39); and PI3K activation and Akt activation were increased in renal cell carcinoma and were associated with poor prognosis (16, 32). Furthermore, in vitro studies suggested that inhibition of PI3K by specific inhibitor could inhibit renal cell carcinoma (14); Overexpression of PTEN in renal cell carcinoma cell lines inhibits cell migration (5). So, these results...
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... demonstrated that dysregulation of PTEN/PI3K/Akt signaling pathway played an important role in the cell growth and migration of renal cell carcinoma.

In light of the importance of microRNA-21 and PTEN/PI3K/Akt signaling pathway in renal cell carcinoma, we postulated that cordycepin-induced apoptotic cell death and migration inhibition in renal cell carcinoma might be through microRNA-21-mediated regulation of PTEN/PI3K/Akt signaling pathway. To verify this postulation, microRNA-21 mimic and siRNA specific to PTEN were used to transfect Caki-1 cells and then effects of cordycepin on cell death and migration were studied. Results indicated that microRNA-21 mimic and PTEN siRNA both significantly suppressed cordycepin-induced apoptotic cell death of Caki-1 cells. Inhibition of cell migration induced by cordycepin in Caki-1 cells was also significantly attenuated by both microRNA-21 mimic and PTEN siRNA transfections.

In summary, findings in this study suggested that cordycepin treatment induced apoptotic cell death and decreased cell migration in renal cell carcinoma through regulating microRNA-21/PTEN/PI3K/Akt pathway.

CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no competing interests.

REFERENCES


