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Original Research Article

Cynaropicrin inhibits lung cancer proliferation by targeting EGFR/AKT signaling pathway

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Abstract

Purpose: *To investigate the anti-proliferative effect of cynaropicrin on lung cancer cell lines, and the underlying molecular mechanism.*
Methods: *The effect of cynaropicrin treatment on the viabilities of H1975 and H460 cells was measured using Cell Counting Kit-8. Apoptosis was analysed by annexin-V/FITC staining, while protein expressions were assayed by western blotting.*
Results: *Treatment of H1975 and H460 cells with cynaropicrin at doses of 0.25 – 2.0 μM led to a marked reduction in their viability ($p < 0.05$). In cynaropicrin-treated H1975 and H460 cells, there was significant increase in apoptosis, when compared to control cells. Caspase-3 and caspase-9 levels were also significantly increased in H1975 and H460 cells on treatment with cynaropicrin at doses of 0.25 and 2.0 μM while treatment with cynaropicrin at doses of 0.25 - 2.0 μM significantly down-regulated the mRNA expression of CCND1 in the two cell lines ($p < 0.05$). Cynaropicrin markedly inhibited mRNA and protein expressions of EGFR, and also downregulated AKT in H1975 and H460 cells ($p < 0.05$). However, cynaropicrin significantly increased the expressions of miR-202 and miR-370.*
Conclusion: *Cynaropicrin exerts anti-proliferative and proapoptotic effects on H1975 and H460 lung cancer cells via deactivation of EGFR/AKT signaling pathway. Moreover, it upregulated the expressions of miR-202 and miR-370 in these cells. Thus, cynaropicrin has potentials for the treatment of lung cancer.*

Keywords: *Cynaropicrin, Anti-proliferation, Pro-apoptosis, Caspase-3, Artichoke*

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INTRODUCTION

Despite advancements in diagnosis and treatment technologies, lung cancer still remains a leading cause of cancer-related mortalities worldwide [1,2]. Poor prognosis of lung cancer is evident in 17 % average 5-year survival of the affected patients [1,2]. Lung cancer is characterized by excessive proliferation potential and rapid metastasis to distant organs [3]. An understanding of the regulatory mechanism involved in lung cancer is important in the development of efficient therapy for the disease.

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MicroRNAs (miRNAs) act as important biomarkers and therapeutic targets for several types of cancers [4]. Various studies have found that the expressions of miR-202 and miR-370 are downregulated in pulmonary carcinoma cells [5-7]. Overexpression of miR-202 leads to arrest of cell cycle, followed by activation of apoptosis through cyclin D1 (CCND1) inhibition in cancer cells [8,9]. Overexpression of miR-202 in pulmonary cancer cells has been associated with inhibition of proliferation through suppression of the expression of signal transducer and activator of transcription (STAT3) [8,9]. The tumor inhibitory potential of miR-370 in pulmonary cancer cells is associated with downregulation of epidermal growth factor receptor (EGFR) [7].

Cynaropicrin belongs to the sesquiterpene lactone class of compounds with a 5-7-5 fused tricyclic skeleton [10]. The important pharmacophore believed to be present in cynaropicrin is γ-butyrolactone ring which is responsible for several biological activities. Cynaropicrin was originally isolated from artichoke (*Cynara scolymus* L.) [10]. Plants of the artichoke family are known for their significant pharmacological properties and numerous health benefits [10]. Studies have shown that the proliferation of human macrophages such as U937 cells, and Jurkat T leukocyte cells is selectively inhibited by cynaropicrin [11]. It has been reported that the cytotoxicity of cynaropicrin in U937 cells was mediated through arrest of the cell cycle at G1/S phase, followed by apoptosis activation [11]. Subsequently, it was shown that cynaropicrin exhibited pro-apoptotic effect via induction of PKCδ cleavage, leading to the view that PKCδ and ROS might be important targets in its anti-tumor potential [11]. The present study investigated the anti-proliferative potential of cynaropicrin in lung cancer cells, as well as the associated molecular mechanism.

EXPERIMENTAL

Cell culture

Lung cancer cell lines (H1975 and H460) were supplied by the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured at 37 ˚C in Dulbecco's modified Eagle's medium (DMEM, Waltham, MA, USA) under 5 % CO_2 atmosphere in an incubator. The medium also contained 10 % fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin).

Cell viability assay

The effect of cynaropicrin (at doses of 0.25 - 2.0 μM) on the viabilities of H1975 and H460 cells was determined using Cell Counting Kit-8 (CCK-8; Inc, Kumamoto, Japan). The cells were seeded in 96-well plates, each at a density at 1 x 10^5 cells/well and incubated with varying concentrations of cynaropicrin (0.25 to 2.0 μM) for 48 h. The incubation was followed by addition of CCK-8 reagent (10 μL) to each well. Thereafter, the plates were incubated for additional 2 h at 37 ˚C, followed by absorbance reading in a microplate spectrophotometer (SPECTRAMax 190; CA, USA). The readings were used for determination of cell proliferation according to the manufacturer's instructions.

Apoptosis assay

The cells were grown on coverslips in 6-well plates and treated with cynaropicrin at concentrations of 0.25 - 2.0 μM for 48 h. Thereafter, the coverslips were rinsed with PBS, followed by washing thrice with 1X binding buffer. Then, the cells were stained with annexin-V/FITC for 40 min in the dark, and washed with 1X binding buffer. Finally, the cells were fixed with 2 % formaldehyde for 25 min, and fluorescence values were determined using Olympus-IX71inverted microscope connected to FITC.

Western blot analysis

Treatment of H1975 and H460 cells with cynaropicrin at doses of 0.25 and 2.0 μM for 48 h was followed by lysis using RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM sodium chloride and 1 % Nonidet P-40). The lysates were centrifuged at 4 ˚C for 20 min at 13,000 g, and the protein contents of the supernatants were estimated using Bradford method (Beyotime, Haimen, China). Then, 20-μg protein samples were resolved on 12 % SDS-polyacrylamide gel electrophoresis, and transferred subsequently to PVDF membranes. Non-specific binding sites in the membranes were blocked by incubation with 5 % skimmed milk at room temperature for 25 min. Thereafter, the membranes were incubated overnight at 4˚C with the primary antibodies anti-CCND1, anti-EGFR, anti-p-AKT, anti-AKT, anti-caspase-3, anti-caspase-9 and anti-β-actin. After washing the membranes with PBS, they were incubated for 2 h with horse radish peroxidase-linked secondary antibody at room temperature. Finally, the protein bands were visualized using enhanced chemiluminescence. The protein expressions were normalized to that of β-actin which served as internal control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cynaropicrin-treated cells using RNAiso Plus (Takara Bio). The RNA extract was reverse-transcribed to complementary DNA (cDNA). The PCR was carried out using 20 µL of reaction mixture consisting of 10 µl PowerUp™ SYBR™ Green Mix, strands of cDNA (2 µl) and 1 µl of each primer (10 µM). The ABI-7300 RT-PCR system (Thermo Fisher Scientific, Inc.) was used for PCR in the following steps: denaturation for 8 min at 94 ˚C, then 39 cycles for 8 s at 94 ˚C, and 59 ˚C for 1 min. The PCR signals were normalized with GAPDH, and gene expression levels were calculated using $2^{-\Delta\Delta Cq}$ method [12].

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of three experiments conducted independently. The data were statistically analysed using GraphPad Prism 6.0 (GraphPad Software, Inc, La Jolla, CA, USA). Comparison of the data was made using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Anti-proliferative effect of cynaropicrin on lung cancer cells

As shown in Figure 1, treatment of H1975 and H460 cells with cynaropicrin at doses of 0.25 to 2.0 µM led to marked reductions in their viabilities ($p < 0.05$). The viability of H460 cells decreased more than that of H1975 cells on treatment with cynaropicrin at doses of 0.25 - 2.0 µM for 48 h. The treatment with cynaropicrin at doses of 0.25 and 2.0 µM decreased H1975 cell viabilities to 88 and 26 %, respectively, while the viability of H460 cells was suppressed to 84 and 21 %, on treatment with 0.25 and 2.0 µM cynaropicrin, respectively.

Cynaropicrin induced apoptosis in H1975 and H460 cells

Cynaropicrin significantly induced apoptosis in H1975 and H460 cells, relative to control cells (Figure 2). Treatment with cynaropicrin at doses of 0.25 and 2.0 µM increased H1975 cell apoptosis to 10.54 and 64.32 %, respectively at 48 h. In H460 cells, apoptosis increased to 12.08 and 68.65 %, on treatment with cynaropicrin at doses of 0.25 and 2.0 µM, respectively. Moreover, the expression levels of caspase-3 and caspase-9 were increased significantly in H1975 and H460 cells on treatment with cynaropicrin at doses of 0.25 and 2.0 µM. These results are shown in Figure 2.

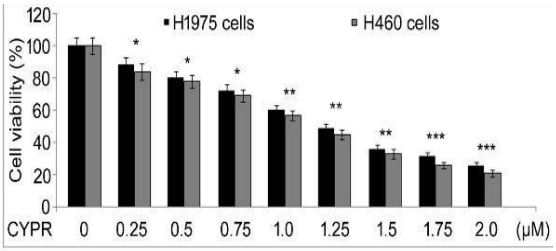

Figure 1: Effect of cynaropicrin on viabilities of H1975 and H460 cells. Cynaropicrin treatment at doses in the range of 0.25 to 2.0 µM for 48 h was followed by measurement of cell viability using Cell Counting Kit-8 assay. *$P < 0.05$; **$p < 0.01$ vs. control cells

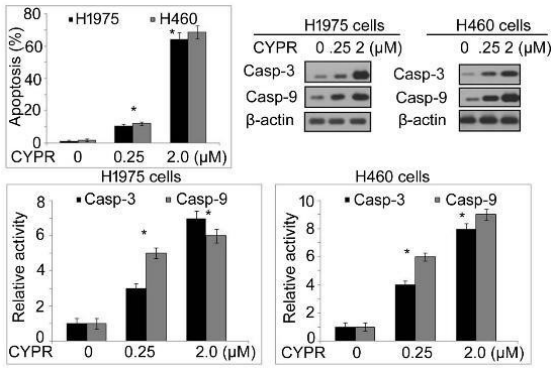

Figure 2: Effect of cynaropicrin on apoptosis of H1975 and H460 cells. Cynaropicrin treatment for 48 h at doses of 0.25 and 2.0 µM was followed by determination of apoptosis using flow cytometry. Caspase protein expression levels were assayed with western blotting. *$P < 0.05$, vs. control cells

Cynaropicrin targeted CCND1 expression in lung cancer cells

In H1975 and H460 cells, cynaropicrin treatment at doses of 0.25 and 2.0 µM for 48 h was followed by assay of CCND1 expression. Cynaropicrin at doses of 0.25 and 2.0 µM significantly downregulated mRNA expression of CCNDI in H1975 and H460 cells ($p < 0.05$; Figure 3). The protein expression of CCND1 was also suppressed in H1975 and H460 cells on treatment with 0.25 and 2.0 µM cynaropicrin.

Cynaropicrin targeted EGFR expression and p-AKT activation

The expression of EGFR and activation of p-AKT in cynaropicrin-treated H1975 and H460 cells were determined after 48 h. Cynaropicrin treatment at doses of 0.25 and 2.0 µM

significantly inhibited the mRNA and protein expressions of EGFR in H1975 and H460 cells. The activation of AKT in H1975 and H460 cells was also significantly suppressed on treatment with 0.25 and 2.0 μM cynaropicrin. These results are shown in Figure 4.

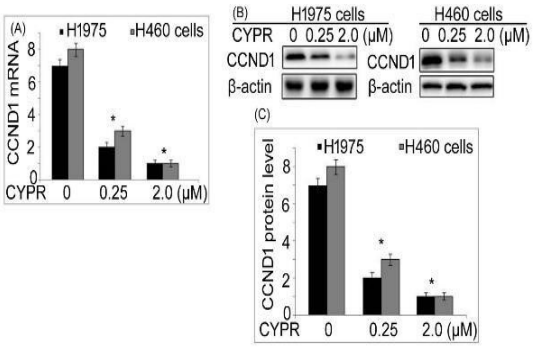

Figure 3: Effect of cynaropicrin on CCND1 expression in H1975 and H460 cells. (A) mRNA expression levels of CCNDI after cynaropicrin treatment at doses of 0.25 and 2.0 μM for 48 h. (B and C) CCND1 protein expression, as assayed with western blotting. **P* < 0.05 vs. control cells

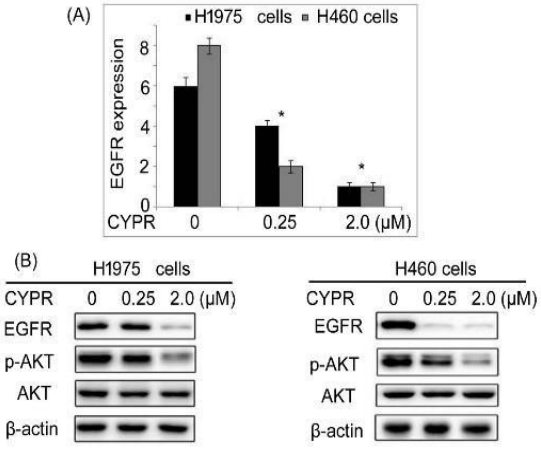

Figure 4: Effect of cynaropicrin on EGFR expression and AKT activation. (A) mRNA expression of EGFR in H1975 and H460 cells exposed to cynaropicrin at doses of 0.25 and 2.0 μM for 48 h. (B) Protein expression levels of p-AKT and EGFR, as assayed using western blotting. **P* < 0.05 vs. control cells

Cynaropicrin elevated the expressions of miR-202 and miR-370

The expressions of miR-202 and miR-370 were significantly increased in H1975 and H460 cells treated with cynaropicrin at doses of 0.25 and 2.0 μM for 48 h (Figure 5).

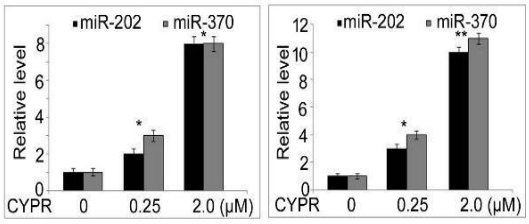

Figure 5: Effect of cynaropicrin on expressions of miR-202 and miR-370. The H1975 and H460 cells were exposed to cynaropicrin at doses of 0.25 and 2.0 μM for 48 h, after which miR-202 and miR-370 levels were assayed using RT-PCR. **P* < 0.05 vs. control cells

DISCUSSION

Cynaropicrin has been shown to selectively target proliferation of human macrophages such as Jurkat T leukocyte cells and U937 cells [12]. It has been reported that the cytotoxicity of cynaropicrin in U937 cells was mediated via arrest of the cell cycle at G1/S phase, and activation of apoptosis [12]. Cynaropicrin exhibits pro-apoptotic effect via induction of PKCδ cleavage, leading to the idea that PKCδ and ROS might be important targets for its anti-tumor potential [12]. In the present study, cynaropicrin induced cytotoxicity against H1975 and H460 cells in a concentration-dependent manner. Moreover, the cytotoxicity of cynaropicrin on H460 cells was greater at all tested concentrations than its toxicity on H1975 cells. In cynaropicrin-treated H1975 and H460 cells, apoptosis was increased significantly, relative to the control cells. Moreover, cynaropicrin markedly upregulated the expressions of caspase-3 and caspase-9 in H1975 and H460 cells.

Lung carcinogenesis is greatly influenced by the EGFR/AKT pathway which is regulated by some miRNAs [13]. In non-small cell lung carcinomas, miR-133a lowers the activation of AKT and suppresses the expression of EGFR [13]. The miR-145-mediated inhibition of migratory ability and induction of apoptosis of non-small cell lung carcinomas have been linked to downregulation of the EGFR/AKT pathway [14]. In the present study, cynaropicrin treatment of H1975 and H460 cells led to significant reduction in mRNA and protein expressions of EGFR. Moreover, AKT activation in H1975 and H460 cells was downregulated on exposure to cynaropicrin for 48 h. These results indicate that cynaropicrin exhibited cytotoxicity against H1975 and H460 cells via downregulation of the EGFR/AKT signaling pathway.

Studies have demonstrated the tumor-suppressive potential of miR-202, and revealed its downregulation in various types of cancers such as pulmonary cancer, prostate cancer, osteosarcoma and breast carcinomas [8,15-17]. In addition, miR-202 downregulation has been reported in bladder carcinoma tissues and cell lines [17]. Increases in miR-202 levels suppress proliferative capacity, colony forming potential and migratory property [18]. The downregulation of miR-202 has been reported in lung cancer cells, while its over-expression has been shown to increase the sensitivity of the cells to cisplatin via deactivation of Ras/mitogen-activated protein kinase pathway [18]. Similarly, miR-370 downregulation has been reported in pulmonary carcinoma cells, while its overexpression decreased proliferation, colony formation and metastatic potential [7].

In the present study, miR-202 expression was significantly increased in H1975 and H460 cells on treatment with cynaropicrin. The cynaropicrin treatment also significantly increased miR-370 levels in H1975 and H460 cells. Overexpression of miR-370 led to inhibition of CCND1/CCND kinase pathway in pulmonary carcinoma cells via upregulation of the expression of p21 [7,19]. In the current study, cynaropicrin treatment significantly downregulated mRNA expression of CCNDI in H1975 and H460 cells. The CCND1 protein expression was also suppressed in H1975 and H460 cells on treatment with cynaropicrin.

CONCLUSION

Cynaropicrin treatment of lung cancer cells produces anti-proliferative and pro-apoptotic effects via deactivation of EGFR/AKT signaling pathway. Furthermore, the expressions of miR-202 and miR-370 are downregulated in H1975 and H460 cells following treatment with cynaropicrin. Thus, cynaropicrin has potential potentials for the management of lung cancer.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xuechao Xu, Yixin Wan, Hong Wang, Hongyan Tao and Huirong Huang performed the experimental work, carried out the literature survey and analysed and compiled the data. Wenjun Li designed the study and wrote the paper. All the authors read the paper thoroughly and approved it for publication.

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