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Chaetoglobosin G inhibits proliferation, autophagy and cell cycle of lung cancer cells through EGFR/MEK/ERK signaling pathway

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Chaetoglobosin G (CG) is a fungal secondary metabolite and shows anti-tumor effects. However, the mechanisms behind the anti-tumor effect is still unclear. In this study, we evaluated the anti-proliferation effect of CG on human NSCLC A549 cells and explored the underlying mechanisms. The anti-proliferation effect of CG on A549 cells was evaluated by MTT. The targets of CG were screened through transcriptome sequencing. A flow cytometer was used to detect cell cycle and apoptosis. Western blotting was used to analyze apoptosis, cell cycle and autophagy related protein expression. Our results showed that CG had a dose-dependent inhibitory effect on proliferation of A549 cells. Transcriptome sequencing analysis found that CG obviously induced cell cycle arrest. Flow cytometry analysis and western blot showed that CG induced G2/M arrest with p21 protein upregulation and cyclinB1 protein downregulation. Western blot analysis also indicated that p-EGFR, EGFR, p-MEk and p-ERK protein expressions decreased and autophagy protein LC3II expression increased, indicating that CG can promote autophagy through EGFR/MEK/ERK/LC3 pathway. Moreover, CG can induce apoptosis with bcl-2 protein decrease. In conclusion, this study indicated that CG obviously inhibited A549 cell proliferation, and its mechanism may induce autophagy of A549 cells through EGFR/MEK/ERK/LC3 pathway to upregulate the expression of P21, thus lead to G2/M phase arrest to exert an anti-tumor role.

1. Introduction

Lung cancer is a common clinical malignancy with high morbidity and mortality (Ferlay et al. 2019). It includes non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), while 80%~85% is NSCLC (Siegel et al. 2017). Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) are exhibiting significant efficacy and safety, and are thus regarded as first-line drugs for NSCLC patients with EGFR-sensitive mutations and no resistance genes (Kujtan et al. 2019; Shah et al. 2020). However, there is a lack of effective therapeutic drugs for NSCLC patients with wide-type EGFR, negative ALK fusion gene or unknown mutation status. Therefore, it is still of great significance to find new anti-tumor drugs for these patients.

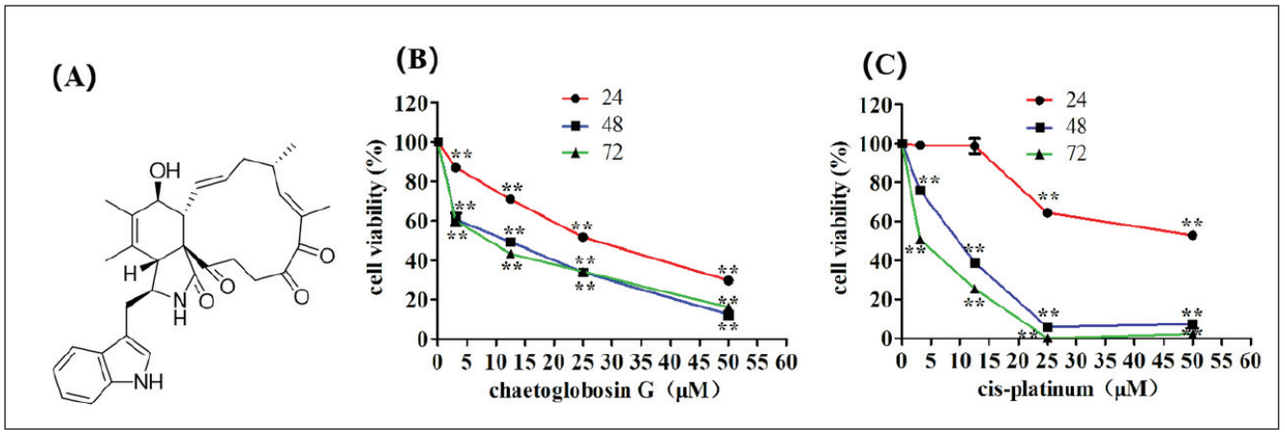

Fig. 1: Inhibition effect of CG and cis-platinum on A549 cells. (A) Structure of CG. (B) CG inhibited A549 cell proliferation at different concentrations and different time. (C) cis-platinum inhibited A549 cell proliferation at different concentrations and different time. ***P*<0.01 vs control.

Chaetoglobosins are fungal secondary metabolites belonging to the cytochalasan alkaloids, which contain a 10-(indol-3-yl) group, a macrocyclic ring and a perhydroisoindolone moiety (Huang et al. 2016). Many pharmacological actions have been shown, including anti-tumor (Ali et al. 2013), antimycotic (Jiang et al. 2017), anti-bacterial (Flewellin et al. 2015), anti-inflammatory (Ruan et al. 2018), fibrinolytic (Shinohara et al. 2000) and anti-HIV activities (Chen et al. 2015). We have previously reviewed the biological activities of chaetoglobosins, and found that chaetoglobosin G (CG) was only reported to show anti-tumor activities on breast cancer (BC1) cells, cholangiocarcinoma KKU-100 cells and HCT116 cells with IC50 values of 10.46 μM, 29.85 μM and 21.89 μM (Chen et al. 2020). However, mechanisms of anti-tumor effect of CG have not been reported.

In this study, we investigated the anti-tumor effects and molecular mechanism of CG on NSCLC A549 cells. It was found that CG inhibited A549 cell proliferation, and its mechanism may induce autophagy of A549 cells through the EGFR/MEK/ERK/LC3 pathway to upregulate the expression of P21, thus lead to G2/M phase arrest to exert an anti-tumor role.

2. Investigations and results

2.1. CG inhibited the proliferation of A549 cells

Cell viability was assessed by MTT assay. As shown in Fig. 1B, CG inhibited the proliferation of A549 cells in a dose-dependent manner. The results also show that CG suppressed the proliferation of A549 cells at 24 h and 48 h in a time-dependent manner. However, the inhibition of CG on A549 cells did not increase at 72 h compared to 48 h. As shown in Fig. 1C, cis-platinum as positive drug showed the inhibition on A549 cells in a dose- and time-dependent manner.

2.2. RNA-seq analysis screened targets of CG inhibiting A549 cell proliferation

To further explore the targets of CG inhibiting A549 cell proliferation, mRNA change was analyzed by RNA-seq. As shown in Fig. 2, CG treatment significantly inhibited cell cycle compared to control.

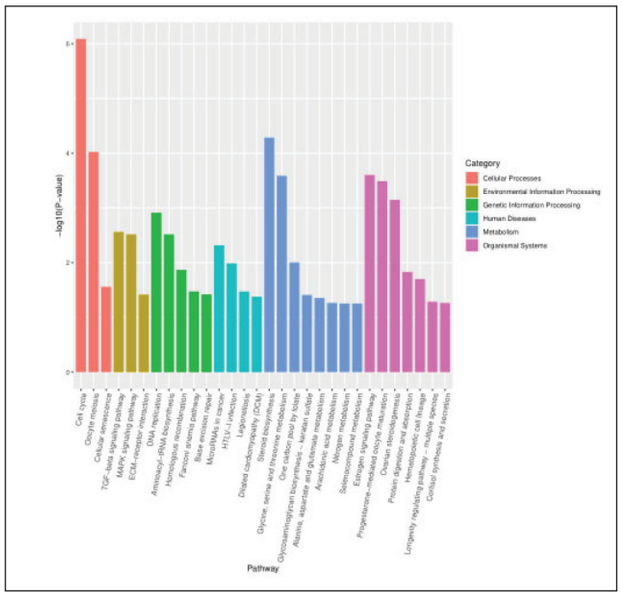

Fig.2: KEGG enrichment analysis diagram in RNA-seq.

2.3. CG inhibited proliferation of A549 cells by inducing G2/M phase arrest

Based on the result of RNA-seq analysis, we used flow cytometry and western blot to further analyze the effect of CG on A549 cells. The cell cycle distribution is shown in Figs. 3A and B, after CG treatment, the percentages of G0/G1 phase and S phase were obviously decreased and the percentage of G2/M phase was significantly increased. Western blot results showed that CG treatment upregulated the expression level of p21 protein and downregulated the expression level of cyclin B1 protein compared to control (Fig. 3C). These results suggested that CG induced G2/M phase arrest in A549 cells.

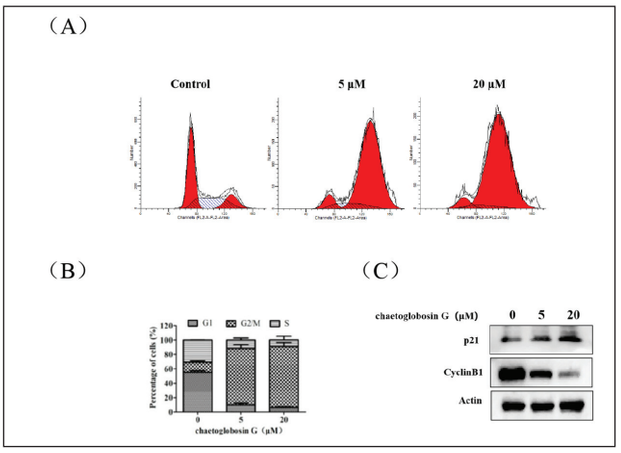

Fig. 3: Effect of CG on A549 cell cycle distribution. (A) Cell cycle was analyzed by flow cytometry in A549 cells. (B) Quantification of A549 cells at various phases of the cell cycle. (C) The G2/M phase related protein expression was detected by using western blot.

2.4. CG induced autophagy through EGFR/MEK/ERK signaling pathway

Accumulating evidence demonstrated that the MEK/ERK signaling pathway is involved in the cell autophagy process by upregulating LC3 protein expression. Moreover, MEK/ERK signaling pathway is an important downstream signaling pathway of EGFR. Therefore, this study detected the protein expression of EGFR/MEK/ERK signaling pathway by western blot. Results showed that CG treatment downregulated the expression of EGFR, p-EGFR, p-MEK and p-ERK proteins, and upregulated the expression of LC3II protein, suggesting CG induced autophagy through EGFR/MEK/ERK signaling pathway.

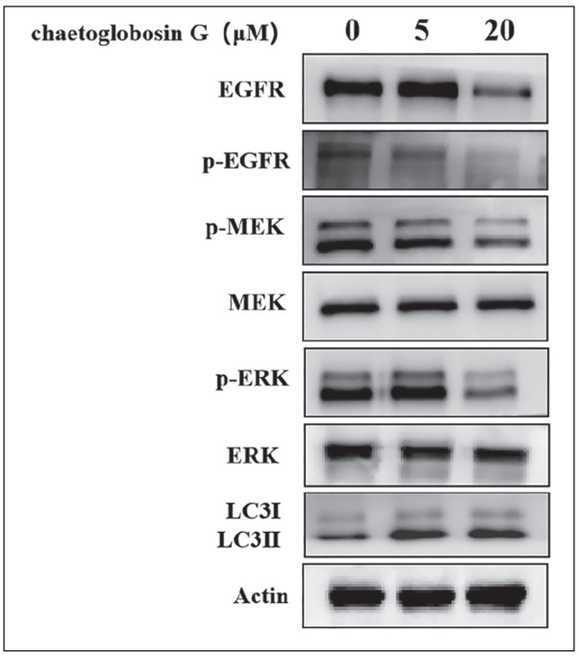

Fig. 4: Effect of CG on expression of autophagy-related proteins in A549 cells.

2.5. CG inhibited proliferation of A549 cells by inducing apoptosis

Apoptosis is another important process of cell death. This study used flow cytometry and western blot to analyze the effect of CG on cell apoptosis. As shown in Figs. 5A and B, after CG treatment, the apoptotic rate was obviously increased compared to control. Western blot results showed that CG treatment decreased the expression of bcl-2 protein (Fig. 5C). These results suggested that CG induced cell apoptosis.

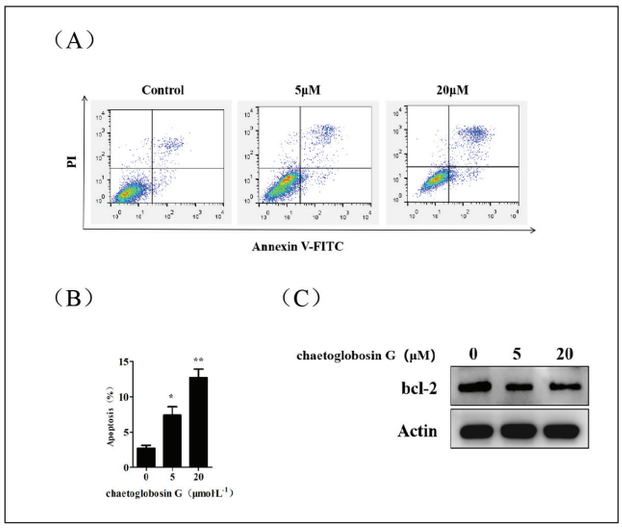

Fig. 5: CG induced A549 cell apoptosis. (A) CG inhibited apoptosis of A549 cells at 48h. (B) Statistical result of apoptotic rate of CG-induced A549 cells. (C) CG inhibited bcl-2 protein expression at 48h. *P<0.05 vs control.

3. Discussion

Chaetoglobosins are secondary metabolites of fungi, and more than 100 chaetoglobosins and their analogues have been discovered from various fungi, such as *Chaetomium globosum* (Jiang et al. 2017; Li et al. 2014), *Chaetomium subaffine* (Jiang et al. 2017), *Phomopsis sp* (Udagawa et al. 1979) and *Chaetomium elatum* (Thohinung et al. 2010). We isolated CG from the fungus *Chaetomium madrasense* 375. CG has been reported to inhibit the proliferation of several tumor cells, including breast cancer (BC1) cells (Thohinung et al. 2010), cholangiocarcinoma KKU-100 cells (Thohinung et al. 2010) and HCT116 cells (Zhang et al. 2010), but mechanisms of the anti-tumor effect of CG have not been studied so far.

In this study, CG showed a dose-dependent inhibitory effect on A549 cells with no EGFR-sensitive mutation, negative ALK fusion gene and low PD-L1 expression. To further explore the targets of CG, mRNA change was analyzed by RNA-seq and the results showed that CG treatment significantly inhibited cell cycle compared to control. Cell cycle arrest inhibited tumor growth (Hanahan et al. 2011). Then flow cytometry was used to verify the RNA-seq result and revealed that CG induced G2/M phase arrest. G2/M phase is activated by cyclin B1 (Gao et al. 2014) and regulated by cylin-dependent kinase inhibitor p21 (Mendoza et al. 2002). Western blot result showed that CG treatment upregulated p21 protein expression and downregulated Cyclin B1 protein expression, which were consistent with the results of flow cytometry. These results showed that CG inhibited proliferation by inducing G2/M phase arrest.

According to literature reports, autophagy inducers can upregulate p21 expression (Li et al. 2012) and over-expression of p21 protein can also induce autophagy (Capparelli et al. 2012), indicating that p21 may be a regulatory factor between cell cycle and autophagy. Subsequently, this study evaluated the effect of CG on autophagy. LC3 protein has been demonstrated to be a very important autophagy-related protein (Li et al. 2020). The result showed that CG treatment significantly upregulated LC3II protein expression, indicating that CG induced autophagy. Moreover, it is known that the EGFR/MEK/ERK signaling pathway is involved in the cell autophagy process by upregulating LC3 protein expression (Corcino et al. 2019; Wu et al. 2020). The study showed that CG significantly decreased the expression of EGFR, p-EGFR, p-MEK adn p-ERK protein. These results showed that CG induced autophagy by inhibiting the EGFR/MEK/ERK/LC3 signaling pathway.

In conclusion, CG inhibited A549 cell proliferation and induced autophagy of A549 cells through the EGFR/MEK/ERK/LC3 pathway to upregulate the expression of P21, thus leading to G2/M phase arrest to exert an anti-tumor role. The research results provide an experimental basis for developing new anti-tumor drugs derived from cytochalasan, especially tryptophan-derived cytochalasan.

4. Experimental

4.1. Reagents

CG (purity≥98%; MW, 506,) was isolated from the fungus *Chaetomium madrasense 375* by Zhengzhou Key Laboratory of Medicinal Resources Research. MTT was purchased from Sigma-Aldrich Co. Protein extraction kit and Annexin V-FITC/PI apoptosis detection kit were purchased from Keygen Biotech Co., Ltd. Cell cycle detection kit was purchased from New Cell & Molecular Biotech Co., Ltd. ERK, p-ERK and p-MEK antibodies were purchased from Cell Signaling Technology, Inc. MEK, Bcl-2, EGFR, p-EGFR, LC3, p21, cyclin B, actin antibodies and secondary antibodies were purchased from Shanghai Abways Biotechnology Co., Ltd.

4.2. Cell culture

The human NSCLC A549 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin and incubated with 5% CO_2 at 37 °C.

4.3. Cell viability assay

Cells were seeded in 96-well plates (1×10^4 cell/well). After 24 h, cells were treated with different concentrations of CG for 24 h, 48 h and 72 h. Subsequently, cell viability was assessed by MTT assay (Chen et al. 2014).

4.4. RNA-seq analysis

Cells were seeded in 10 cm^2 culture dish (3×10^5 cell/cm^2). After 24 h, cells were treated with 20 μM CG for 48 h. 48 h later, the cell supernatant was discarded and cells were washed with PBS. Subsequently, cells were lysed with Trizol, and the lysates were collected for RNA-seq analysis.

4.5. Cell cycle assay

Cells were seeded in 6-well plates (3×10^5 cell/well). After 24 h, cells were treated with different concentrations CG for 48 h. 48 h later, cells were collected and washed with PBS. Subsequently, cells were fixed with 70% ethanol at -20 °C overnight. The following day cells were treated with cell cycle detection kit and analyzed with flow cytometry.

4.6. Apoptosis assay

Cells were seeded in 6-well plates (3×10^5 cell/well). After 24 h, cells were treated with different concentrations CG for 48 h. 48 h later, cells were collected and washed with PBS. Cells were suspended in 500 μl binding buffer with 5 μl Annexin V and 5 μl PI for 15 min at room temperature. Then, cells were analyzed with flow cytometry.

4.7. Western blot analysis

Cells were seeded in 6-well plates (3×10^5 cell/well). After 24 h, cells were treated with different concentrations CG for 48 h. 48 h later, cells were collected and washed with PBS. The cellular proteins were extracted with protein extraction kit. The proteins were separated in 10% SDS-PAGE gels by different molecular weights. And the separated proteins were transferred to PVDF membrane by wet rotation. PVDF membranes were blocked with 5% skim milk for 1 h and incubated with primary antibodies at 4 °C overnight. The following day, the PVDF membranes were washed with TBST and incubated with secondary antibody for 1h. Protein bands were imaged with BLT GelView 6000Plus.

4.8. Statistical analysis

Statistical analysis was performed by Graphpad Prsim 5. All results were described as mean±SD. P<0.05 was considered to indicate a statistically significant difference.

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