Mulberrofuran G inhibits proliferation and migration by inactivating JAK2/STAT3 signaling in lung cancer
Mulberrofuran G inhibits proliferation and migration by inactivating JAK2/STAT3 signaling in lung cancer cells

Hai-Yuan-Bo Guo1, Xiao-Xiao Liu2, Xiao-Yong Zhu3 and Zhe Yu4

1Department of Medical Oncology, Pinghu Second People’s Hospital, Pinghu City, Zhejiang Province 314200, China; 2Department of Medical Oncology, Xinchang People’s Hospital of Zhejiang Province, Shaoxing City, Zhejiang Province 312500, China; 3Department of Second Ward of Medical Oncology, Zhiwu People’s Hospital of Zhejiang Province, Shaoxing City, Zhejiang Province 311800, China; 4Department of Respiratory Medicine, HwaMei Hospital, University of Chinese Academy of Sciences, Ningbo City, Zhejiang Province 315099, China.

Abstract

The present study has investigated how mulberrofuran G affects proliferation, invasion, and migration of the lung adenocarcinoma and squamous cell carcinoma. Four different concentrations of mulberrofuran G (1, 5, 10, and 100 μmol/L) were used to simulate human lung adenocarcinoma cells (A549 cells) and squamous-cell carcinoma (NCI-H226 cells). The results showed that mulberrofuran G significantly inhibited the proliferation, invasion, and migration of both A549 and NCI-H226 cells, with a dose-effect relationship. The IC50 inhibited the growth of A549 cells and NCI-H226 cells by mulberrofuran G were 22.5 and 30.6 μmol/L, respectively. It strengthened the expression of CDK4 and MMP9 but significantly weakened the expression of p27 in both A549 cells and NCI-H226 cells. Furthermore, the expression of p-JAK2 and p-STAT3 was significantly down-regulated in drug treatments. In conclusion, mulberrofuran G could inhibit proliferation, migration, and invasion of lung cancer cells via inactivating JAK2/STAT3 signaling.

Introduction

Lung cancer is the leading cause of cancer mortality, with estimating 2.1 million new diagnosed cases (approximately 11.6% of all types of cancer cases) and 1.8 million deaths (about 18.4% of all types of cancer deaths) (Bray et al., 2018). Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) are two types of lung cancer. Among the NSCLC, adenocarcinoma of the lung and squamous cell carcinoma of the lung are two major subtypes.

Chemotherapy is the most common method of lung cancer treatment. Despite this, poor prognosis and drug resistance are still inescapable, especially in patients with advanced lung cancer. It is well-known that poor clinic outcome of the solid malignant tumor is attributable to cancer invasion and metastasis (Gupta and Massagué, 2006). Hence, a comprehensive assessment of how to distinguish the key molecular and genetic pathways and how the pathways work in the invasion and metastasis of cancer is a pressing issue.

The JAK/STAT signaling pathway is comprised of Janus kinases (JAKs), signal transducer and activator of transcription proteins (STATs) and binding receptor (Aaronson and Horvath, 2002). The pathway mediates signals transferring from outside to nucleus of a cell. Many studies have investigated the potential roles of JAK/STAT signaling pathway in lung cancer, hematopoietic cancer, primary mediastinal large B-cell lymphoma, and breast cancer (Haque et al., 2018; Fortschegger et al., 2021; Li et al., 2021; Szyladowski et al., 2021).

Mulberrofuran G is isolated from the bark of Morus alba (Li et al., 2018). A recent study has indicated that mul-
berrofuran G has a neuroprotective effect in cerebral ischemia by suppressing NOX4 protein expression and NOX4-induced reactive oxygen species (ROS) generation and endoplasmic reticulum stress (Hong et al., 2017). However, elevated NOX4 activity is associated with drug resistance in cancer (Shanmugasundaram et al., 2017). Moreover, mulberrofuran G is a dual inhibitor of protein tyrosine phosphatase 1B (PTP1B) and α-glucosidase enzymes (Paudel et al., 2018). The deficiency or inhibition of PTP1B mediates the metastasis process in lung cancer (Chen et al., 2020). However, this knowledge gap is of considerable concern given the effect of mulberrofuran G on lung cancer.

Therefore, the study aimed to illustrate how mulberrofuran G impacts the proliferation, invasion, and migration of lung cancer cells and to evaluate the hypothesis of whether or not mulberrofuran G regulated these processes via the JAK2/STAT3 signaling pathway.

Materials and Methods

Preparation of mulberrofuran G solution

Mulberrofuran G (CAS 87085-00-5, HPLC≥98%) was purchased from Shanghai Tauto Biotech Co., Ltd. and dissolved in phosphate-buffered saline to make the solutions with 4 final concentrations of 1, 5, 10, and 100 μmol/L.

Cell lines and cell culture

The human lung adenocarcinoma cell line (A549 cells) and human lung squamous cell carcinoma cell line (NCI-H226 cells) were purchased from the Cell Bank of Type Culture Collection of The Chinese Academy of Sciences (China). A549 cells and NCI-H226 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640, Thermo Fisher Scientific) medium containing 10% fetal bovine serum (Thermo Fisher Scientific) at 37°C in a humidified air containing 5% CO2 incubator (Thermo Fisher Scientific). Cell passage was carried out by the usual procedure and the third generation cells were used for the subsequent experiments.

CCK8 assay

A549 cells (1×10^4 cells/well) and NCI-H226 cells (1×10^4 cells/well) in the logarithmic growth phase were seeded into 96-well plates and permitted to adhere overnight, respectively. The cells were then administrated with 100 μL 1, 5, 10 and 100 μmol/L mulberrofuran G. The control group was treated with an equal volume of phosphate-buffered saline. After treatment for 24 hours, 100 μL of medium containing 10% cck8 was replaced, and continuously cultured for 2 hours at 37°C in a humidified air containing 5% CO2. The optical density at 450 nm was measured by a microplate reader (PT-3502PC) and used as the positive index of cell viability.

Cell colony assay

A549 cells and NCI-H226 cells (approximately 2×10^5 cells) were suspended in 1, 5, 10 and 100 μmol/L mulberrofuran G containing 0.33% agar, RPMI 1640 medium, 10% fetal bovine serum, respectively. Thereafter, the mixture containing 0.5% agar, RPMI 1640 medium, and 10% fetal bovine serum were administrated in an upper layer with a 6-well plate. Followed by an incubation period of 10 days, the colonies of cells (>0.1 mm in diameter) were counted and photographed by an inverted microscope. Each treatment had three replicates and was repeated three times.

Scratch assay

A549 cells and NCI-H226 cells were seeded into 6-well plates, respectively. After incubated for 6 hours at 37°C, 1, 5, 10 and 100 μmol/L mulberrofuran G were used to treat the adhered cells. When the confluence rate of cells chamber.

**Box 1: Transwell Invasion Assay**

**Principle**
The most characteristic readout of a migration assay is the change of the cell-covered area (gap closure) over time. The cells should be monitored immediately after the gap creation, in most cases by using phase contrast microscopy at different time points (e.g., after 6 and 12 hours).

**Requirements**

Crystal violet, Fetal bovine serum (10%), Incubator with 5% CO2, Human lung adenocarcinoma cell line (A549 cells) suspensions (1×10⁶ cells), Human lung squamous cell carcinoma cell line (NCI-H226 cells) suspensions (1×10⁶ cells), RPMI 1640 medium, Mulberrofuran G, Paraformaldehyde, Phase contrast microscopy, Phosphate-buffered saline, Transwell chamber

**Procedure**

**Step 1:** 0.1 mL cell suspensions containing 1, 5, 10, and 100 μmol/L mulberrofuran G were seeded into the Transwell

Reference

Pijuan et al., 2019

Reference (video)

Chen, 2018
reached 95% or more, using a p200 pipette tip generated the 'scratch' by scraping the monolayer in a neat and straight line (Liang et al., 2007). The plate was then washed with phosphate-buffered saline. Fresh RPMI 1640 medium containing 10% fetal blood serum was added and cultured at 37°C in a humidified air containing 5% CO₂. The plates were examined at 0 and 24 hours after being scratched. The ability of migration was evaluated by the formula: wound closure = (Pair distance\textsubscript{0}\textdegree hour − Pair dis-tance\textsubscript{24}\textdegree hour) /Pair distance\textsubscript{0}\textdegree hour.

**Western blot**

After administrating A549 cells with 22.5 μmol/L mulberrofuran G and administrating NCI-H226 cells with 30.6 μmol/L mulberrofuran G for 24 hours, the cells were collected and lysed in RIPA buffer for 30 min. The protein samples were collected by low-temperature centrifugation. The concentration of each protein sample was then determined by the BCA method. After 10% SDS-PAGE electrophoresis separation, the gel was transferred to nitrocellulose membranes, blocked with 5% skim milk and probed with a primary antibody (ABCAM) at 4°C overnight (Mahmood and Yang, 2012; Pyo et al, 2019). After washing with TBST, this was followed by probing with the HRP-labeled secondary antibody (ABCAM) for 1 hour at room temperature. The band intensity was then analyzed by an automatic chemiluminescence fluorescence image analysis system (Tanon 5200 Multi) and Image J software. The markers of cell cycle included p27, CDK4, CDK6, RB1; the MMP9 was used as the marker of metastasis. β-actin was used as an internal control.

**Statistical Analysis**

All treatments were carried out in triplicates. All values were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS v20.0 software. The comparison between the two sample means was evaluated by t test. The comparison between the treatment and the control was performed by one-way ANOVA. The pairwise comparisons between groups were carried out by the least significant difference method.

**Results**

**Proliferation of lung cancer cells**

To evaluate the effect of mulberrofuran G on the proliferation of lung cancer cells, A549 cells and NCI-H226 cells were administrated with various concentrations of mulberrofuran G for 24 hours. CCK8 assay results (Figure 1A) indicated that mulberrofuran G significantly inhibited the proliferation of lung cancer cells (p<0.05), with a positive dose-inhibition relationship. The IC\textsubscript{50} of growth inhibition of A549 cells and NCI-H226 cells were 22.5 μmol/L and 30.6 μmol/L, respectively. The two inhibitory concentrations were used in subsequent experiments. Compared with the control, with increased mulberrofuran G, the number of A549 cells and NCI-H226 cells clones were significantly decreased (p<0.05, Figure 1B). No difference in both response variables was detected between A549 cells and NCI-H226 cells. Consequently, it may point out that the mulberrofuran G obviously suppressed the proliferation of lung adenocarcinoma and lung squamous cell carcinoma cells.

**Invasion and migration of lung cancer cells**

To evaluate the effect of mulberrofuran G on the invasion and migration of A549 cells and NCI-H226 cells, the Transwell invasion assay and scratch assay were performed. Results (Figure 2A) indicated that the number of invasive A549 cells and NCI-H226 cells was significantly decreased (p<0.05, Figure 2B). With increased mulberrofuran G concentration, the number of invasive cells showed a decreasing trend. The scratch assay results showed that the cell migration rate of A549 cells and NCI-H226 was significantly weakened by mulberrofuran G (Figure 2B), with a negative linear relationship. No difference in both response variables was detected between A549 cells and NCI-H226 cells. Consequently, it may point out that mulberrofuran G inhibited the invasion and migration of lung adenocarcinoma and lung squamous cell carcinoma cells.

![Figure 1: Effects of mulberrofuran G on the proliferation of A549 cells and NCI-H226 cells. (A) CCK8 assay; (B) Cell colony assay](image-url)
Next, Western blot analysis was performed to evaluate the expression levels of related markers of cell cycle and metastasis. For both A549 cells and NCI-H226 cells, mulberrofuran G significantly down-regulated the expression levels of CDK6 and CDK4, whereas significantly up-regulated the expression levels of p27 and RB1. Further, mulberrofuran G significantly weakened the metastasis-related marker MMP9 in A549 cells (Figure 3A).

JAK2/STAT3 signaling pathway

To illuminate how mulberrofuran G impacted cell proliferation, invasion and migration, A549 cells and NCI-H226 cells were administrated by 22.525 μmol/L and 30.591 μmol/L MG, respectively. The expression levels of JAK2/STAT3 signaling markers were then investigated by a Western blot (Figure 3B). After treatment for 24 hours, there was no difference in JAK2 and STAT3. However, the levels of p-JAK2 and p-STAT3 in A549 cells and NCI-H226 cells were significantly lower than that in the control group (p<0.05), which was similar to the effect of JAK2/STAT3 inhibitor AG490. When cells were co-treated with both JAK2/STAT3 inhibitor AG490 and mulberrofuran G, the levels of p-JAK2 and p-STAT3 were the lowest. All the results suggested that mulberrofuran G regulated the proliferation, invasion, and migration of lung cancer cells via inactivating JAK2/STAT3 signaling pathway.

Discussion

Due to the highly invasive and metastatic capacity of tumor cells, the overall survival time of patients with lung cancer is poor; especially patients aged ≥ 75 years (Heist et al., 2017; Cerfolio et al., 2018; Driessen et al., 2018). Better understanding the invasion and migration of tumor cells is the main event prolonging the survival time of individuals with cancer. The present study focuses on the effect of mulberrofuran G on cells proliferation, invasion, and migration of lung adenocarcinoma and squamous cell carcinoma. Previous literature has illuminated that mulberrofuran G plays crucial regulatory roles in several cellular processes (e.g.,
signaling regulation in ROS and protein tyrosine phosphatase) (Hong et al., 2017; Paudel et al., 2018). The proliferations of lung adenocarcinoma and lung squamous cell carcinoma cells were significantly linearly suppressed by mulberrofuran G. By increasing mulberrofuran G, the growth of lung adenocarcinoma and lung squamous cell carcinoma cells were increasingly down-regulated. A recent study has suggested that mulberrofuran G has variable activities, such as anti-Alzheimer’s disease, anti-inflammatory, fungicidal, anti-cancer, antibacterial, and anti-tyrosinase properties (Koirala et al., 2018). Hence, this study points to experimental evidence that mulberrofuran G has anti-cancer activity, which provides new insight into the clinical treatment of lung cancer.

Next, the expression level of cell cycle markers was evaluated after treated with various concentrations of mulberrofuran G. Mulberrofuran G prominently weakened the expression levels of CDK6 and CDK4, but strengthened the expression levels of p27 and RB1. At its essence, dysregulation in the cell cycle (e.g. G2/M phase) is associated with the occurrence and development of lung cancer (Ramalingam et al., 2017). The inhibitor p27 regulates the activity of cyclin-dependent kinases (including CDK4 and CDK2); dual inhibition of CDK4 and CDK2 further induces down-regulation of tyrosine phosphorylation of p27Kip1 (CDKN1B) (Patel et al., 2018). CDK4 and CDK6 inactivate phosphorylations of the central cell cycle/tumor suppressor Rb to initiate G1 phase (Sherr, 1995; Bartek et al., 1996). Degradation of p27Kip1 inhibits cell cycle transition at G1/S boundary and decreases tumor growth (Singh et al., 2018). The deregulation of CDK4 in cancer decreases emerging of E2F-dependent transcription of cyclin E and influences DNA synthesis (Colleoni et al., 2017). Consequently, the proliferation of lung cancer cells is attributable to the p27-mediated down-regulation of CDK4/6 complexes.

Tumor metastasis is the leading cause of mortality in individuals with cancer. The effect of mulberrofuran G on the metastasis of A549 cells and NCI-H226 cells was evaluated. Transwell invasion assay and scratch assay indicated that mulberrofuran G inhibited the invasion and migration of lung adenocarcinoma and lung squamous cell carcinoma cells, with a negatively linear dose-effect relationship. Western blot analysis showed mulberrofuran G significantly down-regulated MMP9 in A549 cells. The regulator MMP-9 plays an important role in the metastasis of cancer cells (Vandooren et al., 2013). Accumulating evidence indicates that up-regulation of MMP9 enhances migration and invasion of cancer cells (Khan et al., 2017; Zhang et al., 2017). The present results suggested that the metastasis of lung cancer cells is linked to inhibition of MMP9 mediated by mulberrofuran G.

Notably, it has been reported that the JAK2/STAT3 signaling pathway is associated with inflammatory response (Weng et al., 2017) and regulated the expression levels of interleukin 6 (IL-6) and MMP-10 in a human lung adenocarcinoma cell (Zhang et al., 2009). The present study showed that mulberrofuran G significantly down-regulated p-JAK2 and p-STAT3 in A549 cells and NCI-H226 cells, which is similar to the effect of JAK2/STAT3 inhibitor AG490. The JAK2/STAT3-specific inhibitor AG490 blocked STAT3 phosphorylation (Liu et al., 2014). The cell growth and migration mediated by JAK2/STAT3 signaling pathway in NSCLC have been reported in several cancers (Liu et al., 2018; Sun et al., 2018). STAT3 is a negative regulator of inflammation (Welte et al., 2003). The activation of STAT3 further enhances the TGF-β/Smad signaling (Zhang et al., 2009) and suppresses p53 transcription (Niu et al., 2005). Mulberrofuran G may have a similar function to AG490. JAK2/STAT3 signaling pathway has protective effects of epidermal growth factor (EGF) receptor activation (Tang et al., 2018). JAK/STAT3 pathway is required to sustain EGF/EGFR-induced epithelial-mesenchymal transition in cancer (Colomiere et al., 2009; Liu et al., 2014). JAK2/ STAT3 pathway is required to strengthen the anti-angiogenic property of anlotinib (Liang et al., 2019)

**Conclusion**

The anti-cancer effect of mulberrofuran G via inhibition of JAK2/STAT3 signaling pathway to regulate the proliferation, invasion, and migration of lung adenocarcinoma and lung squamous carcinoma cells.

**Financial Support**

Self-funded

**Conflict of Interest**

Authors declare no conflict of interest

**References**

Aaronson DS, Horvath CM. A road map for those who don’t know JAK-STAT. Science 2002; 296: 1653-55.


Cerfolio RJ, Ghanim AF, Dylewski M, Veronesi G, Spaggiari L, Park BJ. The long-term survival of robotic lobectomy for non


Haque I, Ghosh A, Acup S, Banerjee S, Dhar K, Ray A, Sarkar S, Kambhampati S, Banerjee SK. Leptin positive breast cancer cell viability and migration is media-


