Toxicology and Applied Pharmacology 356 (2018) 1–7

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Polyphyllin I modulates MALAT1/STAT3 signaling to induce apoptosis in gefitinib-resistant non-small cell lung cancer

Qi Yang[a,1], Wenyu Chen[a,1], Yufeng Xu[b,1], Xiaodong Lv[a], Ming Zhang[a], Hao Jiang[c,*]

[a] Department of Respiration, The First Hospital of Jiaxing (The First Affiliated Hospital of Jiaxing University), Jiaxing, Zhejiang 314000, PR China
[b] Department of Oncology, The First Hospital of Jiaxing (The First Affiliated Hospital of Jiaxing University), Jiaxing, Zhejiang 314000, PR China
[c] Department of Oncology, Zhejiang Hospital, Hangzhou, Zhejiang 310013, PR China

ARTICLE INFO

Keywords:
Polyphyllin
MALAT1
STAT3
Gefitinib-resistance
NSCLC

ABSTRACT

Non-small cell lung cancer (NSCLC) patients harboring EGFR mutation who initially respond to EGFR-TKI will gradually develop acquired resistance. There is still a challenge to treat EGFR-TKI resistant NSCLC patients. Polyphyllin I (PP I), a steroidal saponin isolated from Paris polyphylla., has been exhibited antitumor activities against various carcinomas. However, its mechanism in treating EGFR-TKI resistant NSCLC has not been well elucidated. In this study, we found that PP I suppressed the cell viability and induced apoptosis of gefitinib-resistant NSCLC cells and xenograft models. These therapeutic efficacies were associated with down-regulated level of MALAT1, leading to inactivation of STAT3 signaling pathway. The cell viability inhibition and apoptosis inducing in gefitinib-resistant NSCLC triggered by PP I were abolished by MALAT1 overexpression, while the cell viability inhibition and apoptosis inducing triggered by PP I were potentiated by MALAT1 knockdown. These findings suggest that, in vitro and in vivo, PP I inhibits the viability and induces apoptosis of gefitinib-resistant NSCLC by down-regulating MALAT1 and inactivating STAT3 signaling pathway. Thus, PPI could serve a promising therapeutic agent for the treatment of gefitinib-resistant NSCLC.

1. Introduction

Non-small cell lung cancer (NSCLC) remains the leading cause of morbidity and mortality in the world (Siegel et al., 2017). Epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), such as gefitinib or erlotinib, shows an encouraging response rate to NSCLC with activating mutations in exons 19 or 21 of EGFR (Rosell et al., 2009). Unfortunately, patients harboring EGFR mutation who initially respond to EGFR-TKI will gradually develop acquired resistance (Kim et al., 2014), that becomes the most important limiting factor for NSCLC treatment. The mechanism involved in acquired resistance to EGFR-TKI has been investigated, including EGFR T790 M secondary mutation (Pao et al., 2005), activation of bypassing signaling such as c-Met, HER2, AXL, IGF-1R, PIK3CA and BRAF (Wu et al., 2017; Tian et al., 2016; Ahsan, 2016; Eng et al., 2015; de Langen & Smit, 2017), but there is 40% of all cases remaining unclear. Currently, the third-generation EGFR-TKI is the main treatment strategy for patients with acquired T790 M mutation, but there is still a challenge to treat T790 M-negative patients.

Long non-coding RNAs (lncRNAs) are > 200 nucleotides in length, non-protein coding transcripts. Increasing evidence has demonstrated that differentially expressed lncRNAs are involved in EGFR-TKI resistance and some lncRNAs play critical roles in gefitinib-resistant lung cancer cells (Fu et al., 2018; Liu et al., 2018; Wang et al., 2017a; Ma et al., 2017; Pan et al., 2016). Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is first found in NSCLC that plays functional roles in the tumor progression and development of chemotherapeutic resistance (Wang et al., 2017b; Chen et al., 2017; Fang et al., 2018). The recent studies have shown that MALAT1 contributed to NSCLC development and cisplatin resistance via modulating STAT3 activation (Fang et al., 2018; Li et al., 2017). It has been revealed that STAT3 might play important role in gefitinib resistance (Phan et al., 2016; Shou et al., 2016; Li et al., 2015; Makino et al., 2017), and lncRNA such as MALAT1 may be potential target to reverse EGFR-TKIs resistance.

Polyphyllin I (PS I), a steroidal saponin isolated from *Paris polyphylla.*, has been exhibited antitumor activities as well as the polyphyllin family members in a number of cancer cells (Wang & Fei, 2017; Zheng et al., 2017; Zhu et al., 2016; Zhao et al., 2016; Song et al., 2016; Zhao et al., 2015; Jiang et al., 2014a; Jiang et al., 2014b). Recent study

* Corresponding author at: Department of Oncology, Zhejiang Hospital, No.12 Lingyin Road, Hangzhou, Zhejiang 310013, PR China.
E-mail address: zjhoncol@163.com (H. Jiang).
[1] Contributed equally to this work.

https://doi.org/10.1016/j.taap.2018.07.031
Received 26 May 2018; Received in revised form 18 July 2018; Accepted 31 July 2018
Available online 01 August 2018
0041-008X/

has shown that PP I could overcome erlotinib resistance via IL-6/STAT3 pathway inhibition (Lou et al., 2017), however, the underlying mechanism of PP I against acquired gefitinib-resistant NSCLC by modulating lncRNA and STAT3 is still unexplored. In the present study, we aim to evaluate the anti-tumor activity of PP I in acquired gefitinib-resistant NSCLC and the underlying mechanism associated with lncRNA and STAT3 modulation. The results indicated that PP I inhibits the viability and induces apoptosis of gefitinib-resistant NSCLC by down-regulating MALAT1 and suppressing STAT3 phosphorylation.

2. Materials and methods

2.1. Chemicals and reagents

PP I ($C_{44}H_{70}O_{16}$) was obtained from the Zhejiang Institute for Food and Drug Control (batch No. 111590–201,103, Hangzhou, China). MTT and DMSO were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Annexin V Apoptosis Detection Kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Primary antibodies against STAT3, p-STAT3, cleaved PARP, cleaved caspase-3, cleaved caspase-9 were purchased from the Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture

Human NSCLC PC-9 cells and the gefitinib-resistant PC-9-ZD cells were obtained from the Laboratory of Biochemistry and Molecular Biology (Tongji University, Shanghai, China) (Ji et al., 2009). PC-9-ZD cells were more resistant to gefitinib than their parental PC-9 cells (Ji et al., 2009). The cells were cultured in DMEM medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA) in a humidified atmosphere at 37 °C containing 5% CO_2.

2.3. Cell viability assay and colony forming assay

The cell viability under treatment of PP I was evaluated by MTT assay. PP I was dissolved in dimethylsulfoxide (DMSO) with a final DMSO concentration < 0.25% (v/v). The PC-9-ZD cells were seeded in 96-well plates (1×10^4/well) overnight and treated with different doses of PP I for 48 h. DMSO concentration < 0.25% (v/v) was set as the control. MTT solution (5 mg/ml) was added to each well and further incubated for 4 h. Then the formazan was resuspended with DMSO and the absorbance was measured at 490 nm by a multi scanner auto reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cells were seeded in 6-well plates at a density of 1×10^3 cells/well and incubated for 24 h. The cells then were treated with PP I for 48 h. The supernatant was removed and replaced by complete medium and cells were cultured for 2 weeks. The colonies were then fixed, stained and scored which contained > 50 cells. The experiments were performed in triplicate.

2.4. Annexin V-FITC/PI apoptosis analysis

The PC-9-ZD cells were seeded in 6-well plates (5×10^4 cells/well) overnight and then treated with different doses of PP I for 48 h. DMSO concentration < 0.25% (v/v) was set as the control. Cells were harvested and washed in PBS, then stained with Annexin V-FITC and PI in the dark for 15 min. Samples were analyzed using FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) to detect apoptosis. Apoptotic cells were defined as Annexin V-FITC-positive and PI-negative cells. The experiments were performed in triplicate.

2.5. Western blot analysis

The PC-9-ZD cells were harvested after treatment. Protein expression of STAT3, p-STAT3, cleaved PARP, cleaved caspase-3 and cleaved caspase-9 were detected by Western blot, with the protocol previously described (Fang et al., 2018). primary antibodies against STAT3, p-STAT3, cleaved PARP, cleaved caspase-3, cleaved caspase-9 (1:1000) and HRP-conjugated secondary antibody (1:10,000) were used.

2.6. qRT-PCR analysis

Total RNAs were extracted from cells, and the MALAT1 expression was detected with the protocol previously described (Fang et al., 2018). The primes of MALAT1: forward, 5′-CTTCCCTAGGGGATTTCAGG-3′, reverse, 5′- GCCCACAGGAACAAGTCCTA-3′. β-actin was used as endogenous control. The level of RNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Cell transfection

The cDNA encoding MALAT1 was PCR-amplified and subcloned into the pLenti-GIII-CMV-Puro vector (Applied Biological Materials Inc., Richmond, BC, Canada). The sh-RNA specifically targeting MALAT1 were synthesized by Hibio Tech Co., Ltd. (Hangzhou, China). The sh-RNA sequence for MALAT1 was 5′-GCAGCCCGAGACTTCTGTAAA-3′. The PC-9-ZD cells were seeded in 6-well plates (3×10^5 cells/well) with the density of 80% for transfection. Then the transfection was performed using Lipofectamine 2000 kit (Invitrogen) with plasmid DNA/shRNA (4 μg/250 μl) according to the manufacturer's instructions.

2.8. In vivo study

BALB/c nude mice (4 week old, male, weighing 18–22 g) were purchased from Zhejiang Academy of Medical Sciences (Hangzhou, China). The protocol and all procedures of animal experiments used in this study were approved by the ethics committee of Zhejiang Hospital (Hangzhou, China). The experiment was performed according to the NIH guidelines for animal care and use. Briefly, the PC-9-ZD cells were subcutaneously transplanted into nude mice. When the tumor volume reached 200 mm^3, the mice were randomly assigned to two groups ($N = 3$ for each group). PPI group was intraperitoneally injected with 100 μl PP I (5 mg/kg), and control group was injected with saline including DMSO (1/10000 *V*/V) for 4 weeks, once a day. Tumor volumes were measured using the formula $0.5 \times \text{length} \times \text{width}^2$. Mice were sacrificed at day 28 and the tumor samples were embedded in paraffin for further use.

2.9. Terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) assay

The tissue sections (4 μm) were deparaffinized and rehydrated. In situ apoptosis detection kit (Abcam Co., Cambridge, MA, USA) was used to detect apoptotic cells according to manufacture's instructions. The positive cells were identified and analyzed by fluorescence microscope (Olympus Corporation, Tokyo, Japan).

2.10. Immunohistochemistry

Paraffin-embedded tumor tissues were deparaffinized, hydrated and blocked with endogenous peroxidase, and then were treated with the protocol previously described (Fang et al., 2018). The sections were incubated with antibody against p-STAT3 and STAT3, followed by secondary antibody incubation. Positive cells were calculated under 200 × magnification.

2.11. Statistical analysis

All experiments were repeated in triplicate. Data were presented as a mean ± standard deviation. Statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance and SNK-q test were used. *p*-values < .05 was considered