Paclitaxel aggravating radiation-induced pulmonary fibrosis is associated with the down-regulation of the negative regulatory function of Spry2

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Paclitaxel limits the negative regulatory function of Spry2

ABSTRACT

Paclitaxel (PTX) is capable of aggravating radiation-induced pulmonary fibrosis (RIPF), but the mechanism is unknown. Spry2 is a negative regulator of receptor tyrosine kinase-related Ras/Raf/ERK pathway. This experiment was aimed at exploring whether the aggravation of RIPF by PTX is related to Spry2. The RIPF model was established with C57BL/6 mice by thoracic irradiation, and PTX was administered concurrently. Western blot was used to detect the expression level of ERK signaling molecules and the distribution of Spry2 in the plasma membrane/cytoplasm. Co-IP and immunofluorescence were used to observe the co-localization of Spry2 with the plasma membrane and tubulin. The results showed that PTX-concurrent radiotherapy could aggravate fibrotic lesions in RIPF, down-regulate the content of membrane Spry2 and up-regulate the levels of p-c-Raf and p-ERK in lung tissue. It was found that knockdown of Spry2 in fibroblast abolished the up-regulation of p-c-Raf and p-ERK by PTX. Both Co-IP results and immunofluorescence staining showed that PTX increased the binding of Spry2 to tubulin and that microtubule depolymerizing agents could abolish PTX’s inhibition of Spry2 membrane distribution and inhibit PTX’s up-regulation of Raf/ERK signaling. Both Nintedanib and ERK inhibitor were effective in relieving PTX-exacerbated RIPF. Taken together, the mechanism of PTX’s aggravating RIPF was related to its ability to enhance Spry2’s binding to tubulin, thus attenuating Spry2’s negative regulation on Raf/ERK pathway.

Key words: Paclitaxel (PTX), Radiation-induced pulmonary fibrosis (RIPF), Spry2, ERK, Tubulin

Significance statement

This study revealed that PTX concurrent radiation therapy exacerbates RIPF during the treatment of thoracic tumors, which is associated with PTX restraining Spry2 and upregulating the Raf/ERK signaling pathway, and provided drug targets for mitigating this complication.

INTRODUCTION

Radiation-induced lung injury is a common and serious complication of radiation therapy for...
thoracic tumors, which reduces pulmonary ventilation and is even life-threatening, thus making an important factor in determining tumor radiation dose and affecting patient prognosis (Hanania et al., 2019; Roy et al., 2021). In recent years, with the advancement of radiotherapy technology, the lung injury caused by radiotherapy has been reduced, but on the other hand, with more extensive adaptability of thoracic radiotherapy, the incidence of RIPF is still at a high level. RIPF represents the highest incidence in radiotherapy of lung cancer (5%-25%), followed by mediastinal lymphoma (5%-10%) and breast cancer (1%-5%) (Ebert et al., 2015; Hanania et al., 2019).

Paclitaxel (PTX) and its derivatives are commonly used in treating breast cancer (Untch et al., 2016; Abu Samaan et al., 2019), lung cancer (Fossella et al., 2000; Villaruz and Socinski, 2016) and esophageal cancer (Shitara et al., 2018) and other thoracic tumors, and often given concurrently with thoracic radiotherapy (Bradley et al., 2015; Liang et al., 2017). Studies have shown that the main long-term adverse reaction of PTX-concurrent radiotherapy is RIPF, of which the incidence of Grade II and III fibrosis could reach as high as 20% (Zhang et al., 2007; Dai et al., 2011), but the mechanism is not yet clear.

The core pathogenic mechanism of RIPF is the excessive activation of fibroblasts (FBs) (Thannickal et al., 2004; Henderson et al., 2020). Ras/Raf/ERK pathway serves an important signaling pathway for the proliferation and activation of FBs (Foglia et al., 2019; Ng et al., 2019; Wu et al., 2020), and targeting the Ras/Raf/ERK signaling pathway against liver fibrosis is underway in preclinical and clinical trials (Foglia et al., 2019). Levels of TGF-β1 (Wang et al., 2017; Thakur et al., 2021) and PDGF (Abdollahi et al., 2005; Dadrich et al., 2016) are often detected elevated in RIPF lung tissue, leading to receptor tyrosine kinases (RTKs) phosphorylation activation (Tsai et al., 2012), and further activating Ras/Raf/ERK through Grb2 and SOS (Gross et al., 2001;
Nussinov et al., 2020). The Sprouty family (Spry1-4) is the kind of widely-existing intracellular negative regulator of the RTK-related Ras/Raf/ERK signaling pathway (Gross et al., 2001; Masoumi-Moghaddam et al., 2014). Among Spry1-4, Spry2 protein demonstrates the strongest activity (Ahn et al., 2010). It is capable of binding to the SH3 domain of Grb2, thereby inhibiting the binding of Grb2 to SOS, consequently blocking the Ras/Raf/ERK signaling (Masoumi-Moghaddam et al., 2014; Yim et al., 2015). Spry2 protein binds to microtubules at rest (none of the other three members of the Sprouty family can bind to the cytoskeleton (Lim et al., 2000)). And two regions located respectively at the N-terminus (123-177) and the C-terminus (195-221) mediate the binding of Spry2 to microtubules (Lim et al., 2000). When stimulated by growth factors, Spry2 will detach from microtubules, undergoing membrane translocation and phosphorylation modification, and bind with Grb2 to inhibit the Ras/Raf/ERK pathway (Yim et al., 2015). Studies have shown that inhibiting Spry2 expression can upregulate Ras/ERK pathway activity and aggravate liver fibrosis (Huang et al., 2019b; Zhu et al., 2020).

PTX can specifically bind to β-tubulin, thereby blocking cell mitosis (Yang and Horwitz, 2017). Studies have found that PTX can up-regulate ERK activity in cells, which is related to the resistance of tumor cells to PTX (Seidman et al., 2001; Huang et al., 2019a). Overexpression of Spry2 can abolish PTX-induced c-Raf activation (Ahn et al., 2010), but there are no relevant studies yet to see clearly whether PTX induces Raf/ERK activation by downregulating the negative regulation of Spry2.

In this work, it was found that administration of PTX concurrently with irradiation could aggravate RIPF in mice, with down-regulation of membrane Spry2 and up-regulation of Raf/ERK activity in lung tissue at the same time. We explored the mechanism and found that PTX could
increase the binding of Spry2 with tubulin, thereby inhibiting the negative regulation of Spry2 on Raf/ERK signal pathway.

MATERIALS AND METHODS

Reagents

Antibodies (Supp. Reagents 1), cell culture reagents (Supp. Reagents 2), and other reagents (Supp. Reagents 3) used were described in the Supplementary Material-Reagents.

Mouse Models of Radiation-induced Fibrosis

C57BL/6 mice, SPF grade, male, 6-8 weeks old, were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. (SCXK (Zhe) 2019-0001) (Hangzhou, China). Studies have reported that a sex difference was observed in the C57BL/6 mice exposed to whole thoracic irradiation (Jackson et al., 2016), so we chose a single gender (male) to avoid the influence of this variable. A linear accelerator (Clinac 600 C/D; Varian Medical Systems, CA, USA) created an X-ray IR field of 3.8 × 1.2 cm² to cover the entire chest of each mouse with a total dose of 18 Gy. Pulmonary fibrosis gradually developed within 4-6 months after irradiation. The irradiated mice were randomly divided into RIPF group, RIPF+PTX (1 mg/kg, 5 mg/kg, 10 mg/kg) groups, and unirradiated normal mice comprised the control group, with 6 mice per group. PTX was administered intraperitoneally, once a week for 10 times, and the dosing schedule was shown in Fig. 1A. All animal experiments were institutionally approved and performed in accordance with the guidelines of Institutional Animal Welfare & Ethics Committee of Fujian Medical University (FJMU IACUC 2021-0465).

Pulmonary ventilation function test
The DSI Buxco® PFT Pulmonary Function Testing System (DSI, MN, USA) was used and the parameters adjusted according to the manufacturer's instructions. After the mice were anesthetized, tracheotomy was performed and a tracheal cannula was inserted, then the tracheal cannula was connected to the pulmonary function detection system, with FinePointe® software collecting the data of pulmonary ventilation function.

**Hydroxyproline assay**

100 μl of 12 M concentrated HCl was added to 100 μl of lung homogenate, capped tightly, and hydrolyzed at 120 °C for 3 h. Then 4 mg of activated charcoal mixed in and centrifuged at 10,000 ×g, the supernatant such obtained was transferred to a 96-well plate, evaporated to dryness by heating at 60 °C. The crystalline residue obtained was then dissolved and colored and analyzed at 560 nm with a microplate reader (ThermoFisher Scientific Inc., USA).

**Real-time polymerase chain reaction (RT-PCR)**

Total RNA was extracted from cells and lung tissue by using Trizol reagent (Invitrogen) and reverse transcribed. Then the real-time PCR analysis was done by using Hieff® qPCR SYBR® Green Master Mix (Yeasen Biotechnology Co., Ltd., Shanghai, China) and LightCycler®96 (Roche Diagnostics Corp., IA, USA). The primers were shown in (Supp. Table 1). β-actin (ACTB) was used as endogenous control.

**Western blot**

Total cell proteins were prepared with RIPA Lysis Buffer, and membrane proteins were extracted with Minute™ Plasma Membrane Protein Isolation and Cell Fractionation Kit (Invent Biotechnologies, Inc., Beijing, China). Then proteins were separated with 10% SDS-PAGE, then electrotransfer, antibody incubation, and imaging were executed as previously described (Guo et al.,...
2020). Antibodies used (anti-α/β-tubulin, #2148, anti-Na,K-ATPase, #3010, anti-Spry2, #14954, anti-Phospho-c-Raf (Ser338), #9427, anti-c-Raf, #53745, anti-p44/42 MAPK (Erk1/2), #4695, anti-Phospho-p44/42 MAPK (Thr202/Tyr204), #4370) were purchased from Cell Signaling Technology (MA, USA), and all antibodies were diluted at 1:1000. The experiment was repeated at least 3 times.

**Knockdown of Spry2 by siRNA**

siRNAs were designed and synthesized by Hanheng Biotechnology Co., Ltd. (Shanghai, China). NIH3T3 were transfected with si-Spry2s by using Lipofectamine™ RNAiMAX Transfection Reagent (ThermoFisher Scientific), and cultured for 24h for qPCR detection and 48h for Western blot detection. Si-Spry2-2 showed the highest knockdown efficiency (reaching 60%) and was used in subsequent experiments. The sequences of si-Ctrl and si-Spry2 were shown in (Supp. Table 2).

**Co-immunoprecipitation**

Total cell proteins were extracted and 20 μL of protein A/G Magnetic Beads (MedChemExpress, Shanghai, China) was added to the protein solution, incubated at 4 °C for 1 h, then the supernatant was collected by centrifugation so as to remove nonspecific binding. The supernatant was then incubated overnight with 10 μL of diluted Spry2 primary antibody at 4 °C, and subsequently 80 μL of protein A/G magnetic beads were added, incubated at 4 °C for 2 h. The immunoprecipitated complexes were thereby collected by centrifugation, and the protein was denatured for Western blot detection. The experiment was repeated at least 3 times.

**Immunofluorescence staining**

Fixed Cells and tissue sections were blocked at room temperature for 1 h, and then incubated overnight with primary antibody at 4 °C before they were rinsed and incubated with
fluorescein-conjugated secondary antibody in the dark for 1-2 h. Once again rinsed, they were examined under a fluorescence microscope (Zeiss, DE) to obtain imaging.

**Enzyme-linked immunosorbent assay (ELISA)**

The level of PDGF in mouse lung tissue was detected by ELISA. The Duoset ELISA kit for mouse PDGF was purchased from the R&D system (Minneapolis, MN, USA). Lung tissues of mice were collected before and 7d, 14d, 2m and 4m after thoracic irradiation, and three mice were collected at each time point. Then the level of PDGF in lung homogenate was detected according to manufacturer’s instruction, with lung homogenate from 3 mice in duplicate. The OD450 of each well was read with a microplate reader (Multiskan FC, ThermoFisher Scientific Inc.) and the concentration was obtained by converting the OD450 values against a standard curve.

**Sirus Red assay**

200 µl of sample or standard was mixed with 1 ml of 0.1% Sirus red F3B (Sigma-Aldrich) in saturated picric acid and placed on a shaker for 1 h at room temperature. Then the supernatant was carefully removed after centrifugation. Subsequently 1 ml of 0.1 M HCl was added, well mixed, centrifuged to remove the supernatant, then 0.5 ml of 0.5 M NaOH was added in for a whirling vortex. Thus the solution was transferred to a 96-well plate, and read at 550 nm with a microplate reader.

**Statistical analysis**

GraphPad Prism 8 software was adopted and one-way or two-way ANOVA analysis of variance was used for testing. P<0.05 indicated that the results were significant.

**RESULTS**
PTX could aggravate the pathological fibrotic changes and reduce pulmonary ventilation function of RIPF

C57BL/6 mice received 18 Gy thoracic irradiation, and concurrently were given 1, 5, and 10 mg/kg PTX respectively (Fig. 1A). The administration schedule of PTX is derived from the clinical treatment schedule (Supp. Table 3). The lung tissues were collected 4 months after irradiation, and it was found that septal thickening and excessive collagen deposition were even further aggravated in the RIPF+10 mg/kg PTX group compared with the RIPF group (Fig. 1B, 1C, 1D, 1E, Supp. Fig. 1). Dynamic compliance (Cdyn), pulmonary resistance (RI), forced vital capacity (FVC) and maximum inspiratory flow (PIF) were tested, and the results showed that compared with the RIPF group, the pulmonary ventilation function (FVC, PIF) and lung compliance (Cdyn) in the RIPF+10 mg/kg PTX group got further decreased with a risen pulmonary resistance (PIF, Fig. 1F).

Activation of FBs in lung tissue was detected after PTX-concurrent irradiation, accompanied with up-regulation of Raf/ERK activation and down-regulation of membrane Spry2 levels

Compared with the RIPF group, 10 mg/kg PTX treatment groups presented even higher levels of α-SMA (marker for FBs activation, Fig. 2A, 2B), higher mRNA levels of fibronectin, collagen I and collagen III (Fig. 2C), and higher levels of p-c-Raf and p-ERK (Fig. 2D).

By detecting the expression levels of total Spry2 (Fig. 2E) and membrane Spry2 (Fig. 2F), it was found that compared with the RIPF group, the level of total Spry2 in the 10 mg/kg PTX treatment group was not significantly changed, but that of membrane Spry2 was significantly down-regulated, with PTX 10mg/kg having a stronger downregulation effect than PTX 5mg/kg (Supp. Fig. 2). Consistent with Western blot results, immunofluorescence staining showed that compared with the normal group, the expression of Spry2 in RIPF lung tissue increased, and the
degree of its co-localization with membrane was higher. While in the 10 mg/kg PTX treatment group, the co-localization of Spry2 with tubulin increased (Fig. 2G).

**PTX reduced Spry2 membrane translocation by promoting microtubule aggregation, thereby upregulating the activation of Raf/ERK signaling pathway**

Cell viability experiments showed that 50 nM PTX had a strong killing effect on tumor cells (A549 and MCF-7), but had no significant effect on the viability of FBs (NIH3T3) (Fig. 3A). Referring to the dosage of PTX *in vitro* experiments (Supp. Table 4), we determined that the dosage of PTX was 50nM. The dynamic change of PDGF content in lung tissue after irradiation was detected, and it was found that PDGF remained at a high level from 7 days to 2 months after thoracic irradiation (Fig. 3B). Therefore, PDGF was used to stimulate FBs, and it was observed that the level of p-ERK reached the peak 3 h after treatment with 5 ng/ml PDGF (Fig. 3C). The dose of PDGF was determined by previous experiments (Supp. Fig. 3A). Treated with PTX 50 nM for 3 h, FBs were then stimulated with PDGF for another 3 h. The results showed that PDGF could up-regulate the activation of Raf/ERK pathways, and PTX pretreatment could further enhance the levels of p-c-Raf and p-ERK in cells (Fig. 3D). We knocked down Spry2 in NIH3T3 cells by siRNA (Fig. 3E, 3F). The experimental results showed that Spry2 knockdown abolished the effect of PTX on enhancing the phosphorylation of Raf/ERK stimulated by PDGF (Fig. 3G), confirming that PTX mainly regulates the activation of Raf/ERK pathway through Spry2.

PDGF stimulation can enhance the expression of total Spry2 in FBs, and also significantly increase the level of membrane Spry2 (Fig. 3H, 3I). PTX alone has no effect on total Spry2 and membrane Spry2 (Supp. Fig. 3B-C). PTX+PDGF had no significant effect on total Spry2 (Fig. 3H), but significantly reduced the membrane Spry2 content (Fig. 3I). Co-IP assay results showed that
PDGF stimulation could reduce the binding of Spry2 to tubulin compared with resting cells, while PTX significantly increased the binding of Spry2 to tubulin (Fig. 3J). Immunofluorescence staining result was consistent with this observation, PTX+PDGF resulted in tubulin aggregation and increased co-localization of Spry2 with tubulin (Fig. 3K).

Nocodazole is a microtubule-depolymerizing agent (Lin et al., 2022). Contrary to the effect of PDGF+PTX, PDGF+Nocodazole upregulated the membrane Spry2 content (Supp. Fig. 4B-C). Subsequently, we used Nocodazole for pretreatment and then administered PTX treatment. The results showed that the membrane Spry2 level (Fig. 4A-B) and microtubule binding level of Spry2 (Fig. 4C) in the PDGF+PTX+Nocodazole group were similar to those in the PDGF control group, indicating that PTX and Nocodazole overcame each other. The immunofluorescent staining revealed that Nocodazole pretreatment caused an increased co-localization of Spry2 with cell membrane (Fig. 4D). In addition, the microtubule depolymerizer abolished the up-regulation of p-c-Raf and p-ERK by PTX (Fig. 4E), indicating that the up-regulation of Raf/ERK phosphorylation activation caused by PTX was directly related to PTX’s enhancing Spry2 binding to microtubules.

PTX promoted FBs activation by upregulating activity of Raf/ERK signaling pathway

Compared with PDGF, combination of PTX and PDGF could significantly increase the activation of FBs (Fig. 4E-F) and the mRNA expression levels of ECM proteins (Fig. 4G), and consequently increased the amount of collagen secreted by FBs (Fig. 4H). Both Nocodazole (50 nM) and the ERK inhibitor PD98059 (10 μM) (Zelivianski et al., 2003; Rao et al., 2014) could effectively inhibit PTX promoting FBs activation, migration and collagen secretion (Fig. 4F-H; Supp. Fig. 5). Studies have shown that the IC50 value of PD98059 in cell experiments is 10 μM (Dudley et al., 1995).

Nintedanib alleviated PTX’s aggravating RIPF
Nintedanib is a targeted inhibitor of multiple RTKs, including VEGF, FGF, and PDGF receptors (Richeldi et al., 2014). It is approved by the U.S. Food and Drug Administration for the treatment of idiopathic pulmonary fibrosis. In this study, Nintedanib was used as a positive control drug for anti-pulmonary fibrosis. We added Nintedanib to the PTX-concurrent radiotherapy dosing regimen (Fig. 5A). The results showed that with 30 mg/kg or 60 mg/kg (Wollin et al., 2014; Wollin et al., 2015) Nintedanib administered, the pulmonary fibrosis lesions in mice were seen to be alleviated and pulmonary ventilation function improved, with the therapeutic effect of Nintedanib 60 mg/kg better than that of 30 mg/kg (Fig. 5B-F; Supp. Fig. 6A-B). In addition, compared with RIPF+PTX group, RIPF+PTX+Nintedanib (60 mg/kg) treatment significantly reduced the levels of p-c-Raf and p-ERK in lung tissue (Fig. 5G).

ERK inhibitor effectively relieved PTX-exacerbated RIPF

Nintedanib was used as a positive control drug to determine whether ERK inhibitor could alleviate RIPF aggravated by PTX (Fig. 6A). The results showed that 10 mg/kg PD98059 (Hong et al., 2016) intragastric administration could effectively alleviate the characteristics of fibrotic lesions aggravated by PTX such as collagen increment (Fig. 6B-E; Supp. Fig. 7A-B), and also effectively improve lung function. Compared with PTX combined with Nintedanib, PTX combined with ERK inhibitors presented similar therapeutic effect (Fig. 6B-F; Supp. Fig. 7A-B). The levels of Spry2 in lung tissues were determined, and it was found that either PTX alone or in combination reduced membrane Spry2 levels compared with the RIPF group (Fig. 6G-H). Nintedanib is capable of targeted inhibition of RTKs, so it can lower the occurrence of RTK-induced membrane translocation and phosphorylation of Spry2 (membrane translocation of Spry2 is necessary for its phosphorylation (Hanafusa et al., 2002)). However, compared with PTX alone, neither PTX
combined with Nintedanib nor PTX combined with PD98059 significantly downregulated the level of membrane Spry2 (Fig. 6H). This may be due to the fact that PTX administration inhibited the membrane translocation of Spry2 and upregulated the activity of Raf/ERK pathway, and the subsequent administration of Nintedanib or PD98059 directly downregulated the excessive activity of Raf/ERK pathway through their target inhibition, without significantly further downregulating the already inhibited Spry2 function.

**DISCUSSION**

This study is the first to show that PTX enhances Spry2 binding to microtubules and reduces Spry2 membrane translocation when stimulated by growth factors. Spry2 binds to microtubules at rest, but detaches from microtubules when stimulated by growth factors, translocates to the cell membrane and binds to its effectors such as Grb2 at the cell membrane to negatively regulate the Ras/Raf/ERK pathway (Masoumi-Moghaddam et al., 2014). Under the stimulation of growth factors or cytokines, many signaling proteins are transferred to the cell membrane site. The most common domain responsible for membrane localization of these proteins is the Pleckstrin Homology (PH) domain, which mediates proteins in localizing to the cell membrane by binding to PIP2 or PIP3. For instance, the PH domain-containing AKT will rapidly translocate from the cytoplasm to the membrane if stimulated by the growth factor (Agamasu et al., 2017). However, the PH domain (or C2 domain, which mediates the binding of proteins such as PKCs to diacylglycerols (Brandman et al., 2007)) is not contained in the Spry2 protein structure. Studies have shown that N-terminal sequences ∆123–177 and C-terminal sequences ∆195–221 of Spry2 mediate its binding to microtubules, while C-terminal sequences ∆178–237 mediates Spry2 membrane binding (Lim et al., 2000) with the mechanism of its membrane binding remained to be further elucidated. PTX is capable of exerting a
potent microtubule aggregation-promoting effect that disrupts normal microtubule dynamics. Our study showed that PTX inhibited the detaching of Spry2 from microtubules by promoting microtubule aggregation, which could be abrogated with the use of microtubule depolymerizing agents. Thus, it was indicated that the binding of Spry2 to microtubules might result in the Δ195–221 sequence (belonging to the membrane-bound sequence) not being exposed, and the amount of membrane binding reduced, thus limiting its negative feedback ability.

In this work, it was showed that the aggravation of RIPF by PTX was associated with the attenuated Spry2 negative regulation as well as the increased activation of Raf/ERK pathway and FBs. PTX is capable of exerting a radiosensitizing effect on tumor cells, which is based on the fact that PTX arrests hyperproliferative tumor cells in the G2/M phase by promoting the aggregation and stabilization of microtubules (Jin et al., 2007; Yang and Horwitz, 2017). Therefore, PTX is often administered concurrently with radiotherapy to exert a synergistic anti-tumor effect, which however may aggravate the damage to normal tissues caused by radiotherapy. Clinical cases (Zhang et al., 2007; Dai et al., 2011) as well as our study have shown that PTX can aggravate RIPF, the damage to normal lung tissue from thoracic irradiation. In light of the observation that ERK is the main signaling pathway that promotes FBs activating into more vigorous myofibroblasts (Foglia et al., 2019; Ng et al., 2019; Wu et al., 2020), and that PTX can up-regulate ERK activity (Seidman et al., 2001; Huang et al., 2019a), also with the findings that overexpression of Spry2 can abolish PTX-induced c-Raf activation (Ahn et al., 2010), we explored the mechanism by which PTX aggravates RIPF. Our results showed that PTX attenuated the negative regulation of Spry2 on the Raf/ERK pathway downstream of RTK by increasing the binding of Spry2 to microtubules, which in turn promoted FBs activation, migration ability, and secreting more ECM proteins, ultimately
aggravating RIPF. Studies have shown that PTX can up-regulate Raf/ERK phosphorylation by activating FoxM1/PHB1 (Huang et al., 2019a). Based on the specific effects of PTX in promoting microtubule aggregation and inhibiting microtubule depolymerization, we found that PTX could up-regulate the activity of Raf/ERK pathway by interfering with Spry2’s microtubule-binding activities, suggesting that the up-regulation of ERK activity by PTX is the effect of multiple pathways. In our experiments, PTX couldn’t significantly up-regulate the AKT pathway, which was consistent with the report of Patterson et al. (Patterson et al., 2006). However, some studies showed that Spry2 inhibited the EGF-stimulated PI3K/AKT pathway by dephosphorylating PTEN and increasing its activity at the plasma membrane (Edwin et al., 2006). Our results showed that PTX did not significantly change the level of p-PTEN, which might be in that the main function of Spry2 in cells varied with cell types and the stimulating growth factors (Masoumi-Moghaddam et al., 2014).

RIPF causes severe damage to lung function, so we need seek ways to alleviate the exacerbation of RIPF caused by PTX-concurrent chemoradiotherapy. Nintedanib is a multi-target RTK inhibitor for the treatment of IPF (Wollin et al., 2019). We found that Nintedanib effectively inhibited the up-regulation of p-ERK caused by PTX and alleviated the PTX-aggravated fibrotic lesions in RIPF. Based on our findings that PTX exacerbated RIPF mainly by upregulating ERK activation, we therefore investigated whether ERK inhibitors could alleviate RIPF exacerbated by PTX. Our results showed that ERK inhibitors presented a similar ability to alleviate PTX-related RIPF as Nintedanib. With the potential to be good antitumor drugs, several small-molecule ERK inhibitors are under clinical research (Samatar and Poulakakos, 2014). Tumor cells that demonstrate resistance to PTX often render up-regulated ERK activity (Huang et al., 2019a), so it is reasonable
to speculate that the combination of PTX and ERK inhibitors may well enhance the anti-tumor effect. Our experimental results even showed that the combination of PTX and ERK inhibitors could also obtain other benefits: Alleviating RIPF exacerbated by PTX and reducing the risk of RIPF.

In conclusion, PTX enhances the binding of Spry2 to microtubules and attenuates the negative regulation of Spry2 on Raf/ERK signaling pathway, thereby aggravating RIPF complications of thoracic radiotherapy. The use of ERK inhibitors and Nintedanib can effectively alleviate the aggravating effect of PTX on RIPF. This study was the first to explore the mechanism by which PTX aggravates RIPF and proposes effective drug targets to alleviate this adverse effect.

**Abbreviations**

α-SMA, alpha-smooth muscle actin; Cdyn, dynamic compliance; ECM, extracellular matrix; ERK, extracellular signal regulated kinase; FBs, fibroblasts; FVC, forced vital capacity; Grb2, growth factor receptor-bound protein 2; HE, hematoxylin-eosin staining; PDGF, platelet derived growth factor; PIF, peak inspiratory flow; PTX, paclitaxel; RIPF, radiation induced pulmonary fibrosis; RI, lung resistance; RTK, receptor tyrosine kinase; SOS, son of sevenless.
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Authorship Contributions

Participated in research design: Chun Chen.

Conducted experiments: Jianxing Zheng, Jiandong Wu, Lingfeng Xie and Yihao Huang.

Performed data analysis: Chun Chen and Jinsheng Hong.

Wrote or contributed to the writing of the manuscript: Jianxing Zheng, Chun Chen.

Footnotes

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Declaration of Competing Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are stored in an institutional repository and will be shared upon request to the corresponding author.
References


Figure legends

Figure 1. PTX significantly aggravated collagen deposition and pulmonary ventilation function of RIPF.

[A] Schematic diagram of animal experiments. PTX was administered in three doses, 1mg/kg, 5mg/kg and 10mg/kg, ip, once a week, 10 times in total. 2 times before and 8 times after the irradiation. The experiment was terminated 4 months after thoracic irradiation (n=6). [B and C] Mice lung tissues were collected at 4 months post irradiation. Then, collagen deposition in lung tissue was observed by Masson staining. Representative results are shown (scale bar: B: 500 μm; C: 50 μm). [D] Integrated Optical Density (IOD) was measured by Image Pro Plus software (n=6). [E] The content of collagen in lung tissue was determined by hydroxyproline assay (n=6). [F] Before euthanasia, the pulmonary ventilation functions of mice were determined by PFT system (n=6).

*P<0.05, **P<0.01. Error bars represent SD. PTX1: PTX 1 mg/kg; PTX5: PTX 5 mg/kg; PTX10: PTX 10 mg/kg. Cdyn, dynamic compliance; RI, lung resistance; FVC, forced vital capacity; PIF, peak inspiratory flow.

Figure 2. PTX induced a further enhancement of Raf/ERK signaling activation and reduced membrane Spry2 in RIPF lung tissue.

Lung tissue was collected from mice 4 months after PTX treatment combined with thoracic irradiation. [A] The expression of α-SMA (activation marker of FBs) in lung tissue was assayed by Western blot, and pixel value was analyzed by using ImageJ software (n=3). [B] PTX 10mg/kg group with significant difference was selected. The expression of α-SMA and vimentin (pan-marker for FBs) in lung tissue was assayed by immunofluorescent staining (scale bar: 20 μm). [C] The
mRNA levels of collagens and fibronectin in the lung tissues were determined by RT-PCR (n=6).

[D] The levels of the indicated signal proteins in lung tissues 4 months after irradiation were measured by Western blot. [E and F] The levels of total (E) and membrane (F) Spry2 was assayed by Western blot (n=3-4). [G] Fluorescence triple staining was used to detect the co-localization of Spry2 with cell membrane (DiO: membrane dye) and tubulin. The degree of co-localization of Spry2 with membrane and with tubulin was comparable in the Normal group. In RIPF group, there was a significant increase in the co-localization of Spry2 with membrane, while in the RIPF+PTX 10mg/kg group, there was a significant aggregation of tubulin, and the co-localization of Spry2 with tubulin was increased compared to the RIPF group. The arrows indicate typical images for each group. *P<0.05, **P<0.01. Error bars represent SD. PTX1: PTX 1 mg/kg; PTX5: PTX 5 mg/kg; PTX10: PTX 10 mg/kg.

Figure 3. In vitro, PTX upregulated the activity of Raf/ERK signaling pathway through regulating Spry2.

[A] The antiproliferative effect of PTX on Tumor cells (A549 and MCF-7) and FBs (NIH3T3) was assayed by CCK-8 method (n=5). [B] The dynamic levels of PDGF in lung tissue at multiple time points after irradiation were detected by ELISA (n=3). [C] The level of p-ERK in FBs at different time points after PDGF (5 ng/ml) stimulation was determined by Western blot. [D] FBs was stimulated with PDGF, and the effect of PTX (50 nM) on the levels of p-c-Raf and p-ERK were measured by Western blot (n=3). [E and F] NIH3T3 were transfected with si-Spry2s and control siRNA (si-Ctrl), and cultured for 24h for qRT-PCR detection [F] and 48h for Western blot detection [E]. *P<0.05, **P<0.01 vs Normal. Error bars represent SD. Si-Spry2-2 showed the highest
knockdown efficiency (reaching 60%) and was used in subsequent experiments. [G] After treating Spry2-knockdown cells with PDGF and PTX, the levels of p-c-Raf and p-ERK were measured by Western blot (n=3). [H and I] Effect of PTX on the levels of total (H) and membrane (I) Spry2 were determined by Western blot. [J and K] The binding of Spry2 to tubulin was determined by Co-IP (J) (n=3) and immunofluorescence staining (K) (scale bar: 5 μm). *P<0.05, **P<0.01.

Figure 4. Microtubule depolymerizer down-regulated the PTX-enhanced binding of Spry2 to tubulin, increased the amount of membrane Spry2, and inhibited the activation of Raf/ERK pathway.

FBs were treated with Nocodazole (50 nM, 3 h) and PTX (50 nM, 3 h) successively, and then stimulated with PDGF for 3 h. [A and B] The levels of total (A) and membrane (B) Spry2 was assayed by Western blot (n=3). [C and D] Nocodazole downregulated the binding of Spry2 to tubulin was determined by Co-IP (C) and Immunofluorescence (D) (scale bar: 5 μm). [E] Nocodazole downregulated the p-c-Raf and p-ERK levels were assayed by Western blot. [F] Nocodazole and ERK inhibitor (PD98059, 10 μM treated for 3 h) abolished PTX-induced α-SMA expression (n=3). [G and H] Nocodazole and ERK inhibitor blocked PTX-induced ECM protein production were measured by RT-PCR (G) (n=6) and Sirus Red method (H) (n=6). *P<0.05, **P<0.01. Error bars represent SD. Noco: Nocodazole; PD98: PD98059.

Figure 5. Nintedanib effectively relieves RIPF aggravated by PTX.

[A] Schematic diagram of animal experiments. The irradiated mice received PTX 10 mg/kg with or without Nintedanib 30 mg/kg or 60 mg/kg (po, once every 3 days, a total of 19 times). The
experiment was terminated 4 months after thoracic irradiation (n=6). [B and C] Collagen deposition in lung tissue 4 months post irradiation was observed by Masson staining. Representative results are shown (scale bar: B: 500 μm, C: 50 μm). [D] IOD was measured by Image Pro Plus (n=6). [E] The content of collagen in lung tissue was determined by hydroxyproline assay (n=6). [F] Before euthanasia, the pulmonary ventilation functions were determined by PFT system (n=6). [G] The protein levels of p-c-Raf and p-ERK in lung tissue was assayed by Western blot (n=3). *P<0.05, **P<0.01. Error bars represent SD. Nin30: Nintedanib 30 mg/kg; Nin60: Nintedanib 60 mg/kg.

**Figure 6.** ERK inhibitor effectively alleviates the RIPF aggravated by PTX.

[A] Schematic diagram of animal experiments. The irradiated mice received PTX 10 mg/kg with PD 98059 10 mg/kg (po, once every 3 days, a total of 19 times) or Nintedanib 60 mg/kg. The experiment was terminated 4 months after thoracic irradiation (n=6). [B and C] Collagen deposition in lung tissue at 4 months post irradiation was observed by Masson staining. Representative results are shown (scale bar: B: 500 μm; C: 50 μm). [D] IOD was measured by Image Pro Plus. (n=6). [E] The content of collagen in lung tissue was determined by hydroxyproline assay. (n=6). [F] Before euthanasia, the pulmonary ventilation functions were determined by PFT system (n=6). [G and H] The levels of total (G) and membrane (H) Spry2 was assayed by Western blot. *P<0.05, **P<0.01. Error bars represent SD.
Fig. 3 Zheng, et al.
Fig. 4 Zheng, et al
Fig. 5 Zheng, et al.
**Fig. 6 Zheng, et al.**

**A**

- **Injection**
- **Irradiation**
- **Detection point**

Day 1 8 12 15 16 19 22 25 28 29 31 64 67 70 132

- **PTX**
- **Nintedanib**
- **PD98059**

**B**

- **Normal**
- **RIPF**
- **RIPF+PTX**
- **RIPF+PTX+PD98**
- **RIPF+PTX+Nin60**

**C**

- **Normal**
- **RIPF**
- **RIPF+PTX**
- **RIPF+PTX+PD98**
- **RIPF+PTX+Nin60**

**D**

- Masson (panorama)
- Masson (enlarged)

**E**

- Masson area sum
- Hydroxyproline (μg/mg)

**F**

- Cdyn
- RI
- FVC
- PIF

**G (Total)**

- Spry2
- GAPDH

**H (Membrane)**

- Spry2
- Na,K-ATPase

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