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Original Article

The role of JAK/STAT3 signaling pathway on apoptosis of lung adenocarcinoma cell line PC-9 induced by icotinib

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Abstract: Objective: The aim of this study is to estimate the role of JAK/STAT3 signaling pathway on apoptosis of lung adenocarcinoma induced by icotinib. Methods: EGFR mutation was detected in lung adenocarcinoma cell line PC-9 by ARMS assay; The inhibitory rates of cell proliferation of PC-9 cells which were exposed to different concentrations of icotinib (0~100 μMol/L) for different time (24~72 h) respectively were evaluated by MTT assay; Apoptosis of PC-9 cells exposed to different concentrations of icotinib (0, 0.1, 1 and 10 μMol/L) for 48 h were evaluated by TUNEL assay; JAK2, STAT3, Bcl-2, Bax mRNA expressions were evaluated by Real-time PCR assay; The protein levels of P-STAT3 and IL-6 were evaluated by Western-blot assay. Results: Human lung adenocarcinoma cell line PC-9 had an exon 19 deletion mutation in EGFR gene; Followed by treatment of icotinib, the proliferation of PC-9 cells were all inhibited significantly, especially in 48 and 72 h ($P<0.01$) in all concentrations; The inhibitory rates of cell proliferation in different treating time had statistical significance ($P<0.01$); Cell apoptosis in different concentrations were increased significantly ($P<0.05$); Along with the increasing concentrations, gene expression levels of JAK2, STAT3 and Bcl-2 decreased significantly ($P<0.05$), Bax increased significantly ($P<0.05$), JAK2/STAT3 ratios increased significantly ($P<0.01$), and Bcl-2/bax ratios decreased significantly ($P<0.01$); P-STAT3 and IL-6 protein levels were inhibited significantly in higher concentration. Conclusions: JAK/STAT3 signaling pathway participates in apoptosis of PC-9 cells induced by icotinib. The most likely mechanism is icotinib inhibited the gene expression levels of JAK2, STAT3 and Bcl-2, so with the P-STAT3 and IL-6 protein levels, and mediated gene Bax overexpression.

Keywords: Icotinib, JAK/STAT3 signaling pathway, apoptosis, lung adenocarcinoma

Introduction

Lung cancer is currently the most frequent human tumour with the highest morbidity and mortality in the world [1]. Because of its non-specific clinical symptoms and strong tendency of metastasis, many patients are in advanced stages when they were diagnosed. In spite of many available treatments, the prognosis is still poor without significant development [2]. Therefore the study of lung cancer is still focus on the therapy. Currently, comprehensive treatment based on chemotherapy is still the popular therapy on advanced non-small cell lung cancer (NSCLC), but chemotherapy seems to enter a bottleneck period with a limited curative effect [3, 4]. We need to find new ways to treat NSCLC. With the pathogenesis of lung cancer is further studied and many related molecular targets have been found in succession, the molecular targeted therapy has become more and more important in recent years. Icotinib, a new type of EGFR tyrosine kinase inhibitor founded by China independently, attracts our eyeballs for its similar efficacy and better safety compared with other EGFR targeted drugs [5]. STAT3 is recognized as proto-oncogenes and the intersection of multiple signal transduction pathways related to tumor, which involves in a variety of tumor initiation and progression. As we all know, JAK/STAT3 is a classic way of STAT3 signal transduction and

Table 1. EGFR gene mutation detection of PC-9 cells

Exon	Item	Result
18	G719X	negative
19	Deletion	positive
20	S768I	negative
20	Insertion	negative
20	T790M	negative
21	L858R	negative
21	L861Q	negative

transcriptional activation. A variety of drugs play an anticancer role by inhibiting the JAK/STAT3 signal transduction pathway. But the Role of JAK/STAT3 signaling pathway on apoptosis of lung adenocarcinoma cell line PC-9 induced by icotinib is unclear now.

Materials and methods

Source of cell and drug

The human lung adenocarcinoma cell line PC-9 was purchased from American Type Culture Collection (ATCC). Icotinib was a gift from Zhejiang BetaPharma Co., Ltd.

Main reagents

DMEM (High Glucose); The fetal bovine serum, 0.25% Trypsin-EDTA, DMSO (Hyclone); AmoyDX TYMS Gene Expression Analysis Kit (Amoy Diagnostics Co., LTD.); MTT (Sigma); In Situ Cell Death Detection Kit (Roche); Primers Designing of JAK2, STAT3, Bcl-2, Bax and β-actin (Pioneer Technology Biotechnologies Co., Ltd.); TRIzol reagent (Invitrogen); PrimeScript RT reagent Kit (TaKaRa); SYBR Premix Ex TaqII (TaKaRa); Human Phospho-STAT3 Antibody (R&D); Rabbit Anti-human IgG/HRP (Zhong Shan Golden Bridge Biotechnologies Co., Ltd.); Tris, SDS, Glycine, Glycerol, APS, TEMED, Urea, PMSF, Bradford protein assay kit (Bio-Rad); Polyvinylidene Fluoride Membrane (Xi'an Wolsen Bio-technology Co., Ltd.); 75% ethyl alcohol, chloroform, isoamyl alcohol and so on other reagents are domestically produced analytically pure reagents.

Cell culture and detection of EGFR gene mutation

lung adenocarcinoma cell line PC-9 was cultured in DMEM medium with 10% FBS, at 37°C, 5% CO_2 incubator for 2-3 days and then digestion by 0.25% Trypsin-EDTA. Cells at exponential phase were digested, centrifuged and suspended for DNA extraction. Then we detected EGFR gene mutation by ARMS assay with AmoyDX TYMS Gene Expression Analysis Kit.

Cell proliferation detection by MTT analysis

Briefly, 200 μL of cell suspension containing an initial population of 5×10^3 PC-9 cells was added to three 96-well plates. The cells were allowed to attach and spread typically for 24 h to reach a stable baseline before the addition of particle suspension, and the old culture medium was aspirated from the cells. Subsequently, 100 μl of treatment medium containing different concentrations of icotinib (0 μMol/L (control), 0.001 μMol/L, 0.01 μMol/L, 0.1 μMol/L, 1 μMol/L, 10 μMol/L, 100 μMol/L) were added to each well. The E-plates were continued to incubate for 24, 48 and 72 h. 20 μl of medium containing 5 mg/ml MTT was added to E-plats at the corresponding time points and continued to incubate for 4 h, the medium was aspirated from the cells followed by the addition of 150 μl DMSO with oscillating for 10 min. Absorbance in each well was detected by enzyme-labeled instrument at 490 nm wavelength. The wells containing PBS was background reading and each test was run three times.

Detection of cell apoptosis by TUNEL analysis

Cells were incubated in 24-well plates with an initial population of 3×10^4 PC-9 cells and treated with icotinib at various concentrations (0 μMol/L (control), 0.1 μMol/L, 1 μMol/L, 10 μMol/L) for 48 h. Cell apoptosis was detected by operating instructions of in situ cell death detection kit. Six views were selected randomly in low magnification for count. The test was run three times.

Real-time fluorescence quota PCR analysis

The treatment medium containing different concentrations of icotinib (0 μM (control), 0.1 μM, 1 μM, 10 μM) was added to PC-9 cells for 48 h. Subsequently, total RNA was extracted from cells and identified. Then RNAs were transformed to cDNAs by reverse transcriptase and specific primers of JAK2, STAT3, Bcl-2 and Bax were used for Real-time PCR amplification.

Western-blot analysis

After exposure to icotinib at different concentration (0 μMol/L, 0.1 μMol/L, 1 μMol/L, 10

Table 2. Cells growth inhibitory detection by MTT analysis

Concentration (μMol/L)	24 h	48 h	72 h
0 (control)	0.00±0.00**	0.00±0.00**	0.00±0.00**
0.001	5.45±0.81	10.08±0.95**	16.51±1.36**
0.01	6.23±1.04	19.47±1.40**	31.53±0.80**
0.10	9.91±1.50*	30.42±1.19**	53.92±0.96**
1	18.75±2.26**	42.49±1.83**	71.03±1.08**
10	29.49±2.27**	61.66±1.48**	83.43±1.01**
100	42.72±1.29**	79.36±1.12**	94.28±1.08**

At same time, comparisons among the different concentration, *P<0.05, **P<0.01; In same concentration (except the control group), comparisons among the different time, P<0.01.

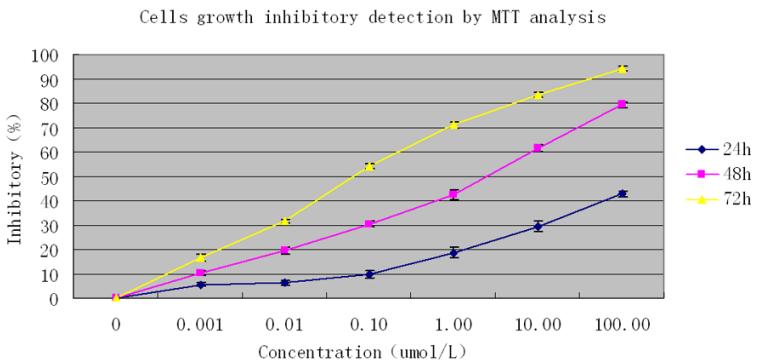

Figure 1. Cells growth inhibitory detection by MTT analysis.

μMol/L) for 48 h. PC-9 cells were lysed in 150 μl of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4, 0.5 μg/ml leupeptin, 1 mM PMSF). Subsequently, lysates were centrifuged at 12,000 rpm for 5 min at 4°C. Protein concentrations of the supernatants were determined by using BCA Protein Assay kit. Samples containing 60 μg of total protein were boiled and separated by SDS-PAGE (10% SDS tricine gel), then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA and incubated overnight at 4°C with monoclonal anti-phospho-STAT3, anti-IL-6, antiβ-actin. Post-incubation with HRP-conjugated secondary antibody (1:1000) for 2 h at room temperature, membranes were washed extensively with 0.1% Tween20 in TBS, and antigenic bands were visualized by ECL system (Syngene) according to the manufacturer's protocol. The grey scale values were analyzed by Gelpro Analyzer 4.0 (Media Cybernetics, Inc.).

Statistical analysis

The results are expressed as the mean value and standard error of the mean. Statistical significance was analyzed by one-way analysis of variance using SPSS 16.0 (SPSS, Chicago, IL). $p<0.05$ was considered statistically significant.

Results

The normal cellular morphology

Under inverted microscope, PC-9 cells were irregularly shaped and adherently grew well. The junction between cells is tight. Big, irregular nucleus with multiple nucleoli could be seen in the cytoplasm.

The detection result of EGFR mutation

As exons 18, 19, 20 and 21 mutations were regarded as the most common EGFR mutations, these four exons were detected by ARMS assay in PC-9 cells in present research. The result showed that only exon 19 deletion mutation in EGFR gene occurred steadily in PC-9 cells which indicated better efficacy of EGFR-TKI. The exon 18, 20, 21 were wild type without mutation (**Table 1**).

Growth inhibitory effect of icotinib on PC-9 cells

The MTT result showed that the proliferation of PC-9 cells were changed significantly followed by treatment with icotinib at different concentrations ($P<0.01$). By the time of exposure to icotinib (24 h, 48 h, 72 h), the inhibitory rate of cells proliferation at the same concentration also has statistical significance especially in 48 and 72 h ($P<0.01$). The calculations of the IC_{50} value at 48 h and 72 h exposure periods were 2.80 μMol/L and 0.08 μMol/L respectively (**Table 2** and **Figure 1**).

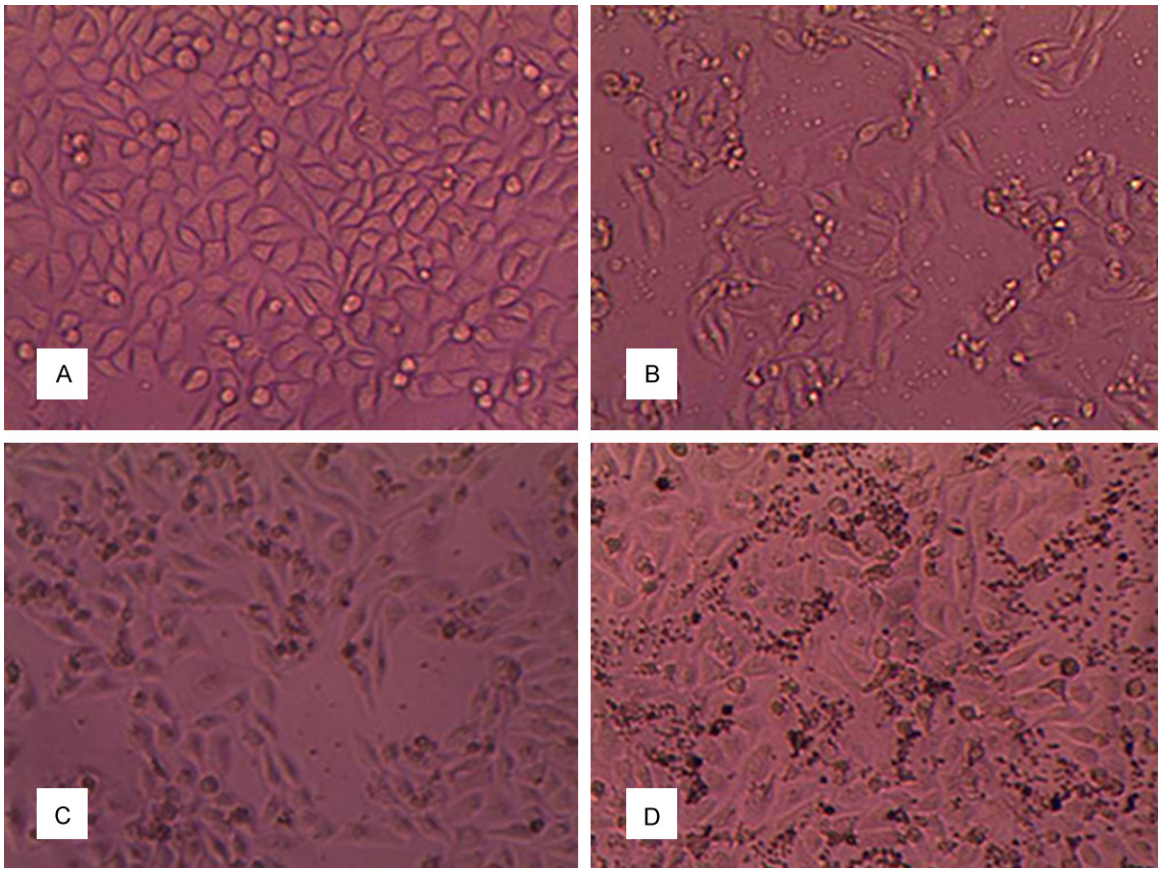

Figure 2. The morphological change of PC-9 cells (magnification ×100) treated with icotinib at various concentrations. A. PC-9 cells exposure to 0 μMol/L icotinib for 48 h. B. PC-9 cells exposure to 0.1 μMol/L icotinib for 48 h. C. PC-9 cells exposure to 1 μMol/L icotinib for 48 h. D. PC-9 cells exposure to 10 μMol/L icotinib for 48 h.

Table 3. Apoptosis of PC-9 cells treated with icotinib at various concentrations by TUNEL analysis

	Concentration (umol/L)			
	0 (control)	0.1	1	10
Cells apoptosis	2.55±0.68	5.78±1.08	11.06±1.64	24.56±2.11

Comparisons among groups, P<0.05.

The morphological change of PC-9 cells treated with icotinib at various concentrations

After exposed to icotinib at various concentrations (0, 0.1, 1 and 10 μMol/L) for 48 h, PC-9 cells were observed about morphological change under inverted microscope. The results showed that with the increase of concentration, the junctions between cells were looser, the volumes of cells were smaller, more chromatins were condensed and more karyopyknosis occurred. In addition, the higher the drug concentration, the earlier apoptotic body appeared and the more cell debris can be seen (**Figure 2**).

Apoptosis effect of icotinib on PC-9 cells

Apoptotic bodies labeled with GFP/FITC were confirmed by fluorescence microscope. The result showed that there were more apoptotic bodies labeled with green in higher drug concentration and a concentration-dependent manner can be observed, which was agreed with the results of the morphological change in recent research (**Table 3**, **Figures 3** and **4**).

Gene expression level detection by real-time fluorescent PCR

Gene expression levels of JAK2 and STAT3 were inhibited significantly with increasing concentrations ($P<0.05$); JAK2/STAT3 ratio was increased significantly in different concentrations ($P<0.01$). That means the inhibitive effect on STAT3 by icotinib is higher than that on JAK2;

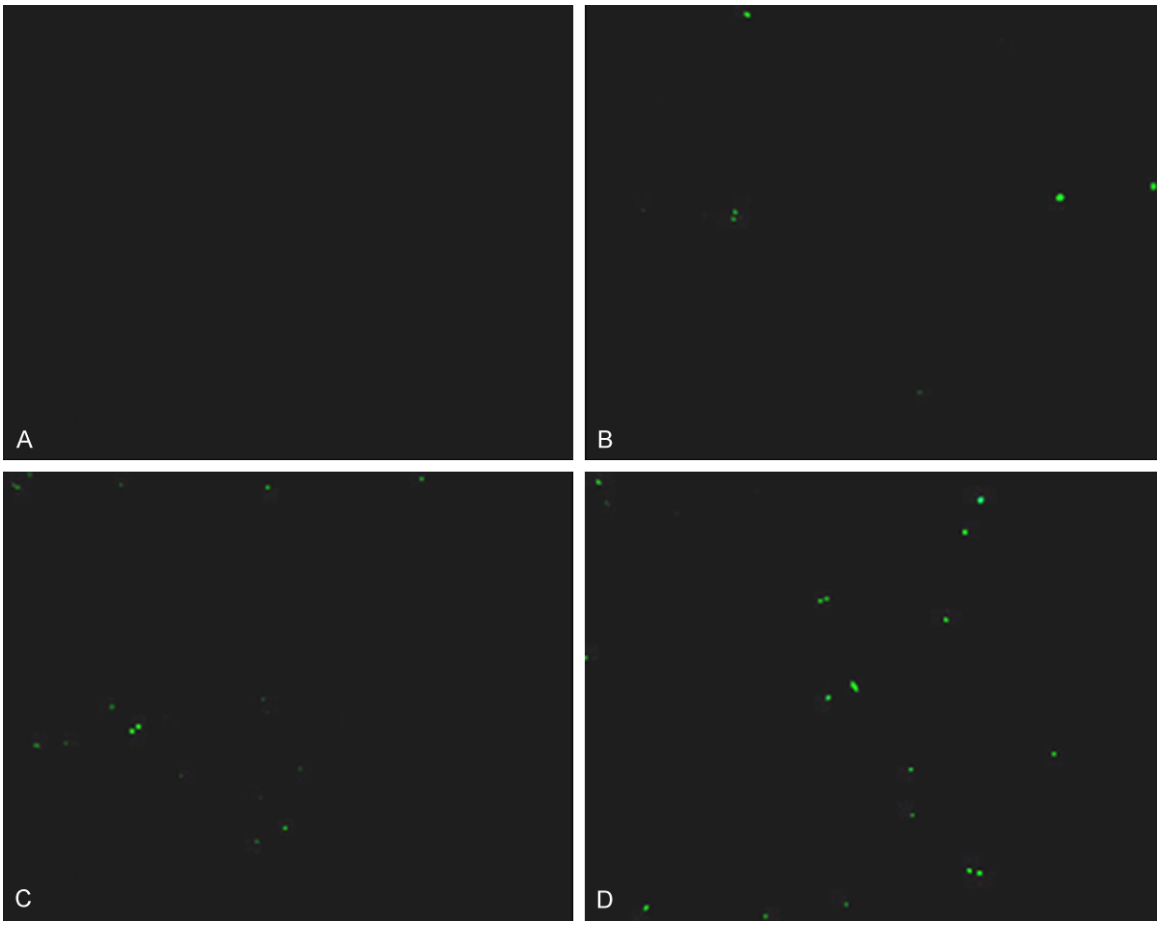

Figure 3. Apoptosis of PC-9 cells (magnification ×100) treated with icotinib at various concentrations by TUNEL analysis. A. PC-9 cells exposure to 0 μMol/L icotinib for 48 h. B. PC-9 cells exposure to 0.1 μMol/L icotinib for 48 h. C. PC-9 cells exposure to 1 μMol/L icotinib for 48 h. D. PC-9 cells exposure to 10 μMol/L icotinib for 48 h.

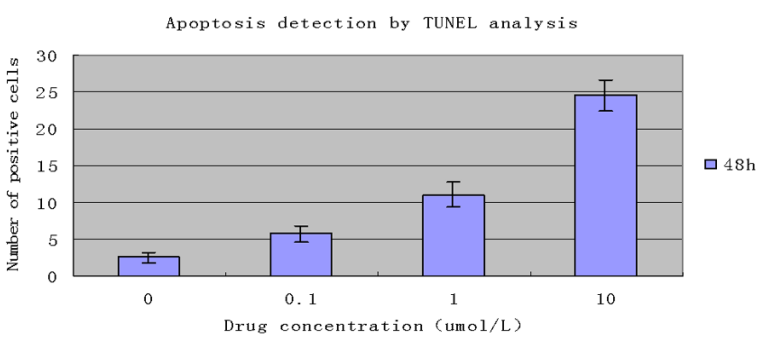

Figure 4. Apoptosis of PC-9 cells treated with icotinib at various concentrations by TUNEL analysis.

Bcl-2 expression level was inhibited in high concentration ($P<0.05$); Bax expression level was increased significantly in higher concentration ($P<0.05$); Bcl-2/Bax ratio decreased significantly in higher concentration ($P<0.01$). Therefore, icotinib had a negative effect on the expression of Bcl-2 gene and a positive effect on that of Bax gene (**Table 4**, **Figure 5**).

Proteins expression level detection by western-blot analysis

The results of grey analysis demonstrated the reducing trends of P-STAT3 and IL-6 protein levels as drug concentration increased (**Figure 6**). IL-6 was an important downstream molecule of JAK/STAT3 signaling pathway. The overexpression of these two proteins indicated the activation of JAK/STAT3 pathway.

Discussion

It's reported that [5], patients with EGFR mutation accounted for about 71.1% of advanced

Table 4. JAK2/STAT3 related genes' $2^{-\Delta\Delta CT}$ value of PC-9 cells treated with icotinib at various concentrations for 48 h

Gene ($2^{-\Delta\Delta CT}$)	Concentration (μMol/L)			
	0 (control)	0.1	1	10
JAK2	1.000±000	0.852±0.062	0.743±0.064	0.490±0.069*
STAT3	1.000±000*	0.588±0.099*	0.412±0.051*	0.140±0.026*
Bcl-2	1.000±000*	0.768±0.052	0.691±0.041	0.225±0.030*
Bax	1.000±000*	2.057±0.094	2.267±0.097	4.593±0.112*
Bcl-2/Bax	1.000±000*	0.373±0.013*	0.305±0.007*	0.049±0.006*
JAK2/STAT3	1.000±000*	1.464±0.143*	1.809±0.097*	3.506±0.173*

Comparisons among same gene group, $P<0.05$, $^*P<0.01$.

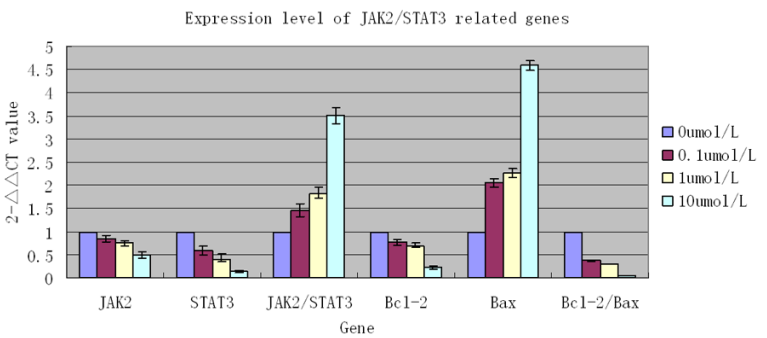

Figure 5. JAK2/STAT3 related genes' expression level of PC-9 cells treated with icotinib at various concentrations for 48 h.

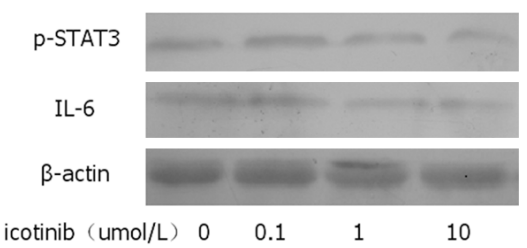

Figure 6. Effects of icotinib on P-STAT3 and IL-6 protein expressions. The expression of these proteins was analyzed in cells following treatment with various concentrations of icotinib (0-10 μMol/L).

NSCLC, of which exon 19 deletions were approximately 32%; The ORR and DCR of patients with EGFR mutation after treatment with icotinib were 54.1% and 93.5% respectively. The clinical efficacy was ideal. Human lung adenocarcinoma cell line PC-9 with an exon 19 deletion mutation in EGFR gene, which was chosen in present study, was sensitive to icotinib. The result of MTT analysis showed that icotinib had an obviously concentration- and time-dependently inhibitory effect, which was consistent with literature reports [6-8]. The PC-9 cells growth curve was previously monitored after treatment with icotinib by real-time cell electronic sensing (xCELLigence) system and the IC_{50} value of 24 h, 48 h, 72 h was less than 1.25 μMol/L [8]. The present study showed that the calculation of the IC_{50} value at 48 h and 72 h exposure periods was higher at 2.80 μMol/L and 0.08 μMol/L respectively [8]. This discrepancy is possibly due to experimental conditions, as the IC_{50} value of human lung adenocarcinoma cell line A431 and HCC827, which were sensitive to icotinib, has been reported at 0.04 and 0.15 μMol/L, similar to our study [6]. The IC_{50} value of icotinib was also similar to that of gefitinib as previously reported, although with some discrepancy [6-8]. In a recent study, IC_{50} value of gefitinib was about 0.05 μMol/L, which was lower than that in our study [9, 10]. However, whether the curative effect of icotinib is superior to that of gefitinib remains inconclusive due to lack of parallel experiment in our current study.

Icotinib has been shown to promote apoptosis of lung adenocarcinoma cell by flow cytometric analysis with 7-AAD and Annexin V-FITC, and this effect was concentration-dependent [11]. Here, we confirmed that this concentration-dependent effect by another common apoptosis detection method-TUNEL analysis.

It was proved that the suppression of STAT3 activity could sensitize gefitinib-resistant non-small cell lung cancer cells [11]. But the role of STAT3 in icotinib inducing lung adenocarcinoma cell apoptosis has not been reported. The present experiment observed the role of JAK/STAT3 signal pathway by detecting expression levels of gene JAK2, STAT3, Bcl-2, Bax and key active proteins-P-STAT3 and IL-6. The results showed that a concentration-dependent reduction in mRNAs of JAK2 and STAT3, so with P-STAT3 and IL-6 proteins. These meant that the excitation of JAK/STAT3 signal pathway was inhibited

significantly. It was also revealed that JAK2/STAT3 ratio was increased significantly with the drug concentration increased, which meant the inhibitive effect of icotinib on STAT3 was stronger than that on JAK2, which may be associated with the suppression of EGFR expression and phosphorylation. EGFR-mutated cell lines usually have a high expression and phosphorylation of EGFR. The downstream pathways, including EGFR-STAT3, are activated to promote proliferation of tumor cells [7]. By inhibiting EGFR-STAT3 signaling pathway, icotinib can promote lung carcinoma cell apoptosis.

Bcl-2 and Bax both belong to Bcl-2 gene family. They are representative regulatory proteins of mitochondrial apoptotic pathway and fruitful discussed. Bcl-2/Bax ratio, to a certain extent, is a decisive factor of cellular fate. In present research, Bcl-2 expression had positive correlation with drug concentration, while Bax expression had negative correlation with it. So Bcl-2/Bax ratio decreased along with increasing drug concentration. The cellular sensitivity to apoptosis signal was enhanced and apoptosis was promoted after icotinib stimulating cells. Meanwhile, the promoting effect was concentration-dependent, similar to JAK2 and STAT3. Icotinib can promote apoptosis of lung adenocarcinoma cell by inhibiting activation of JAK/STAT3 signal pathway and the expression of Bcl-2 and Bax gene.

STAT3 signal pathway plays an important role in tumorigenesis and tumor progression. Many drugs play a role of anti-tumor by inhibiting this pathway, such a celecoxib, which inhibiting this pathway mediated by IL-6 [12]. Therefore, STAT3 may be a promising molecular target of lung cancer therapy. The expression level of P-STAT3 was previously shown to be negatively correlated with apoptosis of tumor, which was consistent with our observation [13]. Our study also showed that P-STAT3 protein level was inhibited significantly in high concentration as previously reported [7]. The changing tendency of P-STAT3 protein was consistent with that of P-EGFR protein, which implied that the expression level of P-STAT3 could become an important molecular marker on whether using EGFR-TKI as Non-small cell lung cancer therapy [14].

Combination therapy based on chemotherapy has positive significance for patients with malignant tumor, but the occurrence of drug resistance is one of the most common and difficult problem to be solved for the failure of chemotherapy. Although there are some new antitumor drugs developed and widely used in clinical, there is still an inevitable question-the emergence of drug resistance. This is also the key factor which limits the drug effect. So, clarify antitumor drug resistant mechanism and looking for reversal agents of oncology drug resistance are important tasks of anti-tumor drugs research. Tumor proliferation, apoptosis, invasion and metastasis are result of synergy of several multi-signaling pathways. Therefore, a targeted therapy of single target or inhibitor of single pathway may be meaningless if there is a compensatory pathway. STAT3 is intersection of multiple signal transduction pathways, and can be activated by multi-signaling pathways. So, it has a closely related to the emergence of oncology drug resistance. In vitro study [15], STAT3 was highly activated in gefitinib-resistant lung cancer cells with T790M mutation. Chiu [11] considered that the suppression of STAT3 activity sensitized gefitinib-resistant non-small cell lung cancer cells. Byers [16] thought JAK/STAT3 was a compensatory pathway that allowed NSCLC survival. This study showed that icotinib could promote apoptosis of lung adenocarcinoma cell by inhibiting the activation of JAK/STAT3 signal transduction pathway and the expression of Bcl-2 gene, IL-6 protein while promoting the expression of Bax gene. Consequently, we suspected that abnormal activation of STAT3 may help NSCLC survive from icotinib by regulating Bcl-2 family. Similarly, combination with suppression of STAT3 activity will enhance pharmic antitumor effect.

Disclosure of conflict of interest

None.

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