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FoxM1 inhibition enhances chemosensitivity of docetaxel-resistant A549 cells to docetaxel via activation of JNK/mitochondrial pathway

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Abstract

Docetaxel is recommended as a second-line chemotherapy agent for the non-small-cell lung cancer (NSCLC); however, drug resistance greatly limits its efficiency. Forkhead box M1 (FoxM1), an oncogenic transcription factor, is believed to be involved in the chemoresistance of various human cancers; whereas the association of FoxM1 with acquired docetaxel-resistance in NSCLC remains unclear. In the present study, we investigated the involvement of FoxM1 in the docetaxel-resistant human lung adenocarcinoma A549 cells (A549/DTX). Our results showed that FoxM1 expression was significantly increased in the A549/DTX cells compared with that in the parental A549 cells. FoxM1 siRNA silencing promoted the cytotoxic and pro-apoptotic effect of docetaxel in A549/DTX cells, which was possibly mediated through inducing the activation of c-Jun N-terminal kinases/mitochondrial signaling pathway. Our results suggest a critical role of FoxM1 in docetaxel-resistance of the A549 cells and form the basis for the development of combined therapy of docetaxel and FoxM1 depletion in treating NSCLC.

Key words: non-small-cell lung cancer, docetaxel, FoxM1, chemoresistance

Introduction

Lung cancer is one of the leading causes of cancer-related deaths in humans. It is still increasing both in the prevalence and mortality worldwide [1]. The non-small-cell lung cancer (NSCLC) accounts for 80%–85% of lung cancers; while the incidence of the small-cell lung cancer has been decreased over the last two decades [2,3]. Radical surgery remains the cornerstone of treatment for early-stage NSCLC; however, for advanced-stage of this disease, chemotherapy has been the optimal treatment choice [4,5]. Docetaxel, a semi-synthetic taxane, was the first agent to show efficacy in the second-line treatment of NSCLC, and has since become a mainstay of NSCLC [6]. Unfortunately, it appears that cancer cells can acquire resistance to docetaxel during treatment, which has greatly limited the life-expanding effect of this chemotherapeutic agent [7]. Understanding the molecular mechanism underpinning such chemoresistance is fundamental to the development of therapeutic strategies to overcome this adverse effect.

Forkhead box M1 (FoxM1) is a transcription factor with a conserved forkhead/winged-helix 100-amino-acid DNA-bind domain that is responsible for the binding of Fox proteins to its consensus sites [8]. FoxM1 was identified to play an important role in cell cycle by promoting the G1-S transition through multiple mechanisms [9]. In addition to being a cell cycle transcription factor, FoxM1 also has the function of increasing resistance of cancer cells to apoptosis, inducing replicative immortality, stimulating angiogenesis, contributing to invasion and metastasis, and enabling genomic instability and inflammation [10,11]. Recent studies have revealed that FoxM1 is overexpressed in a board range of solid tumors including pancreatic, prostate, liver, breast, and lung carcinomas, which is closely associated with the poor treatment outcome in these diseases [12–15].

It has been shown that overexpression of FoxM1 could lead to an enhanced docetaxel resistance in gastric cancer cells [16]. FoxM1 protein was previously reported to be essential for cell proliferation during the progression of human lung cancer; however, the association of this protein with acquired docetaxel-resistance in NSCLC has not been elucidated.

In this study, the involvement of FoxM1 in the docetaxel-resistance of human lung adenocarcinoma A549 cells was investigated for the first time. In addition, the underlying molecular mechanisms of such effect were also explored.

Materials and Methods

Chemicals and reagents

Docetaxel, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and SP600125 were purchased from Sigma-Aldrich (St Louis, USA). Annexin V-FITC and PI apoptosis detection kit was obtained from BD Biosciences (San Jose, USA). The primary antibodies against FoxM1, p-c-Jun N-terminal kinases (JNK), JNK, Bax, Bcl-2, cytochrome c, β-actin, and prohibitin, as well as the HRP-conjugated secondary antibody were purchased from Santa Cruz biotechnology (Santa Cruz, USA). Caspase-3 and Caspase-9 activity assay kits were obtained from BioVision (Milpitas, USA).

Cell line and culture

The human lung adenocarcinoma A549 cells and the docetaxel resistant derivative A549/DTX cells were provided by Dr Yunying Xie's laboratory (Institute of Medicinal Biotechnology, Chinese Academy of Medical Science & Peking Union Medical College, Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml stretomycin, and 2 mM glutamine. Cell cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. A549/DTX cells were cultured in complete DMEM medium without docetaxel for 3 d before being used in experiments.

Real time-PCR analysis

Total cellular RNA was isolated from cultured cells using Trizol reagent (Life Technologies, Carlsbad, USA). Quantitative analysis of mRNA was performed with SYBR green PCR Master Mix (TaKaRa, Shiga, Japan) using ABI7500 sequence detection system (Life Technologies). The primers for FoxM1 and β-actin were used as previously reported to insure the binding efficiency and specificity of PCR reaction [17]. Each sample was run in duplicate. β-actin was used as an endogenous control.

Western blot analysis

Cell lysate was run on a 10% SDS polyacrylamide gel and then transferred to a polyvinylidene difluoridepolyvinylidenefluoride membrane. The membrane was then blocked with 5% bovine serum albumin in Tris-buffer saline with Tween 20 for 1 h, followed by incubation with primary antibody overnight and then with secondary antibody for 1 h at room temperature. After extensive wash, membrane was detected using an ECL detection system (Amersham, Buckinghamshire, UK). The photographic density of the band was quantitated by a gel documentation and analysis system (Alpha Imager 2000; Alpha Innotech, San Leandro, USA). β-actin was used as an endogenous control.

Cell viability assay

Cell viability was determined by MTT method as previously described [18]. Cells (1×10^4 cells/well) were plated in 96-well plate and cultured overnight. After treatment with indicated drugs for 24 h, 10 μl of MTT stock solution (5 mg/ml) was added and the culture was continued for 4 h at 37°C. Then the medium was removed and 150 μl DMSO was added to each well and shaken carefully. The absorbance was measured at 570 nm with an ELISA reader (ELX800; Bio-TEK, Winooski, USA). Cell viability was expressed as a percentage of the value against the untreated control group.

Cell apoptosis assay

Cell apoptosis was done by Annexin V-FITC/PI double staining using flow cytometry analysis. Cells (5×10^5 cells/well) were plated in 6-well plates and cultured overnight. After treatment with indicated drugs for 24 h, cells were washed with pre-chilled phosphate buffer saline (PBS) at 4°C. The pellet (1×10^5 cells/ml) was re-suspended in binding buffer. With the addition of Annexin V-FITC and PI, cell suspension was incubated for 15 min at room temperature in the dark. After filtration, the suspension of each group was processed to the FASCcan Flow Cytometer (Becton-Dickinson, San Jose, USA) and data was analyzed with the CellQuest software (Becton-Dickinson).

Cytochrome c release assay

The cytosol and mitochondrial fractions were isolated from cells using Apo Alert Cell Fractionation Kit (Clontech, Mountain View, USA) as described previously [19]. The expression of cytochrome c was determined by western blot analysis using the monoclonal antibody against cytochrome c as described previously [19]. β-actin was used for normalization of cytosol fraction and prohibitin was used for normalization of mitochondrial fraction.

Caspase activity assay

For detection of caspase activation, cells were lysed in caspase assay buffer and incubated with caspase-3 substrate DEVD-AFC or caspase-9 substrate LEHD-AFC at 37°C for 30 min. The AFC fluorescence absorbance was quantified using a spectrofluorometer (Molecular Devices, Sunnyvale, USA). Then the activity was determined by measuring the relative fluorescent intensity at 505 nM following excitation at 400 nm.

Statistical analysis

Statistical analyses were conducted using the GraphPad Prism 5.0 and SPSS 16.0 software packages. Data were presented as the mean ± SE and were analyzed using the Student's *t*-test. Significant difference was set at $P < 0.05$.

Results

FoxM1 silencing in A549/DTX cells

The up-regulation of FoxM1 has been previously reported to be closely associated with the chemoresistance of various human cancers including lung cancer; however, it still needs to be further explored in detail. In this study, the expression of FoxM1 in A549 and A549/DTX cells was first evaluated. As shown in **Fig. 1**,

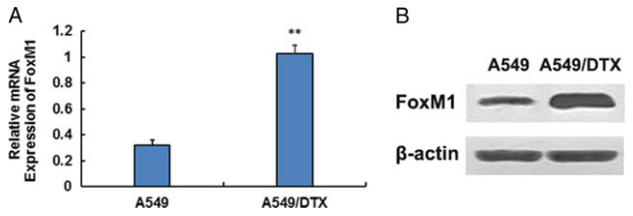

Figure 1. The expression of FoxM1 in A549 and A549/DTX cells The relative mRNA and protein levels of FoxM1 were assessed by RT-PCR (A) and western blot analysis (B), respectively. All experiments were conducted independently three times and values are expressed as the mean ± SE. $^{**}P < 0.01$ vs. A549.

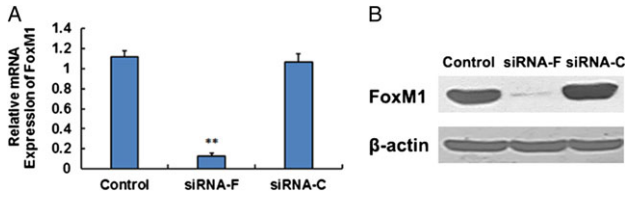

Figure 2. FoxM1 silencing in A549/DTX cells The relative mRNA and protein expression levels of FoxM1 in A549/DTX cells with or without FoxM1 knockdown were assessed with RT-PCR (A) and western blot analysis (B), respectively. A non-targeting scrambled siRNA was used as the negative control in these experiments. All experiments were conducted independently three times and values are expressed as the mean ± SE. $^{**}P < 0.01$ vs. control.

FoxM1 expression in A549/DTX cells was ~4.2-fold higher than that in parental A549 cells at both transcriptional and translational levels, which suggested that FoxM1 expression might be correlated with docetaxel resistance. To further explore the role of FoxM1 in docetaxel resistance, small interfering RNA technology was used to knockdown FoxM1 expression in A549/DTX cells. As shown in **Fig. 2**, FoxM1 silencing was achieved at both mRNA and protein levels in A549/DTX cells transfected with FoxM1 siRNA compared with cells transfected with scrambled siRNA.

FoxM1 silencing sensitizes A549/DTX cells to cytotoxicity induced by docetaxel

After exposure to docetaxel, the viability of A549/DTX cells with or without FoxM1 knockdown was assessed with MTT assay. As shown in **Fig. 3**, docetaxel exhibited a mild cytotoxic effect on the growth of A549/DTX cells (IC_{50} = 52.17 μM); however, such effect was much more potent in the FoxM1-knockdown cells (IC_{50} = 8.34 μM). These results indicated that FoxM1 silencing sensitized A549/DTX cells to docetaxel through inducing cell growth inhibition.

FoxM1 silencing sensitizes A549/DTX cells to apoptosis induced by docetaxel

After exposure to docetaxel, apoptosis of A549/DTX cells with or without FoxM1 knockdown was assessed by dual-staining with Annexin V-FITC/PI. As shown in **Fig. 4**, the percentage of apoptotic cells was mildly increased in A549/DTX cells treated with docetaxel (25 μM, 18.76% ± 4.17%); however, the percentage of apoptotic cells in FoxM1-knockdown A549/DTX cells was increased to 39.12% ± 5.66% ($P < 0.05$). These results indicated that FoxM1 silencing sensitized A549/DTX cells to docetaxel through inducing cell apoptosis.

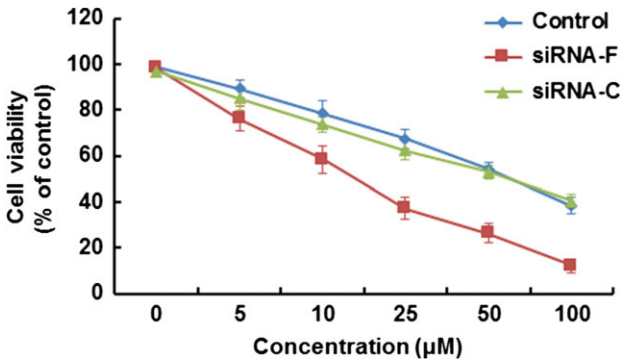

Figure 3. FoxM1 silencing promotes the cytotoxic effect of docetaxel on A549/DTX cells A549/DTX cells with or without FoxM1 knockdown were treated with docetaxel (0–100 μM) for 24 h and cell viability was then assessed with MTT assay. All experiments were conducted independently three times with three replicates in each experiment and values are expressed as the mean ± SE.

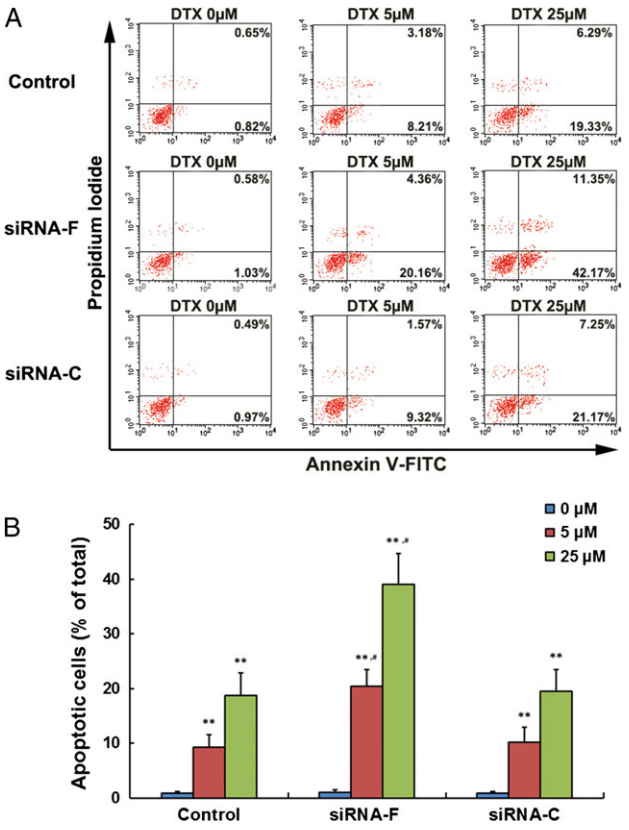

Figure 4. FoxM1 silencing promotes the pro-apoptotic effect of docetaxel on A549/DTX cells A549/DTX cells with or without FoxM1 knockdown were treated with docetaxel (0, 5, and 25 μM) for 24 h. (A) Flow cytometry analysis of cell apoptosis using Annexin V-FITC/PI dual-staining. (B) The densitometric analysis of the percentage of apoptotic cells. All experiments were conducted independently three times with three replicates in each experiment and values are expressed as the mean ± SE. $^{**}P < 0.01$ vs. 0 μM, $^{\#}P < 0.05$ vs. control.

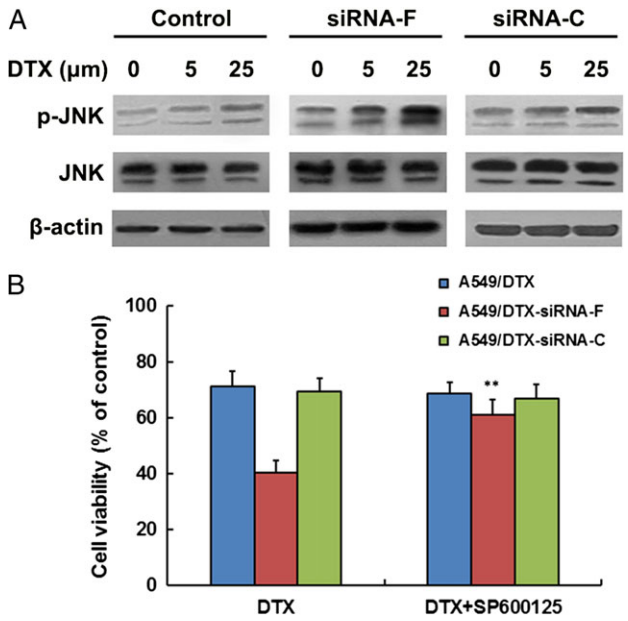

Figure 5. FoxM1 silencing promotes the pro-apoptotic effect of docetaxel on A549/DTX cells through activating the JNK signaling pathway (A) A549/DTX cells with or without FoxM1 knockdown were treated with docetaxel (0, 5, and 25 μM) for 24 h and the expression of the JNK was assessed by western blot analysis. (B) A549/DTX, A549/DTX-siRNA-F, and A549/DTX-siRNA-C cells were treated with docetaxel (25 μM) or docetaxel (25 μM) plus SP600125 (10 μM) and the cell viability was assessed with MTT assay. $^{**}P < 0.01$ vs. docetaxel treatment alone. All experiments were conducted independently three times with three replicates in each experiment, and representative images were shown.

FoxM1 silencing enhances docetaxel-induced A549/DTX cell apoptosis via activating the JNK/mitochondrial pathway

To determine which pathway was involved in this cellular process, further experiment was conducted. The mitochondrial and MAPKs pathways were previously described to be involved in docetaxel-induced cancer cell apoptosis [20,21]. Therefore, the expression of related proteins was analyzed by western blot analysis. As shown in **Fig. 5**, after exposure to docetaxel, JNK but not Erk1/2 or p38 was significantly phosphorylated in FoxM1-knockdown A549/DTX cells compared with that in control. Moreover, the blockade of JNK with SP600125 in A549/DTX-siRNA-F cells significantly reversed docetaxel-induced cell growth inhibition, suggesting that JNK pathway was an essential signaling pathway participated in the regulation of FoxM1 in docetaxel resistance. In addition, FoxM1 knockdown also enhanced A549/DTX cell apoptosis through augmenting docetaxel-induced increase of Bax/Bcl-2 ratio, Bax mitochondrial translocation and activation of caspases-3, -9 as well as the decrease of cytochrome c in mitochondria compared with that in control (**Fig. 6**). These results indicated that FoxM1 silencing enhanced docetaxel-induced A549/DTX cell apoptosis via activating the JNK/mitochondrial pathway.

Discussion

The standard first-line treatment for the advanced or metastatic NSCLC was Platinum-based chemotherapy; however, nearly all patients exposed to the first-line chemotherapy eventually received second-line chemotherapy [22,23]. Docetaxel is currently the

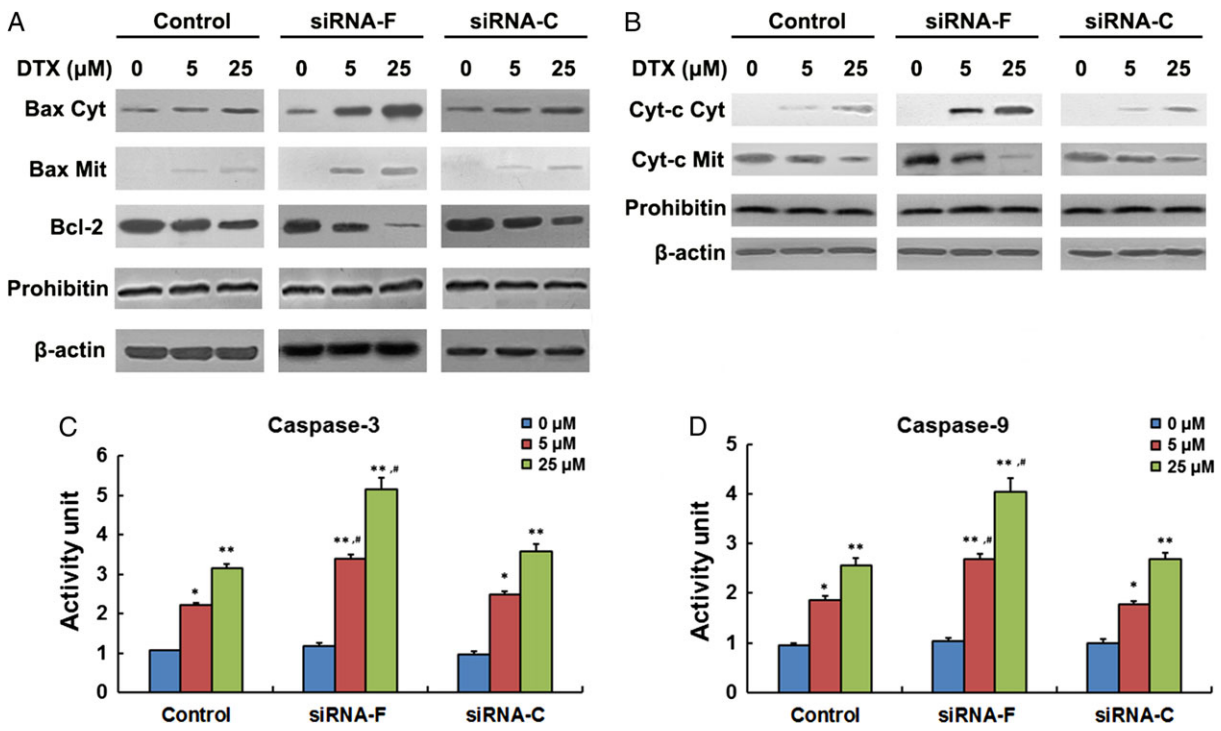

Figure 6. FoxM1 silencing promotes the pro-apoptotic effect of docetaxel in A549/DTX cells through activating the mitochondria-dependent signaling pathway A549/DTX cells with or without FoxM1 knockdown were treated with docetaxel (0, 5, and 25 μM) for 24 h. (A) Assessment of cytosolic and mitochondrial Bax and Bcl-2 protein levels by western blot analysis. (B) Determination of the levels of cytosolic cytochrome c (Cyto-c Cyt) and mitochondrial cytochrome c (Cyto-c Mit) by western blot analysis. (C,D) Assessment of the activities of caspase-3 and caspase-9 by fluorometric assay. All experiments were conducted independently three times with three replicates in each experiment and values are expressed as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 μM, and $^{\#}P < 0.05$ vs. control.

standard second-line chemotherapeutic agent for NSCLC, but drug resistance greatly limits its efficiency [6,24]. FoxM1, an oncogenic transcription factor, is closely associated with the occurrence, development and prognosis of various human cancers including NSCLC. In addition to being a cell cycle transcription factor, FoxM1 has been confirmed to be involved in DNA damage and apoptosis pathway, which suggests that FoxM1 plays an important role in multidrug resistance [16,25]. FoxM1 overexpression in NSCLC was recently reported to be associated with cisplatin resistance [17]. However, to the best of our knowledge, the role of FoxM1 in chemoresistance of lung cancer cells to docetaxel has not been explored. In the present study, we found that the expression of FoxM1 was significantly up-regulated in docetaxel-resistant human lung adenocarcinoma A549 cells, and that knockdown of FoxM1 sensitized A549/DTX cells to cytotoxicity induced by docetaxel, with an IC_{50} of 8.34 μM. Moreover, the percentage of apoptotic cells induced by docetaxel was increased from 18.76% ± 4.17% to 39.12% ± 5.66% after FoxM1 knockdown, suggesting that FoxM1 silencing sensitized A549/DTX cells to docetaxel through inducing cell apoptosis. Our findings revealed that FoxM1 plays a critical role in chemoresistance to docetaxel, and that FoxM1 depletion may contribute to the improvement of lung cancer therapy.

To gain insights into the molecular mechanism underpinning the effect of FoxM1 on docetaxel resistance in A549/DTX cells, the involvement of apoptosis-related signaling pathways was investigated focusing on the intrinsic pathway. JNKs (also known as stress-activated protein kinases) respond to diverse extracellular stimuli and environmental stresses. Increasing evidence has indicated that JNK plays a major role in chemoresistance to docetaxel of lung cancer cells; however, the relationship between FoxM1 and the JNK pathway in such process has never been reported [26]. FoxM1 has been shown to act as an upstream regulator of the JNK signaling pathway, and transcriptionally activate JNK1 to control cell cycle progression and apoptosis [27]. Based on the previous studies, we hypothesized that FoxM1 mediated sensitivity to docetaxel in A549 cells via the JNK/mitochondrial pathway. Our findings showed that when the expression of FoxM1 was down-regulated, p-JNK level was significantly increased, which was accompanied by an enhanced sensitivity to docetaxel. Moreover, the blockade of JNK with SP600125 in A549/DTX-siRNA-F cells significantly reversed docetaxel-induced cell growth inhibition, suggesting that JNK pathway was an essential signaling pathway participated in the regulation of FoxM1 in docetaxel resistance. In the mitochondria-initiated intrinsic pathway, the activated JNK can stimulate cytochrome c release from mitochondria into cytosol through an analogous pathway which involves the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2, and activates caspase-3 and caspase-9 [28]. In the present study, FoxM1 silencing was found to enhance docetaxel-induced A549/DTX cell apoptosis through augmenting the increase of Bax/Bcl-2 ratio, the Bax mitochondrial translocation, the activation of caspase-3 and caspase-9, as well as the decrease of cytochrome c in mitochondria.

In summary, FoxM1 was shown to be a critical mediator of docetaxel sensitivity in NSCLC. With FoxM1 silencing, the acquired drug resistance to docetaxel was reversed, demonstrating that inactivation of FoxM1 was essential for reversing docetaxel resistance. In addition, FoxM1 silencing effectively attenuated drug resistance to docetaxel via activating the JNK/mitochondrial pathway. However, the underlying mechanisms involved in this process still remain unclear. Therefore, appropriate combination of docetaxel application and FoxM1 depletion might be a promising therapeutic approach in treating NSCLC.

Funding

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