Downregulated Mucin 1 alleviates paclitaxel resistance in non-small cell lung cancer cells

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Abstract. Multidrug resistance of non-small cell lung cancer (NSCLC) is a common clinical problem, which is one of the main reasons leading to the failure of chemotherapy. Therefore, how to overcome or prevent drug resistance has become a hot and difficult issue in clinical research. The present study was designed to investigate the expression patterns, functions and underlying mechanisms of MUC1 in regulating paclitaxel-resistant cell line A549/PR in NSCLC. RT-qPCR and western blot was performed to determine the mRNA and protein level, respectively. CCK-8 was conducted to determine the cell viability of A549/PR cells. Moreover, flow cytometry assay was applied to examine the apoptosis rate of A549/PR. Herein, the MUC1 was over-expressed in clinic NSCLC tissues and A549/PR cells. Silence of MUC1 could obviously suppress the proliferation and promote apoptosis of A549/PR cells in treatment of paclitaxel through up-regulating the expression of Bax and Caspase-3, and down-regulating the expression of Bcl-2, suggesting that chemotherapy combined with the modulation of MUC1 might be characterized as a promising therapeutic approach to overcome paclitaxel-resistance in NSCLC in the future.

Introduction

Lung cancer has the highest morbidity and mortality of all malignant tumors and is a serious threat to human health. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung malignancies and is the main pathological type of lung cancer (1). NSCLC has a young age of onset and is characterized by increasing morbidity and mortality rates. The current treatment strategies for patients with NSCLC include surgery, radiotherapy, immunotherapy and chemotherapy, as well as a combination of these treatment modalities (2). With the development of imaging techniques and molecular biology techniques, great progress has been made in the early diagnosis and treatment of patients with NSCLC. Of note, epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors may be used to treat patients with NSCLC with EGFR mutations, and have been demonstrated to improve prognosis and prolong the survival time of the aforementioned patients (3). However, the 5-year survival rate of patients with NSCLC remains low and the 5-year recurrence rate following complete surgical resection of stage I NSCLC is as high as 50%. The majority of patients are diagnosed in the advanced stages of the disease, and present with even higher recurrence and metastasis rates (4).

Certain biological indicators, including histological and tumor-node-metastasis staging, number of lymph node metastases and molecular immune markers, such as EGFR mutation status, currently serve as prognostic predictors and guide treatment strategies in patients with NSCLC (5). However, chemotherapy resistance is one of the key factors leading to poor prognosis in patients with NSCLC and remains a challenging clinical problem (6,7). Despite surgical intervention, radiotherapy and targeted therapies, the majority of patients require standardized treatment with paclitaxel. Paclitaxel is a type of taxane, and exerts its cytotoxic effect predominantly by disrupting microtubule assembly. Paclitaxel inhibits microtubule depolymerization and leads to cell cycle arrest in the G2/M phase, resulting in apoptosis or necrosis of tumor cells (8,9). However, in clinical treatment, the majority of patients develop resistance to paclitaxel chemotherapy, which eventually leads to treatment failure and affects the survival time of patients (10,11). The mechanisms of paclitaxel resistance are multifaceted and complex, and include changes in cell membrane dynamics, increased drug metabolism, alterations in DNA repair mechanisms, cell cycle dysregulation and decreased apoptosis and autophagy (12,13). Therefore, investigating the mechanisms of paclitaxel resistance may improve the outcome of patients with NSCLC.

Improvements in molecular biology techniques have allowed the identification of oncogenes and tumor suppressor genes, which are an area of current interest in lung cancer research and may provide a valuable basis for individualized treat-

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ment and prognosis evaluation of patients with NSCLC (14). Mucin 1 (MUC1) is a member of the membrane-bound mucin family of glycoproteins. MUC1 is located in the apical surface of epithelial cells in the mammary glands, pancreas and gastrointestinal, respiratory and urogenital tracts, and under physiological conditions is not detected by the immune system. MUC1 is characterized by polar expression, apical distribution and rich glycosylation (15). MUC1 is ubiquitously expressed on the cell surface in several types of cancer, including esophageal and gastric cancer. Furthermore, MUC1 loses polarity and become hypoglycosylated in cancer cells and may serve as a diagnostic marker or a therapeutic target (16,17). MUC1 is spontaneously hydrolyzed to produce two subunits, the α and β subunits. The α subunit, also known as MUC1-N, is characterized by 20-200 variable number of tandem repeats (VNTRs), which are serine-, threonine- and proline-rich and may be modified by O-glycosylation. The β subunit, also known as MUC1-C, is composed of MUC1-extracullar domain, MUC1-transmembrane and MUC1-cytoplasmic tail (CT). MUC1-CT is highly conserved and can interact with phosphoinositide 3-kinase (PI3K), C-terminal Src kinase (CSK) and nuclear factor κB (NF-κB) signaling pathways to regulate cell activity (18-20). Previous studies have reported that MUC1 is upregulated in several types of cancer, such as esophageal squamous-cell carcinoma (16,21). The high expression level of MUC1 in lung cancer has been closely correlated with early recurrence, poor prognosis and a high metastatic potential (22). Furthermore, MUC1 knockdown suppressed lung cancer growth and invasion by inhibiting cell proliferation and inducing apoptosis (23). However, despite these findings, the association between MUC1 and paclitaxel resistance in patients with NSCLC remains unclear. Therefore, the aim of the present study was to explore the role of MUC1 in paclitaxel-resistant lung cancer cell line A549/PR, and to investigate the associated mechanism.

Materials and methods

NSCLC tissue collection. A total of 30 patients with NSCLC patients were recruited at The General Hospital of Western Theater Command from February 23, 2016 to January 1, 2018. Tumor and adjacent non-cancerous tissues were collected, snap frozen in liquid nitrogen and stored at -80˚C. The present study was approved by the Ethics Committee of The General Hospital of Western Theater Command and all patients provided written informed consent.

Cell culture and transfection. A549 cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and A549/PR cells were purchased from Nanjing KeyGen Biotech, Co., Ltd. The two cell types were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). All cells were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C.

Paclitaxel-resistant NSCLC cell lines A549/PR were treated with 0.5 μM of paclitaxel (Glpbio) in the culture for 3 months (24). Then cells were maintained in 1 μM of PTX to maintain this drug-resistant phenotype. Cellular assays were carried out when the cells were in the logarithmic growth phase.

Two small interference (si) RNAs targeting MUC1 and a scrambled siRNA were designed and synthesized by Ribobio. The 50 nM siRNAs were transfected into A549/PR cells ($5x10^4$ cells) using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions when cells reached 50% confluence. The sequences of MUC1 siRNA and its NC were as follows: MUC1 siRNA, 5'-AAGACTGATGCCAGTAGCACT-3'; NC, 5'-AATTCTCCGAACGTGTCACGT-3'. After 72 h transfection, reverse-transcription quantitative PCR (RT-qPCR) and western blotting were used to assess the silence effect.

Cell proliferation assay. Cell proliferation was evaluated using the Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technology). Briefly, A549/PR cells transfected with MUC1 siRNA or NC siRNA were cultured in a 96-well plate ($3x10^3$ cells/well). The cells were incubated for 0, 12, 24, 48 or 72 h at 37˚C and a total of 10 μl CCK-8 solution was added per well. The cells were subsequently incubated for 4 h and the optical density at a wavelength of 450 nm was measured using a microplate reader (Peiou).

Cell apoptosis analysis. Cell apoptosis was analyzed with an Annexin V Apoptosis Detection kit I (BD Biosciences). A total of 48 h following transfection, A549/PR cells were digested and washed twice in pre-chilled PBS. The cells were subsequently incubated with fluorescein isothiocyanate annexin V and propidium iodide for 15 min in the dark. Apoptosis was analyzed using a flow cytometer (FACScan; BD Biosciences) using FlowJo v7.6.1 (FlowJo LLC).

RT-qPCR. At 48 h post-transfection, the total RNA was extracted from tissue specimens and transfected A549/PR cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA was reversed transcribed into cDNA using the TaqMan Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using the Fast Start Universal SYBR Green Master mix (Roche Applied Science) and a 7500 Real-time system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primers were used: MUC1 forward, 5'-TCCTTTCTCTGCCCAGTCTG-3' and reverse, 5'-GTGTGGTAGGTGGGGTACTC-3', GAPDH forward, 5'-GGACCTGACCTGCCGTCTAG-3' and reverse, 5'-GTAGCCCAGGATGCCCTTGA-3'. GAPDH was used as the reference gene.

Western blotting. At 48 h post-transfection, the total protein was extracted from tissue specimens and transfected A549/PR cells according to the manufacturer's protocol. Total protein was quantified using a bicinchoninic acid assay kit (Vazyme) and 50 μg protein/lane was separated via SDS-PAGE on a 10% gel. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes and blocked in 5% skim milk in TBST for 1.5 h at 25˚C. The membranes were incubated with primary antibodies against MUC1 (cat.

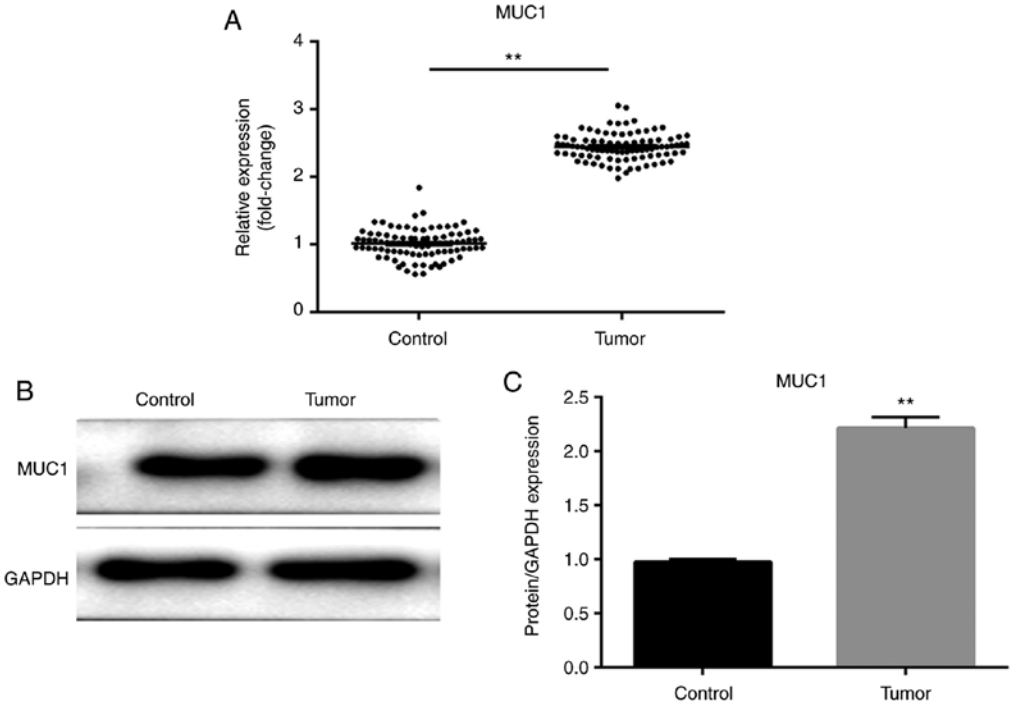

Figure 1. MUC1 was up-regulated in clinic NSCLC tissues. MUC1 mRNA and protein expressions in a total of 30 NSCLC tissues and matched adjacent normal tissues were examined by (A) RT-PCR and (B and C) western blot assays. The results were illustrated as the mean ± SD of three independent experiments and each was performed in triplicate. **P<0.01 vs. control group.

no. 14161), BCL2 associated X apoptosis regulator (Bax; cat. no. 2774), BCL2 apoptosis regulator (Bcl-2; cat. no. 2872), Caspase-3 (cat. no. 9662) and GAPDH (cat. no. 8884) at 4˚C overnight. All antibodies were used at a 1:1,000 dilution and were purchased from Cell Signaling Technology, Inc. Membranes were washed three times with TBST. Following the primary incubation, membranes were incubated with secondary antibodies (1:1,000) for 2 h at 25˚C. Protein bands were visualized using an Enhanced Chemiluminescence Detection system. GAPDH was used as the loading control.

Statistical analysis. GraphPad Prism software (v5; GraphPad Software, Inc.) was used to perform all the statistical analysis. The Student's t-test was used to compare two groups and the one-way analysis of variance (ANOVA) followed by Tukey's test was used for the comparison of multiple groups. P<0.05 was considered to indicate a statistically significant difference. All quantitative data are expressed as the mean ± SD.

Results

MUC1 is upregulated in NSCLC tissues. RT-qPCR and western blotting were performed to detect the expression levels of MUC1 in NSCLC and adjacent noncancerous tissues. As shown in Fig. 1A-C, the mRNA and protein expression levels of MUC1 were upregulated in NSCLC tissues compared with adjacent noncancerous tissues (P<0.01), suggesting that MUC1 may promote the progression and development of NSCLC.

MUC1 is upregulated in A549/PR cells. In order to further explore the role and underlying mechanisms of MUC1 in paclitaxel resistance in NSCLC, at 72 h post-transfection, the expression level of MUC1 was measured by RT-qPCR and western blot respectively. The mRNA expression level of MUC1 was significantly increased in A549/PR cells compared with A549 cells (P<0.01; Fig. 2A). The effect of MUC1 upregulation in NSCLC was further investigated by transfecting A549/PR cells with NC siRNA or MUC1 siRNA. The results revealed that the MUC1 siRNA significantly inhibited the mRNA and protein expression levels of MUC1 in A549/PR cells compared with the control (P<0.01; Fig. 2B-D).

Silencing of MUC1 suppresses the proliferation of A549/PR cells following treatment with paclitaxel. Paclitaxel resistance in control and transfected cells A549/PR cells was investigated using the CCK-8 assay. As shown in Fig. 3A and B, the expression level of MUC1 was significantly decreased in MUC1-siRNA group and there was no significant difference between the proliferation rate of A549/PR cells and A549/PR cells transfected with NC siRNA following paclitaxel treatment. However, the proliferation rate of A549/PR cells transfected with MUC1 siRNA was significantly decreased compared with the untransfected cells and cells transfected with NC siRNA (P<0.01). These data suggested that the inhibition of MUC1 significantly enhanced paclitaxel sensitivity in A549/PR cells.

Inhibition of MUC1 promotes apoptosis of A549/PR cells following treatment with paclitaxel. The apoptotic rates of A549/PR cells transfected with NC siRNA or MUC1 siRNA following treatment with paclitaxel were determined using flow cytometry. There were no significant differences between the apoptotic rates of the untransfected cells and cells transfected with NC siRNA following treatment with paclitaxel (P<0.05;

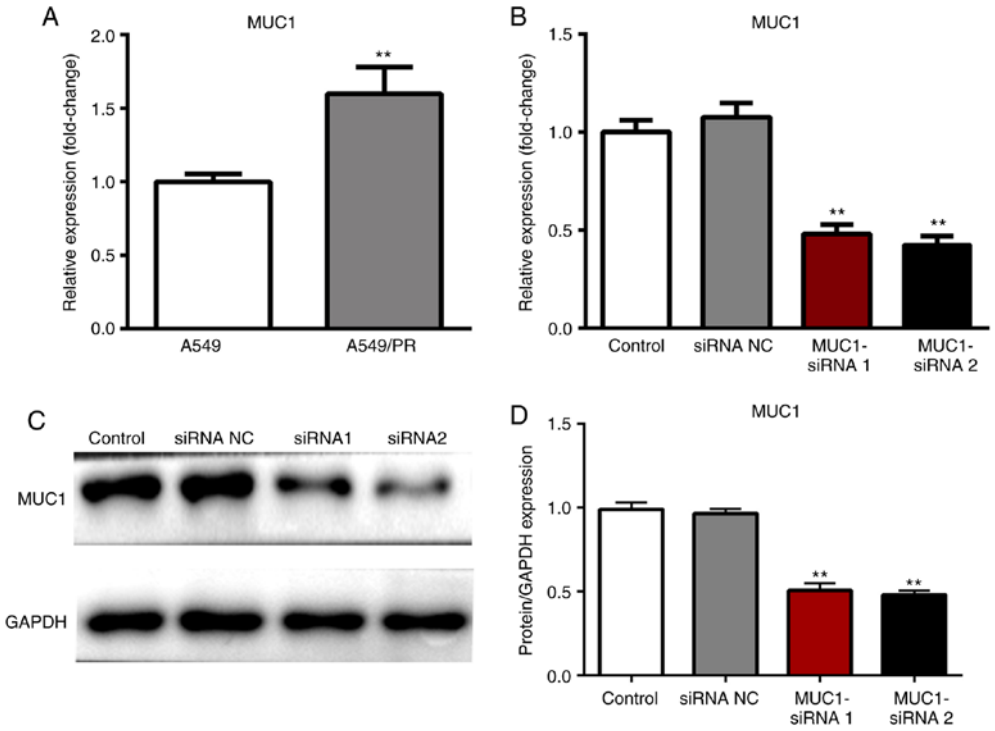

Figure 2. MUC1 was over-expressed in A549/PR cells. (A) MUC1 mRNA expression in A549/PR cells and A549 cells was examined by RT-PCR assay at 72 h post-transfection. **P<0.01 vs. A549 group. Transfection efficiency was assessed by (B) RT-PCR and (C and D) western blot assays at 72 h post-transfection with MUC1-siRNA in A549/PR cells. **P<0.01 vs. control or siRNA NC groups.

Fig. 4A and B). However, cells transfected with MUC1 siRNA exhibited significantly increased apoptosis compared with the untransfected cells or cells transfected with NC siRNA (P<0.01). The data suggested that inhibition of MUC1 promoted apoptosis of paclitaxel-treated A549/PR cells.

MUC1 regulates the expression of Bcl2/BAX and caspase-3. Western blotting was performed to evaluate the protein expression levels of apoptosis-associated proteins in A549/PR cells transfected with NC siRNA or MUC1 siRNA following treatment with paclitaxel. The protein levels of Bax, Bcl-2 and caspase-3 were not significantly different between untransfected cells and cells transfected with NC siRNA following treatment with paclitaxel (P>0.05; Fig. 5). The mRNA and protein levels of Bax and caspase-3 were significantly upregulated while Bcl-2 was significantly downregulated in cells transfected with MUC1 siRNA compared with untransfected cells or cells transfected with NC siRNA (P<0.01). The results further suggested that the inhibition of MUC1 promoted apoptosis of paclitaxel-treated A549/PR cells.

Discussion

The increase in environmental pollution and smoking in recent years has resulted in >500,000 new cases of lung cancer and >400,000 lung cancer-associated mortalities annually. Lung cancer has the highest incidence and mortality rates among all tumors. Approximately ~80% of lung cancer cases are NSCLC. The majority of patients with NSCLC are diagnosed in the advanced stages of the disease and have inoperable tumors. Chemotherapy is therefore the main treatment strategy for patients with advanced NSCLC (25,26). However,

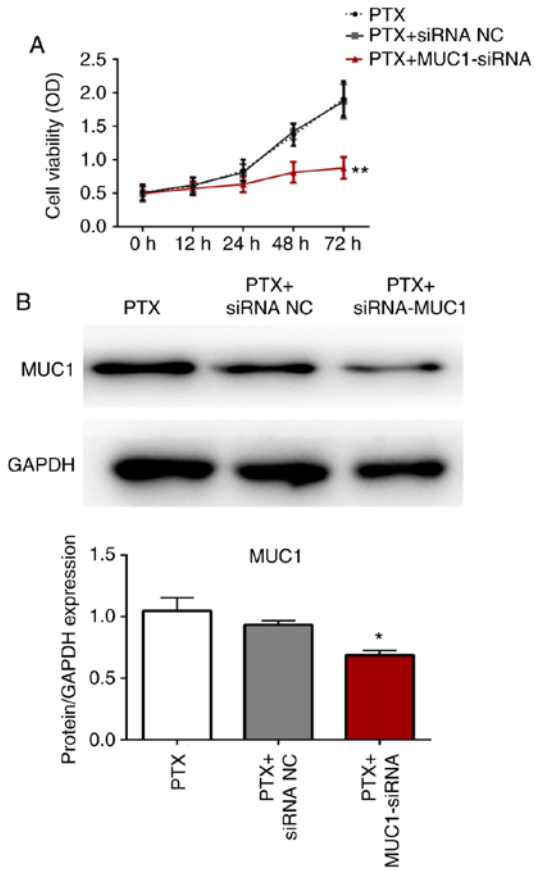

Figure 3. Silence of MUC1 suppressed the cell viability of A549/PR cells in treatment of paclitaxel. (A) The viabilities of A549/PR cells transfected with MUC1-siRNA for 0, 12, 24, 48 or 72 h, respectively, were assessed by CCK-8 assay. **P<0.01 vs. PTX + siRNA NC group. (B) The expression level of MUC1 at 72 h post-transfection. *P<0.05 vs. PTX + siRNA NC group. PTX, paclitaxel.

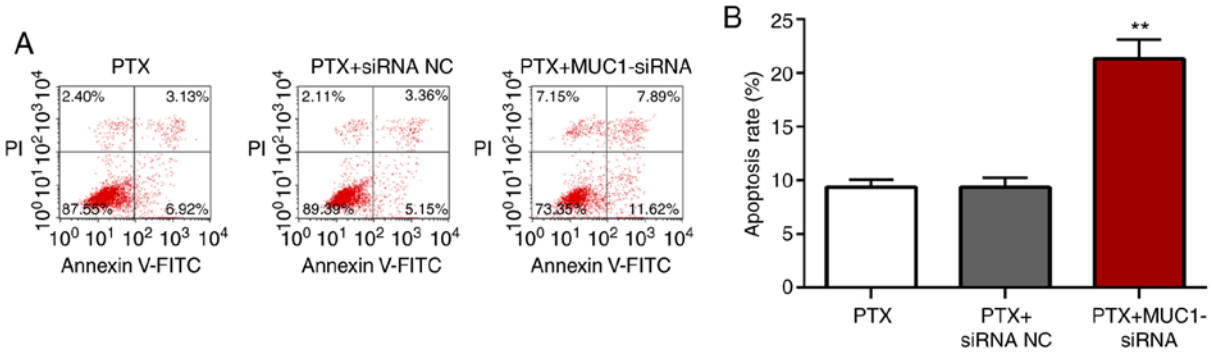

Figure 4. Inhibition of MUC1 promoted apoptosis of A549/PR cells in treatment of paclitaxel. (A) Apoptosis assays were performed by flow cytometry analysis after transfection with MUC1-siRNA for 48 h in A549/PR cells. (B) Quantified results of A were presented. **P<0.01 vs. PTX + siRNA NC group. PTX, paclitaxel.

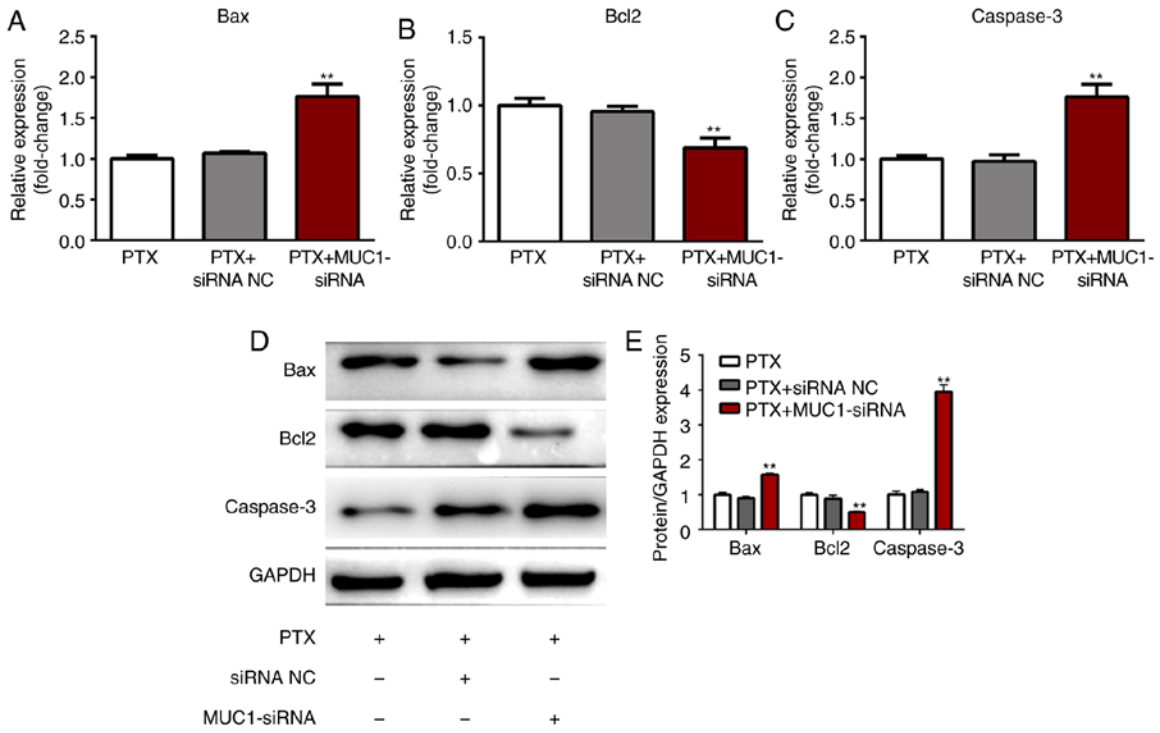

Figure 5. Downregulated MUC1 inhibited the expression of Bcl-2 and increased the expression of Bax and caspase-3. The expression of Bcl-2 in A549/PR cells transfected with MUC1-siRNA was significantly decreased both in (A-C) mRNA and (D and E) protein level, while the expression of Bax and caspase-3 was increased. **P<0.01 vs. PTX + siRNA NC group. PTX, paclitaxel; Bax, BCL2-associated X protein; Bcl2, B-cell lymphoma-2.

the efficacy rate of combined chemotherapy for NSCLC is 14-40%, and may result in relapse. This phenomenon is closely associated with multidrug resistance in NSCLC (27). Drug resistance in tumors involves several mechanisms, and may lead to multidrug resistance (28,29). Tumors with multidrug resistance are a common clinical problem, and often result in chemotherapy failure (27). Therefore, there has been increased interest in overcoming or preventing drug resistance. The main mechanisms of drug resistance in lung cancer include increased expression of multidrug resistance genes such as P-glycoprotein, glutathione transferases, decreased expression of topoisomerase and promoting DNA damage repair and anti-apoptotic ability of cancer cells (30).

Paclitaxel was originally isolated from the bark of Taxus pacificus and has cytotoxic effects on many types of cancer cells (31). Paclitaxel is a tubulin-binding drug and has exhibited better prospects; however, it is less cost-effective than other drugs (32). Additionally, paclitaxel is associated with drug resistance and cross-resistance in cancer cells. The mechanisms underlying paclitaxel resistance are complex and are not fully understood. Paclitaxel resistance may occur due to upregulation of P-glycoprotein, abnormal expression of microtubule regulatory proteins or post- translational changes expression of drug-resistant genes, abnormal signal transduction and cell death pathway regulation, and alterations in tubulin subtypes, proteins that regulate tubulin dynamics and paclitaxel binding sites (33). The elucidation of the pathways involved in paclitaxel resistance may allow the identification of suitable patients and guide the selection of treatment strategies. Furthermore, the emergence of drug resistance may be avoided or reversed, thereby improving the efficacy of chemotherapy.

The mucin family consists of high molecular weight glycoproteins that protect and lubricate epithelial cells under physiological conditions. MUC1, a member of the mucin family, is a transmembrane heterodimer glycoprotein that is expressed at low levels in the proximal side of secretory epithelial cells in the mammary glands and respiratory, gastrointestinal and urogenital tracts. In epithelial tumors, MUC1 expression is upregulated and loses its polar distribution. Furthermore, MUC1 has been shown to serve an important role in tumor proliferation, invasion, metastasis, and chemotherapy resistance. MUC1 is highly expressed in >90% of breast cancer cases and is associated with the occurrence and development of breast cancer by interacting with PI3K/AKT, MAP, NF-κB, Wnt, signal transducer and activator of transcription, tumor protein P53 and estrogen receptor (ER) α signaling pathways (34). Following the stimulation of breast cancer cells with EGF, the MUC1 intracellular segment was phosphorylated, CSK and MAPK signaling pathways were activated and cell proliferation was enhanced. In 17 β-estradiol-stimulated breast cancer cells, MUC1 enhanced ERα-mediated transcription and promoted the survival and growth of breast cancer cells. Ren *et al* (35), revealed that silencing MUC1 enhanced the sensitivity of A549 and ZR-75-1 cells to cytotoxic drugs. Horn *et al* (36), found that murine breast cancer DA3 cells overexpressing MUC1 increased the expression of interstitial markers, decreased the expression of epithelial markers and enhanced the ability of cells to produce extracellular matrix. In addition, previous studies have revealed that MUC1 was upregulated in NSCLC tumors, and that downregulation of MUC1 inhibited the progression of the disease (37,38). In the future, we will conduct further experiments concerning the effect of MUC1 on paclitaxel resistance in H1299 or H1975 cell lines.

In the present study, MUC1 was upregulated in clinical NSCLC tissues and A549/PR cells. MUC1 silencing significantly suppressed the proliferation and promoted apoptosis of paclitaxel-treated A549/PR cells by regulating Bax, Bcl-2 and caspase-3 expression. The results obtained in the present study suggested that the modulation of MUC1 may serve as a promising therapeutic approach to overcome paclitaxel resistance in NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HYX, HG and HL designed the experiments and performed the statistical analysis. DL, WWY and LZ were involved in the study design, data acquisition and manuscript revision. PC, XMS, ZHL and GJW were in charge of writing the manuscript and data analysis. TZ collected all the samples and patients' clinical data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The General Hospital of Western Theater Command and all patients provided prior written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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