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Paclitaxel promotes lung cancer cell apoptosis via MEG3-P53 pathway activation

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ABSTRACT

Paclitaxel (PTX) is a first-line chemotherapy drug for advanced non-small cell lung cancer (NSCLC). The long-chain non-coding RNA maternally expressed gene 3 (MEG3) is a recognized tumor suppressor. This study aimed to explore the effects of PTX on the expression of MEG3 and its anti-tumor mechanism in lung cancer cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed to determine cell proliferation. Quantitative polymerase chain reaction was used to determine the levels of MEG3 expressions. Western blot and immunofluorescence were used to detect protein levels. Small interfering RNA or pCDNA-MEG3 transfection was used to downregulate or upregulate MEG3 expression. Dichlorof luorescein diacetate was used to detect intracellular reactive oxygen species. Flow cytometry was used to analyze apoptosis. PTX significantly inhibited the proliferation of NSCLC cells and increased the expressions of MEG3 and P53. The downregulation of MEG3 attenuated PTX-induced cytotoxicity, whereas upregulation of MEG3 induced cell death and increased P53 expression. The inhibition of P53 caused no effect on the upstream MEG3 expression. Our results suggest that the MEG3-P53 pathway is involved in the apoptosis of A549 cells induced by PTX.

1. Introduction

Lung cancer is one of the most common causes of cancer-related deaths worldwide [1]. Among the leading cancer types of the new cancer cases and deaths by sex in 2018, lung cancer is the second major cause of estimated new cases and the first major cause of estimated new deaths in both men and women in the USA [2]. At present, non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancer cases [3], and its 5-year survival rate is ~10%–15% [4].

Paclitaxel (PTX) is effective in lung cancer, ovarian cancer, and breast cancer. Nowadays, PTX plays an important role in the first-line treatment of advanced NSCLC [5]. PTX exerts an anti-tumor effect by promoting microtubule polymerization, interfering with cellular functions (particularly normal cell division), and promoting apoptosis [6]. However, other anti-tumor mechanisms of PTX remain undiscovered.

Long-chain non-coding RNAs (lncRNAs) are broadly defined as endogenous cell non-coding RNA molecules that are longer than 200 nucleotides. lncRNAs serve as signals, decoys, guides, and scaffolds for the cellular components to perform multiple functions [7]. Maternally expressed gene 3 (MEG3), the product of which is expressed as a non-coding RNA, is expressed in normal tissues and is absent in several human tumors [8]. Previous studies have demonstrated that MEG3 can inhibit tumor cell proliferation and induce apoptosis in vitro [9]. MEG3 exerts an anti-tumor effect through accumulation of the P53 protein and activation of the p53 pathway [10]. As an RNA-based tumor suppressor, MEG3 is involved in the etiology, progression, and chemosensitivity of cancer by affecting the activities of tumor protein p53, mouse double minute 2 homolog, growth differentiation factor-15, retinoblastoma, and other key cell cycle regulators [11]. The differences in MEG3 expression levels in various cancers indicate the possibility of using MEG3 expression levels for cancer diagnosis and

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prognosis.

Studies have revealed that several lncRNAs can be regulated by chemotherapeutic agents, such as cisplatin and PTX [12]. However, few reports discussed the relationship between PTX and MEG3. Therefore, the present study aimed to explore the effects of PTX on the MEG3-P53 pathway in NSCLC cell lines and its potential mechanism.

2. Materials and methods

2.1. Reagents and antibodies

PTX was purchased from Aladdin. Dulbecco's Modified Eagle Medium (DMEM)/F12 was purchased from Gibco. Fetal bovine serum (FBS) was purchased from Biological Industries. Trypsin, crystal violet, 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) kit, dichlorof luorescein diacetate (H2DCF-DA), and P53 antibody were purchased from Beyotime Biotechnology. Trizol was purchased from Ambion. RevertAid First Strand complementary DNA (cDNA) Synthesis Kit was purchased from Thermo. SYBR Green Polymerase Chain Reaction (PCR) kit was purchased from Bimake. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Signalway Antibody. Goat anti-mouse IgG horseradish peroxidase horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Santa Cruz Biotecnology. Polyvinylidene difluoride (PVDF) membrane was purchased from Immobilon. 0.3% triton-X was purchased from Vetec. 4,6-diamidino-2-phenylindole (DAPI) was purchased from Vector. siRNAs and pCDNA-MEG3 were synthesized by GenePharma (Shanghai, China). Apoptosis detection kit was purchased from BD (Material Number: 559763). Lipofectamine 3000 transfection reagent was purchased from Invitrogen. P53 inhibitor pifithrin-α (PFTα) was purchased from Selleckchem.

2.2. Cell culture

A549 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (which contained 100 U/mL penicillin and 100 μg/mL streptomycin) in a humidified incubator at 37 °C with 5% CO_2.

2.3. Assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion

The cells were seeded into a 96-well plate (Corning) at a density of 5×10^3 cells/well in 200 μl culture medium. After treatment, the cells were incubated in 200 μl DMEM/F12 containing 0.5 mg/ml MTT at 37 °C for 4 h. Afterward, the supernatant was removed, and the cells were lysed in 200 μl dimethyl sulfoxide (DMSO) for 10 min at 37 °C. Optical density (OD) values were detected at 490 nm. The obtained values were presented as folds of the control group.

2.4. RNA extraction and quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted using Trizol reagent according to the manufacturer's protocol. The purity and concentration of RNA were determined using NanoDrop ND-1000 spectrophotometer according to the OD260/280 reading. Total RNA (500 ng) was reverse-transcribed to cDNA in a final volume of 20 μl; random primers and oligo dT primers were used under standard conditions using the RevertAid First Strand cDNA Synthesis Kit (Thermo). The purity and concentration of cDNA were determined using the NanoDrop ND-1000 spectrophotometer according to the OD260/280 reading. Then, qPCR was performed using the SYBR Green PCR kit. The reaction settings were as follows: 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Final extension was performed at 72 °C for 7 min. qPCR results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The qPCR method results were analyzed relative to the threshold cycle (Ct) value and were converted to fold-change values according to the rules of $2^{-\Delta\Delta Ct}$. The primers for MEG3 were F: 5′-ATCATCCGTCCACCTCCTT-3′ and R: 5′-TGGCTGCTTTGTATGTTGGT-3′. GAPDH was used as the housekeeping gene (F: 5′-TGGTGCCAAAAGGGTCATCTCC-3′ and R: 5′-GCCAGCCCCAGCATCAAA GGTG-3′).

2.5. Western blot analysis

Cells were washed thrice with cold phosphate-buffered saline (PBS) and lysed with radio immunoprecipitation assay lysate containing phenylmethylsulfonyl fluoride on ice for 45 min. The samples were centrifuged for 15 min at 12,000 rpm at 4 °C, and the supernatants were collected. Proteins (60 μg for each extract) were resolved via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electro-blotted to polyvinylidene difluoride membrane, and blocked in 5% non-fat milk at room temperature. The membranes were incubated with primary antibodies overnight at 4 °C. The membranes were washed with Tris Buffered Saline with Tween® 20 and probed with *horseradish peroxidase*-conjugated anti-mouse IgG.

2.6. Immunofluorescence

For immunofluorescence staining, the cells were fixed with 4% paraformaldehyde for 10 min, washed thrice with cold PBS, and permeabilized in 0.3% triton-X for 10 min. After washing with PBS, the cells were blocked in 1% *bovine serum albumin* (BSA) for 30 min and incubated overnight at 4 °C with P53 antibody (1:500 v/v, antibody: BSA) After washing thrice (5 min) with PBS, the cells were incubated with the secondary antibody IgG conjugated with Texas Red at a 1:1000 dilution for 1 h at room temperature. 4′,6′-Diamino-2-phenylindole was used to label the nuclei, and the cells were observed for fluorescent images under a fluorescence microscope (Nikon).

2.7. siRNAs and pCDNA-MEG3 transfection

MEG3-siRNAs and pCDNA-MEG3 plasmid were synthesized by GenePharma. The MEG3-siRNAs used in the present study were mixtures of 3 siRNAs whose sequences were 5′-CCCUCUUGCUUGUCUUACUTT-3′, 5′-GCUCAUACUUU GACUCUAUTT-3′, and 5′-GAUCCCACCAACAUACAAATT-3′. The A549 cells were plated in a six-well plate (1×10^5/well). siRNAs, pCDNA-MEG3, and negative controls were transfected with Lipofectamine 3000 for 48 h according to the operation manual.

2.8. Detection of intracellular reactive oxygen species (ROS)

H2DCF-DA was used to detect the intracellular generation of ROS following the reference [13]. After treatment, the A549 cells were incubated with 25 μM H2DCF-DA for 30 min. After washing thrice with cold PBS, the cell images were acquired immediately via fluorescence microscopy. The intracellular ROS intensity was measured via the ImagePro Plus software.

2.9. Apoptosis assay

The A549 cells were seeded in six-well plates (Corning) for 24 h

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