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Original article

Downregulation of FOXP3 inhibits cell proliferation and enhances chemosensitivity to cisplatin in human lung adenocarcinoma

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ABSTRACT

Our study aimed to investigate the biological role of FOXP3 expression in human lung adenocarcinoma (LAD) tissues and evaluate its involvement in cell proliferation and chemosensitivity to cisplatin in LAD cells. Paraffin-embedded tissues from 50 LAD patients were collected to detect FOXP3 and Ki-67 expression using immunohistochemistry (IHC). Downregulation of FOXP3 in A549 cells was performed using siRNA transfection. Real-time PCR or western blot assay was performed to analyze FOXP3 expression in A549 cells. Cell proliferation and cisplatin cytotoxicity test were assessed by CCK-8 assay. The expression of FOXP3 was significantly associated with lymph node metastasis and TNM stage of LAD patients. The FOXP3 expression was positively correlated with Ki-67 labelling index(LI)in LAD tissues. The downregulated expression of FOXP3 by siRNA transfection significantly inhibited cell proliferation and enhanced chemosensitivity to cisplatin in A549 cells. The expression of FOXP3 was significantly upregulated following cisplatin treatment in A549 cells. Our study indicates that FOXP3 may potentially be a novel molecular target in combating drug resistance in the chemotherapy of LAD.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide and its overall 5-year survival rate was less than 16% [1]. Because lung cancer does not show any symptoms in early stage, most patients with lung cancer are in advanced stage when diagnosed [2]. Thus, chemotherapy treatment is the main strategy which may improve average survival and quality of life in lung cancer patients [3,4], but the resistance to chemotherapeutic drug which is associated with tumor recurrence and metastasis becomes one of the main factors affecting patient prognosis [5]. Non-small cell lung cancer (NSCLC) constitutes 80–85% of lung cancers and is further divided into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Among the three histological subtypes, lung adenocarcinoma (LAD) accounts for almost 40% of (NSCLC) and is the most common form of NSCLC [1]. The exploration of new marker predicting the chemotherapy response is important to improve the prognosis of patients with LAD.

Forkhead box P3 (FOXP3) is considered to be the specific marker of regulatory T cells (Tregs) and plays a crucial role in the development and function of Tregs [6,7]. Tumor-infiltrating Tregs have been shown to be associated with tumor progression and poor prognosis [8–10]. Furthermore, certain studies have reported that Tregs could promote tumor progression by the resistance to conventional chemotherapy [11,12]. Recent studies showed that FOXP3 is also expressed in tumor cells and its expression in different kinds of tumor cells may play different roles in tumor progression. FOXP3 expression has been reported to be increased in tumor cells and promote tumor progression in colorectal cancer [13], hepatocellular carcinoma [14], thyroid carcinoma [15], cervical cancer [16] and oral squamous cell carcinoma [17]. Conversely, its decreased expression in tumor cells has been reported to be a tumor suppressor gene and play anti-tumorigenic role in breast cancer [18], prostate cancer [19] and glioblastoma [20]. Our previous study has demonstrated that FOXP3 was over-expressed in NSCLC cells and play a pro-tumorigenic role in NSCLC [21]. In addition, we also reported that FOXP3 could reduce the sensitivity to chemotherapeutic drugs Adriamycin (ADM) and mitomycin C (MMC) in mouse Lewis lung cancer (LLC) cells [22]. Although FOXP3 has been implicated in the development of NSCLC, its involvement in the tumorigenicity and drug resistance to cisplatin in LAD has not been well studied.

In this study, we aimed to investigate the biological function of FOXP3 and the chemosensitivity to cisplatin in human LAD. Firstly, we investigate the associations between FOXP3 expression and

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clinicopathological parameters. The correlation between FOXP3 and Ki-67 expression was analyzed. After knockdowning FOXP3 expression in LAD cells, we further observed the effects of FOXP3 downregulation on cell proliferation and cisplatin sensitivity of LAD cells. Moreover, the effects of cisplatin on the expression of FOXP3 were also investigated. These findings may provide a novel mechanism underlying LAD progression.

2. Materials and methods

2.1. Patients

Formalin-fixed paraffin-embedded (FFPE) samples from 50 patients who were diagnosed with LAD without chemotherapy and 15 normal FFPE lung samples from patients with benign lesions were involved in the present study. All cases were surgically resected at the General Hospital of China National Petroleum Corporation (CNPC) in Jilin between 2009 and 2012. All of the experiments were approved by the Ethics Committee of CNPC.

2.2. Immunohistochemical staining

Tissue sections were first deparaffinized in xylene and rehydrated in a series of graded alcohols. After antigen retrieval, endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide for 10 min followed by incubation with primary antibody FOXP3 (1:100, ab20034; Abcam, Cambridge, MA, USA) or Ki-67 (Ready to use, ZM-0166; Beijing Zhongshan Golden Bridge Biotechnology, Co., Beijing, China) overnight at 4 ˚C. After washing with PBS for three times, the sections were incubated with horse-radish peroxidase (HRP)-conjugated secondary antibody (PV6000, ZBGB-BIO) for 30 min. Then, immunostaining was performed using diaminobenzidine (DAB).

2.3. Immunohistochemical scoring

All slides were assessed using a double-blind method. A total of 10 fields were observed and 500 tumor cells were counted at 400× magnification in each slide. FOXP3 expression was assessed as cytoplasmic or nuclear staining of tumor cells. The final score of each specimen was assessed by multiplication of the score of the stained cells and the color intensity. The stained cells: 0 for ≤5%, 1 for 6–25%, 2 for 26–50%, 3 for 51–75% and 4 for ≥76%. Staining intensity: 0 = no stain, 1 = weak staining, 2 = moderate staining, 3 = strong staining. A score of 0–2 was considered negative, 3–4 was weak expression, 6–8 was moderate expression and 9–12 was high expression [21]. Ki-67 labelling index (LI) was assessed as a percentage of total counted cells with nuclear staining. The Ki-67 LI was scored as follows: low, LI < 10%; moderate, 10% ≤ LI < 30%; high, LI ≥ 30%. A score of LI < 10% was considered negative, LI ≥ 10% was considered positive [23].

2.4. Cells culture

The human lung adenocarcinoma line A549 was obtained from the American Type Culture Collection (ATCC, Shanghai, China). Cells were cultured at 37 ˚C with 5% CO_2 in complete DMEM medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Sijiqing Co., Zhejiang, China).

2.5. siRNA transfection

The A549 cells (2.5×10^5) were seeded in six-well plate with antibiotics-free DMEM for 12 h and then washed with serum-free DMEM. The FOXP3 siRNA (sc-43569) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection was done using Lipofectamine 2000 (Invitrogen Life Technologies, Shanghai, China) following the manufacturer's instructions. The medium with DMEM containing 10% FBS was changed 6 h later. Transfected cells were harvested at 48 h for real-time quantitative PCR (RT-qPCR) or 72 h for western blot.

2.6. Real-time quantitative PCR

RNA isolation and reverse transcription were performed using RNAiso Plus kit and PrimeScript™ RT reagent Kit (both from TaKaRa Biotechnology co., Dalian, China). The expression of FOXP3 mRNA was examined by RT-qPCR with SYBR Premix Ex Taq™ II (Takara) and a PikoReal 24 Real-Time PCR system (Thermo Fisher Scientific, America). The PCR reaction was performed using FOXP3 primers: 5′-CAC AAC ATG CGA CCC CTT TCA CC-3′ (forward) and 5′-AGG TTG TGG CGG ATG GCG TTC TTC-3′ (reverse); GAPDH primers: 5′-ATG GGG AAG GTG AAG GTC G-3′ (forward) and 5′-GGG TCA TTG ATG GCA ACA ATA TC-3′ (reverse). In Brief, a 5 μl aliquot of 1:10 diluted cDNA was mixed with 10 μl SYBR Premix Ex Taq II and 0.4 μM of the designated primers. The reaction condition was: 95 ˚C for 15 min and 40 cycles of 95 ˚C for 10 s and 60 ˚C for 10 s. Aliquots of the PCR products were analyzed by melting curves. FOXP3 mRNA levels were normalized to GAPDH expression and the amplifiation results for qRT-PCR were analyzed using the $2^{-\Delta\Delta Ct}$ method. Each experiment was conducted three times and each sample was tested in triplicate.

2.7. Western blot analysis

Cells were washed with PBS and then incubated in RIPA buffer (Beyotime, Jiangsu, China) containing protease inhibitor on ice for 30 min, and centrifuged at 15,000*g* for 15 min at 4 ˚C. Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% BSA for 1 h at room temperature and then incubated with primary monoclonal antibodies anti-FOXP3 (1:500, ab20034; Abcam) or GAPDH (1:1000, AF0006; Beyotime) and gently shaken at 4 ˚C overnight. The next day, the membrane was washed five times with TBST buffer and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:2000, Beyotime) for 1 h at room temperature. The immunoreactive proteins were detected using a chemiluminescence reagent (BeyoECL Star Kit, Beyotime). Each experiment was conducted three times

2.8. CCK-8 assay

Cell proliferation was detected as follows: After undergoing transfection with siRNA, cells were seeded at 5×10^3 per well in 96-well plates and incubated for 24 h, 48 h, and 72 h, respectively. Then, the cultures were added 10 μl of CCK-8 solution (Beyotime) at different time point and incubated at 37 ˚C for 2 h. Triplicate wells were used in each group.

Cytotoxicity assay is used to evaluate the effect of chemotherapeutic drug cisplatin. In brief, transfected cells were seeded at 1×10^4 per well in 96-well plates and incubated overnight. Then media was renewed with fresh media, and different concentrations of cisplatin (Hansoh Pharmaceutical Group Co., Jiangsu, China) were added into wells and further incubated for 48 h. Each well was added 10 μl of CCK-8 solution and continuously incubated for 2 h, absorbance at 490 nm was measured using an immunoreader. Survival rate (100%) = [OD of treated group/OD of control group] × 100%. The IC_{50} value was defined as the concentration of drug eliciting 50% cell death and calculated using CalcuSyn software (version 2.0, Biosoft, Cambridge, UK). Each experiment was conducted three times and each sample was tested in triplicate.