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COPB2 promotes cell proliferation and tumorigenesis through up-regulating YAP1 expression in lung adenocarcinoma cells

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ABSTRACT

Lung adenocarcinoma is the most common subtype of non-small cell lung cancer and responsible for more than 500,000 deaths per year worldwide. In this study, we aimed to explore the effects of COPB2 in the progression of lung adenocarcinoma and its underlying mechanism. The mRNA and protein levels of COPB2 in tumor tissues and cell lines were determined by qRT-PCR and western blotting analysis. siRNAs and over-expressed vector targeting COPB2 were used to down-regulate and up-regulate COPB2 expression in lung adenocarcinoma cell lines H1975. Cell apoptosis rate, proliferation and tumorigenesis of H1975 cells were determined by flow cytometry analysis, MTT assay and in vivo xenotransplantation assay, respectively. Western blotting and immunofluorescence assays were performed to evaluate the effects of COPB on the expression and subcellular location of YAP. Results showed COPB2 was significantly up-regulated in lung adenocarcinoma tissues and cell lines, which showed a close correlation with advanced clinical symptoms, such as tumor differentiation, TNM stage and the occurrence of lymph node metastasis and distance metastasis. Besides, the overall survival time of patients with high expression of COPB2 was shorter than that of patients with low COPB2 expression. After knockdown of COPB2, cell apoptosis rate was increased, whereas cell proliferation was decreased. Compared with that in the normal lung cell line H1688 cells, YAP1 expression was obviously increased in H1975, and overexpression of COPB2 translocated YAP1 from cytoplasm to nuclear, whereas knockdown of COPB2 showed the opposite effect. Overexpression of COPB2 enhanced cell proliferation, tumorigenesis and inhibited cell apoptosis. However, these effects were abolished when down-regulated YAP1 expression on the base of COPB2 overexpression. In conclusion, the increased expression of COPB2 was significantly correlated with the progression of lung adenocarcinoma. Up-regulation of COPB2 inhibited cell apoptosis and promoted cell growth and tumorigenesis through up-regulating YAP1 expression in lung adenocarcinoma.

1. Introduction

Lung adenocarcinoma, the most common subtype of non-small cell lung cancer, is responsible for more than 500,000 deaths per year worldwide [1]. Most often, tumors are discovered as locally advanced or metastatic disease. Despite the improvements in molecular diagnosis and targeted therapies, the average of 5 year survival rate for lung adenocarcinoma is ~15% [2]. While epidermal growth factor receptor (EGFR)-targeted agents may result in dramatic responses, they demonstrate efficacy in only a fraction of patients and resistance to these agents frequently develops [3]. Recent findings show that there are many pathways activated during lung cancer progression [4], hence, the identification of molecular targets hitting multiple targets for lung cancer is urgently needed.

Coatomer protein complex subunit β (COPB) is one of the seven nonclathrin-coated vesicular coat subunits that form the coatomer complex I (COPI), which plays a role in membrane transport between endoplasmic reticulum and Golgi apparatus [5]. COPB serves as the sole chlamydial translocation pore and COPB2, the main member of COPB family, is comprised of 906 amino acids and is capable of association with the inclusion membrane [6,7]. Two recent studies reported that some subunits (including COPB2) in COPI were correlated with the cell proliferation of malignant tumors [8,9], suggesting that COPI might play a crucial role in the development and progression of cancers.

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However, the function of COPB2 in lung adenocarcinoma remains unclear.

Cancer cell proliferation is a critical process in tumor development [10,11], and thus, anti-growth therapy is considered to be an important approach for lung adenocarcinoma treatment. Hippo pathway plays a critical role in organ size control by regulating cell growth, proliferation, and apoptosis [12–14]. Emerging evidences have demonstrated that Yes-associated protein 1 (YAP1), the downstream effector of tumor-suppressor Hippo pathway, functions as an oncoprotein and contributes to lung tumorigenesis [15]. However, the relationship between COPB2 and YAP1 in lung adenocarcinoma remains unclear.

In the current study, we explored the effects and underlying mechanism of COPB2 in the progression of lung adenocarcinoma. We firstly investigated the expression pattern of COPB2 in lung adenocarcinoma, and the association between COPB2 expression and the clinicopathological characteristics and survival overall of patients with lung adenocarcinoma. We then explored the effect of COPB2 on the proliferation, apoptosis and tumorigenesis of lung adenocarcinoma cells. Finally, we investigated the relationship of COPB2 and YAP1, and the effects of COPB2/YAP1 on cell proliferation, apoptosis and tumorigenesis in lung adenocarcinoma.

2. Material and methods

2.1. Patients

145 patients with lung adenocarcinoma were collected from The First affiliated Hospital of Nanjing Medical University, Tumor Hospital of Jiangsu Province and The Old Hospital in Jiangsu Province. The relative expression level of COPB2 in each patient was determined. The patients with high/low expression level of COPB2 were selected, and the expression of COPB2 in tumor tissues and the adjacent tissues were determined.

This study was approved by the ethics committee of Shanghai Eastern Hepatobiliary Surgery Hospital affiliated to the Second Military Medical University and consent forms were assigned with all patients or their relatives.

2.2. Immunohistochemistry

Twenty lung adenocarcinoma tissue samples from 145 lung adenocarcinoma patients were chosen for immunohistochemistry randomly, which were embedded in paraffin and sectioned. Sections (4 μm thick) were deparaffinized, rehydrated with PBS (pH 7.4), incubated with 3% H_2O_2 for 10 min, and then the antigen was retrieved in 0.1% trypsin (M/V) for 10 min at 37 °C. Samples were blocked with 5% BSA for 30 min at room temperature, then monoclonal antibody of rabbit anti-COPB2 (No. PA5-77106, Thermo Fisher Scientific, USA) was applied at the concentration of 1:150, at 4 °C overnight. Next, they were incubated with second antibody conjugated Diaminobenzidine (DAB) at room temperature for 15 min and then washed with PBS. DAB was applied for 5 min and the cell nucleus was dyed with Harri's hematoxylin solution. One representative area was shown in Fig. 1A.

The expression levels of COPB2 were scored by the extent and intensity of the staining. The extent of staining was scored by the percentage of the positively stained area. Stained area in each region of extent was scored using the following scale: 0 for a percentage $< 5\%$, 1 for 5–25%, 2 for 25–50%, 3 for 50–75%, and 4 for > 75%. The staining intensity was scored as 0, 1, 2 and 3 for the representation of negative (no staining), mild (weak), intermediate (distinct) and intense (strong) staining, respectively. The staining intensity and stained area percentage were multiplied to make a weighted score.

2.3. Cell culture

Lung adenocarcinoma cell lines H1299, A549, SK-MES-1, H1688 and H1975 were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were routinely cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified cell incubator with an atmosphere of 5% CO_2 at 37 °C. Cells growing at an exponential rate were used for the experiments.

2.4. Plasmids, small interfering RNA, and transfection

A plasmid of human COPB2 gene (No. SC117165), small interfering RNAs targeting human COPB2 and its negative control siRNA (No. SR306142) and YAP1 (No. SR323110)were purchased from OriGene (MA, USA). The empty vector obtained from OriGene (MA, USA) was used as the negative control of COPB2 and YAP1 plasmids. The cells were transfected with siRNAs, plasmids, and their controls using INTERFERin® transfection reagent (Polyplus, France) according to the manufacturer's instructions. In total, 2×10^5 cells were transfected with 110 pmoles of siRNA or 2 μg of DNA. The transfection efficiency was detected by western blotting 48 h and 24 h after transfection and confirmed by the real-time polymerase chain reaction (RT-PCR).

The sequences of siRNA targeting human COPB2 are listed as follows:

SR306142A – rCrUrCrArUrArCrGrArArGrArArArUrUrGrArArArUrUrCrAGC;

SR306142B – rUrGrCrUrUrUrGrGrArCrUrArUrGrArGrArArArCrUrUrCTT;

SR306142C – rGrGrArGrCrArGrArArArArGrUrArUrCrUrArCrGrGrGrCrGrGCT.

2.5. RNA isolation and qRT-PCR

Total RNA was extracted and isolated from tissue samples or cell lines using the TRIzol reagent. After isolation, the concentration of RNA was assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and the samples were stored at −80 °C for further use. Genes were amplified by specific oligonucleotide primers, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study were as follows: COPB2-F, 5′-GTGGGGACAAGCCATACCTC-3′, and COPB2-R, 5′-GTGCTCTCAAGCCGGTAGG-3′ (amplification efficiency was 98%, amplification size was 211); YAP1-F, 5′- TGACCCTC GTTTTGCCATGA-3′, and YAP1-R, 5′-GTTGCTGCTGGTTGGAGTTG-3′ (amplification efficiency was 100%, amplification size was 125); GAPDH-F, 5′-CATCACCATCTTCCAGGAGCG-3′, and GAPDH-R, 5′-TGA CCTTGCCCACAGCCTTG-3′ (amplification efficiency was 100%, amplification size was 443).

2.6. Western blotting

Cells were washed thrice with PBS and transferred to buffer containing 25 mM HEPES, 2.5 mM EDTA, 0.1% Triton X-100, 1 mM PMSF and 5 μg/mL leupeptin. Total protein concentration was determined with a UV spectrophotometer using a modified Bradford assay (Beckman Coulter, Fullerton, CA, USA). Protein (15 μL) were separated on SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA). The membrane was washed by TBST (50 mM Tris, 150 mM NaCl and 2% Tween-20; pH 7.5) for 3 times and each for 10 min at room temperature, and incubated at 4 °C overnight with primary antibodies: COPB2 (No. PA5-77106, Thermo Fisher Scientific, USA) and YAP1 (No. ab52771, Abcam, CA, USA). Then incubation with the secondary antibodies was performed at room temperature for 1 h. Membrane was washed again with TBST and incubated in SuperECL Plus detection reagent (Nanjing KeyGEN Biotech, KGP1123, China), which produced a chemiluminescence signal that was detected by exposure to X-ray film. Images were