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Downregulation of ribosomal protein S6 inhibits the growth of non-small cell lung cancer by inducing cell cycle arrest, rather than apoptosis

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ABSTRACT

Ribosomal protein S6 (rpS6), a component of the small 40S ribosomal subunit, has been found to be associated with multiple physiological and pathophysiological functions. However, its effects and mechanisms in non-small cell lung cancer (NSCLC) still remain unknown. Here, we showed that expressions of total rpS6 and phosphorylation rpS6 (p-rpS6) were both significantly overexpressed in NSCLC. Further survival analysis revealed the shortened overall survival (OS) and relapse-free survival (RFS) in p-rpS6 overexpressed patients and confirmed it as an independent adverse predictor. Stable downregulation of rpS6 in lung adenocarcinoma A549 and squamous cell carcinoma H520 cell lines was then achieved by two specific small hairpin RNA (shRNA) lentiviruses separately. Subsequent experiments showed that downregulation of rpS6 dramatically inhibited cell proliferation *in vitro* and tumorigenicity *in vivo*. Moreover, loss of rpS6 promoted cells arrested in G0-G1 phase and reduced in G2-M phase, along with the expression alterations of relative proteins. However, no notable change in apoptosis was observed. Collectively, these results suggested that rpS6 is overactivated in NSCLC and its downregulation suppresses the growth of NSCLC mainly by inducing G0-G1 cell cycle arrest rather than apoptosis.

Introduction

Lung cancer remains one of the most lethal human cancers worldwide, with 80% being non-small cell lung cancer (NSCLC) [1]. Despite prominent recent advances in the introduction of new therapeutic agents for NSCLC, its outcome is still dismal with a 5-year survival rate of approximately 15% [1]. It is now widely accepted that NSCLC develops from bronchial or alveolar epithelial cells with multiple genetic mutations, which finally leads to dysfunctions of various proteins. Ribosome, consisting of several kinds of RNA (rRNA) and structural proteins, is one of the most important intracellular organelles, with the essential function of synthesizing proteins in accordance with mRNA instructions. Of all known ribosomal proteins, ribosomal protein S6 (rpS6), a component of the 40S ribosomal subunit, is by far the most extensively studied one, and has been functionally implicated in the stimulation of protein translation efficiency, mainly by combining with the mRNAs rich in 5′-terminal oligopyrimidine tracts (TOP mRNAs) [2,3]. Although still in controversy, the most recognized mechanism for rpS6 activation is the mTOR-dependent phosphorylation at the evolutionary conserved C-terminal serine residues of Ser235, Ser236, Ser240, Ser244 and Ser247, in response to different growth factor stimulations [2–5]. Importantly, the biological effect of rpS6 phosphorylation was found by the persistent small size of embryonic fibroblasts from the rpS6 knock-out mice, in which all rpS6 phosphorylatable serine residues were substituted by alanines [6]. Further studies provided more

Abbreviations: rpS6, ribosomal protein S6; NSCLC, non-small cell lung cancer; shRNA, small hairpin RNA; sh-rpS6, shRNA for rpS6 knockdown; NC, negative control for rpS6; ADC, adenocarcinoma; SCC, squamous cell carcinoma; FACS, fluorescence activated cell sorting; RT-PCR, real-time PCR; SA-β-gal, senescence-associated β-galactosidase; OS, overall survival; RFS, relapse-free survival; HR, hazard ratio; CI, confidence interval.

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evidence to stress the rpS6 phosphorylating as a critical event in the cell survival, glycometabolism and many other bioactivities [2,7,8]. In line with the physiological effects, anomalous overactivation of rpS6 is frequently observed in numerous tumors [9–15], especially phosphorylated at the site of Ser235/236 [11,13–15]. Studies even showed that rpS6 dephosphorylation suppressed the proliferation of pancreatic cancer [14] and breast cancer [16] cell lines. Moreover, the antiproliferative efficacy of mTOR inhibitors that prevent the phosphorylation of rpS6 is currently being evaluated as a new treatment for several benign and malignant tumors [17,18]. These suggest that rpS6, mainly with the phosphorylated activated form, might be a powerful biomarker in tumors. However, an obvious inverse finding that the high level of rpS6 phosphorylation indicated a low mouse embryonic cell proliferation was also observed to contradict the traditional viewpoint [2]. As for NSCLC, the expression, biological functions and basic mechanisms of rpS6 are even little known. Here, in this study we firstly detected the expressions of total rpS6 and its phosphorylation (p-rpS6, Ser235/236) in NSCLC clinical tissues *via* immunohistochemical (IHC) stainings and Western blot assays. Their clinical relevance and prognostic impacts on the overall survival (OS) and relapse-free survival (RFS) in NSCLC patients were evaluated as well. Secondly, two small hairpin RNA (shRNA) lentiviruses specifically silencing rpS6 were constructed to stably inhibit the expression of rpS6/p-rpS6 in adenocarcinoma A549 cell line and squamous cell carcinoma H520 cell lines. Thirdly, the effects of rpS6 on the growth and senescence of NSCLC cell lines were detected both *in vitro* and *in vivo*, followed by mechanisms explorations, including cell cycle distribution and cell apoptosis.

Methods

Patient information and tissue specimens

A cohort of 131 surgically resected primary NSCLC patients and their paraffin-embedded archived tumor samples, together with 68 adjacent normal lung tissues, were collected in the Department of Pathology of West China Hospital of Sichuan University (WCHSU, Chengdu, China) from 2006 to 2007, for the immunohistochemical stainings (IHC) for total rpS6 and p-rpS6 (Ser235/236). Clinical information was obtained by chart review and the survival data were procured by telephone interviews. Histopathological diagnosis, histological gradings and clinical stages were determined according to the WHO classification for NSCLC and the International Union Against Cancer's tumor–node–metastasis system [19–21]. There were 50 squamous cell carcinomas (SCC), 62 adenocarcinomas (ADC) and 19 other types, mainly including adenosquamous carcinomas and large-cell carcinomas in the study. Another group of 36 fresh NSCLC tissues and controls from the tumor resection surgeries in the Department of Thoracic Surgery of WCHSU in 2009 were also gathered up for the Western blot analysis, to confirm the results of IHC. All patients in the whole study were adjuvant therapy-free before surgeries. Prior approval from the Ethics Committee of Sichuan University, China and patients' consents were obtained as well.

Immunohistochemical (IHC) stainings

Envision immunohistochemical staining kit (DAKO) was used for the IHC detections [22]. Total rpS6 (CST, #2217, 1:100 dilution) and p-rpS6 (Ser235/236, CST, #4858, 1:400 dilution) were performed for the patients' specimens and xenografted tumors; while Ki-67 was used in the xenografted tissues (CST, #9027, 1:400 dilution). The known colorectal cancer tissues were utilized as positive controls; whereas a negative control was obtained by replacing the primary antibody with phosphate buffered saline (PBS) (0.01 mol/l, pH = 7.4). IHC images were read semiquantitatively, including both the staining intensities and positive cells' proportions. Ten fields were randomly selected at 200× magnification. The intensities were classified into four levels from 0 to 3. That was 0 indicating invisible positive stainings, 1 for weak stainings, 2 for moderate and 3 for strong stainings. The proportion of positive cells was scored from 0 to 3 as well, which meant that 0 indicated none of the tumor cells positively stained, 1 for less than 20% positive cells, 2 for 20% to 50% positive cells and 3 for over 50% positive cells. Scores for the intensity and proportion were multiplied to get the IHC scores. In the final statistical analysis, scores of 0–2, including the completely negative ones or slightly weak expressions, were classified as negative; whereas scores of 3–9 were divided into the positive group (3~5 scores for moderate and 6~9 scores for strong expressions).

Western blot

Western blot for the total proteins from patients' tissues, xenografted tumors and cultured cells was carried out according to the procedures described previously [23]. Controls were referred to β-actin (CST, #4970, 1:1000 dilution). The main antibodies in Western blot included total rpS6 (CST, #2217, 1:1000 dilution), p-rpS6 (Ser235/236, CST, #4858, 1:2000 dilution), $p16^{Ink4A}$ (CST, #2407, 1:1000 dilution), $p21^{Cip1}$ (CST, #2947, 1:1000 dilution), $p27^{Kip1}$ (CST, #3688, 1:1000 dilution), and $p57^{Kip2}$ (CST, #2557, 1:1000 dilution), total Rb (CST, #9313, 1:1000 dilution), p-Rb (Ser780, CST, #3590, 1:1000 dilution), cyclin D1(CST, #2978, 1:1000 dilution), cyclin A (CST, #4656, 1:2000 dilution), cyclin E (SAB, #29030, 1:1000 dilution), Bcl-xl (CST, #2764, 1:1000 dilution), Bax (CST, #5023, 1:1000 dilution), caspase 3 (CST, #9665, 1:1000 dilution), caspase 8 (CST, #4927, 1:1000 dilution) and cyto-c (CST, #11940, 1:1000 dilution). Expressions of each protein were analyzed with the Biometrics digitized image software and recorded as integrated densities (ID). Ratios of $ID_{(each\ protein)}$ to $ID_{(\beta\text{-actin})}$ were the final results for the protein expressions.

Cell lines and cell culture

NSCLC cell lines, including A549 for adenocarcinoma, H520 for squamous carcinoma and H460 for large cell carcinoma, and human bronchial epithelial cell line (HBE) purchased from the American Type Culture Collection (ATCC) were used in the study. As per the manufacturers' instructions and previous reports [24,25], NSCLC cell lines were maintained in RPMI-1640 (Hyclone, USA) supplemented with fetal bovine serum (FBS, Gibco, USA), 2 mmol/l L-glutamine, 1000 U/ml penicillin and 1 mg/ml streptomycin (Life Tech, UK). HBE was cultured in keratinocyte-serum free medium with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract and supplemented with 0.005 mg/ml insulin, 500 ng/ml hydrocortisone, penicillin and streptomycin (Life Tech, UK). All cells were grown in a sterile humidified incubator with 5% CO_2 at 37 °C.

Construction of small hairpin RNA (shRNA) lentivirus and cell transfection

The lentiviral packaging plasmid of pGCsil with the marker of green fluorescent protein (GFP) was bought from Genechem, Shanghai, China. The two targeted rpS6 knockdown sequences and their negative control (NC) were 5′-GCAGAA TATGCTAAACTTT-3′ (sh1), 5′-TGAACGCAAACTTCGTACT-3′ (sh2) and 5′-TTCT CCGAACGTGTCACGT-3′ respectively. Cell transfections and the stable-transfected clones' selections were conducted as previous researches [26,27]. Briefly, cells were seeded as 150,000 cells/well on six-well culture dishes until 30% of confluence, and then transfected with lentivirus at the optimal multiplicity of infection (MOI). The GFP under fluorescence microscopy was observed to demonstrate the progress of transfections. Nearly five days after the initial transfection, cells were collected for the selection by fluorescence activated cell sorting (FACS) to obtain the stably transfected cells.

Quantitative real-time PCR (RT-PCR) analysis

Total RNA was abstracted using the RNAsimple Total RNA Kit (Tiangen, China) as described by the manufacturer. β-actin was used for controls. Genomic DNA was amplified by real-time RT-PCR using the specific rpS6 gene amplifying primers. The forward and reverse PCR primers for rpS6 were 5′-CCTATTTCCCATGATTCCTTCATA-3′ and 5′-GTAATACGGTTATCCACGCG-3′ respectively; whereas for β-actin the forward primer was 5′-CCATCACCAT CTTCCAGG-3′, and reverse was 5′-ATGAGTCCTT CCACGATAC-3′. Samples were amplified in an RT-PCR System (Applied Biosystems) as the following steps: denaturation at 94 °C for 30 s, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and another 72 °C for 6 min. The final relative expressions of rpS6 mRNA were compared in $2^{-\Delta\Delta Ct}$ [27,28].

Xenografted tumor model

The experiments involving animals were also approved by the Ethics Committee of Sichuan University, China. Only A549 with rpS6 knockdown by the first shRNA was selected for the xenografted tumor model. Six to eight week-old BALB/c-nu/nu nude female mice 18–22 grams in weight were obtained from the Animal Centre of Sichuan University, China. Each of them was injected with 3×10^5 cells subcutaneously in the right flank and divided into the three groups: A549 with sh1-rpS6 (A549 + sh1-rpS6), A549 with sh-NC (A549 + sh-NC) and A549 without any inference (A549). There were six mice in each group. The xenografted tumors were measured by calipers for the dimensions twice a week after 7 days of injection and volumes were calculated using the formula $V = \pi/6 \times \text{length} \times \text{width}^2$ for the tumor growth curves. At the end of the experiment (on the 28th day after the cell implantation), all mice were euthanized to measure the tumor weights and other parameters, such the expressions of rpS6/p-rpS6, Ki-67 and TUNEL in paraffin-embedded tissues [29]. The rest of xenografted tissues were stored at –80 °C until use.