Original Article

Ribosomal protein s15a (RPS15A) knockdown inhibits thyroid cancer cell proliferation through induction of apoptosis

Jian-Hua Wu, Zi-Hao Cai, Jie Wang, Zhen-Xin Cai, Zhu-Chao Zhou

Department of General Surgery, Huashan Hospital, Fudan University, Shanghai 200040, China

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Abstract: Thyroid cancer has become one of the most common endocrine tumors with a growing incidence. In this study, we identified Ribosomal protein s15a (RPS15A), which mediates the interaction of mRNA and ribosome in the early stage of translation, as an anti-apoptotic protein in the thyroid cancer cells. Knockdown of RPS15A significantly inhibited the proliferation of TT cells, a medullary thyroid cancer cell line with high malignancy. Two different shRNA sequences targeting RPS15A could both specifically down-regulate its endogenous expression, as determined by Real-Time PCR and Western Blot. The inhibited proliferation was related to the cell cycle arrest at G0/G1 phase and an elevated apoptosis in response to RPS15A knockdown determined by flow cytometry. Mechanically, we found that RPS15A-silencing promoted apoptosis through enhancing the expression of bad, bax and p53, whereas through decreasing the levels of survivin and bcl-2. Finally, through the data-mining public Oncomine microarray datasets, we identified that RPS15A was upregulated in thyroid cancer tissues in the TCGA Thyroid dataset. In conclusion, RPS15A plays an essential role in thyroid cancer cells and may be a potential therapeutic target in thyroid cancer.

Keywords: RPS15A, shRNA, thyroid cancer, cell proliferation, apoptosis, bcl-2

Introduction

Thyroid cancer is one of the most common endocrine malignancies and is rapidly increasing in incidence [1, 2], which is classified into papillary, follicular, medullary and anaplastic carcinoma [3]. At the time of diagnosis, neck lymph node metastases occur in about 50% of patients and distant metastases were detected in 20% of cases [4-6]. Fine-needle aspiration (FNA) biopsy is a useful clinical examination for thyroid tumors (papillary, medullary and anaplastic), but its accuracy of diagnosis remains to be improved [7]. The radiotherapy is effective but the appropriate use and dosage of radioactive iodine is still controversial [8]. Thus, it is urgently needed to identify novel therapeutic targets in early stage of thyroid tumor, which might provide fundamental information for future new therapy for thyroid cancer.

Ribosomal protein s15a (RPS15A), as a highly conserved protein, is a component of the small 40S subunit of ribosomes, which are responsible for catalyzing protein synthesis. It has been also observed that RPS15A is abundantly expressed in some parts of plants such as flower buds and young leaves, but less abundant in mature tissues [9, 10]. As the part of 40S ribosomes, RPS15A has been shown to promote mRNA and ribosome interactions in early stage of translations by interacting with eukaryotic initiation factor 4F (eIF-4F) in yeast [11]. Recent evidence showed that RPS15A play important roles in human carcinoma. The expression of RPS15A is different in situ and invasive breast tumors [12]. It has been shown that RPS15A is highly expressed in astrocytoma, prostate cancer and colorectal cancer in a meta-analysis [13]. In hepatic cells, the overexpression of RPS15A promotes the cells proliferation and down-regulated RPS15A suppressed the G0/G1 phase of cell cycle, which indicated RPS15A is involved with the hepatic cancer cell growth [14]. In addition, RPS15A stimulates the tumor cell proliferation by response to the transforming growth factor-beta 1 (TGF-beta 1) in lung cancer A549 cell lines [15]. All of this reminded...
us that RPS15A plays key roles in the regulation of cell proliferation. However, the function of RPS15A in human thyroid cancer has not yet been determined.

In this study, we knocked down the expression of RPS15A by lentivirus-mediated short hairpin RNA (shRNA) in human thyroid cancer cell lines TT. Then we investigated the biological function of RPS15A in TT cell growth, as well as the underlying mechanism. Our study demonstrates the oncogenic role of RPS15A in thyroid cancer.

Materials and methods

Cell culture

The thyroid medullary carcinoma cell line TT and the human embryonic kidney cell line 293T (HEK293T) were obtained from the Cell Bank of Chinese Academy Science (Shanghai, China). TT cells were cultured in F-12K, a Kaighn’s modification of Ham’s F-12 medium, which containing 0.2922 g/L L-glutamine (21127022, Gibco, Cambrex, MD, USA), with 10% FBS (fetal bovine serum) (S1810, Biowest, Nuail, France). HEK293T cells were cultured in DMEM (SH30243.01B+, Hyclone, Logan, UT, USA) with 10% FBS (fetal bovine serum) (S1810, Biowest, Nuail, France). Both of the cell lines were maintained at 37°C in incubator (311, Thermo) with 5% CO₂.

Lentivirus-delivered short hairpin RNA (shRNA) transduction

According to the sequence of RPS15A (NM_001019), two RPS15A shRNAs sequences were designed to knock down RPS15A expression: sequence 1 (S1): 5’-GTGCAACTCAAAAGACCTGGAACTCGAGTTCCAGGTCTTTGAGTTGCACTTTTT-3’, sequence 2 (S2): 5’-GCATGGTTACATTGGCGAATTCTCGAGAATTCGCCAATGTAACCATGCTTTTT-3’. A scrambled shRNA (5’-GCGGAGGGTTTGAAAGAATATCTCGAGATATTTCTTTCAAACCCTCCGCTTTTTT-3’) was used as control shRNA. Then these oligos were cloned into the pFH-L vector (Shanghai Hollybio, China). The recombinant lentivirus was collected 48 h post infection. TT cells were plated in a six-well plate and added prepared lentiviral supernatant at a multiplicity of infection (MOI) of 60. After infection for 12 h, cells were collected and observed under a fluorescence microscope to count the number of green fluorescent protein (GFP)-positive cells. The down-regulated expression of RPS15A was further confirmed by qPCR and western blotting analysis.

Quantitative PCR (qPCR) analysis

After infected with lentivirus for 5-6 days, the expression of RPS15A was detected by performing quantitative PCR in TT cells, using BioRad Connet Real-Time PCR platform (CFX-96, Biorad, California, USA) according to the manufacturer’s protocol (1708882AP, Biorad, California, USA). RPS15A was amplified using primers: Forward: 5’-TGACGTGCAACTCAAAGAC-3’ and Reverse: 5’-CCAGAGGGTTTGAAAGAATATC-3’. The housekeeping gene actin was used as reference and amplified using primers: Forward: 5’-GAAAGGTTGAACGCAACTA-3’ and Reverse: 5’-AAAGGTTGAACGCAACTA-3’. The primers of downstream target genes were as follows: Bad (Forward): 5’-GGACATCGGAAAGAC-3’ and Bad (Reverse): 5’-GGACATCGGAAAGAC-3’. Bax (Forward): 5’-TGGGC TGGACATTGGAGAATTCGCCAATGTAACCATGCTTTTT-3’ and Bax (Reverse): 5’-GACCTCCCCGACCAAAAG-3’. p53 (Forward): 5’-GACTGACATTTCCATCTCCTTG-3’ and p53 (Reverse): 5’-CTCTGACAGCAACCTTTG-3’. survivin (Forward): 5’-AATGAGGCTTCTGGGCTAT-3’ and survivin (Reverse): 5’-GGCTTTGCTGGCTCTCTTG-3’. The reaction system (170888, BioRad, California, USA) was 20 ml mixture including 2×SYBR premix ex taq 10 ml, forward and reverse primers (2.5 mM) 0.8 ml, cDNA 5 ml and ddH₂O 4.2 ml. The program was performed as follows: Initial denaturation at 95°C for 1 min; denaturation at 95°C for 5 s; annealing extension at 60°C for 20 s (a total of 40 cycles). And absorbance value was read at the extension stage. The 2-ΔΔCT method was used to analyze the mRNA expression of genes.

Western blotting

After lentivirus infection for 5-6 days, TT cells were lysed by 2×SDS Lysis Buffer (2×SDS Sample Buffer (100 mM Tris-Hcl (pH 6.8), 10 mM EDTA (Sangon, Shanghai, China), 4% SDS (SB0485-500 g, Sangon, Shanghai, China), 10471 Int J Clin Exp Med 2016;9(6):10470-10478)
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10% Glycine) and centrifuged for 5 min in 4°C, 12000 rpm, to obtain supernatant for the total protein. The total protein was quantified using BCA (bicinchoninic acid) method. Total 30 µg protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane. Then the PVDF membrane was washed by Tris-buffered saline Tween (TBST) and incubated with blocking buffer (5% skimmed milk) for at least 1 h at room temperature. Proteins were probed with primary antibody (rabbit anti-human RPS15A antibody, AP4804a, abgent, San Diego, USA, dilution: 1:1000; and rabbit anti-human cyclin D antibody, sc-25765, Santa Cruz Biotechnology, Inc., CA, USA, dilution: 1:200; rabbit anti-human Bcl-2 antibody, 2876, Cell signaling, Massachusetts, USA, dilution: 1:500; rabbit anti-caspase-3, #9661, Cell signaling, dilution: 1:500; rabbit anti-human GAPDH anti-body, 10494-1AP, Proteintech Group, Inc. Chicago, USA, dilution: 1:50000, at 4°C overnight. Then, membranes were washed in TBST for 10 min three times and incubated with secondary antibody (goat anti-rabbit IgG-HRP (SC-2054, Santa Cruz, California, USA, dilution: 1:5000) at room temperature for 2 h. The signals were detected by enhanced chemiluminescence detection kit (Millipore, Billerica, MA). GADPH was used as the internal standard.

Cell proliferation assay

After lentivirus infection for 5 days, TT cells were seeded in 96 well plate at a density of 5×10³ cells per well. MTT (3-(4,5)-dimethyl-thiahiazol-(z-y1)-3, 5-di-phenytetrazoliumromide) assay was performed to determine the effect of RPS15A on cell proliferation in a period of 5 days. At each time point, 20 µl of 5 mg/ml MTT (M2128, Sigma, St. Louis, USA) was added into per well. After incubation for 4 h, 100 µl acidified isopropanol (10% SDS, 5% isopropanol (80190218, Sinopharm Chemical Regant Co. Ltd, Shanghai, China) and 0.01 mol/L HCl) per well was added to stop reaction. The absorbance values were detected at 595 nm on a microplate reader (Epoch).

Cell cycle analysis

After lentivirus infection for 6 days, TT cells were seeded in 6 cm dishes with 6×10⁵ cells per dish. After cultured in 37°C (5% CO₂) for 40 h, the TT cells were digested by trypsin (SH30042, Hyclone, Logan, UT, USA), washed by PBS and fixed with 75% cold ethanol (10009269, Sinopharm Chemical Regant Co. Ltd, Shanghai, China) overnight at 4°C. Finally, 500 µl PI (Propidium Iodide) solution (20×, C1052, Beyotime Biotechnology, Shanghai, China) was added in the cell suspension after centrifugation and placed in 37°C for 1 h in dark. Cell cycle was detected by flow cytometry (FACS Calibur, BD Biosciences). The factions of cells in different cell cycle phases were determined.

Flow cytometric apoptosis assay

After lentivirus infection for 7 days, approximately 6×10⁵ cells per dish cells was plated in 6-well plates and determined by flow cytometry using Annexin V-APC/7-AAD. According to the Annexin V/7-AAD double staining kit manufacturer’s instructions, the TT cell was harvested and washed twice with PBS. The TT cells were resuspended in 500 µl binding buffer and then added 5 µl Annexin V-APC and 5 µl 7-AAD. The percentage of apoptotic cells was determined on flow cytometry (FACS Calibur, BD Biosciences).

Oncomine database analysis

To determine the expression pattern of RPS15A in thyroid cancer, TGCA dataset in Oncomine data (https://www.oncomine.org) was used. We compared RPS15A gene expression in thyroid cancer tissues according to the standard procedures as previously [16]. We eliminated one data in each group which were considered to be exceptional data according Pauta criterion.

Statistical analysis

We performed all experiments at least thrice. Statistical analysis was performed using Graphpad Prism software. Quantitative data were expressed as mean ± standard deviation. Comparisons between groups were conducted by Student’s t test. The P-value < 0.05 was considered as statistically significant.

Results

Lentivirus-delivered shRNA down-regulated RPS15A expression in cells

To suppress the expression of RPS15A in thyroid cancer cells, the stably expressing RPS15A-
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shRNA lentivirus (shRPS15A(S1) or shRPS15A(S2)) was transduced to TT cells at a multiplicity of infection (MOI) of 60. The shRNA (shCon) infected cells were used as negative control. After lentivirus infection for 120 h, we recorded the GFP-expressing in TT cells and found most of cells were observed to be GFP-positive expression (Figure 1A), suggesting a satisfying infection efficiency. To further measure the knockdown efficiency, we compared the mRNA and protein levels of RPS15A among different treatment groups (shCon, shRPS15A(S1) or shRPS15A(S2)). Our data revealed the RPS15A mRNA level was successfully suppressed by either shRPS15A(S1) or shRPS15A(S2) in TT cells compared with the shCon group, corresponding to a knockdown efficiency of 99.1% and 98.7% respectively (P < 0.01, Figure 1B). Western blot also confirmed the absolute silencing of RPS15A expression in TT cells (Figure 1C). In this way, our constructed shRPS15A(S1) and shRPS15A(S2) could specifically down-regulate endogenous RPS15A expression.

Knockdown of RPS15A inhibited proliferation of TT cells

To investigate the effect of RPS15A knockdown on cell proliferation, MTT assay was performed.
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in TT cells after RPS15A knockdown. Compared with the shCon group, the proliferation rate of the shRPS15A(S1) group or the shRPS15A(S2) group was significantly suppressed ($P < 0.001$, Figure 2). What’s more, shRPS15A(S1) suppressed TT cell growth more obviously than that in shRPS15A(S2). These results indicated that depletion of RPS15A decreased proliferation of TT cells.

Knockdown of RPS15A arrested cell cycle at G0/G1 phase

To further determine the mechanism underlying RPS15A knockdown suppressed cell growth, we performed the flow cytometry to detect the cell cycle progression (Figure 3A). As shown in Figure 3B, a small percentage of cells remain blocked in G0/G1 phase in TT cells after shRPS15A(S1) lentivirus infection ($P < 0.001$). Cyclin D1 is a key cell cycle regulator required for G1/S transition [16]. As shown in Figure 3C, Western blot analysis showed the expression of Cyclin D1 was obviously down-regulated in shRPS15A(S1) infected TT cells, which suggested that RPS15A knockdown could induce G0/G1 phase arrest via suppression of Cyclin D1.

Effect of RPS15A on TT cell apoptosis

According to the effect of RPS15A on cell growth, shRPS15A(S1) has obvious inhibition on TT cell growth than shRPS15A(S2). Thus, TT cells infected with shRPS15A(S1) was chosen to investigate whether RPS15A-silencing caused any apoptosis effects using flow cytometric apoptosis analysis. As shown in Figure 4A, shRPS15A(S1) caused significant change in the profile of Annexin-APC/7-AAD-stained cell populations. An obvious early apoptosis rate was observed in shRPS15A(S1) groups compared with that in shCon groups in TT cells. The quantification of gene expression associated with apoptosis was analyzed by qRT-PCR. As shown in Figure 4B, the level of bad, bax and p53 in shRPS15A(S1) infected cells were sig-
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Significantly increased and survivin decreased significantly. In addition, Western blot analysis showed knockdown of RPS15A down regulated expression of bcl-2.

Database analysis reveals RPS15A is overexpressed in thyroid cancer

RPS15A mRNA levels in human thyroid cancer were investigated between thyroid cancer tissues and normal thyroid tissues using datasets form the publicly available Oncomine database. We found that RPS15A expression was significantly upregulated in thyroid gland carcinoma tissues (n = 44, P = 0.0011) compared with paired normal thyroid gland tissues (n = 96) using TCGA thyroid dataset (Figure 5).

Figure 4. Knockdown of RPS15A promoted apoptosis in TT cell line. (A) Quantification of apoptotic cells in TT cells by flow cytometry, including early apoptotic cells (Annexin V+/7-ADD-) and late apoptotic cells (Annexin V+/7-ADD+). Expression of mRNA and proteins related to apoptosis in TT cells after RPS15A knockdown, as determined by qRT-PCR (B) and Western blot analysis (C). Beta-actin was used as control gene and GAPDH was used as control protein. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5. Upregulation of RPS15A copy number in thyroid cancer tissues revealed by data-mining of TCGA dataset from the Oncomine database. Gene expression in 44 thyroid cancer tissues and 96 normal thyroid tissues were analyzed according to the standard procedures as previously. One datum was eliminated in each group, which was considered to be exceptional data according Pauta criterion.

Discussion

Translational control, which depend on ribosome formation, plays a critical role in the regulation of gene expression in eukaryotes and affects many essential cellular processes, including proliferation, apoptosis and differentiation [17]. It has been reported that the ribosome biogenesis play essential roles in malignant process and a plenty of oncogenes or tumor suppressors have been identified in human cancers, indicating the important role of the protein synthesis machinery in the regulation of malignant progression of cancer [18-22]. However, the biological role of ribosome formation in thyroid cancer is unknown.

RPS15A is a component of the 40S ribosome subunit and is highly evolutionary conserved. Recently, tumorigenesis promoting effects of 40S ribosomal subunit biogenesis were identified in previous studies in other cancer types [23, 24]. Aberrant RPS15A expression has been observed in lung, invasive breast, astrocytoma, prostate and colorectal cancer tissues [12, 13, 25]. And RPS15A was reported to stimulate the cell growth in human hepatic cancer by regulating G1/S transition [14, 26]. In lung cancer A549 cell line, RPS15A was found to stimulate the tumor cell proliferation by response to the transforming growth factor-beta 1 (TGF-beta 1) [15]. Hence, it is conceivable that RPS15A is involved in carcinoma cell
growth and cell cycle control. But the effect of RPS15A in thyroid cancer is not clear.

Thyroid cancer is a common endocrine cancer with high morbidity, lethality, and with a growing incidence, which calls urgent need for mechanisms that modulate the carcinogenesis. There are four histopathological forms of thyroid cancer. Despite only 5% of the proportion, medullary thyroid carcinoma (MTC) carries a worse prognosis than other differentiated thyroid cancers [27]. We found RPS15A was overexpressed in thyroid cancer tissues, as determined by Oncomine database analysis. To further evaluate its role in MTC, we knocked down the expression of RPS15A in TT cells. Functional analysis showed knockdown of RPS15A by two different shRNAs both significantly suppressed cell proliferation might through inducing cell cycle arrest in the G0/G1 phase. Our results were consistent with a previous research showing that knockdown of RPS15A inhibited cell proliferation and arrested cell cycle at G0/G1 phase in hepatocellular carcinoma [26] and lung cancer cells [25]. Furthermore, we found that RPS15A knockdown suppressed the expression of Cyclin D1 in TT cells. Cyclin D1 regulates G1 to S phase transition through CDKs (cyclin-dependent kinases) and was overexpressed in various carcinomas [28-31]. Based on this result, we could conclude knockdown of RPS15A arrested cell cycle at G0/G1 phase via suppressing Cyclin D1.

Subsequently, flow cytometry analysis further demonstrated knockdown of RPS15A promoted cell apoptosis in TT cells. Western blot analysis confirmed RPS15A-silencing markedly up-regulated the expression levels of bad, bax and p53, as well as down-regulated the expression of survivin and bcl-2. The transcription factor p53, as a tumor suppressor, is usually phosphorylated and activated on DNA damage, which was responsible for leading to growth arrest and cell death induction [32]. It has been reported that bad, bax and bcl-2 are involved in TGF-β-mediated apoptosis [33-35]. Bax, as a pro-apoptotic factor, has been shown to serve as a transcriptional target for p53. Bcl-2 could counteract and suppress the activity of bax [36-38]. As a similar pro-apoptotic protein, bad could also promote cell apoptosis [39]. Survivin has been identified as an inhibitor of apoptosis, which was down regulated in TT cells after RPS15A knockdown [40]. These data suggest RPS15A regulating cell growth by controlling apoptosis signaling pathways.

Collectively, these results indicated that RPS15A was overexpressed in thyroid cancer tissues. Functional experiment indicated that RPS15A plays a crucial role in cell proliferation of thyroid cancer. These findings will provide a preliminary experiment basis for RPS15A as a potential molecular target against thyroid cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhu-Chao Zhou, Department of General Surgery, Huashan Hospital, Fudan University, No. 12 Wulumuqi Zhong Road, Shanghai 200040, China. Tel: 86-21-52889353; E-mail: zhuchaoprof@163.com

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