
Maiyu Xu a,1, Yi Wang b,1, Lei Chen a, Bujian Pan a, Feng Chen a, Yang Fang c, Zhengping Yu d, Gang Chen d,⁎

a Department of Hepatobiliary Surgery, Wenzhou Central Hospital, Wenzhou 325000, China
b Environmental and Public Health School of Wenzhou Medical University, Wenzhou 325000, China
c Department of Anus & Intestine Surgery, Wenzhou Central Hospital, Wenzhou, Zhejiang 325000, China
d Department of Hepatobiliary Surgery, The First Affiliated Hospital, Wenzhou Medical University, Wenzhou 325000, China

A R T I C L E   I N F O

Article history:
Accepted 26 November 2013
Available online 12 December 2013

Keywords:
RPS15A
shRPS15A
Growth
Hepatocellular carcinoma

A B S T R A C T

Ribosomal protein s15a (RPS15A) is a highly conserved protein that promotes mRNA/ribosome interactions early in translation. Recent evidence showed that RPS15A could stimulate growth in yeast, plant and human lung carcinoma. Here we report that RPS15A knockdown could inhibit hepatic cancer cell growth in vitro. When transduced with shRPS15A-containing lentivirus, we observed inhibited cell proliferation and impaired colony formation in both HepG2 and Bel7404 cells. Furthermore, cell cycle analysis showed that HepG2 cells were arrested at the G0/G1 phase when transduced with Lv-shRPS15A. In conclusion, our findings provide for the first time the biological effects of RPS15A in hepatic cancer cell growth. RPS15A may play a prominent role in heptocarcinogenesis and serve as a potential therapeutic target in hepatocellular carcinoma.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Hepatocellular carcinoma (HCC) is a worldwide clinical and social issue. Actually, it is the sixth most common cancer and the third cause of cancer-related death (Parkin et al., 2005). The incidence of HCC is increasing in the general population of patients with cirrhosis (Bosetti et al., 2008; Jemal et al., 2008) as well as some subgroups of patients, such as those with human immunodeficiency virus (HIV) infection or thalassemia (Ioannou et al., 2013; Mancuso et al., 2005; Merchante et al., 2013; Sahasrabuddhe et al., 2012). The incidence of HCC increases with advancing age, with a median age at onset of about 70 years old in developed countries and there is a male preponderance, with a gender ratio of about 2.4 (El-Serag and Mason, 1999; Parkin et al., 2005).

Worldwide and also in China, HCC is mainly caused by chronic hepatitis B virus and hepatitis C virus infection. The hepatitis B virus \( \times \) protein (HBxAg), a multifunctional protein, serves many important roles in hepatocellular carcinogenesis. It was reported that HBxAg promotes hepatic cell proliferation through up-regulation of ribosomal protein s15a (RPS15A) (Lian et al., 2004), indicating that RPS15A may play a key role in hepatic cancer cell growth. This gene encodes a ribosomal protein which is a component of the 40S ribosomal subunit, the small subunit of eukaryotic ribosomes. It has been identified that RPS15A promotes mRNA/ribosome interactions early in translation and facilitates translation by interacting with eukaryotic initiation factor 4F (eIF-4F) as is encoded by CDC33 in yeast. Mutation in CDC33 caused growth arrest at the G1–S transition which phenomenon was reversed by overexpression of RPS15A (Lavoie et al., 1994), implicating that RPS15A could overcome G1 arrest and promote cell cycle progression. There is evidence that RPS15A is abundantly expressed in the actively dividing tissues of Brassica napus (oilseed), such as the apical meristem, flower buds and young leaves, but less abundant in a mature stem and fully expanded leaves (Bonham-Smith and Moloney, 1994; Bonham-Smith et al., 1992), further indicating that RPS15A may promote cell growth. In addition, RPS15A was also reported to be a responsive gene of transforming growth factor-beta 1 (TGF-beta 1) and promotes cell proliferation in the human lung carcinoma cell line A549 (Akiyama et al., 2000).

Despite the reports that RPS15A stimulated growth in yeast, plants and human lung carcinoma (Akiyama et al., 2000; Bonham-Smith and Moloney, 1994; Bonham-Smith et al., 1992; Reed, 1980), its functional role in human HCC remains unknown. Herein, we demonstrated that knockdown of RPS15A by lentivirus-mediated short
hairpin RNA (shRNA) could inhibit hepatic cancer cell proliferation and colony formation in vitro. Moreover, RPS15A depletion suppressed cell cycle progression via G0/G1 phase arrest. Collectively, RPS15A may modulate hepatic cancer growth and play a prominent role in heptocarcinogenesis. We propose RPS15A as a potential therapeutic target in HCC.

Fig. 1. RPS15A knockdown by a lentivirus-mediated RNAi system. (A) Relative RPS15A mRNA level in six hepatic cancer cell lines (HepG2, Bel7402, Bel7404, SK-HEP-1, HEP3B and SMMC-7721). (B, C) Fluorescence photomicrographs of HepG2 and Bel7404 cells infected by lentivirus. Pictures were taken 96 h after infection at a magnification of 100×. (D, E) Identification of RPS15A knockdown efficiency via RT-qPCR in HepG2 and Bel7404 cells (Con: no lentivirus treatment; Lv-shCon: control lentivirus; Lv-shRPS15A: lentivirus containing shRNA targeting RPS15A). **p < 0.01, ***p < 0.001 compared to Lv-shCon.
2. Materials and methods

2.1. Cell lines and cell culture

Hepatic cancer cell lines HepG2, SK-HEP-1 and Hep3B were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Hepatic cancer cell lines Bel7402, Bel7404, and SMMC-7721 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Cambrex, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, SV30087.02, Hyclone, Logan, UT) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO2.

2.2. RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNAs of cultured cells were extracted respectively using the Trizol solution (Invitrogen, Carlsbad, CA) at 24 h after transfection. The first-strand complementary DNA was synthesized from total RNA (1000 ng) using the PrimeScript RT Master Mix Perfect Real Time (TaKaRa, Shiga, Japan). RPS15A mRNA expression was evaluated by real-time PCR using the SYBR Premix Ex Taq™ Perfect Real Time (TaKaRa, Shiga, Japan) on an ABIPRISM 7500 Real-Time System. β-actin was applied as the input reference. Primers were designed based on cDNA sequences to amplify RPS15A (NM_001019.4) and β-actin (ACTB, NM_001101.3). The primers used were as follows:

**RPS15A:**
Forward, 5′-TGAGTGCCAACTCAAGGACC-3′
Reverse, 5′-CCAGATCCAGGCAATT-3′

β-actin:
Forward, 5′-GGCAATCCGCAAGAGAC-3′
Reverse, 5′-AAAGGCTGTAACGCAACTA-3′

Relative mRNA was determined by using the formula $2^{-\Delta \Delta CT}$ (CT; cycle threshold) where $\Delta CT = CT$ (target gene) — $CT$ (β-actin).

2.3. Construction of RPS15A short hairpin RNA-expressing lentivirus

The non-silencing small interference RNA (siRNA) (5′-TCTTCCGAAC GTGTCACGT-3′) was used as a control that does not target any gene in human as determined by screening with NCBI RefSeq. RPS15A short hairpin RNA (shRNA) was 5′-GCATGTTACATTGCCGAATCTCGAGAAT TCGCAATGTAACCATGCTTTTT-3′ that targets nucleotides 225–243 of human RPS15A mRNA (NM_001019.4). The stem–loop–stem oligos were synthesized, annealed, and ligated into the Nhe I/Pac I-linearized pFV-L vector (Hollybio, Shanghai, China). The vector employs the H1 RNA polymerase III promoter, which drives consecutive expression of active shRNA. The generated plasmids were named as pFV-L-shRPS15A or pFV-L-shCon. For the transfection, HepG2 andBel7404 cells (1 × 10^5) were seeded in 10-cm dishes and cultured for 36 h to reach 90% confluence, respectively. At 2 h before transfection, the medium was replaced with FBS-free DMEM. The plasmid mixture containing pFV-L-shRPS15A (or pFV-L-shCon) and pSVSVG-I/pCMVΔR8.92 packaging vectors, as well as Lipofectamine 2000 (Invitrogen, Carlsbad, CA) were added to the cells. At 5 h after incubation, the medium was replaced with DMEM containing 10% FBS. Lentiviral particles (Lv-shRPS15A or Lv-shCon) were harvested at 48 h after transfection and purified by ultra-centrifugation according to previous reports (Sakoda et al., 1999; Soneoka et al., 1995). As the lentivirus carries a green fluorescence protein (GFP) reporter driven by the CMV promoter, the viral titer was determined by counting GFP-expressing cells under fluorescence microscopy 96 h after infection as described in previous reports (Tiscornia et al., 2006).

2.4. MTT proliferation assay

To detect cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed 3 days after lentivirus infection. Briefly, HepG2 and Bel7404 cells were washed with phosphate-buffered saline (PBS) and suspended at a final concentration of 2 × 10^5 per ml and dispensed into 96-well plates. The plates were incubated for 1 to 5 days at 37 °C in a humidified CO2 incubator. On each day, 100 ml of MTT (5 mg/ml) was added and incubated at 37 °C for 4 h, and then 10% dimethylsulfoxide (100 ml) was added to each well. The absorbance at 595 nm was measured using a synergy 2 multi-mode microplate reader (Bio Tek Instruments, Winooski, VT, USA).

2.5. Colony formation assay

HepG2 and Bel7404 cells (a total of 1500 or 500 cells per well) were seeded into six-well plates 3 days after lentivirus infection. The medium was changed at three-day intervals. After 14 days of culture at 37 °C, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were then stained with freshly prepared diluted crystal violet for 10 min, washed with water and air-dried. Cell survival was measured according to the usual criterion of 50 cells or more per colony (Puck and Marcus, 1956). The total number of colonies was counted using light microscope and fluorescent microscope.

2.6. Fluorescence-activated cell sorting analysis (FACS)

Cell cycle distribution of Lv-shRPS15A- or Lv-shCon-infected cells was analyzed by flow cytometry assay following PI staining as described (Tricoli and Bracken, 1993). In brief, 96 h after lentivirus infection, three groups of HepG2 cells (non-infected, infected with Lv-shCon and Lv-shRPS15A) were synchronized by serum starvation for 72 h. A total of 2 × 10^5 cells were seeded in each well of six-well plates and the
complete medium containing 10% FBS was then added to each culture. Cells were allowed to attach overnight and collected. After washing with ice-cold PBS, cells were suspended in about 0.5 ml of 70% cold alcohol and kept at 4 °C for 30 min. The cells were then treated with 100 mg/ml of DNase-free RNase and incubated for 30 min at 37 °C. Propidium iodide (PI, 50 mg/ml; Sigma-Aldrich) was added directly to the cell suspension. The suspension was filtered through a 50-mm nylon mesh, and total of 1 × 10⁴ stained cells were analyzed by a flow cytometer (FACSCalibur, BD Biosciences).

2.7. Statistical analysis

All data were expressed as mean ± SD of three independent experiments, in which each assay was performed in triplicate. The Student’s t-test was used to evaluate the differences and p < 0.05 was considered statistically significant.

3. Results

3.1. Expression of RPS15A was suppressed by infection with Lv-shRPS15A in hepatic cancer cells

To investigate the expression level of RPS15A in hepatic cancer cell lines, qPCR was performed to detect RPS15A mRNA level in diverse hepatic cancer cell lines, including HepG2, Bel7402, Bel7404, SK-HEP-1, Hep3B and SMMC-7721. As depicted in Fig. 1A, all six cell lines expressed RPS15A whereas the highest level was observed in HepG2 cells, followed by Bel7404 cells. We chose HepG2 cells along with Bel7404 cells for subsequent investigation of the role of RPS15A in hepatic cancer growth.

To suppress RPS15A expression, lentivirus stably expressing RPS15A-specific shRNA (Lv-shRPS15A) was infected to HepG2 and Bel7404 cells (multiplicity of infection 50), respectively. Lentivirus expressing

![Knockdown of RPS15A inhibited the colony-forming ability of HepG2 and Bel7404 cells.](image)
negative shRNA (Lv-shCon) was employed as negative control. More than 80% of GFP-expressing cells was observed in both cell lines after 96 h indicating successful lentivirus infection (Fig. 1B and C). To further validate the knockdown efficiency, we measured RPS15A mRNA level in each group. Our results showed that the expression of RPS15A mRNA in Lv-shRPS15A infected cells was significantly suppressed, corresponding to 59.6% and 52.1% suppression in HepG2 and Bel7404 cells, respectively, compared with control cells (p < 0.01, Fig. 1D and E). In all, RPS15A shRNA-expressing lentivirus was successfully constructed.

3.2. Knockdown of RPS15A inhibits the proliferation of hepatic cancer cells

To examine the role of RPS15A in hepatic cancer cell proliferation, MTT assay was performed to detect cell growth once a day continuously for 5 consecutive days. It was shown that both HepG2 cells and Bel7404 cells infected with Lv-shRPS15A presented a slower proliferative rate (Fig. 2A and B). In fact, compared with cells infected with Lv-shCon, HepG2 cell proliferation was reduced by up to 46.4% on day 4 and 60.8% on day 5 (p < 0.001). Consistently, Bel7404 cell proliferation was inhibited by up to 44.5% on day 4 and 44.3% on day 5 (p < 0.001). There was no significant difference in Lv-shCon infected cells and controls. RPS15A depletion significantly decreased the proliferation of both HepG2 and Bel7404 cells.

3.3. Knockdown of RPS15A suppresses the colony-forming ability of hepatic cancer cells

Plate colony formation assay was then conducted to investigate the effect of RPS15A knockdown on in vitro tumor formation. In line with MTT assay results, colony numbers in Lv-shRPS15A infected cells were significantly reduced by comparing with the control or Lv-shCon group. HepG2 cells showed no colony after RPS15A knockdown (Fig. 3A and B). Similarly, the number of a colony was only 39.6% in Lv-shRPS15A infected Bel7404 cells significantly lower than the observed control group (Fig. 3C and D). These results revealed that knockdown of RPS15A had a significant negative effect on hepatic cancer cell growth.

3.4. RPS15A depletion affects cell cycle progression in HepG2 cells

To see the underlying mechanism of inhibition of cell growth, cell cycle progression was assayed by flow cytometry. As shown in Fig. 4A, cell percentage in distinct cell cycles (G0/G1 phase, S phase and G2/M phase) was observed as significantly different in three groups. Lv-shRPS15A infected HepG2 cells were mostly distributed in the G0/G1 phase (55.45%) and least distributed in the G2/M phase (19.82%) (p < 0.001 vs. con or Lv-shCon) (Fig. 4B), indicating cell cycle arrest in the G0/G1 phase. These results suggested that knockdown of RPS15A suppressed cell growth by inducing cell cycle arrest.

4. Discussion

HCC constitutes 85–90% of primary liver cancers and causes significant morbidity and mortality (El-Serag and Rudolph, 2007). Ranking as the third leading cause of cancer deaths, the prognosis of HCC is among the bleakest, with a 5-year survival rate of only 5% for untreated cancer (Li et al., 2013). The effects of RPS15A dysregulation in yeast, plants and human lung carcinoma have been elucidated. In the present study, we found that RPS15A, a ribosomal protein that functions to promote mRNA/ribosome interaction, was aberrantly expressed in several human hepatic cancer cell lines. Gene knockdown using shRNA represents an excellent tool to assess the functional importance of cancer.
related genes in vitro. Previous studies showed that shRNA-targeted MAP4K4 or COMMD7 could remarkably inhibit HCC growth (Liu et al., 2011; Zheng et al., 2012). Therefore, in order to investigate the role of RPS15A in HCC, we employed lentivirus-mediated shRNA to knock down RPS15A in hepatic cancer cells. When infected with Lv-shRPS15A, HepG2 and Bel7404 cells were observed to have dampened cell proliferation and impaired colony formation, together with arrested cell cycle in the G0/G1 phase. Our results indicate that RPS15A may play a prominent role in regulating hepatic cancer cell growth.

The identification of RPS15A as a critical factor for hepatic cancer cell growth is of great biological importance. Specific factors that exclusively expressed in liver cancers are badly needed to be exploited for early diagnosis and prevention of liver cancers. Seven plasma microRNAs were reported to have considerable clinical values in diagnosing early-stage HCC (Zhou et al., 2011). And here we propose that RPS15A might be one of these factors due to its promotion of hepatic cancer cell growth. Our research might broaden the understanding of the role of RPS15A in human HCC.

There are two possible mechanisms whereby RPS15A may contribute to hepatic cancer cell growth: HBxAg-dependent manner and cell cycle-mediated manner. HBxAg was reported to contribute to the development of HCC through the up-regulation of the expression of RPS15A in hepatititis B-related HCC (Li, A.W., et al., 2011). HBxAg could enhance TGFβ1 signaling in tissue culture (Lee et al., 2001), while TGFβ1 could target RPS15A gene and promote cell growth (Akiyama et al., 2000). HBxAg probably facilitates RPS15A-mediated cell growth through the enhancement of TGFβ1 signaling. However, up-regulation of RPS15A by HBxAg was only reported in hepatititis virus B-related HCC. Except for the HBV, there exists other risk factors for the development of HCC. Thus there might be an alternative way for RPS15A-mediated HCC cell growth. Further investigations will be needed.

In conclusion, the Lv-shRPS15A was established to knockdown RPS15A expression and inhibit HCC cell proliferation along with G0/G1 phase arrest. To the best of our knowledge, this is the first study to examine the effect of RPS15A in HCC. Our findings suggest that RPS15A may promote HCC cell growth, and knockdown of RPS15A could be a potential therapeutic approach in HCC. Further investigation regarding the regulatory mechanism of RPS15A in HCC may help to better understand liver cancer progression.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors are thankful for the financial support from the National Natural Science Foundation (81201953), the Natural Science Foundation of Zhejiang Province (Y2090538), the Education Department Foundation of Zhejiang Province (Y201120049) and Wenzhou Municipal Science and Technology Foundation (H20100069).

References


