Research paper

Over-expressed RPL34 promotes malignant proliferation of non-small cell lung cancer cells

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ABSTRACT

Ribosomal protein L34 (RPL34) was reported to be involved in the regulation of cell proliferation of prokaryotes, plant and animal cells. In the present study, we analyze the expression and function of RPL34 in NSCLC. Immuno-histochemical analysis, qPCR and Western blot were used to detect the expression of RPL34 in NSCLC tissues and cells lines. Flow cytometry was used to detect cell activity of NSCLC cell line H1299 under lentivirus-mediated RNAi on RPL34. Cell proliferation and colony formation assays were used to analyze the role of RPL34 in NSCLC cell proliferation. We found that expression of ribosomal protein RPL34 was significantly up-regulated in NSCLC tissues compared to adjacent normal tissues. Lentivirus-mediated shRNA knockdown of RPL34 in NSCLC cell line H1299 resulted in a strong decrease of proliferation, and a moderate but significant increase of apoptosis and S-phase arrest. These data indicate that over-expressed RPL34 may promote malignant proliferation of NSCLC cells, thus playing an important role in development and progress of NSCLC.

1. Introduction

Mammalian ribosomes are constituted of a 60S large subunit and a 40S small subunit, in which main components are ribosomal RNA (rRNA) and ribosomal proteins. Ribosomal proteins are named as RPL (ribosomal proteins in large subunit) or RPS (ribosomal proteins in small subunit) according to the size of subunits they derived from. The main function of ribosomal proteins is participating in the self-assembly of ribosomes and protein synthesis. Recently, ribosomal proteins were found to be involved in other cell functions besides ribosomal functions, including regulation of cell cycle (Nosrati et al., 2015; Gou et al., 2010) and even malignant transformation of normal cells (Wang et al., 2011). Some ribosomal proteins were found to play important roles in the development and progress of cancer cells (Khalaileh et al., 2013; Donati et al., 2011).

Ribosomal proteins play diverse roles in cancer cells. RPL6 (Du et al., 2005), RPL13 (Kobayashi et al., 2006), RPS13 (Guo et al., 2011) and RPL15 (Hsu et al., 2011; Wang et al., 2006; Wang et al., 2001) were highly expressed in gastrointestinal cancers, increased proliferation of cancer cells and played an important role in development and malignance of cancer. In gastric cancer cell line, RPL6 promoted cell cycle and cancer cell proliferation by up-regulating the expression of Cyclin E (Gou et al., 2010). RPS13 promoted the growth of gastric cancer cells by down-regulating the expression of p27 (Kip1) (Guo et al., 2011). Phosphorylated RPS3 activated anti-apoptotic protein TRAF2 and enhanced chemoresistance in non-small cell lung cancer cells (Yang et al., 2013). RPS3 could be secreted into extracellular in homodimers. And the secretion was positively correlative with degree of drug-resistance and malignance of cancer cells (Kim et al., 2013). RPL19 was highly expressed in some prostate cancers and colorectal cancers, and it was studied as a prognostic biomarker for these two cancers (Bee et al., 2011; Yang et al., 2010; Huang et al., 2008; Bee et al., 2006). In addition, RPS15A (Xu et al., 2013), RPL26 and RPL29 (Li et al., 2012) were also found to be associated with cancer cells proliferation. On the contrary, expression of some ribosomal proteins was down-regulated in cancers, for example RPS8, RPS12, RPS18, RPS24, RPL13a, RPL18, RPL28, RPL32 and RPL35 (Kasai et al., 2003). Some of these ribosomal proteins acted as suppressors of cancer. Down-regulation of these ribosomal proteins promotes cancer development.

RPL34 which is constituted by 110 amino acids is a conserved ribosomal protein from Archaea to eukaryotes. The protein folding pattern of RPL34 is different from most of the other ribosomal proteins. There is a zinc finger motif binding a zinc ion in RPL34 (Klinge et al., 2011), which suggests the distinction of RPL34. Besides the role as a ribosome-constituting protein, RPL34 was reported to involve in other cell functions. Devitt and Stafstrom (1995) reported that accumulation
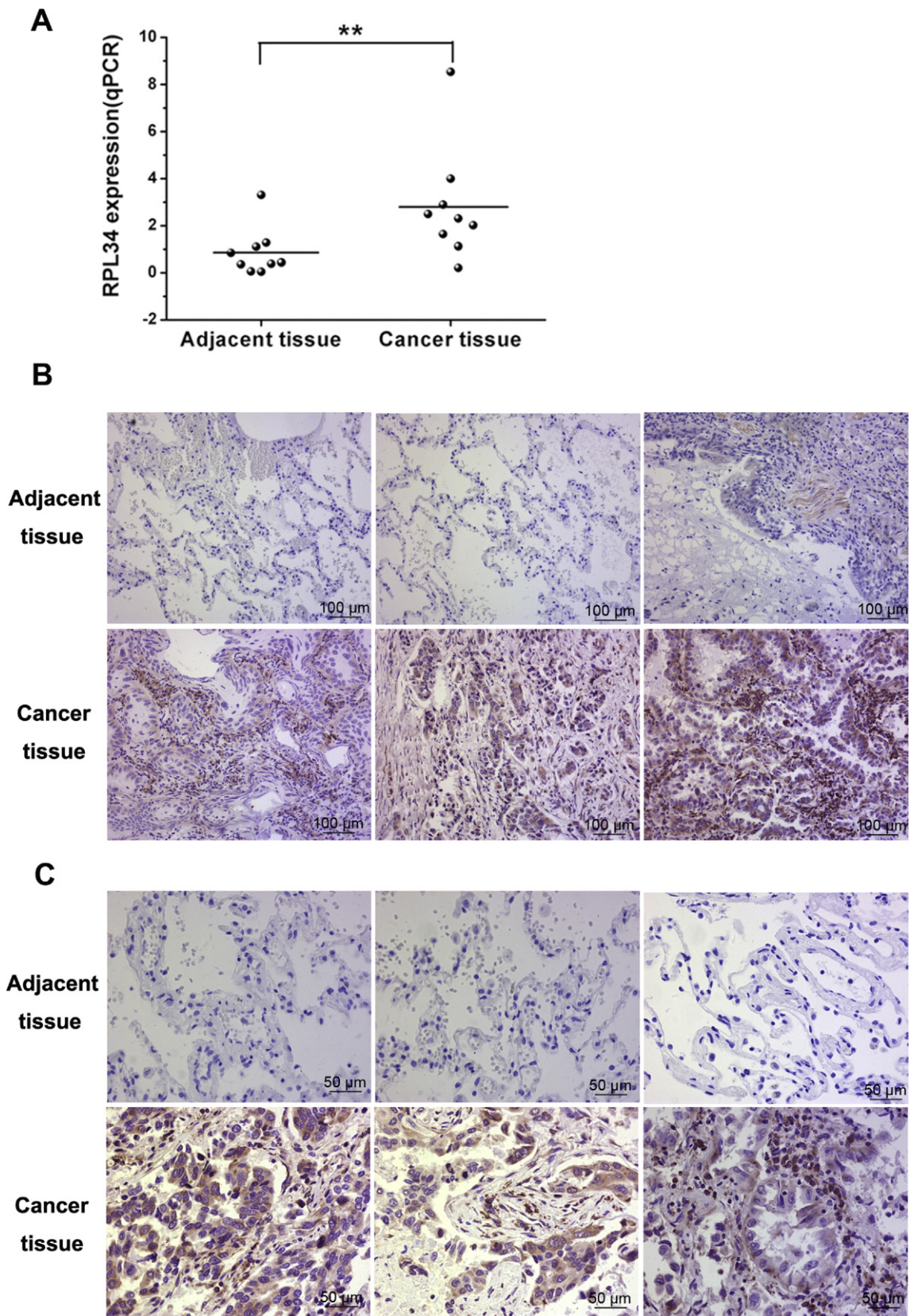
Fig. 1. Over-expression of RPL34 in NSCLC cancer tissue compared to adjacent tissue. (A) RPL34 expression in 9 pairs of liquid-nitrogen-frozen tissue samples. (B) Representative immunohistochemical staining of RPL34 in adjacent and cancer tissues from 32 patients. Brown staining tumor cells are RPL34 positive (magnification: 200×). (C) Representative immunohistochemical staining of RPL34 in adjacent and cancer tissues from 32 patients. Brown staining tumor cells are RPL34 positive (magnification: 400×). **Significant difference (p < 0.01).

Table 1
The expression pattern of RPL34 in NSCLC samples revealed in immunohistochemistry analysis.

Staining grade[a]	Cancer tissue		Adjacent tissue	
	Cases	Percentage	Cases	Percentage
<5%	3	9.38%	21	65.63%
5%–25%	6	18.75%	8	25.00%
26%–50%	10	31.25%	2	6.25%
51%–75%	8	25.00%	1	3.13%
75%–100%	5	15.63%	0	0.00%

[a] Samples were defined as "RPL34 positive" when the proportion of tumor cells positive for RPL34 (staining rate) was more than 50%.

of RPL34 mRNA was correlated with cell proliferation and RPL34 might involve in cell cycle regulation during growth-dormancy cycles in pea axillary buds. Dai et al. (1996) found that expression of *rpl34* was much higher in actively growing tissues than that in normal tissues of tobacco leaf, which indicated that RPL34 was involved in cell proliferation in plant tissues. Moorthamer and Chaudhuri (1999) found that RPL34 bound and inactivated cyclin-dependent kinase Cdk4 and Cdk5 in Hela cell. In addition, it's reported (Panagiotidis et al., 1995) that RPL34 regulated biosynthesis of polyamines in *Escherichia coli*. On the other hand, elevated levels of intracellular polyamines up-regulated the expression of RPL34. As small biomolecules with positive charge, polyamines were associated with chromatin stability, gene

transcription and protein translation. Therefore, RPL34 may play roles as mediator in the feedback regulation between polyamines and cell functions. In conclusion, RPL34 was reported to be involved in cell proliferation in prokaryotes, plant and animal cells.

So far, there is no report about the role of RPL34 in NSCLC. In the present study, we showed for the first time that over-expressed RPL34 promotes malignant proliferation and apoptosis-resistance in NSCLC cells.

2. Methods

2.1. Cell lines, reagents and antibodies

Human NSCLC cell lines H1299 and embryonic kidney cell line 293 T were purchased from the American Type Culture Collection (ATCC, USA) and cultured either in DMEM medium (293 T) or RPMI-1640 medium (H1299) containing 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin at 37 °C with 5% CO_2 and 95% humidity. MTT was purchased from Genview (Cat. No. JT343). Rabbit RPL34 polyclonal antibody used for immunohistochemical assay and Western blot was purchased from Abcam (Cat No.ab129394). Mouse anti-Bcl-2 mAb was purchased from Abcam (Cat No. ab117115). Anti-GAPDH mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Annexin V-APC apoptosis detection kit was purchased from eBioscience (San Diego, CA, USA).
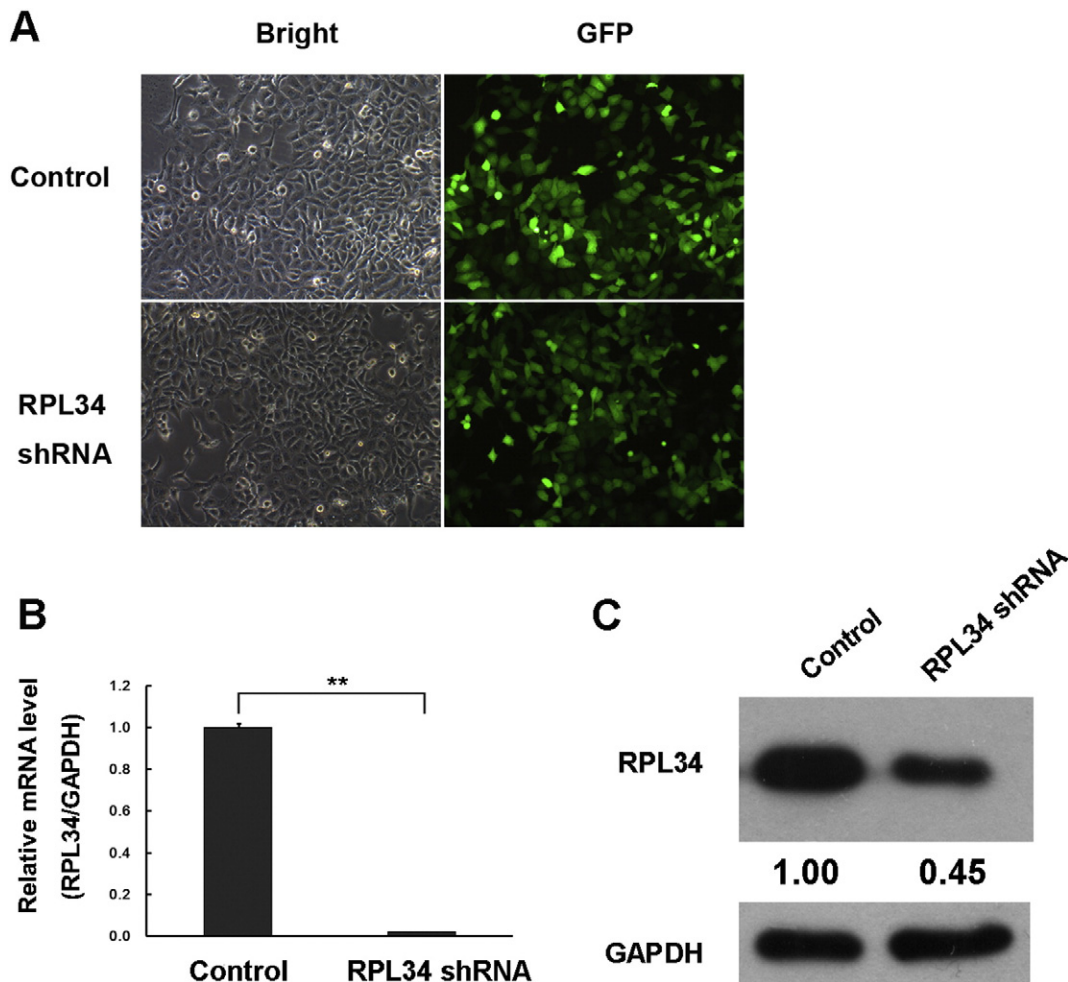

Fig. 2. Lentivirus-mediated RPL34 shRNA significantly suppressed the expression of RPL34. (A) The infected efficiency of control lentivirus and Lv-shRPL34 in H1299. Cell showed green fluorescence indicated lentivirus infection. (B) mRNA level of RPL34 in H1299 was significantly down-regulated in Lv-shRPL34 infected group (RPL34 shRNA) compared to control shRNA lentivirus infected group (control). (C) Western bolt signal of RPL34 by anti-RPL34 antibody in H1299 transfected by Lv-shRPL34 (RPL34 shRNA) or lentivirus mediated control shRNA (control). The relative expression level of RPL34 was shown in the figure, which was detected by gray value analysis of the western-blot strips. ** Significant difference from control groups ($p < 0.01$).

2.2. Lentivirus-mediated shRNA delivery

Sequences of RPL34 shRNA were inserted into the pGCSIL-GFP lentivirus shRNA expression system (Genechem). The shRNA containing vectors were co-transfected together into 293 T cells with pHelper1.0 and lentiviral helper plasmid pHelper2.0 using Lipofectamine 2000 (Invitrogen) to generate the respective lentiviruses. Viral stocks collected from 293 T cells were used to infect H1299 cells. The RNAi target of RPL34 was: TGCTGTAAGACCTAAAGTT. The sequence inserted into pGCSIL-GFP to form RPL34 shRNA was: CCGGGACCTAAAGTTCTTATGA GATTTCAAGAGAATCTCATAAGAACTTTAGGTCTTTTTG. The mRNA and protein levels were measured at 72 h after cells being infected.

2.3. qPCR analysis

Total RNA was isolated with TRIzol reagent (Invitrogen, USA) and reverse transcribed with PrimeScript® RT reagent Kit (Takara, Japan). cDNA was normalized with GAPDH. Real-time PCR was performed by three-step methods using SYBR® Premix Ex TaqTM II kit (Takata, Japan) with 55 °C annealing temperature and 40 amplification cycles. Individual test was carried out in triplicate. GAPDH was used as internal control. The relative amount of each cDNA were analyzed by means of $2^{-\triangle\triangle Ct}$. Primers for real-time PCR for RPL34 and GAPDH were as follow: RPL34-F:GTTTGACATACCGACGTAGGC,RPL34-R:GCACACATGGAACCAC CATAG;GAPDH-F:TGACTTCAACAGCGACACCCA,GAPDH-R:CACCCTGTTG CTGTAGCCAAA.

2.4. Western blot

Western blot was done to detect the expression of RPL34 in H1299. 20 μg of protein samples prepared from H1299 transfected with RPL34 shRNA lentivirus or control shRNA lentivirus was subjected to SDS-PAGE, transferred to PVDF membranes (Millipore, Kankakee, IL, USA) and detected with rabbit anti-RPL34 (1:500, Abcam) followed by horseradish peroxidase conjugated goat anti-rabbit IgG (1:5000, Santa Cruz). Western blot of Bcl-2 was detected by mouse mAb (1:5000, Abcam) followed by horseradish peroxidase conjugated anti-mouse IgG (1:5000, Santa Cruz). GAPDH, chosen as internal control, was detected with mouse anti-GAPDH (1:5000, Santa Cruz). The protein signals were detected using SuperSignal™ West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) and analyzed in Tanon GIS 1D (Shanghai, China) software. All densitometric values were normalized against the relative GAPDH value.

2.5. Colony formation assay

Cells were seeded into six-well plates (200 cells/well) (in three duplicate wells) and cultured at 37 °C in 5% CO_2. After two weeks, the cells were fixed with paraformaldehyde for 30 min and then stained with GIEMSA for 10 min. ddH$_2$O was used to wash the cells three times to obtain a clean background. The number of colonies and the cell number in each colony were counted and statistically analyzed.
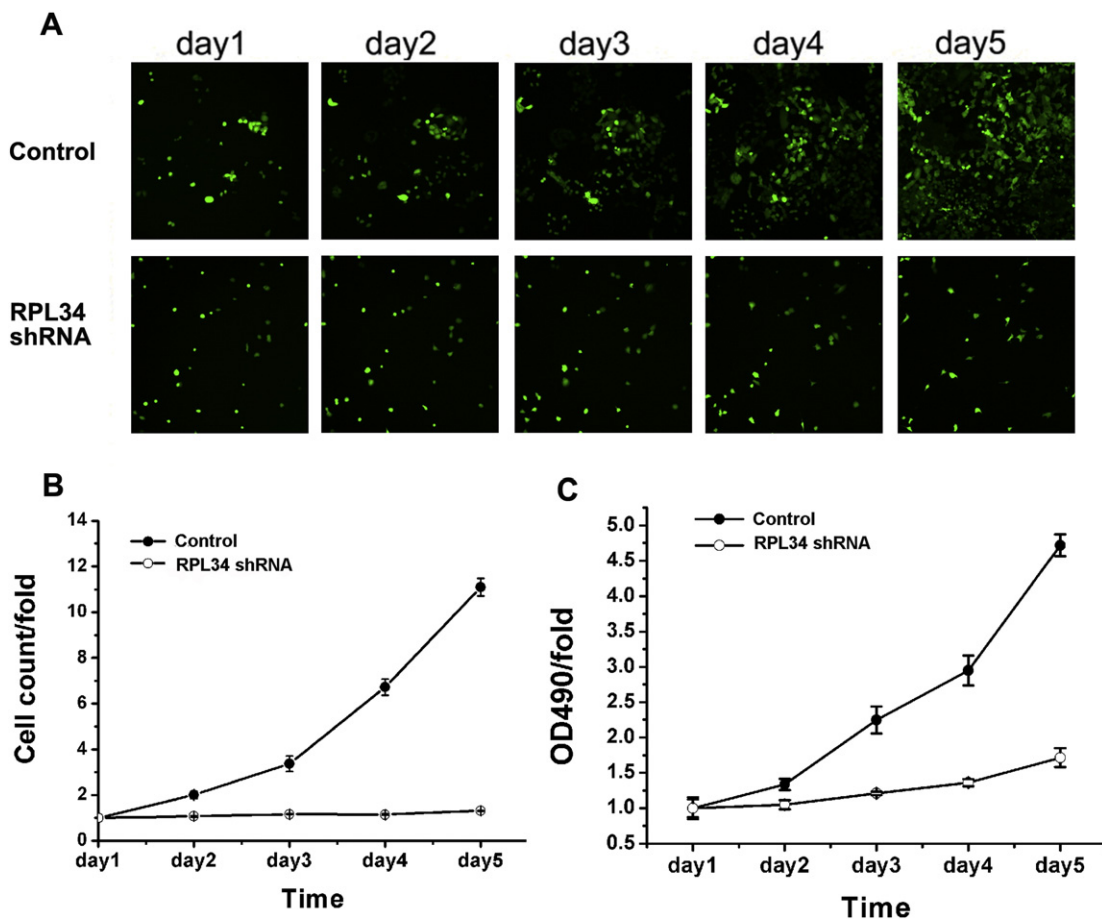

Fig. 3. Down-regulation of RPL34 inhibits proliferation of NSCLC cells H1299. (A) Pictures of lentivirus transfection of nonsense shRNA (control) and RPL34 shRNA (RPL34 shRNA) in different times after transfection. (B) Cell growth was measured by multiparametric high content screening (HCS). Proliferation of H1299 was significantly inhibited when RPL34 was down-regulated.(C) MTT assay showed that proliferation of H1299 was significantly inhibited when RPL34 was down-regulated.

2.6. Immunohistochemical staining

Tissue sections (5-μm thick) were dewaxed, followed by quenching the endogenous peroxidase with 3% H_2O_2 in methanol for 30 min. Prior to staining, non-specific binding was blocked by incubation with 10% BSA in PBS at 37 °C for 1 h. Tissue sections were incubated with specific antibodies in PBS containing 1% BSA at 4 °C overnight, followed by incubation with a horseradish peroxidase-conjugated anti-mouse or rabbit antibody. Color was then developed by incubation with an Immuno Pure Metal Enhanced Diaminobenzidine (DAB) Substrate kit (Pierce). After incubation, tissue sections were washed three times with PBS for 10 min. Tissue sections were finally counterstained with hematoxylin. For determination of RPL34, cytosolic staining of yellowish or brownish granules was graded as follows: 0 for background staining, 1 for faint staining, 2 for moderate staining and 3 for strong staining. In addition, positive staining areas in entire tissue section were graded as follows: 0 for <5%, 1 for 5–25%, 2 for 26–50%, 3 for 51–75%, and 4 for 76%–100%. Combining these two parameters, 0–2 and ≥ 3 were considered negative and positive staining respectively.

2.7. Flow cytometric analysis

Cells were harvested by centrifugation at 1200 rpm for 5 min after 7 days of infection. The pellets were washed twice with cold PBS, fixed with chilled 70% ethanol, centrifuged at 1500 rpm for 5 min to discard ethanol and re-suspended with PBS sequentially. The suspensions were filtrated through a 38 μm pore membrane and centrifuged at 1200 rpm for 5 min. The cells were stained with propidium iodide (PI) or Annexin V at 4 °C for 30 min in dark for flow cytometric analysis. Each experiment was conducted in triplicate.

2.8. Cell proliferation assay

Cell growth was measured via multiparametric high content screening (HCS). Briefly, H1299 cells at 10 days after infection with either control or RPL34 shRNA lentivirus were seeded at 2000 cells per well in 96-well plates, incubated at 37 °C with 5% CO_2 for 5 days. Plates were scanned with ArrayScan™HCS (Cellomics Inc.) every day. The system is a computerized, automated fluorescence-imaging microscope that automatically identifies stained cells. MTT assay was conducted as below. H1299 cells at 10 days after infection with either control or RPL34 shRNA lentivirus were seeded at 2000 cells per well in 96-well plates, then incubated at 37 °C with 5% CO_2 for 5 days. 5 wells each group were added in MTT and incubated for 4 h at 37 °C with 5% CO_2. Then standard procedure was conducted as described by the manufacturer and OD value was tested at a wavelength of 490 nm (Tecan infinite).

2.9. Statistical analysis

One-way ANOVA and Student's t-test were used for raw data analysis. Statistical analysis was performed using SPSS12.0 software package. All values in the text and figures are expressed as mean ± s.d. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Up-regulated expression of RPL34 in NSCLC

RPL34 expression in 9 pairs of liquid-nitrogen-frozen tissue samples (NSCLC tissues and adjacent normal tissue) from 9 patients was detected with qPCR. As shown in Fig. 1A, RPL34 expression in mRNA level was 3.25 times in NSCLC tissue (with a mean mRNA expression level of 2.8) of that in adjacent normal tissue (with a mean mRNA expression level of 0.86). The results showed that RPL34 was over-expressed in NSCLC cells in mRNA level. To study the expression of RPL34 in NSCLC cells in

protein level, immunohistochemical analysis was performed on 32 pairs of paraffin-embedded cancer and adjacent normal tissues from patients with medical records. As shown in Table 1, RPL34 positive staining was 40.63% (13/32) in lung cancer tissues and 3.13% (1/32) in adjacent non-cancerous tissues, $p < 0.001$. Representative immunohistochemical staining results were shown in Fig. 1B (magnification: 200) and Fig. 1C (magnification: 400). The results showed that expression of RPL34 in cancer tissues was much higher than adjacent normal tissues, while there was no significant difference in the expression of other control ribosomal proteins (data not shown). Thus, the elevation of RPL34 expression may play an important role in the pathogenesis of human NSCLC.
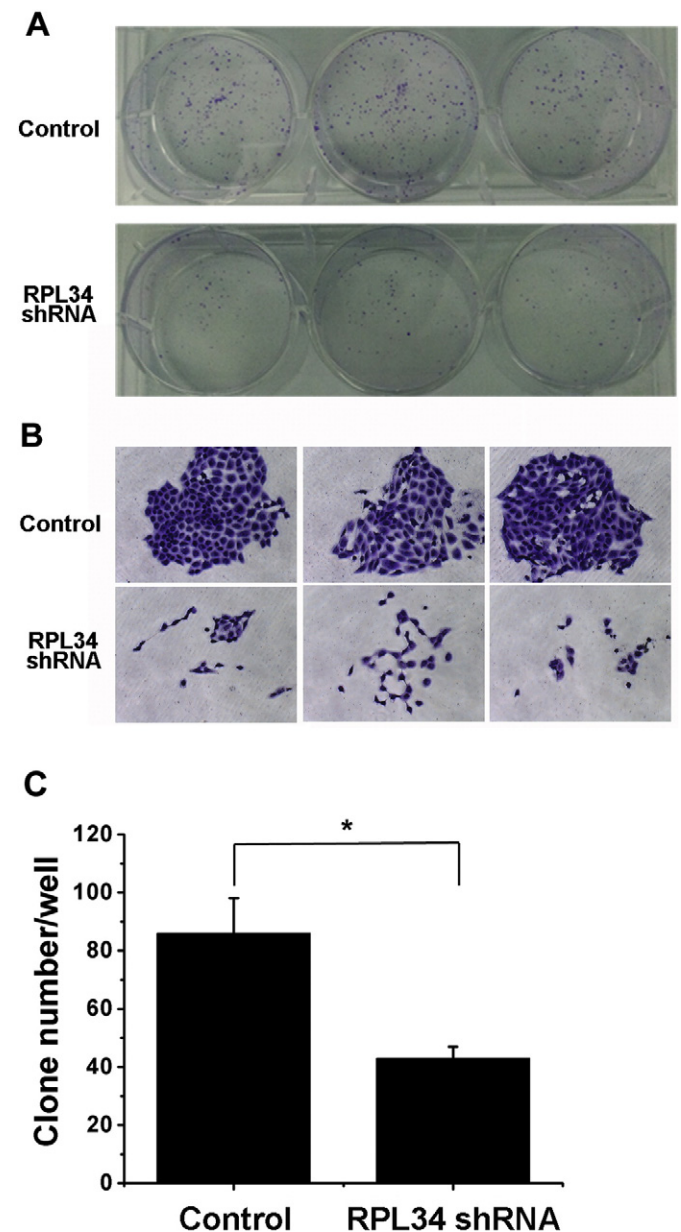

Fig. 4. Down-regulation of RPL34 inhibits colony formation of NSCLC cells H1299. (A) Colony formation of H1299 infected with control lentivirus (control) and Lv-shRPL34 (RPL34 shRNA) in a 6-well plate 2 weeks after seeded. (B) Representative colony of H1299 infected with control lentivirus (control) and Lv-shRPL34 (RPL34 shRNA) 2 weeks after seeded. (C) Clone number per well shown in histogram. Results were shown as mean ± S.D.* Significant difference from control groups (p < 0.05).

3.2. Lentivirus-mediated shRNA silencing specifically inhibits the expression of RPL34in mRNA and protein level

In order to study the role of RPL34 in NSCLC, lentivirus-mediated shRNA of RPL34 in H1299 was conducted. RPL34 shRNA and control shRNA lentivirus were constructed. The infection efficiency of lentivirus was monitored by detection of GFP expression after 3 days of transduction. As shown in Fig. 2A, over 90% of the cells were infected. qPCR and Western blot were used to analyze the knockdown efficiency of RPL34 in H1299 cells. As shown in Fig. 2B, RPL34 mRNA level was reduced by about 90% following RPL34 shRNA lentivirus as compared to control group (p < 0.001). RPL34 protein level was also significantly decreased by shRNA lentivirus treatment (Fig. 2C). Expression of RPL34 in RPL34 shRNA group is about 45% of that in control group. The results showed that lentivirus-mediated shRNA silencing specifically inhibits the expression of RPL34 in mRNA and protein level.

3.3. Inhibition of RPL34 significantly suppresses growth of NSCLC cells in vitro

To study the effect of RPL34 down-regulation on cell proliferation, cell growth was measured via multiparametric high content screening (HCS) at different times after RPL34 shRNA lentivirus infection. As shown in Fig. 3B, proliferation of H1299 was significantly inhibited when RPL34 was down-regulated. The same result was also observed in MTT assay, as shown in Fig. 3C.

To study the long-term effect of RPL34 shRNA lentivirus on cell growth, we studied the colony formation capacity of H1299 cell line with lentivirus treatment. After 72 h of lentivirus infection, cells were allowed to grow for 14 days to form colonies (Fig. 4). The number of colonies in RPL34 shRNA treated H1299 was 40 ± 3.8, which was strikingly lower than control group with 84.4 ± 12.2 colonies (p < 0.05). The results revealed that down-regulation of RPL34 significantly decreased proliferation and colony formation of NSCLC cells in vitro.

3.4. Inhibition of RPL34 moderately induced S phase arrest in NSCLC cells in vitro

To study the impact of Lv-shRPL34-mediated knockdown of RPL34 on cell cycle of NSCLC cells, H1299 cells were subjected to flow cytometry assay 7 days after infection. The proportion of different cell cycle phases was quantitated by PI staining followed by flow cytometry analyses. As shown in Fig. 5, cells in RPL34 shRNA groups displayed more proportion in S phase compared to that in the control groups (47.9 to 38.9%, p < 0.01). The result indicated that RPL34 may have some impact on cell cycle, but the impact was moderate.

3.5. Inhibition of RPL34 moderately increases apoptosis of NSCLC cells in vitro

To study the effect of RPL34 knockdown in NSCLC cells, Annexin V staining and flow cytometry were carried out. As shown in Fig. 6A and Fig. 6B, cell apoptosis rate in RPL34 shRNA group was about 1.46 times to that in control group. RPL34 knockdown moderately but significantly increases apoptosis of H1299, which indicated that RPL34 plays an important role in H1299. In consistence with the observation, the expression of Bcl-2 in H1299 infected with shRPL34 lentivirus was decreased (shown in Fig. 6C).
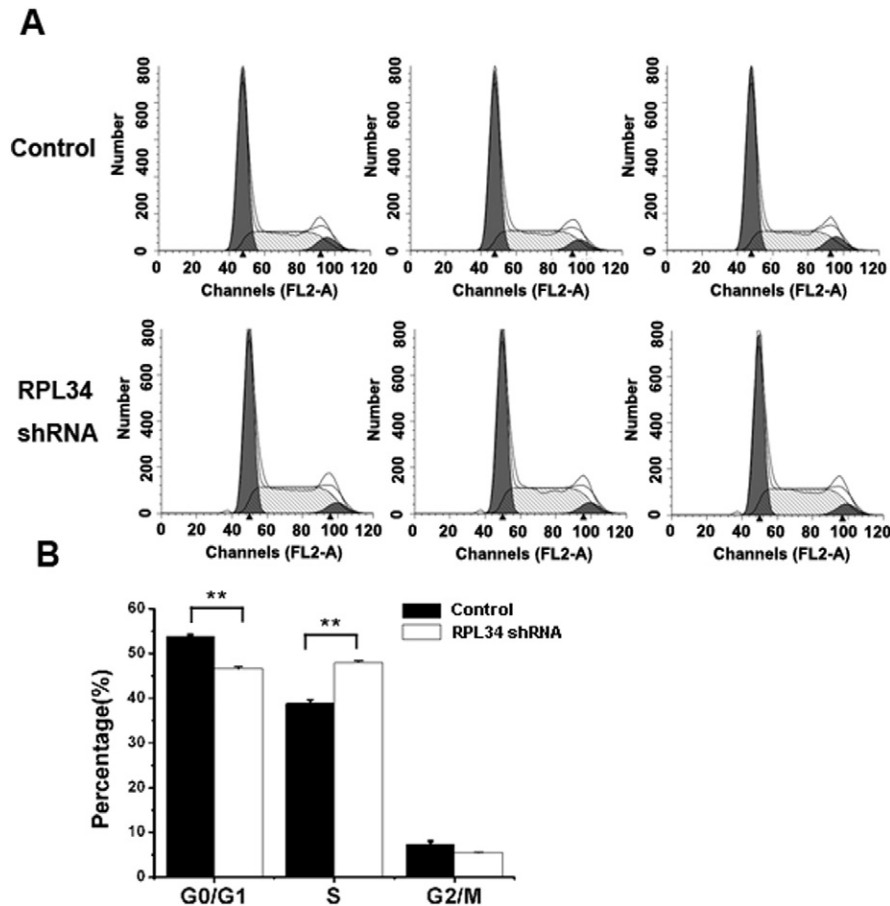

Fig. 5. Knockdown of RPL34 induced moderate S phase arrest in H1299.The proportion of different cell cycle phases was quantitated by PI staining followed by flow cytometry analyses. Control and RPL34 shRNA: lentivirus transfection of nonsense shRNA and RPL34 shRNA. ** Significant difference from control groups (p < 0.01).
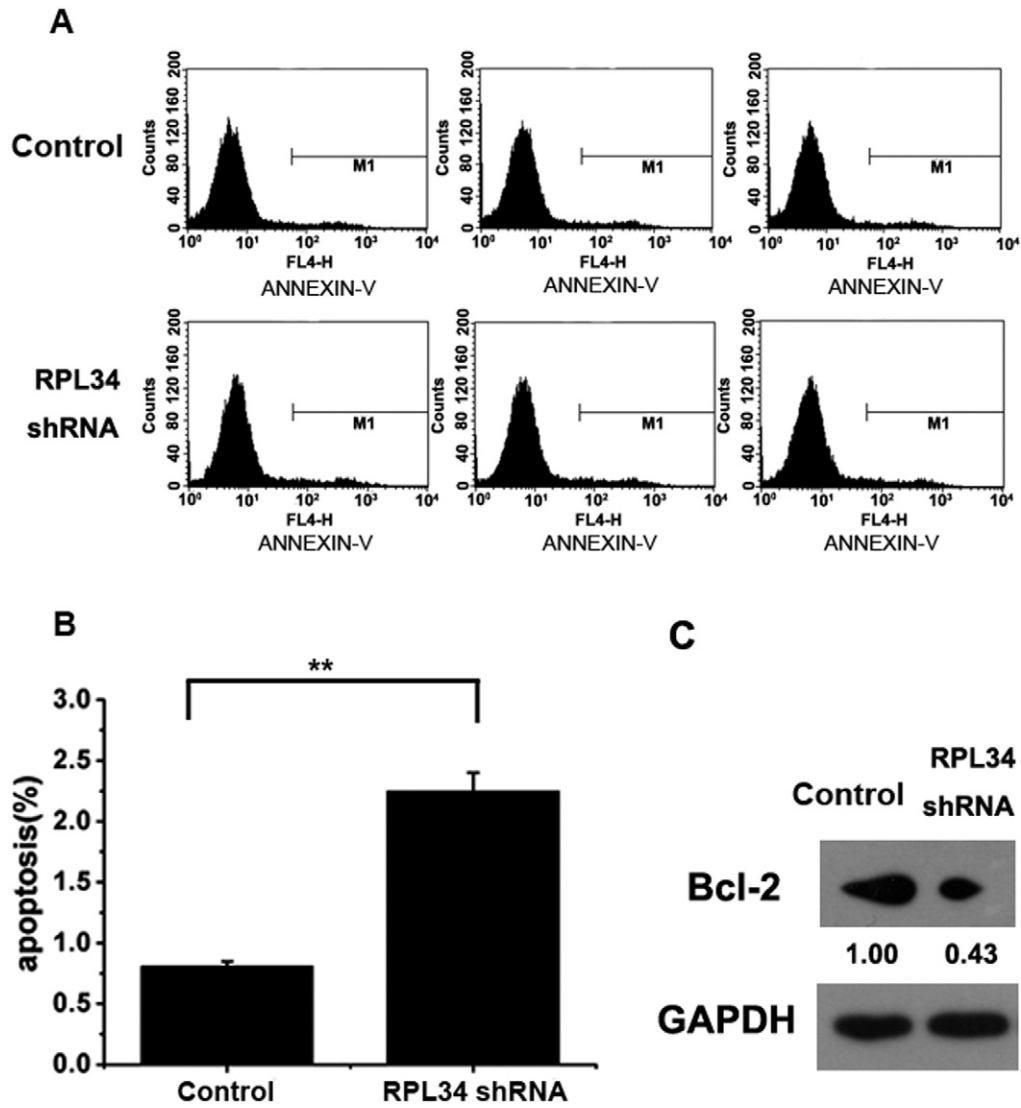
Fig. 6. Down-regulation of RPL34 increases apoptosis of NSCLC cells H1299 in vitro. (A) Cell death was determined by annexin V staining and flow cytometry. (B) RPL34 shRNA cultures showed a moderate but significant increase in apoptosis compared with control. (C) Expression of Bcl-2 protein in H1299 was detected with Western blot in H1299 treated with control shRNA and RPL34 shRNA. The relative expression level of RPL34 was shown in the figure, which was detected by gray value analysis of the western-blot strips. ** Significant difference from control groups (p < 0.01).

4. Discussion

The function of ribosome in tumor cells was regulated abnormally (Belin et al., 2009). For example, the transcription of rRNA was up-regulated by oncogene MAPK/ERK and MYC, and down regulated by tumor suppressor genes pRB and p53 (Belin et al., 2009). Besides the aberrant regulation of ribosome function in cancers, the outside-ribosome function of ribosomal proteins was also involved in the development and progress of some cancers (Khalaileh et al., 2013; Donati et al., 2011). Therefore, it's not surprising to find ribosomal protein RPL34 involved in cancer development and progression.

RPL34 was a rarely-reported ribosomal protein. So far, there is no report on the role of RPL34 in cancers. In the present study, we found that ribosomal protein L34 (RPL34) was highly expressed in clinical non-small cell lung cancer (NSCLC) sections compared to sections of adjacent tissues. The result led us to think that the over expression of ribosomal proteins was to meet the urgent need of protein synthesis by NSCLC that is in rapid proliferation. But after checking the expression of other ribosomal protein, we found that there is not an overall elevating expression of ribosomal proteins, because we found that the expression of other ribosomal proteins was not significantly higher than adjacent tissue. In order to study the function of RPL34 in NSCLC, we conducted a lentivirus mediated shRNA of RPL34 in H1299 cell lines.

When RPL34 was knocked down by RNAi, there was a strong impact on cell proliferation and colony formation of H1299, which means that RPL34 is very important for the growth of NSCLC cancer. We also found that apoptosis of H1299 was significantly increased when RPL34 was knocked down though the impact was relatively moderate compared to that on cell proliferation. Minor but significant S phase arrest was also observed in cell cycle test on H1299 after RPL34 knock-down. In conclusion, over-expressed RPL34 may mainly increase cell proliferation and therefore plays an important role in the development and/or progression of NSCLC. RPL34 was also involved in the apoptosis-resistance and cell cycle in NSCLC in some way.

RPL34 is a conserved ribosomal protein from Archaea to eukaryotes. The protein folding pattern of RPL34 is different from most of the other ribosomal proteins. There is a zinc finger motif binding a zinc ion in RPL34 (Klinge et al., 2011), which suggests the distinction of RPL34. Besides the role as a ribosome-constituting protein, RPL34 was reported to be involved in other cell functions in prokaryotes, plant and animal cells. We have a hypothesis that NSCLC may take advantage of the distinctive feature and "conserved" cell functions outside of ribosome of RPL34 to

promote cell growth and apoptosis-resistance, thus playing an important role in the development and progress of NSCLC. RPL34 may be a good molecular diagnostic target and prognosis biomarker for NSCLC. But more studies are needed to reveal the detailed mechanism in the following study.

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