# The effect of ribosomal protein S15a in lung adenocarcinoma

Yifan Zhang, Guangxin Zhang, Xin Li, Bingjin Li, Xingyi Zhang

**Background:**  RPS15A (ribosomal protein S15A) promotes mRNA/ribosome interactions in translation . It is critical for the process of eukaryotic protein biosynthesis. Recently, aberrantly expressed RPS15A was found in the virus hepatitis and malignant tumors. However, the role of RPS15A has not been fully revealed on the development of lung cancer. **Method:**  In this study, a tissue microarray (TMA) of primary lung adenocarcinoma tissue specimens was carried out. Furthermore, to further investigate the function of RPS15A in lung cancer, RPS15A-specific short hairpin RNA (shRNA) expressing lentivirus (Lv-shRPS15A) was constructed and used to infect H1299 and A549 cells. **Result:**  Our data showed that RPS15A expression was increase in tumor tissues. Furthermore, the knockdown of RSP15A inhibited cancer cell growth and induced apoptosis in the cancer cells. Gene expression profile microarray also revealed that the P53 signaling pathway was activated in Lv-shRPS15A-infected cancer cells. **Conclusion:**  Taken together, our results demonstrate that RPS15A is a novel oncogene in non-small cell lung cancer and may be a potential therapeutic target in lung cancer.

1    **The effect of ribosomal protein S15a in lung adenocarcinoma**

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24    **Abstract**

25    **Background:** RPS15A (ribosomal protein S15A) promotes mRNA/ribosome interactions in

26    translation. It is critical for the process of eukaryotic protein biosynthesis. Recently, aberrantly

27    expressed RPS15A was found in the virus hepatitis and malignant tumors. However, the role of

28    RPS15A has not been fully revealed on the development of lung cancer.

29    **Method:** In this study, a tissue microarray (TMA) of primary lung adenocarcinoma tissue

30    specimens was carried out. Furthermore, to further investigate the function of RPS15A in lung

31    cancer, RPS15A-specific short hairpin RNA (shRNA) expressing lentivirus (Lv-shRPS15A) was

32    constructed and used to infect H1299 and A549 cells.

33    **Result:** Our data showed that RPS15A expression was increase in tumor tissues. Furthermore,

34    the knockdown of RSP15A inhibited cancer cell growth and induced apoptosis in the cancer cells.

35    Gene expression profile microarray also revealed that the P53 signaling pathway was activated in

36    Lv-shRPS15A-infected cancer cells.

37    **Conclusion:** Taken together, our results demonstrate that RPS15A is a novel oncogene in non-

38    small cell lung cancer and may be a potential therapeutic target in lung cancer.

39

40    **Key words:** RPS15A, Tissue microarray, shRNA, Lung adenocarcinoma, P53 pathway

**Introduction**

Lung cancer is the most prevalent malignant tumor and the leading cause of cancer-related death in the world (Jemal et al. 2011). The principal histological forms of lung cancer have been well established. Approximately 85% of lung cancer cases are non-small cell lung cancer (NSCLC). Adenocarcinoma is the most commonly diagnosed type (Butnor & Beasley 2007). Despite advances in surgical techniques and other therapeutic strategies, most patients diagnosed with lung cancer ultimately succumb to this disease within 5 years (Miller 2005; Paleari et al. 2008). Therefore, comprehensive understanding of the molecular mechanisms underlying lung cancer progress is important for the development of optimal anti-cancer therapy (Hensing et al. 2014).

Tumorigenesis involves multistep development to acquire certain malignant capabilities, such as, sustaining proliferative signaling, resisting cell apoptosis, activating invasion and metastasis et al (Hanahan & Weinberg 2011). Underlying these abilities of tumor cells, rapid *de novo* biosynthesis of functional ribosome is essential for cancer cells to aggressively grow and obtain multiple malignant phenotypes. Ribosomes are composed of diverse ribosomal RNAs in eukaryotic organs. So far, most ribosomal proteins have been identified to bind to certain regions of ribosomal RNA and perform catalytic functions. Many ribosomal protein shave various extra-ribosomal functions, such as DNA repair, transformation, development, apoptosis, and transcription (Lee et al. 2010; Nishiura et al. 2013; Nosrati et al. 2014). Some studies show that these proteins may play a role in human tumor development and progression. For example, depletion of ribosomal protein L26 and L29 suppress the proliferation of human pancreatic cancer PANC-1 cells (Li et al. 2012), while RPL22 expression is highly associated with NSCLC (Yang et al. 2013).

Human ribosomal protein S15a (RPS15A) is a highly conserved cellular gene that maps to human chromosome 16p12.3 locus (Chan et al. 1994; Schaap et al. 1995). It promotes mRNA/ribosome binding in translation via the interactions with the cap-binding subunit of eukaryotic initiation factor 4F (eIF-4F) (Linder & Prat 1990). In yeast, G1/S cell cycle phase arrest induced by *cdc33* (encoding eIF-4F in yeast) mutation could be reversed by RPS15A over-expression, these findings suggest that RPS15A may play a role in cell cycle transition (Lavoie et al. 1994). RPS15A over-expression also facilitates hepatocellular growth via promoting cell cycle transition and accelerates tumor formation *in vitro* (Lian et al. 2004), whereas RPS15A mRNA down-regulation inhibited hepatic cancer cell growth (Xu et al. 2014). However, the role of RPS15A in lung cancer has not been completely studied.

The present study was aimed to investigate whether RPS15A is involved in the development and

79 progression of lung cancer. A tissue microarray was carried out to determine the expression of
80 RPS15A. To examine its functional role in lung cancer progress, an RPS15A-specific small
81 interfering RNA (siRNA)-lentiviral vector was constructed to block RPS15A expression in
82 human lung adenocarcinoma H1299 and A549 cells. Furthermore, the impacts of RPS15A
83 silencing on the growth of the cancer cells were examined by MTT assay and colony formation
84 assay. In addition, the effects of RPS15A knockdown on the apoptosis of H1299 and A549 cells
85 were determined by flow cytometry analysis. To further explore the potential molecular
86 mechanisms, we also performed a human whole genome oligo microarray followed by a KEGG
87 pathway enrichment analysis.
88

90    **Materials and methods**

91

92    **Tissue microarray and immunohistochemical staining**

93

94    Tumor samples were collected from 75 patients with lung adenocarcinoma at the Department of

95    Thoracic Surgery, The Second Affiliated Hospital of Jilin University, China,from July 2005 to

96    December 2011. All the tissue samples were obtained from surgery with informed consent and

97    with institutional review board approval of the hospital. Non-tumor samples from the

98    macroscopic tumor margin were isolated atthe same time and used as the matched adjacent non-

99    neoplastic tissues. Expression of RPS15A protein was detected using immunohistochemical

100   analysis on commercially available tissue microarrays (TMAs) from Shanghai Zhuoli

101   Biotechnology Co., Ltd. (Shanghai, China), which contained a total of 150 tissue samples of

102   tumor or nontumor lung tissues from 75 patients. There were 44 male patients and 31 female

103   patients aging from 32 to 80 years, with an average age of 58 years old. The tumors were

104   classified according to the tumor nodes metastasis (TNM) stage revised by the International

105   Union Against Cancer in 2002.

106

107   **Immunohistochemical staining and scoring**

108

109   To detect RPS15A expression in lung cancer, archival paraffin-embedded tumor samples were

110   used to build up tissue microarray (TMA) blocks for immunohistochemical (IHC) staining.

111   Immunohistochemical staining was performed using the Vectastain Elite ABC Kit (Vector

112   Laboratories, Burlingame, CA) according to the manufacturer's protocol. Briefly, TMA sections

113   were deparaffinized and hydrated in xylene, ethanol and water. After heat-induced antigen

114   retrieval procedures, sections were incubated overnight at 4 °C with anti-RPS15A primary

115   antibody(1:50 dilution; Abcam). After the primary antibody was washed off, the ABC detection

116   system using biotinylated anti-rabbit IgG. The slides were counterstained with haematoxylin and

117   mounted inxylene mounting medium for examination.Negativecontrols were treated identically

118   but with the primary antibodyomitted.Three researchers evaluated immunoreactivity

119   independently. The percentageof positive tumor cells was determined by each observer,and the

120   average of three scores was calculated. The proportion of immunopositive cells were categorized

121   as following: intensityof staining: none (0), mild (1), moderate (2), strong (3);percentage of the

122   positive staining: 0 (-), <15 % (+), 15–50 % (++), >50 % (+++). To obtain final statistical results,

123   - and + groups were considered as negative.

124

125   **Cell lines**

126

127 Lung adenocarcinoma cell lines H1299 and A549, lung squamous cancer cell line SK-MES-1, as
128 well as small cell lung cancer cell line H1688 (Cell Bank of Chinese Academy of Sciences,
129 Shanghai, China) and human embryonic kidney (HEK) 293T cell line (American Type Culture
130 Collection, ATCC, Manassas, VA, USA) were maintained in DMEM (Hyclone, Logan, UT,
131 USA) with 10% FBS (Hyclone) and penicillin/streptomycin at 37℃ in humidified atmosphere of
132 5% $CO_2$.
133
134 **Construction and infection of RPS15A short harpin (shRNA)-expressing lentivirus**
135
136 To permit robust inducible RNAi-mediated RPS15A silencing in tumor cells, RPS15A-specific
137 shRNA containing lentiviral vector was constructed. The RNAi was designed based on
138 conservative cDNA fragments within the coding region of human RPS15A gene (NM_001019)-
139 targeting sequence (5'-GCAACTCAAAGACCTGGAA -3') of oligonucleotides. The sequences
140 were annealed and ligated into the AgeI/EcoRI (NEB, Ipswich, MA, USA)-linearized pGCSIL-
141 GFP vector (Shanghai Genechem Co. LTD., Shanghai, China). The lentiviral-based shRNA-
142 expressing vectors were confirmed by DNA sequencing. Recombinant lentiviral vectors and
143 packaging vectors were then cotransfected into 293T cells using Lipofectamine 2000 (Invitrogen,
144 Carlsbad, CA, USA), according to the manufacturer's instructions for the generation of
145 recombinant lentiviruses Lv-shRPS15A and negative control Lv-shCon. The culture supernatants
146 containing lentiviral particles expressing Lv-shRPS15A and Lv-shCon were harvested and ultra-
147 centrifuged 48 h after transfection, respectively. H1299 and A549 cells were infected with the
148 lentiviruses at multiplicity of infection (MOI) of 10 and 20, respectively.
149
150 **Quantitative real-time PCR analysis**
151
152 In brief, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The reverse
153 transcription reactions were carried out following the protocol of the M-MLV Reverse
154 Transcriptase (Promega Corp., Madison, WI). Real-time quantitative PCR analysis was
155 performed using SYBR Master Mixture Kit (TaKaRa, Dalian, China). The primer sequences for
156 PCR amplification of RPS15A were 5′-CTCCAAAGTCATCGTCCGGTT-3′ and 5′-
157 TGAGTTGCACGTCAAATCTGG-3′. GAPDH was applied as an internal control. The primer
158 sequences of GAPDH were 5′-TGACTTCAACAGCGACACCCA-3′and 5′-
159 CACCCTGTTGCTGTAGCCAAA-3′. $2^{-\triangle\triangle CT}$ method was adopted to calculate the relative
160 expression levels of RPS15A by subtracting CT values of the control gene from the CT values of
161 RPS15A.
162
163 **MTTproliferation and colony formation assay**

164

165   Briefly, exponentially growing cancer cells were inoculated into 96-well plates with $2\times10^4$ cells

166   per well. After incubation for 24, 48, 72, 96 and 120 h, 10 µl of sterile MTT (5 mg/ml) was

167   added into each well. Following incubation at 37℃ for 4 h, the reaction was blocked by adding

168   100µl of dimethyl sulfoxide. The formazan production was determined by measurement of the

169   spectrometric absorbance at 490 nm. The values obtained are proportional to the amount of

170   viable cells and each experiment was repeated three times. In colony formation assay, H1299 and

171   A549 cells infected with Lv-shRPS15A or Lv-shCon were seeded in six-well plates with $5\times10^2$

172   cells per well and cultured at 37℃ with 5% $CO_2$ for 14 days. The cell colonies were washed

173   twice with PBS, fixed in 4% paraformaldehyde for 30 min and stained with Giemsa for 20 min.

174   Individual colonies with more than 50 cells were counted under a fluorescence microscope.

175

176   **Apoptosis assay by fluorescence-activated cell sorting (FACS) analysis**

177

178   In apoptotic cell detection, expotentianlly growing H1299 and A549 cells were seeded in six-

179   well plates. After 48 h, cells were collected and washed with pre-chilled PBS (4℃). Then, the

180   cells were centrifuged at 1500 rpm for 5 min. After discarding the supernatant, the pellet was

181   resuspend with binding buffer. The cells were then incubated with 5 µl Annexin V-APC for 15

182   min in the dark. After fitration of the cell suspension and the analysis of apoptotic cells was

183   performed by FACS can (Becton– Dickinson).

184

185   **Gene expression profile microarray**

186

187   Briefly, A549 cells were seeded in a six-well plate at a density of $4\times10^5$ cells per well and

188   infected by Lv-shRPS15A and Lv-shCon at MOI of 20, respectively. After 96h, the total RNAs

189   were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and were used for cDNA

190   synthesis and labeling, microarray hybridization and then followed by flour-labeled cDNA

191   hybridizing their complements on the chip (Affymetrix, Santa Clara, CA, USA). The resulting

192   localized concentrations of fluorescent molecules were detected and quantified by GeneChip

193   Scanner 3000 (Affymetrix). Finally, the data were analyzed by Expression Console Software

194   (Affymetrix) with default RMA parameters. Data are representative of three separate assays.

195

196   **Gene ontologyannotation and KEGG pathway enrichment analysis**

197

198   The gene ontology analysis was performed to functionally annotate the differentially expressed

199   genes according to the Gene Ontology database (http://www.geneontology.org/). The pathway

200   enrichment analysis was performed according to the KEGG (Kyoto Encyclopedia of Genes and

201    Genomes) database. The Fisher's exact test and $\chi^2$ test were applied to classify the significant GO
202    categories and pathways, and the FDR was calculated to correct the $P$-value by multiple
203    comparison tests. A P-value <0.05 and an FDG <0.05 were set as thresholds to select significant
204    GO categories and pathways.
205
**Western Blotting assay**
207
208    In brief, A549 cells were collected and lysed with precooled lysis buffer after 96 h of infection.
209    Total protein was extracted from the cells and determined by the BCA method. Protein (20 μg)
210    was loaded onto a 10% SDS-PAGE gel. The gel was run at 30 mA for 2 h and transferred to
211    poly- vinylidene difluoride membrane (Millipore, Billerica, MA, USA). The resulting membrane
212    was blocked in 5% non-fat dry milk blocking buffer and then probed with rabbit anti-P21
213    (1:2,000 dilution; Abcam, Cambridge, MA; Cat. ab7960), mouse anti-TP53I3 (1:500 dilution;
214    Abcam, Cambridge, MA; Cat. ab123917), rabbit anti-SESN2 (1:500 dilution; Abcam,
215    Cambridge, MA; Cat.ab57810) and mouse anti-GAPDH (1:6,000; Santa Cruz Biotechnology,
216    Inc., Sana Cruz, CA) overnight at 4 ℃. The protein level of GAPDH was used as a control and
217    detected by an anti-GAPDH antibody. The membrane was washed three times with Tris-buffered
218    saline Tween-20 (TBST), followed by incubation for 2 h with anti-rabbit and anti-mouse IgG at a
219    1:5,000 dilution (Santa Cruz Biotechnology, Inc.). The membrane was developed using
220    enhanced chemiluminescence (Amersham, UK). Bands on the developed films were quantified
221    with an ImageQuant densitometric scanner (Molecular Dynamics, Sunny-Vale, CA, USA).
222
**Statistic analysis**
224    Data were expressed as mean ± SD. Comparisons were performed by    two-sided    independent
225    Student's test, one-way ANOVA analysis and$\chi^2$ test using SPSS software for Windows version
226    23.0 (SPSS, Chicago, USA). Kaplan-Meier survival curves were plotted and log rank test was
227    done. Statistical significance was accepted when $P$<0.05. All experiments carried out in this
228    study                were                repeated                three                independenttimes.

229 **Result**

230

231 **RPS15A was significantly overexpressed in lung adenocarcinoma tissues**

232

233 We detected the expression of RPS15A protein in a tissuemicroarray (TMA) of primary lung

234 adenocarcinoma and adjacent normallung tissue specimens using immunohistochemical staining

235 with an anti-RPS15A antibody.RPS15A immunostaining was primarily detected in cytoplasm.

236 Representative examplesof RPS15A protein expression in lung cancer and normal lung

237 samplesare shown in Fig. 1a. The immunopositive rates of RPS15A in lung adenocarcinoma and

238 normal lung tissues were 66.7%(50/75) and 42.7%(32/75), indicating that RPS15A was highly

239 expressed in lung cancer in comparison with adjacent normal tissues, as shown in Table.1

240 ($P<0.001$). The correlation between RPS15A expression and different clinicalpathological

241 factors in lung adenocarcinoma is shown in Table 2.No significant correlation was found

242 between RPS15A expression and age, grade, TNM stages, tumor size and lymph node metastasis

243 ($P<0.05$). Kaplan-Meier survival analysis of overall prognosis between patients with higher

244 RPS15A expression and patients with low RPS15A expression was carried out. No significant

245 correlation between RPS15A expression and overall prognosis was found, as shown in Fig 1b.

246

247 **Efficacy of lentivirus-mediated RNAi targeting RPS15A**

248

249 Quantitative real-time PCR was performed to detectthe fundamental expression of RPS15

250 mRNA in several lung cancer cell lines, including lung adenocarcinoma cell lines H1299, A549

251 and H1975, lung squamous cancer cell line SK-MES-1, as well as small cell lung cancer cell line

252 H1688. As shown in Fig. 2a, RPS15A expression levels were quite obvious in the cancer cell

253 lines. Consequently, loss of function assay was applied through RPS15A knockdown. H1299 and

254 A549 cell lines were selected for subsequent studies. To downregulate RPS15A expression,

255 lentivirus stably expressing RPS15A-specific shRNA (Lv-shRPS15A) was infected to H1299

256 and A549 cells. Lentivirus expressing negative shRNA (Lv-shCon) was used as negative control.

257 More than 80% of GFP-expressing cells were observed under fluorescence microscope after 72 h

258 (Fig. 2b). The silencing effect of lentivirus mediated RPS15A RNAi on RPS15A expression in

259 H1299 and A549 cells was examined through real-time PCR and Western Blotting assay. The

260 expression level of RPS15A in the Lv-shRPS15A infected cells was significantly lower than that

261 in the Lv-shCon infected cells (Fig. 2c,2d 2e and 2f). Therefore, these data indicated the high

262 efficacy of lentivirus mediated RPS15A silence in lung cancer cells.

263

264 **RPS15A silence inhibited NSCLC cell growth*in vitro***

265

266    To investigate the role of RPS15Ain the proliferation of lung cancer cells, the proliferative
267    abilities of Lv-shCon and Lv-shRPS15A infected H1299 and A549 cells was determined by
268    MTT assay. During the 120 hour incubation period, the growth of Lv-shRPS15A infected cells
269    was significantly slower than that of Lv-shCon infected cells at the time points of 48 h, 72 h, 96
270    h and 120 h (Fig. 3a and3b). To investigate the effect of RPS15A downregulation on tumor
271    formation, colony formation assay was performed. Quantitative analysis of colonies showed that
272    after incubation for 10 days, the number of colonies in Lv-shRPS15A infected cells was
273    significantly lower than that in the Lv-shCon infected cells (Fig. 3c, 3d, 3e and 3f). Therefore,
274    the low colony-forming efficiency of Lv-shRPS15A infected H1299 and A549 cells
275    demonstrated that RPS15A silencing inhibited the colony forming ability of lung cancer cells *in*
276    *vitro*.

277

278    **RPS15A silencetriggered cell apoptosis *in vitro***

279

280    To determine whether RPS15A knockdown induces apoptosis in H1299 and A549 cells,
281    Annexin V-APC staining was performed and the percentage of apoptotic cells was detected by
282    flow cytometry. As shown in Fig. 4a,4b, 4c and 4d, Lv-shRPS15A infected cells exhibited
283    significantly higher proportion of apoptotic cells than that of Lv-shCon infected cells, especially
284    in Lv-shRPS15A infected A549 cells. This suggested that downregulation of RPS15A expression
285    might trigger apoptosis in lung cancer cells, which contributed to the cell growth suppression.

286

287    **RPS15Aknockdown activated P53 signaling pathway *in vitro***

288

289    To explore the potential downstream mechanismsunderlying the actions of RPS15A silencing, a
290    cDNA microarray assay was performed to compare the differential gene expression profiles
291    between Lv-shRPS15A infected and Lv-shCON infected A549 cancer cells.Microarray analysis
292    demonstrated that 885 genes were upregulated and 566 genes were downregulated significantly
293    in Lv-shRPS15A infected cancer cells when compared to Lv-shCON infected cells.To further
294    classify the function of these genes, GO analysis was carried out. The most significant GO
295    categories of biological processes included protein, biopolymer and cellular
296    macromoleculemetabolic processes.Of these genes, the most enriched GO in terms of molecular
297    function involved DNA binding, cation and ion bindingand enzyme regulator activity. KEGG
298    pathway enrichment analysis revealed that certain signaling pathways participated in the tumor
299    inhibition induced by RPS15A knockdown,of whichP53 signaling pathway members were most
300    evidently annotated.The top 20 significantly perturbed pathways are listed in Table 2. To
301    confirm the P53 pathway activation, the expressions of key factors of P53 signaling pathway

302 were determined through western blotting. Consistent with microarray results, P21 and TP53I3

303 expressions were upregulated by RPS15A knockdown, while SESN2 expression was

304 downregulated, as shown in Fig.4e.

305

307   **Discussion**

308

309   Therapeutic drugs targeting cancer-related molecules can specifically inhibit malignant cells,
310   causing minimal adverse off target reactions because of the well-defined mechanisms (Chen et al.
311   2013). Therefore, identification of molecular factors responsible for pulmonary carcinogenesis
312   and elucidation of their underlying mechanisms of proliferation are urgently needed for novel
313   therapeutic targets.

314

315   Earlier genetic studies in zebrafish suggested that many ribosomal protein genes are haploid-
316   sufficient suppressor gene that are highly related to tumorigenesis (Amsterdam et al. 2004).
317   Ribosomal proteins are abundant in most cells and well known for their functional role in the
318   assembly of ribosomal subunits at the early stages of translation. In addition, ribosomal proteins
319   have been found to regulate cell proliferation, apoptosis, DNA repair and gene transcription.
320   Currently, ribosomal proteins are emerging as novel regulators of cancer cell growth, whose
321   mutations and changes of expression level are highly relevant to human malignancies. RPS15A,
322   as a component of the 40S ribosomal subunit, has been found to facilitate the binding of capped
323   mRNA to the ribosomal subunit 40S in translation initiation. Furthermore, it has been reported
324   that downregulation of its mRNA expression in hepatocellular carcinoma cells significantly
325   inhibits tumor growth, suggesting that RPS15A may play a role in human carcinogenesis. Yet,
326   up to now, little is known about its function in NSCLC cells.

327

328   To determine the expression levels of RPS15A in lung adenocarcinoma tissues, TMAs was
329   performed and revealed that RPS15A was highly expressed in lung cancer tissue. Therefore, we
330   hypothesized that RPS15A may play an important role in the proliferation of lung cancer. In this
331   regard, a lentivirus-mediated RNAi system was applied to inhibit RPS15A mRNA expression in
332   human lung adenocarcinoma H1299 and A549 cells *in vitro*. Lentivirus expressing RPS15A-
333   specific shRNA was constructed and used to infect H1299 and A549 cells. The efficiency of
334   lentivirus-induced silencing of endogenous RPS15A was confirmed by qPCR and western
335   blotting assay. To determine the impact of RPS15A knockdown on the lung cancer growth*in
336   vitro*, an MTT assay and colony formation assay were carried out. Asa result, downregulation of
337   RPS15A expression greatly impaired the proliferation and colony-forming ability of H1299 and
338   A549 cells. Furthermore, flow cytometry analysis data showed that RPS15A silencing induced
339   apoptosis as characterized by the prominent presence of apoptotic cancer cells.

340

341   To elucidate the downstream mechanisms underlying RPS15A silence in lung cancer, we carried
342   out a human whole genome oligomicroarray and KEGG pathway enrichment analysis. The data
343   revealed that the P53 signaling pathway was activated significantly in Lv-shRPS15A infected

344    A549 cells. The P53 pathway has been well known for its anticancer function through initiating
345    apoptosis, cell cycle arrest, maintaining genomic stability, angiogenesis inhibition etc(Levine &
346    Oren 2009). To confirm the P53 signaling activation, we determined the expression of
347    P21,TP53I3and SESN2 by using western blotting. P21(P21), which is tightly controlled by the
348    tumor suppressor protein p53, is a potent cyclin-dependent kinase inhibitor that binds to cyclin-
349    CDK2 or -CDK4 complexes, thus functioning as a regulator of cell cycle progression at G1 (el-
350    Deiry et al. 1993).TP53I3 is induced by the tumor suppressor p53 and is thought to beinvolved in
351    p53-mediated cell death(Polyak et al. 1997). SESN2 is also known for itsfunction in
352    theregulation of cell growth and survival in cellular response to different stressconditions (Hay
353    2008).
354
355    Ribosomal proteins are often classified as cell growth associated molecules due to their key role
356    in protein synthesis(Eid et al. 2014; Lian et al. 2004; Wang et al. 2014). Our finding that
357    RPS15A downregulation inhibits NSCLC cell growth is also supported by previous studies that
358    RPS15A knockdown also inhibits hepatic cancer cell growth(Xu et al. 2014). Like other
359    ribosomal proteins, RPS15A has been found to involve extra-ribosomal functions in cell growth,
360    apoptosis and cell cycle transition.In this study, our findings that RPS15A induced apoptosis and
361    cell cycle phase arrest in lung cancer A549 cells further supported that P53 signaling is critical
362    for the regulation of apoptosis and cell cycle transition. However, to date, the issue of whether
363    and how RPS15A interacts with other regulators remains poorly studied, and further
364    investigation is warranted to elucidate the detailed mechanisms underlying the action of RPS15A.
365    Taken together, our study demonstrated that RPS15A might serve as an upstream modulator of
366    P53 signaling pathway.
367
368    In conclusion, RPS15A expression was increase in tumor tissues. Furthermore, the knockdown
369    of RSP15A inhibited cancer cell growth and induced apoptosis in the cancer cells. Gene
370    expression profile microarray also revealed that the P53 signaling pathway was activated in Lv-
371    shRPS15A-infected cancer cells. Therefore, our findings demonstrate that RPS15A is a novel
372    oncogene in non-small cell lung cancer and may be a potential therapeutic target in lung cancer.
373
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375
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379
380    **References**
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432

434 **Figure Legend**

435

436 **Figure 1.** Immunostaining of RPS15A in lung adenocarcinoma and adjacent normal tissues with
437 tissue microarray. (a)Three representative cases with different expression status of RPS15A,
438 ranging from negative, mild and strong expression were taken at $100\times$ and $400\times$ magnification
439 in lung cancer and normal tissues.(b)Kaplan-Meier survival analysis of overall prognosis
440 between patients with higher RPS15A expression and patients with low RPS15A expression.
441 Log-rank test was used to statistically calculate the difference.

442

443 **Figure 2.** Lentiviral mediated RPS15A downregulation. (a) Relative RPS15A mRNA level in
444 lung cancer cell lines (H1299, A549, SK-MES-1, H1688 and H1975). (b) Fluorescence
445 photomicrographs of H1299 and A549 cells 72h after lentivirus infection. (c, d) RPS15A mRNA
446 and protein expressions were dramatically downregulated in Lv-shRPS15A infected cells
447 evidenced by real-time PCR and Western blotting assay. **$P<0.01$ versus Lv-shCon.

448

449 **Figure 3.** The proliferation of H1299 and A549 cells was inhibited after Lv-shRPS15A infection
450 determined by MTT assay (a, b). The colony formation abilities of H1299 and A549 cells were
451 determined by colony formation assay after Lv-shRPS15A infection. (c, d) Images of
452 coloniesand statistical analysis of the number of colonies. (e, f) Images of colonies recorded
453 under microscope. **$P<0.01$ versus Lv-shCon.

454

455 **Figure 4**. RPS15A knockdown induced apoptotic cells were determined by flow cytometry
456 analysis after Annexin V-APC staining.(a, b) Histograms of FACS analysis. (c, d) Percentage of
457 apoptotic cells. **$P<0.01$ versus Lv-shCon. (e) Key factors of P53 signaling pathway, such as
458 P21,TP53I3and SESN2, were examined in Lv-shRPS15A infected A549 cells by using western
459 blotting method. The protein level of GAPDH was employed as a control.

460

461 **Table 1.**RPS15A expression in 75 lung adenocarcinoma and adjacent normal tissue specimens

462

463 **Table 2.**Correlation between RPS15A expression and clinicopathological factors in 75 lung
464 adenocarcinoma patients specimens.

465

466 **Table 3.**KEGG pathway enrichment analysis revealed that P53 signaling pathway members were
467 most evidently annotated. Top 20 significantly perturbed pathways are listed.

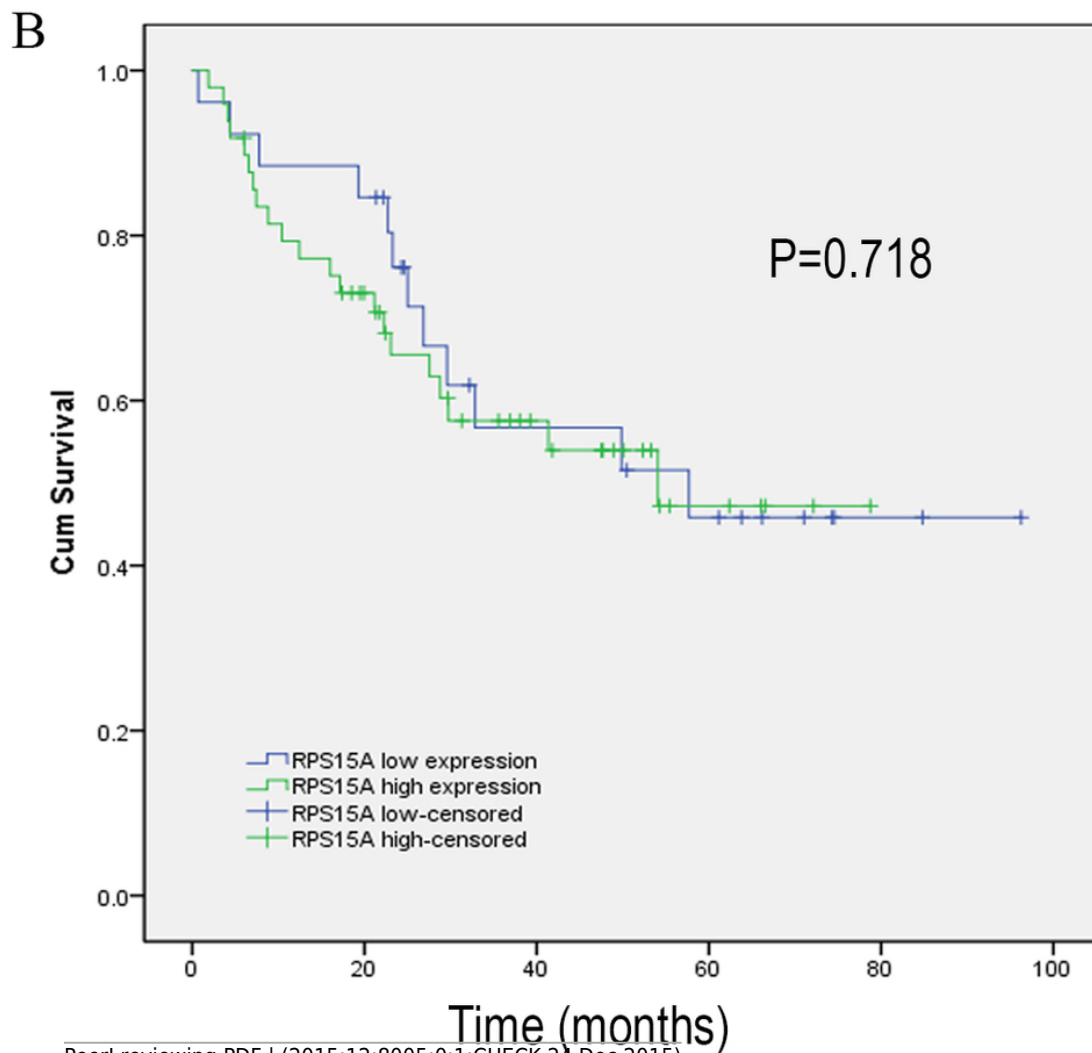
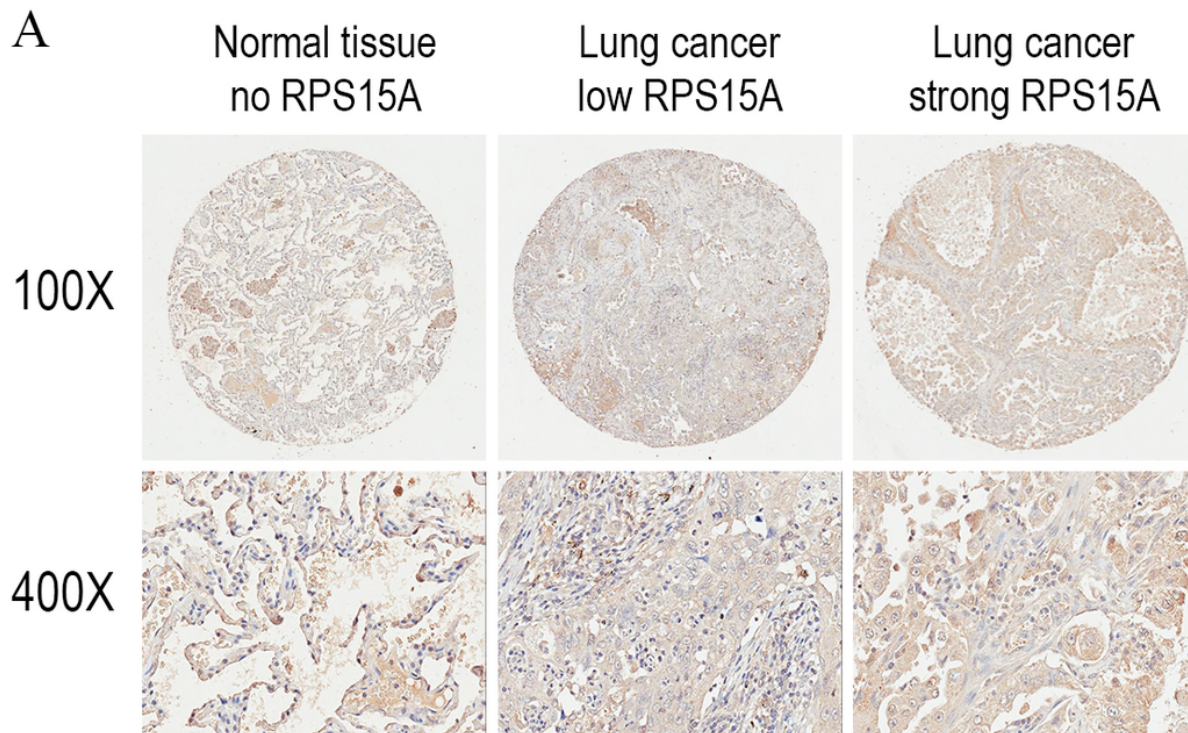
468

469

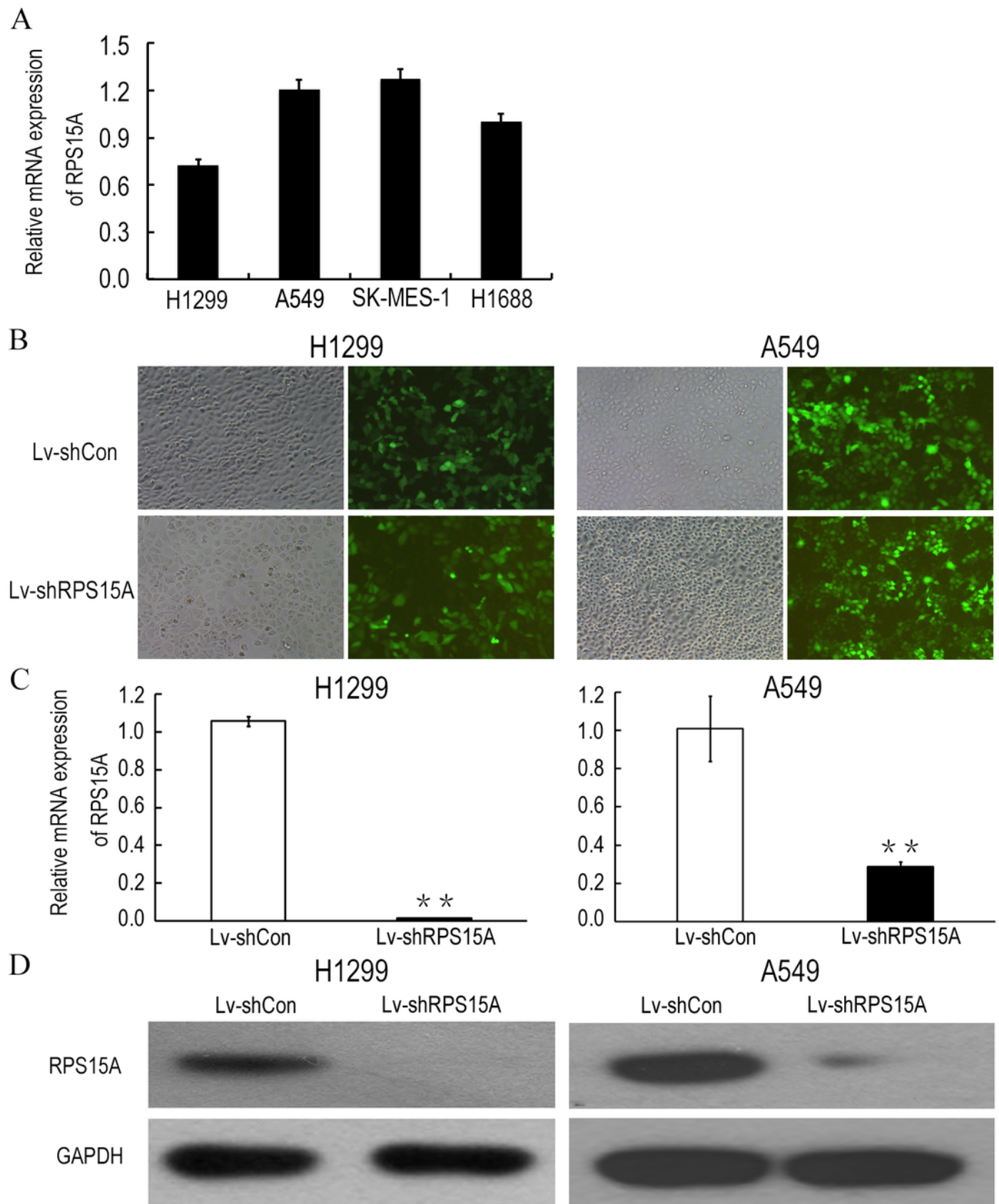
# 1

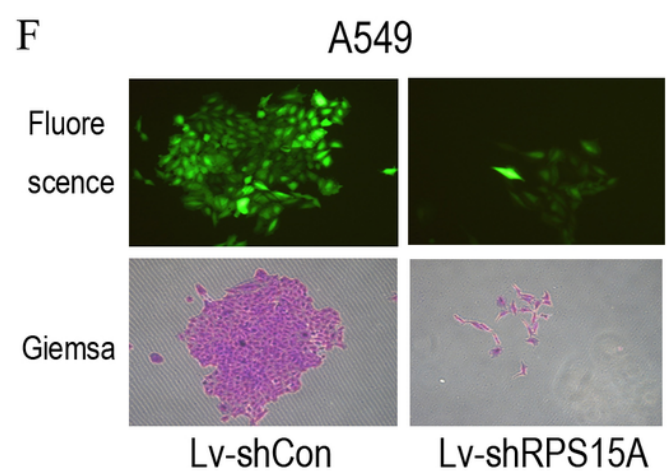
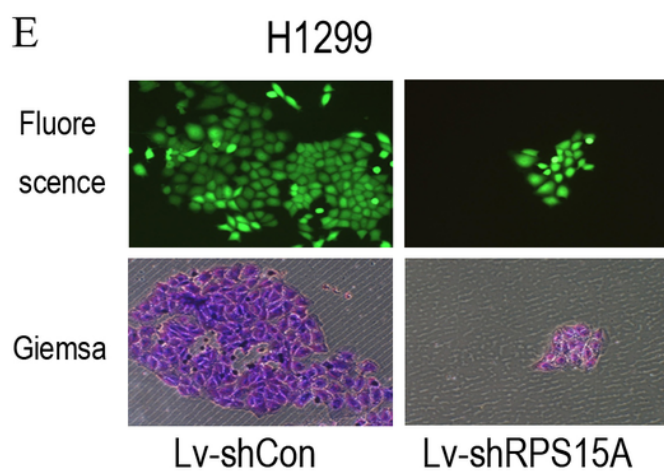
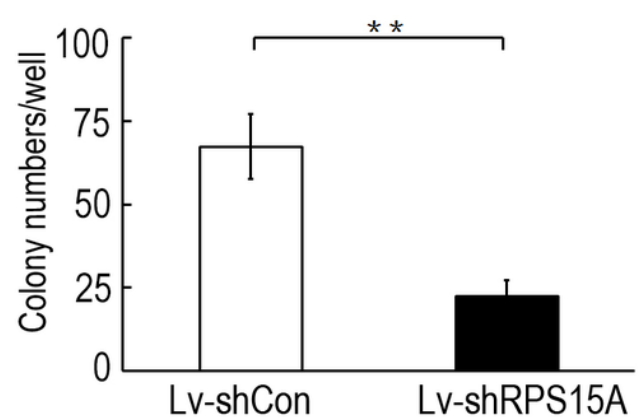
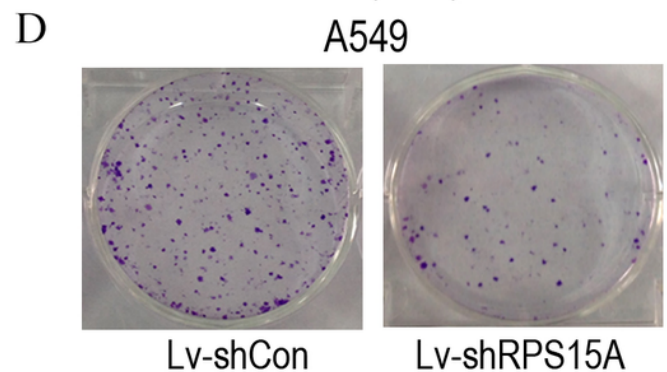
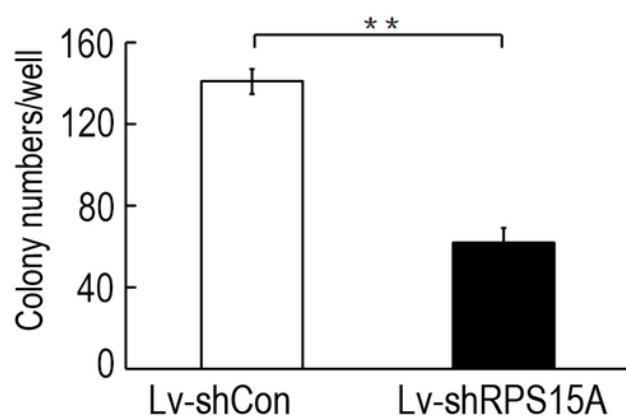
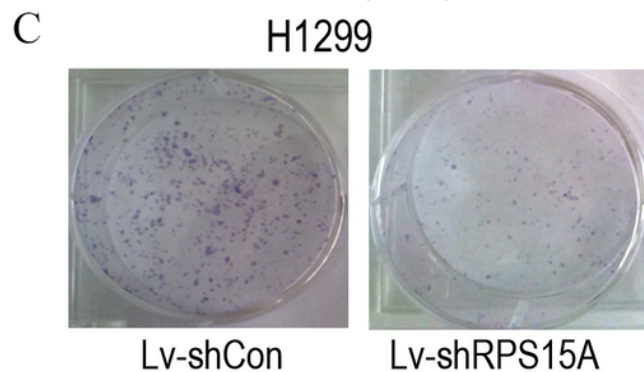
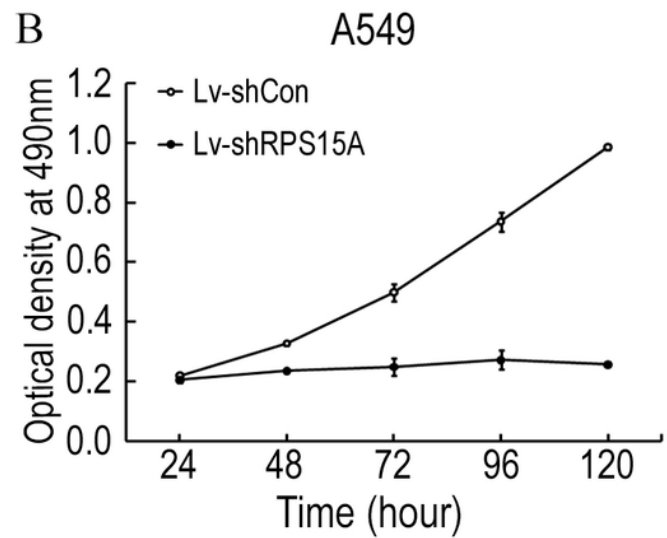
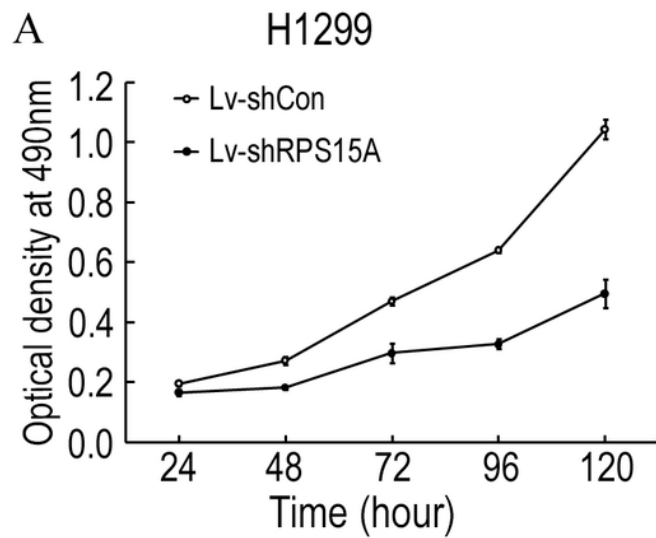
Immunostaining of RPS15A with tissue microarray

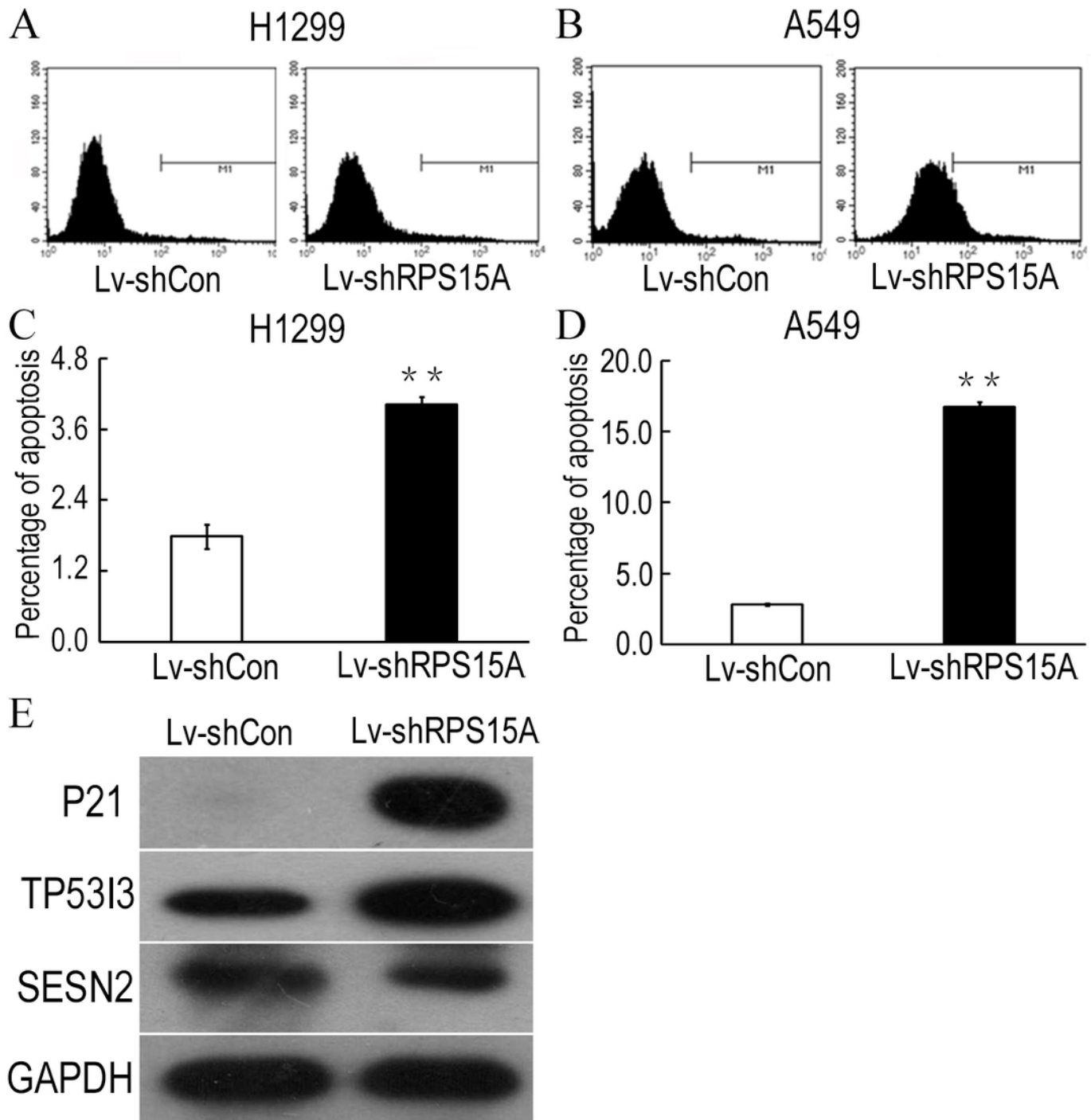
Immunostaining of RPS15A in lung adenocarcinoma and adjacent normal tissues with tissue microarray. (a) Three representative cases with different expression status of RPS15A, ranging from negative, mild and strong expression were taken at 100 × and 400 × magnification in lung cancer and normal tissues. (b)Kaplan-Meier survival analysis of overall prognosis between patients with higher RPS15A expression and patients with low RPS15A expression. Log-rank test was used to statistically calculate the difference.

# 2

Lentiviral mediated RPS15A downregulation.

Lentiviral mediated RPS15A downregulation. (a) Relative RPS15A mRNA level in lung cancer cell lines (H1299, A549, SK-MES-1, H1688 and H1975). (b) Fluorescence photomicrographs of H1299 and A549 cells 72h after lentivirus infection. (c, d) RPS15A mRNA and protein expressions were dramatically downregulated in Lv-shRPS15A infected cells evidenced by real-time PCR and Western blotting assay. **P<0.01 versus Lv-shCon.

# 3

The proliferation of H1299 and A549 cells

The proliferation of H1299 and A549 cells was inhibited after Lv-shRPS15A infection determined by MTT assay (a, b). The colony formation abilities of H1299 and A549 cells were determined by colony formation assay after Lv-shRPS15A infection. (c, d) Images of coloniesand statistical analysis of the number of colonies. (e, f) Images of colonies recorded under microscope. **$P<0.01$ versus Lv-shCon.

RPS15A knockdown induced apoptotic cells

RPS15A knockdown induced apoptotic cells were determined by flow cytometry analysis after Annexin V-APC staining.(a, b) Histograms of FACS analysis. (c, d) Percentage of apoptotic cells. **$P<0.01$ versus Lv-shCon. (e) Key factors of P53 signaling pathway, such as P21,TP53I3and SESN2, were examined in Lv-shRPS15A infected A549 cells by using western blotting method. The protein level of GAPDH was employed as a control.

RPS15A expression in 75 lung adenocarcinoma and adjacent normal tissue specimens .

1  **Table 1** RPS15A expression in 75 lung adenocarcinoma and adjacent normal tissue specimens

2  *P values were obtained with the $\chi^2$ test, P<0.001.

| Histological types | Number | RPS15A expression | | | | p* |
|---|---|---|---|---|---|---|
| | | - | + | ++ | +++ | |
| **Cancer** | 75 | 11 | 19 | 15 | 35 | 0.000 |
| **Normal tissue** | 75 | 5 | 38 | 26 | 6 | |

Correlation between RPS15A expression and clinicopathological factors in 75 lung adenocarcinoma patients specimens.

1   **Table 2** Correlation between RPS15A expression and clinicopathological factors in 75 lung
2   adenocarcinoma tissue specimens

| Variables | All patients | RPS15A expression | | p* |
|---|---|---|---|---|
| | | Negative | Positive | |
| **Total** | 75 | 26 | 49 | |
| **Age(y)** | | | | |
| **≤60** | 48 | 14 | 25 | 1.000 |
| **>60** | 27 | 12 | 24 | |
| **Gender** | | | | |
| **Male** | 43 | 19 | 24 | 0.053 |
| **Female** | 32 | 7 | 25 | |
| **TNM stage** | | | | |
| **I~II** | 47 | 20 | 36 | 0.788 |
| **III~IV** | 28 | 6 | 13 | |
| **Tumor size** | | | | |
| **≤3cm** | 8 | 1 | 7 | 0.249 |
| **>3cm** | 67 | 25 | 42 | |
| **Lymph node metastasis** | | | | |
| **Yes** | 28 | 8 | 20 | 0.458 |
| **No** | 47 | 18 | 29 | |

3   *P values were obtained with the $\chi^2$ test

KEGG pathway enrichment analysis revealed that P53 signaling pathway members were most evidently annotated. Top 20 significantly perturbed pathways are listed.

1    **Table 3** Top 20 highly expressed signaling pathways induced by RPS15A silencing