**MiR-339 depresses cell proliferation via directly targeting S-phase kinase-associated protein 2 mRNA in lung cancer**

Hong Ren¹, Yueqiao Zhang¹ & Hongzhou Zhu²

¹ Department of Radiology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China
² Department of Interventional, Zhejiang Cancer Hospital, Hangzhou, China

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Correspondence
Hongzhou Zhu, Department of Interventional, Zhejiang Cancer Hospital, 38 Guangji Road, Hangzhou 310022, China.
Tel: +86 571 8812 2222
Email: zhuhongzhouzj@163.com

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**Abstract**

**Background:** S-phase kinase-associated protein 2 (Skp2) takes great part in the development of multiple tumors. However, the post-transcriptional modulation mechanism of Skp2 remains unclear. Here, we present a new regulatory micro-RNA of Skp2, miR-339, which directly targets Skp2 to inhibit cell proliferation in lung cancer.

**Methods:** The expression of miR-339 or Skp2 in lung cancer samples was tested by real time-PCR. The correlation between miR-339 and Skp2 in lung cancer samples was analyzed by Pearson’s correlation coefficient. The effect of miR-339 or anti-miR-339 on Skp2 was evaluated by immunoblotting. The luciferase reporter gene assay was used to test the targeting of miR-339 on Skp2. 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide and colony formation analysis were applied to examine the function of miR-339 targeting Skp2 in lung cancer cells.

**Results:** The negative correlation of miR-339 with Skp2 was found in clinical human lung cancer tissues. Furthermore, Skp2 expression was obviously abated by miR-339 in lung cancer A549 cells. Mechanistically, we used bioinformatics to predict that miR-339 could target the 3'-untranslated region of Skp2 mRNA. Luciferase reporter gene assay demonstrated that miR-339 could decrease the luciferase activities of the 3'-untranslated region vector of Skp2. In terms of function, ectopic miR-339 expression significantly suppressed cell proliferation in lung cancer. Overexpressed Skp2 accelerated miR-339-bated proliferation of lung cancer cells. MiR-339 inhibitor promoted cell proliferation in lung cancer, but Skp2 RNA interference reversed miR-339 inhibitor-driven cell proliferation.

**Conclusion:** MiR-339 targets the 3'-untranslated region of Skp2 mRNA to depress the proliferation of lung cancer cells.

**Introduction**

As a well-known F-box protein, S-phase kinase-associated protein 2 (Skp2) can function as a classic oncogene in cancer cell survival via inducing some factors’ degradation.¹⁻⁷ A cascade composed of some F-box proteins including Skp2 has an effect on the cell growth of lung cancer.⁸ Highly expressed Skp2 is associated with a poor prognosis, and promotes tumor development including metastasis and invasion in non-small cell lung cancer.⁹⁻¹¹ Skp2 suppression makes lung cancer cells sensitive to drugs.¹² Overexpressed Skp2 is able to depress the radiation-induced bystander effects in esophageal carcinoma.¹³ Skp2 destabilizes Twist to regulate castration-resistant prostate cancer progression.¹⁴ Skp2 is stabilized by mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation in augmentation of gastric cancer.¹⁵ Skp2 is involved in N-myc downstream regulated gene 1-regulated c-Myc degradation in the maintenance of lung cancer cell stem-like properties.¹⁶ It has been reported that YAP can induce Skp2 acetylation through the AKT pathway to enhance tumorigenesis.¹⁷ Skp2 can be post-transcriptionally regulated by Bcl-2-associated athanogene 3 in promotion of ovarian cancer cell proliferation.¹⁸ MYC is capable of forming a bidirectional
cross-talk with CDK2, p27, or Skp2 to accelerate tumor development. Mechanical tensions-induced YAP promotes oncogenesis through Skp2 in breast cancer. In colorectal cancer, some oncogenes including Skp2 together with microRNAs (miRNAs) and tumor suppressor genes form the co-regulatory networks. Yet, the post-transcriptional regulation mechanism of Skp2 remains to be further investigated.

MiRNAs are capable of inhibiting transcription or translation of target genes. MiR-339 can serve as a tumor suppressor miRNA in many types of cancers. MiR-339 can suppress cell migration and invasion in breast cancer. MiR-339 targeting PRL-1 is involved in the development of colorectal cancer. MiR-339 can target Skp2 to inhibit gastric cancer metastasis. MiR-339 plays a great part in cell invasion in liver cancer. A report has shown that miR-508 can target Skp2 to inhibit gastric cancer metastasis. However, whether miR-339 is able to target Skp2 in lung cancer is unexplored.

We are interested in whether miR-339 has an effect on post-transcriptional regulation of Skp2 in lung cancer. We reveal that miR-339 restrains lung cancer proliferation through directly targeting Skp2 3′-untranslated region (3′ UTR). We present a novel perspective for understanding how lung cancer development by Skp2 is modulated.

**Methods**

**Cell line**

Ten percent fetal bovine serum was added into Dulbecco’s modified Eagle’s medium (Gibco, Carlsbad, CA, USA) to culture human lung cancer cell line A549. Cells were contained in a 5% CO2 incubator at 37°C.

**RNA isolation and real-time polymerase chain reaction**

Using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), total RNA of tissues or cells was extracted and SuperScript™ IV Reverse Transcriptase (ThermoFisher Scientific, Waltham, MA, USA) was utilized to perform reverse transcription. To detect miR-339 expression, total RNA was polyadenylated by poly (A) polymerase (Ambion, Austin, TX, USA). The real-time PCR was applied by TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China). PCR reaction was evaluated using melting curve analysis. Relative transcription alteration was evaluated as $^{\Delta\Delta}C_{\text{T}}$. Glycerinaldehyde 3-phosphate dehydrogenase was used to normalize Skp2. The level of miR-339 was normalized to U6 expression. The primers were listed: Skp2 forward, 5′-CGCTGCCCACGATCATTTAT-3′, reverse, 5′-TGCAACTTGGAAACACTGAC-3′; glyceraldehyde 3-phosphate dehydrogenase forward, 5′-AACGGATTG GTCGTATTG-3′, reverse, 5′-GGAAGATGTGTTGGA TT-3′; miR-339 forward, 5′-GGGTGCTGTCGTGGAG-3′, reverse, 5′-CTCGTTCGGCAGCAG-3′, reverse, 5′-AACGCTTC AATTGGGCT-3′.

**Plasmids construction**

Full-length Skp2 was amplified and cloned into pcDNA3.1 vector (primers: forward, 5′-CGGAATTCACACAG GAACGACCTCCAGGAGATTCCAC-3′, reverse, 5′- CCGCTGAGTCTAGACACGGGCTTTTGCAGTG-3′). Wild type (wt) or mutated (mut) miR-339 targeting site in Skp2 3′ UTR was cloned into pGL3-control vector (primers: wt forward, 5′-GCTCTAGAGTGTCTGATGATTTCATGTC TTAGAACAGGGAAAAT-3′, reverse, 5′-GGGCCC GGTCTTATAAGGTATCTTGAAATTAGGCT-3′; mutant forward, 5′-GCTCTAGAGTGTCTCTTTCTTCTTAGAC AAGGGAAAAT-3′, reverse, 5′-GGGCCC GGTCTTATAAGGTATCTTGAAATTAGGCT-3′). Wild type (wt) or mutated (mut) miR-339 targeting site in Skp2 3′ UTR was cloned into pGL3-control vector (primers: wt forward, 5′-GCTCTAGAGTGTCTGATGATTTCATGTC TTAGAACAGGGAAAAT-3′, reverse, 5′-GGGCCC GGTCTTATAAGGTATCTTGAAATTAGGCT-3′; mutant forward, 5′-GCTCTAGAGTGTCTCTTTCTTCTTAGAC AAGGGAAAAT-3′, reverse, 5′-GGGCCC GGTCTTATAAGGTATCTTGAAATTAGGCT-3′).

**siRNAs, miRNA, and miRNA inhibitor transfection**

Transient transfection of cells was performed using Lipofectamine 2000 (Invitrogen). MiR-339, control, inhibitor, and siSkp2 were synthesized by Ribobio (Guangzhou, China). siSkp2-1, 5′-CUAUCGAACUCAGUUATT-3′; siSkp2-2, 5′-GCAAGGGAGUGACAAA-3′.

**Luciferase reporter gene assays**

Seeded cells on 24-well plates were cultured overnight. Lipofectamine 2000 (Invitrogen) was applied to transfect the indicated luciferase reporters and/or miRNA (inhibitor). Renilla luciferase activities were utilized to normalize Firefly luciferase activities. Luciferase activity was analyzed using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) and monitored by a luminometer (Promega).

**Immunoblotting**

Radioimmunoprecipitation assay buffer was used to isolate total protein. After running in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, all protein was transferred to polyvinylidene fluoride membranes (Millipore, USA). Five percent skim milk was applied to block the membranes for two hours and then incubated in primary antibodies: anti-Skp2 antibody (Santa Cruz Biotechnology, USA) or anti-β-actin antibody (Abcam,
Cambridge, MA, USA) followed by the corresponding secondary antibody.

**Proliferation assays**
3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) and colony formation assays were used to evaluate cell proliferation ability. A549 cells seeding on 96-well plates at 1000 cells/well were transfected. After transfection, cell proliferation was evaluated by MTT assay once per day for three days. For colony formation analysis, post-forty-eight hours after transfection with miR-339, miR-339 + Skp2, anti-miR-339, anti-miR-339 + siSkp2-1, or negative control seeded cells on six-well plates (1000 cells/well) were cultured for two weeks until visible colonies formed. The colonies were fixed with 4% paraformaldehyde and stained with methylene blue. Colonies were counted by three observers separately.

**Patient samples**
The Sir Run Run Shaw Hospital (Hangzhou, China) provided 32 lung cancer tissues harvested from lung cancer patients by surgery (Table S1). Written consent was banked with tissues, and the study protocol has been approved by the Research Ethics Board.

**Statistical analysis**
Every experiment was performed in triplicate. Data were recorded as mean ± SD. Student’s t-test was utilized to compare different groups’ differences. A P-value <0.05 was determined significant. The correlation between miR-339 and Skp2 in lung cancer samples was evaluated by Pearson’s correlation coefficient.

**Results**

**Low level of miR-339 is related to highly expressed Skp2 in clinical lung cancer tissues**
Skp2 abundance is frequently found in multiple cancers. A high level of Skp2 is involved in the development of many cancers. Tumor suppressor, miR-339, has an effect on the progression of many cancers. To explore the relationship of miR-339 and Skp2 in lung cancer development, we examined the expression of miR-339 and Skp2 in 32 clinical lung cancer samples. Our data showed that a low level of miR-339 was related to a high level of Skp2 in clinical lung cancer samples (Pearson’s correlation coefficient of −0.7982, P < 0.001) (Fig 1a). In addition, Skp2 expression was confirmed in lung cancer samples by real time-PCR assay. Abundant Skp2 expression in lung cancer tissues was observed (Fig 1b). Our data hint that deregulated miR-339 is related to upregulated Skp2 in clinical lung cancer tissues.

**MiR-339 is able to control Skp2 expression in lung cancer cells**
Then, we tried to evaluate the effect of miR-339 on Skp2 expression lung cancer cells. We examined the Skp2 level in miR-339 or anti-miR-339-treated cells using real time-PCR and immunoblotting. As shown in Figure 2a, miR-339 decreased the mRNA and protein levels of Skp2. Furthermore, the expression of Skp2 was elevated in miR-339-depleted cells (Fig 2b). Our data imply that miR-339 is able to control Skp2 expression in lung cancer. Meanwhile, miR-339 and anti-miR-339 transfection was evaluated by real time-PCR (Fig 2a,b). Collectively, our data support that miR-339 is capable of depressing Skp2 in lung cancer cells.
MiR-339 directly targets Skp2 3’UTR to inhibit its expression

Next, we wanted to investigate the Skp2 post-transcriptional regulation in lung cancer. MiRNA.org was applied to predict the miRNAs that could target Skp2. MiR-339 was a potential regulatory miRNA of Skp2. To test the effect of miR-339 on Skp2, we cloned the wild-type (wt) or mutant (mut) 3’UTR of Skp2 into pGL3-control plasmid (pGL3-Skp2-wt and pGL3-Skp2-mut) (Fig 3a). The pGL3-Skp2-wt or pGL3-Skp2-mut plasmid combined with miR-339 or inhibitor (anti-miR-339) was transfected into A549 cells. We found that ectopic miR-339 expression significantly decreased the luciferase activities of pGL3-Skp2-wt in lung cancer A549 cells. However, miR-339 lost the inhibitory effect on pGL3-Skp2-mut containing the seed region mutation in cells.
(Fig 3b). In addition, miR-339 inhibitor (anti-miR-339) was capable of decreasing the miR-339 level to enhance the luciferase activities of the pGL3-Skp2-wt. Nevertheless, pGL3-Skp2-mut cannot be affected by anti-miR-339 introduction (Fig 3c). Our data indicate that miR-339 inhibits Skp2 expression via directly targeting its mRNA 3'UTR in lung cancer cells.

**MiR-339 targeting Skp2 contributes to cell proliferation inhibition in lung cancer**

To further evaluate the function of miR-339 targeting Skp2 in lung cancer, we performed MTT and colony formation analysis. We first evaluated the effect of two different siRNAs targeting Skp2 on the expression of Skp2 (Fig 4a). Then, we found that the miR-339-overexpressed cells showed more highly proliferative ability, while ectopic Skp2 expression was able to recover the miR-339-suppressed cell proliferation. Furthermore, anti-miR-339 transfection showed the inhibitory effect on cell proliferation, but Skp2 siRNA could breach the acceleration of cell proliferation induced by anti-miR-339 (Fig 4b). Taken a further step, colony formation assay showed that the colony numbers were less in miR-339-treated cells compared with that in the control group. Anti-miR-339 was able to enhance the colony formation in the cells. Notably, Skp2 siRNA could destroy the anti-miR-339-augmented colony formation in A549 cells (Fig 4c). Our data support that miR-339 can bate Skp2 to restrain cell proliferation in lung cancer.

**Discussion**

Lung cancer is the leading cause of cancer-related death and the most prevalent malignancy in the world. Skp2 is a classic oncogene during the development of cancers...
involving some factors’ degradation. Highly expressed Skp2 can serve as a poor prognosis marker in non-small cell lung cancer. The progression of castration-resistant prostate cancer is modulated by Skp2 via destabilizing Twist. Lung cancer cells become sensitive to drugs for Skp2 inhibition. Bcl-2 associated athanogene 3 can post-transcriptionally modulate Skp2 in ovarian cancer growth. However, the post-transcriptional regulation mechanism of Skp2 remains unexplored.

Previous studies have shown that miR-339 plays key roles as a tumor suppressor miRNA in many types of cancers. In breast cancer, miR-339 has an inhibitory effect on cell migration and invasion. MiR-339 targeting PRL-1 partakes in colorectal cancer progression. Non-small cell lung cancer metastasis staging is related to miR-339 level. MiR-339 plays a great part in cell invasion in liver cancer. We first evaluated the negative correlation between miR-339 and Skp2 in clinical lung cancer tissues. Furthermore, our data showed that the level of Skp2 could be decreased by miR-339 using real time-PCR and immunoblotting, further indicating that miR-339 is able to target Skp2. To investigate how miR-339 regulates Skp2 in lung cancer, we predicted which miRNA might target Skp2 by microRNA.org analysis. MiR-339 was a potential regulatory miRNA of Skp2 for its tumor suppressor role in multiple cancers. However, whether miR-339 is able to target Skp2 to regulate its expression in lung cancer remains unclear. To further clarify the regulation of miR-339 on Skp2, we cloned the wild-type (wt) and mutant (mut) 3’UTR of Skp2 into pGL3-control plasmid. We showed that ectopic miR-339 expression significantly decreased the luciferase activity of pGL3-Skp2-wt in lung cancer A549 cells. MiR-339 inhibitor was capable of decreasing the miR-339 level to enhance the luciferase activities of the pGL3-Skp2-wt. It suggests that miR-339 can inhibit Skp2 expression through directly targeting its mRNA 3’UTR in lung cancer cells. In terms of function, miR-339 owned the ability by which miR-339 targets Skp2 to bate the cell proliferation in lung cancer in vitro. Our finding that miR-339 is capable of targeting the key oncoprotein, Skp2, provides new evidence for the tumor suppressor role of miR-339 in cancers.

For the first time, we present a novel role of miR-339 in post-transcriptional regulation of Skp2 for Skp2 inhibition, resulting in decreased cell proliferation in lung cancer. We gained novel insight into the mechanism of lung cancer growth induced by Skp2.

Disclosure
No authors report any conflict of interest.

References
1. Yu ZK, Gervais JL, Zhang H. Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins. Proc Natl Acad Sci U S A 1998; 95: 11324–9.
2. Carrano AC, Eytan E, Hershko A, Pagano M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol 1999; 1: 193–9.
3. Spruck C, Strohmaier H, Watson M et al. A CDK-independent function of mammalian Csk1: Targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. Mol Cell 2011; 7: 639–50.
4. Kamura T, Hara T, Kotoshiba S et al. Degradation of p57Kip2 mediated by SCF-Skp2-dependent ubiquitylation. Proc Natl Acad Sci U S A 2003; 100: 10231–6.
5. Tedesco D, Lukas J, Reed SI. The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF(Skp2). Genes Dev 2002; 16: 2946–57.
6. Huang H, Regan KM, Wang F et al. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. Proc Natl Acad Sci U S A 2005; 102: 1649–54.
7. Hao Z, Huang S. E3 ubiquitin ligase Skp2 as an attractive target in cancer therapy. Front Biosci (Landmark Ed) 2015; 20: 474–90.
8. Xu J, Zhou W, Yang F et al. The beta-TrCP-FBXW2-SKP2 axis regulates lung cancer cell growth with FBXW2 acting as a tumour suppressor. Nat Commun 2017; 8: 14002.
9. Hung WC, Tseng WL, Sheia J, Chang HC. Skp2 overexpression increases the expression of MMP-2 and MMP-9 and invasion of lung cancer cells. Cancer Lett 2010; 288: 156–61.
10. Osoegawa A, Yoshino I, Tanaka S et al. Regulation of p27 by S-phase kinase-associated protein 2 is associated with aggressiveness in non-small-cell lung cancer. J Clin Oncol 2004; 22: 4165–73.
11. Yokoi S, Yasui K, Mori M, Iizasa T, Fujisawa T, Inazawa J. Amplification and overexpression of SKP2 are associated with metastasis of non-small-cell lung cancers to lymph nodes. Am J Pathol 2004; 165: 175–80.
12. Huang T, Yang L, Wang G et al. Inhibition of Skp2 sensitizes lung cancer cells to paclitaxel. Onco Targets Ther 2017; 10: 439–46.
13. Wang XC, Zhang TJ, Guo ZJ et al. Overexpression of SKP2 inhibits the radiation-induced bystander effects of esophageal carcinoma. Int J Environ Res Public Health 2017; 14: 155.
14. Ruan D, He J, Li CF et al. Skp2 deficiency restricts the progression and stem cell features of castration-resistant prostate cancer by destabilizing twist. Oncogene 2017; 36: 4299–310.
15. Geng Q, Liu J, Gong Z et al. Phosphorylation by mTORC1 stabilizes Skp2 and regulates its oncogenic function in gastric cancer. Mol Cancer 2017; 16: 83.
16 Wang Y, Zhou Y, Tao F et al. N-myc downstream regulated gene 1 (NDRG1) promotes the stem-like properties of lung cancer cells through stabilized c-Myc. *Cancer Lett* 2017; **401**: 53–62.

17 Zhang S, Chen Q, Liu Q et al. Hippo signaling suppresses cell ploidy and tumorigenesis through Skp2. *Cancer Cell* 2017; **31**: 669–84 e667.

18 Yan J, Liu C, Jiang JY et al. BAG3 promotes proliferation of ovarian cancer cells via post-transcriptional regulation of Skp2 expression. *Biochim Biophys Acta* 2017, 1864; 1668–78.

19 Hydbring P, Castell A, Larsson LG. MYC modulation around the CDK2/p27/Skp2 Axis. *Genes (Basel)* 2017; **8**: 174.

20 Jang W, Kim T, Koo JS, Kim SK, Lim DS. Mechanical cue-induced YAP instructs Skp2-dependent cell cycle exit and oncogenic signaling. *EMBO J* 2017; **36**: 2510–28.

21 Slattery ML, Herrick JS, Mullany LE et al. The co-regulatory networks of tumor suppressor genes, oncogenes, and miRNAs in colorectal cancer. *Genes Chromosomes Cancer* 2017; **56**: 769–87.

22 Wu ZS, Wu Q, Wang CQ et al. MiR-339-5p inhibits breast cancer cell migration and invasion in vitro and may be a potential biomarker for breast cancer prognosis. *BMC Cancer* 2010; **10**: 542.

23 Zhou C, Liu G, Wang L et al. MiR-339-5p regulates the growth, colony formation and metastasis of colorectal cancer cells by targeting PRL-1. *PLoS One* 2013; **8**: e63142.

24 Li Y, Zhao W, Bao P et al. miR-339-5p inhibits cell migration and invasion in vitro and may be associated with the tumor-node-metastasis staging and lymph node metastasis of non-small cell lung cancer. *Oncol Lett* 2014; **8**: 719–25.

25 Jansson MD, Damas ND, Lees M, Jacobsen A, Lund AH. miR-339-5p regulates the p53 tumor-suppressor pathway by targeting MDM2. *Oncogene* 2015; **34**: 1908–18.

26 Wang YL, Chen CM, Wang XM, Wang L. Effects of miR-339-5p on invasion and prognosis of hepatocellular carcinoma. *Clin Res Hepatol Gastroenterol* 2016; **40**: 51–6.

27 Duan X, Bai J, Wei J, Li Z, Liu X, Xu G. MicroRNA-508-5p suppresses metastasis in human gastric cancer by targeting S-phase kinase-associated protein 2. *Mol Med Rep* 2017; **16**: 2163–71.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

**Table S1** Clinical characteristics of lung cancer samples.