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ORIGINAL ARTICLE

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

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Keywords
Lung cancer; miR-339; proliferation; S-phase kinase-associated protein 2.

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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 microRNA 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 acethylation 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.[19] Mechanical tensions-induced YAP promotes oncogenesis through Skp2 in breast cancer.[20] In colorectal cancer, some oncogenes including Skp2 together with microRNAs (miRNAs) and tumor suppressor genes form the co-regulatory networks.[21] 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.[22] MiR-339 targeting PRL-1 is involved in the progression of colorectal cancer.[23] In non-small cell lung cancer, miR-339 is related to tumor metastasis staging.[24] MiR-339 is able to target MDM2 to affect the p53 pathway.[25] MiR-339 plays a great part in cell invasion in liver cancer.[26] A report has shown that miR-508 can target Skp2 to inhibit gastric cancer metastasis.[27] 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% CO_2 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 $2^{-\Delta\Delta Ct}$. glyceraldehyde 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′-TGCAACTTGGAACACTGAGACA-3′; glyceraldehyde 3-phosphate dehydrogenase forward, 5′-AACGGATTTG GTCGTATTG-3′, reverse, 5′-GGAAGATGGTGATGGGA TT-3′; miR-339 forward, 5′-GGGTCCCTGTCCTCCA-3′, reverse, 5′- TGCGTGTCGTGGAGTC-3′; U6 forward, 5′-CTCGCTTCGGCAGCACA-3′, reverse, 5′- AACGCTTC ACG AATTTGCGT-3′.

Plasmids construction

Full-length Skp2 was amplified and cloned into pcDNA3.1 vector (primers: forward, 5′-CCGGAATTCACACAG-GAAGCACCTCCAGGAGATTCCAGAC-3′, reverse, 5′-CCGCTCGAGTCATAGACAACTGGGCTTTTGCAGTGT CAG-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′-GCTCTAGAGTGTCTCTTCT TTAGAACAGGGAAAAT-3′, reverse, 5′-GGGGGCCGGC CGCTCTTTAAGGTATCTTGAAATTATAGGCT-3′; mut forward, 5′- GCTCTAGAGTGTCTGATGATTTCATGTC CCAAAAT-3′, reverse, 5′-GGGGGCCGGCC GCTCTTTA AGGTATCTTGAAATTATAGGCT-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′-CCUAUCGAACUCAGUUAUATT-3′; siSkp2-2, 5′- GCAAAGGGAGUGACAAA-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.

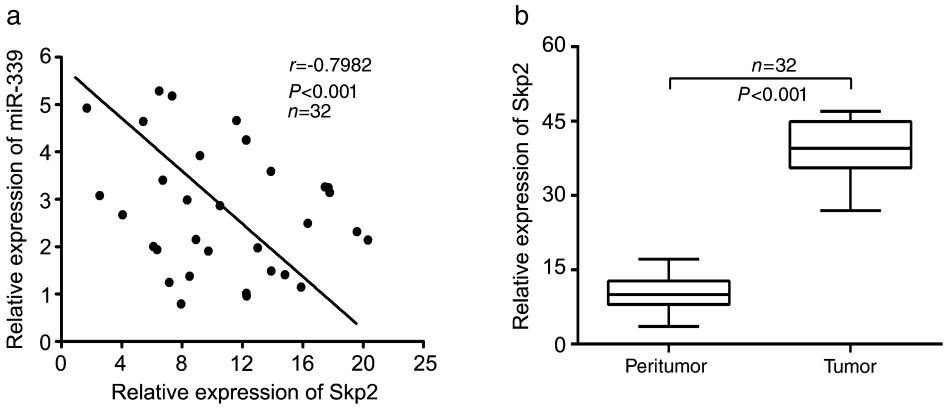

Figure 1 A low level of miR-339 is related to highly expressed S-phase kinase-associated protein 2 (Skp2) in clinical lung cancer tissues. (**a**) The relationship between miR-339 and Skp2 was investigated using real time-PCR in 32 clinical lung cancer samples (Pearson's correlation coefficient, $R = -0.7982$). (**b**) In clinical samples, the Skp2 level was evaluated by real time-PCR. ***$P < 0.001$; Student's *t*-test.

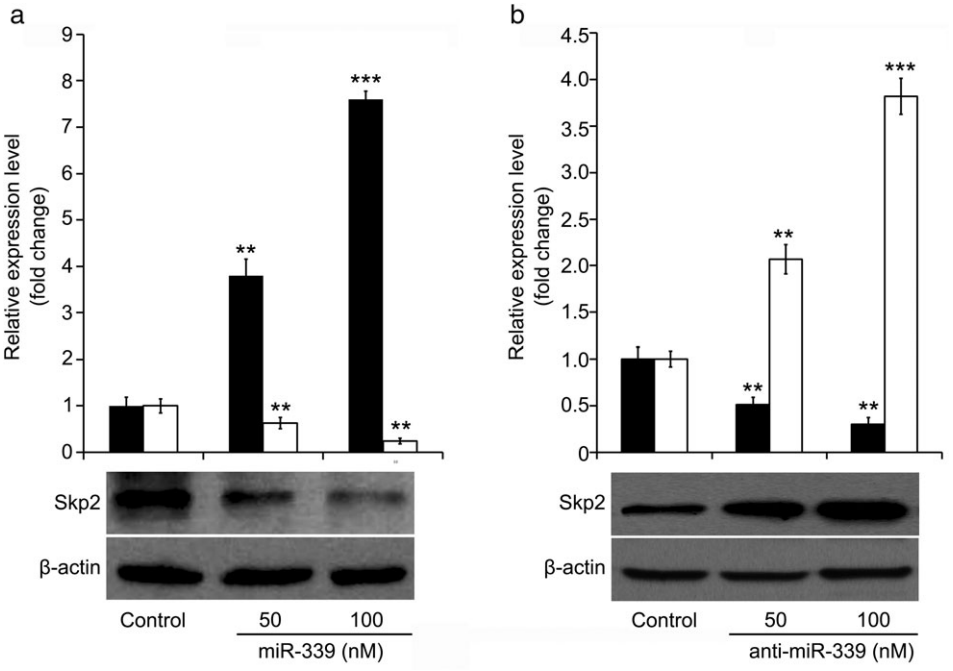

Figure 2 MiR-339 is able to control S-phase kinase-associated protein 2 (Skp2) expression in lung cancer cells. (**a**, **b**) Skp2 levels were analyzed in miR-339 (or anti-miR-339)-treated A549 cells by real time-PCR assay and immunoblotting. The transfection of miR-339 or anti-miR-339 was confirmed by real time-PCR analysis. ***$P < 0.001$; **$P < 0.01$; Student's *t*-test. (■) miR-339 and (□) Skp2.

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

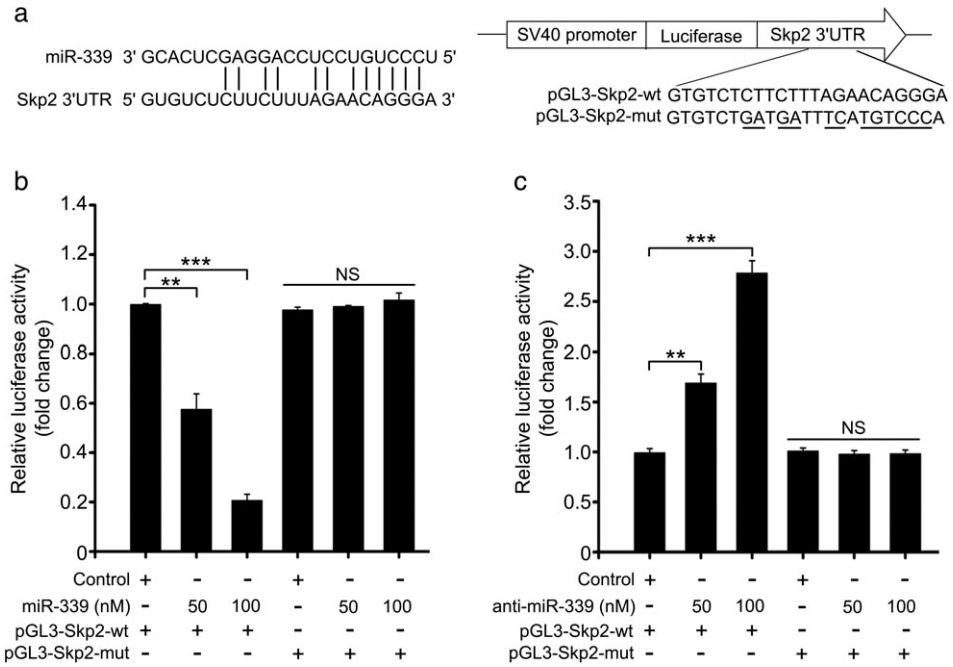

Figure 3 MiR-339 directly targets S-phase kinase-associated protein 2 (Skp2) 3′-untranslated region (3′UTR) to inhibit its expression. (**a**) The binding site of miR-339 within Skp2 3′UTR is shown. The wild-type (wt) or mutant (mut) 3′UTR of Skp2 (wt or mut) is cloned into pGL3-control plasmid. (**b**, **c**) The effect of miR-339 or inhibitor (anti-miR-339) on pGL3-Skp2-wt or pGL3-Skp2-mut was analyzed by luciferase reporter gene analysis in A549 cells. ***$P < 0.01$; **$P < 0.01$; NS, not significant; Student's *t*-test.

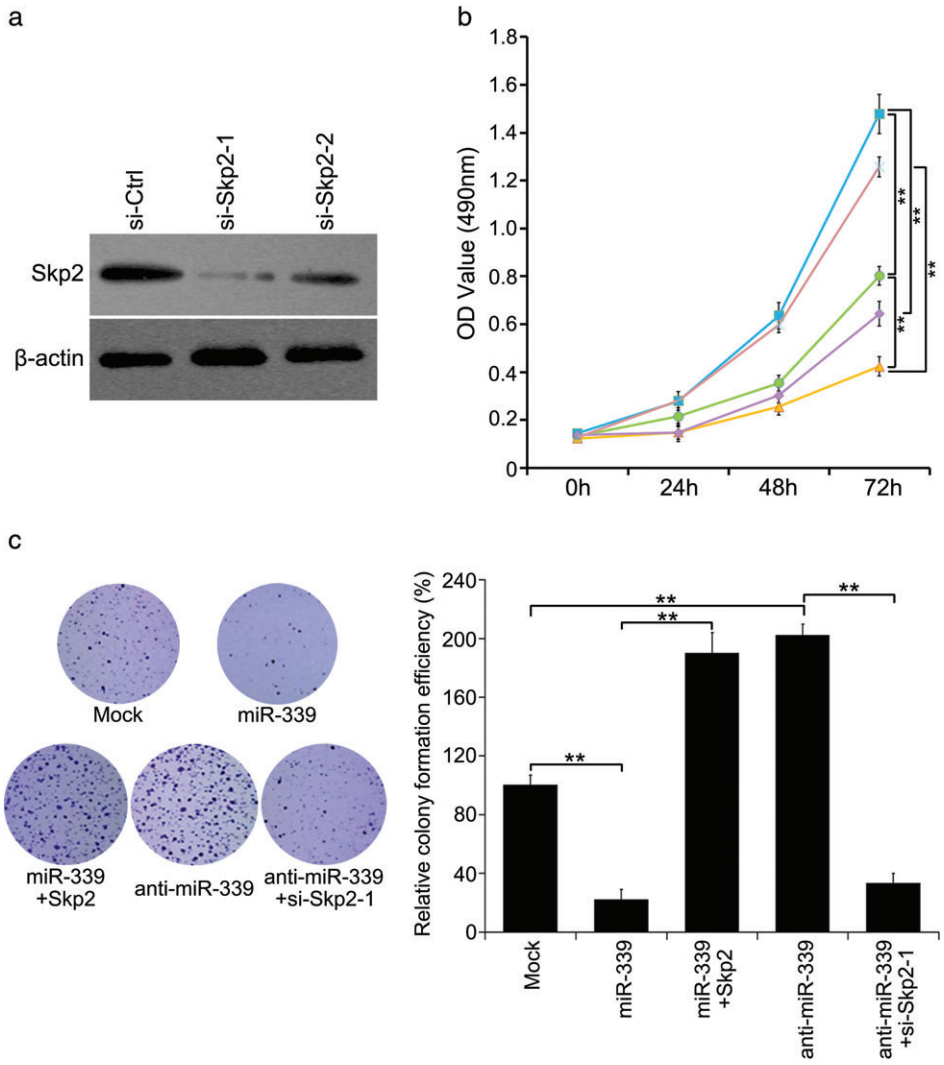

Figure 4 MiR-339 targeting S-phase kinase-associated protein 2 (Skp2) contributes to cell proliferation inhibition in lung cancer. (**a**) Interference efficiency of siSkp2–1 or siSkp2–2 was evaluated by immunoblotting in A549 cells. (**b**, **c**) Proliferation abilities of miR-339, miR-339/Skp2, anti-miR-339, or anti-miR-339/siSkp2–1-treated A549 cells were detected by MTT and colony formation analysis. ** $P < 0.01$; Student's *t*-test. (–●–) Mock, (–▲–) miR-339, (–■–) anti-miR-339, (–◆–) anti-miR-339+si-Skp2-1, and (–✳–) miR-339+Skp2.

(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.[1–7] Highly expressed Skp2 can serve as a poor prognosis marker in non-small cell lung cancer.[9–11] The progression of castration-resistant prostate cancer is modulated by Skp2 via destabilizing Twist.[14] Lung cancer cells become sensitive to drugs for Skp2 inhibition.[12] Bcl-2 associated athanogene 3 can post-transcriptionally modulate Skp2 in ovarian cancer growth.[18] 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.[22] MiR-339 targeting PRL-1 partakes in colorectal cancer progression.[23] Non-small cell lung cancer metastasis staging is related to miR-339 level.[24] MiR-339 plays a great part in cell invasion in liver cancer.[26] 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.

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Supporting Information

Additional Supporting Informationmay be found in the online version of this article at the publisher's website:

Table S1 Clinical characteristics of lung cancer samples.

