

MiR-516a-5p inhibits the proliferation of non-small cell lung cancer by targeting HIST3H2A

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Abstract

The dysregulation of microRNAs (miRNAs) is associated with the pathogenesis of non-small cell lung cancer (NSCLC). However, the mechanisms by which miR-516a-5p contributes to NSCLC remain unclear. The association between miR-516a-5p expression and the clinicopathological characteristics and prognosis in patients with NSCLC was analyzed by The Cancer Genome Atlas (TCGA) data set. The targets of miR-516a-5p were identified by bioinformatic analysis and luciferase report assay. MTT and soft agar assays were conducted to investigate the function of miR-516a-5p in NSCLC cells. We found that the expression of miR-516a-5p was decreased in NSCLC tissues and associated with the age, pathological stage, and tumor size, acting as an independent prognostic factor of tumor recurrence in patients with NSCLC. Restoration of miR-516a-5p inhibited the cell viability and anchorage-independent growth of NSCLC cells, but its inhibitor had the opposite effects. Histone cluster 3 H2A (HIST3H2A) was further identified as a direct target of miR-516a-5p and displayed a negative correlation with miR-516a-5p expression in NSCLC tissues. Overexpression of HIST3H2A reversed the anti-proliferation effects induced by miR-516a-5p and acted as an independent prognostic factor of poor survival in patients with NSCLC. Altogether, our findings demonstrate that miR-516a-5p may function as a tumor suppressive factor in NSCLC cells by targeting HIST3H2A and might represent a potential indicator of tumor recurrence in patients with NSCLC.

Keywords

HIST3H2A, miR-516a-5p, NSCLC, proliferation

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Introduction

Lung cancer is one of the most common malignancies with the highest morbidity and mortality worldwide.¹ Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancer cases, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. The incipient symptom of NSCLC is unobvious, and most of them are diagnosed in advanced stage due to their unlimited growth and distant metastasis.² Thus, identification of the molecular biomarkers related to the progression of NSCLC is critical for improving their outcome.

MicroRNAs (miRNAs), a subtype of small non-coding RNAs, can repress the expression of their target mRNAs by binding to their 3'-untranslated

region (3'UTR). The dysregulation of miRNAs is associated with the growth and metastasis of NSCLC.³ They act as oncogenic factors⁴ or tumor

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suppressors⁵ involved in the pathogenesis of NSCLC. On one hand, miR-19b promotes the growth and apoptosis escape,⁶ and miR-661 facilitates the invasion and metastasis of NSCLC by inhibiting the RB transcriptional co-repressor 1.³ On the other hand, miR-183/-200c/-1258 suppresses the proliferation and invasion of NSCLC cells.⁷

There is little knowledge about the role of miR-516a-5p in cancers. Decreased expression of miR-516a-5p predicts a poor survival in neuroblastoma.⁸ But, miR-516a-5p expression is increased in abdominal aortic aneurysm and promotes the proteolytic degradation.⁹ In this study, we found that the decreased expression of miR-516a-5p was associated with the age, pathological stage, and tumor size, acting as an independent prognostic factor of tumor recurrence in patients with NSCLC. Re-expression of miR-516a-5p inhibited the proliferation of NSCLC cells by targeting Histone cluster 3 H2A (HIST3H2A) and might represent a potential marker for tumor recurrence in patients with NSCLC.

Materials and methods

Clinical data

The data including 262 NSCLC tissues, 23 pair-matched normal tissues, overall survival (OS) time and status, recurrence time and status as well as miR-516a-5p, and HIST3H2A expression levels were downloaded from The Cancer Genome Atlas (TCGA) database (<https://genome-cancer.ucsc.edu>). The protocols used in our study were approved by the Ethics Committee of Shanghai Chest Hospital. The pathological diagnosis for these tissues was conducted by two independent pathologists.

Identification of the target genes of miR-516a-5p

The target genes of miR-516a-5p were identified using the prediction tool TargetScanHuman7.1 (http://www.targetscan.org/vert_71/), and according to the cumulative weighted text score, five target genes of miR-516a-5p were selected for further investigation.

Cell culture

NSCLC cell lines (A549 and NCI-H460) were purchased from Chinese Academy of Sciences Cell Bank and were cultured in Dulbecco's Modified

Eagle medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37°C.

Quantitative real-time PCR

Total RNA of NSCLC cells was extracted using TRIzol. Reverse transcription was performed by M-MLV and cDNA amplification using the SYBR Green Master Mix kit (Takara, Otsu, Japan). Total RNA for miRNAs was isolated using a High Pure miRNA isolation kit (Roche, Indianapolis, USA) and RT-PCR using a TaqMan MicroRNA Reverse Transcription kit (Life Technologies, Carlsbad, USA). Data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$). Three experiments were performed for each clone.

Western blot analysis

NSCLC cell lines were harvested and extracted using lysis buffer. Cell extracts were boiled in loading buffer, and equal amount of cell extracts was separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Separated protein bands were transferred into polyvinylidene fluoride membranes. The primary antibodies against HIST3H2A (AA 2-130; 4A Biotech, Beijing, China) and β -actin (ab16039, Rabbit polyclonal antibody; Abcam, Cambridge, MA, USA) were diluted at a ratio of 1:1000 according to the instructions and incubated overnight at 4°C.

Luciferase reporter assay

NSCLC cell lines were seeded into 96-well plates in combination with the co-transfection with a mixture of 60 ng of luciferase, 6 ng of pRL-CMV Renilla luciferase reporter, and miR-516a-5p mimic or inhibitor. After 48 h of incubation, the luciferase activities were examined by a dual-luciferase reporter assay (Promega, Madison, WI, USA).

Plasmid, miR-516a-5p mimic, and inhibitor

Plasmid-mediated pcDNA3.1-HIST3H2A (HIST3H2A), miR-516a-5p mimic, and inhibitor and the control vectors were purchased from GenePharma (Shanghai, China). NSCLC cell lines were planted in 6-well plates 24 h prior to HIST3H2A plasmid, miR-516a-5p mimic, or inhibitor transfection with 50%–70% confluence and then were transfected with

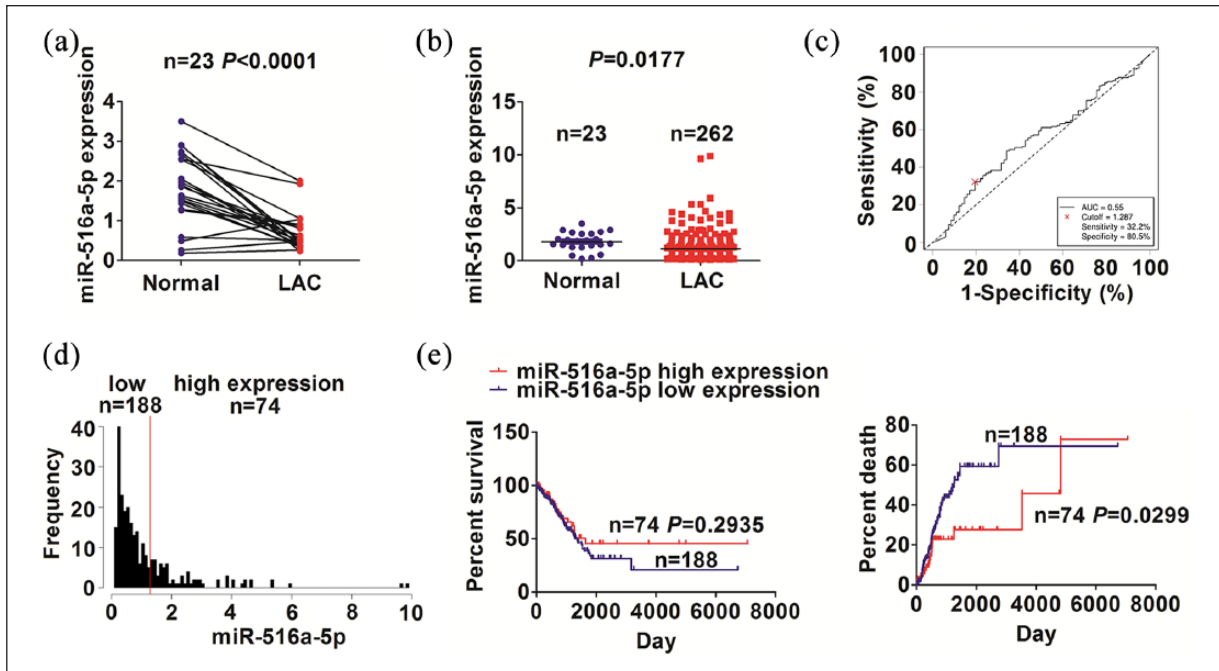


Figure 1. The expression of miR-516a-5p was associated with tumor recurrence in NSCLC patients. (a, b) TCGA analysis of the expression levels of miR-516a-5p in paired and unpaired NSCLC tissues. (c) ROC curve was used to obtain a cut-off value of miR-516a-5p in NSCLC. (d) The grouping NSCLC patients was determined by the cut-off value of miR-516a-5p. (e) Kaplan–Meier analysis of the association of miR-516a-5p expression with OS and tumor recurrence in NSCLC patients.

Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacture instructions.

MTT, agar assays, and statistical analysis

MTT, agar assays, and statistical analysis were performed as previously described.^{5,10}

Results

Downregulation of miR-516a-5p expression was associated with tumor recurrence in patients with NSCLC

TCGA cohort indicated that miR-516a-5p expression levels were decreased in paired ($n=23$, Figure 1(a)) and unpaired NSCLC tissues ($n=262$, Figure 1(b)) as compared with the adjacent normal tissues ($n=23$). In light of the OS time, survival status, and miR-516a-5p expression levels, a cut-off value of miR-516a-5p in NSCLC (Figure 1(c)) was acquired, and divided the patients into high-expression group and low-expression group (Figure 1(d)). We found that low expression of miR-516a-5p was associated with the age ($P=0.024$), pathological stage ($P=0.025$), and tumor size ($P=0.011$), but had no association with other factors in patients

with NSCLC (each $P > 0.05$, Supplementary Table S1). Kaplan–Meier showed that the patients with low miR-516a-5p expression had a higher tumor recurrence rate, but had no difference in OS, as compared with those with high miR-516a-5p expression (Figure 1(e)). Univariate and Multivariate Cox regression analyses uncovered miR-516a-5p expression as an independent prognostic factor of tumor recurrence in patients with NSCLC ($P=0.024$, Table 1).

MiR-516a-5p suppressed the proliferation and anchorage-independent growth of NSCLC cells

The transfection efficiency of miR-516a-5p mimic in A549 and NCI-H460 cell lines was confirmed by Quantitative real-time PCR (qRT-PCR) analysis (Figure 2(a)). We then found that miR-516a-5p reduced the cell viability (Figure 2(b)) and anchorage-independent growth (Figure 2(c)), as compared with the miRNA negative control (miR-NC) group. Proliferating cell nuclear antigen (PCNA) protein expression, indicated by Western blot, was decreased by miR-516a-5p, as compared with the miR-NC group in A549 and NCI-H460 cell lines (Figure 2(d)).

Table 1. Cox regression analysis of miR-516a-5p expression as a tumor recurrence predictor.

Variables	Univariate Cox regression analysis		Multivariate Cox regression analysis	
	RR (95% CI)	P-value	RR (95% CI)	P-value
Age (years)				
≥60 versus <60	1.416 (0.857–2.341)	0.174	NA	NA
Gender				
Male versus Female	0.771 (0.492–1.206)	0.254	NA	NA
Pathological stage				
III/ IV versus I/II	1.555 (0.927–2.606)	0.094	NA	NA
T stage				
T3 + T4 versus T1 + T2	2.007 (1.081–3.728)	0.027	2.032 (1.092–3.781)	0.025
N staging				
Positive versus Negative	1.878 (1.213–2.906)	0.005	1.909 (1.232–2.958)	0.004
M stage				
Positive versus Negative	0.986 (0.632–1.538)	0.951	NA	NA
miR-516a-5p expression				
High versus Low	0.546 (0.313–0.950)	0.032	0.526 (0.301–0.919)	0.024

RR: risk ratio; CI: confidence interval; NA: not analyzed.

In addition, the transfection efficiency of miR-516a-5p inhibitor in A549 and NCI-H460 cell lines was verified by qRT-PCR analysis (Figure 2(e)). We found that miR-516a-5p inhibitor favored the cell viability (Figure 2(f)) and anchorage-independent growth (Figure 2(g)), as compared with the NC group. PCNA protein expression was increased by miR-516a-5p inhibitor as compared with the NC group in A549 and NCI-H460 cell lines (Figure 2(h)).

HIST3H2A was identified as a direct target of miR-516a-5p in NSCLC cells

According to the cumulative weighted scores, five targets of miR-516a-5p were identified and their expression levels were estimated in paired and unpaired NSCLC tissues, indicating that only HIST3H2A possessed a significantly increased expression in paired and unpaired NSCLC tissues (Supplementary Figure S1) and exhibited a negative correlation with miR-516a-5p expression in NSCLC tissues ($r = -0.181$, $P = 0.038$; Figure 3(a)).

Luciferase reporter vector containing the wild type (WT) or mutant (Mut) 3'UTR of HIST3H2A (Figure 3(b)) was co-transfected with miR-516a-5p mimic into A549 and NCI-H460 cell lines, indicating that the luciferase activity of WT 3'UTR of HIST3H2A was reduced by miR-516a-5p, but that of Mut 3'UTR of HIST3H2A was unaffected as compared with the miR-NC group (Figure 3(c)). qRT-PCR and Western blot analysis showed that

miR-516a-5p decreased the expression levels of HIST3H2A, as compared with the miR-NC group in A549 and NCI-H460 cells (Figure 3(d)).

The transfection efficiency of HIST3H2A plasmid in A549 and NCI-H460 cells was determined by qRT-PCR and Western blot analysis (Figure 3(e)). We found that overexpression of HIST3H2A promoted the cell viability and reversed the anti-proliferation effects induced by miR-516a-5p in NSCLC cells (Figure 3(f)).

Upregulation of HIST3H2A expression was associated with poor survival in patients

HIST3H2A expression levels were found dramatically increased in paired ($n = 56$) and unpaired NSCLC tissues ($n = 398$) (Supplementary Figure S2A). A cut-off value of HIST3H2A was acquired in NSCLC (Supplementary Figure S2B) and divided the patients into high-expression and low-expression groups (Supplementary Figure S2C). We found that high expression of HIST3H2A had no association with clinicopathological factors in NSCLC (each $P > 0.05$, Supplementary Table S2). Kaplan–Meier demonstrated that the patients with high HIST3H2A expression possessed a poorer survival, but had no difference in tumor recurrence, as compared with those with low HIST3H2A expression (Supplementary Figure S2D). Univariate and Multivariate Cox regression analyses revealed HIST3H2A expression as an independent prognostic factor of poor survival in patients with NSCLC ($P = 0.027$, Supplementary Table S3).

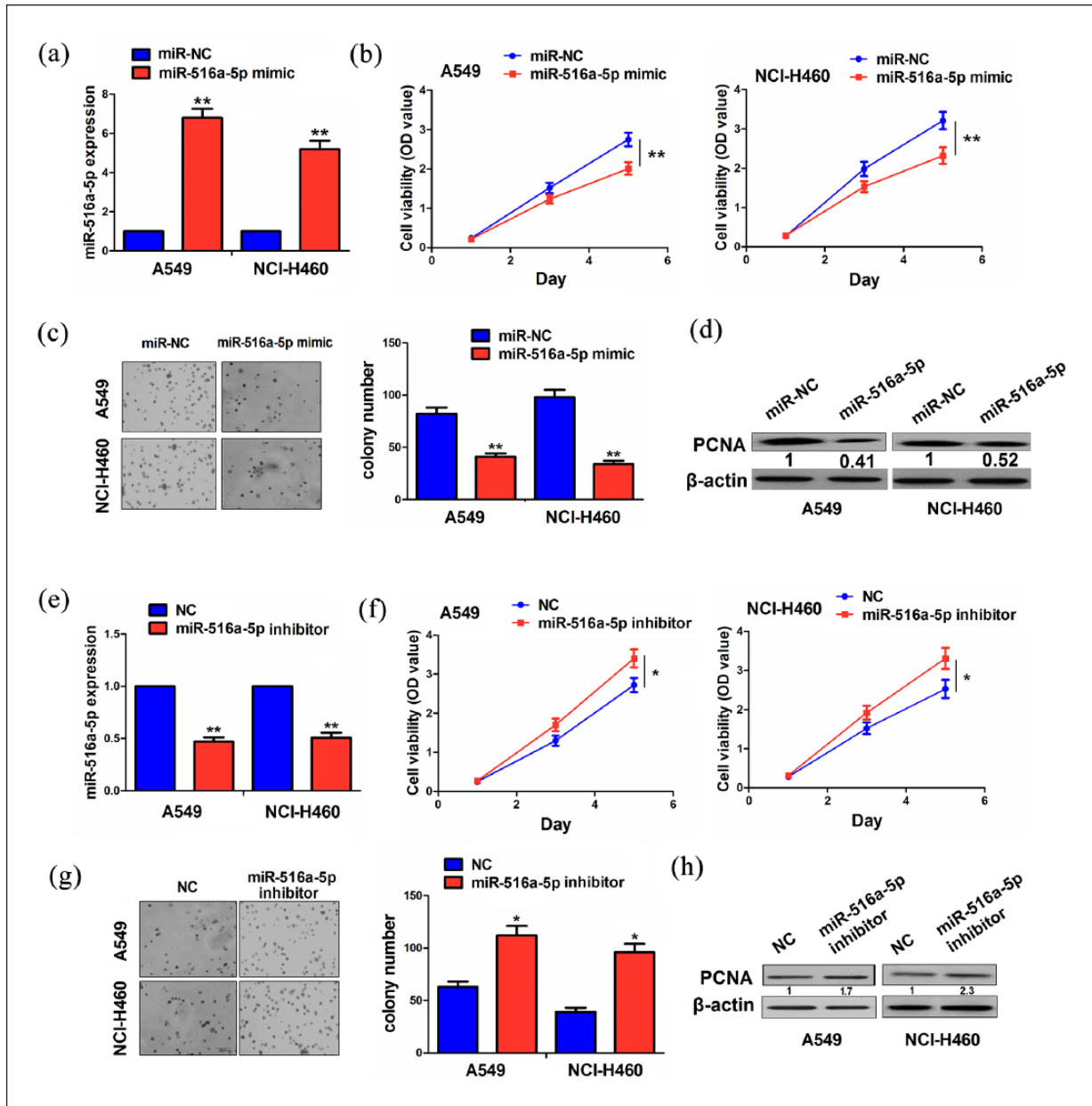


Figure 2. miR-516a-5p inhibited the proliferation and anchorage-independent growth in NSCLC cells. (a) qRT-PCR analysis of the transfection efficiency of miR-516a-5p mimic in A549 and NCI-H460 cell lines. (b) MTT analysis of the effects of miR-516a-5p mimic on cell viability. (c) Soft agar analysis of the effects of miR-516a-5p mimic on anchorage-independent growth. (d) Western blot analysis of the effects of miR-516a-5p mimic on PCNA protein expression. (e) qRT-PCR analysis of the transfection efficiency of miR-516a-5p inhibitor in A549 and NCI-H460 cell lines. (f) MTT analysis of the effects of miR-516a-5p inhibitor on the cell viability. (g) Soft agar analysis of the effects of miR-516a-5p inhibitor on anchorage-independent growth. (h) Western blot analysis of the effects of miR-516a-5p inhibitor on PCNA protein expression. Data were the means \pm SEM of three experiments. * $P < 0.05$; ** $P < 0.01$.

Discussion

Loss of miR-516a-5p expression is associated with a poor prognosis in patients with neuroblastoma.⁸ ERK inhibits the activity of miRNAs, but miR-516a-5p is unaffected by this signaling.¹¹ Herein, we found that low expression of

miR-516a-5p was associated with the age, pathological stage, and tumor size and acted as an independent prognostic factor of tumor recurrence in NSCLC patients, indicating miR-516a-5p as a potential marker for tumor recurrence in NSCLC.

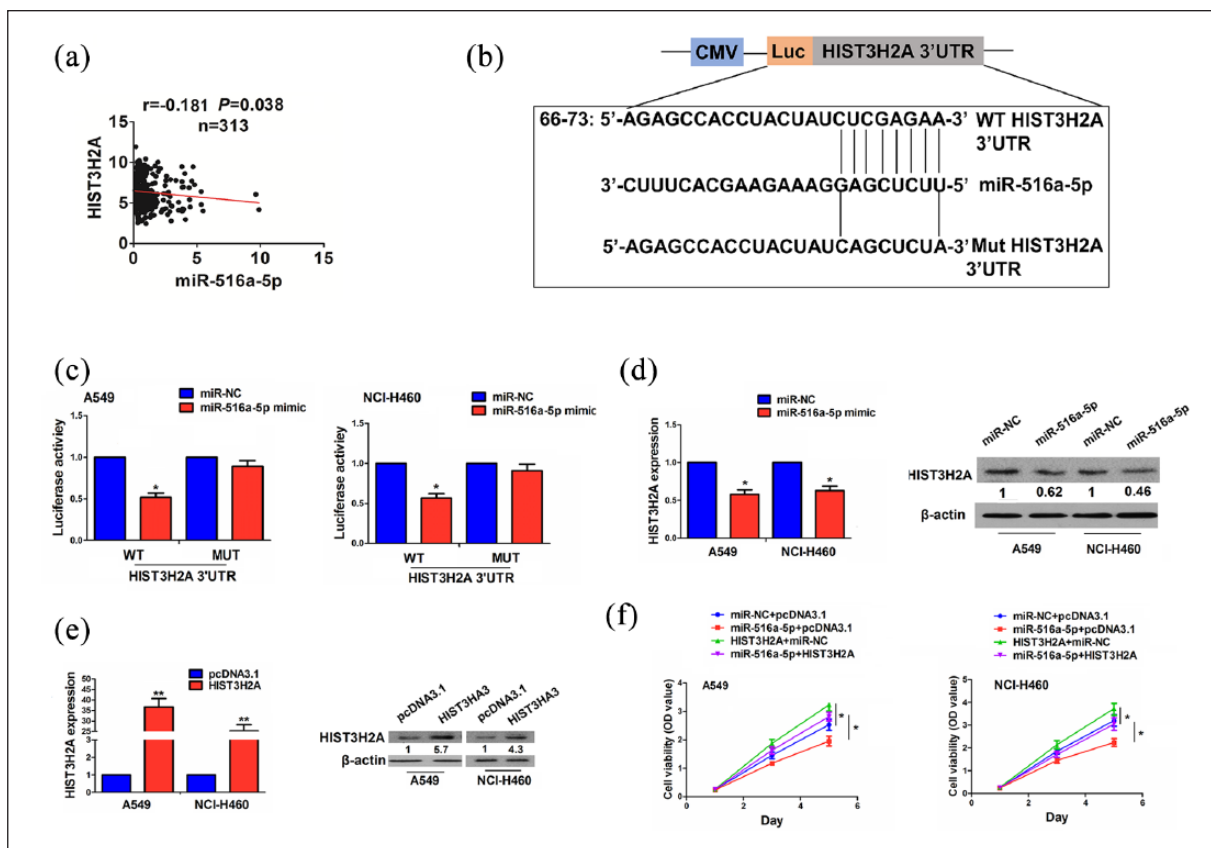


Figure 3. Identification of HIST3H2A as a direct target of miR-516a-5p in NSCLC cells. (a) Pearson correlation analysis of the correlation of miR-516a-5p with HIST3H2A expression in NSCLC tissues. (b) The binding sites of miR-516a-5p with WT or Mut 3'UTR of HIST3H2A. (c) The luciferase activities of WT or Mut 3'UTR of HIST3H2A after the co-transfection with miR-516a-5p mimic and WT or Mut 3'UTR of HIST3H2A in NSCLC cells. (d) qRT-PCR and Western blot analysis of the effects of miR-516a-5p on HIST3H2A expression. (e) qRT-PCR and Western blot analysis of the transfection efficiency of HIST3H2A plasmid in A549 and NCI-H460 cell lines. (f) MTT analysis of the cell viability after co-transfection with the miR-516a-5p mimic and HIST3H2A plasmid in NSCLC cells. Data were the means \pm SEM of three experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

MiR-516a contributes to the growth of ovarian cancer cells.¹² But, we found that miR-516a-5p inhibited the proliferation of NSCLC cells, but miR-516a-5p inhibitor had the opposite effects, suggesting that miR-516a-5p might act as a tumor suppressor in NSCLC cells.

HIST3H2A was further identified as a direct target of miR-516a-5p and had a negative correlation with miR-516a-5p expression in NSCLC. High expression of HIST3H2A was associated with a poor survival in patients with NSCLC. We further confirmed that miR-516a-5p downregulated the expression of HIST3H2A, which reversed the anti-proliferative effects induced by miR-516a-5p in NSCLC cells, indicating that miR-516a-5p might inhibit the proliferation of NSCLC cells by targeting HIST3H2A.

Taken together, our findings demonstrated that low expression of miR-516a-5p acted as an independent prognostic factor of tumor recurrence in patients with NSCLC and miR-516a-5p repressed the proliferation of NSCLC cells by targeting HIST3H2A.

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X.-Y.Y. and L.X. contributed equally to this article.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

This study was approved by the Hospital's Protection of Human Subjects Committee.

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Supplemental material

Supplemental material for this article is available online.

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