


ORIGINAL ARTICLE

MiR-520b restrains cell growth by targeting HDAC4 in lung cancerKe Jin^{1*}, Weixin Zhao^{2,3*}, Xuan Xie¹, Yuejiang Pan¹, Kexi Wang¹ & Huizhong Zhang¹ 

1 Department of Thoracic Surgery, Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China

2 Department of Radiation Oncology, Fudan University Shanghai Cancer Center, Shanghai, China

3 Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China

Keywords

Growth; HDAC4; lung cancer; miR-520b.

Correspondence

Huizhong Zhang, Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, No. 135, Xingang Xi Road, Guangzhou, 510275, China.

Tel: +86 20 8133 2295

Fax: +86 20 8133 2295

Email: zhanghzdr@163.com

*Ke Jin and Weixin Zhao contributed equally to this work.

Received: 21 June 2018;

Accepted: 4 July 2018.

doi: 10.1111/1759-7714.12825

Thoracic Cancer 9 (2018) 1249–1254

Abstract

Background: MicroRNAs (miRNAs) function as tumor promoting or tumor suppressing factors in many cancers. MiR-520b contributes to progression in head-neck and liver cancers, spinal osteosarcoma, and glioma; however, the association of miR-520b with lung cancer progression remains unknown. In this investigation, we explore the effect of miR-520b targeting HDAC4 on lung cancer growth.

Methods: The regulation of miR-520b or its inhibitor on HDAC4 expression was analyzed using Western blot analysis. After treatment of miR-520b or its inhibitor, miR-520b and HDAC4 levels were examined using quantitative real time-PCR. The modulation of miR-520b on HDAC4 was investigated by luciferase reporter gene assay. Cell proliferation evaluation was performed using colony formation and methyl-thiazolyl-tetrazolium assays. The correlation between miR-520b and HDAC4 in human clinical samples was verified using Pearson's correlation coefficient.

Results: An obvious decrease in HDAC4 expression was observed in lung cancer A549 cells treated with different doses of miR-520b. The miR-520b inhibitor enhanced HDAC4 expression in lung cancer cells. Bioinformatics predicted the targeting of miR-520b on HDAC4. MiR-520b directly targeted the 3' untranslated region of HDAC4. The introduction of miR-520b obviously inhibited cell proliferation in vitro. Anti-miR-520b was capable of accelerating lung cancer cell proliferation; however, HDAC4 knockdown destroyed anti-miR-520b-induced cell proliferation. Finally, a negative correlation between miR-520b and HDAC4 was observed in clinical human lung cancer samples.

Conclusion: MiR-520b decreases HDAC4 expression to control cell proliferation in lung cancer.

Introduction

MicroRNAs (miRNAs) can sequence dependently induced messenger RNA (mRNA) cleavage or translational repression of genes.^{1,2} Changes in miRNA expression profiles are frequently found in human cancers.³ MiRNAs play key roles in many cellular processes, including differentiation, development, proliferation, and tumorigenesis.^{4–6} Research has shown the roles miRNA promoters or suppressors play in the development of cancers.^{7–11} A previous study showed

that miR-520b/e inhibits CD46, resulting in the sensitivity of cells to complement attack in breast cancer.¹² MiR-520b can inhibit the cell proliferation and migration of liver and breast cancers.^{13–15} The MiR-520b/ATG7 axis plays a significant role in the sensitivity of liver cancer cells to doxorubicin by apigenin.¹⁶ Glioma progression can be affected by miR-520b targeting MBD2.¹⁷ The MiR-520b/CD44 axis plays an important role in the inhibition of head-neck cancer stemness.¹⁸ In colorectal cancer, miR-520b, as a tumor suppressor, can target *DCUN1D1*.¹⁹ Cell growth and

metastasis can be suppressed by miR-520b-targeting Frizzled-8 in spinal osteosarcoma.²⁰ MiR-520b/e is reported to be involved in cell proliferation and migration in gastric cancer.²¹ However, the association of miR-520b with lung cancer development has not previously been explored.

As one of the class IIa histone deacetylases, HDAC4 exists in different transcriptional corepressor complexes. HDAC4 can restrain the expression of p21 by involving Sp1 in cancer.²² HDAC4 can also enhance cell growth by inhibiting p21 in colon cancer.²³ Gastric cancer progression can be enhanced by HDAC4 via p21 inhibition.²⁴ STAT1/HDAC4 augmentation makes human A549 lung cells resistant to etoposide via increased MDR1.²⁵ High levels of HDAC4 are found and contribute to oncogenesis in liver cancer.²⁶ However, whether HDAC4 is involved in miR-520b-associated lung cancer progression remains unknown.

In the current investigation, we examined miR-520b in lung cancer to clarify its association with progression. Our findings reveal that HDAC4 is a new member of miR-520b target genes in lung cancer. We first prove the tumor suppressor effect of miR-520b by inhibiting HDAC4. We then present evidence for the potential use of miR-520b-targeting HDAC4 for the treatment of lung cancer.

Methods

Cell line

Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) was applied to cultured human lung cancer A549 cells supplemented with 10% fetal bovine serum and placed in a 5% CO₂ incubator at 37°C.

RNA extraction and quantitative real-time PCR

RNA of tissues or cells was acquired by extraction with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using ImPro-II Reverse Transcriptase (Promega, Madison, WI, USA), following the manufacturer's instructions. To test the miR-520b level, poly (A) polymerase (Ambion, Foster City, CA, USA) was used to polyadenylate total RNA. Quantitative real time-PCR was performed using Fast Start Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH, Mannheim, Germany). HDAC4 was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). U6 was used to normalize miR-520b. The primers used in this study are presented in Table S1.

Transfection

The cells were grown on 6-well, 24-well, or 96-well plates for 24 hours. Lipofectamine 2000 (Invitrogen) was used as a reagent to perform transient transfection of miRNAs or small interfering RNAs (siRNAs). MiR-520b, control (Ctrl), miR-520b inhibitor, and si-HDAC4 were purchased from RiboBio (Guangzhou, China).

Western blot analysis

Ice-cold phosphate buffered saline was applied to wash the cells and a rubber policeman to harvest cultured cells. The total protein was isolated by radioimmunoprecipitation assay buffer. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, polyvinylidene fluoride membranes containing total protein (Millipore, Billerica, MA, USA) were incubated in 5% skim milk solution of phosphate buffered saline, followed by primary antibodies: anti-HDAC4 (Invitrogen) or anti-β-actin (Abcam, Cambridge, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were applied for two hours and were then developed on photographic membranes.

Luciferase reporter gene assays

Luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The lung cancer cells grew on 24-well plates overnight. A combination of luciferase vectors with miRNA/inhibitor were transfected using Lipofectamine 2000 (Invitrogen). Firefly luciferase activities were normalized through renilla luciferase activities.

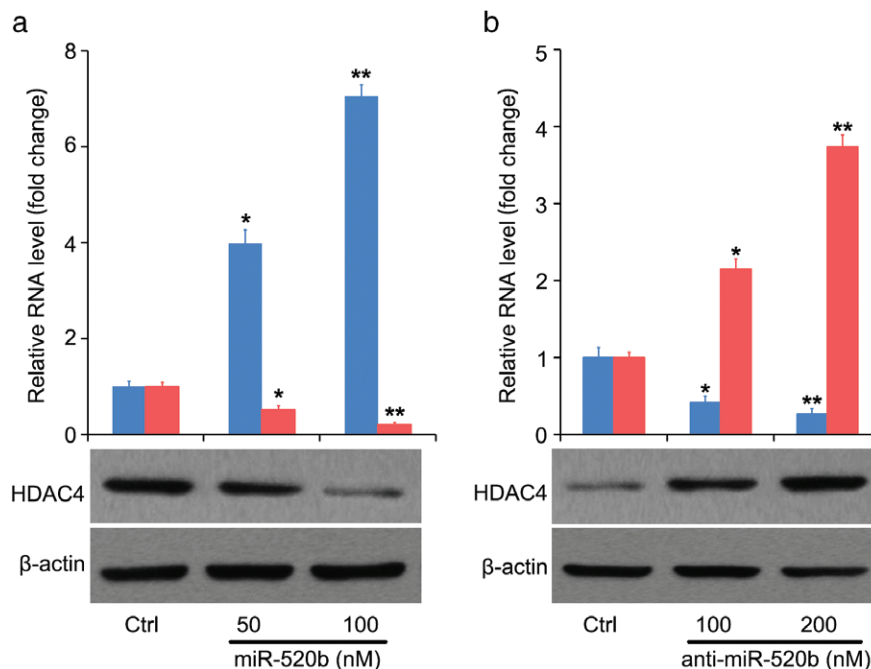
Proliferation analysis

A549 cells were cultured in 96-well plates (1000 cells in 100 μL medium/well). After transfection, methyl-thiazolyl-tetrazolium assay was used to assess cell proliferation. Forty-eight hours later, the transfected cells, including miR-520b, anti-miR-520b, anti-miR-520b + si-HDAC4-2, and Ctrl, were seeded on six-well plates (1000 cells per well) for two weeks in colony formation assay. Three different investigators counted the fixed and stained colonies.

Patient samples

Sun Yat-sen Memorial Hospital (Guangzhou, China) provided 26 lung cancer tissue samples (Table S2). The patients granted consent for the use of their tissue samples in this study. The Research Ethics Board at the Sun Yat-sen Memorial Hospital (Guangzhou, China) approved the study protocol.

Figure 1 MiR-520b reduces the HDAC4 level in lung cancer cells. **(a,b)** The level of HDAC4 of miR-520b/anti-miR-520b-treated A549 cells was tested using quantitative real time-PCR and Western blot analysis. MiR-520b/anti-miR-520b transfection was assessed through quantitative real time-PCR analysis. Student's *t* test: ***P* < 0.01; **P* < 0.05. **a:** (■) miR-520b and (■) HDAC4. **b:** (■) anti-miR-520b and (■) HDAC4.



Statistical analysis

Each assay was repeated three times. All data is reported as mean \pm standard deviation. The differences between groups were compared by student's *t* test and are shown as ***P* < 0.01, **P* < 0.05, or not significant (NS). A correlation between miR-520b and HDAC4 in human clinical lung samples was analyzed using Pearson's correlation coefficient.

Result

MiR-520b reduces the HDAC4 level in lung cancer cells

MiR-520b plays a key role during the development of liver, gastric, head-neck, and colorectal cancers. To show the effect of miR-520b on lung cancer, we used TargetScan (<http://www.targetscan.org/>) to predict miR-520b target genes. Our point of interest for the study was HDAC4. HDACs can silence tumor suppressive genes and apoptosis inducers to promote oncogenesis. Thus, we sought to determine whether miR-520b could modulate HDAC4 expression in lung cancer cells. Firstly, we applied quantitative real time-PCR and Western blot assays to study the modulation of miR-520b on HDAC4 in lung cancer cell lines transfected with miR-520b or anti-miR-520b. We observed that miR-520b markedly inhibited the level of HDAC4 (Fig 1a), while anti-miR-520b increased the level of HDAC4 in lung cancer cells (Fig 1b). Transfection of miR-520b and anti-miR-520b was then evaluated by quantitative real time-PCR analysis (Fig 1). Overall, miR-520b can restrain HDAC4 expression in lung cancer.

MiR-520b downregulates HDAC4 expression by directly targeting its 3' untranslated region

We then sought to clarify how miR-520b affects HDAC4 in lung cancer. Firstly, pGL3-control vector containing the wild type (wt) or mutant (mut) binding site of HDAC4 with miR-520b (pGL3-HDAC4-wt and pGL3-HDAC4-mut) was cloned (Fig 2a,b). Combinations of miR-520b/anti-miR-520b with pGL3-HDAC4-wt/pGL3-HDAC4-mut were transfected into lung cancer cells. MiR-520b overexpression reduced pGL3-HDAC4-wt luciferase activity in a dose dependent manner, whereas miR-520b failed to suppress the luciferase activity of pGL3-HDAC4-mut (Fig 2c). Furthermore, anti-miR-520b could increase pGL3-HDAC4-wt activity by reducing the endogenous miR-520b level of lung cancer cells. However, anti-miR-520b had no effect on the luciferase activity of pGL3-HDAC4-mut (Fig 2d). Collectively, miR-520b has an inhibitory effect on HDAC4 by binding its 3' untranslated region (UTR).

MiR-520b-modulated HDAC4 involves cell proliferation

Based on these findings, we investigated the association between the miR-520b/HDAC4 axis with lung cancer cell growth by methyl-thiazolyl-tetrazolium and colony formation assays. The silencing efficiency of the two different siRNAs targeting HDAC4 in lung cancer cells is shown in Figure 3a. In further experiments, our data showed that miR-520b-treated cells grew slower than control-treated

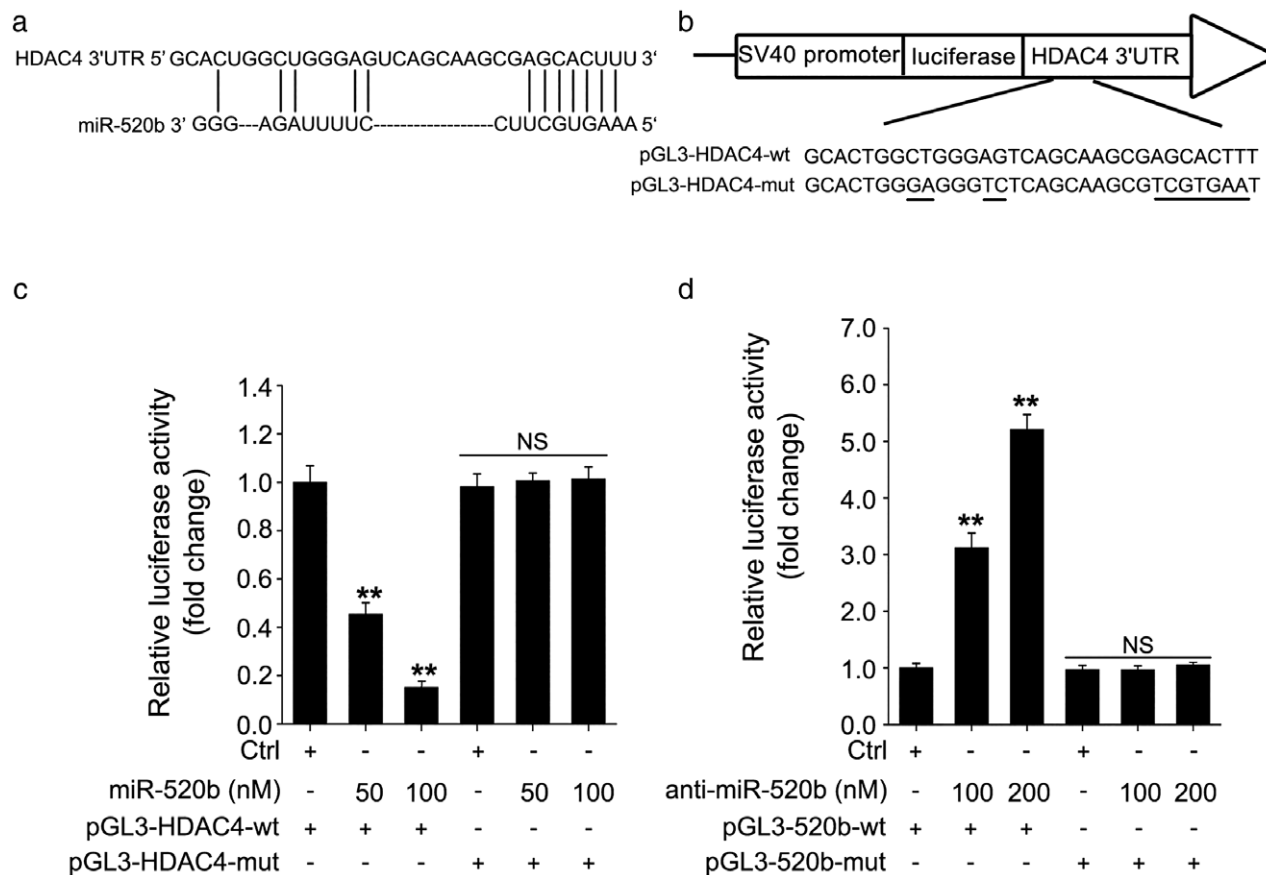


Figure 2 MiR-520b downregulates HDAC4 expression of HDAC4 by directly targeting its 3' untranslated region (UTR). (a,b) The binding of HDAC4 3'UTR with miR-520b is presented. The wild type (wt) or mutant (mut) binding site of HDAC4 with miR-520b is constructed. (c,d) In A549 cells, the regulation of miR-520b/anti-miR-520b on pGL3-HDAC4-wt or pGL3-HDAC4-mut was examined using luciferase reporter system. Student's *t* test: ***P* < 0.01; **P* < 0.05; NS, not significant.

cells. Anti-miR-520b was obviously capable of enhancing cellular proliferative ability, but HDAC4 RNA interference could reverse anti-miR-520b-upregulated cell growth (Fig 3b). Colony formation assay showed fewer colony numbers in miR-520b-transfected cells than in the control. On the other hand, anti-miR-520b was capable of increasing the colony formation of the cells. Interestingly, HDAC4 silence abrogated the colony formation increase induced by anti-miR-520b (Fig 3c). Our findings imply that miR-520b is capable of downregulating HDAC4, leading to the suppression of cell proliferation in lung cancer.

MiR-520b level is reversely related to HDAC4 level in human clinical lung cancer samples

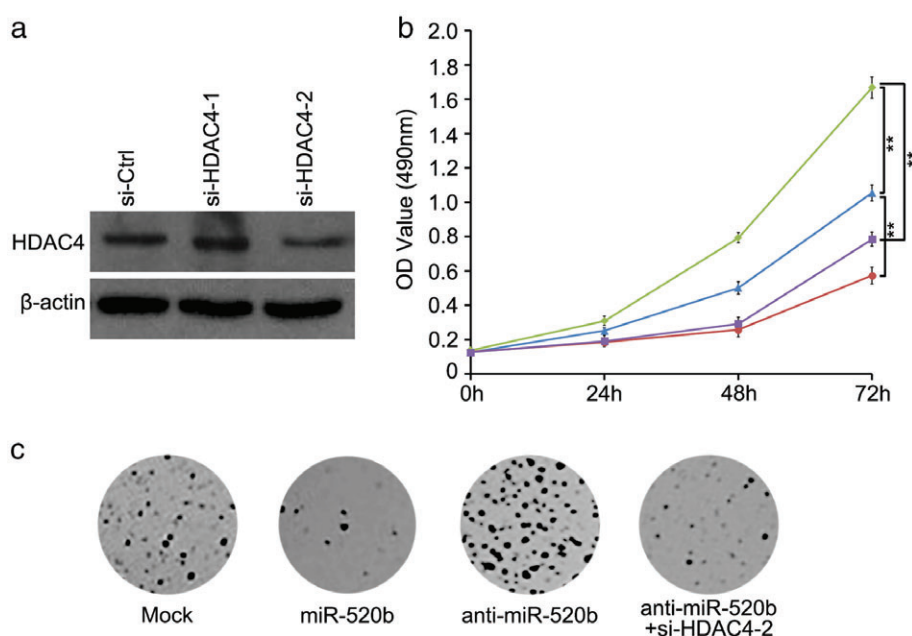
MiR-520b can affect the progression of multiple cancers. HDACs can silence some tumor suppressive genes and apoptosis inducers, leading to the promotion of cancer development. Finally, we used 26 pairs of human lung

cancer and noncancerous samples to evaluate miR-520b and HDAC4 levels. MiR-520b was downregulated in 26 lung cancer tissues (Fig 4a). Furthermore, our data manifested a reverse correlation of miR-520b and HDAC4 in lung cancer samples ($R^2 = 0.7069$, $P < 0.01$) (Fig 4b). Our data indicate that lower miR-520b is related to higher HDAC4 in human lung cancer.

Discussion

Accumulating evidence has revealed that miRNAs play a significant role in cancer development, serving as oncogenes or tumor suppressor genes.⁷⁻¹¹ MiR-520b targets HBXIP, IL-8, MEKK2, and CCND1 to suppress the proliferation and migration of liver and breast cancer cells.¹³⁻¹⁵ MiR-520b targeting MBD2 is involved in glioma development.¹⁷ MiR-520b-downregulated CD44 can reduce the phenotype of head-neck cancer stemness.¹⁸ In spinal osteosarcoma, miR-520b-modulating Frizzled-8 depresses cell growth and metastasis.²⁰ MiR-520b/e inhibits cell proliferation and

Figure 3 MiR-520b-modulated HDAC4 is involved in cell proliferation. (a) RNA interference of small interfering (si)-HDAC4-1 or si-HDAC4-2 was assessed using Western blot assay. (b,c) Cell proliferation of miR-520b, anti-miR-520b, or the (anti-miR-520b + si-HDAC4-2)-treated group was assessed by methylthiazolyl-tetrazolium and colony formation assays. Student's *t* test: $^{**}P < 0.01$. (—▲) Mock, (—●) miR-520b, (—◆) anti-miR-520b, and (—■) anti-miR-520b+si-HDAC4-2.



migration in gastric cancer.²¹ Despite these findings, whether miR-520b can function in lung cancer development has not been investigated.

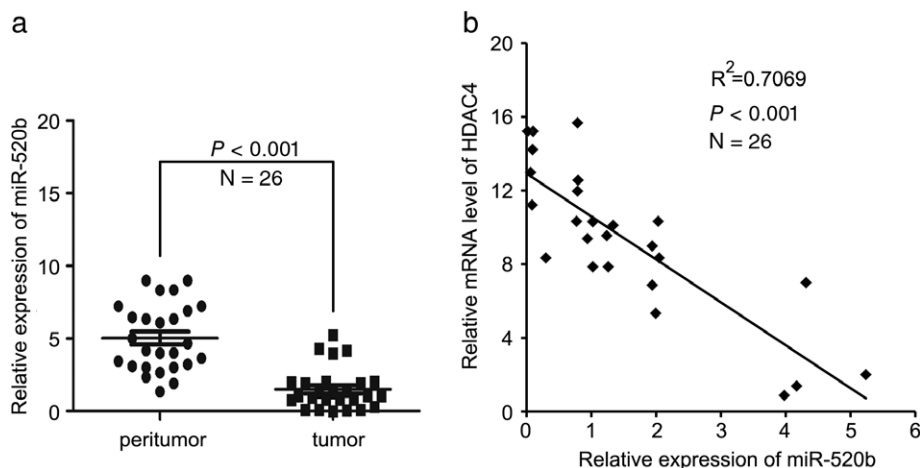
HDAC4 can inhibit p21 to enhance cell growth in colon cancer.²³ HDAC4-inhibited p21 is involved in the development of gastric cancer.²⁴ STAT1/HDAC4-elevated MDR1 confers resistance of human lung A549 cells to etoposide.²⁵ Overexpressed HDAC4 can promote liver cancer.²⁶ However, it is unknown whether HDAC4 is involved in miR-520b-associated lung cancer.

In our current investigation, we used TargetScan to predict miR-520b target genes and observed that HDAC4 could be a candidate. Quantitative real time-PCR and Western blot analysis showed that HDAC4 expression was reduced by miR-520b. To show the modulation of miR-520b on HDAC4 in cells, vectors containing wt and mut binding sites

of HDAC4 with miR-520b were cloned. MiR-520b introduction obviously reduced pGL3-HDAC4-wt luciferase activity. The inhibitor of miR-520b decreased the endogenous miR-520b levels, which subsequently increased pGL3-HDAC4-wt activity. This finding implies that HDAC4, as a target gene of miR-520b, is restrained by miR-520b in lung cancer. Thus, miR-520b could reduce lung cancer cell proliferation by inhibiting HDAC4. Our results confirm a negative correlation between miR-520b and HDAC4 in human clinical samples, indicating that miR-520b directly targets HDAC4 to inhibit lung cancer growth.

Herein, we provide evidence of miR-520b in lung cancer progression. MiR-520b is capable of post-transcriptionally modulating the expression of HDAC4, leading to suppressed lung cancer cell proliferation. Our findings present novel evidence of miR-520b-mediated lung cancer growth.

Figure 4 The level of miR-520b is reversely related to the level of HDAC4 in human clinical lung cancer samples. (a) The level of HDAC4 in human clinical lung samples was analyzed using quantitative real time-PCR. (b) A correlation between miR-520b and HDAC4 in 26 pairs of clinical lung cancer tissues was evaluated by Pearson's correlation coefficient ($R^2 = 0.7069$). Student's *t* test: $^{***}P < 0.001$; $^{**}P < 0.01$. mRNA, messenger RNA.



Disclosure

No authors report any conflict of interest.

References

- 1 Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281–97.
- 2 Bartel DP, Chen CZ. Micromanagers of gene expression: The potentially widespread influence of metazoan microRNAs. *Nat Rev Genet* 2004; **5**: 396–400.
- 3 Lu J, Getz G, Miska EA et al. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834–8.
- 4 Chen CZ. MicroRNAs as oncogenes and tumor suppressors. *N Engl J Med* 2005; **353**: 1768–71.
- 5 Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004; **303**: 83–6.
- 6 Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 2005; **6**: 376–85.
- 7 Budhu A, Jia HL, Forgues M et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology* 2008; **47**: 897–907.
- 8 Dong Q, Meng P, Wang T et al. MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. *PLoS One* 2010; **5**: e10147.
- 9 Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; **6**: 857–66.
- 10 Calin GA, Croce CM. Chromosomal rearrangements and microRNAs: A new cancer link with clinical implications. *J Clin Invest* 2007; **117**: 2059–66.
- 11 Kent OA, Mendell JT. A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. *Oncogene* 2006; **25**: 6188–96.
- 12 Cui W, Zhang Y, Hu N et al. miRNA-520b and miR-520e sensitize breast cancer cells to complement attack via directly targeting 3'UTR of CD46. *Cancer Biol Ther* 2010; **10**: 232–41.
- 13 Zhang W, Lu Z, Kong G et al. Hepatitis B virus X protein accelerates hepatocarcinogenesis with partner survivin through modulating miR-520b and HBXIP. *Mol Cancer* 2014; **13**: 128.
- 14 Hu N, Zhang J, Cui W et al. miR-520b regulates migration of breast cancer cells by targeting hepatitis B X-interacting protein and interleukin-8. *J Biol Chem* 2011; **286**: 13714–22.
- 15 Zhang W, Kong G, Zhang J, Wang T, Ye L, Zhang X. MicroRNA-520b inhibits growth of hepatoma cells by targeting MEKK2 and cyclin D1. *PLoS One* 2012; **7**: e31450.
- 16 Gao AM, Zhang XY, Hu JN, Ke ZP. Apigenin sensitizes hepatocellular carcinoma cells to doxorubic through regulating miR-520b/ATG7 axis. *Chem Biol Interact* 2018; **280**: 45–50.
- 17 Cui S, Liu L, Wan T, Jiang L, Shi Y, Luo L. MiR-520b inhibits the development of glioma by directly targeting MBD2. *Am J Cancer Res* 2017; **7**: 1528–39.
- 18 Lu YC, Cheng AJ, Lee LY et al. MiR-520b as a novel molecular target for suppressing stemness phenotype of head-neck cancer by inhibiting CD44. *Sci Rep* 2017; **7**: 2042.
- 19 Xiao J, Li G, Zhou J et al. MicroRNA-520b functions as a tumor suppressor in colorectal cancer by inhibiting defective in cullin neddylation 1 domain containing 1 (DCUN1D1). *Oncol Res* 2018; **26**: 593–604.
- 20 Wang J, Pang W, Zuo Z, Zhang W, He W. MicroRNA-520b suppresses proliferation, migration, and invasion of spinal osteosarcoma cells via downregulation of frizzled-8. *Oncol Res* 2017; **25**: 1297–304.
- 21 Li S, Zhang H, Ning T et al. MiR-520b/e regulates proliferation and migration by simultaneously targeting EGFR in gastric cancer. *Cell Physiol Biochem* 2016; **40**: 1303–15.
- 22 Mottet D, Pirotte S, Lamour V et al. HDAC4 represses p21 (WAF1/Cip1) expression in human cancer cells through a Sp1-dependent, p53-independent mechanism. *Oncogene* 2009; **28**: 243–56.
- 23 Wilson AJ, Byun DS, Nasser S et al. HDAC4 promotes growth of colon cancer cells via repression of p21. *Mol Biol Cell* 2008; **19**: 4062–75.
- 24 Kang ZH, Wang CY, Zhang WL et al. Histone deacetylase HDAC4 promotes gastric cancer SGC-7901 cells progression via p21 repression. *PLoS One* 2014; **9**: e98894.
- 25 Kaewpiboon C, Srisuttee R, Malilas W et al. Upregulation of Stat1-HDAC4 confers resistance to etoposide through enhanced multidrug resistance 1 expression in human A549 lung cancer cells. *Mol Med Rep* 2015; **11**: 2315–21.
- 26 Tsai CL, Liu WL, Hsu FM et al. Targeting histone deacetylase 4/ubiquitin-conjugating enzyme 9 impairs DNA repair for radiosensitization of hepatocellular carcinoma cells in mice. *Hepatology* 2018; **67**: 586–99.

Supporting Information

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

Table S1. List of primers used in this paper.

Table S2. Clinical characteristics of lung cancer samples.