

MicroRNA-20a promotes non-small cell lung cancer proliferation by upregulating *PD-L1* by targeting *PTEN*

JIAOMEI GONG^{1*}, YONG SHEN^{2*}, FUGUO JIANG³, YAN WANG¹, LULU CHU¹,
JINQI SUN¹, PENGXIAO SHEN¹ and MAOCAI CHEN¹

¹Department of Clinical Laboratory, Second Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450013;

²Department of Clinical Laboratory, Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, Henan 450008;

³Department of Clinical Laboratory, Jiaozuo People's Hospital, Jiaozuo, Henan 454000, P.R. China

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Abstract. Non-small cell lung cancer (NSCLC) remains one of the most common malignant tumors worldwide. The aim of the present study was to investigate the possibility of microRNA-20a (miR-20a) as a biomarker and therapeutic target for the diagnosis and treatment of NSCLC. Bioinformatics prediction, together with functional validation, confirmed miR-20a bound to programmed death ligand-1 (PD-L1) 3'-untranslated region to upregulate PD-L1 expression. Both miR-20a and PD-L1 could promote the proliferation of NSCLC cells. The expression level of PD-L1 was controlled by PTEN; however, further upstream regulation of PD-L1 expression was largely unknown. The present study showed that miR-20a could not restore the inhibition of PD-L1 expression levels by PTEN. Knockdown of PTEN expression upregulated the expression level of PD-L1 and promoted the proliferation of NSCLC cells. PTEN negatively regulated the Wnt/ β -catenin signaling pathway by inhibiting β -catenin and Cyclin D1. Interestingly, PTEN could reverse miR-20a-mediated proliferation of NSCLC cells and the inhibitory effect was similar to that of XAV-939. miR-20a promotes the proliferation of NSCLC cells by inhibiting the expression level of PTEN and upregulating the expression level of PD-L1. It is suggested that miR-20a could be used as a biomarker and therapeutic target for the treatment of NSCLC.

Introduction

Lung cancer is the leading cause of cancer-associated death among men and the second leading cause among women

worldwide (1). Non-small cell lung cancer (NSCLC), including adenocarcinoma, squamous cell carcinoma and large cell carcinoma, represents ~85% of all lung cancer cases, with an overall 5-year survival rate of <15% (2,3). Most patients with NSCLC are diagnosed with advanced stage tumors owing to the inadequate screening methods and the late onset of clinical symptoms; consequently, most patients have a poor prognosis (4). Therefore, new screening regimens and technologies, as well as new tumor markers to maximize the detection of early NSCLC, are key goals for solving the high mortality rate of NSCLC.

Tumorigenesis and progression are highly related to gene regulation. Recently, some studies have found that microRNA (miRNA/miR) levels can be used for the early diagnosis of NSCLC (5). miRNA is a family of endogenous, single-stranded, non-coding small RNA molecules, which are ~20-24 nucleotides (6). They bind to the 3'-untranslated region (3'-UTR) of the target mRNA and participate in the fine-tuning of various biological processes as a key regulator of gene expression (6,7). An increasing number of studies have showed that miRNAs serve a vital role in the progression of NSCLC (6,7). A previous study reported that hsa-miR-338 may have a tumor inhibitory effect in the progression of NSCLC (8). miR-126 targeting PI3K inhibits cell proliferation, migration and invasion in the A549 cell line by regulating the PTEN/PI3K/AKT pathway (9). Nevertheless, the mechanism of miRNA in NSCLC is still unclear.

The inhibitors of programmed cell death protein 1 (PD-1) and its ligand programmed death ligand-1 (PD-L1) have been the focus of tumor immunotherapy in recent years (10). The protein expression level of PD-L1 is upregulated in numerous types of tumor cells (11). PD-L1 binds to PD-1 on T cells and subsequently inhibits the proliferation, and activation of these cells. The T cell response is a prominent part of the antitumor immune response via the direct killing of target tumor cells or via indirect inhibition by cytokines (12). PD-1/PD-L1 immune checkpoints play an important role in immune regulation by delivering inhibitory signals to maintain the balance in T cell activation, tolerance and immune-mediated tissue damage (13).

In the present study, it was shown that the expression level of miR-20a in NSCLC cell lines was upregulated. miR-20a promoted the proliferation of human NSCLC cells by directly

Correspondence to: Professor Maocai Chen, Department of Clinical Laboratory, Second Affiliated Hospital of Zhengzhou University, 2 Jingba Road, Zhengzhou, Henan 450013, P.R. China
E-mail: acai85@126.com

*Contributed equally

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targeting PTEN to promote the expression level of PD-L1 and activating the Wnt/ β -catenin pathway. These results demonstrated that miR-20a may be a potential therapeutic target in NSCLC.

Materials and methods

Cell culture, transfection and proliferation assay. The cell lines (BEAS-2B, A549 and H1299) were purchased from American Type Culture Collection. The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C, and 5% CO₂. The cells were detected for mycoplasma contamination using routine PCR.

The miR-20a mimics, miR-Con mimics, anti-miR-20a mimics, anti-miR-Con mimics, PTEN-short hairpin RNA (shRNA) and knockdown (KD)-PTEN-shRNA were obtained from Shanghai GenePharma Co., Ltd. The shRNA sequence for the PTEN gene was as follows: sh-PTEN: 5'-CCGGCCACAAATGAAGGGATATAA ACTCGAGTTTATATCCCTTCATTTGTGGTTTTT G-3'. Flag-PD-L1/pcDNA3.0, Flag-PTEN/pcDNA3.0 and Flag/pcDNA3.0 plasmids were from our laboratory. Flag plasmids and miRNA mimics (concentration of 50 nM) were transfected into A549 cells and H1299 cells using SuperFectin II (Shanghai Pufei Biotechnology Co., Ltd.) and 24 h after transfection, the cells were processed as described for each experiment. A549 and H1299 cells were transduced with the lentivirus at multiplicity of infection of MOI 20 for 48 h, and then selected with puromycin (2 mg/ml). Independent stable clones were evaluated by Western blotting.

Cell proliferation was determined using the Cell Counting Kit (CCK)-8 (GlpBio Technology) at 37°C for 72 h. The absorbance was detected by enzyme labeling instrument (450 nm) every 24 h; the XAV-939 (cat. no. HY-15147; 10 μ M) was purchased from MedChemExpress.

Reverse transcription-quantitative (RT-qPCR). Total RNA was isolated from A549 cells and H1299 cells using TRIzol® (Thermo Fisher Scientific, Inc.). RT-qPCR analysis of miRNA or mRNA was performed as previously reported (14). The following primer sequences were used: PD-L1 forward, 5'-CCTACTGGCATTGCTGAACGCAT-3' and reverse, 5'-ACCATAGCTGATCATGCAGCGGTA-3'; PTEN forward, 5'-GATGAGGCATTATCCTGTACACA-3' and reverse, 5'-CTCTTCAGATACTCTTGTGCTGT-3' and β -actin forward, 5'-ACCATTGGCAATGAGCGGT-3' and reverse, 5'-GTCTTTGCGGATGTCCACGT-3'.

Western blotting. Total protein extracts of A549 cells and H1299 cells were prepared using Keygen Protein Extraction Reagent (cat. no. KGP250; Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's instructions. The proteins were quantified by BCA Protein Assay kit (Beyotime Institute of Biotechnology) and the mass of protein loaded per lane was 25 μ g. The protein was fractionated using 10% SDS-PAGE for 2 h at 110 V and transferred onto the PVDF membranes. Membranes were blocked in 5% skim milk powder diluted with Tri-buffered saline Tween-20 (20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20) at room

temperature for 1 h, and immunostained with the following primary antibodies at 4°C overnight: PD-L1 (1:1,000; cat. no. 28076-1-AP), PTEN (1:5,000; cat. no. 22034-1-AP), β -catenin (1:1,000; cat. no. 51067-2-AP), Cyclin D1 (1:1,000; cat. no. 26939-1-AP), β -actin (1:2,000; cat. no. 20536-1-AP) and GAPDH (1:10,000; cat. no. 10494-1-AP), and were all purchased from ProteinTech Group, Inc. GAPDH and β -actin were analyzed to show equal protein loading. The secondary antibodies used were goat-rabbit IgG (1:10,000; cat. no. sc-2004) and goat anti-mouse IgG (1:10,000; cat. no. sc-2005) (both purchased from Santa Cruz Biotechnology, Inc.). The blots were detected with an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.) and exposed in a Molecular Imager® ChemiDoc XRS system (Bio-Rad Laboratories, Inc.).

Bioinformatics prediction. TargetScan 7.1 software (http://www.targetscan.org/vert_71/) was used to predict the potential target genes of microRNA-20a. The search terms 'Human' and 'microRNA-20a' were used.

Luciferase activity assay. The interaction between miR-20a and PD-L1 was determined using a luciferase activity assay. The PD-L1 3'-UTR containing miR-20a binding site was subcloned into the luciferase reporter plasmid vector (Promega Corporation). The 3'-UTR luciferase vector was co-transfected with miR-20a mimics or mimics control (miR-Con) or anti-miR-20a mimics into the A549 or H1299 cells using SuperFectin™ II (Shanghai Pufei Biotechnology Co., Ltd.) and *Renilla* luciferase reporters were used as an internal control. A luciferase activity assay was performed after 48 h using the Dual-Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's protocol.

Statistical analysis. All statistical analyses were performed using SPSS v22.0 (IBM Corp.) statistical package for windows. All results were expressed as the mean \pm SD and the experiments were performed at least 3 times. The statistical differences between categorical data were evaluated using a Fisher's exact test. Comparisons between continuous variables were analyzed using non-parametric Mann-Whitney U test (2 groups) or one-way Analysis of Variance test (>2 groups). All tests were two-sided and $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

PD-L1 is expressed in NSCLC cell lines and promotes NSCLC cell proliferation. In order to gain insight into the function of PD-L1, RT-qPCR was performed to verify the transcriptional level of PD-L1 in the NSCLC cell lines (A549 and H1299). Compared with that in the human normal lung epithelial cell line (BEAS-2B), the mRNA expression levels of PD-L1 in the A549 and H1299 cell lines were upregulated (Fig. 1A). Consistent with the mRNA expression levels, the PD-L1 protein expression levels in the A549 and H1299 cell lines were significantly higher compared with that in the BEAS-2B cell line (Fig. 1B and C).

To examine the contribution of endogenous PD-L1 in NSCLC cell proliferation, overexpression of PD-L1

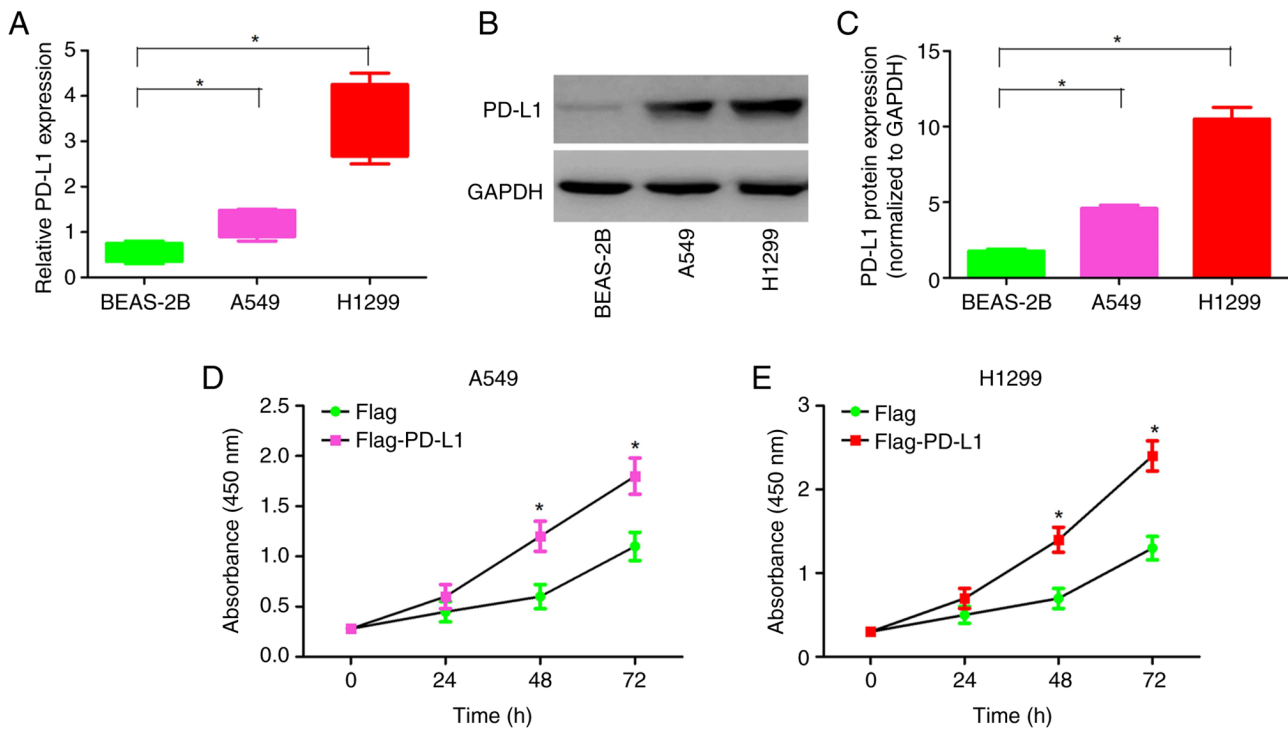


Figure 1. PD-L1 promotes the proliferation of non-small cell lung cancer cells. (A) PD-L1 mRNA expression levels in BEAS-2B, A549 and H1299 cells lines were analyzed using reverse transcription-quantitative PCR. (B) Western blot analysis was performed to determine the PD-L1 protein expression levels in the BEAS-2B, A549 and H1299 cells lines and the results were subsequently (C) quantified. Cell Counting Kit-8 assay was performed to determine cell proliferation in the (D) A549 and (E) H1299 cell lines (Flag Group and Flag PD-L1 Group at 48 or 72 h, respectively). The results are presented as the mean \pm SEM from three independent experiments. * $P < 0.05$. PD-L1, programmed death-ligand 1.

was performed in the A549 and H1299 cell line (Fig. S1). Overexpression of PD-L1 enhanced the proliferation of the A549 and H1299 cell lines (Fig. 1D and E). These results indicate that PD-L1 may play the role of oncoprotein in NSCLC and induce the proliferation of NSCLC cells.

miR-20a regulates PD-L1. miRNAs regulate gene expression after transcription by binding to the 3'-UTR of mRNAs. miRNAs play an important role in the immune response (6,7). Bioinformatics analysis showed that PD-L1 was a potential target gene of miR-20a (Fig. 2A). Luciferase reporter plasmid, with PD-L1 3'-UTR and miR-20a mimics or inhibitor were co-transfected into the A549 and H1299 cell lines. The results confirmed that miR-20a mimics significantly enhanced the activity of PD-L1 in the A549 cell line (Fig. 2B). Consistent with the results from the A549 cells, the luciferase activity of the PD-L1 reporter gene was inhibited by anti-miR-20a inhibitors in the H1299 cell line (Fig. 2C).

In order to verify that the PD-L1 expression levels were regulated by miR-20a, the NSCLC cell lines were transiently transfected with miR-20a mimics or anti-miR-20a inhibitors. The RT-qPCR results showed that miR-20a mimics increased the mRNA expression levels of PD-L1 in the A549 and H1299 cell lines (Fig. 2D and E). At the same time, anti-miR-20a inhibitors inhibited the mRNA expression levels of PD-L1 in A549 and H1299 cells (Fig. 2D and E). Consistent with the results at the transcriptional level, miR-20a promoted the expression level of PD-L1 protein in both the A549 and H1299 cell lines. When miR-20a inhibitors were used, the PD-L1 protein expression levels were downregulated in both

the A549 and H1299 cells (Fig. 2F). Taken together, these results indicate that miR-20a may regulate the expression level of PD-L1 by binding to the 3'-UTR of PD-L1.

miR-20a enhances NSCLC cell proliferation. To investigate the biological role of miR-20a in NSCLC cells, a CCK-8 assay was performed and the effect of miR-20a on the proliferation of NSCLC cells was evaluated. miR-20a mimic, miR-Con mimic, anti-miR-20a mimic and anti-miR-Con mimic were transfected into A549 cells (Fig. S2) and H1299 cells (Fig. S3), respectively, and the expression level of miR-20a was detected. miR-20a mimics increased cell proliferation, while miR-20a inhibitor decreased cell proliferation in the A549 cell line (Fig. 3A). Similar results were found in the H1299 cell line (Fig. 3B). In summary, these results suggest that miR-20a enhanced NSCLC cell proliferation.

miR-20a inhibits the transcription and protein expression level of PTEN in NSCLC cells. When miR-20a mimics were highly expressed in A549 cells and H1299 cells, the expressions of PTEN were downregulated (Fig. 3C and D). At the protein expression level, miR-20a mimics significantly inhibited PTEN in both the A549 and H1299 cell lines (Fig. 3E). These results suggest that miR-20a may directly target the tumor suppressor, PTEN and inhibit PTEN transcription and protein expression levels in the A549 and H1299 cell lines.

miR-20a enhances PD-L1 expression by repressing PTEN. PD-L1 protein expression level was decreased in the PTEN overexpressing A549 cell line. In addition, in cells transfected

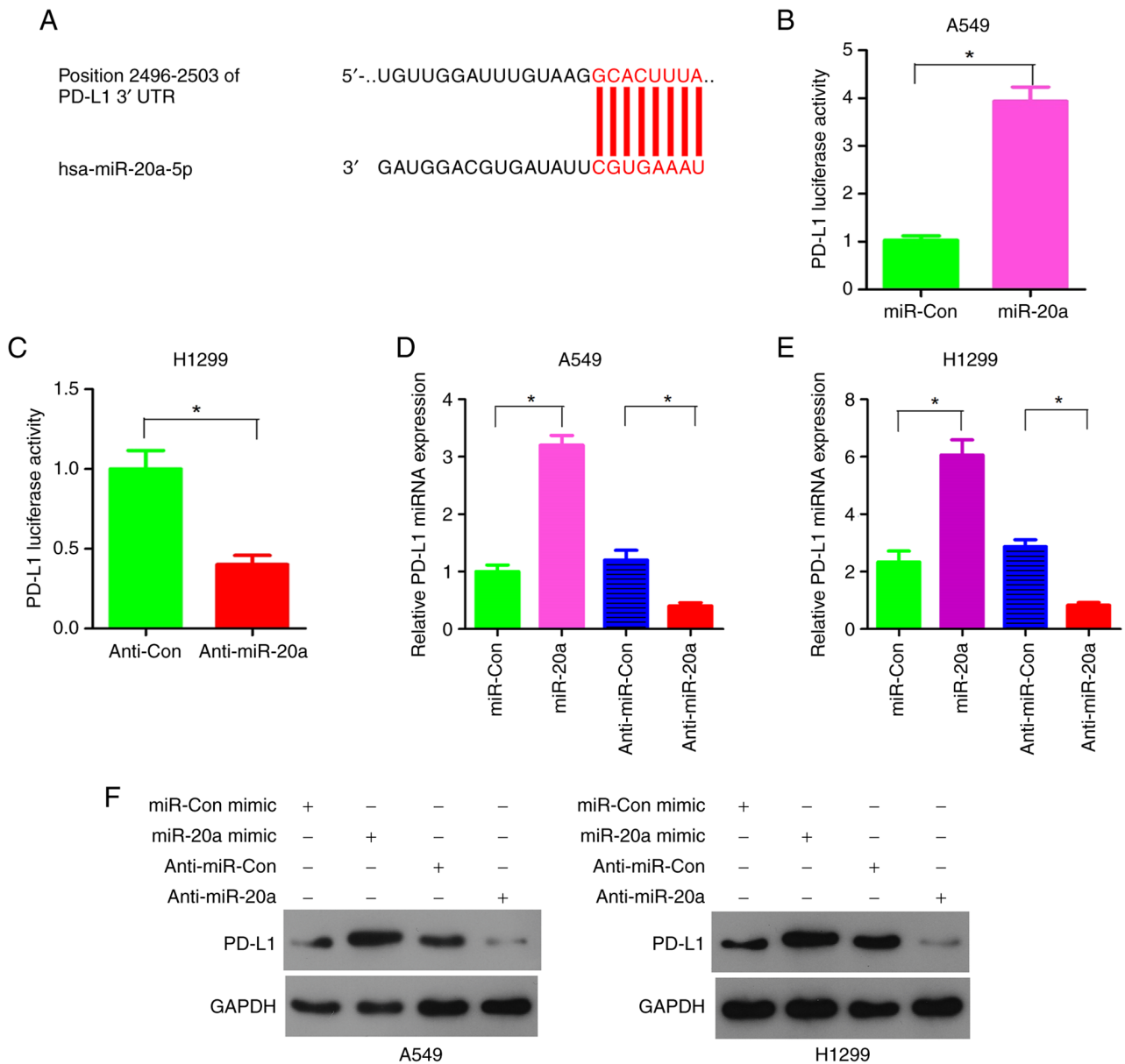


Figure 2. miR-20a regulates PD-L1. (A) Predicted miR-20a binding site in the 3'-UTR of PD-L1. Luciferase activity of PD-L1 3'-UTR in the (B) A549 and (C) H1299 cell lines. Relative PD-L1 mRNA expression levels following transfection with miR-Con mimics, miR-20a mimics, anti-miR-20a and anti-miR-Con in the (D) A549 and (E) H1299 cell lines. (F) The PD-L1 protein expression level was measured in the A549 and H1299 cell lines stably transfected with miR-Con mimics, miR-20a mimics, anti-miR-20a and anti-miR-Con. The results are presented as the mean \pm SEM from three independent experiments. * $P < 0.05$. PD-L1, programmed death-ligand 1; miR, microRNA; Con, control; UTR, untranslated region.

with miR-20a mimics and PTEN overexpression vector, the protein expression level of PD-L1 was lower compared with that in the control cells (Figs. 4A and B, and S4). Furthermore, the expression level of PD-L1 was increased following knockdown of PTEN expression, while the protein expression level of PD-L1 was further increased following transfection with miR-20a mimics and knockdown of PD-L1 (Figs. 4A and B, and S4). The proliferation rate of the A549 cell line, transfected with PTEN overexpression vector was the slower compared with that in control A549 cell line, while knockdown of PTEN in the A549 cell line was fastest compared with that in the other groups (Fig. 4C). Similar results were observed with the H1299 cell line (Fig. 4D). These findings indicated that PTEN may be an inhibitor of PD-L1, by affecting the proliferation of NSCLC cells.

miR-20a promotes NSCLC cell proliferation by targeting PTEN to activate the Wnt/ β -catenin pathway. A previous study has shown that there was a synergistic effect between the knockdown of PTEN expression and the activation of Wnt/ β -catenin pathway (15). Thus, we hypothesized that miR-20a may enhance the proliferation of NSCLC cells by regulating the PTEN/Wnt/ β -catenin pathway. Therefore the protein expression levels of β -catenin and Cyclin D1 were analyzed. The results revealed that they were both inhibited by the overexpression of PTEN in the A549 and H1299 cell lines (Fig. 5A). XAV-939 is a potent tankyrase inhibitor that targets the Wnt/ β -catenin signaling pathway. XAV-939 stabilizes Axin by inhibiting tankyrase 1 and tankyrase 2, thereby stimulating β -catenin degradation (16). The miR-20a mimics were transfected into A549 and H1299 cells respectively and

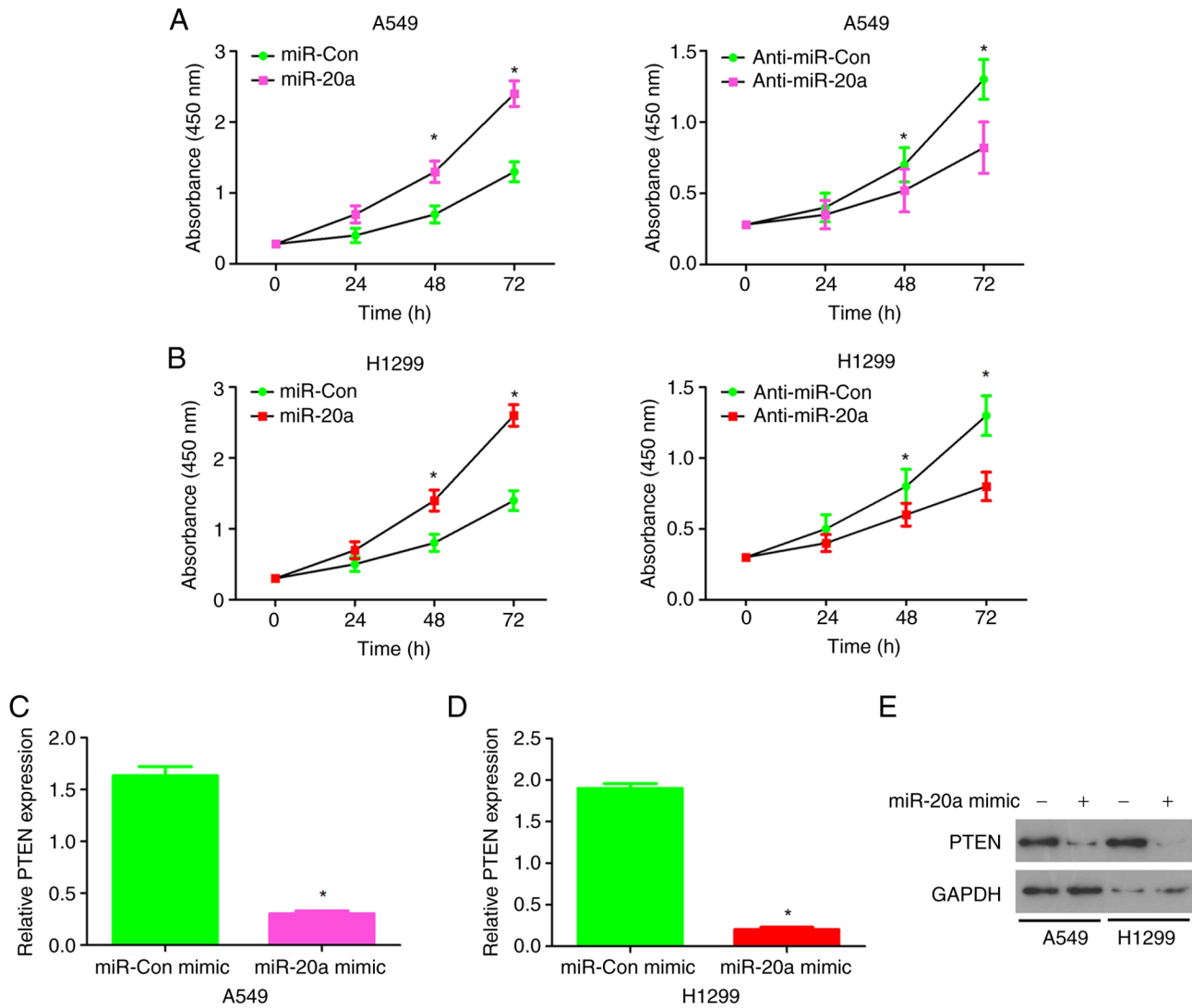


Figure 3. miR-20a inhibits PTEN and promotes the proliferation of non-small cell lung cancer cells. Cell Counting Kit-8 assay of the (A) A549 and (B) H1299 cells transfected with miR-20a mimic or anti-miR-20a or their respective negative controls. * $P < 0.05$. Relative PTEN mRNA expression level was measured after miR-Con mimics or miR-20a mimics transfection in the (C) A549 and (D) H1299 cell lines. * $P < 0.05$. (E) PTEN protein expression levels were measured using western blot analysis in the A549 and H1299 cells stably transfected with miR-20a mimics or the negative control. miR, microRNA; Con, control.

the proliferation ability of A549 and H1299 cells was analyzed with or without XAV-939 treatment. Compared with the cells treated with miR-20 alone, the proliferation ability of A549 or H1299 cells treated with miR-20a combined with XAV-939 or PTEN was significantly lower, but still higher than that of untransfected and untreated cells (Fig. 5B and C). Additionally, it was found that the inhibitory effect of PTEN was similar to that of XAV-939, as the proliferation rate was similar in cells transfected with miR-20a mimics and PTEN overexpression vector (Fig. 5B and C). In conclusion, these results indicated that miR-20a may enhance the proliferation of NSCLC cells by targeting PTEN and the activating Wnt/ β -catenin pathway.

Discussion

NSCLC is one of the most common malignant tumors (17). However, the high mortality rate of NSCLC has not decreased, mainly due to the lack of early diagnosis, leading to the loss of surgical opportunity, as patients with NSCLC are found at

an advanced stage (18). Therefore, early diagnosis is the key to reduce mortality. A number of studies have reported that some important functional miRNAs could play a role in NSCLC cells. For example, miR-196b-5p-mediated downregulation of TSPAN12 and GATA6 promotes tumor progression in NSCLC (19). miR-7-5p suppresses tumor metastasis of NSCLC by targeting NOVA2 (20).

miR-20a belongs to the miR-17-92 cluster and is located on chromosome 13q31.1 (21). Previous studies have shown that miR-20a is upregulated in liver cancer and breast cancer, indicating that miR-20a could play a key role in tumorigenesis and progression (21,22). However, miR-20a could act as a tumor suppressor in other types of tumor, including endometrial and liver cancer (21,23). These findings indicated that the function of miR-20a may vary between different cell types. In a previous study, using samples from patients with adenocarcinoma from China, miR-20a-induced WTX deficiency promoted gastric cancer progression by regulating the PI3K/AKT signaling pathway (24). A previous study has demonstrated that the high

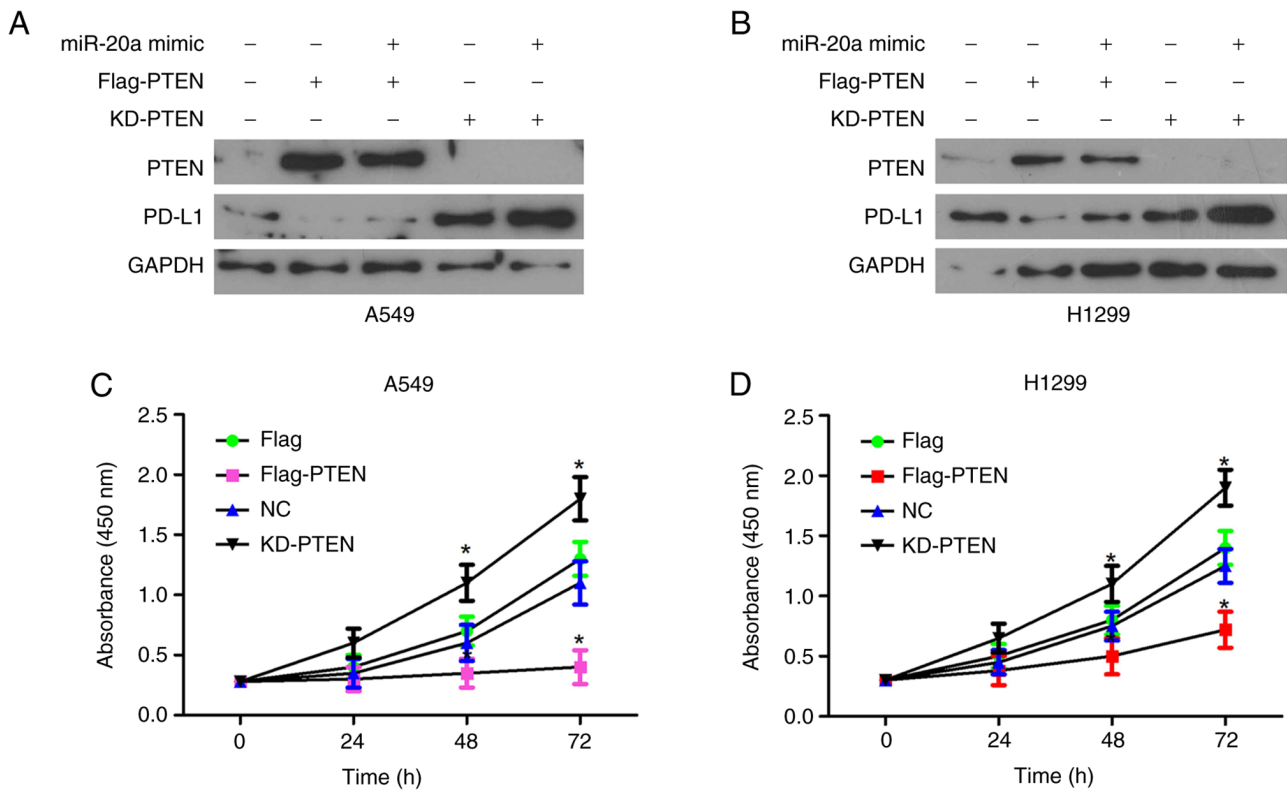


Figure 4. Knockdown of PTEN promotes the expression level of PD-L1 and non-small cell lung cancer cell proliferation. PD-L1 and PTEN protein expression levels were measured in the (A) A549 and (B) H1299 cell lines following transfection with PTEN overexpression or KD expression vectors and with or without miR-20a mimics. Cell Counting Kit-8 assay was performed to determine cell proliferation in the (C) A549 and (D) H1299 cell lines transfected with PTEN overexpression or KD expression vectors and their respective controls. * $P < 0.05$ (Flag Group and Flag-PTEN Group at 48 or 72 h, respectively; NC Group and KD-PTEN Group at 48 or 72 h, respectively). PD-L1, programmed death-ligand 1; miR, microRNA; Con, control; KD, knockdown.

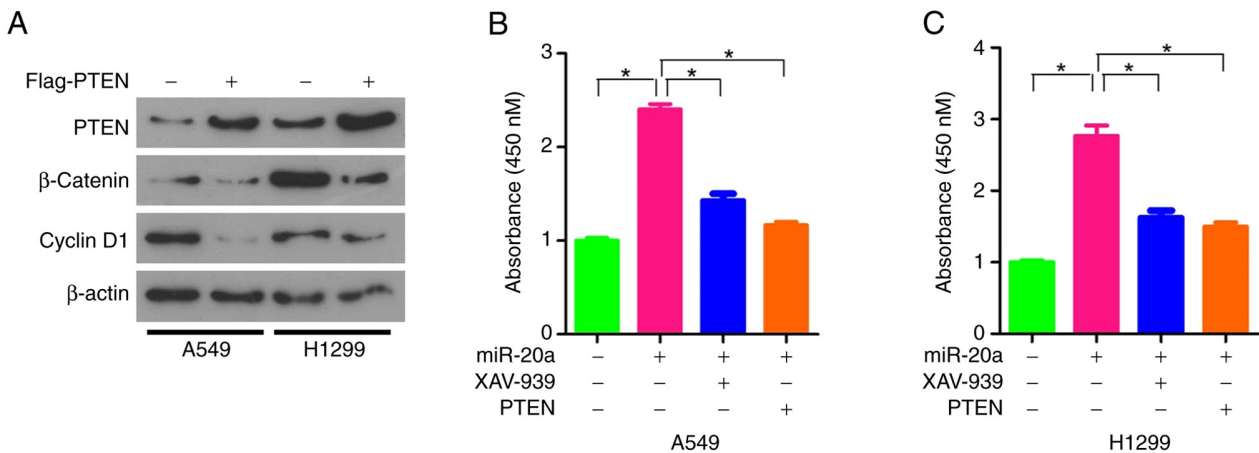


Figure 5. PTEN inhibits the Wnt/ β -catenin signal pathway and inhibits non-small cell lung cancer cell proliferation. (A) Expression levels of proteins associated with the Wnt/ β -catenin signaling pathway were measured in the A549 and H1299 cell lines transfected with PTEN overexpression vector. Cell Counting Kit-8 assay was used to measure cell proliferation in the (B) A549 and (C) H1299 cell lines transfected with or without miR-20a mimic and PTEN overexpression vector, and treated with or without XAV-939 inhibitor. * $P < 0.05$. miR, microRNA.

expression level of plasma miR-20a was associated with shorter disease free survival (DFS) and overall survival (OS) in patients with NSCLC, which was an independent risk factor (25). Consistent with this finding, the present study revealed that miR-20a promoted the proliferation of NSCLC cells.

To further determine how miR-20a acted as an oncogene, the present study confirmed that PD-L1 was a potential target for miR-20a and miR-20a promoted the expression level of

PD-L1. The PD-1/PD-L1 axis is responsible for cancer immune escape and has a marked effect on cancer therapy (26). Blocking inducible PD-L1 expression, upon tumor-antigen specific T cell infiltration, was the key event leading to the response to anti-PD-1 or anti-PD-L1 antibody therapy in patients with NSCLC (27). The suppression of PD1/PD-L1 immune checkpoint provided a promising new method for the treatment of NSCLC. A number of studies showed that PD-L1 was highly expressed in NSCLC

cells (27,28). The use of drugs to block PD1/PD-L1 immune checkpoint (such as atezolizumab) could prolong the survival time in patients with advanced NSCLC (29). The present study found that miR-20a promoted the expression level of PD-L1 by inhibiting the expression level of PTEN, which in turn promoted the proliferation of NSCLC cells.

PTEN is a key tumor suppressor gene and one of the most frequently mutated genes in human tumors. The expression level of PTEN was downregulated in numerous tumor types (30-32). A previous study described the association between the expression of PD-L1 and PTEN (31). In other cases, knockdown or inhibition of PTEN resulted in increased PD-L1 expression level in breast and prostate cancer (31,32). The expression level of PD-L1 in prostate, breast and lung cancer was dependent on PI3K and regulated by PTEN (27,29). However, this association was context-dependent, as the regulation of PD-L1 expression was controlled by a number of factors and pathways (33). A previous study has shown that the increased expression level of PD-L1 could directly mediate the activation of the β -catenin/TCF/LEF transcriptional complex (34). The Wnt/ β -catenin signal pathway played an important role in regulating the growth and metastasis of glioblastoma cells (34). Another study also confirmed that the Wnt/ β -catenin signaling pathway could promote the growth and progression of numerous cancers, including NSCLC (35). The Wnt/ β -catenin signaling pathway could be activated by frizzled and low-density lipoprotein receptor-related protein 5/6. Dishevelled was recruited and phosphorylated to induce the dissociation of glycogen synthase kinase-3 beta (GSK-3 β) from axon proteins (36). The inhibition of GSK-3 β expression led to the inactivation of the degradation complex (36). The phosphorylation and degradation of β -catenin were inhibited and accumulated stably in the cytoplasm (36). A previous study showed that active Wnt/ β -catenin signal transduction could lead to T cell rejection and resistance to the treatment of anti-PD-L1/anti-CTLA-4 monoclonal antibodies in melanoma (37). XAV-939 is an effective inhibitor of the Wnt/ β -catenin signal pathway. The present study found that PTEN inhibited the expression of β -catenin and cyclin D1. The inhibitory effect of PTEN on cell proliferation was similar to that of XAV-939.

In summary, the present study showed that miR-20a promoted the proliferation of NSCLC cells by inhibiting the expression level of PTEN and enhancing the expression level of PD-L1. These findings suggest that miR-20a could be used as a biomarker and therapeutic target in the treatment of NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JG and MC designed the research. JG, YS, FJ, YW and LC performed the experiments. JG and YS wrote the manuscript. JS and PS provided materials and performed data analysis. JG, YS and MC confirm the authenticity of all raw data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Second Affiliated Hospital of Zhengzhou University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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