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MIR-301b-3p Promotes Lung Adenocarcinoma Cell Proliferation, Migration and Invasion by Targeting DLC1

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Abstract

Background: miR-301b-3p is reported in various human cancers for its abnormal expression, while the role and molecular mechanisms in lung adenocarcinoma (LUAD) remain unclear, and this is the focus of the present study. **Materials and Methods:** TCGA database was consulted to know gene expression in LUAD tissue. CCK-8, colony formation assay and Transwell assay were applied to identify the role of target genes in regulating LUAD cell biological properties. Bioinformatics analysis plus dual-luciferase assay were performed to validate the potential connection between genes. **Results:** miR-301b-3p and DLC1 were the target genes of this study and respectively differentially up-regulated and down-regulated in LUAD. Functional experiments indicated that miR-301b-3p contributed to cancer cell proliferation, migration and invasion, while this effect was reversed with overexpressed DLC1 which was identified as a direct target of and regulated by miR-301b-3p. **Conclusions:** Collectively, miR-301b-3p was identified to actively function on LUAD malignant progression by suppressing DLC1 expression. This discovery provides a novel therapeutic strategy for LUAD patients, which helps improve the survival of patients.

Keywords

miR-301b-3p, DLC1, lung adenocarcinoma, proliferation, migration

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Highlight

1. miR-301b-3p boosts the progression of lung adenocarcinoma;
2. DLC1 was targeted and regulated by miR-301b-3p in lung adenocarcinoma cells;
3. DLC1was involved in regulation of miR-301b-3p on the malignant progression of lung adenocarcinoma.

Introduction

As a heterogeneous disease, lung cancer is regarded as the most common cause of cancer-related deaths predicted to take up 18.4%.[1,2] It is estimated that approximately 1.8 million lung cancers are newly diagnosed worldwide per year.[3] Lung adenocarcinoma (LUAD) serves as the most prevalent type of lung cancer having severely jeopardized human life and health due to a high rate of incidence and mortality.[4] As data revealed, more than 60% of LUAD patients were diagnosed at stage III or IV, making the treatment efficacy of surgical resection and chemoradiotherapy keeping low.[5,6] In view of the situation, it is noteworthy that with the genetic test growing faster, novel therapeutic targets and biomarkers might be promising in improving survival of LUAD patients to some extent.

Recently, non-coding RNA has been revealed to be involved in regulating the progression of human cancers. LINC00301, a long non-coding RNA (lncRNA), for example, is mediated by FOXC1 in non-small cell lung cancer (NSCLC) and then facilitates tumor progression and triggers an immune-suppressing microenvironment by regulating HIF1α pathway.[7] PDIA3P another lncRNA is identified to play a part in oral squamous

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cell carcinoma progression via targeting Cyclin D2 by interacting with miR-185-5p.[8] In addition, microRNAs (miRNAs) have also received a great deal of attention as a cancer promoter or a tumor suppressor in cancer. A study reported that miR-629-3p in LUAD can induce cell proliferation and inhibit cell apoptosis via down-regulating surfactant protein C (SFTPC).[9] Reversely, miR-450b-5p in LUAD serves as an anti-tumor molecule by interacting with EZH2.[10] Currently, in the field of tumor treatment, miRNAs are emerging as promising therapeutic targets and there have been 2 major applications that work: (1) miRNA antagonists for inhibition of oncomiRs, such as antisense oligonucleotides, antagomirs and miRNA sponges; (2) miRNA mimics for amplifying expression of tumor suppressor miRNAs, such as double-stranded synthetic miRNAs and miRNA expression vectors.[11] Given the above findings and achievements, miRNAs can be linked to malignant progression of LUAD with their roles as oncogenes or tumor suppressors, the way of which is likely to be beneficial for survival improvement. As reported, miR-301b-3p can be an oncogene in diverse human cancers. Fan H *et al*. showed that miR-301b-3p is highly expressed in gastric cancer (GC), and knockdown of miR-301b-3p can significantly inhibit GC cell proliferation, induce cell cycle arrest in G1 phase and potentiate cell apoptosis.[12] As well, miR-301b-3p can facilitate tumor growth of hepatocellular carcinoma (HCC), and it is closely associated with tumor size and advanced tumor-node metastasis when being overexpressed.[13] While in NSCLC, miR-301b-3p is seen to present with elevated expression.[14] Considering the achievements abovementioned, miR-301b-3p may have a promoting effect on occurrence and development of LUAD.

Materials and Methods

Bioinformatics Analysis

TCGA database (https://portal.gdc.cancer.gov/) was consulted to obtain expression profiles regarding mature miRNA (normal: n = 46, tumor: n = 521) and mRNA (normal: n = 59, tumor: n = 535) of LUAD. Differential mRNAs (DEmRNAs) were acquired from differential analysis with the aid of the "edgeR" package ($|logFC| > 2$, padj < 0.01). Potential targets of miR-301b-3p were searched on TargetScan (http://www.targetscan.org/vert_72/) and miRDB (http://mirdb.org/) databases, and candidate targets were identified from the predicted results and the differentially down-regulated mRNAs in TCGA. Pearson correlation analysis was performed to identify the mRNA of the highest correlation coefficient.

Cell Lines and Cell Transfection

The cell lines used in this were as below: human lung epithelial cell line BEAS-2B (BNCC254518) and 16HBE (BNCC338044), human LUAD cell lines H1975 (BNCC100301), HCC78 (BNCC338064), PC9 (BNCC340767) and SPCA1 (BNCC101697). All the cell lines were ordered from BeNa Culture Collection (Beijing, China), and maintained under the following condition: Dulbecco's Modified Eagle Medium-H (DMEM-H; 11965092; Gibco, Shanghai, China) with an addition of 10% fetal bovine serum (FBS), 37 °C, 5% CO_2.

miR-301b-3p mimic and its negative control (NC mimic) were accessed from Guangzhou Ribo Bio Co., LTD and transiently transfected into human LUAD cell line PC9 by Lipofectamine RNAiMAX Reagent (Life Technologies, Grand Island, NY). The cells following transfection were harvested and subjected to expression analysis and functional experiments. Plasmids with DLC1 sequence (oe-DLC1) were constructed using lentiviral vector pLVX-IRES-neo (Clontech, USA), and then dripped into PC9 cells for infection.

qRT-PCR

Total RNA was extracted from selected cells using the TRIzol Kit (10296010, Invitrogen, Carlsbad, CA, USA) according to instructions, and then reversely transcribed into cDNA on M-MLV (Takara, Otsu, Japan). Subsequently, the cDNA was amplified using the SYBR Green Master Mix kit (Takara). The expression levels of miR-301b-3p and DLC1 mRNA were analyzed on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA) and assessed with U6 and β-actin as an internal reference, respectively. Relative expression was compared by $2^{-\Delta\Delta Ct}$. All primers used in qRT-PCR were synthesized by BGI Co., Ltd (Shenzhen, China), and sequenced as follows: miR-301b-3p Forward: 5'-CAGTGCTCTGACGAGGTTG-3', Reverse: 5'-TGTCCCAGATGCTTTGACA-3'; DLC1 Forward: 5'-CCGCCTCATACGA-3', Reverse: 5'-TTCTCCCCCATATACTA-3'; U6 Forward: 5'-CTCGCTTCGGCAGCATA-3', Reverse: 5'-AACGATTCACGAATTTGCGT-3'; β-actin Forward: 5'-CCTGGCACATAAT-3', Reverse: 5'-GCTGATCCACTGCTGCTGCAGAA-3'. The experiment was independently conducted in triplicate.

Western Blot

Total proteins from cells were respectively obtained and concentrated by RIPA lysis buffer added with phenylmethylsulfonyl fluoride (PMSF; R0010; Solarbio Science & Technology, Beijing, China) and the BCA protein assay kit (20201ES76; Yeasen Company, Shanghai, China). The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvinylidene fluoride (PVDF) membranes (FFP 36; Beyotime, Shanghai, China). 5% bovine serum albumin (BSA) was used to block the membranes for 2 h at 37 °C. Primary rabbit polyclonal antibodies composed of DLC1 (ab126257, 1:1000), MMP2 (ab92536, 1:3000), MMP7 (ab207299, 1:1000), MMP9 (ab76003, 1:10000) and GAPDH (ab22555, 1:2000) were added onto the membranes for incubation overnight at 4°C. PBST (phosphate buffered saline buffer + 0.1% Tween-20) was used to wash the membranes 3 times with 10 min each time. Following that, horseradish peroxidase (HRP)–labeled secondary antibody goat anti-rabbit IgG H&L (ab6721, 1:3000) was added onto the membranes for 1 h of incubation

at room temperature and then the membranes were washed with PBST 3 times. Images of protein bands were observed and captured under the optical luminometer (GE, USA). All antibodies used in this assay were from Abcam, Cambridge, UK.

CCK-8

The viability of PC9 cells was assayed by the CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan). Transfected PC9 cells were seeded into 96-well plates at a density of 3×10^3 cells/well. According to the protocol, 10 μmol/L of CCK-8 solution was added at 0, 24, 48, 72 and 96 h, respectively. After 2 h of incubation at 37°C, the absorbance of cells was read at 450 nm in wavelength by a microplate reader. Each experiment was conducted at least 3 times.

Colony Formation Assay

Transfected PC9 cells in different groups were digested with 0.25% trypsin and seeded into 6-well plates at a density of 4×10^2 cells/well. Then, the cells were grown in DMEM containing 10% FBS and 5% CO_2 for 2 weeks at room temperature. The mediums were discarded when colonies were visible to the naked eyes. The colonies were fixed in 95% methanol, stained with 0.1% crystal violet for 10 min, and then rinsed with PBS. Cell colonies (more than 50 cells in a colony) were counted at the end.

Transwell Assay

For examining cell migration and invasion, a 24-well Transwell chamber (8 mm in aperture, BD Biosciences, NJ, USA) was used. For migration assay, 5×10^4 PC9 cells (after 48 h of transfection) re-suspended by 100 μl serum-free mediums were planted into the upper chamber, whereas 500 μl of DMEM containing 20% FBS was added into the lower chamber as a chemotactic agent. The non-migrated cells were softly wiped off using a cotton swab after 24 h of incubation at 37°C, while the cells in the lower chamber were fixed in 95% methanol for 15 min and stained with 0.1% crystal violet for 15 min at room temperature. The migrated cells were counted and images were captured using the IX 71 inverted microscope (Olympus Corporation, Tokyo, Japan). Five fields in the view of each chamber were randomly selected for cell count. Procedures for cell invasion assay were similar to those of migration assay, except that the Transwell chamber was coated with Matrigel (BD Biosciences) before examination.

Dual-Luciferase Reporter Gene Assay

Amplified wild type (WT) and mutant (MUT) DLC1 3'UTR were inserted into downstream polyclonal sites of the luciferase reporter gene on pmirGLO vector to construct luciferase reporter vectors DLC1-3'UTR-WT and DLC1-3'UTR-MUT. Renilla luciferase expression vector pRL-TK (TaKaRa, Dalian, China) was taken as an internal reference. miR-301b-3p mimic and NC mimic were co-transfected with DLC1-3'UTR-WT or DLC1-3'UTR-MUT into 293 T cells (BNCC100530). The activity of luciferase was detected by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

All data were processed by GraphPad Prism 8.0 software (GraphPad Software, Inc., La Jolla, CA). Measurement data were expressed in Mean ± standard deviation (SD). Difference between 2 groups was analyzed in *t* test. $P < 0.05$ was considered statistically significant.

Results

miR-301b-3p Is Up-Regulated in LUAD

miR-301b-3p is recognized as an oncogene in several studies.[13,15,16] This study sought to make an attempt to explore the mechanism of miR-301b-3p affecting the progression of LUAD. We firstly used TCGA-LUAD data to analyze miR-301b-3p expression, finding that miR-301b-3p in LUAD tissue was remarkably up-regulated than that in normal tissue (Figure 1A). Thereafter, we verified miR-301b-3p expression at the cellular level. As revealed by qRT-PCR, miR-301b-3p was significantly up-regulated in LUAD cell lines (H1975, HCC78, PC9, SPCA1) compared to that in normal lung epithelial cell lines (BEAS-2B, 16HBE) (Figure 1B), among which the highest miR-301b-3p expression level was observed in PC9 cell line. Therefore, PC9 cell line was selected for subsequent experiments. Collectively, these findings validated that miR-301b-3p was differentially up-regulated in LUAD tissue and cells.

miR-301b-3p Promotes LUAD Cell Proliferation, Migration and Invasion

To investigate the effect of miR-301b-3p on the malignant progression of LUAD, miR-301b-3p mimic or NC mimic was transfected into PC9 cells. qRT-PCR was conducted for detection of transfection efficiency, and it was found that miR-301b-3p expression was increased in PC9 cells transfected with miR-301b-3p mimic (Figure 2A). We then performed a series of *in vitro* experiments. As revealed by CCK-8 assay, up-regulation of miR-301b-3p facilitated PC9 cell viability (Figure 2B). Colony formation assay showed that the colony formation ability was evidently higher in the miR-301b-3p mimic group than that in the NC mimic group (Figure 2C). Transwell assay found that overexpressing miR-301b-3p significantly promoted PC9 cell migratory and invasive abilities (Figure 2D). Taken together, these findings illustrated that miR-301b-3p could markedly promote LUAD cell proliferation, migration and invasion.

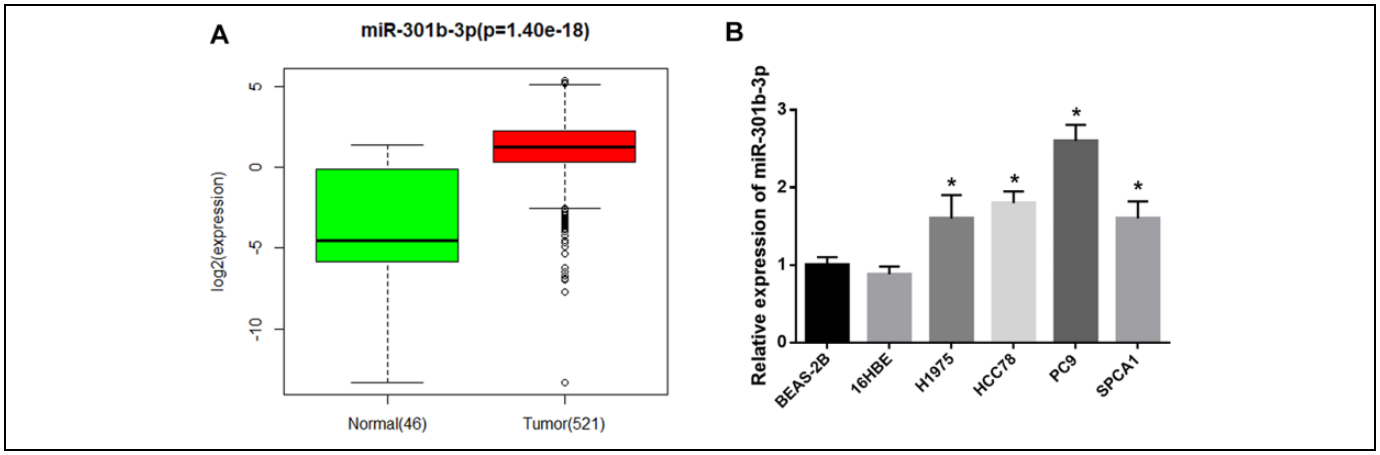

Figure 1. miR-301b-3p is up-regulated in LUAD tissue and cells (A) Box plots of miR-301b-3p expression in normal group and tumor group in TCGA-LUAD dataset (the green box represents the normal group and the red box represents the tumor group); (B) The expression of miR-301b-3p in human normal lung epithelial cell lines (BEAS-2B, 16HBE) and LUAD cell lines H1975, HCC78, PC9, SPCA1; *$p < 0.05$.

miR-301b-3p Targets and Down-Regulates DLC1 Expression

From the above experiments, we had found that miR-301b-3p promoted LUAD cell proliferation, migration and invasion. To further explore the mechanism of miR-301b-3p in LUAD, we combined gene expression analysis with target prediction method to obtain the target gene of miR-301b-3p. From the differential analysis on mRNA expression data of TCGA-LUAD using "edgeR" package, a total of 2,501 DEmRNAs were obtained, among which 1,973 mRNAs were up-regulated, and 528 mRNAs were down-regulated (Figure 3A). TargetScan and miRDB databases were used for target gene prediction for miR-301b-3p, and 9 candidate genes were obtained from the intersection between the predicted target genes of miR-301b-3p and the down-regulated DEmRNAs (Figure 3B). Pearson correlation analysis was carried out on the 9 candidate genes and miR-301b-3p, finding that DLC1 was extraordinarily negatively correlated with miR-301b-3p with the highest Pearson correlation coefficient (Figure 3C). Besides, gene expression analysis elucidated that DLC1 was remarkably lowly expressed in LUAD tumor tissue (Figure 3D), and similar trend could be observed in LUAD cell lines as revealed by cell experiments (Figure 3E). To further verify whether DLC1 is a direct target of miR-301b-3p in LUAD, TargetScan database was used to predict the binding sites of miR-301b-3p on DLC1, while dual-luciferase assay was carried out for verification (Figure 3F). The results showed that the luciferase activity was significantly inhibited in the DLC1-3'UTR-WT group with overexpression of miR-301b-3p, while no obvious change was observed in the DLC1-3'UTR-MUT group. We could conclude that there was a binding relationship between miR-301b-3p and DLC1. Besides, qRT-PCR and western blot uncovered that overexpressing miR-301b-3p evidently suppressed mRNA and protein expression of DLC1 (Figure 3G-H). Taken together, these findings validated that miR-301b-3p targeted and inhibited DLC1 expression in LUAD cells.

miR-301b-3p Regulates LUAD Cell Proliferation, Migration and Invasion by Targeting DLC1

To further investigate the mechanism of miR-301b-3p/DLC1 related to the malignant progression of LUAD cells, we divided PC9 cells into 3 groups for transfection: NC mimic + oe-NC group, miR-301b-3p mimic + oe-NC group and miR-301b-3p mimic + oe-DLC1 group. qRT-PCR was firstly conducted to examine the expression level of miR-301b-3p/DLC1 in different groups, showing that overexpressing miR-301b-3p significantly decreased DLC1 mRNA and protein expression in the miR-301b-3p mimic + oe-NC group relative to the NC mimic + oe-NC group. Besides, DLC1 mRNA and protein expression exhibited significant elevated expression in the miR-301b-3p mimic + oe-DLC1 group relative to those in the miR-301b-3p mimic + oe-NC group, while miR-301b-3p expression showed no marked changes (Figure 4A-C). A series of *in vitro* experiments were then performed to explore the effect of the miR-301b-3p/DLC1 regulatory axis on the biological function of LUAD cells. As indicated by CCK-8 assay and colony formation assay, the viability and colony formation ability were considerably increased in the miR-301b-3p mimic + oe-NC group than those in the NC mimic + oe-NC group, while restored when miR-301b-3p and DLC1 were simultaneously overexpressed (Figure 4D-E). We also detected cell invasive and migratory abilities in different groups. Transwell assay found that overexpressing miR-301b-3p significantly promoted LUAD cell migration and invasion, while overexpressing DLC1 simultaneously remarkably decreased their abilities (Figure 4F). In addition, overexpression of miR-301b-3p remarkably increased the protein levels of metastasis markers MMP2, MMP7 and MMP9, while overexpression of DLC1

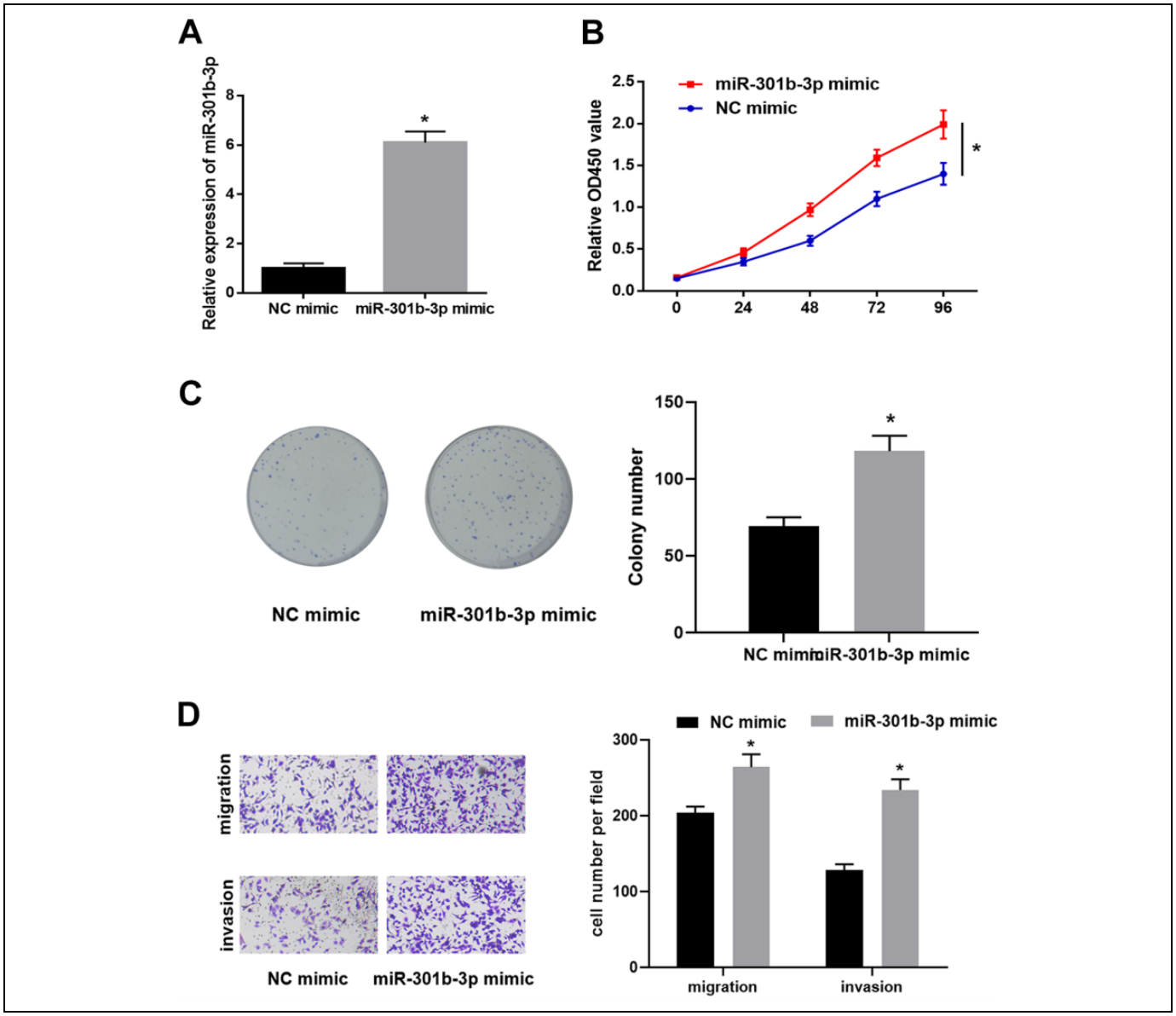

Figure 2. Overexpressing miR-301b-3p promotes LUAD cell proliferation, migration and invasion (A) The transfection efficiency of miR-301b-3p mimic in PC9 cells was detected by qRT-PCR; (B) Cell viability, (C) colony formation ability, (D) cell migratory and invasive abilities (100×) in 2 groups were examined by CCK-8 assay, colony formation assay and Transwell assay, respectively; *$p < 0.05$.

restored the levels of these 3 proteins, which once again confirmed the conclusion of the Transwell assay (Figure 4G). Taken together, these findings suggested that miR-301b-3p could target and inhibit DLC1 to affect the malignant progression of LUAD cells.

Discussion

In recent years, the promoting effect of miR-301b-3p on the malignant progression of various human cancers including gastric cancer,[12] liver cancer[17] and high grade ovarian serous carcinoma[16] has been reported in many studies. Here, we found that miR-301b-3p expression was significantly increased in tumor tissue than that in normal tissue according to TCGA-LUAD dataset, and similar trend was observed in LUAD cell lines. Functional analysis demonstrated that overexpressing miR-301b-3p could promote LUAD cell proliferation, migration and invasion. These findings are consistent with the expression pattern and functional role of miR-301b-3p in cancers in current existing studies.

Prior to our study, previous studies also elucidated the molecular mechanism of miR-301b-3p in cancers. Zheng H *et al*. reported that hypoxia-induced up-regulation of miR-301b-3p can promote cell proliferation, migration and invasion of prostate cancer cells by targeting lipoprotein receptor-related protein 1B (LRP1B).[15] Man X *et al*. revealed that USP13 is a target of miR-301b-3p, and overexpressing miR-301b-3p decreases USP13 expression, resulting in the down-regulation

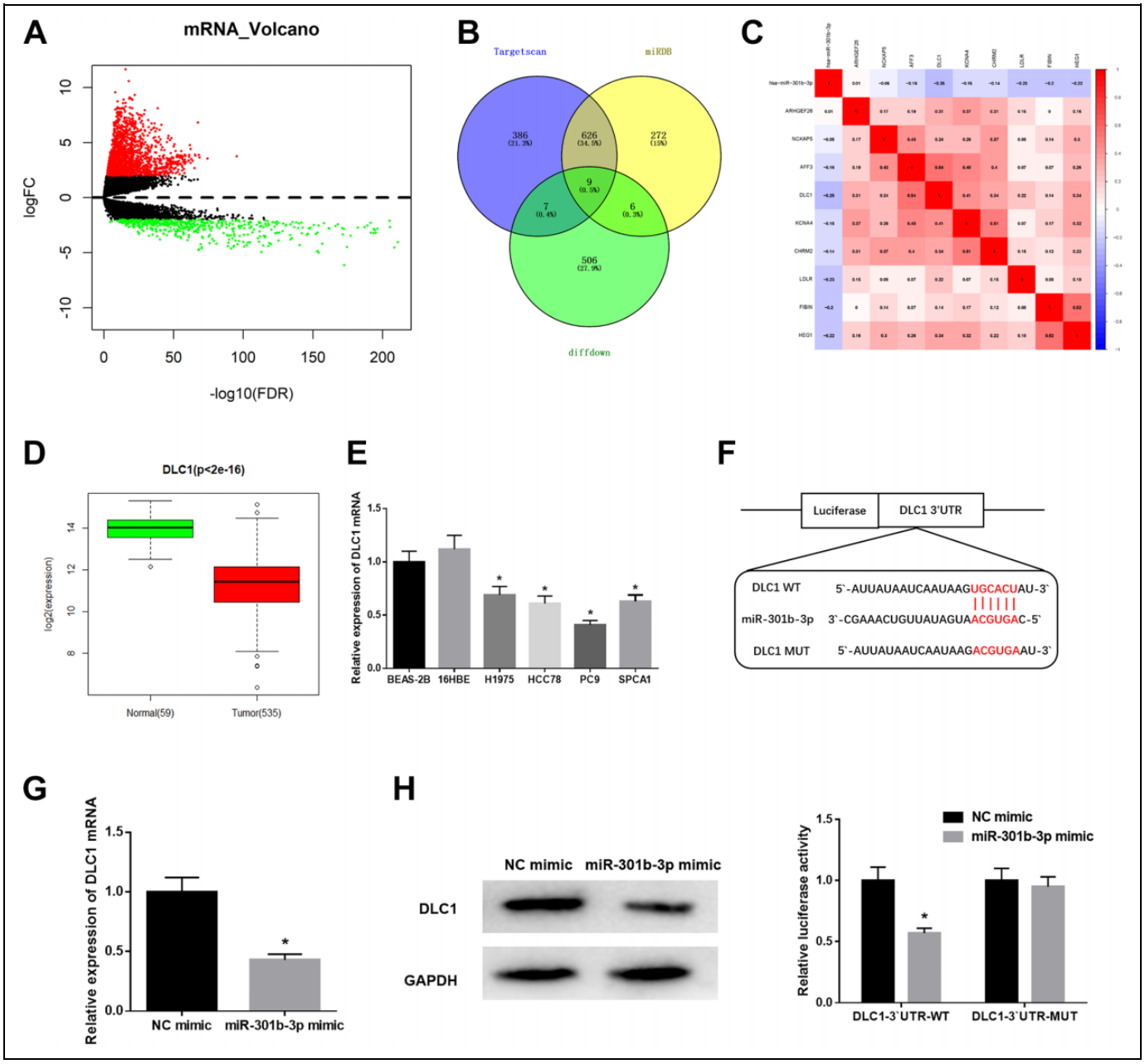

Figure 3. miR-301b-3p targets and inhibits DLC1 expression (A) Volcano plot of DEmRNAs in normal group and tumor group in TCGA-LUAD dataset. Red dots refer to differentially up-regulated genes and green dots refer to differentially down-regulated genes; (B) Venn diagram of predicted target genes of miR-301b-3p on TargetScan and miRDB database and down-regulated DEmRNAs; (C) Pearson correlation analysis of miR-301b-3p and 9 candidate target genes; (D) Box plots of DLC1 expression in normal group and tumor group in TCGA-LUAD; (E) The expression level of DLC1 mRNA in human normal lung epithelial cell lines (BEAS-2B, 16HBE) and LUAD cell lines (H1975, HCC78, PC9, SPCA1) was detected by qRT-PCR; (F) The binding site sequence of miR-301b-3p on DLC1 3'UTR was predicted by TargetScan database and was verified by dual-luciferase assay; Relative expression of (G) DLC1 mRNA and (H) DLC1 protein after overexpressing miR-301b-3p were assessed by qRT-PCR and Western blot, respectively; $*p < 0.05$.

of PTEN protein expression, ultimately promoting the occurrence of bladder cancer.[18] These results suggest that miR-301b-3p may promote the malignant progression of LUAD by regulating the expression of its target genes. In this study, we scientifically combined gene expression analysis with target prediction method and found that DLC1 was a target gene of miR-301b-3p. DLC1 was initially found to be a deleted or downregulated gene in primary HCC, and to exert its tumor suppressor role mainly through the Rho-GTPase activating protein (RhoGAP) domain.[19,20] Accumulating studies have elucidated that DLC1 acts as a metastasis suppressor gene and is associated with the occurrence and development of various cancers including lung cancer,[21] breast cancer[22] and kidney cancer.[23] Yang X *et al*. discovered that DLC1 plays an

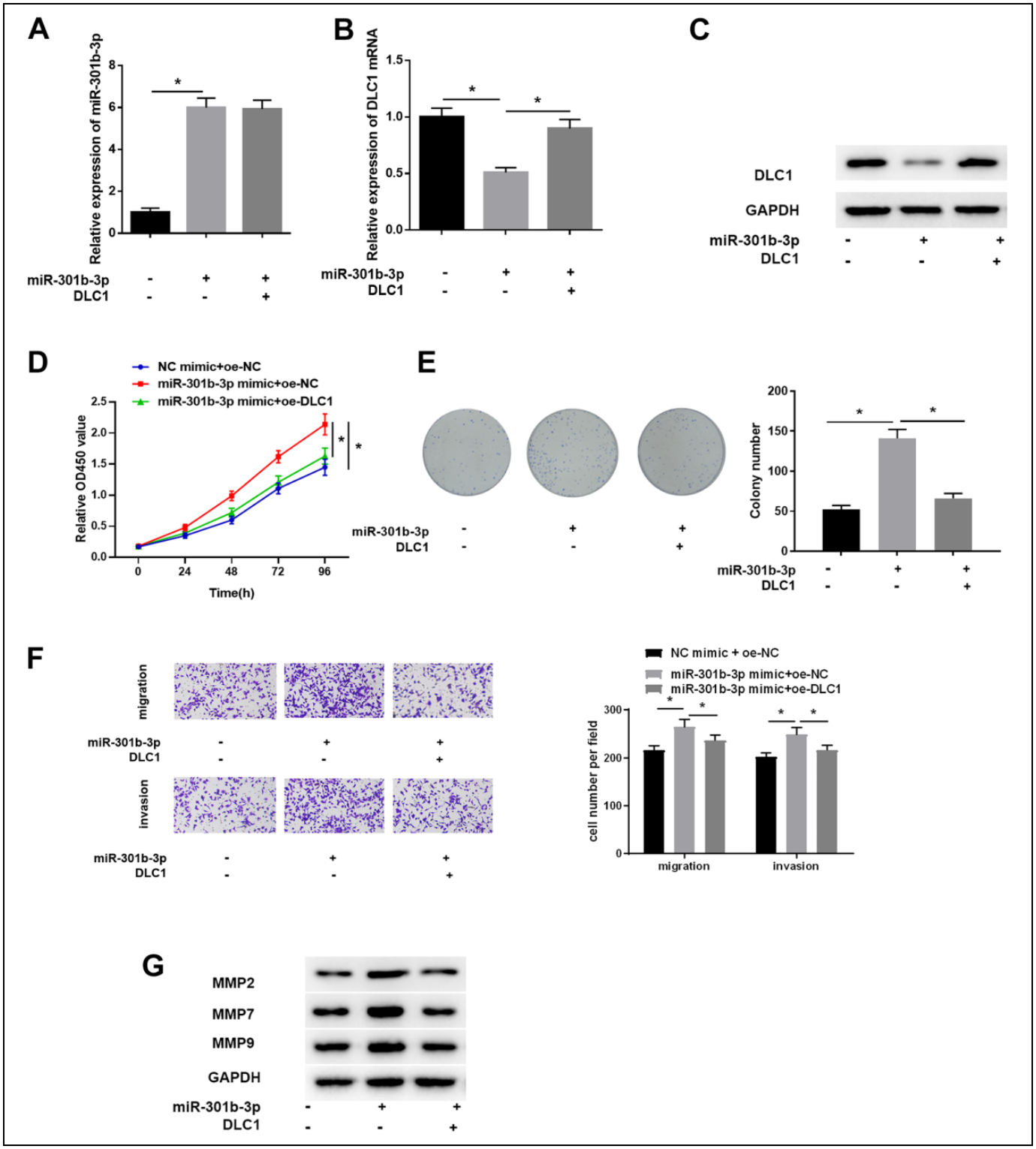

Figure 4. miR-301b-3p regulates LUAD cell proliferation, migration and invasion by targeting DLC1 (A) The expression of miR-301b-3p in 3 groups (NC mimic + oe-NC group, miR-301b-3p mimic + oe-NC and miR-301b-3p mimic + oe-DLC1) was detected by qRT-PCR; (B) DLC1 mRNA and (C) protein expression in 3 groups were detected by qRT-PCR and western blot, respectively; (D) Cell viability, (E) colony formation ability, (F) cell migratory and invasive abilities in 3 groups were examined by CCK-8 assay, colony formation assay and Transwell assay (100×), respectively; (G) Expression of metastasis markers MMP2, MMP7, MMP9 proteins in 3 groups was detected by western blot; *$p < 0.05$.

inhibitory role in cell migration, invasion, colony formation and anchorage-independent growth of aggressive lung cancer cells *in vitro*.[24] Besides, it is reported that DLC1 expression is significantly correlated with the prognosis of LUAD patients.[25] The abovementioned studies collectively demonstrate that DLC1 may function as a tumor suppressor in the malignant progression of LUAD. Our study showed that DLC1 was differentially down-regulated in LUAD tissue and cells. Besides, DLC1 had a high negative correlation and a targeting relationship with miR-301b-3p. Rescue experiments illustrated that miR-301b-3p promoted cell proliferation, migration and invasion of LUAD cells by targeting to down-regulate DLC1 expression.

In summary, we first reported that miR-301b-3p functions as an oncogene in LUAD cells, and there is a targeting relationship between miR-301b-3p and DLC1. Besides, we elucidated the mechanism by which miR-301b-3p facilitates the malignant progression of LUAD by targeting DLC1, which is crucial for the development of targeted therapies for LUAD and provides reference and data support for the target selection. However, the limitation of the study is that there is no in-depth research on the downstream signaling pathways associated with DLC1 in the malignant progression of LUAD. In the following studies, we will continue to work at this direction so as to improve the survival of LUAD patients.

Authors' Contributions

Haitao Liu contributed to the study design. Xingjie Ma conducted the literature search. Niu Niu acquired the data. Haitao Liu wrote the article. Junjie Zhao performed data analysis and drafted. Chao Lu reviewed the article. Fan Yang and Weibo Qi gave the final approval of the version to submitted.

Availability of Data and Material

The data and materials in the current study are available from the corresponding author on reasonable request.

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.

Ethical Statement

Our study did not require an ethical board approval because it did not contain human or animal trials.

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