

miR-301b-3p promotes lung adenocarcinoma cell proliferation, migration and invasion by targeting DLC1

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Research

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

Posted Date: May 20th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-27752/v1>

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Abstract

Background

miR-301b-3p has been reported to be abnormally expressed in various human cancers including lung cancer. However, the underlying role and molecular mechanisms in lung adenocarcinoma (LUAD) remain unclear. This study aimed to elucidate the underlying mechanisms of miR-301b-3p in LUAD.

Methods

Based on TCGA database, we found that miR-301b-3p was prominently up-regulated in LUAD tissues. A series of functional experiments including CCK-8 assay, colony formation assay and Transwell assay uncovered that the up-regulation of miR-301b-3p facilitated LUAD cell proliferation, migration and invasion abilities. Bioinformatics analysis and dual-luciferase reporter gene assay demonstrated that Deleted in Liver Cancer 1 (DLC1) was negatively regulated by miR-301b-3p, and it was extremely lowly expressed in LUAD tissues and cells.

Results

Rescue experiments suggested that overexpressing DLC1 restored the promoting effect of miR-301b-3p on LUAD cell proliferation, migration and invasion.

Conclusions

Taken together, our study elucidated that miR-301b-3p promoted LUAD cell proliferation, migration and invasion by targeted suppressing DLC1 expression. The discovery of the mechanism provides a novel therapeutic strategy for LUAD patients, which helps to improve the survival of patients.

Background

As a heterogeneous disease, lung cancer is considered to be the most common cause of cancer-related deaths, leading to 18.4% of all cancer deaths. An estimate of 1.8 million are newly diagnosed as lung cancer worldwide per year. Lung adenocarcinoma (LUAD) is the most common type of lung cancer and a disease that severely jeopardizes human life and health because of a high rate of incidence and mortality. As data revealed, more than 60% of LUAD patients are diagnosed at stage III or IV, making the treatment efficacy of surgical resection and chemoradiotherapy low. It is noteworthy that exploration of the therapeutic targets and biomarkers for LUAD could improve patient's survival to some extent with the rapid development of gene detection technology.

In recent years, microRNAs (miRNAs) have received a great deal of attention as a cancer promoter or a tumor suppressor in cancers. Studies have shown that miR-629-3p can promote LUAD cell proliferation and inhibit cell apoptosis via down-regulating surfactant protein C (SFTPC). miR-133 suppresses LUAD cell metastasis by targeting Flotillin 2 (FLOT2) via Akt signaling pathway. Currently, there are two approaches concerning miRNA-based therapies: (1) the inhibition of oncomiRs by miRNA antagonists, such as antisense oligonucleotides, antagomirs and miRNA sponges; (2) amplifying the expression of tumor suppressor miRNAs by miRNA mimics, such as double-stranded synthetic miRNAs and miRNA expression vectors. Collectively, these findings suggest that miRNAs could act as oncogenes or tumor suppressors to affect the malignant progression of LUAD, which can be used to improve patient's survival. miR-301b-3p has been reported as an oncogene in various human cancers. Fan H *et al.* showed that miR-301b-3p is highly expressed in gastric cancer (GC), and the knockdown of miR-301b-3p significantly inhibits GC cell proliferation, induces cell cycle arrested in G1 phase and potentiates cell apoptosis. miR-301b-3p also facilitates tumor growth in hepatocellular carcinoma (HCC), and clinical association analysis found that high level of miR-301b-3p is closely associated with tumor size and advanced tumor-node metastasis. Besides, miR-301b-3p has been observed to exhibit an elevated expression in non-small cell lung cancer (NSCLC). Taken together, miR-301b-3p may have a promoting effect on the occurrence and development of LUAD.

Despite the promise of miRNA mimics acting as clinical therapeutic agents for LUAD, identification of the optimal target miRNA remains one of nonnegligible challenges. In the present study, we used gene expression analysis combined with target prediction method to explore the direct target of miR-301b-3p in LUAD and the relevant molecular mechanism of miR-301b-3p affecting the malignant progression of LUAD.

Methods

1. Bioinformatics Analysis

The expression profiles of mature miRNA (normal: n = 46, tumor: n = 521) and mRNA (normal: n = 59, tumor: n = 535) in TCGA-LUAD dataset were downloaded from TCGA database (<https://portal.gdc.cancer.gov/>). The expression of miR-301b-3p was analyzed based on the obtained data. "edgeR" package was employed to perform differential analysis ($|\logFC| > 2$, $\text{padj} < 0.01$) on mRNAs, and differentially expressed mRNAs (DEmRNAs) of LUAD were obtained. TargetScan (http://www.targetscan.org/vert_72/) and miRDB (<http://mirdb.org/>) databases were applied to predict the targets of miR-301b-3p, and candidate target genes were identified from the intersection of predicted targets of miR-301b-3p and the down-regulated DEmRNAs. Pearson correlation analysis was performed on candidate target genes and miR-301b-3p, and the mRNA showed the highest correlation coefficient was selected as the target gene for the study.

2. Cell Lines And Cell Transfection

Human lung epithelial cell line BEAS-2B (BNCC254518) and human LUAD cell lines H1975 (BNCC100301), HCC78 (BNCC338064), PC9 (BNCC340767) and SPCA1 (BNCC101697) were all purchased from BeNa Culture Collection (Beijing, China). All cell lines were cultured in Dulbecco's Modified Eagle Medium-H (DMEM-H; 11965092; Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) in an incubator at 37 °C with 5% CO₂.

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). Cells (after 48 h of transfection) were harvested and subjected to expression analysis and functional experiments. Overexpression plasmids oe-DLC1 and oe-NC were established using lentiviral expression vector pLVX-IRES-neo (Clontech, USA), and then dripped into PC9 cells for infection.

3. qRT-PCR

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

4. Western Blot

Total proteins were isolated from tissues and cells by RIPA lysis buffer with phenylmethylsulfonyl fluoride (PMSF; R0010; Solarbio Science & Technology, Beijing, China), and the total protein concentration was determined by a 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. DLC1 rabbit polyclonal antibody (ab126257, 1:1000, Abcam, Cambridge, UK) and GAPDH rabbit polyclonal antibody (ab22555, 1:2000, Abcam) 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 three times with 10 min each time. Horseradish peroxidase (HRP)-labeled secondary antibody goat anti-rabbit IgG H&L (ab6721,

1:3000, Abcam) was added onto the membranes for 1 h of incubation at room temperature and then the membranes were washed with PBST three times. Images of the protein bands were observed and captured under an optical luminometer (GE, USA).

5. CCK-8

The viability of PC9 cells was assayed by a 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 $\mu\text{mol/L}$ 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 was read at 450 nm in wavelength by a microplate reader. Each experiment was conducted at least three times.

6. 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. Transfected cells were grown in DMEM containing 10% FBS and 5% CO_2 for two 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) were counted at the end.

7. Transwell Assay

24-well Transwell chambers (8 mm in aperture, BD Biosciences, NJ, USA) were used for cell migration and invasion assay. 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 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 an IX 71 inverted microscope (Olympus Corporation, Tokyo, Japan). Five fields in the view of each chamber were randomly selected for cell count. Cell invasion assay procedures were similar to those of migration assay, except that the Transwell chambers were coated with Matrigel (BD Biosciences).

8. 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 internal reference. miR-301b-3p mimic and NC mimic were co-transfected with DLC1-3'UTR-WT or DLC1-

3'UTR-MUT into 293T cells (BNCC100530). The activity of luciferase was detected by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

9. Statistical Analysis

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

Results

1. miR-301b-3p Is Up-regulated In LUAD

miR-301b-3p has been recognized as an oncogene in several studies. 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 was remarkably up-regulated in LUAD tissues than that in normal tissues (Fig. 1A). Thereafter, we verified miR-301b-3p expression at 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 line (BEAS-2B) (Fig. 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 up-regulated in LUAD tissues and cells.

2. 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 miR-301b-3p mimic transfected PC9 cells (Fig. 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 (Fig. 2B). Colony formation assay showed that the colony formation ability was evidently higher in miR-301b-3p mimic group than that in NC mimic group (Fig. 2C). Transwell assay found that overexpressing miR-301b-3p significantly promoted PC9 cell migration and invasion abilities (Fig. 2D). Taken together, these findings illustrated that miR-301b-3p could markedly promote LUAD cell proliferation, migration and invasion.

3. miR-301b-3p Targeted 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. “edgeR” package was employed to perform differential analysis on mRNA expression data of TCGA-LUAD. A total of 2,501 DEmRNAs were obtained, among which 1,973 mRNAs were up-regulated, and 528 mRNAs were down-regulated (Fig. 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 predicted target genes of miR-301b-3p and down-regulated DEmRNAs (Fig. 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 (Fig. 3C). Besides, gene expression analysis elucidated that DLC1 was remarkably lowly expressed in LUAD tumor tissues (Fig. 3D), and similar trend could be observed in LUAD cell lines (Fig. 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 (Fig. 3F). The results showed that the luciferase activity was significantly inhibited in DLC1-3'UTR-WT group upon overexpression of miR-301b-3p, while no obvious change was observed in 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 (Fig. 3G and 3H). Taken together, these findings validated that miR-301b-3p targeted inhibited DLC1 expression in LUAD cells.

4. miR-301b-3p regulates LUAD cell proliferation, migration and invasion by targeting DLC1

To further investigate the mechanism of miR-301b-3p/DLC1 in the malignant progression of LUAD cells, we divided PC9 cells into three 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 expression in miR-301b-3p mimic + oe-NC group relative to NC mimic + oe-NC group. Besides, DLC1 mRNA exhibited a significant elevated expression in miR-301b-3p mimic + oe-DLC1 group relative to that in miR-301b-3p mimic + oe-NC group, while miR-301b-3p expression showed no marked changes when miR-301b-3p and DLC1 were simultaneously overexpressed (Fig. 4A and 4B). 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 miR-301b-3p mimic + oe-NC group than those in NC mimic + oe-NC group, while PC9 cell proliferation ability was restored when miR-301b-3p and DLC1 were simultaneously overexpressed (Fig. 4C and 4D). We also detected cell invasion and migration 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 (Fig. 4E). Taken together, these findings suggested that miR-301b-3p could targeted 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, liver cancer and high grade ovarian serous carcinoma has been reported in many studies. Here, we found that miR-301b-3p expression was significantly increased in tumor tissues than that in normal tissues according to TCGA-LUAD dataset, and similar trend could be observed in LUAD cell lines. Functional analysis demonstrated that overexpressing miR-301b-3p could promote LUAD cell proliferation, migration and invasion abilities. These findings are consistent with the expression pattern and functional role of miR-301b-3p in cancers in current studies.

Prior to our study, previous studies also have elucidated the molecular mechanism of miR-301b-3p in cancers. Zheng H *et al.* reported that hypoxia-induced up-regulation of miR-301b-3p promoted cell proliferation, migration and invasion of prostate cancer cells by targeting lipoprotein receptor-related protein 1B (LRP1B). Man X *et al.* revealed that USP13 was a target of miR-301b-3p, and overexpressing miR-301b-3p decreased USP13 expression, resulting in the down-regulation of PTEN protein expression, ultimately promoting the occurrence of bladder cancer. 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. 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, breast cancer and kidney cancer. Yang X *et al.* discovered that DLC1 plays an inhibitory role in cell migration, invasion, colony formation and anchorage-independent growth of aggressive lung cancer cells *in vitro*. Besides, it is reported that DLC1 expression is significantly correlated with the prognosis of LUAD patients. The abovementioned studies demonstrate that DLC1 may function as a tumor suppressor in the malignant progression of LUAD. Our study showed that DLC1 was down-regulated in LUAD tissues and cells. DLC1 had a high negative correlation and a targeted relationship with miR-301b-3p. Rescue experiments illustrated that miR-301b-3p promoted cell proliferation, migration and invasion abilities of LUAD cells by targeted down-regulating DLC1 expression.

In summary, we first reported that miR-301b-3p functioned as an oncogene in LUAD cells, and there was a targeted relationship between miR-301b-3p and DLC1. Besides, we elucidated the mechanism of miR-301b-3p in LUAD. miR-301b-3p facilitated 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 was 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.

Conclusions

miR-301b-3p promoted LUAD cell proliferation, migration and invasion by targeted suppressing DLC1 expression. The discovery of the mechanism provides a novel therapeutic strategy for LUAD patients, which helps to improve the survival of patients.

Abbreviations

LUAD: lung adenocarcinoma; DLC1: Deleted in Liver Cancer 1; miRNAs: microRNAs; SFTPC: surfactant protein C; FLOT2: Flotillin 2; GC: gastric cancer; HCC: hepatocellular carcinoma; NSCLC: non-small cell lung cancer; DEmRNAs: differentially expressed mRNAs; HRP: Horseradish peroxidase; WT: wild type; MUT: mutant; SD: standard deviation; LRP1B: lipoprotein receptor-related protein 1B; RhoGAP: Rho-GTPase activating protein;

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare no conflicts of interest.

Funding

This study was supported by 2019Jiaxing Key Discipline of Medicine-Thracic Surgery(Supporting Subject) and Jiaxing Key Laboratory of Precision Treatment for Lung Cancer.

Authors' contributions

HT contributed to the study design. XJ conducted the literature research. NN acquired the data. HT wrote the article. JJ performed data analysis and drafted. CL and FY revised the article. WB gave the final approval of version to be submitted.

Acknowledgements

Not applicable

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Figures

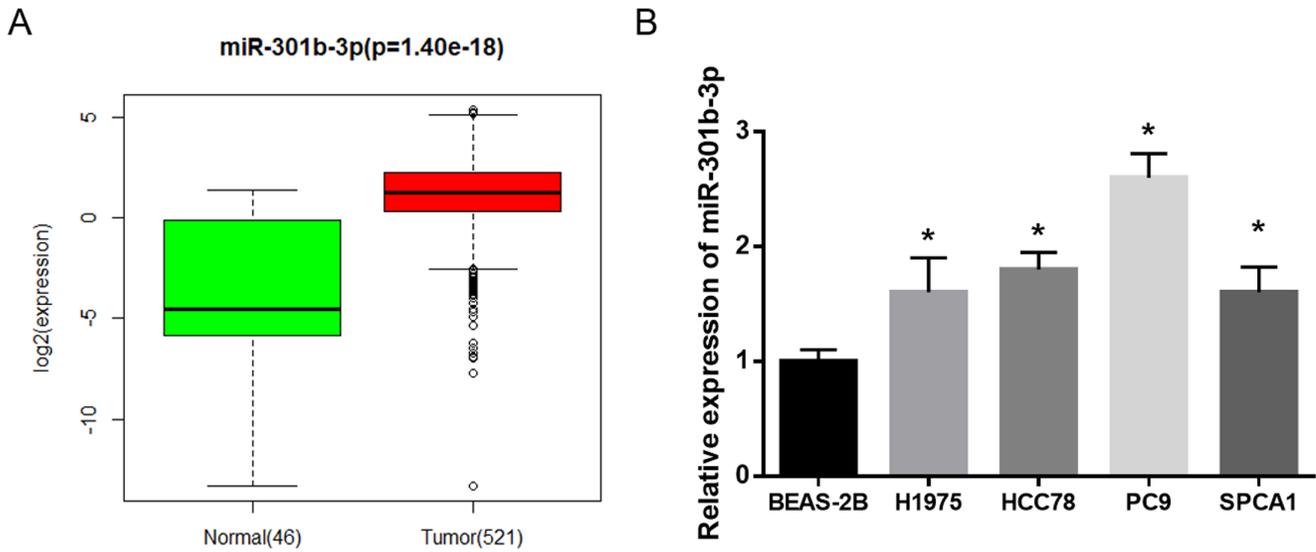


Figure 1

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

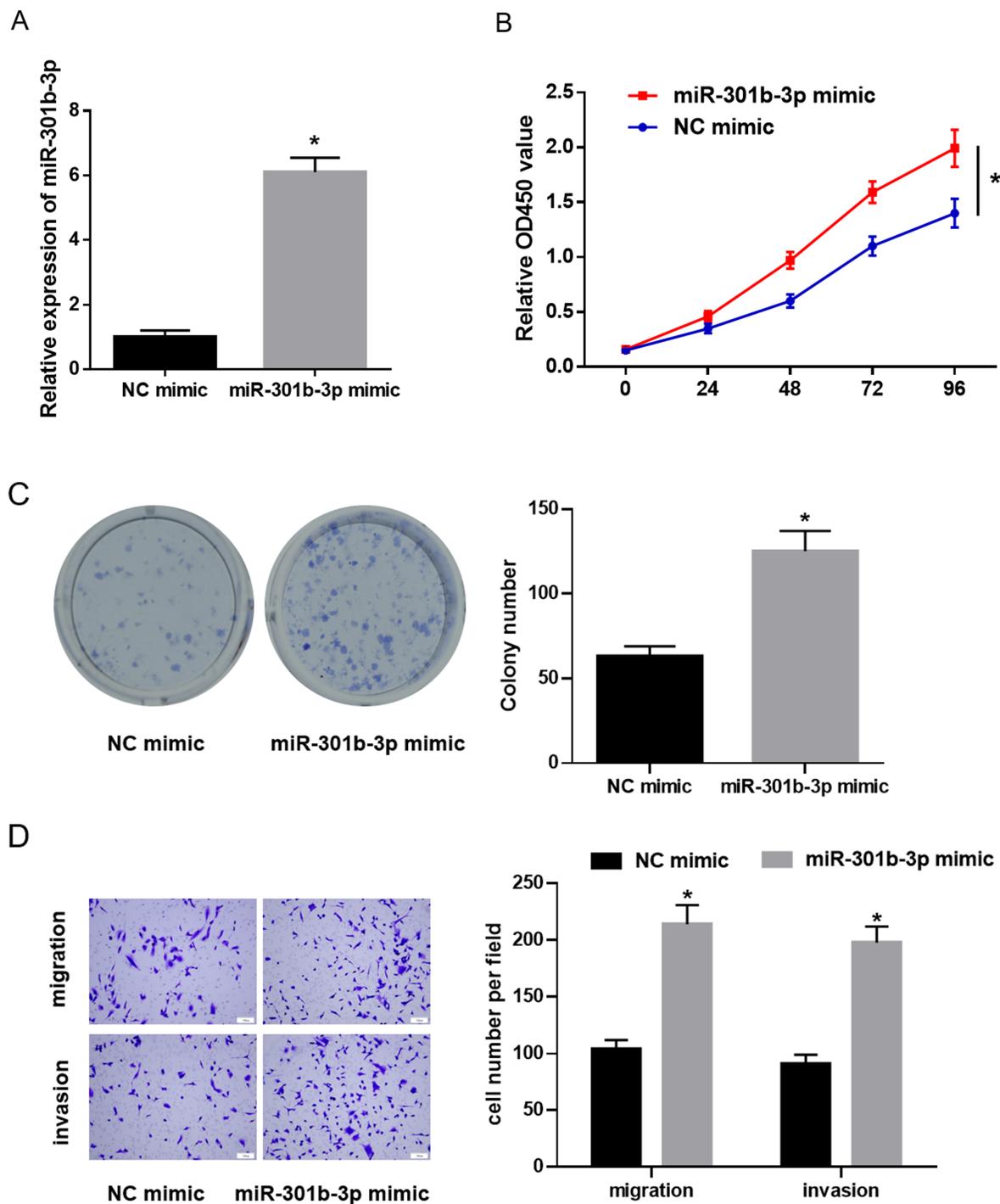


Figure 2

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 migration and invasion abilities (100×) in two groups were examined by CCK-8 assay, colony formation assay and Transwell assay, respectively; * $P < 0.05$.

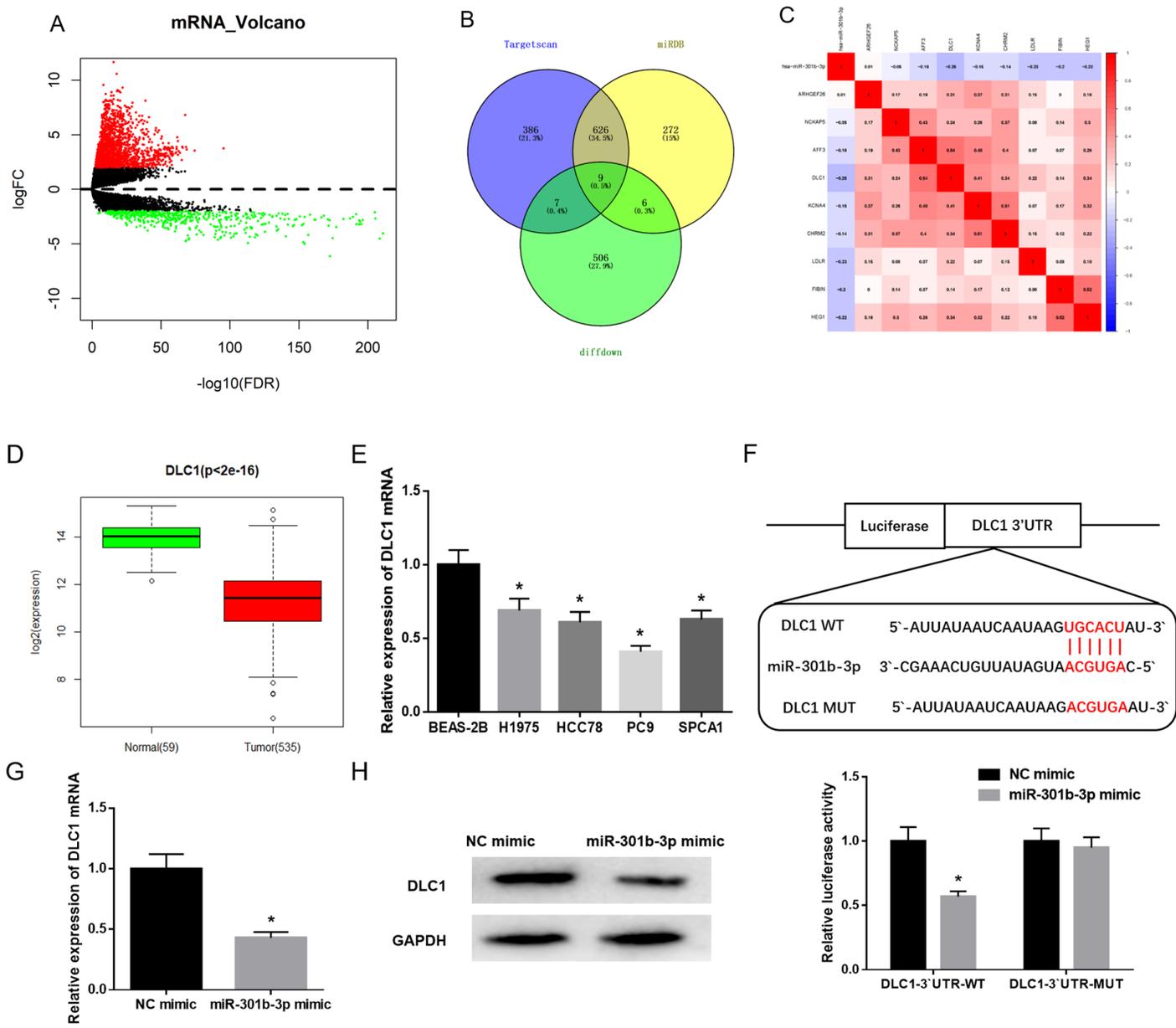


Figure 3

miR-301b-3p targeted inhibits DLC1 expression. (A) Volcano plot of DEmRNAs in normal group and tumor group in the TCGA-LUAD dataset. Red refers to up-regulated genes and green refers to down-regulated genes; (B) Venn diagram of predicted target genes of miR-301b-3p 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 the TCGA-LUAD; (E) The expression level of DLC1 mRNA in human normal lung epithelial cell line BEAS-2B 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; * P<0.05;

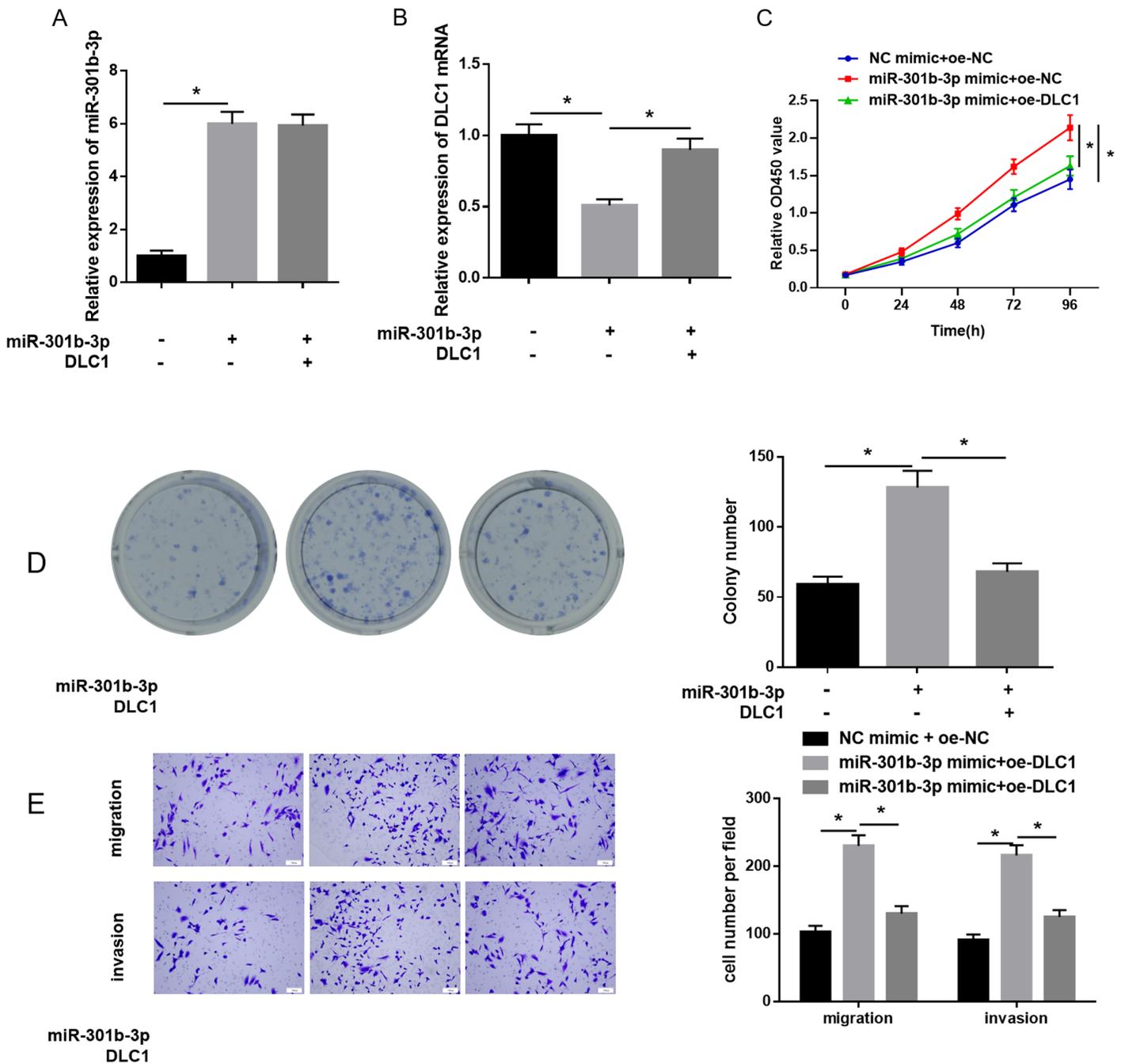


Figure 4

miR-301b-3p regulates LUAD cell proliferation, migration and invasion by targeting DLC1. The expression of (A) miR-301b-3p and (B) DLC1 mRNA in three groups were detected by qRT-PCR; (C) Cell viability, (D)

colony formation ability, (E) cell migration and invasion (100×) abilities in three groups were examined by CCK-8 assay, colony formation assay and Transwell assay, respectively; * $P < 0.05$.