Abstract. Lung cancer is the most common cause of cancer-associated mortality. MicroRNAs (miRNAs), as oncogenes or tumor suppressor genes, serve crucial roles not only in tumorigenesis, but also in tumor invasion and metastasis. Although miRNA-let-7a (let-7a) has been reported to suppress cell growth in multiple cancer types, the biological mechanisms of let-7a in lung adenocarcinoma are yet to be fully elucidated. In the present study, the molecular roles of let-7a in lung adenocarcinoma were investigated by detecting its expression in lung adenocarcinoma tissues and exploring its roles in the regulation of lung cancer cell proliferation. Let-7a expression was identified to be downregulated in lung adenocarcinoma tissues compared with normal tissues. Overexpression of let-7a effectively suppressed cancer cell proliferation, migration and invasion in H1299 and A549 cells. Let-7a also induced cell apoptosis and cell cycle arrest. Furthermore, let-7a significantly inhibited cell growth by directly regulating cyclin D1 signals. This novel regulatory mechanism of let-7a in lung adenocarcinoma provides possible avenues for future targeted therapies of lung cancer.

Introduction

The increasing rate of cancer morbidity has become a serious public health issue worldwide (1). It has been reported that there were 14.1 million newly diagnosed cancer cases, and 8.2 million cases of cancer-associated mortality in 2012 (2). Lung cancer is among the most common malignant tumors and the leading cause of cancer-associated cases of mortality worldwide (3). In lung cancer, non-small cell lung cancer (NSCLC) accounts for ~80% of all clinical cases, with a poor prognosis and a 5-year survival rate as low as 15% (4). Treatment of lung cancer usually includes a combination of surgery, radiation therapy and chemotherapy. Although considerable advances have been made in lung cancer research, there is still an urgent need to improve understanding of the molecular pathogenesis of lung cancer, particularly to identify novel therapeutic targets (5).

MicroRNAs (miRNAs) are a class of highly conserved non-coding RNAs of ~20-22 nucleotides in length (6). The regulatory roles of miRNAs in gene expression are associated with their pairing to the 3' untranslated region (3'UTR) of their target mRNAs (7-9). Following processing by the consecutive actions of Drosha and Dicer ribonucleases, miRNAs are subsequently loaded onto RNA-induced silencing complexes to interact with their target mRNAs and induce post-transcriptional silencing (2,9,10). Numerous studies have indicated that miRNAs perform their functions by regulating various biological processes, including cell proliferation,
deregulated in multiple types of malignant cells. For instance, overexpression of let-7a was demonstrated to suppress the proliferation, migration and invasion of gastric cancer cells by downregulating the expression of pyruvate kinase muscle isozyme M2 (11,14).

As a dysplastic disease, cancer formation is largely attributed to the deregulation of cell proliferation, which is strictly controlled by checkpoints of the cell cycle. G1/S and G2/M are the classic cell cycle checkpoints (11,15). As a member of the cyclin family, cyclin D1 is an important regulator of cell proliferation. Cyclin D1 reaches a peak level at the G1 stage, indicating that it is involved in the checkpoint of G1/S. Therefore, overexpression of cyclin D1 may lead to transition through the G1/S checkpoint and promotion of cell proliferation, which may eventually lead to the formation of cancer (16).

Although it has been observed that certain miRNAs are unconventionally expressed in lung cancer and associated with poor outcomes, the role of let-7a in lung cancer has not yet been fully elucidated (17). The present study aimed to investigate the effects of let-7a on cell proliferation, apoptosis, migration and invasion in A549 and H1299 cells.

Materials and methods

Lung adenocarcinoma tissues. The present study was conducted between August 1, 2014 and July 31, 2015 at the Inpatient Department of Medical Oncology, Yantai Shan Hospital, The Teaching Hospital of Binzhou Medical University (Yantai, China). The experiments were performed according to the relevant guidelines of the Code of Ethics of the World Medical Association for experiments involving humans and the Medical Ethics Committee of Binzhou Medical University (18).

A total of 20 patients (10 males and 10 females), who were pathologically diagnosed with lung adenocarcinoma for the first time and had not yet received chemotherapy, were included in the present study. Fresh lung adenocarcinoma and control tissues were obtained from the patients who underwent surgery. Written informed consent was obtained from all patients prior to the collection of lung tissue samples. The control and lung adenocarcinoma tissues were collected from the same patients, and control samples were normal adjacent control tissues.

Quantitative polymerase chain reaction (qPCR). Lung adenocarcinoma cells were incubated with let-7a-mimics or let-7a-inhibitor and harvested 48 h after miRNA treatment. Small RNA was isolated using RNAiso for Small RNA reagent (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed as previously described (14). The primers used to amplify cyclin D1 (GenePharm Co., Ltd.) were as follows: Forward, 5'-CGC GGA TCC AAG AGA AGA GGG ACA CAG CC-3' and reverse, 5'-CGC TTCAGATCTTACCTCGTGGAG-3'. Total RNA was isolated using TRIzol reagent (Takara Biotechnology Co., Ltd.). The primers used for amplifying cyclin D1 (GenePharm Co., Ltd.) were as follows: Forward, 5'-CTGGGCAACTAGTCCCTTGA-3' and reverse, 5'-GGATCGCATCACTGGGAG-3'. The amplification template was human genomic DNA. Then, cyclin D1-3' UTR was inserted into the pcDNA3.1-GFP-neo (+) expression vector.

Cell culture and transfection. Lung adenocarcinoma cell lines (A549 and H1299) were obtained from the Shanghai Institute of Cell Biology (Shanghai, China). HBE 135E6E7 cells were obtained from the American Type Culture Collection (human bronchus epithelial; ATCC® CRL-2741; Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO2. Cells (2x10⁵) were seeded into 6-well plates. Let-7a mimics, let-7a inhibitors, negative controls and si-cyclin D1 were synthesized by Shanghai GenePharma Co., Ltd. The target sequence of si-cyclin D1 was as follows: Sense, 5'-CAAACAGUAUACUCCGAA-3' and antisense, 5'-UUGCGGGAUACUGUU-3'. Transfection was performed in triplicate at ~60% confluence using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol (19).

MTT assay. Cell proliferation assays were conducted using a modified colorimetric MTT assay as previously described (20). All procedures were repeated three times. Cell colony formation rate was assessed using a plate colony formation assay. The plate was gently washed and stained with crystal violet. Then, the number and size of colonies was analyzed.

Apoptosis assays. An apoptosis assay was performed 48 h after the oligonucleotides were transfected into lung adenocarcinoma cells. The assay was performed using Annexin V-FITC/PI (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol (19).

Cell cycle analysis by flow cytometry. A cell cycle assay was performed 48 h after transfection. The cells were collected using a Cell Cycle Detection kit (Nanjing KeyGen Biotech,
Western blot analysis. Western blot analysis was performed as previously described (22). The antibodies used were as follows: Rabbit antibodies against GAPDH (1:800; cat. no. AP0063), Rb (1:800; cat. no. BS1311), p-Rb (1:800; cat. no. BS4164), Bcl-2 (1:800; cat. no. BS1511), Bax (1:800; cat. no. BS2538; all from Bioworld Technology, Inc., St. Louis Park, MN, USA) and caspase-9 (1:1,000; cat. no. 9502), caspase-8 (1:1,000; cat. no. 9748) and caspase-3 (1:1,000; cat. no. 9662; all from Cell Signaling Technology, Inc., Danvers, MA, USA). GAPDH was used as an internal reference.

Cell migration and invasion assays. An invasion assay was performed using a modified two-chamber plate with a pore size of 8.0 μm (23) as previously described (14). The Transwell migration assay was conducted according to the same protocol as the invasion assay, with the exception that the cell suspension was added into the upper chamber directly, without Matrigel.

Statistical analysis. SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used to analyze the significance of all results. One-way analysis of variance (ANOVA) was used to analyze the differences among three or more groups. A post-hoc test of ANOVA was conducted by performing a Tukey's test. Correlations were calculated with Spearman's rank correlation coefficient. Group means were compared using an unpaired, two-sided, Student's t-test. Wilcoxon signed-rank test was used to compare the expression of let-7a and cyclin D1 in para-carcinoma and carcinoma tissues. Array data of cyclin D1 were downloaded from data link(s): https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13213. Overall survival was determined using Kaplan-Meier survival analysis by a log-rank test. P<0.05 was considered to indicate a statistically significant difference (11,23). All experiments were performed in triplicate, and all data are expressed as the mean ± standard deviation.

Results

Let-7a is downregulated in human lung adenocarcinoma tissues. Although let-7a has previously been investigated in other types of cancer (24), the molecular mechanism of let-7a in lung adenocarcinoma remains unclear. In the present study, we first collected the clinicopathological features of patients with lung adenocarcinoma (Table I). Lung cancer tumor-node-metastasis (TNM) staging was based on the 8th edition of the Union for International Cancer Control (UICC) (25). The number of nuclear fission images or proliferation index, necrosis range, invasion status and other parameters were used to determine the lung cancer grade according to the tumor structure and cell atypia in H&E stained sections (26-29). Then, let-7a expression was analyzed in lung adenocarcinoma tissues to investigate the roles of let-7a in lung cancer. The current results indicated that let-7a levels were significantly lower in lung adenocarcinoma tissues (n=20) compared with para-carcinoma tissues (2 cm from the para-carcinoma tissues, n=20, Fig. 1A; P<0.001).
Figure 1. let-7a is markedly downregulated in human lung adenocarcinoma tissues. (A) Let-7a expression in lung adenocarcinoma tissues (n=20) and para-carcinoma tissues (n=20) was revealed by qRT-PCR. *P<0.001, vs. para-carcinoma. (B) Overall survival was determined through Kaplan-Meier survival analysis. The low survival rate of cancer patients was related to high cyclin D1 expression. Kaplan-Meier survival analysis was performed to determine the significance of cyclin D1 expression in the outcome of lung cancer patients from the GSE13213 dataset. (C) We detected the expression of cyclin D1 protein in lung adenocarcinoma, and found that the expression of cyclin D1 was considerably higher in lung adenocarcinoma tissues (n=10) than that in para-carcinoma tissues. (D) The mean protein expression levels of cyclin D1 in tumor and normal para-carcinoma tissues. *P<0.001. (E) The mRNA levels of cyclin D1 in para-carcinoma and carcinoma tissue samples obtained from the 20 patients with lung cancer, *P<0.0001.

Figure 2. Let-7a is markedly downregulated in A549 and H1299 cells. (A) Let-7a expression in A549, H1299 and control HBE cells was revealed by qRT-PCR. *P<0.001. (B and C) The levels of let-7a were detected after let-7a-mimic and let-7a-inhibitor transfection in A549 and H1299 cells. *P<0.05, vs. the scrambled control group.
current results indicated that let-7a is involved in the progression of lung adenocarcinoma.

Kaplan-Meier survival analysis was performed to determine the significance of cyclin D1 expression in affecting the outcome of lung cancer patients from the GSE13213 dataset. The poor survival time of patients was not significantly correlated with age (P=0.360) or sex (P=0.278). Notably, patients with high cyclin D1 levels exhibited short survival time (P=0.003; Fig. 1B) through a log-rank test analysis, suggesting that higher cyclin D1 levels increase the risk of lung adenocarcinoma. Then, the expression of cyclin D1 was evaluated in lung adenocarcinoma tissues. The results indicated that the expression of cyclin D1 was considerably higher in lung adenocarcinoma tissues (n=10) compared with para-carcinoma tissues (2 cm from the para-carcinoma tissues, n=10, Fig. 1C).

Subsequently, we assessed the protein and mRNA expression levels of cyclin D1 in para-carcinoma and carcinoma tissue samples, and our results revealed that cyclin D1 levels were significantly higher in lung adenocarcinoma tissues (n=20) than those in para-carcinoma tissues (Fig. 1D and E; P<0.0001). These results indicated that lower levels of let-7a and higher levels of cyclin D1 may be associated with the development of lung adenocarcinoma.

**Let-7a is downregulated in lung adenocarcinoma cell lines.** Let-7a levels were detected in lung adenocarcinoma cell lines (A549 and H1299) and control human bronchus epithelial (HBE) cell line by qPCR. Notably, let-7a levels were reduced in the two lung adenocarcinoma cell lines compared with the HBE cells (Fig. 2A; P<0.001). Let-7a-mimic oligo treatment
led to significant upregulation of let-7a compared with the control group (P<0.05). Conversely, the expression of let-7a was significantly reduced in lung adenocarcinoma cell lines following let-7a-inhibitor transfection (P<0.05; Fig. 2B and C).

**Let-7a directly targets cyclin D1.** To elucidate the molecular mechanisms of let-7a in the suppression of the growth of lung adenocarcinoma cells, the putative target gene of let-7a was identified. It was indicated that cyclin D1 was a target gene of let-7a with high probability using TargetScan software online (Fig. 3A; http://www.targetscan.org/vert_71/). Then, a cyclin D1-3’ UTR GFP plasmid was constructed to transfect cells with let-7a. The results indicated that GFP fluorescence intensity and positive rate decreased significantly in let-7a-treated cells compared with the control treatment (P<0.05; Fig. 3B), indicating that cyclin D1-3’ UTR was directly targeted by let-7a. Next, the effects of let-7a on cyclin D1 expression in lung cancer cell lines were investigated by western blot analysis. Notably, the expression of cyclin D1 was significantly reduced in lung adenocarcinoma cell lines following overexpression of let-7a (P<0.05). Conversely, the let-7a inhibitor resulted in the significant upregulation of cyclin D1 compared with the control group (P<0.05; Fig. 3C-F). Cyclin D1 is a positive regulator of cell cycle progression (11), and these results indicated that let-7a suppresses lung cancer cell growth by regulating the pathway of cyclin D1.

**Let-7a inhibits cellular proliferation and colony formation.** To further assess the potential inhibitory roles of let-7a in lung adenocarcinoma, the effects of let-7a on cell proliferation and colony formation were studied. An MTT assay indicated that the proliferation of lung adenocarcinoma cells in the let-7a-treated groups was significantly decreased compared with the scrambled oligo-treated cells (P<0.05). In contrast, the let-7a-inhibitor caused a significant increase in cell proliferation compared with the scrambled oligo treatment (P<0.05; Fig. 4A and B).

Figure 4. Upregulation of let-7a suppresses cell proliferation and colony formation. (A and B) The MTT assay assessed the viability of A549 and H1299 cells that were transfected with let-7a-mimics and let-7a-inhibitor, respectively. *P<0.05 vs. the scrambled control. (C) The effects of let-7a on the colony formation of A549 cells. *P<0.01 vs. the scrambled control. (D) The effects of let-7a on the colony formation of H1299 cells. *P<0.01 vs. scrambled control.
A colony formation assay was performed to evaluate the long-term effect of let-7a on cell proliferation. Compared with the scrambled control treatment, a smaller size and a decreased number of clones were observed in the let-7a-treated cells, suggesting that the upregulation of let-7a significantly suppressed the colony formation ability of A549 and H1299 cells (Fig. 4C and D).

Let-7a regulates cell apoptosis and apoptosis-associated proteins. Cell apoptosis was detected following transfection of lung adenocarcinoma cells with let-7a mimics and controls. Cell apoptosis was significantly increased in let-7a-treated A549 and H1299 cells, indicating that let-7a overexpression induced lung adenocarcinoma cell apoptosis (P<0.05; Fig. 5A and B). To further characterize the

Figure 5. The effects of let-7a on cell apoptosis and gene expression. (A) Cell apoptosis in A549 cells was determined by flow cytometry. *P<0.05. (B) The effects of let-7a on apoptosis were analyzed in H1299 cells. *P<0.05. (C) Let-7a significantly downregulated Bcl-2 expression and upregulated the expression of Bax, cleaved-caspase-3, -8 and -9, compared with the scrambled control treatment in A549 cells. The let-7a-inhibitor increased the Bcl-2 levels and reduced Bax, and decreased cleaved-caspase-3, -8 and -9. (D) The expression of Bcl-2, Bax and cleaved-caspase-3, -8 and -9 was detected in the H1299 cell line. GAPDH was used as an internal control.
possible mechanisms of let-7a in inducing cell apoptosis, the apoptotic genes, Bcl-2 (as an anti-apoptotic protein) and Bax (as a pro-apoptotic protein), were studied. It was determined that let-7a treatment significantly downregulated Bcl-2 expression and upregulated the expression of Bax and cleaved caspase-3, -8 and -9, compared with the control treatment in A549 and H1299 cells. In contrast, the opposite trends for these proteins were observed in cells transfected with the let-7a-inhibitor (Fig. 5C and D). These findings demonstrated that the Bcl-2/Bax pathways and the activation of caspase-3, -8 and -9 were constitutively downregulated by let-7a overexpression, and that the Bcl-2 family proteins are involved in the apoptosis induced by let-7a.

**Let-7a functions as an inhibitor of cell cycle progression.** The effects of let-7a on cell cycle regulation were evaluated by flow cytometric analysis (30). The results indicated that the distribution of the cell cycle in A549 and H1299 cells was notably affected by let-7a overexpression compared with the scrambled group. The cell cycle profile indicated that 77.02% of A549 cells and 79.01% of H1299 cells were arrested at the G0/G1 phase at 48 h after let-7a treatment, compared with 68.09% of NC-transduced A549 cells and 68.95% of NC-transduced H1299 cells. However, the let-7a inhibitor-transduced cells exhibited a smaller percentage of arrest at the G0/G1 phase and increased cells inhibited in the G2/M phase compared with let-7a treatment (Fig. 6A and B). These experiments demonstrated that let-7a exerts an inhibitory effect on cell cycle progression. To investigate the potential molecular mechanism of let-7a in cell cycle arrest, the expression of cell cycle molecules, namely cyclin D1, Rb and p-Rb, was detected. Let-7a treatment significantly
decreased the expression of cyclin D1 and p-Rb, but increased the Rb protein expression compared with the controls. The opposite trends were observed in let-7a inhibitor-transduced cells (Fig. 6C and D). Therefore, let-7a induces G0/G1 arrest in A549 and H1299 cells, which may be mediated by downregulation of cyclin D1 and upregulation of Rb.

**Let-7a inhibits cell migration and invasion.** Cyclin D1-associated factors may affect the migration and invasion potential of breast cancer cells (31). In the present study, Transwell experiments were performed to detect whether let-7a inhibits lung cancer cell migration and invasion by influencing cyclin D1-associated factors. The number of migrated cells in let-7a-treated A549 and H1299 cells was significantly reduced compared with the controls (P<0.0001; Fig. 7A and B), while the let-7a inhibitor increased the number of migrated cells. There was no significant increase in the rate of migration in A549 and H1299 cells co-transfected with let-7a-inhibitor and si-cyclin D1 (P<0.001; Fig. 7E and F). Furthermore, an invasion assay demonstrated that invasive ability was significantly decreased in let-7a-transfected A549 and H1299 cells compared with the control groups (P<0.0001; Fig. 7C and D), and the effects of let-7a on invasion could be reversed by knockdown of cyclin D1 using siRNA (P<0.001; Fig. 7G and H). These results indicated that let-7a inhibits cell migration and invasion by targeting cyclin D1.
Discussion

As small non-coding RNAs, miRNAs are able to influence numerous biological processes through post-transcriptional regulation of gene expression (18). Over the past two decades, numerous studies have indicated miRNA changes are associated with cancer biology, including cancer formation, development and metastasis (18,32). Let-7 miRNAs are underexpressed in the blood of patients with NSCLC (33) and expressed at lower levels in lung cancer (34). Similarly, the current results indicated that the expression of let-7a in lung adenocarcinoma tissues is reduced, suggesting that loss of let-7a may be a key factor in lung cancer development.

Gain-of-function and loss-of-function experiments were performed to observe the effects of let-7a on the biological characteristics of A549 and H1299 cells. It was determined that let-7a treatment inhibits proliferation and induces apoptosis of lung adenocarcinoma cells, via a decrease of cyclin D1. It was previously demonstrated that cyclin D1/cyclin-dependent kinase 4 may interact with filamin A and impact the migration and invasion potential of breast cancer cells (31). In the present study, it was determined that migration and invasion abilities were reduced following let-7a upregulation in A549 and H1299 cells.

Aberrant expression of cyclin D1 is observed frequently in a variety of tumor types, and cyclin D1 is regarded as a prognostic marker in multiple cancer types (35,36). Recently, researchers have aimed to explore the roles of miRNAs in regulating cyclin D1. Lower expression of miR-138 increases cyclin D1 expression, which serves a key function in the pathogenesis of OLP mucosal disease (37). MiRNA-520a-3p induces breast cancer cell apoptosis by directly targeting cyclin D1 (38). In the present study, it was revealed that let-7a, as a novel miRNA, directly suppresses cyclin D1-associated signals, which effectively inhibits cell growth and induces cell apoptosis. As downstream factors of cyclin D1, Bel-2 was
decreased, whereas Bax was increased, in let-7a-treated A549 and H1299 cells. These phenomena may be associated with the inhibitory effect of let-7a on lung cancer cell proliferation. The regulation of Bcl-2 promotes the mitochondria to release cytochrome c, which further induces cell apoptosis through caspase signals (39). The current results demonstrated that let-7a could induce lung adenocarcinoma cell apoptosis by reducing Bcl-2 and upregulating cleaved caspase-3, -8 and -9.

Although it was revealed in the present study that let-7a inhibits the malignant behavior of lung cancer cells by targeting cyclin D1, the mechanisms of let-7a remain to be elucidated. The regulatory networks between miRNAs and mRNAs indicate that their interactions are complex. Therefore, it is necessary to assess the roles of other miRNAs in the regulation of cyclin D1 in lung adenocarcinoma. Further investigations are warranted in order to extend these findings, and in vivo studies are required to provide more convincing data about the roles of cyclin D1 and let-7a in lung adenocarcinoma (31).

In summary, the present study demonstrates novel roles of let-7a in lung cancer. Let-7a significantly inhibits cell proliferation and enhances apoptosis of lung adenocarcinoma cells by directly regulating cyclin D1 signals. The current findings suggest that let-7a may be a novel therapeutic target in patients with lung cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SYX and CZ conceived and designed the study. WZ, JXH, RMH, QZ, JQG, YJL, NX, LYL and PYW performed the experiments. WZ, JXH and SYX wrote the paper. CZ and SYX reviewed and edited the manuscript. 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 are appropriately investigated and resolved.

Ethics approval and consent to participate

The experiments were performed according to the relevant guidelines of the Code of Ethics of the World Medical Association for experiments involving humans and the Medical Ethics Committee of Binzhou Medical University. Written informed consent was obtained from all patients prior to the collection of lung tissue samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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