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ORIGINAL ARTICLE

Effect of miR-375 on non-small cell lung carcinoma invasion, migration, and proliferation through the CIP2A pathway

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Abstract

Objective The aim of this study was to study the effect of miR-375 on non-small cell lung carcinoma (NSCLC) invasion, migration, and proliferation through the CIP2A pathway.

Methods We constructed a stable over-expressing cell line with lentivirus as the experimental group (Lv-miR-375) and transfected the empty vector as the negative control group (Lv-NC). The expression level of miR-375 was detected using real-time fluorescence quantitative PCR (qRT-PCR).Western blots were used to detect the expression levels of cancerous inhibitor of PP2A (CIP2A), MYC, protein kinase B (AKT) and p-AKT in Lv-NC- and Lv-miR-375-transfected cells. Transwell assays were conducted to detect the cell invasion and metastasis ability, and the cell counting kit-8 (CCK8) was used to detect cell proliferation.

Results qRT-PCR showed that miR-375 was overexpressed in NSCLC. Compared to the Lv-NC-transfected cells, the western blot results showed that CIP2A, MYC and p-AKT were highly expressed in Lv-miR-375-transfected cells. Transwell assays showed that the invasion and migration ability of Lv-miR-375-transfected A549 cells was significantly higher than that of Lv-NC-transfected cells. CCK8 experiments showed that compared to Lv-NC-transfected cells, the cell proliferation ability of the Lv-miR-375-transfected cells increased.

Conclusion MiR-375 could promote the invasion, migration, and proliferation of NSCLC A549 cells via the CIP2A pathway. MiR-375 is expected to become a new target for the treatment of NSCLC, and may become an important biomarker for the diagnosis, prognosis, and treatment of the disease.

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Key words: miR-375; invasion; migration; non-small cell lung cancer (NSCLC); CIP2A

Lung cancer is one of the malignant tumors with the fastest increase in morbidity and mortality and the greatest threat to people's health and life. Worldwide, lung cancer is the leading cause of cancer death in men and the second leading cause of cancer deaths in women [1]. Lung cancer is divided into two major groups, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [2]. SCLC accounts for approximately 20% of lung cancers. It has a high degree of malignancy and early metastasis, and is sensitive to chemotherapy and radiotherapy. The initial remission rate is high, but it is prone to secondary drug resistance and relapse. Chemotherapy is the mainstay. NSCLC includes three major histological subtypes, lung squamous cell carcinoma (SCC), lung adenocarcinoma (ADC), and large cell lung cancer (LCLC), accounting for approximately 80% of lung cancers. Cell division is slower, and the diffusion shift is relatively late [3]. Nevertheless, while current research on the biological characteristics of different histological subtypes of NSCLC is expanding, its basic molecular mechanism is not yet clear. For example, smoking is more risky for SCC than ADC [4].

Micro-RNA is an endogenous small RNA with a length of approximately 19–25 nucleotides. As a short non-coding RNA, its main function is to regulate the

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expression level of mRNA. miRNAs can be used as proto-oncogenes or tumor suppressors, and participate in various processes including proliferation, apoptosis, metabolism, and differentiation of cells through targeted binding to different transcripts [5]. miRNAs are expressed in specific tissue and developmental stages under normal physiological conditions, but abnormal expression of miRNAs can lead to a series of pathological states, such as tumorigenesis and metastasis [6–7]. miRNA regulates the function of tumor cells by regulating the expression of functional proteins. For example, miR-206 promotes breast cancer proliferation by inhibiting estrogen receptor alpha (ERα) while miR-34a downregulates E2 factor transcription factor 2 (E2F2) expression to regulate the cell cycle and apoptosis [8–9].

CIP2A, as an oncogenic protein during the malignant transformation and progression of cancer cells, has been shown to have a certain relationship with the efficacy of many drugs in cancer treatment. Oncoprotein CIP2A, also known as KIAA1524 or P90, was named in 2007 and is a cancerous inhibitor of PP2A due to its effect on cancer cells. The stability of PP2A and MYC is controlled to form a "carcinogenic connection" [10].

In this study, we found that miR-375 regulated the expression of downstream protein kinase B (AKT), MYC, p-AKT and other related proteins through the CIP2A/PP2A signaling pathway, inhibiting the cancer phenotype of lung cancer, and affecting cell invasion, proliferation, apoptosis, and the cell morphology process. We found that the CIP2A gene is a direct binding target of miR-375. Thus, it has become a topic of great interest to explore whether and how miR-375 regulates ADC cells through the CIP2A signaling pathway. By up-regulating miR-375 in a lentivirus-transfected A549 cell line, we found that a series of phenotypes, including cell invasion, proliferation, and apoptosis were changed, and that CIP2A and its downstream signaling proteins were also changed.

Material and methods

Cell culture and transfection

The human lung adenocarcinoma cell line, A549 (Jikai Gene Company), was cultured in Roswell Park Memorial Institute (RPMI) 1640 (Solarbio Company) medium containing 10% fetal bovine serum (FBS, Solarbio Company), 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C under 5% CO_2. After the cells were grown to logarithmic growth phase, they were incubated at 37 °C for 10 h according to the instructions of the transfection kit. They were then transfected with a multiplicity of infection of 25 of miR-375-expressing lentivirus (Lv-miR-375) (Jikai Gene Company) and control lentivirus (Lv-NC) (Jikai Gene Company); then, the transfected A549 cells were stained.

RNA extraction and real-time fluorescence quantitative PCR (qRT-PCR)

After extraction of total cellular RNA with RNAIso plus, the reverse transcription reaction was performed using the PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time). The reverse transcription primer for miR-375 was 5′-GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGACACTGCC-3′. The RT-PCR primer sequences were: upstream primer 5′-GGACAGCAGGCACAGACA-3′, and downstream primer 5′-CAGTGCAGGGTCCGAGGT-3′. The PCR primers for the *CIP2A* sequence were: upstream primer 5′-ATGCAAACTTGCTGCTGATG-3′, and downstream primer 5′-ATCAAACGTGGGTCCTGAAG-3′. The U6 small nuclear RNA was used as an endogenous reference for miRNA and *β-ACTIN* was used as an endogenous reference for the target gene mRNA. The reaction was performed using SYBR green, with the following conditions: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 34 s, and fluorescence detection at 74°C for 3 s. The difference between the two was compared with the $2^{-\Delta\Delta Ct}$ values, and 3 replicates were used for each group of experiments.

Western Blot

A western blot assay was used to detect protein expression levels. The total protein lysate was extracted using instruction manual and stored at –80°C for later use. According to the kit instructions, the protein concentration was determined using a bicinchoninic acid assay, diluted, and mixed with 5× loading buffer. Proteins were denatured at 95°C for 10 min, and a 50 μg sample was loaded to separate proteins by electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and blocked with 5% skim milk powder in a shaker for 1 h at room temperature. Membrane strips were placed in one of the following primary antibodies and incubated overnight at 4°C: AKT (1:1000, Cell Signaling Technology (CST), USA), p-AKT (1:1000, CST, USA), MYC (1:1000, CST, USA), CIP2A (1:1000, CST, USA), and β-ACTIN (1:3000, CST, USA). Membranes were washed three times with Tris-buffered saline, 0.1% Tween 20 (TBST), 10 min/wash, and incubated with the secondary antibody for 2 h at room temperature. Membranes were washed three times with TBST, 10 min/wash, soaked in enhanced chemiluminescent (ECL) solution, and the strip was placed in the cartridge and exposed in the darkroom. Bands were analyzed using grayscale analysis with Photoshop CS6, and statistics were conducted on the grayscale analysis values.

Transwell Assay

For the invasion test, Matrigel® and serum-free medium were diluted 1:9. The dilution (100 μL) was added to the Transwell chamber and placed in a 37 °C incubator for 4 h. Then, 50,000 cells each of Lv-NC- and Lv-miR-375-transfected cells were added to the Transwell chamber, 20% FBS containing RPMI 1640 was added to the lower chamber, and the Transwell chamber was incubated at 37 °C for 48 h. The chamber was removed from the incubator, the culture solution from the upper chamber was removed, and the cells in the upper chamber were scraped off with a sterile cotton swab. After fixing for 10 min with methanol, cells were stained with crystal violet for 10 min and then photographed. This experiment was repeated three times.

For the migration test, 50,000 cells each of Lv-NC- and Lv-miR-375-transfected cells were added to the Transwell chamber. The experimental procedure was followed by an invasive experiment. This experiment was repeated three times.

Cell Counting Kit-8 (CCK8) assay

To measure cell proliferation, 3000 cells of the Lv-NC- and Lv-miR-375-transfected cells were seeded into 96-well plates, and the CCK8 reagent was added at 24, 48, and 72 h, respectively, and then detected 4 h later. This experiment was repeated three times.

Statistical analysis

GraphPad Prism 5 software was used for statistical analysis. Statistical results are expressed as the mean ± standard deviation. Comparisons among groups for the RT-PCR and western blots were performed using one-way analysis of variance. The test level was $P = 0.05$ and $P < 0.05$ was considered significant. All experiments were repeated three times.

Results

MiR-375 was highly expressed in NSCLC

As shown in Fig. 1a, compared to normal human lung epithelial cells, BEAS-2B, the expression levels of miRNA-375 were significantly upregulated in transfected A549 cells ($P < 0.01$). As shown in Fig. 1b, compared to Lv-NC-transfected cells, the expression of miRNA-375 was significantly increased in Lv-miR-375-transfected cells ($P < 0.01$).

MiR-375 promoted invasion and metastasis of NSCLC

Compared to Lv-NC-transfected cells, the metastasis ability (Fig. 2a and 2c, $P < 0.01$) and invasive ability (Fig. 2b and 2d, $P < 0.01$) of Lv-miR-375-transfected cells was increased. This experiment demonstrated that miR-375 promoted the invasion and metastasis ability of NSCLC.

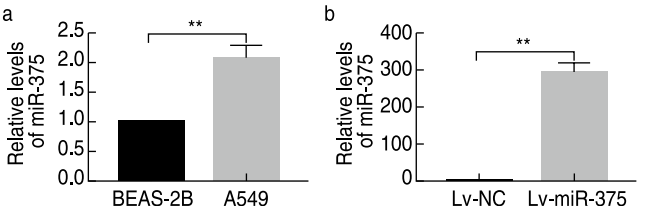

Fig. 1 Expression levels of miR-375 are upregulated in NSCLC. (a) Relative levels of miR-375 in BEAS-2B and A549 cell lines. (b) Relative levels of miR-375 in Lv-NC- and Lv-miR-375-transfected cells. $^{**}P < 0.01$

MiR-375 promoted the proliferation of NSCLC

Compared to Lv-NC-transfected cells, the proliferation ability of cells was increased in Lv-miR-375-transfected cells (Fig. 3; $P < 0.01$). This experiment demonstrated that miR-375 promoted the proliferation ability of NSCLC.

Western blot

Western blot analysis of CIP2A, MYC, AKT, p-AKT and β-ACTIN protein expression in A549 cells after transfection with Lv-NC and Lv-miR-375 demonstrated that miR-375 promoted the expression of CIP2A, p-AKT/AKT and MYC protein levels. Compared to Lv-NC-transfected cells, the expression level of CIP2A (Fig. 4a and 4d, $P < 0.01$), p-AKT/AKT (Fig. 4a and 4b, $P < 0.01$) and MYC (Fig. 4a and 4c, $P < 0.01$) was increased in Lv-miR-375-transfected cells.

Discussion

Since miR-375 was discovered, it has gradually become a hot topic in the field of cancer research. miRNAs can degrade mRNA or inhibit protein translation at the post-transcriptional level through complete or incomplete complementary pairing with the 3′ non-coding region of target mRNAs. In ontogenesis, cell differentiation, and proliferation, it plays an important role in life activities, such as apoptosis. miRNAs bind to complementary sequences in the 3′ untranslated region (UTR) of their target mRNAs and induce mRNA degradation or translational inhibition [11]. Because of these mechanisms, miRNAs can regulate the expression of target genes, so miRNAs can have an anti-tumor role. Genes also play an important role, including those that drive apoptosis, those that inhibit cell proliferation and those that regulate drug efflux mechanisms [12–14]. miRNAs may act as oncogenes or tumor suppressors in the occurrence and development of tumors. By inhibiting the role of tumor suppressor genes or regulating cell apoptosis, and promoting tumorigenesis and development, miRNAs can also act as oncogenes [15–16].

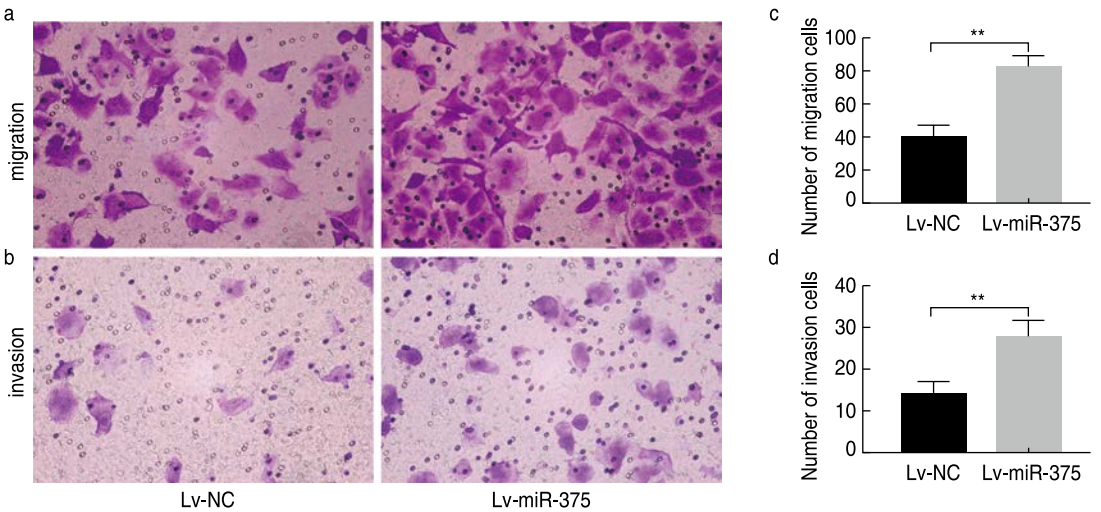

Fig. 2 MiR-375 promotes the invasion and metastasis ability of NSCLC. (a and c): Transwell migration assays in A549 cells after transfection with Lv-NC and Lv-miR-375. (b and d): Transwell invasion assays in A549 cells after transfection with Lv-NC and Lv-miR-375. $^{**}P < 0.01$

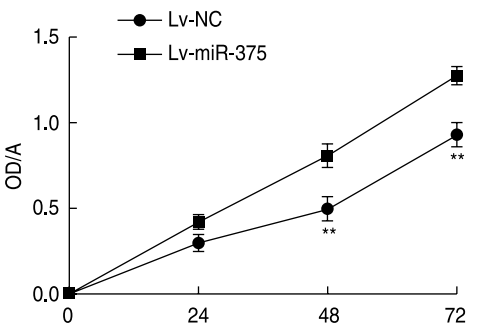

Fig. 3 MiR-375 promotes the proliferation ability of NSCLC. $^{**}P < 0.01$

The cancerous inhibitor of protein phosphatase 2A (CIP2A) is a recently identified human oncoprotein that inhibits c-MYC protein degradation in many cancer cells. CIP2A inhibits the degradation of proto-oncogenes in tumor cells by inhibiting the phosphorylation of the proto-oncogene serine 62 (S62) to regulate the expression of stable proto-oncogene proteins by inhibiting the serine of protein phosphatase 2 (PP2A). With a threonine phosphatase function, CIP2A can activate oncogenic proteins, such as proto-oncogenes (MYC), extracellular signal-regulated kinase (ERK) and AKT. Previous studies have shown that CIP2A is the main molecule that induces apoptosis of bortezomib-induced apoptosis in head and neck squamous cell carcinoma (HNSCC), triple negative breast cancer (TNBC), leukemia cells, and hepatocellular carcinoma (HCC) [17–20]. Recent studies have proposed a comprehensive interrelated CIP2A regulatory network (with an oncogenic correlation) that was established through direct interaction of multiple key cell proteins/transcription factors or CIP2A components of major oncogenic pathways or indirect CIP2A-PP2A completed interactions [21].

Related studies have found that miR-375 can influence tumor proliferation and apoptosis expression [22–24]. Therefore, we suspect that miR-375 may be an important target for the treatment of lung cancer and could improve prognosis. We decided to study miR-375 after consulting

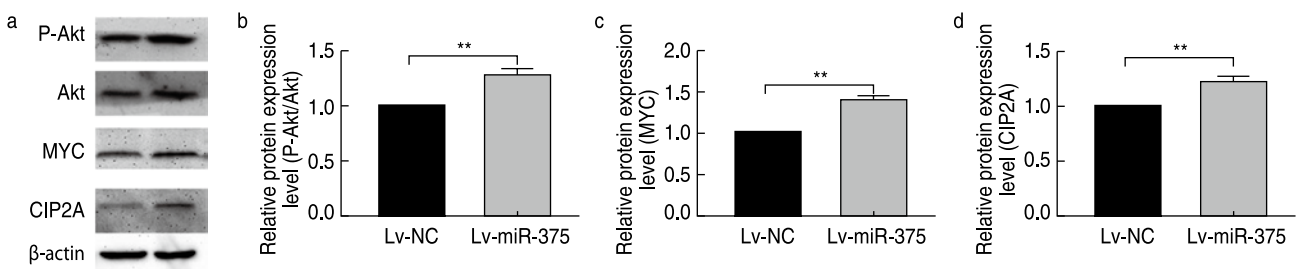

Fig. 4 MiR-375 increases the expression of CIP2A, p-AKT/AKT, and MYC. (a) Western blot assays for CIP2A, p-AKT, AKT, MYC, and β-ACTIN in A549 cells after transfection with Lv-NC and Lv-miR-375; (b) Analysis of p-AKT and AKT western blot results; (c) Analysis of CIP2A western blot results; (d) Analysis of MYC western blot results. $^{**}P < 0.01$

relevant domestic and foreign literature. After screening, miR-375 was highly expressed in the NSCLC A549 cell line. Among all types of lung cancer, NSCLC accounts for approximately 85%, and the 5-year survival rate is 15% [25]. When we up-regulated miR-375 in A549 cells, we found that cell invasion, metastasis, and proliferation were increased, apoptosis was reduced, and CIP2A and MYC were elevated with increasing levels of miR-375. miR-375 could affect lung cancer invasion, proliferation, and apoptosis ability through CIP2A, MYC and the epithelial-to-mesenchymal transition. We identified CIP2A as a novel miR-375 target that was regulated by post-transcriptional control through multiple binding sites on the CIP2A coding region and thus stabilized the oncogenic MYC. However, the specific mechanisms involved in miR-375, its effect on NSCLC, and the many uncertainties in the application of targets to the clinic require further scientific research.

In this experiment, miRNA-375 was over-expressed in the lung cancer cell line A549 by recombinant lentivirus transfection. A quantitative detection of the target protein was performed using western blot analysis. At the same time, Transwell assays were used to test the invasion and migration of cells. The proliferation of cell lines was determined by CCK-8. Compared to Lv-NC-transfected cells, these experiments have confirmed that the ability of cells to invade, metastasize and proliferate was increased in Lv-miR-375-transfected cells. This study further investigated the effect and interaction of miR-375 on the CIP2A pathway in lung cancer A549 cells. Western blot analysis detected the expression of major proteins, including MYC and p-AKT in the CIP2A pathway, and showed that miR-375 could significantly up-regulate MYC and p-AKT protein expression.

Taken together, these results suggested that miR-375 promoted invasion, migration, and proliferation of NSCLC via the CIP2A pathway. Therefore, miR-375 is expected to become a new target for neoplastic diseases and drug treatment. It is also likely to become a very important biomarker for early diagnosis, prognosis, and treatment of diseases.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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