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ORIGINAL ARTICLE

CLK2 promotes occurrence and development of non-small cell lung cancer

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Summary

Purpose: *To explore the role of CDC-like kinase 2 (CLK2) in the development and progression of lung cancer and its regulatory mechanism.*

Methods: *Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay was used to detect the expressions of micro ribonucleic acid (miR)-573 and CLK2 in non-small cell lung cancer (NSCLC) cell lines or tissues. The cell proliferative ability after overexpression of CLK2 was determined via cell counting kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assays. It was verified using dual-luciferase reporter assay and gain-loss assay that CLK2 was the target gene of miR-573, which was regulated by miR-573. According to the reverse assay, the effect of CLK2 on the proliferation of NSCLC cells was regulated by miR-573.*

Results: *In qRT-PCR, the expression of CLK2 in NSCLC tissues and cell lines significantly rose. The CLK2 expression was increased in patients with stage III-IV NSCLC and metastasis. According to survival analysis, highly-expressed CLK2 indicated a worse prognosis. The receiver operating characteristic (ROC) curves showed that CLK2 possessed the potential as a biomarker. It was found using the bioinformatics prediction that CLK2 was a potential target of miR-573. The results of dual-luciferase reporter assay confirmed that there was a binding relation between the two, and up-regulation of miR-573 could obviously inhibit the expression of CLK2. In qRT-PCR, the miR-573 expression in lung cancer tissues obviously declined, which was significantly negatively correlated with the expression of CLK2. CCK-8 and EdU assays manifested that the proliferation of lung cancer cells could be markedly enhanced through up-regulating CLK2. Finally, the results of reverse assay showed that up-regulating miR-573 could partially reverse the promoting effect of CLK2 on cell proliferation.*

Conclusions: *Highly-expressed CLK2 significantly enhances the proliferation of lung cancer cells, thereby promoting the occurrence and development of lung cancer, which may be regulated by miR-573.*

Key words: *lung cancer, miR-573, CLK2, proliferation*

Introduction

As one of the most common malignant tumors in the world, lung cancer accounts for about 13% of all tumors, and it is histologically divided into small cell lung cancer (SCLC) and non-SCLC (NSCLC). Lung cancer has increasing morbidity and mortality rates in recent years. Lung cancer patients have a 5-year survival rate lower than 20%, and it is even lower (about 2%) in advanced patients [1,2]. Among all types of lung cancer, NSCLC accounts for 75-80%. Due to lack of typical clinical manifestations, the early screening of NSCLC is more difficult, and most patients have already been in the late stage at the time of initial diagnosis. In addition, poor prognosis is also a severe problem in the treatment of this disease [3]. At present, gene therapy of tumors has gradually become a research

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hotspot, and exploring proto-oncogenes and tumor suppressor genes related to NSCLC also provides a new research direction for lung cancer in diagnosis and treatment [4]. Therefore, it has important scientific significance and clinical value to seek effective markers for early diagnosis and prognosis of NSCLC.

CDC-like kinase (CLK) is a kind of bispecific protein kinase first discovered, and it can catalyze the serine residues of proteins and also phosphorylate the tyrosine and serine/threonine residues of substrate proteins. The CLK family contains four subtypes, namely CLK1, CLK2, CLK3 and CLK4. The proteins encoded by these four subtypes all have a highly-conserved gene sequence at C-terminus, which mainly regulate intracellular signal transduction through phosphorylating the tyrosine and serine/threonine residues of substrate proteins [5,6]. It has been found that in eukaryotes, the CLK family can regulate many biological activities in the organism via regulating the structure and function of many cells. However, there are few studies on the specific regulatory mechanism and functions of the CLK family in a variety of human diseases [7].

It has been demonstrated that CLK2 is an oncogene in breast cancer, and its down-regulation inhibits the growth of breast cancer in cell culture and xenograft models, and suppresses migration and invasion of tumor cells. The deletion of CLK2 leads to up-regulation of genes related to epithelial-mesenchymal transition (EMT) and the migration of mesenchymal splicing variants of multiple genes, such as ENAH (MENA) [8]. It can be seen that CLK2-targeted therapy may be used to regulate EMT splicing mode and suppress breast cancer growth. According to another study, CLK2 regulates the cell cycle in glioblastoma through the FOX3a/p27 pathway, thereby participating in the progression of the tumor. All these findings indicate that CLK2 may be closely associated with the tumor.

There have been no reports about CLK2 in NSCLC yet, and its expression in NSCLC and its association with the prognosis of NSCLC patients remain unclear. In this paper, therefore, the expressive abundance and clinical significance of CLK2 in NSCLC were studied, and its regulatory mechanism was preliminarily explored.

Methods

Clinical specimens

All NSCLC tissue specimens were obtained upon the consent of patients or their families, and the informed consent was signed. A total of 32 cases of carcinoma and para-carcinoma tissues were stored at -80°C. No patients underwent conventional therapy before the operation. According to the code of ethics of the *Declaration of Helsinki,* the Institutional Review Board of the Yantai Yuhuangding Hospital Ethics Committee approved this study.

Cell culture and transfection

Human lung cancer cell lines (SPC-A1, H1650, H1299 and A549) and human bronchial epithelial cell line (HBE) were bought from Shanghai Fudan Cell Bank (Shanghai, China). The cells were routinely cultured and passaged in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a 5% CO_2 incubator under saturated humidity at 37°C, and the old medium was replaced once every 3 d. When the cells covered the bottom of the culture flask in a single layer at a density of 70-80%, they were digested with 0.25% trypsin and subcultured at 1:3. Shanghai GenePharma Co., Ltd. (Shanghai, China) synthesized the miR-573 mimics and CLK2 overexpression plasmids and the corresponding negative control sequences. Before transfection, the cells were seeded into 6-well plates. Upon reaching 60-70% confluence, the original medium was replaced with serum-free medium, and then the cells were transfected using Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At 6 h after transfection, the original medium was replaced with the complete medium, and the cells continued to be cultured for subsequent experiments.

Cell counting kit-8 (CCK-8) proliferation assay

The transfected cells were resuspended in the complete medium, inoculated into a 96-well plates (1×10^5 cells/well), with 3 replicates in each group, and cultured in the incubator for 1-3 d. Twenty-four h later, CCK-8 reagent (Beyotime, Shanghai, China) was added onto the plates along the well wall, shaken and cultured at 37°C for 2 h. Finally, the optical density of each well was measured at 450 NM using an automatic enzyme-linked immunometric meter.

5-Ethynyl-2'- deoxyuridine (EdU) proliferation assay

EdU kits were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China), specifically as follows: The cells in the logarithmic growth phase were harvested, seeded into 96-well plates (4×10^3-1×10^5 cells/well), and cultured till the normal phase, followed by transfection. Medium was used to dilute EdU solution (reagent A) at 1000:1, and 50 μM of EdU medium prepared was incubated in each well for 2 h. Then, the medium was discarded, and the cells were washed with phosphate buffered saline (PBS), fixed with 50 μL of 4% paraformaldehyde in each well, and decolorized in 50 μL of glycine (2 mg/mL) in each well for 5 min. Then, the glycine solution was discarded, and the cells were washed with PBS, and stained with 100 μL of 1×Apollo® staining solution at room temperature in the dark in the shaker for 30 min. Then, the staining solution was discarded, and the cells were washed in 100 μL of penetrant (PBS with 0.5% Triton X-100) for 2-3 times (10 min/time), and then the penetrant was discarded. Afterwards the cells were cul-

tured with 100 μL of 1×Hoechst33342 solution at room temperature in the dark in the shaker for 30 min in each well and then the reaction solution was discarded, and the cells were washed with PBS, followed by observation under a fluorescence microscope.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissues and cells, and stored at -80°C, followed by qRT-PCR: 1 μg of RNA was taken, added with primers and reverse transcriptase, and transcribed (37°C for 20 min, and 95°C for 5 min). qRT-PCR was conducted according to the instructions of qRT-PCR kits (TaKaRa, Tokyo, Japan): The target gene sequences were searched in the NCBI database, and ABI (Applied Biosystems, Foster City, CA, USA) synthesized the primers. With glyceraldheyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the specifically amplified target genes were relatively quantified using $2^{-\Delta\Delta ct}$. The primer sequences were as follows: MiR-573: Forward 5'-ACACTCCAGCTGGGCTGAAGTGATGTGTAA-3'; Reverse 5'-TGGTGTCGTGGAGTCG-3'. CLK2: Forward 5'-AATATTTTTACCGGGGTCGC-3'; Reverse 5'-AGCCGCTTAGCTGGTTCATA-3'. U6: Forward 5'-ATACAGAGAAAGTTAGCACGG-3'; Reverse 5'-GGAATGCTTCAAAGAGTTGTG-3'. GAPDH: F 5'-AATCCCATCACCATCTTCC-3'; Reverse 5'-GTCCTTCCACGATACCAA-3'.

Dual-luciferase reporter assay

Based on the predicted binding site sequences, Shanghai GenePharma Co., Ltd. (Shanghai, China) constructed the CLK2-WT and CLK2-MUT luciferase reporter vectors. CLK2-wild type (WT) or CLK2-mutated (MUT) vectors and miR-573 mimics or miR-NC were transfected into A549/H1299 cells, respectively, using Lipofectamine™ 3000, and cultured for 1 d. Then, the firefly luciferase intensity and Renilla luciferase intensity were determined using the dual-luciferase reporter assay kits. The ratio of the two was used to indicate the activation degree of CLK2, reflecting the binding relation between CLK2 and miR-573.

Statistics

Measurement data were expressed as mean ± standard deviation (SD). Analysis of variance was used for comparison of means among the groups. T-test was performed for the comparison of statistically significant means between two groups. SPSS 20.0 software (IBM, Armonk, NY, USA) was used for statistical analyses. P<0.05 was considered to be statistically significant.

Results

CLK2 had a high expression in NSCLC tissues and cell lines

The CLK2 expression in NSCLC and para-carcinoma tissues was detected using qRT-PCR. It was found that the expression of CLK2 in lung cancer tissues was significantly higher than that in control group (Figure 1A). The expression of CLK2 in stage III-IV lung cancer patients was significantly

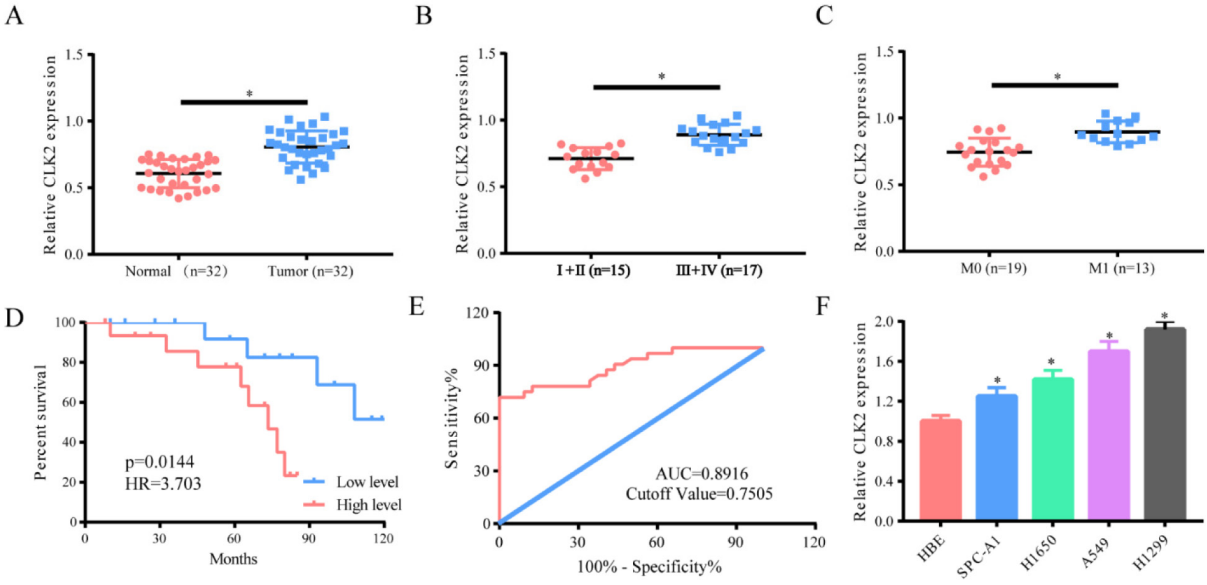

Figure 1. CLK2 had a high expression in NSCLC tissues and cell lines. **A:** According to qRT-PCR, the CLK2 expression in 32 cases of lung cancer tissues significantly rose compared with that in the control group. **B:** The CLK2 expression in stage III-IV lung cancer patients significantly rose compared with that in stage I-II. **C:** The expression of CLK2 significantly rose in patients with metastasis compared with that in those without metastasis. **D:** According to survival analysis, highly-expressed CLK2 indicated a poor prognosis (p=0.0144, HR=3.703). **E:** The ROC curves showed that CLK2 possessed the potential as a tumor marker (AUC=0.8916, cut-off value=0.7505). **F:** The CLK2 expression in lung cancer cell lines was generally high (*p<0.05).

higher than that in stage I-II (Figure 1B), and it was significantly higher in metastatic patients than that in those without metastasis (Figure 1C), indicating that CLK2 may be a player in the progression of NSCLC. Then, according to survival analysis, highly-expressed CLK2 indicated a poor prognosis of lung cancer (p=0.0144, HR=3.703, Figure 1D). The receiver operating characteristic (ROC) curves showed that CLK2 possessed the potential as a tumor marker (AUC=0.8916, cut-off value=0.7505, Figure 1E). Besides, the CLK2 expression in lung cancer cell lines was also detected. The results

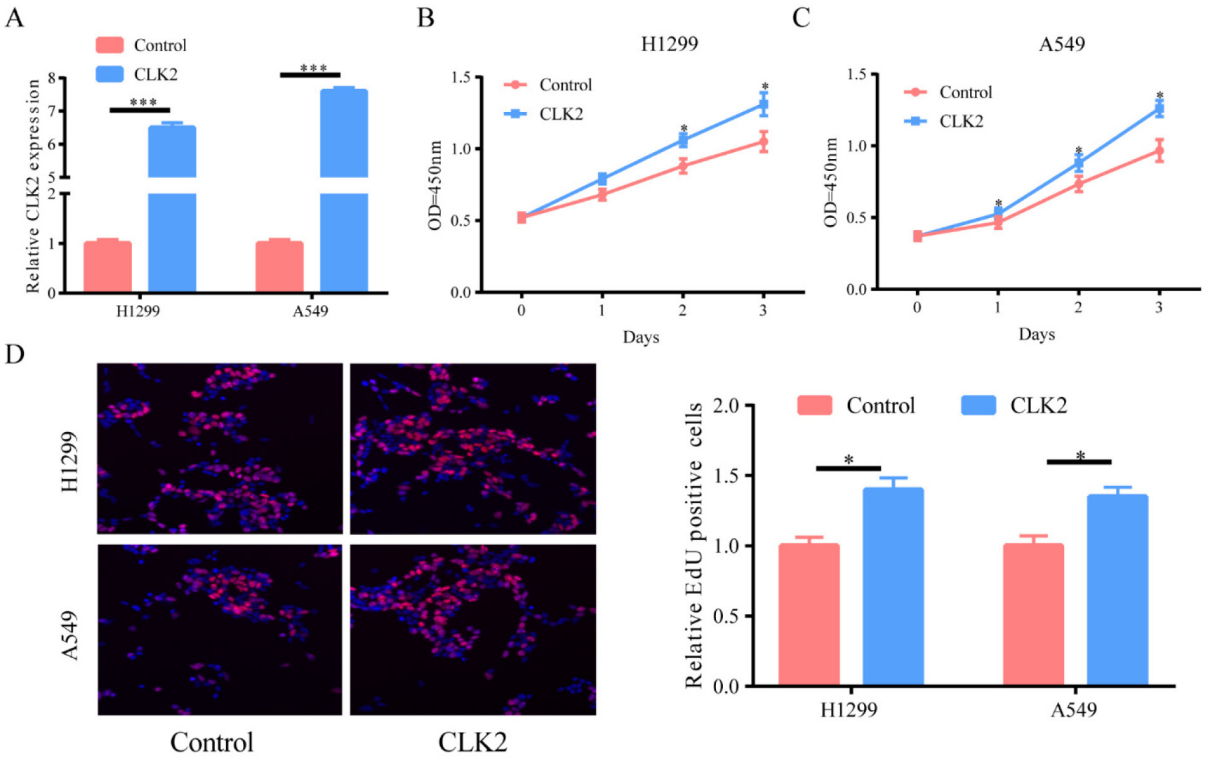

Figure 2. Up-regulation of CLK2 obviously promoted the proliferation of lung cancer cells. **A:** After transfection of A549 and H1299 cells with CLK2 overexpression plasmids, the expression of CLK2 obviously rose. **B-D:** After up-regulation of CLK2, the proliferation ability of the two kinds of cells was greatly enhanced (magnification: 200×) (*p<0.05).

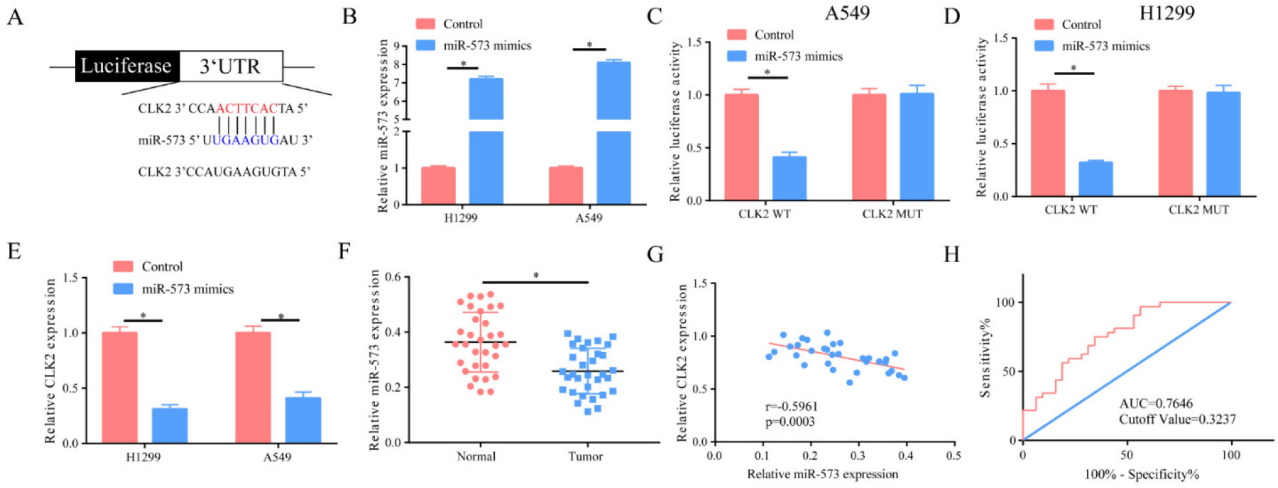

Figure 3. MiR-573 regulated CLK2. **A:** CLK2 and miR-573 had potential binding sites. **B:** After transfection with miR-573 mimics into cells, the expression of miR-573 markedly rose. **C and D:** According to dual-luciferase reporter assay, there was a binding relation between miR-573 and CLK2. **E:** After up-regulation of miR-573 in A549 and H1299 cells, the CLK2 expression remarkably declined. **F:** The expression of miR-573 remarkably declined in lung cancer tissues. **G:** The expressions of miR-573 and CLK2 in lung cancer had a negative correlation (r=-0.5961, p=0.0003). **H:** The ROC curve showed that miR-573 also had the potential as a diagnostic marker (AUC=0.7646, cut-off value=0.3237) (*p<0.05).

revealed that the CLK2 expression in lung cancer cell lines generally rose (Figure 1F), especially in A549 and H1299 cells. Therefore, the two kinds of cells were selected for subsequent functional experiments.

Up-regulation of CLK2 obviously promoted the proliferation of lung cancer cells

In order to explore the effect of CLK2 on the function of lung cancer cells, CLK2 overexpression plasmids were transfected into A549 and H1299 cells to steadily increase its expression (Figure 2A). CCK-8 and EdU assays were used to determine the effect of CLK2 on the proliferation of lung cancer cells. The results manifested that after up-regulation of CLK2, the proliferation ability of A549 and H1299 cells was greatly enhanced (Figure 2B-2D), indicating that CLK2 may work through promoting the proliferative ability of lung cancer cells.

CLK2 was regulated by miR-573

According to increasing evidence, miRs regulate genes. To further explore the mechanism of CLK2, bioinformatics analysis was performed which showed that CLK2 was a potential target gene of miR-573, having potential binding sites (Figure 3A). Prior to dual-luciferase reporter assay, the transfection efficiency of miR-573 mimics was first detected. The stably high expression of miR-573 was confirmed (Figure 3B). Then, according to dual-luciferase reporter assay, there was a binding relation between miR-573 and CLK2 (Figure 3C,3D). Furthermore, after the expression of miR-573 was up-regulated in A549 and H1299 cells, qRT-PCR confirmed that the expression of CLK2 remarkably declined in cells (Figure 3E). The above findings indicate that CLK2 is the target gene of miR-573 and regulated by miR-573. Finally, the miR-573 expression was detected in lung cancer tissues. As shown in Figure 3F, the miR-573 expression remarkably declined in lung cancer tissues, which had a negative correlation with the expression of CLK2 in cancer tissues (r=-0.5961, p=0.0003, Figure 3G). At the same time, the ROC curve of miR-573 was also analyzed and confirmed that miR-573 also had the potential as a diagnostic marker (AUC=0.7646, cut-off value=0.3237, Figure 3H)

The promoting effect of CLK2 on cell proliferation was regulated by miR-573

In order to further explore whether miR-573 regulates the role of CLK2, reverse assay was conducted. The results of CCK-8 and EdU assays manifested that in the case of up-regulation of miR-573 alone, the proliferation ability of A549 cells was

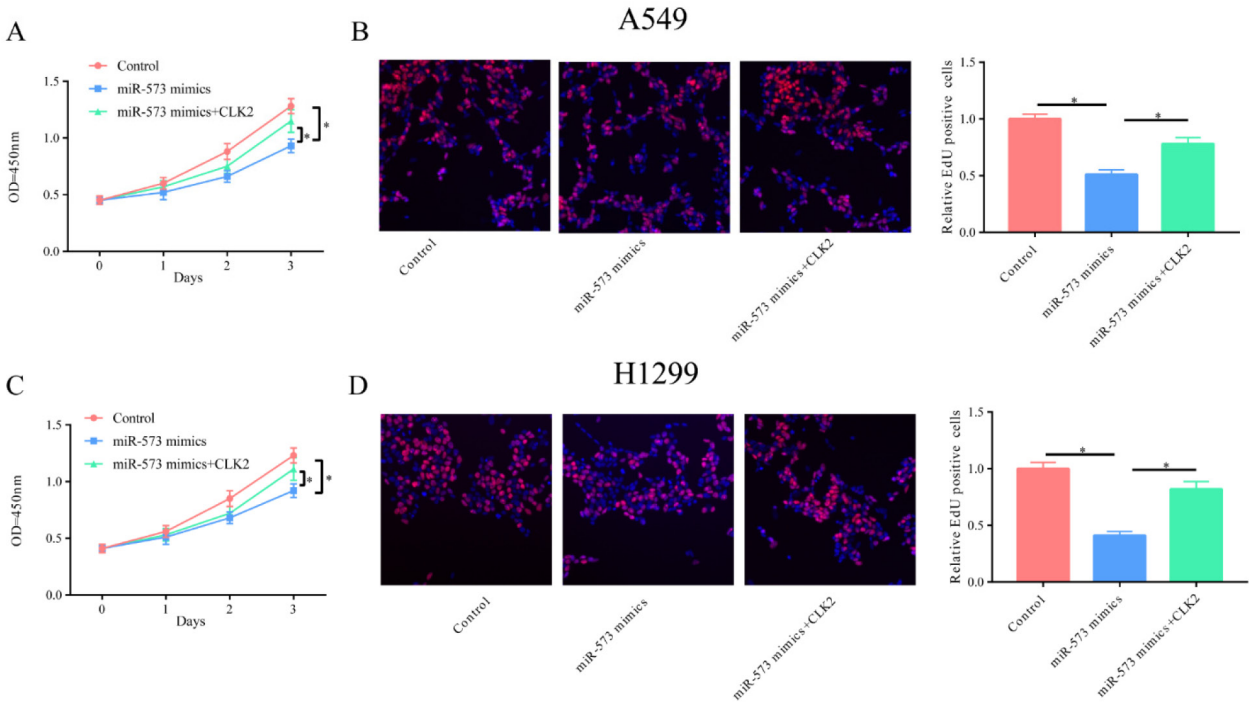

Figure 4. MiR-573 regulated the promoting effect of CLK2 on cell proliferation. **A and B:** Up-regulation of miR-573 in A549 cells could markedly inhibit the cell proliferation, and reverse the promoting effect of CLK2 on cell proliferative ability. **C and D:** The results of CCK-8 and EdU assays manifested that the promoting effect of CLK2 on H1299 cell proliferation could be partially reversed by miR-573. (magnification: 200×) (*p<0.05).

markedly inhibited. In the case of up-regulation of both CLK2 and miR-573, the promoting effect of CLK2 on cell proliferation was partially reversed (Figure 4A,4B). Meanwhile, the same results were obtained in H1299 cells (Figure 4C,4D), which showed that miR-573 regulates the promoting effect of CLK2 on cell proliferation.

Discussion

The development and progression of cancer is a complex pathophysiological process involving multiple factors and stages, such as the activated oncogenes, inactivated tumor suppressor genes, and abnormal cell signal transduction and cell cycle regulation, cell invasion, metastasis and apoptosis [8,9]. There are many reasons for the poor prognosis of lung cancer patients. Early detection, early diagnosis, and early treatment are still the best preventive measures for lung cancer patients [10,11]. Surgical resection, radiotherapy and chemotherapy are currently common clinical treatment means, but the prognosis and quality of life of cancer patients remain greatly improved [12]. With in-depth research on the pathogenesis of lung cancer in recent decades, the newly discovered targeted drugs have provided new ideas for the lung cancer treatment. However, the pathogenetic process of lung cancer still cannot be effectively clarified by its known pathogenesis. Therefore, further studying the pathogenesis of lung cancer and developing new targeted drugs have important practical significance for the treatment and prognosis of lung cancer patients. In the present study, it was found that the expression of CLK2 significantly rose in NSCLC tissues, and it had certain diagnostic potential. According to further functional experiments, CLK2 might be a player through promoting the proliferation of lung cancer cells, so it may become a new therapeutic target for this disease.

With the deepening of studies on non-coding RNAs in recent years, the mechanism of tumor occurrence and development has been gradually explored from the functional genes (coding proteins) to non-coding RNAs. The mechanism of the occurrence and development of a variety of tumors including NSCLC has been increasingly explained at the molecular level. As important regulatory factors, miRs have become a research hotspot about the prevention and treatment of malignant tumors [13,14]. A class of endogenous non-coding single-stranded small RNAs constitutes miRs, with interspecies conservation and tissue specificity. Through specific base pairing with target mRNAs, miRs can lead to degradation and translational suppression of target mRNAs, thereby regulating genes [15,16]. For example, it is reported that the expression of miR-21 in liver cancer cells is 9-fold higher than that in normal cells, and the introduction of exogenous miR-21 can inhibit the function of PTEN, thus significantly enhancing the proliferation of liver cancer cells [17]. MiR-148a is a prognostic factor inhibiting migration and invasion via Wnt1 in NSCLC [18]. Moreover, miR-4326 facilitates the proliferative ability of lung cancer cells through tumor suppressor APC2 [19].

In view of this, the potential mechanism of CLK2 was explored. It was first found through bioinformatics analysis that the target gene of miR-573 might be CLK2. According to dual-luciferase reporter assay, there was a binding relation between the two, and miR-573 regulated the expression of CLK2. The results of qRT-PCR revealed that miR-573 had a greatly lower expression in NSCLC tissues. Therefore, it is speculated that miR-573 may regulate the role of CLK2 in NSCLC. To verify the above conjecture, reverse assay was performed. In the cell function assay it was found that miR-573 had an inhibitory effect on the proliferation of lung cancer cells. Up-regulation of both CLK2 and miR-573 partially suppressed the promoting effect of CLK2 on the proliferation of lung cancer cells, indicating that miR-573 regulates such an effect of CLK2. Therefore, the above conjecture was confirmed.

Conclusions

In conclusion, CLK2 can promote the occurrence and development of NSCLC via enhancing the proliferative ability of lung cancer cells, and its role in NSCLC may be regulated by miR-573. The research results offer new research directions to the diagnosis of NSCLC in the future, and also provide a potential therapeutic strategy. However, whether CLK2 has other regulatory mechanisms for the lung cancer cell phenotype remains to be further studied.

Conflict of interests

The authors declare no conflict of interests.

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