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Original Article

LncRNA *LOC285758* Induced Non-Small Cell Lung Cancer Development through Up-Regulating CDK6 by Sponge Adsorption of miRNA-204

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Abstract

Background: Non-coding RNA played one pivotal role in NSCLC in terms of pathogenesis and progression. We aimed to determine the LncRNA, which can be one new potential target for NSCLC treatment and its possible mechanisms from Jan 2017 to Aug 2020.

Methods: Gene *LOC285758*, which produced new cells in tumor cellular system, was knocked out. Its specific effects were tested in terms of cellular phenotype. *LOC285758* was chosen to target for miRNA as well as downstream mRNA targeted by miRNA, which verified the combination predicted before. Specific impacts brought from miRNA on NSCLC cells were examined. At last, dynamic impacts produced through miRNA and *LOC285758* on mRNA expression and NSCLC cellular phenotype were examined.

Results: *LOC285758* expression was up-regulated in tissues and cells from NSCLC. Knocking out gene *LOC285758* could repress cellular survival and migration of A549 and H292 cells. miRNA-204 was repressed via *LOC285758* targeting. miRNA-204 over-expressing repressed invasion ability of NSCLC cells and CDK6 targeted by miRNA-204. CDK6 knocking out suppressed survival and migration of NSCLC cells. The influence brought from gene *LOC285758* knocking out could be reversed through suppressing miRNA-204, causing up-regulated CDK6 as well as *LOC285758* expression in NSCLC tissues. miRNA-204 was negatively correlated with CDK6 as well as *LOC285758*, respectively. Nonetheless, CDK6 possessed the positive relationship with *LOC285758*.

Conclusion: An axis of lncRNA *LOC285758*/miRNA-204/CDK6 can modulate NSCLC cells in terms of migration as well as survival.

Keywords: Cancer; Micro RNAs; Cellular

Introduction

Lung tumors, including SCLC (Small Cell Lung Cancer) as well as NSCLC (Non-small Cell Lung Cancer), are the most common malignant tumors in both mortality rate and incidence rate (1,2). NSCLC takes the 80% share of the all lung tumors, implying high incidence rate and bad prognosis (3). Besides, 5-year survival ratio is relatively low in patients with NSCLC. Thus, targeting

therapy of NSCLC is extremely pivotal (4). However, the pathogenesis mechanisms of NSCLC remains unclear.

LncRNAs refer to the non-coding RNA with length over 200 nucleotides (5). Owning to their major impacts on lives activities, they have been studied in a lot among genetics studies (6). Several researches have already explained roles of lncRNAs in cancer pathogenesis, angiogenesis and metastasis, which acting as pro-caner or anti-cancer genes, extensively take part in metastasis and progression in NSCLC8 (7,8).

MicroRNAs (miRNAs) are endogenous non-coding RNA over 22 nucleotides, exerting post-transcriptive effects among animals and plants (9). Mature miRNA can recognize mRNA through matching complementary bases and can block or degrade mRNA translation further according to match situation (10). miRNAs participate in tumor progression, virus defense, cellular apoptosis and so on through various mechanisms (11-13). miRNA-204 is a new miRNA found recently, taking part in tumor pathogenesis and progression. For cervical cancers, miRNA-204 mediated the cellular invasion and proliferation abilities through targeting for EphB214. miRNA-204 influences EMT in gastric cancers through targeting for Snail15 (14,15). In light of the basis of researches, we revealed the biology effects on NSCLC pathogenesis and progression from lncRNA *LOC285758*. LncRNA *LOC285758* sponges' miRNA-204 induced non-small cell lung cancer development as the expression of up-regulating CDK6 by ceRNA.

Methods and Materials

Ethics and clinical samples

From Jan 2017 to Aug 2020, 30 pairs of NSCLC and peripheral normal tissue specimens (3cm by NSCLC edge) were taken from Chinese Medicine Hospital in Yan Tai city.

All the testers received written consents in accordance with the "Helsinki Declaration" and all the patients joining in this study were approved by the committees from Chinese Medicine Hospital in Yan Tai city (No.S1613). The specimens were kept under -80 ℃ until being used.

Cellular transfection and culture

Normal pulmonary epithelium (BEAS-2B) was purchased from Lifeline Cell Tech (FC-0094; Oceanside, CA, USA) and was cultured within K keratinocyte medium Complete Kit (LL-0007; DermaLife, Oceanside, CA, USA). NSCLC cellular systems, H292 and A549, came from ATCC (Manassas, VA, USA). BEAS-2B, A549 and H292 cells were cultured in 1:1 mixture containing Dulbecco enhanced Eagle's medium with 10% FBS as well as Ham's F12 medium(DMEM/F12).

Cells with gene *LOC285758* silenced were gained through si-*LOC285758*-1/2/3 (GenePharma, Shanghai, China) transfection. Over-expression and repression of miR-204 in cells was produced through miR-204 transfection or antisense miR-204 carriers (GenePharma). Cells with gene *CDK6* silenced were produced through si-CDK6-1/-2/-3 (GenePharma, Shanghai, China) transfection. Liposome 3000 reagents (Thermo Fisher Scientific, Waltham, MA, USA) were used for all transfection.

RT-qPCR

Total RNAs from target tissues and cells were extracted using Trizol reagents (Invitgen, CA, USA), according to directions from manufacturers. GAPDH acted as the endogenous control, and SYBR green PCR Master Mix (QIGEN) was used to detect mRNA expression. Hairpin-it TM miRNAs qPCR(Takara, Japan) was used to examine mRNA expression. U6 acted as internal control. The $2^{-\Delta\Delta CT}$ method was used to proceed data.

CCK-8 assay

CCK-8 kits (Beyotime, Shanghai, China) was employed to detect cellular activity according to manufactures instructions. OD values were measured under 450nm wavelength.

Transwell

Cells (5×10^5) were coated on the top side of polycarbonate Transwell filter. In the Transwell migration tests, cells suspended in culture medium containing no serum, and culture medium with serum was used in the bottom chamber. Cells were incubated under 37 ℃ for two days. Cells that did not migrate in the top chamber were removed via cotton swabs. Cells migrated under membrane surfaces were fixed and dried, then dyed with crystal violet. Cellular numbers were counted via microscope.

Dual-luciferase reporter experiments

Wild type or mutant one *LOC285758* or 3'UTR were cloned on the downstream of Renilla psiCHECK2 carriers (Promega, Madison, WI, USA) to verify the conjugation of miR204 and 3'UTR or *LOC285758*. The downstream part was named as wt-CDK6 3'UTR, wt-*LOC285758*, mut-CDK63'UTR and mut-*LOC285758*. Then, 293T cells were co-transfected with miR-204 Mimics/miR-204 repressors and 2 different kinds of dual-luciferase reporter carriers, which were examined via dual-luciferase reporter carrier.

Western blotting

Cells and tissues were lysed using RIPA buffer (Thermo-Fisher Science, Waltham, MA, USA). SDS-PAGE was used to separate proteins. Then proteins were transferred on PVDF membrane (MilliPore Billerica, Massachusetts state, USA). After block within fat-free milk, these membranes were incubated with the CDK6 and GAPHD primary antibodies (1: 1500, Santa Cruz, CA, USA) under 4 °C overnight. Later, the secondary goat anti-mouse IgGs antibodies (1: 1000, Santa Cruz, CA, USA) were incubated with the membranes together. At last, ECL detector (Thermo-Fisher Scientific, Waltham, MA, USA) were adopted to detect blotting.

Statistical analysis

All data were analyzed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) and presented as mean ± standard deviation (SD). For statistical analysis, Two-tailed Student's t-test were used between different groups. $P<0.05$ were considered statistically significant.

Results

Expression of lncRNA LOC285758 was up-regulated while miR-204 was down-regulated in NSCLC tissues and cells

Compared with peripheral tumor tissues and normal pulmonary epithelial cells, expression of lncRNA *LOC285758* was up-regulated while miR-204 expression was down-regulated in NSCLC tissues of all 30 patients (Fig. 1). Cell experiments showed similar results. Up-regulation of lncRNA *LOC285758* and down-regulation of miR-204 were also observed in normal pulmonary epithelium BEAS-2B and NSCLC cells (H292 and A549).

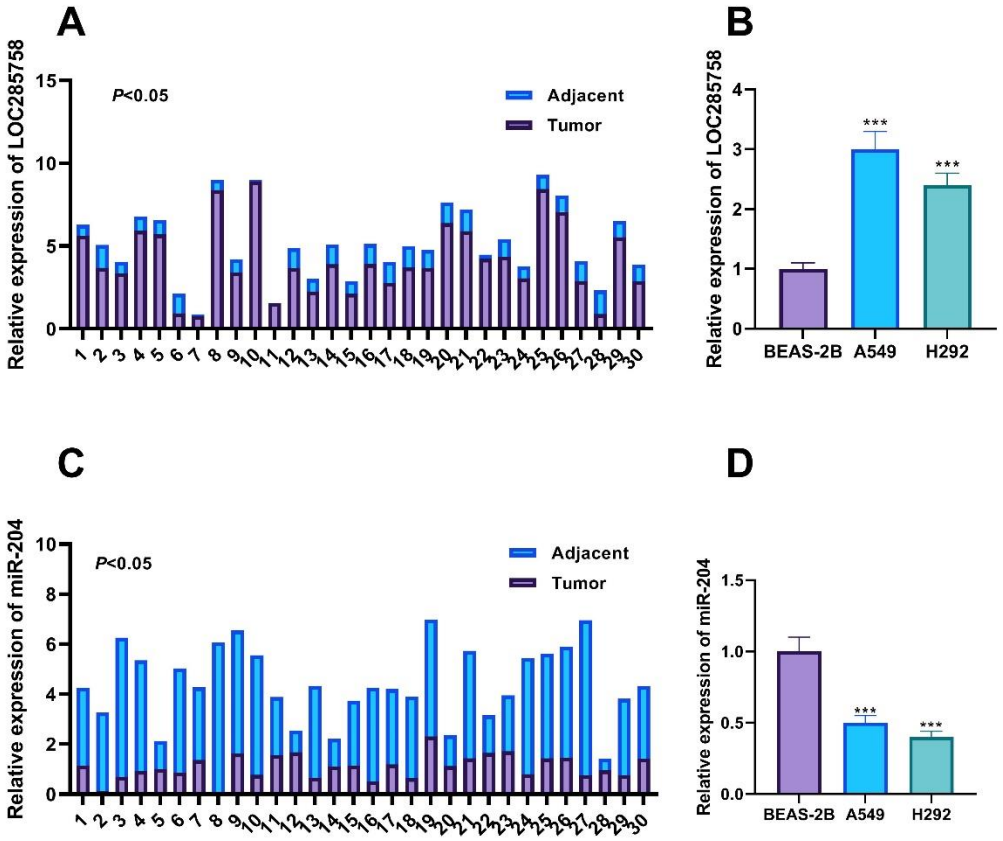

Fig. 1: Expression of miR-204 and LOC285758 in NSCLC tissues and cells. (A) Detecting *LOC285758* expression in NSCLC tissues and adjacent tissues via qRT-PCR. (B) Examining *LOC285758* expression in normal pulmonary epithelium BEAS-2B and NSCLC cells(H292 and A549) via qRT-PCR. (C) Measuring miR-204 expression in NSCLC tissues as well as tissues adjacent via qRT-PCR. (D) Measuring miR-204 expression in normal pulmonary epithelium BEAS-2B and NSCLC cells(H292 and A549) via qRT-PCR

Effects of lncRNA LOC285758 on NSCLC cellular proliferation and metastasis

The real-time fluorescence PCR showed that transfection of si-*LOC285758*-1/-2/-3 into H292 and A549 cells demonstrated the effectiveness of the decreased expression of *LOC285758* (Fig. 2A). After transfection of si-*LOC285758*, H292 and A549 cells displayed reduced migrating ability and cellular activity (Fig. 2B). Meanwhile, gene *LOC285758* silencing greatly repressed NSCLC cellular viability and migration (Fig. 2C).

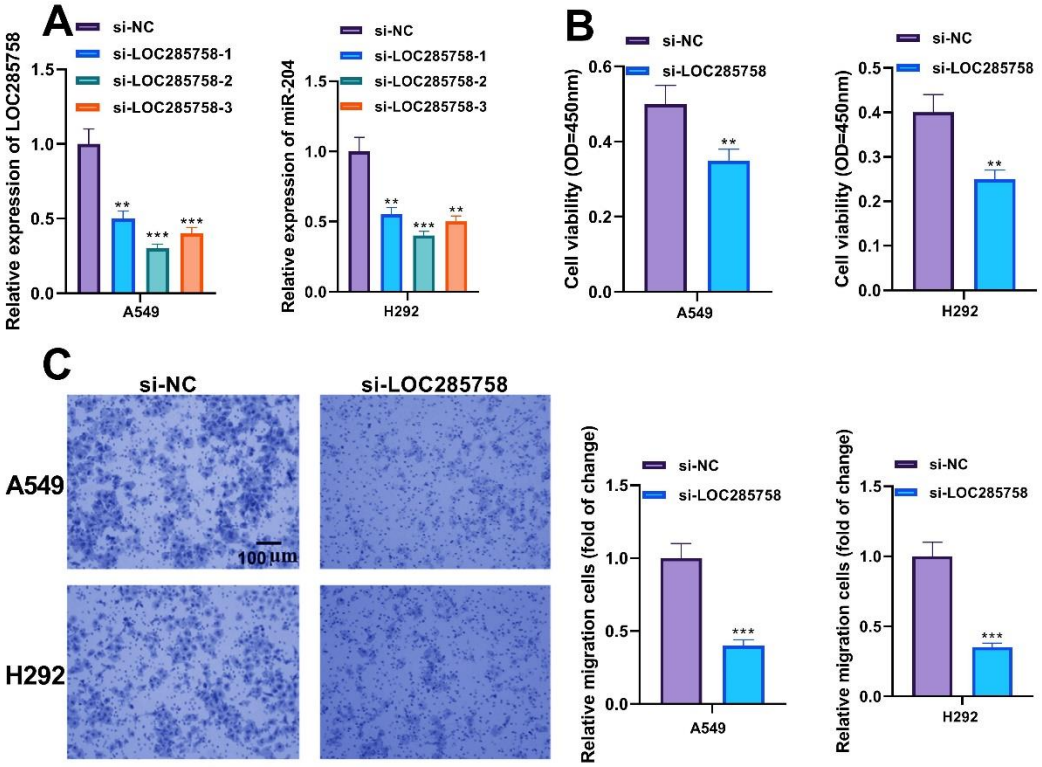

Fig. 2: The impacts on NSCLC cellular migration as well as proliferation from *LOC285758* (A) knocking gene *LOC285758* down in H292 and A549 cells through si-*LOC285758*-1/-2/-3 transfection and detecting their transfection efficiency. Then screened out the possessed high efficiency recombinant plasmid si-*LOC285758*-2. (B-C) si-*LOC285758* was adopted to transfect H292 as well as A549 cells, and measuring their cellular viability and migrating ability, respectively. **Compared with Negative Control group, $P<0.01$; **Compared with Negative Control group, $P<0.001$

lncRNA LOC285758 combined to miR-204 and miR-204 inhibited tumors in terms of NSCLC

Gene *LOC285758* silencing within H292 and A549 cells resulted in consistently expressed miRNA-204 (Fig. 3A). The expressions of miRNA-204 were positively correlated with the expression of *LOC285758* in H292 and A549 cells (Fig. 3B). RT-qPCR results showed an effective transfection efficiency of miRNA-204/anti-miRNA-204 carriers in H292 and A549 cells (Fig.3C). Besides, we also detected miRNA-204 binding sites to *LOC285758* through on-line tools (Fig. 3D) and verified via luciferase experiments. Luciferase activity of the carriers of wt-*LOC285758* had a negative correlation with expression of miRNA-204. Potential miRNA-204 combination sites mutated, suppressing the changes of luciferase activity (Fig. 3D).

Then, we employed miRNA-204/anti-miRNA-204 to transfect H292 as well as A549 cells These results showed over-expression of miRNA-204 siginificantly down-regulated cellular migrating ability and cellular viability, while repression of miRNA-204 up-regulated them (Fig. 3E-F).

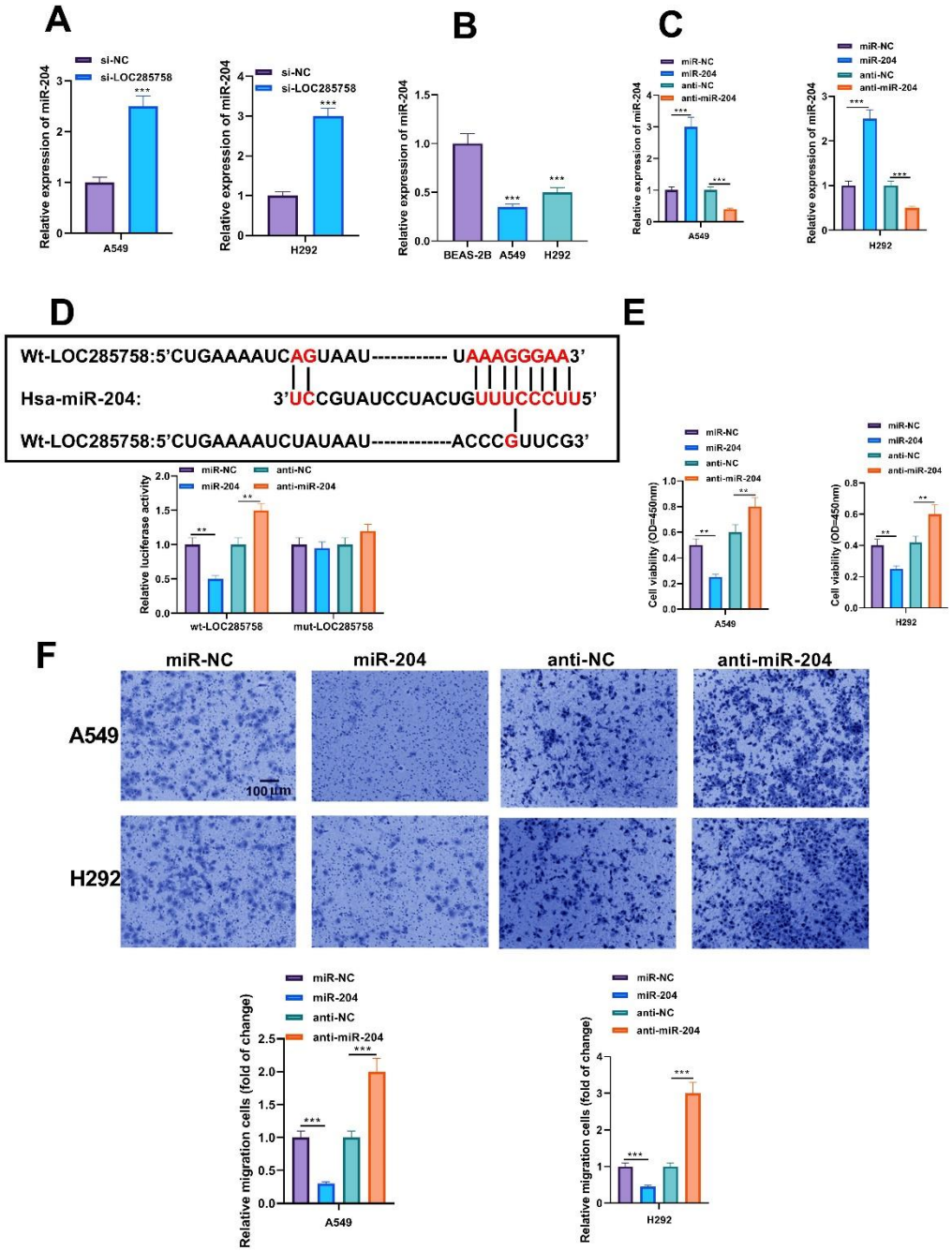

Fig. 3: miR-204 was the direct aimed site for *LOC285758*, working as one anti-cancer factor. (A) Measuring miR-204 expression after using si-*LOC285758* to transfect H292 as well as A549 cells through qRT-PCR. (B) miR-204 expression in H292 as well as A549 cells. (C) Transfection of miR-204 or anti-miR-204 was capable to make miR-204 over-expression or repression in H292 and A549 cells. (D) Using wild types and mutant types of *LOC285758* luciferae reporter carriers to co-transfect 293T cells to measure luciferase activity. (E) miR-204 or anti-miR-204 were used for H292 and A549 cellular co-transfection, and examine their cellular viability. (F) anti-miR-204 and miR-204 were used for H292 and A549 cellular co-transfection,then measuring their cellular migrating ability detection.

$^{**}P<0.01$, $^{***}P<0.001$

miR-204 can combine with CDK6 as well as negatively modulate its expression

To verify the possible mechanism of miRNA-204 and *LOC285758* in NSCLC, our research analyzed possible targets downstream to miRNA-204. CDK6 protein level and mRNA expression were down-regulated in H292 and A549 cells transfected with si-*LOC285758* (Fig. 4A-B). These observations implied that *LOC285758*/miRNA-204 axis down-regulated CDK6 to affect NSCLC. Over-expression of miRNA-204 prominently down-regulated mRNA and protein level of CDK6, while repressing miRNA-204 greatly up-regulated them in both H292 and A549 cells (Fig. 4C-4D). Next, we studied the predicting combination of CDK6 3'UTR and miRNA-204. Luciferase reporter experiments verified this point. We employed CDK6 3'UTR mutant type and wild type carriers to co-transfect 923T cells and examined their luciferase activity. Luciferase activity in wild type CDK6 3'UTR decreased after over-expression of miRNA-204. Mutated miRNA-204 combination site can eliminate the changes in luciferase activity (Fig.4E).

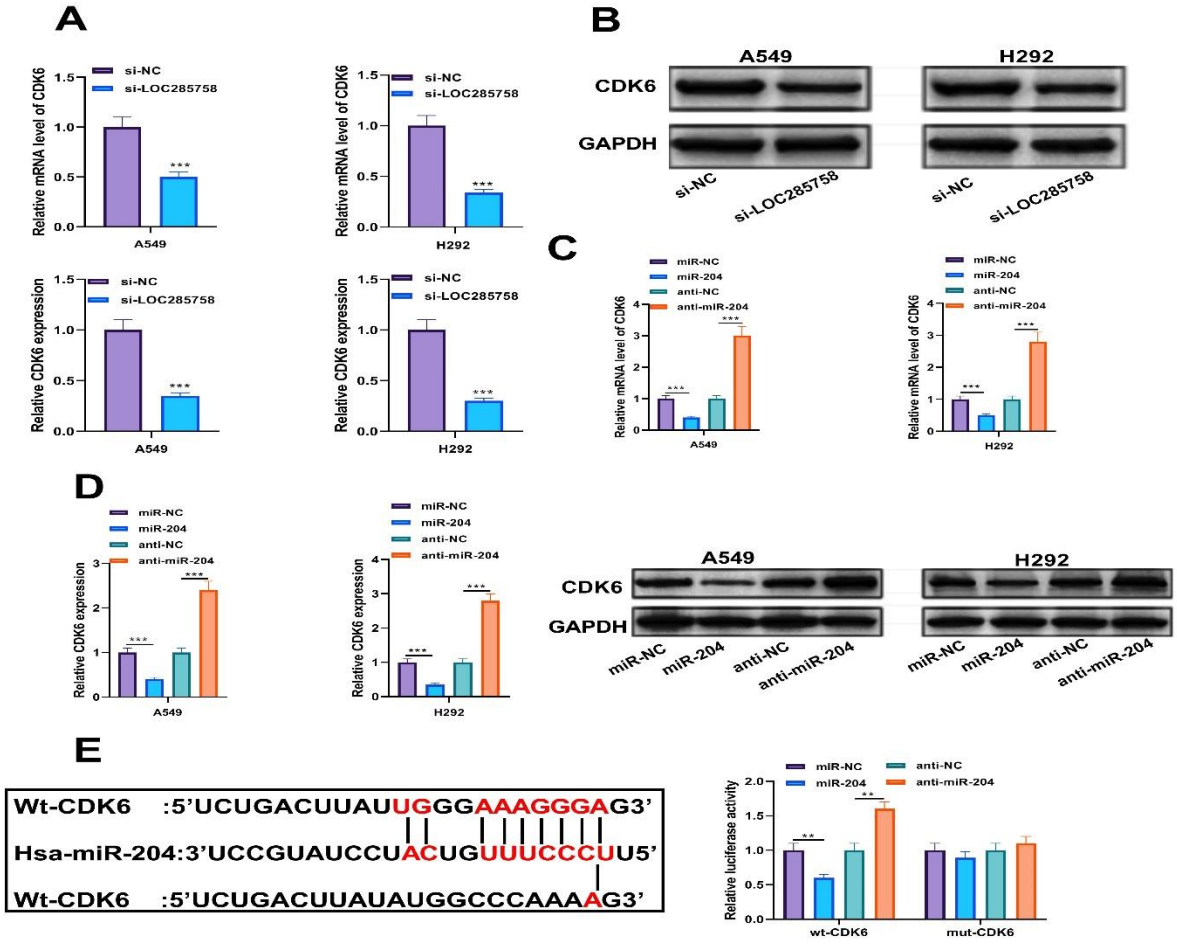

Fig. 4: miR-204 aimed at CDK6 and inhibited its expression. (A) Transfecting H292 and A549 cells through si-*LOC285758*, then testing their CDK6 expression. (B) Using Western blotting to detect CDK6 protein level after transfecting H292 and A549 cells through si-*LOC285758*. (C) Using qRT-PCR to examine CDK6 protein level after transfecting H292 and A549 cells through anti-miR-204 and miR-204. (D) Using immunoblotting to examine CDK6 protein level after transfecting H292 and A549 cells through anti-miR-204 or miR-204. (E) Measuring luciferase activity of 293T cells after co-transfecting with wild and mutant type of luciferase reporter carrier for CDK6 3'UTR. $^{**}P<0.01$, $^{***}P<0.001$

Dynamic effects of lncRNA LOC285758 and miRNA-204 on CDK6 and NSCLC cells

Then we explored effects of *LOC285758*/miRNA-204 axis regulating mRNA and protein levels of CDK6 as well as cellular phenotype of NSCLC. Silencing *LOC285758* inhibited miRNA-204 and ultimately resulted in a decreased CDK6 (Fig.5A-B). Similarly, *LOC285758* knockout significantly reduced the migration and viability of NSCLC cells, while inhibition of miRNA-204 had the opposite effect (Fig.5C-D). Next, we verified the association between CDK6, miRNA-204, and *LOC285758* expression in tumor adjacent tissues and NSCLC tissues of 30 patients. Expression of miRNA-204 was negatively correlated with CDK6 and *LOC285758* within tissues while CDK6 was positively related with *LOC285758* (Fig.5E-G).

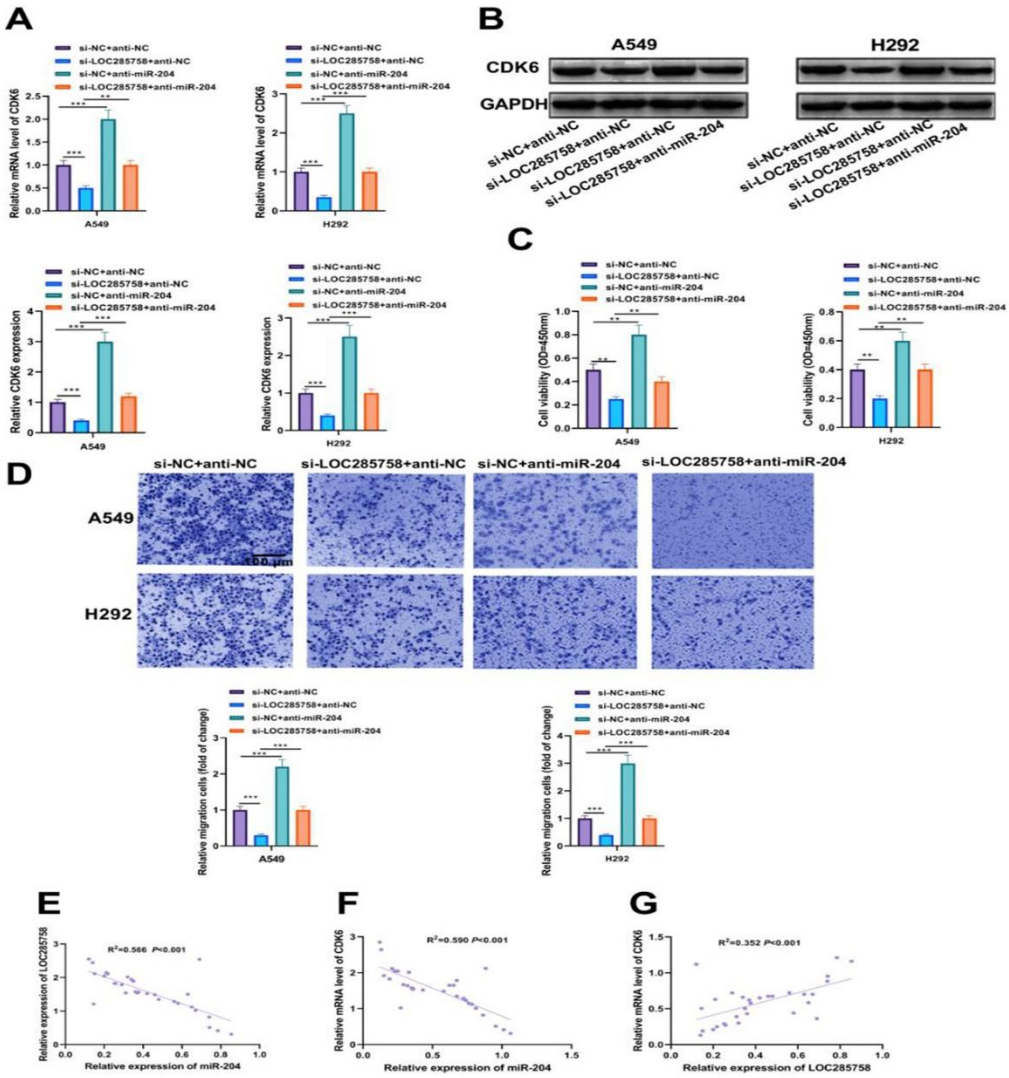

Fig. 5: The dynamic influence on CDK6 expression from miR-204 and *LOC285758* (A) the expression of CDK6 mRNA after co-transfecting anti-miR-204 and si-LOC285758 into H292 and A549 cells; (B) the CDK6 protein expression after co-transfecting anti-miR-204 and si-LOC285758 into H292 and A549 cells; (C-D) cellular viability and migrating ability after co-transfecting anti-miR-204 and si-LOC285758 into H292 and A549 cells; (E-G) The correlation of *LOC285758*, miR-204 and CDK6 expression was analyzed using Pearson correlation coefficient.

$^{**}P<0.01$, $^{***}P<0.001$

Discussion

In this research, we found that compared with normal tissue samples, *LOC285758* expression was prominently up-regulated in NSCLC tissue samples and cells. Silencing *LOC285758* could greatly repress cellular migrating as well as viability of H292 and A549 cells. *LOC285758* directly aimed at miR-204, and more miR-204 expressed in H292 and A549 cells with silenced *LOC285758*. lncRNA *LOC285758* and miR-204a had strong association with NSCLC. *LOC285758* had a promoted effect of NSCLC, and down-regulated *LOC285758* could inhibit the invasive progression of NSCLC. .miR-204 over-expression inhibited cellular migrating ability as well as viability of NSCLC. Silencing gene *LOC285758* down-regulated CDK6 mRNA and protein in NSCLC cells. Knocking out gene CDK6 prominently repressed NSCLC cellular migrating and viability. However, the impacts brought from knocking gene *LOC285758* out were greatly reversed through repression of miR-204. In the NSCLC tissue group, more CDK6 as well as *LOC285758* expressed and less miR-204 expressed. miR-204 was negatively correlated with CDK6 and *LOC285758*, respectively, while CDK6 had positively correlated with *LOC285758,* which verified axis of *LOC285758*/miRNA-204/CDK6 had influence on NSCLC.

Some researchers found LncRNA *LOC285758* could induce invasion of AML through down-regulating miR-204 (16). The extent of malignancy in glioma was correlated with LncRNA *LOC285758*, which might act as a bio-marker in diagnosing glioma (17). However, silencing *LOC285758* was regarded as repressing NSCLC cellular migrating and viability. In our study, more *LOC285758* expressed in NSCLC tissue samples and cellular systems, and knocking *LOC285758* out was capable to prominently repress cellular migrating ability as well as viability, further indicating pro-tumor influence on NSCLC from *LOC285758*.

As ceRNAs, IncRNAs were capable to competitively aim for miRNAs to modulate aim RNA expression (18). CDK6 might be downstream factor of miR-204, and knocking out miR-204 can improve CDK6 expressing in NSCLC cells. Thus, our research screened out *LOC285758* as a potential IncRNAs aiming at miR-204 and CDK6. miR-204 exerted the effects of cancer repression on many cancers. In prostate gland cancer cells, miR-204 linking to bcl2 could induce cell apoptosis (19). miR-204 inhibited proliferating ability of ovary cancer cells through down-regulating USP47 (20), and over-expressing miR-204 greatly inhibited cancer cellular migrating ability as well as viability of NSCLC. More importantly, miR-204 aimed for CDK6 as well as *LOC285758* at the same time, implying that it is possible that miR-204 took part in the influence of CDK6 and *LOC285758* on NSCLC.

As we all know, tumor pathogenesis is an extremely complicated process with many steps (21). The changes on inheritance as well as epigenetics triggered normal cells transform to tumor (22-23). CDK6 was not only a cyclin dependent kinase, but also a transcriptional regulator factor (24), which could be modulated by several miRNAs and took part in tumor metastasis and development (25).

Conclusion

In our study, knocking out CDK6 prominently repressed cancer cellular migrating ability as well as viability, further indicating its pro-tumor effects on NSCLC. Besides, down-regulating *LOC285758* in NSCLC cells could repress up-regulating CDK6 via miR-204, implying that CDK6 could be affected through *LOC285758* combing to miR-204. *LOC285758* inhibited miR-204 to reverse the effects on NSCLC cellular migrating ability as well as viability, implying that *LOC285758* plays an improtant role as one ceRNA to remove the CDK6 repression from miR-204. This provides a new strategy for the clinical treatment of NSCLC.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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