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Original Article

Linc01614 Regulates the Proliferation, Apoptosis, and Chemotherapy Resistance in Esophageal Squamous Cell Carcinoma by Targeting Mir-4775

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Abstract

Background: Esophageal squamous cell carcinoma (ESCC), a widely known esophageal disease, severely affects people's health. Numerous investigations demonstrated that long non-coding RNAs (lncRNAs) performed key jobs inside a wide scope of organic cycles and stand out in malignant growth. Our study planned to investigate the roles and mechanisms of linc01614 in ESCC.

Methods: A Total of 60 ESCC tissue samples including 30 patients with cisplatin sensitivity and 30 patients with cisplatin resistance, who received DDP-based treatment, were obtained from Zhuhai People's Hospital, Zhuhai City during 2021. These tissues were frozen and saved in a -80 ℃ ultra-low temperature freezer. We performed CCK-8, clone formation, flow cytometry assays to determine the effect of linc01614 on ESCC progression, and explored the specific mechanism of linc01614 in ESCC cell proliferation, apoptosis, and chemotherapy resistance.

Results: linc01614 expression was upregulated in ESCC tissues and cells compared with non-tumor tissues and human normal esophageal epithelial cells (Het-1A). Knockdown of linc01614 repressed cell expansion, chemotherapy opposition, and advanced cell apoptosis in ESCC. Besides, linc01614 regulated the expression of miR-4775 as a competitive endogenous RNA (ceRNA).

Conclusion: The linc01614/miR-4775 axis played an important role in ESCC progression and drug resistance, revealing that linc01614 is a promising target in ESCC treatment.

Keywords: linc01614; Apoptosis; Drug resistance

Introduction

As one of the leading diseases affecting human health, esophageal cancer (EC) has risen to be the sixth major reason for cancer-related deaths, according to cancer statistics (1). ESCC is the dominating subtype of EC, as it represents over 90% of EC cases. Although radiotherapy, chemotherapy and medical procedure have taken impressive steps in the clinical treatment of ESCC, it is as yet challenging to defeat the chemotherapy resistance and ESCC metastasis (2-4). Nowadays, targeted

treatment has gotten expanding consideration as a significant and successful ESCC treatment strategy. In like manner, understanding the molecular basis of ESCC progression and chemoresistance might be useful for ESCC treatment.

Long non-coding RNAs (lncRNAs, >200 nucleotides) are a type of mature RNAs without protein-coding abilities (5). Various investigations have identified that dysregulated lncRNAs in various diseases is associated with aberrant proliferation, migration, invasion, apoptosis, or chemotherapy resistance (6). Moreover, lncRNAs filled in as a valuable biomarker for ESCC finding and therapy (6,7). Linc01614 participated in the regulation of carcinogenesis and is used as a biomarker for cancer diagnosis, for example, knockdown of linc01614 prevented gastric cancer progression through targeting miR-217 to inhibit the expression of FOXP1 (8). For glioma malignant progression, linc01614 was a promoter after the activation of nuclear transcription factor SP1 (9). In non-small cell lung cancer, linc01614 was identified as the most aberrantly expressed lncRNA (10). In addition, linc01614 was regarded as an important biomarker for prognostic prediction in patients with bosom cancer (11,12). In any case, the articulation and organic capacity of linc01614 in ESCC are hazy.

Subsequently, we designed to investigate the function of linc01614 in the movement of ESCC and ideally gave another objective to ESCC therapy.

Materials and Methods

Clinical tissues sample

A Total of 60 ESCC tissue samples including 30 patients with cisplatin sensitivity and 30 patients with cisplatin resistance, who received DDP-based treatment, were obtained from Zhuhai People's Hospital, Zhuhai City during 2021. These tissues were frozen and saved in a -80 ℃ ultra-low temperature freezer. Thirty-two men and 28 women (range, 40-78 years) were included in the study. Lymph node metastasis was detected in 25 of them, and 15 were diagnosed with grade I or II ESCC. None of ESCC patients received radiotherapy, chemotherapy or immunotherapy before surgery. Samples were independently diagnosed by two pathologists.

This study obtained approval from Zhuhai People's Hospital (ZH20230117). All patients marked informed assent.

Cell culture and treatment

ESCC cell lines (TE-13, EC9706, KYSE30, and KYSE140) and human normal esophageal epithelial cells (Het-1A) were gotten from Shanghai Institute of Biochemistry and Cell Biology. Cell was kept up with RPMI-1640 (Invitrogen, CA, USA) enhanced with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin/streptomycin at 37 °C in 5% CO_2. To observe the impacts of cisplatin on cell expansion and cell apoptosis, EC9706 and KYSE30 cells were incubated with various groupings of cisplatin (0, 1, 2, 4, 8 and 16 μM) for 48 h.

Cell transfection

Short hairpin RNA (shRNA) focusing on linc01614 (sh-linc01614), miR-4775 mimic, miR-4775 inhibitor, and their corresponding controls were planned and integrated by GenePharma (Shanghai, China). As indicated by the producer's guidelines, cell was transfected by utilizing Lipofectamine 3000 (Invitrogen, CA, USA).

Establishment of ESCC model

Male nude mice aged at 8 weeks (Model Animal Research Center, Nanjing, China) were used to establish model in the experiment. According to previous study (13), EC9706 cells (5×106) were injected subcutaneously into the forelimbs of nude mice to produce 2 groups of mice (sh-NC, sh-01614). Tumor volume was assessed weekly for 5 weeks. Different groups of xenografts were isolated and weighed after 5 weeks. The nude mice were exposed to a 12-h light/dark cycle and received standard food and running water free of charge in a temperature-controlled room. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH

publication, 8th edition, 2011) and received ethics committee approval.

qRT-PCR assay

RNA was collected using TRIzol reagent (Invitrogen). According to the manufacturer's protocol of PrimeScript RT reagent Kit (Takara, Japan), 500 ng RNA was reverse transcribed to cDNA. An SYBR Green Mix kit (Vazyme, Nanjing, China) was used to perform a qRT-PCR assay on the Real-time PCR system (Applied Biosystems, Darmstadt, Germany). The levels of linc01614 and miR-4775 were normalized by the housekeeping gene GAPDH or small nuclear RNA U6, and analyzed by quantitative real-time PCR. The primer of GAPDH, U6, linc01614, and miR-4775 were displayed in Table 1.

Table 1: Primers was used for qRT-PCR

Gene name	*Forward primer (5'-3')*	*Reverse Primer (5'-3')*
GAPDH	GCCATGGCTACGGTTCAG	CAGAACCAGCGGTTAGGC
U6	GCTTCGGCAGCACATATACT	AACGCTTCACGAATTTGCGT
miR-4775	GCGCGTTAATTTTTTGTTTCG	AGTGCAGGGTCCGAGGTATT
linc01614	TGACATAATCTGGGTTCTT	TCAACCTCAGATGTCCCT

Cell viability assay

Viabilities of EC9706 and KYSE30 cells in various groups were quantified by the methyl thiazolyl tetrazolium (MTT) assay. Briefly, transfection-treated EC9706 and KYSE30 cells were plated into 96-well plates and the viability was measured after treating with MTT solution (Sigma, MO, USA). The optical density was measured at 490 nm.

Nuclear/cytosol fractionation

Cellular fractionation and qPCR analysis of Linc-01614 in the cytosolic and nuclear fraction of KYSE/DDP cells were performed by RiboBio. Briefly, cells were fractionated by lysis using specific lysates of different strength according to the difficulty of cell membrane and nuclear membrane lysis. Linc-01614 levels in the cytosolic RNA and nuclear RNA were determined by qPCR using GAPDH and U6 as internal references gene.

Colony formation assay

EC9706 and KYSE30 cells (1×10^3) were cultivated on six-well plates. Following 10 days of incubation, cells were treated with methanol for 15 min and colored using 0.5% crystal violet liquid for 20 min. Finally, the colonies in various groups were photographed and recorded.

Cell apoptosis analysis

The cell apoptosis ratio in different groups was measured by flow cytometry (14). EC9706 and KYSE30 cells (1×10^5) treated with sh-NC, sh-linc01614 or miR-4775 were collected, suspended in 100 µl binding buffer and incubated with Annexin V and PI at room temperature for 15 min. 400 µl binding buffer was supplemented into cell suspension. The marked cells were examined by utilizing FACS flow cytometry (Leica).

Western blot analysis

Protein was extracted through RIPA lysis buffer containing 0.5% protease inhibitors. Protein concentration was quantified by the BCA Kit (Thermo-Scientific). The primary antibody contained Bax (1:1000), Bcl-2 (1:1500), GAPDH (1:5000), the secondary antibody included goat anti-rabbt-(1:5000) and goat anti-mouse (1:5000) HRP antibody were gotten from Abcam. The densitometry of every protein band was evaluated by Image J programming.

Bioinformatics analysis

The binding sites of LINC_00355 and miR-15a-5p were predicted by bioinformatics analysis. (LncBase v.2, http://carolina.imis.athena-innova-

tion.gr/diana_tools/web/index.php?r=lncbasev2/index-predicted).

Luciferase reporter assay

Based on previous studies (14), the wild-type (WT) or mutant (MUT) sequences of miR-4775-conjugated linc01614 3' untranslated region (3'UTR) were expanded and embedded into the pGL3 luciferase vector (General Biol, Anhui, China) to obtain linc01614-WT and linc01614-MUT. The constructed luciferase vectors were transfected into EC9706 and KYSE30 cells along with miR-NC or miR-4775 mimics. After 48-h post-transfection, luciferase activity was estimated by a dual-luciferase reporter assay system (Promega) as instructed.

TUNEL staining

Tunel staining were performed as described previously (15). In brief, after paraffin section samples were deparaffinized and hydrated, the samples were biotin-labeled with tunel solution, then incubated with DAB for 15 min, then in hematoxylin solution for 15 min, and finally observed under a light microscope (Olympus, Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry (IHC) was carried out as previously described with minor modifications (16). Treated sample sections were incubated with Ki-67 primary antibody (1:100) at 4 °C overnight, the secondary antibody was incubated at room temperature for 1 h, washed with 1×TBST, incubated with diaminobenzidine chromogen for 5 min, and reconstituted with hematoxylin. stained nuclei. Observe the sections under a microscope.

Statistical analysis

Statistical comparisons were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL). Statistical data were shown as the mean ± standard deviation. The unpaired Student's *t*-test was utilized to examine significant differences. $P<0.05$ was considered statistically significant.

Results

linc01614 is highly expressed in ESCC tissues and cells

In Fig. 1A, linc01614 expression was elevated in drug resistance patients compared with cisplatin-sensitive patients.

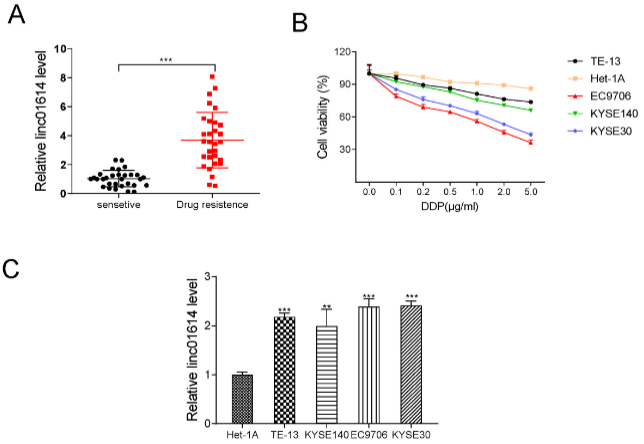

Fig. 1: The expression pattern of linc01614 in ESCC tissues and cell lines. A. The expression levels of linc01614 in drug sensetive patients and cisplatin-resistant patients detected by RT-PCR assay. **B.** linc01614Cell viability was detected by CCK-8 assay in ESCC cell lines (TE-13, KYSE140, EC9706 and KYSE30) and Het-1A . C. linc01614 expression in ESCC cell lines (TE-13, KYSE140, EC9706 and KYSE30) and Het-1A. The * represented significant differences compared with corresponding control group (***$P < 0.001$)

linc01614Furthermore, cell viability of ESCC cells gradually decreased with increasing cisplatin (DDP) concentration, especially in KYSE30 and EC9706 cells (Fig. 1B). Consistently, the expression of linc01614 was markedly increased in ESCC cell lines (TE-13, KYSE140, EC9706, and KYSE30) compared with Het-1A, especially in EC9706 and KYSE30 (Fig. 1C).

Knockdown of linc01614 inhibits the proliferation and chemotherapy resistance, promotes cell apoptosis in ESCC

To decide the roles of linc01614 in the ESCC process, linc01614 shRNA was utilized to transfect EC9706 and KYSE30 cells. qRT-PCR analysis illustrated that the expression of linc01614 in the sh-linc01614 group was diminished compared with control group in ESCC cells (Fig. 2A). Silencing of linc01614 restrained cell proliferation in EC9706 and KYSE30 cells (Fig. 2B, 2C). Cisplatin is an extensively used chemotherapeutic agent for ESCC (17). Knockdown of linc01614 obviously promoted cell apoptosis in EC9706 and KYSE30 cells. Cisplatin further enhanced the positive effects of sh-linc01614 on cell apoptosis (Fig. 2D). Besides, the protein level of pro-apoptosis proteins (Bax, caspase-3 and caspase-9) was increased in the sh-linc01614 group compared to sh-NC in EC9706 and KYSE30 cells (Fig. 2E). Similarly, cisplatin further promoted the impact of linc01614 on ESCC apoptosis-related proteins (Fig. 2E).

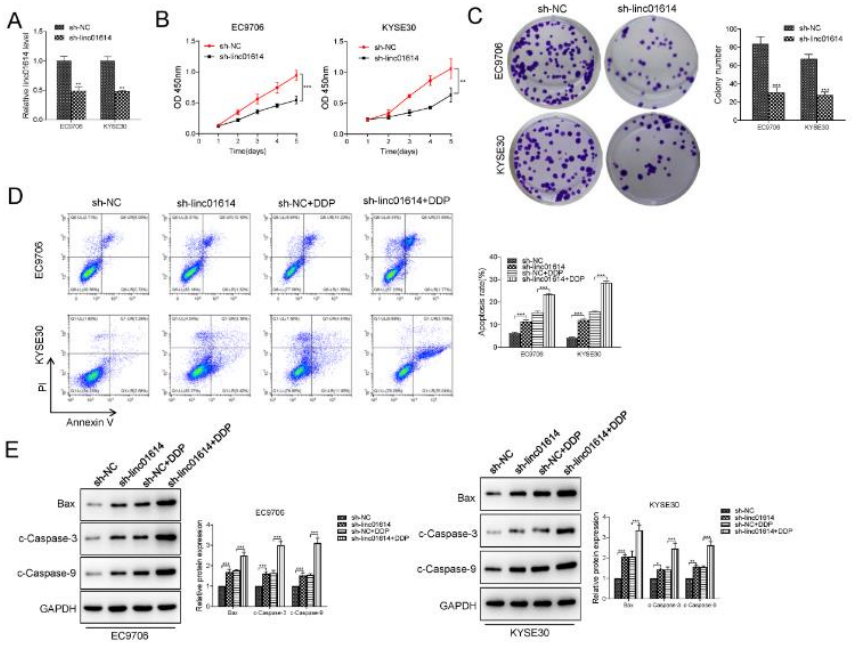

Fig. 2: The biological effect of linc01614 on ESCC cells. A. The knockdown efficiency of linc01614. EC9706 and KYSE30 cells were treated with sh-linc01614 and sh-NC. **B-C.** MTT assay and Colony formation analysis detected cell proliferation. **D**. Apoptosis tested/measured by flow cytometry. **E.** The protein levels of Bax, caspase-3 and caspase-9 were analyzed by western blot assay. The * represented significant differences compared with corresponding control group ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

linc01614 capacities as s a molecular sponge for miR-4775

To explore the specific molecular mechanism of linc01614 in ESCC, we previously localized linc01614 at the cellular location. As displayed in Fig. 3A, linc01614 was dispersed in both cytoplasm and nucleus, yet chiefly limited in the cytoplasm. miR-4775 was anticipated to be a possible

objective of linc01614 by bioinformatics, and the presence of binding sites for both is displayed in Fig. 3B. Moreover, luciferase assay results announced that miR-4775 mimic reduced the luciferase activity in the linc01614-WT group, but the luciferase activity in linc01614-MUT group had no significant change (Fig. 3C). In Fig. 3D, the deletion of linc01614 resulted in the upregulation of miR-4775 expression, which implied that the interaction of linc01614 with miR-4775 could suppress the expression of miR-4775. Besides, we found the expression of miR-4775 in drug resistance patients was down-regulated compared with the cisplatin-sensetive patients (Fig. 3E). Moreover, miR-4775 was adversely associated with linc01614 expression in ESCC tissue (Fig. 3F).

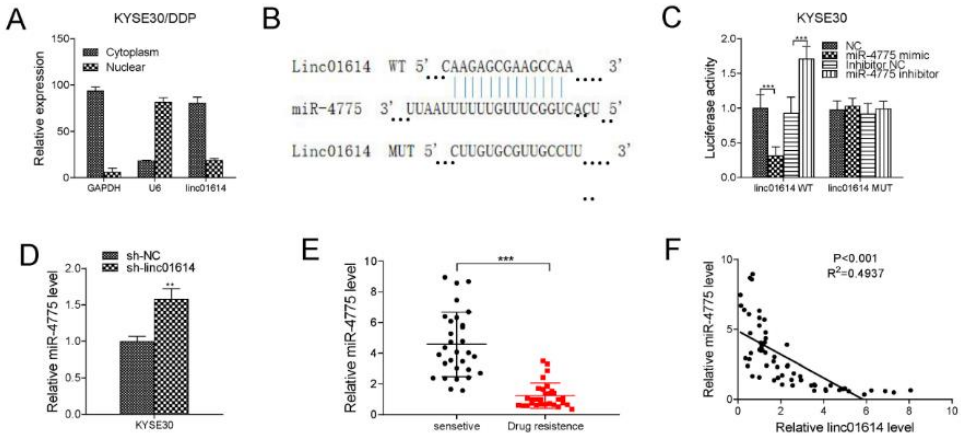

Fig. 3: linc01614 is a sponger of miR-4775 in ESCC cells. A. Relative expression levels of GAPDH, U6 and linc01614 were analyzed in the cytoplasm and nucleus of EC9706 or KYSE30 cells. **B.** The predicted binding sites of miR-4775 in linc01614 were displayed. **C.** Luciferase reporter assay. **D.** Relative expression levels of miR-4775 in ESCC tissues and cell. **E.** MiR-4775 expression levels. **F.** qRT-PCR analysis was performed to confirm the correlation between miR-4775 and linc01614. The * represented significant differences compared with corresponding control group ($^{***}P < 0.001$)

Upregulation of miR-4775 hindered the viability and advanced apoptosis in EC9706/DDP and KYSE30/DDP cells

Next, stable cisplatin-resistant cell lines (EC9706/DDP, KYSE30/DDP) were constructed to explore the function for miR-4775. First, EC9706/DDP and KYSE30/DDP cells were stimulated with NC-mimic and miR-4775 mimic, we found that miR-4775 mimic fundamentally expanded miR-4775 expression (Fig. 4A). MTT assay demonstrated decreased proliferation of EC9706/DDP and KYSE30/DDP cells in the miR-4775 group in contrast to with the NC-mimic group (Fig. 4B). Additionally, flow cytometry and western blot revealed that upregulation of miR-4775 dramatically prompted cell apoptosis and apoptosis-related protein expression (Fig. 4C-4D).

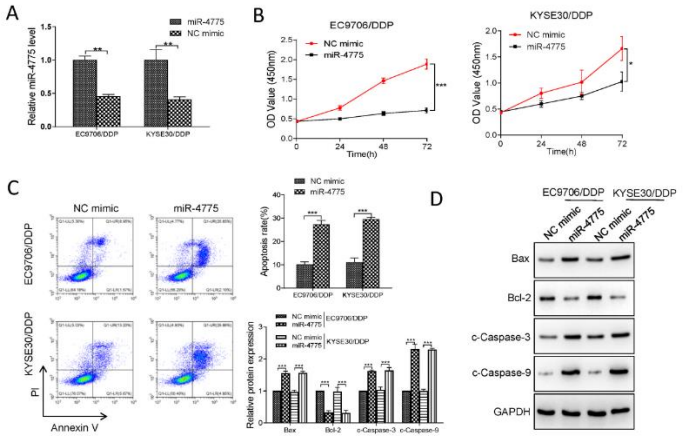

Fig. 4: The biological effect of miR-4775 on ESCC cells. A. The expression of miR-4775. **B.** Cell viability detected by MTT analysis. **C.** Flow cytometry measured the apoptosis. **D.** The expression of apoptosis-related protein. The * represented significant differences compared with corresponding control group ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

linc01614 promotes ESCC progression and enhances drug resistance via modulating the miR-4775 signaling axis

To investigate the regulatory mechanism in which linc01614 impacted ESCC progression, we transfected EC9706 or KYSE30 cells with sh-NC, sh-linc01614, sh-linc01614+inhibitor NC or sh-linc01614+miR-4775 inhibitor. Functionally, MTT assay and colony formation assay demonstrated that silencing of linc01614 essentially repressed the proliferation of EC9706 or KYSE30 cell. However, the downregulation of miR-4775 reversed the inhibitory impact caused by sh-linc01614 (Fig. 5A,5B). Moreover, the positive effects of sh-linc01614 on apoptosis were weakened through miR-4775 inhibitor (Fig. 5C). In Fig. 5D, downregulation of miR-4775 expression effectively reversed the inhibitory effect of cell resistance to cisplatin mediated by the knockdown of linc01614.

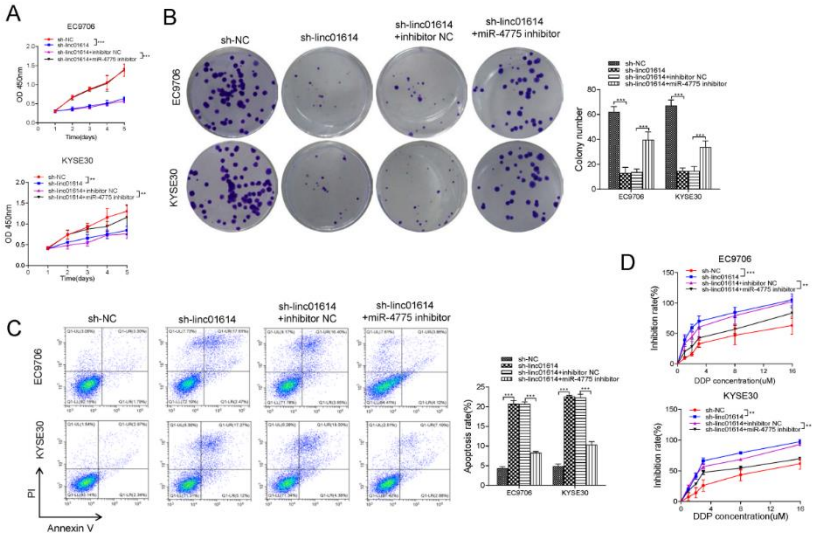

Fig. 5: linc01614 regulates ESCC progression and drug resistance by modulating miR-4775 expression. sh-linc01614, miR-4775 inhibitor and their corresponding control were transfected into EC9706 and KYSE30 cells. **A.** MTT assay checked the cell viability. **B.** Cell proliferation was detected by colony formation assay. **C.** Cell apoptosis was detect-

ed by flow cytometry. **D.** The inhibition rate of cell viability was assessed in EC9706 and KYSE30 cells treated with 0, 1, 2, 4, 8 and 16 μM cisplatin for 48h. The * represented significant differences compared with corresponding control group (**$P < 0.01$, ***$P < 0.001$)

Knockdown of linc01614 inhibits the ESCC progression in vivo

Given the inhibitory effect of linc0614 in vitro, we further examined its effect on tumor growth in vivo. As shown in Fig. 6A-6C, knockdown of linc01614 significantly inhibited tumor growth. TUNEL staining results showed that sh-linc01614 significantly promoted ESCC cell apoptosis, and immunohistochemistry showed that Ki67 protein level was significantly reduced in sh-linc01614-transfected cells (Fig. 6D).

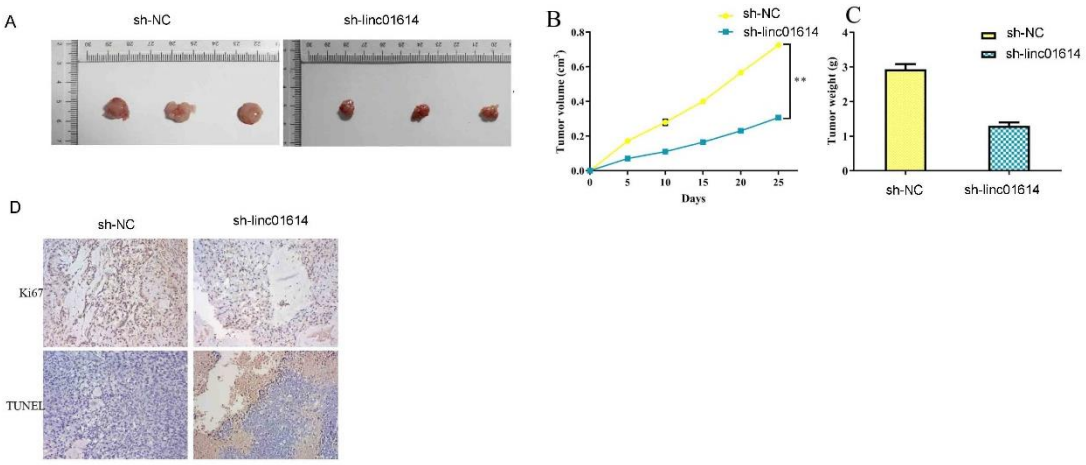

Fig. 6: Sh-linc01614 inhibits the ESCC progression in vivo. A. Tumor growth of experimental mice models after injected with EC9706 cells transfected with sh-LINC01614. **B, C.** Tumor volume and weights of the mice at the end of 25 days. **D.** Representative Ki67 and TUNEL level in subcutaneous tumors of the mouse model. The * represented significant differences compared with corresponding control group (**$P < 0.01$, ***$P < 0.001$)

Discussion

linc01614 was firstly discovered in lung adenocarcinoma patients, and it showed that down-regulated linc01614 inhibited lung adenocarcinoma progression. In bosom tumor, NSCLC and glioma, linc01614 expression showed a positive correlation with cancer progression. In addition, according to high-throughput data with large sample from public databases, linc01614 expression was enhanced in most malignant tumors such as bladder carcinoma, thyroid carcinoma, stomach adenocarcinoma, rectum adenocarcinoma, kidney renal papillary cell carcinoma and liver hepatocellular carcinoma (18). In this research, linc01614 expression was dramatically boosted in ESCC tissues or cell lines in contrast to adjoining typical tissues or Het-1A. The down-regulated linc01614 was always associated with the growth inhibition of ESCC cells, induced G1 arrest, and inhibited the expression of apoptosis-related protein. All results exhibited that linc01614 promoted the ESCC progression.

Chemoresistance is an impediment to the fruitful therapy of cancer (19). The reasons for chemotherapy resistance are extremely complex, and anomalous changes of lncRNAs have been accounted to correlate with chemoresistance. Exosomal H19 derived from carcinoma-associated fibroblasts contributes to tumor development and chemoresistance (20). In colorectal cancer, lncRNA CRNDE promotes colorectal cancer cell proliferation and chemoresistance via miR-181a-5p (21). In colon cancer, lncRNA KCNQ1 oppo-

site strand/antisense transcript 1 participates in regulating the chemoresistance of oxaliplatin (22). Knockdown of LncRNA PCAT-1 promoted the cisplatin chemosensitivity in esophageal cancer (23). In ESCC, the expression of lncRNAs was altered along with the chemoresistance, including lncRNA PART1, lncRNA CCAT1, lncRNA POU3F3, lncRNA TUG1, linc00337 and lincROR (24-29). Our findings illustrated that the levels of linc01614 was markedly upgraded after cisplatin treatment in ESCC cells. Moreover, inhibition of linc01614 reversed cisplatin resistance in ESCC cells, which suggests that linc01614 participated in chemoresistance in ESCC.

Further, we explored the cellular position of linc01614 and the large cytoplasmic localization of linc01614 was found in ESCC cells. LncRNAs exert their function as ceRNA to bind with miRNA. In this paper, we verified that miR-4775 was the target of linc01614. Accumulating evidence has indicated the association of miR-4775 with many human tumor progressions. In colorectal cancer, miR-4775 promotes invasion and metastasis (30). miR-4775 was a tumor suppressor in lung cancer and glioma (31,32). Nevertheless, the mechanism of how miR-4775 controls ESCC is tricky. The data in this research discovered that miR-4775 in ESCC cell lines and tissues was low expressed and negatively regulated by linc01614. Downregulation of miR-4775 expression reversed the inhibitory effect on ESCC progression and drug resistance caused by the knockdown of linc01614. Hence, miR-4775 could act as a basic cancer silencer in ESCC.

Conclusion

Taken together, all these discoveries exhibited the capacity of two players (linc01614/miR-4775 hub) in ESCC and offered a new goal for the treatment of ESCC.

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 interest.

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