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Prognostic value of long noncoding RNA LINC00924 in lung adenocarcinoma and its regulatory effect on tumor progression

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Summary. Purpose. Long non-coding RNAs (lncRNAs) have been used in the study of tumor biomarkers in recent years. However, the prognostic role of lncRNA LINC00924 (LINC00924) in lung adenocarcinoma (LUAD) has not yet been concluded. Therefore, this study investigates the prognostic value of LINC00924 in LUAD and its regulatory effect on tumor progression.

Patients and methods. The LUAD tissues and adjacent normal tissues of 128 subjects were extracted, and the expressions of LINC00924 and miR-196a-5p in tissues and cells were detected by RT-qPCR. The prognostic value of LINC00924 in LUAD patients was obtained by Kaplan-Meier analysis and multivariate Cox regression test. The cell counting kit-8 (CCK-8) and Transwell assay were used to detect the effect of overexpression LINC00924 on LUAD cells.

Results. In LUAD tissues and cells, LINC00924 expression was down-regulated and miR-196a-5p expression was up-regulated compared with the normal control group. High expression of LINC00924 inhibited the proliferation level, migration ability and invasion situation of LUAD cells, which was more conducive to the survival and prognosis of LUAD patients. Bioinformatics studies indicated that overexpression of LINC00924 inhibited the development of LUAD by targeting miR-196a-5p, while miR-196a-5p mimic effectively weakened the inhibition.

Conclusion. LINC00924 sponges of miR-196a-5p may be considered as a potential prognostic biomarker for LUAD.

Key words: Non-small cell lung cancer, circTADA2A, miR-214-3p, EIF4A3, MAPK8

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Introduction

Lung adenocarcinoma (LUAD) is the most common subtype of lung cancer, which is non-small cell carcinoma, accounting for about 40% of lung cancer cases (Denisenko et al., 2018; Zhong et al., 2021). Unlike other types of lung cancer, LUAD is more likely to occur in women and non-smokers. Evidence suggests that lung cancer is the leading cause of cancer-related death, and LUAD patients are often in advanced stage of tumor at the time of diagnosis, resulting in poor treatment outcomes and prognosis, with a 5-year survival rate of less than 20% (Roointan et al., 2019; Li et al., 2021; Liu et al., 2021). Therefore, there is an urgent need to find more therapies, such as reliable prognostic biomarkers that can help patients with disease intervention.

Long noncoding RNAs (lncRNAs) are a group of RNAs longer than 200 nucleotides and have no coding ability (Zhu et al., 2014). It has been reported that abnormally expressed lncRNAs are associated with the occurrence and development of various diseases and serve as diagnostic and prognostic markers (Li et al., 2017; Zhu and Han, 2021). LncRNAs have bright prospects in the prediction, diagnosis and prognosis of lung cancer. For example, Zhen and his companions found that lncRNA DANCR can favor the progression of lung cancer through sequestering miR-216a (Zhen et al., 2018). In addition, LINC00924 has been confirmed to be associated with gastric cancer (Fang et al., 2021), hepatocellular carcinoma (Yu et al., 2022) and thyroid cancer (Rao et al., 2020), but there are no reports on the influence of LINC00924 expression on the progression of LUAD and the prognosis of patients.

MicroRNAs (miRNAs) are a class of non-coding single-stranded RNA molecules about 22 nucleotides in length, which are involved in the regulation of gene expression (Cai et al., 2020; Jin et al., 2020b). According to the literature, lncRNAs may act as miRNA sponges to regulate its functional expression (Assmann et al., 2019).

LncRNAs can be regulated by multiple miRNAs, and miRNAs can also have several target genes (Gan et al., 2021). LncRNAs and miRNAs are closely correlated and have a wide range of biological functions, so it is reasonable to speculate that the abnormal expression of LINC00924 interferes with miR-196a-5p, which is worthy of further study.

Therefore, the expression of LINC00924 in LUAD tissues and cells was measured, and the effects of LINC00924 on the survival and prognosis of LUAD patients were analyzed in this study. Furthermore, the regulation of LUAD progression by LINC00924 targeting miR-196a-5p was investigated.

Materials and methods

Clinical date of patients

A total of 128 LUAD patients participated in this experiment, including the high expression group (n=60) and the low expression group of LINC00924 (n=68), which were grouped according to the average expression of LINC00924 in patients. Patients with LUAD diagnosed in China-Japan Union Hospital of Jilin University from January 2015 to March 2017 were selected as the research subjects, and the required tissues were collected and stored in liquid nitrogen. The clinical information of all patients is shown in Table 1.

LUAD patients participating signed the informed consent under the supervision of the ethics committee to understand the relevant content. Five years of observation were conducted through telephone interviews, online communication and face-to-face diagnosis to obtain clinical information of patients in a timely manner.

Cell culture and transfection

LUAD cell lines NCI-H2170, NCI-H226, NCI-H520, SK-MES-1 and normal cells BEAS-2B were all obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA). The above cells were routinely cultured at 37°C in a humidified environment with 5% CO_2. With the help of Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA), pcDNA3.1-LINC00924 or LINC00924+miR-196a-5p mimic or negative control were transfected into NCI-H2170 or NCI-H520 cells, respectively, and analyzed by RT-qPCR method to evaluate transfection efficiency.

RT-qRCR assays

Total RNA in tissues and cells was extracted with TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and the PrimeScript RT Master Mix Kit and SuperScript II RT Kit (TaKaRa, Kusatsu, Japan) were introduced to complete RNA to cDNA transcription. The SYBR® Premix Ex Taq™ II Kit (Takara, Tokyo, Japan) was involved in the reaction, while cDNA amplification was performed on the Thermal Cycler Dice™ real-time system. The expression of LINC00924 was normalized to GAPDH, while the expression of miR-196a-5p was normalized to U6, and the data were handled with the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation level, migration ability and invasion situation assays

After transfection, NCI-H2170 and NCI-H520 cells were seeded into 96-well plates at a density of 4×10^4 cells/well, followed by adding cell counting kit-8 (CCK-8) solution at 0h, 24h, 48h, 72h and 96h, and cultured for 2h. The cell proliferation level was indicated by the OD value at 450 nm. Transwell assay was used to conduct cell invasion experiments. Transfected cells were cultured in serum-free medium and the density was adjusted to 2×10^4 cells/well. Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was added to the upper chamber using 300 μg/mL, and medium containing 10% FBS was loaded into the lower chamber. Cultured at 37°C for 24h, the invaded cells were sequentially fixed and stained (0.1% crystal violet solution), and finally cell counts were performed in 5 random fields under a microscope. Similarly, the cell migration experiments were performed in the same manner as described above, but there was no need to add Matrigel in the upper chamber.

Table 1. Basic clinical information of patients with lung adenocarcinoma.

Items	Patients (n=128)	LncRNA LINC00924		significance
		High expression (n=60)	Low expression (n=68)	
Age				0.868
≤60	63	30	33	
>60	65	30	35	
Gender				0.743
Male	77	37	40	
Female	51	23	28	
Tumor size (cm)				0.058
≤5	61	33	26	
>5	67	27	42	
Smoking status				0.572
Non-smoker	61	27	34	
Smoker	67	33	34	
Differentiation				0.176
Well, Moderate	73	38	35	
Poor	55	22	33	
Lymph node metastasis				0.002
Negative	78	45	33	
Positive	50	15	35	
TNM stage				0.001
I, II	79	46	33	
III, IV	49	14	35	

Luciferase report

WT-LINC00924 (wild type) or MUT-LINC00924 (mutant type) were used to co-transfect miR-196a-5p or the negative control with pmirGLO Vector by Lipofectamine 3000 transfection reagent. After 48h, luciferase activity of LUAD cells was measured on SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, CA, USA), and the relationship of LINC00924 to miR-196a-5p was analyzed.

Statistical analysis

To ensure the accuracy of the experiment, each group of data was obtained from at least three experiments, and were processed by SPSS 20.0 and GraphPad 5.0. Measurement data were formed as mean ± standard deviation, and differences between groups were tested by Student's t test. The Kaplan-Meier method was used to evaluate the survival of patients with LUAD. Pearson's chi-square test was chosen to detect the correlation between LINC00924 expression and clinical parameters of LUAD patients. $P<0.05$ was considered the difference to be statistically significant.

Table 2. Multivariate Cox analysis of clinical characteristics in relation to overall survival.

Indicators	Multivariate analysis		
	HR	95% CI	*P*
LncRNA LINC00924	3.636	1.735-7.620	0.001
Age	1.207	0.654-2.227	0.547
Gender	1.073	0.582-1.977	0.821
Tumor size	1.358	0.742-2.486	0.320
Smoking status	1.256	0.698-2.260	0.448
Differentiation	1.495	0.804-2.780	0.204
Lymph node metastasis	1.659	0.834-3.303	0.149
TNM stage	2.280	1.152-4.512	0.018

Results

LINC00924 was underexpressed and miR-196a-5p was overexpressed

The expression of LINC00924 in LUAD tissues and normal tissues of 128 subjects were detected via RT-qPCR. In Figure 1A, LINC00924 was lowly expressed in tumor tissues compared to normal tissues. The expression of miR-196a-5p was measured and it was found that the expression of miR-196a-5p was obviously increased in tumor tissues (Fig. 1B). LINC00924 was inversely correlated with miR-196a-5p by Spearman correlation analysis as shown in Figure 1C.

Clinical correlation of LINC00924 expression with LUAD patients

LINC00924 high expression group (n=60) and low expression group (n=68) were classified according to the average expression of LINC00924 in tumor tissues. The basic clinical information of 128 patients with LUAD is presented in Table 1. The results showed that LINC00924 expression was correlated with lymph node metastasis ($P=0.002$) and TNM stage ($P=0.001$), but was not affected by factors such as age, gender, tumor size, smoking status and differentiation ($P>0.05$).

Prognosis of patients with LUAD

The Kaplan-Meier survival curve combined with log-rank test was used to analyze the effect of abnormal expression of LINC00924 on the survival of LUAD patients. Figure 2 suggests that patients with LINC00924 high expression of LUAD had a higher overall survival than LINC00924 low expression (log-rank test $P=0.004$). Table 2 illustrates the prognostic value of LINC00924 in LUAD by multivariate Cox regression analysis. LINC00924 was identified as a biomarker associated with prognosis with LUAD patients (HR=3.636, 95%CI: 1.735-7.620, $P=0.001$).

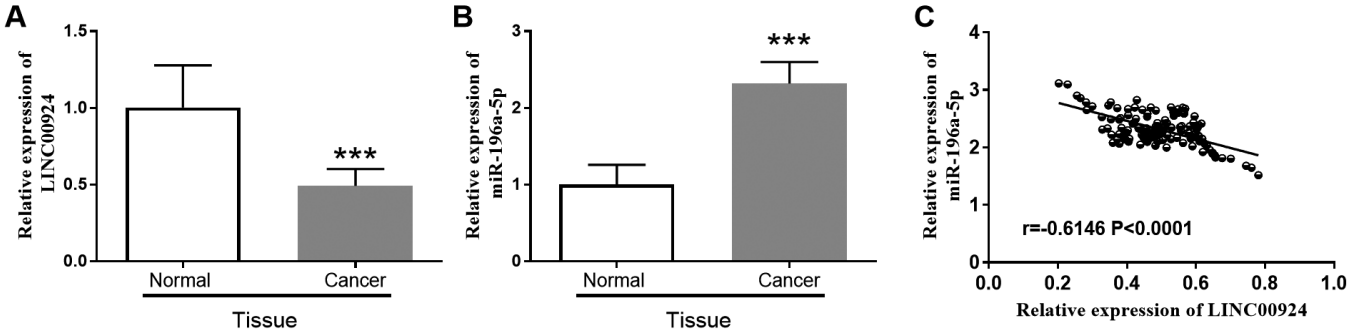

Fig. 1. Relative LINC00924 and miR-196a-5p expression in LUAD tissues. **A.** The expression level of LINC00924 in LUAD tissues and the corresponding normal tissues. **B.** The expression level of miR-196a-5p in LUAD tissues and the corresponding normal tissues. ***P<0.001. **C.** The expressions of LINC00924 and miR-196a-5p were negatively correlated. r=-0.6146, *P*<0.0001.

Upregulation of LINC00924 negatively affected proliferation level, migration ability and invasion situation of LUAD cells

High expression of LINC00924 was more beneficial to the survival of patients, suggesting that the upregulation of LINC00924 may have a corresponding impact on the progression of LUAD. LINC00924 expression was down-regulated in LUAD cells (NCI-H2170, NCI-H226, NCI-H520, SK-MES-1) compared to the control cells (BEAS-2B) in Figure 3A. NCI-H2170 or NCI-H520 cells were transfected with pcDNA3.1 or pcDNA3.1-LINC00924, and the transfection effect was evaluated by the expression level of LINC00924 (Fig. 3B). The proliferation level of NCI-H2170 and NCI-H520 cells were detected via CCK-8 method. Figure 3C,D show that high expression of LINC00924 significantly inhibited the cell proliferation. Besides, the migration and invasion ability of NCI-H2170 and NCI-H520 cells were also markedly decreased compared with pcDNA3.1 transfected cells by Transwell assay (Fig. 4A,B).

MiR-196a-5p acted as the target of LINC00924

To further clarify the mechanism, bioinformatics database (https://starbase.sysu.edu.cn/) was used to predict the potential targets of LINC00924 in advance. The results are shown in Figure 5A, LINC00924 directly targets miR-196a-5p, which has binding sites. According to the binding sites, WT-LINC00924 and MUT-LINC00924 were constructed, and LUAD cells were co-transfected with control group or mimic NC or miR-196a-5p mimic or inhibitor NC or miR-196a-5p inhibitor. From the luciferase activity in Figure 5B, NCI-H520 cells transfected with WT-LINC00924 showed inhibition in miR-196a-5p mimic, while there was no significant change in cells transfected with MUT-LINC00924. In

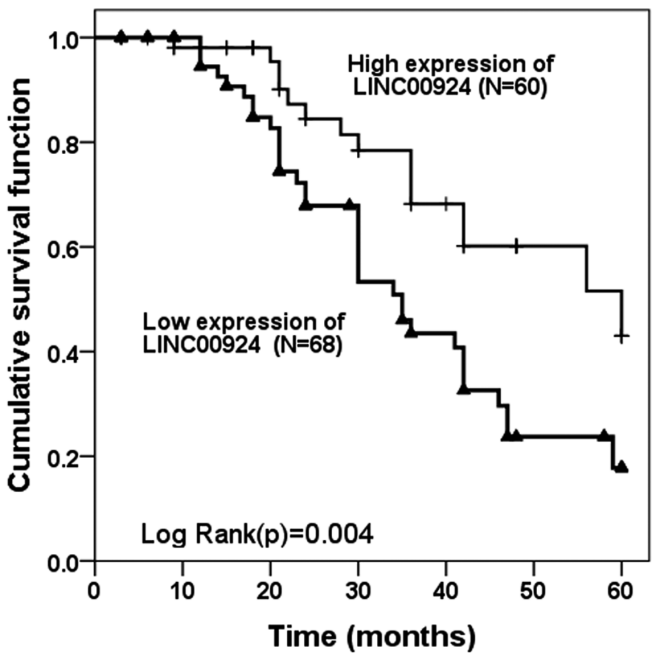

Fig. 2. Kaplan-Meier curves for LUAD patients with high expression of LINC00924 and low expression of LINC00924 (log-rank test P=0.004).

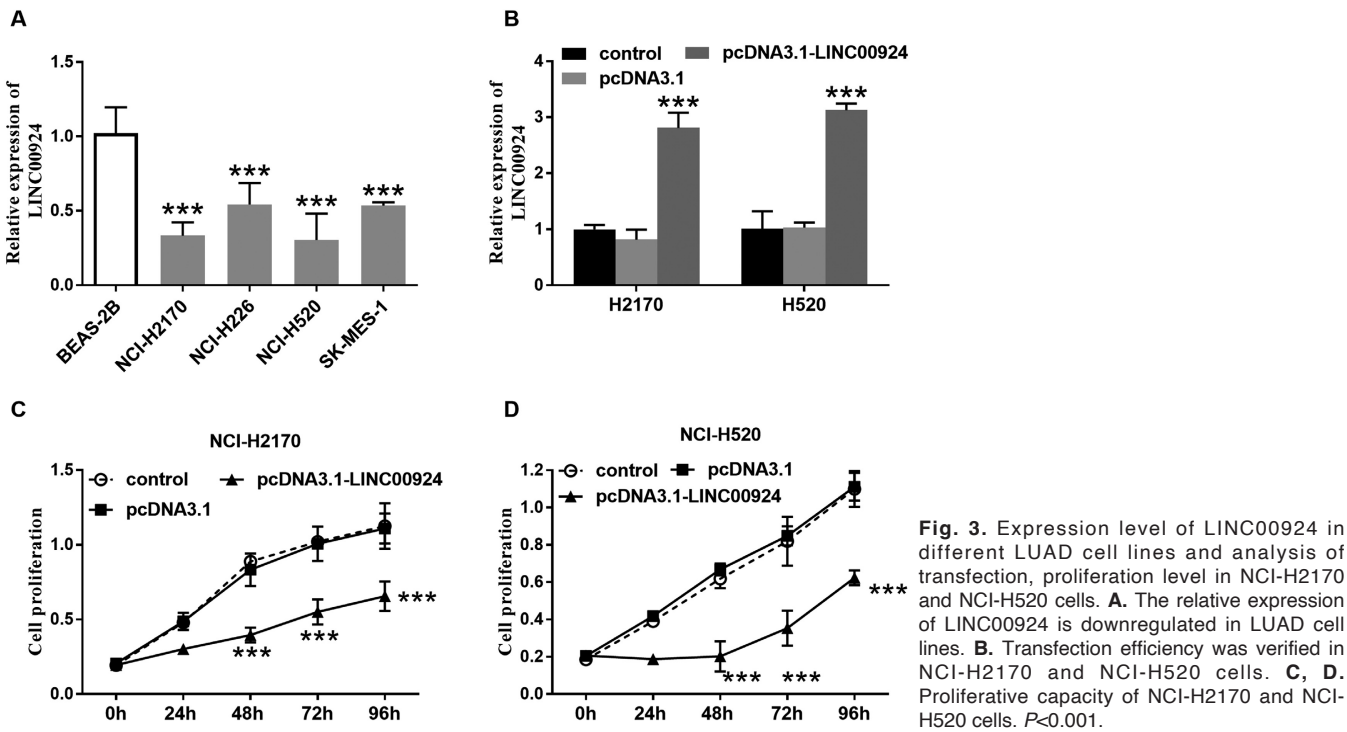

Fig. 3. Expression level of LINC00924 in different LUAD cell lines and analysis of transfection, proliferation level in NCI-H2170 and NCI-H520 cells. **A.** The relative expression of LINC00924 is downregulated in LUAD cell lines. **B.** Transfection efficiency was verified in NCI-H2170 and NCI-H520 cells. **C, D.** Proliferative capacity of NCI-H2170 and NCI-H520 cells. P<0.001.

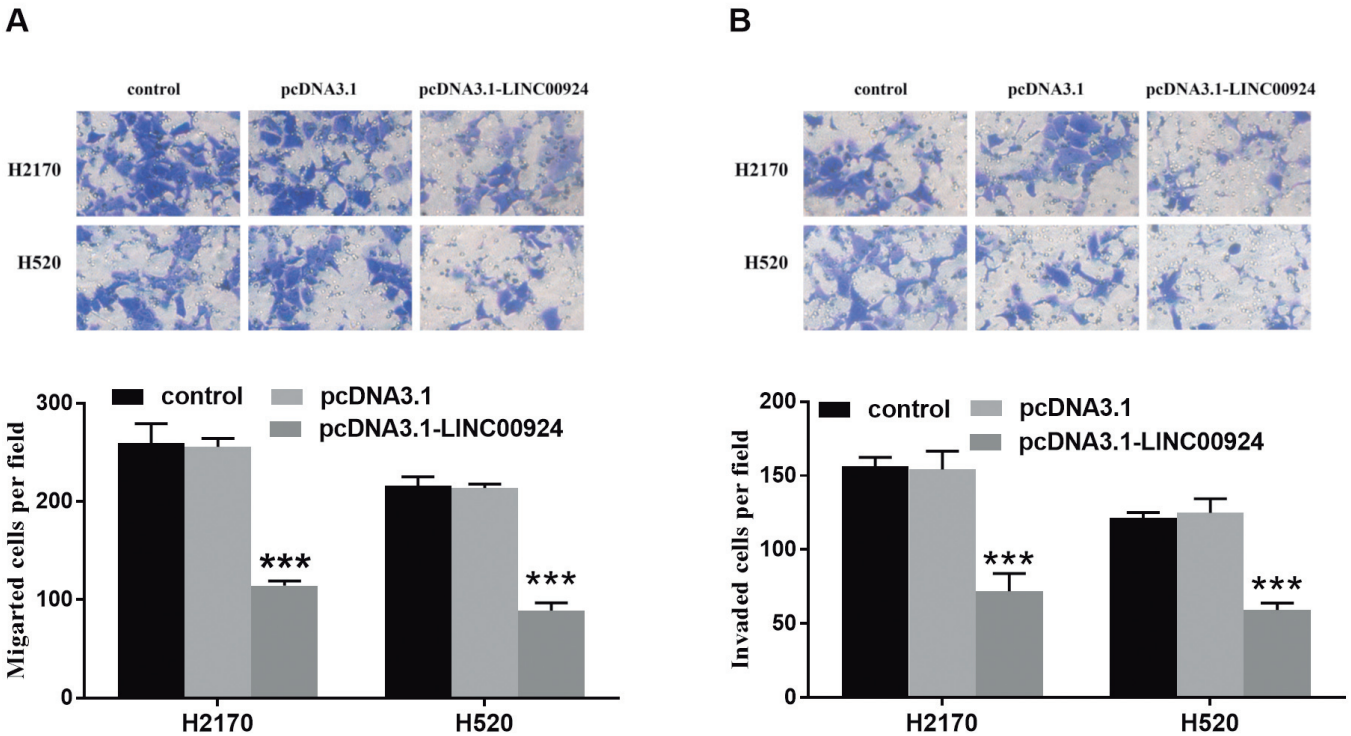

Fig. 4. Migration and invasion ability of LUAD cells after overexpression of LINC00924. **A.** Migratory ability of NCI-H2170 and NCI-H520 cells. **B.** Invasive ability of NCI-H2170 and NCI-H520 cells. ***P<0.001. x 200.

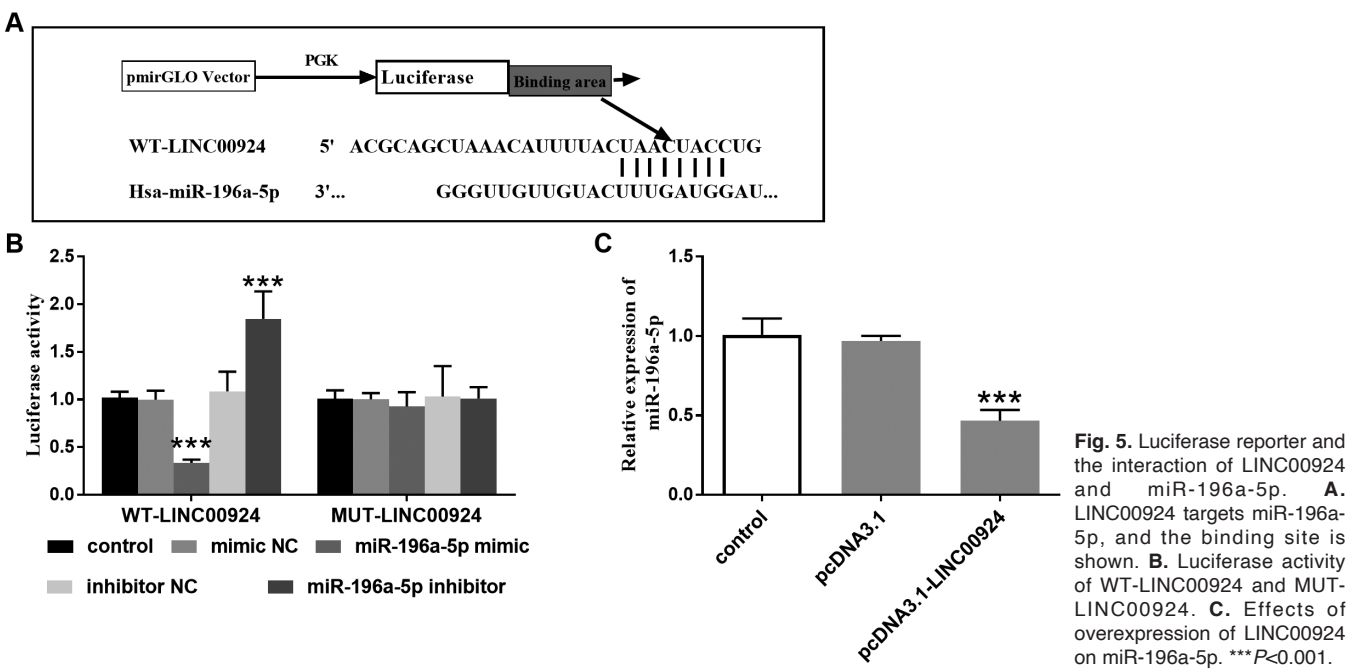

Fig. 5. Luciferase reporter and the interaction of LINC00924 and miR-196a-5p. **A.** LINC00924 targets miR-196a-5p, and the binding site is shown. **B.** Luciferase activity of WT-LINC00924 and MUT-LINC00924. **C.** Effects of overexpression of LINC00924 on miR-196a-5p. ***P<0.001.

addition, the expression level of miR-196a-5p was inhibited by pcDNA3.1-LINC00924 (Fig. 5C).

miR-196a-5p mimic rescued the inhibitory effect of LINC00924 on LUAD cells

LUAD cells were co-transfected with control group, pcDNA3.1, pcDNA3.1-LINC00924, LINC00924+miR-NC or LINC00924+miR-196a-5p mimic, and the transfection efficiency was verified by the expression level of miR-196a-5p (Fig. 6A). Figure 6B,C demonstrate that co-transfection with miR-196a-5p mimic rescued the inhibitory effect of LINC00924 on cell proliferation level in NCI-H2170 and NCI-H520 cells. Similarly, miR-196a-5p mimic reversed the inhibitory effect of LINC00924 overexpression on migration ability and invasion situation of NCI-H2170 and NCI-H520 cells (Fig. 7A,B).

Discussion

The application of lncRNAs in the research of many diseases and tumors is a hot direction in recent years, which is also confirmed by a large quantity of literature

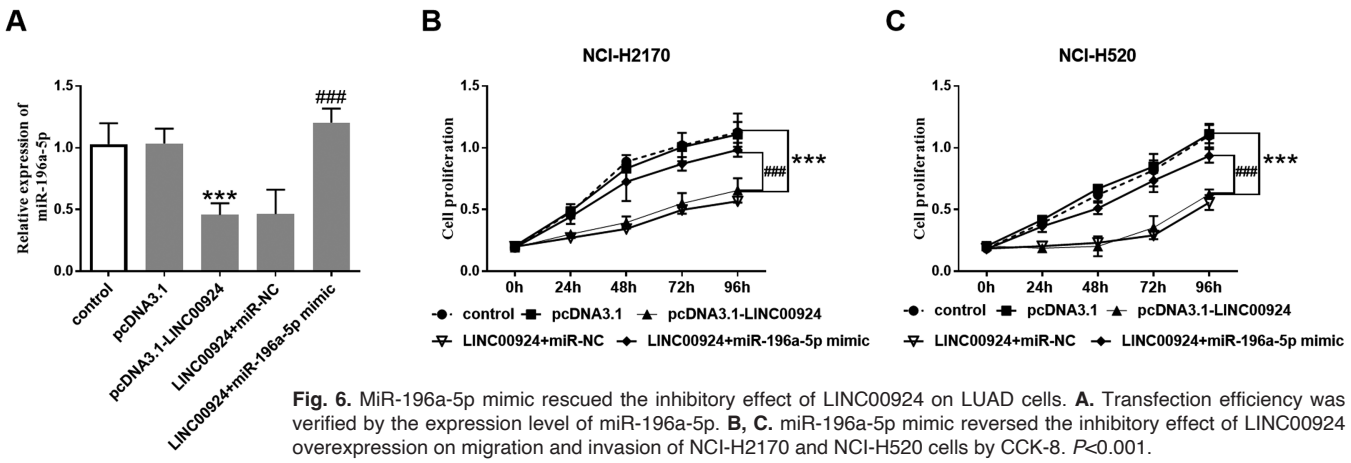

Fig. 6. MiR-196a-5p mimic rescued the inhibitory effect of LINC00924 on LUAD cells. **A.** Transfection efficiency was verified by the expression level of miR-196a-5p. **B, C.** miR-196a-5p mimic reversed the inhibitory effect of LINC00924 overexpression on migration and invasion of NCI-H2170 and NCI-H520 cells by CCK-8. *P*<0.001.

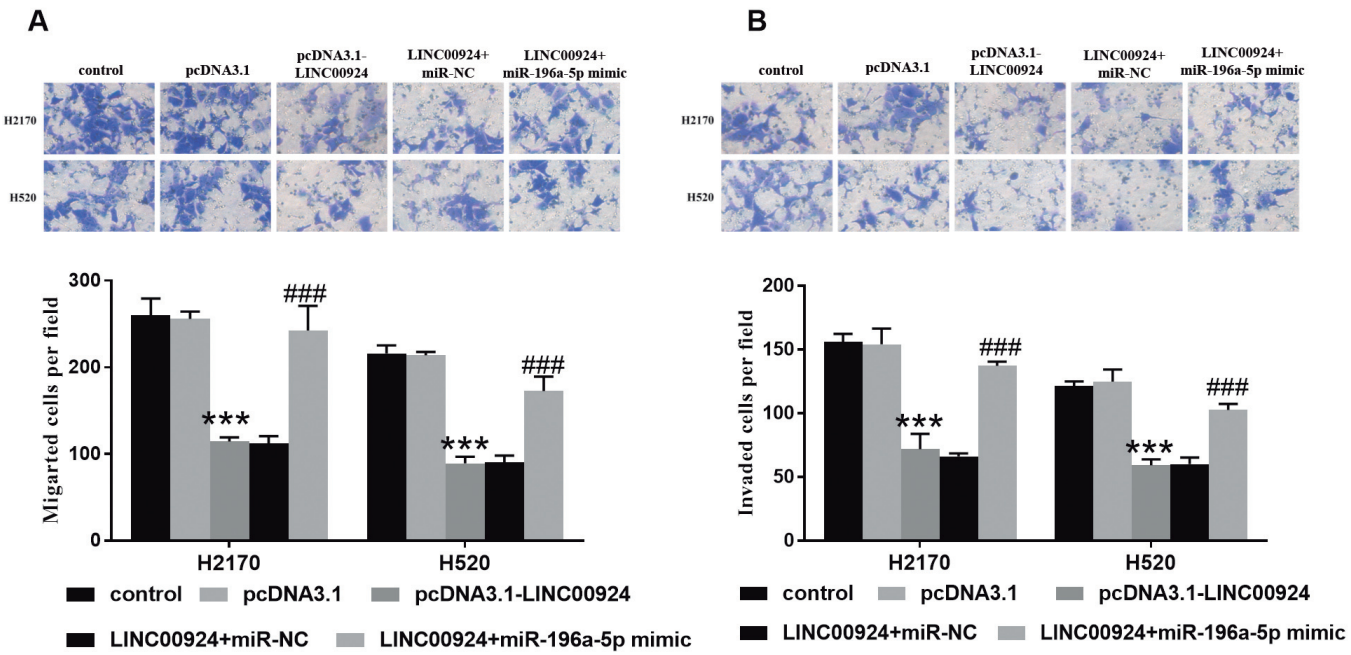

Fig. 7. Effects of miR-196a-5p mimic on migration and invasion levels of LUAD cells. **A.** miR-196a-5p mimic reversed the inhibitory effect of LINC00924 overexpression on NCI-H2170 and NCI-H520 cell migration. **B.** miR-196a-5p mimic reversed the inhibitory effect of LINC00924 overexpression on NCI-H2170 and NCI-H520 cell invasive. ***P<0.001 vs pcDNA3.1, ###P<0.001 vs LINC00924+ miR-NC. x 200.

and reports. In the process of fighting lung cancer, it has been found that non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancer, which is a persistent disease with poor prognosis and high mortality (Dai et al., 2020; Feng et al., 2022). Zhou and colleagues regulated the miR-30c-5p/SOX9 axis through lncRNA DLEU2 to promote the proliferation and invasion of NSCLC cells, thus DLEU2 was considered as a new diagnostic target for NSCLC (Zhou et al., 2019). Liu et al found that downregulation of lncRNA-XIST could trigger pyroptosis mediated by the miR-335/SOD2/ROS signaling pathway to inhibit NSCLC progression (Liu et al., 2019). In LUAD, one of the subtypes of NSCLC, there are also many differentially expressed lncRNAs can be used as potential biomarkers for diagnosis and prognosis. For example, DGCR5 has been shown to promote LUAD progression and act as a therapeutic target for the prognosis of LUAD patients by inhibiting miR-22-3p (Dong et al., 2018).

Previous exploration of LINC00924 revealed that central lncRNAs including LINC00924 may serve as key target genes for risk assessment of Hirschsprung's disease (Niu et al., 2020). Besides, lncRNAs such as LINC00924 can be chosen as candidate biomarkers to participate in the diagnosis or prognosis of thyroid cancer, and regulate cell migration, hormone levels, and RNA metabolism (Rao et al., 2020). The expression level of LINC00924 in LUAD and the regulation of its abnormal expression on the proliferation level, migration ability and invasion situation of cells were determined by RT-qPCR, CCK-8 method and Transwell method in this study. Compared with the control group, the expression of LINC00924 was down regulated in LUAD, while the overexpression of LINC00924 had a negative effect on the viability of LUAD cells. This result was consistent with the findings of LINC00924 in hepatocellular carcinoma (Yu et al., 2022), indicating that overexpression of LINC00924 had the function of inhibiting LUAD and might serve as a researchable target.

In the discussion of LUAD treatment and prognostic factors, we learned that Lymph node metastasis and TNM stage are associated with high expression of LINC00924. Kaplan-Meier survival curve and multivariate Cox regression analysis also confirmed the prognostic value of LINC00924 in LUAD patients. In the study of ferroptosis-related lncRNAs, Lu et al. demonstrated that 12 lncRNAs related to ferroptosis have good robustness and predictive ability for the treatment of LUAD through the above method (Lu et al., 2021). Similarly, the reports of immune-related 8-lncRNA signatures used to improve the prognosis of LUAD also showed that PR-lncRNA signatures can develop more LUAD anti-cancer treatment strategies to better predict the survival risk of patients (Chen et al., 2021). It is worth noting that lncRNA PTTG3P (Huang et al., 2020), hypoxia-associated lncRNA (Shao, et al., 2021a), and pyroptosis-related lncRNA (Huang et al., 2022) have all been confirmed to play a reference role in the prognostic value of LUAD.

LncRNAs were known to play biological functions to regulate the occurrence and development of diseases by targeting miRNAs and affecting their expression (Ni et al., 2021; Shao et al., 2021b). In this study, bioinformatics analysis confirmed the existence of binding sites between LINC00924 and miR-196a-5p. And the expression of miR-196a-5p was increased in LUAD, while high expression of LINC00924 inhibited the expression of miR-196a-5p. Interestingly, miR-196a-5p was identified as a biomarker for NSCLC, and miR-196-5p was overexpressed in tissues and serum and affected the pathogenesis of NSCLC (Bao et al., 2018; Jin et al., 2020a). In addition, miR-196-5p is closely related to a variety of lncRNAs and plays a key role in tumors. For example, lncRNA SNHG3 targeting miR-196a-5p promotes cell growth, suggesting poor survival in osteosarcoma (Chen et al., 2019). LncRNA NEAT1 promoted colorectal cancer cell proliferation level, and migration ability by sponge-sucking miR-196a-5p (Zhong et al., 2018). That is to say, LINC00924 targeting miR-196a-5p to inhibit the process of LUAD has certain research significance and value.

Conclusion

In conclusion, LINC00924 was decreased in LUAD tissues and cells, while miR-196a-5p was up-regulated. High expression of LINC00924 could effectively inhibit the proliferation, migration and invasion of LUAD cells, which was beneficial to patient survival and prognosis, possibly achieved by targeting miR-196a-5p. Therefore, LINC00924 might serve as a potential prognostic biomarker for LUAD, providing a theoretical basis for the treatment and survival of patients in the future.

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Disclosure. The author reports no conflicts of interest in this work.

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