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LncRNA WT-AS inhibits metastatic ability of non-small cell lung cancer by regulating KLK13

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of long non-coding RNA (lncRNA) WT-AS on the invasiveness and migration of non-small cell lung cancer (NSCLC) cells, and to explore the underlying molecular mechanism of lncRNA WT-AS in the pathogenesis of NSCLC.

PATIENTS AND METHODS: LncRNA WT-AS expression in 50 pairs of NSCLC tissues and adjacent ones was studied by quantitative real-time polymerase chain reaction (qRT-PCR) analysis, and the correlations of WT-AS with clinicopathological indicators and prognosis of NSCLC patients were analyzed. Meanwhile, NSCLC expression levels in NSCLC cell lines were also evaluated by qPCR assay. In addition, WT-AS overexpression and knockdown models were constructed using lentivirus in NSCLC cell lines A549 and H1299, respectively. Thereafter, transwell and cell wound healing assays were carried out to assess the implication of WT-AS in biological functions of NSCLC cells. Furthermore, the interaction between WT-AS and KLK13 was determined via Luciferase assay.

RESULTS: The results showed that WT-AS expression in NSCLC was remarkably lower than that in normal tissues adjacent to the cancer. Univariate analysis suggested that compared with patients with high expression of WT-AS, patients in low expression group showed higher incidence of metastasis and lower survival rates. Overexpression of WT-AS suppressed cell invasion and metastasis capacity, while the opposite result was observed in WT-AS knockdown group. KLK13 expression showed an increase in NSCLC cell lines and tissues, which was negatively correlated with WT-AS level. Meanwhile, Luciferase assay confirmed the binding between WT-AS and KLK13. Western blotting revealed that KLK13 expression was remarkably elevated in EC tissues and was positively correlated with TRIM62. In addition, it was also found that WT-AS and KLK13 had a mutual regulatory effect, which together affect the malignant progress of NSCLC.

CONCLUSIONS: This study shows for the first time that LncRNA WT-AS interacts with KLK13 to serve as a negative regulator of NSCLC progression.

Key Words:
LncRNA WT-AS, KLK13, NSCLC, Metastasis.

Introduction

Lung cancer, as one of the most common respiratory diseases with a very high mortality rate, ranks first in incidence and mortality rates[1,2]. According to the latest global cancer statistics, lung cancer is the most common type of male cancer patients, and it is the number one killer of male cancer patients[3,4]. The most common pathological type of lung cancer is non-small cell lung cancer (NSCLC), which accounts for 80-85% of all types of lung cancer[4]. In recent years, with the continuous development of medical technology, such as surgical resection, radiotherapy, chemotherapy, molecular targeted therapy, etc., some progress has been made in the treatment of lung cancer[5,6]. However, due to the lack of effective early diagnosis methods, most lung cancer patients have entered the middle and advanced stages at the time of diagnosis, and thus lost the best surgical opportunity[6,7]. Although these treatments have achieved certain effects, there are also many disadvantages, such as large side effects, easy recurrence, and poor patient tolerance[8,9]. Therefore, finding new tumor diagnostic markers and therapeutic targets is crucial for the diagnosis and treatment of lung cancer[10-12].

Non-coding RNA (ncRNA) plays a pivotal regulatory role in the occurrence of lung can-

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cer[10-13]. Competing endogenous RNA (ceRNA) is a network regulator that has been widely studied in tumors, which contains long non-coding RNA (lncRNA), microRNA, mRNA, pseudogene transcription products, circular RNA, etc[14-16]. Among which, lncRNA is a type of RNA that is longer than 200 bp without ability of encoding proteins and is mainly located in the nucleus or cytoplasm. At first, lncRNA has always been considered as a gene "noise" and has no biological function. Several lncRNAs have been verified to have certain biological properties in many diseases[17,18] and function in transcription level, post-transcription level and translation level, such as RNA processing, genome rearrangement, chromosome modification, X chromosome silencing, etc[17,18]. Currently, researches have demonstrated that lncRNAs are abnormally expressed in various tumor tissues to affect tumor progression[19,20].

There are currently few studies on LncRNA WT-AS, especially on the relationship between LncRNA WT-AS and NSCLC[21,22]. Bioinformatics suggests that KLK13 is one of the potential target genes of LncRNA WT-AS. Therefore, in this study, the expressions of WT-AS and KLK13 in 50 pairs of NSCLC tissues and adjacent tissues were analyzed, and their effects on the biological functions of NSCLC cells were explored. The purpose of this study was to investigate whether lncRNA WT-AS is implicated in malignant progression of NSCLC cells through modulating KLK13.

Patients and Methods

Patients and NSCLC Samples

A total of 50 NSCLC patients in our hospital were collected as the research object, and lung cancer tissues and normal tissues adjacent to the cancer (5 cm or more from the edge of the cancer tissues) were surgically removed and immediately stored in a -80°C refrigerator for subsequent research. The selection criteria for NSCLC are as follows: (l) all NSCLC patients were confirmed by pathological tissue section, (2) all NSCLC patients were initially diagnosed, without any treatment before diagnosis, (3) NSCLC staging was performed according to UICC 7th edition TNM staging system, (4) the patient's pathological characteristics were complete, and (5) the selected patients had no other malignant tumors. The exclusion criteria for this study are as follows: (l) patients whose pathological tissue sections could not be diagnosed, (2) patients with congenital lung hypoplasia, (3) patients with cardiovascular disease, or (4) patients combined with other lung diseases, etc. This investigation was approved by the Ethics Committee of the hospital, and the patients signed the informed consent.

Cell Lines and Reagents

Five human-derived NSCLC cell lines A549, H1299, PC-9, H358, SPC-A1 and one normal human bronchial epithelial cell line BEAS-2B (American Type Culture Collection (ATCC) (Manassas, VA, USA)) were cultured Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with fetal bovine serum (FBS, Life Technologies Company, Gaithersburg, MD, USA) in an incubator at 37°C with 5% CO_2.

Transfection

When cell density reached to 30-50%, transfection was carried out with the control sequences (NC or Anti-NC) and the lentivirus (WT-AS or Anti-WT-AS) containing WT-AS overexpression or knockdown sequences (GenePharma, Shanghai, China) according to the manufacturer's instructions.

Transwell Assay

A 24-well plate with an 8 mm chamber was used. The invasion experiment was performed by diluting a matrix gel with DMEM medium without FBS overnight at a ratio of 1: 6. 24 h after transfection, cells were prepared into cell suspensions and seeded in the upper chamber. The migrated cells were counted after washing with crystal violet and under a microscope. 5 fields of view were randomly selected.

Cell Wound Healing

After transfection for 48 hours, cells were resuspended in medium without FBS to adjust the density to 5 x 10^5 cells/mL. The density of the plated cells was determined according to their size (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the cells reached 90% or more the next day. After the stoke, cells were rinsed gently with phosphate-buffered saline (PBS) for 2-3 times and observed again after incubation in low-concentration serum medium for 24 h.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs in cells or tissues were isolated using TRIzol method (Invitrogen, Grand Island, NY), which were reversely transcribed to cDNA following the instructions of TaKaRa RNA PCR™ Kit (AMV). A 15 μL of PCR system including cDNA and 0.4 mol/L primers was subjected to amplification on a PCR Reactor, with three replicates per sample. Data obtained from three independent experiments were calculated by the formula $RQ = 2^{-\Delta\Delta Ct}$ and analyzed using the ABI Step One software. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal reference. Primers used in qRT-PCR were synthesized and their sequences are shown in Table I: LncRNA WT1-AS: 5'-GCCTCTCTGTCCTCTTCTTTGT-3' (forward) and 5'-GCTGTGAGTCCTGGTGCTTAG-3' (reverse); KLK13: 5'-CCCCAAATGGGTCCTCACTG-3' (forward) and 5'-GGGGTAATTCACAGCTTCCAC-3'; GAPDH: 5'-GCTCTCTGCTCCTCCTGTTC-3' (forward) and 5'-GACCAAATCCGTTGACTC-3'.

Western Blotting

Protein extraction in cells was performed using a radioimmunoprecipitation assay (RIPA) kit (Beyotime, Shanghai, China). The protein samples were separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). Primary antibodies against KLK13 and GAPDH were incubated on the membrane to determine protein levels in the cells. After incubation of the corresponding secondary antibody, Image J software (NIH, Bethesda, MD, USA) was used for protein quantification. All antibodies were purchased from CST (Danvers, MA, USA).

Luciferase Assay

A549 and H1299 cells were co-transfected with the reporter plasmid and KLK13 overexpression plasmid using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After that, the Luciferase activity of each group was measured after 48 h of transfection.

Statistical Analysis

Statistical analyses were performed with Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA). Kaplan-Meier analysis was used for analyzing patients' survival. The relationship between the factors affecting the prognosis of NSCLC was analyzed using the Cox proportional hazard model. $p<0.05$ suggested that the difference was statistically significant.

Results

LncRNA WT-AS Was Lowly Expressed in NSCLC

Figure 1A shows that WT-AS expression in NSCLC tissues was remarkably lower than that in adjacent ones ($p<0.05$). In addition, 12 pairs of above tissue samples were randomly selected to exhibit their WT-AS expression (Figure 1B), indicating that lowly-expressed lncRNA WT-AS may stunt the malignant progression of NSCLC. In addition, the same tendency was observed *in vitro*, which was, compared to normal human bronchial epithelial cells BEAS-2B, WT-AS was remarkably lowly expressed in NSCLC cell lines (Figure 1C). Among them, the expression was lower in H1299 and higher in A549 cell line, so these two cells were selected for subsequent transfection.

LncRNA WT-AS Was Correlated With Metastasis Incidence and Survival of NSCLC Patients

According to qPCR results of WT-AS expression, the 50 pairs of tissue samples were divided into high- and low-expression groups. Table II shows that the associations between the low expression of WT-AS or the high expression of KLK13 with the incidence of metastasis were

Table I. Primers used in qPCR reaction.

Gene	Primers
LncRNA WT-AS	Forward: 5'-GCCTCTCTGTCCTCTTCTTTGT-3' Reverse: 5'-GCTGTGAGTCCTGGTGCTTAG-3'
KLK13	Forward: 5'-CCCCAAATGGGTCCTCACTG-3' Reverse: 5' -GGGGTAATTCACAGCTTCCAC-3'
GAPDH	Forward: 5'-GCTCTCTGCTCCTCCTGTTC-3' Reverse: 5'-GACCAAATCCGTTGACTC-3'

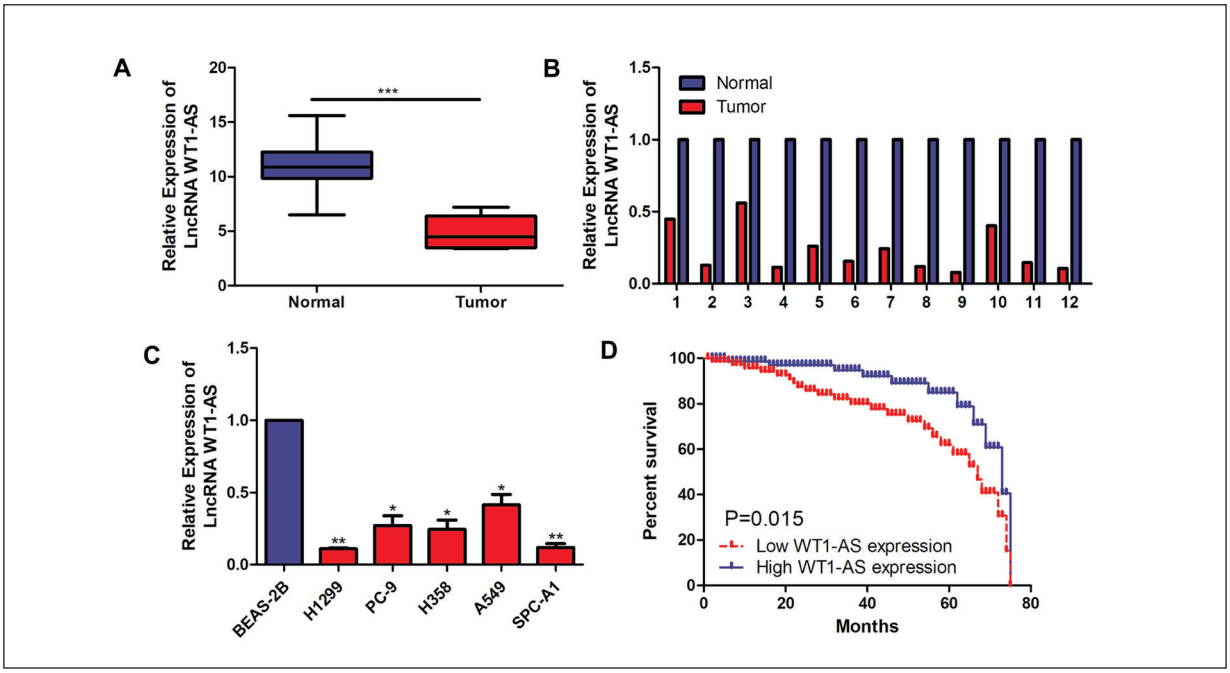

Figure 1. WT1-AS is underexpressed in NSCLC tissues and cell lines. **A, B,** qRT-PCR is used to detect the differential expression of WT1-AS in NSCLC tumor tissues and normal tissues adjacent to the tumor. **C,** qRT-PCR is used to detect the expression level of WT1-AS in NSCLC cell lines. **D,** Kaplan Meier survival curve of NSCLC patients based on WT1-AS expression is shown, and the prognosis of patients with a low expression of WT1-AS is significantly worse than that with a high expression of WT1-AS. Data are average ± SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

significant. In addition, Kaplan-Meier survival curve revealed that NSCLC patients with low expression of WT-AS had a lower survival rate than those with higher expression, suggesting that the lowly expressed WT-AS predicts poor prognosis of NSCLC patients ($p<0.05$, Figure 1D).

Table II. Association of LncRNA WT1-AS and KLK13 expression with clinicopathologic characteristics of Non-small cell lung cancer.

Parameters	No. of cases	WT1-AS expression		p-value	KLK13 expression		p-value
		High (%)	Low (%)		High (%)	Low (%)	
Age (years)				0.248			0.556
< 60	20	12	8		13	7	
≥ 60	30	13	17		17	13	
Gender				0.396			0.564
Male	25	14	11		16	9	
Female	25	11	14		14	11	
T stage				0.083			0.077
T1-T2	30	18	12		21	9	
T3-T4	20	7	13		9	11	
Lymph node metastasis				0.037			0.010
No	33	20	13		24	9	
Yes	17	5	12		6	11	
Distance metastasis				0.021			0.018
No	30	19	11		22	8	
Yes	20	6	14		8	12	

WT-AS Inhibited Cell Invasiveness and Migration in NSCLC

To test the influence of WT-AS on NSCLC cell invasiveness and migration ability, WT-AS overexpression and knockdown models were constructed in A549 and H1299 cells, respectively, and the transfection efficiency was verified through qPCR analysis. The results of qPCR showed that the transfection WT-AS overexpression vector increased the expression level of WT-AS, but the transfection WT-AS knockdown vector decreased the expression level of WT-AS (Figure 2A). Over-expression of WT-AS attenuated the invasive, as well as the migratory abilities of A549 cells, while knockdown of WT-AS enhanced those of H1299 cells, measured by cell scratch test and transwell experiment (Figure 2B-2C).

KLK13 Is a Direct Target of WT-AS

To further verify the targeting between KLK13 and WT-AS, the WT-AS sequence was cloned into the Luciferase reporter plasmid pmirGLO, and the mutation vector pmirGLO-WT-AS-mut was also constructed. pmirGLO-WT-AS-WT, pmirGLO-WT-AS-mut or pmirGLO and KLK13 were co-transfected into A549 and H1299 for Luciferase reporter experiment. The results revealed that over-expression of KLK13 markedly attenuated the Luciferase activity ($p<0.05$) in pmirGLO-WT-AS-WT group, but no significant changes were detectable in mutant ($p>0.05$) or empty control group ($p>0.05$), further demonstrating that WT-AS can be targeted by KLK13 (Figure 3A). Meanwhile, KLK13 expression was significantly decreased after over-expression of WT-AS but increased after knockdown of WT-AS, measured by Western blotting analysis (Figure 3B). KLK13 expression also showed an elevation in NSCLC cell lines as compared to normal human bronchial epithelial cells BEAS-2B (Figure 3C). Similarly, NSCLC tissue samples showed a higher expression of KLK13 than the corresponding normal tissues (Figure 3D). In NSCLC tissues, the mRNA expression levels of WT-AS and KLK13 were negatively correlated (Figure 3E). In addition, Kaplan-Meier survival curve revealed that the expression of KLK13 was negatively relevant to the prognosis of NSCLC patients ($p<0.05$; Figure 3F).

KLK13 Modulated WT-AS Level in NSCLC

To further understand how WT-AS and KLK13 inhibit the malignant progression of NSCLC cells, we performed co-transfection of WT-AS and KLK13 overexpression plasmid or knockdown sequences in NSCLC cell lines A549 and H1299. It was found that overexpression of KLK13 reversed the increased WT-AS gene expression induced by overexpression of

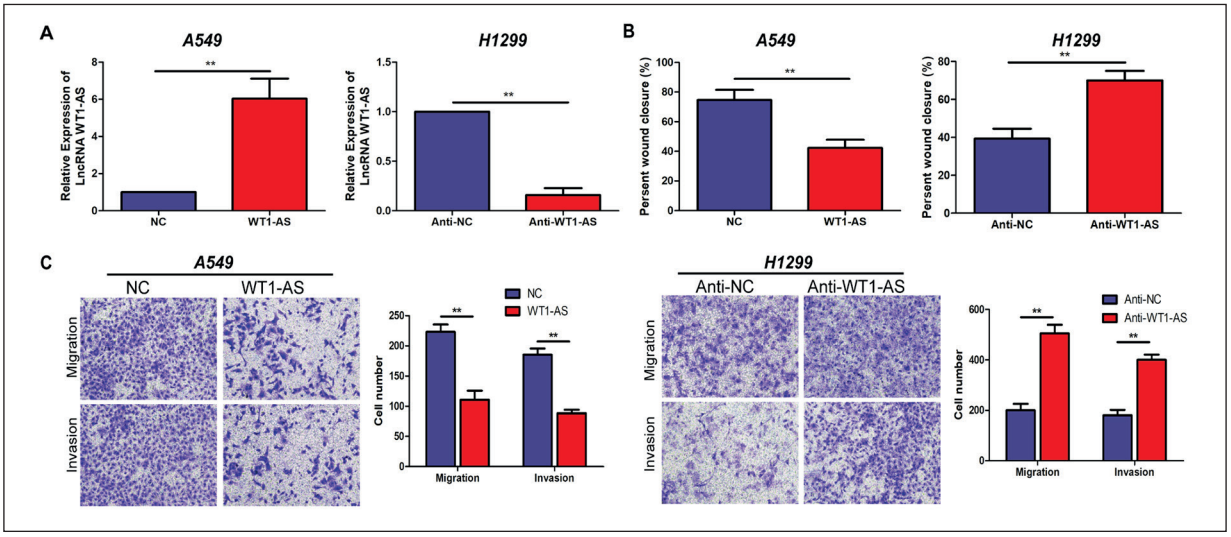

Figure 2. WT1-AS can inhibit the invasion and migration of NSCLC cells. **A,** qRT-PCR verifies the transfection efficiency of WT1-AS after transfection of WT1-AS overexpression vector in A549 cell line and WT1-AS knockdown vector in H1299 cell line. **B,** The cell wound healing test is used to detect the crawling ability of NSCLC cells after transfecting A549 and H1299 cell lines with WT1-AS overexpression or knocking down the vector, respectively. **C,** Transwell assay tests the ability of NSCLC cells to invade and migrate after A549 and H1299 cell lines are transfected with WT1-AS overexpression or knockdown vectors, respectively (magnification: 40 ×). Data are average ± SD, ** $p<0.01$.

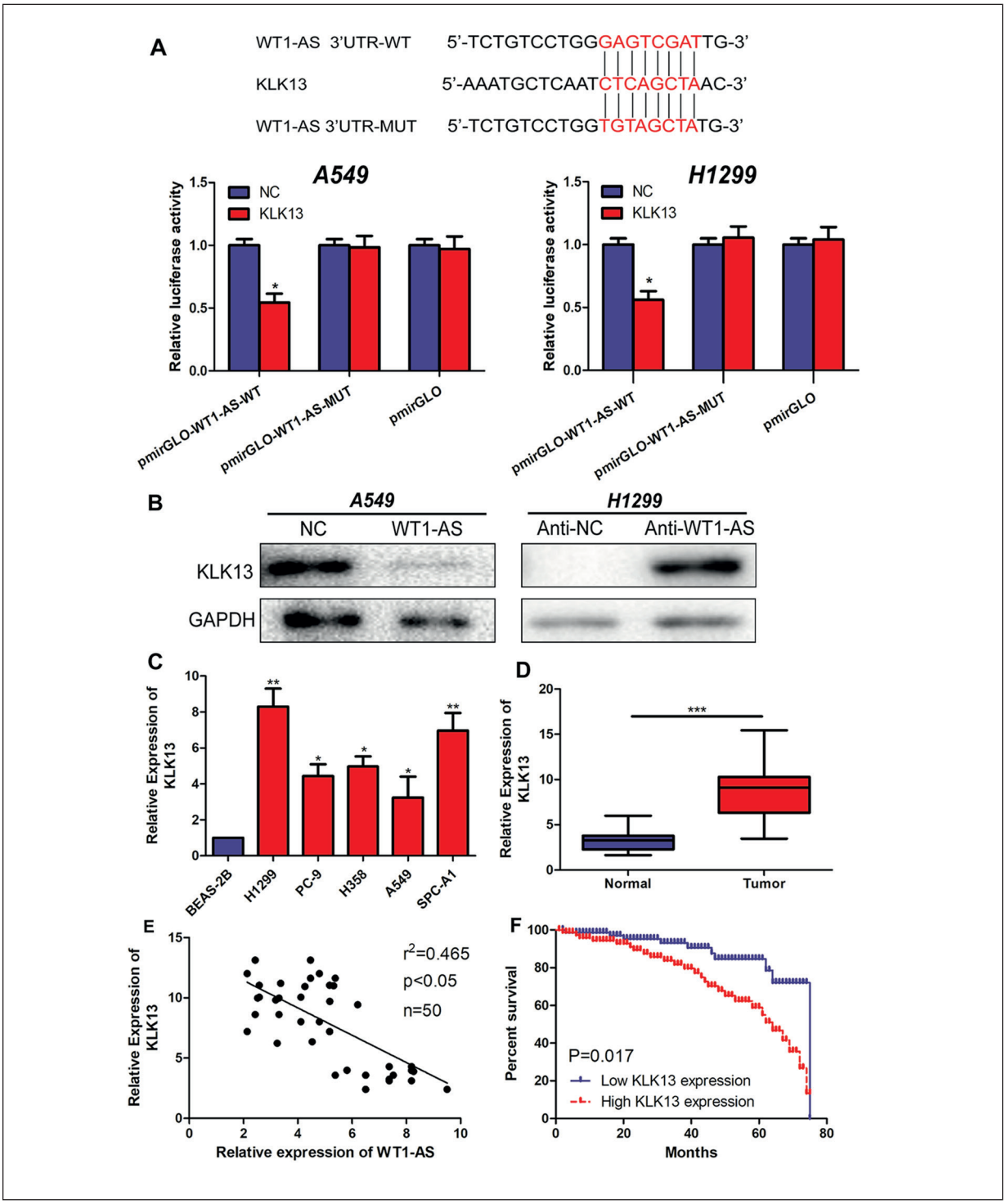

Figure 3. WT1-AS directly targets KLK13. **A,** Dual-Luciferase reporter assay verifies the direct targeting of WT1-AS and KLK13. Overexpression of KLK13 can significantly reduce the Luciferase activity (p<0.001) of the wild-type WT1-AS vector, but not the mutant vector (p>0.05) or Luciferase activity of the empty vector (p> 0.05); **B,** Western blotting experiments verify KLK13 expression after transfection of WT1-AS overexpression vector in A549 cells and transfection of WT1-AS knockout vector in H1299 cell line. **C,** qRT-PCR is used to detect the expression level of KLK13 in NSCLC cell line. **D,** qRT-PCR is used to detect the difference in KLK13 expression in NSCLC tumor tissue and normal tissue adjacent to the cancer. **E,** There is a significant negative correlation between the expression levels of WT1-AS and KLK13 in NSCLC tissues. **F,** Kaplan Meier survival curve of patients with NSCLC based on KLK13 expression is shown, and the prognosis of patients with a high expression of KLK13 is significantly worse than that with a low expression of KLK13. Data are average ± SD, * p<0.05, ** p<0.01, *** p<0.001.

WT-AS, while knockdown of KLK13 enhanced the inhibited WT-AS mRNA expression caused by transfection of anti-WT-AS. On the contrary, the opposite result was observed in KLK13 protein expression (Figure 4A, 4B). Subsequently, transwell assay demonstrated that silencing KLK13 could offset the promotion effect of silenced WT-AS on the invasion and metastasis of NSCLC cells, while overexpression of KLK13 could offset the inhibitory effect of overexpression of WT-AS on cell invasiveness and metastasis (Figure 4C).

Discussion

Tumor occurrence and development are complex pathophysiological processes involving multiple factors and stages, including activation of oncogenes, inactivation of tumor suppressor genes, abnormal cell signal transduction, abnormal cell cycle regulation, cell invasion, metastasis and apoptosis[9-11]. In recent decades, malignant tumors have become the largest killer threatening human health on a global scale[1,12], among which, lung cancer is one of the malignant tumors with the highest morbidity and mortality worldwide[1-3]. There are many reasons for poor prognosis of lung cancer patients, so early detection, early diagnosis and early treatment are still the best preventive measures[4-6]. Surgical resection, radiotherapy and chemotherapy are common treatment methods in clinical practice, but there is still much room for improvement in the prognosis and quality of life of cancer patients[7-9]. Currently, with in-depth research on the pathogenesis of lung cancer, the discovery of new targeted drugs has provided new ideas for its treatment[9,10]. However, the known pathogenesis of lung cancer is still unable to effectively elucidate its progression mechanism, so it is of great practical significance to further study the pathogenesis of lung cancer and develop new targeted drugs[11,12].

With the emergence of genomics and large-scale gene sequencing, it has been found that there are about 20,000 protein-coding genes in human, but the total gene proportion is less than 2%, that is, about 98% of the genes cannot encode proteins, which is called non-coding RNA (ncRNA)[13,14]. LncRNA plays a pivotal role in the pathogenesis of cancer, and thus become another research hotspot after miRNA[15,16]. Although they do not have a functional open reading frame (ORF), some lncRNAs are implicated in both regulatory and non-dependent protein coding functions[16,17]. In terms of cell function, they

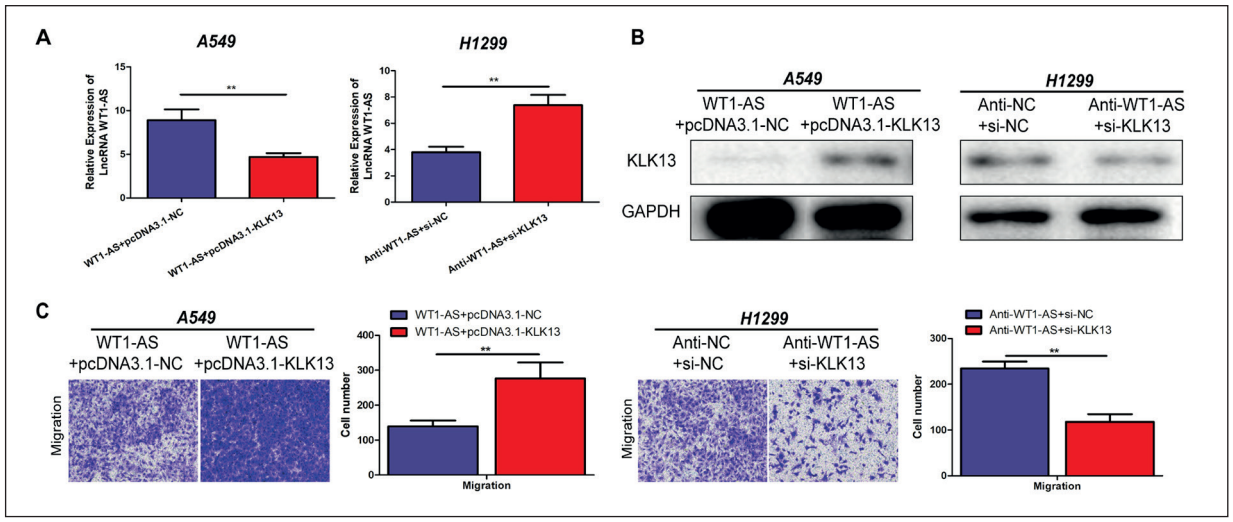

Figure 4. WT1-AS regulates the expression of KLK13 in NSCLC cells. **A,** qRT-PCR is used to detect the expression levels of WT1-AS in A549 and H1299 cell lines after co-transfection of WT1-AS and KLK13 overexpression or knockdown of the vector, respectively. **B,** Western blotting is used to detect KLK13 expression levels in A549 and H1299 cell lines after co-transfection of WT1-AS and KLK13 overexpression or knockdown of the vector, respectively. **C,** Transwell invasion assay examines the invasion ability of NSCLC cells after co-transfection of WT1-AS and KLK13 overexpression or knockdown of the vector in A549 and H1299 cell lines (magnification: 40 ×). Data are average ± SD, ** $p<0.01$.

can affect the proliferation, invasion, metastasis and apoptosis of tumor cells[17]. Abnormal expression of lncRNA can promote the occurrence of a variety of cancers, including NSCLC[17,18]. Therefore, the identification of differentially expressed lncRNAs in NSCLC and the analysis of their function can contribute to the advancement of diagnosis and treatment level and the prognosis improvement[13,19]. In this study, the expressions of LncRNA WT-AS and KLK13 in 50 NSCLC tissues and adjacent tissues were first verified, and it was found that LncRNA WT-AS expression was remarkably down-regulated while KLK13 was up-regulated. Low expression of WT-AS was positively correlated with the incidence of distant metastasis and poor prognosis of NSCLC patients. These results suggest that WT-AS acts as a cancer-inhibiting gene while KLK13 serves as an oncogene in NSCLC. To further explore the impact of lncRNA WT-AS on the biological function of NSCLC, WT-AS overexpression/knockdown models were constructed in NSCLC cells, and transwell and cell scratch experiments showed that WT-AS could inhibit the invasiveness and migration ability of NSCLC. However, the specific molecular mechanism remains unclear.

LncRNA can regulate various functions of cells through different molecular mechanisms, such as chromatin modification, transcriptional regulation and post-transcriptional regulation[13,18]. They have been shown to act as "molecular sponges" for mRNA and serve as regulatory mRNA targets for ceRNA[18,19]. In this study, the binding of KLK13 to WT-AS was verified through Luciferase reporter assay. The KLKs family, located at 19q13.3-13.4, contains a total of 15 serine protease genes and encodes a group of secreted serine proteases[23,24]. KLK mutation or enzyme activity increase is associated with many diseases, including neurodegenerative changes, skin inflammation, and cancer, etc[25]. Among them, KLK13, also known as KLK-l4, is a 10-kb DNA sequence with five exons and four introns[26]. KLK13, alone or in combination with other markers, can be used to predict the prognosis of multiple tumors[27,28]. It was found that KLK13 was highly expressed in NSCLC tissues than in adjacent ones and could promote the invasiveness and migration ability of NSCLC cells. The above findings suggest that LncRNA WT-AS may lead to a rapid decrease in KLK13 concentration, thus inhibiting the malignant progression of lung cancer.

Conclusions

In summary, LncRNA WT-AS expression is remarkably down-regulated in NSCLC and correlated with metastasis incidence and poor prognosis of NSCLC patients. This describes a novel mechanism of action for LncRNA WT-AS inhibiting the malignant progression of NSCLC through negative regulation of KLK13.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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