Long Noncoding RNA DNAH17-AS1 Promotes Tumorigenesis and Metastasis of Non-Small Cell Lung Cancer via Regulating miR-877-5p/CCNA2 Pathway

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Abstract

Background: A growing number of studies have revealed that long noncoding RNAs (lncRNAs) can function as important oncogenes or tumor suppressors. This study aimed to investigate the regulatory role of lncRNA DNAH17 antisense RNA 1 (DNAH17-AS1) on non-small cell lung cancer (NSCLC) and the underlying molecular mechanisms.

Methods: RT-PCR was used to examine the expression of DNAH17-AS1, miR-877-5p and CCNA2 in NSCLC specimens and cell lines. The diagnostic and prognostic values of DNAH17-AS1 expression in NSCLC patients were statistically analyzed. We also evaluated the effects of DNAH17-AS1 on the proliferation, migration, invasion and apoptosis of H1299 and 95D cells. Bioinformatic analysis and luciferase reporter assays were carried to confirm the molecular binding.

Results: The expression of DNAH17-AS1 and CCNA2 mRNA was distinctly upregulated in NSCLC specimens and cell lines, while miR-877-5p expression was significantly decreased. DNAH17-AS1 could be used to distinguish NSCLC specimens from adjacent non-tumor tissues. Clinical assays revealed that high DNAH17-AS1 was associated with TNM stage, distant metastasis and shorter overall survival and disease-free survival. Functional assays indicated that knockdown of DNAH17-AS1 suppressed the proliferation, migration and invasion of H1299 and 95D cells, and promoted apoptosis. Mechanically, DNAH17-AS1 served as competing endogenous RNA (ceRNA) for miR-877-5p to positively recover CCNA2.

Conclusion: We identified a novel NSCLC-related lncRNA, DNAH17-AS1 which may exert an oncogenic function via serving as a sponge for miR-877-5p to upregulate CCNA2. Our study presents novel insights into NSCLC progression and provided a prospective therapeutic target for NSCLC.

Background

Lung cancer is still the major cause of mortality related to cancer worldwide regarding the incidence and mortality worldwide and is responsible for approximately 1.62 million newly diagnosed cases and 1.43 million deaths every year^{1,2}. The proportion of people diagnosed with non-small cell lung cancer (NSCLC) occupies about 80% of those diagnosed with lung cancer³. In China, NSCLC population has presented a quick uptrend in the last five years⁴. Despite some improvements in early diagnosing and treating NSCLC patients, NSCLC still presents a poor long-term prognosis because of frequent cancer metastasis and high recurrence rate^{5,6}. On that account, it is necessary to more deeply understand the mechanism of NSCLC as well as identify related novel biomarkers.

As the high-throughput DNA sequencing technology and assay-based technology develop, a lot of new long non-coding RNAs (lncRNAs) have been identified⁷. lncRNAs generally comprise non-protein-coding RNAs with 200 nt- to 100 kb-long transcripts, which do not have an open-reading frame or the protein-coding ability⁸. As proved by studies recently, lncRNA can regulate expression of various genes, before,

during and after the transcription, thereby remarkably affecting different biological activities, such as embryonic development, growth of cell and tumorigenesis^{9,10}. An increasing number of reports indicated that lncRNAs dysregulation could provide a new insight for better understanding the abnormal cancer activities regarding metabolism, proliferation, and metastasis^{11,12}. Based on recent studies, lncRNAs could play the role of molecular sponges, thus competitively hindering miRNA¹³. These findings stressed on the potential effects of lncRNA as novel therapeutic targets and tumor-related biomarkers for predicting tumor patients' diagnosis and prognosis^{14,15}.

Long non-coding RNA DNAH17 antisense RNA 1 (DNAH17-AS1) can be found in the 17q25.3, and is a kind of lncRNA related to tumors. Studies reported its high expression and the ability to promote tumor progression in pancreatic carcinoma and colorectal cancer^{16,17}. Nevertheless, its expression pattern as well as potential function in other tumors was still not well understood. The study firstly reported the upregulation of DNAH17-AS1 expression in NSCLC specimens and further explored its biological activity and value for NSCLC patients.

Methods

Patients and Tissue Sample

We obtained the tumor tissues with paired adjacent normal tissues from 147 patients with NSCLC at the Department of The Second Affiliated Hospital of Xi'an Medical University from 2012 to 2015. All cancers were confirmed as NSCLC by the medical examination of hematoxylin and eosin. No patient had received chemotherapy or radiation therapy before surgery. The obtained fresh tissue samples were quickly frozen in the liquid nitrogen. Our present study has obtained the approval of the Medical Ethics Committee of The Second Affiliated Hospital of Xi'an Medical University, and the written informed consents of all participators prior to experiments.

Cell culture and Cell transfection

NSCLC cell lines A549, SPC-A1, H1299, 95D, and SK-MES-1 as well as normal human bronchial epithelial cells (16HBE) were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). We cultured cells in RPMI 1640 medium (GIBCO, Hangzhou, Zhejiang, China) added with 10% FBS (GIBCO, Hangzhou, Zhejiang, China). Cultures underwent incubation at 37 °C in a humidified atmosphere containing 5% CO₂.

We seeded cells into 6-well plates and used the Lipofectamine 2000 (Invitrogen, Nanjing, Jiangsu, China) to transfect them with miR-877-5p inhibitor, miR-877-5p mimics, or normal control (NC). Recombinant plasmid overexpressing DNAH17-AS1 with the empty pcDNA3.1 vector (GenePharma, Pudong, Shanghai, China), were transfected into cells in logarithmic growth phase. For inhibiting endogenous DNAH17-AS1 expression, siRNA targeting DNAH17-AS1 and the corresponding control RNA (s-NC) were provided by Shanghai GenePharma, Pudong, Shanghai, China).

Bioinformatics analysis

The expression pattern of CCNA2 and its survival assays in NSCLC patients was analyzed using GEPIA website or UALCAN program^{18,19}. MiRDB, TargetScan, starbase and mirWalk predicted the target genes of miR-877-5p. DAVID program or Co-LncRNA helped to perform the Gene ontology (GO) and KEGG analysis. The putative targets of DNAH17-AS1 were predicted by the starbase v2.0²⁰.

Quantitative real time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Grand Island, NY, USA) helped to extract the total RNA of the frozen specimens and cells following the instruction of manufacturer. UV spectrophotometer helped to detect the purity and concentration exhibited by the total RNA at 260 nm. Superscript III transcriptase (Invitrogen, Grand Island, NY, USA) assisted in reverse transcribing RNA into cRNA. An ABI7500 system together with SYBR Green PCR Master Mix (Takara, Dalian, Liaoning, China) were applied to the qRT-PCR for measuring the expression level of DNAH17-AS1, miR-877-5p and CCNA2. We used GAPDH and U6 as an endogenous control. The relative expressions of genes were determined by the $2^{-\Delta Ct}$ methods. The qRT-PCR specific primers were purchased from Ambion (Xuhui, Shanghai, China). Table 1 lists the primer sequences.

Table 1
Sequence of the primers used in this study.

Names	Primer sequences (5'-3')
DNAH17-AS1: Forward	TGCTGGACGGAAGCACCATC
DNAH17-AS1: Reverse	CTCAACGGCTGATCAACGCCA
miR-877-5p: Forward	GTAGAGGAGATGGCGCAGG
miR-877-5p: Reverse	CTCTACAGCTATATTGCCAGCCAC
CCNA2: Forward	CGCTGGCGGTACTGAAGTC
CCNA2: Reverse	GAGGAACGGTGACATGCTCAT
GAPDH: Forward	GGGAGCCAAAAGGGTCATCT
GAPDH: Reverse	GAGTCCTTCCACGATACCAAC
U6: Forward	GCGCGTCGTGAAGCGTTC
U6: Reverse	GTGCAGGGTCCGAGGT

Cell proliferation assays

Cell Counting Kit-8 (CCK-8) assay kits (Dojindo, Haidian, Beijing, China) assisted in conducting the cell proliferation assays. Briefly, cells received 24 h, 48 h, 72 h and 96 h of transfection by using the plasmid. Each well was added with CCK-8 (10 μ L). Cells were incubated for one hour at 37 $^{\circ}$ C, then a microplate reader absorbance test plate (Molecular Devices, Shenzhen, Guandong, China) assisted in measuring

each well's spectrophotometric absorbance at 450 nm at various time points. The relative absorbance at 450 nm decided the number of cells. We conducted all experiments in triplicate.

When performing the colony formation assay, experimenters placed 600 GC cells in total in a fresh six-well plate as well as maintained them in the media that contained 10% FBS (replaced each three days). Twelve days later, methanol and 0.1% crystal violet (1 mg/ml) helped to fix and stain colonies, respectively. Number of visible colonies was counted by experimenters.

5-Ethynyl-2'-deoxyuridine (EdU) assays

An EdU Apollo-DNA kit (RIBOBIO, Xicheng, Beijing, China) was applied to the 5-Ethynyl-2-deoxyuridine incorporation assays for determining the proliferation of cells. Experimenters seeded about 6.0×10^3 cells/well in a 96-well plate, then used si-NC and si-DNAH17-AS1#1 and si-DNAH17-AS1#2 to transfect them. After being transfected, cells received 2 h of incubation by EdU at 37 °C. 4% polyformaldehyde that contained PBS was used to fix cells. Finally, a fluorescence microscopy assisted in visualizing these cells. Experiments were conducted repeatedly for no less than three times.

Flow cytometric analysis

48 hours after being transfected with trypsinisation, H1299 and 95D cells transfected with DNAH17-AS1#1, si-DNAH17-AS1#2 or si-NC were harvested. FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Hangzhou, Zhejiang, China) was firstly adopted to double stain cells by using propidium iodide (PI) and FITC-Annexin V based on the users' handbook, then flow cytometry (BD Biosciences, Hangzhou, Zhejiang, China) was employed to analyze stained cells under the assistance of CellQuest software (BD Biosciences, Hangzhou, Zhejiang, China).

Caspase activity detection

Caspase activity detection kits (Beyotime, Nanjing, Jiangsu, China) helped assess caspase 3/9 activities in DNAH17-depleted NSCLC cells according to the manufacturer's protocol.

Wound healing assay

Cells in each group were implanted into 6-well culture plates with the density of 4.0×10^6 cells/well. With the density of cell up to 80 to 90%, a 100 µl pipette tip was used to made a scratch in the monolayer in the middle of the well. Experimenters kept the tip perpendicular to the bottom of the well for maintaining a straight gap. Experimenters used solutions to wash away the detached cells and removed them three times each day. At 0 and 48 h incubation, the H1299 and 95D cell lines were photographed using the inverted microscope (Olympus, Shenzhen, Guangdong, China) and the scratch area was assessed using Image J software.

Transwell invasion assay

Matrigel (1:8, BD, Lanzhou, Gansu, China) was coated on transwell inserts' upper chamber (with the pore size of 8 µM, Nalge Nunc Intl., NY, USA) so as to perform the for invasion assays. Experimenters placed 3

$\times 10^4$ cells into the upper chamber in RPMI-1640 (0.3 ml, without serum), and placed RPMI-1640 that contained 10% FBS in the lower chamber for being used as a chemoattractant. Cells that still were in the upper chambers were removed, then 0.3% crystal violet dye was used to stain these in lower chambers and PBS was used to wash them. A light microscope ($\times 40$) assisted in counting the obtained images. Each assay was repeated three times.

Subcellular fractionation

The PARIS Kit (Life Technologies, Haidian, Beijing, China) assisted in separating the cytosolic and nuclear fractions of the two kinds of cells. After the extraction of total RNA from both fractions, RT-PCR was further carried out to examine DNAH17-AS1 expression ratios between the nuclear fraction and the cytoplasmic fraction. GAPDH and U6 were used as the controls.

Luciferase reporter assays

Experimenters synthesized the full fragments or mutant of DNAH17-AS1 that contained putative miR-877-5p-binding sites in the DNAH17-AS1, followed by cloning them into downstream of the firefly luciferase gene in the pGL3 plasmids (Promega Corporation, Hangzhou, Zhejiang, China), with the name of pGL3-DNAH17-AS1-wild type (Wt) and pGL3-DNAH17-AS1-mutant (Mut). Also, PCR helped to amplify the 3'UTR of CCNA2 that contained predicted miR-877-5p-binding sites or mutant sites, which were then inserted into pGL3 plasmids, with the name of pGL3-miR-877-5p-3'UTR-Wt1, pGL3-miR-877-5p-3'UTR-Mut, pGL3-miR-877-5p-3'UTR-Wt2 and pGL3-miR-877-5p-3'UTR-Mut2. Cells grew in the 96-well plate, followed by being co-transfected using Lipofectamine 2000 (Invitrogen, Suzhou, Jiangsu, China). Cells were harvested 48 h after being transfected and the Dual-Luciferase reporter assay system (Promega, Guangzhou, Guangdong, China) was employed for measuring the luciferase activity following manufacturer's protocol.

Statistical analysis

SPSS software, version 19.0 (SPSS, Chicago, IL, USA) assisted in performing the statistical analysis. Chi-square test or student's t-test helped to analyze the difference between the two groups. The multi-group comparison was performed using one-way analysis of variance. The paired comparison was performed by SNK approach. Receiver-operating characteristic (ROC) curves assisted in evaluating the performance of CCNA2 and DNAH17-AS1 to discriminate NSCLC specimens from normal lung tissues. The survival rate was calculated with the Kaplan-Meier method and the calculated survival rates were compared with the log-rank test. The significance of survival variables was analyzed using the Cox multivariate proportional hazards model. The value of $p < 0.05$ was considered with statistical significance.

Results

DNAH17-AS1 Upregulation in NSCLC patients and its diagnostic value

In recent years, growing studies have revealed that lncRNAs were frequently dysregulated in different types of tumors, including NSCLC²¹. To explore whether DNAH17-AS1 expression was dysregulated in NSCLC, we collected 147 pairs of tumor specimens together with matched normal tissues (the control group) from 147 patients. As shown in the RT-PCR in Fig. 1A, NSCLC specimens exhibited a higher DNAH17-AS1 expression related to the control group ($p < 0.01$). We also observed that advanced-stage tumor tissues exhibited a higher level of DNAH17-AS1 than early-stage tumor specimens (Fig. 1B). Then, we examined the levels of DNAH17-AS1 in several cell lines, finding that five NSCLC cell lines saw obviously upregulated DNAH17-AS1 expression relative to 16HBE cells (Fig. 1C). Therefore, it is suggested to Fig. out the possible diagnostic value of DNAH17-AS1 for NSCLC patients. As presented in Fig. 1D, according to the ROC assays, high DNAH17-AS1 expression had an AUC value of 0.7400 (95% CI: 0.6831 to 0.7969) for NSCLC. The sensitivity and specificity of DNAH17-AS1 expressions for distinguishing HCC samples from normal samples was 63.21%/80.67%, indicating DNAH17-AS1 as an indicator for the diagnosis of NSCLC patients. Combining the above discussion, DNAH17-AS1 presented a high expression level in NSCLC and may serve for positively regulating NSCLC.

High DNAH17-AS1 expression means poor prognosis in NSCLC patients

Then, we explored the clinical significance exhibited by DNAH17-AS1 expression for NSCLC patients. The median expression value of DNAH17-AS1 in NSCLC tissues was chosen as a cutoff value and used to assign the 147 patients with NSCLC to group with a high expression ($n = 74$) and group with a low expression ($n = 73$). Based on Table 2, we observed that high DNAH17-AS1 was associated with TNM stage ($p = 0.002$) as well as distant metastasis ($p = 0.014$). However, DNAH17-AS1 expression status was not associated with other clinical factors (All $p > 0.05$). For further figuring out if DNAH17-AS1 expression can help to predict the prognosis of NSCLC patients, the study applied the log-rank test to the Kaplan-Meier analysis, for assessing the impact of DNAH17-AS1 expression on the survival of patients. As shown in Fig. 1E and 1F, group with a high expression exhibited an obviously poorer OS ($p = 0.0120$) and DFS ($p = 0.0028$) relative to group with a low expression. Moreover, multivariate assays were performed to determine whether DNAH17-AS1 was an independent factor for prognostic prediction in NSCLC patients. Importantly, our group observed that expression of DNAH17-AS1 could be used to independently predict the prognosis of patients in terms of OS (HR = 2.817, 95% CI: 1.314–4.457, $p = 0.013$) and DFS (HR = 3.117, 95% CI: 1.438–4.895, $p = 0.007$) (Table 3). Accordingly, DNAH17-AS1 can be used as a biomarker to predict the prognosis of NSCLC patients.

Table 2
Correlation of DNAH17-AS1 expression with clinicopathological features of NSCLC.

Clinicopathological features	Number of cases	DNAH17-AS1 expression		<i>p</i> -value
		High	Low	
Age (years)				0.215
< 60	75	34	41	
≥ 60	72	40	32	
Gender				0.815
Male	94	48	46	
Female	53	26	27	
Tumor size (cm)				0.335
< 3	93	44	49	
≥ 3	54	30	24	
Histologic type				0.360
SCC	79	37	42	
AD	68	37	31	
TNM stage				0.002
I + II	97	40	57	
III + IV	50	34	16	
Distant metastasis				0.014
Yes	103	45	58	
No	44	29	15	

Table 3
Cox regression analysis of factors associated with overall survival in 147 NSCLC patients.

Variables	Overall survival		Disease-free survival	
	HR (95%CI)	<i>p</i> -value	HR (95%CI)	<i>p</i> -value
Age (years)	1.352(0.673–2.138)	0.138	1.542(0.783–2.327)	0.113
Gender	1.234(0.572–1.895)	0.482	1.347(0.668–2.137)	0.218
Tumor size (cm)	1.542(0.713–2.228)	0.218	1.238(0.682–2.145)	0.195
Histologic type	0.986(0.658–2.138)	0.138	1.113(0.732–2.138)	0.213
TNM stage	2.896(1.328–4.768)	0.011	3.018(1.427–5.018)	0.021
Distant metastasis	3.018(1.487–4.831)	0.006	3.275(1.528–5.328)	0.001
DNAH17-AS1 expression	2.817(1.314–4.457)	0.013	3.117(1.438–4.895)	0.007

DNAH17-AS1 depletion hindered NSCLC cells regarding the proliferation, migration and invasion

Based on the findings mentioned above, we hypothesized that DNAH17-AS1 may influence NSCLC cells in terms of the growth and metastasis. For figuring out the biological function exhibited by DNAH17-AS1 in NSCLC, siRNA was employed to knock down endogenous DNAH17-AS1 in H1299 and 95D cells. DNAH17-AS1 expression presented a downtrend in H1299 and 95D cells after transfection with DNAH17-AS1 siRNA (Fig. 2A). Then, we performed CCK-8 and colony formation assays for exploring the function possessed by DNAH17-AS1 in NSCLC cells, which demonstrated that DNAH17-AS1 expression depletion could hinder NSCLC cell proliferation (Fig. 2B and 2C). Also, based on the EdU assay, the silencing of DNAH17-AS1 greatly hindered NSCLC cell proliferation (Fig. 2D). Besides, we also determined the potential function of dysregulated DNAH17-AS1 expression on cellular apoptosis. As presented in Fig. 2E, we observed that DNAH17-AS1 depletion induced cell apoptosis markedly. Moreover, a mechanistic investigation showed silencing DNAH17-AS1 expression resulted in an obvious increase in the caspase 3/9 activities (Fig. 2F). On the other hand, as indicated by the transwell and wound healing assays, si-DNAH17-AS1 transfected NSCLC cells showed an obviously hindered invasion and migration relative to si-NC transfected NSCLC cells (Fig. 3A and 3B). Overall, our findings indicated that DNAH17-AS1 knockdown suppressed NSCLC cells in terms of the proliferation, migration and invasion, but facilitated the apoptosis.

DNAH17-AS1 functions as a ceRNA for miR-877-5p in NSCLC cells

It has been proved that a lot of cytoplasmic lncRNA can competitively bind microRNAs, thus playing the role of a competing endogenous RNA (ceRNA)¹³. Then, we used subcellular fractionation which revealed that both the nucleus and cytoplasm saw the expression of DNAH17-AS1, and the nucleus saw a larger

proportion (Fig. 4A). Then, ChipBase, LncRNAdb, and StarBase were used to investigate the interaction between DNAH17-AS1 and its potential targeting miRNAs. In Fig. 4B, miR-877-5p was indicated as a target gene of DNAH17-AS1. Then, we searched TargetScan, Starbase and miRDB to identify the potential targeting genes of miR-877-5p, finding 204 genes in all the above tools (Fig. 4C). Subsequently, we performed KEGG analysis which revealed that the 204 genes were positively associated with tumor-related pathways, suggesting miR-877-5p as an important regulator in tumor progression (Fig. 4D and 4E). Dual luciferase reporter assays also revealed a direct interaction between miR-877-5p and DNAH17-AS1 in H1299 cells (Fig. 4F). Previous studies have found the low expression of miR-877-5p in many types of tumors, like NSCLC, and its oncogenic roles were also functionally identified. The study paid attention to examining the levels of miR-877-5p in our cohort and cell lines. As expected, our group observed an obvious decrease in the miR-877-5p expression in NSCLC specimens as well as cell lines (Fig. 4G and 4H). For further confirming the possibility of miR-877-5p to bind to predicted target sites in DNAH17-AS1, luciferase reporter assays were conducted, which revealed that cotransfection with miR-877-5p mimics and wt-DNAH17-AS1 reporter plasmid but not the mut-DANCR could distinctly suppress the luciferase activity in H1299 and 95D cells (Fig. 4I and 4J). Moreover, knockdown of DNAH17-AS1 was observed to promote the expression levels exhibited by miR-877-5p (Fig. 4K), and miR-877-5p overexpression could suppress the expression exhibited by DNAH17-AS1 (Fig. 4L), which also support the fact that DNAH17-AS1 can play the role of a ceRNA for miR-877-5p in NSCLC cells. Overall, the data indicate that DNAH17-AS1 competitively sponged miR-877-5p, forming the RNA-induced silencing complex (RISC).

DNAH17-AS1 regulates CCNA2 expression via competitive interaction with miR-877-5p

To explore the potential targeting genes of miR-877-5p, we identified 1874 possible candidates using ENCORI and also collected the top 600 overexpressed genes in lung cancer based on TCGA. A total of 186 overlapping targets were identified by Venn diagram (Fig. 5A). The expression pattern of 186 genes was also shown in heat map (Fig. 5A). Among these potential genes, our attention focused on CCNA2 due to its distinct upregulation in NSCLC and several different types of tumors (Fig. 5E-5D). Moreover, survival assayed based on TCGA datasets using GEPIA tools revealed that patients with high CCNA2 expression exhibited a shorter OS ($p < 0.0001$) and DFS ($p < 0.0001$) than those with low CCNA2 expression (Fig. 5E). In addition, we also confirmed the distinct overexpression of CCNA2 mRNA in NSCLC specimens from our cohort, which was consistent with the above results. According to ROC assays, CCNA2 was high CCNA2 expression had an AUC value of 0.7693 (95% CI: 0.7157 to 0.8229) for NSCLC (Fig. 5G). Moreover, we performed the Kaplan-Meier methods to analyze the influence of CCNA2 expression on survivals of our cohort, finding that NSCLC patients with high CCNA2 expression level had poor OS ($p = 0.0038$) and DFS ($p = 0.0005$) than those with low CCNA2 expression level (Fig. 5H). Previously, the potential of CCNA2 as a tumor promotor had been confirmed in several tumors, including NSCLC^{22,23}. Then, we performed cellular experiments to explore whether CCNA2 was a target of miR-877-5p in NSCLC cells. The predicting binding sites using starbase program analysis were presented in Fig. 5I. Further luciferase reporter assays demonstrated that miR-877-5p overexpression led to remarkable

inhibition of CCNA2 wild-type (wt) luciferase reporters (Fig. 5J). Overall, our findings demonstrated that miR-877-5p targeted CCNA2 in H1299 cells. Then, we explore the possible regulatory association between DNAH17-AS1 and CCNA2 expression. To our interest, we observed that overexpression of DNAH17-AS1 was able to distinctly reduce the mRNA levels of CCNA2, while knockdown of DNAH17-AS1 exhibited an opposite result (Fig. 6A). what's more, overexpression of DNAH17-AS1 could reduce the mRNA levels of CCNA2 which was decreased by miR-877-5p mimics. (Fig. 6B). To further confirm whether DNAH17-AS1 exhibited its tumor-promotive roles via regulating miR-877-5p/CCNA2 axis, we performed rescue experiments which indicated overexpression of DNAH17-AS1 could promote the colony formation, migration and invasion of H1299 and 95D cells stimulated by miR-877-5p mimics (Fig. 6C-6E). Overall, our findings revealed that DNAH17-AS1 promoted the progression of NSCLC cells via sponging miR-877-5p to increase CCNA2 expression.

Discussion

NSCLC acts a common malignancy in China and has a poor prognosis and low survival rate²⁴. With the development of targeted therapies, the clinical outcome of NSCLC patients may be improved. Nevertheless, targeted treatments may not be well applied in practice as there is no sensitive or specific biomarkers for NSCLC^{5,25}. On the other hand, the diagnosis schemes remained to be systematically optimized²⁶. In recent years, more and more studies reported the great potential of lncRNAs used as novel biomarkers for patients with various tumors^{27,28}. A novel lncRNA related to NSCLC was identified in the study, i.e, DNAH17-AS1 which presented a high expression in both NSCLC specimens and cell lines. The diagnostic study revealed that high DNAH17-AS1 expression in the tumor specimens enabled the discrimination of NSCLC patients from non-tumor tissues with an AUC of 0.7698, suggesting it as a potential diagnostic biomarker for NSCLC. A clinical study revealed that increased DNAH17-AS1 could lead to TNM stage, shorter survivals and distant metastasis, indicating DNAH17-AS1 as a positive regulator in the clinical progression of NSCLC. More importantly, multivariate assays confirmed the clinical value of DNAH17-AS1 used as an independent factor for NSCLC patients. These findings suggested DNAH17-AS1 as an oncogene in the progression of NSCLC. Then, we further performed functional assays for studying how DNAH17-AS1 affects the cellular progress of NSCLC cells. As expected, we observed that DNAH17-AS1 knockdown hindered H1299 and 95D cells in terms of their proliferation, migration and invasion, and meanwhile facilitated apoptosis. Our results revealed that DNAH17-AS1 could be a potential biomarker as well as a new target specific to NSCLC treatment.

A growing number of studies performed recently have reported the dysregulation of lncRNAs in NSCLC, and the obvious association between aberrant lncRNA expressions and carcinogenesis^{27,29}. To be specific, studies reported the high expression of lncRNA KCNQ10T1 in NSCLC, which promoted the proliferation and metastasis regarding A549 and H460 cells by regulating miRNA-129-5p/JAG1 axis³⁰. lncRNA UCA1, an overexpressed lncRNA in NSCLC, was found to contribute gefitinib resistance via regulating miRNA-143/FOSL2 axis in NSCLC³¹. Geng and his group reported that lncRNA SNHG6 was distinctly upregulated in NSCLC and predicted a poor prognosis in NSCLC patients. With regard to the

function, lncRNA SNHG6 knockdown could regulate ETS1 signaling, thereby suppressing NSCLC cell proliferation and invasion³². The findings provided a novel clue for the exploration of the potential mechanisms regarding NSCLC oncogenesis and progression. DNAH17-AS1 is a kind of lncRNA that has been identified latest, while few reports revealed its potential effects. In pancreatic carcinoma, DNAH17-AS1 was found to be overexpressed and promote pancreatic carcinoma cells' proliferation and migration by upregulating PPME1 expression via sponging miRNA-432-5p, indicating that DNAH17-AS1 acted as tumor promotor in this tumor¹⁶. In this study, we also found the distinct upregulation of DNAH17-AS1 in NSCLC, which was consistent with its expressing trend in pancreatic carcinoma. Then, by the use of loss-of-function assays, we confirmed that knockdown of DNAH17-AS1 played a negative role in the cellular progress of H1299 and 95D cells, which was also consistent with previous findings. Our findings, together with previous findings, revealed that DNAH17-AS1 may be an oncogene in pancreatic carcinoma and NSCLC.

As proved by a lot of evidence, lncRNAs have the function of suppressing the expression level as well as the biological activities exhibited by miRNAs through being a competitive endogenous RNA^{33,34}. The localization of DNAH17-AS1 in H1299 and 95D cells was confirmed first, for figuring out its potential regulating mechanisms over the downstream effectors in NSCLC. It has been confirmed that being a microRNA sponge, cytosolic lncRNAs exhibited their function by the modulation of mRNA stability and the localization of proteins. Then, we performed subcellular fractionation, finding the existence of more DNAH17-AS1 in the cytoplasm. Subsequently, bioinformatics analysis indicated miR-877-5p may be a potential target of DNAH17-AS1. Moreover, KEGG assays revealed that the potential targeting genes of miR-877-5p was positively associated with tumor-related pathways. Previous studies have reported miR-877-5p expression was distinctly down-regulated and acted as an oncogene in several tumors, including NSCLC. We also provided evidence that miR-877-5p was highly expressed in both NSCLC specimens from our cohort and cell lines, which was consistent with previous findings³⁵. Then, luciferase reporter gene assays assisted in confirming the regulatory role of DNAH17-AS1 in miR-877-5p. Further functional experiments also provided evidence that knockdown of DNAH17-AS1 suppressed the miR-877-5p expression, while overexpression of miR-877-5p had an opposite result. Overall, DNAH17-AS1 may promote tumor via decreasing miR-877-5p expression.

CCNA2, also known as cyclin A2, is located on chromosome: 4 and belongs to the highly conserved cyclin family³⁶. It has been demonstrated that CCNA2 exhibited a functional role in the progression of tumor metastasis^{37,38}. Previous findings revealed that CCNA2 was highly expressed in several tumors and also triggered EMT and promotes the metastasis of tumor cells through activation of the phosphatidylinositol 3-kinase (PI3K) pathways^{39,40}. In lung cancer, CCNA2 was also found to contribute to tumor progression the integrin $\alpha\beta3$ signaling⁴¹. Based on the bioinformatics analysis in the study, CCNA2 expression may be a targeting gene of miR-877-5p. We also observed the obvious upregulation of CCNA2 expression in NSCLC specimens based on the TCGA datasets, which was confirmed in our cohort. In addition, survival assays revealed that patients with high CCNA2 exhibited a shorter OS and DFS in patients from TCGA datasets, which was consistent with our findings. A diagnostic study also confirmed the potential

diagnostic value of CCNA2 in NSCLC patients. These findings suggested CCNA2 as an oncogenic gene. More importantly, Luciferase assay further confirmed that miR-877-5p was directly regulated in the 3'UTR of CCNA2 mRNA. Thus, as indicated by our results, miR-877-5p suppressed the progression of NSCLC cells via targeting CCNA2. To further explore the association among DNAH17-AS1, miR-877-5p and CCNA2, we performed rescue experiments, finding that DNAH17-AS1 could downregulate miR-877-5p to enhance expression of CCNA2 and promote NSCLC cells regarding the proliferation, the migration, and the invasion. In spite of this, the study did not conduct a sufficient discussion about other potential targets and mechanism of regulatory actions. Besides, it is necessary to recruit more NSCLC patients for further demonstrating the diagnostic and prognostic values of DNAH17-AS1.

Conclusion

DNAH17-AS1, a novel NSCLC-related lncRNA, has been identified in the paper. It presents an upregulation in NSCLC tissues and is characterized as a novel tumor promotor. DNAH17-AS1 can play the role of a molecular sponge of miR-877-5p upon upregulating CCNA2 levels. Our results suggest the potentiality of DNAH17-AS1 as a biomarker for NSCLC treatments and provide a new insight for understanding the molecular mechanisms associated with NSCLC.

Declarations

Authors' contributions

Guarantor of integrity of the entire study: Rui-jun Jing and Li-juan Du. Definition of intellectual content: Long-jun Mao. Literature research: Li-juan Du. Clinical studies: Li-juan Du and Long-jun Mao. Experimental studies: Rui-jun Jing and Li-juan Du. Statistical analysis: Long-jun Mao. Manuscript preparation: Long-jun Mao. Manuscript editing: Li-juan Du. Manuscript review: Rui-jun Jing. All authors have read and approved the manuscript.

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Xi'an Medical University. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients and healthy volunteers provided written informed consent prior to their inclusion within the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The upregulation of DNAH17-AS1 expression and its clinical significance in NSCLC. (A) Relative expression of DNAH17-AS1 in NSCLC tissues in comparison with adjacent non-tumor tissues using RT-PCR. (B) Advanced NSCLC specimens showed a higher level of DNAH17-AS1. (C) RT-PCR showed the expression levels of DNAH17-AS1 in six cells (A549, SPC-A1, H1299, 95D, and SK-MES-1) and 16HBE cells. (D) ROC assays for DNAH17-AS1 as a diagnostic marker for NSCLC patients. (E, F) Kaplan-Meier curves for OS (E) and DFS (F) and DNAH17-AS1 expression in group of 147 NSCLC patients. **p < 0.01.

Figure 2

Down-regulation of DNAH17-AS1 restricts the proliferation and promoted apoptosis capacity of NSCLC cells. (A) qPCR analysis of DNAH17-AS1 expression levels following treatment of H1299 and 95D cells with siRNAs against DNAH17-AS1. (B) CCK-8 assays were performed to determine the proliferation of NSCLC cells. (C) Colony-formation assays were carried out to determine the proliferation of si-DNAH17-AS1-transfected NSCLC cells. (D) Cell proliferation was evaluated using EdU incorporation assays. (E)

Annexin V/PI staining and flow cytometry analysis were used to explore the effects of DNAH17-AS1 on apoptosis. (F) The activities of caspase 3/9 were determined. * $p < 0.05$, ** $p < 0.01$.

Figure 3

DNAH17-AS1 suppression inhibited the migration and invasion of NSCLC cells. (A) The migration ability of H1299 and 95D cells treated with si- DNAH17-AS1-#1 and DNAH17-AS1-#2 was assessed by wound healing assays. (B) Transwell assays were used to determine the effects of DNAH17-AS1 knockdown on H1299 and 95D cells migration and invasion. * $p < 0.05$, ** $p < 0.01$.

Figure 4

DNAH17-AS1 acts as a sponge for miR-877-5p in the cytoplasm. (A) Relative DNAH17-AS1 expressions in nuclear and cytosolic fractions of H1299 and 95D cells. Nuclear controls: U6; Cytosolic controls: GAPDH. (B) Predicted miR-877-5p-binding sites in the DNAH17-AS1 sequence. The numbers show the distance in nucleotides from the transcriptional start site of DNAH17-AS1. (C) Venn analysis of the potential targeting genes of miR-877-5p using "TargetScan", "Starbase" and "miRDB". (D, E) KEGG pathways for

the 204 potential targeting genes. (F) Dual luciferase reporter assays for the determination of the miRNAs that directly interacted with DNAH17-AS1. (G) RT-PCR assays for the determination of miR-877-5p expression in NSCLC patients from our cohort. (H) RT-PCR assays for the determination of miR-877-5p expression in five NSCLC cell line and 16HBE cells. (I, J) Dual luciferase reporter assays suggested that co-transfection with miR-877-5p distinctly reduced the luciferase activity of the reporter containing wild-type DNAH17-AS1 in H1229 and 95D cells. (K) Knockdown of DNAH17-AS1 promoted the expression of miR-877-5p H1229 and 95D cells. (L) Overexpression of miR-877-5p suppressed the levels of DNAH17-AS1 in H1229 and 95D cells. * $p < 0.05$, ** $p < 0.01$.

Figure 5

CCNA2 was identified as a direct target of miR-877-5p in NSCLC cells. (A) Venn diagram and Heat Map of the potential targeting genes which was overexpressed in TCGA datasets. (B) CCNA2 expression was distinctly upregulated in NSCLC specimens compared to normal lung tissues based on TCGA datasets. (C) NSCLC specimens with advanced stages showed a high level of CCNA2 using TCGA datasets. (D) The expression pattern of CCNA2 in different types of tumors based on TCGA datasets. (E) The influence of

CCNA2 expression on the OS and DFS of NSCLC patients based on the clinical data from TCGA datasets. (F) RT-PCR assays for the determination of CCNA2 expression in our cohort. (G) ROC assays for CCNA2 as a diagnostic marker for NSCLC patients. (H) Kaplan-Meier analysis was used to showed the OS and DFS rates of our cohort (147 NSCLC patients). (J) Starbase algorithm predicted the potential binding sites between 3'UTR of CCNA2 mRNA and miR-877-5p. (J) Luciferase reporter assays were used to prove that miR-877-5p can target CCNA2. * $p < 0.05$, ** $p < 0.01$.

Figure 6

Overexpression of miR-877-5p abrogates the promotive effects of DNAH17-AS1 upregulation on the proliferation and invasion of NSCLC cells. (A) the influence of dysregulated DNAH17-AS1 expression on the levels of CCNA2 mRNA in H1299 and 95D cells. (B) CCNA2 mRNA level in H1299 and 95D cells following overexpression of DNAH17-AS1 and/or overexpression of miR-877-5p. (C-E) The cell viability (C) Colon formation (D) and invasive ability (E) of H1299 and 95D cells were assessed after transfection with pcDNA-DNAH17-AS1 and/or miR-877-5p mimics. * $p < 0.05$, ** $p < 0.01$.