LINC00942 Promotes Tumor Proliferation and Metastasis in Lung Adenocarcinoma via FZD1 Upregulation

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Abstract

Background: Long non-coding RNAs (lncRNAs) have been reported to play important roles in the progression of human cancers. Herein, bioinformatic analysis identified that LINC00942 was a highly overexpressed lncRNA in lung adenocarcinoma (LUAD). The present study aimed to explore the roles and possible molecular mechanisms of LINC00942 in LUAD. Methods: First, on the basis of TCGA database, the expression and prognosis of LINC00942 were analyzed in LUAD tissues. Then, si-LINC00942 was transfected into A549 and H1299 cells to knockdown the expression of LINC00942. Cell viability was detected by MTT assay. Flow cytometry was used to analyze cell apoptosis. The expressions of PCNA, Bax, Bcl-2, and wnt/b-catenin pathway proteins were detected by western blotting. Dual-luciferase reporter assay was used to evaluate the regulatory relationship between LINC00942 and miR-5006-5p, or miR-5006-5p and FZD1. Results: We discovered that LINC00942 was up-regulated in LUAD tissues compared with adjacent tissues. Besides, we found the increased LINC00942 expression was associated with poor survival. In addition, silencing of LINC00942 suppressed the proliferation, migration, invasion and facilitated the apoptosis of A549 and H1299 cells. Moreover, silencing of LINC00942 repressed the expression of PCNA, Bcl-2, and enhanced Bax expression in A549 and H1299 cells. Mechanically, LINC00942 exerted its effects via enhancing Wnt signaling. LINC00942 functioned as competing endogenous RNA (ceRNA) by binding to miR-5006-5p, upregulating the expression of FZD1, which was a direct target of miR-5006-5p. Conclusion: Our findings indicated that LINC00942/miR-5006-5p/FZD1 axis played important roles in LUAD growth through enhancing Wnt signaling. LINC00942/miR-5006-5p/FZD1 axis might serve as a potential biomarker and therapeutic target for LUAD treatment.

Keywords
LUAD, LINC00942, miR-5006-5p, FZD1

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Introduction

Lung cancer (LC) is a type of malignant tumors with high morbidity and mortality rates across the world. Among the urban populations of China, LC is the leading cause of cancer-associated fatalities.1 Despite the advancements in clinical diagnosis and therapeutic strategies, the prognosis of patients with lung adenocarcinoma (LUAD) remains dismal, mainly due to late diagnosis, metastasis, and a high rate of postoperative recurrence.2 Since the molecular mechanisms underlying the initiation and progression of LUAD remain unclear, identifying potential LUAD-promoting molecules will be crucial for understanding of the LUAD pathogenesis and for the discovery of new therapeutic targets.3

lncRNAs, longer than 200 nucleotides, are endogenous transcripts that do not encode proteins.4 LncRNAs act a crucial role in numerous biological activities, including stem cell
pluripotency, cell differentiation, cell cycle regulation, and cancer pathogenesis by regulation of the gene expression through various mechanisms.\textsuperscript{5,6} Accumulating evidence indicates that lncRNAs function as a critical factor in LUAD development, metastasis, and prognosis.\textsuperscript{7} Moreover, past evidence have demonstrated that several lncRNAs, including lncRNA LCAT1, lncRNA DLEU2, and lncRNA AFAP1-AS1, were aberrantly expressed in LUAD and modulated the LUAD initiation and progression.\textsuperscript{8-10} However, the roles and mechanisms of action of most lncRNAs in LUAD remain unclear.

LINC00942, a recently discovered lncRNA, was found to be correlated with autism spectrum disorder.\textsuperscript{11} Sun et al. uncovered that LINC00942, as an oncogene, exerted its functions in breast cancer initiation and progression.\textsuperscript{12} Bioinformatics analyses of publicly available data revealed that LINC00942 was overexpressed in LUAD. In the present study, we verified and characterized the upregulation of LINC00942 in LUAD and confirmed the hypothesis that LINC00942 is a critical lncRNA participating in the progression of LUAD.

Materials and Methods

Patients

We studied 58 paired LC and adjacent non-tumor tissues (located >5-cm away from the tumor) from patients who underwent surgery at our hospital. None of the patients received any preoperative therapy before the operation. We validated all the samples after their histopathological evaluation by 2 pathologists in a double-blind manner, followed by immediately freezing the samples in liquid nitrogen and stored at −80°C until further use. All the subjects provided their informed consent, and our study protocol was approved by the Review Board of the Hospital Ethics Committee.

Bioinformatics Analysis

The gene expression profiles obtained 479 LC patients and 54 Normal specimens were downloaded from the TCGA database (https://cancergenome.nih.gov/). The gene expression profiles were normalized using the scale method provided in the limma R package.\textsuperscript{13} Next, the overall survival (OS) of 479 profiles were normalized using the scale method provided in base (https://cancergenome.nih.gov/). The gene expression profiles obtained 479 LC patients and 54 Normal specimens were downloaded from the TCGA data-

Cell Transfection

We transfected LINC00942 siRNA and its corresponding negative control (NC) (Gene Pharma, China) into A549 and H1299 cells for 48 h for the loss-of-function experiments using Lipofectamine2000 (Invitrogen, USA) following the manufacturer’s protocol. We co-transfected the miR-5006-5p inhibitor (RiboBio, China) into A549 and H1299 cells with LINC00942 siRNA. After 8 h transfection, we replaced the medium with RPMI-1640 medium containing 10% FBS.

qRT-PCR

We isolated total RNA from the LUAD cells using the TRIZOL reagent (Invitrogen, USA) strictly following the manufacturer’s protocol. Subsequently, we reverse-transcribed the RNA into cDNA using a reverse transcription reagent kit. After that, we assayed for the relative expression in an ABI7500 PCR system (ABI, USA) using the SYBR\textsuperscript{®} Green Master Mix (Takara, Japan). The qRT-PCR reaction conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 34 s. We used the $2^{-\Delta\Delta C_{t}}$ method to calculate the relative expression.

MTT

We re-suspended the transfected cells in RPMI-1640 with 10% FBS, seeded into 96-well plate (5 $\times$ 10\textsuperscript{3}), and incubated at 37°C with 5% CO\textsubscript{2} for 24-72 h. Next, we added 15-μl of MTT solution (5 mg/mL; Sigma, USA) into cells. After incubating the cells for 4 h, we supplemented 150 μl of DMSO to each well. The mixture was incubated on a shaker at low speed for 10 min to dissolve the crystals sufficiently. We measured the OD490 absorbance values for the samples using the microplate reader at 0, 24, 48, and 72 h.

Apoptosis Analysis

At the end of the 48-h culturing period for the transfected cells, we washed the cells, digested and stained with Annexin V/PI (BD, USA) for 15 min at the room temperature, as per the manufacturer’s protocol. We analyzed cell apoptosis using flow cytometry (BD, USA).

Western Blotting

We assayed the protein lysate concentrations using the BCA kit (Thermo Fisher, USA). First, we separated all the protein lysates using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. Subsequently, we sealed the PVDF membrane with 5% skimmed milk powder for 1 h, and then incubated with primary antibodies including GAPDH, PCNA, Bax, Bcl-2, β-catenin, p-β-catenin (Ser33/37/Thr41), c-Myc, and CyclinD1 overnight at 4°C (we purchased all the primary antibodies from CST, USA, and used them in 1:1000 dilution). Subsequently, we washed the membranes thrice with...
Tris-buffered saline containing 0.1% Tween 20 (TBST) and then incubated with the corresponding secondary antibody for 1 h. Finally, we added freshly prepared electrochemiluminescence (ECL) luminescent solution to analyze the gray values. We used GAPDH as the control.

**Dual-Luciferase Reporter Assay**

We used the StarBase (http://starbase.sysu.edu.cn/) to predict the regulation of miR-5006-5p by LINC00942. We synthesized the wild type (LINC00942-WT) or mutant (LINC00942-MUT) fragments of LINC00942 with the binding site of miR-5006-5p. After that, we cloned the wild type or mutant sequence into the pGL3 luciferase reporting vector (Promega, USA). We seeded the HEK-293 T cells (5 × 10⁵) in 24-well plates for 24 h. We then co-transfected pMir-LINC00942-WT or pMir-LINC00942-MUT with pRL-TK Renilla luciferase vector and miR-5006-5p mimics into the HEK-293 T cells using Lipofectamine 2000 (Invitrogen). Similarly, we co-transfected pMir-FZD1-WT or pMir-FZD1-MUT with pRL-TK Renilla luciferase vector, and miR-5006-5p mimics into the HEK-293 T cells. We normalized the relative luciferase activity to Renilla luciferase activity 48 h following transfection.

**Colony Formation Assay**

After 48 h culture of the transfected cells, we washed the cells and digested. Then we seeded 800-1000 A549 or H1299 cells into each well of the six-well plate and supported in a medium containing 10% FBS for 10 d. After that, we fixed the colonies with methanol and stained using 0.1% crystal violet. We used an inverted microscope to count the number of clones.

**Transwell**

We used the transwell chambers either coated with Matrigel or uncoated to observe cell migration and invasion in A549 and H1299 cell lines. After 48 h culture of the transfected cells, we washed the cells and digested. Briefly, we added 1 × 10⁵ cells to the top chamber in FBS-free medium with the bottom chamber containing 600 µl RPMI-1640 with 10% FBS. After 24 h incubation, we washed the membranes, fixed and stained using crystal violet. In the invasion assay, we coated the top chamber with matrigel prior to the cells.

**Statistical Analysis**

We performed survival analysis available in the R package "survival" using the Kaplan–Meier curve (K–M curve) method. A log rank test was performed to compare the survival times between 2 groups, and p < 0.05 was considered to represent the statistical significance. All the data were representative of 3 independent experiments and expressed as mean ± SD. The differences between 2 groups were analyzed using the t test, and those among more than 2 groups were analyzed by using the analysis of variance (ANOVA) test with the SPSS v22.0. P < 0.05 was considered as statistically significant.

**Results**

**The Expression of LINC00942 Increased in LUAD**

To reveal which IncRNA participates in the progression of LUAD, we analyzed the LUAD dataset of TCGA. These results demonstrated that LINC00942 was overexpressed in the LUAD tissues (n = 479) than in the normal tissues (n = 54) (Figure 1A). Moreover, the analysis of the TCGA dataset revealed that the expression of LINC00942 was positively associated with shorter OS period in LUAD patients (Figure 1B). To verify these results, the expression of LINC00942 in 58 paired NSCLC tissues and the adjacent tissues were analyzed by qRT-PCR. Our results revealed that the expression of LINC00942 was markedly up-regulated in NSCLC tissues than that in the adjacent tissues (P < 0.05, Figure 1C). Furthermore, we divided the LUAD patients into the high and low LINC00942 expression groups based on the median of LINC00942 expression to analyze the 5-year survival rate. The findings revealed that patients with elevated levels of LINC00942 showed a lower 5-year-OS rate than those with low levels of LINC00942 (P < 0.05, Figure 1D). Taken together, these findings indicated that LINC00942 was highly expressed in the LUAD tissues and was associated with worse prognosis in LUAD patients.

**Silencing LINC00942 Suppressed the Proliferation and Promoted the Apoptosis of LUAD Cells**

Downregulation of the LINC00942 expression using si-LINC00942 transfection revealed significant reduction in the expression of LINC00942 in A549 and H1299 cells (P < 0.05, Figure 2A, B). The MTT results demonstrated that cell viability in si-LINC00942-arm was markedly lower than that in si-NC and Mock arms (P < 0.05, Figure 2C, D). Apoptosis rates of the A549 and H1299 cells were markedly higher than that in the si-NC-arm (P < 0.05, Figure 2E-H). Besides, the expressions of PCNA and Bcl-2 were decreased while Bax expression was increased significantly in LINC00942-silenced A549 as well as H1299 cell lines (P < 0.05, Figure 2I, J). These results demonstrated that depletion of LINC00942 repressed LUAD cell proliferation while facilitated LUAD cell apoptosis.

**Silencing LINC00942 Inhibited the Migration and Invasion of LUAD Cells**

To explore the oncogenic role of LINC00942 in LUAD metastasis, we assessed the impacts of LINC00942 on LUAD cell migration and invasion. LINC00942 knockdown markedly suppressed the migration and invasion of A549 as well as H1299 cells (Figure 3A, B, D, and E). To investigate the LINC00942-mediated LUAD cancer cell migration at the molecular level, we assayed for protein expression of the EMT markers using western blotting. The results showed that LINC00942-knockdown resulted into the down-regulation of the mesenchymal markers: N-cadherin and Vimentin. However, the epithelial marker: E-cadherin in A549 and H1299...
cells was markedly up-regulated than in si-NC (Figure 3C, F). Hence, these results verified that LINC00942 promoted LUAD cell mobility.

**LINC00942 Induces LUAD Cell Progression by Enhancing FZD1 Activation and Wnt Signaling Pathway**

Given the up-regulation and oncogenic function of LINC00942 in LUAD cells, to explore the subsequent signaling pathway of LINC00942, we assessed the changing factors after LINC00942 knockdown in LUAD cells. We found that the expression of FZD1 and its downstream target genes β-catenin, c-myc, and cyclin D1 were markedly decreased after LINC00942 knockdown (Figure 4A, B), suggesting that LINC00942 potentially affected the LUAD cell progression by enhancing FZD1 activation in correlation with the Wnt signaling.

**LINC00942 Increases FZD1 Expression by Sponging miR-5006-5p**

Recent evidence reveal that IncRNAs participated in cancer progression by functioning as competing endogenous RNAs (ceRNAs). Since LINC00942 knockdown decreased both FZD1-mRNA and protein concentrations, we hypothesized that LINC00942 acted as a miRNA sponge to modulate FZD1 expression. To test this hypothesis, we first conducted the bioinformatics analyses using the DIANA. Consequently, miR-5006-5p was speculated to target LINC00942. Moreover, the putative complementary sequences at LINC00942 were speculated to target miR-5006-5p, including one 8-mer site (Figure 5A).

Next, we investigated whether LINC00942 interacted with miR-5006-5p. As per the pMir-reporter plasmid, we created luciferase reporters with the 5’-500nt of LINC00942, which comprised of the wild-type (WT) or deletion mutated (MUT) miR-5006-5p targeting site. We co-transfected these reporters with miR-5006-5p mimics into the HEK-293 T cells. We identified that miR-5006-5p mimics markedly reduced the activity of the reporter of the WT LINC00942-construct (Figure 5B). In addition, the expression of miR-5006-5p was substantially up-regulated in A549 and H1299 cells after the knockdown of LINC00942 (P < .05, Figure 5C, D). Moreover, the expression of LINC00942 was markedly increased in A549 and H1299 cells on miR-5006-5p inhibition (P < .05, Figure 5E, F).

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**Figure 1.** LINC00942 was up-regulated and predicted a poor prognosis in LUAD patients. A, LINC00942 relative mRNA expression levels in LUAD and non-tumor tissues in the TCGA dataset. B, Kaplan-Meier analyses of the overall survival of the TCGA cohort patients with high LINC00942 expression versus those with low LINC00942 expression. C, The relative expression levels of LINC00942 were assayed using qRT-PCR and normalized against an endogenous control (GAPDH) in paired LUAD tissues (n = 58). D, Kaplan-Meier analyses of the overall survival of patients with LUAD based on LINC00942 expression.
To assess whether FZD1 is the target of MiR-5006-5p, we conducted the bioinformatics analyses using the TargetScan. The FZD1 mRNA’s 3’-untranslated region (3’-UTR) possesses a single site matching the miR-5006-5p. Next, we created the luciferase reporters with the FZD1 mRNA’s 500nt of the 3’-UTR, which has the WT or MUT MiR-5006-5p targeting site. We co-transfected these reporters with miR-5006-5p mimics into the HEK-293 T cells. The miR-5006-5p mimics markedly reduced the reporter activity of the FZD1 mRNA-WT 3’-UTR-construct (Figure 5G). This finding implies that the miR-5006-5p inhibits the 3’-UTR of the FZD1 mRNA. We also noted that miR-5006-5p mimics decreased endogenous FZD1 protein levels in A549 and H1299 cells. However, miR-5006-5p inhibitor demonstrated an opposite effect in A549 and H1299 cells (Figure 5 H, I). MiR-5006-5p inhibitor could attenuate the reduction of FZD1 protein induced by siLINC00942 A549 and H1299 cells. (Figure 5 J, K). Therefore, LINC00942 regulated FZD1 expression by targeting miR-5006-5p.

**Figure 2.** LINC00942 knockdown suppressed the proliferation, elevated the apoptosis of LUAD cancer cells. A and B, LINC00942 was knocked down in A549 and H1299 cells. C and D, The impacts of LINC00942 knockdown on proliferation ability were measured by MTT in A549 and H1299 cells. E and H, The effects of LINC00942 knockdown on apoptosis were measured by AnnexinV/PI staining in A549 and H1299 cells. I and J, The effects of LINC00942 knockdown on the expression of PCNA, BAX, Bcl-2 were measured by western blotting.

MiR-5006-5p Inhibition Attenuated the Biological Functions of LINC00942 Knockdown

To further confirm that LINC00942 exerted its biological effects by regulating miR-5006-5p, we silenced the LINC00942 expression together with miR-5006-5p inhibition in A549 and H1299 cells. We discovered that miR-5006-5p inhibitor attenuated the inhibition of cell proliferation (Figure 6A, B), increased cell apoptosis (Figure 6C-F), the decrease of cell migration induced by LINC00942 silence (Figure 6G, H, I). Altogether, these results revealed that miR-5006-5p was a potential functional downstream-target of LINC00942.
Discussion

LC continues to be a significant worldwide health concern associated with high rates of morbidity and mortality.\textsuperscript{15} Despite the advancements in the technology on the prevention of metastatic recurrence following LC therapies, urgent researches are warranted to assess the molecular changes involved in LC progression and to search for potential prognostic biomarkers in LC.\textsuperscript{16}

Accumulating evidence has revealed the important roles of lncRNAs in various types of cancer, especially their impacts on cancer biological processes, such as proliferation,\textsuperscript{17} apoptosis,\textsuperscript{18} cell cycle, metastasis\textsuperscript{19} and therapeutic resistance.\textsuperscript{20} In the present study, we screened the profiles of abnormally expressed lncRNAs in LUAD tissues obtained from the TCGA dataset and identified LINC00942 as a potential oncogenic driver for LUAD. Notably, LINC00942 was upregulated in LUAD samples, and elevated LINC00942 expression predicted poor OS in LUAD patients, which together imply that LINC00942 is a potential prognostic indicator for LUAD patients. We used the loss-of-function assays to demonstrate that LINC00942 promoted LUAD cell proliferation and invasion, which further suggest that LINC00942 functioned as an oncogenic lncRNA in LUAD.

Our results demonstrated the key roles exerted by LINC00942 in the progression of LUAD. However, the mechanism by which LINC00942 promoted the proliferation and metastasis of LUAD remains unclear. LncRNAs can modulate gene expression through numerous manners, such as transcriptional and post-transcriptional processing.\textsuperscript{21-23} However,

Figure 3. LINC00942 knockdown inhibited the metastatic potential of LUAD cancer cells. A and B, Representative images and bar graphs depicting the migration and invasion abilities of LINC00942-silenced A549 cells. C, The effects of LINC00942 knockdown on the expression of metastasis related protein Vimentin, N-cadherin, E-cadherin in the A549 cells. D and E, Representative images and bar graphs depicting the migration and invasion abilities of LINC00942-silenced H1299 cells. F, The effects of LINC00942 knockdown on the expression of metastasis related protein Vimentin, N-cadherin, E-cadherin in the H1299 cells.
Figure 4. LINC00942 activated the expression of downstream targets of the Wnt pathway via FZD1. A and B, The protein expression of FZD1, p-β-catenin, p-β-catenin, c-myc and cyclin D1 after siLINC00942 treatment in A549 and H1299 cells by western blot.

Figure 5. LINC00942 increases FZD1 expression by sponging miR-5006-5p. A, Illustration of the base pairing between LINC00942 and miR-5006-5p predicted with DIANA tools and between LINC00942 and FZD1 predicted by TargetScan. B, Luciferase activity indicated miR-5006-5p targeting LINC00942. C and D, The expression levels of miR-5006-5p were determined by qRT-PCR after silencing LINC00942 in A549 and H1299 cells. E and F, The expression levels of LINC00942 were determined by qRT-PCR after inhibition of miR-5006-5p in A549 and H1299 cells. G, Luciferase activity indicated miR-5006-5p targeting FZD1. H and I, The protein levels of FZD1 were determined by Western blot analysis after overexpression of miR-5006-5p or silencing miR-5006-5p in A549 and H1299 cells. J and K, Western blotting showed the total FZD1 proteins expression after transfection of siLINC00942 or both siLINC00942 and miR-5006-5p inhibitor in A549 and H1299 cells.
recent evidence have revealed that lncRNAs function as ceRNAs to modulate miRNAs, which in turn regulate the expression of target genes. For example, lncRNA THAP9-AS1 augmented pancreatic ductal adenocarcinoma cell growth, resulting in a poor clinical outcome as a result of sponging miR-484 and modulation of the YAP expression. MIR17HG elevated the expression of NF-κB through antagonistic sponging of miR-375. Lnc-TALC induced TMZ resistance through competitive binding to miR-20b-3p and promoting c-Met expression in glioblastoma. Herein, we noted common miR-5006-5p response elements with LINC00942 and recognized that FZD1 and LINC00942 facilitated FZD1 expression through sponging miR-5006-5p. Moreover, FZD1 was experimentally verified as a target of miR-5006-5p. Functional inhibition of miR-5006-5p could effectively relieve the reduced expression of FZD1 protein level actuated by LINC00942 knockdown in LUAD cells, which implies that LINC00942 functioned as a ceRNA.

Frizzled class receptor 1 (FZD1), a member of the “frizzled” gene family, is a receptor for Wnt signaling pathway. It has indicated that FZD1 could be involved in the regulation of cell proliferation in cellular microenvironment. Reportedly, FZD1 is abnormally expressed in various cancers, such as breast cancer, pancreatic ductal adenocarcinoma, leukemia. However, the influences of FZD1 on LUAD has not yet been expounded. In our paper, we found that FZD1 had some impacts on LUAD cells. Through downregulation of FZD1, miR-135b reversed chemoresistance of NSCLC cells. Nevertheless, the relationship between FZD1 and miR-5006-5p has not been reported. In our study, we discovered that FZD1 was a target of miR-5006-5p and, for the first time, was negatively regulated by miR-5006-5p in LUAD cells. Our findings may offer some theoretical insights for LUAD treatment. The Wnt/β-catenin signaling pathway includes a series of evolutionarily conserved, lipid-modified glycoproteins that function at short and long distances to modulate the processes related to the

Figure 6. MiR-5006-5p inhibition restored the biological function of LINC00942 knockdown. A and B, The proliferation inhibition induced by LINC00942 knockdown was restored by miR-5006-5p inhibitor. C and F, The increase of apoptosis induced by LINC00942 knockdown was attenuated by miR-5006-5p inhibitor. G and I, The decrease of migration induced by LINC00942 knockdown was attenuated by miR-5006-5p inhibitor.
proliferation, survival, differentiation, polarity and dryness. The dysregulation of the Wnt/β-catenin signaling pathway modulates crucial cancer biological processes, such as proliferation, metastasis, metabolism, and cancer cells’ stemness. As is well known, Wnt/β-catenin signaling pathway modulates crucial cancer biological processes, such as proliferation, survival, differentiation, polarity and dryness. The dysregulation of the Wnt/β-catenin signaling pathway modulates crucial cancer biological processes, such as proliferation, survival, differentiation, polarity and dryness.

Numerous lncRNAs regulate the LUAD cell proliferation, migration and apoptosis. Li et al. indicated that lncRNA MIR100HG could induce cetuximab resistance via Wnt/β-catenin signaling pathway. Li et al. reported that the up-regulation of lncRNA GATA6-AS1 hinders LNM as well as EMT via FZD4 through the Wnt/β-Catenin signaling pathway. In conclusion, we demonstrated that LINC00942 is up-regulated in LUAD tissues, which is connected with the prognosis of LUAD patients. LINC00942 promotes LUAD malignant phenotypes through Wnt/β-catenin signaling pathway. In addition, LINC00942 promotes LUAD cell progression by sponging miR-5006-5p and FZD1 expression. Our cumulative findings thus provide the first evidence of the presence of LINC00942/miR-5006-5p/FZD1 axis as a potential therapeutic target for LUAD.

Authors’ Note
Ronghua Wang, and Xiuyun Wang, are co-first authors. This study was authorized by the ethical committee of Dongying People’s Hospital (DYYX-2020-015) and all patients provided written informed consent.

Declaration of Conflicting Interests
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