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ITGA9-AS1 up-regulates ITGA9 by targeting miR-4765 and recruiting HNRNPU to affect the proliferation and apoptosis of non-small cell lung cancer cells

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ABSTRACT

Long non-coding RNAs (lncRNAs) have been regarded as promising biomarkers in the regulation of various biological and pathological processes of non-small cell lung cancer (NSCLC). LncRNAITGA9-AS1 has been reported to be down-regulated in elderly patients with lung cancer, but how it may influence NSCLC remains to be identified. Therefore, we aim to explore the specific mechanism involving ITGA9-AS1 and ITGA9 in NSCLC. A functional assay was conducted to verify ITGA9-AS1's proliferative effects on NSCLC cells. Mechanism experiments with bioinformatics predictions were performed to explore the interaction of ITGA9-AS1 and ITGA9 in NSCLC cells. ITGA9-AS1 inhibited NSCLC cell proliferation while enhancing cell apoptosis. It up-regulated ITGA9 by competitively sponging miR-4765, and it stabilizedITGA9 mRNA by recruiting a RNA-binding protein (RBP)-HNRNPU (heterogeneous nuclear ribonucleoprotein U) in NSCLC cells. ITGA9-AS1 suppressed NSCLC progression by the up-regulation of ITGA9 via targeting miR-4765 and recruiting HNRNPU.

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Introduction

Originating in bronchial mucosa epithelium or alveolar epithelium, in terms of lung cancer subtypes, 85% of lung cancers are non-small cell lung cancer (NSCLC) (1). NSCLC is featured with high morbidity and mortality, and as the early symptoms are not obvious, it is easy to be ignored by the elderly and finally leads to a poor prognosis (2). The improvement of NSCLC treatment requires exploring effective therapeutic targets.

To find more targeted diagnostic and prognostic indicators for NSCLC patients, it is necessary to focus on the elderly. Long non-coding RNAs (lncRNAs), with 200 nucleotides in length are mainly derived from gene introns (3). In NSCLC, lncRNA-XIST knockdown promotes cell apoptosis and inhibited proliferation (4), through regulation of the miR-30c-5p/SOX9 axis, lncRNA DLEU2 modulates cell proliferation and invasion (5), and lncRNA FENDRR suppresses the progression (6). By referring to a related document, a total of eight lncRNAs (LOC284632, LINC00324, LINC00703, LINC00662, ITGA9-AS1, HOXA11-AS LINC00869, and DHRS4-AS1) built a prognostic formula which had a good ability in predicting prognosis in elderly NSCLC patients (7). Among these lncRNAs, LOC284632 and LINC00869 lack database and literature support, while LINC00703, LINC00662, LINC00324, HOXA11-AS1and DHRS4-AS1 have already exerted research outcomes (8-12). As for the down-regulated lncRNA ITGA9-AS1, it has been reported to be a potential diagnosis or prognosis marker of tamoxifen resistance (13), but how it may function in the progression of cancer, especially NSCLC remains unclear, which leads to our present study.

As a complementary form of mRNA, antisense RNA consists of 19-23 nucleotides (14-17). They have been discovered to have a good effect on the regulation of its parent gene (15). For example, lncRNA ZEB1-AS1 acts as a regulator of the miR-141-3p/ZEB1 axis during lung fibrosis (16), and gastric cancer progression is supported by LNC RNA GAS6-AS1 by upregulating GAS6 expression (18). In NSCLC, FAM83A-AS1 is found to be markedly up-regulated and positively correlated with its cognate sense counterpart, FAM83A (19), and natural antisense transcript TMPO is regulated by lncRNA TMPO-AS1 to promote the progression of NSCLC (20). As the parent gene of ITGA9-AS1, ITGA9 has been reported to regulate diverse biological functions in cancer development (21), in NSCLC cells, we also examined the relationship between ITGA9-AS1 and its parent gene ITGA9.

In NSCLC, lncRNAs are found to function through competing endogenous RNAs (ceRNAs), and this mechanism has been shown to function in cytoplasmic lncRNAs (22,23). Also, as RBPs are post-transcriptional regulators, their interactions withlncRNAs in regulating NSCLC malignancy have been discussed (24). Through our investigations, we figured out the specific localization of ITGA9-AS1 in NSCLC cells and then explored whether ITGA9-AS1 may exert its function in NSCLC cells via interacting with certain RBPs or the ceRNA pattern.

Here, we aim to explore the ITGA9-AS1 function in the NSCLC development and further verify the molecular mechanism involving ITGA9-AS1 and ITGA9 in NSCLC.

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Materials and Methods

Cell lines

Human NSCLC cell lines (NCI-H1299, A549, PC9, NCI-H1975), bronchial epithelial cell line BEAS-2B and embryonic kidney HEK-293T cell line were used in this study. American Type Culture Collection provided these cell lines (ATCC, Manassas, VA, USA) and cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). At 37°C, the above cell lines were preserved in a humid incubator with 5% CO_2.

Quantitative reverse transcription real-time polymerase chain reaction (RT-qPCR)

Following the instructions of the TRIzol reagent, RNA samples were isolated from A549 and PC9 cells and an RT-qPCR reaction was performed with ChamQ Universal SYBR qPCR Master Mix following a $2^{-\Delta\Delta Ct}$ method. Controls for loading were GAPDH and U6 in relevant assays.

Cell transfection

SiRNA targeting ITGA9 (si-ITGA9-1/2/3), and HNRNPU (si-HNRNPU -1/2/3) were utilized to silence ITGA9 or HNRNPU expression. For ITGA9-AS1 and ITGA9 overexpression, pcDNA3.1-ITGA9-AS1 and pcDNA3.1-ITGA9-AS1 were constructed by synthesizing the entire sequence and subcloning it into pcDNA3.1 vector. MiR-4765 mimics were used to overexpress its expression. Transfections were conducted with Lipofectamine 2000 (Invitrogen).

Cell counting kit-8 (CCK-8) assay

CCK-8 analysis was used to determine cell viability. A cell suspension of 100 liters was prepared in 96-well plates, and they were then pre-cultivated. After 24 hours, 48 hours, or 72 hours of incubation, 10L of CCK-8 was added to each sample. Finally, the absorbance at 450nm was measured via a microplate reader after 24, 48, or 72 hours.

5-ethynyl-20-deoxyuridine (EdU) incorporation assay

In 96-well plates, logarithmic growth stage cells were seeded after 48 hours of plasmid transfection. Diluting EdU solution by 1:1000 yielded 50M EdU culture medium. We cultivated each well for 2 hours with an EdU assay kit, then removed the culture medium. The stained cells were visualized under a fluorescence microscope, and the proliferation of the cells was calculated using EdU-positive cells.

Transferase-mediated dUTP nick-end labeling (TUNEL) assay

In 96-well plates containing NSCLC cells, 4% paraformaldehyde was used to fix them, 0.1% Triton X100 was used to permeabilize them, and a TUNEL kit was used to stain them. DAPI-stained nuclei were blue, while TUNEL-stained nuclei were green. In order to determine apoptotic cells, the ratio of TUNEL-positive cells to DAPI-stained cells was calculated using a fluorescence microscope.

Flow cytometry for apoptotic cells

Annexin V-FITC/PI was used to double-stain A549 and PC9 cells. Flow cytometry was used to determine whether NSCLC cells were undergoing apoptosis.

Western blot assay

RIPA buffer was used to extract total proteins from NSCLC cell lines, and after SDS-PAGE, in 10% skim milk, proteins were transferred to PVDF membranes. Overnight at 4°C, the membranes were cultivated with primary antibodies (HNRNPU, ITGA9-AS1, ITGA9), followed by one hour of cultivation with secondary antibodies. After washing in TBST and adding secondary antibodies, ECL Substrate was used.

Subcellular fractionation

According to the user guide, A549 and PC9 cells were fractionated using the PARISTM Kit. The cellular distribution of ITGA9-AS1, U6 and β-actin was detected separately in the cell nucleus and cell cytoplasm by RT-qPCR and considered cytoplasmic and nuclear controls, respectively β-actin and U6.

Fluorescence in situ hybridization (FISH) assay

FISH probes designed for ITGA9-AS1 were used as directed. DAPI was used to counterstain nuclei for confocal laser scanning microscopy. In A549 and PC9 cells, ITGA9-AS1 is localized subcellular.

Immunofluorescence (IF) assay

In the IF assay, A549 and PC9 cells were grown on coverslips, fixed with 4% paraformaldehyde, and blocked with 3% bovine serum albumin. In the next step, HNRNPU and ITGA9-AS1 antibodies were probed overnight at 4°C on coverslips. After adding fluorescent-conjugated secondary antibodies for 2 h, the nucleus of the cell was stained by DAPI for 5 minutes. Using confocal laser scanning microscopy, images were captured.

RNA immunoprecipitation (RIP) assay

A RIP assay was performed in A549 and PC9 cells using the Imprint® RNA Immunoprecipitation Kit and an anti-IgG control antibody. Cell lysates from NSCLC cells were incubated with antibodies against AGO2 or IgG. In the end, RT-qPCR was used to analyze the precipitated RNAs.

RNA pull-down assay

Incubation of biotinylated ITGA9-AS1-probes and streptavidin magnetic beads and cell extracts were used as probes for ITGA9 3' UTR. As a control, the Bio-NC probe was used. Finally, the enrichment of several miRNAs (miR-1236-5p, miR-4765 and miR-6810-3p) was analyzed by RT-qPCR. The Bio-NC, Bio-miR-4765-Wt, and Bio-miR-4765-Mut groups were used for determining the binding ability of miR-4765 to ITGA9-AS1/ITGA9 3' UTR. For verifying the binding ability between HNRNPU and ITGA9-AS1/ITGA9 3’ UTR, western blot analysis was used to examine the enrichment of HNRNPU in different groups (Input, anti-sense and ITGA9-AS1 sense/ITGA9 3’UTR sense).

Dual-Luciferase reporter assay

To construct pmirGLO+ITGA9-AS1-Wt/Mut or pmirGLO+ITGA9-AS1-Wt/Mut, we subcloned the ITGA9-AS1 fragment or the ITGA9 3' UTR containing wild-type and mutant miR-4765 binding sites. A miR-4765 mimic or miR-4765 mimic NC was transfected into HEK-293T cells. respectively.48 hours after transfection,

luciferase activity was detected using a dual-luciferase reporter assay.

Statistical analysis

To ensure accuracy, each experiment was repeated three times, and experimental data were presented as means ± SD. We used GraphPad Prism to analyze the data. The $p<0.05$ seems statistical significance.

Results

ITGA9-AS1 positively regulates ITGA9

As we have discussed before that ITGA9-AS1 was down-regulated in elderly patients with lung cancer and we wanted to verify whether ITGA9-AS1 could regulate its host geneITGA9, we first clarified the relationship between ITGA9-AS1 and ITGA9 in NSCLC cells. According to the UALCAN database (http://ualcan.path.uab.edu/index.html), ITGA9-AS1 and ITGA9 were obviously down-regulated in NSCLC. Through GEPIA2 (http://gepia2.cancer-pku.cn/#index) prediction, a high correlation was observed between ITGA9-AS1 and ITGA9 expression in NSCLC (Figure 1A). Then, the expression of ITGA9-AS1 in NSCLC cell lines (NCI-H1299, PC9, NCI-H1975, A549) and BEAS-2B cell line, which showed thatITGA9-AS1expressed at a significantly low level in NSCLC cells (Figure 1B). As ITGA9-AS1 expression was the lowest in A549 and PC9 cells, in the following experiments, they were selected. Then, we evaluated the ITGA9 in these two NSCLC cells compared with BEAS-2B cells, and ITGA9 was down-regulated in NSCLC cells (Figure 1C). By transfecting pcDNA3.1-ITGA9-AS1 and pcDNA3.1-ITGA9 into NSCLC cells respectively, we obtained favorable overexpression efficiency of ITGA9-AS1 and ITGA9 via RT-qPCR (Figure 1D-E). ITGA9 expression was increased by the overexpression of ITGA9-AS1 in A549 and PC9 cells, while ITGA9 overexpression could not affect ITGA9-AS1 expression (Figure 1F). In a word, ITGA9-AS1 positively regulates ITGA9 in NSCLC cells.

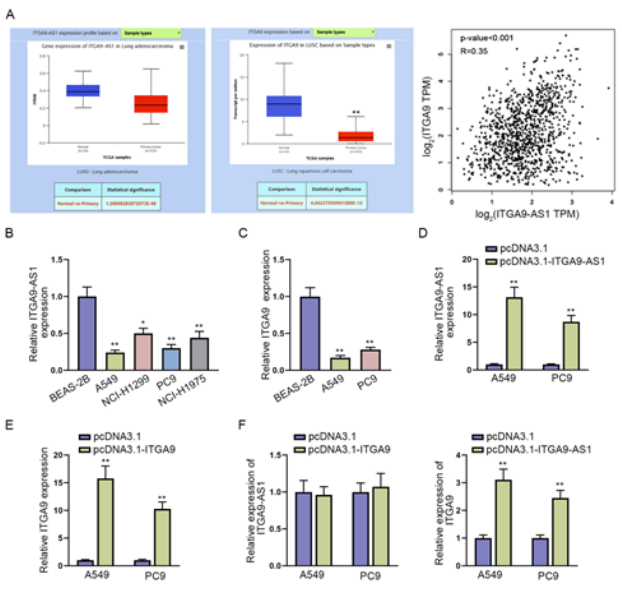

Figure 1. ITGA9-AS1 positively regulates ITGA9. (A) In LUAD, ITGA9-AS1 and ITGA9 were detected using UALCAN and LUSC tumor samples, and the GEPIA2 database was utilized to display the correlation between ITGA9-AS1 and ITGA9 in NSCLC. (B) RT-qPCRexamine the expression of ITGA9-AS1 in NCI-H1299,A549,PC9,NCI-H1975and BEAS-2B. (C) The expression of ITGA9 in A549 and PC9 compared toBEAS-2B cells was detected via RT-qPCR. (D-E) pcDNA3.1-ITGA9-AS1 and pcDNA3.1-ITGA9 were respectively transfected into A549 and PC9 cells and RT-qPCR was used to examine the overexpression efficiency of ITGA9-AS1 and ITGA9. (F) Using RT-qPCR to respectively examine the effect of ITGA9 overexpression on ITGA9-AS1expression and the up-regulation of ITGA9-AS1 on ITGA9 expression in A549 and PC9 cells. $^{*}P<0.05$, $^{**}P<0.01$.

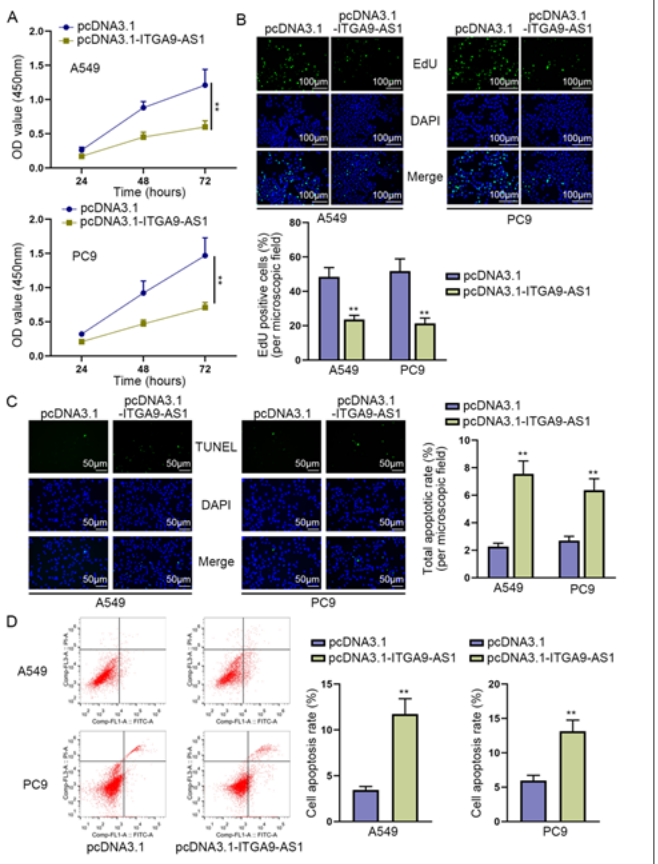

Figure 2. ITGA9-AS1 suppresses cell proliferation and induces cell apoptosis in NSCLC. (A-B) A549 and PC9 cells were examined for viability and proliferation after ITGA9-AS1 overexpression using CCK-8 and EdU assays. (C-D) TUNEL and flow cytometry assays were carried out to assess the influence of ITGA9-AS1 overexpression on NSCLC cell apoptosis. $^{**}P<0.01$.

ITGA9-AS1 suppresses cell proliferation and induced cell apoptosis in NSCLC

After verifying the positive correlation between ITGA9-AS1 and ITGA9, the effects of ITGA9-AS1 on NSCLC progression were further explored. First, CCK-8 and EdU assays showed that ITGA9-AS1 overexpression suppressed the OD value of A549 and PC9 cells as well as the EdU-positive cells, indicating that ITGA9-AS1 overexpression suppressed the viability and proliferative ability of NSCLC cells (Figure 2A-B). TUNEL-positive cells and cell apoptosis rate declined by the up-regulation of ITGA9-AS1, which suggested that ITGA9-AS1 overexpression induced the apoptosis of NSCLC cells (Figure 2C-D). In conclusion, ITGA9-AS1 inhibits NSCLC cell proliferation and facilitates cell apoptosis.

ITGA9-AS1 suppressed the malignant progression of NSCLC via ITGA9

In this part, we tried to figure out whether ITGA9-AS1

may exert its suppressive role in NSCLC cells via regulating ITGA9. We first transfected three siRNAs targeting ITGA9 (si-ITGA9-1/2/3) to obtain silenced ITGA9 expression in A549 cells (Figure 3A). si-ITGA9-1/2 was chosen for further investigations. Next, related functional assays were conducted to assess the proliferation as well apoptosis of NSCLC cells with pcDNA3.1, pcDNA3.1-ITGA9-AS1, pcDNA3.1-ITGA9-AS1+si-NC and pcDNA3.1-ITGA9-AS1+si-ITGA9-1 plasmids. CCK-8 and EdU assays that the declined proliferative ability of A549 cells caused by ITGA9-AS1 overexpression was reversed by ITGA9 silencing (Figure 3B-C). TUNEL and flow cytometry assays showed that ITGA9 inhibition reversed the promoting effect of ITGA9-AS1 overexpression on NSCLC cell apoptosis (Figure 3D-E). To conclude, ITGA9-AS1 suppresses the malignant properties of NSCLC cells via up-regulating ITGA9.

ITGA9-AS1 up-regulates ITGA9 expression by competitively sponging miR-4765

In this part, we continued to explore how ITGA9-AS1 regulated ITGA9 in NSCLC cells. At first, we conducted FISH and subcellular fractionation assays to identify the localization of ITGA9-AS1 and the distribution of ITGA9-AS1 in A549 and PC9 cells was shown in Figure 4A, with a higher concentration in the cytoplasm, which indicated that ITGA9-AS1 may exert its role via competing endogenous RNA (ceRNA) pattern. Compared to the control anti-IgG group, ITGA9-AS1 and ITGA9 were enriched in the anti-AGO2 group, suggesting that they coexist in RNA-induced silencing complex (RISC). (Figure 4B). ThroughmiRDB-microRNA target prediction database (http://mirdb.org/), 109 potential miRNAs that ITGA9 may bind to were selected, and through DIANA tools (http://diana.imis.athena-innovation.gr/DianaTools/), the downstream 188 miRNAs of ITGA9-AS1

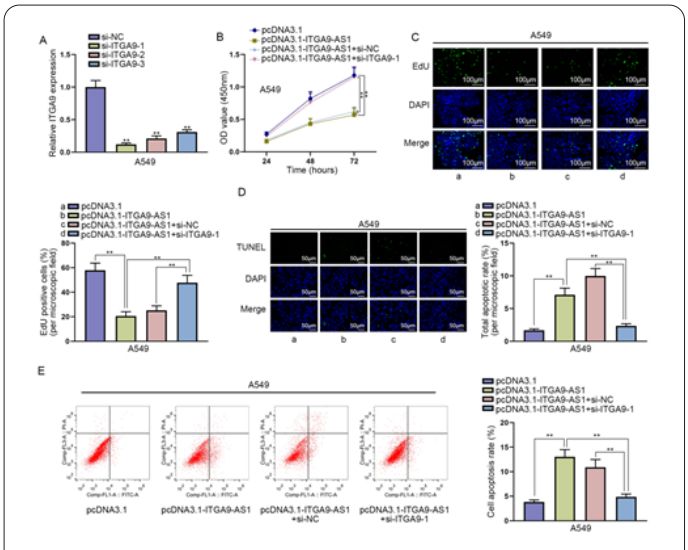

Figure 3. ITGA9-AS1 suppresses the malignant progression of NSCLC via ITGA9. (A) Three siRNAs targeting ITGA9 (si-ITGA9-1/2/3) were transfected into A549 cells and RT-qPCR examine the interference efficiency of ITGA9. (B-C) CCK-8 and EdU assays assess the proliferation of A549 cells in different groups transfected with pcDNA3.1,pcDNA3.1-ITGA9-AS1,pcDNA3.1-ITGA9-AS1+si-NC and pcDNA3.1-ITGA9-AS1+si-ITGA9-1 plasmids. (D-E) TUNEL and flow cytometry detect the apoptosis of A549 cells under different transfection conditions. $^{**}P< 0.01$.

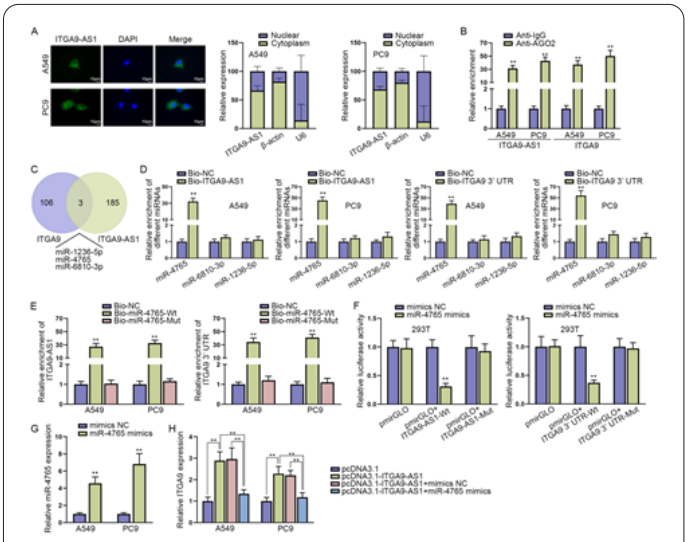

Figure 4. MiR-4765 is competitively sponged by ITGA9-AS1 to upregulate ITGA9 expression. (A) FISH and subcellular fractionation assays identify the localization of ITGA9-AS1 in NSCLC cells. (B) RIP assay examines the enrichment of ITGA9-AS1 and ITGA9 in the anti-AGO2 and control anti-IgG group. (C) miRDBdatabase to predict potential miRNAs that may bind to ITGA9 and DIANA website was utilized to select the downstream miRNAs of ITGA9-AS1 (selection condition: score>0.8). Three miRNAs were finally displayed in the intersection region. (D) RNA pull-down assay with RT-qPCR examines the enrichment of miRNAs candidates (miR-1236-5p, miR-4765 and miR-6810-3p) in bio-ITGA9-AS1 and bio-ITGA9 3' UTR group respectively in A549 and PC9 cells. (E) RNA pull-down assay measured the enrichment of ITGA9-AS1 and ITGA93' UTR in bio-miR-4765-Wt and bio-miR-4765-Mutand bio-NCgroups. (F) Dual-luciferase reporter assay detected the luciferase activity of HEK-293T cells transfected with miR-4765 mimics and miR-4765 NC in different groups. (G-H) RT-qPCR examine the overexpression of miR-4765 in A549 and PC9 cells, and a rescue assay was conducted to examine the expression of ITGA9 in pcDNA3.1,pcDNA3.1-ITGA9-AS1,pcDNA3.1-ITGA9-AS1+mimics NC and pcDNA3.1-ITGA9-AS1+miR-4765 mimics groups. $^{**}P<0.01$.

(selection condition: score>0.8) were predicted. Combined with the above prediction, three miRNAs were displayed in the intersection region, which was miR-1236-5p, miR-4765 and miR-6810-3p (Figure 4C). After that, the results of the RNA pull-down assay with RT-qPCR showed that only miR-4765 displayed the highest enrichment in the bio-ITGA9-AS1 group and bio-ITGA93' UTR group (Figure 4D). Moreover, the mutant type miR-4765 could not be enriched in the ITGA9-AS1 or ITGA9 3' UTR (Figure 4E). Dual-luciferase reporter assay showed that pmirGLO-ITGA9-AS1-Wt and pmirGLO-ITGA9 3' UTR-Wt cells transfected with miR-4765 mimics had decreased luciferase activity, while their corresponding mutant groups displayed no obvious changes (Figure 4F). The binding ability among miR-4765, ITGA9-AS1 and ITGA9 in NSCLC cells. The overexpression efficiency of miR-4765 in A549 and PC9 cells was examined via RT-qPCR, and it was then uncovered thatITGA9 expression was increased by ITGA9-AS1 overexpression, while this effect was partially countervailed by the co-transfection of miR-4765 mimics (Figure 4G-H). In a word, ITGA9-AS1 competitively binds to miR-4765 to elevate ITGA9 expression.

ITGA9-AS1 recruitsHNRNPU to stabilizeITGA9 expression

As we discovered in Figure 4H that miR-4765 overexpression only partially reverse the increasedITGA9 expression caused by ITGA9-AS1 overexpression, we then explored whether there were other regulatory mechanisms underlying ITGA9-AS1 on the regulation of ITGA9. Previous studies have validated that lncRNA can regulate the downstream mRNA by recruiting certain proteins in LUAD (25), so we decided to make an exploration in this direction. Through the prediction of the *cat*RAPID website (http://service.tartaglialab.com/page/catrapid_group), three potential RBPs of ITGA9-AS1 with the highest binding scores were selected, which were HNRNPU, UPF1 and DHX9 (Figure 5A). It was shown from RIP assay with RT-qPCR that ITGA9-AS1 was enriched in the HNRNPU antibody at the highest level compared with the other two RBPs in A549 cells (Figure 5B). ITGA9-AS1 was also enriched in the anti-HNRNPU group in PC9 cells, so we chose HNRNPU for further studies (Figure 5C). By referring to related documents, we learned that HNRNPU could combine with the 3' UTR of mRNA in cytoplasm so as to stabilize its expression (26,27). Therefore, we speculated that HNRNPU may also combine with the 3' UTR of ITGA9 in our research. Through the bioinformatics website (http://pridb.gdcb.iastate.edu/RPISeq/index.html), a significantly high interaction probability between HNRNPU and the 3' UTR of ITGA9 mRNA was presented (Figure 5D). Then, it was shown from the Immunofluorescence assay that ITGA9-AS1 and HNRNPU were co-localized in the cytoplasm of NSCLC cells (Figure 5E). The degradation process of mRNA takes place in the cytoplasm, which was consistent with the above literature, and this result confirmed our expectation. After that, usingRT-qPCR to detect HNRNPU after ITGA9-AS1 was overexpressed in A549 and PC9 cells, which showed that HNRNPU expression was not affected by ITGA9-AS1 overexpression (Figure 5F). Three siRNAs targeting HNRNPU (si-HNRNPU-1/2/3) were transfected into NSCLC cells, and we obtained a favorable interference efficiency of HNRNPU (Figure 5G). It was shown from the RT-qPCR result that ITGA9 expression declined after HNRNPU interference in A549 and PC9 cells (Figure 5H). HNRNPU could be pulled down by ITGA9-AS1 and ITGA9 3'UTR, indicating the binding ability between HNRNPU and ITGA9-AS1/ITGA9 (Figure 5I-J). At last, we used RT-qPCR to test the stability of ITGA9 under50mMα-amanitin treatment, and it was shown from the result that the stability of ITGA9 mRNA declined in the si-HNRNPU#1 group compared with the β-action group (Figure 5K). In conclusion, ITGA9-AS1 stabilizes ITGA9 mRNA by recruiting HNRNPU.

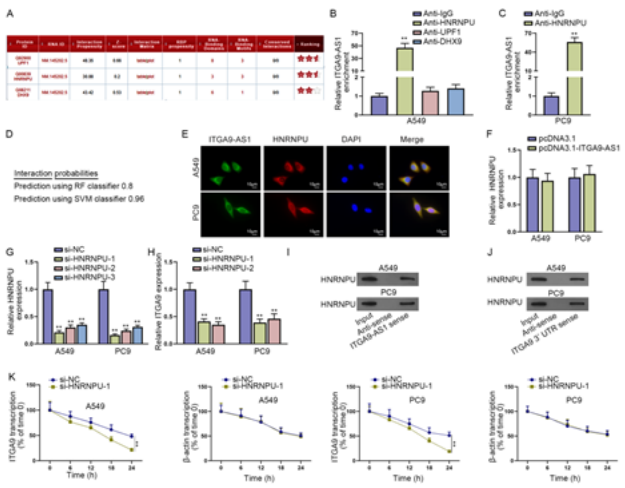

Figure 5. ITGA9-AS1 recruitsHNRNPU to stabilizeITGA9 expression. (A) Through the prediction of the *cat*RAPID website, potential RBPs of ITGA9-AS1 with the highest binding scores were selected. (B) RIP assay with RT-qPCR detected the enrichment of ITGA9-AS1 in antibodies of RBPs (HNRNPU, UPF1 and DHX9). (C) RIP assay with RT-qPCR verified the enrichment of ITGA9-AS1 in the anti-HNRNPU group in comparison with the control IgG group. (D) The interaction probability between HNRNPU and the 3' UTR of ITGA9 mRNA was presented via the bioinformatics website. (E) Immunofluorescence assay identified the co-localization of ITGA9-AS1 and HNRNPU in NSCLC cells. (F) RT-qPCR was used to detect the expression of HNRNPU after ITGA9-AS1 was overexpressed in A549 and PC9 cells. (G) Three siRNAs targeting HNRNPU (si-HNRNPU-1/2/3) were transfected into NSCLC cells and then RT-qPCR was used to detect the interference efficiency of HNRNPU. (H) RT-qPCR detect ITGA9 after HNRNPU was reduced in A549 and PC9 cells. (I-J) RNA pull-down assays and western blot examine the enrichment of HNRNPU under different treatment conditions. (K) RT-qPCR tested the stability of ITGA9 under50mMα-amanitin treatment in si-HNRNPU-1 and the control β-action group. $^{**}P<0.01$.

Discussion

Despite a poor prognosis, the most common type of lung cancer is NSCLC, which accounts for 85% of all lung cancers (28). When NSCLC is diagnosed, most patients are at advanced stages with a poor prognosis because of unobvious clinical symptoms and ineffective screening programs (29). To provide effective therapeutic options for NSCLC, it is therefore important to have a thorough understanding of its mechanism. The crucial role of lncRNA in the progression of cancers which include NSCLC (30-32). According to early research, ITGA9-AS1 was discovered to be down-regulated in elderly patients with lung cancer and it could predict prognosis in elderly NSCLC patients (7), but its function in NSCLC has not been studied. Through a series of functional assays, it was verified that ITGA9-AS1 expressed at a low level in NSCLC cells, and it exerted an inhibitory impact on the malignant development of NSCLC cells. Those research showed the potential use of ITGA9-AS1 in the treatment of NSCLC.

Previous researches have elucidated the regulation of antisense RNA on its host gene, and a number of cancers have been reported to be accelerated by antisense lncRNAs, including NSCLC (19,33). Through bioinformatics prediction and RT-qPCR, it was verified that ITGA9-AS1, together with ITGA9, was down-regulated in NSCLC cells, and ITGA9-AS1 positively regulated ITGA9 expression. What's more, it was verified that ITGA9-AS1 induced NSCLC cell apoptosis and suppressed cell proliferation by up-regulating ITGA9.

The experimental results manifested that the majority of ITGA9-AS1 was found in the cytoplasm of NSCLC cells. To regulate gene expression, cytoplasmic lncRNAs interact with miRNAs through ceRNA mechanisms. For example, LINC01123promotes cell proliferation inNSCLC (34), the lncRNA TMPO-AS1 regulates ERBB2 via miR-204-3p to facilitate proliferation and metastasis of

NSCLC cells (35) and cisplatin resistance is regulated in NSCLC cells by lncRNA XIST, which acts as a sponge for microRNA-520 through ceRNA (23). In the current study, we verified that ITGA9-AS1 elevated ITGA9 mRNA by competitively binding to miR-4765 in NSCLC cells, and it was verified by rescue assay that the addition of miR-4765 mimics could reverse the increased ITGA9 expression caused by ITGA9-AS1 overexpression.

Due to the fact that miR-4765 overexpression could not fully achieve the rescue effect, we continued to explore the other regulatory mechanism underlying the ITGA9-AS1/ITGA9 axis. Previous studies have elucidated that lncRNAs can regulate their downstream mRNAs by recruiting proteins in lung cancer (25,30). In this study, HNRNPU was screened as a potential RBP of ITGA9-AS1 in NSCLC cells. As it has been illustrated to stabilize the expression of mRNA by combining with its 3' UTR region (26,27), we made the speculation that HNRNPU may combine with the 3' UTR of ITGA9 so as to stabilize its expression. By conducting a series of mechanism experiments, it was verified that ITGA9-AS1 recruited HNRNPU to stabilize ITGA9 mRNA in NSCLC cells.

Conclusion

Our study indicated that down-regulated ITGA9-AS1 recruited HNRNPU to stabilize the 3' UTR of ITGA9 mRNA in the cytoplasm of NSCLC cells, and it competitively sponging miR-4765 to up-regulate ITGA9, thereby inhibiting the malignant progression of NSCLC. Though more efforts should be made to the support clinical significance of ITGA9-AS1, our findings may shed some light on the therapeutic strategies of NSCLC.

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