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Original Article

Circular RNA La-related protein 4 inhibits cell proliferation, migration, and invasion and sponges microRNA-367 in non-small cell lung cancer

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Abstract: This study aimed to investigate the effect of circular RNA (circRNA) La-related protein 4 (LARP4) on inhibiting the malignant progression of non-small cell lung cancer (NSCLC) cells. CircRNA LARP4 expression in a human normal lung epithelial cell line and NSCLC cell lines was detected by reverse transcription-quantitative polymerase chain reaction. CircRNA LARP4 overexpression and control overexpression plasmids were transfected into NCI-H1650 cells; circRNA LARP4 knockdown and control knockdown plasmids were transfected into A549 cells. CircRNA LARP4 and microRNA (miR)-367 expressions, cell proliferation, apoptosis, migration, and invasion after transfection were investigated. Moreover, the luciferase reporter assay was used to explore the interaction between circRNA LARP4 and miR-367. CircRNA LARP4 was decreased in NSCLC cell lines (including A549, NCI-H1650, NCI-H1299, and HCC827 cells) compared to BEAS-2B cells. In NCI-H1650 cells, circRNA LARP4 overexpression inhibited cell proliferation, migration, and invasion, while promoting apoptosis. In A549 cells, circRNA LARP4 knockdown increased cell proliferation, migration, and invasion, while decreasing apoptosis. Moreover, miR-367 was decreased by the overexpression of circRNA LARP4, but increased by the knockdown of circRNA LARP4; and further luciferase reporter assay revealed that circRNA LARP4 could directly bind to miR-367. In conclusion, circRNA LARP4 is downregulated, suppresses cell proliferation, migration, and invasion, and acts as a sponge of oncogene miR-367 in NSCLC.

Keywords: Circular RNA La-related protein 4, non-small cell lung cancer, cell proliferation, cell motility, microRNA-367

Introduction

Lung cancer is the most common cause of cancer-related death, resulting in an estimated 1.7 million deaths each year [1]. Non-small cell lung cancer (NSCLC) is the major subtype of lung cancer (accounting for 85%) [2]. Although NSCLC can be treated surgically at the initial stage, most patients have locally advanced or metastatic disease when diagnosed [3]. Current treatment strategies for these patients mainly include targeted therapy (including epidermal growth factor inhibitor and vascular endothelial growth factor inhibitor), immune therapy, chemotherapy, and radiotherapy, which have greatly improved survival of NSCLC patients [4]. However, the prognosis of NSCLC patients is still not satisfactory and their 5-year overall survival is as low as 15% [3]. Therefore, further exploring the biology of NSCLC to provide potential therapeutic targets is critical.

Circular RNA (circRNA) La-related protein 4 (LARP4) is one of the newly discovered circRNAs, which is reported to be a tumor suppressor in several carcinomas [5-7]. For example, circRNA LARP4 overexpression inhibits cell proliferation while promoting apoptosis in gastric cancer cells as well as hepatocellular carcinoma cells, and it hampers cell motility in esophageal squamous cell carcinoma cells [6-8]. circRNA LARP4 is found in human lung tissues, according to Tissue-Specific CircRNA Database (http://gb.whu.edu.cn/TSCD/). Moreover, circRNA LARP4 was detectable in NSCLC cell lines, and its expression was decreased in

NSCLC cell lines compared to a normal lung epithelial cell line in our preliminary study. Based on the aforementioned information, we hypothesized that circRNA LARP4 also acted as a tumor suppressor in NSCLC. However, the role of circRNA LARP4 in NSCLC remains unclear. Therefore, the aim of this study was to investigate the effect of circRNA LARP4 on inhibiting NSCLC cell malignant progression.

Methods

Cell culture

Human NSCLC cell lines (including A549, NCI-H1650, NCI-H1299, and HCC827) and human normal lung epithelial cells BEAS-2B were all purchased from American Type Culture Collection (ATCC) (Manassas, USA). The A549 cells and the BEAS-2B cells were cultured in 90% Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). The NCI-H1650, NCI-H1299, and HCC827 cells were cultured in 90% RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). All cells were cultured in a humid atmosphere of 5% CO_2, 95% air at 37°C. The relative expressions of circRNA LARP4 in the cells after culture were determined by reverse transcriptional-quantitative polymerase chain reaction (RT-qPCR).

Cell transfection

The pCD25-ciR vector (Geneseed Biotech Co., Ltd., China) was used for the construction of circRNA LARP4 overexpression (Circ(+)) plasmids and negative control (NC(+)) plasmids; and the pGPH1 vector (Geneseed Biotech Co., Ltd., China) was used for the construction of circRNA LARP4 knockdown (Circ(-)) plasmids and negative control (NC(-)) plasmids. The Circ(+) plasmids and the NC(+) plasmids were transfected into NCI-H1650 cells, and the Circ(-) plasmids and the NC(-) plasmids were transfected into the A549 cells, using Lipofectamine 3000 kit (Thermo Fisher Scientific, USA). After incubation for 24 hours (h), the relative expressions of circRNA LARP4 in these transfected cells were detected by RT-qPCR assay.

Cell proliferation, apoptosis, migration, and invasion assays

At 0 h, 24 h, 48 h, and 72 h post-transfection, cell proliferation in the transfected cells were determined using Cell Counting Kit-8 (Sigma, USA) according to the instructions of the manufacturer, and quantitative analysis was performed by a microplate reader according to the optical density (OD) value. At 48 h post-transfection, cell apoptosis rate was detected using Annexin V-FITC Apoptosis Detection Kit (Sigma, USA) according to the instructions of the manufacturer. At 48 h post-transfection, cell migration was assessed by Wound Healing assay; and cell invasion was determined by the Transwell assay using invasion chambers (BD Biosciences, USA), which was performed as described in a previous study [9].

Reverse transcriptional-quantitative polymerase chain reaction (RT-qPCR) assay

The RT-qPCR assay was conducted according to previous research with some modifications [10]. Briefly, total RNA extraction was conducted with TRIzol reagent (Invitrogen, USA). For circRNA LARP4 detection, digestion of linear RNA was performed with RNase R enzyme (Epicentre, USA) according to the manufacturer's instructions; while for detection of other RNAs, linear RNA digestion was not performed. Subsequently, the reverse transcription was conducted with PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara, Japan), and the qPCR was performed with QuantiNova SYBR Green PCR Kit (Qiagen, German) according to manufacturers' guidance. GAPDH was set as the internal reference of circRNA, and U6 was set as the internal reference of microRNA (miRNA/miR). The relative expression of circRNA and miRNA was calculated by the $2^{-\Delta\Delta Ct}$ formula. The primers were listed as follows: circRNA LARP4: forward: 5'-ATTGTGCCTCAGTCTTGGTCTC-3', reverse: 5'-AGTTCCTTTAGATGCTACCTGCTAT-3'; GAPDH: forward: 5'-GAGTCCACTGGCGTCTTCAC-3', reverse: 5'-ATCTTGAGGCTGTTGTCATACTTCT-3'; miR-367-5p: forward: 5'-ACACTCCAGCTGGGACTGTTGCTAATATGC-3', reverse: 5'-TGTCGTGGAGTCGGCAATTC-3'; U6: forward: 5'-CGCTTCGGCAGCACATATACTA-3', reverse: 5'-ATGGAACGCTTCACGAATTTGC-3'.

Luciferase activity assay

Luciferase reporter assay was performed with Dual-Luciferase® Reporter (DLR™) Assay System (Promega, USA). CircRNA LARP4 wild type (WT) plasmid and mutant (Mut) plasmid were constructed using the pGL4 vector (Promega, USA). MiR-367 overexpression (miR-

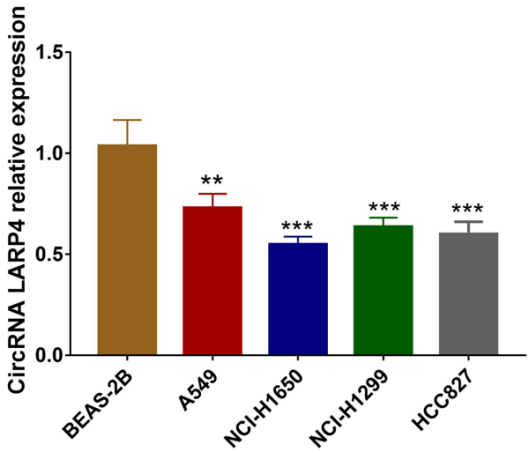

Figure 1. Relative expression of circRNA LARP4 in a human normal lung epithelial cell line and NSCLC cell lines. **: *P*<0.01; ***: *P*<0.001; circRNA: circular RNA; LARP4: La-related protein 4; NSCLC: non-small cell lung cancer.

367(+)) plasmid and negative control (NC(+)) plasmid were constructed using the pcDNA3.1 vector (GenePharma, China). CircRNA LARP4 WT or Mut plasmid and miR-367(+) or NC(+) plasmid were co-transfected into 293T cells (ATCC, USA) using Lipofectamine 3000 (Thermo, USA), resulting in four groups: WT&miR-367(+) cells, WT&NC(+) cells, Mut&miR-367(+) cells, and Mut&NC(+) cells. At 24 h after transfection, cells were lysed and firefly luciferase luminescence was detected according to the Dual-Luciferase® Reporter Assay System Protocol.

Statistical analysis

Data are presented as mean and standard deviation. Comparison between two groups was determined by unpaired t-test, and multiple comparisons were determined by Dunnett's test. *P* value <0.05 was considered significant, and is displayed as *P<0.05, **P<0.01, ***P<0.001, while the *P* value >0.05 was marked as NS (not significant) in all figures.

Results

CircRNA LARP4 expression in human NSCLC cells and human normal lung epithelial cells

The relative expression of circRNA LARP4 was measured in human normal lung epithelial cell line BEAS-2B and human NSCLC cell lines (including A549, NCI-H1650, NCI-H1299 and HCC827). Data revealed that circRNA LARP4 was decreased in A549 (*P*<0.01), NCI-H1650 (*P*<0.001), NCI-H1299 (*P*<0.001), and HCC827 (*P*<0.001) (**Figure 1**). We chose NCI-H1650 cells and A549 cells for further investigation.

Effect of circRNA LARP4 on cell proliferation and apoptosis

After transfection into NCI-H1650 cells, circRNA LARP4 was increased in Circ(+) cells compared to NC(+) cells (*P*<0.001) (**Figure 2A**). Moreover, cell apoptosis was increased at 48 h (*P*<0.001) (**Figure 2B**, **2C**), while proliferation was decreased at 48 h (*P*<0.05) and 72 h (*P*<0.01) (**Figure 2D**) in Circ(+) cells compared to NC(+) cells. In A549 cells, circRNA LARP4 was decreased in Circ(-) cells compared to NC(-) cells (*P*<0.001) (**Figure 2E**). Moreover, cell apoptosis was decreased at 48 h (*P*<0.01) (**Figure 2F**, **2G**), while proliferation was increased at 48 h (*P*<0.05) and 72 h (*P*<0.01) (**Figure 2H**) in Circ(-) cells compared to NC(-) cells.

Effect of circRNA LARP4 on cell migration and invasion

In NCI-H1650 cells, cell migration rate (*P*<0.05) (**Figure 3A**, **3B**) and invasive cell count (*P*<0.01) (**Figure 3C**, **3D**) were inhibited in Circ(+) cells compared to NC(+) cells. In A549 cells, cell migration rate (*P*<0.01) (**Figure 3E**, **3F**) as well as invasive cell count (*P*<0.01) (**Figure 3G**, **3H**) were augmented in Circ(-) cells compared to NC(-) cells.

Effect of circRNA LARP4 on miR-367 expression

In NCI-H1650 cells, miR-367 expression was suppressed in the Circ(+) cells compared to NC(+) cells (*P*<0.001) (**Figure 4A**). In A549 cells, it was increased in Circ(-) cells compared to NC(-) cells (*P*<0.001) (**Figure 4B**). Therefore, circRNA LARP4 was able to negatively regulate miR-367 in NSCLC cells.

CircRNA LARP4 as a sponge of miR-367

CircRNA LARP4 had a very strong potential to bind miR-367, and the probable binding site was shown in **Figure 5A**. The relative luciferase activity was decreased in WT&miR-367(+) cells compared to WT&NC(+) cells (*P*<0.001), while no difference was found in the relative lucifer-

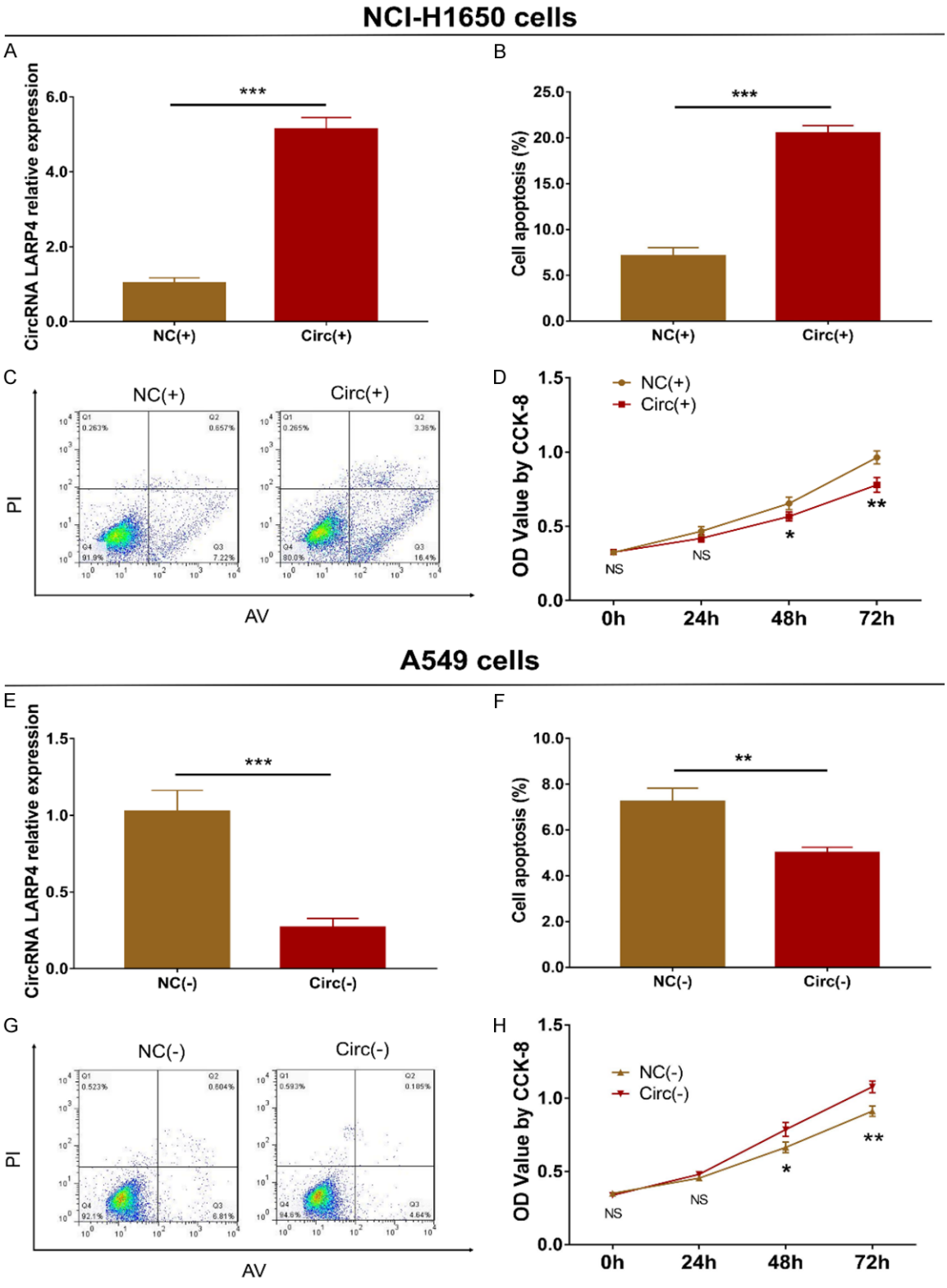

Figure 2. Regulation of cell proliferation and apoptosis by circRNA LARP4. A: Comparison of circRNA LARP4 relative expression between Circ(+) and NC(+) cells. B: Comparison of apoptosis rate between Circ(+) and NC(+) cells. C: Example figure of apoptosis detection in NCI-H1650 cells. D: Comparison of cell proliferation between Circ(+) and NC(+) cells at 0 h, 24 h, 48 h, and 72 h. E: Comparison of circRNA LARP4 relative expression between Circ(-) and NC(-) cells. F: Comparison of apoptosis rate between Circ(-) and NC(-) cells. G: Example figure of apoptosis detection in A549 cells. H: Comparison of cell proliferation between Circ(-) and NC(-) cells at 0 h, 24 h, 48 h, and 72 h. NS: not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; circRNA: circular RNA; LARP4: La-related protein 4.

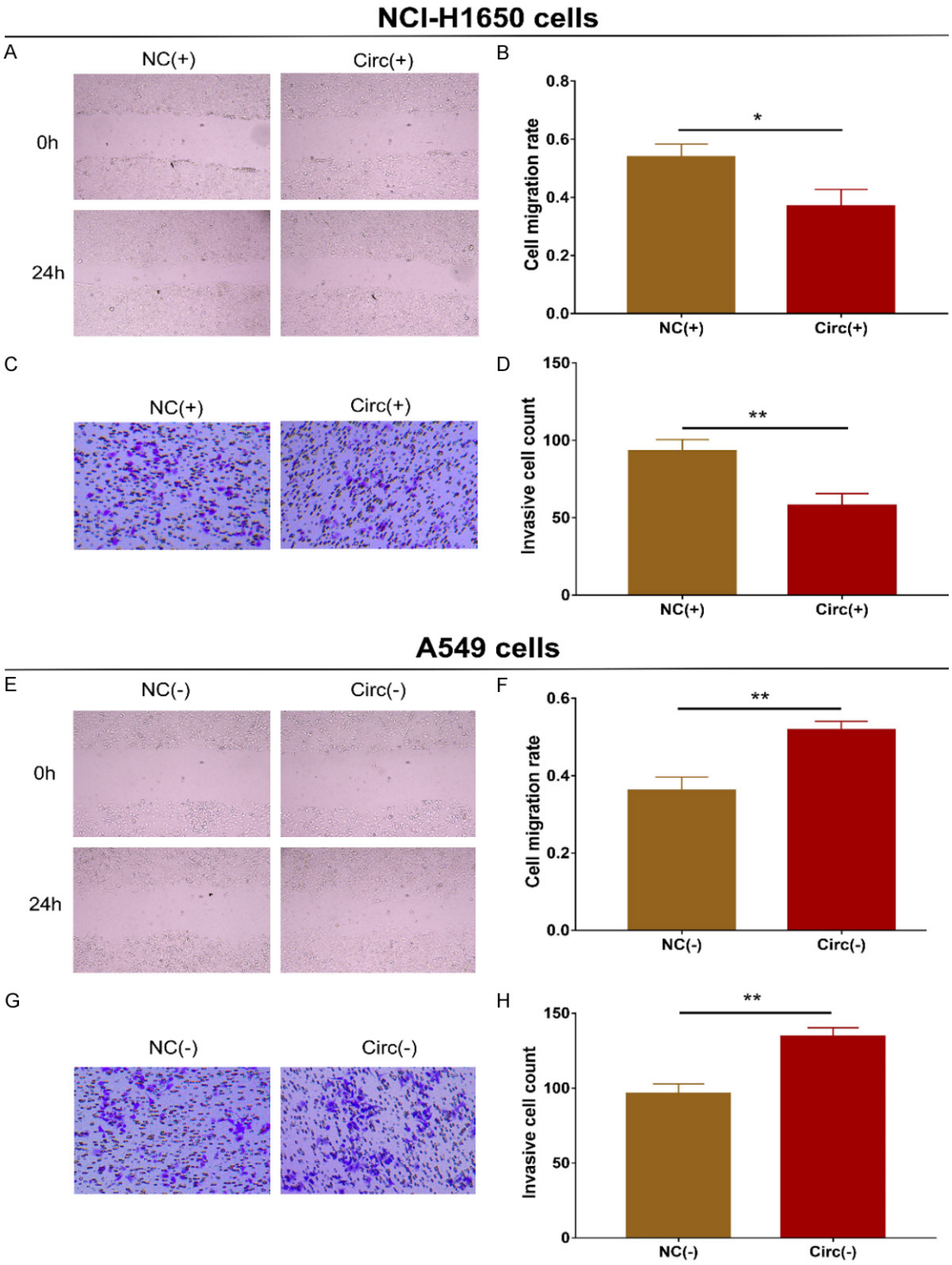

Figure 3. Regulation of cell migration and invasion by circRNA LARP4. A: Example figure of cell migration of NCI-H1650 cells. B: Comparison of migration rate between Circ(+) and NC(+) cells. C: Example figure of cell invasion of NCI-H1650 cells. D: Comparison of invasive cell count between Circ(+) and NC(+) cells. E: Example figure of cell migration of A549 cells. F: Comparison of migration rate between Circ(-) and NC(-) cells. G: Example figure of cell invasion of A549 cells. H: Comparison of invasive cell count between Circ(-) and NC(-) cells. *: $P<0.05$; **: $P<0.01$; circRNA: circular RNA; LARP4: La-related protein 4.

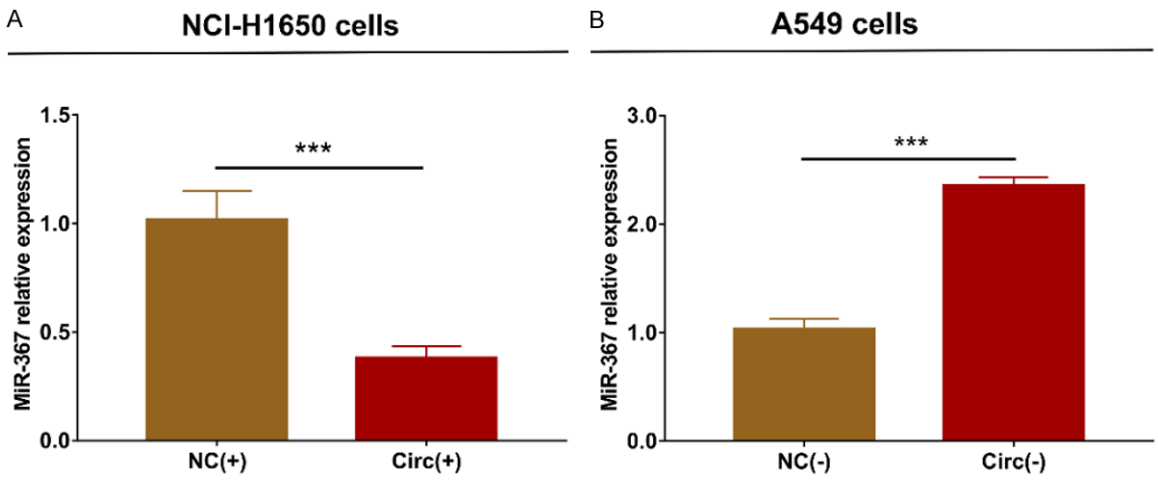

Figure 4. Regulation of miR-367 by circRNA LARP4. A: Comparison of miR-367 relative expression between Circ(+) and NC(+) cells. B: Comparison of miR-367 relative expression between Circ(-) and NC(-) cells. ***: *P*<0.001; miR: microRNA; circRNA: circular RNA; LARP4: La-related protein 4.

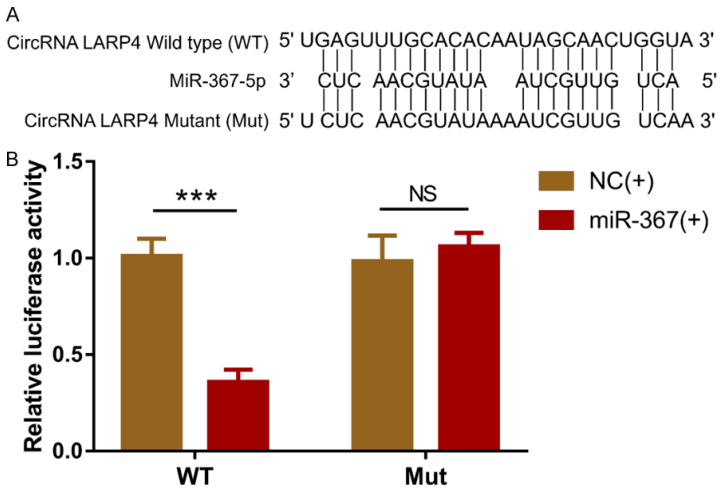

Figure 5. Potential of circRNA LARP4 as the sponge of miR-367. A: The binding site of wild type or mutant circRNA LARP4 with miR-367. B: Relative luciferase activity of WT&miR-367(+) cells, WT&NC(+) cells, Mut&miR-367(+) cells, and Mut&NC(+) cells. NS: not significant; ***: *P*<0.001; miR: microRNA; circRNA: circular RNA; LARP4: La-related protein 4; WT: wild type; Mut: mutant.

ase activity between Mut&NC(+) cells and Mut&miR-367(+) cells (*P*>0.05) (**Figure 5B**).

Discussion

CircRNA LARP4 relative expression and its regulatory effect on cellular function have been reported in several carcinomas according to previous studies. For example, in gastric cancer cell lines, circRNA LARP4 inhibits cell proliferation as well as invasion by sponging miR-424 [6]. In nasopharyngeal carcinoma cells, the relative expression of circRNA LARP4 is decreased compared to normal cells, and the overexpression of circRNA LARP4 represses cell proliferation, migration, and invasion by targeting Rho-associated kinase 1 [11]. In ovarian cancer cells, the overexpression of circRNA LARP4 impairs cell proliferation and motility through sponging miR-513b, and further regulates LARP4 expression [12]. Therefore, these previous studies demonstrate that circRNA LARP4 is a tumor suppressor in several solid tumors.

Previous studies suggest that circRNA LARP4 suppresses the progression of several cancer cells (including esophageal squamous cell carcinoma cells, osteosarcoma cells, and ovarian cancer cells) [8, 10, 12]. Moreover, circRNA LARP4 exists in human lung tissues according to Tissue-Specific CircRNA Database (http://gb.whu.edu.cn/TSCD/), and its low expression was observed in NSCLC cell lines compared to a normal lung epithelial cell line in our small-scale preliminary research. Based on this information, we hypothesized that circRNA LARP4 was also a suppressor in NSCLC cells. However, no relevant previous study had been conducted before. Therefore, we performed this study and found that circRNA LARP4 was insufficiently

expressed in several NSCLC cell lines (including A549, NCI-H1650, NCI-H1299, and HCC827) compared to normal human lung epithelial cells. Possible explanations of our data might be: (1) As a miRNA sponge, the insufficient expression of circRNA LARP4 might increase the relative expression of several miRNAs (such as miR-424 and miR-367) to promote the malignant transformation of normal cells; (2) Decreased circRNA LARP4 might suppress p53 and p21 expression, which might induce tumorigenesis of NSCLC [13]. In support of that, its depletion was found in NSCLC cell lines.

Regarding the effect of circRNA LARP4 on tumor cellfunction, previous studies demonstrated that its overexpression inhibits cell proliferation, migration, and invasion, while promoting apoptosis in ovarian cancer cells and esophageal squamous cell carcinoma cells [8, 12]. However, no relevant previous study had been conducted in NSCLC cells to the best of our knowledge. In the present study, we found that the overexpression of circRNA LARP4 suppressed cell proliferation, migration, and invasion, while promoting apoptosis in NSCLC cell lines. Possible explanations for these data might be that: (1) CircRNA LARP4 might sponge several miRNAs to suppress the proliferation and motility of NSCLC, such as miR-424 (as in gastric cancer [11]), miR-1323 (as in esophageal squamous cell carcinoma [8]), as well as miR-367 (displayed in the luciferase assay of this study). (2) CircRNA LARP4 might promote the expression of LARP4 (as in ovarian cancer [12]) to inhibit the migration and invasion of NSCLC cells. (3) CircRNA LARP4 might suppress several survival/epithelial-mesenchymal transition (EMT)-related pathways such as the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway to promote apoptosis while suppressing the cell migration and invasion of NSCLC cells.

miR-367 is one of the members of the miR-302/367 cluster, which acts as a regulator in the self-renewing and reprogramming of diverse cells [14]. Moreover, the role of miR-367 in cancer cells has been reported in previous studies. In osteosarcoma cells, miR-367 promotes cell proliferation, migration, and invasion by suppressing DOC-2/DAB2 interactive protein [15]. In pancreatic ductal adenocarcinoma cells, miR-367 increases EMT through targeting the tumor growth factor-β signaling [16]. In NSCLC cells, one study displays that miR-367 increases cell cycle by suppressing F-box and WD repeat domain-containing 7 in NSCLC cells [17]; and another reveals that miR-367 promotes the self-renew ability of NSCLC stem-like cells by maintaining the Wnt signaling via the regulation of Lin-28 homolog B/Let-7 [18]. Therefore, miR-367 enhances the progression of several cancers including NSCLC. In this study, we further performed the luciferase assay and found that circRNA LARP4 was able to act as a sponge of miR-367 in NSCLC cells.

In conclusion, circRNA LARP4 is downregulated, suppresses cell proliferation, migration, and invasion, and sponges oncogene miR-367 in NSCLC. This study provides a new perspective on circRNA LARP4 as a therapeutic option for NSCLC.

Disclosure of conflict of interest

None.

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