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Circular RNA hsa_circ_0012673 facilitates lung cancer cell proliferation and invasion *via* miR-320a/LIMK18521 axis

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Abstract. – OBJECTIVE: Lung cancer is one of the most malignant tumors with high morbidity and mortality in the world. The incidence and mortality of lung cancer were increased per year in many countries over the past 50 years. The increasing studies had shown that circular RNA (circRNA) was involved in the progression of lung cancer. Therefore, it was significant to seek the molecular mechanism of circ_0012673 in lung cancer.

MATERIALS AND METHODS: Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to estimate the expression levels of circ_0012673, miR-320a and LIM domain kinase 1 (LIMK1) in lung cancer tissues and cells. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazol-3-ium bromide (MTT), flow cytometry and transwell assays were recruited to evaluate proliferation, apoptosis and mobility of lung cancer cells, respectively. The relative protein expression levels of Vimentin, N-cadherin, E-cadherin and LIMK1 were determined with Western blot assay. The relationships among circ_0012673, miR-320a and LIMK1 were analyzed by starBase database, dual-luciferase reporter assay, and Pearson's correlation.

RESULTS: Circ_0012673 was overexpressed in lung cancer tissues and cell lines. Loss-of-functional experiment confirmed that knockdown of circ_0012673 constrained proliferation, motility and Epithelial-Mesenchymal Transition (EMT), but induced apoptosis by targeting miR-320a. Furthermore, LIMK1 was a target of miR-320a in lung cancer cells. Elevated LIMK1 could abolish the overexpression of miR-320a induced effects on lung cancer cells. Mechanistically, circ_0012673 contributed to lung cancer progression through mediating miR-320a /LIMK1 pathway.

CONCLUSIONS: Circ_0012673 was a tumor-promoter in lung cancer via acting as competing endogenous RNA to regulate LIMK1 expression by binding miR-320a.

Key Words
Circular RNA, Circ_0012673, MiR-320a, LIMK1, Lung cancer.

Introduction

Lung cancer is one of the main causes of cancer-death globally, leading to millions of deaths per year[1]. Non-Small Cell Lung Cancer (NSCLC) accounts for 85% of all lung cancer and roughly 83% of the patients with NSCLC used to die within 5 years[2,3]. The serious invasion and distant metastases are considered to be the major contributors to death caused by NSCLC. Therefore, a better understanding of NSCLC biology and novel targeted therapies is urgently needed for this cancer.

Currently, increasing reports have confirmed that circular RNA (circRNA) played significant roles in the processes of multiple cancers[4,5]. CircRNAs, covalently-closed loops, have no potential to translate into proteins[6]. However, circRNAs participated in a variety of biological process as differentiation[7], apoptosis[8], and metastases[9]; importantly, circRNAs could function as microRNA sponges to regulate transcription and splicing of gene[10], which attracted the attention of many researchers. Zhang et al[11] reported that circular RNA ZFR-mediated NSCLC cell proliferation, migration and invasion was dependent on upregulating cullin 4B via sponging miR-101-3p. CircCRIM1 had an anti-oncogenic role in NSCLC and it sponged miR-93 and miR-182 to impede invasion and metastasis of lung adenocarcinoma cancer cells[12]. Besides, mounting evidence suggested a variety of circRNAs was aberrantly expressed in lung cancer, including circ_0012673[13]. Consequently, dysregulated circRNAs were therapeutic targets for lung cancer by acting as tumor suppressors or promoters[14,15], but the study of circ_0012673 in lung cancer was extremely lacking.

The downregulation of miR-320a was observed in colorectal cancer[16] and breast cancer[17].

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Similar results were observed in NSCLC and primary squamous cell lung carcinoma[18,19], suggesting miR-320a may play an anti-oncogene role in lung cancer by mediating proliferation and invasion of lung cancer cells. In addition, LIM domain kinase 1 (LIMK1), serine-threonine protein kinase, has been described to participate in Epithelial-Mesenchymal Transition (EMT) process by affecting actin cytoskeleton[20]. LIMK1 was upregulated in cancer tissues, and this was consistent with the results that LIMK1 played an oncogene role in prostate cancer[21], oral squamous cell carcinoma[22], and colorectal cancer[23]. Importantly, to our knowledge, the relationship among circ_0012673, miR-320a and LIMK1 so far in lung cancer has not been expounded.

Therefore, we initially measured the abundance of circ_0012673 in lung cancer tissues and cell lines. StarBase database and dual-luciferase activity assay were employed to seek the target gene of circ_0012673; additionally, a series of functional experiments were used to probe the regulatory mechanism and biological function of circ_0012673/ miR-320a/ LIMK1 axis in the process of lung cancer.

Materials and Methods

Patient Tissues

Forty human lung cancer tissues and matched normal tissues (located more than 5 cm far away from the lung cancer tissues) were derived from patients with lung cancer undergoing surgical resection at Shengli Oilfield Central Hospital. When human tissue samples were received, they were rapidly frozen in liquid nitrogen and long-time conservation at -80°C for subsequent study. This study was approved by the Ethics Committee of Shengli Oilfield Central Hospital and all of the patients with lung cancer did not receive any treatment and provided written informed consent prior to this research.

Cells Culture

The cells used in this research (BEAS-2B normal human lung epithelial cell line; A549 and H23 human lung cancer cell lines) were acquired from the American Type Culture Collection (ATCC Manassas, VA, USA). Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Biochrom, Berlin, Germany), including 10% (v/v) fetal bovine serum (FBS; Solarbio, Beijing, China), 1% penicillin/streptomycin (Solarbio), was employed to cultivate above cells in a constant humidity environment at 37°C with 5% CO_2.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

The RNA extraction kit (Solarbio) was applied to extract total RNA from lung cancer tissues or cells according to the references and then dissolved in RNase-free water. The quality and integrity of RNA were checked by Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) and gel electrophoresis, correspondingly. Thereafter, 5 μg of the RNA was used to synthesize complementary DNA using High-capacity cDNA Reverse Transcription kit (Bio-Rad, Hercules, CA, USA) and All-in-One miRNA cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) referring to the manufacturer's procedures. SYBR Green Master Mix (Invitrogen) was utilized to quantify the transcript levels of circ_0012673, miR-320a and LIMK1 on Thermal Cycler CFX6 System (Bio-Rad) based on the formula $2^{-\Delta\Delta C}$, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or endogenous small nuclear RNA U6 as a reference control.

The sequences of primers were displayed as follows:

Circ_0012673 (sense, 5'- CGTGTTGCCTGAGCTTGATG -3'; antisense, 5'- GGCCGTACTTGTGGGATGAT -3');

miR-320a (sense, 5'-GCCGAGAAAGCTGGGTTGAG-3'; antisense, 5'- CTCAACTGGTGTCGTGGA-3');

LIMK1 (sense, 5'-AGACCTCAACTCCCACAA-3'; antisense, 5'- CTCAGGTGCCATCCAGT-3');

GAPDH (sense, 5'-TCCCATCACCATCTTCCAGG-3'; antisense, 5'- GATGACCCTTTTGGCTCCC-3');

U6 (sense, 5'-CTCGCTTCGGCAGCACA-3'; antisense, 5'- AACGCTTCACGAATTTGCGT-3').

Cell Transfection

Specific small interfering RNA (siRNA) objecting circ_0012673 (si- circ_0012673) and siRNA scrambled control (si-NC), circ_0012673-overexpression plasmid (circ_0012673) and overexpressed plasmid of LIMK1 (LIMK1) as well as their negative control (pcDNA), miR-320a mimic (miR-320a) and its negative control (miR-NC), miR-320a inhibitor (anti-miR-320a) and its negative control (anti-miR-NC) were approved by GeneCopoeia (Rockville, MD, USA). Lipofect-

amine 2000 (Invitrogen) was employed for plasmids or oligonucleotides transfection as per user's guidebook. Then, transfected cells were incubated at 37°C for 48 h.

3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl-2H-Tetrazol-3-Ium Bromide (MTT) Assay

The proliferation capacity of transfected A549 and H23 cells was assessed by MTT assay. The transfected cells were cultivated into 96-well culture plates (4×10^3 cells/well). After incubation in medium at 37°C for 24 h, 48 h, or 72 h, MTT (Promega, Madison, WI, USA) was added in each well and cells were incubated for another 4 h. After supernatant liquids were removed, 150 μL of dimethyl sulfoxide (DMSO) was administrated to cells to dissolve formazan crystals. The cell viability was examined by detecting optical density at wavelength of 490 nm each well on a microplate reader (Applied Biosystems, Foster City, CA, USA).

Flow Cytometry

Cell apoptosis was monitored by flow cytometry analysis. After A549 and H23 cells were transfected for 24 h, the medium was replaced with phosphate buffer saline (PBS) buffer solution, and then 5 μL of Annexin V labeled with fluorescein isothiocyanate (FITC) plus 5 μL of propidium iodide (PI) were added to each well for 30 min in dark condition at room temperature. The rate of cell apoptosis was examined using an Annexin V-FITC/PI apoptosis detection kit (KeyGen, Nanjing, China), and Annexin V positive cells served as apoptotic cells.

Transwell Assay

In the migration assay, A549 and H23 cells in medium without FBS were injected into the upper chamber of transwell 24-well chamber with 8 μm pore filter. Subsequently, the lower chamber was added with RPMI-1640 medium with 10% FBS. 24 h later, the cells that remained on the upper chamber were scraped with a cotton swab, whereas migrated cells under the bottom of the transwell membrane were fastened and stained by 95% ethanol and 0.1% crystal violet, respectively. A microscope was used to compute numbers of migrated cells in three randomly selected fields. In addition, the protocols of invasion assay were similar to migration, but upper chamber was attached with a matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA) before cell seeding.

Western Blot Assay

Lung cancer tissues and cells were lysed with Radio-Immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) for total protein isolation. Then sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to segregate proteins based on relative molecular mass and then the bands were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk solution, membranes were reacted with monoclonal antibodies, including LIMK1 (1:500 dilution, Proteintech, Chicago, IL, USA), Vimentin (1:1500 dilution, Proteintech), N-cadherin (1:1500 dilution, Proteintech) and E-cadherin (1:1500 dilution, Proteintech), respectively, with GAPDH (1:3000 dilution, Proteintech) as the internal control. After that, the secondary antibody (1:2000 dilution, Proteintech) was used to interact with membranes for 1 h at room temperature. The protein signal was visualized and analyzed by ECL Chemiluminescence Detection System (Solarbio) according to the operation manual.

Dual-Luciferase Reporter Assay

The potential binding sites between miR-320a and circ_0012673 or 3'untranslated region (UTR) of LIMK1 were predicted by starBase (http://starbase.sysu.edu.cn/). The sequences of wild type circ_0012673 and LIMK1 as well as matched mutant forms were cloned by PCR and then embedded into PGL3 vector (Promega, Madison, WI, USA) to generate luciferase reporters, named as WT-circ_0012673, MUT-circ_0012673, LIMK1 3'UTR-WT, or LIMK1 3'UTR-MUT, respectively. A549 and H23 cells were co-transfected with luciferase reporters along with miR-320a or miR-NC with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in line with the manufacturer's manuals. Dual-Luciferase Assay Kit (GeneCopoeia, Rockville, MD, USA) was applied to analyze luciferase activity, with Renilla luciferase as the transfection control.

Statistical Analysis

All data were presented as mean ± standard deviation. SPSS 21.0 software (IBM Corp. Armonk, NY, USA) and Pearson's correlation analysis were introduced for statistical analyses. $p < 0.05$ indicated a significant difference. The significance of difference between groups or among multiple groups was analyzed by Student's *t*-test or one-way analysis (followed by Tukey's test) of variance, respectively.

Results

Circ_0012673 was Highly Expressed in Lung Cancer Tissues and Cells

Originally, RT-qPCR was implemented to check the relative expression of circ_0012673 in lung cancer tissues and neighboring non-tumor tissues, a remarkable increase of circ_0012673 level was witnessed in lung cancer tissues compared with healthy tissues (Figure 1A). Consistently, when in comparison with BEAS-2B cells, circ_0012673 was overexpressed in A549 and H23 cells (Figure 1B). Conclusively, these data implicated that circ_0012673 was highly related to lung cancer.

Absence of circ_0012673 Impaired Development of Lung Cancer

We knocked down the expression of circ_0012673 to probe the functional effect of si-circ_0012673 in lung cancer; consequently, A549 and H23 cells were introduced with si-NC or si-circ_0012673. As indicated in Figure 2A, circ_0012673 was obviously decreased in cells introduced with si-circ_0012673 in comparison with si-NC group. MTT assay confirmed that proliferation of A549 and H23 cells was both overly declined in the si-circ_0012673 group compared with the si-NC group (Figure 2B-2C). In addition, the high apoptosis rate was noticed in A549 and H23 cells transfected with si-circ_0012673 relative to si-NC group by flow cytometry analysis (Figure 2D). In consistent with the trend of MTT assay, migration and invasion of A549 and H23 cells were dramatically restrained by si-circ_0012673 (Figure 2E-2F). Western blot assay indicated that the levels of Vimentin and N-cadherin were reduced, whereas E-cadherin was reinforced in A549 and H23 cells treated with si-circ_0012673, suggesting EMT process in A549 and H23 cells was impeded by silencing circ_0012673 (Figure 2G-2H). These results further verified that circ_0012673 exerted a function of tumor promoter in lung cancer.

Circ_0012673 Adversely Regulated miR-320a Expression in Lung Cancer Cells

We attempted to figure out the relationship between miR-320a and circ_0012673. As displayed in Figure 3A, circ_0012673 possessed binding sites with miR-320a. Afterwards, the interacted relationship between miR-320a and circ_0012673 was evaluated by dual-luciferase reporter assay. A549 and H23 cells were co-transfected with WT-circ_0012673 and miR-320a declined luciferase activity; however, the luciferase activity of cells co-transfected with MUT-circ_0012673 and miR-320a has no difference compared with control group (Figure 3B-3C). Interestingly, the reduction of miR-320a could be observed in lung cancer tissues and cells when compared with non-tumor tissues and BEAS-2B cells, separately (Figure 3D-3E). Importantly, circ_0012673 was negatively correlated with miR-320a expression in lung cancer tissues (Figure 3F). The gain-of-function experiments were used to analyze the relationship between miR-320a and circ_0012673. We observed the expression level of miR-320a in A549 and H23 cells was enhanced by silencing circ_0012673; accordingly, overexpression of circ_0012673 resulted in opposite effect on miR-320a expression (Figure 3G-3H). Taken together, circ_0012673 regulated miR-320a expression in lung cancer cells with negative feedback.

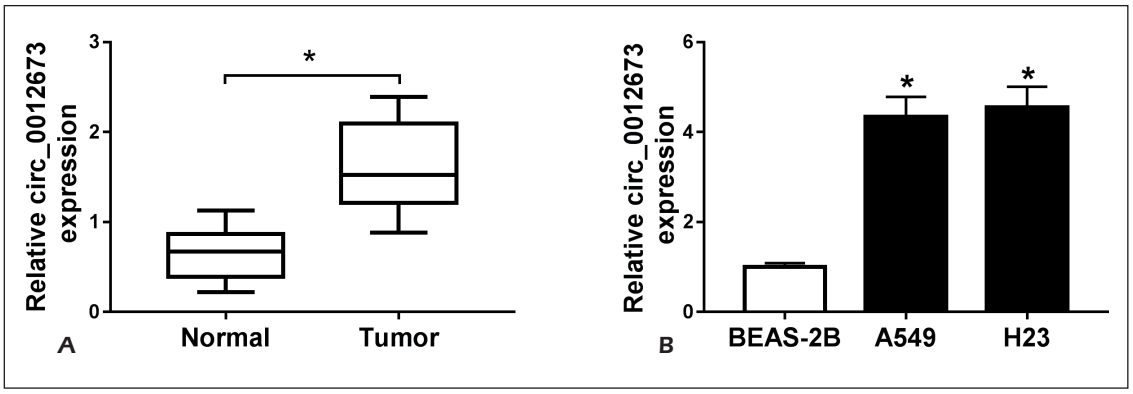

Figure 1. The expression level of circ_0012673 in lung cancer tissues and cells. (**A**-**B**) The relative abundance of circ_0012673 in lung cancer tissues and adjacent non-tumor tissues as well as in BEAS-2B, A549 and H23 cells, was analyzed with RT-qPCR. *$p<0.05$.

Knockdown of miR-320a Could Abolish the Effects of circ_0012673 Silencing on Proliferation, Apoptosis, Migration, Invasion and EMT of Lung Cancer Cells

Whether circ_0012673 mediated cell progression through regulating miR-320a expression? As shown in Figure 4A-4B, RT-qPCR revealed that knockdown of circ_0012673 increased miR-320a expression, but this action was eliminated by depletion of miR-320a. The results of MTT analysis presented that inhibition effect on cell proliferation by absence of circ_0012673 was attenuated by knockdown of miR-320a (Figure 4C-4D). Inversely, flow cytometry assay confirmed that apoptosis rate was dramatically upregulated by the transfection with

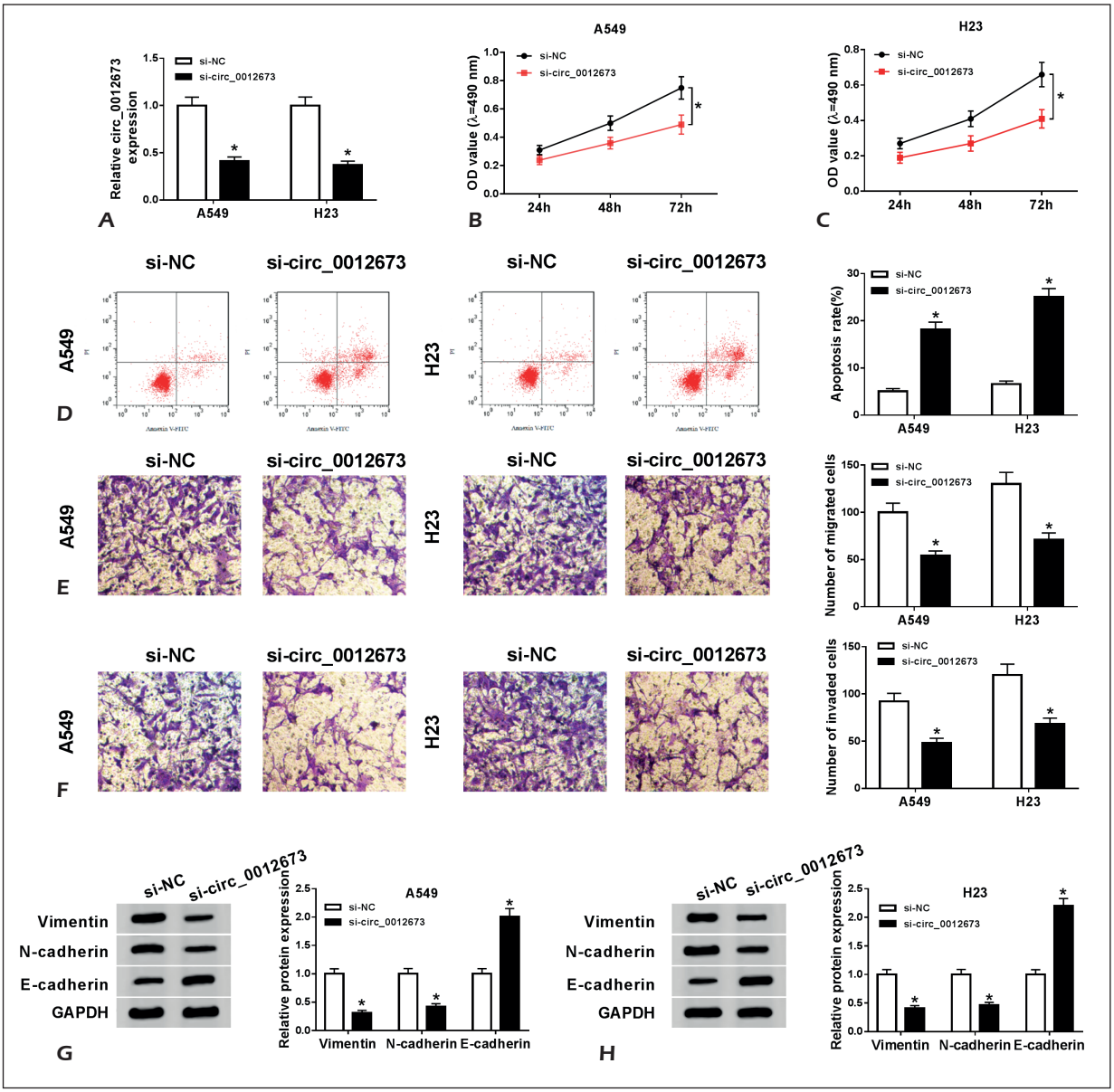

Figure 2. The influences of circ_0012673 silencing on proliferation, apoptosis, mobility and EMT of lung cancer cells. (A-H) A549 and H23 cells were introduced with si-NC or si-circ_0012673. **A**, The interference efficiency of si-circ_0012673 was measured by RT-qPCR assay in transfected A549 and H23 cells. **B**-**C**, The cell viability of A549 and H23 cells was examined with by MTT assay post-transfection. **D**, Apoptosis of treated A549 and H23 cells was determined by flow cytometry assay. **E**-**F**, Transwell assay was used to detect migration and invasion of A549 and H23 cells post-transfection (100 ×). **G**-**H**, The relative protein expression levels of Vimentin, N-cadherin and E-cadherin in transfected A549 and H23 cells were estimated with Western blot assay. *p<0.05.

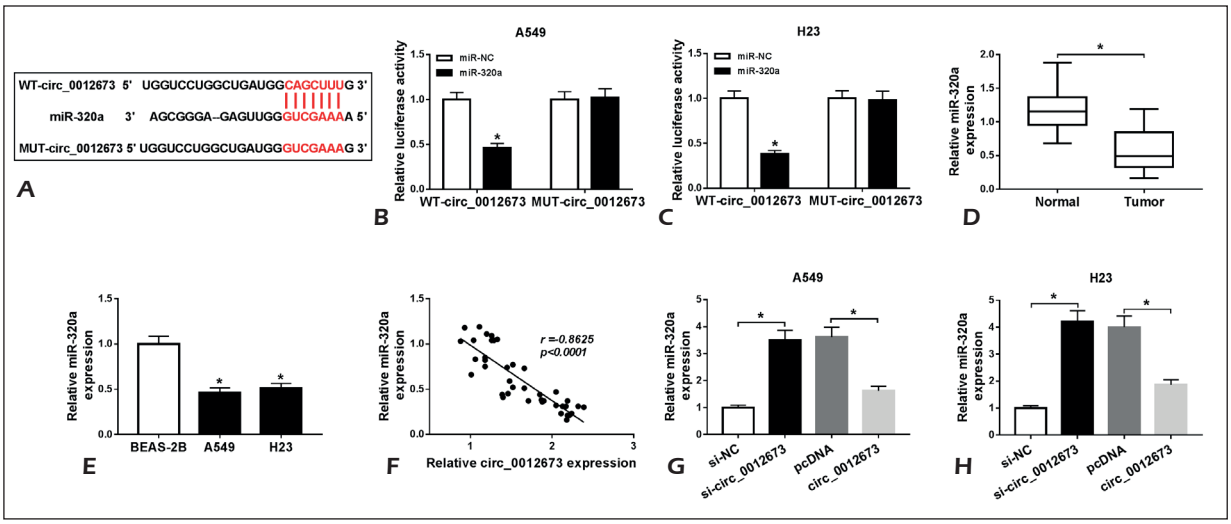

Figure 3. Circ_0012673 sponged miR-320a in lung cancer cells. **A**, Binding sequences between miR-320a and circ_0012673 was predicted by starBase. **B-C**, The interacted relationship between miR-320a and circ_0012673 was confirmed by dual-luciferase reporter assay. **D-E**, RT-qPCR was applied to assess the expression level of miR-320a in lung cancer tissues and adjacent normal tissues as well as in BEAS-2B, A549 and H23 cells. **F**, The relationship between miR-320a and circ_0012673 expression was evaluated by Pearson's correlation analysis. **G-H**, The expression level of miR-320a in A549 and H23 cells infected with si-NC, si-circ_0012673, pcDNA, or circ_0012673 was tested by RT-qPCR assay. *$p<0.05$.

si-circ_0012673, then partly resumed by the transfection of miR-320a inhibitor in A549 and H23 cells (Figure 4E-4F). The transwell assay demonstrated that the capabilities of migration and invasion were remarkably constrained with knockdown of circ_0012673; however, the migrated and invaded cells were augmented by adding miR-320a inhibitor in A549 and H23 cells (Figure 4G-4J). Finally, Western blot analysis displayed that silencing of circ_0012673 downregulated the protein levels of Vimentin and N-cadherin, meanwhile, it enhanced the protein level of E-cadherin, which could be inverted by infection with miR-320a inhibitor (Figure 4K-4L). Altogether, these data implicated that knockdown of circ_0012673 repressed proliferation, migration, invasion and EMT but induced apoptosis in lung cancer cells by regulating miR-320a.

MiR-320a Directly Interacted with LIMK1 in Lung Cancer Cells

MiR-320a possibly bound to 3'UTR of LIMK1 was found by bioinformatics online software starBase (Figure 5A). Moreover, miR-320a mimic repressed luciferase activity in A549 and H23 cells co-transfected with luciferase reporter (LIMK1 3'UTR-WT) embracing binding region sites, whereas co-transfection of LIMK1 3'UTR-MUT and miR-320a had no change on the luciferase activities (Figure 5B-5C). We also found LIMK1 was overexpressed in lung cancer tissues and cells when contrasted to neighboring normal tissues and BEAS-2B cells, respectively (Figure 5D-5G). What's more, the mRNA and protein expression levels of LIMK1 were lower in A549 and H23 cells transfected with miR-320a than that in cells transfected with miR-NC (Figure 5H-5I). Additionally, LIMK1 was negatively correlated with miR-320a expression in lung cancer tissues (Figure 5J). The results implied that LIMK1 was a target of miR-320a in lung cancer cells.

The Effects of miR-320a Overexpression on Proliferation, Apoptosis, Migration, Invasion and EMT of Lung Cancer Cells Could be Weakened by Enhancement of LIMK1

As described in Figure 6A-6D, we found that overexpression plasmid of LIMK1 reversed the reduction effects of miR-320a mimic on mRNA expression of LIMK1; parallel results of protein expression of LIMK1 in treated A549 and H23 cells was observed. In addition, the suppression effect of miR-320a mimic on cell proliferation (Figure 6E-6F), the reinforce effect of miR-320a mimic on cell apoptosis (Figure 6G-6H), and the decline effect of miR-320a mimic on migration and invasion of lung cancer cells (Figure 6I-6L), were inverted by transfecting overexpression plasmid of LIMK1. Moreover, miR-320a mimic

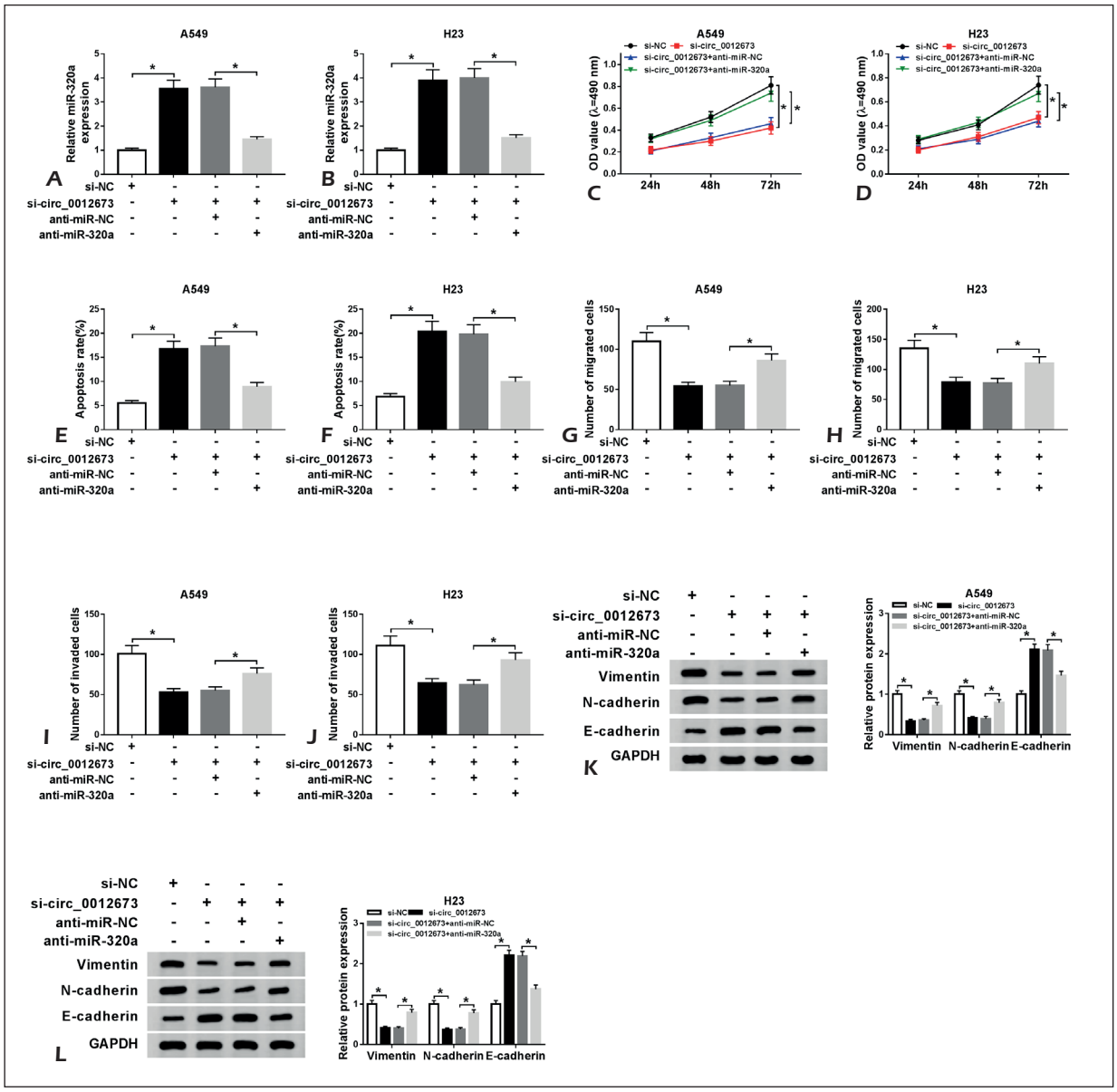

Figure 4. Knockdown of circ_0012673 mediated impacts on proliferation, apoptosis, migration, invasion and EMT of lung cancer cells could be abolished by silencing miR-320a. **A-L**, A549 and H23 cells were transfected with si-NC, si-circ_0012673, si-circ_0012673+anti-miR-NC, or si-circ_0012673+anti-miR-320a. **A-B**, RT-qPCR assay was employed to quantify the expression level of miR-320a in treated A549 and H23 cells. **C-D**, The MTT assay was performed to observe cell viability of A549 and H23 cells after transfection. **E-F**, The flow cytometry assay was conducted to monitor apoptosis of treated A549 and H23 cells. **G-J**, The migration and invasion of A549 and H23 cells were analyzed with transwell assay post-transfection. **K-L**, The Western blot assay was introduced to measure protein expression levels of Vimentin, N-cadherin and E-cadherin in transfected A549 and H23 cells. *p<0.05.

strengthened protein level of E-cadherin and attenuated the expression of N-cadherin and Vimentin to block EMT, but overexpression plasmid of LIMK1 reversed these effects (Figure 6M-6N). Collectively, the data suggested that miR-320a played important roles in proliferation, apoptosis, migration, invasion and EMT of lung cancer cells by regulating LIMK1 expression.

Circ_0012673 Regulated miR-320a/LIMK1 Axis in Lung Cancer

As presented in Figure 7A-7D, RT-qPCR and Western blot assays revealed that deficiency of miR-320a partially limited the suppression effects on mRNA and protein expression levels of LIMK1 in A549 and H23 cells caused by circ_0012673 knockdown. Considering all these

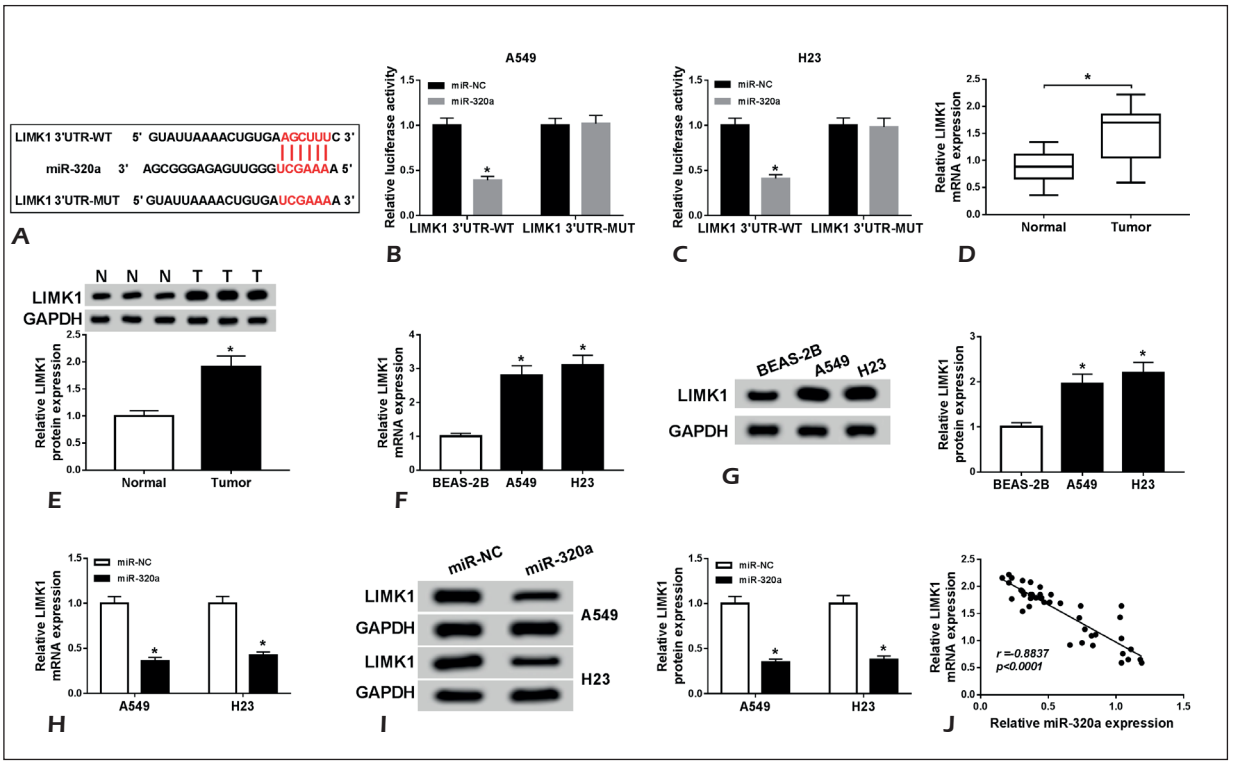

Figure 5. LIMK1 was a direct target of miR-320a in lung cancer cells. **A**, Online bioinformatics software starBase predicted there was a potential binding site between LIMK1 and miR-320a. **B-C**, Dual-luciferase reporter assay was used to verify the interaction between LIMK1 and miR-320a. **D-I**, The mRNA and protein expression levels of LIMK1 in lung cancer tissues and neighboring normal tissues, BEAS-2B, A549 and H23 cells as well as A549 and H23 cells introduced with miR-NC or miR-320a, were examined by RT-qPCR and Western blot assays, respectively. **J**, Pearson's correlation analysis was recruited to analyze the relationship between LIMK1 and miR-320a expression in lung cancer tissues. *p<0.05.

data, we concluded that circ_0012673 regulated LIMK1 expression in lung cancer cells by sponging miR-320a.

Discussion

Circ_0012673 was much more enriched in lung cancer tissues and cell lines relative to normal tissues and normal human lung epithelial cell, respectively. Simultaneously, the following loss-of-function experiment uncovered that knockdown of circ_0012673 effectively suppressed lung cancer cell growth, mobility, as well as EMT, but enhanced apoptosis, indicating circ_0012673 was a tumor promoter in lung cancer. Wang et al[13] confirmed that circ_0012673 was a molecular sponge to target miR-22 in lung adenocarcinoma by bioinformatics tools and dual-luciferase activity assay. Therefore, we wondered if there was a functional link between circ_0012673 and miR-320a in lung cancer. As we expected, the results of dual-luciferase activity assay revealed the interacted relationship between circ_0012673 and miR-320a was existed; besides, repression of miR-320a could weaken impacts of circ_0012673 silencing on proliferation, apoptosis, migration, invasion and EMT of lung cancer cells.

What's more, circRNA may have a key role in tumor via competitively binding to miRNAs, which was known as regulatory model in tumorigenesis[24,25]. Occasionally, EMT process was closely associated with cancer metastasis[26]. In addition, the loss of cell polarity and intercellular adhesion were considered as major characteristic for EMT, which was necessary to mesenchymal migratory and invasive[27]. Currently, the studies have reported that EMT participate to the development of lung cancer. Yuan et al[28] revealed that upregulation of cyclooxygenase 2 enhanced cisplatin resistance of lung cancer cells by affecting EMT pathway. Notch signal path facilitated the development of non-small cell lung cancer by strengthened EMT by affecting related-factors

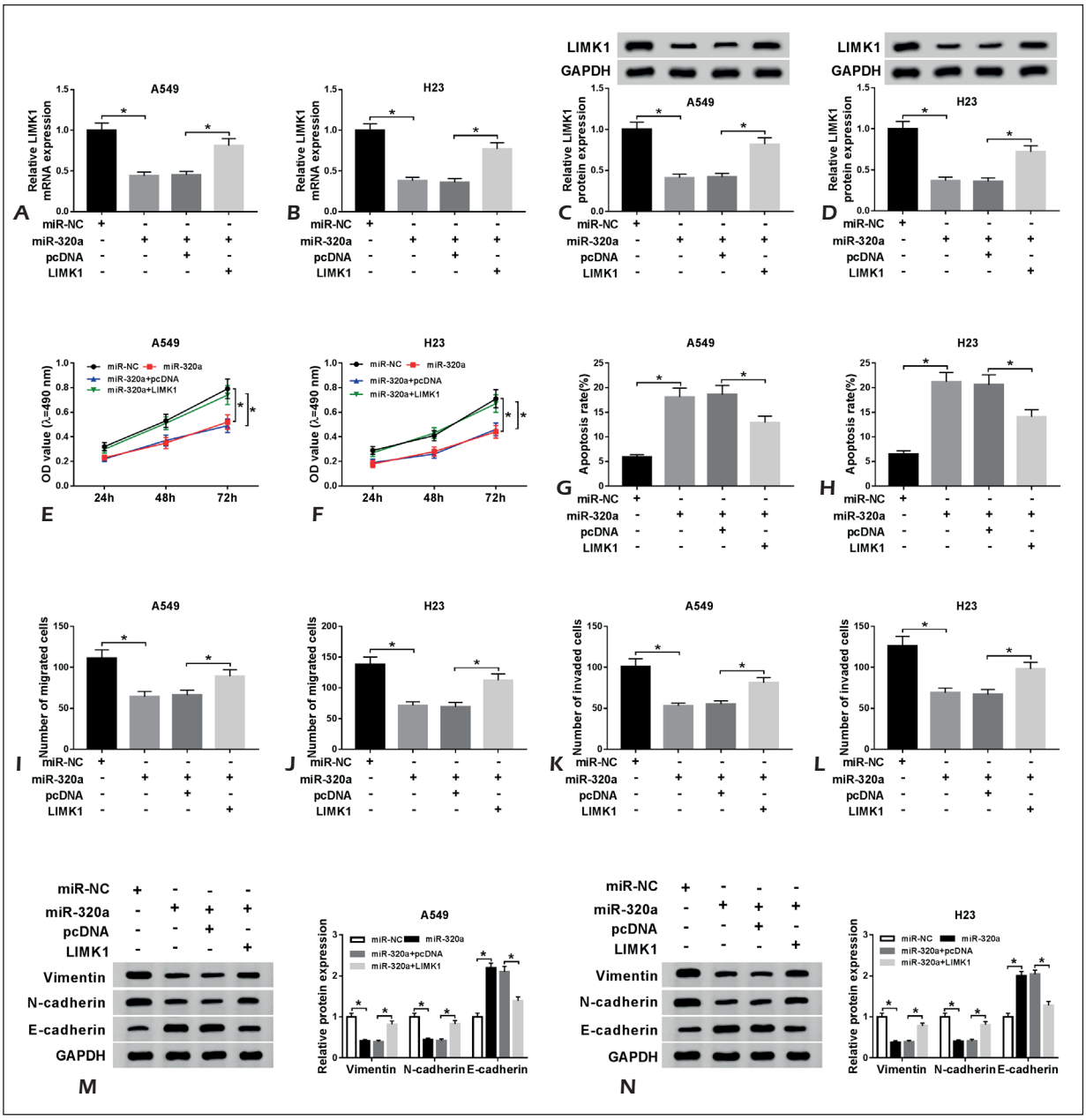

Figure 6. MiR-320a regulated proliferation, apoptosis, migration, invasion and EMT of lung cancer cells by affecting LIMK1. **A-N**, A549 and H23 cells were transfected with miR-NC, miR-320a, miR-320a+pcDNA, or miR-320a+LIMK1. **A-D**, The expression abundance of LIMK1 in treated A549 and H23 cells was examined by RT-qPCR and Western blot assays. **E-F**, The MTT assay was utilized to check cell viability of A549 and H23 cells post-transfection. **G-H**, The flow cytometry assay was exploited to calculate apoptosis rate of treated A549 and H23 cells. **I-L**, The migration and invasion abilities of A549 and H23 cells were computed with transwell assay. **M-N**, The Western blot assay was executed to assess expression levels of EMT-associated proteins in transfected A549 and H23 cells. *p<0.05.

expression. Consistently, lncRNA NBR2 impeded the process of EMT in lung cancer by mediating the Notch pathway[29]. In this paper, knockdown of circ_0012673 impeded EMT process by declining N-cadherin and Vimentin and enhancing E-cadherin expression.

Furthermore, miR-320a was reduced in lung cancer tissues and cells and was negatively correlated with circ_0012673 expression, indicating miR-320a was anti-oncogene gene in lung cancer. Noteworthy, miR-320a could target different mRNA to affect cellular behavior as cell growth,

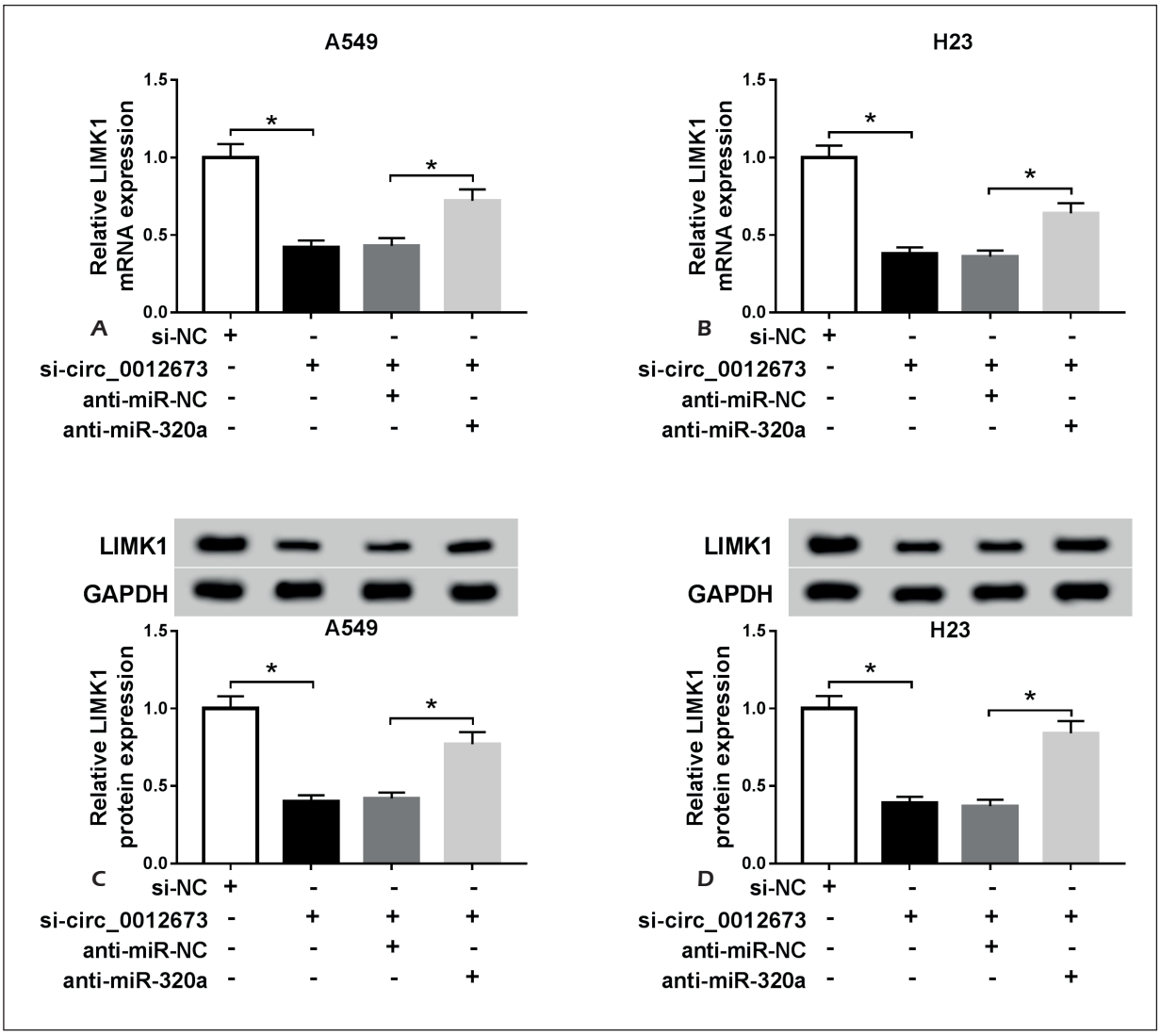

Figure 7. Circ_0012673 regulated LIMK1 expression in lung cancer cells by sponging miR-320a. **A-D**, The mRNA and protein expression levels of LIMK1 in A549 and H23 cells introduced with si-NC, si-circ_0012673, si-circ_0012673+anti-miR-NC, or si-circ_0012673+ anti-miR-320a were determined by RT-qPCR and Western blot assays, respectively. *p<0.05.

migration, and invasion in various human cancers. Evidence elucidated that B cell-specific Moloney murine leukemia virus integration site 1[30], neuropilin 1[31], and β-catenin[32] were target gene of miR-320a. Regarding different regulatory pattern of miR-320a in diverse tumors, a molecular association between miR-320a and LIMK1 in the present study was demonstrated. Using starBase database, we established that miR-320a has a potential binding site on 3'UTR of LIMK1 mRNA. In view of the results of dual-luciferase activity assay that LIMK1 was a direct target of miR-320a in lung cancer cells, we conducted functional experiments and found that miR-320a regulated proliferation, apoptosis, migration and invasion of lung cancer cells by regulating LIMK1. LIMK1 was upregulated in lung cancer and cells in current results. Furthermore, LIMK1 was negatively correlated with miR-320a in lung cancer tissues. By the way, because cell migration and invasion were connected with EMT, we further performed Western blot assay to demonstrate EMT concurrent in this study by quantizing mesenchymal characteristic marker and epithelial features marker. In the present study, the reinforced impact of LIMK1 on the mobility of lung cancer cells was confirmed, and this was identical with the results of Tan et al[33]. Conversely, the inhibition of LIMK1 expression that suppressed NSCLC cell migration was pre-

viously verified by Chen et al[34]. MiR-320a mimic resulted in considerable suppression of cell proliferation, mobility and reinforcement of apoptosis, which could be rescued after re-expression of LIMK1. Collectively, mechanism analysis experiments revealed that circ_0012673 promoted lung cancer process by acting as a sponge for miR-320a to increase LIMK1 expression and regulate growth, apoptosis, mobility and EMT of lung cancer cells.

Conclusions

These findings proved that circ_0012673 could act as miR-320a sponge to enhance LIMK1 expression to mediate proliferation, apoptosis, migration, invasion and EMT of lung cancer cells, thereby providing a promising therapeutic target for lung cancer treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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