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Original Article

miR-370 impacts the biological behavior of lung cancer cells by targeting the SMAD1 signaling pathway

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Abstract: Background: MicroRNAs (miRNAs) have been identified to play a role in the development and progression of lung cancer (LC). As of now, the expression and function of miR-370 in LC are still under investigation. Accordingly, this study explores the role and mechanism of miR-370 in LC. Methods: MiR-370 mimics or inhibitors were used to transfect A549 and NCI-H460 cells to overexpress or inhibit miR-370. The colony formation test and Cell Counting Kit-8 were conducted to detect the cell proliferation activity, and transwell test and wound healing test were conducted to evaluate the cell invasion and migration activities. In addition, the downstream target genes of miR-370 in LC were verified by dual luciferase reporter assay and western blot. Results: Compared to normal tissues and cell lineslines, the miR-370 expression in LC tissues and cells was decreased greatly. Compared to the negative control group, the up-regulation of miR-370 greatly intensified the apoptosis of NCI-H460 cells and weakened the migration, proliferation, and invasion of the cells. However, compared to the inhibitor-negative control group, the downregulation of miR-370 caused the opposite results. Additionally, SMAD family member 1 (SMAD1) was identified as a direct target of miR-370 in LC and could be inhibited by miR-370. Its overexpression restored the impact of miR-370 mimics on LC cells. Conclusion: With low expression in LC tissues and cell lineslines, miR-370 is a tumor suppressor that weakens the growth, invasion as well as migration of LC cells by inhibiting SMAD1 expression. Our results may provide novel insights for the biological treatment of LC.

Keywords: MiR-370, SMAD1, lung cancer

Introduction

Lung cancer (LC) is the leading cause for cancer associated deaths in the world [1]. According to statistics, there were over 2.2 million new cases of LC, and the death toll exceeded 1.8 million in 2020 [2]. Approximately 85% of LCs can be classified as non-small cell LC (NSCLC) which has an unfavorable prognosis [3]. Moreover, NSCLC usually has entered an advanced phase at diagnosis. During this phase, LC cells fast pass lymph or blood and affect other organs, resulting in a high mortality globally [4]. The diagnosis, surgical techniques and new treatments of LC have made progress in last several years, but LC is often accompanied by the mutation and overexpression of epithelial growth factor receptor (EGFR) [5]. A high EGFR level is able to activate downstream PI3K/AKT as well as MAPK/ERK, then accelerate the proliferation as well as metastasis of LC [6]. Accordingly, it is of value to understand the molecular pathogenesis of LC to develop novel therapeutic strategies.

MicroRNA (miR), a member of non-coding RNA, widely exists in human body [7]. MiR, approximately 19-22 nucleotides long, can change the mRNA stability as well as translation efficiency of the target gene through base pairing with 3' untranslated region (UTRs) [8]. It plays an essential role in regulating various cell functions, such as cell proliferation and migration [9]. Moreover, its imbalance can trigger the formation and metastasis of cancer [10]. Reportedly, miR is implicated in the development of various tumors and has the potential to be a therapeutic target [11]. For instance, miR-425-5p suppresses the growth of LC cells via down-regulating TFIIB-related factor 2 [12].

MiR-375 lowers the dryness of gastric cancer cells through triggering iron death [13]. MiR-370 is strongly bound up with tumor growth and metastasis, with aberrant expression in many tumors. Furthermore, miR-370-3p, a new biomarker, can accelerate the progress of breast cancer (BC) through targeting FBLN5 [14]. miR-370 in LC has been rarely reported, and only two reports described the research of miR-370 in LC [15, 16].

This study firstly found the ability of miR-370-3p to regulate SMAD family member 1 (SMAD1) and improve the growth as well as metastasis of LC cells through inhibiting SMAD1, which offers a new target for the treatment of LC.

Methods and data

Cell incubation

Human lung adenocarcinoma cell lines (A549, NCI-H460) and normal human BEAS-2B from the Cell Bank of CAS (Shanghai, China) were subjected to incubation with 10% fetal bovine serum (FBS)-contained DMEM (HyClone, Los Angeles, USA).

Cell transfection

Negative control (NC)-mimics, miR-370 mimics (miR-370-mimics), NC-inhibitor and miR-370-inhibitor were offered by GenePharma (Shanghai, China). SMAD1 plasmid (pcDNA3.1-SMAD1) and NC plasmid (pCDNA3.1-NC) were also synthesized through GenePharma. Under the manufacturer's instructions, transfection of A549 and NCI-H460 cells was conducted by Lipofectamine 2000 (Invitrogen, Shanghai, China). NC-mimics sense: UUCUCCGAACGUGUCACGUTT, antisense: ACGUGACACGUUCGGAGAATT; miR-370 mimics sense: GCCUGCUGGGGUGGAACCUGGU, antisense: CAGGUUCCACCCCAGCAGGCUU; NC-inhibitor: CAGUACUUUUGUGUAGUACAA; miR-370-3p inhibitor: ACCAGGUUCCACCCCAGCAGGC.

Colony formation test

A total of 1000 cells were transferred to a 6-well plate, followed by 6-week incubation to form colonies. The colonies were dyed with 0.1% crystal violet (Beyotime Biotechnology, Shanghai, China) in 50% methanol as well as 10% glacial acetic acid for analysis.

Cell counting kit (CCK)-8 test

Cells in a 96-well plate (1×10^3 cells/hole) were given 0, 24, 48 and 72 h incubation, respectively. Totally 10 μL CCK-8 reagent (Dojindo, Kumamoto, Japan) was put in each well at every temporal point. Cells treated by 30 h continuous incubation were determined for optical density through microplate reader (Infinite M200 PRO, TECAN, Switzerland) at 450 nm.

Cell invasion test

A Matrigel invasion kit (Corning, USA) was used for cell invasion assay. A Transwell insert was rehydrated for at least 2 h. After resuspension in serum-free medium, transfected cells (2×10^4 cells) were added to the upper compartment, and 10% FBS-contained complete medium to the lower one as chemotactic agent. After 24 h, the cells were immobilized with methanol, followed by staining with crystal violet. Then, cotton swabs were used to wipe the upper surface to remove non-invasive cells, and the invaded ones were counted under a microscope. At least 5 visual fields were selected from every compartment, and the cells were counted to evaluate cell invasion characteristics. All the measurements were independently conducted in triplicate.

Wound healing assay

In the wound healing assay, both A549 and NCI-H460 cells were transferred to a 6-well plate until they grew to 100% confluence. The wound was produced in the middle of the cell monolayer and evaluated under phase contrast microscope at a specified time point. The measurement of wound width was conducted from four locations. The relative wound width was the ratio of the final and initial actual wound widths.

RT-qPCR

The extraction of total RNA was performed using a Trizol kit (Ambion, USA) following the manufacturer's instruction. Poly (A) Tail was added to miRNA, and cDNA was obtained with a reverse transcription kit (Fermentas, Canada) according to Oligomer (DT) 18 primer and M-MLV reverse transcriptase. The relative expression of miR-370 and Smad1mRNA were quantified through RT-qPCR. Synthesis of prim-

ers was conducted by Invitrogen. The reaction conditions: 95°C for 10 min, 95°C for 10 seconds, 60°C for 20 s and 72°C for 35 s. The 2-ΔΔCt method was adopted for the calculation of relative expression [17]. The assay was carried out in triplicate. The upstream sequence of miR-370: 5'-TGGATGCAATAATCACGCATGG-3', the downstream sequence of miR-370: 5'-GCCUGCUGGGGUGGAACCUGGU-3'; The upstream sequence of SMAD1: 5'-AACCGTTTGAAGTGTAA-3', the downstream sequence of SMAD1: 5'-AAACAAACTGGGAAAGA-3'; the upstream sequence of GPADH: 5'-GGGAAACTGTGGCGTGAT-3'; the downstream sequence of GPADH: 5'-GAGTGGGTGTCGCTGTTGA-3'; the upstream sequence of U6: 5'-CTCGCTTCGGCAGCACA-3', the downstream sequence of U6: 5'-AACGCTTCACGAATTTGCGT-3'.

Western blotting (WB)

The protein was isolated through 10% SDS-PAGE, followed by transferring onto a polyvinylidene fluoride membrane (Bio-Rad, Shanghai, China). After immersion in 3% BSA in PBS containing 0.05% Tween-20, the membrane was mixed with rabbit polyclonal anti-SMAD1 antibody (1:1000; ab154658, Abcam, Shanghai, China), anti-Bax antibody (1:1000; ab32503; Abcam, Shanghai, China), anti-Bcl-2 antibody (1:1000; ab182858; Abcam, Shanghai, China), anti-SMAD1 antibody (1:1000; ab32351; Abcam, Shanghai, China) and rabbit monoclonal anti-GPADH (1:5000; ab181602; Abcam, Shanghai, China). The secondary antibody was coupled with horseradish peroxidase. Chemiluminescence was used for visualizing impressions.

Cell apoptosis assay

At 48 hours after transfection, Annexin V FITC apoptosis kit (DOJINDO) was adopted for ratio measurement of living cells and apoptotic cells in different treatment groups in accordance with the manufacturer's guidelines. With trypsin, the cells were released followed by washing in PBS. Then, the cells were treated by resuspension in Annexin V-binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and by 10-min staining with Annexin V-FITC. Subsequently, the cells were treated by another 10-min staining via propidium iodide in dark (indoor temperature). Thereafter, BD FACSCanto II (BD Biosciences) was used for analyzing the cells within 1 h. Lastly, 10,000 cells were acquired, and the data were analyzed using FlowJo 7.0 (Tree Star, San Carlos, Canada).

Dual luciferase reporter (DLR) assay

Through Targetscan (https://www.targetscan.org/vert_72/) [18] and miRDB (http://mirdb.org/) [19], the potential targets of miR-370 were forecasted. The SMAD1 3'-UTR sequence with the forecasted binding site of miR-370 or the mutated binding site was inserted into psiCHECK™-2 vector (Promega, Madison, WI, the States). A549 cells were co-treated with psiCHECK-2-SMAD1-WT or psiCHECK-2-SMAD1-MUT together with miR-370 mimics/miR-NC. After 48 h of transfection, the luciferase activity was determined through a DLR gene detection kit (Promega, Madison, WI, the States) following the corresponding protocol.

Statistical analyses

GraphPad 8 was used for graphic drawing and data processing. The inter-group comparison of data (mean ± sd) was performed by independent-samples t test, and multi-group comparison by one-way ANOVA (expressed by F). The repeated measurement variance was used for expression analysis at multiple time points (marked as F), the LSD-t test for post-hoc paired t test, and Bonferroni for post Hoc test. $P<0.05$ indicated a significant difference.

Results

miR-370 was downregulated in LC

We first analyzed the miR-370 expression in NSCLC based on the TCGA and found it was lowly expressed in NSCLC (**Figure 1A**, $P<0.05$). Then, we quantified miR-370 in A549 and NCI-H460 cells via RT-qPCR, and again found its low level in LC cells (**Figure 1B**, $P<0.001$).

miR-370 regulated the growth and metastasis of LC cells

To determine the impact of miR-370 on the function of LC cells, we transfected miR-370-mimics/NC-mimics into A549 cells, and transfected miR-370-inhibitor/NC-inhibitor into NCI-H460 cells. The transient transfection plasmid was successfully established by

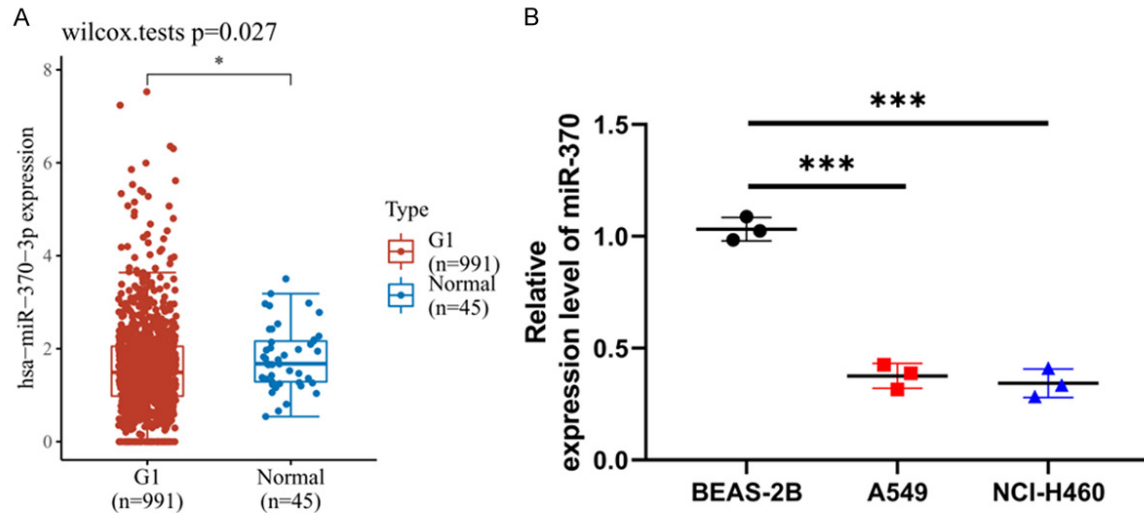

Figure 1. Expression of miR-370 in LC. A. Expression of miR-370 in LC patients. B. Expression of miR-370 in LC cells. Note: miR: microRNA; LC: Lung Cancer, ***P<0.001.

RT-qPCR, and the cell detection results showed that we successfully knocked out and overexpressed miR-370 in LC cells (**Figure 2A**, P<0.01). CCK-8 and cell colony assays revealed significantly decreased cell viability and cell colony count after miR-370-mimic transfection in contrast to those after NC-mimic transfection, and significantly increased cell viability and cell colony count after miR-370-inhibitor transfection in contrast to those after NC-inhibitor transfection (**Figure 2B**, **2C**, P<0.001). Then, Transwell test and wound healing assay revealed a significantly decreased number of invaded cells and migrated cells after miR-370-mimics transfection in contrast to those after NC-mimics transfection, and found opposite results after miR-370-inhibitor transfection (**Figure 2D**, **2E**, P<0.05). Finally, the Flow cytometry showed a significantly higher apoptosis rate after miR-370-mimics transfection than after NC-mimics transfection, and a notably lower apoptosis rate after miR-370-inhibitor transfection (**Figure 3A**, P<0.05). In addition, the WB assay showed changes of apoptotic protein after transfection (**Figure 3B**, P<0.05).

SMAD1 was found to be the target of miR-370

To further understand the function of miR-370, we forecasted its potential targets. Through Targetscan and miRDB online websites, SMAD1 was predicted to be a potential target of miR-370 (**Figure 4A**). To verify the relationship of SMAD1 with miR-370, we first analyzed the SMAD1 expression in NSCLC through online websites. According to the analysis of TCGA and GTEx, SMAD1 had high expression in NSCLC (**Figure 4B**, P<0.0001). Then, RT-qPCR and WB were adopted for the quantification of SMAD1, and SMAD1 was found to also have high expression in LC cells (**Figure 4C**, P<0.001). The DLR assay revealed notable suppression of miR-370-mimics on the fluorescence activity of SMAD1-WT (**Figure 4D**, P<0.001). Thus, SMAD1 was a binding target of miR-370. Furthermore, we found that the SMAD1 expression was inhibited in A549 cells after miR-370-mimic transfection but increased in NCI-H460 cells after miR-370-inhibitor transfection, and the WB experiment showed consistent results (**Figure 5**, P<0.05). These results revealed that SMAD1 is a target of miR-370 and is regulated by miR-370.

miR-370-mimic reversed the promoting effect of up-regulation of SMAD1 on the growth and metastasis of LC

To further understand the impact of SMAD1 on the growth of LC cells, we first constructed a stable SMAD1 overexpression (pCDNA3.1-SMAD1), and then transfected it into A549 cells (**Figure 6A**, P<0.05). The CCK-8 assay revealed notably enhanced proliferation activity of A549 cells after transfection of pcDNA3.1-SMAD1 than that after transfection of pcDNA3.1-NC (**Figure 6B**, P<0.05). Transwell test and wound healing assay revealed a signifi-

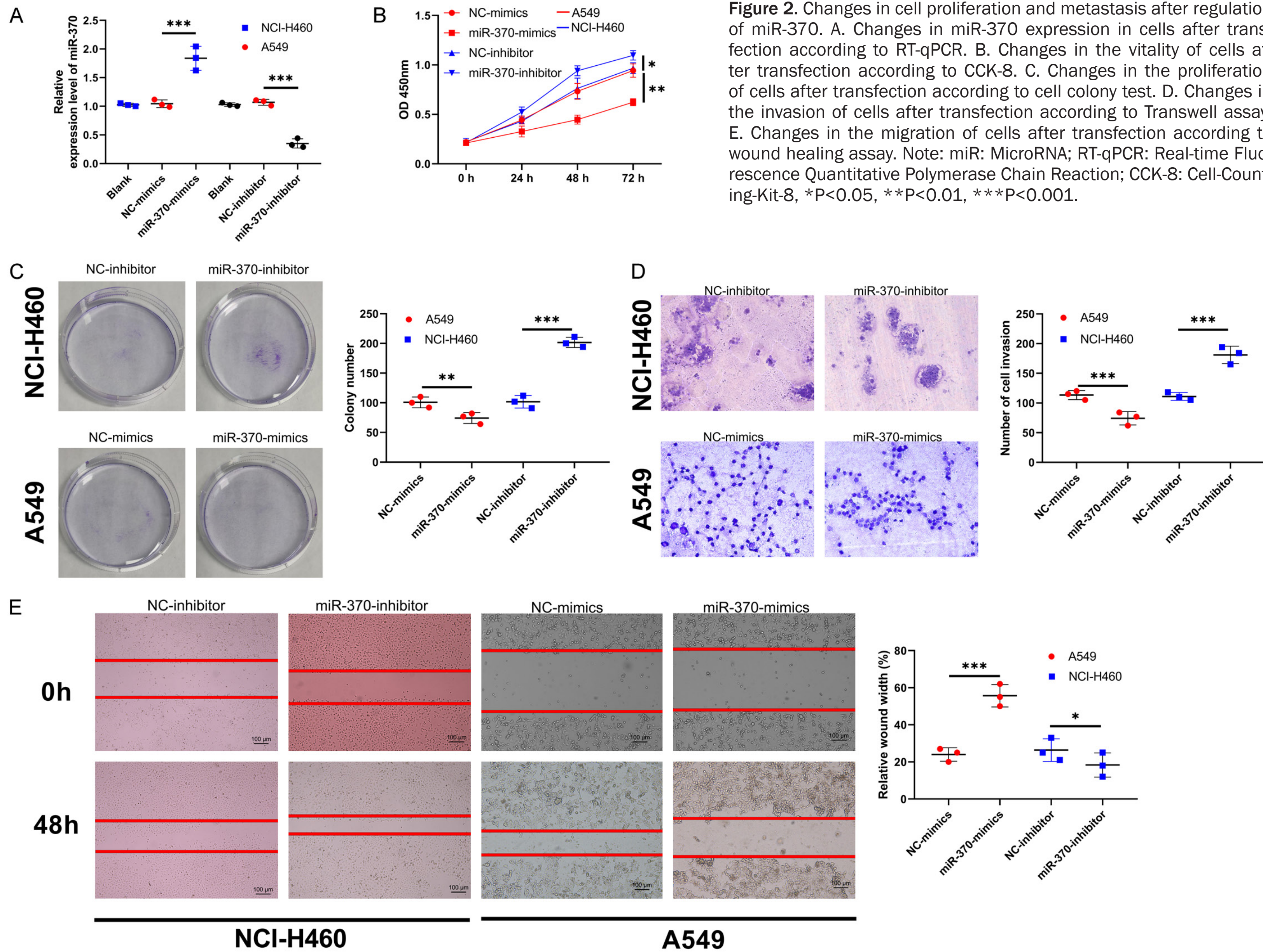

Figure 2. Changes in cell proliferation and metastasis after regulation of miR-370. A. Changes in miR-370 expression in cells after transfection according to RT-qPCR. B. Changes in the vitality of cells after transfection according to CCK-8. C. Changes in the proliferation of cells after transfection according to cell colony test. D. Changes in the invasion of cells after transfection according to Transwell assay. E. Changes in the migration of cells after transfection according to wound healing assay. Note: miR: MicroRNA; RT-qPCR: Real-time Fluorescence Quantitative Polymerase Chain Reaction; CCK-8: Cell-Counting-Kit-8, *P<0.05, **P<0.01, ***P<0.001.

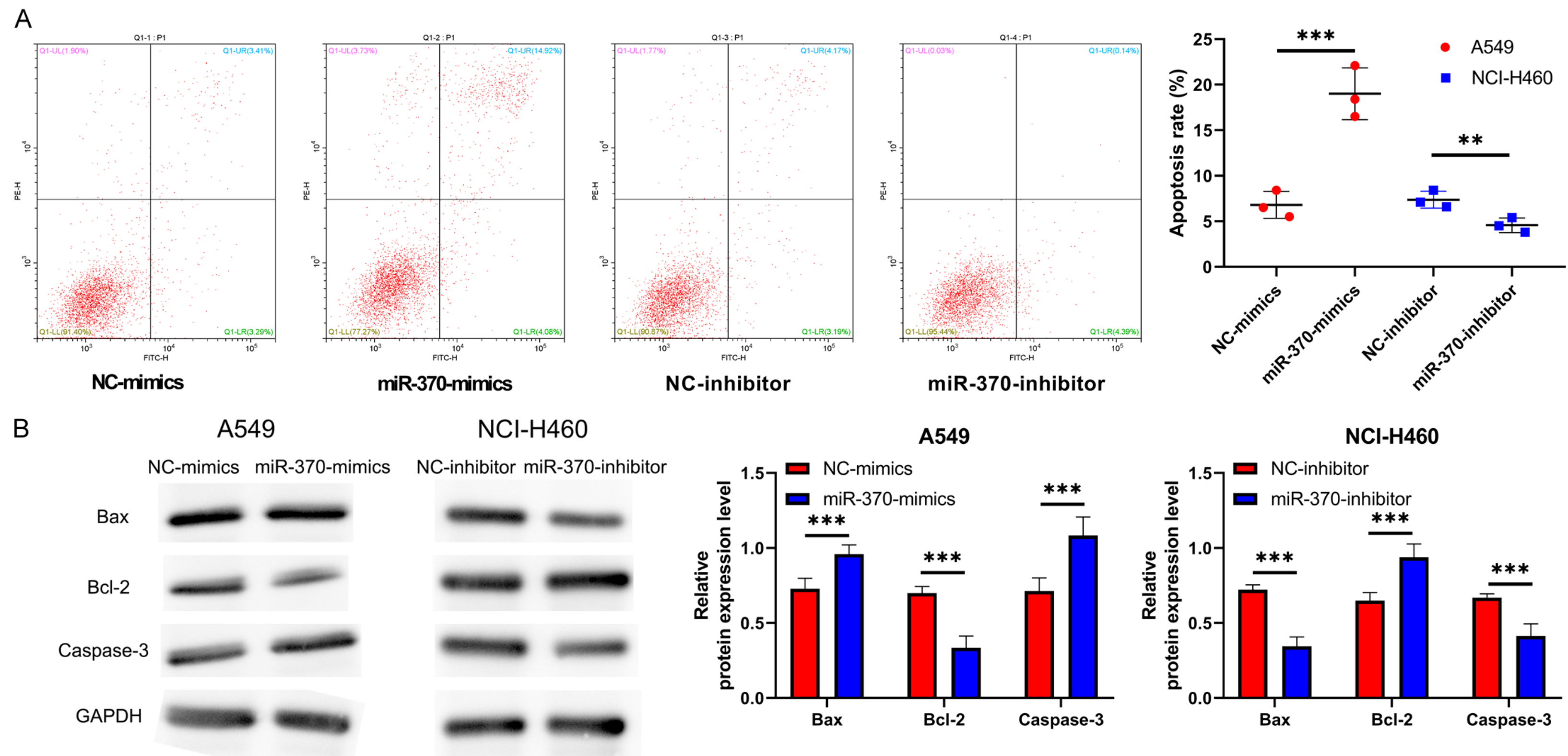

Figure 3. Changes in cell apoptosis after regulation of miR-370. A. Changes in apoptosis of cells after transfection according to Flow cytometry. B. Changes in apoptosis-associated proteins after transfection according to western blot. Note: miR: MicroRNA; WB: western blot, **P<0.01; ***P<0.001.

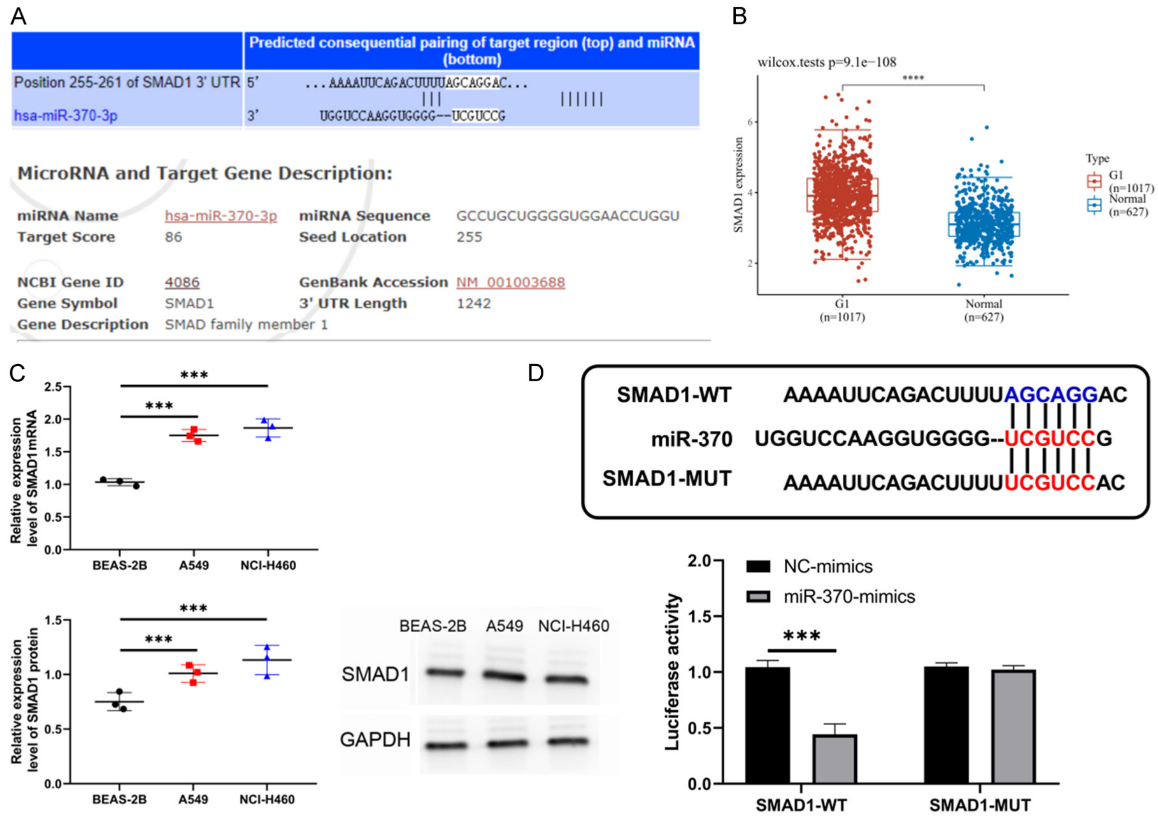

Figure 4. Targeting association between miR-370 and SMAD1. A. Prediction of targets of miR-370 by Targetscan and miRDB. B. Analysis of SMAD1 expression in NSCLC by TCGA and GTEx. C. Detection of SMAD1 expression in LC cell lines by RT-qPCR and western blot. D. Analysis of the targeting association between miR-370 and SMAD1 by DLR assay. Note: miR: MicroRNA; LC: Lung Cancer; RT-qPCR: Real-time Fluorescence Quantitative Polymerase Chain Reaction; SMAD1: SMAD Family Member 1; DLR: Dual Luciferase Reporter; WT: Wild Type; MUT: Mutant; WB: western blot, ***P<0.001, ****P<0.0001.

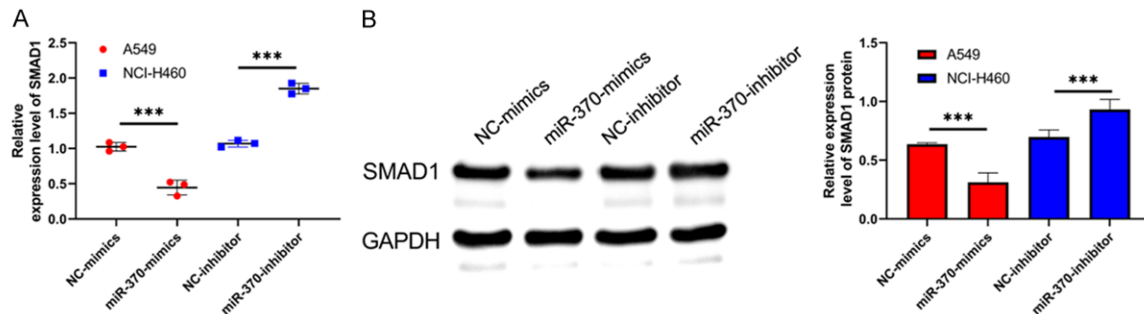

Figure 5. Verification of the regulatory association between miR-370 and SMAD1. A. Detection of SMAD1 expression in transfected cells by RT-qPCR. B. Detection of SMAD1 protein in transfected cells by WB. Note: miR: MicroRNA; RT-qPCR: Real-time Fluorescence Quantitative Polymerase Chain Reaction; SMAD1: SMAD Family Member 1; WB: western blot, ***P<0.001.

cantly increased number of invaded cells and migrated cells after transfection of pcDNA3.1-SMAD1 in contrast to those after transfection of pcDNA3.1-NC (**Figure 6C**, **6D**, P<0.05). Additionally, Flow cytometry and WB revealed significantly suppressed apoptosis of A549 cells after transfection of pcDNA3.1-SMAD1 in contrast to that after transfection of pcDNA3.1-NC (**Figure 7A**, **7B**, P<0.05). Whereas, the results were reversed after co-transfec-

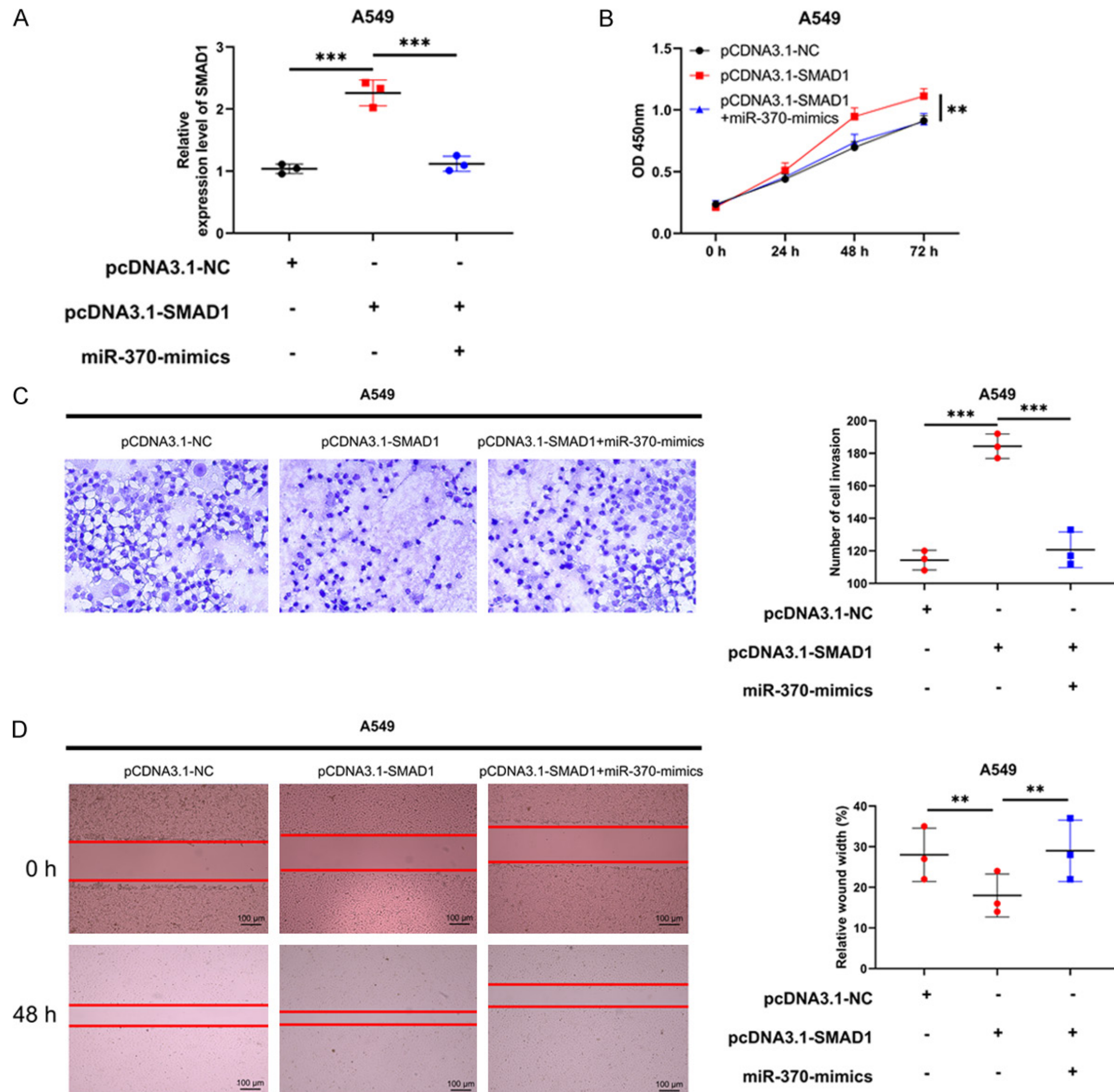

Figure 6. miR-370 regulates tumor cell growth and invasion by SMAD1. A. Relative expression of SMAD1 in transfected cells according to RT-qPCR. B. Change in cell viability after transfection according to CCK-8 assay. C. Change in cell invasion activity after transfection according to Transwell assay. D. Change in cell migration activity after transfection according to wound healing assay. Note: miR: MicroRNA; RT-qPCR: Real-time Fluorescence Quantitative Polymerase Chain Reaction; CCK-8: Cell-Counting-Kit-8; CCK-8: SMAD Family Member 1, **P<0.01, ***P<0.001.

tion of miR-370-mimics and pcDNA3.1-SMAD1. The results indicate involvement of miR-370 in the growth and metastasis of LC by SMAD1.

Discussion

Lung cancer (LC), has the highest morbidity and mortality worldwide [20]. LC is one of the primary causes of tumor-associated death, and a better treatment for it is yet to be found [21]. This study verified the low miR-370 expression in LC through experiments, and also confirmed its participation in the development and apoptosis of LC through SMAD1, which offers a theoretical basis for the development of LC treatment.

Over the past few years, with various high-throughput sequencing platforms used to analyze the expression of genome-wide miRNA genes, the aberrant expression of miRNA has been noted to be strongly bound up with tumors and can be a tumor biomarker [22]. miRNA can regulate the translation and expression of tar-

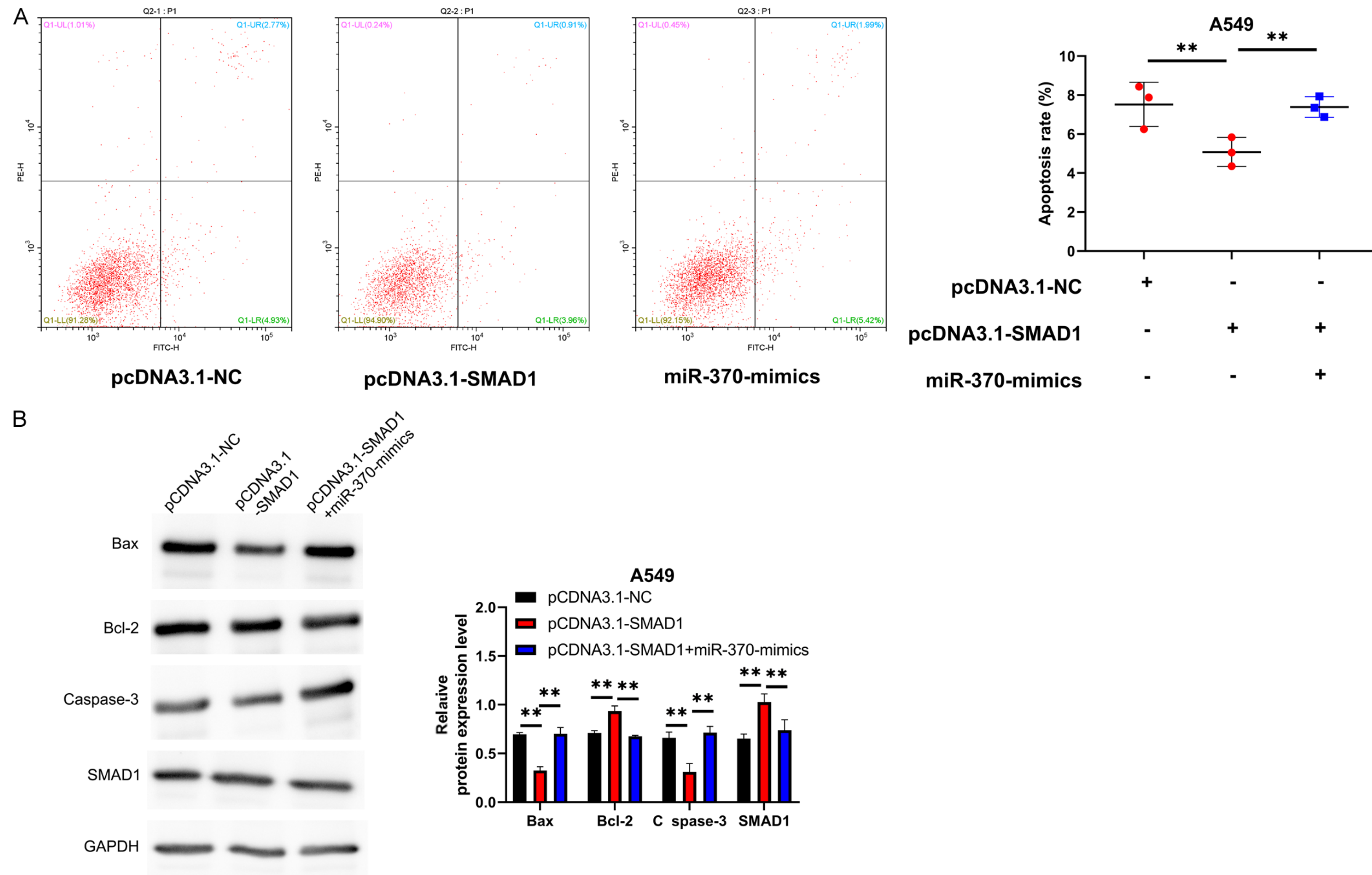

Figure 7. MiR-370 impacted tumor apoptosis through SMAD1. A. Change in apoptosis rate after transfection according to flow cytometry. B. Change in apoptosis-associated proteins after transfection according to WB. Note: miR: MicroRNA; SMAD1: SMAD Family Member 1; WB: western blot, **P<0.01.

get genes post-transcriptionally through forming an RNA-induced silencing complex and complementary binding with the 3' end noncoding region of target gene miRNA [23]. Generally, a single miRNA is able to regulate target genes, either oncogenes or tumor suppressor genes, and take part in the development as well as progression of tumors [24]. Increasing research has revealed a strong association of miR with the development of various tumors [25]. For example, miR-370 is lowly expressed in colon cancer. Up-regulating miR-370 can in a targeted manner, suppress MDM4 expression and accelerate the apoptosis of colon cancer cells [26]. According to another study, low miR-370-3p expression is bound up with the unfavorable prognosis of BC patients, and its upregulation can inhibit the metastasis of BC [27]. In addition, in the study of Sun et al., miR-370 was found to promote liver cancer cell death through Akt/Foxo3a signaling pathway [28]. The detailed mechanism of miR-370 in LC is yet to be reported. Therefore, our study analyzed the related mechanism of miR-370 in LC. We found low miR-370 expression in NSCLC samples according to TCGA and in LC cell lines, which indicated miR-370 was a tumor suppressor gene. According to prior research [29], miR-370/UQCRC2 axis can promote tumor occurrence by regulating epithelial-mesenchymal transition of gastric cancer. However, whether miR-370 has the same regulatory effect in LC is still under investigation. Accordingly, we carried out assays and found suppressed proliferation and vitality of LC cells under miR-370 upregulation. According to prior research by Peng et al. [30], KCNK15-AS1 can regulate miR-370 and inhibit the growth of LC by up-regulating miR-370, which is similar to results in the present study. Unlike in their study, we further analyzed the ability of miR-370 to cause LC metastasis. According to our study, up-regulation of miR-370 could accelerate the apoptosis of LC cells and weaken their invasion and migration, which indicated the involvement of miR-370 in the development of LC and its potential to be a therapeutic target.

SMAD protein is regarded as the central regulator of TGF-β and BMP signaling pathways. It is able to regulate cell growth as well as differentiation [31]. According to previous research [32], SMAD1 protein has high expression in LC tissues, with an impact on the incidence of LC. Additionally, according to the research by Qu et al. [33], miR-155 down-regulated SMAD1 and SMAD5 in A549 cells, and thus regulated the cyclin-dependent kinase inhibitor p21 [34]. As a crucial member of the SMAD protein family, SMAD1 can mediate the signal of bone morphogenetic protein, which is implicated in a series of biological activities, such as cell morphogenesis, apoptosis, growth, development and the immune response [35]. This study found a targeted association between SMAD1 and miR-370 and verified the ability of miR-370 in regulating SMAD1 through experiments. Later, we further revealed through rescue experiments that after up-regulating the expression of SMAD1 in LC cells, the cell growth and metastasis were strengthened, and the apoptosis rate was weakened. These results were reversed after co-transfection of miR-370-mimics and pcDNA3.1-SMAD1 into A549 cells. Qi et al. [36] found that miR-345 inhibited the proliferation, migration, and invasion of human prostate cancer through targeting Smad1. Fan et al. [37] reported that miR-26a inhibited epithelial-mesenchymal transition and invasion of head and neck cancer cells through Smad1. This indicates that multiple miRs can regulate the Smad1 gene to participate in tumorigenesis. However, this study only analyzed the mechanism of miR-370 and Smad1 in LC. Whether inhibiting miR-370 will affect the expression of other miRs needs further study.

This study has revealed the molecular mechanism of targeting SMAD1 and miR-370 in the proliferation as well as invasion of LC cells, but it still has some limitations. First, this study did not collect any clinical samples, and did not use online bioinformatics tools to analyze the association of miR-370 with LC clinical data, so the association still needs further exploration, and further research on the survival of patients is also needed. Second, this study was only conducted in vitro, and whether the same mechanism exists in animal models needs verification by in vivo experiments. Finally, although this study has confirmed the expression of SMAD1 and miR-370 in LC, whether they can be used as diagnostic markers of LC remains unclear. Therefore, we hope to perform clinical and animal assays in the future to supplement and verify the conclusions.

In conclusion, overexpressing miR-370 in LC tissues and cell lines can suppress the invasion, growth, and migration of LC cells through inhibiting SMAD1 expression.

Disclosure of conflict of interest

None.

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