ORIGINAL RESEARCH

Prognosis Value of microRNA-3677-3p in Lung Adenocarcinoma and Its Regulatory Effect on Tumor Progression

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Purpose: Lung adenocarcinomas (LUAD) was the most common subtype of lung cancer, and may result in a poor prognosis. This study was designed to explore the role of miR-3677-3p in LUAD and discuss in what way it functions in LUAD.

Materials and Methods: We used RT-qPCR method to detect the expression levels of miR-3677-3p in 105 pairs of LUAD tissues and noncancerous tissues, as also as in LUAD cells. We used χ^2 test to analyze the correlation between miR-3677-3p level and the clinical data. The prognosis significance of miR-3677-3p was inferred with Kaplan–Meier and multivariate Cox regression assays. Biological functions of LUAD cells were accessed by cell counting kit-8, transwell migration and invasion assay. The target gene of miR-3677-3p was investigated by luciferase activity assay.

Results: miR-3677-3p represented an ascendant expression in LUAD tissue specimens and cells. miR-3677-3p expression was associated with the TNM stage and with solitary metastasis. Over-expression of miR-3677-3p can shorten the overall survival period of LUAD patients when compared with low expression. Knockdown of miR-3677-3p suppressed the biology function of NSCLC cells including proliferation, migration, and invasion. KLF12 was a target gene of miR-3677-3p.

Conclusion: miR-3677-3p represents as a potential prognostic biomarker for LUAD. miR-3677-3p can promote LUAD progression by targeting *KLF12*.

Keywords: miR-3677-3p, lung adenocarcinomas, prognosis, *KLF12*

Introduction

Carcinoma of the lung is one of the major causes in cancer-related mortality. To make matters worse, incidence and mortality of lung cancer are rising.[1] In China, lung cancer ranked the first commonly diagnosed cancer in males (21.9% of total cases), and second female (13.3% of total cases) in 2018, which is expected to rise because of aging and growing population.[2] Histologically, lung cancer is classed into two main types as small cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). In these types, NSCLC accounts for 85% of all lung cancer cases, while its main subtype, lung adenocarcinoma (LUAD), comprises around 40%.[3] Despite there were improvements in outcomes of lung cancer due to recent treatment advances, the 5-year survival only improved by small account and remains largely influenced by the stage when first diagnosed.[4] Therefore, more research should be focused on screening reliable prognosis factors for LUAD.

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Cancers are characterized as complex heterogeneous diseases and are usually associated with genetic and epigenetic aberrations.[5] MicroRNAs (miRNAs), though small in length (about 19–24 nucleotides), can function as gene expression regulators in the posttranscriptional process by binding to target sequences in messenger RNA (mRNA) complementarily, thereby regulating the production of the protein.[9] Advanced technologies lead to simultaneous detection of multiple genes over some time points, and revealed the alterations of miRNA expression are the rule rather than exceptional in human cancer.[10] Because of the ability in cellular processes regulation, miRNAs were hypothesized to predict cancer prognosis, and then many of them have been verified successfully as prognostic markers that predict cancer outcome.[10] Till now, a lot of reports supported the clinical significance of miRNAs as prognostic biomarkers in various cancers. For instance, in gastric cancer, a five-miRNA signature can accurately predict the prognosis of gastric cancer.[11] Similarly, a 7-miRNA signature has been identified to be associated with survival in nivolumab-treated lung cancer patients.[12] microRNA-3677-3p (miR-3677-3p) has been investigated as an oncogene of breast cancer,[13] a useful factor to predict tumor mutation burden level in endometrial cancer,[14] and a prognostic miRNA for hepatocellular carcinoma.[15] However, the prognostic power of miR-3677-3p in LUAD has not been validated.

Kruppel Like Factor 12 (*KLF12*), a family of DNA binding transcriptional regulators, has been reported to be downregulated in lung carcinoids, especially lower in samples with positive nodal status and with necrosis.[6] *KLF12* was involved in the regulation of many cellular processes, so its dysregulation led to different roles in different neoplasm, as tumor-suppressive or -oncogenic gene.[7,8] More importantly, *KLF12* was found to act as a tumor suppressor in lung carcinoids and low expression of *KLF12* has been verified to be associated with shorter survival in lung cancer.[7] It has been reported that *KLF12* overexpression can abrogate the proangiogenic effect of miR-141 on human umbilical vein vascular endothelial cells in small cell lung cancer.[16]

The focus of this study was on the prognostic value of miR-3677-3p in LUAD and regulation in cancer cellular processes. We first determine the expression of miR-3677-3p in LUAD tissues and cells, then evaluated its potential prognostic significance based on the clinical data. In addition, we accessed the regulatory effect on tumor progression, and explored the preliminary mechanism by which miR-3677-3p regulated *KLF12*.

Materials and Methods
Tissues and Cells
This study was approved by the Ethics Committees of Liaoning Cancer Hospital and Institute, and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent before inclusion. Patients were selected from LUAD patients receiving surgical treatment at Liaoning Cancer Hospital and Institute from April 2012 to October 2015. The patients who received any type of therapy for cancer before surgery were excluded. Based on this, 105 paired tissues with adequate clinical and histological characteristics were collected in the study according to the criteria proposed by the IASLC/ATS/ERS in 2011.[17] TNM stage was determined according to the 7th edition of the American Joint Committee on Cancer (AJCC) cancer staging manual.

Four kinds of human LUAD cells, A549, HCC827, NCI-H441, and NCI-H1734, and an immortalized human bronchial epithelial cell line (BEAS-2B) were obtained from ATCC (Rockville, MD, USA) and cultured in RPMI medium 1640 (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA). All cells were maintained at the condition of 5% CO_2 and 37°C.

Cell Transfection
miR-3677-3p inhibitor (miR inhibitor), miR-3677-3p inhibitor control (inhibitor NC), the miR-3677 sequence (miR mimic) and mimic NC, siRNA negative control (si-NC), siRNA targeted to *KLF12*, pcDNA3.1 control, and pcDNA3.1-*KLF12* was synthesized by Ribobio. Co., (Guangzhou, China). Firstly, cells were seeded at 50% confluence without antibiotics one day before transfection. The transfection was conducted using Lipofectamine 3000 (Invitrogen) according to the manufacturer's direction. After transfection, the cells were incubated for 24 h or 48 h for the next step.

Dual-Luciferase Reporter Assay
3′-UTR regions from *KLF12* containing the predicted seeding sequences were used to construct the dual-luciferase reporter vectors of WT-*KLF12*, and the mutant one that lacks GGGUGCU was inserted into the pGL3.0 vector which expressed firefly luciferase as MUT-*KLF12*.

A549 cells were transfected with WT-*KLF12* or MUT-*KLF12*, together with miR inhibitor or inhibitor NCby Lipofectamine 3000 transfection reagent. 48 h later, cell lysates were harvested and then reseeded into 96-well plates. The plasmid expressing Renilla luciferase was used as the internal reference. Luciferase activity was measured with a SpectraMax iD5 Multi-Mode Microplate Reader (Molecular Devices, USA).

RNA Isolation and Real-Time Quantitative PCR (RT-qPCR) Assay

Total RNA was extracted from LUAD tissue homogenates and cultured cells by the RNeasy Mini Kit (Qiagen, China). For target *KLF12* mRNA detection, genomic DNA was removed RNA was isolated using the RNase-Free DNase Kit (Qiagen, China), and then reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad, USA) based on the manufacturer's recommendations. For miR-3667-3p detection, mirVana miRNA isolation kit (Life Technologies, USA) was used following the manufacturer's instructions. Sample cDNA was amplified and quantified in a Thermal Cycler Dice™ Real-Time System III (Takara, Japan). Levels of *KLF2* were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and levels of miR-3677-3p were normalized to U6. Data were analyzed via $2^{-\Delta\Delta Ct}$.

Cell Proliferation Assay

The proliferation rate was measured using a cell proliferation assay kit (CCK-8, Dojindo Corporation, Japan) following the manufacturer's handbook. Briefly, cells (4×10^3 cells) cultured in a 96-well plate were added with 10 μL of CCK-8 solution every 24 h in 72 h and then incubated for 2 h in a CO_2 incubator. The subsequent absorbance change was measured using a microplate reader (Molecular Devices, USA) at 450 nm.

Cell Migration and Invasion Assays

The migration and invasiveness of LUAD cancer cells were assessed by the number of cells that migrated or invaded through uncoated transwell chambers (Corning, USA) or Matrigel (Corning, USA)-coated ones. LUAD cells (1×10^5 cells/well) were seeded in the upper chambers without serum and incubated for 24 h. The lower chambers were filled with medium with 10% FBS. Cancer cells that migrated or invaded to the lower surface were fixed with 70% ethanol, stained, and five random fields of the A549 and HCC827 cells' image were counted under a light microscope (Nikon, Japan).

Western Blot Analysis

Briefly, A549 cells were homogenized in ice-cold RIPA buffer mixing with PMSF (MP Biomedicals, Solon, OH, USA). After 30 min of incubation on ice and brief sonication, protein concentrations were determined using BCA protein assay kit (Thermo Scientific, USA). The assay was performed as described previously,[18] with KLF12 primary antibody and GADPH primary antibody (as control). Immunoreactive proteins were measured using eZwest Lite Auto Western Blotting System (GenScript, Nanjing, China).

Target Gene Identification

To predict target genes regulated by miR-3677-3p, we used miRDB, miRWalk, and TargetScan methods. The predicted target genes of miR-3677-3p supported by all the three methods were subjected to literature retrieval and the first downregulated gene in LUAD was selected for further analysis.
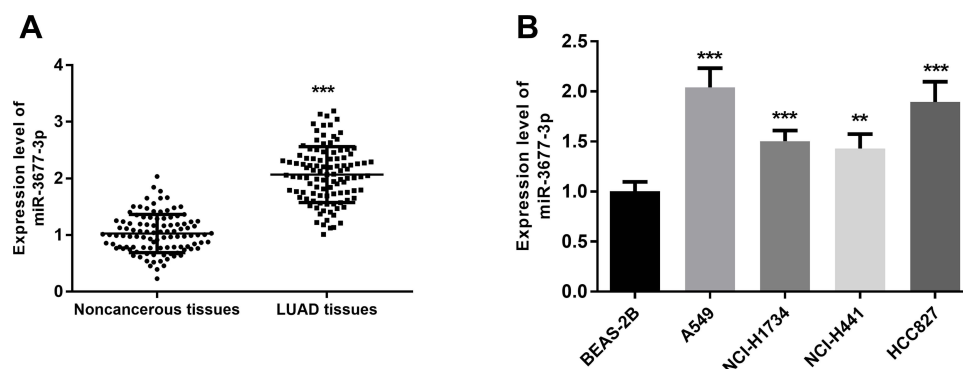

Figure 1 Expression level of miR-3677-3p in LUAD tissue samples and cells. (**A**) miR-3677-3p is upregulated in LUAD tissue samples and non-cancerous samples; (**B**) expression level of miR-3677-3p in NSCLC cell lines (A549, HCC827, NCI-H441, and NCI-H1734). **$P < 0.01$, ***$P < 0.001$.

Table 1 Correlation of the miR-3677-3p Expression with Clinical Characteristics in LUAD

Parameters	Cases (n = 105)	miR-3677-3p Expression		P
		Low (n = 48)	High (n =57)	
Age				
≤ 55	51	22	29	0.606
> 55	54	26	28	
Gender				
Female	57	24	33	0.419
Male	48	24	24	
Tumor size				
≤ 3 cm	56	30	26	0.084
> 3 cm	49	18	31	
\Smoking status				
Non-smoker	52	27	25	0.206
Smoker	53	21	32	
Differentiation				
Well, Moderate	53	25	28	0.762
Poor	52	23	29	
Lymph node metastasis				
Negative	65	36	29	0.011
Positive	40	12	28	
TNM stage				
I, II	68	36	32	0.044
III, IV	37	12	25	
Solitary metastasis				
Negative	93	46	47	0.032
Positive	12	2	10	

Statistical Analysis

Data were expressed as means ± standard deviation (SD) from at least 3 independent experiments. The statistical differences were analyzed by the one-way analysis of variance (ANOVA) or the two-tailed Student's t-tests. χ^2 test was used to compare the association between miR-3677-3p expression and clinical data of patients. Kaplan–Meier curve with Log rank test and multivariate Cox regression assay was performed to access the prognostic significance of miR-3677-3p. $P < 0.05$ indicates a statistical significance.

Results

Expression of miR-3677-3p and Correlation with Clinicopathologic Features in LUAD

miR-3677-3p expression in 105 human LUAD tissues and their adjacent noncancerous tissues was investigated by RT-qPCR. In contrast to the weak expression of miR-

3677-3p in normal tissues and cells, a high level of miR-3677-3p was detected in LUAD tissues and cells ($P<0.01$ or $P<0.001$, Figure 1). The enrolled 105 patients with LUAD were grouped into low miR-3677-3p expression (n = 48) and high miR-3677-3p expression (n = 57) according to the mean of miR-3677-3p expression value in the enrolled resected tumor tissues. Table 1 showed the association between events of poor prognosis and miR-3677-3p expression. miR-3677-3p expression was associated with TNM stage ($P = 0.044$), and with solitary metastasis ($P = 0.032$).

Multivariate Analysis and Kaplan–Meier Plotter for Prognoses of Patients with LUAD

Multivariate Cox regression was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) of the association between miR-3677-3p expression and overall survival. As shown in Table 2, the analysis revealed that

Table 2 Multivariate Cox Analysis of Clinical Characteristics Correlated to Overall Survival

Characteristics	Multivariate Analysis		
	HRs	95% CIs	P
miR-3677-3p	2.575	1.166–4.368	0.016
Age	1.325	0.724–2.424	0.361
Gender	1.395	0.768–2.534	0.274
Tumor size	1.099	0.602–2.005	0.758
Smoking status	1.324	0.711–2.467	0.376
Differentiation	1.082	0.573–2.042	0.808
Lymph node metastasis	1.867	0.961–3.627	0.066
TNM stage	2.327	1.093–4.954	0.028
Distant metastasis	3.450	1.150–10.348	0.027

Abbreviations: HRs, hazard ratios; Cis, confidence intervals; TNM, tumor, node, metastasis.

up-regulation of miR-3677-3p was a significant independent prognostic factor for LUAD patients (HR, 2.575; 95% CI, 1.166–4.368, $P = 0.016$). To predict the prognosis of patients with LUAD, a Kaplan–Meier Plotter was built to predict overall survival difference among patients with different miR-3677-3p level. The patients with high miR-3677-3p expression tended to exhibit a shorter overall survival compared with that in patients with low expression (Figure 2, Log rank test $P = 0.026$).
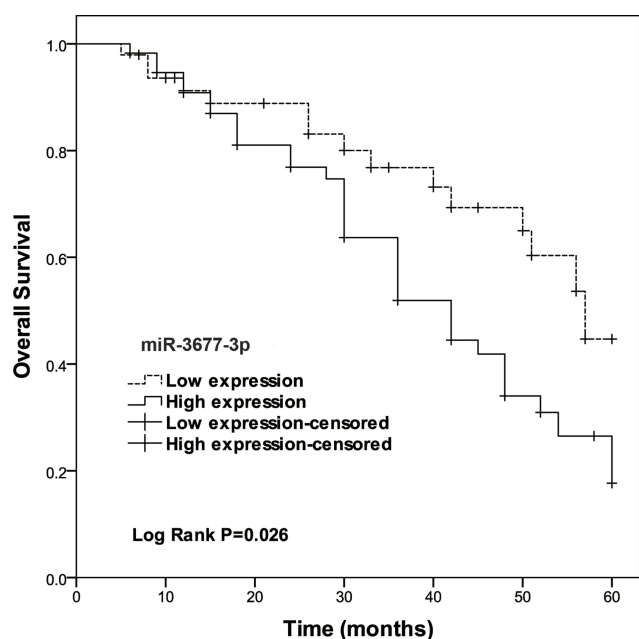
Figure 2 Comparison of 5-year survival curves of LUAD patients with high and low expression of miR-3677-3p by Kaplan–Meier plotter. (Log rank test $P= 0.026$).

miR-3677-3p Expression Influence LUAD Cell Proliferation, Migration, Invasion

The role of miR-3677-3p in LUAD cell proliferation, migration, and invasion was analyzed by transfecting A549 and HCC827 cells with miR-3677-3p inhibitor and mimics. miR-3677-3p inhibitor and mimics successfully resulted in a notable reduction or addition in the miR-3677-3p levels ($P < 0.001$; Figure 3A and C). The results of the CCK-8 assay indicated that A549 and HCC827 cell proliferation was significantly inhibited by the miR-3677-3p inhibitor and promoted by the miR-3677-3p mimics ($P < 0.01$, $P < 0.001$; Figure 3B and D). The Transwell assay evaluating the migration capability revealed that miR-3677-3p inhibitor suppressed cell migration, while mimic promotes migration ($P < 0.001$; Figure 4A and B). Similarly, the data from the transwell assay demonstrated that the invasion ability in A549 and HCC827 cells was significantly suppressed by miR-3677-3p inhibitor and increased by miR-3677-3p mimic ($P < 0.01$, $P < 0.001$; Figure 4C and D). These mentioned findings suggested that miR-3677-3p promotes the proliferation, migration, and invasion of LUAD.

miR-3677-3p Targeted the 3′-UTR of KLF12

Bio-informatics prediction suggests that *KLF12* (Gene ID: 11278) ranked first in the overlapped gene list from miRDB, miRWalk, and TargetScan. Combined with the downregulation of *KLF12* in LUAD, miR-3677-3p may target the 3′-UTR of *KLF12* in LUAD. A possible direct link between miR-3677-3p and *KLF12* 3′-UTR was examined using TargetScan (Figure 5A). The expression of *KLF12* in LUAD tissues was downregulated ($P < 0.01$ or $P < 0.001$; Figure 5B), negatively correlated with miR-3677-3p expression ($P < 0.001$; Figure 5C). Besides, *KLF12* was downregulated in LUAD cells ($P < 0.01$; Figure 5D), and silence or overexpression of miR-3677-3p can lead to an increase or reduction of KLF12 mRNA level ($P < 0.001$; Figure 5E). The luciferase activity results indicate that was transfection of the miR-3677-3p was lower after mimic transfected than in the control groups ($P < 0.001$; Figure 5F). By using Western blot analysis, significant decrease in *KLF12* was found in miR-3677-3p-overexpressed A549 cells, while increase in *KLF12* was found in miR-3677-3p-knockdown A549 cells, compared to untreated cells (Figure 5G).
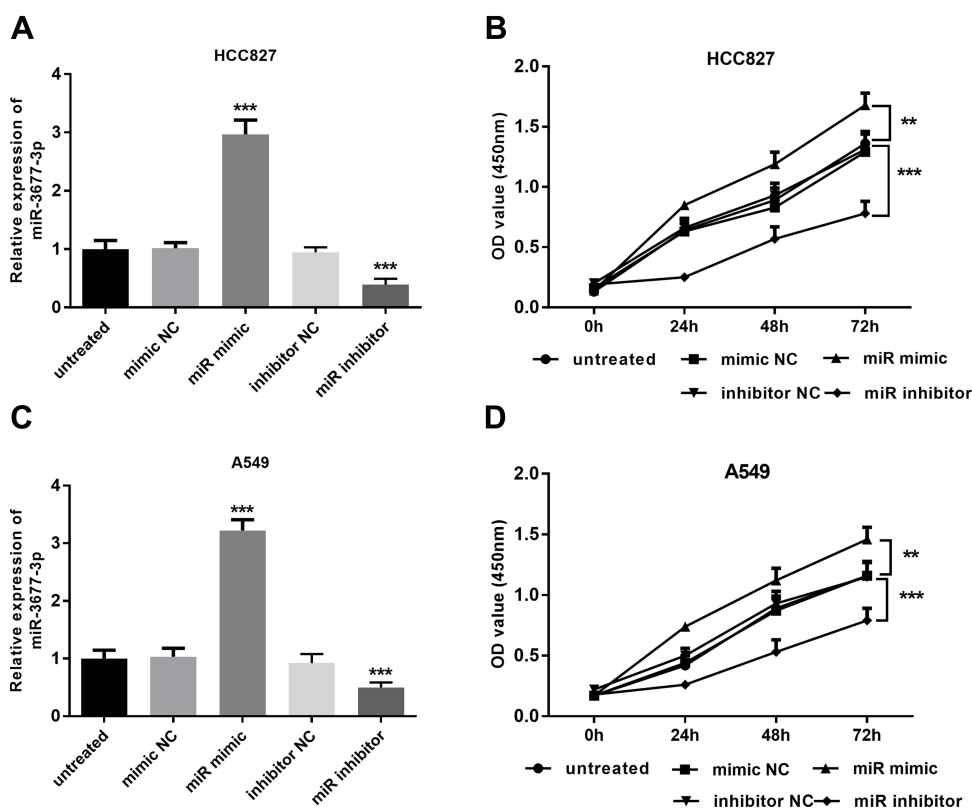
Figure 3 Proliferation of A549 and HCC827 cells were suppressed after miR-3677-3p silence and boosted by miR-3677-3p overexpression when compared with untreated cells. (**A** and **C**) Expression level of miR-3677-3p was determined by RT-qPCR; (**B** and **D**) proliferative capacity was measured by CCK-8. $^{**}P < 0.01$, $^{***}P < 0.001$.
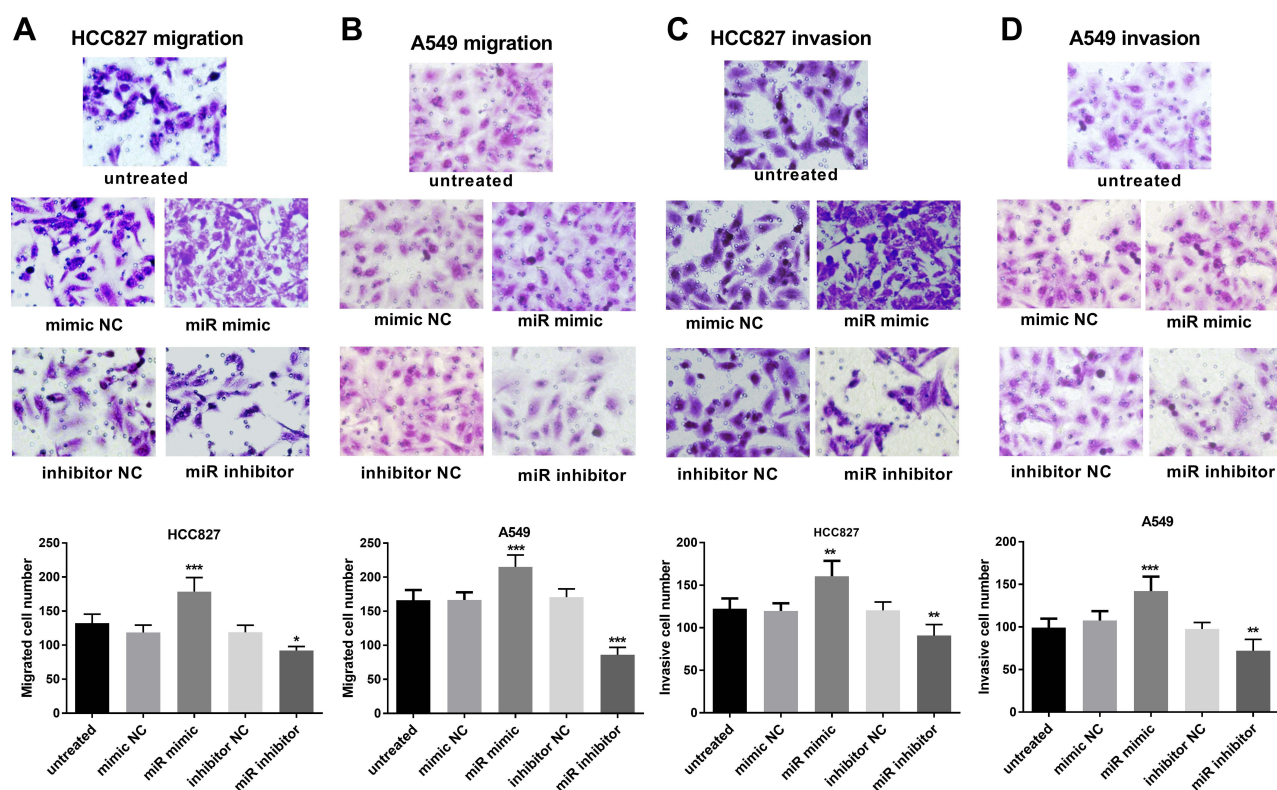

Figure 4 Migration, and invasion of A549 and HCC827 cells were suppressed after miR-3677-3p inhibition and enhanced by miR-3677-3p overexpression when compared to untreated cells. (**A** and **B**) Migratory ability was measured by transwell assay; (**C** and **D**) invasive ability was measured by modified transwell assay. $^{*}P < 0.01$, $^{**}P < 0.01$, $^{***}P < 0.001$.
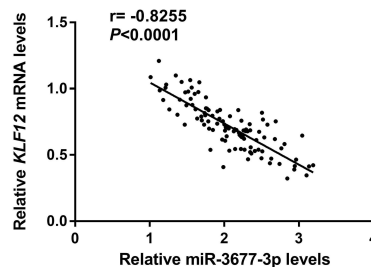
A

Position 107-113 of KLF12 3' UTR 5' ...AAAGGGCAUCACCAUCCCACGAU ...

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has-miR-3677-3p   3'   CCGGCACCGGUCUCGGGUGCUC
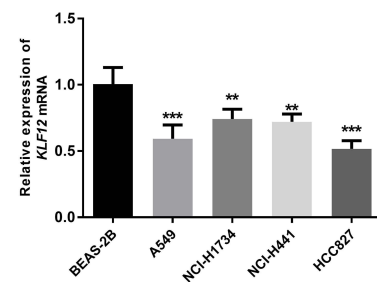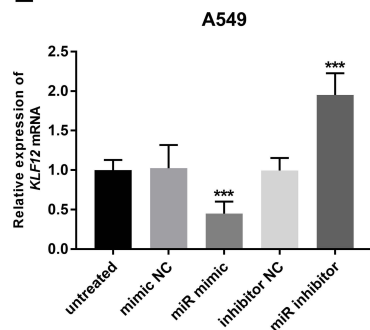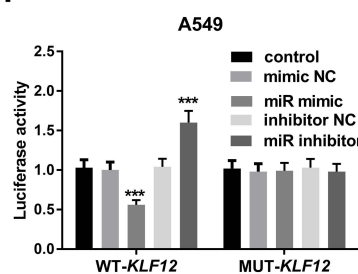MUT-KLF12   5'   AAAGGCCAUGACCUU GGGUGCUU
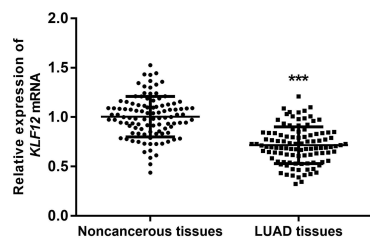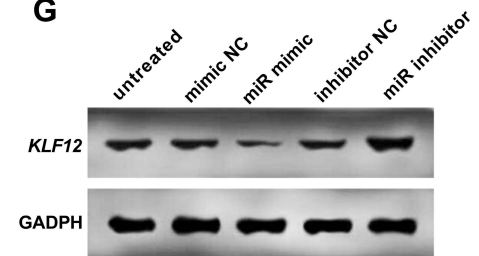

Figure 5 *KLF12* is a target of miR-3677-3p. (**A**) Predicted miR-3677-3p target sequence in the 3'-UTR of *KLF12*; (**B**) *KLF12* mRNA were decreased in LUAD tissues; (**C**) *KLF12* expression was negatively correlated with expression of miR-3677-3p. (**D**) *KLF12* mRNA were decreased in LUAD cells; (**E**) *KLF12* mRNA level was influenced by miR-3677-3p silence or overexpression. (**F**) Dual-luciferase reporter assay was performed in A549 cells. (**G**) Western blot analysis of protein levels of *KLF12* and GADPH. ****** $P < 0.01$, ******* $P < 0.001$.

These findings were further supported by rescue experiments using miR-3677-3p and siRNA of *KLF12* ($P < 0.001$; Figure 6A). Notably, inhibition of miR-3677-3p and *KLF12* can restore the decreased cell proliferation because of which miR-3677-3p inhibition ($P < 0.01$; Figure 6B). Similarly, the suppression of cell migration and invasion caused by miR-3677-3p silence ascended after *KLF12* inhibition ($P < 0.01$; Figure 6C and D).

Discussion

Recent studies have reported that miR-3677 is abnormally expressed in several malignant tumors and has a relation with cancer survival rate.[19] For instance, miR-3677-3p has a higher expression in hepatocellular carcinoma tissues than adjacent non-tumor tissues and is negatively correlated with the overall survival of patients with

hepatocellular carcinoma.[20–22] In colon cancer patients, miR-3677 also represents an elevated expression and is verified to be an independent prognostic miRNA for patients' survival based on the 5-year survival rate.[23] Our study provides evidence for miR-3677-3p upregulation in LUAD tissues and cells. Furtherly, upregulation of miR-3677-3p expression was associated with the TNM stage and with distant metastasis in LUAD patients. Multivariate Cox regression revealed miR-3677-3p can act as a significant independent prognostic factor for LUAD patients. Kaplan–Meier Plotter verified the association between miR-3677-3p expression and overall survival, presenting as LUAD patients with higher miR-3677-3p expression result in a short survival. Another study about the identification of microRNAs as prognostic markers in LUAD patients also suggested that miR-3677 can develop
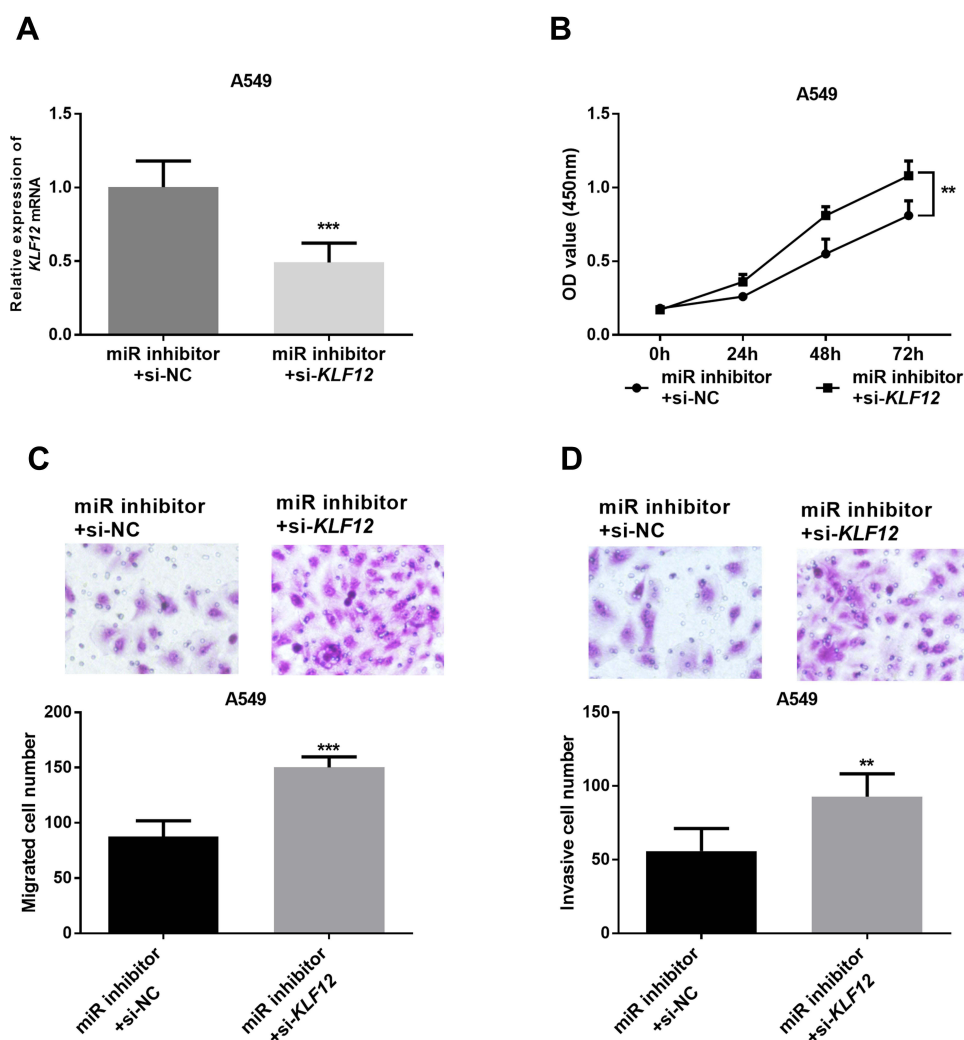
Figure 6 *KLF12* inhibition can rescue the suppression of cell proliferation, migration, invasion caused by miR-3677-3p. (**A**) RT-qPCR was performed to evaluate the expression level of *KLF12* mRNA. (**B**) Interfering *KLF12* reversed the effect of miR-3677-3p inhibitor on cell proliferation; (**C** and **D**) *KLF12* inhibition reversed the effect of miR-3677-3p inhibitor on cell migration and invasion. **$P < 0.01$, ***$P < 0.001$.

to predict prognostic markers for LUAD according to their prognostic potential CWx scores.[24] Combined with these results, miR-3677-3p has the potential to be a predictive factor for the prognosis of LUAD patients.

Cancers involve a set of essential cellular traits for tumor maintenance and malignant transformation, including sustained proliferative signaling and activation of invasion and metastasis.[25] It is reported that miR-3677 acts oncogenic activity in breast cancer, and promoting breast cancer cell proliferation, migration, and invasion by inhibiting *TLE3* expression.[26] Since we found miR-3677-3p was involved in a high-expression-related poor prognosis in LUAD patients, this indicates its oncogenesis function in LUAD. Thus, we further performed a series of experiment to study the cellular function and observed that miR-3677-3p upregulation can promote

cell proliferation, migration, and invasion of LUAD cells. By contrast, inhibiting miR-3677-3p expression via miR-3677-3p inhibitor can weaken the proliferation, migration, and invasion of LUAD cells. This influence of miR-3677-3p on cell function was also found in hepatocellular carcinoma, presenting as influence in the proliferation and cell cycle.[27] Moreover hypoxia-induced miR-3677-3p up-regulation contributed to hepatocellular carcinoma malignancy and invasiveness by mediating *SIRT5* inhibition, which could increase hepatocellular carcinoma cell proliferation, migration, and invasion in hypoxic microenvironments.[28] In this study, we found miR-3677-3p can function as a tumor promoter to benefit the proliferative capacity, migratory and invasive ability of LUAD cells, which provide clues to develop a new therapeutic target.

As mentioned above, miR-3677-3p may play considerable roles in regulating LUAD progression, formation, or metastasis.[29] But it is not known enough in the underlying molecular mechanisms. The most common mechanism of miRNAs function is binding to its target mRNA, leading to mRNA destabilization, degradation, and the resulting decrease in mRNA expression levels.[30] For example, through targeting the 3′-UTR region of GSK3β or SIRT5, miR-3677-3p could inhibit *GSK3β* or *SIRT5* expression and play a crucial role in hepatocellular carcinoma.[27,28] Another study using the bioinformatics analysis found miR-3677-3p putatively targets 3′-UTR of *SphK1* (at the position of 235–242) and promotes OS cell progression in vitro.[31] We performed the bioinformatics analysis, and found there are ten target sites between *KLF12* and miR-3677-3p at 3′-UTR. Hence we deducted that miR-3677-3p might promote LUAD progression by modulating *KLF12*. The inverse expression level of miR-3677-3p and *KLF12* primarily implied the binding, and the luciferase activity change with or without miR-3677-3p verified the direct binding relationship between miR-3677-3p and *KLF12*. Further, the rescue experiment showed *KLF12* can reverse the effect of miR-3677-3p on cell function. It has been reported that *KLF12* could act as a tumor suppressor in the human lung cancer.[7] In addition, miR-137, miR-141, and miR-200a-3p could target *KLF12* to act as tumor promoters in different cancers.[32–34] Based on these studies, miR-3677-3p may target *KLF12* and play roles in LUAD partly at least. Our study is currently the only one to verify the interaction between miR-3677-3p and *KLF12*, especially in LUAD.

Conclusion

In summary, miR-3677-3p was up-regulated in LUAD tissues and cells and was correlated with clinicopathological parameters, including TNM stage and distant metastasis. miR-3677-3p knockdown could alter the capabilities of proliferation, cell migration, and invasion in LUAD cell lines. miR-3677-3p might act as a tumor promoter in LUAD by targeting *KLF12*.

Disclosure

The authors report no conflicts of interest in this work.

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