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miR-3615 suppresses proliferation and induces apoptosis in lung adenocarcinoma cells by targetting CALML4

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Abstract

Introduction and Objective. MicroRNAs (miRNAs) are essential in the modulation of cellular activities. Studies suggest that miR-3615 may possess a tumour-suppressive function in lung adenocarcinoma (LUAD); however, its precise role and molecular mechanisms in LUAD progression remain incompletely understood. The aim of the study is to systematically investigate the biological functions and underlying mechanisms of miR-3615 in LUAD.

Materials and Method. The expression of miR-3615 in LUAD cell lines was quantified by real-time quantitative PCR (RT-qPCR). Subsequently, its functional effects on proliferation, migration, invasion, and apoptosis were evaluated in A549 and H1299 cells using the CCK-8 assay, Transwell assay, and flow cytometry, respectively. Potential target genes of miR-3615 were predicted and functionally annotated using bioinformatics methods. The binding interaction of miR-3615 with the CALML4 gene was demonstrated via a dual-luciferase reporter assay.

Results. miR-3615 expression was notably downregulated in LUAD cell lines compared with normal cells. Overexpression of miR-3615 inhibited the proliferation, migration, and invasion capabilities of LUAD cells while promoting apoptosis. Integrated bioinformatics analysis involving target prediction and functional enrichment identified CALML4 as a key candidate target gene of miR-3615. CALML4 was confirmed as a direct target of miR-3615. The effects of miR-3615 mimic on LUAD cell proliferation, migration, invasion, and apoptosis were partly attenuated by CALML4 overexpression.

Conclusions. miR-3615 exerts its functional impact on LUAD cells by directly downregulating CALML4, thereby curbing proliferation, migration, and invasion and promoting apoptosis. The elucidated miR-3615/CALML4 axis advances the theoretical foundation for studying LUAD development.

Key words

proliferation, migration, LUAD, apoptosis, invasion, miR-3615, CALML4

INTRODUCTION

Lung cancer represents the leading cause of cancer-related mortality worldwide [1]. Among all lung cancer cases, approximately 85% are classified as non-small cell lung cancer (NSCLC). The major histological subtypes of NSCLC include lung adenocarcinoma (LUAD), squamous cell carcinoma, and large cell neuroendocrine carcinoma. Of these, LUAD is the most prevalent, representing over 50% of total lung cancer diagnoses [2]. Primary treatment modalities for LUAD in current practice are surgery, chemotherapeutic agents, and radiotherapy; however, these approaches lack specificity and often cause damage to normal tissues while targeting tumours [3]. Although the discovery of oncogenic driver genes and the application of immunotherapy in recent years have advanced treatment strategies, patient prognosis continues to be poor [4]. Therefore, the identification of viable therapeutic targets remains a priority to curb disease advancement, and address the ongoing challenge of limited long-term survival. Unravelling the molecular basis of LUAD pathogenesis remains fundamental to devising targeted interventions with greater efficacy and precision.

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Non-coding RNAs, particularly microRNAs (miRNAs), represent a long-standing focus of cancer research, with their dysregulated expression playing key regulatory roles in tumourigenesis [5]. Studies have shown that miR-3615 exhibits distinct expression patterns and functions across different cancer types. In hepatocellular carcinoma, tumour tissues demonstrate upregulated miR-3615 expression. This elevated expression shows a significant association with adverse clinical outcomes, including reduced overall survival, higher TNM stage, and increased serum AFP levels, supporting its role as a marker of unfavourable prognosis [6]. Furthermore, serum levels of miR-3615 are significantly downregulated in patients with Barrett's esophagus and early-stage adenocarcinoma, demonstrating its potential as an auxiliary diagnostic marker [7]. In LUAD, existing studies have reported decreased expression of miR-3615 in tumour tissues [8], and it may possess diagnostic value for early detection [9]. How miR-3615 precisely modulates processes like proliferation, migration, invasion, and apoptosis in LUAD at the molecular level remains largely uncharacterized. Elucidating its overarching regulatory network demands systematic study.

The biological functions of miRNAs are primarily achieved through the regulation of their downstream target genes [10]. CALML4 is a cancer-associated protein, and bioinformatics analysis predicts CALML4 as one of the potential downstream

targets of miR-3615 [11]. Studies have shown that in LUAD harbouring EGFR mutations, osimertinib treatment can induce the upregulation of CALML4 expression and promote the formation of a drug-tolerant persister state via activation of the RSPH1-CALML4-GSTA1 signaling axis [12]. Therefore, CALML4 may represent a downstream mechanism through which miR-3615 exerts its regulatory functions.

This study seeks to examine the expression patterns and biological roles of miR-3615 in LUAD, and to systematically uncover the molecular mechanisms by which it modulates proliferation, migration, invasion, and apoptosis in LUAD cells. The results are anticipated to offer novel insights and potential therapeutic targets for LUAD.

Supplementary Table 1. Primer sequences used in this study

Name	Sequences (5′-3′)	
miR-3615	Forward	TCTCTCGGCTCCTCGCGG
	Reverse	GTGCAGGGTCCGAGGT
CALML4	Forward	AGAAGAAAGGTTACGTCATGGC
	Reverse	TCCAGGAAGGGTGATCTTGTG
U6	Forward	CGTTTTACTTCCTCATACAGCAC
	Reverse	GCACCAAGAGACCTGTGACA
GAPDH	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT

MATERIALS AND METHOD

Cell culture. Human normal lung epithelial cells BEAS-2B and LUAD cells A549, H1299, H1975 and H441 were purchased from the American Type Culture Collection (ATCC). All cells were cultured in DMEM complete medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2.

Cell transfection. A549 and H1299 cells were seeded in 12-well plates at a density of 1×10^4 cells per well. Transfection was performed when cell confluence reached 70 – 80%. Cells were transfected with mimic negative control, miR-3615 mimic, inhibitor negative control, miR-3615 inhibitor, overexpression negative control, or the CALML4 overexpression plasmid (oe-CALML4), all synthesized by Shanghai GenePharma Co., Ltd. Transfection was carried out using Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA). Following transfection, cells were cultured for an additional 48 hours at 37 °C for subsequent experiments.

RT-qPCR. Total RNA was purified from serum and cells by means of Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was then synthesized using a reverse transcription kit (Applied Biosystems, Foster, CA, USA). PCR amplification was conducted on the Roche PCR system (Roche, Basel, Switzerland) with corresponding primers. The PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative expression levels were calculated with the $2^{-\Delta\Delta CT}$ method, and U6 and GAPDH were employed as endogenous controls for miR-3615 and CALML4. The primer sequences are shown in Supplementary Table 1.

Cell proliferation assay. Proliferation was analyzed via CCK-8 assay. A549 and H1299 cells (1×10^4 cells/well) were cultured in 96-well plates. After 24, 48, and 72 hours, 10 μL of CCK-8 solution was added to each well, and cells were incubated for 2 hours at 37 °C. Absorbance was read at 450 nm on a microplate reader (Multiskan, Thermo, Waltham, MA, USA).

Transwell migration and invasion assays. Transwell assays were performed to evaluate cell migration and invasion. At 48 hours post-transfection, cells were harvested and resuspended in serum-free medium. Subsequently, 5×10^4 cells were added to the upper chamber of a Transwell insert. The lower chamber contained complete medium supplemented with 10% fetal bovine serum as a chemoattractant. After 24 hours of incubation, non-migratory cells on the upper membrane surface were removed with a cotton swab. Cells that had traversed the membrane were fixed, stained with 0.5% crystal violet, and quantified microscopically. For the invasion assay, the procedure was identical except that the Transwell membrane was pre-coated with Matrigel matrix prior to cell seeding.

Cell apoptosis assay. Cells were harvested after the corresponding treatments or transfections. A549 and H1299 cells were resuspended at a concentration of 5×10^5 cells/mL and stained with Annexin V-FITC (BD Biosciences) and propidium iodide, respectively, for 10 minutes. Apoptosis was then evaluated by collecting the stained cells. Annexin V-positive cells were analyzed using a FACSCalibur flow cytometer and quantified with CellQuest Software (Becton-Dickinson, San Jose, CA, USA).

Prediction and functional annotation of target genes for miR-3615. Potential target genes of miR-3615 were predicted using the miRWalk (http://mirwalk.umm.uni-heidelberg.de/), miRTarget (https://mirtarget.com/), and TargetScan (https://www.targetscan.org/vert_72/) databases. The results were illustrated in a Venn diagram, and the overlapping gene set from the intersection was selected for further investigation. This candidate target gene set was imported into the DAVID bioinformatics resource platform for functional annotation. The Gene Ontology database was utilized for functional enrichment analysis of the gene set, and the Kyoto Encyclopedia of Genes and Genomes database was employed for signalling pathway analysis to predict highly annotated GO categories and significantly enriched regulatory pathways. Additionally, the candidate target genes were submitted to the STRING database to perform a protein-protein interaction (PPI) network analysis.

Dual luciferase reporter assay. A549 and H1299 cells were plated into 24-well plates 24 hours before transfection. Reporter plasmids containing either the wild-type or mutated putative target 3′UTR sequence were supplied by Genomeditech (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was employed to co-transfect the cells with the indicated plasmids together with miR-3615 mimics, inhibitors, or their corresponding negative controls. After transfection for 48 hours, the luciferase activity was determined by the dual luciferase reporter assay system (Promega, Madison, WI, USA).

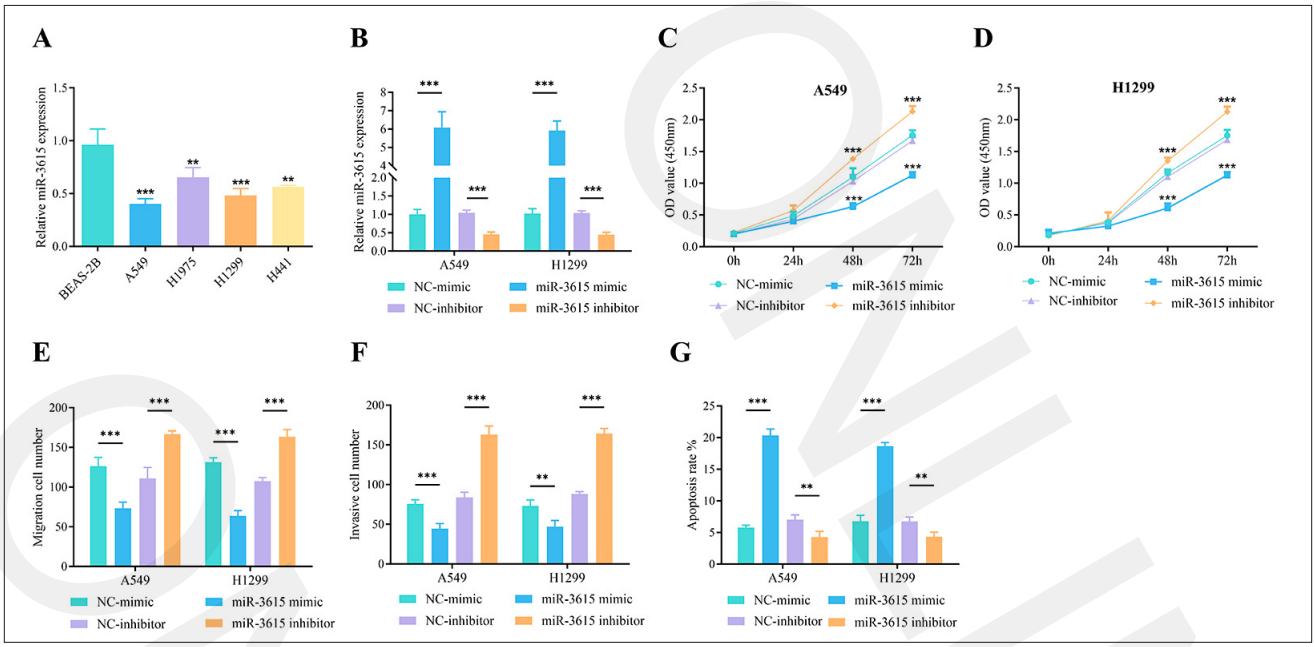

Figure 1. miR-3615 is downregulated and exerts tumour-suppressive functions in LUAD. (A) Expression levels of miR-3615 in LUAD cell lines (H441, H1299, H1975, A549), and normal human bronchial epithelial cell line BEAS-2B were determined by RT-qPCR. (B) Transfection efficiency of the miR-3615 mimic and inhibitor in A549 and H1299 cells was verified by RT-qPCR. (C, D) Effects of the miR-3615 mimic or inhibitor on the proliferation of A549 (C) and H1299 (D) cells assessed using the CCK-8 assay. (E, F) Effects of the miR-3615 mimic or inhibitor on the migration (E) and invasion (F) capabilities of A549 and H1299 cells evaluated by Transwell assays. (G) The impact of the miR-3615 mimic or inhibitor on apoptosis in A549 and H1299 cells was analyzed by flow cytometry. All the experiments were independently repeated three times (n=3). $^{**}P < 0.01$, $^{***}P < 0.001$

Statistical analyses. Data are expressed as mean ± SD. Differences between two groups were analyzed with the independent samples t-test. For comparisons involving multiple groups, one-way ANOVA with Tukey's *post-hoc* test was applied. Prior to ANOVA, the normality of data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated by Levene's test. All data met the assumptions of normal distribution and equal variances Statistical analyses were carried out using SPSS 24.0 and GraphPad Prism 9.0.

RESULTS

miR-3615 is downregulated in LUAD and inhibits cell proliferation, migration and invasion, while inducing apoptosis. RT-qPCR was performed to determine miR-3615 expression in normal BEAS-2B cells and multiple LUAD cell lines (A549, H1975, H1299, H441), aiming to clarify its biological function in LUAD. The expression of miR-3615 was significantly suppressed in all LUAD cell lines relative to BEAS-2B controls, an effect that was most prominent in A549 and H1299 cells **(Fig. 1A)**. Consequently, A549 and H1299 cells were used in subsequent experiments. To determine the function of miR-3615, these cells were transfected with miR-3615 mimic, inhibitor, or corresponding negative controls. RT-qPCR results confirmed successful overexpression and knockdown, with the mimic notably elevating and the inhibitor reducing miR-3615 levels **(Fig. 1B).** Functional analyses revealed that upregulation of miR-3615 markedly suppressed cellular proliferation **(Fig. 1C, D),** migration **(Fig. 1E),** and invasion **(Fig. 1F)** of A549 and H1299 cells, and promoted apoptosis **(Fig. 1G).** Conversely, inhibition of miR-3615 enhanced cell proliferation, migration, and invasion, while suppressing apoptosis **(Fig. 1C–G).** The data suggest a tumour-suppressive activity of miR-3615 in the context of LUAD.

Bioinformatics analysis of miR-3615. To explore the molecular mechanisms through which miR-3615 achieves its regulatory outcomes, three online databases were employed to predict its potential target genes. miRWalk predicted 2,983 targets, TargetScan predicted 1,096, and miRTarget predicted 443. The intersection of these predictions yielded 90 candidate target genes for further analysis **(Fig. 2A)**. GO enrichment analysis of the candidate genes revealed that their encoded proteins are mainly associated with molecular functions, including protein binding (e.g., to MDM2/MDM4 family proteins) and enzymatic activity **(Fig. 2B)**. At the cellular component level, these proteins were predominantly localized to the nucleus and perinuclear region **(Fig. 2C)**. With respect to biological processes, they were mainly associated with transcriptional regulation, metabolic reprogramming (particularly lipid metabolism), stress response, proliferation-related signaling pathways (including the JNK pathway), and developmental regulation **(Fig. 2D)**. KEGG pathway analysis further indicated significant enrichment of these genes in pathways including the oxytocin signalling pathway, glioma pathway, gastric acid secretion pathway, phosphatidylinositol signalling system, and aldosterone synthesis and secretion **(Fig. 2E)**. Subsequently, a PPI network was built via the STRING database for the 90 candidate genes, resulting in a network of 90 nodes and 90 edges **(Fig. 2F)**.

Based on the integrated bioinformatics analysis, several candidate genes were selected, and their expression changes upon miR-3615 modulation were examined. RT-qPCR results demonstrated that only CALML4 expression was significantly regulated by miR-3615 **(Fig. 2G)**. Therefore, CALML4 was identified as the target gene for subsequent investigation.

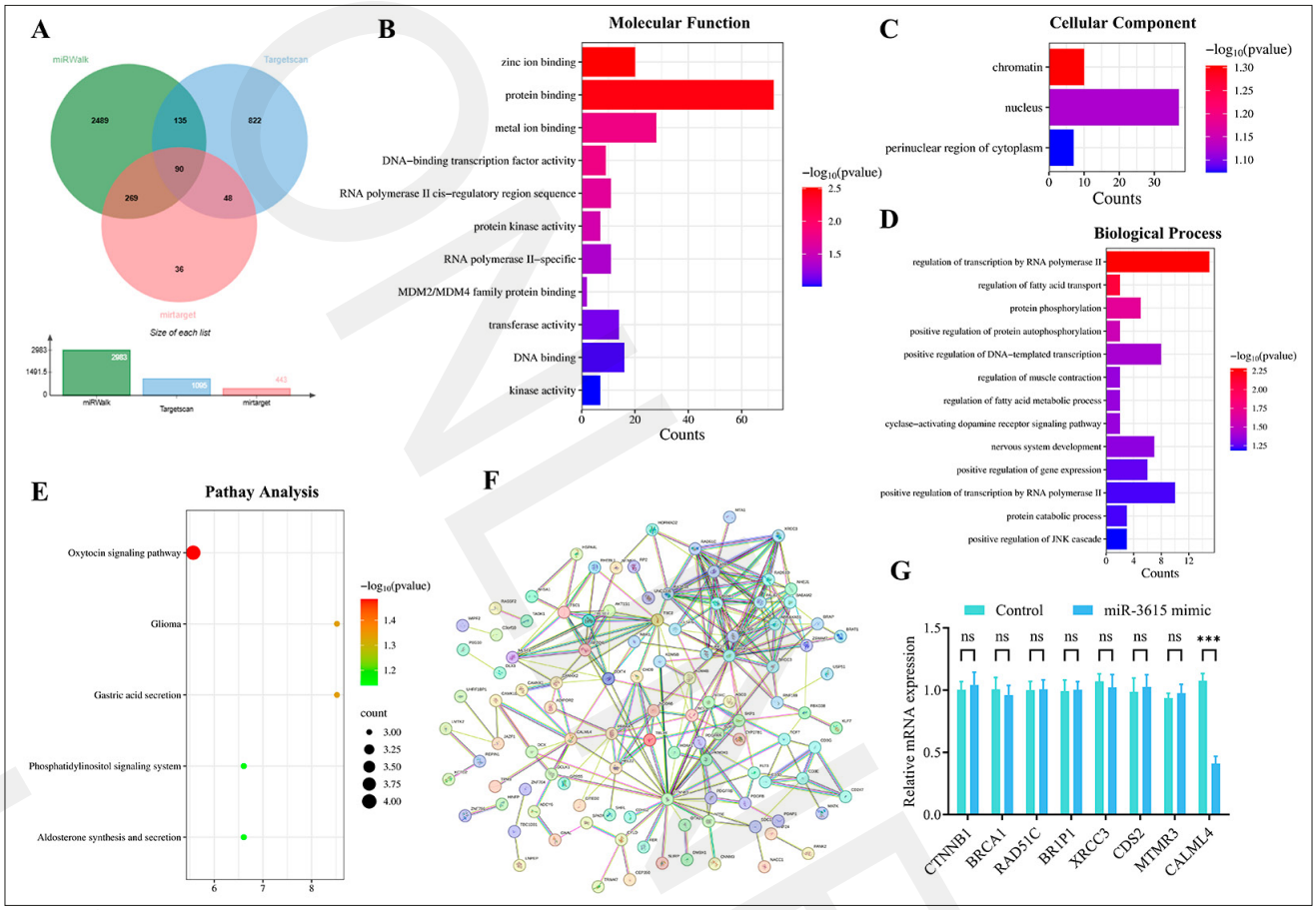

Figure 2. Bioinformatics analysis of potential target genes of miR-3615. (A) Venn diagram of potential target genes of miR-3615 predicted using 3 databases: miRWalk, TargetScan, and miRTarget. **(**B-D) GO enrichment analysis showing significantly enriched molecular function (B), cellular component (C) and biological processe (D) of the target genes. **(**E) KEGG pathway enrichment analysis. **(**F) Protein-protein interaction (PPI) network of the target genes. **(**G) Changes in mRNA levels of candidate target genes after miR-3615 overexpression were detected by RT-qPCR. All the experiments were independently repeated 3 times (n=3).
$^{***}P < 0.001$

miR-3615 Directly Targets CALML4. To verify whether CALML4 is directly regulated by miR-3615, its expression level in LUAD cells was first examined. RT-qPCR analysis revealed a marked upregulation of CALML4 in various LUAD cell lines relative to normal BEAS-2B cells, with the most substantial increase observed in A549 and H1299 cells **(Fig. 3A)**. Functional analyses conducted in A549 and H1299 cells further revealed that overexpression of miR-3615 (miR-3615 mimic) significantly reduced CALML4 expression, while inhibition of miR-3615 (miR-3615 inhibitor) upregulated its expression **(Fig. 3B)**. Bioinformatics analysis indicated the presence of a conserved sequence complementary to miR-3615 within the 3'UTR region of CALML4 **(Fig. 3C)**. The dual-luciferase reporter assay showed that luciferase activity driven by the CALML4-WT vector was significantly suppressed upon transfection with the miR-3615 mimic, while the miR-3615 inhibitor markedly elevated it, but had no significant effect on the CALML4-MUT vector, in both A549 and H1299 cells **(Fig. 3D, E)**. These data establish that miR-3615 directly targets CALML4 and downregulates its expression through specific binding to the 3'UTR region.

CALML4 mediates the effects of miR-3615 on LUAD cells. To confirm that the regulatory effects of miR-3615 on LUAD cell proliferation, migration, invasion, and apoptosis are mediated through CALML4, rescue experiments were conducted in A549 and H1299 cells. RT-qPCR analysis demonstrated that overexpression of CALML4 effectively reversed the downregulation of CALML4 expression induced by miR-3615 overexpression **(Fig. 4A)**. At the functional level, CCK-8 assays indicated that co-expression of CALML4 rescued the proliferation inhibition induced by miR-3615 overexpression **(Fig. 4B, C)**. Transwell assays further indicated that CALML4 overexpression also rescued the impaired cell migration and invasion resulting from miR-3615 upregulation **(Fig. 4D, E)**. Flow cytometric analysis of apoptosis revealed that the increased apoptosis induced by miR-3615 overexpression was similarly counteracted by CALML4 overexpression **(Fig. 4F)**. Collectively, the data show that CALML4 upregulation counteracts miR-3615-driven modulation of LUAD cell malignancy and apoptosis, confirming that miR-3615 acts mainly via direct targeting and downregulation of CALML4.

DISCUSSION

Lung cancer is still among the foremost causes of cancer mortality across the globe. Its fatality rate continues to be significant, despite developments in treatment options and genetic research [1]. Given the incomplete understanding of the molecular mechanisms in lung cancer types like LUAD, further elucidation of its pathogenesis and the identification of useful biomarkers are urgently required. Acting as key

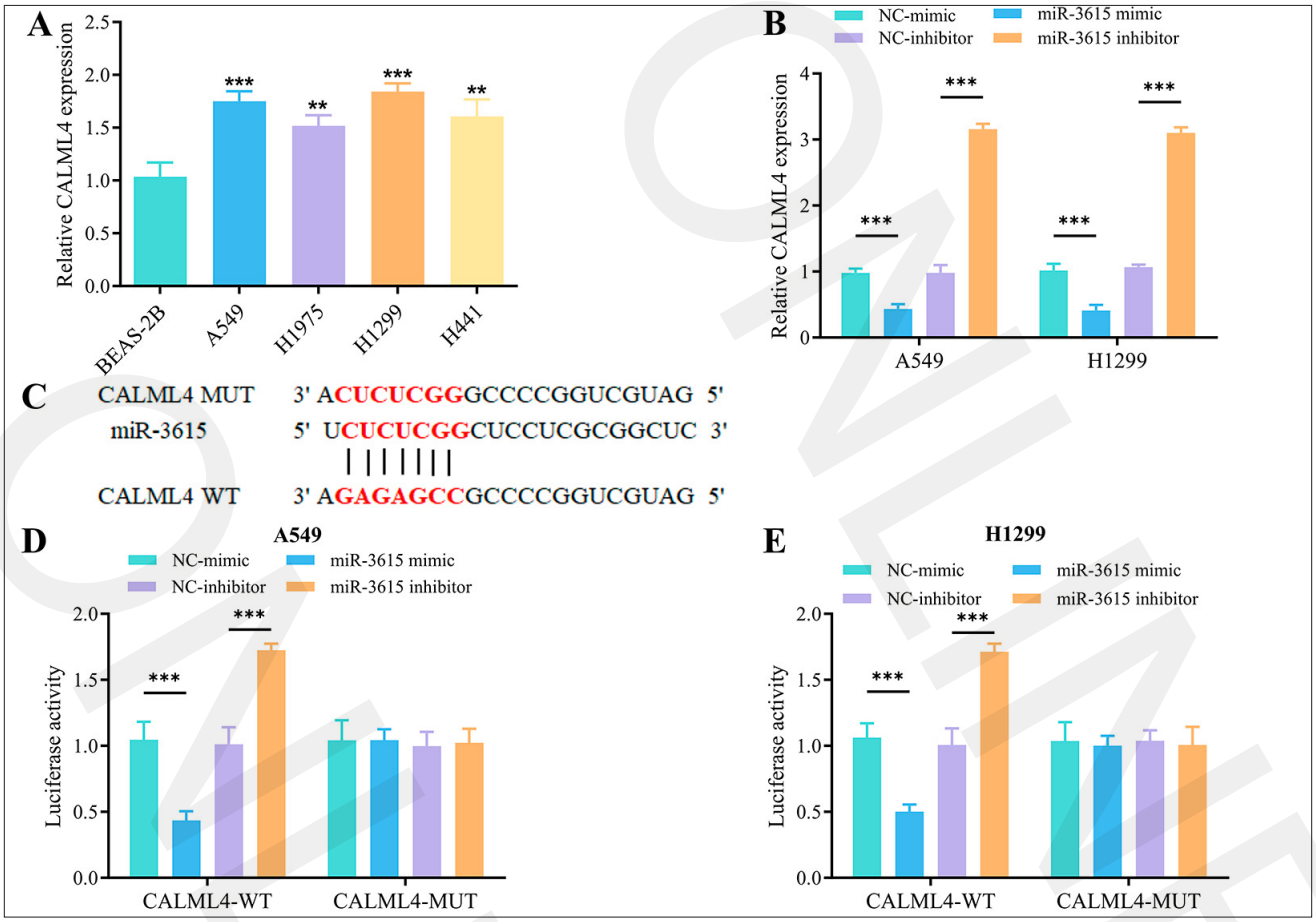

Figure 3. miR-3615 directly targets CALML4. (A) Expression levels of CALML4 in LUAD cell lines and BEAS-2B cells were determined by RT-qPCR. (B) Changes in CALML4 mRNA levels in A549 and H1299 cells following transfection with the miR-3615 mimic or inhibitor were detected by RT-qPCR. (C) Predicted binding site sequence for miR-3615 within the 3'UTR region of CALML4. (D, E) Direct targeting of CALML4 by miR-3615 was validated by dual-luciferase reporter assays in A549 (D) and H1299 (E) cells. All the experiments were independently repeated 3 times (n=3).
$^{**}P < 0.01$, $^{***}P < 0.001$

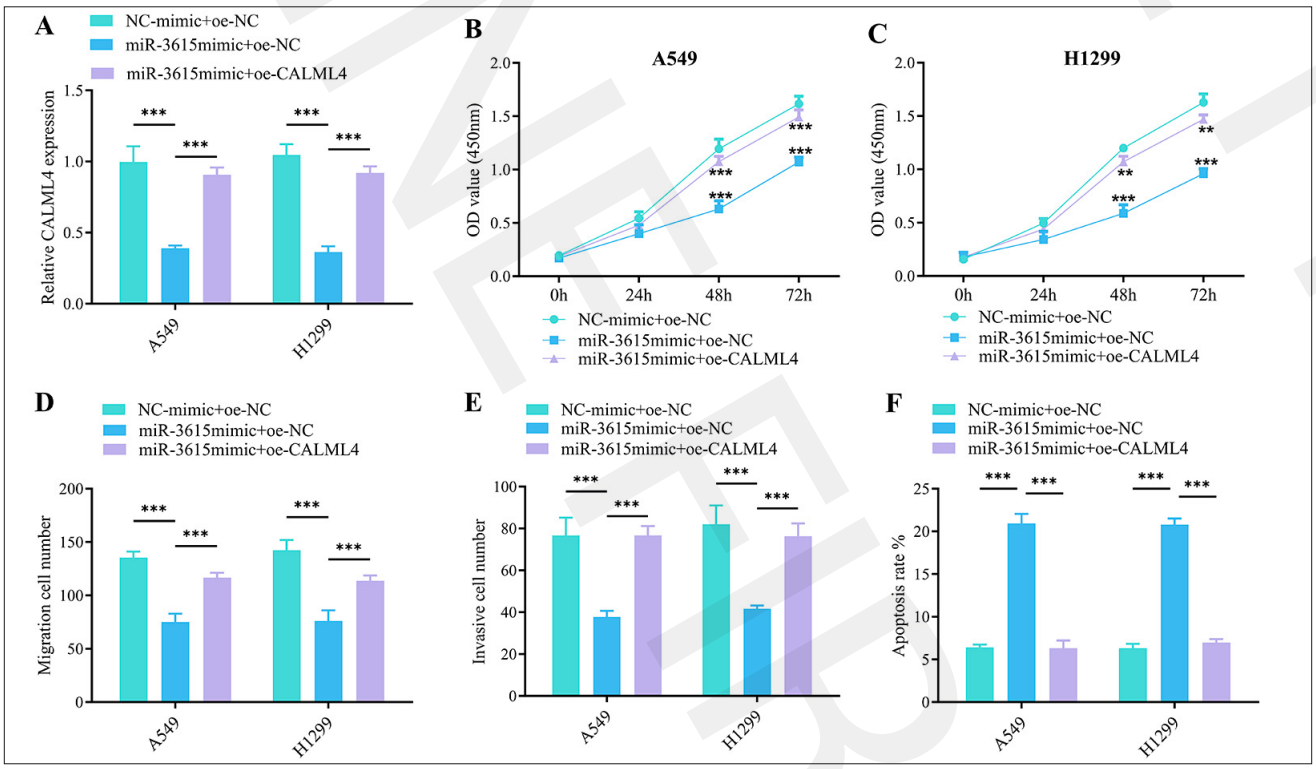

Figure 4. CALML4 mediates the tumour-suppressive effects of miR-3615 in LUAD cells. (A) Restoration of CALML4 expression was confirmed by RT-qPCR in miR-3615-overexpressing A549 and H1299 cells co-transfected with the CALML4 overexpression plasmid (oe-CALML4). (B-E) Overexpression of CALML4 reversed the inhibitory effects of miR-3615 overexpression on cell proliferation (B, C), migration (D), and invasion (E), as well as its promoting effect on apoptosis (F). All the experiments were independently repeated 3 times (n=3).
$^{**}P < 0.01$, $^{***}P < 0.001$

post-transcriptional regulators, miRNAs exert influence on tumourigenesis by disrupting cellular signalling and regulating vital biological processes [13]. Research has demonstrated that miRNAs exhibit distinct expression profiles in lung cancer tissues compared to normal lung tissue. This positions them as promising biomarkers for applications such as screening at-risk individuals, enabling earlier detection, and guiding more precise treatment approaches [14]. Despite advances in understanding miRNA dysregulation in LUAD, novel miRNAs and their functional mechanisms in tumour progression warrant further exploration.

Prior research has documented reduced expression of miR-3615 in LUAD tissue samples [8]. Extending this observation, it was verified that miR-3615 levels are likewise low in a panel of LUAD cell lines. Malignant cell proliferation is a central driver of tumourigenesis and disease progression. It was seen that upregulating miR-3615 notably suppresses LUAD cell proliferation, whereas its knockdown enhances proliferative activity. This suggests that miR-3615 may similarly inhibit tumour growth *in vivo*, an effect analogous to the function of miR-144-3p in LUAD [15]. Approximately 90% of LUAD-related deaths are associated with distant metastasis. The study also demonstrated that increasing miR-3615 levels leads to significant suppression of LUAD cell migration and invasion, whereas its knockdown promotes these processes. Apoptosis, a classic form of Type I programmed cell death, is regulated by both intrinsic and extrinsic pathways, and serves as a vital mechanism for maintaining cellular homeostasis. Its aberrant expression is intimately connected to the pathogenesis of diverse tumours [16].

In the current study, overexpression of miR-3615 promoted apoptosis in LUAD cells, while knockdown of miR-3615 inhibited apoptosis. Collectively, the results indicate that miR-3615 acts as a tumour suppressor in LUAD, potentially restraining malignant phenotypes by negatively regulating tumour growth and metastatic progression.

The primary mechanism of miRNA action involves binding to the 3′-UTR of target mRNAs, which subsequently results in transcript degradation or blockade of translation, thereby playing crucial roles in gene expression regulation, cell growth, and differentiation [17]. Based on this mechanism, GO enrichment analysis was used in this study to predict the biological functions associated with the potential target genes of miR-3615. Analysis revealed that these genes show significant enrichment in functional categories, including transcriptional regulation, metabolic reprogramming (particularly lipid metabolism), stress, and proliferation-related signalling pathways, including JNK, and developmental regulation. Lipid metabolism, a key process in energy supply, immune regulation, and signal transduction, is significantly associated with tumourigenesis and disease progression across various cancers, such as thyroid and breast cancers, when dysregulated [18]. KEGG pathway analysis also showed marked enrichment of the target genes in several pathways, such as the oxytocin signalling pathway, glioma pathway, gastric acid secretion pathway, phosphatidylinositol signalling system, as well as aldosterone synthesis and secretion. Among these, the phosphatidylinositol signalling system originates from the cell membrane with phosphatidylinositol as a core messenger, and its aberrant activation is closely associated with tumour metabolic reprogramming [19].

Research indicates that tumour cells frequently exploit lipid metabolism to meet energy demands, produce cellular membranes, and consequently drive malignant progression, including proliferation, invasion, and metastasis [20]. This suggests a significant link between lipid metabolism and the regulation of the tumour microenvironment. Further analysis through PPI network construction, combined with the GO and KEGG results, led to the selection of eight candidate genes for experimental validation. The data demonstrated that only CALML4 expression was significantly regulated by miR-3615. The current study systematically analyzed the functional and pathway characteristics of miR-3615 and its potential downstream target genes using bioinformatics methods, providing new clues and research directions for further exploration of its mechanisms in LUAD.

CALML4, a calmodulin-related protein within the EF-hand calcium-binding family, functions in the regulation of various signalling pathways by binding to different target proteins [21]. CALML4 has been implicated in previous studies as a key factor associated with persistent tolerance to EGFR-TKIs in LUAD, where it can promote the metabolic clearance of targeted drugs and contribute to disease progression by fostering an immunosuppressive microenvironment [12]. The current study confirms a direct targeting relationship wherein miR-3615 negatively regulates CALML4 expression. Furthermore, CALML4 overexpression counteracts the influence of miR-3615 on malignant phenotypes and apoptosis in LUAD cells. Consequently, CALML4 is identified as a crucial downstream target responsible for mediating the tumour-suppressive role of miR-3615 in LUAD. Consistent with these findings, miR-1247-3p has been reported to directly bind the 3'UTR of STAT5A mRNA and thereby function as a potential regulator of LUAD cell migration and chemoresistance [22]. miR-3648 promotes the proliferation, migration, and invasion of LUAD cells by inhibiting SOCS2 [23].

In summary, these studies collectively reinforce the pivotal role of miRNA-mediated regulatory networks in orchestrating LUAD progression via direct targeting of key effector genes.

Limitations of the study. This study has two limitations. First, Min et al. utilized a mouse xenograft tumour model to conduct *in vivo* analysis which demonstrated that overexpression of MARCKSL1-2 alleviated DTX resistance in LUAD tumours [24], providing an important reference for clinical translational values. The current study, however, only completed functional validation at the cellular level, and the corresponding animal model for *in vivo* experiments has not yet been established. Subsequent experiments in mouse models are required to further confirm the *in vivo* biological functions of miR-3615. Second, the specific regulatory mechanisms of miR-3615 and its downstream target CALML4 in the progression of LUAD have not been fully elucidated. KEGG pathway enrichment analysis suggests that CALML4 may be associated with the PI3K/AKT signalling pathway. Previous studies have confirmed that the PI3K/AKT signalling pathway plays critical roles in tumour microenvironment remodelling, lipid metabolism regulation, and drug sensitivity [25]. However, whether it mediates the miR-3615/CALML4 axis involvement in the pathological process of LUAD remains to be further investigated. Involvement of the mechanism of the PI3K/

AKT signalling pathway therefore represents an important direction for future research.

CONCLUSION

Decreased miR-3615 expression may serve as a biomarker associated with LUAD progression. miR-3615 overexpression inhibits proliferation, migration, and invasion while stimulating apoptosis, effects primarily mediated by its direct targeting and downregulation of CALML4, thereby contributing to its tumour-suppressive role.

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