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ORIGINAL RESEARCH

MiR-499a-5p Promotes the Proliferation and Migration of Uterine Leiomyoma Cells by Activating the Wnt/β-Catenin Signaling Pathway

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Objective: This study aimed to investigate the functional role and underlying mechanism of miR-499a-5p in the progression of uterine leiomyoma (ULM).

Methods: Expression levels of miR-499a-5p were analyzed in ULM tissues and cells. Functional assays were conducted to evaluate the effects of miR-499a-5p knockdown or overexpression on cellular proliferation, migration, apoptosis, and cell cycle progression. The involvement of the Wnt/β-catenin signaling pathway was assessed using pathway-specific protein markers and lithium chloride (LiCl) as a chemical activator.

Results: miR-499a-5p was significantly upregulated in ULM tissues and cells. Its knockdown inhibited proliferation and migration, induced apoptosis, and caused cell cycle arrest at the G0/G1 phase. Additionally, downregulation of miR-499a-5p suppressed the activation of the Wnt/β-catenin pathway, an effect that was reversed by LiCl treatment.

Conclusion: miR-499a-5p facilitates the development of uterine leiomyoma by activating the Wnt/β-catenin signaling pathway. These findings suggest that miR-499a-5p may serve as a promising molecular biomarker and a potential therapeutic target for ULM management.

Keywords: miR-499a-5p, uterine leiomyoma, Wnt/β-catenin signaling pathway, proliferation, migration

Introduction

Uterine leiomyoma (ULM), also known as fibroids, is the most common benign tumor of the female pelvis and frequently affects women of reproductive age.[1] While many cases remain asymptomatic, others lead to complications such as menorrhagia, pelvic pain, and infertility.[2] Hysterectomy is currently the most definitive treatment for ULM, but it compromises fertility and is associated with long-term health risks.[3] Although pharmacological therapy can relieve symptoms, its efficacy is limited, and there is no high-quality evidence demonstrating that drug treatment can eliminate ULM.[4] Therefore, identifying novel therapeutic targets with better safety profiles remains a pressing need in clinical gynecology.

The development and growth of ULMs are known to be hormone-dependent, primarily influenced by estrogen and progesterone signaling, which stimulate smooth muscle proliferation and extracellular matrix accumulation.[5] While estrogen and progesterone are recognized as key drivers of leiomyoma growth, the investigation of estrogen-independent molecular mechanisms has emerged as a complementary approach to identify novel therapeutic targets beyond the scope of endocrine regulation.[6–8]

With the advancement of high-throughput sequencing technologies, numerous non-coding RNAs have been identified, among which microRNAs (miRNAs) have gained considerable attention. These small RNAs (~22 nucleotides) regulate gene expression post-transcriptionally by binding to the 3′ untranslated region (3′-UTR) of target mRNAs, leading to transcript degradation or translational inhibition.[9,10] Accumulating evidence has demonstrated that miRNAs play critical roles in cell proliferation, apoptosis, migration, and tumorigenesis, and they have been explored as diagnostic and prognostic biomarkers.[11,12] Among them, miR-499a-5p displays context-dependent effects in human

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cancers. Its upregulation has been associated with tumor progression and poor prognosis in pancreatic cancer,[13] whereas its downregulation in cervical cancer suppresses malignancy through inhibition of eIF4E.[14] Furthermore, exosome miR-499a-5p has been shown to suppress endometrial cancer growth and metastasis by targeting VAV3.[15] These findings highlight the diverse and tissue-specific roles of miR-499a-5p across tumor types.

In gynecologic oncology, preoperative differentiation between benign uterine fibroids and malignant uterine sarcomas remains a significant clinical and diagnostic challenge. Uterine sarcomas often present with overlapping symptoms and imaging features, and are frequently diagnosed only after surgical resection for presumed benign lesions. This diagnostic uncertainty has been widely recognized, and recent analyses underscore the importance of developing reliable preoperative tools, including imaging-based risk models and molecular classifiers, to improve the identification of uterine sarcomas prior to surgery.[16] In the context of ULM, several miRNAs have been implicated in pathogenesis. For instance, miR-129 was found to be downregulated in ULM and its overexpression promoted apoptosis and inhibited proliferation via TET1 suppression.[17] However, to date, the role of miR-499a-5p in ULM remains unexplored. Therefore, this study investigated the expression and functional role of miR-499a-5p in uterine leiomyoma using clinical specimens and in vitro assays. Its effects on cell proliferation, apoptosis, migration, and cell cycle were evaluated, along with its regulatory association with the Wnt/β-catenin signaling pathway. These findings may contribute to understanding the molecular pathogenesis of ULM and support miR-499a-5p as a potential diagnostic biomarker and therapeutic target.

Materials and Methods

Clinical Sample Collection

Between May and August 2024, ULM tissues and matched adjacent myometrium tissues (MM) were collected from patients undergoing surgery at our hospital. All patients were histologically diagnosed with ULM and had not received hormonal therapy within three months prior to surgery. Written informed consent was obtained from all participants before enrollment. This study was approved by the Medical Ethics Committee of the Affiliated Suzhou Hospital of Nanjing Medical University (Approval No. NJSU-20240155-KY) and conducted in accordance with the principles of the Declaration of Helsinki.

Isolation and Culture of ULM Cells

Fresh ULM tissues were collected under aseptic conditions, minced into approximately 1 mm^3 tissue fragments, and passed through a 40-mesh sieve. The resulting homogenate was digested with 1% type IV collagenase at 37 °C for 30 min in a shaking water bath. After centrifugation (1000 r/min, 10 min, 4 °C), the supernatant was removed and the cell pellets were resuspended in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) culture medium supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin–streptomycin (P/S; Gibco, USA). Cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

Cell Transfection

Third-passage ULM cells in good condition were used for transfection. When cell confluency reached 80%–90%, they were transfected with negative control (NC inhibitor/mimics), miR-499a-5p inhibitor, or miR-499a-5p mimics (Guangzhou RiboBio, China) using Lipofectamine 2000 (Thermo Fisher Scientific, USA) following the manufacturer's instructions. 6 h post-transfection, cells were cultured in fresh complete medium with or without Lithium chloride (LiCl), and harvested after 48 h for subsequent assays.

qRT-PCR

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, USA), and RNA concentration and purity were assessed via NanoDrop. Furthermore, Reverse transcription was performed using a random primer reverse transcription kit (Thermo Fisher Scientific, USA), and qRT-PCR was conducted using the SYBR GREEN kit (TaKaRa, Japan). U6 was used as the internal control, and gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All samples were tested in six replicates. Primer sequences are shown in Table 1.

Table 1 Primer Sequences Used for qRT-PCR

Genes	Primer Sequences (5′–3′)
miR-499a-5p	F: 5′- ACAGACTTGCTGTGATGTTC-3′
	R: 5′- GAACATGTCTGCGTATCTC-3′
U6	F: 5′- CTCGCTTCGGCAGCACAT-3′
	R: 5′- TTTGCGTGTCATCCTTGCG-3′

CCK-8 Method

The treated ULM cells were collected, digested regularly, seeded into 96-well plates at a density of 5×10^3 cells/well in DMEM/F12 medium with 10% FBS and incubated for 24 h. Subsequently, 10 μL of CCK-8 solution was added per well, and cells were incubated for another 2 h. Absorbance was measured at 490 nm using a microplate reader. Each group included five replicate wells per time point.

Colony Formation Assay

The treated ULM cells were digested, suspended in 0.35% agarose-containing DMEM complete medium, and seeded into 6-well plates (1×10^3 cells/well). After 14 min of incubation at 37 °C with 5% CO_2, colonies were fixed with methanol and stained with 0.1% crystal violet. The number of colonies was then counted after photographs were taken with an inverted microscope.

Apoptosis Assay

Cells from each group were harvested using trypsin digestion and washed twice with precooled sterile PBS. The cell concentration was adjusted to 5×10^5 cells/mL. A 200 μL aliquot of the cell suspension was incubated with 10 μL of Annexin V-FITC and 10 μL of propidium iodide (PI, 20 mg/L) in the dark at room temperature for 10 min Subsequently, 500 μL of PBS was added to each sample. Apoptotic cells were quantified by flow cytometry. The apoptotic index was calculated as follows: Apoptotic index (%) = apoptotic cells/(apoptotic cells + viable cells) × 100%.

Cell Cycle Analysis

ULM cells in the logarithmic growth phase were seeded into 6-well plates and cultured until they reached appropriate confluency. The cells were then serum-starved for 24 h, followed by treatment for an additional 24 h. Each group included three parallel wells. After treatment, cells were collected, fixed with precooled 70% ethanol, and stored overnight at 4 °C. Following centrifugation, 400 μL of RNase A (100 mg/L) and PI staining solution (100 mg/L) were added, mixed thoroughly, and stained at room temperature for 30 min in the dark. A flow cytometer was used for detection and analysis.

Wound Healing Assay

ULM cells were seeded into six-well plates and cultured to confluence. A straight-line scratch was created using a sterile 10 μL pipette tip across the cell monolayer. Detached cells and debris were removed by washing with PBS. After incubation in serum-free medium for 24 h, wound closure was imaged using an inverted microscope. The scratch area was calculated using Image J software.

Western Blot

The transfected cells were rinsed twice with precooled PBS and lysed in 80 μL of lysis buffer. After centrifugation at 12,000 r/min for 15 min at 4 °C, the supernatant was collected. Protein concentration was determined using a BCA kit (Thermo Fisher Scientific, USA). Equal amounts of protein (20 μg) were mixed with 1 × loading buffer, boiled for

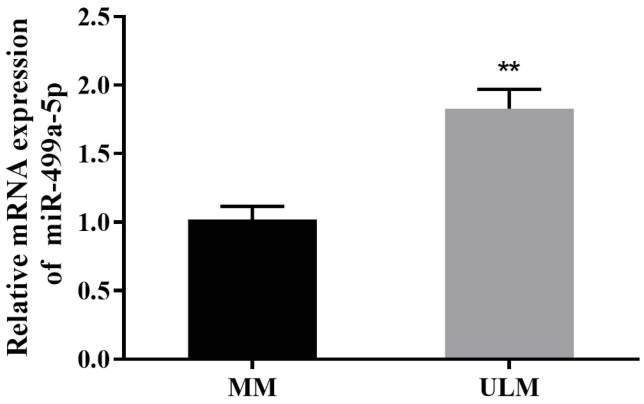

Figure 1 Expression level of miR-499a-5p in uterine leiomyoma (ULM) tissues and matched myometrial (MM) tissues. **p < 0.01 vs MM group.

denaturation, and subjected to SDS-PAGE. Proteins were transferred onto PVDF membranes, blocked with 5% non-fat milk for 1 h, and incubated with primary antibodies overnight at 4 °C. After washing, membranes were incubated with secondary antibodies for 1 hour at room temperature. Following additional washes, signals were developed using chemiluminescence reagents and visualized with a gel imaging system. Band intensities were quantified using ImageJ software, and β-actin was used as an internal control.

Statistical Analysis

Statistical analyses were performed using SPSS 26.0. Data were first assessed for normality using the Shapiro–Wilk test. For normally distributed data, comparisons between two groups were conducted using Student's *t*-test, while multiple group comparisons were performed using one-way analysis of variance (ANOVA). All data are presented as mean ± standard deviation (SD), and a p-value < 0.05 was considered statistically significant.

Results

Upregulation of miR-499a-5p in Uterine Leiomyoma Tissues

qRT-PCR analysis revealed that miR-499a-5p expression was significantly higher in ULM tissues compared to the MM tissues (Figure 1), suggesting its potential involvement in ULM pathogenesis.

Knockdown of miR-499a-5p Inhibits the Malignant Process of Uterine Leiomyoma Cells

We investigated the function of miR-499a-5p in ULM cells by knocking down or overexpressing miR-499a-5p and its effect on the malignant process of ULM cells. The results showed that miR-499a-5p levels were reduced in cells after knockdown, but significantly increased in cells after overexpression (Figure 2A), indicating successful cell transfection. Functional assays demonstrated that miR-499a-5p knockdown markedly suppressed cell proliferation (Figure 2B), reduced colony-forming ability (Figure 2C), and inhibited migration (Figure 2D). In contrast, miR-499a-5p overexpression enhanced these malignant behaviors. Cell cycle and apoptosis analysis showed that miR-499a-5p knockdown induced G0/G1 arrest and increased apoptosis, whereas overexpression promoted S-phase entry and reduced apoptosis (Figure 2E and F). These findings indicate that miR-499a-5p facilitates the proliferative and invasive behavior of ULM cells while inhibiting programmed cell death.

Knockdown of miR-499a-5p Inhibits Activation of the Wnt/β-Catenin Signaling Pathway

To better understand how miR-499a-5p functions, we examined the proteins involved in the Wnt/β-catenin signaling pathway. The results indicated that knockdown of miR-499a-5p led to a significant reduction in β-catenin, Cyclin D1, and

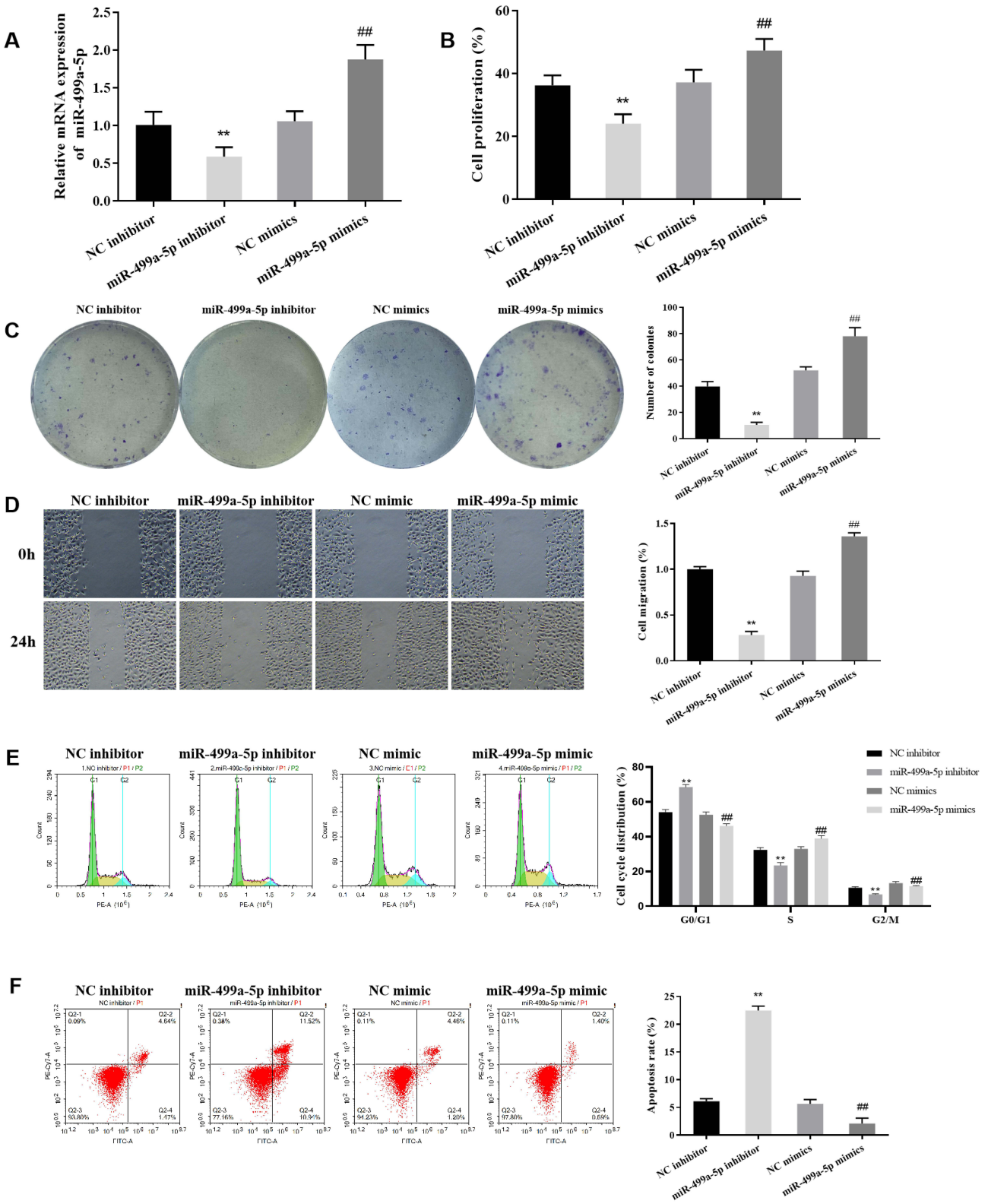

Figure 2 Effect of miR-499a-5p knockdown on the malignant progression of ULM cells. (**A**) qRT-PCR analysis of miR-499a-5p in treatment groups; (**B**) CCK-8 assay assessing cell proliferation in treatment groups; (**C**) cell colony formation assay evaluating cell viability in treatment groups; (**D**) wound healing assay assessing cell migration ability; (**E**) flow cytometry analysis of cell cycle distribution in treatment groups; (**F**) flow cytometry analysis of apoptosis in treatment groups. $^{**}p < 0.01$ vs NC inhibitor group; $^{\#\#}p < 0.01$ vs NC mimics group.

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c-myc protein levels, while overexpression elevated their expression (Figure 3). This supports a mechanistic role for miR-499a-5p in ULM pathogenesis through modulation of canonical Wnt signaling components.

LiCl Reverses the Inhibitory Effect of miR-499a-5p Knockdown on the Wnt/β-Catenin Pathway

The relationship between miR-499a-5p and Wnt/β-catenin pathway was further confirmed by the addition of LiCl, a Wnt/β-catenin pathway activator. The results indicated that LiCl treatment reversed the inhibitory effects of miR-499a-5p knockdown on β-catenin, Cyclin D1, and c-Myc protein expression (Figure 4). This finding suggested that miR-499a-5p can influence the function of the Wnt/β-catenin pathway.

Discussion

MiR-499a-5p has been implicated in a wide range of human diseases with tissue-specific functions. For instance, Liu et al found that miR-499a-5p overexpression suppressed osteosarcoma cell proliferation via the GSK-3β/PPM1D axis,[18] and downregulation of miR-499a-5p has been reported in oral squamous cell carcinoma, osteosarcoma, and lung adenocarcinoma.[19] These findings have led to its classification as a tumor suppressor in several cancers. Conversely, in other contexts, miR-499a-5p may promote disease progression. Zong et al showed that it enhanced the aggressive

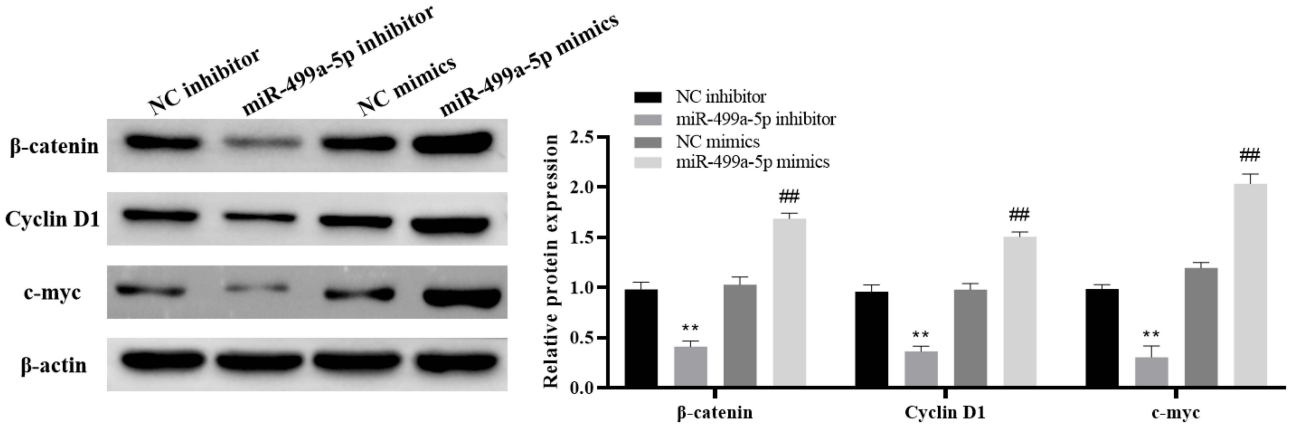

Figure 3 Effect of miR-499a-5p knockdown on the Wnt/β-catenin signaling pathway. $^{**}p < 0.01$ vs NC inhibitor group; $^{\#\#}p < 0.01$ vs NC mimic group.

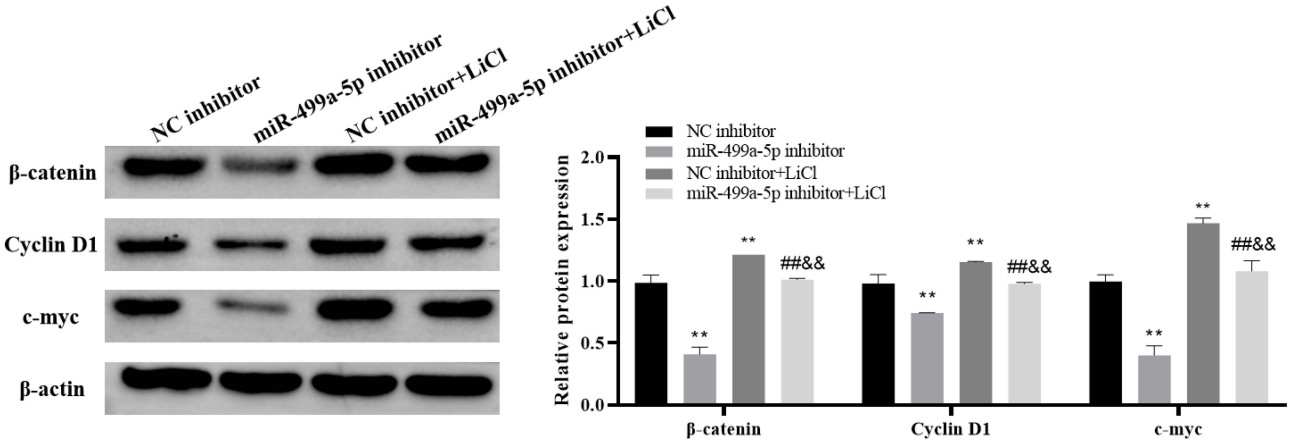

Figure 4 The regulatory relationship between miR-499a-5p and the Wnt/β-catenin pathway in ULM cells. $^{**}p < 0.01$ vs NC inhibitor group; $^{\#\#}p < 0.01$ vs NC inhibitor + LiCl group; $^{\&\&}p < 0.01$ vs miR-499a-5p inhibitor + LiCl group.

behavior of glioma cells,[20] and Liu et al demonstrated that miR-499a-5p promoted invasion and metastasis of colorectal cancer via FOXO4 and PDCD4.[21] Additionally, miR-499a overexpression has been linked to poor prognosis in a range of diseases including myocardial infarction and hematological malignancies.[22]

In this study, we found that miR-499a-5p was significantly upregulated in ULMtissues and cells. Its inhibition suppressed proliferation, colony formation, and migration while inducing apoptosis. These results suggest that miR-499a-5p promotes the proliferative and migratory capacity of ULM cells, consistent with its regulatory role in fibroid growth. Although uterine fibroids are benign tumors, the regulation of their growth and progression via miRNA pathways remains clinically relevant, particularly for patients seeking fertility preservation. Several studies have highlighted the promise of miRNAs as biomarkers or adjunctive diagnostic tools in benign gynecologic conditions, including ULM.[23,24] While our study does not establish immediate clinical applicability, it provides foundational data for future translational investigations.

The Wnt/β-catenin signaling pathway plays a pivotal role in animal development and tissue homeostasis. Aberrant activation of this pathway has been implicated in a variety of human pathologies.[25] Acting as a morphogen, Wnt/β-catenin signaling influences cell fate decisions in a concentration-dependent manner.[26] It is closely associated with the regulation of cell proliferation, migration, the cell cycle, and senescence, and is essential for the proliferation and differentiation of stem and progenitor cells during embryogenesis and adult tissue maintenance. Moreover, dysregulated Wnt/β-catenin signaling has been shown to drive tumorigenesis in various cancer types.[27] Recently, it was discovered that the Wnt/β-catenin signaling pathway is regulated by miRNAs. For example, Ren et al discovered that overexpression of miR-454-3p in breast cancer cells significantly activates the Wnt/β-catenin signaling pathway by inhibiting the activity of pre-nuclear mRNA domain 1A (RPRD1A), which improves breast cancer cell migration.[28] Liu et al discovered that miR-221/222 activates Wnt/β-catenin signaling to promote breast cancer invasiveness.[29] Furthermore, Mandal et al discovered that a decrease in Wnt/β-catenin signaling pathway activity affects miR-499a-5p expression.[30] However, the functional relationship between miR-499a-5p and Wnt/β-catenin signaling in uterine leiomyoma has not been previously reported. In this study, knockdown of miR-499a-5p led to a marked reduction in the expression of β-catenin, Cyclin D1, and c-Myc, suggesting that miR-499a-5p positively regulates Wnt/β-catenin pathway activity in ULM. LiCl, a known Wnt pathway activator, has been shown to enhance β-catenin accumulation and modulate various biological processes including apoptosis, gene expression, and inflammation.[31] To further validate the regulatory role of miR-499a-5p, we co-treated ULM cells with LiCl following miR-499a-5p knockdown. This intervention successfully reversed the inhibitory effects on β-catenin pathway proteins, confirming that miR-499a-5p promotes fibroid cell proliferation and migration through activation of the Wnt/β-catenin signaling cascade.

Despite these insights, our study has several limitations. First, the sample size was relatively small and derived from a single institution. Second, all functional assays were performed in vitro using primary cultured ULM cells, and in vivo validation is needed to confirm biological relevance. Third, off-target effects of transfection reagents cannot be fully excluded. Lastly, while estrogen signaling is a well-established driver of fibroid growth, our study did not examine its interaction with miR-499a-5p. This aspect warrants future investigation.

In summary, we demonstrate that miR-499a-5p promotes proliferation and migration in uterine leiomyoma cells via activation of the Wnt/β-catenin signaling pathway. These findings contribute to a broader understanding of miRNA-mediated, estrogen-independent regulatory mechanisms in fibroid pathogenesis and offer a potential molecular target for future therapeutic exploration.

Conclusions

In conclusion, this study demonstrated that miR-499a-5p is significantly upregulated in ULM tissues and cells. Functional experiments revealed that silencing miR-499a-5p suppressed cell proliferation, migration, and cell cycle progression while promoting apoptosis, through inhibition of the Wnt/β-catenin signaling pathway. These findings suggest that miR-499a-5p may serve as both a molecular biomarker and a potential therapeutic target for the clinical management of ULM.

Data Sharing Statement

The data underlying this article will be shared on reasonable request to the corresponding author.

Ethic Statement

This study was approved by the Medical Ethics Committee of the Affiliated Suzhou Hospital of Nanjing Medical University (Suzhou Municipal Hospital) (NJSU-20240155-KY). All patients provided informed consent prior to participation. This study complies with the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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