miR-24-3p Promotes Cell Proliferation, Migration, and Invasion of Human Cervical Cancer Cells through/via Targeting AMOTL2 and Attenuating the YAP/Hippo Pathway

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Abstract

Background

miR-24-3p promotes the development of the majority of malignancies. However, its function in cervical cancer is not clearly elucidated so far.

Methods

In this study, cell proliferation, migration, and invasion were measured by the CCK8 and transwell assays. Bioinformatic methods were used to predict the target genes of miR-24-3p, verifying by luciferase reporter assay and western blotting. The target genes set was also used for KEGG pathway enrichment analysis.

Results

Then we observed higher miR-24-3p level in cervical cancer cells and faster growth of tumor in a xenograft model. The function assays demonstrated that miR-24-3p promoted proliferation, migration, and invasion of cervical cancer cells in vitro. It was confirmed that miR-24-3p directly targeted AMOTL2 and the recovery of AMOTL2 reversed the function of miR-24-3p in cervical cancer cell line CaSki. Besides, miR-24-3p suppressed the Hippo signaling pathway in CaSki and SiHa cells.

Conclusions

In conclusion, our results reminded that miR-24-3p could boost the migration and proliferation of cervical cancer cells via down-regulating AMOTL2 and attenuating YAP/Hippo signaling pathway activity.

1. Introduction

Cervical cancer with significant morbidity among female genital malignancies is one of the most common malignancies worldwide¹. New cases of cervical cancer were approximately 569,847 people and more than half of them died in 2018². Although now we have regular screening and prevention strategies, the available treatment schemes still pose a threat to fertility. Recurrent and metastatic cervical cancer is generally incurable and it has a poor the prognosis at the same time. Therefore, it is urgently needed to identify distinctive markers of cervical cancer for ascertaining diagnostic and treatment strategies.

miRNA consisting of roughly 22 nucleotides is a class of endogenous non-coding single-stranded small RNA that can inhibit or activate target genes at the transcriptional or post-transcriptional level³, which exerts a significant function on proliferation, apoptosis, invasion, and epithelial-mesenchymal transition (EMT) of cancer cells⁴. miR-24-3p as a member of miR-24 family plays an oncogenic role during the development of multiple kinds of tumors⁵⁻⁷. For example, miR-24-3p and miR-27a-3p improve proliferation of glioma cells with regulating MXI1 cooperatively⁸. miR-24-3p overexpression promote the migration of MCF7 cells and mediate the tamoxifen resistance in breast cancer^{9,10}. No wonder that some miRNAs can exert disputed functions in different cancers, a recent research reveals that miR-24-3p

decelerates the progression by downregulating LAMB3 in Pancreatic ductal adenocarcinoma (PDAC)¹¹. Nonetheless, it remain to figure out the explicit function of miR-24-3p in cervical cancer .

, Angiomotin (AMOT), angiomotin-like 1 (AMOTL1), and angiomotin-like 2 (AMOTL2) make up the motin family, their protein sequences are similar. According to yeast two-hybrid screening, angiomotin was regarded as an angiostatin-binding protein to modulate endothelial cell migration^{12,13}. The motin family is also known as AMOTs, functional studies have demonstrated that AMOTs are regulatory factors in the Hippo signaling pathway, they interact with multiple core proteins including Merlin, LATS1/2, MST1/2, and YAP1¹⁴. Since AMOTL1 and AMOT2 reserve the LPTY motif¹⁵, they can direct interact with YAP1 through protein-protein mode of action to regulate YAP1 cytoplasm-to-nucleus translocation¹⁶. YAP1 has been confirmed to promote the proliferative and migrated ability of cervical carcinoma cells¹⁷. However, few studies have focused on the function of AMOTL2 in human cervical cancer. AMOTL2 has been shown to be a key factor in the normal movement of embryonic cells¹⁸ and is associated with the migration of endothelial cells¹⁹. Meanwhile, AMOTL2 is a underlying target of miR-24-3p, as predicated by miRDB, Targetscan, mitarbase, and starbase. Therefore, it is quite interesting to clearly elucidate the relationship among miR-24-3p, AMOTL2, and YAP1 during cervical cancer progression.

In this study, we identified that miR-24-3p is significantly upregulated in SiHa and CaSki cells and is a critical promoter of cell proliferation, migration, and invasion. Importantly, we demonstrated that miR-24-3p downregulation suppressed proliferation and migration through directly targeting AMOTL2. Moreover, miR-24-3p attenuated the YAP1/Hippo pathway. Overall, our data indicated that miR-24-3p might be a potential target for cervical cancer therapy.

2. Methods And Materials

2.1 Cell Culture

Human cervical cancer cell lines CaSki and SiHa were stored in school of laboratory medicine (Chongqing medical university, China), and C33A were purchased from Chinese Academy of Sciences (Shanghai, China). 10% fetal bovine serum (Biological Industries, Israel) was added to Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA). All cells were cultured in prepared medium and were maintained at 37°C in a cell incubator containing 5% CO₂.

2.2 Cell Transfection

Small interfering RNA (siRNA) for AMOTL2 were purchased from Biomics (Jiangsu, China). Synthesized NC mimics, NC inhibitors, miR-24-3p mimics, and miR-24-3p inhibitors were purchased from GenePharma (Shanghai, China). Cells were transfected with 100 μM mimics or inhibitors for twenty-four hours. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, USA) was used to enhance the transfection efficiency, then we collected and assayed transfected cells. The siRNA sequences are shown in Table I.

2.3 RNA Purification and qRT-PCR (quantitative Real-time PCR)

Total RNA of cells was extracted by TRIzol (Invitrogen; Thermo Fisher Scientific, USA.) method. Reverse transcription was implemented using PrimeScript™ RT reagent Kit (TaKaRa, Japan) to obtain cDNA templates, and the subsequent reaction procedures were used: 37°C for 15 min, 85°C for 5 sec. Then, qPCR was accomplished with 2×SYBR Green qPCR Master Mix (Bimake; Shanghai, China). GAPDH was used as internal controls for AMOTL2, ANKRDD1, CYR61, and CTGF, and U6 served as internal controls for miR-24-3p. The thermocycling conditions of qPCR were utilized: 95°C for 1 min, 95°C for 15 sec maintaining 40 cycles, 55°C for 30 sec and 70°C for 30 sec. We adopted the $2^{-\Delta\Delta C_q}$ method to calculate the RNA expression level. Primers are shown in Table II.

2.4 Prediction and Analysis of miRNA Target Genes

The target genes belonging to miR-24-3p were prognosticated by online prediction software, such as mirtarbase (<http://mirtarbase.mbc.nctu.edu.tw/index.php>), TargetScan (<http://www.targetscan.org/>), starBase (<http://starbase.sysu.edu.cn/index.php>), miRDB (<http://www.mirdb.org/>), then the intersection of targets were captured with Venn (<http://bioinformatics.psb.ujent.be/webtools/Venn/>) diagram. In addition, pathogenic genes related to cervical cancer are found in Genecards (<https://www.genecards.org/>) and were uploaded to the Metascape (<http://metascape.org/>) database for KEGG signal pathway analysis. Prediction of lncRNAs regulating miR-24-3p was finished by starBase and DIANA in the Venn diagram.

2.5 Luciferase Reporter Assays

To construct luciferase reporter plasmids for AMOTL2, we firstly synthesized the seed region of AMOTL2 containing the wild-type or mutant sequences and cloned them into the pGL6-miR luciferase plasmid (Beyotime; Shanghai, China). Later, we cotransfected miR-24-3p mimics or inhibitors (50 nM) and different luciferase reporter plasmids (400 ng) into HEK293T cells for 48 h using Lipofectamine 2000, and pRL-SV40 luciferase plasmid selected as Renilla luciferase plasmid was transfected with 10 ng in all group. Renilla and Firefly luciferase activities were detected with Dual Luciferase Reporter Assay System (Promega, USA), which also showed the relative luciferase expression.

2.6 Cell Proliferation Assay

Cell Counting Kit-8 (Bimake; Shanghai, China) was utilized to test the cell proliferation on the basis of manufacturer's protocol. 2000 cells were seeded in 96-well plates for 24 h, then the NC inhibitor, NC mimic, miR-24-3p inhibitors or mimics was transfected respectively. 24 and 48 h later, we added 10 μ l CCK-8 and cultured the cells for 1 h. Finally, the absorbance was measured at 450 nm. Three independent experiments were conducted.

2.7 Cell Migration and Invasion Analysis

We adopted Transwell chambers (Corning, USA) to fulfill the two analyses. In the first place, 600 μ l DMEM was added to the lower chamber and 200 μ l serumfree medium with transfected cell were seeded into the upper chamber. After incubation at 37°C in a cell incubator containing 5% CO₂, the cells in the upper chambers were removed with a cotton swab meticulously, the membranes were fixed with paraformaldehyde and were cleaned with phosphate buffered saline. Finally, we utilize 0.1% crystal violet to staine,. For migration assay, the number of transfected cells was 4×10^4 , the time for incubation is 24 h, besides, 600 μ l DMEM with 15% fetal bovine serum was added to the lower chamber. For invasion assay, the number of transfected cells was 8×10^4 , the time for incubation is 48 h, in addition, 600 μ l DMEM with 20% fetal bovine serum was added to the lower chamber, and a layer of diluted basement membrane Matrigel (Solarbio; Beijing, China) was precoated on the transwell polycarbonate membrane.

2.8 Western Blotting

The proteins were extracted from cervical cancer cells and lysed in RIPA (Radio Immunoprecipitation Assay) buffer. The protein concentration was measured by the BCA Protein Assay Kit. The equal 35 μ g proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. 5% bovine serum albumin (BSA) dissolved in TBST buffer in order to block the nonspecific binding sites in PVDF membrane. Then, the PVDF membranes were incubated with a primary antibody in Primary Antibody Dilution Buffer overnight at 4°C, membranes were washed with TBST three times for 30 min. Next secondary antibodies were incubated with the membrane for 1 h at room temperature, and the membranes were washed three times in the same way.

Electrochemical luminescence signals were detected using the Supersignal West Pico Chemiluminescent substrate kit (Millipore, Billerica, MA, USA). Primary Antibody Dilution Buffer, RIPA buffer, and BCA Protein Assay Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

The fowolling antibidiies were used: AMOTL2 (1:500, Santa Cruz Biotechnology, CA, USA), p-YAP1 (1:500; Bioss, Beijing, China), YAP1 (1:1000; Bimake, Shanghai, China), and β -actin (1:500; Affinity Biosciences, USA). HRP-conjugated anti-rabbit or -mouse secondary antibody (1:5000; Beijing Zhongshan Golden Bridge Biotechnology; Beijing, China).

2.9 Immunohistochemistry

For IHC analysis, Paraffin-embedded tumor tissue was deparaffinized and the antigen of tumor tissue was retrieved by citric acid buffer (PH6.0). Primary antibodies against p-YAP1 (Bioss, Beijing, China), p-LATS1/2 (Affinity Biosciences, USA), and AMOTL2 (Cusabio, China) were diluted at 1:100 and were incubated with the antigen of tumor tissue overnight at 4°C, then secondary antibodies were incubated with the sections, horseradish peroxidase (HRP) labeled streptomycin solution played a amplification role, the sections were finally reacted with the chromogenic agents. Immunohistochemistry PV-9000 kit (Beijing Zhongshan Golden Bridge Biotechnology; Beijing, China) was utilized for all IHC analyses.

2.10 Xenograft Model

The Institutional Animal Care and Use Committee of Chongqing Medical University granted the implementation of animal studies. We chose 4–6 weeks old female athymic BABL/c nude mice. CaSki cells were transfected with NC antagomir (100 μ M) or miR-24-3p antagomir (100 μ M), respectively. Then 200 μ l phosphate-buffered saline including 4×10^6 CaSki cells were injected subcutaneously into the back flank of nude mice, ten nude mice were divided into two groups randomly. We measured tumor diameters every 7 days for 35 consecutive, next, we taken the tumors from mice and weighted every tumor. The formula of tumor volume was: length $\times$ width² $\times$ 1/2. The sections of tumor were stained with hematoxylin and eosin.

2.11 Statistical Analysis

GraphPad Prism (Prism 5.0, GraphPad Software, San Diego, CA, USA) was used to analyze experimental data, and the student's *t*-test was used to evaluate the differences between two groups. We repeated all the experiments three times, the $p < 0.05$ was considered statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1 Expression of miR-24-3p in cervical cancer cell lines

In the tumor-miRNA-pathway (http://bioinfo.life.hust.edu.cn/miR_path/index.html) online websites, miR-24-3p was shown to be overexpressed in some cancers (Fig. 1A), such as cervical squamous cell carcinoma, endocervical adenocarcinoma (CESC), esophageal carcinoma (ESCA), skin cutaneous melanoma (SKCM), and bladder urothelial carcinoma (BLCA). Moreover, we observed that the mRNA level of miR-24-3p in cervical cancer cell lines SiHa and CaSki cells was higher than that in cervical cancer cell line C33A cells (Fig. 1B). According to the differential expression of miR-24-3p, it may participate in the development of cervical cancer.

3.2 miR-24-3p promoted the proliferation, migration and invasion of cervical cancer cells.

To investigate the biologic function of miR-24-3p in the cervical cancer, we transfected SiHa and CaSki cells with the miR-24-3p mimic or inhibitor. CCK8 assays showed that downregulating and upregulating of miR-24-3p significantly suppressed and enhanced the proliferation of two cell lines, respectively (Fig. 2A, B). Then we investigated whether the miR-24-3p exerted an influence on the migration and invasion of these cells and found that the migratory and invasive abilities of the two cell lines were markedly enhanced by the miR-24-3p mimic but suppressed by its inhibitor (Fig. 2C, D, E, F). In conclusion, miR-24-3p notably promoted proliferation, migration, and invasion of cervical cancer cells *in vitro*.

3.3 AMOTL2 was the direct downstream target of miR-24-3p

miRNAs regulate a variety of biological processes and cellular activities via regulating their target genes. To select putative targets of miR-24-3p, we adopted a comprehensive in silico analysis with dissimilar miRNA target prediction tools (targetscan, miRDB, and miRtarbase) and discovered a gene set including 95 candidate targets (Fig. 3A). Furthermore, another set consisting of 4 target genes was analyzed by starbase (Fig. 3B); AMOTL2 was the intersection of the two sets and the sequences of conserved 3' untranslated regions (3' UTRs) of AMOTL2 was complementary to miR-24-3p across human, chimp, and rhesus. For further study, original and mutant 3' UTRs of AMOTL2 were cloned into the downstream of coding region in the pGL6-miR luciferase reporter vector, respectively (Fig. 3C).

To ascertain whether AMOTL2 is direct target of miR-24-3p, miR-24-3p mimic or miR-24-3p inhibitor was cotransfected with pRL-SV40 into HEK293T cells. The relative luciferase activity was reduced by 37% in mimic group or increased by 34% in inhibitor group in wild-type AMOTL2 3'UTRs-pGL-6 vectors but not in mutant AMOTL2 3'UTRs-pGL-6 vectors (Fig. 3D). miR-24-3p mimics or inhibitors were transfected into SiHa and CaSki cells to identify whether miR-24-3p could reduce AMOTL2 expression, then we discovered that overexpression and inhibition of miR-24-3p contributed to significant reduction and upregulation of AMOTL2 protein level, respectively (Fig. 3E), but the AMOTL2 mRNA level was not changed (Fig. 3F, G). These results suggested that miR-24-3p directly targeted AMOLT2.

3.4 miR-24-3p promoted the proliferation and migration by targeting AMOTL2

We utilized small interfering RNAs (siRNAs) of AMOTL2 by knocking down the expression of AMOTL2 to simulate the effects of miR-24-3p overexpression in cervical cancer cells. qRT-PCR and western blotting confirmed the interference efficiency of AMOTL2 in CaSki. As shown in Figs. 4A and 4B, after being transfected with siAMOTL2s, the siRNA3 significantly suppressed the expression of AMOTL2 and was used in the subsequent experiments. As expected, blocking AMOTL2 expression significantly increased the proliferative (Fig. 4C), migratory, and invasive ability of CaSki cell (Fig. 4D). Next, for proofing whether the revivment of AMOTL2 could rescue the antineoplastic effects of miR-24-3p inhibition, we used CaSki cells co-transfected with miR-24-3p inhibitor and siAMOTL2. Functional assays demonstrated that the suppression of cell proliferation and migration under the miR-24-3p inhibitor were prominently reversed by AMOTL2 down-regulated expression (Fig. 4E, F). Altogether, these data indicated that miR-24-3p exerted carcinogenic effect by suppressing AMOTL2 expression in cervical cancer cells.

3.5 miR-24-3p attenuated the YAP/Hippo signaling pathway

We found 5003 cervical cancer pathogenic genes in Genecards, 36 of which were candidate targets for miR-24-3p (Fig. 5A). The KEGG signaling pathway enrichment analysis in Metascape showed that these 36 target genes were mainly involved in microRNAs in cancer, leishmaniasis, the ErbB signaling pathway, apoptosis, the signaling pathway regulating pluripotency of stem cells, the phospholipase D signaling pathway, the Hippo signaling pathway, and endocytosis (Fig. 5B).

It was substantiated that AMOTL2 interacted with the pivotal effector YAP in the Hippo signaling pathway^{16,20}. Therefore, we explored the impact of miR-24-3p on the Hippo signaling pathway. It is showed that the downregulation of miR-24-3p in CaSki cells increased levels of phosphorylated-YAP (p-YAP) by western blotting. Instead, overexpression of miR-24-3p in CaSki cells led to decreased levels of p-YAP (Fig. 5C). Similar results were observed in SiHa cells. The main downstream targets of YAP included ankyrin repeat domain containing 1 (ANKRD1), connective tissue growth factor (CTGF) and cysteine-rich angiogenic inducer 61 (CYR61). We found that overexpression of miR-24-3p in CaSki cells increased expression of CTGF and Cyr61 at the gene level (Fig. 5D), and downregulation of miR-24-3p in SiHa cells decreased levels of ANKRD1, CTGF, and CYR61 (Fig. 5E). The bioinformatics signaling pathway enrichment analysis results were partly consistent with the experimental results. To sum up, these data indicated that miR-24-3p played a protumor role in cervical cancer through the YAP/Hippo pathway.

3.6 miR-24-3p promoted tumor growth in tumor-bearing mice

In order to investigate the contribution of miR-24-3p to the progression of cervical cancer, we implanted CaSki cells transfected with miR-24-3p antagomir or NC antagomir subcutaneously into nude mice, and the growth of the tumors was monitored every 7 days. After 35 days, the tumor volumes in miR-24-3p antagomir group were observably smaller than those in NC antagomir group (Fig. 6A, B). In accordance with the tumor volumes, the tumor weights of the miR-24-3p antagomir group were significantly lower than NC antagomir (Fig. 6C). Meanwhile, increased AMOTL2, p-YAP1, and p-LATS1/2 were also found in the miR-24-3p antagomir tumors by IHC (Fig. 6D), which was consistent with the aforementioned results in cells. Thus, our results indicated that miR-24-3p exerted oncogenic effect on the development of cervical cancer *in vivo*.

3.7 Prediction and analysis of LncRNAs regulating miR-24-3p

We used starBase and DIANA to predict LncRNAs binding to miR-24-3p, and the results were shown in Fig. 7A. There were eight LncRNAs as sponges of miR-24-3p, and the names of these LncRNAs were shown in Fig. 7B; The expression of LINC00662, NEAT1, and ILF3-AS1 were low in cervical cancer ($p < 0.05$). The survival curve analysis of these three LncRNAs in GEPIA showed that the overall survival time of patients with cervical cancer overexpressing ILF3-AS1 was significantly longer than that of those under-expressing ILF3-AS1 (Fig. 7F). It was suggested that ILF3-AS1 may act as the sponge of miR-24-3p to regulate AMOTL2 and then inhibit the progression of cervical cancer, which will be the main direction of our follow-up research.

4. Discussion

miRNAs can work in a variety of biological processes and participate in the tumor process including uterine cervix carcinoma progression. It has been reported that miR-24 is involved in keratinocyte

differentiation and its expression is regulated by HPV(Human papillomavirus) E6 and E7²¹, suggesting that the miR-24 family may be involved in the cervix carcinoma progression. miR-24-3p which is a member of the miR-24 family could inhibit the malignant progression of lacrimal adenoid cystic carcinoma through the p53/p21 signal pathway, but it promoted the development of varied tumors such as breast, lung, liver, and bladder cancer²²⁻²⁶. However, the specific role and molecular mechanisms of miR-24-3p, which is a well-known cancer-associated miRNA, have not been elucidated in cervical cancer. In our studies, miR-24-3p was upregulated in SiHa and CaSki cervical cancer cells. Inhibition of miR-24-3p remarkably inhibits cell proliferation, migration, and invasion, but overexpression of miR-24-3p has the opposite effect. Moreover, animal experiments revealed that miR-24-3p antagomir could slow the growth of subcutaneous tumor in nude mice. The above results confirmed that miR-24-3p plays a promoting role in the cervical cancer progression.

One of the biggest challenges in formulating miRNA treatment methods is determining the best miRNA candidate or miRNA target for each disease. Compared to experimental verification, bioinformatics can provide a large variety of high-speed predictions of target genes and related pathways. Four online starbases were used to seek the optimal target gene. The bioinformatics screening results showed that miR-24-3p targets 95 genes in miRDB, miRbase, and Targetscan. Biological processes of target genes are enriched in protein autophosphorylation, positive regulation of autophagy, regulation of growth, negative regulation of cell proliferation, DNA damage checkpoint, etc. (Figure S1). Some of these target genes have been linked to cervical cancer; for example, NET1 can promote the angiogenesis of cervical squamous cell carcinoma²⁷; likewise, down-regulating ERBB3 of the miR-24-3p target will significantly reduce the proliferation and invasion of cervical cancer cells²⁸. AMOTL2 was ultimately selected for validation because it is the intersection in all prediction databases.

AMOTL2 is a member of the Amot family of angiomin²⁹. It has been proven to be a key factor for the normal movement of embryonic cells and is also involved in the migration and proliferation of endothelial cells^{18,19}. AMOTL2 plays a role as a pro-oncogene in breast and colorectal cancer^{30,31}, but it participates in tumor growth and invasion by suppressing the activation of the target genes of YAP in gliomas as a tumor suppressor gene³². According to the existing researches, the function of the AMOTs in cervical cancer remains indistinct, and the precise roles and mechanisms of AMOTL2 in cervical cancer are also elusive. In this research, we defined that AMOTL2 was a functional target of miR-24-3p in uterine cervix carcinoma. First, miR-24-3p negatively modulated AMOTL2 protein levels in cervical cancer cells and in vivo. Second, the activity of a luciferase reporter embedded the 3'-UTR sequence of AMOTL2 was markedly reduced by ectopic miR-24-3p expression but was enhanced by inhibition of miR-24-3p. Third, the restoration of AMOTL2 reversed the antitumor effects of miR-24-3p inhibitors. These observations suggested that AMOTL2 was a direct functional target of miR-24-3p and served as a tumor suppressor in tumorigenesis and the progression of cervical cancer.

To elucidate possible molecular mechanisms modulated by miR-24-3p in cervical cancer, it was discovered that 36 candidate targets were cervical cancer pathogenic genes. Moreover, the KEGG

signaling pathway enrichment analysis showed that they are mainly involved in microRNAs in cancer, leishmaniasis, the ErbB signaling pathway, apoptosis, the signaling pathway regulating pluripotency of stem cells, the phospholipase D signaling pathway, the hippo signaling pathway, and endocytosis.

Hippo pathway is cognized as a tumor-suppressor pathway negatively modulating the oncoproteins TAZ and YAP³³. In addition, the Hippo pathway is a conserved signaling cascade and modulate cell growth, homeostasis, fate decision, and regeneration^{34,35}. Moreover, the Hippo pathway has recently been associated with the cervical cancer. Hyperactivation of the YAP1 oncogene actuating the initiation and development of cervical cancer was demonstrated by He et al.³⁶. Accumulated evidence has highlighted the crucial regulatory function of AMOT family proteins in the Hippo signaling pathway^{37–39}. In our study, we further validated that miR-24-3p inactivated the Hippo pathway. Firstly, overexpression of miR-24-3p reduced the p-YAP1 level in western blotting, while downregulation of miR-24-3p exerted the opposite effect. Overexpression of miR-24-3p in CaSki cells augmented the mRNA level of CTGF and CYR61 by qRT-PCR. On the contrary, downregulation of miR-24-3p in SiHa cells depressed the mRNA levels of ANKRD1, CYR61, and CTGF. It indicates that miR-24-3p can inhibit the phosphorylation of YAP and activate transcription of target genes downstream of YAP. However, this requires further investigation of the specific mechanism of AMOTL2 on the YAP/Hippo pathway in cervical cancer.

In order to delve into the mechanism of function of miR-24-3p in cervical cancer, we screened out eight LncRNAs that may regulate miR-24-3p by means of bioinformatics. Among them, LINC00662, NEAT1, and ILF3-AS1 were underexpressed in cervical cancer, and the overall survival time of patients with high expression of ILF3-AS1 was longer than that of patients with low expression of ILF3-AS1, and that reminds us that ILF3-AS1 may be a good prognostic marker for cervical cancer, which will provide favorable laboratorial and theoretical bases to subsequent research.

5. Conclusions

In summary, this study indicates that miR-24-3p promotes cell proliferation, migration, and invasion in human cervical cancer cells, and it inactivates the AMOTL2/YAP1/Hippo signaling pathway, and the LncRNA ILF3-AS1 may be the underlying upstream regulator of miR-24-3p. It provides novel insight into the molecular basis and may be used as a promising therapy for cervical cancer.

6. Abbreviations

EMT
epithelial-mesenchymal transition
PDAC
Pancreatic ductal adenocarcinoma
AMOT
angiomotin
AMOTL1

angiomin-like 1
AMOTL2
angiomin-like 2
siRNA
Small interfering RNA
qRT-PCR
quantitative Real-time PCR
RIPA
Radio Immunoprecipitation Assay
PVDF
polyvinylidene difluoride
BSA
bovine serum albumin
BLCA
bladder urothelial carcinoma
CESC
cervical squamous cell carcinoma, endocervical adenocarcinoma
ESCA
esophageal carcinoma
SKCM
skin cutaneous melanoma
p-YAP
phosphorylated-YAP
ANKRD1
Ankyrin repeat domain containing 1
CYR61
cysteine-rich angiogenic inducer 61
CTGF
connective tissue growth factor

7. Declarations

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Contributions

We appreciate our colleagues for their valuable efforts on this article. Yiyun Huang, He Zha, and Lan Zhou designed the research; Lijun Hu and Lu Lin analyzed the data of the database; Yiyun Huang performed all the experiments; Yan Liu performed the xenograft model; Yiyun Huang, He Zha, and Lan Zhou wrote the manuscript.

Ethics declarations

Ethics approval and consent to participate

All procedures performed in this studies were approved by the Ethics Committee of the Chongqing Medical University.

Consent for publication

Not applicable.

Competing of Interest

The authors declare no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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9. Tables

Table I inhibitor/mimic and siRNA sequences

inhibitor/mimic and siRNA	Sequences (5'-3')
miR-24-3p inhibitor	CUGUCCUGCUGAACUGAGCCA
NC inhibitor	CAGUACUUUUGUGUAGUACAA
miR-24-3p mimic	Forward:UGGCUCAGUUCAGCAGGAACAG Reverse: GUUCCUGCUGAACUGAGCCAUU
NC mimic	Forward:UUCUCCGAACGUGUCACGUTT Reverse: ACGUGACACGUUCGGAGAATT
hs-AMOTL2-si-1	Forward:GAGAGAUUGGAAUCUGCAAdTdT Reverse:UUGCAGAUUCCAAUCUCUCdTdT
hs-AMOTL2-si-2	Forward:CAGUACCCUCAUGUUGUACdTdT Reverse: GUACAACAUGAGGGUACUGdTdT
hs-AMOTL2-si-3	Forward:GUCAGAACAAACUGCGAGAGdTdT Reverse: CUCUCGCAGUUGUUCUGACdTdT
NC	Forward:UUCUCCGAACGUGUCACGUdTdT Reverse: ACGUGACACGUUCGGAGAAAdTdT

Table II Primer sequences

Gene	Primer sequences (5'-3')	
miR-24-3p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACCTGTTC	
U6	RT: CGCTTCACGAATTTGCGTGTTCAT	
miR-24-3p	Forward: GCGTGGCTCAGTTCAGCAG	Reverse: AGTGCAGGGTCCGAGGTATT
U6	Forward: GCTTCGGCAGCACATATACTAAAT	Reverse: CGCTTCACGAATTTGCGTGTTCAT
ANKRD1	Forward: CACTTCTAGCCCACCCTGTGA	Reverse: CCACAGGTTCCGTAATGATTT
CTGF	Forward: AGGAGTGGGTGTGTGACGA	Reverse: CCAGGCAGTTGGCTCTAATC
CYR61	Forward: AGCCTCGCATCCTATAACAAC	Reverse: TTCTTTCACAA GCGGCACTC
AMOTL2	Forward: TAAAGGTGCTCCATGCCAG	Reverse: ATCTCTGCTCCCGTGTTTGG
GAPDH	Forward: CAGCGACACCCACTCCTC	Reverse: TGAGGTCCACCACCCTGT

Figures

Figure 1

The expression of miR-24-3p in various cancers and cervical cancer cells. (A) The tumor-miRNA-pathway analysis of miR-24-3p expression in 34 different kinds of cancer. (B) Relative mRNA levels of miR-24-3p in C33A, CaSki, and SiHa cells were measured by qRT-PCR. **p<0.01, ***p<0.001 vs C33A group.

Figure 2

Effect of miR-24-3p on the proliferation, migration, and invasion of CaSki and SiHa cell lines. (A and B) CaSki and SiHa cell lines were transfected with NC inhibitor, miR-24-3p inhibitor, NC mimic, and miR-24-3p mimic, respectively. Cell viability was evaluated using CCK8 assay at the indicated time points after transfection. (C, D, E, and F) Transwell migration and invasion assays were performed after indicated

transfection in CaSki and SiHa cell lines. The cell numbers of migration and invasion are shown in histograms. **p<0.01, ***p<0.001 vs. control groups.

Figure 3

miR-24-3p directly targeted AMOTL2 3'UTR and suppressed the protein expression of AMOTL2. (A) The Venn diagram indicates the numbers of target genes that overlapped in miRDB, targetscan, and miRtarbase. (B) AMOTL2 was the intersection between aforesaid overlap and predicted targets of

starbase (programNum7). (C)Upper panel: diagram of miR-24-3p putative seed sequences in the 3' UTR of AMOTL2 mRNA across Human, Chimp and Rhesus. Lower panel: a sketch of construction of wild-type or mutant pGL6-AMOTL2 3' UTR vectors. (D) Dual-luciferase reporter assay was used to analyze the effects of miR-24-3p expression on the activities of wild-type and mutant AMOTL2 3' UTR. (E, F, and G) protein or mRNA levels of AMOTL2 in CaSki and SiHa cells were monitored after miR-24-3p mimic or inhibitor transfection by qRT-PCR and Western blot. * $p < 0.05$, ** $p < 0.01$ vs. control groups.

Figure 4

AMOTL2 knockdown imitated the effects of miR-24-3p overexpression in vitro. (A) The inhibition effect of AMOTL2 siRNAs was verified by qRT-PCR. (B, C) The proliferation of CaSki cells transfected with specific siRNA3 (siAMOTL2) was determined with CCK8 assay (B), then the migration and invasion abilities were assessed using Transwell assays C). (D, E, F) CaSki cells were treated with the indicated reagents and cell viability was analyzed by CCK8 assays (D) and cell migration was determined by Transwell assays (E, F). **p<0.01, ***p<0.001 vs. control groups.

Figure 5

miR-24-3p inhibited the YAP/Hippo signaling pathway. (A) The Venn diagram indicates the numbers of targets that overlapped in the intersection of 95 target genes and cervical cancer pathogenic genes in Genecards. (B) The KEGG signaling pathway enrichment analysis of 36 target genes in figure A. (C) The CaSki and SiHa cells were transfected with miR-24-3p inhibitor or mimic for 48 h, and then p-

YAP(Ser127), YAP and β -actin levels were determined using Western blot. (D, E) The CaSki were transfected with miR-24-3p mimic (D) and SiHa cells were transfected with miR-24-3p inhibitor (E), relative mRNA levels of YAP target genes, CTGF, CYR61, and ANKRD1 were measured by qRT-PCR. * $p < 0.05$, ** $p < 0.01$ vs. control groups.

Figure 6

miR-24-3p promoted xenograft tumor growth in vivo. (A, B, C) Mice bearing CaSki of treatment with miR-24-3p antagomir subcutaneous xenografts were killed after 5 weeks (A). The tumor volumes(B) were monitored every week and tumor weights (C)were measured after the mice were sacrificed on day 35. (D) Paraffin sections were stained with hematoxylin and eosin or IHC with anti-AMOTL2, p-YAP(Ser127), and p-LATS1/2 antibodies, respectively. *p<0.05,***p<0.001 vs. control groups.

Figure 7

Prediction and analysis of LncRNAs regulating miR-24-3p. (A) The intersection of LncRNAs was predicted by starBase and DIANA in the Venn diagram. (B) Eight LncRNAs' names in the intersection. (C, D, and E) The different expression of LINC00662, NEAT1, and ILF3-AS1 in cervical cancer and normal tissues. (F) the survival curve of ILF3-AS1 in cervical cancer. * $p < 0.05$.

Supplementary Files

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