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MiR-195-5p inhibits the cell migration and invasion of cervical carcinoma through suppressing ARL2

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) have great effects on the progression of cervical cancer (CC). This study aimed to investigate the role of miR-195-5p in CC and to explain the regulatory mechanism between ARL2 and miR-195-5p.

PATIENTS AND METHODS: Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) was used to detect miR-195-5p levels in CC tissues and cell lines. Transwell assays for cell migration and invasion were also performed. A luciferase reporter assay was used to detect the direct target of miR-195-5p. The protein levels of ARL2 were measured by Western blot analysis.

RESULTS: In CC tissues and cell lines, miR-195-5p expression was decreased. Downregulation of miR-195-5p was associated with higher FIGO stage, deep stromal invasion, and lymph node metastasis. Moreover, over-expression of miR-195-5p inhibited cell migration and invasion in CC. Furthermore, it was observed that miR-195-5p directly targeted ARL2, which affected the suppressive effect of miR-195-5p in CC.

CONCLUSIONS: MiR-195-5p inhibited cell migration and invasion in CC by suppressing ARL2 expression. The miR-195/ARL2 axis may provide a pathway for cell metastasis in CC.

Key Words:
MiR-195-5p, Cervical carcinoma, Migration, Invasion, ARL2.

Introduction

Cervical cancer (CC) is a common gynecologic malignancy for women. The paroxysmal age of carcinoma *in situ* is 30-35 years old, and the invasive cancer is 45-55 years old[1]. In recent years, the incidence of CC has tended to become younger. Although the incidence and mortality of CC have decreased significantly with the widespread use of cervical cytology screening[2,3], some underdeveloped countries are still in urgent need due to underdeveloped medical conditions.

MicroRNAs (miRNAs) play a large role in regulating target gene expression at the post-transcriptional level through mRNA degradation or translation inhibition[4]. It has been reported[5-7] that miRNAs are important regulators of tumor metastasis, such as cell differentiation, migration and invasion. Previous studies have shown that downregulation of miR-195 can be used as a tumor suppressor in non-small cell lung cancer[8], breast cancer[9], colorectal cancer[10], esophageal squamous cell carcinoma[11], and prostate cancer[12]. Nevertheless, the explicit molecular mechanism of miR-195-5p remains ambiguous in CC. ADP-ribosylation factor-like 2 (ARL2) is a GTPase belonging to the ARF family and is involved in vesicle budding and membrane trafficking[13,14]. ARL2 has been shown to be upregulated in pancreatic cancer[15] and colon cancer[16]. In addition, Wang et al[17] have demonstrated that miR-16 directly targeted ARL2, and knockdown of ARL2 led to inhibition of cell proliferation. However, the role of ARL2 has not been elucidated in CC. Here, we found that miR-195-5p was a tumor suppressor in CC by regulating cell migration and invasion. Moreover, ARL2 was indicated as a direct target gene of miR-195-5p. MiR-195-5p showed its effect in CC through suppressing ARL2 expression. Briefly, miR-195/ARL2 axis may provide a pathway for the metastasis of CC cells.

Patients and Methods

Experimental Tissues and Cell Culture

Fifty-two CC tissues and paracancerous tissues were obtained from the First People's Hospital of Fuyang District. Human tissues

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were frozen in liquid nitrogen and stored in a -80°C refrigerator until use. The immortalized HPV-negative skin keratinocyte cells HaCaT and HeLa, SiHa CC cell lines were obtained from the Chinese Center for Type Culture Collection (Wuhan, China). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). This study experiment was approved by Institutional Ethics Committee of the First People's Hospital of Fuyang District.

Cell Transfection

MiR-195-5p mimic and inhibitor or ARL2 siRNA (Yearthbio, Changsha, China) were transferred into HeLa cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturers' protocols.

RNA Extraction and Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). QRT-PCR was performed on ABI PRISM 7500 Sequence Detection System (Thermo Fisher Scientific, Inc., Waltham, MA, USA) by SYBR Premix Ex Taq Master mix (TaKaRa Biotechnology Co, Ltd., Dalian, China) to detect miR-195-5p and ARL2 mRNA expressions. U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for miR-195-5p or ARL2. Expressions of miR-195-5p and ARL2 were analyzed using the $2^{-\Delta\Delta ct}$ method. The primer sequences were as follows: miR-195 forward, 5'-ACACTCCAGCTGGGTAGCAGCACAGAAAT-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; ARL2 forward, 5'-CGAGGATCCCCGGCCAAACTAACAC-3' and reverse, 5'-CGGAATTCGAGACAGCCAGGG CACAG-3'; and GAPDH forward, 5'-TGCACCACCAACTGCTTAGC-3' and reverse, 5'-GGCATG CACTGTGGTCATGAG-3'.

Dual-Luciferase Reporter Assay

ARL2-3'UTR-Wt or ARL2-3'UTR-Mut was inserted into the pGL3 promoter vector for luciferase reporter assay. The cells were then transfected with the vector and miR-195-5p mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After transfection for 24 h, luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA).

Transwell Assay for Migration and Invasion

Transwell chambers (Corning, Corning, NY, USA) were used to assess the migratory and invasive ability of HeLa cells. 5×10^4 transfected cells without FBS were placed in the uncoated top chamber, and the lower chamber was filled with 20% FBS to induce transfected cells to induce migration or invasion of the transfected cells. The cells were then placed in the upper chamber along with the coated membrane for invasion assay. These cells were incubated for migration and invasion assays. The cells were stained with crystal violet (Beyotime Institute of Biotechnology, Shanghai, China).

Western Blot

Protein samples were obtained using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were separated by 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then incubated with 5% skim milk-blocked membranes at room temperature. Next, we incubated membranes with anti-ARL2, anti-GAPDH antibodies overnight at 4°C and then incubated with matched secondary antibodies for 48 h. Finally, protein expression levels were measured by a FluorChem imaging system (Alpha Innotec, San Leandro, CA, USA).

Statistical Analysis

Statistical analysis was analyzed using GraphPad Prism 6.0 (La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD (Standard Deviation). Differences between two groups were calculated by the Student's *t*-test or One-Way ANOVA with Bonferroni post-hoc test. Differences were considered to be statistically significant at $p<0.05$.

Results

MiR-195-5p Levels were Downregulated in CC

To verify alternation of miR-195-5p expression in CC, miR-195-5p expressions were identified in CC tissues and cell lines. And compared with the control group, miR-195-5p expressions were

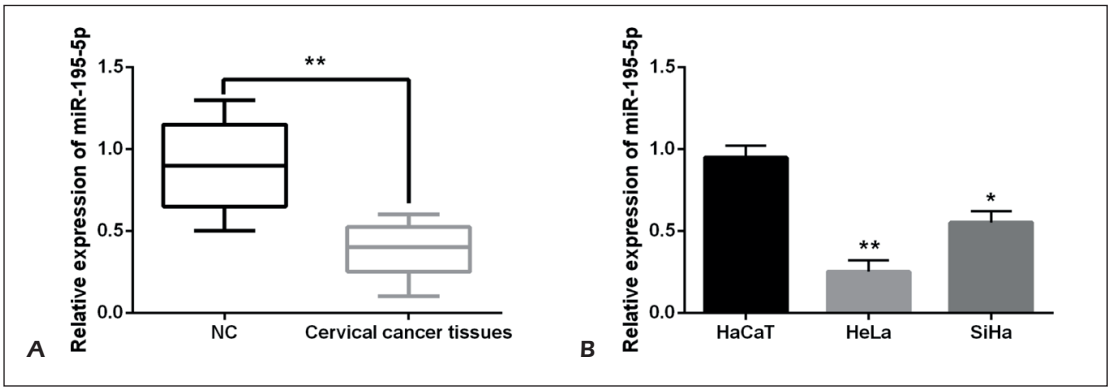

Figure 1. MiR-195-5p was downregulated in human CC tissues and cell lines. **A**, MiR-195-5p expressions were decreased in CC tissues in comparison with the control. **B**, MiR-195-5p levels were decreased in CC cell lines. *p<0.05; **p<0.01.

found to be reduced in CC tissues (p<0.01; Figure 1A). Simultaneously, expressions of miR-195-5p were reduced in HeLa and SiHa cells compared with HaCaT cells (p<0.01; Figure 1B). Furthermore, the expression of miR-195-5p was lower in HeLa cells than that in SiHa cells. Therefore, the HeLa cell line was selected for further study. Additionally, correlations between miR-195-5p expressions and clinicopathological characteristics were detected in 52 CC patients. These patients were divided into two categories (high and low) based on the median expression of miR-195-5p (median, 1.388). We found that low miR-195-5p expression was associated with higher FIGO stage, deep stromal invasion, and lymph node metastasis (Table I). These findings reflected that down-regulation of miR-195-5p may affect metastasis and invasion of CC cells.

MiR-195-5p Inhibited Cell Migration and Invasion in CC

Next, we performed transwell assays to identify the function of miR-195-5p in CC. Expressions of miR-195-5p were observed in HeLa cells with miR-195-5p mimics or inhibitor (p<0.01; Figure 2A, 2B). Moreover, overexpression of miR-195-5p significantly reduced cell migration, while knockdown of miR-195-5p increased cell migration (p<0.01; Figure 2C). The same results as migration were also found in the cell invasion assay (p<0.01; Figure 2D). It was speculated that miR-195-5p suppressed migration and invasion of CC cells.

MiR-195-5p Directly Targeted ARL2 In Vitro

To further investigate the role of miR-195-5p in CC, potential target genes of miR-195-5p were searched by bioinformatics analysis. It predicted that ARL2 was a target gene of miR-195-5p (Figure 3A). Dual-luciferase reporter assay indicated that luciferase activities of wild ARL2 were significantly suppressed by miR-195-5p mimics, whereas luciferase activities of mutant ARL2 were not affected by miR-195-5p mimics (p<0.01; Figure 3B). Furthermore, a decrease in ARL2 expression was identified in HeLa cells with miR-195-5p mimics, and an increased ARL2 expres-

Table I. Relationship between miR-195-5p expression and their clinicopathological characteristics in 52 cervical cancer patients.

Clinicopathological Characteristics	miR-195-5p		*p*-value
	High (n=20)	Low (n=28)	
Age (years)			0.2482
≥ 35	15	19	
< 35	5	13	
FIGO stage			0.006*
IB	13	28	
> IB	7	4	
Tumor size (cm)			0.0768
≥ 4	12	24	
< 4	8	8	
Degree of differentiation			0.0683
Well	11	22	
Moderate and Poor	9	10	
Stromal invasion			0.0012*
< 2/3	8	17	
≥ 2/3	12	15	
Lymph-node metastasis			
Yes	14	25	0.006*
No	6	7	

*p<0.05 was considered significant.

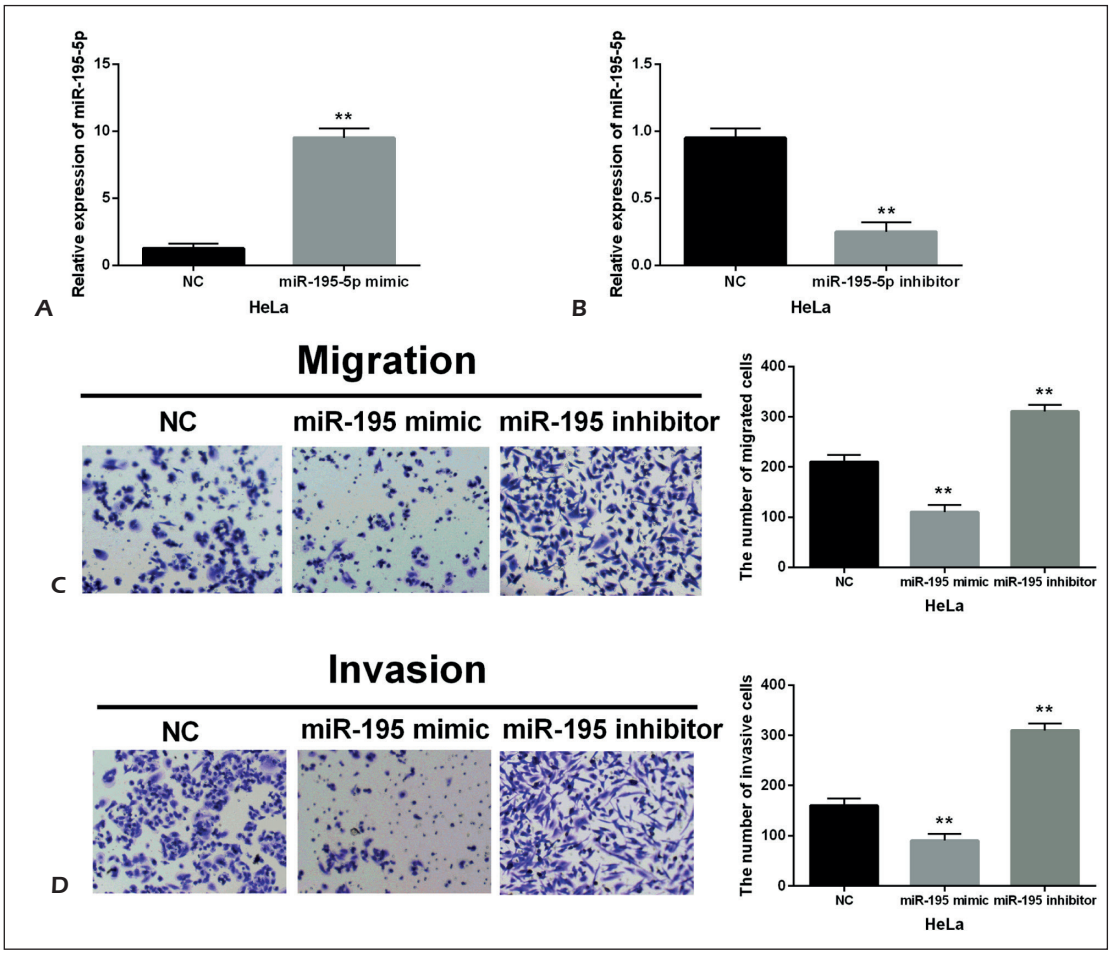

Figure 2. Tumor-suppressing effect of miR-195-5p was identified in CC. **A-B**, MiR-195-5p mimic or in-hibitor was transfected into HeLa cells, and then miR-195-5p level was detected via qRT-PCR. **C-D**, Abnormal miR-195-5p expression regulated migratory and invasive abilities in HeLa cells. ***p*<0.01 (magnification: 40×).

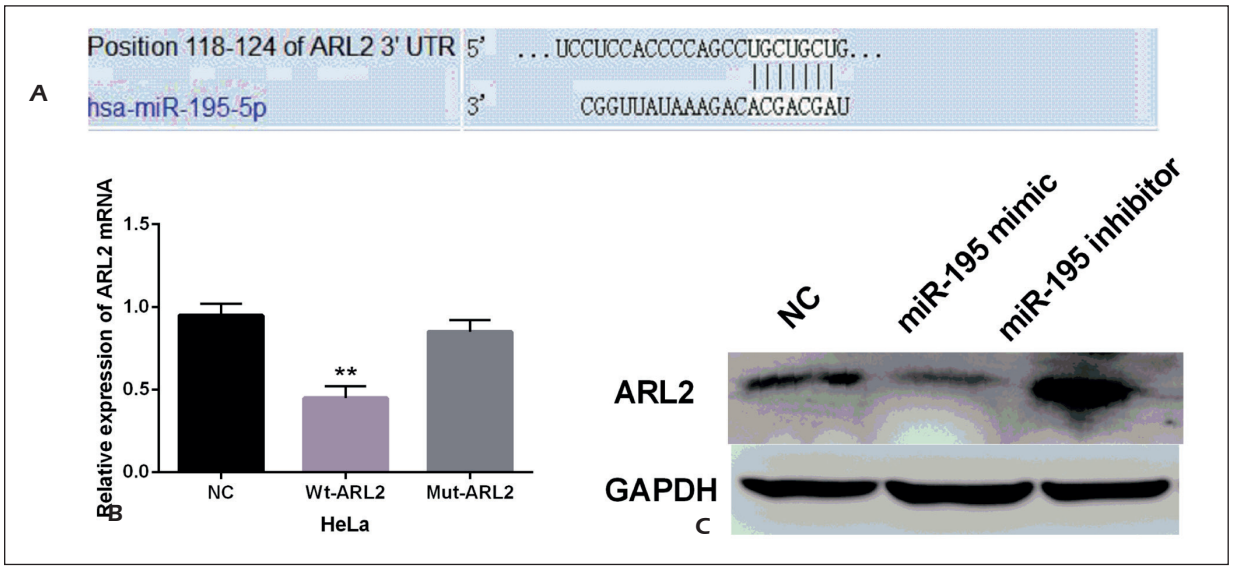

Figure 3. MiR-195-5p directly targeted ARL2 in CC cells. **A**, The binding sites between ARL2 and miR-195-5p. **B**, Luciferase reporter assay. **C**, Protein expressions of ARL2 in cells containing miR-195-5p mimic or inhibitor. ***p*<0.01.

sion was found in cells with miR-195-5p inhibitor (p<0.01; Figure 3C). All these results suggested that miR-195-5p directly targeted ARL2.

ARL2 was Upregulated and Promoted Metastasis in CC

Afterwards, ARL2 siRNA was transfected into HeLa cells to confirm the function of ARL2 in CC. We found that ARL2 expression was decreased compared to the control in cells with ARL2 siRNA (p<0.01; Figure 4A). In addition, ARL2 was upregulated in HeLa and SiHa cells compared to HaCaT cells (p<0.01; Figure 4B). More importantly, ARL2 siRNA was found to inhibit cell migration and invasion, which was similar to miR-195-5p overexpression (p<0.01; Figure 4C, 4D). These findings reflected that ARL2 had a promoting effect on the tumorigenesis of CC.

ARL2 Affected the Tumor-Suppressive Function of miR-195-5p

To verify whether upregulation of ARL2 affects the inhibitory effect of miR-195-5p on cell migration and invasion, ARL2 expression vector was transfected into HeLa cells containing miR-195-5p mimics. Furthermore, mRNA and protein expressions of ARL2 were enhanced by transfection of ARL2 expression vector (Figure 5A, 5B). Besides that, overexpression of ARL2 significant-

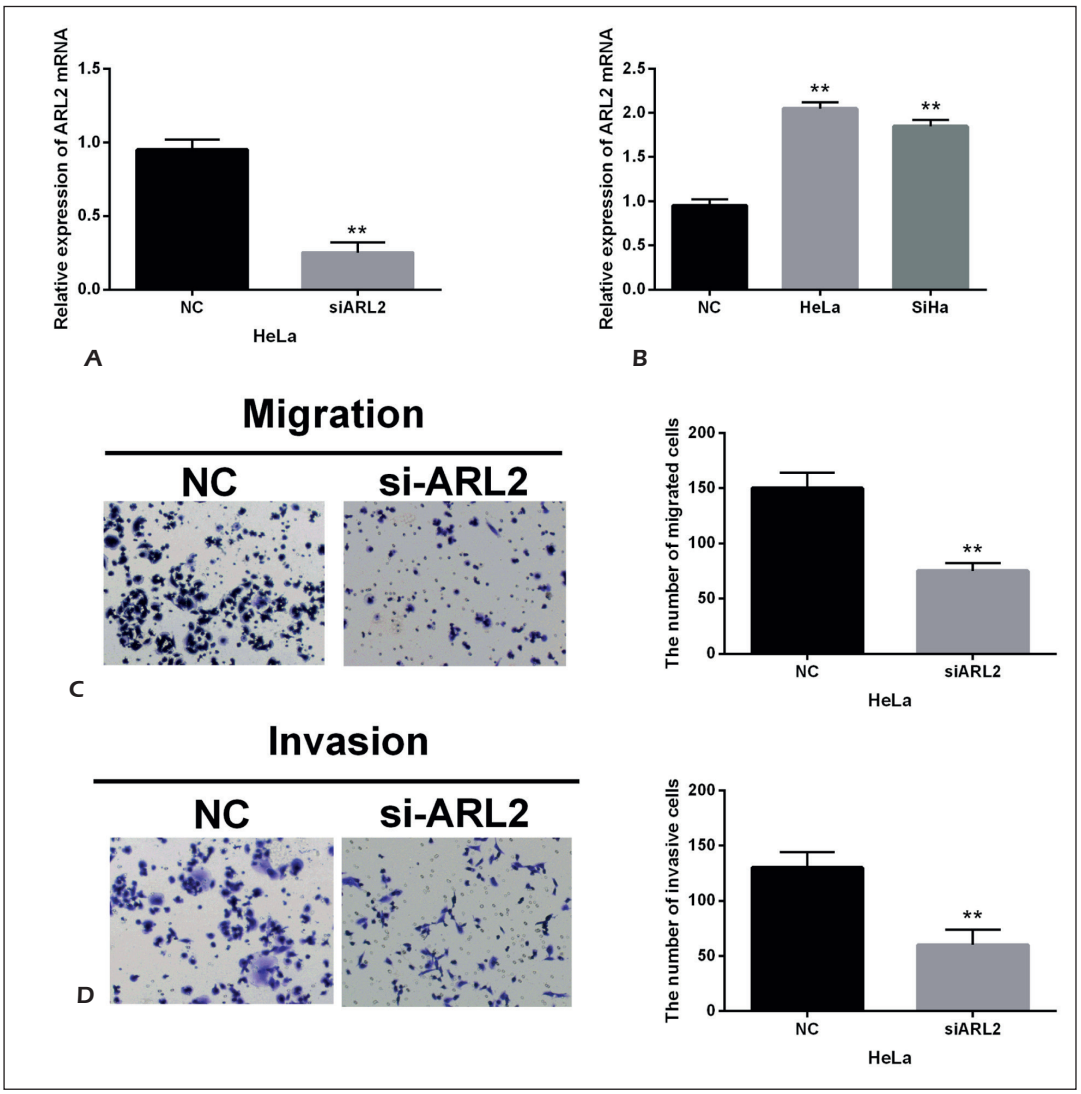

Figure 4. ARL2 siRNA inhibited cell migration and invasion in CC. **A**, ARL2 siRNA suppressed ARL2 expression. **B**, ARL2 expression in CC cell lines was detected by qRT-PCR. **C-D**, Transwell assays in cells with ARL2 siRNA. **p<0.01. (magnification: 40×).

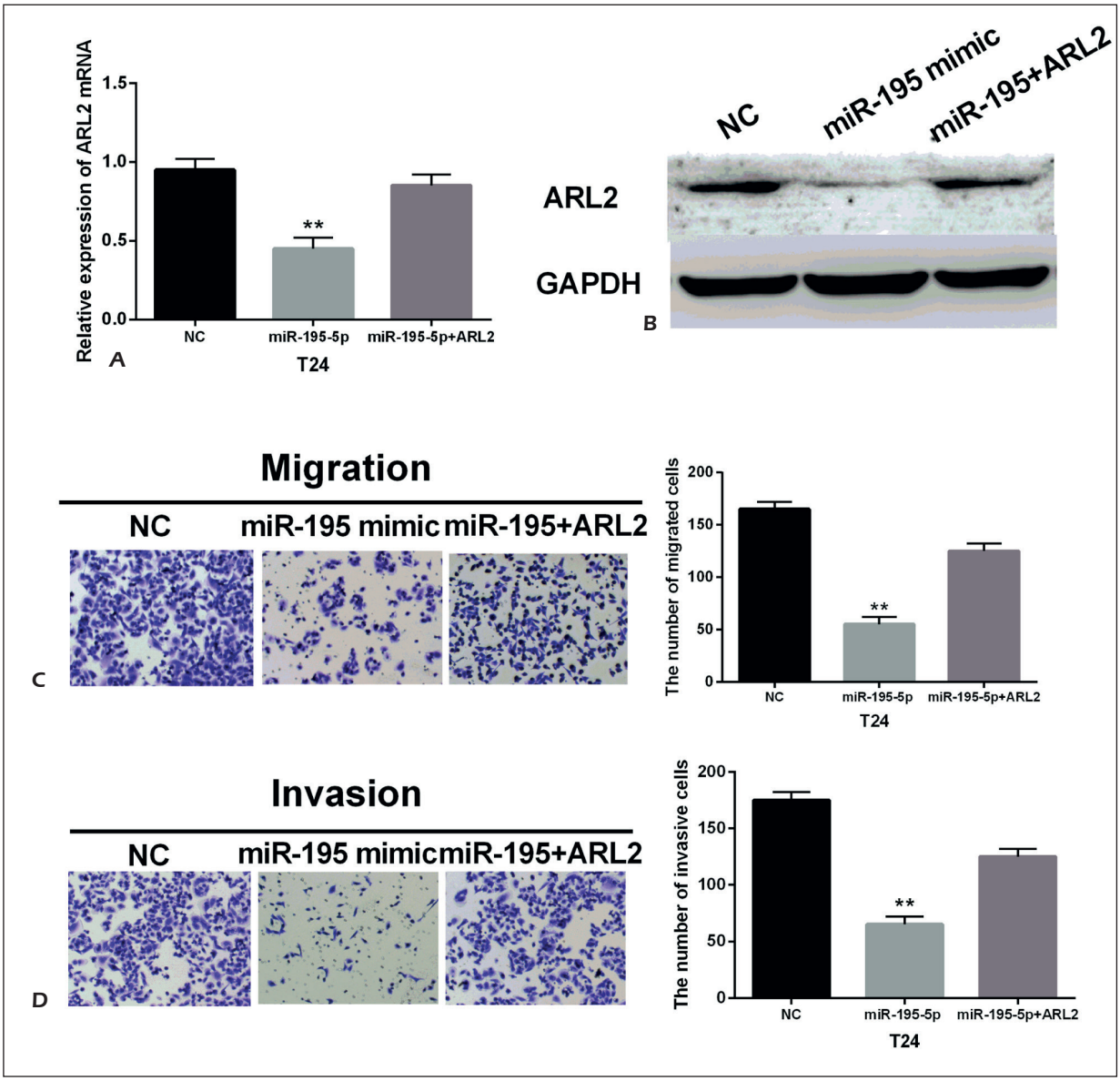

Figure 5. ARL2 affected the tumor-suppressive function of miR-195-5p. **A**, The relative mRNA expression of ARL2 in HeLa cells with miR-195 mimics and ARL2 expression vector. **B**, The protein level in HeLa cells contained miR-195-5p and ARL2 vector. **C-D**, Transwell assay for cell migration and invasion was conducted in cells containing miR-195-5p and ARL2 expression vector (magnification: 40×) $^{**}p<0.01$.

ly rescued tumor suppressive effect of miR-195-5p on cell migration and invasion in CC (Figure 5C, 5D). The observations suggested that overexpression of ARL2 attenuated the inhibitory effect of miR-195-5p in CC.

Discussion

Increasing evidence[18,19] showed that abnormal expression of miRNAs is beneficial to tumor formation and can be used as a biomarker for prediction and prognosis of CC. Although cancer treatments have improved significantly, many patients who have experienced early cancer metastasis generated poor prognosis after surgery[20]. Therefore, miRNAs that affect cell metastasis and invasion can be used as biomarkers for the diagnosis of CC. In the current study, the expression level of miR-195-5p was decreased in CC. Low miR-195-5p expression was dramatically associated with deep stromal invasion and lymph node metasta-

sis. More importantly, miR-195-5p could inhibit cancer metastasis and ARL2 expression. In addition, ARL2 could reverse the inhibitory effect of miR-195-5p in CC. These findings demonstrated that miR-195-5p suppressed cell migration and invasion in CC through inhibiting ARL2 expression. Abnormal expression of miR-195 had been identified in many cancers and affected the development of cancer. For example, miR-195 induced cell apoptosis by targeting ARL2 in human embryonic stem cell-derived neural progenitor cells[21]. In addition, Li et al[22] proved that miR-195 inhibited proliferation of CC cells by suppressing cyclin D1. Zhou et al[23] identified that miR-195 suppressed cell migration and invasion through targeting Smad3 in CC. Our study showed the same results as previous studies, in which miR-195-5p was an inhibitory miRNA and contributed to metastasis in CC. Although some potential targets have been reported, molecular mechanisms of miR-195 remain unknown to some degree. In our study, miR-195-5p directly targeted ARL2 and inhibited its expression by binding to the 3'-UTR of ARL2 in CC. It had also been found that suppression of ARL2 inhibited cells apoptosis in breast cancer[24]. Additionally, knockdown of ARL2 was identified to reduce cell proliferation[25]. Zhou et al[23] demonstrated that miR-195 targeted ARL2 to induce apoptosis in human embryonic stem cell-derived neural progenitor cells. In our study, upregulation of ARL2 abolished the inhibitory effect of miR-195-5p in CC. Same as our findings, Peng et al[26] proposed that miR-214 inhibited ARL2 expression and suppressed growth and invasion of CC cells. However, how ARL2 affects cell metastasis in CC was not investigated in previous studies. Here, we paid attention to the effect of ARL2 on cell migration and invasion in CC.

Conclusions

Briefly, miR-195-5p was downregulated and inhibited cell migration and invasion in CC. Moreover, miR-195-5p directly targeted ARL2. Upregulation of ARL2 weakened the inhibitory effect of miR-195-5p in CC. Our findings will provide a theoretical basis for the treatment of CC.

Conflict of Interests

The authors declare that they have no conflict of interest.

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