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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 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, and affected the suppressive effects of miR-195-5p on 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 targeted 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 differentiation, migration and invasion. Studies have shown that down-regulation 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). C PCR was performed on ABI PRISM Sequence Detection System (Thermo Fisher entific, Inc., Waltham, MA, USA) by SYBR mix Ex Taq Master mix (TaKaRa Biotechnolo Co, Ltd., Dalian, China) to dete 5-5p an ARL2 mRNA expressions. gly dehyde 3-phosphate dehydrogenase PDH as used as a control for miR-19 or of miR-195-5p and 2 were zed using the $2^{-\Delta\Delta ct}$ method mer sequ were as follows: miR-19 orw 5'-ACAC CAGCT-GGGTAGC ACAGA 3' and reverse, 5'-TGGT GTGGAGTC U6 forward, 5'-CTC GCAGCACA and reverse, 5'-AACGC GAA TTGCGT-3'; ARL2 5'- CCGGCCAAACTA- and 5'-CGGAATTCGAGA- AG GGG C AG-3'; and GAPDH for- ACCAACTGCTTAGC-3' and ATG CACTGTGGTCATGAG-3'.

Dua iferase 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 migrat va- sive ability of HeLa cells. 5×10^4 without FBS were placed in the ted top ch ber, and the lower chamber wa with 2 FBS to induce transfected to i nigra or invasion of the trans cells. T then placed in the u han along the coated membran for ssay. These cells were incubated nigrat in n assays. The cells w ned with olet (Beyotime Insti hnology, nghai, China).

W Blot

ein samples we obtained using radioin oprecipita on assay (RIPA) buffer (Beyo Institut Biotechnology, Shanghai, Chi ere separated by 10% Sodium Dodecy ate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then incubated with milk-blocked membranes at room temp e. 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

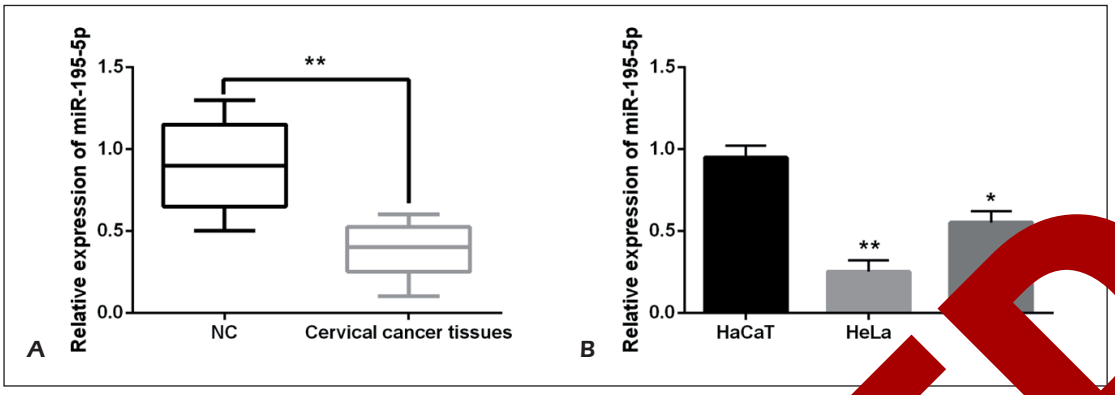

Figure 1. MiR-195-5p was downregulated in human CC tissues and cell lines. **A**, MiR-19 pres were d in CC tissues in comparison with the control. **B**, MiR-195-5p levels were decreased in CC ell , **p<0.0

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-195-5 pressions and clinicopathological characte were detected in 52 CC patients. These pat were divided into two categories (high and l based on the median expression f miR-195- (median, 1.388). We found th iR-195 5p expression was associat th h r FIGO stage, deep stromal invasi d h node metastasis (Table I). Th fin t down-regulation of -195-5p ffect me- tastasis and inva C cells.

MiR-195-5 hibited Migrati nd Invasion

Next, rf d transwe assays to identify the func iR-19 -5p in CC. Expressions 195-5p ed in HeLa cells with p mi nhibitor ($p<0.01$; Figure A, 2 Moreover, verexpression of miR-195-5p i duced cell migration, while knock- 95-5p increased cell migration (p Figure 2C). The same results as migration were ound 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 o natics ana s. It predicted that A 2 was a t ene of miR-195-5p (Figure Dual-lucife porter assay indicated luciferase activ s of wild ARL2 were si antly sup essed by miR-195-5p mimics, wh lucifer activities of mutant ARL2 were y miR-195-5p mimics ($p<0.01$; Figure hermore, a decrease in ARL2 expression was identified in HeLa cells with miR- imics, 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.

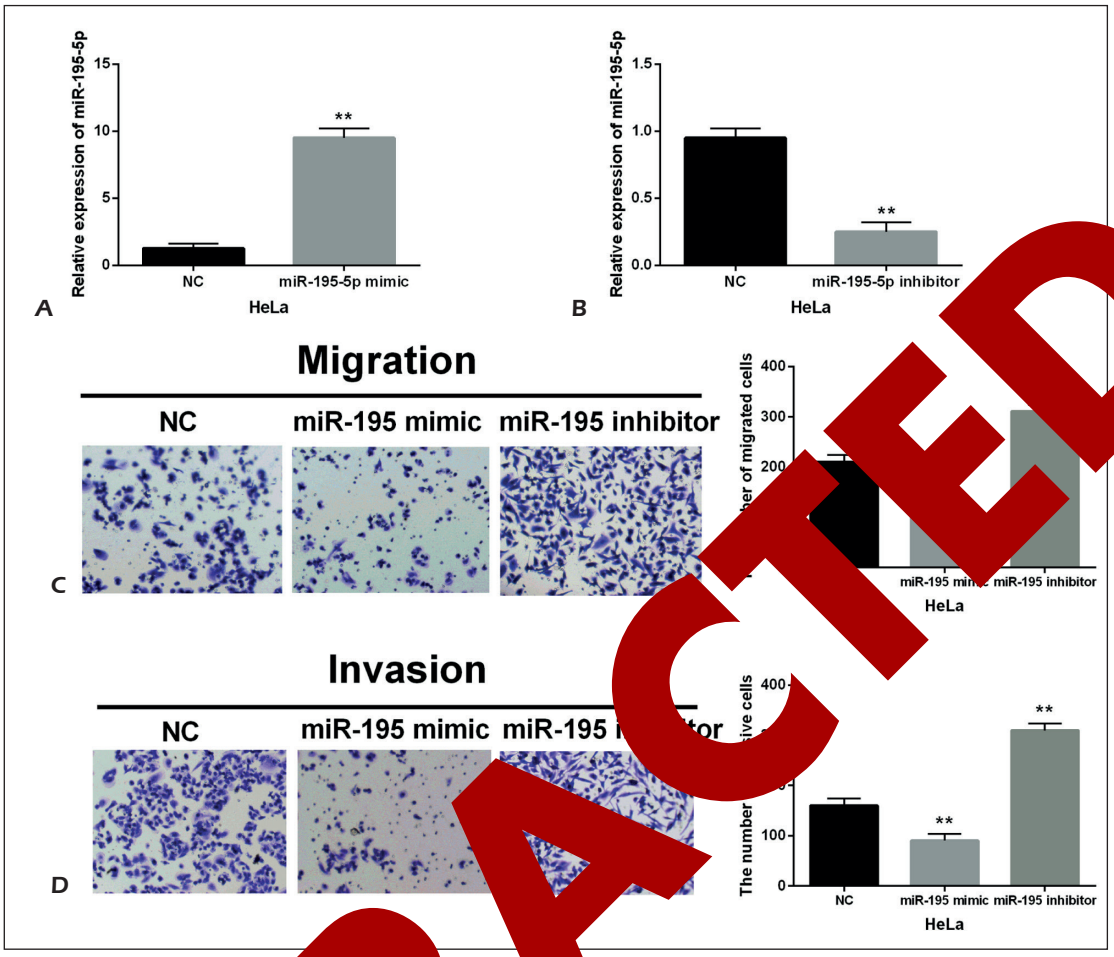

Figure 2. Tumor-suppressing ef miR- p was ide d in CC. **A-B**, MiR-195-5p mimic or in-hibitor was transfected into HeLa cells, and then miR lev detected a qRT-PCR. **C-D**, Abnormal miR-195-5p expression regulated migratory and invasive abili in H agnification: 40×).

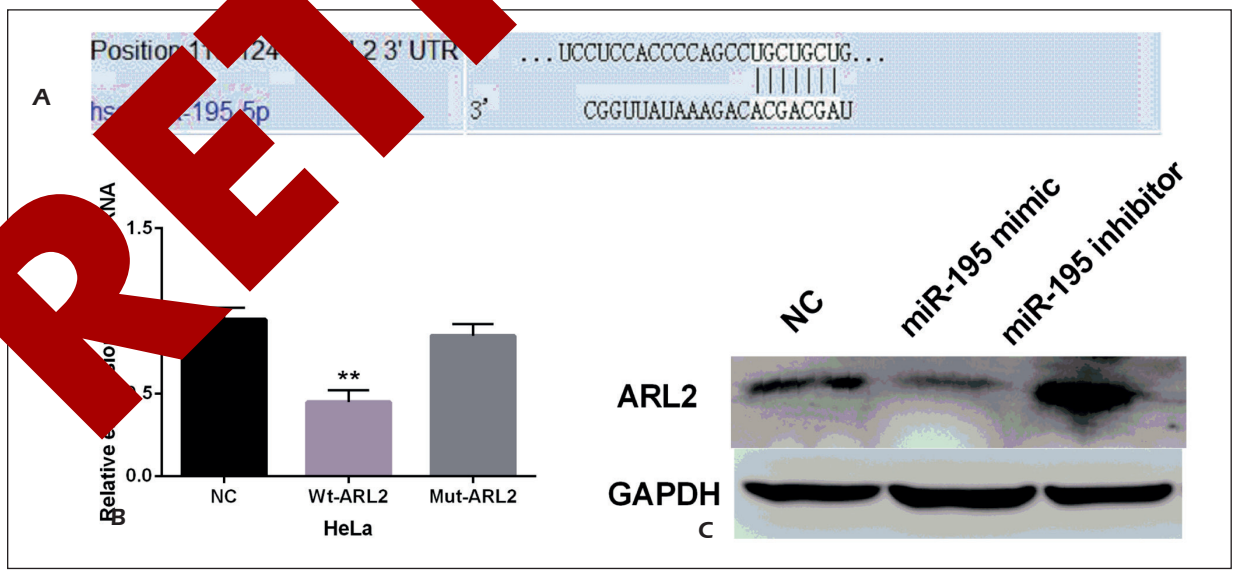

Figure 3. MiR-195-5p directly targeted ARL2 in CC cells. **A**, The binding sites between ARL2 and miR-195-5p. **B**, Luciferase reporter assay. **C**, Protein expressions of ARL2 in cells containing miR-195-5p mimic or inhibitor. **$p<0.01$.

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 [illegible] affects the inhibitory effect of miR[illegible] migration and invasion, ARL2 [illegible]ession ve[illegible] was transfected into HeLa cells [illegible]ining m[illegible] 195-5p mimics. Furtherm[illegible] mR[illegible] pro[illegible] expressions of ARL2 [illegible] enhanced [illegible] tion of ARL2 expre[illegible]ect[illegible] Figure [illegible]B). Besides that, ove[illegible]pre[illegible] RL2 signi[illegible]cant-

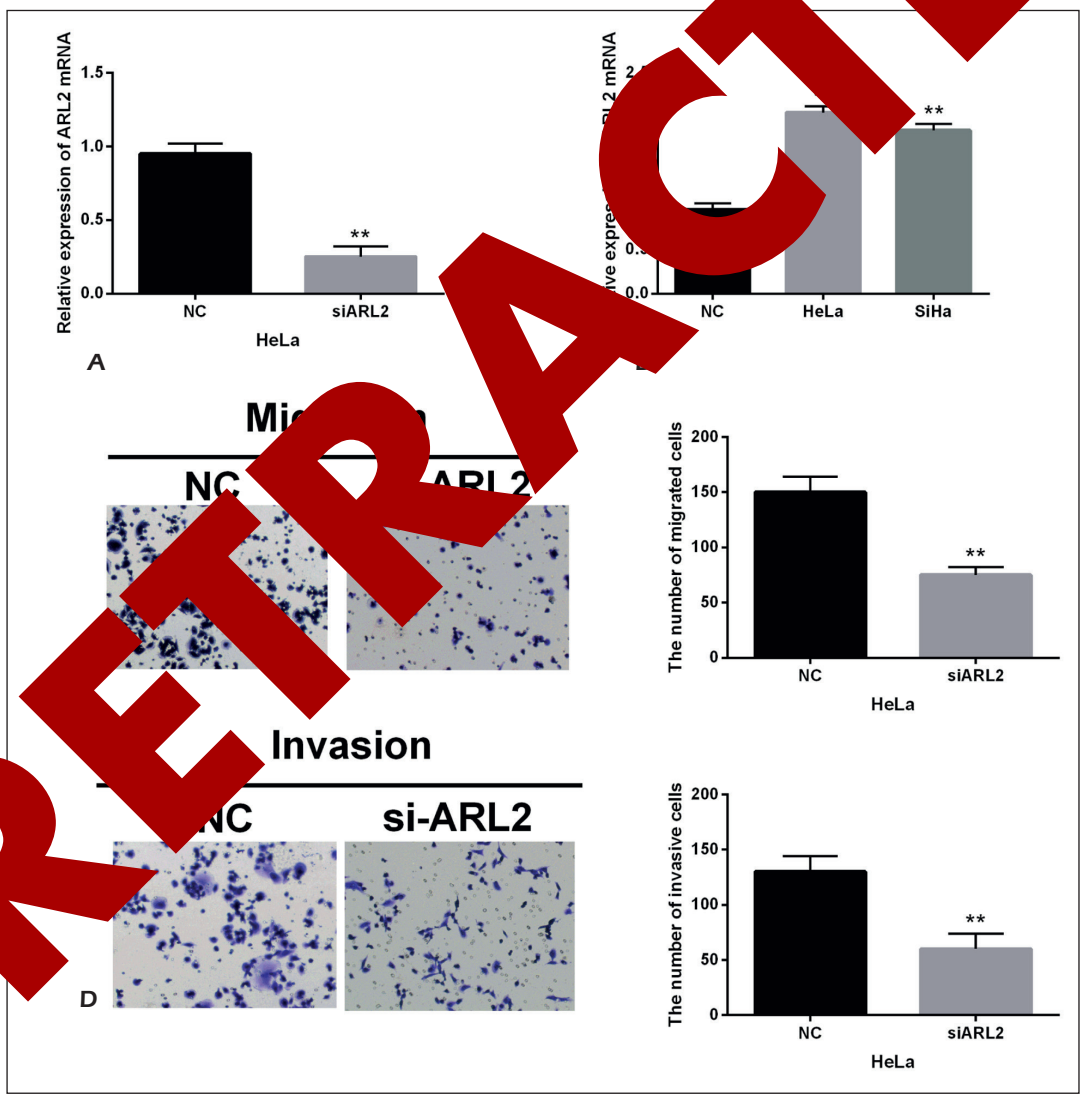

Figure 4. ARL2 siRNA inhibited cell migration and invasion in CC. **A**, ARL2 siRNA suppressed ARL2 expression. **B**, ARL2 expression in CC cell lines was detected by qRT-PCR. **C-D**, Transwell assays in cells with ARL2 siRNA. **p<0.01. (magnification: 40×).

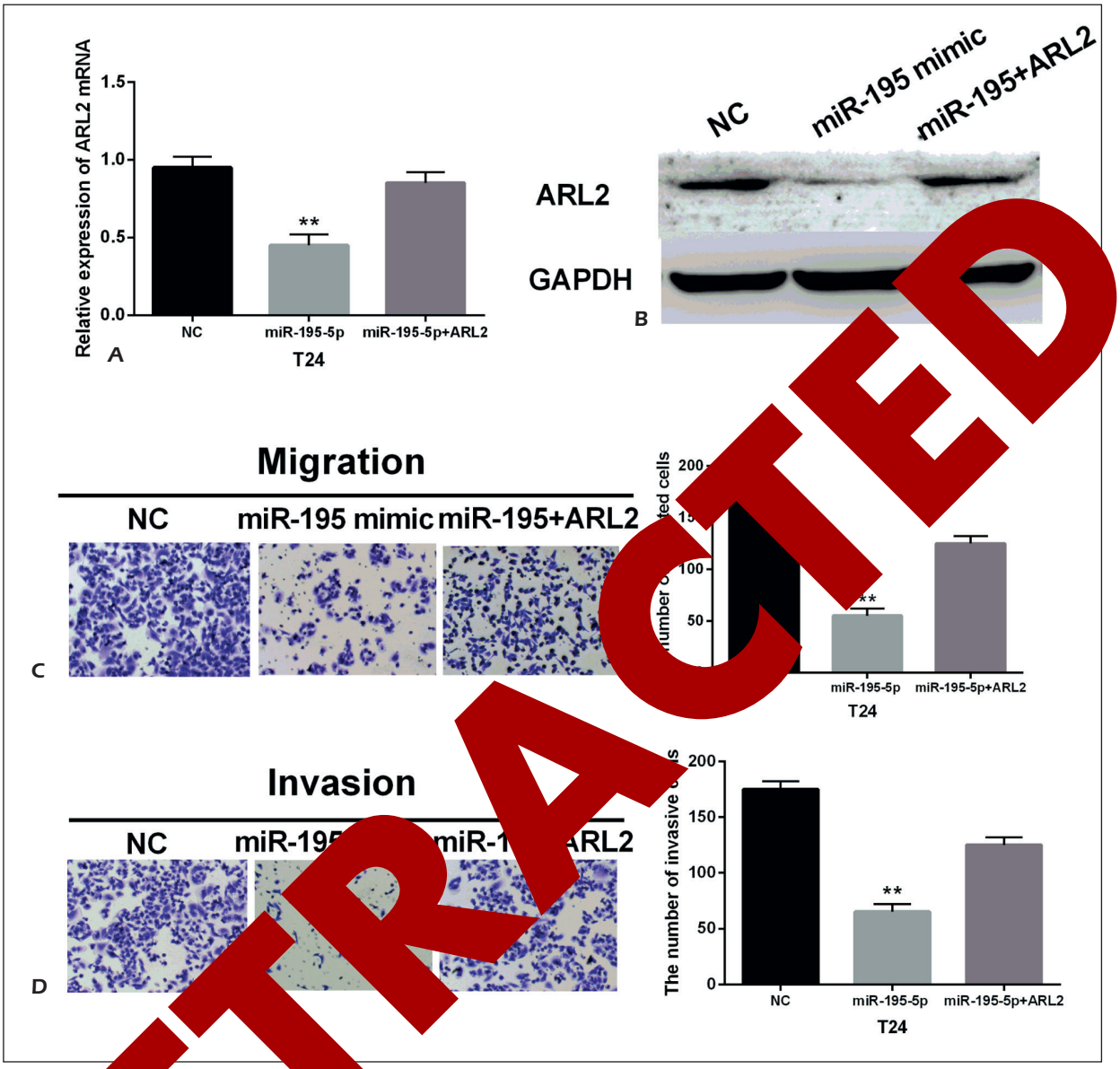

Figure 5 ...2 aff...d the tumor ...pressive function of miR-195-5p. **A**, The relative mRNA expression of ARL2 in HeLa cells with ... ...s and ARL2 expression vector. **B**, The protein level in HeLa cells contained miR-195-5p and ARL2 vector. **C-D**, T... assay... cell migration and invasion was conducted in cells containing miR-195-5p and ARL2 expres-... (magn... ...r ... **$p<0.01$.

...suppressive effect of miR-195-... ...ation and invasion in CC (Figure 5C... The observations suggested that overexpress... 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 ap sis in breast cancer[24]. Additionally, knock of ARL2 was identified to reduce cell pro ation[25]. Zhou et al[23] demonstrated that miR- targeted ARL2 to induce apoptosi human e bryonic stem cell-derived neur tor cell In our study, upregulation ARL olished the inhibitory effect of mi 5p i Same as our findings, Peng [26] p 214 inhibited ARL pression uppressed growth and inva C cells. r, how ARL2 affects ce net in CC s not investigated i vious st Here, we paid attention t effect of AR cell migration and inva

Conclusions

195-5p was downregulated and in cell migration and invasion in CC. More 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.

References

1) Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

2) Zhou J, Liu X, Wang CH, Wang D, Du JJ. Decreased expression of miR-1254 is associated with cancer aggressiveness and p poor outcome in cervical cancer. Eur r-macol Sci 2018; 22: 2997-300

3) Castellsague X, Diaz M, de Sa Munoz N, rero R, Franceschi S, Peeling RW, R, Smith- Snijders PJ, Meijer CJ, B EX. W e hu papillomavirus etiolo ervical ad and its cofactors: ation r scr and prevention. J Natl C 06; 98: 3 315.

4) Wang Z, Ca n L, Cai pregulated expressio croRNA-2 tumor progressio erse progn ediatric osteosarcon ed od Cance 014; 61: 206-210.

5) A ni IA, Ras Nikolova DA, Leupold JH, rn NH, Post gayer H. Mi-croRNA-21 R-21) post-transc nally downregulates tumor suppressor Pdcd4 and stimulates invasion, avasation metastasis in colorectal cancer. gene 27: 2128-2136.

6) Prabhala H, Pan E, Mestdagh P, Muth eruya-Feldstein J, Reinhardt F, Onder TT, Valastyan S, Westermann F, Speleman F, Vandesompele Weinberg RA. miR-9, a MYC/MYCN-activated oRNA, regulates E-cadherin and cancer metastasis. Nat Cell Biol 2010; 12: 247-256.

7) Wang X, Shi Z, Liu X, Su Y, Li W, Dong H, Zhao L, Li M, Wang Y, Jin X, Huo Z. Upregulation of miR-191 promotes cell growth and invasion via targeting TIMP3 in prostate cancer. J BUON 2018; 23: 444-452.

8) Li D, Zhao Y, Liu C, Chen X, Qi Y, Jiang Y, Zou C, Zhang X, Liu S, Wang X, Zhao D, Sun Q, Zeng Z, Dress A, Lin MC, Kung HF, Rui H, Liu LZ, Mao F, Jiang BH, Lai L. Analysis of MiR-195 and MiR-497 expression, regulation and role in breast cancer. Clin Cancer Res 2011; 17: 1722-1730.

9) Wang X, Wang J, Ma H, Zhang J, Zhou X. Down-regulation of miR-195 correlates with lymph node metastasis and poor prognosis in colorectal cancer. Med Oncol 2012; 29: 919-927.

10) Liu B, Qu J, Xu F, Guo Y, Wang Y, Yu H, Qian B. MiR-195 suppresses non-small cell lung cancer by targeting CHEK1. Oncotarget 2015; 6: 9445-9456.

11) Fu MG, Li S, Yu TT, Qian LJ, Cao RS, Zhu H, Xiao B, Jiao CH, Tang NN, Ma JJ, Hua J, Zhang WF, Zhang HJ, Shi RH. Differential expression of miR-195 in esophageal squamous cell carcinoma and miR-195 expression inhibits tumor cell proliferation and invasion by targeting of Cdc42. FEBS Lett 2013; 587: 3471-3479.

12) Cai C, Chen QB, Han ZD, Zhang YQ, He HC, Chen JH, Chen YR, Yang SB, Wu YD, Zeng YR, Qin GQ, Liang YX, Dai QS, Jiang FN, Wu SL, Zeng GH, Zhong WD, Wu CL. miR-195 inhibits tumor progression by targeting RPS6KB1 in human prostate cancer. Clin Cancer Res 2015; 21: 4922-4934.

13) Kahn RA, Volpicelli-Daley L, Bowzard B, Shrivastava-Ranjan P, Li Y, Zhou C, Cunningham L. Arf family GTPases: roles in membrane traffic and microtubule dynamics. Biochem Soc Trans 2005; 33: 1269-1272.

14) Gillingham AK, Munro S. The small G proteins of the Arf family and their regulators. Annu Rev Cell Dev Biol 2007; 23: 579-611.

15) Taniuchi K, Iwasaki S, Saibara T. BART inhibits pancreatic cancer cell invasion by inhibiting ARL2-mediated RhoA inactivation. Int J Oncol 2011; 39: 1243-1252.

16) Long LM, He BF, Huang GQ, Guo YH, Liu YS, Huo JR. microRNA-214 functions as a tumor suppressor in human colon cancer via the suppression of ADP-ribosylation factor-like protein 2. Oncol Lett 2015; 9: 645-650.

17) Wang K, Li P, Dong Y, Cai X, Hou D, Guo J, Yin Y, Zhang Y, Li J, Liang H, Yu B, Chen J, Zen K, Zhang J, Zhang CY, Chen X. A microarray-based approach identifies ADP ribosylation factor-like protein 2 as a target of microRNA-16. J Biol Chem 2011; 286: 9468-9476.

18) Luo S, Li N, Yu S, Chen L, Liu C, Rong J. MicroRNA-92a promotes cell viability and invasion in cervical cancer via directly targeting Dickkopf-related protein 3. Exp Ther Med 2017; 14: 1227-1234.

19) Li S, Yang F, Wang M, Cao W, Yang Z. miR-378 functions as an onco-miRNA by targeting ST7L/Wnt/beta-catenin pathway in cervical cancer. Int J Mol Med 2017; 40: 1047-1056.

20) Chen B, Zhang C, Dong P, Guo Y, Mu N. Molecular regulation of cervical cancer growth and inva-sion by VEGFa. Tumour Biol 2014; 35: 11587-11593.

21) Zhou Y, Jiang H, Gu J, Tang Y, Shen N, Jin Y. MicroRNA-195 targets ADP-ribosylation factor-like protein 2 to induce apoptosis in human embryonic stem cell-derived neural progenitor cells. Cell Death Dis 2013; 4: e695.

22) Li Z, Wang H, Wang Z, Cai H. Mi[illegible]bits the proliferation of human cerv[illegible]s by directly targeting cyclin D1[illegible]our Biol [illegible] 37: 6457-6463.

23) Zhou Q, Han LR, Zhou Y[illegible]i Y. M[illegible] suppre es cervical cancer mi[illegible]n and in[illegible] thr targeting Smad3. I[illegible]ynecol Canc[illegible]6: 817-824.

24) Beghin A, Ma[illegible]EL, [illegible]anqua[illegible]S, Dumontet C. Expres[illegible]f Arl2[illegible]soc[illegible] with p53 localizati[illegible] chemosen[illegible] breast cancer c[illegible]Cycle 200[illegible]074-3082.

25) Be[illegible] X, Hon[illegible] Messana C, Matera EL, Aim [illegible]chon S, [illegible]r D, Dumontet C. ADP [illegible]sylation factor [illegible]Arl2) protein influences [illegible]crotubule dynamics [illegible] breast cancer cells. Exp [illegible]ll Res 200[illegible]313: 473-485.

26) [illegible] R, Me[illegible]Ma R, Wang Q, Wang Y, Sun [illegible]214 down-regulates ARL2 and su[illegible] growth and invasion of cervical cancer cells. Biochem Biophys Res Commun [illegible]017; 484: 623-630.