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# MiR-155 Promotes Proliferation and Epithelial-mesenchymal Transition (EMT) and Inhibits Apoptosis of Cervical Cancer Cells via Regulating ING4

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**KEYWORDS** Apoptosis. Epithelial-mesenchymal Transition. ING4. miR-155. Proliferation

**ABSTRACT** Cervical cancer, the fourth common gynecologic malignancy, causes huge menace to female health. This work studied the role of miR-155 and ING4 in changes in proliferation, and apoptosis in epithelial–mesenchymal transition (EMT) of cervical cancer cells. RNA levels of miR-155 and ING4 were assessed with RT-qPCR. MiR-155 inhibition and ING4 overexpression were achieved through transfection methods, whose expressions were evaluated by RT-qPCR. CCK-8 and flow cytometry measured cell viabilities and apoptosis, respectively. RT-qPCR was also implemented for examining expressions of E-cadherin, Snail and N-cadherin. MiR-155 was highly expressed in cervical cancer cells while its suppression retarded cell viabilities and EMT but enhanced the apoptosis. In contrast, ING4 expressions were significantly decreased in cervical cancer cells. Overexpression of ING4 blocked cell viabilities and EMT but promoted apoptosis. Moreover, overexpressed ING4 sharply inhibited cell viabilities and EMT and enhanced apoptosis. MiR-155 might accelerate proliferation, EMT and suppress apoptosis in cervical cancer cells in vitro while ING4 played an antipodal role. Further studies are needed for gaining comprehensive knowledge of miR-155 and ING4 in the future.

## INTRODUCTION

Cervical cancer, the fourth common gynecologic malignancy, takes about 7.9 percent of all kinds of female cancers and about 500 thousand of new cases per year (Jemal et al. 2011), which also takes the first place among all kinds of gynecologic malignant tumors (Nam et al. 2008). Based on plentiful research, cervical cancer, a disease with multi-factors and multi-steps, might have connections with infections of virus, bacteria and chlamydia and so on (Arbyn et al. 2011). HPV has been determined as a key factor resulting in cervical cancer and other co-factors and molecules also make contributions in cancer developing (Laukkanen et al. 2010). Until now, low cure rate of cervical cancer is ascribed unclear mechanism of occurrence and development. For a better clinical treatment, it is necessary and meaningful to dig out mechanism of occurrence of cervical cancer. During this process, related genes and their functions should be the top priority.

Based on results of whole genome sequencing, about 93 percentage sequences of human genomes can be transcribed and less than 2 percentage of transcripts have abilities to coding proteins while others who have no coding proteins abilities are named non-coding RNAs (Kanyina et al. 2017). Among those noncoding RNAs, microRNA is a noncoding small RNA having about 22 nucleotides in length, which is an important post-transcriptional regulator broadly participated in multiple biological behaviors of cells (Walboomers et al. 1999). Recent years, miRNAs have been discovered to have close correlation with proliferation, apoptosis, invasion and angiogenesis of cervical cancer, regarded as new targets for diagnosing, treating and prognosis evaluating (Wardak 2016). Wang et al. have found that miR-15b, miR-16, miR-146a, miR-155 and miR-223 expressions were increased in

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cervical cancer cells while miR-126 and miR-424 were downregulated (Qin et al. 2012). In other studies, miR-155 was checked to be highly expressed in tissues or cells of cervical cancer, reminding that it could be a potential factor in regulating progression of cervical cancer (Bartel 2004; Ponting and Belgard 2010). Moreover, miR-155 RNA expression was much higher in cervical cancer tissues, which promoted the proliferation by binding LKB1 (González-Quintana et al. 2016). Besides that, upregulated miR-155 leaded to poor prognosis of cervical cancer patients and miR-155 inhibition resulted in lower migratory and invasive capacities of cervical tumor cells (Wang et al. 2008).

Inhibitor of growth 4 (ING4), downregulated in mRNA and protein level in cervical cancer tissues, has been examined as a suppressor of cervical cancer (Witten et al. 2010). Ectopic expression of ING4 restrained cervical cancer cell proliferation and decreased transcription of HPV E6/E7 (Witten et al. 2010). Moreover, ING4 upregulated by irradiation inhibited HIF1$\alpha$ expression in hypoxic conditions and accelerated cell apoptosis, indicating that ING4 might enhanced radiosensitivity of cervical cancer cells (Ma et al. 2021). In hepatocellular carcinoma, overexpression of ING4 inhibited expression of miR-155 while suppression of ING4 caused a reversed result, indicating that ING4 and miR-155 had potential correlations in HCC (Yuan et al. 2016). However, studies between miR-155 and ING4 in cervical cancers were devoid.

### Objective

This study aims to analyze the functions of miR-155 and ING4 and their correlation in progressions of cervical cancer cells in vitro.

## METHODOLOGY

### Cell Culture

HcerEpic (Human normal cervical epithelial cell line) and cervical cancer cell lines (Hela, SiHa and C-33A) were utilized from ATCC (USA). Hela cell line is epithelial cells from 31-year adult. SiHa cells are epithelial cells in grade aII, squamous cell carcinoma from a 55-year adult. C-33A cells extracted from cervical cancer tissues are epithelial cells from 66 years adult. All of them are adherent. Cells were all cultivated in DMEM medium with 10 percent FBS (Gibco™, USA) at $37^0$C, 5 percent $CO_2$ and 50 percent relative humidity. According to speed of cell growth, mediums were changed at appropriate time. After that, cells were digested by 0.25 percent trypsin for passaging or inoculated culturing. C-33A cells was applied for proceeding following tests.

### Cell Transfection

C-33A cells were seeded into 12-well plates for transfection. For overexpressed transfection, after confluence of cells reached 80 percent, Overexpression of ING4 and negative control vector were compounded by pcDNA3.1 vector (Invitrogen™, USA). Overexpressed negative control and overexpressed ING4 were transfected into cells incubated in 12-well plate using Lipofectamine 2000 (Invitrogen, USA). RNA level of ING4 was measured through RT-qPCR 48h after transfection. MiR-155 inhibitor, NC inhibitor, NC mimics and miR-155 mimics were acquired from GenePharma (Shanghai, China). Suppression transfection was processed after cell confluences reached 50 percent. Lipofectamine 2000 was applied as well. NC inhibitor and miR-155 inhibitor were transfected into cells and keep incubating for 48h. After that, RT-qPCR evaluated miR-155 and ING4 RNA expressions.

### RT-qPCR

To collect total RNA, Ezol Total RNA Extraction Reagent (GenePharma, Shanghai, China) was applied for extraction strictly following the manufacturer's instructions. Thereafter, cDNAs were compounded through RNA reverse transcript. Next, real time PCR was conducted with compounded cDNA as the template. Steps were strictly according to specifications of TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems™, USA) while U6 and GAPDH were applied as internal references for measuring expressions of RNAs. Primers' sequences were listed as follows: miR-155, F 5'- ACGCTCAGTTAATGCTAATCGTGATA-3', R 5'- ATTCCATGTTGCCACTGTCTCTG-3'; ING4, F 5'- TTTCAGAGGGAGGGTCCTTT-3', R 5'- GCCAGAGCCTAGATGACCTG-3'; E-cadherin, F 5'- TACACTGCCCAGGAGCCAGA-3', R 5'- TGGCACCAGTGTCCGGATTA-3'; N-cadherin, F 5'- TCAGGCGTCTGTAGAGGCTT-3', R 5'- ATGCACATCCTTCGATAAGACTG-3'; Snail, F 5'- TCGGAAGCCTAACTACAGCGA-3', R 5'- AGATGAGCATTGGCAGCGAG-3' and internal controls:

U 6, F 5'-ATTGGAACGATACAGAGAAGATT-3', R 5'- GGAACGCTTCACGAATTTG-3' and GAPDH, F 5'- CTGGGCTACACTGAGCACC-3', R 5'- AAGTGGTCGTTGAGGGCAATG-3'. RNA expressions were calculated through $2^{-\Delta\Delta Ct}$ methods. Conditions of PCR were set: predenaturation, $95^0$C, 3min; denaturation, $95^0$C, 20s; annealing, $62^0$C, 50s and extension, $72^0$C, 30s, 40 cycles.

### CCK-8

C-33A cells seeded onto 96-well plates with $1\times10^4$ cells/well were grouped and transfected by with NC/miR-155 inhibitor, overexpressed NC, overexpressed ING4, miR-155 inhibitor with oeNC and miR-155 inhibitor with oeING4. Later cells were cultured for 24h, 48h and 72h. Medium without cells were considered as control group. The plate was cultured at $37^0$C, 5% $CO_2$ and 10μl CCK-8 was added in settled time points. Then the plate with CCK-8 were kept incubating for another 2h. Varioskan™ LUX (Thermo Scientific™, USA) was used to evaluate optical density (OD) values in 450 nm wavelengths.

### Flow Cytometry

This assay was applied for measuring apoptosis rate of cells using eBioscience™ Annexin V-FITC Apoptosis Detection Kit (Invitrogen™, USA). After cells in control groups (miRNC inhibitor and oeNC) and experimental groups (miR-155 inhibitor, oeING4 and miR-155 inhibitor with oeING4) were digested by 0.25 percent trypsin and resuspended in Annexin V-FITC binding buffer, Annexin V-FITC was added and incubated without light for 10 min at $25^0$C. After that, 7-AAD (Invitrogen™, USA) with PBS was mixed for staining. Attune NxT Flow Cytometer (Invitrogen™, USA) then determined rate of cell apoptosis.

### Statistical Analysis

All experiments were run in a triplicate and data were displayed as mean±SD. SPSS 19.0 (IBM, USA) and GraphPad Prism 7 (GraphPad, USA) were implemented for data analyzing. Student's t test and one-way ANOVA analyzed comparisons of two groups and multiple groups, respectively. $P<0.05$ was considered significant statistically.

## RESULTS

### *MiR-155*, Upregulated in Cervical Cancer Cell Lines, Promoted Proliferation and Epithelial–mesenchymal Transition (EMT) and Suppressed Apoptosis

Shown by RT-qPCR, miR-155 expressions were much higher in cervical cancer cells than normal HcerEpic and C-33A had highest level of miR-155 (Fig. 1A). Therefore, C-33A cell line was chosen for detections. Suppressed miR-155 was created to gain an overall knowledge about miR-155. After interference, not only miR-155 expression but also cell viabilities were significantly decreased compared with negative control group (Figs. 1B, 1C). Meanwhile, inhibitor of miR-155 caused an obviously growth in cell apoptosis of C-33A cell line (Fig. 1D). EMT of C-33A cells transfected by inhibited miR-155 was checked, revealing that E-cadherin expression was promoted while Snail and N-cadherin expressions were significantly inhibited (Fig. 1E).

### *ING4* Expressed Lower in Cervical Cancer Cells with Suppressing Proliferation and EMT and Enhancing Apoptosis

After functions of miR-155 were checked, ING4 was carried on research. According to data from RT-qPCR, expressions of ING4 were suppressed in cervical cancer cells especially in C-33A cell line (Fig. 1A). Therefore, overexpression of ING4 was put forward to figuring out ING4 functions, showing that level of ING4 was markedly increased (Fig. 2B). Meanwhile, cell viabilities of C-33A cells were obviously declined in oeING4 group and apoptosis rate of C-33A cells was growing much higher compared with negative control group (Figs. 2C, 2D). Same as before, EMT was evaluated as well, indicating that overexpression of ING4 accelerated E-cadherin expression and pronouncedly inhibited Snail and N-cadherin (Fig. 2E).

### Overexpressed *ING4* Repressed Proliferation and EMT but Promoted Apoptosis Via Inhibiting *miR-155*

As miR-155 and ING4 were examined one by one, their correlation was considered. In C-33A cell line, expression of miR-155 was sharply de-

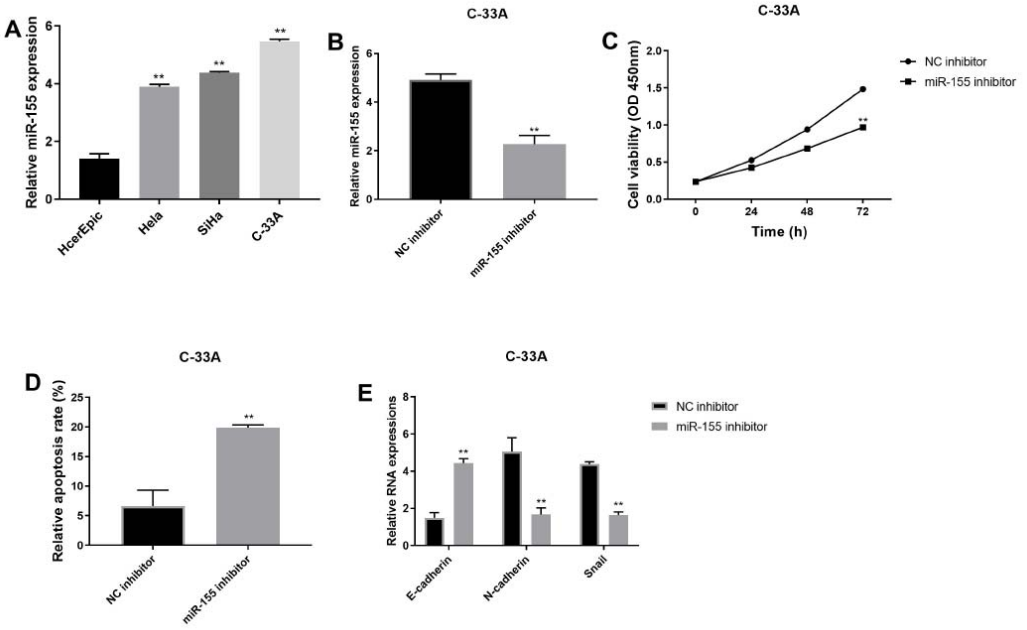


**Fig. 1. MiR-155 was highly expressed in cervical cancer cells with promoting proliferation and EMT and suppressing apoptosis**
**A: Expressions of miR-155 in HcerEpic, Hela, SiHa and C-33A cell lines were assessed using RT-qPCR, P<0.05 versus HcerEpic cell line**
**B: Expressions of miR-155 were checked in C-33A cell line by RT-qPCR after inhibition, P<0.05 versus NC inhibitor group**
**C: CCK-8 measured viabilities of C-33A cells with inhibited miR-155 and NC, P<0.05 versus NC inhibitor group**
**D: Apoptosis rate of cells containing NC/miR-155 inhibitor was evaluated using flow cytometry, P<0.05 versus NC inhibitor group**
**E: E-cadherin, Snail and N-cadherin expressions were validated by RT-qPCR after NC/miR-155 inhibitor transfection, P<0.05 versus NC inhibitor group. Each experiment was run in a triplicate.**

creased with overexpression of ING4 (Fig. 3A). Suppressed miR-155 significantly decreased cell viabilities of C-33A cells which was suppressed by overexpressed ING4 before (Fig. 3B). The apoptosis rate was remarkably upregulated after miR-155 inhibitor joined in overexpressed ING4 group (Fig. 3C). Changes also happened in EMT of C-33A cells, oeING4 and miR-155 inhibitor caused the highest level of E-cadherin compared to oeING4 and oeNC while Snail and N-cadherin were lowest in this group (Fig. 3D).

## DISCUSSION

Cervical carcinoma is a malignant oncology threatening health and life of females. With high proliferation and metastasis abilities, unsatisfied prognosis exists among patients. Therefore, it is urgent to find powerful biomarkers to increase overall survival rate of patients suffering from cervical cancer. According to previous researches, over 50 percent miRNAs were located in chromosome fragile sites or cancer associated genomic regions, revealing important roles of them in progressions of tumors (Zhang et al. 2007). MiR-155 was prompted in cervical cancer tissues and cell lines, which could accelerate progression of cell proliferation and other biological activities (Bartel 2004; González-Quintana et al. 2016). Consistently, in this study, the researchers found that miR-155 expression was higher in cervical cancer cell lines, whose suppression retarded cell viabilities of C-

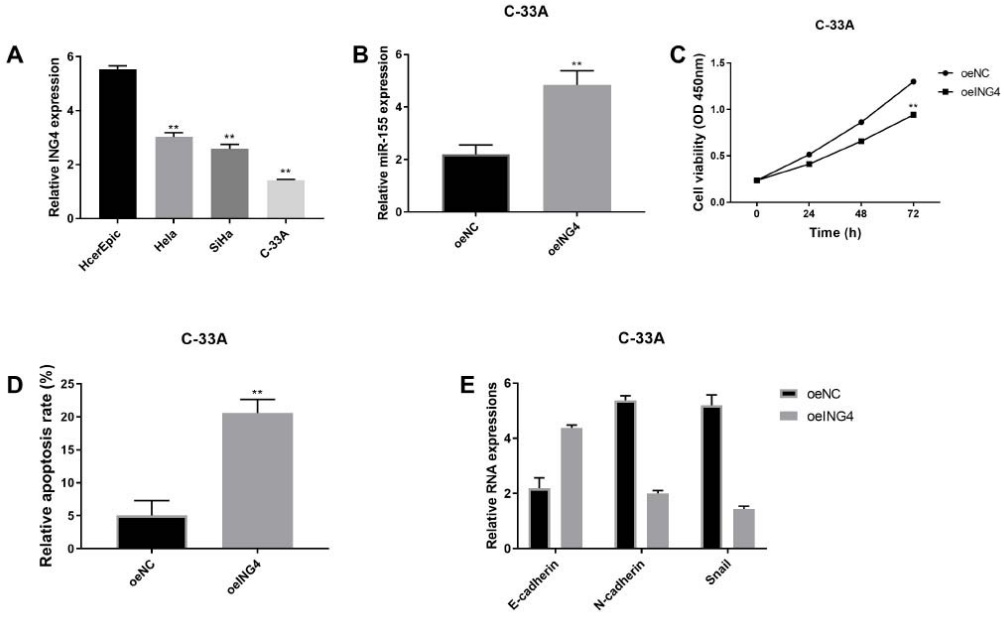


**Fig. 2. ING4 expressed lower in cervical cancer cells with inhibiting proliferation and EMT but increasing apoptosis**
**A: ING4 expression was measured with RT-qPCR in HcerEpic, Hela, SiHa and C-33A cells, P<0.05 versus HcerEpic cells**
**B: Overexpressed NC and ING4 expressions in C-33A cell line were analyzed by RT-qPCR, P<0.05 versus oeNC group**
**C: Cell viabilities of C-33A cell lines with oeNC and oeING4 were examined by CCK-8, P<0.05 versus oeNC group**
**D: Apoptosis rate of C-33A cells containing oeNC and oeING4 were validated through flow cytometry, P<0.05 versus oeNC group**
**E: Expressions of E-cadherin, Snail, and N-cadherin with oeNC and oeING4 were validated with RT-qPCR, P<0.05 versus oeNC group. Each experiment was repeated three times**

33A cells and speeded up cell apoptosis. In metastasis of tumors, EMT is an important step for causing distant migration of tumors (Lao et al. 2014; Runyan and Savagner 2018). EMT also takes an vital part in cancer evolvement and metastasis of cervical cancer (Kalluri and Weinberg 2009). According to results in this study, E-cadherin was upregulated after miR-155 inhibition and N-cadherin and Snail were impeded, reminding that suppression of miR-155 could block EMT process of cervical cancer cells. According to these findings, miR-155 promoted cell viability and EMT of cervical cancer cells and repressed the apoptosis in vitro, indicating that miR-155 might be a potential gene that contributed to progressions of cervical cancer cells.

ING4 was a nominated tumor suppressor which regulated cell progress in tumors including DNA repair, apoptosis, proliferation, cell cycles and so on (Yuan et al. 2016). Though functions of ING4 have been measured in many other cancers, research achievement about ING4 in cervical cancer was still scarce (Qureshi et al. 2015). Fortunately, correlation between miR-155 and ING4 was found in hepatocellular carcinoma, indicating that in cervical cancer there might be a connection between those two biomarkers (Yuan et al. 2016). Hence, ING4 expressions were detected, revealing that the level of ING4 were downregulated in cervical cancer cells. ING4 overexpression decreased cell viabilities in cervical cancer cell line but promoted apoptosis. E-cadherin expression had an increase

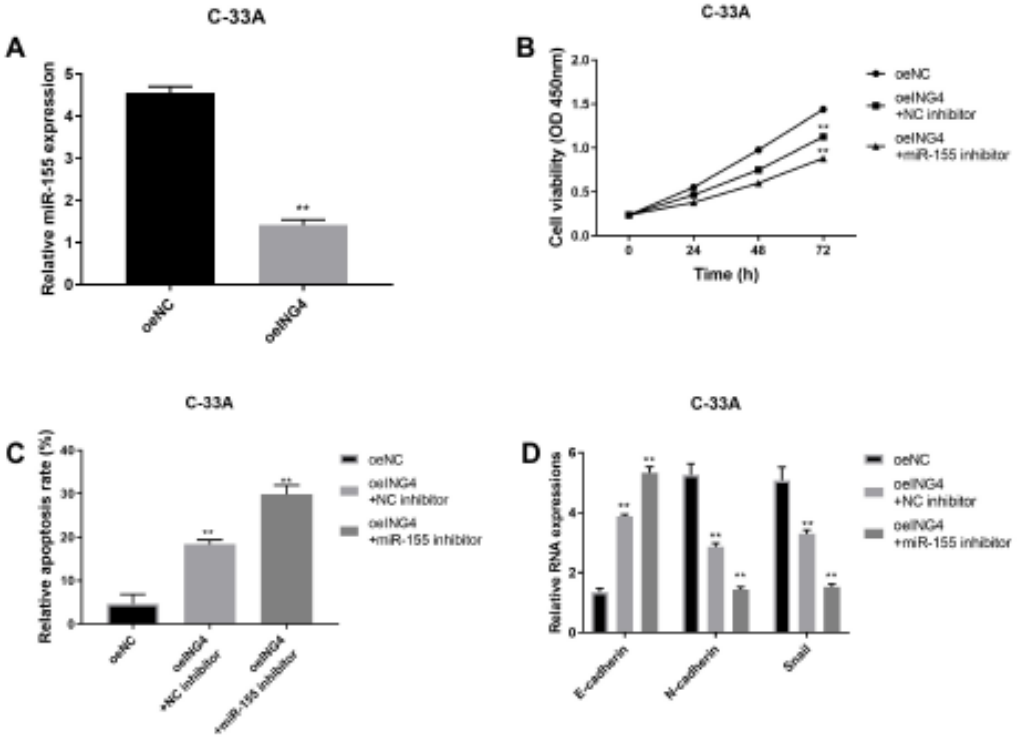


**Fig. 3. Overexpressed ING4 inhibited proliferation and EMT and promoted apoptosis of cervical cancer cells through downregulating miR-155**

**A: Relative RNA expressions of miR-155 with overexpressed NC and ING4 were checked by RT-qPCR, P<0.05 versus oeNC group**

**B: CCK-8 was used to analyze cell viabilities with oeNC, oeING4 with NC inhibitor and oeING4 with miR-155 inhibitor, $^{**}$P<0.05 compared to oeNC group and $^{\#\#}$P<0.05 versus oeING4 with NC inhibitor group**

**C: Flow cytometry evaluated apoptosis rate of C-33A cells containing oeNC, oeING4 with NC inhibitor and oeING4 with miR-155 inhibitor, $^{**}$P<0.05 versus oeNC group and $^{\#\#}$P<0.05 versus oeING4 with NC inhibitor group.**

**D: E-cadherin, Snail and N-cadherin were validated with RT-qPCR in C-33A cells containing oeNC, oeING4 with NC inhibitor and oeING4 with miR-155 inhibitor, $^{**}$P<0.05 versus oeNC group and $^{\#\#}$P<0.05**

while N-cadherin with Snail was declined. Therefore, ING4 acting as a suppressor of cervical cancer cells were determined through these in vitro experiments (Keenen and Kim 2016).

Then, correlations between miR-155 and ING4 were checked, indicating that miR-155 was significantly decreased after ING4 upregulation. Moreover, overexpressed ING4 could produce the lowest level of cell viabilities and the highest level of apoptosis of C-33A cells transfected with miR-155 inhibitor. Similarly, the topmost E-cadherin expression and the lowermost levels of N-cadherin and Snail were created by inhibited miR-155 and ING4 upregulation. Besides that, overexpression of ING4 resumed functions of upregulated miR-155 in promoting cell viability and EMT and suppressing apoptosis. In consequence, ING4 might suppress functions of miR-155 in cervical cancer cells to regulate progression of the tumor.

## CONCLUSION

MiR-155 was highly expressed and facilitated cell viability and EMT but inhibited apoptosis in

cervical cancer cells in vitro while ING4 played a reverse role as miR-155, suggesting that these two could be underlying biomarkers for treating cervical cancer. Whereas, further experiments in vivo and clinical stages are still requested for more knowledge.

## RECOMMENDATIONS

According to this study, miR-155 and ING4 had connections with EMT-related cervical cancer, reminding us of a new regulatory axis in cervical cancer.

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