MiR-299-3p inhibits proliferation and invasion of cervical cancer cell via targeting TCF4

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Abstract. – OBJECTIVE: Many studies have demonstrated that the abnormal microRNAs (miRNAs) expression plays crucial roles in the development of human cancers including cervical cancer (CC). However, the expression and the underlying mechanism of miR-299-3p in CC remain unclear.

MATERIALS AND METHODS: In this study, the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect miR-299-3p expression level in CC cell lines. The cell proliferation assay, colony formation assay, and transwell invasion assay were conducted to investigate the biological functions of miR-299-3p. Followingly, the Luciferase activity reporter and the Western blot assays were conducted to validate the transcription factor 4 (TCF4) as a direct target of miR-299-3p.

RESULTS: MiR-299-3p expression level was reduced in CC cell lines compared with the normal control line. The overexpression of miR-299-3p inhibits CC cell growth and invasion. Furthermore, TCF4 was validated as a direct target of miR-299-3p. In addition, TCF4 overexpression reversed the inhibitory effects of miR-299-3p on CC cell behaviors.

CONCLUSIONS: Taken together, our results illustrated that miR-299-3p acts as a tumor suppressor to inhibit CC cell behaviors by targeting TCF4.

Key Words: Cervical cancer, MiR-299-3p, TCF4, Cell growth, Cell invasion.

Introduction

Cervical cancer (CC) is the most commonly diagnosed cancer type in women, especially in underdeveloped countries¹. The overall survival rate for CC remains quite low although the improvements in combined cancer therapies and early cancer screening methods²³. Therefore, there is a need for new biomarkers identification with the aim to assist in diagnosis.

MicroRNAs (miRNAs) are non-protein-coding RNAs that can regulate target gene expression mainly via 3'-untranslated region (3'-UTR) binding⁴. MiRNAs have been recognized as regulators for almost all cell behaviors and hence play crucial roles in cancer progression⁵. It was reported that miRNAs can function as either tumor promoter or tumor suppressor⁶. To date, there are still many obstacles to put miRNA-based treatment methods into clinical including off-target effects, and many failures to deliver miRNA⁷. However, targeting miRNAs remain promising therapeutic measures for cancer treatment⁷.

MiR-299-3p is reported to function as tumor suppressor in several cancer types. In thyroid cancer, miR-299-3p expression was significantly downregulated in both cancer tissues and cell lines⁸. The overexpression of miR-299-3p could inhibit cancer cell growth but promote cell apoptosis, while the knockdown of miR-299-3p caused the opposite effects on cancer cell behaviors⁹. Dang et al⁹ reported that the low miR-299-3p expression in hepatocellular carcinoma was closely associated with large tumor size, advanced tumor stage, poor overall survival, and disease-free survival of cancer patients. Another study¹⁰ confirmed that miR-299-3p was able to inhibit colon carcinoma progression in vitro and in vivo via targeting Vascular Endothelial Growth Factor A expression. However, it was not reported whether miR-299-3p has a role in regulating the CC progression.
Transcription factor 4 (TCF4) is a key player in the Wnt signaling pathway and could interact with β-catenin\(^1\). High TCF4 expression was found associated with FIGO stage, lymph node metastasis, disease-free survival, and overall survival of epithelial ovarian cancer patients\(^2\).

In this study, the biological roles of miR-299-3p and TCF4 were investigated using CC cell lines. Furthermore, the association of miR-299-3p and TCF4 was explored using Luciferase activity reporter assay and Western blot assay.

**Materials and Methods**

**Cell Line and Cell Culture**

The CC cell lines (SiHa and C33A) obtained from ATCC (Manassas, VA, USA) were incubated at Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum. Human cervical epithelial cell line End1 obtained from ATCC was grown at keratinocyte serum-free medium (K-SFM; Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). The incubation atmosphere was maintained at 37°C with 5% of CO\(_2\).

**Cell Transfection**

MiR-299-3p mimic and negative control (NC-mimic) were synthesized by RiboBio (Guangzhou, China). TCF4 cloned into pcDNA3.1 by GenScript (Nanjing, China) was named as pTCF4. The cell transfection was accomplished using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

**Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)**

The total RNA was isolated from the cultured cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and then quantified using NanoDrop-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the provided protocols. The reverse transcription was conducted using Prime Script RT reagent kit (TaKaRa, Otsu, Shiga, China). qRT-PCR was conducted at ABI 7500 system using SYBR Green Mix (TaKaRa, Otsu, Shiga, China) with the following primers: miR-299-3p: Forward sequence: 5'-ACACTCCAGCTGGGTATGTGGGATGGTAAAC-3', Reverse sequence: 5'-GTGCAGGGTGCGAGGT-3'; U6 snRNA: Forward sequence: 5'-CTC-GCTTCCAGGCACA-3', Reverse sequence: 5'-AACGCTTCAGAATTGCGT-3'. The following procedures were employed: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. The relative miR-299-3p expression level was calculated using the comparative cycle threshold (CT) method with U6 snRNA as an internal control.

**Western Blot**

The total protein was isolated from cultured cells using RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China) and quantified using BCA kit (Beyotime, Haimen, Jiangsu, China) according to the provided protocols. An equal amount of protein sample was separated at 10% SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membrane (Beyotime, Haimen, Jiangsu, China). After being blocked with fat-free milk, the membranes were incubated with primary antibodies (rabbit anti-TCF4: ab217668, rabbit anti-N-cadherin: ab76011, rabbit anti-vimentin: ab193555, rabbit anti-GAPDH: ab181602; Abcam, Cambridge, MA, USA) at 4°C for overnight. Then, the membranes were washed three times with TBST. The membranes were incubated with goat anti-rabbit secondary antibody (ab6721, Abcam, Cambridge, MA, USA) at 37 °C for 4 h. The band signal was developed using BeyoECL kit (Beyotime, Haimen, Jiangsu, China) and analyzed with Image 1.42 software (NIH, Bethesda, MD, USA).

**Cell Proliferation Assay**

The cell viability was investigated using the Cell Counting Kit-8 (CCK-8; Beyotime, Haimen, Jiangsu, China) assay. In brief, 2,000 cells/well were seeded into 96-well plate and grown at the above-mentioned conditions. At the indicated time, CCK-8 reagent was added to each well. After further incubation for 4 h, the optical density at 450 nm was measured using a microplate reader.

**Colony Formation Assay**

The cells were seeded into 6-well plate at the density of 500 cells/well and incubation for 2 weeks. Then, the cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted under a microscope.

**Transwell Invasion Assay**

2 × 10\(^4\) cells in serum-free medium were seeded in the upper chamber precoated with Matrigel (BD Biosciences, San Jose, CA, USA), while the lower chamber was filled with medium contain-
MiR-299-3p/TCF4 axis in CC

After 48 h of incubation, non-invasive cells were removed, while the invasive cells were stained with 0.1 % crystal violet and counted under a microscope.

Target Predictions
Two miRNA target prediction algorithms, TargetScan and miRDB, were used to analyze the potential target of miR-299-3p.

Dual-Luciferase Reporter Assays
The wild-type TCF4 3'-UTR (TCF4-wt) or mutant (TCF4-mt) were constructed by inserting the 3'-UTR sequences into pGL3 vector (Promega, Madison, WI, USA). The cells were co-transfected with Luciferase activity vectors or synthetic miRNAs using Lipofectamine 2000. After incubation for 48 h, the cells were collected to analyze the rel-

![Figure 1. Downregulation of miR-299-3p in CC cell lines (SiHa and C33A) compared with the normal cell line End1. MiR-299-3p: microRNA-299-3p; CC: cervical cancer.](image1)

![Figure 2. Overexpression of miR-299-3p inhibits CC cell growth and invasion. (A) MiR-299-3p expression, (B) cell proliferation, (C) colony formation (200× magnification), (D) cell invasion (200× magnification), and (E) N-cadherin and vimentin expression in CC cell lines transfected with synthetic miRNAs. MiR-299-3p: microRNA-299-3p; CC: cervical cancer; NC-mimic: negative control for miR-299-3p mimic.](image2)
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ative luciferase activity using the Dual-Luciferase activity assay kit (Promega, Madison, WI, USA) with Renilla luciferase activity as control.

Statistical Analysis
The statistical analysis was conducted using the Student’s t-test or One-way analysis of variance and Tukey post-hoc test on SPSS (Chicago, IL, USA). The data were presented as means ± SD from three different experiments. \( p < 0.05 \) was regarded as statistically significant.

Results

Downregulation of MiR-299-3p in CC Cell Lines
We found that miR-299-3p level was significantly reduced in CC cell lines compared with the normal cell line (Figure 1). Our results indicated that miR-299-3p may play a crucial role in the CC progression.

Overexpression of MiR-299-3p Inhibits CC Cell Growth and Invasion
To investigate the role of miR-299-3p in CC, the gain-of-function experiments were performed. qRT-PCR results revealed that the introduction of miR-299-3p mimic significantly increased the expression of miR-299-3p (Figure 2A). CCK-8 assay demonstrated that the overexpression of miR-299-3p inhibits CC cell proliferation compared with NC-mimic transfected cells (Figure 2B). Similarly, the colony formation assays revealed that the cells transfected with miR-299-3p mimic displayed fewer colonies compared with those transfected with NC-mimic (Figure 2C). We next examined the effects of miR-299-3p on CC cell

Figure 3. TCF4 was a target of miR-299-3p. A, Binding region of miR-299-3p and 3'-UTR of TCF4. B, Relative luciferase activity in CC cells transfected with synthetic miRNAs and luciferase vectors. C, TCF4 expression in CC cells transfected with synthetic miRNAs. MiR-299-3p: microRNA-299-3p; CC: cervical cancer; NC-mimic: negative control for miR-299-3p mimic; UTR: untranslated region; wt: wild-type; mt: mutant; TCF4: transcription factor 4.
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Invasion using the transwell invasion assay. As presented in Figure 2D, the overexpression of miR-299-3p led to cell invasion suppression compared with NC-mimic. Western blot showed that miR-299-3p overexpression repressed N-cadherin and vimentin expression in CC cells (Figure 2E).

**MiR-299-3p Directly Targeted TCF4 in CC**

Bioinformatic analyses methods revealed that TCF4 was a potential target of miR-299-3p (Figure 3A). The Luciferase activity reporter assay revealed that the overexpression of miR-299-3p inhibited the Luciferase activity in the cells transfected with TCF4-wt (Figure 3B). Further, the Western blot showed that the TCF4 expression level could be repressed by miR-299-3p mimic transfection (Figure 3C).

**MiR-299-3p Exerts its Regulatory Roles on CC Cell Behaviors Via Targeting TCF4**

We next wondered whether TCF4 was a function target for miR-299-3p. Hence, the pTCF4 and miR-299-3p mimic was co-transfected into the CC cells. Western blot showed that pTCF4 transfection significantly increased the expression level of TCF4 (Figure 4A). CCK-8 assay revealed that TCF4 overexpression increased cell proliferation (Figure 4B). Besides, colony formation assay indicated that pTCF4 introduction increased the colonies generated (Figure 4C). Moreover, the transwell invasion assay suggested that TCF4 overexpression increased cell invasion ability in the CC cells (Figure 4D). In addition, N-cadherin and vimentin expression level were elevated by pTCF4 (Figure 4E). Notably, we found that the expression of TCF4 remarkably reversed the effects of miR-299-3p mimic on CC cell behaviors (Figures 4B-4E).

**Discussion**

To date, multiple miRNAs have been reported to function as crucial roles in CC. For ex-
ample, miR-944 was found elevated expressed in advanced CC and could be regarded as a biomarker to predict the prognosis of cancer patients. MiR-4524b-5p expression was upregulated in relapsed CC tissues, and was able to regulate CC cell migration and invasion by regulating WTX and β-catenin expression. These results indicated an oncogenic role of miR-944 and miR-4524b-5p in CC. On the contrary, miR-374b was found downregulated in CC and correlated with advanced FIGO stage. They also found that the overexpression of miR-374b could regulate cell proliferation and invasion via targeting FOXM1, indicating the tumor suppressive role of miR-374b in CC.

In this report, we first reported that miR-299-3p expression was significantly decreased in CC cell lines compared with the normal cell line. We further overexpressed miR-299-3p expression level in CC cells to explore the biological roles of miR-299-3p. We found that miR-299-3p expression inhibits CC cell proliferation, colony formation, and cell invasion. Western blot assay was used to measure the protein levels of two epithelial to mesenchymal transition (EMT) associated molecules, N-cadherin, and vimentin. We found that miR-299-3p overexpression inhibited the expression of N-cadherin and vimentin.

Previous investigations have identified several downstream targets for miR-299-3p. Hence, we demonstrated that TCF4 was a putative target of miR-299-3p by bioinformatic analyses algorithms. A previous study suggested that TCF4 could be directly regulated by miR-591 to play an oncogenic role in breast cancer. Here, we demonstrated that miR-299-3p could bind with the 3’-UTR of TCF4 using the Luciferase activity reporter assay. Also, we found that the expression of TCF4 could be downregulated by miR-299-3p. More importantly, rescue experiments showed that the overexpression of TCF4 partially reversed the effects of miR-299-3p on CC cell events. Thus, miR-299-3p regulates CC cell behaviors through TCF4.

Conclusions

In summary, we demonstrated that miR-299-3p decreased the expression in CC cell lines compared with the normal cell line. Overall, we concluded that the overexpression of miR-299-3p inhibits CC cell behaviors by targeting the expression of TCF4. Our investigation provided novel insight into the role of miR-299-3p in human cancer.

Conflict of interest
The authors declare no conflicts of interest.

References


