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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 MATERIALS: 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, transwell invasion assay were conducted to investigate the biological function 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 significantly reduced in CC cell lines compared with the normal cell line. The overexpression of miR-299-3p inhibits CC cell growth and invasion. Furthermore, TCF4 was identified 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 illustrate 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 countries[1]. The overall survival rate for CC remains quite low although the improvements in combined cancer therapies and early cancer screening methods[2,3]. Therefore, there is a need for new biomarker identification with the aim to assist diagnosis.

MicroRNAs (miRNAs) are non-protein-coding RNAs that can regulate target gene expression mainly via 3'-untranslated region (3'-UTR) binding. miRNAs have been recognized as regulators for almost all cell behaviors and hence play crucial roles in cancer progression[5]. It was reported that miRNAs can function as either tumor promoter or tumor suppressor[6]. To date, there are still many obstacles to put miRNA-based treatment methods into clinical including off-target effects, and many failures to deliver miRNA[7]. However, targeting miRNAs remain promising therapeutic measures for cancer treatment[7].

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 lines[8]. 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 behaviors[8]. Dang et al[9] 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 study[10] 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.

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Transcription factor 4 (TCF4) is a key player in the Wnt signaling pathway and could interact with β-catenin[11]. High TCF4 expression was found associated with FIGO stage, lymph node metastasis, disease-free survival, and overall survival of epithelial ovarian cancer patients[12].

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., Wa... MA, USA). The incubation atmosph... maintained at 37°C with 5% of CO_2.

Cell Transfection

MiR-299-3p mimic and negative contr... (NC-mimic) were synthesi... RiboBio (Guangzhou, China). TCF... oned ... pcDNA3.1 by GenScript (Nan... China)... named as pTCF4. The cell trans... was ... using Lipofectamine 2000 (... Carlsbad, CA, USA).

Quantitative ...e-Polymer... Chain Reaction (q...PCR)

The tot... RNA was ... from the cultured cel... using TRIZOL ... t (Invitrogen, Carlsb... CA, USA) and then quantified using Nano... p-1000 ...ermo Fisher Scientific Inc., Wa... MA, USA) ac...rding to the provided protoc... everse ...scription was conducted using ... Scri... T reagent kit (TaKaRa, ...Shiga, ...RT-PCR was conducted at AB... 0 syste... ng SYBR Green Mix (TaKaRa, ... Shiga, China) with the following primer... Forward sequence: 5'-ACACTC-...GCTGGGTATGTGGGATGGTAAAC-3', ...erse sequence: 5'-GTGCAGGGTCCGAG-... U6 snRNA: Forward sequence: 5'-CTC-GC... GGCAGCACA-3', Reverse sequence: 5'-AACGCTTCACGAATTTGCGT-3'. The following procedures were employed: 95... s, followed by 40 cycles of 95 °C f... s, and ... °C for 30 s. The relative miR-29... expression level was calculated using the ...rative cycle threshold (CT) method with U6 s... as an internal control.

Western Blot

The total protein ... isolat... from cultured cells using RIPA ... uffe... Beyoti... Haimen, Jiangsu, C...a) a... tified ... BCA kit (Beyotime ...imen, Ji... C...) according to the pr... d protocols. ... amount of protein sa... eparated at 1... SDS-PAGE, and the... nsfer... polyvinylidene difluoride (PVDF) membrane (... me, Haimen, Jiangsu, Chi... ter being blo... with fat-free milk, th... embranes were incubated with primary ...bodies (rabbi... anti-TCF4: ab217668, rabbit ...-N-cadherin... 76011, rabbit anti-vimentin: ...3555, rabbi... ti-GAPDH: ab181602; Abca... mbrido... A, USA) at 4°C for overnight. Ther... anes were washed three times with TBST. The membranes were incubated with ...at anti-rabbit secondary antibody (ab6721, Abbridge, MA, USA) at 37 °C for 4 h. ...e b... d 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 growth 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-

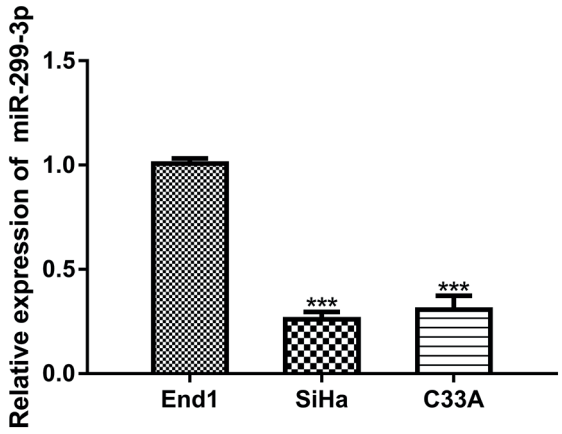


Figure 1. Downregulation of miR-299-3p in CC cell lines (SiHa and C33A) compared with the normal cell line End1. MiR-299-3p: microRNA-299-3p; CC: cervical cancer.

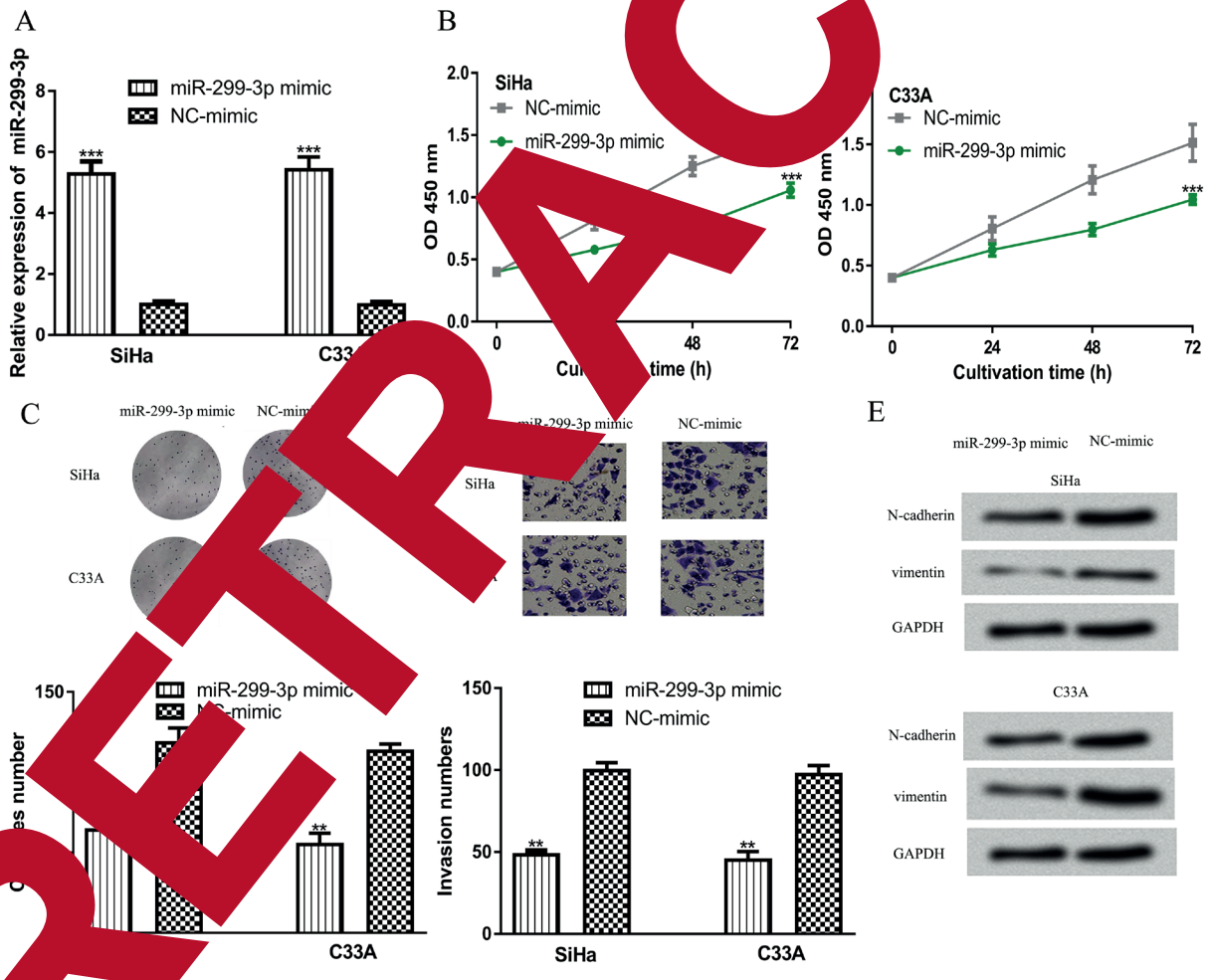
ing FBS. After 48 h of incubation, non-invasive cells were removed, while the invasiv stained with 0.1 % crystal violet an der a microscope.

Target Predictions

Two miRNA target pred on algo Tar getScan and miRDB, w used to ana potential target of miR 3p.

Dual-Luciferase ays

The wild-type F4 (TCF4 or mutant (TCF4-mt re const by rting the 3'-UTR seq s into pGL (Promega, Madison, The cells wer o-transfected with Lu ase a vectors or synthetic miRNAs using Lipofecta 2000. After incubation for cells were c d to analyze the rel-



ure 2. Overexpression of miR-299-3p inhibits CC cell growth and invasion. ***(A)*** MiR-299-3p expression, ***(B)*** cell prolif- ***(C)*** colony formation (200× magnification), ***(D)*** cell invasion (200× magnification), and ***(E)*** N-cadherin and vimentin 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.

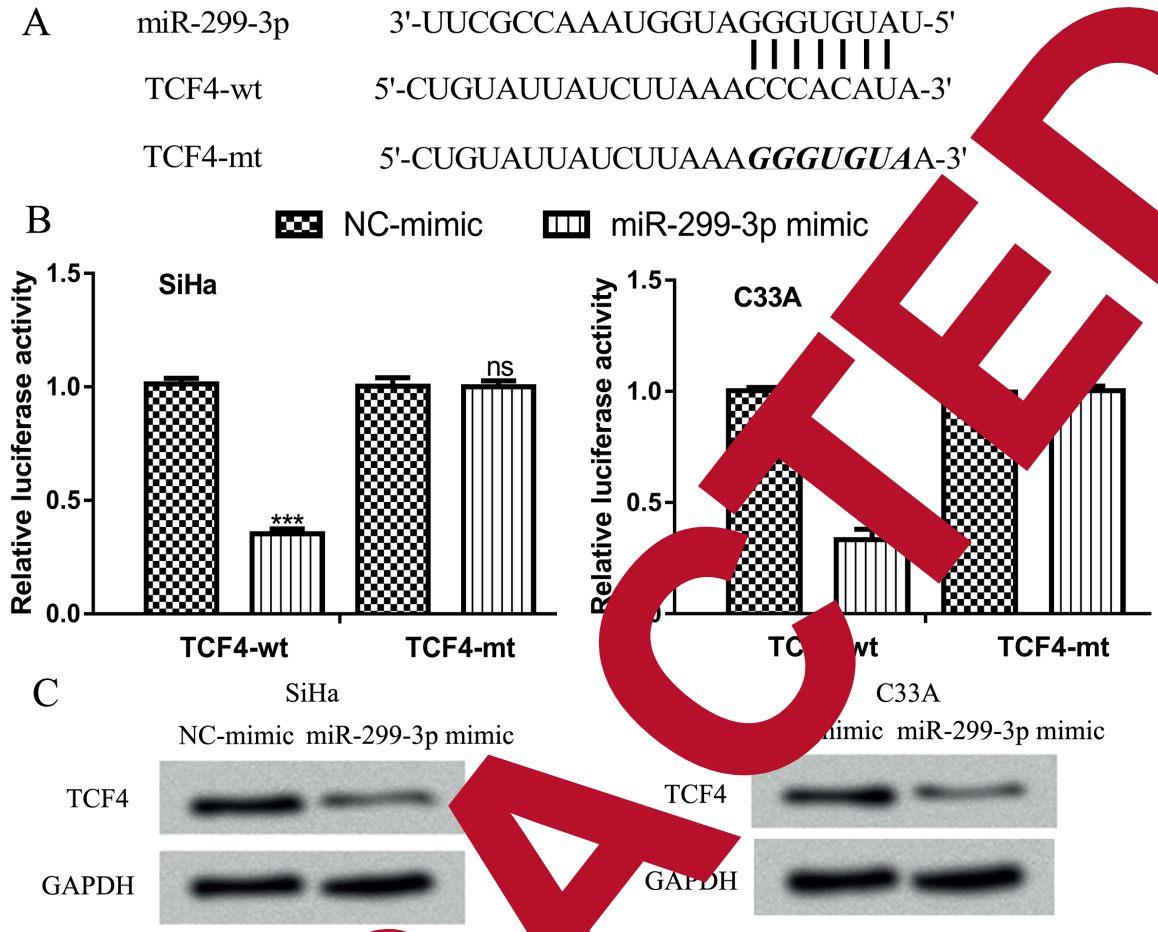


Figure 3. TCF4 was a target of miR-299-3p. *A*, Binding sites of miR-299-3p and 3'-UTR of TCF4. ***B,*** Relative luciferase activity in CC cells transfected with synthetic miRNAs and luciferase vectors. ***C,*** TCF4 expression in CC cells transfected with synthetic miRNAs. MiR-299-3p: microRNA-299-3p; CC: cervical cancer; NC-mimic: negative control for miR-299-3p mimic; UTR: untranslated region; wt: wild-type; mt: mutant-type; TCF4: transcription factor 4.

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). 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

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

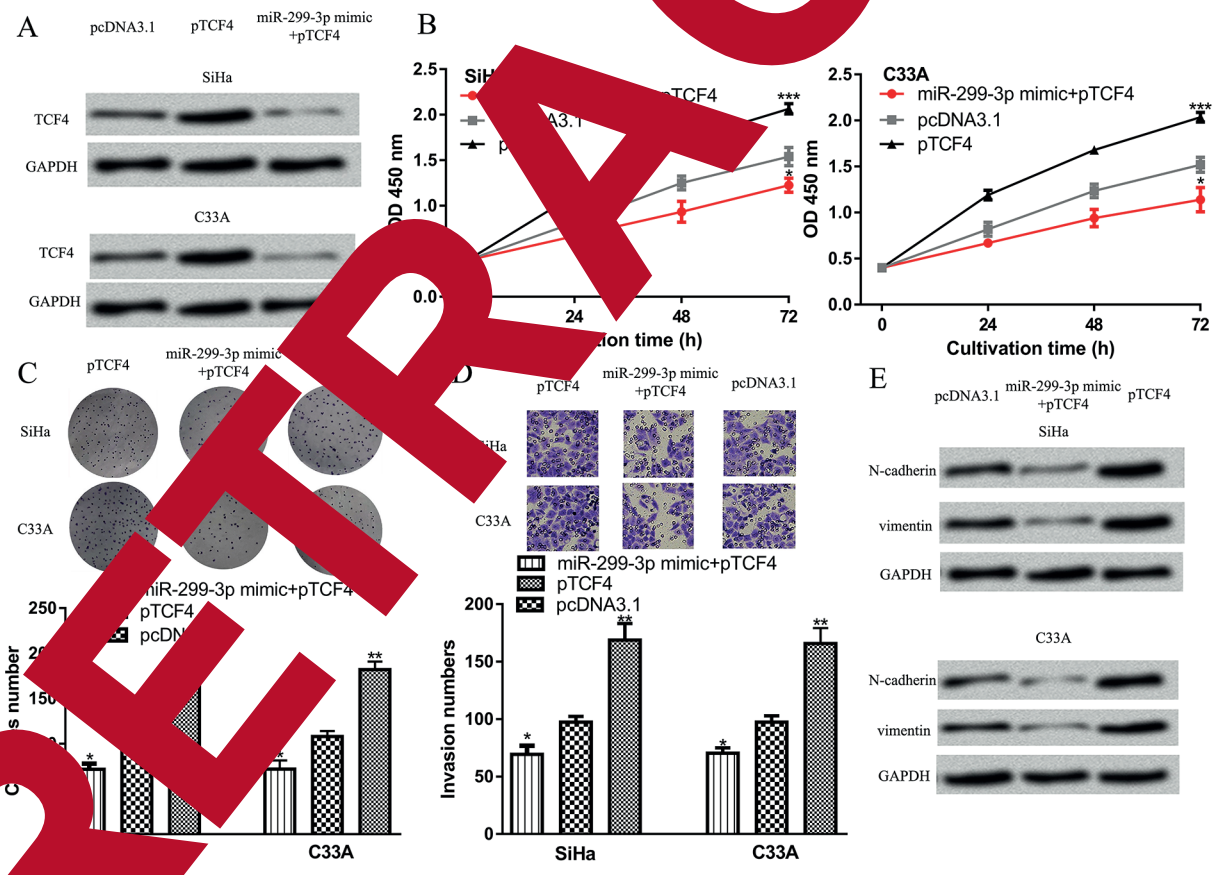
Bioinformatic analyses methods revealed that TCF4 was a potential target of miR-299-3p (Figure 3A). The Luciferase activity reporter assay revealed that the overexpression of miR-299-3p inhibited the Luciferase activity in the cells transfected with TCF4-wt (Figure 3B). Further, the Western blot showed 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 trans[illegible] nificantly increased the expression [illegible] (Figure 4A). CCK-8 assay reve[illegible] that TCF4 overexpression increased cell [illegible] ration (Figure 4B). Besides, colony formatio[illegible] indicated that pTCF4 introduction [illegible] reased [illegible] onies generated (Figure 4C). M[illegible] ver, the trans[illegible] vasion assay suggested [illegible] TCF4 [illegible] verexpre[illegible] increased cell invasi[illegible] lity in [illegible] CC cells (Figure 4D). In addition, [illegible] lhe[illegible] nd vim[illegible] n expression level we[illegible] leva[illegible] TCF4 ([illegible] re 4E). Notably, we fo[illegible] that the [illegible] ion [illegible] TCF4 remarkably re[illegible] d the effects [illegible] 9-3p mimic on CC [illegible] rs (Figures 4[illegible] 4E).

Discu[illegible]n

To date, multi[illegible] miRNAs have been reported to function [illegible] ucial roles in CC[13]. For ex-



[illegible]re 4. Overexpression of TCF4 partially reversed the effects of miR-299-3p on CC cell behaviors. ***(A)*** TCF4 expression, [illegible]l proliferation, ***(C)*** colony formation (200× magnification), ***(D)*** cell invasion (200× magnification), and ***(E)*** N-cadherin a[illegible]tin expression in CC cell lines transfected with pTCF4, pcDNA3.1 or pTCF4 and miR-299-3p mimic. MiR-299-3p: micro[illegible]A-299-3p; CC: cervical cancer; TCF4: transcription factor 4.

ample, miR-944 was found elevated expressed in advanced CC and could be regarded as a biomarker to predict the prognosis of cancer patients[14]. 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[15]. 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[16]. 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[16].

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 tra (EMT) associated molecules, N-cadhe vimentin[17]. We found that miR-299-3p o pression inhibited the expression of N-cad and vimentin.

Previous investigations[8-10] h identifi several downstream targets -299-3p. Hence, we demonstrated th CF4 a putative target of miR-299-3p oinfor ic anal-yses algorithms. A pr stud that TCF4 could be d ecti d by miR-591 to play an onco ic role st cancer[18]. Here, we demon ed that miR p could bind with the f TCF4 usi he Luciferase acti epo say. Also, we found that the ex ession of TC ld be downregulated by -299-3p. More tantly, rescue experi s sho ed that the o erexpression of TCF tially rsed the effects of miR-299-3p C ce ents. T s, miR-299-3p regulates haviо ough TCF4.

nclusions

we demonstrated that miR-299- ecreased the expression in CC cell lines pared with the normal cell line. Overall, we c ed that the overexpression of miR-299-3p i its CC cell behaviors by targeting the expression of TCF4. Our investigation provided novel insight into the role of miR-29 man cancer.

Conflict of interest

The authors declare no conflicts terest.

R nce

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