Am J Cancer Res 2023;13(8):3591-3598
www.ajcr.us /ISSN:2156-6976/ajcr0149770

Original Article

miR-182 promotes cervical cancer progression via activating the Wnt/β-catenin axis

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Received February 20, 2023; Accepted June 26, 2023; Epub August 15, 2023; Published August 30, 2023

Abstract: Cervical cancer (CC) is among the leading causes of cancer-associated mortality in women worldwide; yet the molecular regulators involved in its progression are unclear. This study found that miR-182 was overexpressed in CC tissues when compared with adjacent normal tissues. Moreover, it found that miR-182 expression was significantly positively correlated with distant metastasis in patients with CC. Interestingly, *in vitro* experiments showed that overexpression and inhibition of miR-182 promoted and suppressed the growth of CC cells, respectively. The tumor-promoting effects of miR-182 on CC progression were achieved via the Wnt/β-catenin axis and its downstream genes. Thus, this study revealed the potential of miR-182/β-catenin as an effective new target for CC treatment.

Keywords: Cervical cancer, miR-182, Wnt/β-catenin axis, cancer progression, metastasis

Introduction

Cervical cancer (CC) ranks second among the most common malignancies diagnosed in women worldwide. Its incidence has been on a rise in China where it significantly affects young women; thus, can seriously endanger their quality of life and safety. Nonetheless, the pathogenesis of CC is unclear. To this end, it is urgent to explore novel intervention targets that can be used for the diagnosis and treatment of CC [1].

Numerous studies revealed the critical role of the Wnt/β-catenin axis in the occurrence, development, and prognosis of tumors, including CC [2]. For instance, high expression of the Wnt/β-catenin signaling pathway was reported to accelerate CC cell growth, proliferation, and metastasis [3].

Emerging evidence suggests that microRNAs, short non-coding RNAs, can either promote or suppress CC occurrence and progression [4-6]. For instance, miR-182 upregulation effectively promotes CC cell proliferation and invasion by targeting multiple genes [7, 8]. However, whether miR-182 affects CC progression by directly targeting the key components of the Wnt/β-catenin axis is not yet determined. Thus, we explored the effects of miR-182 on CC cell activity and oncogenicity as well as on the Wnt/β-catenin-related molecular mechanisms. Hopefully, this research can provide new therapeutic targets for CC treatment.

Data and methods

Research subjects

We collected the CC and adjacent tissue specimens of 70 patients with CC diagnosed at The First Affiliated Hospital of Hainan Medical College Hospital between January 2015 and April 2016. For all cases, the diagnosis was confirmed by pathological biopsy after surgical resection. Patients with CC were staged by two clinicopathologists according to the 2018 International Federation of Gynecology and Obstetrics (FIGO) criteria.

The inclusion criteria were as follows: (1) All cases were confirmed by tissue biopsy, which was the gold standard. (2) All patients were diagnosed for the first time. (3) Subjects with complete clinicopathological data and medical records.

The exclusion criteria were as follows: (1) Patients diagnosed with other systemic or local

malignant tumors. (2) Patients with immune system diseases who were not suitable to participate in the trial. (3) Patients with mental disorders. (4) Patients who had previously received radiotherapy or chemotherapy. (5) Subjects with incomplete clinicopathological data and medical records.

All subjects understood and approved the research process and were able to issue corresponding informed consent.

Research methods

Cell culture and transfection: We purchased HeLa and SiHa cells from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS). Then, the cells were placed in a humidified incubator at 37°C with 5% CO_2 and passaged after adding 0.25 mL trypsin. MiR-182 mimics, inhibitors, and controls were transfected. The HeLa cells were transfected with miR-182 inhibitors or mimics using Lipofectamine 2000 (Invitrogen, USA) as per the instructions. The transfection efficiency was determined by western blot after 48 h of cell transfection with 2 μg DNA.

Western blot: The CC cells were lysed and the total proteins were extracted using NP40 buffer. Then, equivalent proteins were transferred to 12% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and separated by electrophoresis. Subsequently, the proteins were transferred to the polyvinylidene difluoride (PVDF) membrane for 1 h at 12 V. Next, the membrane was blocked using 5% nonfat-dried milk for 1-2 hours. The membrane was then incubated with the primary antibody (with a 1000-fold dilution of 5% BSA) overnight at 4°C. After this, the membrane was washed thrice and incubated with the secondary antibody (diluted with 5% nonfat-dried milk for 2 hours at the ambient temperature). Finally, the PVDF membranes were developed with ECL substrate. The antibodies used were anti-β-actin, anti-β-catenin, anti-MYC, and anti-CCND1 and were purchased from Cell Signaling Technology (CST).

Dual-luciferase assay to detect Wnt/β-catenin axis activity: Using the dual-luciferase reporter plasmid system, we determined the luciferase activity of cells after transfection and cultivation for 24 hours. Each group of cells was seeded in 3 duplicate wells, and 0.2 μg of TOP/FOP plasmid and 0.25 μL of Lipofectamine 2000 were added to each well.

Cell proliferation assay: Cell proliferation was evaluated using CCK8 Assay Kit (Abcam) following the manufacturer's instruction.

Real-time PCR: We extracted total RNA from HeLa and SiHa cells in the logarithmic growth phase using the TRIzol reagent. Then, RNA (2 μg) was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. We performed real-time PCR using SYBR Green Real-Time PCR Master Mixes (Thermo Fisher Scientific) according to the manufacturer's instructions. The primers for miR-182 detection were as follows: Upstream primer sequence - 5'-ACACTCCAGCTGGGTTTGGCAATGGTAGAACTCACACT-3'. Downstream primer sequence - 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGTGTGAG-3'. At least three repeated experiments were performed and miR-182 expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Sample size

According to the literature, miR-182 expression in the cervical tissues of patients with CIN was 0.41±0.14, while in those of patients with CC was 0.30±0.10 ($\alpha = 0.05$). The test power was $1-\beta = 0.9$. The sample size in each group was 27 cases as per PASS15.0, while 35 cases considering 20% deletion and detachment.

Statistics

All statistical analyses were performed using SPSS 20.0. All data were represented as mean ± standard deviation (SD). The Kolmogorov-Smirnov test was used to determine the correlation between continuous data and normal distribution. Student's t-test was used to compare the differences between two groups. The χ^2 test was used to determine the inter-group differences for categorical data. A $P < 0.05$ was considered to be statistically significant.

Results and discussion

Results

miR-182 was overexpressed in CC tissues: Of the 70 patients (35 pairs) included in the study, 12 cases had metastasis, while 23 cases had

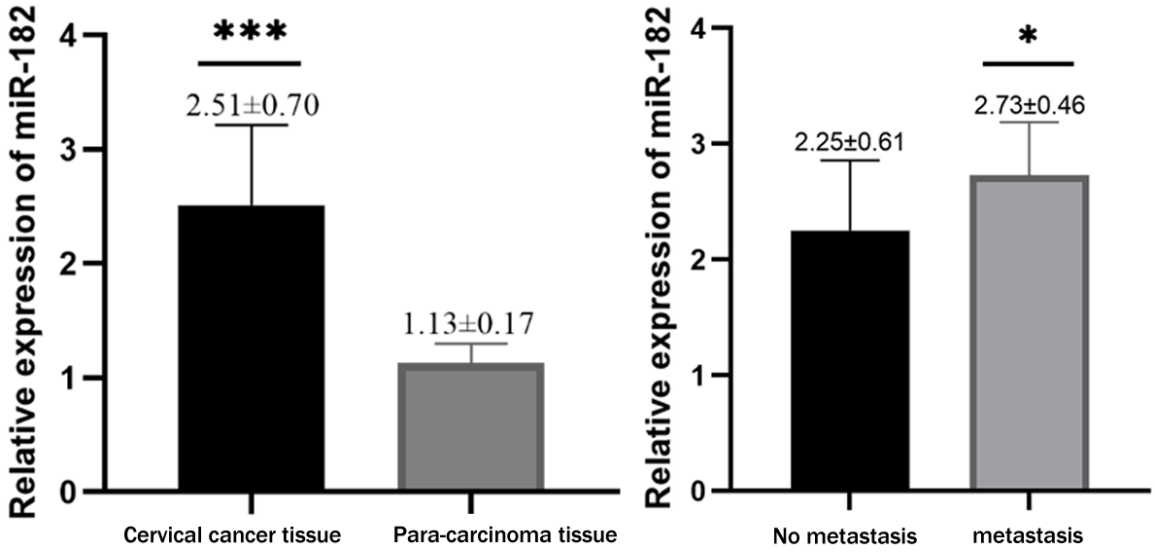

Figure 1. Relative miR-182 expression in cervical cancer (CC) and adjacent tissue specimens. miR-182 was significantly overexpressed in CC tissues compared with adjacent tissues (*left*). miR-182 expression was positively correlated with distant metastasis (*right*).

Table 1. Correlation between miR-182 expression and tumor pathological features ($\bar{x} \pm s$)

Clinicopathological features	Number of cases (n)	miR-182	t/χ^2	*P*
Age			-1.963	0.574
≥ 50 years old	21	2.26±0.58		
< 50 years old	14	2.65±0.58		
FIGO stage			-0.251	0.803
Stage I	24	2.40±0.61		
Stage II	11	2.45±0.61		
Tumor diameter			0.977	0.336
< 4	19	2.51±0.60		
≥ 4	16	2.31±0.60		
With or without lymphatic metastasis			-2.336	0.026
Yes	12	2.73±0.46		
None	23	2.25±0.61		
Vascular invasion				
Yes	12	2.52±0.70		
None	23	2.36±0.56		
Degree of pathological differentiation			-0.013	0.990
Well differentiated	11	2.42±0.63		
Low-moderate differentiated	24	2.41±0.60		

no metastasis. Moreover, miR-182 was significantly overexpressed in CC tissues compared with adjacent tissues ($P < 0.001$) (**Figure 1**). Interestingly, CC tissues from patients with distant metastasis showed significantly higher miR-182 expression than those without metastasis (**Figure 1**).

Relationship between miR-182 expression in CC tissues and tumor pathological characteristics: We found that miR-182 expression in CC tissues was significantly positively correlated with the presence of lymphatic metastasis ($P = 0.026$) (**Table 1**). However, we observed no significant relationship between miR-182 expres-

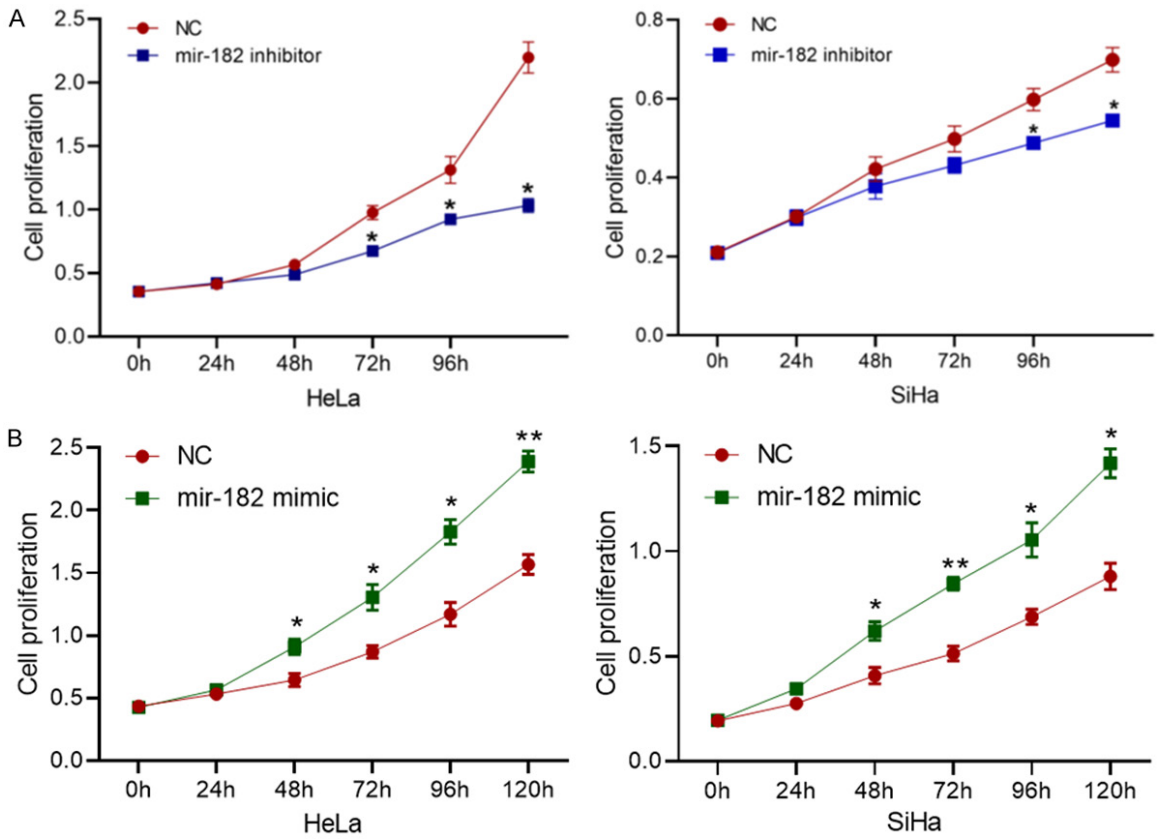

Figure 2. Role of miR-182 on cervical cancer (CC) cell proliferation. A. Treatment with miR-182 inhibitor significantly reduced CC cell proliferation. B. Treatment with miR-182 mimic significantly increased CC cell proliferation. *P < 0.05 and **P < 0.01.

sion and FIGO stage, age stratification, tumor diameter, and vascular invasion (P > 0.05).

Effect of miR-182 on CC cell proliferation: Furthermore, we silenced miR-182 in HeLa and SiHa cells to determine its role in CC cell proliferation. We observed that miR-182 inhibitors significantly reduced the proliferation of HeLa and SiHa cells (**Figure 2A**). In contrast, miR-182 mimics significantly increased the proliferation of CC cells (**Figure 2B**).

Effect of miR-182 on Wnt/β-catenin signaling: miR-182 overexpression significantly increased the TOP/FOP ratio in HeLa and SiHa cells and thereby increased β-catenin protein expression (P < 0.05). Contrastingly, miR-182 inhibition significantly decreased the TOP/FOP ratio and thus significantly suppressed β-catenin protein expression (P < 0.05) (**Figure 3**).

Influence of miR-182 on downstream molecules of the Wnt/β-catenin axis: According to qPCR analysis, the miR-182 mimic group had 33-fold and 3-fold higher *CCND1* expression in HeLa and SiHa cells, respectively, than the control group. Similarly, the miR-182 mimic group had 12-fold and 3-fold higher *MYC* expression in HeLa and SiHa cells, respectively, than the control group (**Figure 4A**, **4B**). In contrast, miR-182 inhibition reduced *CCND1* expression by 70% and 60% in HeLa and SiHa cells, respectively, in the miR-inhibitor group when compared with the control group. In addition, upon miR-182 inhibition, *MYC* expression in HeLa and SiHa cells reduced by 90% and 60%, respectively, in the miR-inhibitor group when compared with the control group (**Figure 4C**, **4D**). Western blot results further validated that miR-182 mimics increased the protein levels of *CCND1* and *MYC*, while miR-182 inhibitor reduced the protein levels of *CCND1* and *MYC* (**Figure 4E**, **4F**). These results indicate that miR-182 could activate the Wnt/β-catenin signaling and thus increase its downstream molecules in CC cells.

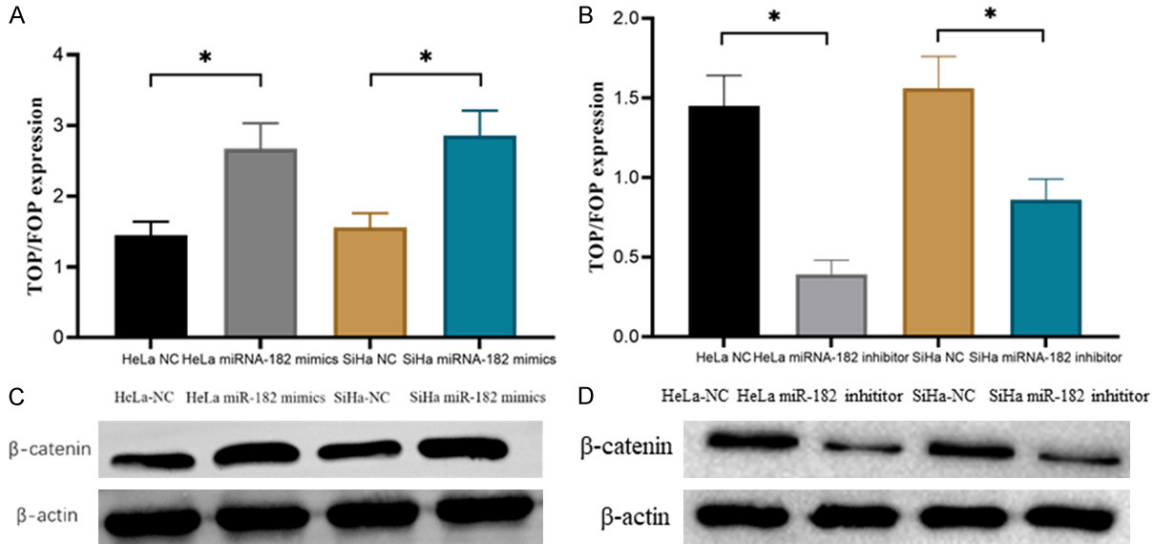

Figure 3. Role of miR-182 on the Wnt/β-catenin signaling: (A) TOP/FOP ratio in miR-182 mimic-transfected CC cell lines. (B) TOP/FOP ratio in miR-182 inhibitor-transfected CC cell lines. (C) β-catenin expression in miR-182 mimic-transfected CC cell lines. (D) β-catenin expression in miR-182 inhibitor-transfected CC cell lines. *$P < 0.05$.

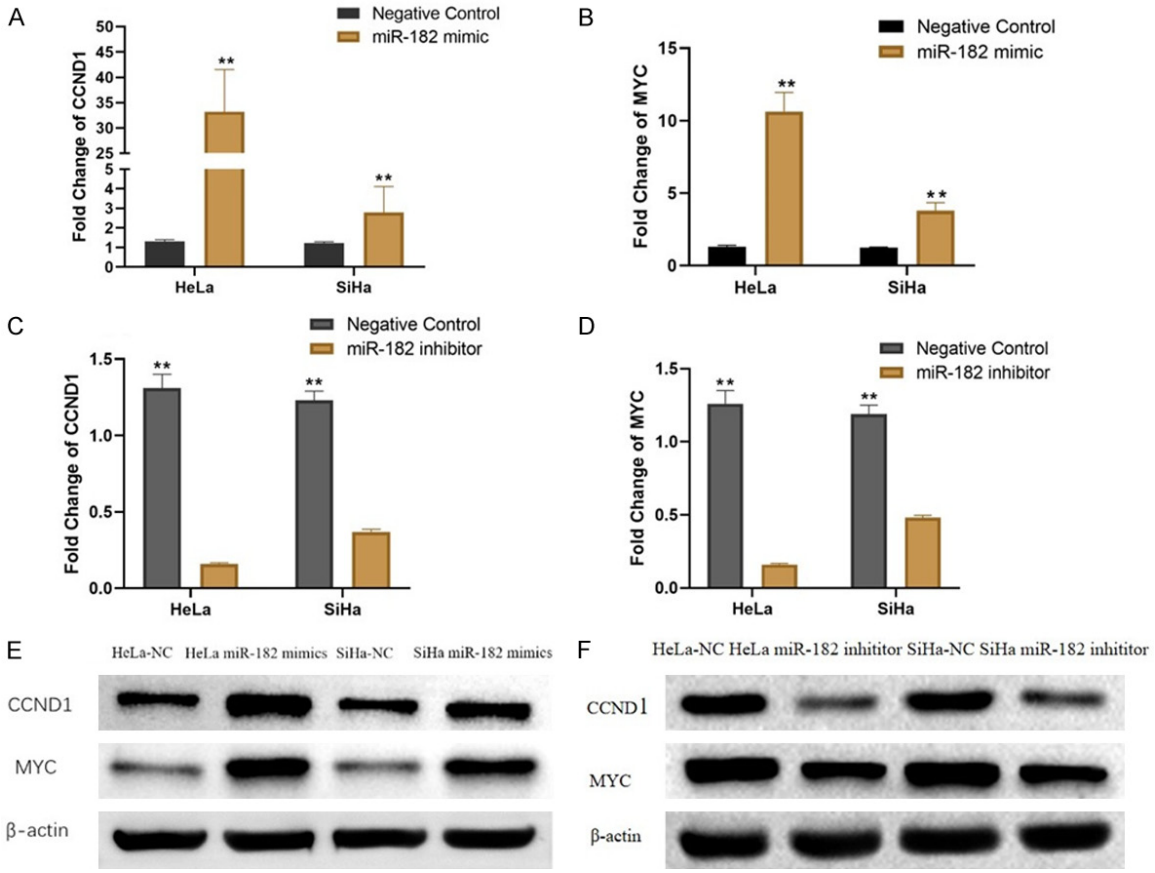

Figure 4. Effect of miR-182 on the downstream molecules of the Wnt/β-catenin axis. A, B. *CCND1* and *MYC* expression in miR-182 mimic-transfected CC cell lines evaluated by qPCR. C, D. *CCND* and *MYC* expression in miR-182 inhibitor-transfected CC cell lines evaluated by qPCR. E. *CCND* and *MYC* expression in miR-182 mimic-transfected CC cell lines evaluated by western blot. F. *CCND* and *MYC* expression in miR-182 inhibitor-transfected CC cell lines evaluated by western blot.

Discussion

CC is a common malignancy in females and its incidence ranks fourth worldwide [9]. According to the 2012 China Cancer Annual Report registration data, it is among the most common malignancies diagnosed in Chinese women and ranks ninth nationally. Clinically, CC is staged based on the FIGO criteria. Radical radiotherapy or hysterectomy and lymph node dissection are generally used for patients at an early stage of the disease. While concurrent chemoradiotherapy is the standard treatment for locally advanced patients with CC. Recently, the application of radiotherapy and chemotherapy has been greatly improved and the radiotherapy technology has also been innovated; yet, there is only little improvement in the survival of patients with CC. Moreover, most patients with CC develop locally advanced stage or have metastases even before diagnosis. Consequently, the 5-year survival rate of patients remains low, the benefits of clinical treatments are limited, and the prognosis is poor. Delayed identification and lack of effective early diagnosis methods are important factors that contribute to the poor efficacy of the treatment modalities and poor prognosis of CC [10]. Therefore, early diagnosis and prompt treatment of CC are key factors that can prolong the survival of patients. However, the key molecules that regulate CC progression remain elusive.

MiRNAs or small non-coding RNAs are widely present in the human body. They regulate approximately one-third of the coding genes and participate in physiological functions, such as regulation of cell proliferation, differentiation, metabolism, apoptosis, etc. [11]. In addition, miRNAs participate in the pathological progression of various cancers. Accumulating evidence indicates their potential as biomarkers for cancer due to their abnormal expression in cancerous tissues, especially in metastatic and invasive cancers, compared with the adjacent counterparts [12]. This study found that miR-182 was overexpressed in CC tissues than the adjacent normal tissues, which was consistent with our hypothesis that miR-182 can promote CC cell proliferation. Additionally, we found that miR-182 was positively correlated with the presence of lymphatic metastasis in CC tissues. Collectively, these results indicate the pro-tumor effects of miR-182.

The Wnt signaling pathway is pivotal as it affects tissue development and homeostasis regulation in adults. β-catenin is the prime effector of this pathway. In the absence of Wnt stimulation, β-catenin predominantly accumulates in the cytoplasm where it undergoes ubiquitin-proteasome-dependent degradation [13]. However, when the Wnt pathway is activated, the “destruction complex” constituted by APC, GSK3β, Axin, and CK1 releases β-catenin. Thereafter, β-catenin translocates to the nucleus, binds to transcription factors Tcf and Lef, and ultimately transcribes downstream genes (such as c-*myc* and *CCND1*) [20, 21]. While aberrant Wnt activation causes nuclear β-catenin accumulation, thereby constitutively activating the cell proliferation and apoptosis-associated proto-oncogenes [14]. Abnormal activation of the Wnt/β-catenin pathway has been reported in osteosarcoma [15], colorectal carcinoma [16], oral squamous cell carcinoma [17], and prostate cancer [18]. Shi and Sheng [19] found that SOX-2, β-catenin, Wnt-1, and Wnt-3a were significantly higher in cervical tissues of high-, middle-, and low-risk human papillomavirus (HPV) groups than normal cervical tissues, and the high-risk HPV 16 group had the highest expression. This indicates that progression from HPV infection to carcinogenesis involves Wnt/β-catenin axis activation. Moreover, Wnt/β-catenin activation is identified as the first step in the complex process of CC, which suggests its pivotal role in CC development [22]. Hence, it is necessary to modulate the activity of this axis to maintain cervical cell homeostasis and normal development. This study identified Wnt/β-catenin as a potential target of miR-182, which explains the protumor effects of miR-182.

HeLa and SiHa are two types of CC cell lines with distinct sources and backgrounds. HeLa is primarily HPV 18, while SiHa is mainly HPV 16 infection. This study found that miR-182 expression was positively correlated with the proliferation of HeLa and SiHa cells. Moreover, we found that miR-182 overexpression markedly increased the TOP/FOP ratio in both HeLa cells and SiHa cells, whereas miR-182 inhibition notably decreased it. These results proved that miR-182 expression was critical in CC. Further, we explored the role of the downstream genes (*MYC* and *CCND1*) and found that the effects of miR-182 overexpression or inhibition were more pronounced in HeLa cells than in SiHa

cells. These findings were similar to the results of Li P [23], suggesting that miR-182 overexpression could activate the Wnt/β-catenin axis, thereby accelerating the occurrence of CC.

In conclusion, we identified a novel mechanism whereby miR-182 promotes CC progression by modulating the Wnt/β-catenin axis and its downstream genes. These findings suggest the potential role of miR-182/β-catenin cascade as a feasible novel target for CC treatment.

Disclosure of conflict of interest

None.

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