MicroRNA-494 promotes cervical cancer proliferation through the regulation of PTEN

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Abstract. The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway appears to be a key regulator in cervical carcinogenesis. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein is principally involved in the homeostatic maintenance of PI3K/Akt signaling and PTEN has been identified to play an important role in the occurrence and development of cervical cancer. MicroRNA (miRNA)-494 has been proven to be involved in the carcinogenesis and development of various types of cancer by directly targeting PTEN. However the role, mechanism and clinical significance of miR-494 in cervical cancer have not been further reported. In the present study, we analyzed the expression of miR-494 in cervical cancer cell lines and clinical specimens by RT-qPCR, and explored the association of miR-494 with PTEN expression and clinicopathological data of cervical cancer patients. The results showed that miR-494 expression was significantly upregulated in human cervical cancer cell lines and tissues. miR-494 upregulation was significantly associated with PTEN downregulation, adverse clinicopathological characteristics, poor overall and progression-free survival and poor prognosis. *In vitro* experiments showed that inhibition of miR-494 suppressed cell proliferation and growth by directly targeting the 3'-untranslated region (3'-UTR) of PTEN mRNA. These findings identified a novel molecular mechanism involved in the regulation of PTEN expression and cervical cancer progression. Results of the present study indicated that miR-494 may have an essential role in the carcinogenesis and progression of cervical cancer and targeting miR-494 may be a promising therapeutic strategy for the treatment of cervical cancer.

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Abbreviations: miR-494, microRNA-494; HR-HPV, high-risk human papilloma virus; PTEN, phosphatase and tensin homolog deleted on chromosome 10; NCEC, normal cervical epithelial cells; WT, wild-type; MT, mutant; 3'-UTR, 3'-untranslated region; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MMAC1, mutated in multiple advanced cancer 1

Key words: miR-494, cervical cancer, proliferation, PI3K/Akt, PTEN

Introduction

Cervical cancer is the fourth most prevalent cause of cancer-related mortality in women worldwide and 12,360 estimated new cases of cervical cancer were diagnosed in 2014, with 4,020 estimated deaths in the USA (1). Significant advances concerning the molecular mechanisms of cervical carcinogenesis have been made during the last several decades (2). However, the detailed mechanisms of cervical cancer initiation and progression have yet to be fully elucidated. Persistent infection with high-risk human papilloma virus (HR-HPV) has been proven to be the main cause of almost all types of cervical cancer. However, a substantial body of evidence shows that HR-HPV infection alone is not sufficient to induce malignant transformation, indicating that other genetic alterations may be involved in cervical carcinogenesis (3). Identification of key factors in cervical cancer is important for the screening, diagnosis and treatment of cervical cancer.

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway appears to be a key regulator in cervical carcinogenesis, as it is activated in >90% of cervical cancer types (4). Akt signaling is the downstream target of HPV oncoproteins which have been identified as major mediators of cervical cancer initiation and development (5). Gene expression profiling also demonstrated that the PI3K/Akt signaling pathway may be of potential therapeutic target in cervical cancer (6). The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein is principally involved in the homeostatic maintenance of the PI3K/Akt signaling pathway (7). Findings of a previous study showed that loss of PTEN resulted in persistent activation of PI3K effectors which has an important impact on various aspects of cancer development such as cell proliferation, cell cycle, cell migration and metastasis (8). It

has been demonstrated that abnormal promoter methylation of the *PTEN* gene was usually identified in cervical cancer and associated with tumor differentiation, lymph-node metastasis and FIGO staging. PTEN was identified to be important in the occurrence and development of cervical cancer (9). Therefore, identification of the molecular regulating PTEN expression may be an attractive strategy for elucidating the underlying mechanism of cervical carcinogenesis.

MicroRNAs (miRNAs) are small non-coding RNAs of 22 nucleotides in length, transcribed from non-protein coding genes or introns, which generally act as negative regulators of gene expression at post-transcriptional levels through mRNA degradation and translation repression (10). Accumulating evidence has shown that the aberrant expression of miRNAs may function as tumor suppressors or oncogenes in cancers according to the role of their target genes (11), which indicates miRNAs have the potential to be diagnostic and prognostic biomarkers for cancer (12). miR-494 has consistently been reported to be aberrantly expressed in various types of cancer. The functional role of miR-494 is extremely complex as it may function as an oncogenic or tumor suppressive miRNA depending on the cellular microenvironments (13-17). More importantly, it has been reported that miR-494 participated in the carcinogenesis and development of colorectal cancers by directly targeting PTEN (18). Aberrant miRNA expression profiles have also been identified in cervical cancer cell lines and cervical cancer tissues (19,20). However, to the best of our knowledge, there are few detailed studies focusing on the role of miR-494 in cervical cancer. Given the complexity of its functionality, it would be of interest to explore the functional roles and relationship of miR-494 and PTEN in cervical cancer carcinogenesis and development.

In the present study, we analyzed the expression of miR-494 in cervical cancer cell lines and clinical specimens, and examined the association of miR-494 with PTEN expression and clinicopathological data of cervical cancer patients. *In vitro* experiments showed that inhibition of miR-494 suppressed cell proliferation and growth by directly targeting the 3'-untranslated region (3'-UTR) of PTEN mRNA. These findings identified a novel molecular mechanism involved in the regulation of PTEN expression and cervical cancer progression. Thus, targeting miR-494 may be a promising therapeutic strategy for the treatment of cervical cancer.

Materials and methods

Patients and tissue specimens. The tissue-based specimen collection and study were approved by the Research Ethics Committee of Xi'an Jiaotong University. All the patients provided written consent and indicated willingness to donate their blood and tissue samples. A total of 89 patients were enrolled in the present study. Clinical and pathological classification and staging were performed according to the International Federation of Gynecology and Obstetrics criteria (21). The clinicopathological information of the patients is shown in Table I. The follow-up information for all participants was updated every 3 months by telephone. Information regarding the death of patients was ascertained from their family. In all 89 snap-frozen cervical cancer samples, the HC2 assay was used to detect the presence of high-risk HPV DNA, including DNA from HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (22). High-risk HPV (HR-HPV) was detected in 79 cases, which gave an overall infection rate of 88.8%.

Cell culture. Primary normal cervical epithelial cells (NCEC) obtained from healthy female cervical tissue were cultured in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA, USA) supplemented with epithelial growth factor, bovine pituitary extract and antibiotics (1% streptomycin and 1% penicillin). The HeLa, C33A, Caski and SiHa cervical cancer cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen). The cells were supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen).

Reverse transcriptase-quantitative PCR (RT-qPCR) assay. The expression of miR-494 in cervical cancer and corresponding adjacent tissues was detected by the RT-qPCR assay. Briefly, total RNA was extracted from tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. miRNA expression levels were quantified using a TaqMan miRNA real-time RT-PCR kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Data were analyzed using 7500 software v. 2.0.1 (Applied Biosystems), with the automatic Ct setting for adapting the baseline and threshold for Ct determination. The universal small nuclear RNA U6 (RNU6B) was used as an endogenous control for miRNAs. Each sample was examined in triplicate and the amount of PCR products produced was non-neoplasticized to RNU6B.

Oligonucleotide transfection. miR-494 inhibitors were chemically synthesized by Shanghai GenePharma (GenePharma, Shanghai, China). When the cells reached 80% confluence, miR-494 inhibitor was transfected into cervical cancer cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were also transfected with scramble oligonucleotide as a negative control (NC). The expression level of mir-494 in the transfected osteosarcoma cells were identified by RT-qPCR.

Luciferase reporter assay. Cervical cancer cells were seeded in 96-well plates at 60% confluence. After 24 h, the cells were transfected with 120 ng of miR-494 expression vector or NC. The cells were transfected with 30 ng of wild-type (WT) or mutant (MT) 3'-UTR of PTEN mRNA. The cells were collected 48 h after transfection, and luciferase activity was measured using a dual luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI, USA).

Cell viability assay. Cells were plated in 96-well plates ($0.5x10^4$ cells/well) and transfected with NC, and miR-494 inhibitors. After 48 h, 10 μl of MTT reagent (5 mg/ml) was added to each well and the cells were incubated at 37˚C for another 4 h. The medium was removed, the cells were solubilized in 150 μl of dimethyl sulfoxide, and colorimetric analysis was performed (wavelength, 490 nm). One plate was analyzed immediately after the cells adhered (~4 h after plating), and the remaining plates were assayed every day for the following 4 consecutive days.

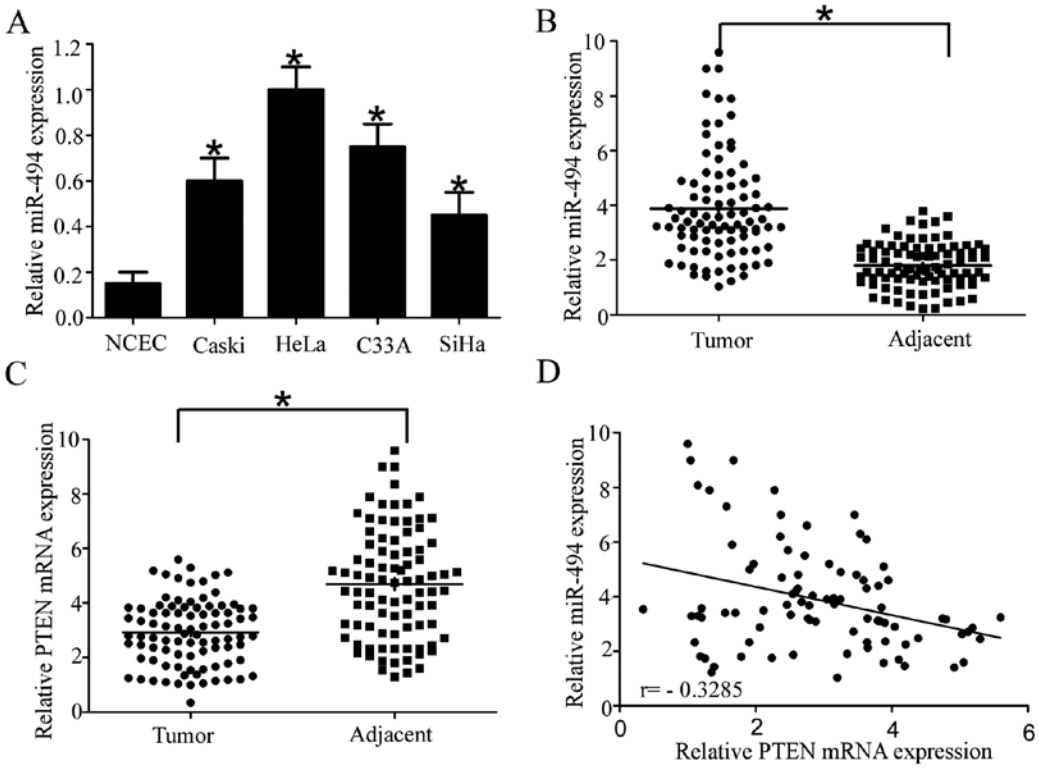

Figure 1. Expression of miR-494 and PTEN is increased in cervical cancer cell lines and tissues. (A) Expression of miR-494 in normal cervical epithelial cells (NCEC) and cervical cancer cell lines (Caski, HeLa, C33A and SiHa). (B) Expression of miR-494 in 89 paired cervical cancer tissues and their adjacent normal tissues. (C) Expression of PTEN mRNA in 89 paired cervical cancer and their adjacent normal tissues. (D) Correlation between miR-494 and PTEN mRNA levels in cervical cancer tissues. Expression levels of miR-494 and PTEN were determined by RT-qPCR and normalized against an endogenous control U6 RNA and β-actin, respectively. *P<0.05. PTEN, phosphatase and tensin homolog deleted on chromosome 10.

Colony formation assay. Briefly, 10 cm dishes were seeded with 500 viable cells in complete medium and allowed to grow for 24 h. The cells were then incubated in the presence of miR-494 inhibitors or NC for up to 48 h. The medium was removed, and the cells were washed in phosphate-buffered saline (PBS) and incubated for an additional 10 days in complete medium. Each treatment was carried out in triplicate. The colonies obtained were washed with PBS and fixed in 4% formalin for 10 min at room temperature and then washed with PBS followed by staining with 0.2% crystal violet.

Soft agar colony formation assay. Cells seeded in a 6-well plate were covered with a layer of 0.6% agar in DMEM medium supplemented with 10% FBS. After transfection for 48 h, the cells were trypsinized, gently mixed with 0.3% agar medium mixture containing selective antibiotics and reseeded in triplicate in a 6-well plate. After 4 weeks, the resistant colonies were stained with 0.2% crystal violet and counted under the microscope.

Flow cytometric analysis of cell cycle. The cervical cancer cells were transfected with NC and miR-101 inhibitors. Forty-eight hours after post-transfection, the cells were trypsinized and analyzed for cell cycle distribution. For cell cycle distribution, the cells of each group were stained with propidium iodide (PI) and analyzed by flow cytometry using FACSCalibur (BD Biosciences, San Diego, CA, USA). For each group, 10,000 events were obtained. The percentage of cells in G1, S and G2 phases of the cell cycle was calculated.

Statistical analysis. Data are presented as mean ±SD. Statistical analysis was performed using IBM SPSS statistical software (version 21.0) (International Business Machines Corporation, Armonk, NY, USA). The differences in characteristics between the two groups were examined by the χ^2 or Fisher's exact tests. P-values were determined from two-sided tests, and statistical significance was based on a P-value of 0.05.

Results

miR-494 is upregulated in cervical cancer cell lines and tissues. To examine the levels of miR-494 expression in cervical cancer, we conducted RT-qPCR to measure miR-494 expression in four cervical cancer cell lines and NCEC. The result showed that miR-494 was markedly increased in the Caski, HeLa, C33A and SiHa cervical cell lines, particularly in HeLa and C33A, compared with NCEC (Fig. 1A). Consistent with the results found in cervical cell lines, miR-494 expression was significantly higher in 89 cervical cancer tissue specimens compared with their adjacent normal tissues (Fig. 1B). By contrast, the expression of PTEN was significantly down-regulated in cervical cancer tissues compared with their normal tissue counterparts (Fig. 1C), which was consistent with previous literature (23). More importantly, statistically significant inverse correlations were revealed by Spearman's correlation analysis between mRNA levels of miR-494 and PTEN in cervical cancer specimens (r=-0.3285; P=0.0017). Taken together, the results suggested that miR-494 played an oncogenic role and PTEN a tumor-suppressor role in cervical

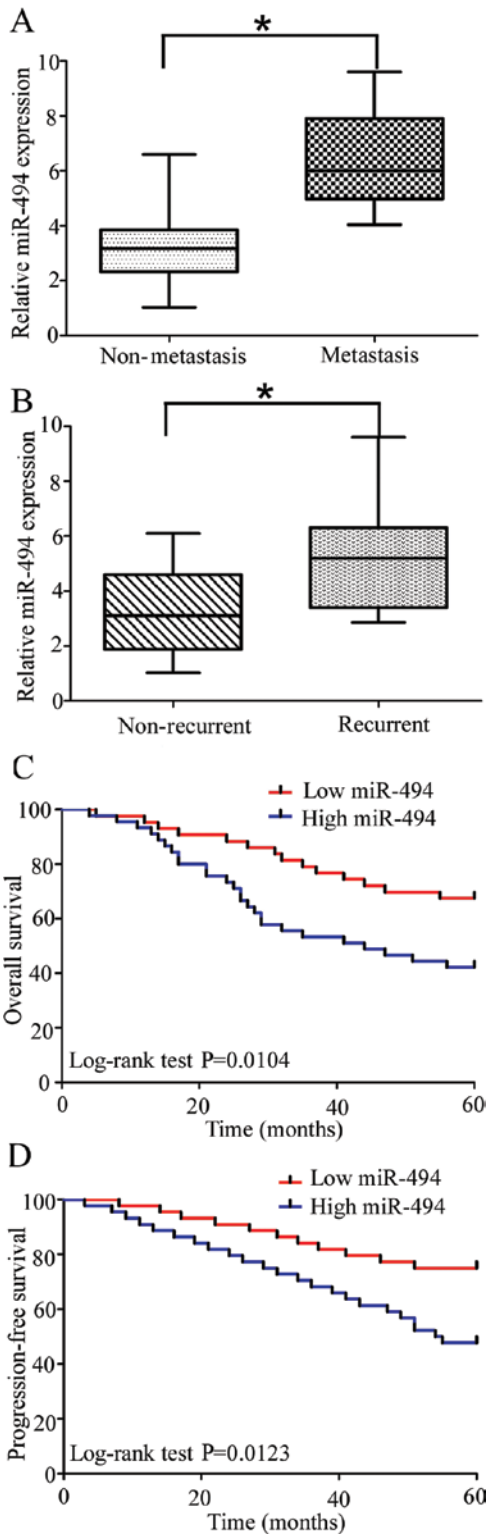

Figure 2. Clinical significance of miR-494 in cervical cancer patients. (A) Comparison of miR-494 levels between metastatic and non-metastatic cervical cancer patients. (B) Comparison of miR-494 levels between recurrent and non-recurrent cervical cancer patients. (C) Overall survival curves for low and high expression of miR-494 in cervical cancer patients. (D) Progression-free survival curves for low and high expression of miR-494 in cervical cancer patients. Expression levels of miR-494 were determined by RT-qPCR and normalized against an endogenous control U6 RNA. *P<0.05.

cancer. Furthermore, miR-494 inversely correlated with PTEN in cervical cancer, which indicated that PTEN was a potential target of miR-494 in cervical cancer.

Table I. Association between miR-494 expression and clinicopathological characteristics.

		miR-494 expression		
Characteristics	No.	Low	High	P-value
Age (years)				0.666
≤35	35	16	19	
>35	54	28	26	
FIGO stage				0.026[a]
IB	58	34	24	
>IB	31	10	21	
HR-HPV				0.091
Yes	79	38	41	
No	10	8	2	
Differentiation				0.833
Well	41	21	20	
Moderate/poor	48	23	25	
Tumor size				0.477
≤4 cm	70	33	37	
>4 cm	19	11	8	
LN metastasis				0.027[a]
Yes	78	35	43	
No	11	9	2	
Stromal invasion				0.045[a]
<2/3	68	38	30	
≥2/3	21	6	15	

[a]Significant difference of clinical factors with overall survival. HR-HPV, high-risk human papilloma virus.

Upregulation of miR-494 is associated with metastasis and recurrence in cervical cancer patients. To explore the relationship between miR-494 and cervical cancer, we investigated the correlation of miR-494 expression with metastasis and recurrence of cervical cancer. Compared with non-metastatic cervical cancer specimens, the miR-494 levels were significantly upregulated in metastatic cervical tissues (Fig. 2A). Moreover, miR-494 levels were significantly higher in the specimens obtained from the patients who suffered cervical cancer recurrence (Fig. 1B). Collectively, these data indicated that significantly upregulation of miR-494 expression was correlated with relapse and metastasis in cervical cancer patents.

miR-494 expression is correlated with clinicopathological characteristics and prognosis in cervical cancer patients. In order to determine the clinical significance of miR-494 in cervical cancer, the 89 patients were divided into two groups based on miR-494 expression levels (low vs. high) with the median expression levels as a cut-off point. The Kaplan-Meier analysis revealed that high miR-494 expression was significantly correlated with reduced overall and progression-free survival in 89 cervical cancer patients (Fig. 2C and D; log-rank test,

Table II. Univariate and multivariate analyses of clinical parameters in relation to overall survival.

	Univariate		Multivariate	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
miR-494 expression	4.143 (1.751-6.397)	0.009[a]	3.279 (1.177-5.192)	0.013[a]
Age (years)	2.145 (0.745-2.451)	0.658	1.784 (0.874-2.175)	0.791
FIGO stage	4.156 (2.209-5.167)	0.017[a]	3.516 (2.124-5.349)	0.009[a]
HR-HPV	3.129 (1.296-4.719)	0.045[a]	2.891 (1.152-4.325)	0.021[a]
Differentiation	1.819 (0.742-2.795)	0.209	2.113 (0.696-2.782)	0.491
Tumor size	1.361 (0.534-1.987)	0.419	1.542 (0.759-2.175)	0.219
LN metastasis	3.714 (1.892-5.562)	0.017[a]	4.115 (1.579-6.123)	0.008[a]
Stromal invasion	1.193 (0.415-1.987)	0.118	1.453 (0.879-2.161)	0.374

CI, confidence interval; HR, hazard ratio; HR-HPV, high-risk human papilloma virus; LN, lymph node. [a]Significant relation of clinical factors with overall survival.

Table III. Univariate and multivariate analyses of clinical parameters in relation to progression-free survival.

	Univariate		Multivariate	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
miR-494 expression	4.891 (2.425-6.257)	0.007*	4.614 (2.895-10.321)	<0.001[a]
Age (years)	1.427 (0.628-2.162)	0.351	1. 891 (0.898-2.477)	0.519
FIGO stage	3.451 (1.679-4.129)	0.029[a]	2.915 (1.789-4.187)	0.011[a]
HR-HPV	3.198 (1.589-5.245)	0.014[a]	2.941 (1.497-4.827)	0.008[a]
Differentiation	1.813 (0.741-2.514)	0.295	2.161 (0.819-3.255)	0.417
Tumor size	1.429 (0.711-2.287)	0.342	1.827 (0.717-3.165)	0.417
LN metastasis	5.104 (1.998-10.179)	0.029[a]	4.219 (2.326-7.619)	0.031[a]
Stromal invasion	1.355 (0.611-2.341)	0.173	1.625 (0.681-2.749)	0.251[a]

CI, confidence interval; HR, hazard ratio; HR-HPV, high-risk human papilloma virus; LN, lymph node. [a]Significant relation of clinical parameters with progression-free survival.

P=0.0104 and P=0.0123, respectively). The patients with a high miR-494 expression tended to have a shorter overall and progression-free survival time when compared to patients with a low miR-494 expression. In addition, upregulation of miR-494 was significantly correlated with FIGO stage, lymph-node metastasis and deep stromal invasion while no significant correlation was observed in other clinicopathological variables (Table I). The, univariate analysis demonstrated that the overall and progression-free survival of cervical cancer patients was associated with FIGO stage, lymph-node status, and HR-HPV and miR-494 expression (Tables II and III). To determine whether the prognostic value of miR-494 was independent of other clinicopathological parameters for poor overall and progression-free survival in cervical cancer patients, a multivariate analysis was performed using a Cox proportional hazard model. The multivariate analysis including miR-494 expression, age, FIGO stage, HR-HPV, differentiation status, tumor size and lymph-node metastasis demonstrated that a high miR-494 expression was an independent prognostic biomarker for poor overall and progression-free survival in cervical cancer patients (Tables II and III; HR=3.279, CI=1.177-5.192, P=0.013 and HR=4.614, CI=2.895-10.321, P<0.001 respectively). Statistically significant results were also obtained for FIGO stage and lymph-node metastasis, where the other parameters were not independent prognostic biomarkers for overall and progression-free survival in cervical cancer patients. Taken together, these results suggest the upregulation of miR-494 was significantly correlated with a worse prognosis and was involved in the progression of cervical cancer.

miR-494 promotes the proliferation of cervical cancer cells. As the relative expression of miR-494 was relatively higher in HeLa and C33A than SiHa and Caski, we chose HeLa and C33A to investigate the physiological function of miR-494 in cervical cancer cells. To analyze the effect of miR-494 on the proliferation of cervical cancer cells, we transfected miR-494 inhibitors into HeLa and C33A cell lines. As shown in Fig. 3A, transfection of miR-494 inhibitors decreased the miR-494 expression

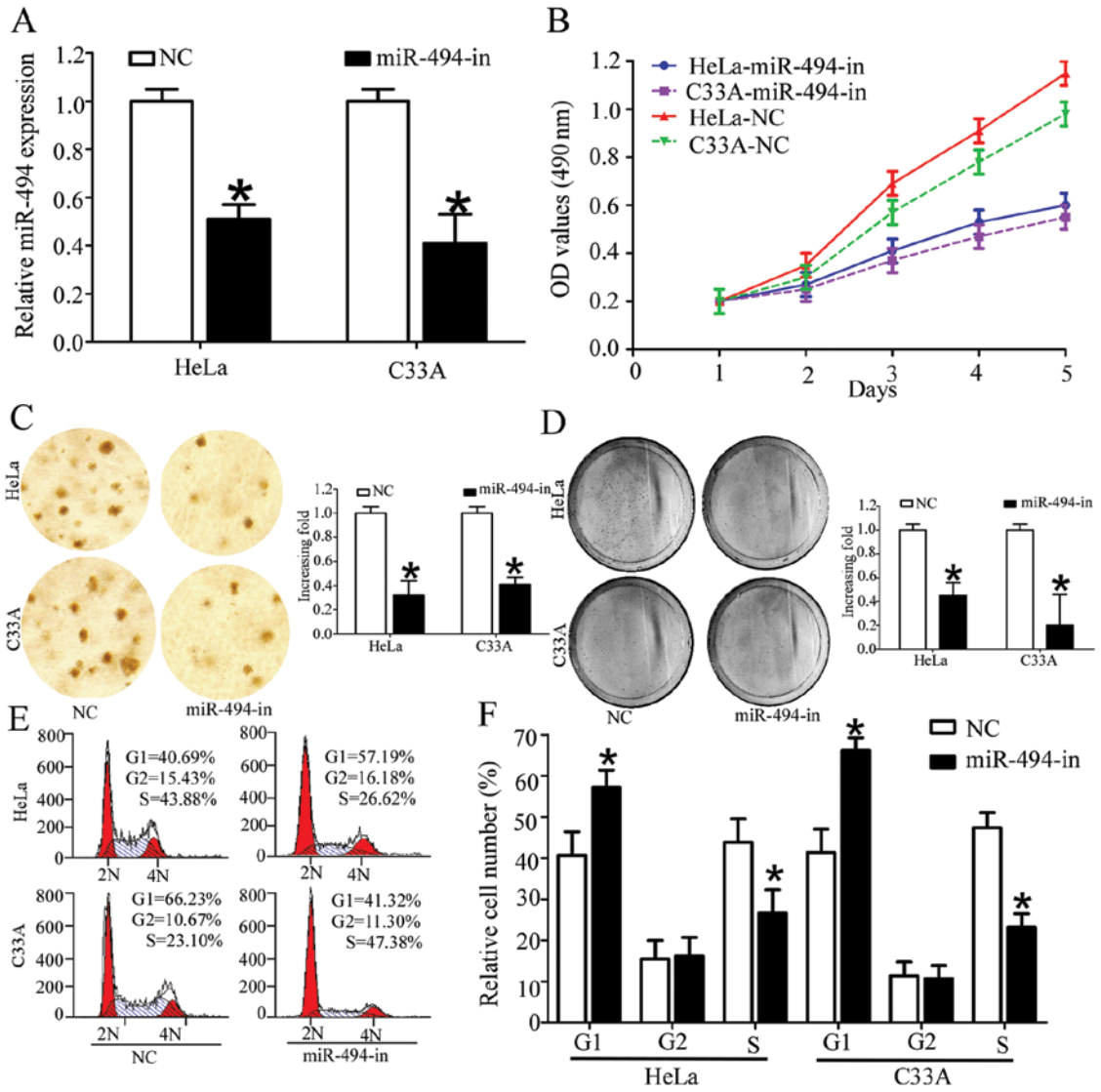

Figure 3. Effects of miR-494 inhibition on cell proliferation and cell cycle of cervical cancer cells. (A) Expression of miR-494 in HeLa and C33A cells transfected with miR-494 inhibitor. (B) Effects of miR-494 inhibition on the cell viability of HeLa and C33A cervical cancer cells. (C) Effects of miR-494 inhibition on soft-agar colony formation ability of HeLa and C33A cervical cancer cells. (D) Effects of miR-494 inhibition on colony formation ability of HeLa and C33A cervical cancer cells. (E and F) Effects of miR-494 on cell cycle of HeLa and C33A cervical cancer cells. Three independent experiments were performed in duplicate. Data are presented as mean ± SD. Two-tailed Student's t-test was used to analyze the significant differences. *P<0.05.

in HeLa and C33A (Fig. 3A). After confirming the efficiency of miR-494 inhibitors, we determined the effects of miR-494 on cell viability using an MTT assay. Cervical cancer cells transfected with miR-494 inhibitors showed a significant decrease in cell viability as compared with the normal control (Fig. 3B). We determined the effect of miR-494 on cell proliferation using colony formation and soft agar colony formation assays. As shown in Fig. 4C and D, inhibition of miR-494 significantly decreased the growth rate of the two cervical cell lines as compared with the normal control (Fig. 4C and D). Taken together, these results indicated that the downregulation of miR-494 suppressed the proliferation of cervical cancer cells.

Effect of miR-494 on cell cycle in vitro. As miR-494 significantly affects cell proliferation in HeLa and C33A cells, we hypothesized that miR-494 functions by affecting the cell cycle of cervical cancer cells. Thus, we investigated the effect of miR-494 on the cell cycle by flow cytometry. The results revealed that overexpression of miR-494 inhibitors markedly increased the number of cells in G1 peak and decreased those in the S peak (Fig. 3E and F). Taken together, these results indicated the inhibition of miR-494 suppressed the proliferation of cervical cancer cells by inducing cell cycle arrest.

Inhibition of miR-494 increases cell cycle inhibitors $p21^{Cip1}$ and decreases cell cycle regulator cyclin D1. As overexpression of miR-494 inhibitors appears tobe closely linked to the proliferation of cervical cancer cells, we further investigated whether the CDK inhibitor $p21^{Cip1}$ or the CDK regulator cyclin D1 could be regulated by miR-494. RT-qPCR and western blot analysis revealed that $p21^{Cip1}$ was upregulated, whereas cyclin D1 was downregulated in cervical cells transfected with miR-494 inhibitors compared with cells transfected with the normal control (Fig. 4A and B). Taken together, these results supported our hypothesis that miR-494 has a critical role in the growth of cervical cancer cells.

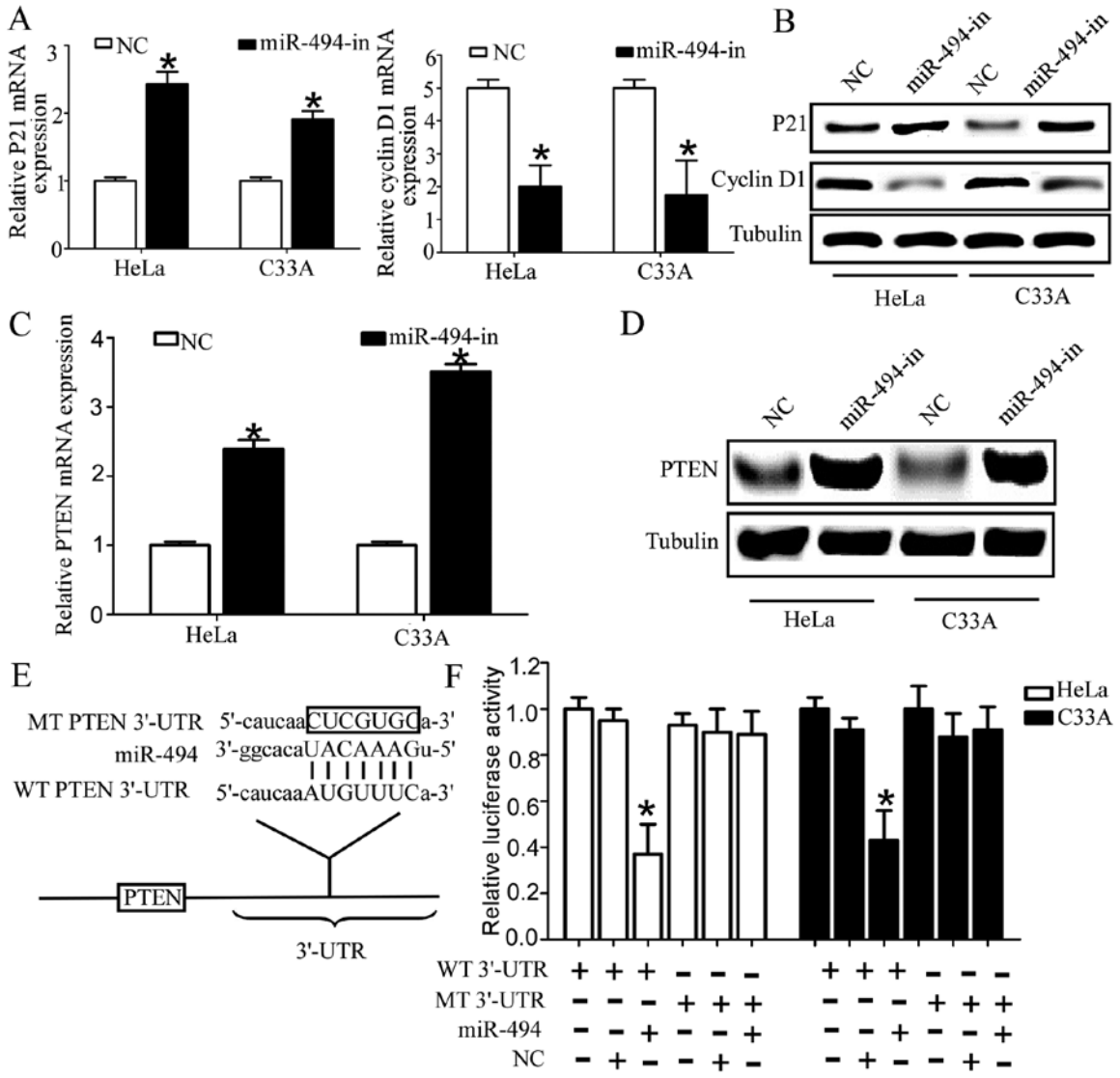

Figure 4. PTEN is the direct target of miR-494. (A) Expression of $p21^{Clip1}$ and cyclin D1 in HeLa and C33A cells following transfection with miR-494 or NC by RT-qPCR. (B) Expression of $p21^{Clip1}$ and cyclin D1 in HeLa and C33A cells following transfection with miR-494 or NC by western blot analysis. Tubulin was used as a loading control. (C) Expression of PTEN in HeLa and C33A cells after transfection with miR-494 or NC by RT-qPCR. (D) Expression of PTEN in HeLa and C33A cells after transfection with miR-494 or NC by western blot analysis. Tubulin was used as a loading control. (E) miR-494 and its putative binding sequence in the 3'-UTR of PTEN, and the diagram of the luciferase reporter plasmids with WT and MT PTEN 3'-UTR. (F) Luciferase assay on HeLa and C33A cells transfected with the plasmids containing WT or MT PTEN 3'-UTR and miR-494 inhibitor oligonucleotides, as indicated. Three independent experiments were performed in duplicate. Data are expressed as means ± SD. Two-tailed Student's t-test was used to analyze the significant differences. *P<0.05. PTEN, phosphatase and tensin homolog deleted on chromosome 10; NC, negative control, WT, wild-type; MT, mutant; 3'-UTR, 3'-untranslated region.

PTEN is the direct target of miR-494 in cervical cancer cells. It has been proven that PTEN was the direct target of miR-494 in multiple solid tumors (24), and loss of protein expression of PTEN was involved in the pathogenesis, proliferation and metastasis of cervical cancer (25,26). Considering the tissue-specific and developmental stage-specific manner of miRNA, we investigated the relationship between PTEN and miR-494 in cervical cancer. In order to confirm PTEN is the target gene for miR-494 in cervical cancer cells, RT-qPCR and western blotting was used to detect the expression of PTEN in HeLa and C33A. As expected, the expression of PTEN at the mRNA and protein level was significantly upregulated in cervical cancer cells transfected with miR-494 inhibitors (Fig. 4C and D). Our previous results demonstrated the mRNA of PTEN was inversely correlated with miR-494 expression (Fig. 1D). Taken together, these results suggested that PTEN was the potential target gene of miR-494 in cervical cancer cell lines and tissues.

Then we performed the luciferase reporter assay to further verify whether miR-494 directly targeted the 3'-UTR of PTEN in cervical cancer cells. The target sequence of wild-type PTEN 3'-UTR (WT 3'-UTR) or the mutant PTEN 3'-UTR (MT 3'-UTR) was cloned into a luciferase reporter vector (Fig. 4E). As shown in Fig. 4F, transfection of miR-494 consistently suppressed the luciferase activity of PTEN WT3'UTR luciferase reporter plasmids in HeLa and C33A cells, whereas point mutations in the miR-494-binding seed region of the PTEN abrogated the repressive effect of miR-494. Taken together, the data suggested that PTEN was a genuine target of miR-494.

Discussion

In the present study, miR-494 expression was significantly upregulated in human cervical cancer cell lines and tissues. miR-494 upregulation was also significantly associated with PTEN downregulation, adverse clinicopathological characteristics, poor overall and progression-free survival, and poor prognosis. In addition, inhibition of miR-494 expression induced cell cycle arrest in G1 stage and inhibited cell proliferation and cell growth in cervical cancer cell lines. Additional *in vitro* studies showed that PTEN was the direct target of miR-494 in cervical cancer cells. Results of the present study show that miR-494 may have an essential role in the carcinogenesis and progression of cervical cancer.

Several miRNAs have been identified as candidate components of oncogene and tumor suppressor networks in cervical cancer, and these miRNAs and their targets play critical roles in the carcinogenesis and progression of cervical cancer. For example, miR-135a/SIAH1/β-catenin signaling functions as an oncogene in the transformation and progression of cervical cancer (27). Similarly, miR-31, miR-155 and miR-1246 are found to promote cervical cancer cell proliferation and function as oncomiRs in cervical cancer (28-30). However, miR-507, miR-99a and miR-99b act as tumor suppressors in cervical cancer and inhibit cervical cancer cell proliferation and cell growth (31,32). Classical tumor suppressor miR-101 induced cell cycle arrest by targeting Fos (33). However the role, mechanism and clinical significance of miR-494 in cervical cancer have not been further reported, since whether miR-494 is an oncogenic or tumor suppressor miRNA remains to be determined.

Accumulating evidence suggested that the functions of miR-494 in cancer development are complicated. Upregulation of miR-494 has been proven to be associated with promotion in cell proliferation and cell growth in H460 lung and breast cancer cells, colorectal cancer, hepatocellular carcinoma and transformed bronchial epithelial cells (18,24,34-36). However, miR-494 functions as a tumor suppressor and induces cell cycle arrest in lung, gastric and prostate cancer, and cholangiocarcinoma (17,37,38). Different tumor microenvironments, cellular contexts, tissue specificity and molecules which miR-494 targeted account for this discrepancy. As the effect of miR-494 in cervical cancer was far from defined, the present study aimed to investigate the potential biological function of miR-494 in cervical cancers. Our results demonstrate that suppression of miR-494 significantly inhibited the cell proliferation and cell growth of HeLa and C33A cells by induction of cell cycle arrest. More importantly, miR-494 was significantly correlated with adverse clinicopathological features, poor survival and prognosis of cervical cancer patients. All these results suggest that miR-494 may function as an oncogenic miRNA in the initiation and progression of cervical cancer. Of note, the present study further supported the hypothesis that various functions of miRNA, including miR-494, in different types of cancer were dependent on the cancer type and cellular context (28).

To address the molecular mechanism involved in miR-494-mediated changes of biological properties, PTEN was selected for further study. The *PTEN* gene, known as mutated in multiple advanced cancer 1 (MMAC1), is a classic tumor-suppressor gene located at chromosome 10q23.31 (39). The PTEN protein is principally involved in the homeostatic maintenance of PI3K/Akt signaling (40). PTEN/PI3K/Akt is highly involved in carcinogenesis and associated with EMT (41), and cell cycle arrest (42,43). The PI3K/Akt signaling pathway is involved in tumor cell proliferation during the development of cervical cancer, and downstream effectors of PI3K/Akt signaling are promising targets for cervical cancer therapy (4). Gene expression profiling also suggests that the PI3K/Akt pathway is a therapeutic target in cervical cancer (6). More importantly, it has been confirmed that PTEN, which counteracts PI3K/Akt activity, is involved in various aspects of cancer development, such as inhibition of cell proliferation, apoptosis, migration and invasion (8,44,45). In particular, PTEN expression intensity is lower in cervical cancer than benign cervical samples (46) and a decreased expression of PTEN was found in invasive cervical cancers (47). From the previous study, we concluded that the PTEN/PI3K/Akt signaling pathway is important in the carcinogenesis and development of cervical cancer, thus identifying the molecules regulating PTEN/PI3K/Akt may be an attractive strategy for the underlying mechanism of cervical carcinogenesis. In the present study, the results supported the hypothesis as, miR-494 was involved in the modulation of PTEN expression in cervical cancer. First of all, miR-494 was inversely correlated with PTEN expression in cervical cancer tissues. Secondly, the mRNA and protein levels of PTEN were significantly upregulated after knockdown of miR-494 expression in cervical cancer cell lines. In addition, luciferase analyses indicated PTEN was the direct target of miR-494 in cervical cancer cells. Taken together, we have demonstrated miR-494 could directly regulate PTEN expression by targeting its mRNA 3'-UTR. Thus, previous findings and our results suggest that, miR-494 functions as an oncogenic miRNA and PTEN/PI3K/Akt regulator in cervical cancer. More specific studies are required to further elucidate the relationship between miR-494 and PI3K/Akt and more specific mechanisms that miR-494 regulated the expression of PTEN.

In summary, to the best of our knowledge, the present study identified for the first time the correlation between miR-494-mediated cervical cancer cell proliferation and downregulation of PTEN. Our findings reveal a crucial role for miR-494 in regulating cell cycle checkpoints and cervical cancer cell proliferation. Understanding the precise role played by miR-494 in inducing tumor cell proliferation may increase our understanding of the biology of cervical cancer and inhibition of miR-494 may be a novel therapeutic strategy in the treatment of cervical cancer.

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