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MicroRNA-9 enhances invasion and migration of cervical carcinomas by directly targeting FOXO1

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Abstract. – OBJECTIVE: Cervical carcinoma is the third frequently diagnosed cancer among women worldwide. Increasing evidence suggests that dysfunctions of miRNAs are involved in human cancers and could act as either tumor suppressors or oncogenes. The purpose of this study is to elucidate pathobiological functions of miR-9 expression by targeting FOXO1 in cervical carcinoma.

PATIENTS AND METHODS: Real-time-PCR was utilized to detect miR-9 and FOXO1 level in cervical carcinoma tissues and cells. Transwell assays were employed to check out the roles of miR-9 on cells invasive and migratory potency. Luciferase reporter and Western blot were applied to verify FOXO1 as a target gene of miR-9.

RESULTS: The results showed that miR-9 was significantly high expressed in cervical carcinoma cell lines and clinical tissues. miR-9 enhanced the ability of migration and invasion of C33A and HeLa cells. FOXO1 was confirmed as the direct target of miR-9, and miR-9 over-expression down-regulated the expression level of FOXO1.

CONCLUSIONS: Our data demonstrate that miR-9 enhances invasion and migration of cervical carcinomas by directly targeting FOXO1. This may lead to a modern therapeutic strategy for the treatment of cervical carcinomas.

Key Words
Cervical carcinomas, FOXO1, Invasion, miR-9, Migration.

Introduction

Cervical carcinoma is the third most frequently diagnosed cancer among women around the world[1]. Cervical carcinoma mortality in developed countries was decreased with advanced healthcare systems[2]; however, it is still high in the developing countries. Previous studies[3] indicate that a significant breakthrough has been made in understanding the molecular mechanism of cervical carcinogenesis. Nevertheless, the detailed molecular mechanisms of cervical carcinoma are still unknown. Hence, determining the primary molecular elements in cervical carcinoma progress may provide potential remedial targets for the prophylaxis and therapy of cervical carcinoma.

MicroRNAs (miRNAs), a kind of endogenous non-coding single-stranded small RNAs with approximately 18-25 nucleotides, could regulate the gene expression[4,5]. miRNAs inhibit translation or induce mRNAs degradation through bind to 3'-UTRs of target mRNAs[6]. They are related to the regulation of multifarious biological processes, containing differentiation, development, cell cycle, and metabolism, as well as kinds of human diseases[7,8]. Increasing evidence suggests that dysfunctions of miRNAs are involved in human cancers and can act as either tumor suppressors or oncogenes[9]. Numbers of miRNAs were participated in the carcinogenesis and promote the progress of the cervical carcinoma, such as miR-376c, miR-10a, miR-720, miR-125a, miR-139-3p, miR-148b[10-15]. Moreover, previous evidence showed that miR-9 was remarkably upregulated both in the early stage invasive squamous cancer cell and human papillomavirus (HPV)-positive cervical tumors[16]. Since its complicated mechanisms are still not known, it might be valuable to detect the function of miR-9 in cervical carcinoma progress.

Forkhead box O1 (FOXO1), one member of the subgroup O of forkhead transcription factors (FOX)[17], has been postulated to have vital roles in all kinds of physiological and pathological processes including DNA repair, cell cycle arrest, energy metabolism,

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apoptosis oxidative stress, and resistance[18]. Reduced expression of FOXO1 is detected in several types of cancers, including lung cancer[19,20], endometrial cancer[21], hepatocellular carcinoma[22], and cervical cance[23], indicating it is a tumor suppressor. Furthermore, FOXO1 was identified as target of several miRNAs, such miR-182[24], miR-196a[23], and miR-21[25]. However, the regulatory mechanism of FOXO1 regulated by miR-9 in cervical carcinoma is still unknown.

We analyzed the oncogenic function of miR-9 by detecting its level in cervical carcinoma and investigated its functions on invasion and migration in cervical carcinoma cells. In addition, our results presented that miR-9 could down-regulate FOXO1 expression by directly binding to the latter 3'-UTR. These results provide a better understanding of molecular mechanism of miR-9/FOXO1 in cervical carcinoma progression and may suggest an original method for targeted treatment.

Patients and Methods

Patients and Tumor Specimens

We obtained 99 paired of cervical carcinomas and corresponding adjacent non-tumor cervical tissues from patients (aged between 28 and 74 years) who received surgery in our hospital between Aug 2011 and Jun 2016. All the samples were obtained after patients' informed consent was signed. This work was approved by the Ethics Committee of People's Hospital of XinJiang Uyghur Autonomous Region. Undergoing hysterectomy cervical carcinomas tissues and adjacent relatively normal cervical tissues were immediately immerged into RNAlater (Ambion, Carlsbad, CA, USA) and stored at -80°C. Sample characteristics are shown in Table I.

Cell Culture and miR-9 Transfection

Two cervical carcinomas cells, C33A and HeLa, and a normal human cervical epithelial cell Ect1/E6E7, were purchased from Chinese Center for Type Culture Collection (Wuhan, China). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (Gibco, Rockville, MD, USA) supplemented with 15% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), along with 100 IU/mL of penicillin and 100 μg/mL of streptomycin. miR-9 mimics or inhibitor and negative controls were purchased from RiboBio (Guangzhou, China). After cells were incubated 24 h at 37°C in a humidified atmosphere, lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was employed to perform the transfection according to the manufacturer's instructions.

Table I. Clinicopathological characteristics of patients with cervical carcinomas.

Characteristics	Total cases (n=99) Cases	miR-9 expression		*p*-value
		Low	High	
Age (years)				
≤ 45	12	4	8	0.9969
> 45	87	30	57	
Tumor size (cm)				
< 4	80	30	50	0.7949
≥ 4	19	9	10	
Lymph node metastases				
Yes	9	1	8	0.0374*
No	90	43	47	
Stromal invasion				
≤ 65%	66	18	48	0.0449*
> 65%	33	16	17	
Differentiation				
Well	10	3	7	
Moderate	65	15	50	0.2229
Poor	24	10	14	
CIN stage				
I	25	13	12	0.0273*
II	35	15	20	
III	39	21	18	

CIN, cervical intraepithelial neoplasia.

RNA Isolation and qRT-PCR

According to the instruction, TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from surgical cervical cancer tissues and cultured cells. The complementary (cDNA) of miRNA-9 was synthesized using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). FastStart Universal SYBR Green Master kit (Roche Diagnostics, Mannheim, Germany) was applied to perform quantitative Real-time PCR (qRT-PCR) with StepOne Plus Real-time PCR System (Applied Biosystems, Waltham, MA, USA). The endogenous snRNA U6 control normalized the expression levels. The miRNA sequence-specific RT-PCR primers and the endogenous control U6 were purchased from HAPK Biotechnology (Shenzhen, China). For mRNA, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Sigma-Aldrich, St. Louis, MO, USA), and detected by qRT-PCR. mRNA quantification used GAPDH as internal controls. Fold changes of expression were calculated by $2^{-\Delta\Delta CT}$ method. All primers were synthesized by RiboBio (Guangzhou, China).

Invasion and Migration Assays In Vitro

For the transwell assays, a total of 2.0×10^5 cells (C33A or HeLa cells), transfected with the miR-9 mimic/inhibitor and negative control, in medium without FBS, were added in the top chamber with or without 150 mg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Medium with 15% FBS was added as the nutritional attractant to the lower chamber. After incubation for 24 h at 37°C, we removed the cells which remained on the upper surface of the membrane. Meanwhile, the cells were fixed on the lower surface of the membrane with 20% methanol and stained with 0.2% crystal violet for 20 min. The stained cells were counted under a light microscope.

Western Blot

Cultured cells were collected, and then lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyongtime, Shanghai, China) to obtain proteins. After quantified, all proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with anti-FOXO1 antibody (Invitrogen, Carlsbad, CA, USA) at 4°C overnight. In addition, the membranes were washed and incubated with secondary antibodies with horseradish peroxidase (HRP)-conjugated (Saierbio, Tianjin, China) for 1 h at 25°C. β-actin was utilized as internal control. Lastly, ECL chemiluminescence system (Amersham, Piscataway, NJ, USA) was applied to detect the reactive proteins.

Luciferase Assay

Wild-type (WT) and mutated-type (MT) FOXO1 3′-UTRs, including the predicted target sites of miR-9, were created and cloned from human genomic DNA. For the luciferase assay, C33A cells and HeLa were seeded in a 24-well plate with a density of 1×10^5 cells before transfection. WT or MT FOXO1 3'UTR reporter constructs were co-transfected with miR-9 into the cells. After 48 h incubation, Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was employed to measure luciferase activities; the corresponding Renilla luciferase activity was used as normalization.

Statistical Analysis

Two groups of experiments were compared using the Student's *t*-test. The relationship between clinicopathological characteristics and miR-9 expression was analyzed by the Pearson χ^2-test. Results are expressed as mean ± SD in triplicate. Statistically significant was marked as p-value < 0.05.

Results

miR-9 and FOXO1 Expression in Cervical Carcinomas Tissues and Cells

To evaluate the expression situation of miR-9 in sample tissues, miR-9 level of cervical carcinomas tissue and corresponding adjacent non-tumor cervical tissue was evaluated by qRT-PCR. The average level of miR-9 in the cervical carcinomas tissues was increased about 2.5-fold than that in the non-tumor cervical tissue, which was consistent with the previous results (Figure 1A). Similarly, in C33A and HeLa cell lines, miR-9 expression was significantly increased in two cervical cancer cells *vs.* normal cells (Figure 1B). Moreover, the expression levels of FOXO1 in cervical carcinomas tissues and adjacent normal tissues were further tested using qRT-PCR. Results showed that expression of FOXO1 was significantly lower in cervical carcinomas tissues than that in the corresponding normal tissues (Figure 1C).

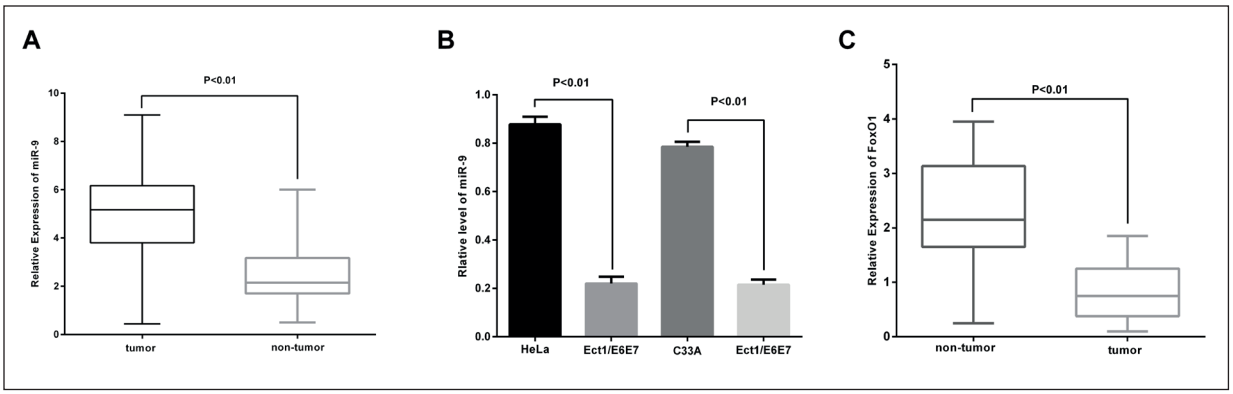

Figure 1. miR-9 was frequently up-regulated and FOXO1 was frequently down-regulated in cervical carcinomas tissues and cells. **A**, qRT-PCR shows that the mean expression level of miR-9 was frequently high expressed in 99 cervical carcinomas tissues vs. their paracancerous tissues. **B**, miR-9 was significantly up-regulated in two cervical carcinomas cell lines compared with normal cervical cell. **C**, qRT-PCR shows that the mean expression level of FOXO1 was frequently down-regulated in 99 cervical carcinomas tissues vs. their adjacent non-tumor tissues.

miR-9 Enhances Cervical Carcinomas Cells Invasion and Migration

Epithelial-mesenchymal transition (EMT), one of the key events involved in invasion and metastasis of tumor cells, detected the influence affected by miR-9 in cervical cells by Western blot. We discovered that miR-9 mimic decreased the expression of epithelial marker-β-catenin and increased the levels of mesenchymal marker-vimentin (Figure 2A). It is probably that miR-9 enhances migration and that the invasion was performed by activation of EMT program in cervical cells.

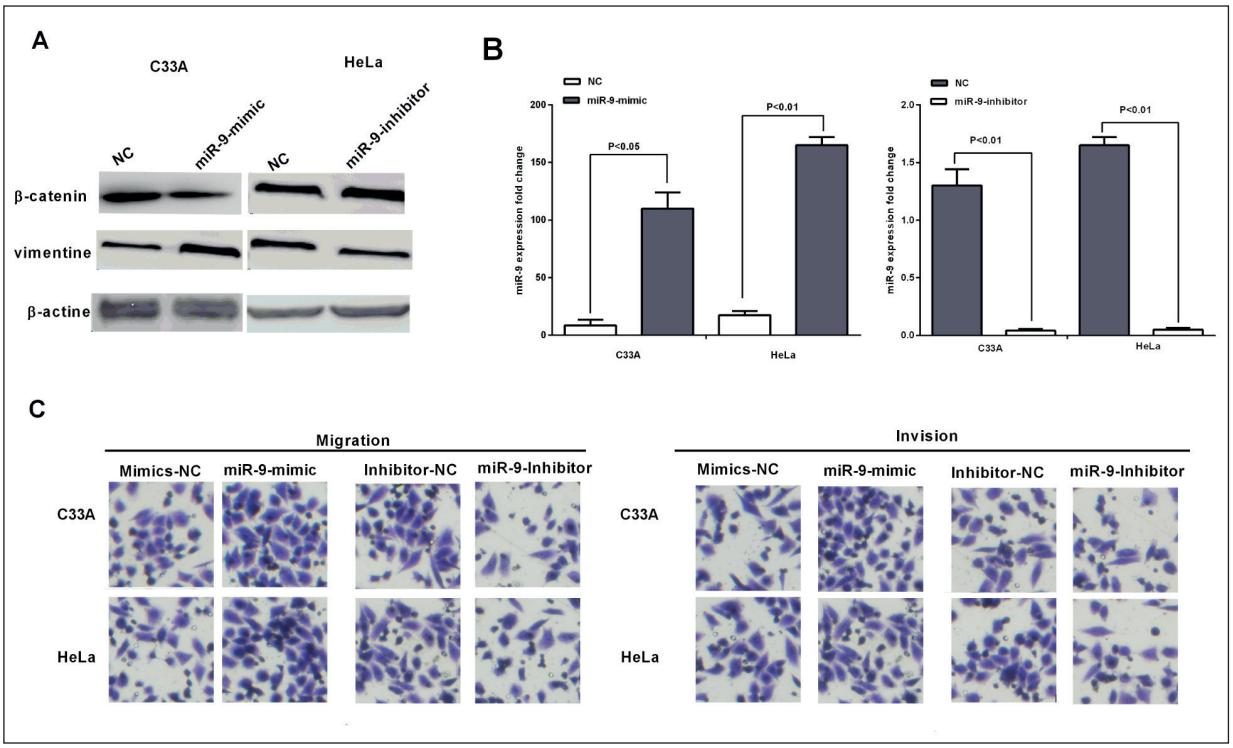

Figure 2. miR-9 promotes cervical carcinomas cell migration and invasion *in vitro*. **A**, miR-9 influence β-catenin and vimentin expression in EMT progress. **B**, C33A and HeLa cells were transfected with miR-9 mimics/inhibitor and the corresponding negative control analyzed by qRT-PCR. **C**, Determination of C33A and HeLa cells migration and invasion transfected miR-9 mimics/inhibitor compared with the control group.

To further investigate the functional roles of miR-9 in cervical carcinomas cells migration and invasion, C33A and HeLa cells were both transfected with miR-9 mimic or inhibitor, and the expression levels of miR-9 were examined by qRT-PCR. The results showed that miR-9 was significantly higher in cell lines C33A and HeLa transfected with miR-9 mimics than that in cells transfected with corresponding negative controls. However, expression level of miR-9 of cell lines transfected with miR-9 inhibitor was relatively lower than cell transfected with corresponding negative control (Figure 2B). Transwell assays with or without matrigel were utilized to measure the effects of miR-9 on the potential migration or invasion of cells. As expected, numbers of cell invasion and migration C33A and HeLa with miR-9 mimic were remarkably increased compared with the corresponding negative control group. However, cell numbers of groups with miR-9 inhibitor were significantly less than its corresponding negative control group (Figure 2C). Taken together, our findings indicate that miR-9 enhances the ability of migration and invasion of C33A and HeLa cells.

FOXO1 Re-Expressed Reverses the miR-9-Induced Promotion of Cell Invasion and Migration

To verify whether FOXO1 re-expression could reverse miR-9-induced phenotypes change, plasmids expressing FOXO1 without (3' – UTR (–)) its 3' – UTR were constructed. The re-introduction of FOXO1 markedly reversed the promotion of cell migration and invasion in the miR-9-expressing cells (Figure 3A and B). In conclusion, these data demonstrate that FOXO1 is an effect of, or related to, the function of miR-9-mediated anti-metastatic activity.

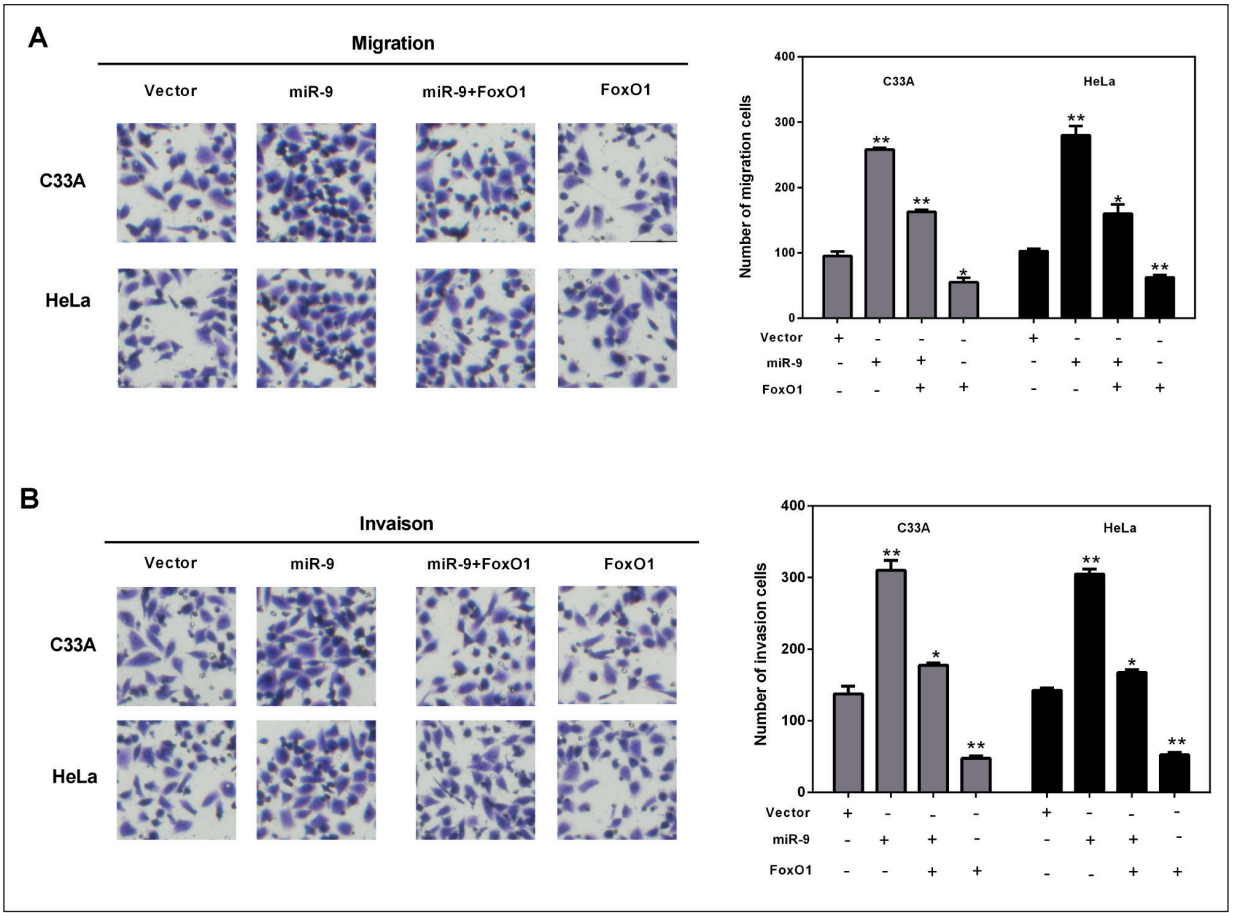

Figure 3. Expression of FOXO1 rescues partial biological effect of miR-9 on migration and invasion of cervical cancer cells. (**A**, **B**) miR-9 ectopic over expression promote cell migration and invasion; the promotion was weakened by FOXO1 in C33A and HeLa cells. Transwell assays were performed after co-transfected miR-9 mimic or foxO1 3′ - UTR. *p<0.05, **p<0.01.

miR-9 Directly Target FOXO1 and Negatively Regulates its Expression

FOXO1 was predicted to be a putative target of miRNA predicted using the TargetScan databases. The putative binding sites of miR-9 at 3' – UTR were from 69 to 75 (Figure 4A). To determine whether miR-9 mediated FOXO1 expression, the wide type plasmids pmirGLO – UTR (WT) and mutant plasmid pmirGLO – UTR (MT) of FOXO1 3' – UTR were constructed based on luciferase reporter assay, and both were transfected into cervical carcinomas cancer cells with or without miR-9 over-expression. The result shows there was no difference in luciferase activity between cervical carcinomas cell lines and normal cells with mutated plasmids. However, miR-9 over-expression significantly reduced luciferase activity in cells transfected with WT in miR-9 precursor transfectants, and the data had statistically significance (Figure 4B). Furthermore, we examined the expression of FOXO1 affected by miR-9. Western blot confirmed that miR-9 over-expressed significantly decreased endogenous FOXO1 expression in both C33A and HeLa cells. β-actin was measured as internal control (Figure 4C). Taken together, our data indicated that FOXO1 was a direct downstream target of miR-9, which could induce EMT progression.

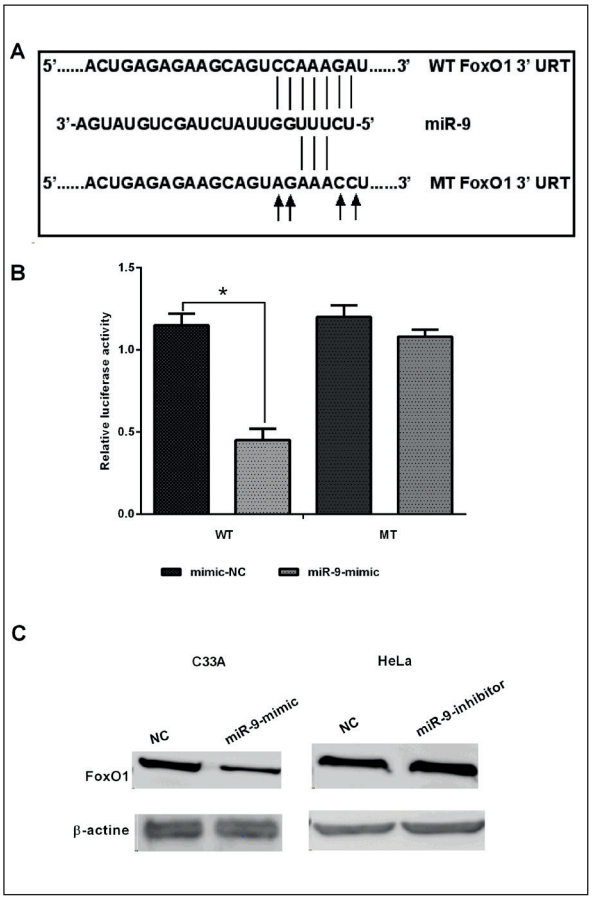

Figure 4. miR-9 downregulates FOXO1 protein expression by directly binding to its 3′ - UTR. **A**, The potential binding sites for miR-9 in the 3′ - UTR of FOXO1 at 69 - 75 bp, the top is the wild-type (WT) 3′ - UTR of FOXO1 and the bottom is mutated (MUT) 3′-UTR of FOXO1, the arrows mean the mutational nucleotides. **B**, Analysis of luciferase activity. C33A cells were cotransfected with miR-9 and WT or MT FOXO1 3′ - UTR luciferase reporter construct. **C**, miR-9 downregulates FOXO1 expression at the protein levels in C33A and HeLa cells. *p<0.05.

Discussion

An increasing number of data have indicated that the miRNAs preternatural expression facilitate tumorigenesis through regulating target genes, thereby acting as potential biomarkers in tumors containing cervical carcinomas[26]. Therefore, identified cancer-specific miRNAs and their involved targets is pivotal for comprehending their effects in tumor migration and invasion; so, could provide critical clues for cervical carcinomas diagnosis and therapy.

Previous studies suggested that miR-9 was frequently abnormal expressed in several cancers and its function was extremely tanglesome as it could act as tumor suppressors or oncogenes, such as esophageal squamous cell carcinoma[27], neuroblastoma cells[28], pediatric acute myeloid leukemia[29], triple negative breast cancer[30], and hepatocellular carcinoma[31]. Furthermore, a precedent microRNA microarray analysis indicated that miR-9 was remarkably over expressed in cervical cancer[5,16]. Song et al[27] reported that HPV-induced miR-9 activation enhanced cell motility by down-regulating multiple targets gene involved in cervical cancer cell migration. In our work, miR-9 was demonstrated to significantly promote invasion and metastasis of cervical carcinomas. miR-9 was overexpressed in HeLa and C33A cells transfected with miR-9 mimic (Figure 2A). Transwell assay showed that the cells number of invasion and migration with miR-9 over-expressed was increased compared with negative control (Figure 2B), and miR-9 could induce EMT progression (Figure 2C).

FOXO1 has been postulated vital roles in all kinds of physiological and pathological processes. Hou et al[23] reported miR-196a down-regulated FOXO1 and mediated cell proliferation in cervical carcinomas. Furthermore, Chen et al[20] reported miR-9 regulated FOXO1 expression to

induce the non-small cell lung carcinoma. We determined FOXO1 as the target of miR-9 in cervical carcinomas, and miR-9 over-expression significantly decreased the luciferase activity of cells transfected with the WT 3'-UTR of FOXO1 in miR-9 precursor transfectants (Figure 4B-C). Furthermore, for the first time, we provide evidence that FOXO1 is an effector related to the function of miR-9-mediated anti-metastatic activity (Figure 3A).

These findings may develop a novel method to explore the biological mechanism of cervical carcinomas. However, there are no studies reporting a deterministic mechanism of miR-9 in cervical carcinomas, and further exploration is required.

Conclusions

We found that miR-9 was deemed to promote cervical carcinomas cell invasion and migration through binding to FOXO1. The ultramodern identified miR-9/FOXO1 axis offers new insight of cervical carcinomas pathogenesis, particularly in regard to migration and invasion, and provide a potential therapeutic target for cervical carcinomas.

Acknowledgments

The authors declared no conflict of interest.

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