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Original Article

Human papillomavirus 16 E6 modulates the expression of host microRNAs in cervical cancer

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ABSTRACT

Objective: Human papillomavirus (HPV) infection is a prerequisite of developing cervical cancer, approximately half of which are associated with HPV type 16. There are reports that HPV can disturb the expression pattern of host miRNAs, but its mechanism is not well understood.
Materials and Methods: In this study, we scanned 11 tumorigenesis related miRNAs in Hela cells that were overexpressed with HPV type 16 E6 protein.
Results: We found the expression of miR-21 was upregulated by HPV type 16 E6 protein and meanwhile, the expression of miR-27a and miR-218 was downregulated. Furthermore, we identified that miR-21 overexpression could promote Hela and U2OS cells proliferation by targeting phosphatase-tensin homolog (PTEN), the result of which can be rescued by miR-21 inhibitor. In addition, E6 overexpression could also promote Hela cell migration and invasion.
Conclusion: Our results indicate that HPV infection and subsequent transformation take place through complex regulatory patterns of gene expression in the host cells, part of which are regulated by the E6 protein.

Introduction

Human papillomavirus (HPV) infection is the major cause of human cervical cancer and persistent infection by one of ~15 genotypes of carcinogenic HPV causes in almost all cases [1]. The most prevalent HPV type found in cervical cancer is HPV 16, which encodes three oncoproteins: E5, E6, and E7. The E6 and E7 oncoproteins can bind to and stimulate the degradation of the tumor suppressors p53 [2,3] and pRb [4], which are essential for cell cycle control.

MicroRNAs (miRNAs) are short noncoding RNAs which modulate gene expression by binding to complementary segments present in the 3′UTR of the mRNAs of protein coding genes. miRNAs play very important roles in maintaining normal human body physiology conditions, and abnormal miRNA expressions have been found related to many human diseases, spanning from psychiatric disorders [5] to malignant cancers [6,7]. Moreover, they play a major role in regulating host gene expression in virus infected cells and cancers caused by virus infection [8–11].

There are no reports that HPV can express its own miRNA, however, it has been confirmed that HPV infection can disturb host miRNA expression and is related to tumorigenesis. Downregulation of human miR-218 in cervical cancer cells was specifically addressed to the HPV 16 E6 oncogene [12], but a wide range interfered miRNAs expression pattern by E6 protein need to be unveiled. Here we scanned the expression of several tumorigenesis related miRNAs when HPV E6 protein was overexpressed. Finally, we found the expression of miR-218 and miR-27a can be reduced by E6, and miR-21 was upregulated. This research gives a clue to explain the mechanism of tumorigenesis induced by HPV infection.

Materials and methods

Cell culture

Hela and U2OS cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/mL penicillin, and 10 mg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO_2.

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miRNA real-time reverse transcription quantitative polymerase chain reaction

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis was used to determine the relative expression level of candidate miRNAs. Total RNA was extracted from tissues and cells, using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression level of miRNAs was detected by TaqMan miRNA RT-Real Time PCR. Single-stranded cDNA was synthesized by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and then amplified by using TaqMan Universal PCR Master Mix (Applied Biosystems) together with miRNA-specific TaqMan MGB probes (Applied Biosystems). The U6 snRNA was used for normalization. Each sample in each group was measured in triplicate and the experiment was repeated at least three times for the detection of miRNAs.

Plasmids transfection

HPV16 E6 full length coding region was amplified by RT-PCR and cloned into pcDNA3.1 vector between the recognition sequence for Hind III and Xho I. E6 expression vector was transfected into cells by the lipofectamine 2000 (Invitrogen) essentially as described by the manufacturer's instruction, pcDNA3.1 vector used as control.

Western blotting

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 30 μg samples were loaded into each lane of 8% polyacrylamide gels. The proteins were separated by electrophoresis and then the gels were blotted onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, St. Albans, Hertfordshire, UK) by electrophoretic transfer. The membrane was incubated with mouse antiE6 monoclonal antibody (Abcam, Cambridge, MA, USA), rabbit antiPTEN monoclonal antibody, (Abcam) or mouse antiβ-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 hour at 37°C. The specific protein antibody complex was detected by using horseradish peroxidase conjugated rabbit anti-mouse or goat antirabbit immunoglobulin G. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control.

Cell proliferation assay

Hela and U2OS cells were seeded in 96-well plates at low density (5×10^3) in dulbecco modified eagle medium (DMEM) culture and allowed to attach overnight. The cells were then transfected with E6 expression vector, with pcDNA3.1 as the control. Twenty microliters methyl thiazolyl tetrazolium (MTT) (3-[4, 5-dimethylthiazol-2-yl] -2, 5-diphenyltetrazolium bromide) (5 mg/mL; Sigma-Aldrich, St.

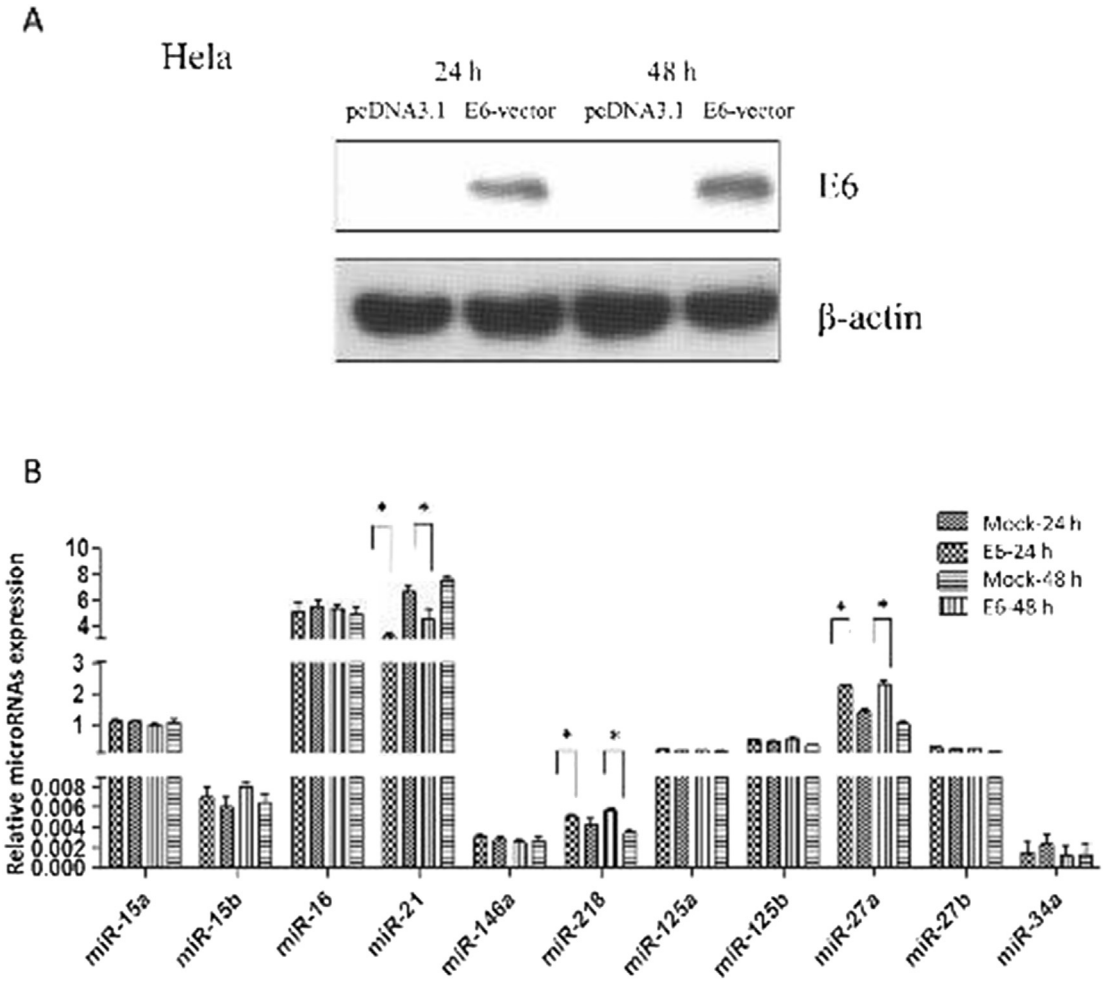

Figure 1. Confirmation of the expression of HPV type 16 E6 protein and scanning the expression of 11 host miRNAs in Hela cells: (A) E6 protein was only detected in E6 expression vector transfected groups 24 hours and 48 hours after transfection; (B) 11 candidate host miRNAs were detected by RT-qPCR. U6 snRNA serves as an internal reference among different samples and helps normalize for experimental error. $^*p < 0.05$. HPV = human papillomavirus; RT-qPCR = reverse transcription quantitative polymerase chain reaction.

Louis, MO, USA) were added into each well after 48 hours of transfection, and the cells were incubated further for 4 hours. The absorbance was recorded at A490 nm with a 96-well plate reader after the dimethyl sulfoxide (DMSO) addition.

Cell migration and invasion assays

Hela cells were transfected with the pcDNA3.1, pcDNA3.1-E6, miR-21inhibitor with pcDNA3.1-E6, or scramble miRNA control with pcDNA3.1-E6. After 48 hours of transfection, the transfected cells were harvested and subjected to the following assays. During the formigration assays, the transfected cells (0.5×10^6 cells/mL) were seeded in the top of an 8.0-mm-pore membrane chamber (Corning CostarCorp., Cambridge, MA, USA). Following a 12-hour incubation period, cells that passed through the membrane and were attached to the bottom of it were fixed and stained with hematoxylin and eosin (Sigma-Aldrich). Cells were scraped and removed from the top of chamber. Membranes were mounted on cover slides, and the cells were counted. Cell migration was quantified by counting the amount of cells passing through the pores from five different fields per sample at 100× selected in a random manner. Cell invasion assays were carried out using modified Boyden chambers in 24-well tissue culture plates at 1×10^5 cells per well (BD Biosciences, San Jose, CA, USA). All experiments were performed in duplicate.

Stable cell line establishment

Stable cell lines expressing Flag-E6 were generated by transfection of Hela cells in six-well plates with 2 μg of the plasmid using lipofectamin-2000 (Invitrogen) according to the manufacturer's protocol. Selection for the plasmid was applied using 0.5 mg/mL of puromycin (Amresco, Solon, OH, USA), beginning 48 hours after transfection. The puromycin-resistant cell population was tested for expression of Flag-E6 by immunoblotting with antiFlag antibody (Abmart, Shanghai, China).

Statistical analysis

MiRNAs expression data were analyzed using SPSS version 16 (SPSS Inc., Chicago, IL, USA), paired *t* test was used and $p < 0.05$ was considered statistical significant.

Results

Confirmation of the overexpression of HPV16 E6 protein

As two time points, 24 hours and 48 hours after plasmid transfection, Hela and U2OS cells were collected to detect the expression of HPV16 E6 protein. As shown in Figures 1A and 2A, E6 protein was overexpressed and was not detected in controls of these two cell lines.

Detection of the expression pattern of 11 tumorigenesis associated miRNAs

We detected 11 selected miRNAs (miR-15a, miR-15b, miR-16, miR-21, miR-146a, miR-218, miR-125a, miR-125b, miR-34a, miR-27a, and miR-27b), the expression of which were disturbed after

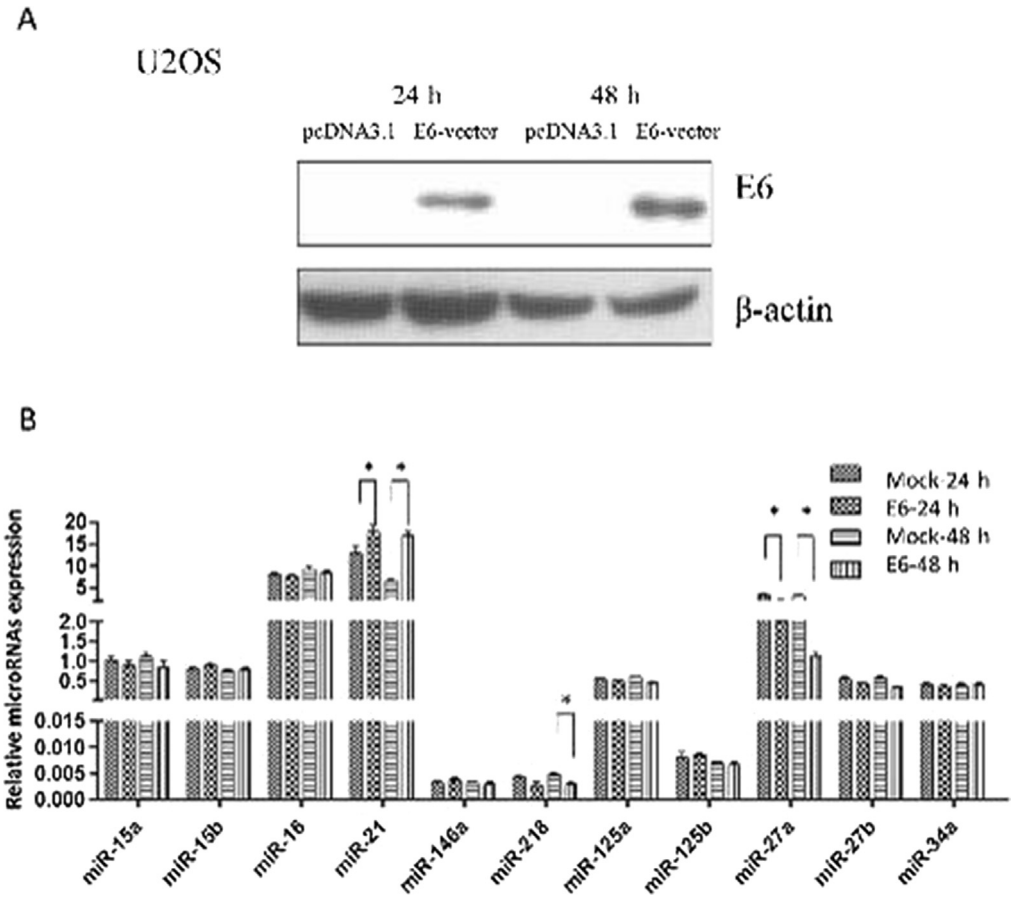

Figure 2. Confirmation of the expression of HPV type 16 E6 protein and scanning the expression of 11 host miRNAs in U2OS cells: (A) E6 protein was only detected in E6 expression vector transfected groups 24 hours and 48 hours after transfection; (B) 11 candidate host miRNAs were detected by RT-qPCR. U6 snRNA serves as an internal reference among different samples and helps normalize for experimental error. $^*p < 0.05$. HPV = human papillomavirus; RT-qPCR = reverse transcription quantitative polymerase chain reaction.

Table 1
Fold change of miR-21, miR-218, and miR-27a by HPV16 E6 protein.

miRNA	Fold change			
	Hela (24 h)	Hela (48 h)	U2OS (24 h)	U2OS (48 h)
miR-21	0.47	1.34	0.38	1.63
miR-218	−0.18	−0.42	−0.56	−0.77
miR-27a	−0.36	−0.88	−0.65	−1.78

HPV infection [11–14], and wanted to know if there are some relations between their disturbance and E6 protein. From the results of RT-qPCR, we found the expression of miR-21 was upregulated, miR-218 and miR-27a were declined, especially 48 hours after E6 expression vector transfection (Table 1).

To further confirm the results that E6 overexpression can disturb miRNAs expression, we established Hela cells that consistently express E6. When the expression of E6 was knocked down by siRNA, the miR-21 was downregulated and miR-218 was overexpressed (Figure 3). These results indicated that the disturbed miR-21 and miR-218 expression was generated by E6.

E6 overexpression repressed phosphatase-tensin homolog (PTEN) expression by upregulating miR-21

MiR-21 is identified as an oncogene which contributes to carcinogenesis in many kinds of cancers. To explore whether the upregulated miR-21, caused by E6 overexpression, can repress its target genes, the expression of PTEN was detected [15,16]. As shown in Figures 4A and 4B, the expression of PTEN was significantly repressed by E6 and the repressed PTEN can be rescued by miR-21 inhibitor in Hela cells. The results indicated that miR-21 upregulation, which is caused by E6 overexpression, is the main factor that repressed PTEN expression. AKT is an important downstream multifunctional molecule that is closely related to cell proliferation and survival. We detected the phosphorylated AKT in the E6 overexpressed Hela cells. As shown in Figure 4C, the amount of P–S473 AKT was upregulated by E6 and partially downregulated by miR-21 inhibitor.

E6 can promote Hela cell proliferation, migration, and invasion

PTEN is a tumor suppressor gene that is frequently mutated in human tumors. During tumor development, mutations and deletions of PTEN occur which inactivates its enzymatic activity leading to increased cell proliferation and reduced cell death. We detected the effect of E6 protein on cell proliferation. As shown in Figure 4D in Hela cells, the cell proliferation can be promoted by E6 overexpression and the result can be rescued by miR-21 inhibitor.

As miR-21 was identified as an oncomir and overexpression of miR-21 can promote migration and invasion of cancer cells [12], we detected the Hela cell migration and invasion ability after E6 expression vector transfection. As shown in Figures 5A and 5B, when E6 is overexpressed in Hela cells, cell migration and invasion were upregulated significantly. When miR-21 inhibitor was cotransfected with E6 expression vector, Hela cell migration ability was repressed significantly.

Discussion

Recently, reports that disturbed expression of several tumorigenesis associated microRNAs is related to HPV infection by different groups, but the results were not consistent [12,13] (miR-

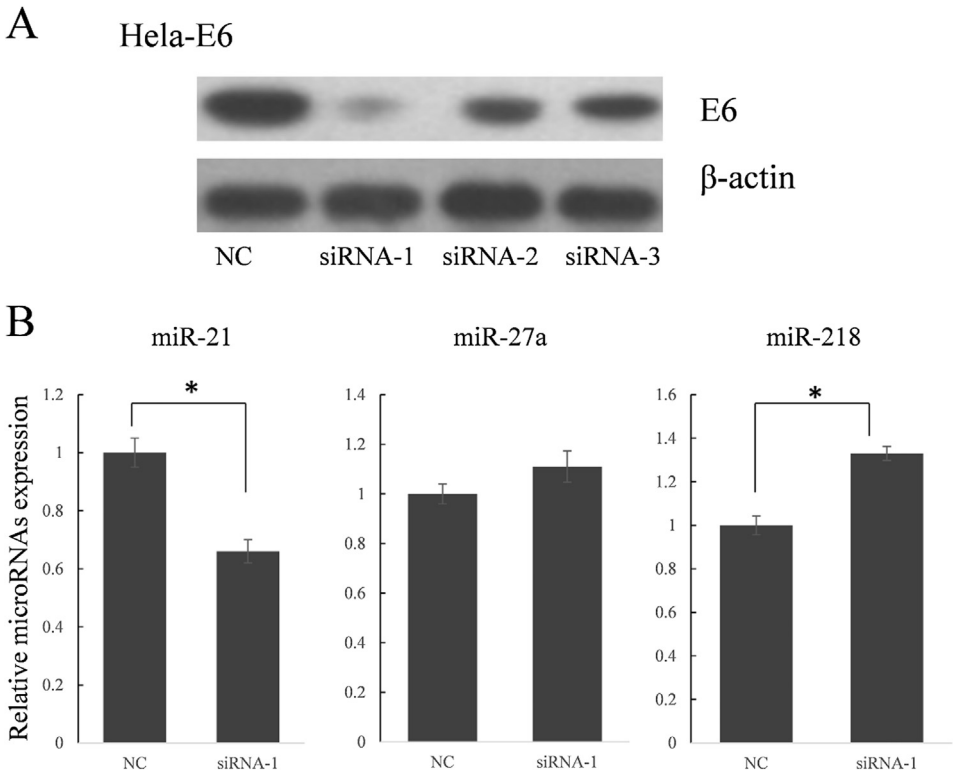

Figure 3. Knockdown the expression of HPV E6 protein and detection of the miR-21, miR-27a, and miR-218 expression: (A) Hela cells that stable express HPV E6 protein were transfected with three E6 specific siRNAs and 48 hours after transfection, E6 expression was detected by Western blots; (B) the expression of miR-21, miR-27a, and miR-218 was detected by RT-qPCR. Results were analyzed by Student *t* test, $p < 0.05$ was considered statistically significant. HPV = human papillomavirus; RT-qPCR = reverse transcription quantitative polymerase chain reaction.

145 for example).Yet one thing is generally accepted, deregulated miRNAs expression contributes to HPV-induced tumors.

In this study, we scanned 11 tumorigenesis associated miRNAs in Hela and U2OS cells that were overexpressed with E6 protein. We found three miRNAs (miR-21, miR-218, and miR-27a) that were significantly interfered by HPV16 E6 protein. MiR-218 is an important tumor suppressor and its suppression was reported not only in cervical cancer tissues, but also in gastric cancer [17], lung cancer [18], head and neck cancer [19], and so on. Martinez et al [12] identified that HPV16 suppress host cell miR-218 expression by E6 protein, thus our research above confirmed that phenomenon.

MiR-21 is considered by most scientists as an oncogenic miRNA and its overexpression was shown in most cancer types analyzed so far [20]. It is also confirmed that miR-21 is overexpressed in HPV induced cervical cancer, but the mechanism is not well understood [21]. Our research indicates that HPV16 E6 protein overexpression can lead to upregulated pri-miR-21 expression and promote Hela and U2OS cells proliferation by repressing PTEN expression, which gives a clue to explaining the mechanism of HPV induced miR-21 overexpression.

The oncogenic role of miR-27a is confirmed by several experimental studies. MiR-27a is significantly upregulated in renal cell

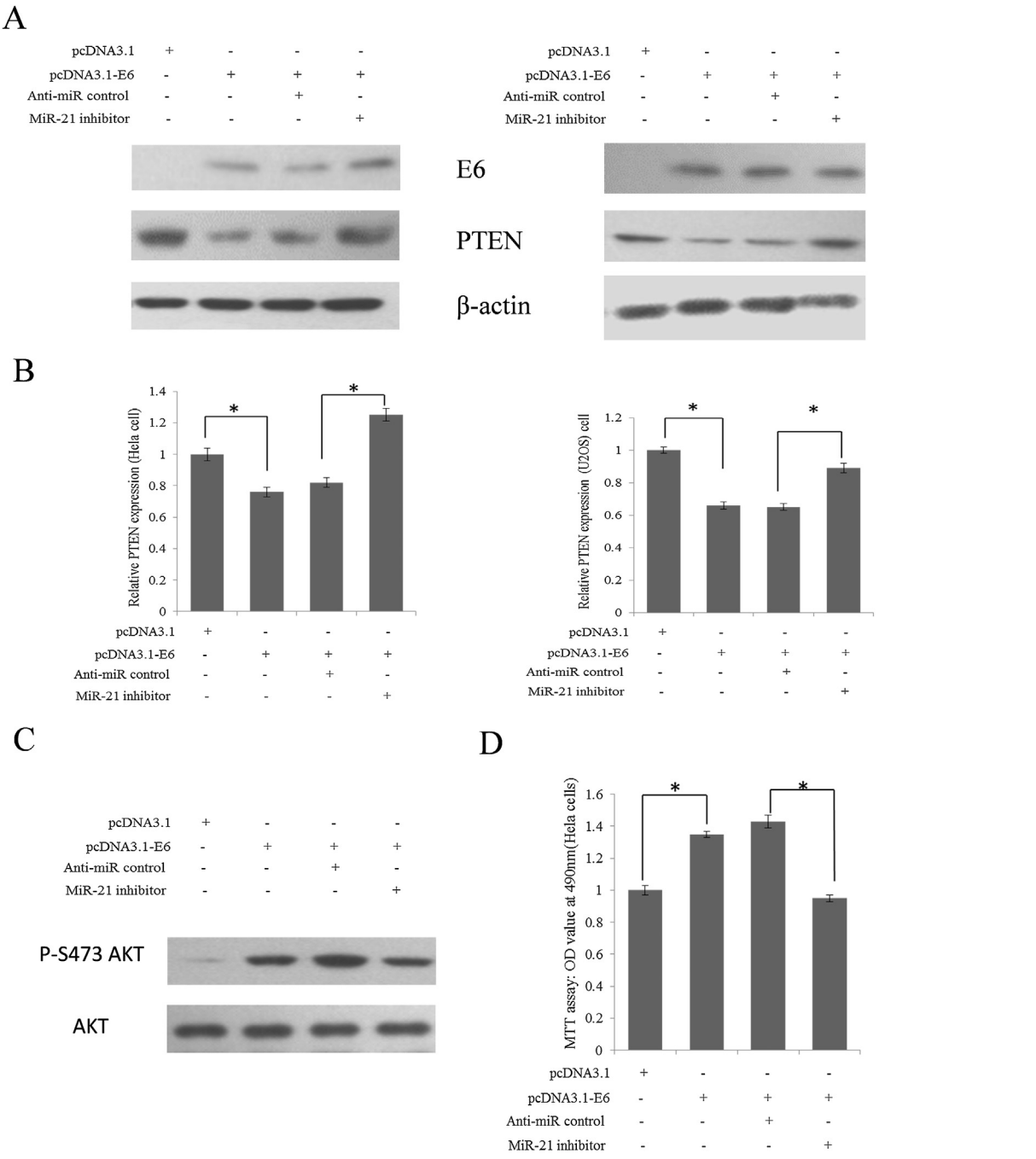

Figure 4. HPV type E6 protein can promote cell proliferation by upregulating miR-21 expression: (A) E6 protein overexpression repressed PTEN expression in Hela and U2OS cells; (B) E6 protein overexpression can repress PTEN mRNA level in Hela and U2OS cells by upregulating miR-21expression; (C) Western blot was used to detect the S473 phosphorylated AKT in the different treated groups of Hela cell lysates, and (D) E6 protein overexpression can promote Hela cells proliferation by upregulating miR-21expression. HPV = human papillomavirus; PTEN = phosphatase-tensin homolog.

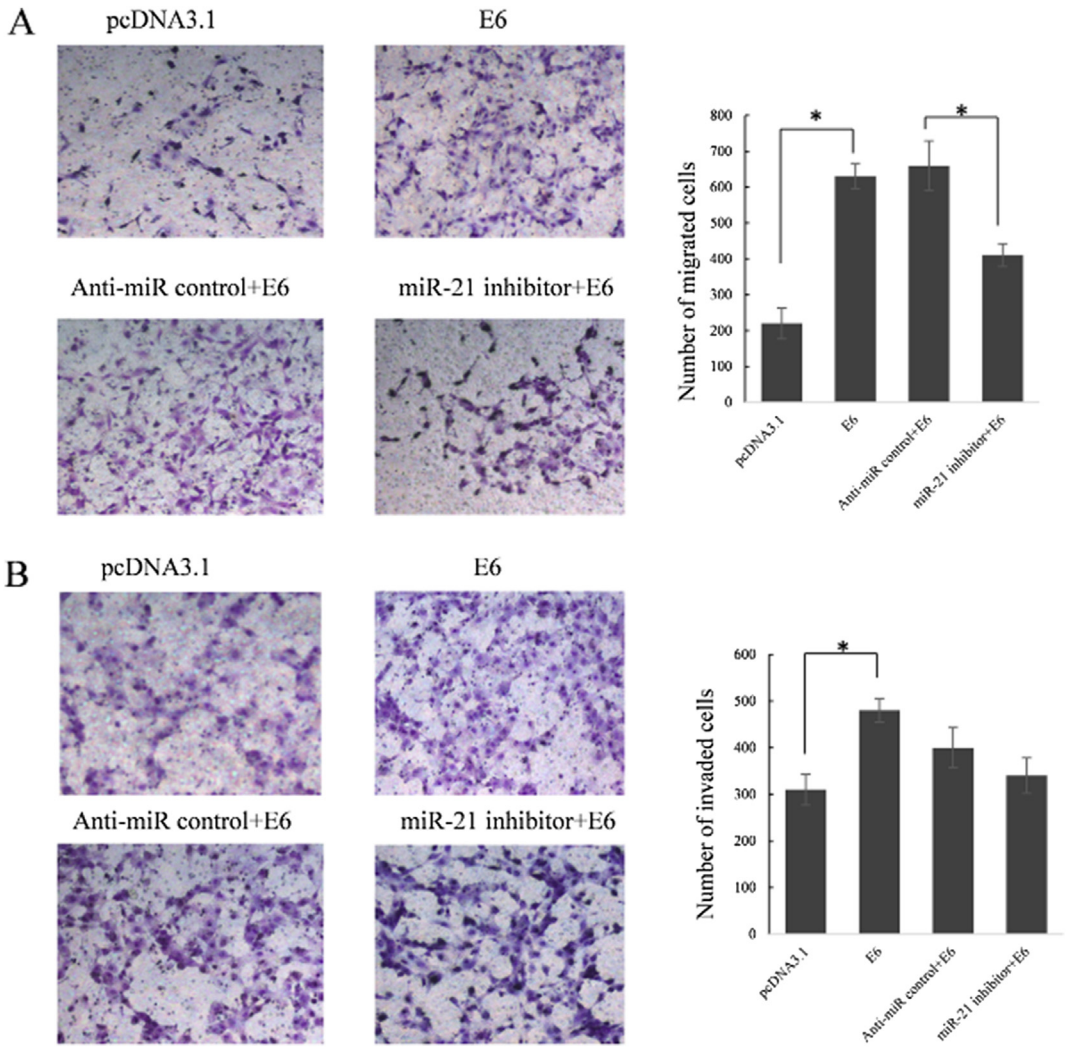

Figure 5. HPV E6 protein promotes Hela cell migration and invasion partially by upregulating miR-21. Hela cells transfected with the pcDNA3.1, pcDNA3.1-E6, miR-21 inhibitor plus pcDNA3.1-E6 or scramble miRNA control with pcDNA3.1-E6 respectively. The transfected cells were harvested and subjected to the following assays, 48 hours after transfection: (A) for migration assays, the transfected cells (0.5×10^6 cells/mL) were seeded in the top of an 8.0-mm-pore membrane chamber (Corning Costar Corp., Cambridge, MA, USA). Following a 12-hour incubation period, cells that passed through the membrane to attach to the bottom of the membrane were fixed and stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA). Cells were scraped and removed from the top of chamber. Membranes were mounted on cover slides, and cells were counted. The cell migration was quantified by counting the amount of cells passing through the pores from five different fields per sample at 100×selected in a random manner; (B) cell invasion assays were carried out using modified Boyden chambers in 24-well tissue culture plates at 1×10^5 cells per well (BD Biosciences, San Jose, CA, USA). All experiments were performed in duplicate.

carcinoma [22], cervical cancer [23], gastric adenocarcinoma [24], and breast cancer [25]. Downregulation of miR-27a also caused a decrease in Bcl-2 expression and increase in the Bax expression, thereby leading the cancerous cells towards apoptosis [26]. In this study, miR-27a was downregulated by HPV E6 protein, which is contrary to the carcinogenesis function of E6. E6 is a multiple function molecule and may regulate the expressions of transcription factors of miR-27a such as RUNX2 [27]. In addition, "miRNA sponge" is also a potential mechanism that E6 reduce the quantity of miR-27a in cells [28].

E6 is known for promoting the degradation of the tumor suppressor p53 and activating telomerase expression and activity. E6 also plays a key role in the deregulation of innate immunity by interfering with the expression of toll-like receptors. Specifically, toll-like receptor 9 transcription is inhibited in cells expressing HPV16 E6 and E7 [3]. In this study, we found that E6 overexpression can upregulate miR-21 expression which have been confirmed suppressing type I IFN production by targeting myeloid differentiation factor 88 (MyD88) and interleukin-1 receptor-associated kinase 1 (IRAK1). Therefore, we can infer that disturbed miRNAs expression may also play a crucial role in the HPV immune evasion progress.

In the present study, we found HPV16 E6 protein can reduce the expression of miR-218 and miR-27a and upregulate the expression of miR-21. This is the first report regarding E6 protein function as a host miRNA modulator and gives an important clue to explain the mechanism of HPV induced carcinogenesis.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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