Original Article

Regulator role of HPV E7 protein on miR-21 expression in cervical carcinoma cells and its functional implication

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Abstract: Cervical cancer is the second leading malignant tumor in women. Human papillomavirus 16 (HPV16) is one risk factor for cervical cancer, with its expressed E7 protein can facilitate the transformation of cervical epithelial cells. MicroRNA-21 (miR-21) is one important tumor growth regulatory factor involving in angiogenesis, tumor invasion and metastasis. This study thus aimed to investigate the role of high-risk HPV16 E7 protein in regulating miR-21 expression in cervical carcinoma and its related functions. Hela cells were transfected with pcDNA-HPV16 E7 expressing vectors. The expression level of E7 was determined by Western blotting, while miR-21 level was quantified by real-time PCR. The alternation of tumor cell proliferation is determined by transfecting miR-21 inhibitor into E7-overexpressing Hela cells. Cell apoptosis was studied by caspase-3 assay, while cell invasion was illustrated in Transwell chamber. The overexpression of HPV E7 protein facilitated the expression of miR-21, which potentiated Hela cell proliferation and invasion. The inhibition of miR-21 in E7-overexpressing Hela cells can inhibit both proliferation and invasion, but without significant effects on caspase-3 activity. HPV16 E7 protein can up-regulate host miR-21 expression, thus elevating cervical carcinoma cell growth, proliferation and invasion. Therefore, E7 protein is one critical factor in occurrence and progression of cervical carcinoma.

Keywords: Cervical carcinoma, Herpes virus 16, E7 protein, MicroRNA, oncogenes

Introduction

As one of the most common malignant tumors in women, cervical cancer maintains a high level of incidence and mortality rate in under-developed countries. The incidence of it locates as the second place of all gynecologic cancers [1, 2]. In Chinese women, the incidence of cervical cancer is more than two times higher than Western countries, with significantly elevated mortality rate [3, 4]. The lack of systematic and effective large-scale screening, as well as the weakness in early intervention, severely compromises the life quality and survival of patients, making cervical cancer as a worldwide healthy issue [5, 6].

High risk subtypes of human papillomavirus (HPV) can infect humans via skin or mucosal contact, and owns a high transmission rate by sexual behavior [7]. HPV16 is one common oncogenic subtype of HPV, as more than 70% infections eventually develop into cervical lesions [8]. The predominant oncogenic protein of HPA is E7 protein with 10 kDa size, containing ~100 amino acids. E7 protein has been shown to facilitate the malignant transformation of cervical epithelial cells and maintain the malignant phenotype [9, 10].

As one type of small RNA molecule regulating biological functions inside cells, microRNA (miR) can negatively regulate gene expression at post-transcriptional level, via the binding onto target mRNA for further degradation. The pluripotent role of miR can regulate body growth as well as environmental acclimation in a wide array [11, 12]. Recent studies have found the involvement of miR in tumor occurrence and progression, especially in ovary, cervical and endometrial cancer, by exerting its oncogenic or tumor suppressor roles [13, 14]. MiR-21 has been shown to be an important tumor growth factor in facilitating tumor angiogenesis, invasion and metastasis, and has been suggested to be correlated with cervical cancer [15, 16]. This study thus investigated the modulatory
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Materials and methods

Cell culture

Hela cells (ATCC cell bank, US) were resuscitated in 37°C water-bath until completely thawing. Following 1 000 g centrifugation (3 min), cells were re-suspended in 1 mL DMEM medium (Hyclone, US) and were cultured in a 37°C humidified chamber with 5% CO₂ perfusion. After 24~48 hours, cell were seeded into culture dish at 1 × 10⁷/mL density, using high-glucose DMEM (containing 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum). Cells were passed every 2~3 days. Log-phased cells were randomly divided into three groups: control group; blank transfection group; and HPV16 E7 transfection group.

HPV16 E7 vector constructs and transfection

Total RNA was extracted from Hela cells by Trizol reagents (Invitrogen, US). Using total RNA as the template, cDNA was synthesized by reverse transcription kit (Invitrogen, US). HPV16 E7 specific primers were designed by Primer-Premier 6.0 software and synthesized (Sangon, China) to amplify the target sequence using cDNA as the template (Forward primer: 5'–TGCCG TTTCG CATGT GCCAT-3'; Reverse primer: 5'–TATAT GCTCT GCCCT TTGTC-3'). PCR conditions were: 95°C 2 min, followed by 35 cycles each containing 94°C denature for 30 sec, 60°C annealing for 50 sec and 72°C elongation for 35 sec. PCR products were purified from agarose gel following electrophoresis and ligated to pcDNA3.1 expressing vector (3:1) using 16-hour incubation at 4°C. JM109 competent cells were used to amplify target DNA fragments for further enzyme digestion.

Log-phased Hela cells were seeded into 6-well plate at 3 × 10⁶/mL under 12-hour incubation until reaching 70~80% confluence. Serum-free medium with lipo2000 reagents (Invitrogen, US) were used to incubate cells for 15 min. Cells were then mixed with serum-free medium containing pcDNA3.1-HPV16 E7 vectors (1 μg/mL) and lipo2000. After 30 min-incubation at room temperature, serum was removed in the plate, followed by PBS washing. Serum-free medium was continuously used for 6-hour incubation, followed by changing normal medium containing serum.

Western blotting

Proteins were extracted from Hela cells by lysis buffer (30 min on ice) and ultrasonic rupture (5 sec, 4 times). After centrifugation at 10 000 g for 15 min, supernatants were transferred to new tubes for quantification. Proteins were separated by 10% SDS-PAGE and were transferred to PVDF membrane (Pall Life, US). Using defatted milk powder to remove non-specific binding sites, rabbit anti-human HPV16 E7 antibody (1:1 000, Cell signaling, US) was added for 4°C overnight incubation. On the next day, goat anti-rabbit secondary IgG conjugated with horseradish peroxidase (HRP) (1:2 000, Cell signaling, US) was added for 30 min-incubation. ECL chromogenic substrates (Amersham Biosciences, US) were added to develop the membrane. After exposure under X-ray, Quantity One software was used to analyze optical density (OD) values of protein bands. All experiments were performed in four times (N=4).

MiR-21 inhibitor transfection

MiR-21 inhibitor (5'-AUCGG GAGGU GCAUU CUA-3') was transfected to HPV16 E7 over-expressing Hela cells using the same lipo2000 using the same procedure as those for vector transfection.

Real-time PCR

Total RNA were extracted from Hela cells, followed by cDNA synthesis. Real-time PCR was performed using specific primers of miR-21
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24-hour time interval. Supernatants were removed after 4 hours, with the addition of 150 μL DMSO to each well. The plate was vibrated for 10 min until complete resolving of crystal violet. Absorbance value at 570 nm was measured by a microplate reader (BD, US) for calculating proliferative rate of all cells.

**Transwell chamber assay**

48 hours after transfection, all cells were cultured in serum-free medium for 24 hours. Transwell chamber (Hyclone, US) was pre-coated with 50 mg/L Matrigel solution on the chamber bottom and upper membrane surface. Supernatants in the plate were removed, followed by the addition of serum-free medium containing 1% bovine serum albumin. Transwell chamber was put into 24-well plate, in which DMEM medium containing 10% FBS was added outside the chamber. 0.1 mL Hela cell suspension was added into each chamber with serum-free medium. All experiments were performed in triplicates. A parallel group was performed using uncoated Transwell chamber. After 48-hour incubation, Transwell chambers were removed and rinsed by PBS. Cells on the upper surface were removed. The whole chamber was fixed in cold ethanol and stained by crystal violet for 30 min. The number of migrated cells on the bottom surface was counted under an inverted microscope. 10 fields were randomly selected from each sample, with triplicates.

**Caspase-3 activity assay**

The activity of caspase-3 in all cells was quantified using test kit (R&D, US) following manual instruction. In brief, cell were firstly digested by trypsin (Sigma, US) and centrifuged at 600 g for 5 min. After discarding the supernatant, cell lysis buffer was added for rupture the cell on ice. After centrifugation at 20 000 for 5 min, 2

**Cell proliferation assay**

Hela cells (5 × 10³/mL) at log-phase were seeded into 96-well plate containing DMEM plus 10% FBS. After 24-hour incubation, 20 μL MTT reagents (Gibco, US) were added to each well at

![Figure 2. MiR-21 expression level. (*) P<0.05 compared to control group; (#), P<0.05 compared to HPV16 E7 group.](image)

![Figure 3. Hela cell proliferation. (*) P<0.05 compared to control group; (#), P<0.05 compared to HPV16 E7 group.](image)

(Forward, 5’-GACTT ACATG TGACC TGCT G-3’; Reverse, 5’-TTCCG GTTCA ACTCT CCTTA-3’) and GAPDH as the internal reference (Forward, 5’-ATCTG GAGTT TACCG CTGG-3’; Reverse, 5’-TACCG ATGTCT GGTAG ACGAT-3’). PCR conditions were: 55°C for 1 min, followed by 35 cycles each containing 92°C denature for 30 sec, 58°C annealing 45 sec and 72°C 35 sec. Standard curve was plotted based on CT values of standards. Semi-quantitative analysis was performed using 2-△Ct method.
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Figure 4. Hela cell invasion after E7 over-expression. A-C showed representative staining images showing number of perforated cells in control, HPV16 E7 and miR-21 inhibitor groups, respectively. D. Quantitative data of perforated cell number in each group. *, P<0.05 compared to control group; #, P<0.05 compared to HPV16 E7 group.

The overexpression of HPV16 E7 protein significantly facilitated Hela cell proliferation (P<0.05, Figure 3). The down-regulation of miR-21 by inhibitor transfection depressed Hela cell proliferation. These results showed the role of HPV E7 protein in potentiating Hela cell proliferation via up-regulating miR-21 expression in tumor cells.

Cell invasion assay

We further used Transwell chamber to examine the effect on Hela cell invasion. Result showed elevated number of perforated cells (128 ± 15) in E7-overexpressing cells compared to controlled Hela cells (78 ± 9, P<0.05, Figure 4). The application of miR-21 inhibitor, however, decreased number of invasive cells (69 ± 12). These results indicated the participation of HPV16 E7 protein in the invasion of Hela cells, by up-regulating miR-21 expression.

Intracellular activity of caspase-3

After overexpression of HP16 E7 protein, the up-regulation of miR-21 had no significant effects on caspase-3 activity (P>0.05, Figure 5). The transfection of miR-21 inhibitor also did not obtain statistical significance. These results collectively rejected the participation of HPV16 E7 in Hela cell apoptosis.

Discussion

The incidence of cervical cancer is increasing in recent years due to multiple factors such as viral infection, unhealthy sexual behaviors and environmental stress. In a demographic view, cervical carcinoma is most commonly occurred in young women between 30~55 years old, with primary cancer at 30~35 years and inlitra-
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tive cancer at 45–55 years. The average age of cervical cancer patients was becoming younger in recent years [17]. Almost 25% of all cervical cancer patients worldwide occurred in China. As most patients were already at late or terminal stage, the treatment strategy is somehow limited [18].

As one important and necessary risk factor for cervical cancer, HPV infection can be divided into high-risk and low-risk groups based on the tumor site and possibility of cancer. More than 99% of cervical carcinoma, including squamous cell injury and gland swelling, caused serum HPV elevation [19]. HPV can enter the body via tiny injury site to infect epithelial basal cells, in which viral DNA may incorporate into host genome, causing further malignant proliferation of epithelial cells, and finally leading to precancerous lesion and infiltrative carcinoma. The most powerful subtypes regarding carcinogenicity included HPV16, HPV18, and HPV16 E7 protein, all of which binds onto pRB protein, leading to the release of transcriptional factor E2F family. Such E2F family number can form complexes with cyclin A, cyclin E, p21 and p27, thus making the entry into S phase of infected cells to facilitate the amplification of viral DNA [20, 21].

As one important regulatory molecule, miR participates in almost all aspects of tumor cells, including growth, proliferation, differentiation, apoptosis, angiogenesis, invasion and metastasis. MiR-21 can negatively regulate tumor-suppressor gene expression, and induce tumor occurrence by modulating cell cycle, as suggested by other tumor observation [15, 16]. The role of HPV16 E7 in cervical cancer, however, remains unknown. This study thus over-expressed HPV E7 protein vector in Hela cells to investigate the modulatory role of E7 protein in cervical cancer. Our results demonstrated the up-regulation of miR-21 by E7 protein. Further studies using miR-21 inhibition on E7 overexpressing cells led to decreased proliferation rate and invasion ability of Hela cells but not for caspase-3 activity. In summary, HPV16 E7 protein can stimulate the expression of miR-21 in host cells, thus facilitating cervical cancer cell growth, proliferation and invasion. This study illustrated the crucial role of E7 in pathogenesis and progression of cervical carcinoma. The exact mechanism of E7 protein in regulating miR-21, however, remained incomplete. Our results should benefit the clinical study of novel drug targets for cervical cancer.

Disclosure of conflict of interest

None.

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