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Original Article

Blocking activity of the HPV18 virus in cervical cancer cells using the CRISPR/Cas9 system

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Abstract: Objective: Specific sgRNA-sequences targeting oncogenes E6 and E7 in HPV18 were designed using the CRISPR/Cas9 system. These sgRNAs knocked out E6 and E7 expressions and were used to study their effects on the proliferation and cell cycle of the cervical cancer HeLa cell line. Methods: Lentivirus vectors targeting E6 and E7 oncogenes were constructed and transfected into HeLa cells. mRNA and protein expression levels of E6 and E7 were measured by RT-PCR and Western blot, respectively. The cell cycle was detected by flow cytometry. A colony formation assay was applied to evaluate the proliferation capacity of the HeLa cells. Results: Three E6 Cas9-sgRNA vectors targeting E6 and three E7 Cas9-sgRNA vectors targeting E7 genes were constructed and transfected into HeLa cells, respectively. RT-PCR results showed that all three E6 and E7 sgRNAs inhibited the expressions of E6 or E7 mRNA, respectively, when compared with the control groups. The inhibition ratios of the three groups of E6-sgRNAs were 28%, 85%, and 19%; the E7-sgRNAs were 86%, 25%, and 27%, respectively ($P<0.05$), with E6-sgRNA2 and E7-sgRNA1 having the greatest inhibitory effects. Western blot results showed that, compared with the control group, the protein expressions of E6 and E7 in the sgRNAs transfected group were also decreased, and E6-sgRNA2 and E7-sgRNA1 had the most inhibitory effects on E6 and E7 proteins. Flow cytometry results showed that the number of cells in G1/G0 was increased by 14.2% in the E6-sgRNA2 transfection group, and by 7.1% in the E7-sgRNA1 transfection group. Colony formation assay results showed that after transfection of E6 or E7 sgRNA plasmids, the HeLa cell colony was reduced significantly compared with the control group. Conclusions: The CRISPR/Cas9 system targeting HPV18 E6 or E7 genes effectively blocked the transcription and expression of oncogenes E6 or E7 in HeLa cells, which resulted in cell cycle arrest and reduced cell proliferation.

Keywords: Cervical cancer, human papillomavirus 18, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9), E6, E7

Introduction

Cervical cancer, one of the most common gynecological malignancies, is a serious threat to women's health and life [1]. Studies have shown that more than 90% of patients with cervical cancer were infected by the human papilloma virus (HPV). The persistent infection of high-risk HPV is a key factor in cervical lesions and cervical cancer [2]. The E6 and E7 proteins are two viral proteins produced by HPV that mediate tumor transformation. They affect carcinogenesis by inhibiting the activities of the tumor suppressor genes p53 and retinoblastoma proteins (Rb) in host cells [3]. Therefore, E6 and E7 genes are potential candidate gene targets for the treatment of cervical cancer [4]. They provide the possibility of clearing the HPV virus and interfering with the progress of cervical cancer by blocking HPV oncogenes and thus reducing viral tumorigenic toxicity.

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) is an immune system that exists in bacteria and archaea to specifically prevent foreign nucleic acid invasion. It consists of direct repeats of a short sequence separated by nonrepetitive sequences of similar size [5]. CRISPR/Cas9 is a new gene fixed-point editing technology that protects bacteria from phages by guide RNA (sgRNA) and the Cas9 enzyme [6]. This system has many advantages, such as the construction of vectors is simple, the application flexible, the targeting accurate, the cytotoxicity lower, and it is very cheap and easy [7].

Our study aimed to investigate the expression of E6 and E7 genes in HeLa cells and their effects on the tumor activities of HeLa cells. The specific sgRNA sequences targeting the HPV18 E6/E7 genes were designed by CRISPR/Cas9 and were transfected into the HeLa cells with lentiviral vectors. Therefore, by blocking HPV oncogenes in vitro, we could better explore the potential of gene therapy in the treatment of cervical cancer.

Materials and methods

Cell line

The human cervical cancer cell line HeLa was purchased from the American Type Culture Collection, which was provided by the Cell bank of the Chinese Academy of Sciences after the recovery. The cells were maintained in a DMEM medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2 or 3 days. Logarithmic growth phase cells were used for the following experiments.

Reagents

The DMEM medium and fetal bovine serum were purchased from BIOTECH. The phosphate buffer, trypsin and penicillin/streptomycin were purchased from Boguang Biotechnology Co., Ltd. The CRISPR/Cas9 plasmid was purchased from Addgene. The E6 and E7 antibodies were obtained from Shanghai Billiton biological science and Technology Co., Ltd. The BsmBI (R0580S) endonucleases were purchased from New England Biolabs. The sgRNA sequences and PCR primers were synthesized from Shanghai Jie Li Biotechnology Co., Ltd. The GAPDH antibody was purchased from Proteintech. The transfection kit, the reverse transcription kit, and the RT-PCR reagents were purchased from Takara Corporation, Japan. The TRIzol was purchased from Invitrogen, and the propidium iodide (PI) was purchased from BD. The relative fluorescence quantitative PCR kit was purchased from Takara Corporation, Japan.

The microplate reader was purchased from the United States BioTek company. The PCR instrument was purchased from Beijing Dongsheng Innovation Biotechnology Co., Ltd., and the flow cytometry was purchased from BD Biosciences.

Construction of plasmids

sgRNA sequences targeting the HPV18 E6 and E7 genes were designed and then synthesized by Shanghai Jie Li Biotechnology Co., Ltd. These sequences are: E6-sgRNA1 (GCA ACA GTT ACT GCG ACG TGA GG), E6-sgRNA2 (AAC TTT CTG GGT CGC TCC TGT GG), E6-sgRNA3 (ACT TTC TGG GTC GCT CCT GTG GG), E7-sgRNA1 (ATA TTG TAA TGG GCT CTG TCC GG), E7-sgRNA2 (TAT GGT TCT GCT TGT CCA GCT GG), E7-sgRNA3 (CAG TAG AGA TCA GTT GTC TCT GG). sgRNA sequences were annealed and inserted into the lentiGuide plasmid that had been digested by BsmBI endonuclease. The lentiGuide and package plasmids were transfected into HEK-293T cells to obtain lentivirus carrying sgRNA sequences. The Lentivirus was stored at -80°C or used freshly for transfection of HeLa cells.

Detection E6 and E7 mRNA levels by RT-PCR

After being transfected with vectors for 48 hours, cells in the 6-well plates were collected. The total RNA in the cells was extracted by TRIzol and the cDNAs were prepared by the reverse transcription Kit. According to the Primer 5.0 software, the following primers were analyzed: E6 upstream primer, 5'-TTGCTTTTC-GGGATTTATGC-3', E6 downstream primer, 5'-CAGGACACAGTGGCTTTTGA-3'; E7 upstream primer, 5'-CAGCTCAGAGGAGGAGGATG-3', E7 downstream primer, 5'-GCCCATTAACAGGTCTT-CCA-3'. The DNA conditions were pre-denaturation at 95°C for 1 min, denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extending at 72°C for 35 seconds, and final elongation at 72°C for 10 minutes. The total number of cycles was 40.

Detection E6 and E7 protein levels by western blot

After being transfected with vectors for 48 hours, cells in the 6-well plates were collected. 200-300 μl of lysate containing cocktail protease inhibitors were added to the cells for 30 minutes and shaken. The supernatant was collected, and a loading buffer was added into the supernatant at 100°C for 5 minutes. Protein samples were loaded on 12% PAGE electrophoresis and then transferred to a PVDF membrane. Primary antibodies (anti-HPV18 E6, anti-HPV18 E7 and anti-β-actin) were incubated at room temperature for 24 hours, and then a sec-

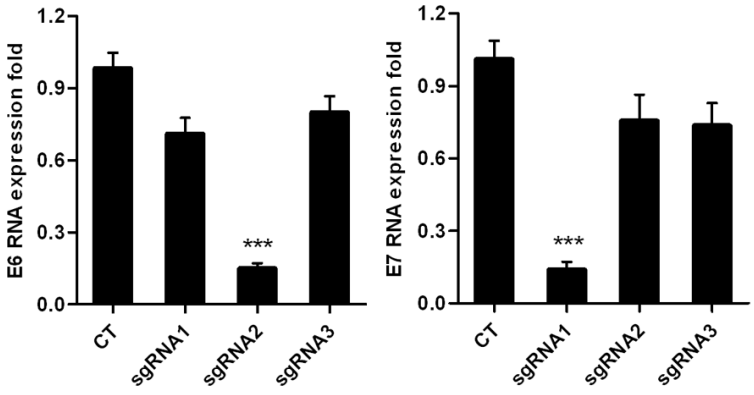

Figure 1. sgRNA vector transfection inhibited the expressions of E6 and E7 mRNA in HeLa cells. CT: control group; sgRNA1/2/3: HeLa cells transfected with E6-sgRNA1, E6-sgRNA2, E6-sgRNA3 or E7-sgRNA1, E7-sgRNA2, E7-sgRNA3 vectors.

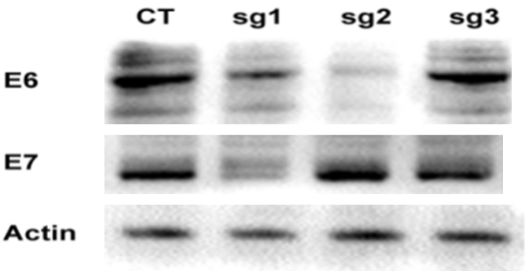

Figure 2. sgRNA vector transfection inhibited the expressions of E6 and E7 proteins in HeLa cells. CT: control group; sgRNA1/2/3: HeLa cells transfected with E6-sgRNA1, E6-sgRNA2, E6-sgRNA3 or E7-sgRNA1, E7-sgRNA2, E7-sgRNA3 vectors.

ondary goat anti-rabbit IgG antibody was incubated at room temperature for another 1 hour before detection.

Detection cell cycle by flow cytometry

After being transfected with E6-sgRNA or E7-sgRNA vectors for 48 hours, the HeLa cells were collected. About 10 μl of propidium iodide (PI) staining solution was added, and the cells were incubated at room temperature for 30 minutes. Flow cytometry was used to detect the cell cycle.

Detection of cell proliferation by the clone formation assay

After being transfected with E6-sgRNA or E7-sgRNA vectors, the cells were diluted into single cell suspensions, with the proportions of individual cells at more than 95%. The single cell suspensions were cultured for 2-3 weeks until the cell clones were visible to the naked eye. The culture medium was discarded, and the clones were subjected to air drying and fixed by methanol for 15 minutes. The methanol fixed clones were stained with crystal violet dye for 10 minutes and the cell clone numbers were examined.

Statistical analysis

The data were expressed by the mean ± standard error and were analyzed by paired t test using Graph Pad Prism software (version 5.0). The comparisons among the different groups were made using the least significant difference test. A difference was considered significant when $P<0.05$.

Results

Inhibition of E6 and E7 mRNA expression by sgRNA transfection

After transfection of different E6-sgRNA (E6-sgRNA1, E6-sgRNA2, E6-sgRNA3) or E7-sgRNA (E7-sgRNA1, E7-sgRNA2, E7-sgRNA3) vectors for 48 hours, the expressions of E6 and E7 mRNA in transfected HeLa cells were lower than those in the control groups (**Figure 1**). And the inhibition ratios of the three groups of E6-sgRNAs were 28%, 85%, and 19%, of E7-sgRNAs were 86%, 25%, and 27%, respectively ($P<0.05$) with E6-sgRNA2 and E7-sgRNA1 having the most inhibitory effects.

Inhibition of E6 and E7 protein expressions by sgRNA transfection

Compared with the control group, the expressions of the E6 and E7 proteins were inhibited by E6-sgRNA (E6-sgRNA1, E6-sgRNA2, E6-sgRNA3) or E7-sgRNA (E7-sgRNA1, E7-sgRNA2, E7-sgRNA3) transfection, respectively (**Figure 2**). And the E6-sgRNA2 vector and E7-sgRNA1 vector showed the greatest inhibitory effects on the E6 or E7 proteins.

Cell cycle arrest of HeLa cells by sgRNA transfection

HeLa cells were transfected with the E6-sgRNA2 vector or the E7-sgRNA1 vector for 48 hours, and flow cytometry was used to detect the cell

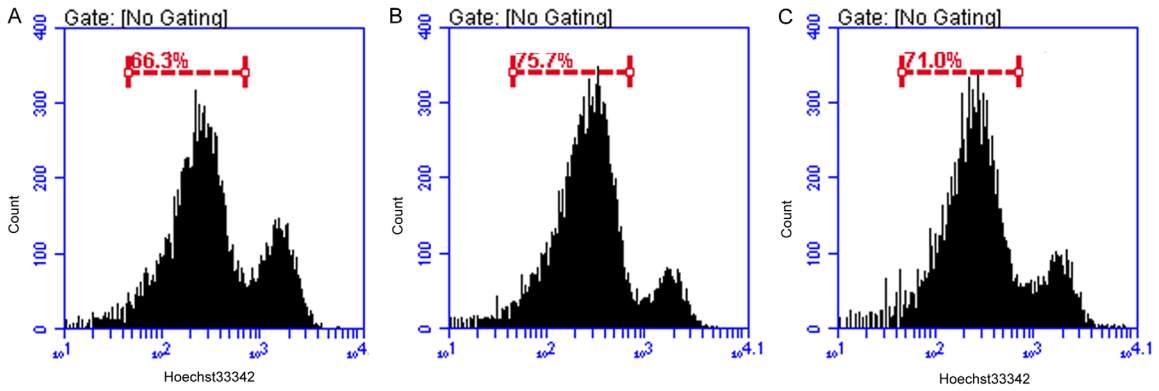

Figure 3. E6-sgRNA2 or E7-sgRNA1 vector transfection induced cell cycle arrest. A: Control group; B: E6-sgRNA2 transfection group; C: E7-sgRNA1 transfection group.

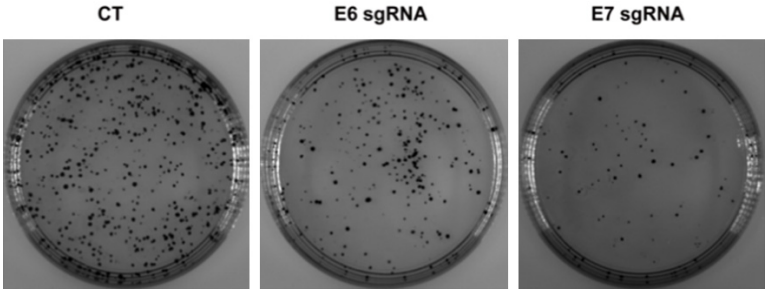

Figure 4. E6-sgRNA2 or E7-sgRNA1 transfection inhibited cell colony formation activity of HeLa cells. CT: control group; E6-sgRNA: E6-sgRNA2 transfection group; E7-sgRNA: E7-sgRNA1 transfection group.

cycle arrest. The results showed that the proportions of G1/G0 (DNA synthesis/quiescent) cells in E6-sgRNA2 and E7-sgRNA1 groups were increased by 14.2% and 7.1% (**Figure 3**), respectively, compared with those in the control group, indicating that the E6-sgRNA2 or E7-sgRNA1 vector transfection induced the cell cycle arrest of the HeLa cells.

Effect on cell cloning by blocking E6 or E7 gene sgRNA transfection

A colony formation assay found that the numbers of cell colonies in both the E6-sgRNA2 transfection and E7-sgRNA1 transfection groups were significantly less than those in the control group (**Figure 4**). These results indicated that the abilities of cell proliferation and cell clone formation were decreased when E6 or E7 gene expressions were blocked by sgRNA transfection.

Discussion

Cervical cancer is caused by many factors, among which HPV infection is the most important. One of the most common types of HPV is HPV18, accounting for 20%-30% of all HPV.

HPV is a circular double-stranded DNA virus. Its early genes are encoded by 5 to 8 frameworks, and their encoding products are E1 to E8 proteins, which are involved in viral replication, transcription, and cell transformation, etc. It is known that multiple receptors play important roles in the process of HPV infection, leading to cervical cancer [8-13]. Gene therapy has been successfully used in mouse models of HPV cervical cancer. However, gene expression in both HPV infected and un-infected cells is controlled by HPV long control regions (LCR), which could be activated in both HPV-16 infected and uninfected cells [14, 15]. Thus, other HPV-specific sequences controlled by HPV-proteins might play a role in the treatment of precancerous lesions and cervical cancer. It was found that the E6 and E7 proteins produced by HPV genes could bind to p53 and Rb proteins, respectively, and cause degradation and inactivation, which then affects the cell cycle and inhibits cell apoptosis. Therefore, E6 and E7 are the key carcinogens in HPV viral DNA.

The CRISPR/Cas9 system is a new gene editing technology. It is the immune system that is present in prokaryotic species. It can prevent foreign nucleic acid invasion specifically. When the system is expressed, its tracrRNA will form double-stranded RNA with the crRNA and bind to the target DNA directly. Also, the Cas9 pro-

tein will cut the positive and reverse chains of target DNA to produce a DSB, and then the cells will activate their native DNA repair systems, including non-homologous end joining (NHEJ) and homologous recombination (HR) repair, to perform DNA repair. In the error-prone NHEJ pathway, the Ku heterodimer binds to the DSB cleavage to form a molecular scaffold to repair proteins. When the complementary strands are subjected to end excision and dislocation repair due to micro homology, indels are formed, leading to frameshift mutations and gene knockouts. CRISPR/Cas9 has been widely applied in the fields of tumor and intractable viral infection. In one study, the CCR5 32 bp sequence in pluripotent stem cells was knocked out by CRISPR/Cas9, and then these CCR5 knockout cells or normal cells were infected with the HIV-1 virus [16]. The results showed that the HIV virus was not detected in the CCR5 knock-out cells, indicating that CRISPR/Cas9 could block HIV infection by knocking out CCR5 expression. When the HBV virus plasmid and sgRNA-HBV-Cas9 plasmid were co-transfected into Huh-7 cells, the expressions of the HBV core and surface antigens were significantly decreased. Furthermore, after these two plasmids were injected into hepatitis-tolerant mice by the tail vein, the expression of the HBV surface antigen was decreased, indicating that CRISPR/Cas9 could inhibit HBV infection *in vivo* [17].

In this study, we blocked the expressions of the E6 and E7 genes using the CRIPSR/Cas9 system, specifically using sgRNA sequences targeting HPV18 E6 or E7 genes. First, the targeted Cas9-sgRNA sequence of HPV18 E6 or E7 genes was constructed and then packaged into lentiviral expression vectors which would be transfected into HeLa cells, so their effects on E6 or E7 expression and cell growth could be detected. The results showed that all three sgRNA1/2/3 targeting E6 or E7 genes could inhibit the expression of mRNAs of E6 and E7, respectively ($P<0.05$). And, E6-sgRNA2 and E7-sgRNA1 vectors showed the greatest inhibitory effects on both the mRNA and protein levels of the E6 and E7 genes. The results of flow cytometry found that E6-sgRNA2 and E7-sgRNA1 transfection induced cell cycle arrests of HeLa cells. Also, a colony formation assay showed that the number of HeLa cell clones in two transfection groups was significantly reduced compared with the control group, which means that E6-sgRNA2 and E7-sgRNA1 transfection decreased the proliferation and the colony formation ability of HeLa cells.

The results of this study showed that oncogenes E6 and E7 of HPV18 in HeLa cells could be successfully inhibited by the CRISPR/Cas9 system, using sgRNA sequences. The proliferation and colony formation abilities of HeLa cells were lessened by the E6-sgRNA2 or E7-sgRNA1 transfection. This provides new ideas and experimental data for the prevention of cervical cancer in patients infected with high risk HPV. However, whether the CRISPR/Cas9 system can edit the HPV virus *in vivo* and block the development of cervical cancer remains to be confirmed. Further studies to explore the CRISPR/Cas9 system in HPV infection are needed before its eventual application in the clinical treatment of refractory cervical cancer.

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Disclosure of conflict of interest

None.

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