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In vitro and *in vivo* growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9

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ABSTRACT

Deregulated expression of high-risk human papillomavirus oncogenes (E6 and E7) is a pivotal event for pathogenesis and progression in cervical cancer. Both viral oncogenes are therefore regarded as ideal therapeutic targets. In the hope of developing a gene-specific therapy for HPV-related cancer, we established CRISPR/Cas9 targeting promoter of HPV 16 E6/E7 and targeting E6, E7 transcript, transduced the CRISPR/Cas9 into cervical HPV-16-positive cell line SiHa. The results showed that CRISPR/Cas9 targeting promoter, as well as targeting E6 and E7 resulted in accumulation of p53 and p21 protein, and consequently remarkably reduced the abilities of proliferation of cervical cancer cells *in vitro*. Then we inoculated subcutaneously cells into nude mice to establish the transplanted tumor animal models, and found dramatically inhibited tumorigenesis and growth of mice incubated by cells with CRISPR/Cas9 targeting (promoter+E6+E7)-transcript. Our results may provide evidence for application of CRISPR/Cas9 targeting HR-HPV key oncogenes, as a new treatment strategy, in cervical and other HPV-associated cancer therapy.

1. Introduction

Cervical cancer is the second most common type of malignancy among women worldwide and a leading cause of mortality among females in developing countries. It affects nearly half a million women each year, claiming a quarter of a million lives [1,2]. According to several epidemiological and biological studies, human papilloma viruses (HPV) infection is the dominant etiological event in CC development [3], HPV DNA can be detected in more than 95% of cervical cancer and intraepithelial neoplasia biopsies [4], demonstrating the strong association of this cancer with HPV infection.

Human papillomaviruses (HPVs) are small DNA viruses with a genome of approximately 8 kb. A small subgroup, types 16, 18, 31, 33, and 45, has been designated high-risk HPVs and found to be associated with more that 90% of cervical cancers [5], of which HPV16 accounts for approximately 50% [6]. The oncogenic function of HPVs has been primarily attributed to E6 and E7, which are essential for malignant transformation and pivotal for maintenance of the malignant phenotype of cervical cancer [7,8]. They are selectively expressed in HPV-related cancer cells to inactively tumor suppressor proteins such as P53 and pRb, leading to cell cycle disorder, telomerase activation, and cell immortalization. E6 is able to induce the degradation of p53, thereby inhibiting p53-dependent signaling, and contributing to tumorigenesis [9,10]. In the case of E7, deregulation of the host cell cycle seems to be a major function of the oncoprotein. It is associated with the retinoblastoma family of proteins: pRb [11,12], p107, and p130 [13,14]), as well as cyclin-dependent kinase inhibitors of p21 [15,16] and p27 [17], and prevents G1 arrest in response to a variety of antiproliferative signals, such as growth factor withdrawal, loss of cell adhesion, and DNA damage [18]. Because of the strong relationship between the expression of this group of HPV oncogenes and cervical cancer carcinogenesis, development of novel approaches directed against these oncogenes may provide a novel block therapy for cervical cancer.

Clustered regularly interspaced short palindromic repeats CRISPR-associated (Cas9) is a recently discovered, site-specific genome editing system that is part of the CRISPR–Cas bacterial acquired immune system, which cleaves foreign DNA [19,20].

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And the CRISPR/Cas9 system requires the design of only a single guide sequence that matches the DNA targeted for cleavage. Since the first report of the use of CRISPR/Cas9 for genome editing in human cells in 2013 [21–23], this technology has been used *in vivo* in human cells and other organisms. Molecular-targeting therapy with Cas9 is expected to prevent the development for disease caused by foreign genes, such as human immunodeficiency virus (HIV) infection, by inhibiting expression of genes [24]. And Fujii et al. [25] indicate the availability of CRISPR/Cas9 system for the large-scale deletion and imply the system is highly efficient for genome modification. Therefore, strategies for achieving specific and selective CRISPR/Cas9 to abrogate E6 and E7 function may be a rational therapeutic approach for treating HPV-positive cervical cancers.

In the hope of applying CRISPR/Cas9 as therapy for HPV-related cancer, in this study, we have designed a CRISPR/Cas9 system that targets E6 and E7. We have shown that a CRISPR/Cas9 system can effectively, specifically and stable suppress HPV-16 E6 and E7 expression *in vitro* and *in vivo*, and also demonstrated that the cervical tumor growth could be inhibited for mouse by CRISPR/Cas9 treatment.

2. Materials and methods

2.1. gRNA expression plasmids

gRNA expression plasmids were constructed according to manufacturer's protocol [26,27]. Briefly, to prepare a 100-bp dsDNA insert fragment containing the target sequence (20 bp) and a protospacer-adjacent motif (PAM) sequence, we used a set of oligonucleotides and generated the fragment using T4 PNK (NEB; Ipswich, MA, USA). The dsDNA fragment was purified and inserted into the BbsI site of a gRNA cloning vector with T4 DNA ligase (NEB). Detailed BLAST searching of human and murine genomes was carried out to identify potential off target binding of HPV gRNAs. Ten sets of oligonucleotides (Table 1) were designed. All oligonucleotides were synthesized and purified by Sangon Biotech Co. (Shanghai, China).

2.2. Cell culture and transfection

The human cervical carcinoma cell lines SiHa and C33-A were selected. Siha cells contain a single copy of HPV16 integrated in the chromosome and express the E6 and E7 oncogenes [28], whereas C33-A cells were negative for HPV. Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 100 ml/l fetal calf serum (Hyclone, Logan, UT, USA), 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37 °C in a humidified atmosphere containing 5%CO_2.

The SiHa cells were seeded into 6-well plates and grown until 60–80% confluency, after which they were transfected with 1 μg of hCas9 expression vector (Plasmid 41815: hCas9 Addgene; Cambridge, MA, USA) and 1 μg of gRNA expression vector using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Two days after transfection, the cells were harvested.

Table 1
The sequences and location of gRNA targeting HPV16 E6 and E7.

Name	Genomic target	Target location
HPV1	CACCGACTAAGGGCGTAACCGAAAT AAACATTTCGGTTA CGCCCTTAGT	23
HPV2	CACCGGTTTCGGTTCAACCGATTT AAACAAATCGGTTG AACCGAAACC	36
HPV3	CACCGACTTTCTGGGTCGCTCCTGT AAACACAGGAGCGA CCCAGAAAGT	E6(115)
HPV4	CACCGCAACAGTTACTGCGACGTG AAACCACGTCGCAG TAACTGTTGC	E6(205)
HPV5	CACCGCTAATTAACAAATCACACAA AAACTTGTGTGATT TGTTAATTAG	E6(386)
HPV6	CACCGTCCGGTTCTGCTTGTCCAGC AAACGCTGGACAAG CAGAACCGGA	E7(682)
HPV7	CACCGACACGTAGACATTCGTACTT AAACAAGTACGAAT GTCTACGTGT	E7(777−)

Sequence design of HPV-16–CRISPR/Cas9.

2.3. Analysis of E6 and E7 mRNA levels by real-time quantitative RT-PCR

The SiHa cells were seed in 6-well plates. For the quantitative real-time RT-PCR, SiHa cells which were transfected CRISPR/Cas9 as described previously. Total RNA was extracted with Trizol reagent (invitrogen), the mRNA expression of E6 and E7 was determined by RT-PCR, similar to previous descriptions [29]. GAPDH was used as an internal reference. The PCR primers were E6 forward. 5′-AATGTTTCAGGACCCACAGG-3′; and reverse, 5′-TCAGGACACAGTGGCTTTTG-3′; E7 forward, 5′-ATGCATGGA GATACACCTA CATTGC-3′; and reverse. 5′-ACAATTCCTA GTGTGCCCAT TAACA-3′.

2.4. Detection of p53 and p21 protein expression

Protein extracts for stably transduced SiHa cells were prepared, Western blotting was performed as described previously [30]. Primary antibodies used were mouse monoclonal anti-p53 (Stata Cruz Biotechnologies), goat polyclonal anti-p21 (Santa Cruz Biotechnologies). The GAPDH (Santa Cruz Biotechnologies) was used as internal control for protein loading and analysis.

2.5. In vitro cell viability assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma–Aldrich, MO, USA) colorimetric assay was used to validate the cell growth. SiHa cells, 15,000 per well, were seeded in 96-well plates on the day prior to transfection. Groups of replicates included cells that were untransfected, mock transfected (CRISPR+Cas9+gRNA empty vector), or transfected with CRISPR+Cas9+gRNA. Cell viability was assessed 3 days after transfection by adding 20 μl of MTT (5 mg/ml) to each well, and incubating at 37 °C for 1 h. Metabolism of the MTT to form the blue formazan was determined by measuring the ratio of optical density at a wavelength of 570 nm, to the background at 690 nm. These CRISPR/Cas9 systems were also transfected into the non-HPV-infected cells to test their specific.

2.6. Mouse tumor model studies

To investigate the effects of CRISPR+Cas9+ (gRNA targeting the promoter, E6 transcript and E7 transcript) on the tumorigenicity of xenograft, and the influence on survival of tumor-burdened animals, twenty female BALB/C nude mice, aged 4–6 weeks, were purchased from the center of experimental animal, the Academy of Military Medical Science (Beijing, China) and were caged individually under specific-pathogen free (SPF) conditions.. SiHa cells were infected with CRISPR/Cas9 as described above and harvested 2 days post-infection. The cells were washed with PBS, counted and resuspended in PBS at 2×10^7/ml. Female mice were injected with 0.1 ml cell suspension subcutaneously to the neck scruff and tumor growth was monitored. Five groups were included in the experiments: the gRNA targeting promoter; E6 transcript; E7 transcript or combined (promoter+E6+E7) transcript targeting CRISPR/Cas9 were applied to the experimental, gRNA empty and hCas9 plasmids was used as negative control. Tumor volume was contin-

uous blindly measured by periodic caliper every 4 days. The tumor volume was calculated according to the formula volume = (length × width^2)/2. Tumor growth was observed in 100% of the mice after 10 weeks. At the end of the experiment, the mice were killed according to UKCCCR guidelines, and each tumor was then excised and weighted.

2.7. Data analysis

The data with mean and SD as presented here were prepared using GraphPad Prism software (version 5.0). Comparisons of treatment outcome were tested for statistical difference by the paired *t* test. All experiments were performed at least three times. A difference was considered significant when $P < 0.05$.

3. Results

3.1. Inhibition of E6 and E7 expression by CRISPR/Cas9

SiHa cells were used to examine the effects of E6 or E7 gene knockdown by CRISPR/Cas9. A total of ten gRNA were designed and their effects on E6 and E7 gene expression were determined by RT-PCR analysis after transfection of each specific CRISPR/Cas9 into cells. As shown in Fig 1, levels E6 and E7 expression in SiHa cells transduced by the CRISPR+Cas9+promoter-1 were reduced by 94% and 91%, compared to the blank, at 48 h. The CRISPR+Cas9+E6-4 showed effective knockdown of HPV16-E6 gene expression 93% of blank levels, at 48 h. Similarly, the CRISPR+Cas9+E7-6 showed effective knockdown of HPV16-E7 gene expression: 94% of blank levels. Therefore, we obtained at least on effective gRNA for each viral oncogene in each type of HPV.

3.2. Induction of p53 and p21 by E6 and E7 targeting CRISPR/Cas9

Two days after infection, SiHa cells infected with CRISPR/Cas9 became more granular, compared to controls (data not shown). We examined the downregulation of E6 or E7 protein expression in the CRISPR/Cas9-transfected cells by analyzing the expression of p53 or P21, since there is no high affinity anti-E6 or anti-E7 antibody capable of detecting low E6 or E7 expression presently available. As shown in Fig 2, levels of p53 and p21 proteins were increased in SiHa cells transduced by the CRISPR+Cas9+promoter-1 and the CRISPR+Cas9+E6-4, compared to the control, when transfected with gRNA empty and hCas9 plasmids. Similarly, by the CRISPR+Cas9+promoter-1 and the CRISPR+Cas9+E7-6, levels of p21 proteins were also up-regulated in SiHa cells. The results were comparable to the RT-PCR.

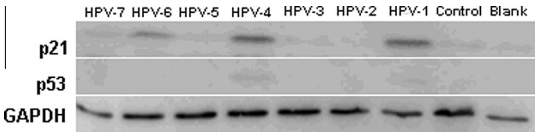

Fig. 2. Effects of the designed CRISPR/Cas9 on E6, E7 related protein expression in SiHa cells, compared with relevant scramble CRISPR/Cas9.

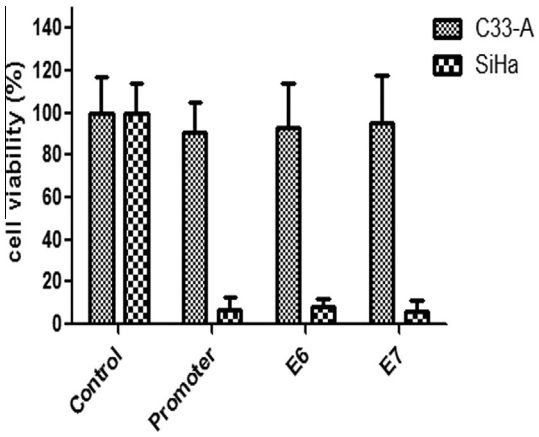

Fig. 3. Effects of the HPV 16 promoter, E6 transcript and E7 transcript targeting CRISPR/Cas9 on cellular proliferation.

3.3. The proliferation of cells transduced by the promoter-targeting and E6/E7-transcript targeting CRISPR/Cas9

HPV16+ (SiHa) and HPV16− (C33-A) cancer cells were transfected with the three CRISPR/Cas9 systems (Promoter-targeting-1; E6-transcript-4; E7-transcript-6) and analyzed for cell viability. As shown in Fig 3, three tested CRISPR/Cas9 systems showed strong growth suppression toward SiHa cells, while they had minimal effect on C33-A cells growth. The results suggested that these CRISPR/Cas9 systems have a strong specific growth inhibition characteristic.

3.4. Inhibition of tumor growth in mice by CRISPR/Cas9

We transfected SiHa cells with the Control (CRISPR+Cas9+gRNA empty), CRISPR+Cas9+promoter-1; CRISPR+Cas9+E6-4; CRISPR+Cas9+E7-6 and CRISPR+Cas9+ (promoter-1 +E6-4 +E7-6), cultured them for 2 days, and subcutaneously inoculated them into BALB/C nude mice at 2×10^7cells. As shown in Fig 4, tumor formation of CRISPR+Cas9+ (promoter-1 +E6-4 +E7-6)-treated cells was

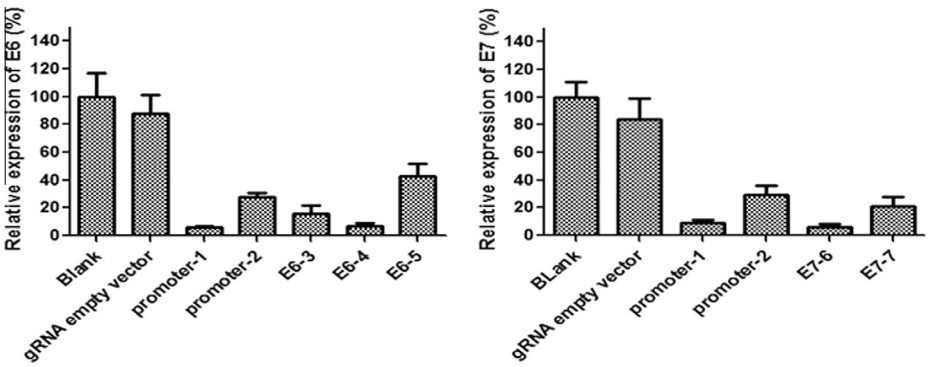

Fig. 1. Knockdown of HPV16-E6 or -E7 gene expression by transfection of CRISPR/Cas9 (Control: gRNA empty vector).

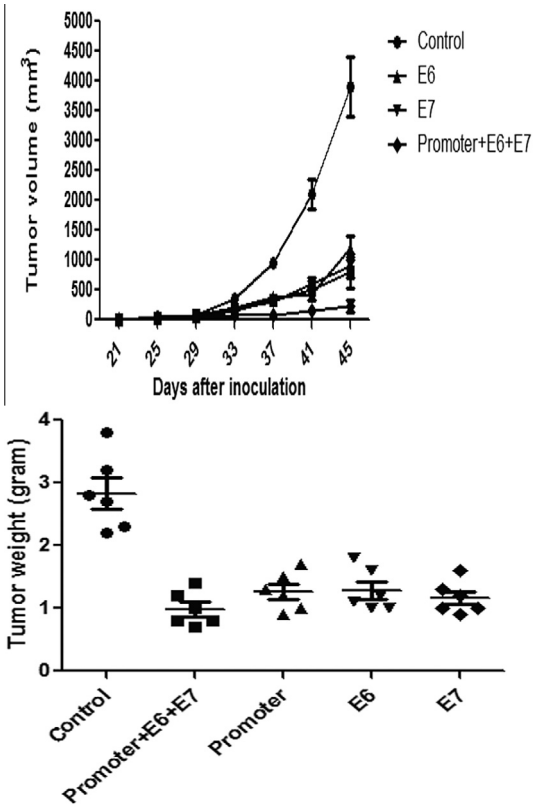

Fig. 4. Suppression of *in vivo* growth of SiHa cells in BALB/c nude mice by CRISPR/Cas9. (A) *In vivo* tumor growth curves of CRISPR/Cas9 systems-treated SiHa cells. The mean tumor volumes ± SD (bars) are shown at the times that tumor measurements were made ($n = 6$). (B) Tumor weight 10 weeks after inoculation. All tumors were excised and weighted.

slower than that of control treated cells. From 21 to 45 days after inoculation, tumors were palpable in all mice ($n = 6$). In a separate experiment, we carefully observed mice for a longer period and found that palpable tumors had formed in all tested mice 10 weeks after inoculation. After euthanasia, we excised and weighed the tumors (Fig 4). Tumor weights in mice inoculated with SiHa cells treated with combined (promoter+E6+E7) transcript targeting CRISPR/Cas9 were significantly lower than those from those mice treated with control ($P < 0.001$).

4. Discussion

Cervical cancer is the second most common cancer in women worldwide though well organized screening has been expanded, and the majority of cases are caused by high-risk types of human papillomavirus, which possess the E6 and E7 oncogenes. Studies have demonstrated that the expression of these oncogenes is indispensable for tumor development and maintenance of malignant phenotypes [31]. Clinical data show that the prognosis of cervical cancer patients remains unoptimistic up to data, with a 5-year survival rate of 9.3–65.8% in those with advanced stage diseases [32], despite the progress of surgery, radiation, and chemotherapy. The development of novel and effective therapy for cervical cancer is still urgently needed.

Targeted genome engineering has been instrumental for the study of biological processes, and its holds great promise for the treatment of disease [33]. Over the last decade, novel genome-editing methods that utilize artificial nucleases, such as zinc finger nucleases (ZENs) and transcription activator like-effector nucleases (TALENs), have been developed [24]. However, ZFNs or TALENs remain somewhat difficult and time-consuming to design, develop, and empirically test in a cellular context [34]. Recently, the clustered, regularly interspaced, short palindromic repeat (CRISPR)–CRISPR-associated (Cas) systems have been developed, Unlike the ZFNs and TALENs, the CRISPR/Cas system offers a few advantages. For example, the system is its amenability to multiplexing allowing for the simultaneous generation of up to five mutations for a single transfection event [35]. In our study, we have indicated the availability of CRISPR/Cas9 system for the large-scale deletion and imply that the system can contribute largely to genomics studies.

It is known that overexpression of HR-HPV E6 and E7 oncogene is essential for malignant transformation and the maintenance of the malignant phenotype of cervical cancer cells. In the present study, we designed several CRISPR/Cas9 against the E6 or E7 viral oncogenes in HPV-16 cells and found three of them effectively inhibit the expression of E6 or E7 in the HPV-infected cervical cancer cells (SiHa cells). Transfected of CRISPR/Cas9 caused significant suppression of cell growth in HPV-positive cells, whereas they had no effect in HPV-negative cells, demonstrating the effectiveness and specificity of these CRISPR/Cas9 systems. As well-characterized targets, p53 and p21 protein as directly inactivated by E6 and E7 respectively, lead to a series of signaling pathway dysfunction. Additionally, recent studies showed that the alteration of additional pathways was equally important for malignant transformation and maintenance in cervical cancer [36–38]. In our experiments, to assess whether the CRISPR/Cas9 we designed blocked the E6-p53 pathway or the E7-p21 pathway, we used Western blot analysis to investigate the expression levels of p53 protein, the target of E6, and of p21 protein, the target of E7. *In vivo*, the designed CRISPR/Cas9 could promote xenograft retardation, and even results in complete loss of tumor growth in tumor-burdened animal models. Thus, both viral proteins, E6 and E7, are believed as therapeutic targets in blocking cervical cancer progress through proliferation inhibition and death inducement of tumor cells.

This is the first report of inhibition of tumor growth derived from cervical cancer cells with a mixture of CRISPR/Cas9 targeting HPV gene. In this study, we have demonstrated that the CRISPR/Cas9 specific to HPV16 oncogenes including targeting the E6-, E7-transcript could effectively, specifically and stably suppress targeted oncogene E6 and E7 expression in cervical cancer and inhibit the cancer cell growth. Using a tumor transplant model, we demonstrated that the designed CRISPR/Cas9 could partially or completely inhibit tumor growth and, furthermore, we observed the tumor growth in tumor burdened mice inoculated by the combined (promoter+E6+E7) transcript targeting CRISPR/Cas9 was much smaller than the gRNA empty vector, demonstrating multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several sites within the mammalian genome. Consistent with our *in vitro* and *in vivo* results, we found that knockdown of HPV E6 or E7 expression by CRISPR/Cas9 has potent anti-tumor effects, as measured by significant suppression of xenograft tumor growth. As the E6 and E7 HPV oncogenes share no sequence homology to human genes, knocking down of these oncogenes may be a highly effective molecular therapy for cervical cancer. These results encourage further investigation of CRISPR/Cas9 is a potential treatment for cervical cancer.

Taken together, CRISPR/Cas9 specific to HPV16 oncogenes including targeting the E6-, E7-transcript effectively knocks down E6 and E7 expression, along with accumulation of p53 and p21 protein, and results in remarkably reduced abilities of proliferation of cervical cancer cells *in vitro*. Consequently, tumorigenesis and growth of transplanted tumors in mouse models were effectively suppressed and survival of tumor burdened mice was prolonged. Our finding may provide important evidences for application of CRISPR/Cas9 targeting HR-HPV key oncogenes, as a new treatment strategy, in cervical and other HPV-associated cancer therapy.

Conflict of interest

The authors declare no conflicts of interest.

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