

## RESEARCH ARTICLE

# A Novel Mutant of Human Papillomavirus Type 18 E6E7 Fusion Gene and its Transforming Activity

Zhi-Xiang Zhou<sup>1\*</sup>, Chen Zhao<sup>1</sup>, Qian-Qian Li<sup>1</sup>, Yi Zeng<sup>1\*</sup>

### Abstract

**Background:** Persistent human papillomavirus (HPV) infection, especially with high-risk types such as HPV16 and HPV18, has been identified as the primary cause of cervical cancer. E6 and E7 are the major onco-proteins of high-risk HPVs, which are consistently expressed in HPV infected tissues but absent in normal tissues and represent ideal therapeutic targets for immunotherapy of cervical cancer. **Materials and Methods:** In this study, the optimized fusion gene HPV18 E6E7 (HPV18 ofE6E7) was constructed according to genetic codon usage for human genes. At the same time, for safety future clinical application, a mutant of HPV18 ofE6E7 fusion gene was generated by site-directed mutagenesis at L52G for the E6 protein and C98G for the E7 protein. **Results:** HPV18-E6E7 mutant (HPV18 ofmE6E7) constructed in this work not only lost the transformation capability for NIH 3T3 cells and tumorigenicity in BALB/c nude mice, but also maintained very good stability and antigenicity. **Conclusion:** These results suggest that the mutant should undergo further study for application as a safe antigen-specific therapeutic vaccine for HPV18-associated tumors.

**Keywords:** Human papillomavirus 18 - E6-E7 - therapeutic vaccine - cervical cancer

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### Introduction

HPV18 is the most prevalent high-risk HPV after type 16 that accounts for the largest number of cervical cancer cases worldwide (Siriaunkgul et al., 2012; Li et al., 2013). Currently, two prophylactic HPV vaccines (Gardasil and Cervarix) both have an excellent safety profile and high clinical efficacy, possibly protecting against up to 75%-80% of all cervical cancers if the vaccine is fully implemented (Nelson et al., 2013). However, these prophylactic vaccines are not predicted to be available in the near future in developing countries due to the expensive price (Farhath et al., 2013). Additionally, there are still no effective countermeasures in clinical trials of cervical cancer in mid and late stage. Therefore the development of therapeutic vaccines targeted to HPV18 of vital importance in cervical cancer treatment.

E6 and E7 are the major onco-proteins of high-risk HPVs (Narisawa-Saito et al., 2007). Since E6 and E7 are consistently expressed in HPV infected tissues but absent in normal tissues, these viral proteins are used as target antigens for developing vaccines and immunotherapeutic strategies against HPV-associated cervical cancer and its precancerous lesions (Cannon et al., 2012). However, HPV E6 and E7 are transforming oncoproteins, one of the dangerous side effects might be the transformation that is not anticipated with vaccine. Appropriate genetic

mutations to HPV18 E6 and E7 are indispensable to ensure the safety of the vaccine.

A point mutational analysis of human papillomavirus type 18 E6 and E7 proteins showed that mutations altering cysteine residues in a -Cys-X-X-Cys- motif decreased or even eliminated the transformation capabilities (Mcintyre et al., 1993). Therefore, to improve the safety and the expression of HPV18 E6E7 based cervical cancer vaccine, a recombinant HPV18 E6E7 fusion gene with the codon-modification for the human gene was constructed. Site-directed mutagenesis at L52G of -Cys-X-X-Cys- motifs in the E6 protein, and at C98G of -Cys-X-X-Cys- motifs in the E7 protein were introduced into HPV18 E6E7 fusion protein. Our work suggested that the optimized and recombinant HPV18 E6E7 mutant not only eliminate their transformation capability into NIH 3T3 cells and tumorigenicity in BALB/c mice, but also retain the very good stability and antigenicity of E6 and E7 proteins.

### Materials and Methods

#### *Plasmid DNA construction*

The optimized HPV 18 E6E7 fusion gene (HPV 18 ofE6E7) with the termination codon of E6 gene removed was optimized on the basis of the codon usage for mammalian cell expression. The HPV18 ofE6E7 was then synthesized and cloned into the pGH vector.

<sup>1</sup>College of Life Science and Bioengineering, Beijing University of Technology, Beijing, China \*For correspondence: [zhouhixiang@bjut.edu.cn](mailto:zhouhixiang@bjut.edu.cn); [zengy@public.bta.net.cn](mailto:zengy@public.bta.net.cn)

To generate pcDNA3.1(+)-HPV18 ofmE6E7 gene, a mutant of the HPV18 ofE6E7 gene at L52G of E6 protein and at C98G of E7 protein with *BamH I/EcoR I* sites was amplified from pGH-HPV 18 ofE6E7 plasmid by Splicing by Overlapping Extension PCR (SOE-PCR) with the primers listed in Table 1. To generate pcDNA3.1(+)-HPV18 ofE6E7 gene, the HPV 18 of the E6E7 gene with *BamH I* and *EcoR I* sites was amplified from pGH-HPV 18 ofE6E7 plasmid by PCR using primers E6Ubam and E7Leco (Table 1). Both PCR products were then digested with *BamH I/EcoR I* enzymes and cloned into the pcDNA3.1(+) vector that was cut by *BamH I/EcoR I*. All plasmid constructions were verified by DNA sequencing.

#### Cell culture

The NIH 3T3 cell line was purchased from American Type Culture Collection (Rockville, MD).

TC-1 cells expressing HPV16 E6 and E7 were kindly provided by Dr. T.C. Wu of Johns Hopkins University. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mmol/L L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were maintained in humidified air containing 5% CO<sub>2</sub> at 37°C.

#### Stable transfections

For stable transfection, pcDNA-HPV18 ofE6E7, pcDNA-HPV18 ofmE6E7 plasmids were transfected into NIH3T3 cells using a Lipofectamine 2000 liposome transfection kit (Invitrogen, USA). The pcDNA3.1 empty vector transfection group and the blank control group (only liposome was added, and no vector DNA transfected) were established. NIH3T3 cells were then selected by antibiotic resistance in cell culture medium containing 500 µg/mL G418. The optimal concentration of G418 was determined by Preliminary experiment. After 3 weeks of culture in 500 µg/mL G418, the remaining cells were then cultured in medium containing 300 µg/mL G418. The fourth week, the remaining cells were isolated with cloning cylinders and transferred into 24-well dishes for the further work..

#### Polymerase chain reaction (PCR)

To identify cell clones that carried the transfected HPV18 ofE6E7 and HPV18 ofmE6E7, the stable NIH3T3 cell line HPV18 ofE6E7 and HPV18 ofmE6E7 were harvested. Cell Genomic DNA was extracted using Cell Genomic DNA Kit (ZOMANBIO, China). PCR was performed using primers: U- 5'-CGGAATTCGCCACCATGGCCAGAT-3' and L- 5'-CGGGATCCTTCACTG CTGGCTGG-3'. Simultaneously, The total RNA was extracted using mirVana miRNA Isolation kit (Carlsbad CA, USA), 1st Strand cDNA was synthesized using Reverse Transcription Kit (M-MLV) (Takara, Dalin, China), PCR was performed using primers:

U- 5'-CGGAATTCGCCACCATGGCCAGAT-3' and L- 5'-CCATA TGTTCACTGCTG GCTGGC-3. All amplified DNA fragments were analyzed using agarose gel electrophoresis.

#### Western Blotting assay

NIH 3T3 cells with stable transfected HPV 18 ofE6E7 or HPV 18 ofmE6E7 genes were digested with lysis buffer which contained 62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 12% glycerol. The cell lysis samples were spun at 13000 r/min for 10 min, the supernatants were collected and boiled for 5 min, then loaded onto the 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein on the gels was electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Sweden), with the nonspecific binding sites blocked by 5% no-fat milk in 0.01 mol/L PBS, pH 7.4, for 2 hours at room temperature. Membrane was then incubated with primary antibodies (goat anti-HPV18 E6, goat anti-HPV18 E7, Santa Cruz) at room temperature for 2 hours. The membranes were washed 3 times with PBS for 10 min each, and then further incubated with Anti-Goat IgG F(c) (Rabbit) Antibody IRDye® 800 Conjugated (1:10,000 dilution) (Rockland, Pennsylvania, USA) for 45 minutes at room temperature. After three rinses (10 min each), the blots were visualized using the Odyssey infrared imaging system (LI-COR Biosciences, California USA).

#### Soft Agar Assay for Colony Formation

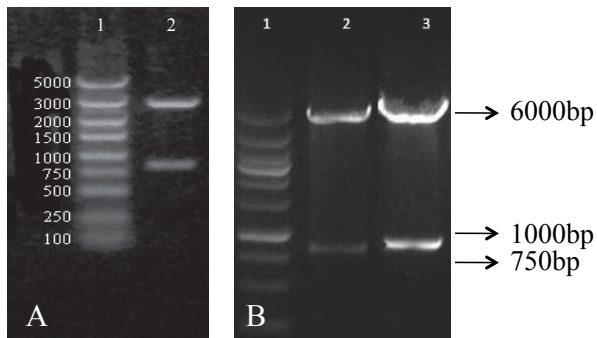
Soft agar assay was determined as described previously (Qiang et al., 2011), The NIH3T3 cell lines (1×10<sup>3</sup>) expressing HPV18 E6E7 modified genes were suspended in 2×DMEM medium containing 20% FBS and mixed 0.7% agarose, subsequently overlaid onto a solidified layer of DMEM medium containing 10% FBS and 0.5% agarose. Cells were cultured for 21 days and photographed. Five representative images from each group were used to quantify foci area from three separate experiments. NIH 3T3 cells and vector-transfected NIH 3T3 cell lines were used as negative control, and the TC-1 cell line was used as the positive control.

#### Tumorigenesis assay and histologic analysis

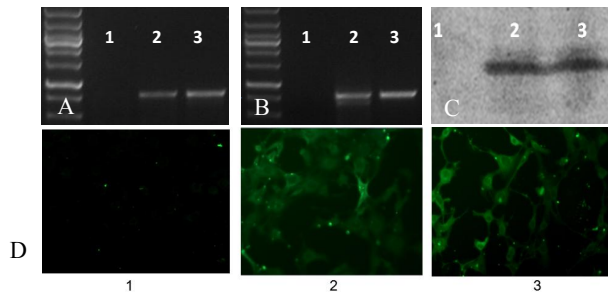
NIH3T3 cell lines stably expressing HPV18 E6E7 modified genes were used in a tumorigenesis assay. Cells were harvested, resuspended at 5×10<sup>7</sup> cells per mL in PBS, and 100 µL of cell suspension was injected subcutaneously into BALB/c Nude mice (5 mice in each group). All animals were monitored for tumor formation once a week for tumor formation. NIH 3T3 cells were used as the control. The animals were sacrificed after 25 days, paraffin sections of xenograft tumors were stained with

**Table 1. Primers used SOE-PCR**

Primers	Sequence(5'-3')
E6Ubam	5'-AAGGATCCGCCACCATGGCCAGATT-3'
E7Leco	5'-CCGGAATTCTTCACTGCTGGCTGG-3'
52U	5'-GGACGGATTCTGGGTGTACAGAGACAG-3'
52L	5'-CCACGAATCCGTCCTTGAAGGCGAACT-3'
98Leco	5'-CCGGAATTCTTCACTGCTGGCTGGCGCACCAGGGTCCCACGAAG-3'



**Figure 1. Construction of The Optimized Recombinant Fusion Gene HPV18 E6E7.** (A) Analysis of the synthetic HPV18 ofE6E7 gene cloned into pGH vector by *Hind* III digestion, 1: DNA Marker DL 6000, 2: pGH-HPV18 ofE6E7; (B) Analysis of the plasmids pcDNA-HPV18 ofE6E7, pcDNA-HPV18-ofmE6E7 by *Bam*H I & *Eco*R I digestion, 1: DNA Marker DL6000, 2: pcDNA-HPV18 ofE6E7, 3: pcDNA-HPV18-ofmE6E7



**Figure 2. Detection of The E6E7 Genes in Stable Transfected NIH 3T3 Cells.** (A) PCR assay; (B) RT-PCR assay; (C) Western blotting; (D) Immunofluorescence imaging, 1: NIH 3T3 cells (control), 2: NIH 3T3-HPV18 ofE6E7, 3: NIH 3T3-HPV18 ofmE6E7; DNA Marker DL6000; anti-HPV18 E6 as primary antibody

hematoxylin and eosin (H&E), representative images from each section were obtained by light microscopic analysis after H&E staining.

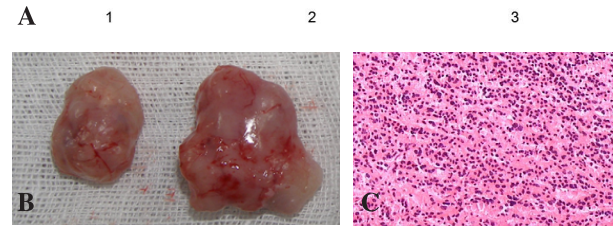
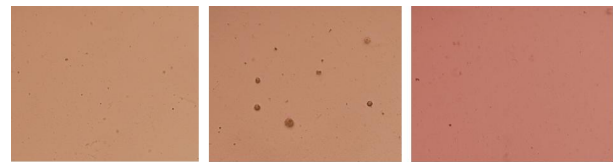
## Results

### Construction of the optimized recombinant fusion gene HPV18 E6E7

In order to have a higher level of expression, usage of this fusion gene was adapted to the codon optimized HPV18 ofE6E7 gene according to the codon bias of *Homo sapiens* genes was synthesized and inserted into vector pGH. DNA sequencing assay suggested that the sequence of the synthetic HPV18 ofE6E7 gene was exactly the same as designed. Furthermore, Restriction endoenzyme analysis of recombinated pGH-plasmid containing optimized HPV18 E6E7 gene produced a target band about 780bp in length (Figure 1A)

### Construction of expression vectors with HPV18 ofE6E7 and HPV18 ofmE6E7

Using pGH-HPV18 ofE6E7 plasmid as template, we spliced together the mutated E6E7 gene HPV18 ofmE6E7 with *Bam*H I & *Eco*R I restriction sites at both ends of



**Figure 3. Identification of transforming activity of E6E7 genes.** (A) Colony formation of NIH 3T3 cell lines transfected with the optimized and recombinant HPV18 E6E7 gene in soft-agar, 1: NIH 3T3 cells (control), 2: NIH 3T3-HPV18 ofE6E7, 3: NIH 3T3-HPV18 ofmE6E7; (B) Tumors from BALB/c nude mice of different groups, tumors in group NIH 3T3-HPV18 ofE6E7 (1) and group TC-1 (2); (C) Micrographs of paraffin section of tumor in BALB/c nude mice in group NIH 3T3-HPV18 ofE6E7

the PCR amplified E6E7 fragment by SOE-PCR, then pcDNA3.1(+) vector was used to build pcDNA-HPV18 ofE6E7, pcDNA-HPV18 ofmE6E7. DNA sequencing verified they had been constructed correctly. Restriction endoenzyme analysis of recombinated pcDNA-plasmid containing optimized and mutated HPV18 E6E7 gene produced a target band about 780bp in length (Figure 1B)

### NIH 3T3 cell lines with stable transfection of HPV18 ofE6E7 and HPV18 ofmE6E7 genes

HPV 18 DNA and RNA were detected in NIH3T3 cell line cells with the stable transfection of the HPV18 ofE6E7 and HPV18 ofmE6E7 genes by PCR using the specific primer set for HPV18 E6/E7 to generate product of 780 bp (Figure 2A, 2B). Furthermore, NIH 3T3 cell lines with the stable transfection of the HPV18 ofE6E7 and HPV18 ofmE6E7 genes were identified by Western blotting and immunofluorescence. As shown in Figure 2C, a distinct band was found at about 36kDa indicating the putative E6E7 protein was detected with the antibody of anti-HPV18 E6. Densities of the Western blotting bands suggested that the expression levels of HPV 18 ofE6E7 and HPV 18 ofmE6E7 were similar in the same amount of cells. The same result was obtained with the antibody against the E7 protein (data not shown). Immunofluorescence imaging suggested that both NIH 3T3-HPV18 ofE6E7 and NIH 3T3-HPV18 ofmE6E7 cells were immunolabeled and exhibited a staining pattern in the cytoplasm (Figure 2D). The results suggested that NIH 3T3 cell lines with the stable transfection of the ofE6E7 and HPV18 ofmE6E7 genes were constructed successfully and both HPV18 ofE6E7 and HPV18 ofmE6E7 genes maintained very good stability and antigenicity.

### Identification of transforming activity

Clone formation test was used to detect oncogenicity in vitro, after growing in soft agarose for 3 weeks, cell colonies were formed in NIH 3T3-HPV18 ofE6E7 cells

and TC-1 cells. However, no colony was observed in NIH 3T3-HPV18 ofmE6E7 cells and NIH 3T3 cells (Figure 3A). Tumorigenicity assays was used to detect oncogenicity in vivo. The results showed that the mice (n=5) injected with either NIH 3T3-HPV18 ofE6E7 or TC-1 cells developed tumors, whereas NIH 3T3-HPV18 ofmE6E7 cells, NIH 3T3 cells did not cause any visible tumors (Figure 3B). The results from in vitro assay and in vivo assay suggested that NIH 3T3-HPV18 ofmE6E7 cell lines had lost its carcinogenicity. In addition, pathological observation of the primary tumors from NIH 3T3-HPV18 ofE6E7 cells revealed a malignant phenotype by hematoxylin and eosin (H&E) staining (Figure 3C). Thus, our findings revealed that engineered HPV18 ofmE6E7 gene losted its transformation activity.

## Discussion

Cellular immune responses have been reported to be effective for tumor cell destruction and control of HPV infection, as HPV-associated cervical lesions are more prevalent in immunosuppressed patients. E6 and E7 are expressed in the HPV positive cancers. E6 proteins induce the rapid degradation of p53 through ubiquitin-dependent proteolysis. The p53 tumor suppressor is not only required for normal cellular proliferation but also functions as a guardian of the human genome (Ham et al., 2014). E7 protein can bind and induce the degradation of the pRb protein and hence the pRb lost it ability as an anti-oncogene (Balsitis et al., 2006; Ham et al., 2014). Since E6 and E7 are consistently expressed in most cervical cancers and their precursor lesions but absent in normal tissues, these viral oncoproteins represent effective targets for the development of specific therapeutic vaccines (Pang et al., 2013). Here, we designed an engineered HPV18 E6 and E7 antigen for use in the therapeutic vaccine construction for the treatment of HPV 18 positive cancer.

Firstly, since the codons used by HPV are different from its host-human gene codons which explains the low expression level of wild-type HPV E6 and E7 genes in human cells (Ohlschlager et al., 2009). This seriously hampers the development of therapeutic vaccines. To improve the expression of HPV18 E6 and E7 proteins, we successfully optimized the wild-type HPV18 E6 and E7 genes according to the codon usage for mammalian cell expression for expecting to increase their expression level in mammalian cells.

Secondly, although therapeutic vaccines that target either HPV E6 or E7 have been previously found to induce good immune responses, the combination of E6 and E7 DNA vaccines may have better anti-tumor potential than either alone (Govan et al., 2005). We fused the HPV18 E6 and E7 gene to include as many epitopes as possible, enabling an improved E6 or E7-specific CTL reaction. Actually, the optimized and fused HPV18 E6E7 gene (HPV 18 ofE6E7) in our work has a high protein expression level and good antigenicity to antibodies against the E6 or E7 protein.

Lastly, since both HPV18 E6 and E7 genes are oncogenes with potential carcinogenicity, genetic mutations at relatively oncogenic sites are necessary

to eliminate their carcinogenicity to ensure their safety when used as vaccines. It reported that a point mutational analysis of human papillomavirus type 18 E6 and E7 proteins showed that mutations of cysteine residues in a -Cys-X-X-Cys- motif decreased or even eliminated the transformation capabilities of E6 and E7 (Mcintyre et al., 1993). Therefore, a mutant was generated by introduction of L52G on the HPV 18 E6 protein and C98G on the HPV 18 E7 protein to generate the HPV18 ofmE6E7 gene. The results showed that the HPV18 ofmE6E7 mutant gene maintained a high protein expression level and good antigenicity to antibodies against the E6 or E7 protein. Furthermore, the mutant of optimized HPV18 E6E7 fusion gene (HPV18 ofmE6E7) constructed in this work lost the transformation activity to NIH 3T3 cells compared to HPV18 ofE6E7 gene.

Taken together, the optimized and recombinated HPV18 E6E7 fusion mutant gene constructed in this work can be taken as a novel candidate antigen of therapeutic vaccines for HPV18-associated tumors.

## Acknowledgements

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