The feasibility of using HPV-RNAi on the inhibition of carcinogenesis in cervical cancer.

Ya-Ching Lu, Ann-Joy Cheng
Graduate Institute of Biomedical Science, Chang Gung University, Taiwan

Abstract

Previous infections with high-risk types (type 16 and 18) of human papillomaviruses (HPVs) are necessary causes of cervical cancer. Giving thought to the importance of cervix and the severe side effects resulting from traditional cancer therapies, this study intends to achieve targeted inhibition of oncogenes in tumor cells based on small interference (siRNA) technique. To this aim, we constructed 7 clones of siRNAs which are sequence-complementally to each target E6 or E7 gene of HPV-16 or HPV-18, as siRNA to 16E6, 16E7, 18E6, and 18E7. The effectiveness of the siRNAs was demonstrated as specific knockdown of E6 or E7 mRNA expression after transfection of these siRNAs into HPV-positive CaSki (HPV-16) or HeLa (HPV-18) cell lines. We found that these HPV-siRNAs significantly reduced cell growth and colony forming ability. Flow cytometry analysis also revealed a significant increase of apoptosis. Besides, there is no effect of these siRNAs in HPV-negative C33A cells. Consistently, an in vivo xenograft study showed that intra-tumor injection of siRNAs reduced tumor growth in Balb/c nude mice. In conclusion, these HPV-siRNAs suppressed cell growth and induced apoptosis in HPV-positive cervical cancer cells and might be a potential modality for further development of adjuvant therapy for cervical cancer.

Keywords: HPV; E6; E7; cervical cancer; siRNA
Introduction

Cervical cancer is the second most common malignancy among women worldwide. It strikes nearly half a million women each year worldwide, claiming a quarter of a million lives (1, 4). Epidemiology evidence has proven that previous infection of high-risk types HPVs is the most important factor for cervical cancer. HPVs DNA can be detected in over 95% of cervical cancer and intraepithelial neoplasia biopsies (15), demonstrating the strong association of this cancer with HPV infection.

Human papillomaviruses (HPVs) are a group of more than 200 viruses (9). They are small, non-enveloped DNA viruses, 55 nm in diameter (10-13). They are called papillomaviruses because certain types may cause warts, or papillomas, which are benign (noncancerous) tumors. HPVs that cause the common warts which grow on hands and feet are different from those that cause growths in the genital area (12). Some types of HPV are associated with certain types of cancer so that they are called high-risk or carcinogenic HPVs (9-14).

High-risk HPVs associated with malignancy have different characteristics compared with those associated with benign lesions. First, the production of E6 and E7 are controlled by the E2 protein in the life cycle of the virus, however in cancer, the E2 gene is disrupted during integration of the viral genome into the host cell. Second, the E6 and E7 proteins of high-risk HPVs have much higher affinity to the tumor suppressor proteins (p53 or Rb) than those of other HPV types (15). The E6 protein binds to p53 tumor suppressor and targets it for acceleration to ubiquitin-mediated degradation (15). The E7 binds to hypophosphorylated Rb protein, resulting the disruption of Rb/E2F complexes and reverse the G1/S suppression check point of cell cycle (15). Therefore, these viral oncogenes are considered to be potential targets for development of gene therapy for HPV-infected malignant diseases.

RNA interference (RNAi) was first described in animals in 1998 and has now been widely used in functional genomics studies. RNA interference (RNAi) is the post-transcriptional silencing of gene expression by double-stranded RNA molecules. RNAi is triggered when a cell encounters a short hairpin (sh) RNA which might be produced from an introduced transgene, a viral intruder or a genetic vector element (23-24). An endogenous enzyme Dicer will then cleave the siRNA into short dsRNAs, forming short interfering RNAs (siRNAs) (24, 29). Subsequently, an RNA-induced silencing complex (RISC) will distinguish between the different strands of the siRNA and degrade the sense strand. The antisense strand is used to target genes for silencing and has several fates depending upon the organism (25-29). The significance of RNAi
is highlighted by the fact that the 2006 Nobel Prize in Physiology or Medicine was awarded to its discoverers, Andrew Fire and Craig Mello. SiRNAs are one group among RNAi system and are powerful in nucleic acid sequence-based gene silencing. SiRNAs are potentially applicable to drug targeting for genes involved in the pathogenesis of human diseases (39).

Although several studies have reported the effects of HPV-siRNA on cervical cancer (A-E), there is either short of simultaneously study on both E6 and E7 oncogenes of high-risk types of HPVs, deficient of HPV-negative controls, or lack of animal study. In this study, we designed 10 clones of HPV-siRNA against E6 or E7 oncogenes of HPV-16 or HPV-18 virus. We compared and investigated their effects on the HPV-infected cervical cancer cells. These siRNA demonstrated high potent and specific tumor growth in vitro and in vivo, while no effect on the HPV-negative cells.

Materials and Methods

Construction of siRNA plasmids

The pTOPO-U6 or pTOPO-U6II vector was used to construct HPV-siRNA plasmid similarly as previously described (Chang et al., 2006, ab), in which a 18~22-nt sense and antisense hairpin oligonucleotide was generated complementary to specific target gene. This hairpin oligonucleotide separated by an 8-nt spacer composed restriction enzyme cutting site which facilitated cloning into vector. Total of 10 HPV-siRNA plasmids were constructed with sequences targeting to HPV16-E6 (3 plasmids, 16E6-249, -164, -161), HPV16-E7 (3 plasmids, 16E7-666, -629, -628), HPV18-E6 (1 plasmids, 18E6-119), and HPV18-E7 (3 plasmids, 18E7-665, -666, -674) genes. A 18E6-Scramble plasmid which has the same nucleotide composition as 18E6-119 but with random sequence was also designed as a control. The selection of the coding sequences for siRNA was empirically determined and analyzed by BLAST research to make sure that they did not have sequence homology with host genes. The specific designed sequences are listed in Table 1.

Cell lines

Cervical cancer cell lines, HeLa (HPV18-positive), CaSki (HPV16-positive) and C33A (HPV-negative) were used in this study. HeLa, Caski and C33A cells were cultured routinely in MEM, RPMI-1640, and DMED medium, respectively, which were all purchased from GIBCO BRL company (Rockville, MD, USA). All mediums were supplemented with 10% FCS and 1% antibiotics, and maintained at 37°C in a humidified incubator containing 5% CO₂.
Plasmid transfection and cell growth assay

For plasmid transfection, cells were seeded at a density of $1 \times 10^6$ in a 100-mm$^2$ dish and cultured for 16 hours. When cells confluence reached 60%, cells were transfected with 6 μg of siRNA plasmid or the vector using Lipofectamine 2000$^{TM}$ reagent (Invitrogen, Carlsbad, CA) in OPTI-MEM medium (Invitrogen, Carlsbad, CA) for 6 hours. After which, the medium was replaced with fresh complete medium and incubated in 37°C, 5% CO$_2$ incubator for 1~3 days. The transfection efficiency is approximately 85% in HeLa and C33A, and 65% in CaSki cells. To determine cell growth, cell numbers and viability were counted daily by staining with 0.25% trypan blue with a hemocytometer.

Colony formation assay

A total of 1000 cells transfected with either HPV-siRNA or vector plasmids were seeded on 6-well plates and allowed to grow without moving for 7~14 days in complete culture medium containing 30% FBS. The number of cell colonies was counted after staining with 5% crystal violet (Sigma) for 15 min and washed with ddH$_2$O. For quantification, Sorenson’s solution (0.03 mM tri-sodium citrate, 0.0195 N HCl, 45% ethanol) was added to each well to elute dye from cells. After incubating for 30 min, the solution was transferred to a new well of a 96-well plate and read with O.D. 570 under room temperature.

Flow cytometry

Cells treated with HPV-siRNA or vector plasmids were harvested and washed with phosphate buffer saline (PBS). The pellets were fixed with ethyl alcohol and PBS at -20°C (EtOH/PBS=3/1). The pellets were then suspended and incubated with 0.5% Triton X-100 and 0.05% RNase in PBS at 37°C for 1 h. After that the pellets were incubated with 40 mg/ml propidium iodide (PI) for 30 min. Samples were analyzed by flow cytometry (Becton Dickinson, San Jose, CA). The distribution of cell cycle phase was determined using Cell Quest Pro and ModiFit softwares.

RNA extraction and RT-PCR analysis

The mRNA expression of E6 and E7 was determined by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted by using TRIzol reagent (GIBCO BRL) following the manufacturer’s instructions. The reverse transcription reaction was performed by incubation of a reaction mixture containing 30 ng RNA, 100 pmole of poly-T oligonucleotide, 4 units of reverse transcriptase (AMV, HT Biotech Ltd, UK), 10 units of RNase inhibitor (CalBiochem, CA, USA)
and 25 mM dNTP in a total of 30 μl reaction buffer at 37°C for 1 hour.

For each PCR run, a master mix was prepared with 1 x TaqMan buffer, 5 mM MgCl₂, 200 μM dNTP, 300 nM of each primer, 1 unit of AmpliTaq Gold DNA Polymerase, and 3 μl of cDNA solution in a total of 30 μl. PCR reactions were carried out with specific primers (Table 2) for a total of 30 cycles at 94°C for 40 sec, 56°C for 40 sec and 72°C for 1 min, using a Tpersonal thermocycler (Biometra). The PCR products were analysed by 2% agarose gel electrophoresis, stained with ethidium bromide, photographed by illuminating with 254 nm UV and the density of each band was determined.

Mice and the xenograft tumor

BALB/c AnN-Foxn1nu/CrlNarl nude mouse was used as our animal model. A 4~6 weeks old nude mouse was subcutaneously injected with 5 x 10⁶ HeLa cells at each side of the upper hind limb region to generate solid tumors at both sides. Three days after tumor cell xenografting, one side of tumor was injected with HPV-siRNA plasmid in 50 μl PBS, following by a booster of 25 μg of the plasmid in 25 μl PBS twice a week for a total of 30 days. The other side of tumor was injected with vector plasmid following the same schedule as the experimental group. The tumor dimensions were measured every 2~3 day with calipers, and calculated as length x width x height.

Statistical Analysis

The two way ANOVA test was used to examine the association of siRNA and control plasmids. All P values were two-sided, and the significance level was set at a P of <0.05.

Results

Inhibition of E6 and E7 expressions by HPV-siRNA

The effects of E6 or E7 gene expression by HPV-siRNAs delivery were examined. CaSki (HPV16 positive) or HeLa (HPV18 positive) cells were used, respectively for determining the specific HPV type. After transfection of the specific siRNAs, cells were harvested at a time course of 6 hr, 12 hr, 18 hr, and 24 hr and subjected to RT-PCR analysis. For quantification, the density of each gene was calibrated with the control vector after normalized with the internal actin level. As shown in the Figure 1A, siRNA of 16E6-164 and 16E6-161 showed effective inhibition for HPV16-E6 gene, with 31% and 32% of expressions at 24 hr compared to the controls. Similarly, 16E7-629 and 16E7-628 showed effective inhibition for HPV16-E7 gene, with 31% and 22% at 24 hr compared to the controls (Figure 1B).
18E6-119 showed effective inhibition for HPV18-E6, with 37% at 24 hr compared to the control (Figure 1C), whereas 18E7-666 and 18E7-674 effective for HPV18-E7 gene, with 37% and 9% at 24 hr compared to the controls (Figure 1D). All of these siRNAs rendered more than 65% suppressions for their specific genes after 24 hr of the treatment. We therefore obtained more than one clone of siRNA for each specific HPV oncogene inhibition.

**High potent and specific suppression of cell growth by HPV-siRNAs**

The potential alteration of cellular phenotype after E6 or E7 knockdown by these siRNAs was next examined. Figure 2 showed the effect of cell growth. As shown in the Figure 2A, consistent with the suppression effect of gene expression (Figure 1A), two of the specific 16E6-siRNA clones (16E6-164, 16E6-161) exhibited significant inhibition on the cell growth, with approximately 70% reduction at day 2 and 85% at day 3, in contrast to minimal effect by the vector control (U6II). Similarly, consistent with the gene expression effect (Figure 1B), two of the specific 16E7-siRNA clones (17E7-629, 16E7-628) exhibited substantial inhibition on the cell growth, with approximately 75% reduction at day 2 and 90% at day 3. For 18E6-siRNA, consistent with the gene expression effect (Figure 1C), clone of 18E6-119 showed substantial inhibition of the cell growth, with 74% and >10% reductions at day 2 and day3 (Figure 2C). For 18E7-siRNA (Figure 2D), two clones of the specific siRNA (18E7-666 and 18E7-674) showed great effect on growth suppression (> 80% reduction at day 2 and > 90% at day 3), whereas 18E7-665 showed partial inhibition on cell growth (30% reduction at day 2 and 47% at day3). These results were also consistent with the gene expression effect (Figure 1D).

To examine whether these siRNAs with high growth suppression ability possess specificity on HPV infected cells, the growth effect by these siRNAs on a non-infected cervical cancer cell line (C33A) were examined. As shown in the Figure 2F for the effect of HPV-16-siRNA, all the 4 clones (16E6-164, 16E6-161, 16E7-629, 16E7-628) showed minimum effect (< 10% inhibition) on cell growth. Similarly, all the 3 clones of HPV-18-siRNA (18E6-119, 18E7-666, 18E7-674) exhibited insignificant effect (< 10% inhibition) on the cell growth (Figure 2F). These results suggested that these siRNAs has high cellular specificity, which only effected on HPV-infected cells.

**High potent and specific suppression of colony forming by HPV-siRNAs**

To validate the cellular results, those siRNAs with high growth suppression ability were selected for further examination on the effect on colony formation after transfection of these specific siRNA plasmids. As shown in the Figure 3A, all the 4
clones of HPV-16-siRNAs (16E6-164, 16E6-161, 16E7-629, 16E7-628) showed significant inhibition (>80) on cell colony formation, compared to minimum level of alteration (<10%) by the vector control plasmid (V-U6II). Effects by the HPV-18-siRNA were shown in the Figure 3B. All 3 clones of the specific siRNAs (18E6-119, 18E7-666, 18E7-674) exhibited substantial effects (>90% inhibition) on colony formation, in contrast to non-effect by the controls (vector or scramble plasmids). The cellular specificity of these siRNA was also confirmed. As shown in the Figure 3C, there is no effect (<5%) on the colony forming ability after transfection of these siRNA on non-HPV-infected cells (C33A).

**Induction of apoptosis by HPV-siRNAs**

Since the target of viral oncogenes E6 and E7 are p53 and pRb which participate on cell cycle and apoptotic regulatory pathways, we examined whether these HPV-siRNA have effect on cellular homeostasis. For this, cells were subjected to flow cytometry analysis after transfection by the specific HPV-siRNAs. As shown in the Figure 4A, the vector transfected control cells has a distinct peak at G1 phase and a smaller peak at G2/M phase, which showed great difference with the other cells transfected by specific HPV-siRNA. It is apparent that both G1 and G2/M phases diminished, whereas apoptotic fraction (M1 phase) increased in the cells transfected by any clone of the 4 HPV-16-siRNAs (16E6-164, 16E6-161, 16E7-629, 16E7-628). At day 3, a >40% of apoptosis was induced by any of the clones. Similarly, substantial increased of the apoptotic fraction cells by any three HPV-18-siRNAs (18E6-119, 18E7-666, 18E7-674) (Figure 4B). At day 3, a >70% of apoptotic cells were found in these cells, contrast to no effect by scramble plasmid (<2%). As for the specificity of these siRNAs, there is no effect (<2% of variation compared to the control) of these HPV-siRNAs on the apoptotic status or cell cycle phases in non-HPV-infected cells (Figure 4C), demonstrating high specificity of these HPV-siRNAs.

**Suppression of tumor growth in vivo by HPV-siRNAs**

To investigate the effects of HPV-siRNA treatment on tumor growth in vivo, we established a xenograft HeLa tumor in BALB/C nude mice. Cancer cells were subcutaneously injected at the both sites of upper portions of the rear limbs. After three days, HPV18-siRNA (18E6-119, 18E7-674) and vector control plasmid were administered to each tumor site of the same mice to compare the effect on tumor formation. Tumor sizes were continuously monitored every 2 to 3 days for a total of 30 days. Figure 5A shows the tumor volumes between groups of 18E6-119 siRNA and vector treated mice. Although each tumor size varied from one another, the tendency showed consistent that siRNA-injected tumors were smaller than control tumors in all
mice. Overall, tumor were shown significant inhibited by the specific HPV-siRNA, with average decrease of the tumor growth to 49% at day 17 (P < 0.001) and 53% at day 30 (P < 0.001). Figure 5B shows the tumor volumes between groups of 18E7-674 siRNA and vector treated mice. Similarly, tumor formation were significant inhibited by the specific HPV-siRNA, with average decrease of the tumor growth to 48% at day 17 (P = 0.0018) and 55% at day 30 (P < 0.001). These results demonstrate the effective suppression of tumor growth in vivo by HPV-siRNAs.

Discussion

Cervical cancer is the second most common cancer in women worldwide nad the majority of the disease is caused by high-risk types of human papillomavirus (HPV-16 and -18), which possess the E6 and E7 oncogenes. The concurrent expression of E6 and E7 protein is a prerequisite for cancer development and maintaining malignant phenotypes. Although in recent years, the pap smear screening test is getting popular to reduce the incidence rate of cervical cancer, the effective therapy for cervical cancer is still urgent for developing countries. Since the E6 and E7 oncogenes of HPVs share no sequence homology to human genes, silencing of these oncogenes theoretically could be high specific and low cytotoxicity in molecular therapies of human cervical cancer (37-39).

In the present study, we established several effective siRNA plasmids against HPV-16 and HPV-18, and all of them exhibited effective inhibition the expressions of E6 or E7 genes on the HPV-infected cervical cancer cells (Figure 1). Transfection of these siRNAs showed comparable and significant suppression of both short term cell viability (Figures 2A-2B) and long term survival (Figures 3A-3B) whereas no effect on the HPV-negative cells (Figures 2C and 3C), demonstrating the effectiveness and high specificity of these siRNAs.

In study the potential effect of the siRNAs on cell cycle status, we found that not only inhibited cell growth, these HPV-siRNAs further rendered an active and specific effect on the induction of cellular apoptosis in the HPV-infected cells (Figure 4). These results are consistent with recently finding in the reduction of E6 or E7 expression (25, 26). The reason of inducing cell apoptosis is not clear so far. Presumably, the accumulation of p53 and pRB and trigger cells to senescence or apoptosis is highly suspected (42).

In xenograft tumor study, we demonstrate that HPV-siRNA effectively suppressed tumor growth (Figure 5A and 5B). Although the siRNA against either E6 or E7 oncogenes contribute to significant results, the E6-siRNA seems render a more effective result. Since E7 and E6 is biocistronic transcribed, the suppression of E6
gene may lead to co-suppression of E7, thus contributing to greater effect on tumor reduction. We further demonstrated that HPV-siRNA reduced cancer metastatic ability (Figure 5C).

All of these results above revealed the specificity and efficacy of siRNAs since the non-HPV infected cell (C33A) and control (vector or scramble plasmid transfected cells) remained unchanged. This suggests that to be a potential therapeutic agent, siRNAs can perform excellent suppression to targeted cancer cells without effects on normal cells.

This study provides preliminary validation of our constructed HPV-siRNA plasmids. So far these HPV-siRNA plasmids have shown significant inhibition in vitro and in vivo. To be a potential therapeutic agent, however, there are still a lot of works to accomplish in the future.

There are a number of major concerns and possible impediments for RNAi-based technology to the widespread application. Two main obstacles of RNAi-based application are delivery system and long-term expression. Many systems such as lipid carriers or viral vectors are still in trials to achieve targeted delivery (30, 35). Recent developments in delivery have also facilitated cell-specific targeting of RNAi reagents through the use of ligands such as receptor-targeting aptamers or ligand-coated nanoparticles (32). For long-term expression of siRNA, viral vector system or some chemical modification are considered to be better approaches (34, 36). All approaches are intent to achieve the most specific and the least cytotoxic effects.

To sum up, further studies must be carried out to reach effective delivery and to better understand the unwanted side effects of RNAi-based therapies. Given the immense interest in RNAi as a potential therapeutic, the following years are expectable to come up with more detailed investigation and increasing range of applications for RNAi-based treatments.

Reference
oncogenes in cervical cancer cells results in increased sensitivity to cisplatin. Molecular Pharmacology Fast Forward 2005;68(5):1311-1319


25. Cheong Weon Cho, Haryoung Poo, Young Sik Cho1, Min Chul Cho, Kyung Ae Lee1, Shin Je Lee, Sue Nie Park, In Ki Kim, Yong Keun Jung, Yong Kyung Choe, Young Il Yeom, In Seong Choe, and Do Young Yoon. HPV E6 antisense induces apoptosis in CaSki cells via suppression of E6 splicing. Experimental and Molecular Medicine 2002;34(2):159-166


38. Yan Kong, Lingfei Ruan, Lili Ma, Youhong Cui, Ji Ming Wang, Yingying Le. RNA interference as a novel and powerful tool in immunopharmacological research. International Immunopharmacology 2007;7:417-426
**Table 1.** Specific sequence design of HPV-siRNAs. The relative position of HPV16 E6 gene in the open reading frame is 83-559, and for HPV16 E7 is 562-858. As to HPV18 E6 is 105-581, and HPV18 E7 is 590-907. The siRNAs target sequence positions are shown in the bracket. All siRNAs sequences have been confirmed by BLAST to ensure no homologous sequence to human genome.

<table>
<thead>
<tr>
<th>Targeted HPV gene</th>
<th>siRNA sequence</th>
</tr>
</thead>
</table>
| 16E6-249 (249-267) | F : 5’TTATGCATAGTATAGAGgaagcttgCTCTATATACTATGCATAA 3’  
R : 5’GGATTTATGCATAGTATAGAGcaagcttcCTCTATATACTATGCATAA 3’ |
| 16E6-164 (160-180) | F : 5’-CTAGGCAAAACACTACATGAggaagcttgATTCATATAGTTGTTTGC-3’  
R : 5’-AAAAAGCACAACACTACATGAgcaagcttcATTCATATAGTTGTTTGC-3’ |
| 16E6-161 (157-174) | F : 5’-CTAGCTTGCAAACACTACATGAggaagcttgATGCATAAATCGCAG-3’  
R : 5’-AAAACTGCAAACACTACATGAgcaagcttcATGCATAAATCGCAG-3’ |
| 16E7-666 (666-682) | F : 5’GAAATAGATGGTCCAGGgaagcttgGCTGGACCATCTATTTC 3’  
R : 5’GGATGAAATAGATGGTCCAGGcaagcttcGCTGGACCATCTATTTC 3’ |
| 16E7-629 (625-641) | F : 5’-CTAGCTCTACTGTGTTATGAGCAgaagcttgTGCTCATAACAGTAGAG-3’  
R : 5’-AAAACTCTACTGTGTTATGAGCAcaagcttcTGCTCATAACAGTAGAG-3’ |
| 16E7-628 (624-645) | F : 5’-CTAGTCTCTACTGTTATGAGCAATTAgaagcttgTAATTGCTCATAACAGTAGAGA-3’  
R : 5’-AAAACTCTACTGTTATGAGCAATTAcaagcttcTAATTGCTCATAACAGTAGAGA-3’ |
| 18E6-119 (119-125) | F : 5’CCAACACGCGCCACCTAgaagcttgTAGGGTCGCCGTGGTG3’  
R : 5’GGATCCAACACGCGCCACCTAcaagcttgTAGGGTCGCCGTGGTG3’ |
| 18E6-Scramble | F : 5’CACCACATACCCGCACGGgaagcttgCCGTGCGGTATGTGGTG3’  
R : 5’GGATCCAACCTACCCGCACGGcaagcttgCCGTGCGGTATGTGGTG3’ |
| 18E7-665 (661-682) | F : 5’-CTAGCTTCTTATGTGTCAGGCAATTAggaagcttgTAATTGCTGACGCAAGAGG-3’  
R : 5’-AAAAACCTCTTATGTGTCAGGCAATTAgcaagcttcTAATTGCTGACGCAAGAGG-3’ |
| 18E7-666 (662-678) | F : 5’-CTAGTTCTTATGTGTCAGGCAATTAggaagcttgTGCTGACGCAAGAGG-3’  
R : 5’-AAAAATCTCTTATGTGTCAGGCAATTAgcaagcttcTGCTGACGCAAGAGG-3’ |
| 18E7-674 (670-686) | F : 5’-CTAGGCAACGCAAATTAAGCGAgaagcttgTCGCTTAAATGCTGCTG3’  
R : 5’-AAAAACACGCAAATTAAGCGAgaagcttcTCGCTTAAATGCTGCTG3’ |
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences</th>
</tr>
</thead>
</table>
| HPV16E6     | F : 5’- AATGGTTTCAAGGACCCCTACGG -3’  
R : 5’- TCAGGACACAGTGCTTTTG -3’ |
| HPV16E7     | F : 5’- TTTGCAACCAGAGACAACAGTGA -3’  
R : 5’- GCCCATTAAACAGGTCTTCCA -3’ |
| HPV18E6     | F : 5’- GCGACCCTACAAGCTACCTG -3’  
R : 5’- GTTGGAGTCGTTCTTCTGCTGT -3’ |
| HPV18E7     | F : 5’- GCATGGACCTAAGGCAACAT -3’  
R : 5’- TGTTGCTTACTGCTGGGATG -3’ |
| Actin       | F : 5’- GGGGGCGCCCCCAGGCACCA -3’  
R : 5’- CTCCTTAATGTACGCACGACAGATTTTC -3’ |
**Figure 1.** The targeted mRNA expression was down-regulated by transfection of each HPV-siRNAs. The silencing ability of each siRNAs was verified by RT-PCR after transfection of 6-24 hrs. A and B are Caski cells transfected by HPV16-E6 and HPV16-E7 siRNAs. The mRNA expression decrease is obvious in si-16E6-164, si-16E6-158, si-16E7-629, and si-16E7-628. C and D are HeLa cells transfected by HPV18-E6 and HPV18-E7 siRNAs. A better inhibition is shown in si-18E6-119, si-18E7-665, and si-18E7-671. U6/U6II vector and 18E6-sc (a scrambled sequence of 18E6-163) are control siRNAs. Note that E7 mRNA expression is confirmed in A and C because E6 and E7 are bicistronic.
Figure 2. Suppression effects of each HPV-siRNAs on targeted cell growth. The X axis represents days and the Y axis represents cell number. A total number of 1x10⁶ cells (Caski/HeLa/C33A) were seeded into a 100-mm² dish. Cell numbers were counted at the third day. A-B show the specific suppression of growth on Caski cells by HPV16-E6 and -E7 siRNAs. C is the control of C33A cells (HPV negative cells) in which siRNAs-transfected cells showed no difference from vector-transfected cells. These results suggested that HPV16 siRNAs can perform specific suppression on targeted cell and showed few biotoxicity on normal cell (C33A). Same results were found in HeLa cells by HPV18 siRNAs, as refer to D-F.
Figure 3. Effects of each HPV-siRNAs on cells colony formation. (Caski/HeLa/C33A) A total of 1000 cells transfected with each HPV-siRNAs plasmids were seeded on 6-well plates and allowed to grow for 7-14 days. Cells treated with HPV-siRNAs formed very few colonies comparing to control and vector cells. The quantitative data were show below (compared to original cells as 100%). These results suggested that these HPV-siRNAs can successfully inhibit target cells on colony forming ability, affecting the long term survival ability of cells. Meanwhile the inhibition ability was not observed in HPV negative cells (C33A), showing that such inhibition is specific and low biotoxic.
Figure 4. Analysis of HPV-siRNAs effects on cell cycle for 3 days by flow cytometry. (A) Caski cells were transfected with various clones of HPV16-siRNAs. (B) HeLa cells were transfected with various clones of HPV18-siRNAs. (C) C33 cells were treated with various clone of HPV16- or HPV18-siRNAs. M1 indicates apoptotic fraction cells.
Figure 5. A nude mouse was subcutaneously injected $5 \times 10^6$ HeLa cells to generate solid tumors at two sites, and then injected specific HPV18-siRNAs plasmids and vector plasmid into tumor respectively. The X axis is the observation time up to 30 days and the Y axis is the tumor size in mm$^3$. Red lines represent tumor injected with vector plasmid and blue lines represent tumor injected with HPV18-siRNA plasmid. A is the result of mice with injection of 18E6-119 siRNA and B is the result of mice with injection of 18E7-670. As in the figure, even though each mouse varied from one another, siRNAs injected tumors were distinctly smaller than vector injected tumors (n=7).