

## Original Article



# Viral Etiology Relationship between Human Papillomavirus and Human Breast Cancer and Target of Gene Therapy

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## Abstract

**Objective** To explore the viral etiology of human breast cancer to determine whether there are novel molecular targets for gene therapy of breast cancer and provide evidence for the research of gene therapy and vaccine development for breast cancer.

**Methods** PCR was used to screen HPV16 and HPV18 oncogenes *E6* and *E7* in the SKBR3 cell line and in 76 paraffin embedded breast cancer tissue samples. RNA interference was used to knock down the expression of HPV18 *E6* and *E7* in SKBR3 cells, then the changes in the expression of cell-cycle related proteins, cell viability, colony formation, metastasis, and cell cycle progression were determined.

**Results** HPV18 oncogenes *E6* and *E7* were amplified and sequenced from the SKBR3 cells. Of the patient samples, 6.58% and 23.68% were tested to be positive for HPV18 *E6* and HPV18 *E7*. In the cell culture models, the knockdown of HPV18 *E6* and *E7* inhibited the proliferation, metastasis, and cell cycle progression of SKBR3 cell. The knockdown also clearly affected the expression levels of cell cycle related proteins.

**Conclusion** HPV was a contributor to virus caused human breast cancer, suggesting that the oncogenes in HPV were potential targets for gene therapy of breast cancer.

**Key words:** Human papillomavirus; Human breast cancer; RNA interference; Gene therapy

*Biomed Environ Sci*, 2016; 29(5): 331-339

doi: 10.3967/bes2016.043

ISSN: 0895-3988

[www.besjournal.com](http://www.besjournal.com) (full text)

CN: 11-2816/Q

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## INTRODUCTION

In 2014, breast cancer was estimated to be the second most common cause of death (15%) and the leading cause of new cancer diagnoses (29%) among women<sup>[1]</sup>. There are many theories about the causes of breast cancer<sup>[2-3]</sup>, including human papillomavirus (HPV)<sup>[4-5]</sup>. The relationship between HPV and many kinds of cancers,

such as cervical carcinoma and head-neck carcinoma, is well established<sup>[6-8]</sup>. The hypothesis that HPVs might contribute to human breast cancer is based on the immortalization of primary mammary epithelial cells by high-risk HPV. However, there are conflicting reports regarding the prevalence of HPV DNA in breast cancer in different countries<sup>[9-14]</sup>. Similarly, there are conflicting reports regarding the presence of an HPV18 oncogene in the SKBR3 breast cancer

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cell line<sup>[5,15]</sup>.

Gene therapy is a novel research field and over 63% of gene therapy clinical trials are conducted for cancer treatment. RNA interference (RNAi) is a mechanism of post-transcriptional gene silencing in plants and animals<sup>[16-17]</sup>. The use of short hairpin RNA (shRNA) to inhibit the expression of defined target genes has cleared therapeutic potential. In the context of breast cancer, identifying a putative target gene is the foundation of efficient gene therapy.

In this study, we aimed to determine whether the *E6* and *E7* oncogenes from HPV16 and HPV18 contribute to the viral etiology of breast cancer in the SKBR3 cell line and primary breast cancer tissue samples. We then explored whether they are novel viral targets for gene therapy for breast cancer, providing evidence for the future research in gene therapy and vaccine development.

MATERIALS AND METHODS

Human Specimens and Cell Culture

Seventy-six paraffin-embedded breast cancer samples were obtained from Peking University People's Hospital. Of the patients included, 31.58% (24/76) were aged 25-45 years, 50.00% (38/76) were aged 46-65 years, 17.11% (13/76) were aged 66-85 years, and 1.32% (1/76) were aged ≥86 years. In terms of the sex distribution, 1.32% (1/76) were males and 98.68% (75/76) were females. Invasive ductal carcinoma was detected in 98.68% (75/76) of the samples, while invasive lobular carcinoma was detected in 1 (1/76) sample. Of the 75 invasive ductal carcinoma samples, 48.00% (36/75) were in stage I, 34.67% (26/75) were in stage II, and 17.33% (13/75) were in stage III. Written consent statements were obtained from all the patients before the study. This study was carried out in accordance with the Code of Ethics of the World Medical Association. The privacy rights of the human subjects were protected. Human breast cancer cells (SKBR3) and human embryonic kidney (HEK) 293 cell lines were obtained from the Cell Resource Center, Peking Union Medical College. The cell line was confirmed free from mycoplasma contamination by PCR and culture assays. The species origin of the cells was confirmed by PCR. The identity of the cell line was authenticated with STR profiling (FBI, CODIS). HEK293 cells were maintained in Dulbecco's Modified Eagle Media (HyClone, Utah, USA)

supplemented with 10% FBS (Gibco, Massachusetts, USA) at 37 °C and 5% CO<sub>2</sub>. The SKBR3 cells were maintained in 1640 (HyClone, Utah, USA) supplemented with 10% FBS (Gibco, Massachusetts, USA) at 37 °C and 5% CO<sub>2</sub>.

Detection of HPV Oncogenes in SKBR3 Cells and Breast Cancer Tissue Samples

**Detection of HPV Oncogenes in SKBR3 Cells**  
Specific primers (Table 1) were designed based on the mRNA sequence of HPV18 *E6*, HPV18 *E7*. The total RNA was extracted from the SKBR3 cells by using Trizol (Invitrogen, Massachusetts, USA). Briefly, each sample was dissolved in 1 mL of Trizol and 0.2 mL of chloroform was added. Then the tube was shaken vigorously for 15 s and centrifuged at 12,000 × g for 15 min. The RNA was extracted by washing the pellet with 1 mL of ice-cold isopropanol followed by cold 75% diethylpyrocarbonate (DEPC) treated ethanol. The ethanol was removed and the tube was briefly air dried. The RNA pellet was resuspended in nuclease-free water. The complementary DNA (cDNA) was synthesized from the total RNA by using the Goscript reverse transcription system (Promega, Wisconsin, USA). The total DNA was extracted by using the QIAamp DNA Blood Mini Kit (Qiagen, Dusseldorf, Germany) from 1×10<sup>6</sup> cells.

Table 1. Primer Sequences

Target	Primer Sequences (5'-3')
HPV16E6	F: GCAAGCAACAGTTACTGCGA R: CAACAAGACATACATCGACC
HPV16E7	F: TGCATGGAGATACACCTCATTTG R: CCCATTAACAGGTCCTCCAAAGT
HPV18E6-322(66-387)	F: CACTTCACTGCAAGACATAGA R: GTTGTGAAATCGTCGTTTTTCA
HPV18E6-155(306-460)	F: AATAAGGTGCCTGCGGTGCC R: TGCCTGCTGGAGTCGTTCC
HPV18E7-125(86-210)	F: AGCAATTAAGCGACTCAGA R: GCTTCACACTTACAACAT
HPV18E7-100(80-179)	F: CGGTTGACCTTCTATGTCACGA R: TGTGTGACGTTGTGGTTCCG
RB-qPCR	F: ATTTGGCGTGCCTCTTGAGG R: GGGTGCTCAGACAGAAGGCG
Tp53-Mutant	F: GGTGACACGCTTCCCTGGATTGGC R: AGGCTGTCAAGTGGGGAACAAGAAGTGG
MDM2	F: TGGAGGGTAGACCTGTGGGC R: GGATTCGATGGCGTCCCTGT
CCNA1	F: TGGGGTCCAGGCAGGTTTTG F: CTGATTCTGTGATCCCCCTGGC
CCND1	F: CAGTAGCAGCGAGCAGCAGAGT R: GCGGCCAGGTTCCAATTGAG
BCL2	F: CAGGATAACGGAGGCTGGGATG R: AGGGCCAACTGAGCAGAGTC
β-Actin-qPCR	F: TGACCCAGATCATGTTTGAGA R: TACGCCAGAGGCGTACAGG

The cDNA from the SKBR3 cells was used to amplify HPV16E6, HPV16E7, HPV18E6-155 (306-460), and HPV18E7-125 (86-210) by using PrimeSTAR HS (TaKaRa, Dalian, China). The PCR reaction conditions were 30 cycles of 98 °C for 10 s, 59 °C for 15 s, and 72 °C for 10 s. Based on the results, the HPV18E7-125 (86-210) primer was optimized to HPV18E7-100 (80-179). The total DNA from the SKBR3 cells was amplified by using the HPV18E7-100 (80-179) primer. The PCR conditions for this reaction were 30 cycles at 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 10 s. To add the poly-A tail after the PCR reaction, 1 µL of ExTaq (TaKaRa, Dalian, China) was added to the PCR reaction and the mixture was maintained 72 °C for 30 min. Following the amplification, the PCR products were cloned into a T-vector and sequenced.

**Detection of HPV Oncogenes in Paraffin-embedded Breast Cancer Samples** DNA was extracted from 76 paraffin-embedded breast cancer samples. Briefly, the samples were deparaffinized by placing them in 1 mL of TES (10 mmol/L Tris-HCl, 1 mmol/L EDTA, and 0.5% SDS) in a 60 °C water bath for 30 min and then centrifuged at 8000 × g for 10 min. The precipitates were dissolved in 500 µL of TET (100 mmol/L Tris-HCl, 1 mmol/L EDTA, 1% TrisX-100, and 500 µg/mL Proteinase K) in a 60 °C water bath for 1 h. The mixture was purified with hydroxybenzene-chloroform-Isoamyl alcohol, and then NaAc (3 mol/L) and alcohol were added to precipitate the DNA. The DNA pellet was resuspended in nuclease-free water.

Dual-PCR was used to determine whether HPV oncogenes are detectable in the breast cancer samples by using primers for HPV16E6, HPV16E7, HPV18E6-322 (66-387), HPV18E7-100 (80-179) and ExTaq (TaKaRa, Dalian, China). The reaction conditions were 94 °C for 2 min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The second round of PCR was conducted in the same manner by using 2.5 µL of the PCR products from the first round PCR reaction as the template. The PCR products were analyzed by using 2% agarose gel electrophoresis and photographed.

### **Assessing Effects of HPV18 E6 or E7 Targeted RNA Interference in SKBR3 Cells**

#### **Constructing shRNA Plasmids and Transfection**

The target sequences were selected from the HPV18 E6 and HPV18 E7 mRNAs. The target sequence of shE6 (targeting HPV18 E6 nucleotides 301-319) was 5'-TTATTAATAAGGTGCCTGC-3'. The sequence of

shE7 (targeting HPV18 E7 nucleotides 82-98) was 5'-CACGAGCAATTAAGCGA-3'. The negative control shRNA sequence (shNC) was 5'-AATATGTAAAGATACTCGCT-3', which was derived from a randomly reshuffled sequence of HPV18 E7. All the shRNA sequences were confirmed by BLAST to share no homology with any part of the human genome.

Both forward and reverse oligos of shRNA was synthesized, including *Bgl*II site (sense of target sequence), Hairpin (antisense of target sequence), and *Hind*III site. The forward and reverse oligos were annealed and cloned into the vector pSUPER RNAi System (OligoEngine, Washington, USA) between the unique *Bgl*II and *Hind*III enzyme sites according to the manufacturer's instructions.

To obtain the maximal RNAi effects, the shRNA plasmid transfections were performed following the manufacturer's instruction. Six microliters of the FuGENE® (Promega, Wisconsin, USA) reagent was mixed with 100 µL of OPTI-MEM (Gibco, Massachusetts, USA) and incubated for 5 min at room temperature. Two micrograms of plasmid was then added and the entire mixture was incubated for 15 min at room temperature. The plasmid mixture was then added to the SKBR3 cells (60% confluence). A *GFP* tag was included in the plasmid to determine the transfection efficiency.

**Real-time Quantitative PCR Analysis** Total RNA was extracted from the SKBR3 cells 72 h after transfection by using Trizol as described above. The reverse transcription reaction was performed by using the GoScript Reverse Transcription System (Promega, Wisconsin, USA). Real-time quantitative PCR was performed by using the GoTaq qPCR Master Mix (Promega, Wisconsin, USA). The level of mRNA expression was assessed by using the threshold cycle (CT) values. The CT values of the expression levels of genes associated with cell proliferation and apoptosis, such as *retinoblastoma (RB)*, *tumor protein p53 mutant (Tp53-mutant)*, *proto-oncogene E3 ubiquitin protein ligase (MDM2)*, *cyclin A1 (CCNA1)*, *cyclin D1 (CCND1)*, and *B-cell CLL/lymphoma 2 (BCL2)*, were normalized with the expression level of  $\beta$ -actin. Previous studies indicated that the *Tp53* mutation was present in the SKBR3 cells. The reaction conditions were 95 °C 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min; then a dissociation stage was added by using the same temperature and time parameters. The level of mRNA expression was quantified in triplicate for every sample. The primer sequences are shown in Table 1.

### **Analysis of in Vitro Cell Viability, Metastasis, and Growth Cycle**

Cell viability was assessed with a colorimetric assay by using Celltiter-Glo Luminescent cell viability assay kit (Promega, Wisconsin, USA) according to the manufacturer's instructions. For the colony formation assay, cells were transfected with shRNA, incubated for 48 h, trypsinized, and then seeded in 6-well plates at a concentration of 2500 cells/well. The cells were incubated at 37 °C for 10 days. After washing twice with PBS, the colonies were fixed with 5% paraformaldehyde for 15 min and stained with Giemsa for 10 min. Visible colonies were photographed and counted.

For the cell metastasis analysis, a 24-well permeable support plate (Corning, Massachusetts, USA) was prepared according to the following protocol: the permeable support was covered with 100 µL diluted matrix coating solution (Corning, Massachusetts, USA) at a final concentration of 300 µg/mL then incubated at 37 °C for 2 h. The upper chamber of the plate was inoculated with  $2.5 \times 10^4$  cells that had been transfected with shRNA, incubated for 48 h, and trypsinized in medium with 2% FBS. The lower chamber contained medium with 20% FBS. After incubation at 37 °C in 5% CO<sub>2</sub> for 12 h, the lower surface of the membrane was washed, fixed, stained, and observed as described, while the upper surface was cleaned with a cotton swab.

For the cell cycle analysis, cells were transfected with shRNA, incubated for 48 h, trypsinized, and then fixed with ice-cold 70% ethanol at 4 °C for 12 h. The cells were then washed twice with PBS, and resuspended in a PI solution and incubated at 37 °C for 30 min. The samples were analyzed by flow cytometry (Becton, Dickinson and Company, New Jersey, USA).

### **Statistical Analysis**

A one-way ANOVA test was used to examine the association between shRNA and control plasmids with SPSS software version 18.0 (SPSS Inc, IL, USA). All the *P*-values were two-sided, and the significance level was set at *P*<0.05.

## **RESULTS**

### **Detection of HPV Oncogenes in SKBR3 Cells and Breast Cancer Tissue Samples**

**Detection and Sequencing of HPV Oncogenes in SKBR3 Cells** HPV18E6-155 (306-460) and HPV18E7-125 (86-210) were amplified and

sequenced from the cDNA of the SKBR3 cells. HPV18E7-100 (80-179) was amplified and sequenced from the DNA of the SKBR3 cells. However, HPV16E6 and HPV16E7 were not detected in the DNA or cDNA of the SKBR3 cells by PCR. The sequence alignment for E6 and E7 with a consensus sequence [Human papillomavirus-18, complete genome (Genbank accession: NC\_001357.1)] are shown in Figure 1A and Figure 1B. The sequencing analysis indicated that there was a T→C mutation at 381 bp of HPV18E6-155 (306-460), an A→G mutation at 147 bp of HPV18E7-125 (86-210), and an A→G mutation at 147 bp of HPV18E7-100 (80-179) (Figure 1A). The mutation in HPV18E7-100 (80-179) was the same as the mutation in HPV18E7-125 (86-210) (Figure 1B). None of the mutations resulted in an amino acid change.

### **Detection of HPV Oncogenes in 76 Paraffin-embedded Breast Cancer Samples**

We then determined whether we could detect HPV oncogenes in primary breast cancer samples with PCR based methods. Eighteen of 76 samples (23.68%) were positive for HPV18 E7 expression by PCR using HPV18E7-100 (80-179) primer. Five of the 76 samples (6.58%) were positive for HPV18 E6 expression by PCR with HPV18E6-322 (66-387) primer. While all the 76 samples were negative for HPV16E6 and HPV16E7 by PCR. Representative results are shown in Figure 2. The sequence alignments of E6 and E7 with a consensus sequence [Human papillomavirus-18, complete genome (Genbank accession: NC\_001357.1)] are shown in Figure 3A and Figure 3B. The sequencing analysis indicated that there were deletions and subtle mutations. The tumors were staged by using the TNM staging scale. More tumor samples were HPV18 E7 positive in stage III (61.54%, 8/13) than in stage I (16.67%, 6/36) or stage II (15.38%, 4/26). While more tumor samples were HPV18 E6 positive in stage III (21.43 %, 3/14) than in stage II (8%, 2/25), the differences were not significant. One case of invasive lobular carcinoma was PCR negative for HPV oncogenes.

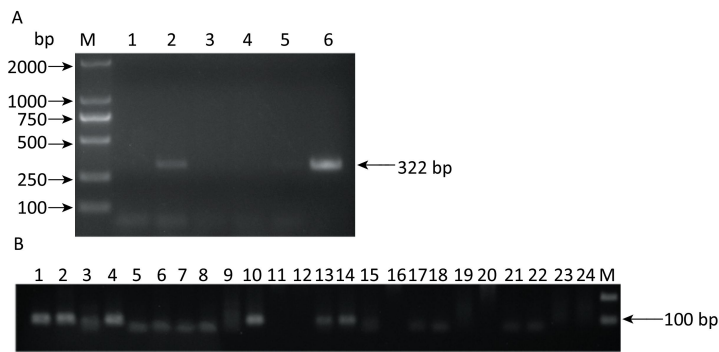
### **Assessing Effects of HPV18 E6 or E7 Targeted RNA Interference in SKBR3 Cells**

#### **Assessing Effects of HPV18 E6 or E7 shRNA on Cell Cycle Related Proteins in SKBR3 Cells**

Successful transfection of SKBR3 cells with the shE6 and shE7 shRNA constructs was demonstrated 48 h after transfection by expression of GFP (Figure 4A). Compared with the shNC, the expression levels of

<b>A</b>		
HPV18- <i>E6</i> (306-460)	AATAAGGTGCCTGCGGTGCCAGAAACCGTTG	31
HPV18- <i>E6</i> (306-460)-SKBR3-mRNA	AATAAGGTGCCTGCGGTGCCAGAAACCGTTG	31
Consensus	aataaggtgcctgcggtgccagaaaccgttg	
HPV18- <i>E6</i> (306-460)	AATCCAGGAGAAAAAATTAGACACCTTAATG	62
HPV18- <i>E6</i> (306-460)-SKBR3-mRNA	AATCCAGGAGAAAAAATTAGACACCTTAATG	62
Consensus	aatccagcagaaaaaacttagacaccttaatg	
HPV18- <i>E6</i> (306-460)	AAAAACGACGATTTCACAACATAGCTGGGCA	93
HPV18- <i>E6</i> (306-460)-SKBR3-mRNA	AAAAACGACGATTTCACAACATAGCTGGGCA	93
Consensus	aaaaacgacgattcacaacatagctgggca	
HPV18- <i>E6</i> (306-460)	CTATAGAGGCCAGTGCCATTCTGTCTGCAAC	124
HPV18- <i>E6</i> (306-460)-SKBR3-mRNA	CTATAGAGGCCAGTGCCATTCTGTCTGCAAC	124
Consensus	ctatagaggccagtgccattctgtctgcaac	
HPV18- <i>E6</i> (306-460)	CGAGCACGACAGGAACGACTCCAACGACGCA	155
HPV18- <i>E6</i> (306-460)-SKBR3-mRNA	CGAGCACGACAGGAACGACTCCAACGACGCA	155
Consensus	cgagcacgacaggaacgactccaacgacgca	
<b>B</b>		
HPV18- <i>E7</i> (80-210)	GTCACGAGCAATTAAGCGACTCAGAGGAAGAAAAC	35
HPV18- <i>E7</i> (80-179)-SKBR3-DNA	GTCACGAGCAATTAAGCGACTCAGAGGAAGAAAAC	35
HPV18- <i>E7</i> (86-210)-SKBR3-mRNA	.....AGCAATTAAGCGACTCAGAGGAAGAAAAC	29
Consensus	agcaattaagcgactcagaggaagaaaac	
HPV18- <i>E7</i> (80-210)	GATGAAATAGATGGAGTTAATCATCAACATTTC	70
HPV18- <i>E7</i> (80-179)-SKBR3-DNA	GATGAAATAGATGGAGTTAATCATCAACATTTC	70
HPV18- <i>E7</i> (86-210)-SKBR3-mRNA	GATGAAATAGATGGAGTTAATCATCAACATTTC	64
Consensus	gatgaaatagatggagttaatcatcaacatttc	
HPV18- <i>E7</i> (80-210)	AGCCCGACGAGCCGAACCAACGTCACACAAATGT	105
HPV18- <i>E7</i> (80-179)-SKBR3-DNA	AGCCCGACGAGCCGAACCAACGTCACAC.....	100
HPV18- <i>E7</i> (86-210)-SKBR3-mRNA	AGCCCGACGAGCCGAACCAACGTCACACAAATGT	99
Consensus	agcccgacgagccgaaccacaacgtcacac	
HPV18- <i>E7</i> (80-210)	TGTGTATGTGTTGTAAGTGTGAAGC	130
HPV18- <i>E7</i> (80-179)-SKBR3-DNA	.....	100
HPV18- <i>E7</i> (86-210)-SKBR3-mRNA	TGTGTATGTGTTGTAAGTGTGAAGC	124
Consensus		

**Figure 1.** The nucleotide sequences of HPV18 *E6* and *E7* PCR products from SKBR3 cells. The sequence alignments of *E6* (A) and (B) *E7* were conducted with a reference strain [Human papillomavirus-18, complete genome (Genebank accession: NC\_001357.1)]. Minor sequence mutations were observed, which resulted in no amino acid change.



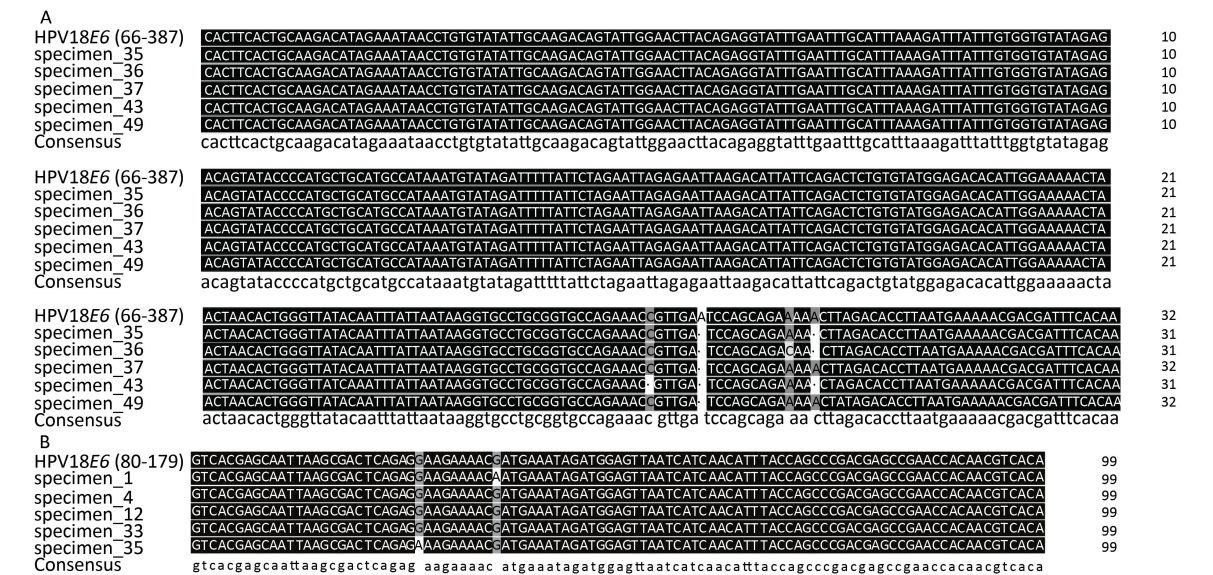
**Figure 2.** Representative results of a PCR based HPV screening in patient samples. (A) HPV18 *E6* PCR products using the HPV18*E6*-322 (66-387) primer. From left to right: lane M is DL2000 DNA marker; lane 1 is negative control; lanes 2-5 are representative results from samples; lane 6 is the positive control; (B) HPV18 *E7* PCR products using the HPV18*E7*-100 (80-179) primer. From left to right: lane 1 is the positive control PCR plasmid containing the full HPV18 sequence, lanes 2-23 are patient samples, lane 24 is the negative control sample (HEK293 DNA), and lane M is the DL2000 ladder marker.

HPV18 *E6* and *E7* significantly decreased by 34.47% and 78.08% ( $P<0.05$ ), respectively, after transfection with their respective shRNA (Figure 4B). The expression levels of cell proliferation and apoptosis proteins such as *RB*, *Tp53*-mutant, *MDM2*, *CCNA1*, *CCND1*, and *BCL2*, were evaluated by fluorescent quantitative PCR (Figure 4B). When the sh*E6* construct was compared with the shNC, the expression of *RB* significantly increased by 93.86% ( $P<0.01$ ). In contrast, the expression levels of *Tp53*-mutant, *MDM2*, *CCNA1*, *CCND1*, and *BCL2* significantly decreased by 54.86%, 63.14%, 7.01%, 18.48%, and 39.11% ( $P<0.05$ ). Similarly, when the sh*E7* construct was compared with the shNC the expression level of *RB* significantly increased by 100.08% ( $P<0.01$ ), and the expression levels of *Tp53*-mutant, *MDM2*, *CCNA1*, *CCND1*, and *BCL2* significantly decreased by 39.29%, 36.05%, 15.23%, 23.14%, and 76.75%, respectively ( $P<0.05$ ).

**Assessing Effects of HPV18 *E6* or *E7* shRNA on SKBR3 Viability, Colony Formation, Metastasis, and Cell Cycle**

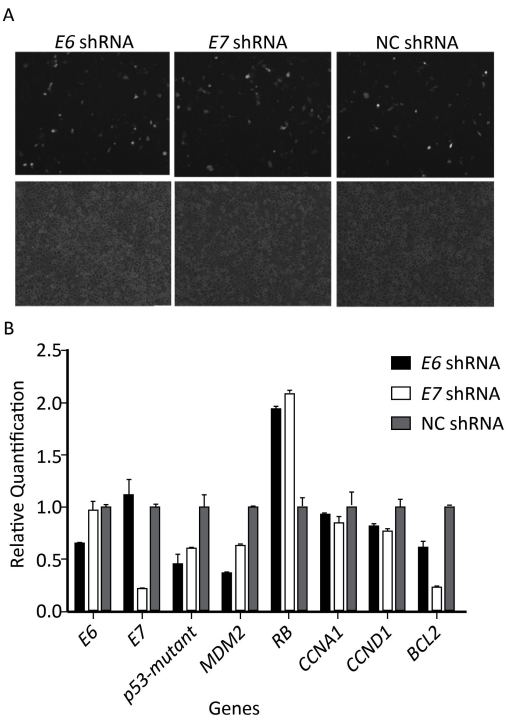
Cell viability was examined by a colorimetric assay based on the change of ATP concentration. Forty-eight hours after transfection with targeted shRNA, the viability of the SKBR3 cells was significantly reduced in both the HPV18 *E6* and

*E7* shRNA transfected groups compared with the shNC group ( $P<0.05$ ; Figure 5). To evaluate the effects on cell colony formation, cells were trypsinized, seeded, and incubated, then stained with crystal violet and counted 48 h after transfection with shRNA. Targeted knockdown of HPV18 *E6* and *E7* significantly reduced the number of SKBR3 cell colony formation by 60.64% and 63.95%, respectively, compared with the shNC group ( $P<0.05$ ; Figure 6 A-B). To determine whether HPV18 *E6* and *E7* were important for the invasive properties of SKBR3 cells, the metastasis abilities of SKBR3 cells transfected with sh*E6* and sh*E7* were analyzed in a transwell experiment. Compared with the shNC group, sh*E6* and sh*E7* significantly reduced the number of cell metastasis through the polycarbonate membrane by 58.70% and 65.41% ( $P<0.01$ ; Figure 6C-D), respectively. Flow cytometry was used to analyze the influence of HPV18 *E6* or *E7* on cell-cycle and apoptosis 48 h after transfection. Sh*E6* and sh*E7* significantly increased the percentage of cells in the G0/G1 phase compared with the shNC group ( $P<0.05$ ). This increase in the number of cells in the G0/G1 phase was coupled with a significant decrease in the percentage of cells in S and G2 phases after 48 h of transfection ( $P<0.05$ ; Figure 7).



**Figure 3.** The nucleotide sequences from the HPV18 *E6* and HPV18 *E7* PCR products from the breast cancer samples. (A) Representative sequence alignments of *E6* with a reference strain [Human papillomavirus-18, complete genome (accession: NC\_001357.1)]; (B) Sequence alignments of *E7* with a reference strain [Human papillomavirus-18, complete genome (accession: NC\_001357.1)].

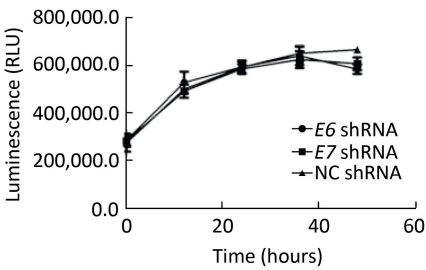




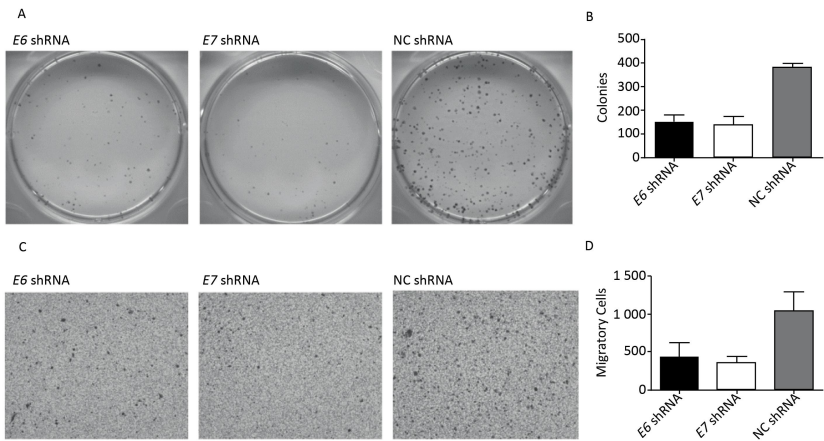
**Figure 4.** Knockdown of HPV18 *E6* or *E7* genes in SKBR3 Cells. (A) Successful transfection of HPV18 *E6* or *E7* shRNA was demonstrated in SKBR3 cells by expression of GFP. Original magnification 100 $\times$ . (B) qRT-PCR was used to quantify the expression of cell proliferation and apoptosis proteins at the RNA level. The values represent the mean from three independent experiments; error bars represent the standard deviation.

DISCUSSION

The role of HPV in breast cancer is not clearly defined. In this study, HPV18 *E6* and *E7* gene segments were observed in the mRNA of the SKBR3 cells, and the gene segments of (or similar to) HPV18 *E7* were present in the DNA of SKBR3 cells. We also demonstrated that HPV18 *E6* and *E7* gene segments were also able to detect the presence of HPV in 76 paraffin-embedded breast cancer samples (6.76% and 23.68%). Finally, we also showed that targeted knockdown of HPV18 *E6* and *E7* using shRNA techniques had an inhibitory effect on breast cancer cell proliferation, metastasis, and cell cycle. These findings suggested that HPV18 *E6* and *E7* might play an important role in the viral etiology of human



**Figure 5.** Effect of HPV18 *E6* or *E7* gene knockdown on the proliferation of SKBR3 cells. The values represents the means from three independent experiments; error bars represent the standard deviation.



**Figure 6.** Effects of HPV18 *E6* or *E7* gene knockdown on colony formation and cell metastasis in the SKBR3 cells. (A) Representative images of cancer cell colonies under a light, bright field microscope. The colonies were stained with crystal violet. Original magnification 10 $\times$ . (B) The number of SKBR3 colonies after HPV18 *E6* or *E7* knockdown compared with the negative control were quantified. (C) Representative images of the migrating SKBR3 cells. Original magnification 100 $\times$ . (D) Quantification of the migratory cells.

breast cancer. Importantly, targeted knockdown of HPV18 *E6* or *E7* might be useful as a potential therapy for breast cancer.

Previous studies have pointed out possible routes for HPV to reach the human breast<sup>[13]</sup>. Firstly, virus particle in the air could invade through the ducts into the breast tissue directly. Secondly, HPV-infected T-cells and B-cells from the mesenteric lymph nodes or HPV-infected cervical cells might enter the breast tissue via the circulatory system and infect the breast. When the breast tissue becomes infected with HPV, virus particles enter the cell, affecting cell cycle progression and eventually causing breast cancer<sup>[18]</sup>. There are conflicting reports about the existence of HPV genes in human breast cancer samples and in the SKBR3 cells. Since the first reports about HPV infection caused breast cancer in 1992<sup>[19]</sup>, there have been controversial reports about the etiology of breast cancer around the world<sup>[10,14,20-21]</sup>. We used primers for HPV oncogenes to screen 76 breast cancer tissue samples from patients. Of these samples, 6.76% were positive for HPV18 *E6* and 23.68% were positive for HPV18 *E7*. We did not find evidence of HPV 16 *E6* or *E7* in breast cancer samples as same as the results from Mexico<sup>[11]</sup> and Australia<sup>[22]</sup> which might be due to the differences in race, primers used, and type of samples<sup>[4]</sup>. Based on our *in vitro* results and the outcome of PCR screen, we believe that HPV is associated with human breast cancers. Furthermore, the detection of HPV18 *E6* and *E7* in multi types of breast cancer suggested that virogene targeted gene therapy would be an effective solution for breast cancer.

In regards to SKBR3 cells, Heng et al. described the presence of the HPV18 *L1* gene found with GP5/GP6 primers by PCR and HPV18 *E6* by in-situ

hybridization<sup>[5]</sup>. In contrast, Peran et al. screened SKBR3 cells by PCR for HPV18 *E6* with two pairs of primer and *E7* with one pair of primer by PCR<sup>[15]</sup>. Given the conflicting data, we chose to use more than 20 pairs of primers for HPV18 *E6* or *E7* (data not shown). Similar to Heng et al., we also found evidence for HPV18 *E6* and *E7* gene segments in the mRNA of SKBR3 cells, and HPV18 *E7*-like gene segments in the DNA of SKBR3 cells<sup>[5]</sup>. However, we could not confirm the existence of HPV18 *E6* in the DNA of the SKBR3 cells because in the PCR analysis, an erroneous band with similar length to the HPV18 *E6* product was always present (not show in data).

Most studies pertaining to gene therapy for breast cancer using RNAi technology have focused on human origin genes, such as *BCL2*<sup>[23]</sup> and *vascular endothelial growth factor A (VEGFA)*<sup>[24]</sup>. Our study has analyzed the development of viral gene therapy in human breast cancer. The knockdown of HPV18 *E6* and *E7* clearly affected the expression levels of some cell cycle related proteins, such as *Tp53*-mutant, *RB*, *MDM2*, *CCDNA1*, *CCND1*, and *BCL2*. The mRNA expression results of *MDM2* and *Tp53*-mutant were consistent with the flow cytometry results, suggesting that the apoptosis was enhanced or the cell cycle was blocked. The mRNA expression results for *CCNA1*, *CCND1*, *BCL2*, and *RB* were consistent with the results of viability, colony formation, and cell metastasis assays, indicating that the proliferation of cancer cells was enhanced. These results suggested that HPV18 *E6* and *E7* might play an important role in the carcinogenic potential of SKBR3 cells and could be used as potential targets for molecular-based therapy for human breast cancer.

Future studies with large sample size are needed to understand the role of HPV in the viral etiology of human breast cancer. Future studies should explore the route of HPV infection in breast cancer and analyze the relationship between HPV infection and breast cancer, and the expression levels of HPV oncogene in relation to human breast cancer.

ACKNOWLEDGEMENTS

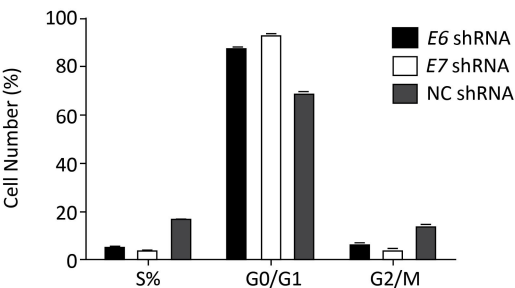
We thank the Peking University People's Hospital for the provision of samples.

Received: December 2, 2015;

Accepted: April 26, 2016

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**Figure 7.** Effects of HPV18 *E6* or *E7* gene knockdown on cell cycle in the SKBR3 cells. For all the panels, the values represent the mean of three independent experiments; error bars represent standard deviation.



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