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Selective targeting of HPV-16 E6/E7 in cervical cancer cells with a potent oncolytic adenovirus and its enhanced effect with radiotherapy in vitro and vivo

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1. Introduction

Cervical cancer is one of the most frequent malignancies in women worldwide, with an estimated approximately 493,000 new cases annually [1]. Although radical hysterectomy is the principal treatment for cervical cancer, patients with advanced stages are mainly treated with combined radiotherapy and chemotherapy or radiotherapy alone [2,3]. However, survival has not improved with current chemotherapy and radiation regimens in patients with recurrent or distant metastatic cervical cancer [3]. In addition, the therapeutic efficacy of above-mentioned methods is limited when cancer cells develop resistance to radiation and chemotherapy [4]. Therefore, these facts underscore an urgent need for the development of more effective and novel therapeutic approaches.

In recent years, a rapid development of the viruses that specifically kill tumor cells while sparing normal cells has been known as oncolytic viruses. The most studied oncolytic adenovirus, known as ONYX-015, carries a E1B-55 kDa-deleted mutant that introduces selectivity for tumor cells [5]. However, survival has not improved with current chemotherapy and radiation regimens in patients with recurrent or distant metastatic cervical cancer [3]. In addition, the therapeutic efficacy of above-mentioned methods is limited when cancer cells develop resistance to radiation and chemotherapy [4]. Therefore, these facts underscore an urgent need for the development of more effective and novel therapeutic approaches.

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mors with dysfunctional p53 [5]. In fact, ONYX-015 has shown encouraging outcome in animal models and clinical trials [6,7]. However, ONYX-015 has demonstrated limited anti-tumor efficacy because the deletion of E1B-55 kDa also decreases replicated potency of the adenovirus in tumors [8]. A second promising adenovirus type 5 (Adv5) mutant, known as Δ24 or dE222–947, with a deletion in the conserved region 2 (CR2) of early region 1A (E1A) protein known to be necessary for retinoblastoma (Rb) proteins binding, has demonstrated much more superior potency to ONYX-015 both in vitro and in vivo [9,10]. In addition to the intrinsic ability to kill infected host cells, oncolytic adenovirus offers several advantages including combination with radiotherapy or chemotherapy to increase therapeutic efficacy and potentially decrease toxic side effects [11,12] and the capacity to deliver therapeutic transgenes [13,14].

Human papillomavirus (HPV) infection has been identified as an etiologic agent in cervical cancer [15]. Infection with high-risk HPV (hr-HPV), particularly HPV types 16 and 18, is causally linked to the development of cervical cancer [16]. Numerous reports have established that hr-HPV strains are oncogenic, which depends on the expression of viral E6 and E7 oncoproteins that are selectively retained in HPV-associated cervical cancers [15,17]. Furthermore, continuous expression of E6 and E7 oncoproteins is necessary for the maintenance of the transformed phenotype of cervical cancer cells [18]. E6 and E7 oncoproteins target pRb and p53 pathways, respectively, which support DNA replication of adenoviruses with the E1A and E1B Genes deleted [19]. Radiation can enhance the expression of HPV16 E6 and E7 oncoproteins in human cervical cancer [20] and then the E6 oncoprotein can degrade p53 and upregulate hypoxia-inducible factor 1α (HIF1α) [21], which may be the likely reason for radiation resistance in HPV-associated cervical cancer. These properties have made E6 and E7 the targets of experimental therapeutics.

In this study, we report the construction of M6, a novel E1A-mutant adenovirus with antisense HPV16 E6 E7 DNA inserted into the deleted 6.7 K/gp19 K region. Theoretically, the mutant would replicate in tumor cells and consequently activate the native E3 promoters to express antisense E6 E7 preferentially in tumor cells. The anti-tumor efficacy of M6 was compared with its parental oncolytic virus Adv5/dE1A. The therapeutic effects of M6 combining with irradiation were also assessed.

2. Materials and methods

2.1. Cell lines

Human cervical cancer cell lines SiHa (HPV16 positive), HeLa (HPV18 positive), C33A (HPV negative), human embryonic kidney cell line HEK293 (adenovirus packaging cell) were obtained from American Type Culture Collection (ATCC, Manassas, USA). Lung-derived primary human microvascular endothelial cells (MVEC) were purchased from Cambrex Bio Science Rockland, Inc. (Rockland, ME). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, USA) containing 10% fetal bovine serum (FBS).

2.2. Adenoviruses

Wild-type adenovirus type 5 (wt-Adv5) was obtained from ATCC. Adv5/dE1A was a wt-Adv5 mutant with a 27-bp deletion of virus genome bases 920–946 corresponding to the amino acid sequence of the E1A protein conserved region 2 (CR2) necessary for pRb binding, which was constructed in our laboratory following described protocols [22,23]. M6 was derived from Adv5/dE1A via replacement of 6.7 K/gp19 K in the E3 region with a fragment of reverse HPV16 E6E7 DNA (bases 214–151 of HPV16 genome). The HPV16 E6E7 DNA was inserted into the deleted E3 region in HEK293 cells. The replication-deficient adenovirus AdEasy-GFP (Ad-GFP) was used as control, which contained a green fluorescent protein (GFP) gene under the control of a cytomegalovirus promoter and a simian virus 40 polyadenylation signal in the deleted E1 region.

2.3. PCR amplification and analysis

The antisense HPV16 E6E7 DNA fragment was amplified for further vector construction by PCR from the plasmids containing cloned full-length genomic DNA of HPV-16 (kindly provided by Dr. Ethel-Michele de Villiers, University of Heidelberg, Heidelberg, Germany). Clal restriction sites were introduced at each end of the amplified fragment. Reverse transcription-PCR (RT-PCR) was performed as described previously [24].

2.4. Western blots

Western blots were done as described previously [25]. Membranes were probed with primary antibodies specific for following proteins: HPV16/18 E6 (sc-460), HPV18 E7 (sc-1590), GAPDH (sc-59540) and β-actin (sc-1616-R) from Santa Cruz Biotechnologies (Calif, USA), HPV16 E7 (MS-768-P0) from NeoMarkers (Fremont, USA). Bands were visualized using an ECL Kit (Pierce, USA).

2.5. In vitro cytopathic effect (CPE) and viral replication assays

The viral CPE and replication assays were performed as described elsewhere [9]. To assess adenovirus replication-dependent cell killing effect, cells were grown to 70% confluence in 6-well plates and infected with M6, Ad-GFP or wt-Adv5 for 90 min at indicated multiplicity of infection (MOI). For CPE assays, the plates were stained with 0.1% crystal violet (Sigma Chemical Co., St. Louis, MO) in 20% ethanol. For viral replication assays, cells were infected at an MOI of 5 with various viruses. Forty-eight hours later, both cells and supernatants were collected, and then frozen and thawed thrice for virus titration analysis. Cell lysates were prepared by three cycles of freezing and thawing and titered on HEK293 cells using the tissue culture infectious dose 50 (TCID50) method according to the instructions in the AdEasy application manual (Quantum Biotechnologies, Montreal, Canada). Results are the mean of four replicates presented as plaque forming units (PFU).
2.6. Quantitative cytotoxicity and cell viability assays

Quantitative cytotoxicity and cell viability assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method. Cells were plated in 96-well tissue culture plates and grown to 70% confluence. For quantitative cytotoxicity assays, cells were infected with M6 at an MOI of 0.1–1000. For cell viability assays, cells were infected with adenoviruses at an MOI of 1. Seventy-two hours later the cells were stained with 5 mg/ml MTT reagent (Sigma). Absorbance of each well was measured at a wavelength of 570 nm and the growth suppression rate was calculated using the following formula: growth suppression rate (%) = [1 - (A570 of experimental wells/A570 of mock control wells)] × 100%. Each assay was performed in triplicate.

2.7. Transwell assays

In vitro invasion was determined in a Matrigel-based Transwell assay essentially as described previously by Pelletier et al. [26]. The upper chambers of the 24-well Transwell plates (Corning Costar, Cambridge, MA) with a pore size of 8 μm were coated with Matrigel (0.7 mg/ml, Sigma) and the lower compartments were filled with serum-free conditioned DMEM of NIH3T3. Twenty-two hours after transfection with adenoviruses at an MOI of 1, 0.5 × 10⁵ cells were seeded in the upper chamber per well and incubated at 37 °C for 24 h. Then, the non-migrated cells on the upper surface of the membranes were gently scraped away with cotton swabs and the migrated cells that had invaded to the lower surface were stained with crystal violet and counted.

2.8. In situ TUNEL assays

For detecting apoptotic cells, tissue slices were analyzed by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay by using the In Situ Cell Death Detection Kit (Boehringer Mannheim, Germany) according to the manufacturer’s instructions. Briefly, the slices were treated with 3% H₂O₂, and then covered with 1 μl each of terminal deoxynucleotidyl transferase (TdT) and digoxigenin-labeled dUTP (DIG-dUTP) in 20 μl TdT buffer at 37 °C for 2 h. The slices were subsequently reacted with biotin-labeled anti-digoxigenin (Anti-DIG-Biotin) for 30 min at 37 °C, followed by washing in phosphate-buffered saline (PBS). Next, the slices were incubated with an avidin and biotinylated horseradish peroxidase macromolecular complex and stained with diaminobenzidine. Finally, the slices were counterstained with hematoxylin. The apoptotic cells were stained brown under the microscope.

2.9. Statistical analysis

All experiments were repeated at least thrice. The data were analyzed with the software package SPSS 12.0.

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**Fig. 1.** Construction and verification of recombinant adenovirus M6. (A) Diagram of constructing recombinant adenovirus M6 containing CR2-deleted mutant E1A and reverse HPV16 E6 and E7 DNA fragment instead of 6.7 K/gp19 K in the E3 region. (B) ClaI restriction enzyme digestion of M6. Two ClaI restriction sites were introduced at both ends of reverse HPV16 E6 and E7 DNA. The insert of the 0.8-kb fragment was verified by ClaI restriction enzyme digestion and agarose gel electrophoresis. (C) RT-PCR amplification of fusion mRNA containing sequences of antisense HPV16 E6 and E7. SiHa cells were infected with M6, Ad-GFP or wt-Adv5 at an MOI of 5. Twenty-four hours after infection, RT-PCR was performed to amplify the fusion mRNA. Amplified fragment showed the expected size of 0.5 kb.

Fig. 2. Selective replication of M6 in cancer cells. (A) E1A-transformed cells (HEK293), cervical cancer cells (SiHa, HeLa and C33A) and normal cells (Q-MVEC) were infected with M6, wt-Adv5 or Ad-GFP at various MOI. (B) Viral replication of M6, wt-Adv5, and Ad-GFP was determined in HeLa, C33A and Q-MVEC cells. Cells were infected at an MOI of 5. Forty-eight hours later, cell lysates were prepared and viral titers were quantified. (C) MTT assays were performed to determine the quantitative cytotoxicity of M6 on HeLa, C33A and Q-MVEC cells. Values and error bars represent the average and s.d. for three independent experiments.

Fig. 3. Effect of M6 transfection on the expression of viral E6 and E7 oncogenes. Cells were infected with Adv5/dE1A and M6 at an MOI of 5 and harvested at 24, 48 and 72 h. The expression of E6 and E7 were measured by RT-PCR amplification in SiHa cell (A) and western blot analysis in SiHa (B) CaSki (C, 72 h) and HeLa (D, 72 h).

\( \chi^2 \) test was performed to compare frequencies between the two groups. \( p \) Value less than 0.05 were considered significant statistically.

3. Results

3.1. Construction of recombinant adenovirus M6 containing antisense E6E7 sequence

To further determine therapeutic effect of targeting E6 and E7 in cervical cancers, we employed an oncolytic adenovirus vector derived from wt-Adv5. E3 transcription unit of Adv5/dE1A genome, corresponding to wt-Adv5 genome nucleotides 28530–29360 known to encode E3 6.7 K and gp19 K protein, was substituted by a fragment of reverse HPV16 E6 and E7 DNA (bases 314–151) with Clal restriction sites introduced at each end to generate M6 (Fig. 1A). To verify the structure of M6, the viral genome DNA was digested with Clal restriction enzymes and separated on a 0.8% agarose gel. The result of electrophoresis showed a 0.6 kb DNA fragment which was identical to the inserted antisense E6E7 sequence in size (Fig. 1B). In M6, antisense E6E7 DNA was fused to the S' portion of the E3 ADP gene and transcribed a single chimeric transcript. Therefore, a RT-PCR was performed to detect the fused mRNA in HEK293 cells 24 h after infection with M6 at an MOI of 5 and amplified products was observed with expected size of 0.5 kb (Fig. 1C).

Fig. 4. Effect of M6 transfection on apoptosis and invasion of cervical cancer cells. (A) Adv5/dE1A transfected and M6-transfected SiHa cell were stained with fluorescent dye Hoechst 33342 and propidium iodide. The typical apoptotic morphology such as nuclear condensation and fragmentation (arrows) in infected SiHa cells was detected by a confocal scanning laser microscope. (B) Apoptosis rates of cervical cancer cells (SiHa, HeLa and C33A) and normal cells (NIH3T3) were analyzed by flow cytometry at 72 h after infection. (C) Matrigel-based Transwell assays were performed to determine the invasive ability inhibition in vitro in both SiHa and HeLa cells. Twenty-two hours after transfection, 0.5 \( \times 10^5 \) cells were seeded in the upper chamber per well and incubated at 37 \(^\circ\)C for 24 h, migrated cells were fixed, stained, and counted.
The cytopathic effect (CPE) assays was performed to determine if M6 was competent to selectively lyse cervical cancer cells in a replication-dependent fashion (Fig. 2A). Three adenoviruses, including M6, wt-Adv5 and Ad-GFP, exhibited complete cytopathic effect with different virus titers (MOI: M6 = 0.1, wt-Adv5 = 0.01, Ad-GFP = 1.0) in HEK293 cells, as an E1A transcription factor-deficient adenoviruses replication permissive cell line. In cervical cancer cell lines, including SiHa, HeLa and C33A, M6 or wt-Adv5 induced complete lyses at lower MOI (from 0.01 to 1.0).

Nevertheless, Ad-GFP did not provoke detectable CPE on cells at an MOI of 10 or 100. In non-proliferating MVEC (Q-MVEC), a normal primary cell line expressing Wild type p53 and pRb, M6 and Ad-GFP could not yield measurable CPE at an MOI of 0.1–100 and replication-deficient showed no detectable cytopathic effect at an MOI below 100. In contrast, wt-Adv5 caused complete cytopathic effect at an MOI of 1.0. Next, we chose two cervical cancer cell lines HeLa and C33A and normal cell line Q-MVEC to test viral replication. As shown in Fig. 2B, M6 selectively replicated in cervical cancer cell lines, but not in normal cell line Q-MVEC, when compared with both Ad-GFP and wt-Adv5 (p < 0.01). The quantitative MTT assay showed that M6 preferentially inhibited the growth of HeLa and C33A cells with a dose-dependent tendency. However, no obvious growth inhibition was detected in Q-MVEC when added M6 at an MOI of 100.

The results above indicted M6 selectively replicated in cervical cancer cells and inhibited their growth in vitro. Further studies were needed to determine whether M6, an oncolytic adenovirus vector inserted sequence complementary to HPV16 viral E6 and E7 mRNA, could inhibit targeting genes expression. To test this, we transfected M6 into cervical cancer cells, SiHa, CaSki and HeLa cells at an MOI of 1 and determined the expression of E6 and E7 oncogenes in HPV16-negative but HPV18-positive cervical cancer HeLa cells (Fig. 3D).

3.3. Effect of M6 transfection on HPV16 viral E6, E7 oncogene expression

To determine whether M6 can facilitate apoptosis, we infected cervical cancer cells with 10

3.4. M6 induce apoptosis and inhibit invasion of HPV16-positive cervical cancer cells

To further determine whether administration of M6 induces apoptosis or increase the anti-tumor efficacy with radiation, M6 or Adv5/dE1A were injected intratumorally into tumor-bearing mice and followed by a single exposure to radiation. A significantly suppression of tumor growth was observed in mice received M6 combined with radiotherapy (30 Gy) when compared with the other four groups including non-treated, radiotherapy only, M6 transfection only and Adv5/dE1A transfection plus radiotherapy (Fig. 6A and B). Immunohistochemistry and TUNEL analysis further showed a momentously reduced expression of E6 and E7 and induced cell apoptosis in M6 transfection plus radiotherapy tumor tissues (Fig. 6C). Kaplan-Meier analysis (Fig. 6D) showed that M6 transfection could improve the survival of tumor-bearing mice in combination with such synergistic inhibition effects of M6 on SiHa cells (Fig. 5A). Our results showed the cell proliferation was inhibited by radiation with a dose-dependent state (Fig. 5A) and M6 transfection significantly augmented the inhibition effect of radiation on SiHa cells proliferation when compared to Adv5/dE1A transfected or non-transfected cells. In addition, Annexin-V/PI flow cytometry analysis was carried out to measure the percentage of early apoptotic cancer cells by radiotherapy (5 Gy) combined with M6 transfection. Compared with Adv5/dE1A transfected or non-transfected SiHa cells, M6 transfection increased the percentage of apoptotic cells by radiotherapy from 8.11% to 58.90%. Both results indicated M6 transfection will lead to additive or synergistic efficacy with radiation.

3.6. The synergistic efficacy of M6 with radiotherapy in vivo

![Image](320x155 to 474x429)

**Fig. 5.** Potentiation of radiotherapy by M6 on HPV16-related cancer SiHa cells. SiHa cells were transfected with either Adv5/dE1A or M6 at an MOI of 5 followed by a single exposure of radiation (from 2.5 to 10 Gy) using a photon linear accelerator at 24 h after transfection. (A) MTT was performed to determine inhibition rates of SiHa cells at 48 h after irradiation. Values and error bars of inhibition rates represent the average and standard deviation. (B) Annexin-V/PI flow cytometry analysis was performed to determine the percentages of early apoptotic SiHa cells at 48 h after irradiation. The percentages of early apoptotic cells were 3.94% ± 0.88% (control), 8.11% ± 1.32% (5 Gy), 9.32% ± 1.56% (Adv5/dE1A, 5 Gy) and 58.9% ± 4.11% (M6, 5 Gy).

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radiotherapy, much superior to that of those treated by Adv5/dE1A plus radiation (p = 0.012) or M6 alone (p = 0.025).

4. Discussion

Gene delivery may provide new therapeutic approaches for the treatment of cancer and recombinant adenovirus is one of the most commonly used vectors for gene therapy especially for cancer treatment [27,28]. Recombinant adenoviruses allow for the introduction of up to 35 kb of DNA with highly efficient for exogenous gene delivery. However, recombinant adenovirus vectors themselves are rarely causing harmful effects on tumor due to non-selective replication in cells. Thus, replication-selective adenovirus is a promising alternative to obviate the problem of side effects of adenovirus vectors [29].

Recently, we report a novel E1A-mutant replication-selective oncolytic adenovirus derived from wild-type adenovirus serotype 5 [22,30,31], which had the 6.7 K/gp19 K region of E3 deleted for insert of interesting gene. The intact E1A associates with pRb through its CR2 domain and releases E2F from pRb/E2F complex, which is a competent transcription factor that promotes the transcription of the genes required for DNA replication and the transition of cells from the G1 phase into the S phase [32,33]. So, E1A is essential for replication of wild-type adenovirus genome DNA. M6 contains a 27-bp deletion in the E1A CR2 region to confer tumor-selective replication in a wide range of tumor cells irrespective of tissue origin and pRb and p53 status. In HPV-associated cervical cancer cells, pRb is functional inactivated by HPV oncoprotein E7 and E2F is free from pRb/E2F complex [28]. Thus the oncolytic adenovirus is able to selectively replicate in HPV-associated cervical cancer cells but not in normal cells. Another bioengineering component in M6 was the insertion of antisense HPV16 E6 E7 DNA into the E3 6.7 K/gp19 K region, which promoted reverse HPV16 E6 E7 cDNA expressing preferentially in HPV-associated cervical cancer cells and consequently silences HPV16 E6 E7. In addition, viral replication of M6 would robustly amplify the copies of antisense HPV16 E6 E7 in HPV-associated cervical cancer cells and allow spread of viral infection to nearby tumor cells. Our in vitro and in vivo experiments verified the specific inhibition of HPV16 E6, E7 expression in M6-transfected SiHa cells, a HPV-positive cervical cancer cells line, and M6-transfection induced apparent apoptosis in SiHa cells.

Cancer invasion and metastasis is a multistep process involving adhesive, migratory, and pericellular proteolytic events. Early study reported by Matsukura [34] indicated that HPV16 was involved in invasive cervical cancers. Recently, Yasmeen et al. [35] reported that high-risk HPV infections could induce cell invasion and metastasis in cervical cancer through regulating inhibitor of DNA binding 1 (Id-1) by E6 and E7. To determine whether antisense RNA delivered by M6 inhibited in vitro invasive ability of HPV16-related cervical cancer, we introduced Matrigel-based Transwell assays, following the infection of SiHa and HeLa cells with M6. The results in our study implied...
M6 significantly inhibited the *in vitro* invasive ability of HPV16-related SiHa cells but not HPV18-related HeLa cells. Besides surgical resection radiotherapy is the most current method in cervical cancer treatments, especially for advanced cases [36]. However, radiotherapy is the dose-limited and over dosage will cause deadly damage for patients [37]. So the new treatments are imperiously needed to be developed for adjuvant therapy of cervical cancer. Development of selective anti-cancer approaches via targeting of specific molecules or DNA is one of the central goals in the field of cancer research [38]. We have reported here a novel strategy of this type using M6 as a potent anti-tumor virus for treatment of primary tumor and disseminated metastases. In fact, oncolytic adenoviruses combined with DNA-damaging agents such as radiation or chemotherapy results in supra-additive cell killing in the treatment of cancer [39]. Moreover, replication-selective viral treatment target oncogenes HPV16 E6 E7 should not lead to cross-resistance or additional side-effects with standard therapies, combinations with radiotherapy might lead to synergistic efficacy [40]. In this experiment, down-regulation of HPV16 E6 E7 expression by oncolytic adenovirus M6 transfection was not sufficient to kill the cells absolutely in SiHa cells as monotherapy, but M6 plus radiation strategy noticeably facilitated radiation-induced anti-tumor effect in SiHa cells. The results observed with tumor growth inhibition and substantially improved survival in cervical cancer-bearing mice model treated by M6 plus radiation is even more encouraging.

In conclusion, we have provided proof of principle that recombinant oncolytic adenovirus M6 containing anti-sense E6E7 sequence selectively replicates in cancer cells and inhibited tumor growth and induced apoptosis of the HPV16-positive cervical cancer cells *in vitro* and *in vivo*. We report that M6 plus radiation treatment achieved noticeable anti-tumor efficacy. It is possible to speculate that M6 may was introduced as an adjuvant or a synergic therapy of radiotherapy for the effective treatment of advanced cervical cancer without cross-resistance or additional side-effects.

**Conflicts of interest**

No potential conflicts of interest were disclosed.

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