The deletion of adenovirus E1B-19KD gene enhanced its cell killing ability against lung cancer cell line A549

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Abstract. Oncolytic Viruses immunotherapy is one of the most frequently used anti-cancer agents because it can significantly reverse local immune suppression and amplify antitumor immunity. Although drugs were already applied in cancer treatment, a new agent may open a new pathway for developing oncolytic therapy. This study intends to improve the adenoviral vector to increase its killing effect on tumor cells for effective and safe cancer gene therapy. We modified the pDC316 plasmid by adding TERT promoter, E1A, and E1B viral genes through homologous recombination and transfection. Then the virus was reassured of its stability through PCR analysis, Western blot, viral titer assays, and CPE assay. In the result, Ad-E1B 55k virus especially showed strong cytotoxicity of more than 50% of killing efficacy in vivo A549 cells. Cell viability in HepG2 is around 80% while in MRC5 cells, the viability is approximately 100%, which showed a tumor-specificity of the viruses. Moreover, only on day 4 has 20% of the efficacy of E1B 55k virus and the viability quickly returned to normal on the next day, which indicates the relative safety of the modified in MRC5 normal cells. In conclusion, the modified virus may serve as another starting point to design a potentially safer, better efficacy, and specificity in cancer immunotherapy. However, a further test of the vector in animal trials is recommended.

Keywords: antitumor. Immunotherapy. oncolytic therapy. Adenovirus. vivo homologous recombination. Adenovirus serotype 5 (Ad5). Western blot.

1. Introduction

According to WHO and CDC, cancer is one of the leading causes of death around the world with approximately 10 million deaths in 2020, among which 136,084 people died of lung cancer [1][2]. Oncolytic Viruses immunotherapy, as one of the neoteric treatments, is a therapeutic approach for cancer treatment that utilizes genetically modified viruses that can selectively target and infect tumor cells [3]. Some have already been implemented into clinical trials or commercialized. Adenovirus-based OVs, known as conditionally replicating adenoviruses (CRAd), are one of the most frequently used anti-cancer agents because of their capability to significantly remove local immune suppression and amplify antitumor immunity [4]. Selective replication of viral agents may lead to improved efficacy over immune-escaped tumor cells by manipulating viral multiplication, lysis of infected cancer cells, and secondary infections to adjacent cells [5]. In 2005, Oncorine (H101), an E1B 55k gene deleted and E3 partially deleted adenovirus, was approved for nasopharyngeal carcinoma in combination with chemotherapy in China [6]. In 2009, oncolytic Herpes Simplex Virus-based therapy, G47Δ, started its first trial for malignant glioblastoma in Japan [7]. This agent is a triple-mutated vector with deletions in the γ34.5 gene, α47 gene, and a lacZ insertion in the ICP6 locus [8]. These cases suggest a likelihood of a stable and promising therapy for eliminating other future solid tumors-as traditional treatment, like chemotherapy or radiation therapy, can neither fundamentally eradicate cancer with mild side effects nor increase the survival rate of patients. Therefore, with previously successful cases, the development of a replication-selective agent with improved potency for lung solid tumor-targeted would be timely and could be applied more widely in anticancer gene therapy.

The virus is designed to have weak toxicity and strong targetability while retaining its replication capability. The telomerase complex is an ideal site for tumor targeting as it is highly expressed in the vast majority of human cancers but not in most host tissues [8]. It consists of an RNA template component, a reverse transcriptase (human telomerase protein/enzyme [hTERT]), and telomerase-associated proteins [10]. The tumor marker enhancer-binding protein-2β (AP-2β) can reactivate

tumor-specific hTERT promoter, which may show a better selectivity for lung cancer [11]. A decrease in cytotoxicity can be achieved by modifying its expression genes, like early region 1 (E1) and early region 3 (E3), which are critical for viral replication in normal cells. It is well established that the E1 gene produces early proteins, including E1A and E1B, which acts as a critical cue for the beginning of the viral replication by up-regulating transcription from the rep gene promoters. The E1 and E3 genes are transferred to shuttle plasmids so that the propagation of the virus can be achieved through homologous recombination of the virus and plasmid vector, thus achieving control over the virus [12]. Studies have shown that the presence of a complete E1A can initiate a cell suicide response to the deregulation of growth control by activating the tumor suppressor p53 while the 19k and 55k gene in E1B provides separate mechanisms that may block the cell suicide pathway of p53 [13]. E1B 19 kDa gene acts as a potent apoptosis inhibitor while the 55kDa gene transcribes protein that possesses a leucine-rich nuclear outlet signal (NES), This signal participates in nuclear plasma shuttling through CRM1-mediated nuclear outlet receptors, which may show a better efficacy for homologous recombination [14]. Therefore, a deletion of E1B 19k gene may give way to P53 tumor suppressor gene during cancer treatment while 55k gene alone is capable for the transcription process.

2. Materials and methods

2.1 Cell line and cell culture

Adenocarcinomic human alveolar basal epithelial cells (A549 cells) and Human Medical Research Council cell strain 5 cells were purchased from Procell. pDC316 plasmid and pBHGloxΔE1,3Cre backbone plasmid were purchased from microbix biosystems Inc. as a whole Ad.MAX™ Adenovirus Expression System. The pBHGloxΔE1,3Cre backbone plasmid is an E1 and E3 region deleted, replication-deficient adenovirus with a total of 34707 bp double-stranded DNA (ds-DNA). pDC316 plasmid is a shuttle vector that has a total of 3913 bp ds-DNA, including Ampr, ori, Ad, ITR, MCMV, SV40 polyA, and LoxP as its key elements. Human embryonic kidney 293 cells (HEK293) cells, which contain E1A and E1B gene sequence, and hepatoma G2 (HepG2), which contains hTERT promoter gene sequence, were imported from Guangzhou Doublle Bio-product Co., Ltd. These cells were cultured at 37°C in a humidified and 5% of CO2 in Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. C11995500BT) or RPMI Medium 1640 basic (Cat. No. C11875500BT) from Gibco supplemented with 10% fetal bovine serum (FBS) (Cat. No. 10100147, YOSHI), 4.5 g/L D-Glucose, L-glutamine, and 110 mmg/L Sodium Pyruvate.

2.2 Preparation for the construction

The initial pBHGloxΔE1,3Cre backbone plasmid was preserved under -80°C for later uses. The modified HEK293 cells (Guangzhou Doublle Bio-product Co., Ltd., China) containing the complete E1A region and E1B region of adenovirus type 5 were used as templates. HEK293 cells extracted its RNA and reverse transcribed it to obtain cDNA. The E1A gene sequence and the promoter sequence of the E1 B gene, as well as the combined sequence of the E1B 55K gene and E1B promoter, were amplified from 293 cells by PCR. The core promoter sequence of hTERT was obtained by PCR amplification of the genomic DNA of HEPG2 cells.

2.3 Construction of pDC316-hTERT non-replicating adenovirus

To obtain a non-reproducible adenovirus, pDC316-hTERT, the genomic DNA of HEPG2 cells (Guangzhou Doublle Bio-product Co., Ltd., China) was extracted, and the core promoter sequence of hTERT was obtained by PCR amplification with the following set: 5'-ATTATTATAGTCAGCTCTAGATTAGGCCGATTCGACCTCTC-3' as the sense primer(hTERT-F) and 5'-CTAATGACTCAGTATATCTCTAGAGGCTTCCCACGTGCGCAGCAGGAC-3' (hTERT-R-pDC316) as the antisense primer. The pDC316 plasmid vector was linearized by XbaI digestion. As both ends of the core sequences of the hTERT promoter contain homologous arms at both ends of pDC316's XbaI restriction site, the hTERT promoter sequence was integrated through

homologous recombination into the XbaI restriction site by utilizing Vazyme's ClonExpress® Ultra One Step Cloning Kit (Cat. No. C115-01), generating the pDC316-hTERT shuttle vector.

2.4 Construction of pDC316-hTERT-E1A-E1B-55k replicating adenovirus

The XbaI digestion linearized the pDC316 plasmid vector for later recombination. The hTERT sequence was amplified by PCR from HepG2 gene sequence DNA. Its N-terminus has a homology arm to the left of the XbaI restriction site of the pDC316 shuttle vector. After extracting RNA from 293 cells, the E1A sequence, the E1B 19k promoter, and the E1B55k gene were separately amplified by polymerase chain reaction (PCR) with the following primer set: 5'-TGCGCACGTGGGAAGCCACACCGGGACTGAAAATGAG-3' as the sense primer(E1A- F) and 5'-CACAGGTTTACACCTTATGGC-3' as the antisense primer(E1A- R) for E1A gene sequence; 5'-GAGGTCAGATGTAACCAAGATTA-3' as the antisense primer(E1B-P-R) for E1B 19k promoter; 5'-TCTTGGTTACATCTGACCTCATGGAGCGAAGAAACCCATC-3' as the sense primer(E1B55K-F) and 5'-CTAATGACTCAGTATATCTCTAGAGCCCACACATTTCAGTACCTC-3' as the antisense primer(E1B55K-R-PDC316) for E1B-55k gene sequence. The E1A sequence has an N-terminus as the homology arm of the c-terminus of the hTERT promoter. The E1B 19k promoter and E1B55k gene sequence were linked together by homologous recombination, where the left side of the E1B 19k promoter contained the homology arm of the E1A C-terminal on the right. The C-terminal of E1B55k genes had the homology arm on the right side of the XbaI restriction site of the pDC316 shuttle vector. By using Vazyme's ClonExpress® Ultra One Step Cloning Kit (Cat. No. C115-01), the hTERT promoter, E1A, and the E1B 55k gene were simultaneously integrated into the linearized pDC316 shuttle vector by homologous recombination through the XbaI restriction site, generating the pDC316-hTERT-E1A-E1B-55k shuttle vector.

2.5 PCR recombination reaction for construction

The recombination reaction proceeded by using Vazyme's ClonExpress® Ultra One Step Cloning Kit (Cat. No. C115-01). Extracting 100ng linearized pDC316, 10ng hTERT fragment, and 20ng E1A fragment and adding them into a solution with 5 μl of 2× ClonExpress Mix. Use pipettes to gently mix the solution, centrifuge briefly, and remove the supernatant to collect the reaction solution on the bottom of the tube. Perform a 5-minute single-fragment recombination reaction at 50 degrees and immediately cool down on ice or reduce to 4 degrees. Thaw the DH5α Competent Cell competent cells (Guangzhou Doublle Bio-product Co., Ltd, China) for cloning on ice. Add 5-10 μl of the recombinant product to 100 μl of competent cells, flick the tube wall for mixing, and stand still on ice for 30 minutes. After heat shock for the solution in a 42°C water bath for 45 sec, immediately cool on ice for another 2 - 3 min. Next, add 900 μl LB liquid medium (without antibiotics) into the solution and shake the bacteria on a shaker for one hour at 37 degrees. (rotation speed 200 - 250 rpm). In the meanwhile, pre-warm the LB solid medium plate that contains brubenzyl resistance in a 37°C incubator. Centrifuge the solution at 5,000 rpm for 5 min and discard 900 μl of the supernatant. Resuspend the bacteria with the remaining medium and spread gently with a sterile spreader bar on the plate containing the correct resistance. Finally, after 12-16 hours of inverted culture in a 37°C incubator, PCR screening and sequencing were performed to obtain the target, pDC316-hTERT. Same process for pDC316-hTERT-E1A-E1B55k, but changes to 15 minutes of multi-fragment recombination reaction.

2.6 Packaging preparation of oncolytic viruses

The rationale of the packaging process is to transfect the shuttle vector and backbone plasmid in normal cells to obtain the P0 generation virus for further amplification. The above-constructed shuttle vectors pDC316-hTERT, pDC316-hTERT-E1A-E1B-55K, and the backbone plasmid pBHGloxΔE1,3Cre of AdMax five adenovirus system were co-transfected into HEK293 cells for virus packaging. The day before transfection, 5×10^5 of HEK293 cells were seeded in a 6 CM culture

dish, with the medium DMEM+10% of FBS, and the cells were cultured overnight at 37°C in a cell incubator that contains 5% CO2 until the cell density had covered about 80% of the overall dish. Later, the backbone plasmids pBHGloxΔE1, 3Cre (3.2μg) were co-transfected with five shuttle plasmids (0.8μg) using TurboFect transfection reagent (Cat. No. R0532). On the second day after transfection, the overgrown cells were passed into T25 cell culture flasks, and continued to be cultured in DMEM medium containing 5% FBS, and repeat the same process if the cells were re-overgrown. During the period, the medium was not changed, but part of the medium was added to the cells. After 5-20 days, the cells were poisoned and presented with green fluorescent protein expression. Observing the flasks, the cells appeared grape-like and began to fall off the wall of the culture flask. After the toxic cells were eluted with the culture medium, centrifuge at 500g for 10 minutes, discard the supernatant, resuspend the cells with 2mL PBS, and place them in a -80°C refrigerator and a 37°C water bath successively for three times. Later, centrifuge at 12000g for another 10 minutes, and collect the supernatant containing the P0 generation virus for further identification of viral genome by Western blot and E1A protein expression by PCR.

2.7 PCR analysis for mutant viral genome

10 μl of 293 cells infected with Adeno-hTERT and Adeno-hTERT-E1A-E1B55k were lysed by 2 μl of Proteinase K for 30 minutes at 50℃ to release viral gene sequence as a template amplification of hTERT E1B55k related fragment. Denaturing the double-stranded template into a single-stranded one under 98℃ for 10 seconds. Annealing the designed primers onto the template to create a hybridization strand to complement each other. This process was done below 10℃ for 30 sec/kb. Extend the primer to synthesize a new strand of DNA along the template by using KOD-Plus-Neo DNA polymerase (TOYOBO CO., LTD. Japan) at 68℃ for 30 sec/kb. Circulating the same process 30 times. Finally, run the agarose gel by electrophoresis with the voltage 120V and current 100mA. Put the gel into the gel imager to observe the length of the genome.

2.8 Identification of protein expression with Western blotting

2×10^5 cells were seeded into 6 wells flasks with an MOI of 1 and cultured for 48 hours. Lysates (50–100g of protein by the Lowry method) were electrophoresed on SDS–7.5% polyacrylamide gels (PAGE) by using Invitrogen Invitrogen™ Mini Gel Tank (Cat. No. A25977) and then electro-blotted onto a nitrocellulose membrane. Blots were stripped and probed with anti-E1A antibody (diluted 1:300 by using mouse anti-E1A monoclonal antibody [mAb], Cat. No. J1316, Invitrogen™) followed by horseradish peroxidase (HRP, ThermoFisher Scientific) conjugated with Goat anti-mouse secondary IgG (diluted 1:3000, Cat. No. A5003). Finally, the blots were visualized by using enhanced chemiluminescence (ECL, ThermoFisher Scientific).

2.9 Amplification of the mutant virus

Once the identifications of genome sequence and protein expression are identified with the model prediction, the P0 generation virus would be amplified quantitatively. 1×10^6 of 293 cells were seeded in T75 cell culture flasks. Add 10ml of DMEM and 10% FBS for culture. After the cell density reached 90% (1×10^7) on the second day, 50 μl of the virus was added to the plated cells. Cells are completely lesioned 48-72 hours after infection. Then use a pipette to collect the solution in a bottle and place it in a -80℃ refrigerator and a 37℃-water bath successively for freeze-thawing three times. Centrifuge at 1200g for 10 minutes to collect 10ml of virus-containing supernatant and save it for later use. Repeat all the above steps for continuous expansion to P3 generation to perform viral titer assays and cytological in vitro functional assays.

2.10 Viral titer assays

293 cells were seeded in 96-well plates at a density of 1×10^4 cells/well. Dilute the cells with 100 μl of DMEM and FBS. Simultaneously dilute the virus we preserved previously 10 times by using the double dilution method. (900 μl of DMEM and FBS with 100 μl of virus solution) Then, add a

diluted virus to 96-well plates plated with 293 cells. Waited for 7 days and observed the number of lesions under the microscope to determine the PFU- As plaque is created during cell lysis, we use PFU as quantification of the number of plaques formed per unit volume of the virus to represent the concentration of viable virus particles in units.

2.11 CPE assays

A549 cells were seeded in 96-well plates with 30 μl of DMEM and FBS at a density of 3000 cells/well. The next day, take the titered virus solution out of the -80℃ refrigerator and dissolve it at 37℃. Preparing the viruses at different MOIs (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4) in multiple centrifuge tubes by adding DMEM to dilute. Completely mixed the virus solution with the vortexer (Scientific Industries, Inc.) and added it to the 96-well plate (16.6 μl/well). Each MOI infection also has 6 secondary holes. After 72 hours of culture, add 100 μl of CCK8 solution (Cat.No.GH620) to each well, put it in the incubator for 4 hours, measure the absorbance value of 490nm wavelength with a microplate reader, calculate the cell survival rate, and draw the cell viability curve.

3. Results

3.1 Models

Two adenoviruses (Ad-TERT and Ad-TERT-E1A-E1B 55k) were developed as described in Material and Method. The Ad-TERT virus was designed to be non-replicable to test the original efficacy of the virus. The Ad-E1A-E1B 55k virus, as previously described, deleted 19k genes but remained a 19k promoter as an activator for 55k genes. Both modified adenoviruses have pDC316 plasmid as background and are homologous recombinant with Ad 5 origin. (Fig.1)

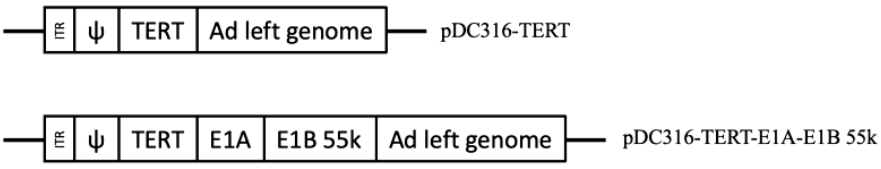


Fig. 1 Model of two modified adenovirus. Both viruses use pDC316 as their plasmid. Ad-TERT deleted the whole sections of E1A and E1B. It does not have a replication function. Ad-TERT-E1A-E1B 55k contains normal E1A genes, but the E1B 19k gene was deleted. It does have a replication function. By deleting the E1B 19k gene, this virus prevents from inhibiting apoptosis.

3.2 Evaluation of genomes of modeled mutant adenovirus

The virus gene sequences were verified by PCR amplification using primers previously described after the construction. (Fig. 2) The expression level of E1A in cells is around 1000 bp and of E1B 55k is around 1600 bp. In conclusion, the E1A and E1B 55k gene were successfully expressed and the genes had sequenced correctly.

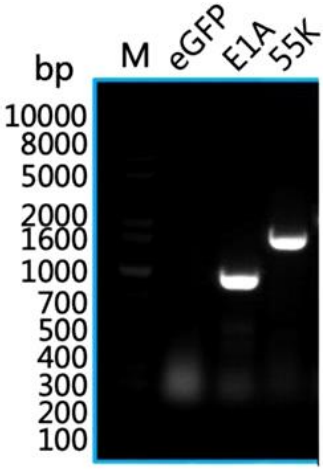


Fig.2 PCR product analysis of E1B 55k mutant adenoviruses. Left marker (TOYOBO KUREHA AMERICA CO., LTD.), 1-kb DNA ladder. eGFP was used as a negative control to exclude false positive results. The presence of each PCR product verified the presence of gene deletion.

3.3 Evaluation of protein expression by WB

To verify the correct sequence necessary for viral replication, proteins expressed by the E1A genome were evaluated by Western blot with an anti-E1A antibody. As shown in Figure 3, the expression of the E1A gene was found in cell lysates derived from A549 cells infected with Ad-E1B 55k adenovirus in contrast to MRC5 cells infected with the virus. Compared with marker control, as expected, bands were produced, which proves the expression of the protein. This result reflects that the E1A has a high expression in tumor cells compared with the expression in MRC5 cells. This indicates that E1A genome can produce the correct protein with tumor specificity and the modified virus has a controlled replication ability.

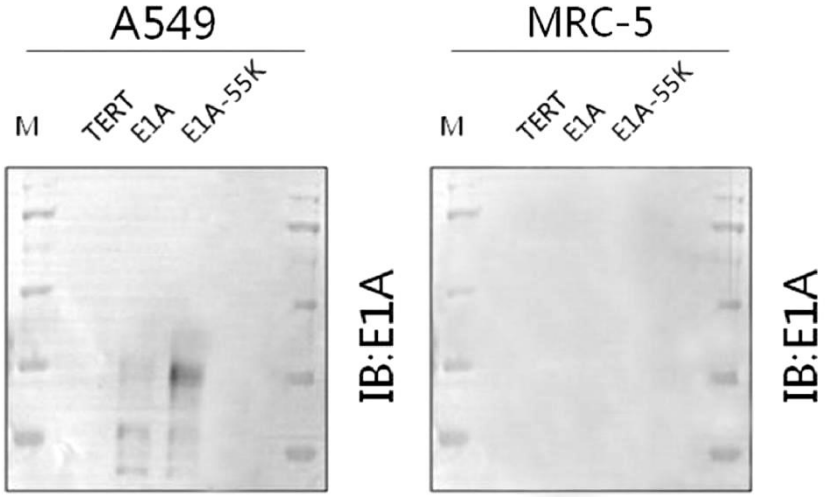


Fig. 3 Detection of E1A protein by WB analysis. 24 hours after infection, total protein from A549 cells infected with Ad- TERT and Ad-TERT-E1A-E1B 55k at a dose of MOI of 1 were analyzed with anti-E1A antibody as described in Material and Method. TERT promoter activates the viral expression in tumor cells, rather than in normal cells.

3.4 Evaluation of CPE for the efficacy of E1B 55k mutant adenovirus

To quantitatively assess adenovirus replication or non-replication dependent cell killing efficacy, cell cytotoxicity with different MOI concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4) was analyzed in vitro CPE assays (Fig. 4). Using the method described previously, graph 4 shows that both types of modified viruses contained cell killing efficacy. Ad-E1B 55k virus especially showed strong cytotoxicity of 50% of killing efficacy in A549 cells at MOI of 0.1. In addition, we examined the killing efficacy of viruses on MRC5 cells, HepG2 cells, and 549 cells with the same procedure above.

The result indicates that this virus has distinct killing suppression effects for human tumor cells and fewer effects against MRC5 cells. On day 5 after infection, cell viability in HepG2 for E1B 55k virus is around 80% while in MRC5 cell, the viability is above 100%, which showed a tumor-specificity of both viruses (Fig 5.A and 5.B). CPE also showed that the 55k virus has better efficacy than the TERT virus that the biggest difference reaches 80% (Fig 5.B and 5.C). In conclusion, CPE proved the strong tumor-specific selective killing functions and species specificity of both viruses for lung cancer (Fig 5). In particular, Ad-E1A-E1B 55k virus has better safety, specificity, and efficacy in tumor cells. It was chosen to enter an intracellular experiment in nude mice to further testify to its specificity to tumor cells, potency to kill infected cells, and safety to avoid severe side effects.

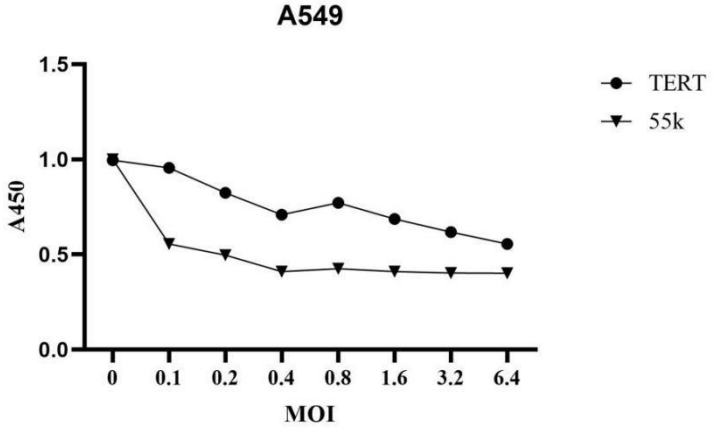
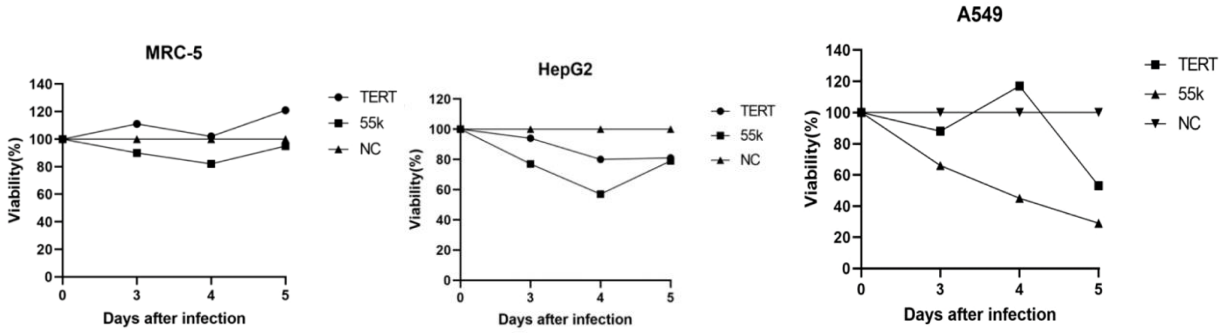


Fig. 4 CPE assays. Each well was measured to implant 3000 cells with DMEM and FBS. 24 hours later, monolayers of A549 cells were infected with Ad-TERT and Ad-E1B 55k at MOI of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4. The virus presented in cells has been amplified and titered by plaque assay. 48 hours later, clean the wells appropriately and add 10 μl of cck8 to testify the viability of A549 cells by Microplate Reader. In particular, adenovirus with MOI of 0.1 has nearly 50% killing efficacy.



(a) (b) (c)

Fig. 5 Viability of different cells with MOI of 1 of Ad-TERT and Ad-E1B 55k virus in days after infection.

All cells were implanted with 3000 cells. 24 hours after celling, both modified adenoviruses were implanted into the wells. Measure the viability on day 3, 4, and 5 to reveal the continuous killing efficacy of the adenovirus toward different cells. Shown in A) reflects the safety of both viruses in MRC5 normal cells. Only on day 4 has 20% of efficacy and the viability quickly increases back to normal on the next day, which indicates that the E1B 55k virus is relatively safe in that it does not cause severe destruction in MRC5 cells. TERT adenovirus instead increases the survival rate of MRC5 cells compared with NC control. HepG2 shown in B) reveals tumor specificity of two modified adenoviruses. Both viruses showed a decrease compared with NC control. The lowest viability of E1B 55k virus is around 60% while cells infected with TERT have around 80% of viability. This data shows a better tumor specificity of viruses in HepG2 cells compared with MRC5 cells. Shown in C) is the killing efficacy of both adenoviruses in A549. The E1B 55k virus has approximately 40% killing efficacy on day 3 and reaches nearly 60% on day 5. TERT adenovirus has

around 10% killing efficacy on day 3 and reaches 40% on day 5. In conclusion, the E1B 55k virus has a better steady-killing efficacy than TERT and should pursue in animal trials.

In conclusion, oncolytic therapy has and will demonstrate a profound impact on drug development for cancer treatment. A new agent with a deletion of the E1B 19k gene revealed a relatively efficient performance for transporting and executing the apoptosis of cancer. Most importantly, the success of developing a new agent indicates a new starting point for future scientists when they develop a more effective oncolytic therapy.

4. Conclusion

Despite the successful testing result, the new agent's efficacy, specificity, and safety haven't reached their maximum and can be improved. Due to the limitation of time and equipment, this study did not do animal trials or modify the skeleton vector. Studies have experimented with P53 tumor immunity suppressor with the deletion of the adenoviral E1b-55kD gene at the adenovirus type 5 site to target viral replication in tumor cells [15]. Proteins formed from the P53 gene can inhibit the formation of tumors by manipulating DNA repair or apoptosis to prevent the propagation of cells with serious DNA damage [16]. Additional obstruction of tumor growth may help to shorten the apoptosis procession. Other studies showed an adjustment on a particular fiber site of the skeleton vector. The tumor specificity of the virus can be achieved by modifying fiber receptors on the vector, like F35, that preferably binding with the CD46 receptor of tumor cells, but not the cellular Coxsackie B Adenovirus Receptor (CAR) receptor of normal cells [17]. As this study proved a relatively safe and steady functions of pDC316-E1A-E1B 55k, a combination of pDC316-E1A-E1B 55k in shuttle vector and Ad5-p53 gene with F35 fiber in and on the skeleton vector may become a new effective, safe and effective drug for future cancer treatment.

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