Research Article

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Prostate Cancer-Specific of DD3-driven oncolytic virus-harboring mK5 gene

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Abstract: Prostate cancer (PCa) is the second most diagnosed cancer in Western male population. In this study, we insert mK5 (the mutational kringle5 of human plasminogen) into a DD3-promoted (differential display code 3) oncolytic adenovirus to construct OncoAd.mK5.DD3.E1A.dE1B, briefly, O^{Ad}.DD3.mK5. DD3 is one of the most prostate cancer specific promoters which can transcriptionally control adenoviral replication. mK5 has been proved to be able to inhibit the tumor angiogenesis and inhibit cell proliferation. Our results suggested that targeting PCa with O^{Ad}.DD3.mK5 elicited strong antitumor effect.

Keywords: Oncolytic adenovirus; mK5; Prostate cancer; Angiogenesis

1 Introduction

Prostate cancer is the most major cause of morbidity and mortality rates for males in Western Societies [1,2]. Now, the rate of prostate cancer is gradually increasing in China. The traditional therapy for patients with the early-stage PCa is androgen deprivation therapy or chemotherapy. However, clinical improvement and tumor regression are temporary, and inevitably some of these patients progresses to androgen-independent and metastatic PCa. Right now, there is no curative treatment for patients with androgen-independent and advanced PCa.

In recent years, the oncology virus has gradually become one of the hotspots for anti-tumor research. The oncolytic adenovirus has been used as a most effective transfer vector with inserted therapeutic gene for tumor cells. It provides a promising treatment to PCa patients. Thus, in this study, we used replicating oncolytic adenovirus vector to express mK5 gene.

mK5, is the mutated K5 (the kringle5 of human plasminogen). Plasminogen is one of the key proenzyme precursors in the process of fibrin and thrombus were dissolved. Plasminogen contains 5 kringles. It has been discovered that kringle5 has more potent antiangiogenic effect [3-5]. Angiogenesis plays a key role in the development of a wide variety of malignant tumors. When the tumor grows, it needs to provide nutrition, oxygenation and should be metabolized by blood vessels. Angiogenesis is a rate-limiting step in tumor progression, so this strategy of targeting antiangiogenesis by inhibiting the formation of new blood vessels is rational [6,7]. On this basis, scientists mutated the kringle5, which replace Leu71 by arginine led to highly enhanced affinity of the ω-amino acid ligands for the LBS of K5 [8]. It showed that mK5 embraced stronger inhibition on angiogenesis [5]. We constructed a DD3-promoted oncolytic adenovirus expressing mK5 (O^{Ad}.DD3.mK5). DD3 promoter to control E1A gene expression shows specific antitumor effect on PCa. The O^{Ad}.DD3.mK5 was constructed to delete E1B containing E1B55KD and E1B19KD. E1B55KD protein facilitates prior transport of late stages viral RNA in tumor cells. Conversely, reduced replication of O^{Ad}.DD3.mK5 in normal cells results from defective exports of late viral RNA. In this paper, we researched the anti-tumor effect of O^{Ad}.DD3.mK5 both in vitro and vivo. And, our results showed that O^{Ad}.DD3. mK5 can powerfully eliminate prostate cancer cell line-LNCaP xenograft tumor in nude mice.

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Analysis and interpretation of date (e.g., statistical analysis, biostatistics, computational analysis): Jiali Hao, Hui Li
Writing, review, or revision of the manuscript: Jiali Hao, Wenjie Xie
Administrative, technical, or material support: Runsheng Li

2 Materials and methods

2.1 Cells and cell cultures

These cell lines Hela, A549, LNCaP, 22RV1, WPMY-1 were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The HEK-293 cell line was obtained from Microbix Biosystems Inc (Toronto, Canada). The human primary prostatic fibroblast and human primary liver fibroblast cell lines were purchased from the iCell Bioscience Inc (Shanghai, China). All cell lines were cultured in manufacturer's instructed medium supplemented with 10% foetal bovine serum (FBS, GIBCO, California, USA).

2.2 Adenovirus construction

The mK5 expression cassette was inserted into a deleted-E1B gene region of plasmid vector (pShuttle-DD3-E1A-dE1B). Oncolytic adenovirus plasmids were generated by shuttle vector and the adenoviral backbone plasmid in E.coli BJ5183 cells [9]. Oncolytic adenoviruses were packaged and amplified in HEK-293 cells, and viruses purified by gradient CsCl centrifugation. The viruses titer was measured using the Quick Titer Adenovirus Titer Immunoassay Kit (Cell Biolabs, San Diego, CA, USA).

2.3 Adenovirus identification

Viral genomic DNA was extracted for viral identification using the Blood Genome Extract Kit according to the manufacturer's instructions (Generay, Shanghai, China). The mK5 gene expression cassette was identified by PCR using mK5 expression cassette primers (forward: GGTACCGCGGCCGCCTCGAG and reverse: CTCGAGATTGCATCGTCGAC). The existence of wild-type contaminants was identified by PCR using E1A-promoter primers (forward: GCGTAATATTTGTCTAGGG and reverse: CGGGTATAAATACACTACAC) and E1B primers (forward: CGTGTAGTGTATTTATACC and reverse: GTGGCAGATAATATGTCT).

2.4 Western blot analysis

Cells were seeded in 6-well plates and infected with O^{Ad}.DD3.mK5 at 5 MOI. After 48h, the cells were harvested. The protein concentration levels were measured by the Lowry assay (BioRad, Hercules, CA, USA) using manufacturer's instructions. Western blot was performed according to standard protocols [10]. Antibodies information as follows: anti-GAPDH (CWBIO, Beijing, China); anti-E1A (Santa Cruz biotechnology, Santa Cruz, CA, USA).

2.5 Quantitative RT-PCR

Total RNA was extracted after 48h being infected with O^{Ad}.DD3.mK5 at 5 MOI using TRIzol (CWBIO, Beijing, China). Single-strand cDNA was synthesized using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). RNA expression was analyzed using SuperReal Premix Plus (TIANGEN, Beijing, China) according to the manufacturer's protocols. Each date was performed in triplicate assay. The sequences of all primers as follows: q-E1A primers (forward: GTGATAGAGTGGTGGGTTTGG and reverse: TCAGGCTCAGGTTCAGACA); q-GAPDH primers (forward: ACCCAGAAGACTGTGGATGG and reverse: TCTAGACGGCAGGTCAGGTC); q-mK5 primers (forward: GTTACGACTACTGTGATG and reverse: CGATCCAGACATGATAAG).

2.6 Proliferation assays

MTT (Amresco, USA) was used to measure cell proliferation. A total of 3000 cells were seeded in 96-well plates and infected with O^{Ad}.DD3.mK5 at a series of MOIs. Cells were cultured appointed times. After 4h of incubation with MTT solution, the medium of 96-well plates was removed and the cells were stained with150 μl DMSO. The absorbance was determined using microplate reader at 595 and 630 nm [11].

2.7 Animal experiments

All animal experiments were performed according to protocols approved by the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. Three or four-week-old female BALB/c nude mice were purchased from the Animal Core Facility (Shanghai, China).

In tumorigenicity assay, 5×10^6 LNCaP cells were mixed with Matrigel (BD, Biosciences) at a 2:1 ratio and subcutaneously injected into the subaxillary regions of each mouse. When the tumors size reached about 80 mm^3, the animals were randomly divided into PBS or O^{Ad}.DD3.mK5 (six mice per group). O^{Ad}.DD3.mK5(2.5×10^8 PFU per mouse) or PBS was intratumorally injected every other day

for four times. The tumor volume of the six mice in each group was measured every three days and estimated using the following equation: (length × width2)/2 [12,13]. At the end of the experiment, mice were killed and the tumors were resected for H&E staining and immunofluorescence staining analyses [14-16].

2.8 Histopathology, IHC and TUNEL assay

Tumor tissues were harvested at the end of experiment and tissues were fixed in 4% paraformaldehyde (PFA) overnight, embedded in paraffin, sliced into 5 μm sections and stained with hematoxylin and eosin (H&E) for histological examination. Tumor tissues were OTC- embedded and sliced into 8μm sections. Frozen tumor sections were fixed in 4% PFA 30-60min, washed with PBS and incubated with 0.5%TritonX-100 to eliminate endogenous peroxidase activity. Then cryosections were blocked with the BSA (5%) and were stained with an anti-CD31 antibody (BD, USA) diluted to 1:400. The TUNEL assays were performed according to the manufacturer's instructions (Beyotime, china) [17,18].

2.9 Statistical analysis

All data are presented as the mean ± standard deviation (S.D). Comparisons between two groups were performed with Student's t-tests and the GraphPad Prism 6.0 software (GraphPad Software, LaJolla, CA, USA).

3 Result

3.1 Construction and identification of O^{Ad}.DD3.mK5

We constructed an oncolytic adenovirus that carried the mK5 gene, whose expression was driven by the differential display code 3 (DD3), one of the most prostate cancer specific promoters. (Figure 1A). PCR analysis of the E1B region and E1A-promoter region were free of wild-type adenovirus contaminations. Similarly, mK5 is proved to be inserted into the designated position by correct primers (Figure 1B-D).

The above results showed that we succeeded in packaging the oncolytic adenovirus O^{Ad}.DD3.mK5. In the subsequent experiments, we will do an in-depth study on its cancer inhibition.

3.2 O^{Ad}.DD3.mK5 exhibits enhanced replication in PCa cell lines

To research the characterization of O^{Ad}.DD3.mK5 in PCa, we compared the abilities of the O^{Ad}.DD3.mK5 to replicate in PCa cell lines and non-PCa cell lines. LNCaP, 22RV1, A549 and Hela cell lines were infected with O^{Ad}.DD3.mK5 at an MOI of 5. The replication efficacy of the adenovirus was estimated by qRT-PCR of adenovirus gene E1A cDNA. We measured more expression of E1A in LNCaP and 22RV1 cells. These results showed that O^{Ad}.DD3.mK5 replicated further effectively in PCa cell lines (Figure 2A-B). It is exhibited that O^{Ad}.DD3.mK5 selectively replicated in PCa cell lines. To further characterize them, the expressions of E1A also was examined in LNCaP, 22RV1, A549 and Hela infected with O^{Ad}.DD3.mK5 (5MOI, 48h) by Western blot. As shown in Figure 2C, the expression of E1A in LNCaP and 22RV1 treated with O^{Ad}.DD3.mK5 were higher than A549 and Hela cell lines. Likewise, the expression of the mK5 gene was measured in LNCaP, 22RV1, A549 and Hela (with O^{Ad}.DD3.mK5 at 5MOI for 48h) by qRT-PCR. We also found that the expression of the mK5 was higher in the PCa cell lines (Figure 2D).

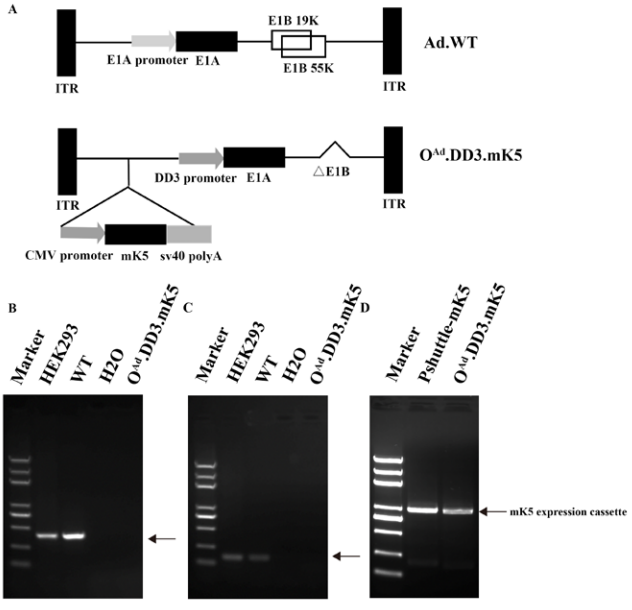

Figure 1: Construction and characterization of O^{Ad}.DD3.mK5. A. Schematic diagram of the O^{Ad}.DD3.mK5. The mK5 expression cassette was inserted into O^{Ad} to construct O^{Ad}.DD3.mK5. ITR, inverted terminal repeats. B-D. Detection of oncolytic adenoviruses by PCR using the E1A-promoter primers (B), E1B primers (C) and mK5 expression cassette primers (D). HEK293, WT and Pshuttle-mK5 were used as positive control. H_2O was used as negative control.

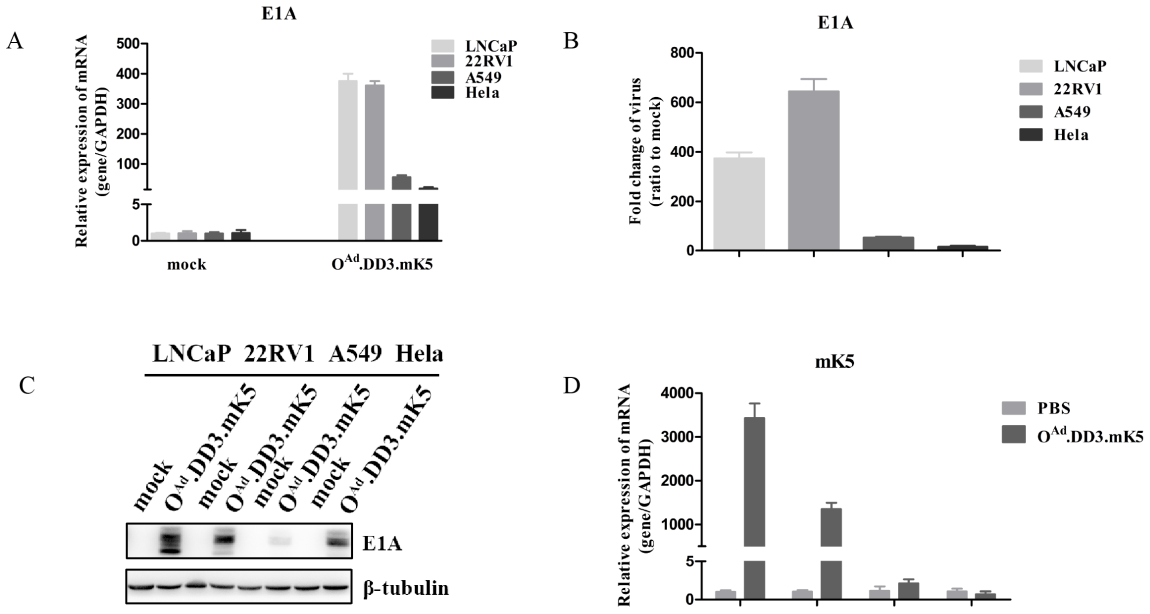

Figure 2: O^{Ad}.DD3.mK5 largely replicated in PCa tumor cells. A. Analysis of E1A in LNCaP, 22RV1, A549 and Hela cell lines treated with O^{Ad}.DD3.mK5 at an MOI of 5 for 48 hours by qRT-PCR. B. Date A are processed as the fold change relative to mock cells. C. Expression of E1A measured by western blot. D. The expression of the mk5 in four cancer cell lines LNCaP, 22RV1, A549 and Hela was detected using qRT-PCR. The qRT-PCR data were normalized to GAPDH RNA. All experiments were repeated three times. The bars represent the mean ± S.D (n = 3).

3.3 O^{Ad}.DD3.mK5 displays specific antitumor activity to prostate cancer cells in vitro

Next, we investigate the cytotoxicity of O^{Ad}.DD3.mK5 in vitro by MTT assay. LNCaP and 22RV1 cells were infected with O^{Ad}.DD3.mK5 at various MOIs from 0.01–20. Cell viability was measured after 4 days (Figure 3A-B). The results showed that O^{Ad}.DD3.mK5 had a stronger antitumor effect in LNCaP and 22RV1 cells in a dose-dependent manner, especially in LNCaP cell. Further, LNCaP and 22RV1 were infected with the O^{Ad}.DD3.mK5 at an MOI of 10 for 4 days (Figure 3C-D). All of these assays came to same conclusion: O^{Ad}.DD3.mK5 had significantly greater antitumor efficacy in LNCaP and 22RV1 cells.

To evaluate the cytotoxicity specificity of O^{Ad}.DD3.mK5, cell viability also was measured in A549 and Hela cells with virus at a series of MOIs. As shown in Figure 3E-F, a rare cytopathic effect of O^{Ad}.DD3.mK5 was observed in A549 and Hela cells (even at an MOI of 50, Hela only was killed a little). These data indicated that O^{Ad}.DD3.mK5 was unable to significantly inhibit growth of non-PCa cells.

3.4 Safety measure of O^{Ad}.DD3.mK5

The above-mentioned data had showed that significant decreases in cell viability occurred after infected O^{Ad}.DD3.mK5 in PCa cells. For safety, we measured the normal prostate mesenchymal cell (WPMY-1). We examined the cell viability under the different treatments via the MTT method (Figure 4A-B). Encouragingly, merely weak slightly cytotoxic effect of O^{Ad}.DD3.mK5 was observed in WPMY-1 cell.

In a similar assay, we also measured the cytotoxicity of O^{Ad}.DD3.mK5 in human primary prostatic fibroblast and human primary liver fibroblast cells (Figure 4C-D). The results showed that O^{Ad}.DD3.mK5 had little killing effect on primary cells.

3.5 O^{Ad}.DD3.mK5 suppresses PCa tumor growth in vivo

To examine the antitumor efficacy of oncolytic adenoviruses in vivo, O^{Ad}.DD3.mK5 and corresponding volumes of PBS were intratumorally injected in LNCaP xenograft. The data showed that O^{Ad}.DD3.mK5 can suppress tumor growth rate compared with PBS. The tumor growth was suppressed on the O^{Ad}.DD3.mK5-treated group in which the tumors of 3 mice (n = 6) completely disappeared about 43 days after injection (Figure 5A). Next, those mice were observed for an additional 120 days and had a slight degree of relapses (Figure 5C), and all the 6 mice were alive at the end of the experiment. We also measured the mouse body weight lasted 160 days. The results showed that the O^{Ad}.DD3.mK5-treated mice had a higher body weight, which

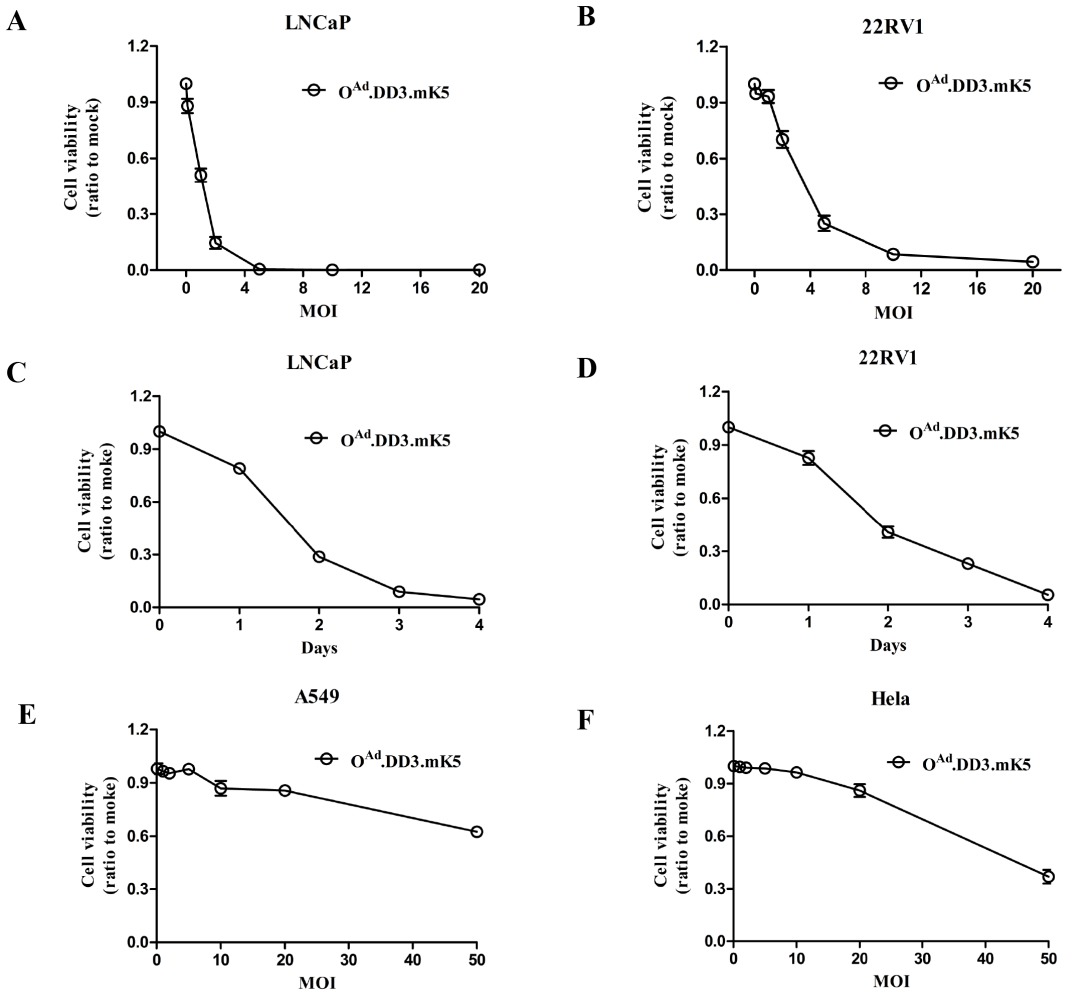

Figure 3: O^{Ad}.DD3.mK5 displays antitumor activity in vitro. A-B. Cell viability of LNCaP and 22RV1 cells infected with O^{Ad}.DD3.mK5 at a series of MOIs on day 4. C-D. Cell viability of LNCaP and 22RV1 cells infected with O^{Ad}.DD3.mK5 for 4 days at 10MOI. E-F. Cell viability of A549 and Hela cells infected with O^{Ad}.DD3.mK5 at various MOIs on day 4. All date were repeated three times. The bars represent the mean ± S.D. (n = 3).

indicated that the injection of O^{Ad}.DD3.mK5 had no significant effect on the living condition of mice (Figure 5B). As shown in Figure 5D, volume of tumors were measured after 160 days treated with O^{Ad}.DD3.mK5 or PBS.

3.6 Immunohistochemistry revealed the antitumor mechanism of O^{Ad}. DD3.mK5

The antiangiogenesis and inhibition of cell proliferation effects of O^{Ad}.DD3.mK5 in LNCaP xenografts were analyzed using CD31 immunofluorescence staining and TUNEL staining, respectively. The number of positive cells for endothelial marker CD31 in untreated mice, and a few positive CD31 cells were observed in treated animals with O^{Ad}.DD3.mK5(Figure 6A-B). These results support a notion that O^{Ad}.DD3.mK5 suppressing tumor growth via inhibiting tumor angiogenesis. A superior percentage of TUNEL positive cells were observed in the O^{Ad}.DD3.mK5 treated tumors than in the PBS groups (Figure 6C-D).

Furthermore, histopathological analysis using H&E staining showed that O^{Ad}.DD3.mK5 induced less levels of cell death and necrosis within the tumor mass than PBS. And, compared to untreated animals or treated animals, tumors from O^{Ad}.DD3.mK5-treated mice tumor tissue contained fewer blood vessels (Figure 6E).

4 Discussion

Prostate cancer is the second most numerous cancer of male. Symptoms of early stage prostate cancer are obscure and the process of prostate cancer can last over ten years. So, when patients were diagnosed with prostate cancer, they also have been developed to an advanced stage in

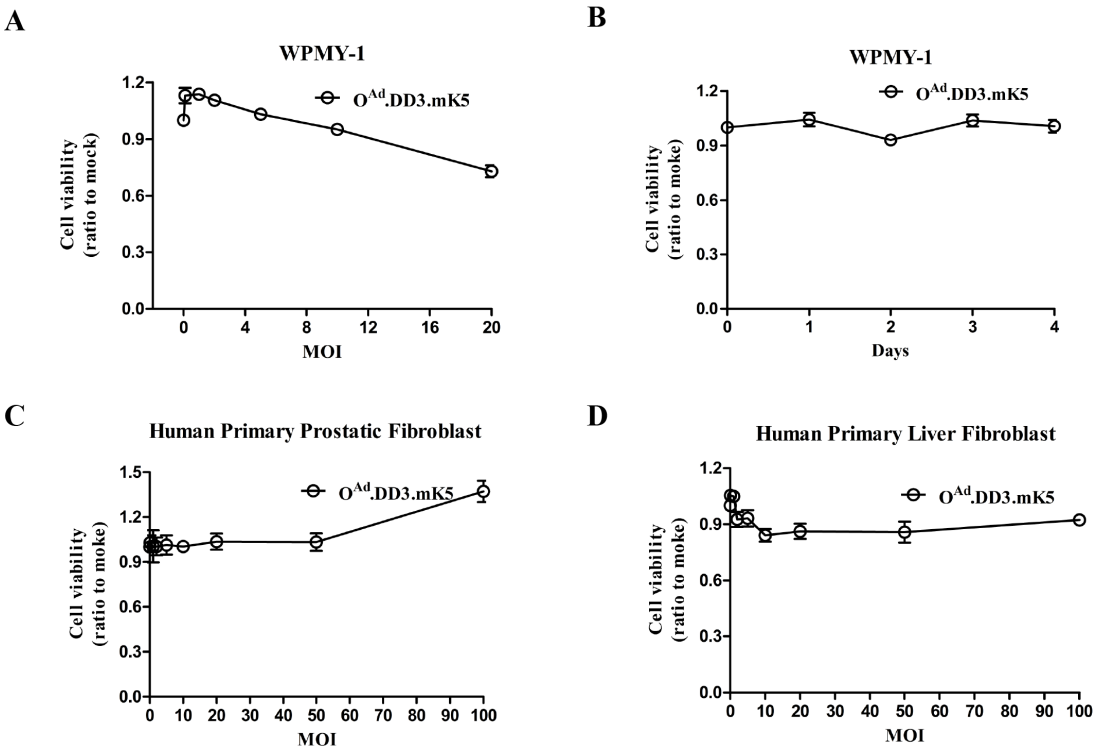

Figure 4: Safety measure of O^{Ad}.DD3.mK5. A. Cell viability of WPMY-1 cell infected with O^{Ad}.DD3.mK5 at a series of MOIs on day 4. B. Cell viability of WPMY-1 cell infected with O^{Ad}.DD3.mK5 at 10MOI for 4 days. C-D. Cell viability of human primary prostatic fibroblast and human primary liver fibroblast cells infected with O^{Ad}.DD3.mK5 at various MOIs on day 4. All date were repeated three times. The bars represent the mean ± S.D. (n = 3).

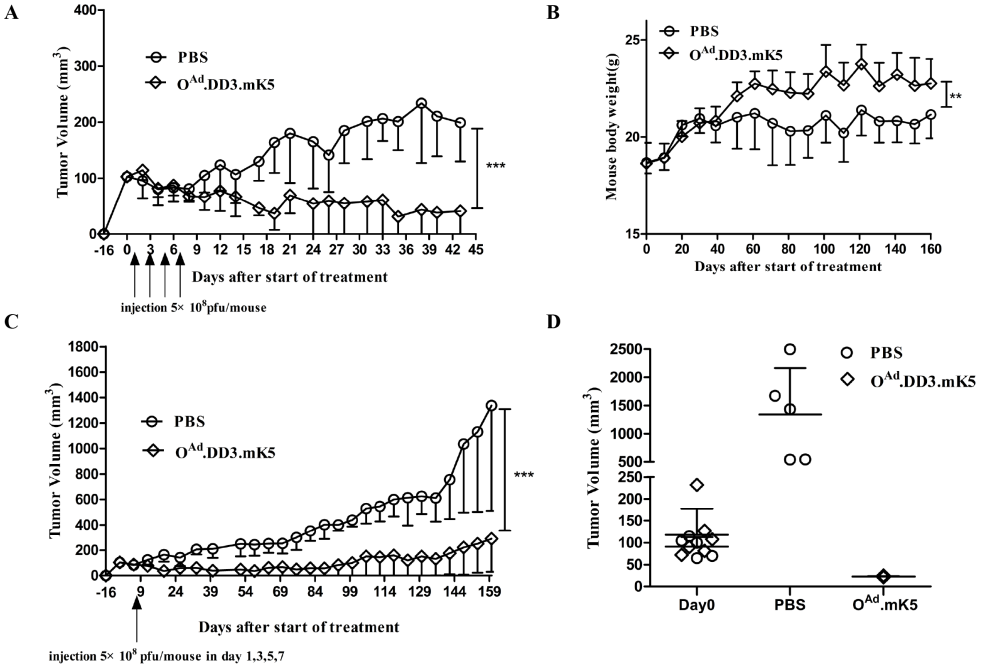

Figure 5: O^{Ad}.DD3.mK5 displays antitumor activity in vivo. A, C. Tumor growth curve of LNCaP xenograft tumors after intratumoral injection of 5×10^8 pfu/mouse adenoviruses . O^{Ad}.DD3.mK5 or PBS were injected 16 days after subcutaneous inoculation of LNCaP cells. B. Body weight of mouse after initial injection. D. Volume of tumors that grew in LNCaP mice to initial volume. After that, mice were treated with PBS or O^{Ad}.DD3.mK5 for 160 days. Data are shown as the mean ± S.D. *P < 0.05, **P < 0.01, ***P < 0.001.

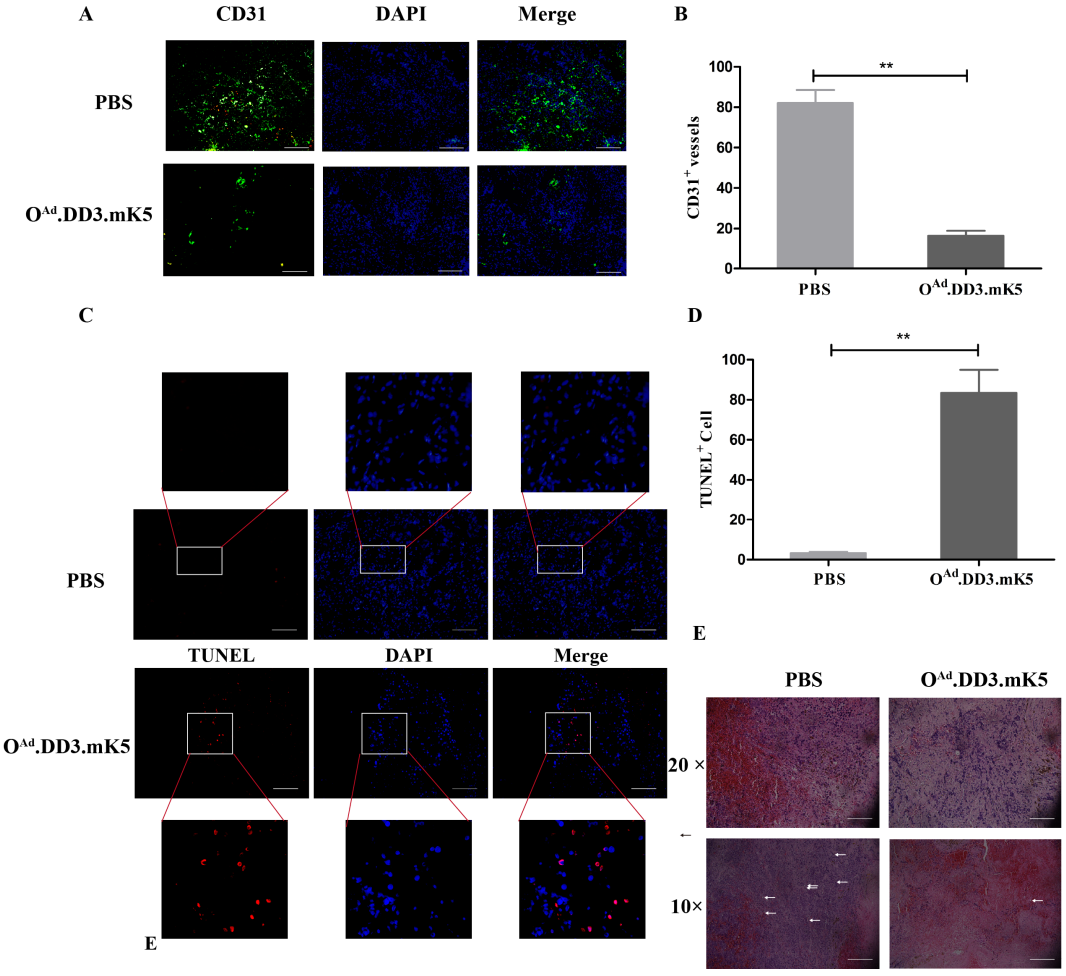

Figure 6: Anti-angiogenic and anti-cell proliferation effects of O^{Ad}.DD3.mK5 in LNCaP mouse model. A.CD31 immunofluorescence staining of tumors tissue isolated from mice after O^{Ad}.DD3.mK5 or PBS treatment. B. Quantification on the positive CD31+ vessels number (n=3). C. Representative images of TUNEL staining from tumor tissue after O^{Ad}.DD3.mK5 or PBS treatment. D. Quantification of the TUNEL staining(n=3). E. H&E staining of the tumors tissue. Arrows indicate the vessels in tumor tissue. Scale bar, 200 μm. All experiments date were repeated three times, and the bars represent the mean ± S.D. (n = 3). **P<0.01.

the majority of instances, which leading to the poor prognosis of patients [19-21]. Limited by the condition that surgical method and chemoradiotherapy are helpless to the advanced prostate cancer patients, the 5-year overall survival rate is lower than 60% [22]. Oncolytic virus has showed the powerful antitumor effect in melanoma, lung cancer and multiple other cancers [23-25]. In this study, we proved a modified oncolytic virus carry a heterologous gene (mK5) can significant killing prostate cancer in vivo or in vitro.

The first time when the concept of oncolytic virus brought up was 1950s. After half a century development, the varies of strategies were put forward to optimize the oncolytic virus therapy. All of these strategies were mainly concentrated on two areas, improve the tumor specificity and the cancer-killing effect [26]. Ad5 is the most common human host virus used as vehicle to carry the heterologous gene to target cancer cells. ZD55 was reported previously, then we exchanged ZD55 E1A promotor to DD3 to improve tumor specificity of O^{Ad}.DD3.mK5. A 214-bp fragment of the DD3 core promoter has a high promoter activity, which highly overexpression in most types of prostate cancer, activated by androgen-receptor sensitively. The specificity of this promoter has been confirmed by the safety test. The DD3 promoter also can improve replication of adenovirus in prostate cancer cell, which significantly enhance the cytotoxicity effect.

Even a lot of the oncolytic virus therapy product have been launched, the clinical effect of virus were still in doubt. Therefore, the “Targeting Gene-Viro Therapy” suggests that oncolytic virus combined with gene therapy may more effective to help the cancer treatment than just inject a simple oncolytic virus alone. K5 play the key role in multiple cancers, mK5 is better than K5[27,28]. The

O^{Ad}.DD3.mK5 showed have more effective to inhibit the angiogenesis and cancer cell proliferation in tumor [18,29]. The immunofluorescence staining and H&E staining also confirmed this effect. In vivo test showed that the O^{Ad}.DD3.mK5 can stop the xenografts tumor growth after injected immediately. However, the mechanism by which O^{Ad}.DD3.mK5 in the inhibition of tumor angiogenesis and proliferation still requires validation with additional experiments

We also try to determine the long-term safety and antitumor effect of O^{Ad}.DD3.mK5 by measuring the tumor size and body weight of mice until 160 days since O^{Ad}.DD3.mK injection. The extended animal experiment was designed to detect whether the tumor would recur after eliminated. The results illustrate O^{Ad}.DD3.mK5 can control the tumor development for an excessively long time, and nearly complete unaffected of mice living condition. But the antiviral system is activated by CD8+ T cell and the nude mice are lack of innate immune system [30]. The treatment effect in the model with whole immune system may different. So, the detailed mechanism and killing effect in immunized model are needed further investigate.

Although O^{Ad}.DD3.mK5 showed significant antitumor effects in our study, there are also some limitations to be solved before O^{Ad}.DD3.mK5 can be further used in clinical trials for prostate cancer patients, especially when it is delivered via intravenous injection. The existing limitations mainly include liver damage caused by virus accumulation and the clearance of viral particles by neutralizing antibodies, immune responses against the virus, and the influence of the tumor microenvironment on viral replication [16]. To address these problems, combined treatment with chemoradiotherapy, immunotherapy, packaging or modification via nanoparticles [17] had been used for the treatment of the oncolytic virus.

In summary, we construct a new oncolytic virus carry the human mK5 gene. These viruses can effective remission the tumor development and kill the prostate cancer cell in vivo and in vitro, which shows to have potential for clinical therapy.

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