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In vitro and *in vivo* antitumor activities of oHSV-BJ-2 oncolytic virus

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ABSTRACT

Objective: To construct an oncolytic herpes simplex virus oHSV-BJ-2 and to evaluate its safety and antitumor efficacy.

Methods: Using the HSV-1 wild-type KOS strain as the backbone, we constructed an oncolytic herpes simplex virus, oHSV-BJ-2. The CCK-8 method was used to detect the killing effect of the virus on eight tumor cell lines, and the *in vivo* tumor inhibitory effect of the virus was evaluated in the BALB/c nude mouse subcutaneous transplant tumor model (PANC-1, SW1990).

Results: oHSV-BJ-2 showed a significant killing effect on seven tumor cell lines in a time- and dose-dependent manner. In the PANC-1 and SW1990 pancreatic cancer xenograft tumor models, intratumoral injection of oHSV-BJ-2 could significantly inhibit tumor growth, with the tumor inhibition rate reaching 70%-80% in the medium and high-dose groups, without a significant impact on the body weight of the mice.

Conclusions: oHSV-BJ-2 has good safety and significant antitumor activity, and could be explored as a new drug candidate for the treatment of pancreatic cancer and other malignant tumors.

KEYWORDS: Oncolytic virus; HSV-1; ICP27; Viral therapy; Tumor specific promoter; *In vitro*; *In vivo*; oHSV-BJ-2; KOS; Mice

1. Introduction

Cancer is one of the leading causes of death worldwide. It exhibits biological characteristics such as abnormal cell differentiation and proliferation, invasive growth, high metastatic potential, and frequent recurrence after treatment. Its pathogenesis involves a complex, multifactorial, and multistep process. Although traditional therapies including surgical resection, chemotherapy, and radiotherapy play crucial roles in oncology treatment, each approach has significant limitations: Surgical intervention requires tumor accessibility and often carries postoperative recurrence risks; Chemotherapy not only

targets cancer cells but also affects normal dividing cells, leading to severe side effects and drug resistance issues; Radiotherapy, while capable of localized radiation delivery, faces limited applicability due to radiation insensitivity in many tumor types[1–3].

In recent years, oncolytic virus therapy has garnered significant attention as an emerging biologic treatment modality due to its unique tumor-selective killing mechanism and favorable safety profile. This therapeutic approach utilizes oncolytic viruses with cytotoxic activity against tumor cells for cancer treatment. Oncolytic viruses selectively replicate and destroy tumor cells while remaining incapable of replication or infection in normal cells, thereby causing no harm to healthy tissues[4].

Summary

Question: Can the newly constructed oncolytic herpes simplex virus oHSV-BJ-2 selectively kill tumor cells while maintaining safety in normal tissues, and what mechanisms support its antitumor activity?

Findings: An oncolytic herpes simplex virus, oHSV-BJ-2, exhibited potent cytotoxicity against seven of eight tested tumor cell lines and significantly inhibited pancreatic tumor growth in PANC-1 and SW1990 xenograft models, achieving tumor inhibition rates of approximately 70%-80% in medium- and high-dose treatment groups.

Meaning: oHSV-BJ-2 represents a promising next-generation HSV-1 oncolytic virus platform with potential therapeutic applications for pancreatic cancer and other solid tumors.

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Herpes simplex virus type 1 (HSV-1) possesses unique advantages among various oncolytic virus vectors: its large genomic capacity contains numerous non-essential genes, providing ample space for exogenous gene insertion[5]; after viral infection of cells, the DNA remains in a free state without insertion-induced mutations, demonstrating excellent safety profiles; clinical availability of antiviral agents such as acyclovir and ganciclovir for HSV infection enables controlled viral therapy processes; additionally, HSV-1 can infect multiple animal species, facilitating evaluation in xenograft tumor models. These characteristics make HSV-1 a pivotal platform for oncolytic virus development. In 2015, the U.S. FDA approved the first oncolytic virus drug, T-VEC, for melanoma treatment—a HSV-1-based modification[6] marking a significant milestone in transitioning oncolytic virus therapy from laboratory research to clinical application.

T-VEC achieves tumor-selective replication by deleting the ICP34.5 neurotoxicity gene[7]. However, recent studies have revealed that certain tumor cells retain specific antiviral mechanisms, which may compromise the replication efficiency of ICP34.5-deficient viruses[8]. Other strategies for HSV-1 tumor-specific replication include using tumor-specific promoters to drive expression of viral essential genes[9] and inserting miRNA target sequences into viral genes to restrict replication in normal cells[10]. These strategies, however, each have limitations: promoter replacement may disrupt temporal regulation of viral gene expression, while pure miRNA target sequence strategies are only applicable to tumor types with specific miRNA expression profiles[11].

ICP27 is an immediate early protein essential for HSV-1 replication, encoded by the *UL54* gene, and plays a critical role in the transition of viral gene expression from early to late stages[12]. Studies have demonstrated that once ICP27 expression is impaired or disrupted, HSV replication capacity is significantly reduced or completely lost[13]. Based on this characteristic, patent CN110684743A proposes a universal oncolytic virus construction strategy: replacing the natural promoter of essential viral genes with exogenous tumor-specific promoters (such as hTERT), so that the essential genes are transcribed and expressed only in tumor cells but not in normal cells, thereby achieving tumor-specific replication of the virus. This strategy retains the integrity of the viral genome and the original expression timing, and theoretically has better replication ability and broad-spectrum antitumor activity.

Based on the abovementioned patent strategy, this study uses the HSV-1 wild-type KOS strain as the backbone, replaces the natural promoter of its essential gene *ICP27* with the human telomerase reverse transcriptase (hTERT) promoter, and connects the cytomegalovirus (CMV) enhancer in series to enhance the transcription activity. At the same time, in order to further enhance the antitumor immune response, a human granulocyte-macrophage colony-stimulating factor (GMCSF) expression cassette was

inserted into the viral genome, driven by the viral late promoter (gD promoter), so that GMCSF is only expressed when the virus replicates (*i.e.*, in tumor cells). This study evaluated the safety and antitumor effect of the recombinant virus in both *in vitro* and *in vivo* studies.

2. Methods

2.1. Cells and viruses

Vero (African green monkey kidney cells), ARPE-19 (human retinal pigment epithelial cells), and all tumor cell lines (FaDu human pharyngeal squamous cell carcinoma cells, PANC-1 human pancreatic cancer cells, A2058 human skin cancer cells, CAL-62 human thyroid cancer cells, KYSE-30 human esophageal squamous cell carcinoma cells, A875 human melanoma cells, SW1990 human pancreatic cancer cells, and KYSE-150 human esophageal squamous cell carcinoma cells) were purchased from ATCC or the Shanghai Cell Bank of the Chinese Academy of Sciences. The Vero-ICP27 cells were constructed and maintained in our laboratory, which stably express the ICP27 protein. The wild-type HSV-1 KOS strain was obtained from ATCC, and the enhanced green fluorescent protein (EGFP)-labeled virus was constructed in our laboratory.

2.2. Construction of recombinant plasmid

In order to replace the ICP27 natural promoter with the hTERT-CMV enhancer and insert the GMCSF expression cassette at the same time, two homology arms and insert fragments were designed.

5'homology arm: approximately 1.0 kb sequence upstream of the ICP27 natural promoter.

3'homology arm: approximately 1.0 kb sequence downstream of the start codon of the ICP27 open reading frame.

Insert sequence 1: hTERT promoter (approximately 456 bp) + CMV enhancer (approximately 66 bp), used to drive ICP27 transcription.

Insert sequence 2: HSV-1 gD promoter + Kozak sequence + GMCSF open reading frame + BGH poly(A), inserted into the non-coding region downstream of the ICP27 open reading frame.

After gene synthesis, the above fragments were sequentially cloned into the pUC19 backbone to obtain the recombinant shuttle plasmid.

2.3. Cell culture

Vero, ARPE-19, and all involved tumor cell lines were routinely cultured and subcultured in DMEM or RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% streptomycin, under constant temperature incubation at 37°C with 5% CO₂.

2.4. Construction and screening of oncolytic virus oHSV-BJ-2

2.4.1. Viral infection and cell transfection

Vero-ICP27 cells were pre-inoculated in a six-well plate and cultured until approximately 70% cell density before proceeding to the next step. Vero-ICP27 cells were infected with EGFP virus at different multiplicity of infection (MOI=0.1, 0.5, 1, and 3) and incubated for 1 h in a 37°C, 5% CO₂ incubator. The culture medium was aspirated from the six-well plate, and 4 µg of recombinant plasmid P1+2 was transfected per well according to the Lipofectamine 2000 protocol, with EGFP plasmid-transfected Vero-ICP27 cells used as controls. The six-well plates were then incubated in a CO₂ incubator for 4 h, after which the transfection solution was aspirated and replaced with serum-containing DMEM complete medium for continued culture for 24 h. Infected or transfected Vero-ICP27 cells were observed, and cells were collected for repeated freeze-thaw cycles to release recombinant viruses.

2.4.2. Screening of oncolytic virus oHSV-BJ-2

The collected viral suspension after construction was diluted and inoculated into Vero-ICP27 cells in a six-well plate, covered with 4 mL of 1.25% methylcellulose medium, and cultured for 3 d in a 37°C incubator with 5% CO₂. Under fluorescence microscopy, viral plaques that did not emit green fluorescence (indicating successful homologous recombination) were selected. The viral plaques were aspirated using a pipette and transferred to centrifuge tubes containing serum-free basic DMEM medium, followed by three cycles of freeze-thaw cycles to release the virus. Subsequent rounds of screening (second and third) were performed by infecting Vero-ICP27 cells with the harvested virus according to the aforementioned protocol.

2.5. Western blot detection

Vero cells (4×10⁵ cells/well) and ARPE-19 cells (6×10⁵ cells/well), inoculated in six-well plates, were infected with oHSV-BJ-2 or KOS at an MOI of 3. Cells were collected after 24 h, and Western blotting and IP cell lysates containing protease inhibitors were added. Ultrasonic disruption was performed at 100 W for 5 s, followed by 1-minute intervals, repeated 3 times. Supernatants were collected by centrifugation at 4°C. Protein concentrations were determined using the BCA method (PierceTM BCA Protein Assay Kit, Thermo Fisher). Then 15 µg of protein was subjected to SDS-PAGE electrophoresis (4%-20% precast gel, BBI), transferred to PVDF membranes, and blocked with 5% skim milk for 1 h. ICP27 antibody (Santa Cruz, sc-69806, diluted 1:250) and β-actin antibody (CST, 3700S, diluted 1:1 000) were incubated separately at 4°C overnight. HRP-labeled secondary antibodies (Abcam, ab205719, diluted

1:1 000) were incubated at room temperature for 1 h, followed by chemiluminescence development.

2.6. Virus plaque formation assay

Vero cells and ARPE-19 cells were seeded in six-well plates at densities of 2×10⁵ and 3×10⁵ per well, respectively, and cultured overnight. Subsequently, cells were infected with 1 mL of oHSV-BJ-2 or wild-type KOS virus at titers of 40, 400, and 4 000 plaque-forming units (PFU), respectively. The virus was adsorbed at 37°C for 1 h, during which the six-well plate was gently shaken every 15 min. After the adsorption was completed, the virus solution was washed off, and 3 mL of DMEM covering medium containing 1.25% methylcellulose and 2% FBS was added to each well. The six-well plate was incubated in a 37°C, 5% CO₂ incubator for 4 d.

Viral plaques were observed at 40× magnification using an inverted phase contrast microscope (Nikon Eclipse Ti2; Nikon Corporation, Tokyo, Japan). Plaques are identified as clear circular areas of cell lysis surrounded by intact cell monolayers. For quantitative analysis, in the 40 PFU-infected group, the number of plaques was manually counted under a microscope, and a total of three independent experiments were performed.

After microscopic observation was completed, the methylcellulose coating was removed, and the cell monolayer was gently washed with PBS. Crystal violet staining was then performed to reveal plaques. Cells were stained with 0.1% crystal violet solution (prepared in 20% ethanol) for 10 min at room temperature. The dye liquor was removed, then the cells were washed with distilled water to remove excess dye liquor. The six-well plate was dried. After staining, the virus plaques in the six-well plate were visible to the naked eye, and the plaques appeared as unstained white circular areas against the background of a purple-stained cell monolayer. All experiments were set up with two replicate wells and repeated at least three times independently.

2.7. CCK-8 cytotoxicity assay

Eight tumor cell lines were seeded in 96-well plates and infected with oHSV-BJ-2 and KOS viruses at different multiplicities of infection (MOI=0.05, 0.10, 0.25, 0.50, and 1.00). After 24, 48, and 72 h, CCK-8 solution was added, and absorbance was measured at 450 nm. Cytotoxicity was calculated relative to controls.

2.8. Animal experiments

4-5 week old BALB/c nude mice (female, 16-18 g) were purchased from Vitong Lever and raised in an SPF-grade animal room, at 20°C-26°C, with a relative humidity of 40%-70%, light and dark cycle of approximately 12 h. Experimental mice were fed *ad libitum*.

5×10^6 PANC-1 or SW1990 cells (200 μ L PBS suspension) were subcutaneously inoculated into the right axilla to establish a transplanted tumor model. When the tumor volume reached 30–80 mm³ (PANC-1) or 100–400 mm³ (SW1990), the mice were randomly divided into 4 groups ($n=6$) according to the tumor volume: control group (stabilizer), low-dose group (5×10^6 PFU), medium-dose group (5×10^7 PFU), and high-dose group (5×10^8 PFU). The stabilizer used in the negative control consisted of 50 g/L lactose, 5 mM Tris-HCl, and 2.88 g/L proline in purified water. For both models, mice received intratumoral injection on days 0, 3, 6, and 9 after grouping (D0, D3, D6, D9), for a total of four injections. The total injection volume was determined as $V/4$ (μ L), where V is the tumor volume in mm³. The volume was distributed among 1 to 4 injection points according to tumor size: 1 point for 30–80 mm³, 2 points for 80–120 mm³, 3 points for 120–180 mm³, and 4 points for >180 mm³. The injection volume per point was calculated as: total injection volume/number of points.

Body weight, tumor long diameter (L), and short diameter (W) were measured every three days. Tumor volume was calculated as $V = 1/2 \times L \times W^2$. Animals were sacrificed 30 days after the first administration, and tumors were dissected and weighed.

2.9. Ethical statement

All animal experiments were approved by the Institutional Animal Care and Welfare Committee of Fuyang Normal University [approval No. FYNU2022AP029, approval date: March 28, 2022; animal facility license No. SYXK (Anhui) 2021-002]. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 8th edition, 2010) and the Chinese National Standard for Laboratory Animal Welfare and Ethical Review (GB/T 35892-2018). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.10. Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism 8.0, employing one-way analysis of variance (ANOVA) followed by LSD *post-hoc* tests for multiple comparisons, with statistical significance set at $P < 0.05$.

3. Results

3.1. Safety evaluation of oHSV-BJ-2 on normal cells

To evaluate the safety of oHSV-BJ-2, the expression of ICP27 protein and viral replication were detected after infection of normal cells (Vero, ARPE-19). Microscopic observation revealed that the majority of cells became rounded and died following infection with wild-type KOS virus, whereas the cell morphology of oHSV-BJ-2-infected cells remained similar to that of the uninfected group (Figure 1A). Western blot analysis demonstrated abundant ICP27 protein expression in KOS-infected cells, whereas no ICP27 protein was detected in oHSV-BJ-2-infected cells (Figure 1B).

Plaque formation assay results demonstrated that KOS infection of Vero and ARPE-19 cells produced clearly visible viral plaques, with plaque numbers increasing in proportion to viral titer. In contrast, no viral plaque formation was observed following oHSV-BJ-2 infection (Figure 2A). Since similar results were observed at all titers tested, representative images from the 40 PFU group are shown in Figure 2A and 2B.

Crystal violet staining further confirmed the absence of white plaque assay in the oHSV-BJ-2-infected group (Figure 2B). Viral plaque counts in the 40 PFU infection group revealed approximately 33 viral plaques in ARPE-19 cells and 28 in Vero cells under KOS infection, whereas no viral plaques were detected in the oHSV-BJ-2-infected group (Figure 2C). These results indicate that oHSV-BJ-2 fails to replicate and proliferate in normal cells, demonstrating an excellent safety profile.

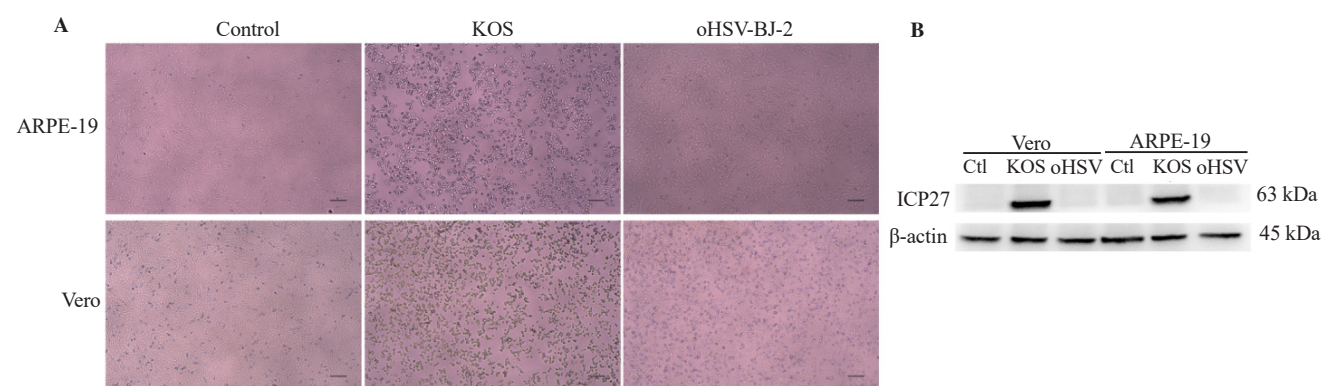


Figure 1. (A) Microscopic observation of Vero cells and ARPE-19 cells infected with oHSV-BJ-2 virus and wild-type virus KOS at MOI=3 for 24 h. (B) Expression levels of ICP27 protein in Vero cells and ARPE-19 cells. The scale represents 100 μ m. Ctl: control; oHSV: oHSV-BJ-2.

3.2. *In vitro* cytotoxic effect of oHSV-BJ-2 on tumor cells

The cytotoxic effects of oHSV-BJ-2 on eight tumor cell lines were evaluated using the CCK-8 assay. Results demonstrated that oHSV-

BJ-2 exhibited significant cytotoxicity against seven tumor cell lines (SW1990, PANC-1, A2058, KYSE-30, KYSE-150, A875, and FaDu), while showing no significant cytotoxic effect on CAL-62 cells (Figure 3). Compared to wild-type KOS, oHSV-BJ-2

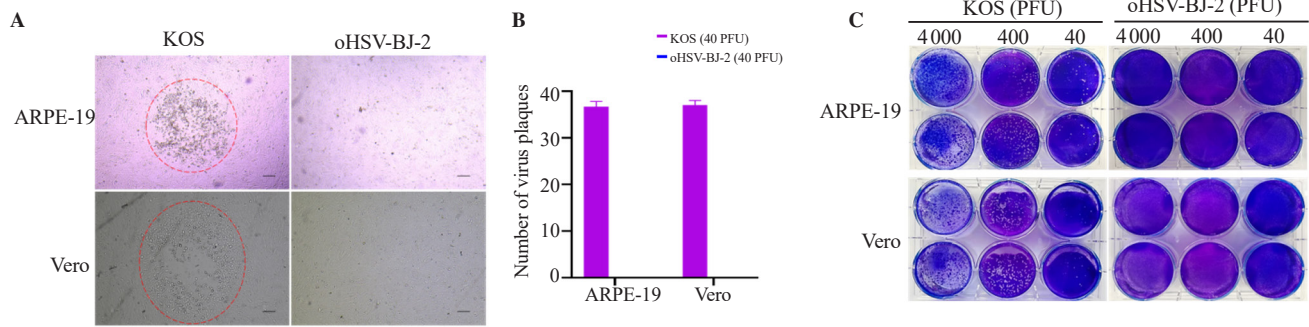


Figure 2. Plaque formation. (A) Microscope observation of viral plaques in Vero cells and ARPE-19 cells; (B) Viral plaque counts (mean $\pm$ SD, $n=3$); (C) Crystal violet staining of Vero cells and ARPE-19 cells. Scale bar = 100 μ m. PFU: plaque-forming units.

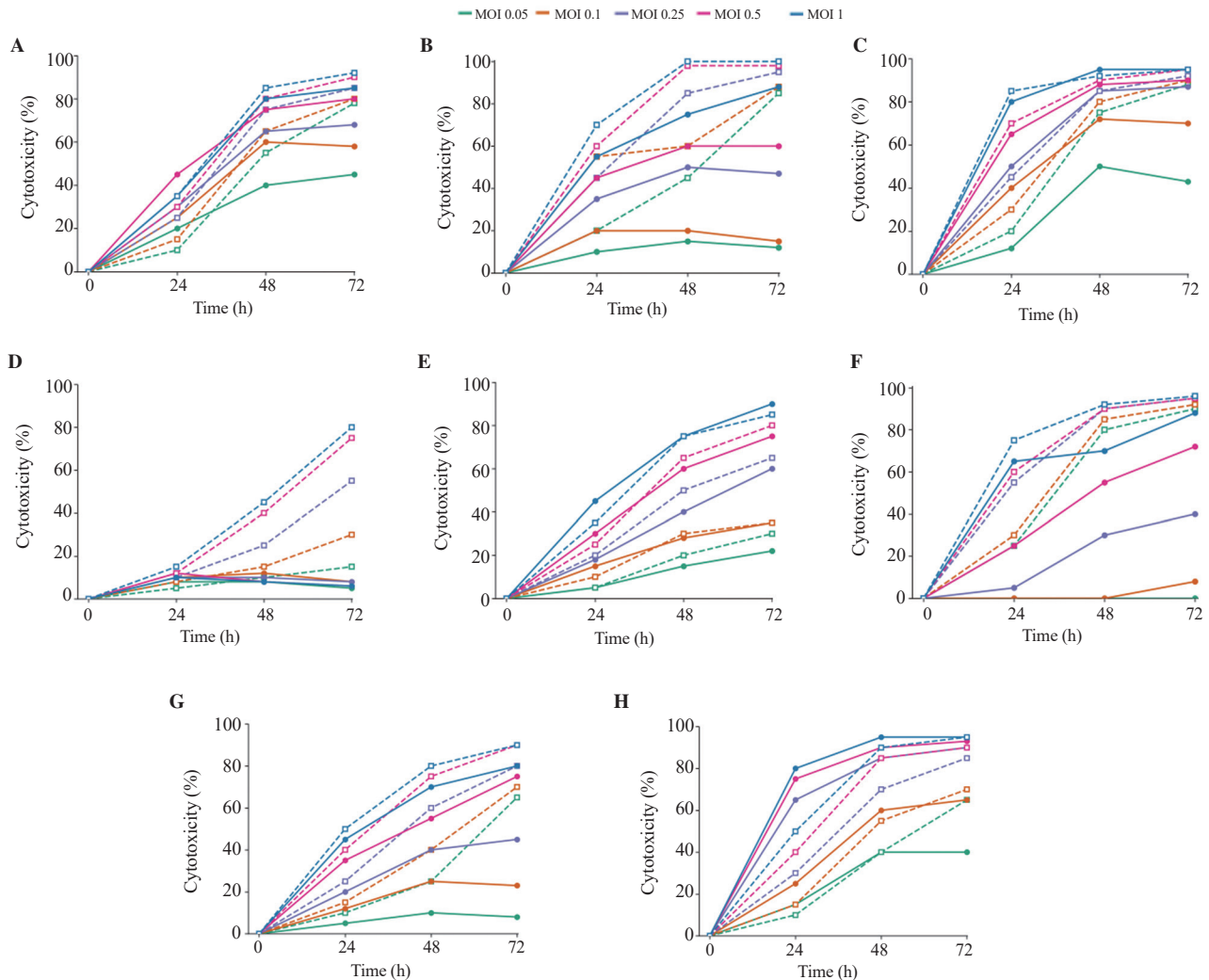


Figure 3. *In vitro* cytotoxic effect of oHSV-BJ-2 on tumor cells. A: SW1990; B: PANC-1; C: A2058; D: CAL-62; E: KYSE-150; F: KYSE-30; G: A875; H: FaDu. Curves represent cytotoxic rate (%) over time (0-72 h) for oHSV-BJ-2 (solid lines) and wild-type KOS (dashed lines) at five MOIs (0.05, 0.1, 0.25, 0.5, and 1). Data are presented as mean $\pm$ SD of three independent experiments and analyzed by one-way ANOVA. MOI: multiplicity of infection.

exhibited slightly weaker cytotoxicity against certain tumor cells (e.g., SW1990 and PANC-1) but demonstrated slightly stronger cytotoxicity against FaDu, A2058, and KYSE-150 at high MOIs. The cytotoxic effects exhibited clear time-dependent and dose-dependent patterns. Statistical analysis using one-way ANOVA showed that killing rates at 24, 48, and 72 h post-infection were significantly higher than at time 0 ($P < 0.05$) for all MOIs tested in the seven sensitive cell lines, confirming the dose- and time-dependent cytotoxic activity of oHSV-BJ-2. Specifically, for both oHSV-BJ-2 and KOS, killing rates at all five MOIs (0.05–1.00) were significantly higher than the time-0 value at 24 h, 48 h, and 72 h post-infection ($P < 0.05$ for all comparisons) in the seven sensitive cell lines (SW1990, PANC-1, A2058, KYSE-30, KYSE-150, A875, and FaDu). In contrast, in CAL-62 cells, killing rates for oHSV-BJ-2 did not differ significantly from time 0 at any MOI or time point, whereas KOS-infected CAL-62 cells showed significantly increased killing rates relative to time 0 at 48 h and 72 h for the 0.25, 0.50, and 1.00 MOI groups ($P < 0.05$), consistent with the resistance of CAL-62 to oHSV-BJ-2 described above.

3.3. *In vivo* inhibitory effect of oHSV-BJ-2 on subcutaneous pancreatic tumor xenografts

Humanized PANC-1 and SW1990 tumor models were established in nude mice to further investigate the anticancer effects of oHSV-BJ-2 virus *in vivo*. After 30 days of administration, there was no significant difference in body weight between the treatment groups and the control group in either model, with normal mental status and behavioral activity, indicating that oHSV-BJ-2 exhibited no systemic toxicity in mice.

In the PANC-1 model, tumor volume was significantly reduced in the treatment groups ($P < 0.05$). Tumor growth was partially inhibited in the low-dose group with an inhibition rate of approximately 50%. Significant tumor growth suppression was observed in the medium-dose and high-dose groups. In the medium-dose and high-dose groups, tumor suppression rates reaching 70%–80%. The differences were compared with the control group ($P < 0.001$), demonstrating dose-dependent effects (Figure 4).

In the SW1990 model, the tumor grew faster than that of PANC-1. Tumors in the control group began to grow from day 6, while

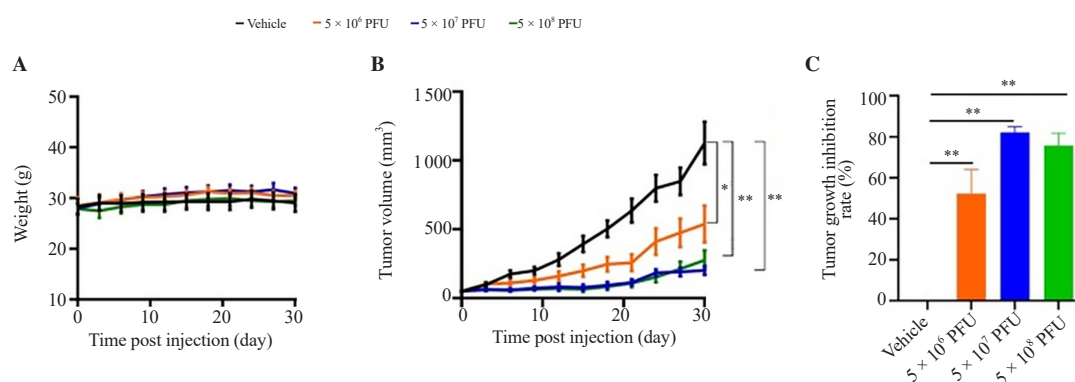


Figure 4. *In vivo* inhibitory effect of oHSV-BJ-2 on subcutaneous pancreatic PANC-1 tumor xenografts. oHSV-BJ-2 oncolytic virus exhibits inhibitory effects on tumor tissue growth in PANC-1 cells. (A) Changes in body weight. (B) Changes in tumor volume. (C) Tumor inhibition rates. * $P < 0.05$, ** $P < 0.001$ compared with the control group.

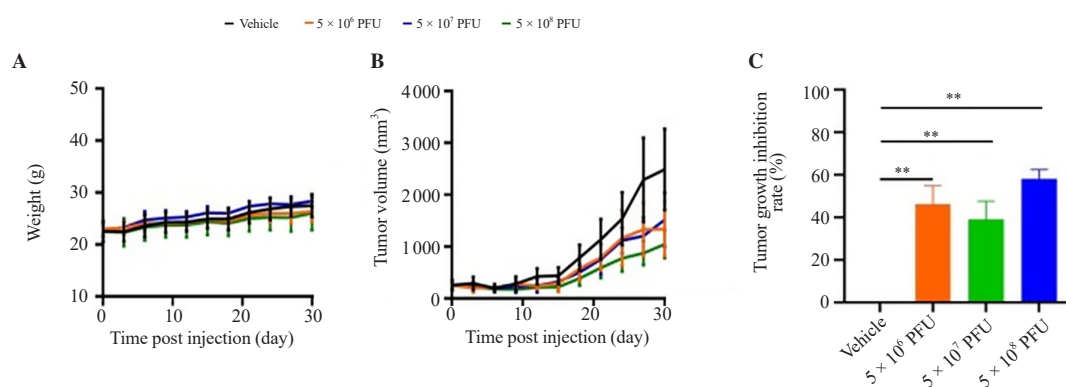


Figure 5. *In vivo* inhibitory effect of oHSV-BJ-2 on subcutaneous pancreatic SW1990 tumor xenografts. (A) Changes in body weight. (B) Changes in tumor volume. (C) Tumor inhibition rates. ** $P < 0.001$ compared with the control group.

tumors in all treatment groups showed only slight growth after day 12. After 30 days of administration, the tumor suppression rate was approximately 40% in low- and medium-dose groups, whereas the high-dose group exhibited a suppression rate exceeding 50% ($P < 0.001$) (Figure 5).

4. Discussion

This study successfully constructed an oncolytic herpes simplex virus oHSV-BJ-2 with ICP27 as the regulatory target, and verified its safety and antitumor effect *in vitro* and *in vivo*. Different from existing oncolytic virus modification strategies, this study uses a post-transcriptional regulatory mechanism to achieve tumor cell-specific replication while ensuring the integrity and expression timing of viral genes.

First, in terms of regulation method, this study belongs to transcriptional level control. The natural promoter of ICP27 is replaced with the hTERT promoter (tandem CMV enhancer), so that ICP27 is only transcribed in tumor cells with high telomerase activity and is almost not transcribed in normal cells. Secondly, this strategy retains the integrity of the viral genome and the original expression timing. No viral genes were deleted, and the coding sequence of the ICP27 protein itself was not changed. This is different from the strategy of T-VEC to delete ICP34.5, which theoretically has a stronger replication ability and broader spectrum tumor killing effect[7,8]. In addition, this study also inserted a GMCSF expression cassette into the viral genome, driven by the viral late promoter gD promoter. Since activation of the gD promoter depends on viral DNA replication and the function of immediate early proteins such as ICP27[14], GMCSF is only expressed when the virus is replicating in tumor cells. This further enhances the antitumor immune response while ensuring safety to normal cells.

Safety assessment results demonstrated that oHSV-BJ-2 infection of normal cells did not express ICP27 protein nor form viral plaques, confirming its safety profile in normal cells. Western blot analysis revealed strong ICP27 protein expression in Vero and ARPE cells infected with wild-type KOS virus, whereas no detectable ICP27 protein was observed after oHSV-BJ-2 infection, indicating that the regulatory sequence exerted the anticipated inhibitory effect in normal cells. Plaque formation assays further provided functional evidence: even at high viral titers (4000 PFU), oHSV-BJ-2 failed to form any viral plaques in normal cells, whereas KOS at the same titer produced abundant viral plaques. These results conclusively demonstrate that regulating ICP27 expression can effectively restrict viral replication outside normal cells[15]. Animal studies showed no differences in body weight between treatment groups and the control group, with normal mental status and behavioral activity, further validating the *in vivo* safety of the virus.

In vitro cytotoxicity experiments demonstrated that oHSV-BJ-2 exhibited broad-spectrum cytotoxic effects against seven tumor cell

lines, with dose- and time-dependent activity. Notably, oHSV-BJ-2 showed no significant cytotoxicity against CAL-62 cells, a human thyroid cancer cell line whose resistance to oHSV-BJ-2 may be associated with insufficient activity of the hTERT promoter in these cells. The expression levels of hTERT vary markedly across different tumor types[15], which may explain the differential sensitivity of tumor cells to oHSV-BJ-2; the reasons for the slightly stronger cytotoxicity against certain cell lines at high MOI remain to be further investigated.

In vivo experiments demonstrated that oHSV-BJ-2 exhibited significant inhibitory effects on both PANC-1 and SW1990 pancreatic cancer xenografts, with tumor suppression rates reaching 70%-80% in medium-to-high dose groups. A clear dose-dependent pattern was observed in the PANC-1 model: the low-dose group showed a tumor suppression rate of approximately 50%, while medium-to-high dose groups achieved rates of 70%-80%, with no statistically significant difference between medium and high doses, suggesting that 5×10^7 PFU may have reached the optimal therapeutic dose. In the SW1990 model, no significant differences were observed across dose groups, which may be attributed to the faster tumor growth rate of SW1990 and the premature death of some control mice at the end of the experiment. The observed differences in tumor growth rates between the two models may be related to the inherent characteristics of the cell lines and variations in blood supply at the inoculation sites, further highlighting the need to consider tumor heterogeneity in clinical applications regarding treatment efficacy.

One limitation of the present study is the absence of a positive control in the *in vivo* experiments. While oHSV-BJ-2 demonstrated significant tumor growth inhibition (70%-80% at medium and high doses) in both PANC-1 and SW1990 subcutaneous xenograft models, we did not include a comparator such as wild-type KOS, gemcitabine, or T-VEC. Therefore, the relative efficacy of oHSV-BJ-2 compared to standard-of-care agents or other oncolytic viruses remains to be determined. We acknowledge this as a limitation of this preliminary proof-of-concept study, and we plan to address it in the follow-up investigation.

Besides, this study has some other limitations. Firstly, a safety evaluation was conducted only in two normal cell lines, and the observation of the nude mice's body weight was made. As HSV-1 is a neurotropic virus, its potential neurotoxicity requires special attention. Future studies should assess viral safety in more normal cell lines and comprehensively evaluate safety indicators such as neurotoxicity, latent activation, and viral shedding in animal models. Secondly, the nude mice used in this study were T-cell-deficient immunodeficient animals, which limited the assessment of virus-induced antitumor immune responses. A key characteristic of oncolytic viruses is their ability to lyse tumor cells, release tumor-specific antigens, and activate the host's antitumor immune response, thereby creating an "*in situ* vaccine" effect[4]. Subsequent studies should further investigate whether oHSV-BJ-2 can induce antitumor immune responses in immunocompetent mouse models and

whether its combination with immune checkpoint inhibitors exhibits synergistic effects[16].

In recent years, combination therapies involving oncolytic viruses and immune checkpoint inhibitors have achieved significant progress in clinical trials. Clinical trials demonstrating the efficacy of T-VEC combined with ipilimumab in melanoma treatment showed superior outcomes compared to monotherapy[16]. The oHSV-BJ-2 constructed in this study retains all genes of HSV-1, theoretically exhibiting enhanced immunomodulatory potential. Future research could explore the synergistic effects of oHSV-BJ-2 when combined with immune checkpoint inhibitors such as PD-1 antibodies and CTLA-4 antibodies.

Conflict of interest statement

The authors declare no conflicts of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

XH performed the experimental study and wrote the original draft. LM contributed to virus purification and the assay of its activity and titer, as well as the final version of the manuscript. YQ participated in virus production and review of experimental data. XFY critically reviewed and revised the research. QQW designed, conceptualized and supervised the experimental study, interpreted the experimental data and acquired funding.

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