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IP6K2 mutations as a novel mechanism of resistance to oncolytic virus therapy

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Abstract

Background Oncolytic virus therapy (OVT) represents a promising frontier in cancer treatment. Despite its efficacy in clinical trials, variability in patient response, particularly resistance development, highlights the need for tailored therapeutic strategies.

Methods The Inositol Hexakisphosphate Kinase 2 (IP6K2) gene knock out was carried by CRISPR/Cas9 system. The evaluation of biomarkers of apoptosis and relevant pathways was conducted to be assessed. Attachment assay was conducted to verify the binding ability of virus to the host cells. Cell proliferation and apoptosis was assessed. Subcutaneous xenograft model was used to evaluate IP6K2 knock out influence in vivo. cBioPortal and TCGA database were applied to analyze genomic alterations in pan-cancer.

Results IP6K2 was essential for effective Herpes Simplex Virus Type1 (HSV-1) replication and subsequent cell apoptosis, acting through the tumor Protein p53 (p53) and Cyclin-Dependent Kinase Inhibitor 1 A (p21) signaling axis. The tumor model demonstrated that tumors lacking IP6K2 exhibited resistance to HSV-1 oncolysis, resulting in diminished therapeutic outcomes. Analysis of cBioPortal and TCGA databases corroborated the potential resistance stemming from IP6K2 mutations across various cancer types, underscoring the necessity for pre-treatment IP6K2 status assessment.

Conclusions This study underscores the role of IP6K2 as potential markers of resistance, which opens avenues for precision medicine approaches in OVT.

Highlights

- First reported that Inositol Hexakisphosphate Kinase 2 (IP6K2) is crucial for effective HSV-1 oncolytic virus therapy.
- CRISPR/Cas9-mediated knockout of IP6K2 resulted in decreased virus replication and apoptosis in cancer cells.

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- Tumors lacking IP6K2 *in vivo* showed significant resistance to HSV-1 oncolysis, leading to reduced therapeutic efficacy.
- The p53-p21 signaling axis mediates the effects of IP6K2 on HSV-1 therapy efficacy.
- cBioPortal and TCGA database analyses confirmed that IP6K2 mutations are associated with resistance to oncolytic virus therapy across multiple cancer types.
- Highlighted the importance of assessing IP6K2 status prior to treatment to optimize oncolytic virus therapy for individual patients.

Keywords HSV-1 oncolytic virus, Inositol hexakisphosphate kinase 2, Cancer treatments, Resistance

Introduction

OVT has undergone rigorous clinical validation, unveiling its dual capacity to meticulously target and lyse cancer cells, thus liberating tumor-specific antigens, while simultaneously eliciting potent anti-tumor immune responses. It has demonstrated remarkable efficacy in clinical trials across various types of tumors, becoming a focal point in clinical research [1, 2].Presently, dozens of HSV-1 oncolytic viruses are in clinical trial stages, with several having entered phase III studies, showing great promise for clinical applications. However, recent clinical studies have indicated that despite observing persistent tumor regression in both injected and non-injected tumor masses following HSV-1 oncolytic virus administration, these responses have not been universal across all cancer patients [3, 4]. These findings suggested that resistance to HSV-1 oncolytic virus therapy remained an issue in a subset of patients.

Viruses, known as acellular life forms, must parasitize within living cells and rely on host proteins to facilitate their replication. The restriction in host proteins essential for HSV-1 can impede the virus's propagation, exemplified by Nectin1's role as a pivotal receptor for HSV-1. Clinical investigations have unveiled a direct association between the expression levels of Nectin1 and the therapeutic efficacy of HSV-1 oncolytic viruses, underscoring the significance of host protein interactions in dictating viral therapeutic outcomes. Therefore, in order to formulate personalized treatment plans for patients with tumors and to determine resistance to HSV-1-like oncolytic viruses, key factors regulating HSV-1 replication must be identified and elucidated.

In the cells infected by HSV-1, cell death represents a basic mechanism for host defense to control viral spread [5]. In the process, cell death induced by HSV-1 infection is triggered or blocked by diverse mechanisms. Infected Cell Protein 4, infected Cell Protein 27, infected Cell Protein 22, Unique Short 3 Protein (ICP4, ICP27, ICP22, US3) and latency-associated transcripts (LAT) of HSV-1 antagonize the cellular apoptosis mechanism, whereas ICP0 and ICP6 are viral apoptotic triggers [6–12]. In addition, the surface glycoproteins D and J(gD, gJ) of HSV-1 are also involved in host cell apoptosis [13, 14]. It's believed that the caspase-3-dependent mechanism is responsible for the cell death induced by HSV-1 in host cells [15, 16]. Since the interactions between host cells and viruses are highly complex, identification of additional cellular factors that participate in HSV-1-dependent cell death should help to clarify the mechanism for HSV-1-induced cell death and viral spread.

Intrinsic apoptotic signaling pathway is triggered by diverse stimuli, such as ER stress, oxidative stress, DNA damage, and virus, etc. All of these stimuli disrupt mitochondrial membrane integrity, consequently inducing cell apoptosis [17, 18] P53, a well-known tumor suppressor, has been shown to sense mitochondrial damage that leads to apoptosis. Furthermore, p53-mediated pathways are involved in immune defense against some viruses, including Epstein-Barr virus, Hepatitis B virus and influenza virus, and so on [19–21]. It has been shown that HSV-1 can upregulate the p53 gene, although the significance remains unclear [22, 23]. IP6K2 encodes a nuclear protein belonging to the inositol phosphokinase (IPK) family. It plays a crucial role in DNA repair, chromosome recombination, and cancer progression [24, 25]. IP6K2 is recognized for its pivotal involvement in the intricate cascade of p53-mediated apoptosis, instigated by p53 activation and consequent upregulation of Phorbol-12-Myristate-13-Acetate-Induced Protein 1 (NOXA) and p53 Upregulated Modulator of Apoptosis (PUMA genes. This orchestrated sequence culminates in caspase-3 activation via mitochondrial signaling pathways, culminating ultimately in the induction of apoptosis [26, 27]. IP6K2 can enhance the cytotoxic actions of several different cell stressors, including chemotherapeutic cisplatin, protein kinase C inhibitor staurosporine, oxidative stresses caused by hydrogen peroxide and hypoxia [28]. By contrast, deletion of IP6K2 facilitates the process of p53-mediated cell survival [27]. In this process, CK2 phosphorylates the IP6K2 and enhances its degradation via ubiquitination. The degradation of IP6K2 prevents the activation of p53-associated cell death, consequently leading to an increase in cell cycle arrest. Despite its known involvement in inositol hexakisphosphate metabolism and the regulation of cell death, the precise role of IP6K2 in the context of HSV-1 oncolytic virus infection remains enigmatic. Therefore, we hypothesize that IP6K2 may play a pivotal role in sensing abnormal cell states, including those during viral infection, thereby

influencing the therapeutic efficacy of HSV-1 oncolytic virus treatments.

To test this hypothesis, we conducted IP6K2 gene deletion analyses and revealed a novel role for this protein in IP6K2-dependent cell death induced by HSV-1. We demonstrated that IP6K2 deletion caused the delay of HSV-1 proliferation in the host cells. IP6K2 might act as a regulator involved in maintaining the homeostasis between the cell survival and cell death, early proliferation and later release of HSV-1. Furthermore, this study offered the initial in vitro and in vivo evidence indicating that IP6K2-deficient tumor cells and mice exhibited reduced sensitivity to HSV-1 oncolytic virus therapy. This suggests that IP6K2 could enhance the efficacy of oncolytic virotherapy by facilitating virus-induced host cell death.

Materials and methods

Ethics statement

All animal experiments were conducted in compliance with the institutional guidelines of the Animal Ethical and Welfare Committee of Fujian Normal University. All animal procedures were approved by the Animal Ethical and Welfare Committee of Fujian Normal University.

Cells culture

BGC-823 cells were purchased from ATCC. Cell were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin and cells were cultured at 37 with 5% CO.The reagents that used for cell cultured were all purchased from Thermo Fisher Scientific (CA, USA) unless specifically indicated.BGC-823$^{IP6K2-/-}$ and BGC-823$^{IP6K2-/}$ cells were generated using the CRISPR/Cas9 tech nology. Briefly, the sgRNAs were synthesized and cloned onto the pX459 plasmid vector that using Lipofectamine 2000 reagent (11,668,019, Invitrogen, USA) according to the manufacturer's instructions. Single-cell clones were obtained by selection in a culture medium containing 5 μg/ml puromycin.

Viruses and virus generation

The HSV-1 oncolytic virus was engineered using the HSV-1-KOS backbone, wherein the ICP47 gene was knocked out. Subsequently, both copies of the ICP34.5 coding sequences were substituted with the EGFP gene, regulated by the human cytomegalovirus promoter. Serial dilutions of the harvested viruses were employed to infect Vero cells. Following three passages of plaque purification in cell culture, recombinant viruses were chosen and isolated based on green fluorescence and confirmed via Sanger sequencing.

Virus titration and replication assay

The virus titer was measured on Vero cells using classical fluorescent plaque assay, as the virus carries a fluorescent protein. The virus titer (PFU/ml) was calculated using the following formula: virus titer = mean plaque number×dilution ratio. Viral replication was assessed by infecting 106 Vero cells with various viral titers and seeding them into 6-well plates. Infected cells were harvested at each time point, and virus titration was conducted.

Designing sgRNAs and genetic editing of BGC-823 cells

The sgRNA sequences targeting IP6K2 and p21 genes were designed and selected using the website http://crispr.mit.edu/. Genetic editing of BGC-823 cells was performed using a sgRNA targeting IP6K2 and p21 genes. A pX459 plasmid vector was transformed with sgRNA inserted into the BbsI restriction enzyme site. For sgRNA hybridization: Combine 1 μl each of sense and antisense primers (100 μM) with 2 μl of 10X Taq polymerase PCR buffer (Takara, Shiga, Japan), and then add 16 μl of ultrapure water to reach a final volume of 20 μl. Proceed to anneal the primers to form heteroduplexes under the specified conditions: 95 °C for 5 min, followed by a gradual decrease in temperature from 95 °C, 5 min, 95 °C to 85 °C at -2.5 °C /s, 85 °C to 25 °C at -0.25 °C /s, and finally incubate at 25 °C for 5 min. BGC-823 cells were transfected with knockout plasmids using Lipofectamine, and 24 h later, puromycin was applied to screen the cells. Following the death of the control cells, the cell clones were isolated. In this study, primers were used as follows:

IP6K2 sgRNA1-5'-CGCATGAGTGCCCCCGACC-3' for BGC cells;

IP6K2 sgRNA2-5'-GGTCGGGGGCACTCATGCG-3' for BGC cells;

p21 sgRNA1- 5'-CGCCCAGCTCCGGCTCCACA-3' for BGC cells;

p21 sgRNA2- 5'-GTGGAGCCCGGAGCTGGGCG-3' for BGC cells;

p21 sgRNA3-5'CAGGGGGAAGGTGGGGTCC for BGC cells;

p21 sgRNA4-5'-GGACCCCACCTTCCCCCTG-3' for BGC cells;

5'-CTAAGGCACTTCCCACGCTA-3' and 5'-C AACTACACTTCTACCAAAAT CAAACT-3' for YZ-BGC-IP6K2;

5'-TAAATCCTTGCCTGCCAGAGT-3' and 5'-ACTC CTTGTTCCGCTGCTAAT-3' for YZ-BGC-p21.

Western blot analysis and antibodies

Cells were washed with ice-cold PBS and gently scraped to collect, followed by lysis in a protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS. Protein concentration was determined using a BCA protein assay kit (P0010S, Beyotime, Shanghai, China). Forty micrograms of total protein were separated by

electrophoresis on a 10% polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad, California, USA). The membrane was blocked with Tris-buffered saline containing 5% BSA at room temperature for 1 h. After blocking, the membrane was incubated with primary antibody overnight at 4 °C. Detection was performed using an IRDye 800CW secondary antibody (1:10,000) and the fluorescence signal was analyzed using the Odyssey CLx Western blot detection system (Westburg, Leusden, Netherlands). Primary antibodies applied in Western blot analysis included Phospho-P53 (Ser15) antibody, Noxa (D8L7u), PUMA, Caspase3, PARP1, IP6K2 Rabbit mAb from (Cell Signaling Technology, MA, USA), p21 mouse monoclonal antibody from (Proteintech, IL, USA)and the secondary antibody was Goat anti-Rabbit IgG H&L (Abcam, Cambridge, UK). GAPDH and ACTIN expression was used as the internal control. Perform western blot assay according to the manufacturer's provided instructions.

Attachment assay

Cells were seeded at 2×10^5 cells per 6 cm dish. HSV-1 was pre-treated with DNase (Takara, Japan) at a concentration of 2 μg/ml prior to infection and subsequently diluted to achieve MOI = 20, 50, and 100 in DMEM (Hyclon, USA). Pre-chilled HSV-1 was applied to both wild-type and BGC-823$^{IP6K2-/-}$ cells and incubated for 1 h at 4 °C. Following incubation, the cells were thrice rinsed with PBS, and genomic DNA was extracted using the EasyPure Genomic DNA Kit (Transgen, China). Quantification of HSV-1 binding copies was conducted using real-time quantitative PCR (qPCR).

Virus assays

HSV-1 levels were quantified in Vero cells using the plaque formation assay. Wild-type and gene knockout BGC-823 cell lines were exposed to HSV-1 for a duration of two hours, followed by a double PBS wash and media renewal. Cell and supernatant collections occurred at designated time points. Subsequent to three freeze-thaw sequences, the specimens were centrifuged at 10,000 revolutions per minute for five minutes. The viral concentration was ascertained employing a conventional plaque assay in Vero cells. Vero cell layers underwent a 48-hour infection with cell culture supernatants. Next, the cells were fixed in 4% formaldehyde, dried, and stained using 1% crystal violet.

qPCR

The quantification of HSV-1 genomic copies was performed through qPCR employing SYBR/ROX (RR82LR; Takara, Shiga, Japan) on an ABI qPCR system (Lifetech, CA, USA). The relative quantification of HSV-1 DNA was determined by calculating the ΔΔCT values utilizing the software provided by the manufacturer. qPCRHSV-1-gD-F: CGCCGTCAGCGAGGATAA, qPCRHSV-1-gD-R: TCTTCACGAGCCGCAGGTA; qPCRHSV-1-gB-F: GTCGG CAAGGTGGTGATGG, qPCRHSV-1-gB-R: G TAGCGAAAGGCGAAGAAGG; qPCRHSV-1-TK-F: C GATGACTTACTGGCGGGTG, qPCRHSV-1-TK-R: GGTCG AGGCGGTGTTGTGT; qPCRHSV-1-ICP0-F: GTGCATGAAAACCTGGATGC, qPCRHSV-1-ICP0-R: TTGCCCGTCCAGATAAAGTC; Alterations in the levels of the various samples were assessed following normalization to the β-actin control.

Cellular proliferation assays

Cell proliferation was assessed using the Cell Counting Kit (CCK) (TransGene Biotech, Beijing, China). Cells (2×10^3 per well) were seeded in 96-well plates with three replicates. CCK (10 μl) was added at the start and after 24, 48, or 72 h, followed by measuring absorbance at 450 nm to calculate the rate of cell proliferation.

Cell cycle and apoptosis detection

Following stimulation, cells were harvested, and treated with 70% alcohol at 4 °C for 2 h. After washing and staining with Propidium Iodide (PI)/RNase Staining Solution (BD Biosciences, CA, USA), apoptosis was assessed using flow cytometry.

Subcutaneous xenograft model

Cancer cells in the logarithmic growth stage were centrifuged, enriched, and suspended in PBS. Then, the cells were inoculated subcutaneously into each mouse at a concentration of 10^6 cells per mouse. The average tumor size reached approximately 100 mm^3 after 10 d of inoculation. Then, the mice were assigned in a randomized manner to either the control group or the treatment group, and the oncolytic virus was diluted in 50 ml of sterile PBS and administered by intratumoral injection at a concentration of 10^7 PFU per mouse once every 3 d. The tumor volume was assessed by applying the formula: (length × width × height) π/6.

Analysis of genome alterations in pan-cancer

Through cBioPortal resources (https://www.cbioportal.org/), we conducted an analysis of the genetic mutation profile for IP6K2 in the TCGA dataset [29]. The gene mutation rate, comprehensive alteration in pan-cancer, copy-number alteration and gene mutation sites were extracted from the "Oncoprint", "Cancer Type Summary", "Plots" and "Mutations" sub modules.

Statistical analysis

The statistical evaluation for comparisons between two distinct groups was performed using the unpaired, two-tailed Student's t-test in Prism 7.0c (GraphPad Software).

Standard deviations (SDs) were depicted by error bars. A p-value < 0.05 was deemed statistically significant.

Results

HSV-1 infection activates the IP6K2 signaling pathway

As the most widely used oncolytic virus, HSV-1 could infect a variety of tumor cells. Here, we selected the BGC-823 gastric adenocarcinoma cell line, which demonstrated sensitivity to HSV-1 infection, as evidenced by the substantial cell death observed after 96 h infection (Fig. 1A). Flow cytometry data showed that HSV-1-induced cell death in BGC-823 cells was significantly more than in the untreated cells (Fig. 1B-C). We also examined biochemical markers of apoptosis and found that HSV-1 virus infection caused stabilization of p53 protein, maturation of caspase 3 and the cleavage of its substrate poly (ADP-ribose) polymerase 1 (PARP1) in host cells (Fig. 1D). Considering the role of IP6K2 in mediating p53-induced apoptosis, we conducted an examination of IP6K2 expression alongside its downstream targets, NOXA and PUMA, subsequent to HSV-1 infection. Our data unveil heightened expression levels of IP6K2, NOXA, and PUMA in cells infected with HSV-1 compared to control cells (Fig. 1E). These observations imply an activation of the apoptotic signaling cascade following HSV-1 infection.

IP6K2 is a critical factor for cell death induced by HSV-1 infection

Since IP6K2 has been considered as a major regulator for the cytotoxic effects induced by various cellular stressors, including cisplatin, staurosporine, hydrogen peroxide and hypoxia [30], we generated a clonal cell line null for IP6K2 gene using the CRISPR/Cas9 system to assess the role of IP6K2 in HSV-1 induced cell death. The success of gene disruption was confirmed by sequencing and Western blotting (Fig. 2A-B). Notably, following HSV-1 infection (MOI = 1), the BGC-823$^{IP6K2-/-}$ cells displayed resistance to cell death caused by the virus, while wild-type BGC-823 cells were clustered and their shapes became round after HSV-1 infection, and died gradually following the time course (Fig. 2C-D).

Viral binding is the first step of HSV-1 infection, and blocking this step can significantly slow down the HSV-1-induced cell death process. In our exploration of the essential role of IP6K2 in HSV-1 binding, we employed a DNA-based

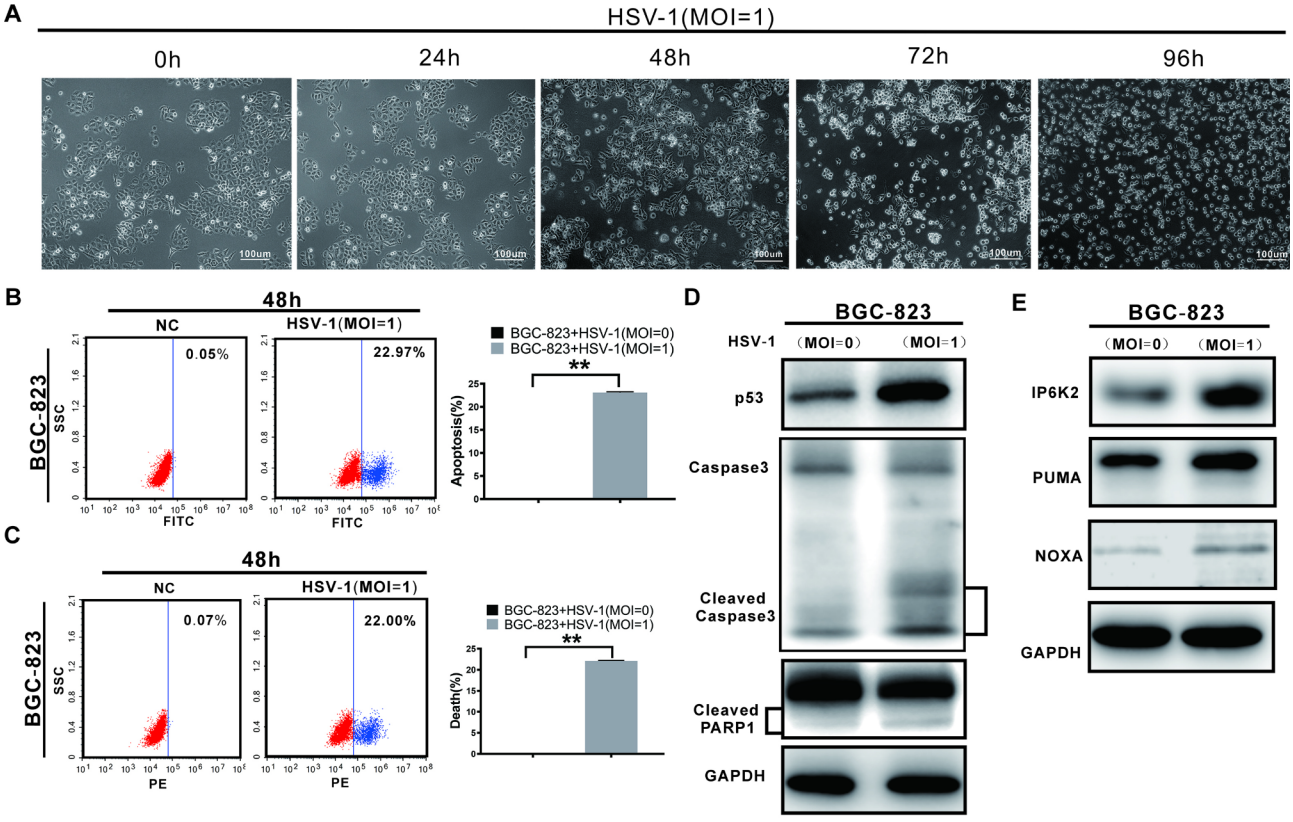

Fig. 1 HSV-1 Infection Triggers IP6K2 Signaling Activation. **A**, Cell mortality was induced by HSV-1 infection. BGC-823 cells were subjected to HSV-1 infection (MOI = 1) at various intervals (0, 24, 48, 72, 96 h). **B**, Flow cytometry analysis was performed to assess HSV-1-induced cell apoptosis following 48 h of infection. **C**, Flow cytometry analysis was performed to assess HSV-1-induced cell death following 48 h of infection. **D**, Effects of HSV-1 infection on the expression of the p53, caspase 3, and PARP1 in BGC-823 cells. **E**, Effects of HSV-1 infection on the expression of the IP6K2, PUMA, and NOXA in BGC-823 cells

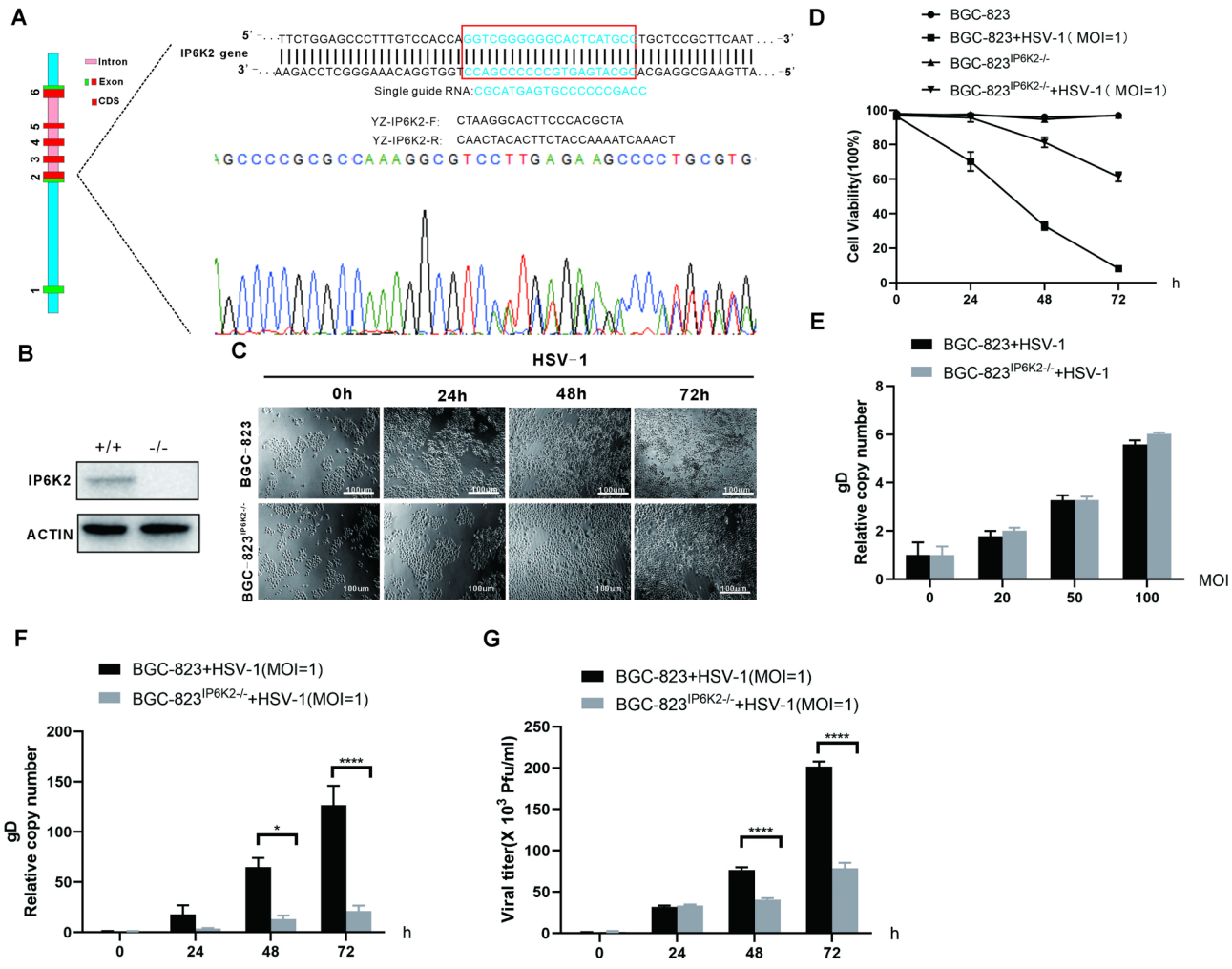

Fig. 2 The dependency of HSV1-induced cell death on IP6K2 was investigated. **A**, Genomic sequencing of the BGC knockout cell line at the targeted locus by IP6K2 sgRNAs was performed using primers flanking the IP6K2 gene. **B**, Detect IP6K2 protein in both wild type and IP6K2 knockout cells. **C**, The restriction of HSV-1 spread due to IP6K2 deficiency was observed by exposing cells to HSV-1 (multiplicity of infection, MOI = 1) and measuring cell activity at various intervals (0, 24, 48, 72 h). **D**, Alterations in cell proliferation curves were monitored by exposing cells to HSV-1. **E**, The viral binding capacity was evaluated by treating cells with HSV-1 (MOI = 20, 50, 100). **F**, Quantification of HSV-1 copy numbers via qPCR at various time points after infection (the amount of the GB gene was used to represent the viral copy numbers). **G**, A delay in HSV-1 dissemination due to IP6K2 deficiency was noted

quantitative binding assay to quantify the presence of HSV-1 on the cell surface. However, our findings indicated that the absence of IP6K2 did not prevent HSV-1 binding to the host cells (Fig. 2E). Next, we tested whether IP6K2 knockout could affect the viral proliferation. When infected with HSV-1, the copy numbers of HSV-1 in BGC-823$^{IP6K2-/-}$ cells were drastically reduced at every time point examined (Fig. 2F). The titers of HSV-1 in IP6K2 KO BGC-823 cells were also markedly lower than that in WT BGC-823 cells 48 and 72 h following infection (Fig. 2G).

In our continued investigation into the influence of IP6K2 on HSV-1's life cycle, we monitored the mRNA levels of viral genes such as ICP0 (immediate-early gene), TK (early gene), and gD (late gene) in both wild type and BGC-823$^{IP6K2-/-}$ cells after infection at intervals of 24, 48, and 72 h. The qRT-PCR results indicated a significant downregulation of mRNA transcription for ICP0, TK, and gD genes in the BGC-823$^{IP6K2-/-}$ cells (Fig. 3A-C). In line with this, western blot data revealed a decrease in the protein levels of these viruses in the IP6K2-deficient cells (Fig. 3D). The findings indicate that the absence of IP6K2 didn't impair HSV-1's ability to bind to the cell membrane. Instead, it appeared to modulate the proliferation of HSV-1 within host cells.

Loss of IP6K2 prevents cell apoptosis in response to HSV-1 infection

IP6K2 plays an essential role in p53-mediated apoptosis [28]. To further explore if the IP6K2 deletion could resist viral invasion by inhibition of cell apoptosis, we evaluated

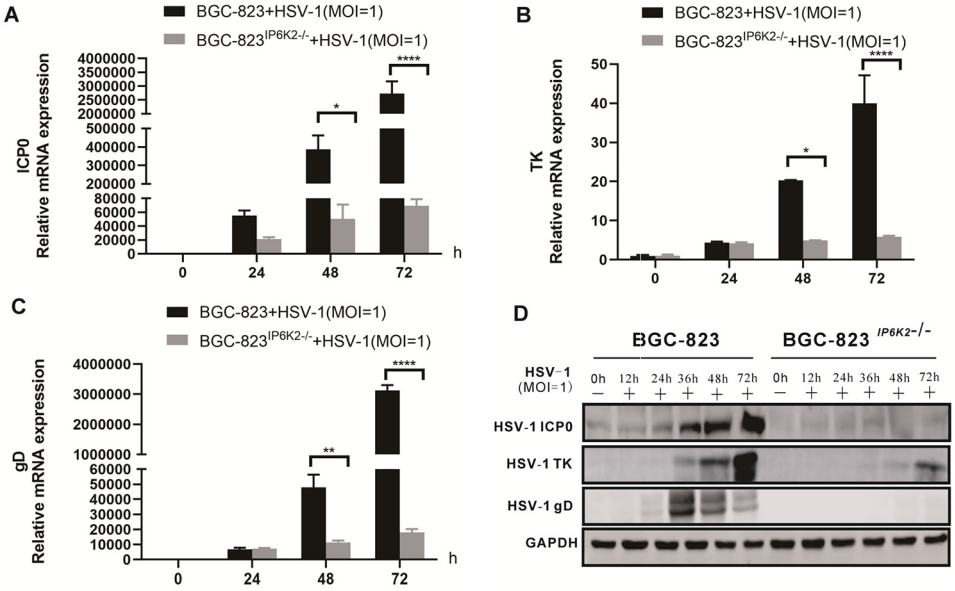

Fig. 3 Influence of IP6K2 deficiency on viral proliferation following HSV-1 Infection. **A-C**, mRNA levels of ICP0, TK, and GD after infection at various time intervals (0, 24, 48, 72 h) were analyzed by qRT-PCR in both wild type and BGC-823$^{IP6K2-/-}$ cells. **D**, Protein expression levels of ICP0, TK, and GD after infection at various time intervals (12, 24, 36, 48, 72 h) were analyzed by Western blot in both wild type and BGC-823IP6K2-/- cells

the apoptosis of the control and BGC-823$^{IP6K2-/-}$ cells utilizing flow cytometry. Our data showed that IP6K2 deletion reduced HSV-1-mediated apoptosis (Fig. 4A-B). Correspondingly, HSV-1-induced cell death in BGC-823$^{IP6K2-/-}$ cells was significantly less than that in the wild type cells (Fig. 4C-D). Since NOXA and PUMA proteins are the downstream effectors of IP6K2, we examined the expression of NOXA and PUMA genes in BGC-823$^{IP6K2-/-}$ cells. The results showed that the expression of NOXA and PUMA proteins in WT cells were much higher than that in IP6K2 KO cells (Fig. 4E). These results further supported the role of IP6K2 in modulating HSV-induced cell apoptosis.

BGC-823IP6K2−/− cells triggered the defense of HSV-1 infection through the p21-mediated cell cycle G1 phase arrest

Under normal culture conditions, the BGC-823$^{IP6K2-/-}$ cells were viable but proliferated at a slightly lower rate comparable to the wild-type cells (Fig. 5A). To delve deeper into the impact of IP6K2 loss on cell cycle dynamics subsequent to HSV-1 infection, we examined cell cycle stages of the wild type and BGC-823$^{IP6K2-/-}$ cells by flow cytometry and found that, relative to WT cells, the content of G1 phase cells was decreased in IP6K2 deficiency cells and reduced by 33.53% as detected by PI staining (Fig. 5B). To investigate the effect of cell cycle changes on viral infection, we prolonged time to test cell death in IP6K2 knockout cells. We found that the deletion of IP6K2 significantly delayed HSV-1-induced cell death, but eventually, BGC-823$^{IP6K2-/-}$ died following the time course (Fig. 5C). These findings imply that alterations in cell status resulting from the deletion of the IP6K2 gene might influence HSV-1-induced cell death. Since IP6K2 deficiency could result in p21-mediated cell cycle G1 phase arrest in response to various stresses,[35] we examined the expression of p21 gene to verify if the HSV-1 proliferation could be prevented by the cell cycle arrest. Western blotting data showed that missing IP6K2 significantly enhanced the expression of p21 protein (Fig. 5D). This result provided evidence that the resistance of BGC-823$^{IP6K2-/-}$ to HSV-1 was associated with p21-mediated cell cycle arrest. Thus, loss of IP6K2 could enhance p21 gene-mediated cell cycle G1 phase arrest in response to HSV-1 infection.

To further explore the p21 role in the defense of HSV-1 infection, we created a clonal cell line with a large fragment deletion of p21 gene by using the CRISPR/Cas9 system (Fig. 5E). Genomic DNA PCR assays showed that the p21 mutant was successfully generated with the deletion from the exon 1 to exon 3 (Fig. 5G). When infected with HSV-1 viruses, the cell viability of p21 KO cells decreased slightly (Fig. 5H). Furthermore, the BGC-823$^{p21-/-}$ cells were clustered and became round after HSV-1 infection, and died faster than wild-type cells (Fig. 5F). These data suggested that p21-mediated cell cycle arrest might be involved in the reversion of HSV-1-induced cell death.

IP6K2 deletion enhances resistance to HSV-1 oncolytic virus therapy in vivo

To further validate whether IP6K2 mutation affects the therapeutic efficacy of HSV-1 oncolytic virus treatment, we

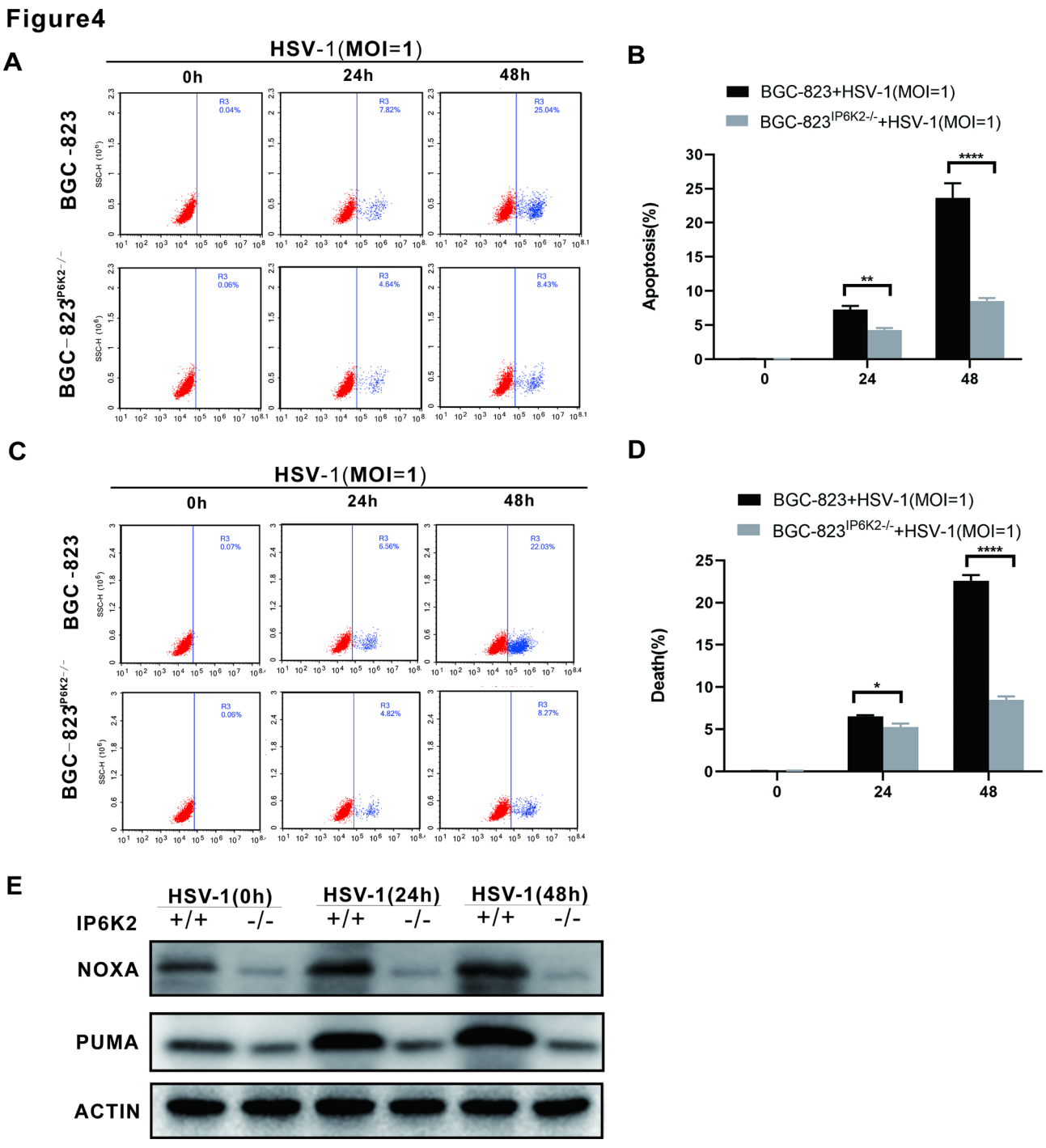

Fig. 4 Loss of IP6K2 prevents cell apoptosis in response to HSV-1 infection. **A**, Effects of IP6K2 on early cell apoptosis following HSV-1 infection. **B**, Quantification of data in A. **C**, Effects of IP6K2 on cell death following HSV-1 infection. **D**, Quantification of data in C. **E**, Effect of HSV-1 infection on the expressions of the NOXA, and PUMA after 48 h with HSV-1 infection

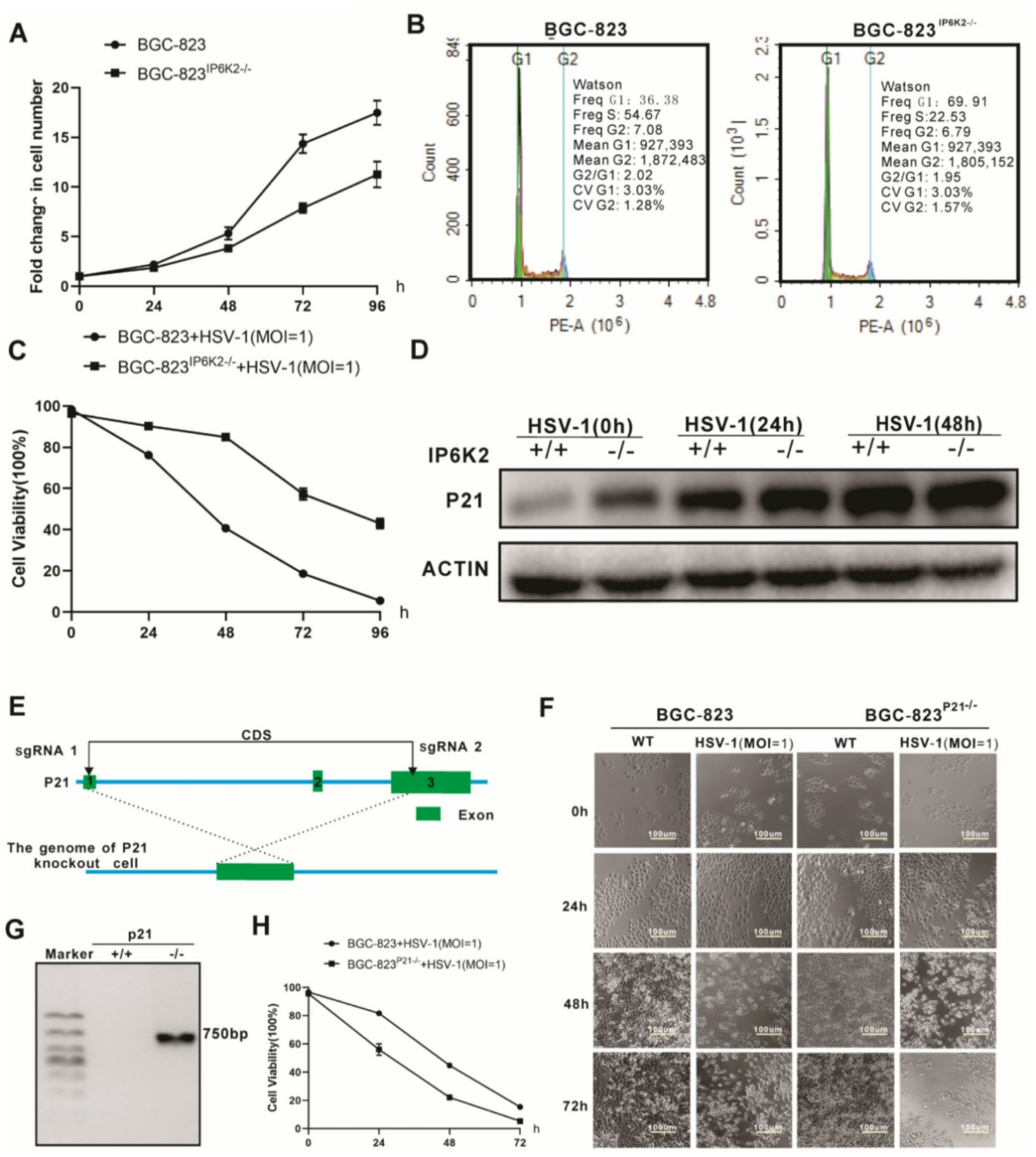

Fig. 5 Loss of IP6K2 promotes the cell cycle G1 phase arrest in response to HSV-1 infection. **A**, Alterations in cell proliferation curves following IP6K2 knockout. **B**, The cell cycle of wild type and BGC-823$^{IP6K2-/-}$ cell were examined by flow cytometry. **C**, Effects of cell proliferation rate on the viral proliferation following HSV-1 infection. **D**, Detection of the expression of p21 protein after HSV-1 infection at different time points. **E**, Schematic diagram of large fragment knockout of the p21 gene. **F**, Cell viability imaging was employed to evaluate the antiviral effect. **G**, The p21 knockout cell line was identified through PCR analysis. **H**, Changes in the cell proliferation curves following HSV-1 infection

established xenograft tumors in two groups of SCID mice by subcutaneously injecting BGC-823 and BGC-823$^{IP6K2-/-}$ cells, respectively, followed by administering 10^7 PFU of HSV-1 oncolytic virus per mouse (Fig. 6A). Compared to tumors with intact IP6K2, the therapeutic effect was significantly inhibited in tumors lacking IP6K2 (Fig. 6B-D). Moreover, we analyzed the levels of HSV-1 oncolytic virus within the tumor microenvironment and found that, relative to tumors with intact IP6K2, the copy number of HSV-1 oncolytic virus was significantly reduced in tumors lacking IP6K2 (Fig. 6E). These data indicated that tumors with IP6K2 mutation exhibit partial resistance to HSV-1-ΔICP34.5 oncolytic virus therapy.

IP6K2 served as a crucial screening gene prior to HSV1 oncolytic virotherapy

As is widely known, viruses are a form of non-cellular life that must parasitize within living cells and rely on certain host proteins for replication and propagation, known as Host Dependency Factors (HDFs). Our data revealed that a deficiency in IP6K2 leaded to failure in HSV-1 infection. Furthermore, animal-level studies indicated that the absence of IP6K2 within tumor cells similarly resulted in the failure of HSV-1 oncolytic virotherapy. According to findings from the cBioPortal database, there were a notable prevalence of IP6K2 gene mutations observed in Diffuse Large B-Cell Lymphoma, Uterine Corpus Endometrial Cacinoma, Stomach Adenocarcinoma (Fig. S1A). The predominant types of mutations identified in pan-cancer analysis were gain and diploid (Fig. S1B). This further substantiated the type, location, and frequency of modifications observed in the IP6K2 gene. R212H/C site mutations were detected in 3 patients with IP6K2 (Fig. S1C). Furthermore, utilizing the TCGA database, we found that IP6K2 exhibited an average mutation frequency exceeding 1.3% in tumor patients. Currently,

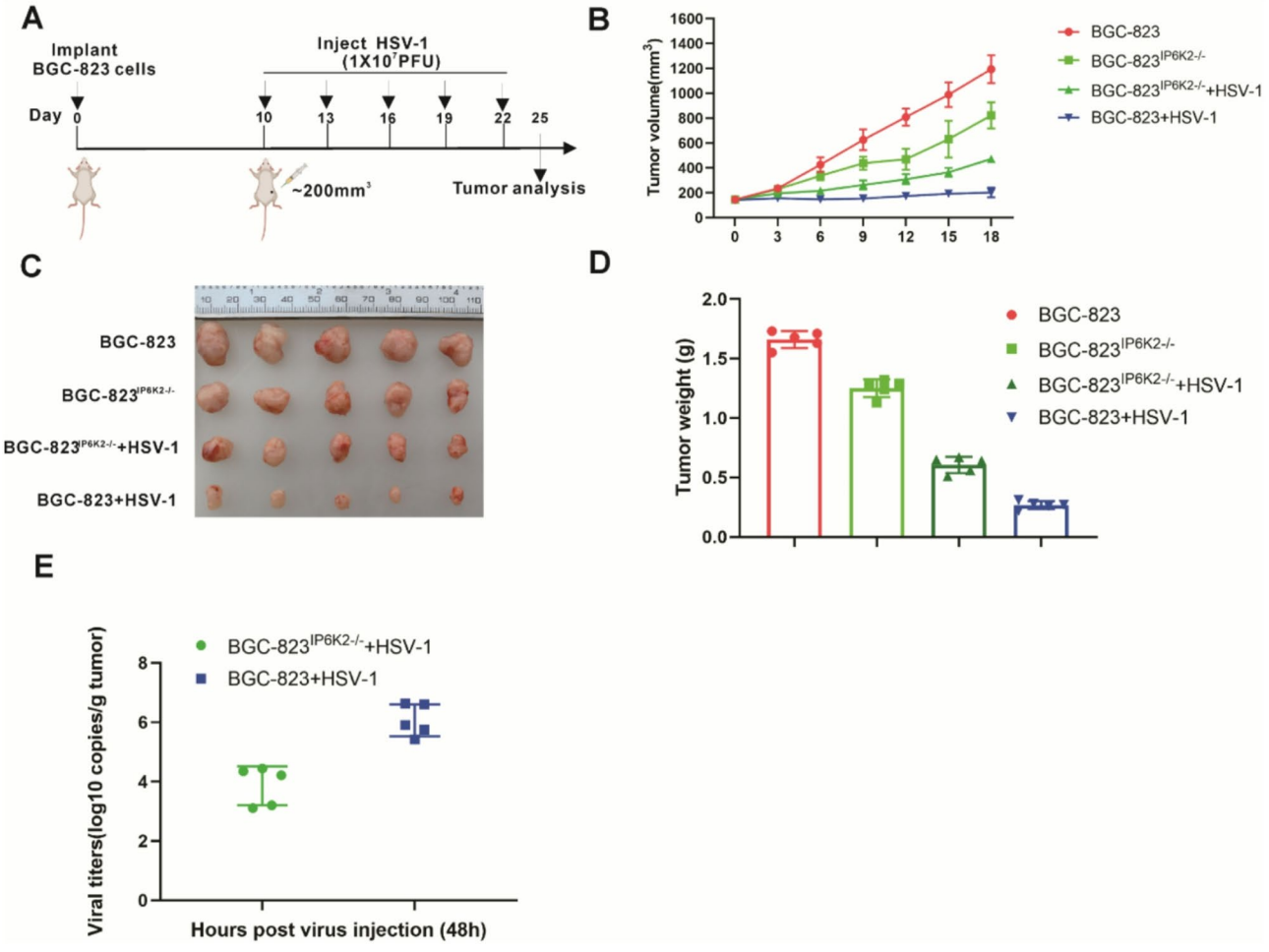

Fig. 6 IP6K2 Deletion Enhances Resistance to HSV-1 Oncolytic Virus Therapy In Vivo. **A**, Schematic representation of murine tumor treatment. Tumor volume measurements and drug administration were performed every three days, with a total of five treatments. **B**, Trends in murine tumor volume change, with error bars representing mean ± SEM ($n = 5$). (*$p < 0.05$, **$p < 0.01$, Two-way ANOVA). **C**, On the third day after the fifth drug administration, tumors on the backs of the mice were excised and photographed. **D**, On the third day after the fifth drug administration, tumors on the backs of the mice were excised and weighed to record the tumor mass. **E**, IP6K2 Deletion suppresses the HSV-1 Oncolytic Virus replication in tumor tissues

only a few receptors such as Nectin1, TNFRSF14, EXT1, and HCFC1 have been reported for HSV-1 HDFs internationally. The TCGA database revealed average mutation frequencies in tumor patients of 2.1%, 1.7%, 7%, and 4% respectively for these receptors (Fig. S2). This suggested a potential therapeutic resistance in a subset of tumor patients undergoing HSV-1 oncolytic virotherapy, emphasizing the necessity for pre-treatment screening of suitable candidates.

Discussion

Oncolytic Viruses (OVs) were initially conceived as a class of tumor-lysing therapeutics. With the advancement of engineering technologies over the past decade, an exciting development has been the incorporation of exogenous genes into these viruses, transforming OVs into potent vectors for gene therapy and immunotherapy. However, studies have identified that the efficacy of direct intra-tumoral injections stands at only 67%, indicating substantial issues with primary resistance. Herpes simplex virus 1 (HSV-1), a member of the alphaherpesvirus family, is capable of establishing both lytic and latent infections [30, 31]. Under certain conditions, the inability of HSV-1 to effectively lyse tumors leads to therapeutic tolerance to HSV-1 oncolytic virotherapy. Our research revealed that the loss of IP6K2 not only significantly inhibited viral proliferation but also promoted therapeutic tolerance in tumor treatment, suggesting that IP6K2 could be a predictive biomarker for the effectiveness of HSV-1 oncolytic virus therapy.

Binding the surface of cell membranes is considered a prerequisite for HSV-1 infection [32]. Our data showed that the absence of IP6K2 did not affect the binding of HSV-1, which might suggest that the absence of IP6K2 did not affect the function of heparan sulfate or viral receptors on the cell surface. Since viruses live in their host cells to optimize their own propagation, the virus has to make a balance between preventing and promoting host cell death. Early HSV-1 infection orchestrates a complex interplay between cell survival and cell-cycle regulation. Initially, HSV-1 suppresses cell death and arrests cell-cycle progression, primarily at the G1/S checkpoint. However, during late infection, there is a shift in this delicate balance, leading to the promotion of cell death by HSV-1. Notably, the HSV-1 immediate-early (IE) gene product, ICP27, plays a crucial role in establishing G1/S arrest during early infection [33]. Furthermore, the immediate-early protein ICP0 plays a key role in inhibiting progression of cells at the G2/M border, while ICP22 was considered to play an important role in the S phase of cell cycle [33–35]. Here, we found that the deletion of IP6K2 could significantly delay HSV-1-induced cell death. Interestingly, we also found that IP6K2 knockout cells grew slightly slower than wild-type cells under normal culture conditions (Fig. 2C) and consequently affected the proliferation of HSV-1. Thus, the absence of IP6K2 might delay virus proliferation by enhancing cell cycle arrest.

HSV-1 can induce apoptosis in many ways to promote viral own propagation. Previous reports indicated that IP6K2 was associated with apoptosis. However, it remained unclear whether IP6K2 induced apoptosis during HSV-1 infection. Here we showed that HSV-1 infection induced host cell apoptosis through IP6K2-dependent pathway. Conversely, IP6K2-deficient cells could inhibit the apoptosis induced by HSV-1. Following HSV-1 infection, a pronounced increase in survival rate was observed in IP6K2 knockout cells when compared to wild-type cells. Furthermore, our comprehensive in vitro and in vivo experimental analyses unveiled a notable decrease in sensitivity to HSV-1 oncolytic virus therapy in tumor cells and mice lacking IP6K2 expression. This suggested that IP6K2 might potentiate the therapeutic efficacy of oncolytic viruses by facilitating virus-induced host cell death.

Based on the aforementioned results, we speculated that, under normal conditions, p53 regulated the expression of the NOXA and PUMA genes in an IP6K2-dependent manner, leading to the activation of caspase-3 through mitochondrial signaling pathways, culminating in apoptosis. When lacking IP6K2, activated p53 could also stimulate the p21 gene expression, leading to cell cycle arrest in the G1 phase, as well as DNA replication is inhibited, so that the damaged cells have sufficient time to be fixed. Our demonstration of the role of IP6K2 in HSV-1-induced apoptosis suggested that IP6K2 might thus act as a regulator involved in maintaining the homeostasis between the cell survival and cell death, early proliferation and later release of HSV-1 (Fig. 7).

Furthermore, the emergence of oncolytic virotherapy as a promising method for cancer treatment has underscored the importance of understanding the complex interactions between viral mechanisms and host cellular factors. Our research into the role of IP6K2 within this framework provided pivotal insights that could significantly improve patient selection and therapeutic outcomes for HSV-1 oncolytic virotherapy. The link between IP6K2 mutations and specific cancers, as evidenced by our analysis of the cBioPortal and TCGA cancer databases, further highlights the gene's significance in the context of these therapies. Particularly, the identification of specific mutation types, such as the R212H/C site mutations, in our study leads to a more nuanced comprehension of how genetic variations in IP6K2 can impact the effectiveness of HSV-1 based treatments. Although the average mutation frequency of IP6K2 in tumor samples is relatively low, not exceeding 1.3%, the potential for high heterogeneity within tumors and the co-occurrence

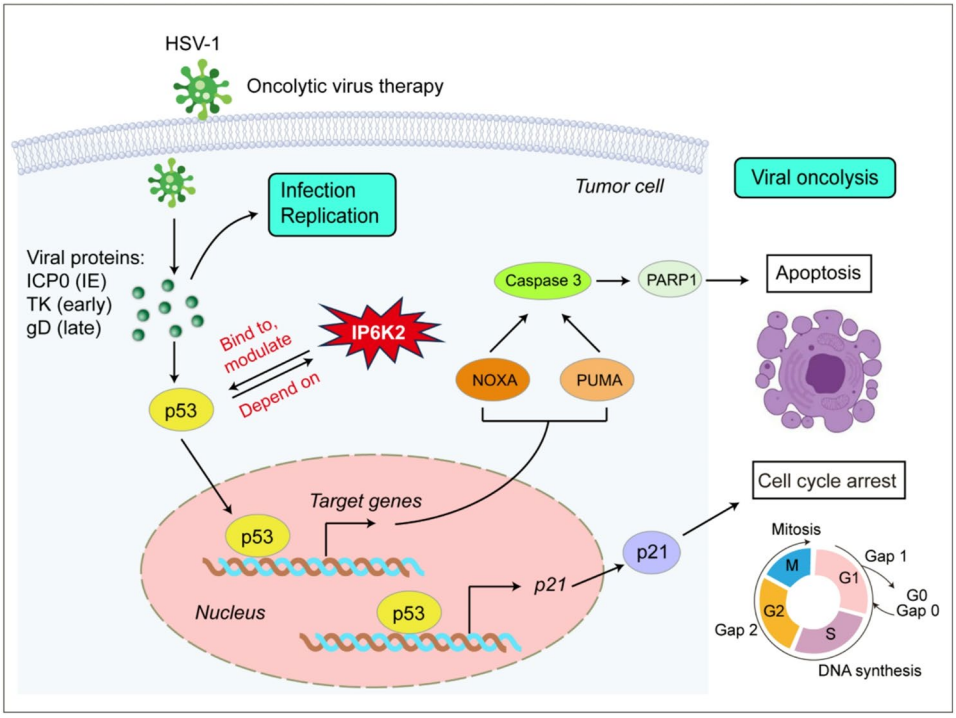

Fig. 7 The regulatory mechanisms of IP6K2 in HSV-1-induced viral oncolysis involve its interaction with the p53 protein. IP6K2 can bind to p53 and regulate its activity. Consequently, the function of p53 and its downstream signaling pathways are dependent on IP6K2. The loss of IP6K2 results in inhibited HSV-1 replication and apoptosis of cancer cells, while also enhancing p21-mediated cell cycle arrest in the G1 phase

of mutations in known HSV-1 Host Defense Factors (HDFs) such as Nectin1, TNFRSF14, EXT1, and HCFC1 suggest that the collective mutation rate across these factors is of considerable importance. This advancement not only deepens our understanding of the essential viral-host interactions for the success of oncolytic virotherapy but also signifies progress towards personalized cancer treatment, which aims to customize therapies according to the genetic makeup of each patient's tumor.

In summary, this study represented a preliminary exploration into a novel regulatory signaling pathway governing sensitivity to oncolytic herpes simplex virus type 1 (HSV-1) therapy at the molecular level. By shedding light on these intricate interactions between oncolytic viruses and tumor cells, our findings contribute to a deeper understanding of the mechanisms underlying virus-based cancer therapy. Investigating the specific molecular mechanisms of IP6K2 in this process would aid in understanding the emergence of tumor cell resistance and might provide new avenues for overcoming drug resistance. On one hand, further exploration into whether IP6K2's regulation of HSV-1 oncolytic virus sensitivity is tumor-specific, as well as clarifying the role of IP6K2 in normal tissues, is essential for assessing its safety as a therapeutic target. On the other hand, further research into how IP6K2 expression and activity differences in patients with various genetic backgrounds affect individual variance in oncolytic virus therapy efficacy could lead to the development of reliable IP6K2 molecular markers. Such markers could potentially help in selecting a responsive patient population, thereby realizing more precise oncolytic virus therapy.

Supplementary Information

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Supplementary Material 1

Author contributions

The study was conceptualized by RT, YS and QC. The experiments were carried out by ZH, YS, and XZ. Data were collected and analyzed by ZJ, DW, XS, XQ and HZ. Data interpretation and manuscript preparation were performed by YS, ZH, and RT. All authors approved the final version of the paper.

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Declarations

Ethics approval and consent to participate
All animal experiments were conducted in compliance with the institutional guidelines of the Animal Ethical and Welfare Committee of Fujian Normal University. All animal procedures were approved by the Animal Ethical and Welfare Committee of Fujian Normal University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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