Targeted Oncolytic Therapy for Hepatocellular Carcinoma with High Specificity and Efficiency

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Abstract. Introduction and hypothesis: Hepatocellular Carcinoma (HCC) represents a significant global health burden, characterized by high mortality rates and limited therapeutic options. Annually, over 500,000 new cases of HCC are diagnosed globally, with the overall five-year survival rate for advanced stages ranging from a mere 5-9%. This study proposes a novel oncolytic virus (OV) approach for HCC treatment, hypothesizing that a combination of alpha-fetoprotein (AFP) promoter/enhancer system for precise targeting of HCC cells while sparing normal liver tissue, Diphtheria Toxin A (DTA) for potent cell lysis, and shGPX4 to overcome treatment resistance through ferroptosis pathway. **Methodology**: An Adeno-Associated Virus 2 (AAV2) vector was engineered to contain an AFP promoter/enhancer-driven DTA gene and a short hairpin RNA targeting GPX4 (shGPX4). The virus was produced in HEK293T cells, purified, and its efficacy was tested on different HCC cell lines (HepG2, Hep3B and Huh7) and normal hepatocyte cell line (MIHA) at various multiplicities of infection (MOIs). Cell viability was assessed using CCK8 assays. Moreover, constructed AAV2's function on anti-resistance is tested on combination treatment with different-dosage Cisplatin. **Results**: The AFP promoter/enhancer was specifically selected based on comprehensive analysis of published dataset, which demonstrated significantly high AFP expression in liver cancer, correlation with disease outcomes, survival outcomes and specific epigenetic regulation in HCC. Further *in vitro* experiments demonstrated the construct AAV2 oncolytic virus has remarkable specificity and therapeutic efficacy. Multiple analysis revealed significant cytotoxicity in high-AFP-expressing HCC cell lines (HepG2 and Hep3B), reducing cell viability to approximately 40% at maximum MOI, while showing minimal impact on normal hepatocytes (MIHA). Moreover, combination therapy studies with cisplatin showed significant synergistic effects, particularly in chemoresistant cells, attributed to the GPX4 suppression mechanism. **Conclusion**: Our novel OV construct shows promising results in selectively targeting and eliminating HCC cells while sparing normal liver tissue. This approach addresses the critical needs for specificity and overcoming treatment resistance in HCC therapy. Further in vivo studies are warranted to fully elucidate its efficacy and safety, potentially leading to more effective and targeted treatments for hepatocellular carcinoma.

Keywords: Hepatocellular Carcinoma, Oncolytic Virus, AFP, therapy resistance and GPX4.

1. Introduction

Hepatocellular Carcinoma (HCC) is considered one of the most prevalent and lethal forms of liver cancer worldwide, primarily due to its limited treatment options and extremely low survival rates[1]. Annually, over 500,000 new cases of HCC are diagnosed globally, with the overall five-year survival rate for advanced stages ranging from a mere 5 to 9 percent[2]. This highlights the deadly nature of HCC. The tumor itself is characterized by a high degree of diversity, both at the tissue and molecular levels. Malignant HCC often displays elevated levels of alpha-fetoprotein (AFP), a biomarker associated with poor prognosis, and is marked by a high rate of chromosomal instability and epithelial to mesenchymal transition (EMT)[3]. The tumor's aggressive nature is further compounded by its tendency for early vascular invasion and a high rate of recurrence after treatment, driven by the dedifferentiation of cancer cells and the presence of cancer stem cell-like properties. These molecular complexities contribute to the rapid growth and metastasis of cancer, often rendering traditional treatments ineffective. Standard therapeutic interventions, including surgical resection, transarterial chemoembolization (TACE), and radiofrequency ablation (RFA), encounter numerous challenges such as tumor recurrence, limited applicability in advanced disease stages, lack of specificity, and

significant side effects. Curative therapies are viable for approximately 30% of early-stage HCC patients[4], and the five-year survival rate of these therapies stands at just 18.1%[5-7]. This underscores the urgent need for a novel therapeutic approach that is highly precise, safe, and capable of preventing tumor recurrence concurrently.

In recent years, oncolytic viruses (OVs) have emerged as a promising avenue in cancer treatments. OVs are genetically engineered viruses designed to selectively target and kill tumor cells while sparing normal cells. They achieve this by recognizing specific receptors on the surface of tumor cells, allowing them to bind and enter these cells. Tumor cells often abnormal signaling pathways, such as the RAS pathway[8], which OVs can exploit to facilitate viral replication and spread. Once inside a tumor cell, the virus replicates and induces cell death. This not only directly eliminates tumor cells but also initiates systemic immune responses. Tumor cells possess mechanisms to evade immune detection, such as secreting factors like IL-10 and TGF-β[9], which promote the presence of immunosuppressive cells in the tumor microenvironment. OVs counteract this by modifying the immune cell landscape around the tumor. For example, OVs can express soluble forms of TGF-B receptors that act as decoys, binding to TGF-B and preventing it from interacting with cell surface receptors. This inhibition of TGF-β signaling reduces its immunosuppressive effects[10].

A specific case highlighting the therapeutic potential of oncolytic viruses (OVs) is the use of T-VEC in treating advanced melanoma. T-VEC, short for Talimogene Laherparepvec, is an engineered oncolytic virus derived from herpes simplex virus type 1 (HSV-1). It is one of the first FDA-approved oncolytic viral therapies for cancer treatment[11]. T-VEC is designed to selectively replicate within tumor cells, causing direct oncolysis, or destruction of the cancer cells[12]. In addition to its lytic activity, T-VEC has been further engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), an immune-stimulatory protein that enhances the recruitment and activation of dendritic cells[13]. These cells are crucial for initiating an anti-tumor immune response, helping the body's immune system recognize and attack cancer cells. Overall, the response rate for patients treated with T-VEC was approximately 26%, while in contrast, the control group, which only received a control treatment for comparison, exhibited a response rate of only 6%[14]. This indicates how OVs have serious potential as a therapy for cancer.

Oncolytic viruses (OVs) represent a promising approach in cancer therapy, however, it should be understood that OVs still exhibit serious side effects. One primary concern is the safety and precision of OV therapies. Despite their potential, OVs are fundamentally viruses and could potentially trigger severe immune responses, including cytokine storms and systemic inflammatory reactions, posing substantial risks to patients. To address this concern, we propose utilizing the adeno-associated virus (AAV), specifically the AAV2 strain, as a vector for improving the delivery and overall safety of OV therapy. AAV2 offers several advantages that enhance the safety profile of OV treatments. As a non-pathogenic and replication-deficient virus, AAV2 has a reduced likelihood of affecting normal cells[15]. The AAV2 strain is engineered with significant deletions in its viral genome, eliminating pathogenic elements while retaining its ability to efficiently deliver therapeutic genes. These modifications result in low immunogenicity[16], significantly reducing the risk of adverse immune responses such as cytokine storms.

Another critical challenge in OV therapy is preventing cancer recurrence. Traditional therapies often fail due to the inherent ability of cancer cells to evade the immune system, leading to the survival of residual tumor cells and potential tumor regrowth or metastasis. This risk of tumor resistance is primarily associated with the epithelial-mesenchymal transition (EMT) process and is further sensitized to ferroptosis, a form of programmed cell death induced by lipid peroxidation[17]. GPX4, an essential enzyme protecting cells from ferroptosis, has been observed at elevated levels in hepatocellular carcinoma (HCC), contributing to the resistance of tumor cells to various forms of oxidative damage, including those induced by oncolytic viruses[18]. To address this challenge, we have incorporated RNA interference (RNAi) targeting the GPX4 pathway into our OV design.

Our approach utilizes AAV2 to design an oncolytic virus with enhanced specificity for HCC cells. This initial method is further refined by incorporating the alpha-fetoprotein (AFP) promoter and

enhancer. HCC is characterized by high levels of AFP expression, a liver-specific biomarker significantly elevated in cancerous cells but minimal in normal adult liver cells[19]. By harnessing this aberrant expression pattern, we can drive the selective expression of therapeutic genes or viral components within HCC cells, ensuring a potent therapeutic effect while minimizing off-target effects and reducing damage to normal liver tissue.

The efficacy of our OV is further enhanced by the incorporation of the diphtheria toxin A (DTA) gene. DTA, the enzymatically active component of the diphtheria toxin, catalyzes the ADP-ribosylation of elongation factor 2 (EF-2), effectively halting protein synthesis within the cell and leading to rapid cell death[20]. The high potency of DTA, even at low concentrations, makes it an exceptionally effective anti-cancer agent.

To mitigate the development of tumor cell resistance during OV treatment, we have integrated short hairpin RNA against GPX4 (shGPX4) into our platform. By suppressing GPX4 expression, shGPX4 disrupts the cancer cells' ability to counteract oxidative stress, rendering them more susceptible to ferroptosis. This approach not only enhances the cytolytic effect of OVs but also directly targets a major factor responsible for tumor recurrence and drug resistance.

In summary, this study aims to develop a novel OV specifically designed for treating HCC while addressing key challenges associated with current treatments. Our focus is on developing a recombinant AAV2 vector through the construction of a plasmid vector encoding the AFP promoter/enhancer-DTA-shGPX4 gene for malignant HCC therapy. This innovative approach combines the specificity of the AFP promoter, the potency of the DTA gene, and the resistance-targeting capability of shGPX4 (Figure 1). By integrating these elements, we aim to create an OV therapy that is safe, effective, and highly selective in targeting HCC, potentially revolutionizing the treatment landscape for this challenging malignancy.

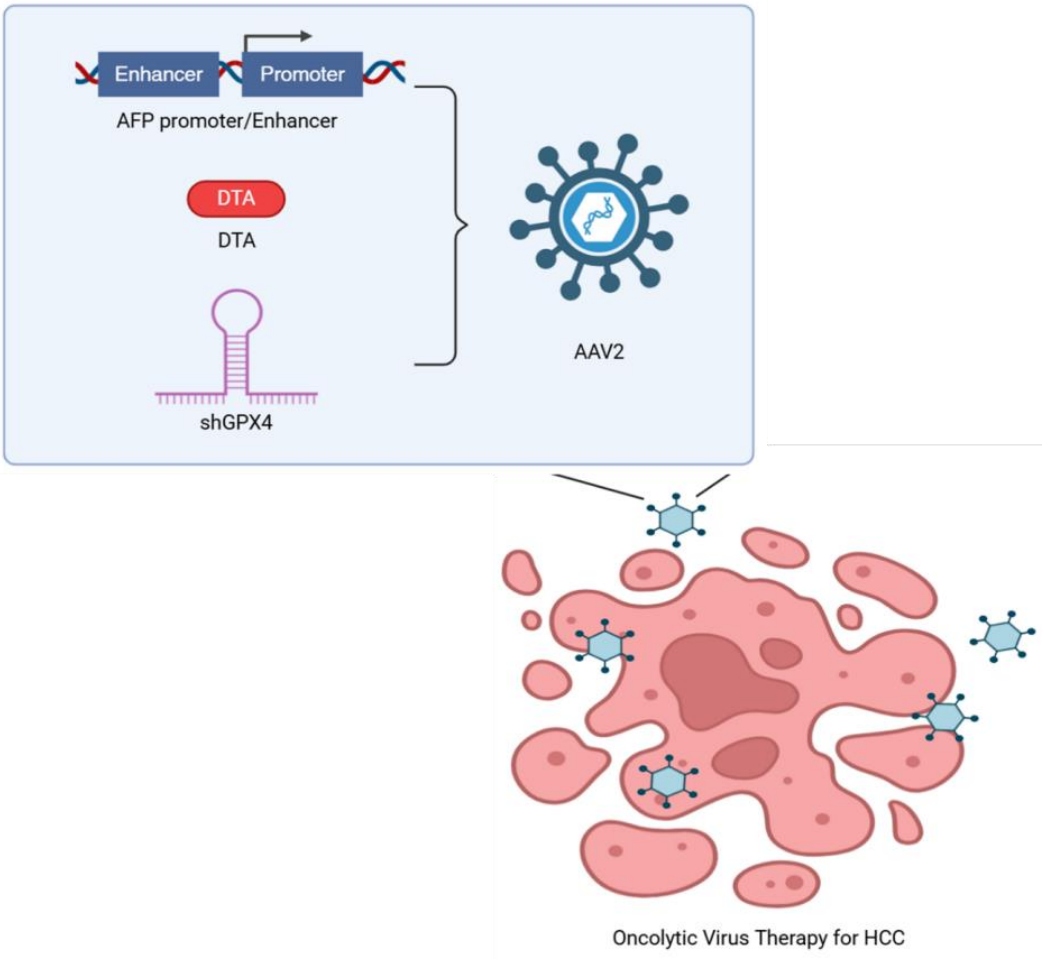

Figure 1. Schematic view of construction of oncolytic virus AAV2-AFP promoter/enhancer-DTA-shGPX4 for malignant HCC therapy.

2. Methodology

2.1. Plasmid Construction

The plasmid for our oncolytic virus was designed to incorporate the AFP promoter/enhancer, DTA gene, and shGPX4 sequence. Sequence information is obtained from NCBI, while shGPX4 sequence was designed by VectorBuilder online tool (https://en.vectorbuilder.com/tool/shrna-target-design.html). The genes were commercially synthesized and cloned into the vector using standard molecular cloning techniques with commercial kits (TakaraBio), including PCR amplification, restriction enzyme digestion, and ligation. The resulting plasmid was subsequently transformed into DH5α competent E. coli cells (Beyotime) for amplification.

2.2. Plasmid Extraction

Plasmid extraction was conducted using the Plasmid Extraction Kit (Tiangen biotech), following all the manufacturer's instructions. Following plasmid extraction, the concentration of the plasmid DNA was quantified using a Nanodrop One Microvolume W-Vis spectrophotometer (Thermo Scientific).

2.3. Plasmid Verification

Plasmid verification was conducted through restriction enzyme analysis and DNA sequencing. The restriction digest patterns were analyzed via 10% agarose gel electrophoresis, while sequencing results were aligned with the theoretical construct to confirm the correct insertion and orientation of all components by *SnapGene*.

2.4. Viral Production

For viral production, we employed a triple transfection method using HEK293T cells (National collection of authenticated cell cultures). The process involved co-transfection of three plasmids: our construct plasmid, an AAV-RepCap plasmid, and a pHelper plasmid.

Transfection was performed using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). HEK293T cells (1×10^5 per well) were seeded in 6-well plate 24 hours prior to transfection, aiming for 70-90% confluence at the time of transfection. On the day of transfection, 12.5 μL Lipofectamine 2000 reagent was diluted in 112.5 μL Opti-MEM medium. Concurrently, the three plasmids were diluted in Opti-MEM as follows: 1.5 μL of AAV Recap, 1.6 μL of AAV vector, and 4.98 μL of AAV Helper were combined with 116.92 μL of Opti-MEM. This mixture was gently combined with the diluted Lipofectamine 2000 reagent at a 1:1 ratio and incubated for 30 minutes to facilitate complex formation.

Following incubation, the growth medium was carefully aspirated from the HEK293T cells and replaced with serum-free Opti-MEM. The DNA-Lipofectamine complexes were then added dropwise to the wells, ensuring uniform distribution across the cell monolayer to maximize transfection efficiency.

Post-transfection, cells were incubated for 48 hours under standard culture conditions. Transfection efficacy was assessed by visualizing GFP expression using a Confocal Laser Scanning Microscope (Olympus, FV4000).

2.5. Viral Purification

Following transfection, cells were lysed, and the viral particles were harvested from the cell lysate. The initial crude viral preparation underwent a series of purification steps, including ultrafiltration and density gradient centrifugation, to yield a refined viral isolate suitable for further experimentation or analysis.

2.6. Immunoblotting Analysis

Purified target oncolytic virus and control rAAV2 viral proteins were diluted in RIPA buffer supplemented with PMSF (Beyotime). A total volume of 20 μg viral suspension was prepared in sample buffer, heated at 95℃, and loaded into SDS-PAGE gels Electrophoresis was performed at 80 V in the stacking gel and 120V in separating gel. Following separation, proteins were visualized by staining with Coomassie Brilliant Blue (Beyotime).

2.7. Cell Viability Assay

Cell viability was measured using Cell Counting Kit 8 following all manufacturer's instructions (Beyotime). CCK-8 provides a convenient way to assess cell viability. The CCK-8 reagent contains a water-soluble tetrazolium salt (WST-8), which is reduced by dehydrogenases in metabolically active cells to form a formazan dye. The amount of formazan produced is directly proportional to the number of living cells, making it a reliable indicator of cell viability.

HepG2, Hep3B, Huh7 and MIHA cells (5000 cell per well) were seeded in 96-well plates and transduced with two distinct viral constructs- target oncolytic virus with either AAV2-AFPpromoter-DTA-shGPX4 and rAAV2 control, and in the combination experiment with different cisplatin (1uM, 5uM and 10uM). The transduction was performed at varying series of infection (MOIs) of 100, 500, 1000, and 2000. Following transduction, the cells were incubated for a period of 72 hours to facilitate viral entry, transgene expression, and the manifestation of consequent cellular responses, including potential cytotoxic effects. After 72 hours of incubation, 10 μL of CCK-8 solution was added to each well. After 2 hours of incubation with CCK-8, the absorbance of the cells at 450 nm was measured by MicroplateReader (Thermo Fisher). The optical density (OD450) values were recorded and used to calculate cell viability as a percentage of untreated control cells.

2.8. Statistical Analysis

All data of cell viability analysis were from six independent experiments and data were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed by *GraphPad Prism* (version 8.0.1). Paired two-tailed Student's t tests were used to analyze the comparisons between groups. $P < 0.001$ was considered statistically significant.

3. Results

3.1. AFP expression and related regulation in human HCC

To make a better understanding of human HCC expression pattern, I made a comprehensive analysis based on the TCGA dataset. AFP was selected as the specific therapeutic target. Significantly higher AFP expression in liver cancer tissues (T) compared to normal tissues (N), confirming AFP as a reliable HCC tumor marker with minimum effect on normal liver (Figure 2a). AFP expression increasing with cancer stage progression (Stage I to IV), ensuring the construct's efficacy across all stages (Figure 2b). The Kaplan-Meier plot reveals worse survival rates in patients with high AFP expression (red) versus low AFP expression (blue), underscoring the correlation between elevated AFP levels and poorer outcomes, further justifying AFP as a therapeutic target (Figure 2c). Then, I searched the correlated AFP enhancer/promoter combination of high-AFP expression HCC cell line HepG2, compared with normal hepatocyte based on EnhancerAtlas 2.0. and I chose the closest HepG2 specific enhancer for my next plasmid construction (Figure 2d).

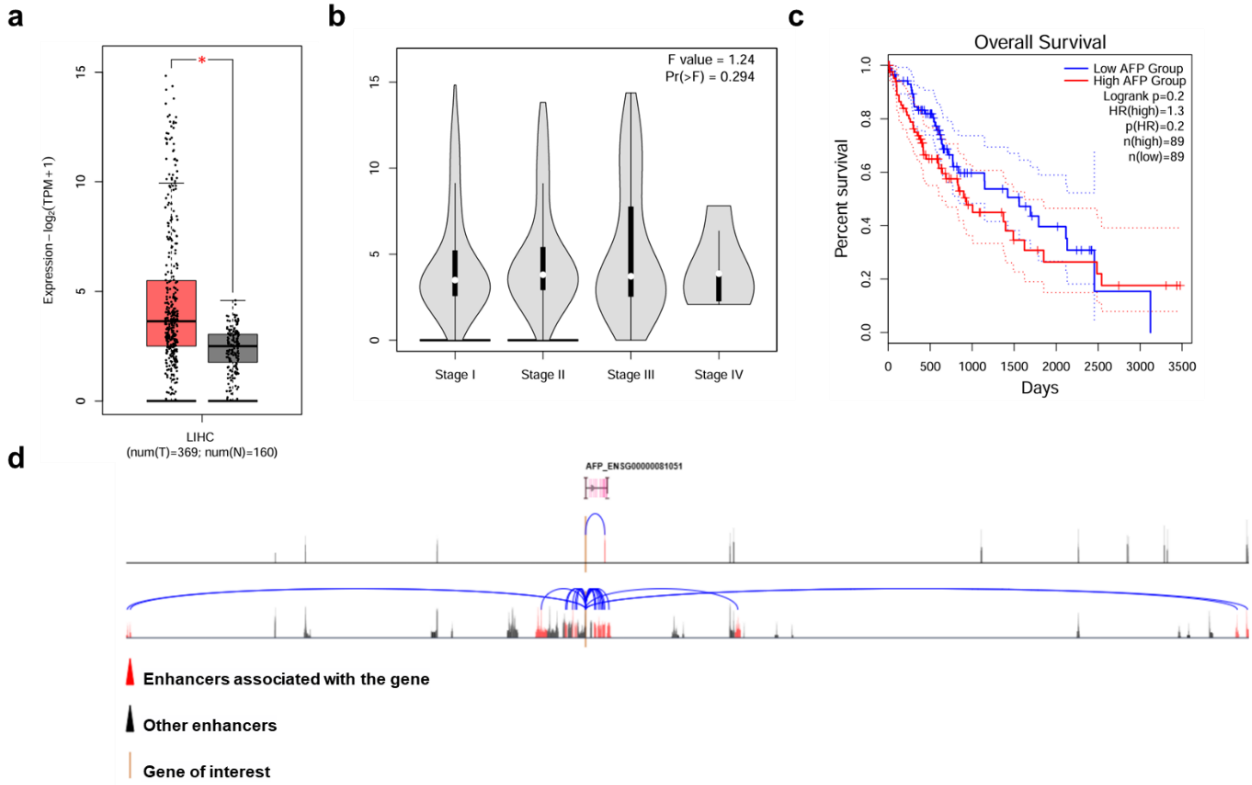

Figure 2. AFP expression and related regulation in human HCC. a,b, AFP gene expression in tumor vs normal tissue(**a**), and different stages(**b**) of HCC patients based on TCGA dataset. **c**, Survival analysis of HCC patients according to AFP expression. **d**, The database analysis AFP enhancer annotation of Hepatocyte and HepG2 (Hepatoblastoma) cell line based on EnhancerAtlas 2.0.

3.2. Successful plasmid construction and detection

To achieve high specificity and safety in targeting HCC, we constructed an AA2 plasmid incorporating three key components: the AFP promoter/enhancer, DTA, and the shGPX4 (Figure 3a). The AFP promoter/enhancer was strategically employed to exploit the elevated AFP expression characteristic of malignant HCC cells, thereby selectively inducing DTA expression and subsequent cytotoxicity in these target cells. Simultaneously, to address the challenge of chemotherapy resistance often associated with EMT and reduced apoptosis in HCC cells, we integrated the shGPX4 construct. This addition facilitates the silencing of the GPX4 gene, potentially enhancing treatment efficacy by promoting the elimination of DTA-resistant HCC cells. The AFP promoter-enhancer sequence, positioned upstream of the DTA gene, ensures that toxin expression is confined to AFP-positive HCC cells, while the shGPX4 construct enables targeted GPX4 knockdown. Importantly, this dual-targeting approach minimizes potential off-target effects on normal liver cells. As these normal hepatocytes typically maintain an epithelial phenotype and express lower levels of AFP, they are less susceptible to both DTA-mediated cytotoxicity and the effects of GPX4 knockdown. This design aims to maximize therapeutic efficacy while preserving the integrity of healthy liver tissue.

We then inserted these target genes into the AAV vector, pAAV2, using restriction enzyme sites MluI and BstEII at the 5’ and 3’ ends, respectfully. Following the digestion of both the pAAV2 vector and the commercially synthesized AFP promoter/enhancer-DTA-ShGPX4 cassette with MluI and BstEII, the DNA fragments were purified and ligated using T4 DNA ligase. The ligation reaction promotes the successful joining of the vector and our AFP promoter/enhancer, DTA gene, shGPX4 construct. To confirm the successful insertion of the target genes into the pAAV2 vector, several colonies were subjected to specific restriction enzyme test, including separate digestions with BamHI or SmaI. The digested plasmids were then analyzed through gel electrophoresis (Figure 3b). The gel electrophoresis results demonstrated that we had successfully inserted the AFP promoter/enhancer,

DTA, and shGPX4 constructs into the pAAV2 vector, as evidenced by the presence of bands of the correct size. Furthermore, we utilized DNA sequencing to verify the accuracy of the gene sequences within our construct (Figure 3c).

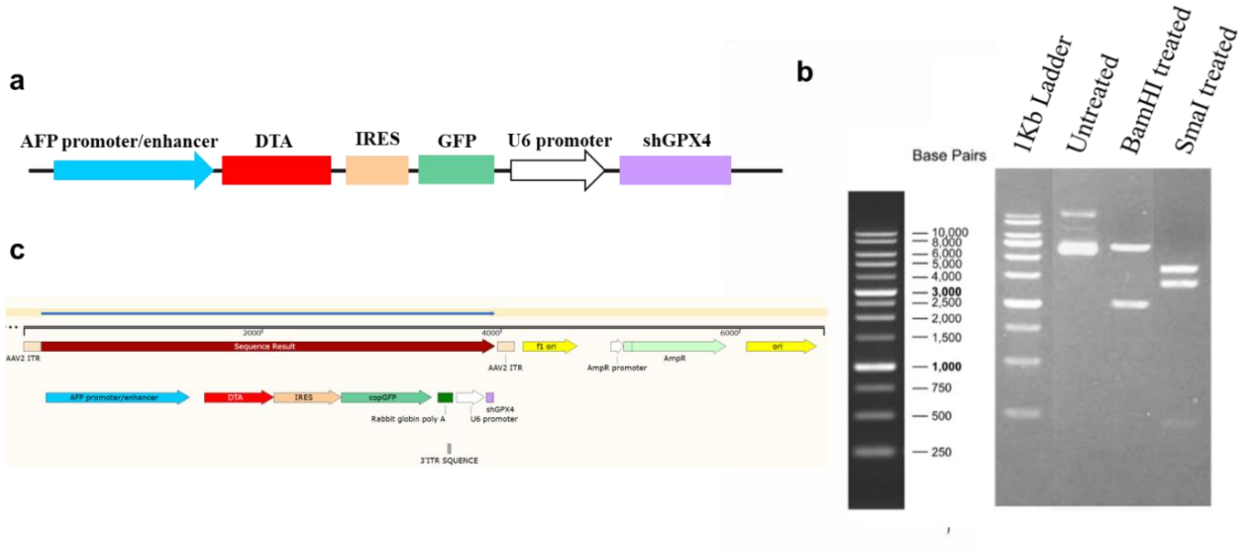

Figure 3. Construction of the functional plasmid AAV2-AFP promoter/enhancer-DTA-IRES-GFP-shGPX4 a, Schemic figure of constructed plasmid AAV2-AFPpromoter/enhancer-DTA-IRES-GFP-shGPX4. **b**, Gel electrophoresis of constructed plasmid following digestion with restriction enzymes BamHI and SmaI. Untreated plasmid was used as the control. **c**, Align analysis of sequencing result with our constructed plasmid mediated by SnapGene. The top blue line reveals our plasmid was constructed correctly, with no mutations.

3.3. Successful viral packaging and validation

To produce recombinant AAV2, a three-plasmid system was employed. This system comprised the following three plasmids: the pAAV2-AFPpromoter/enhancer-DTA-shGPX4 backbone plasmid, which contained the gene of targeting hepatocellular carcinoma (HCC) through AFP promoter/enhancer-driven expression of DTA; the U6 promoter driven expression of shGP4; the AAV-RepCap plasmid, which harbored the AAV replication (Rep) and capsid (Cap) genes essential for viral replication and packaging; and the pHelper plasmid, which supplied adenoviral helper functions, including the E2A, E2B, E4 ORF6, and VA RNA genes. These three plasmids were transfected into HEK293T cells using Lipofectamine 2000 as the transfection reagent, facilitating efficient delivery of the plasmid DNA into the cells. Following this transfection, the cells were incubated for 24 hours. To observe whether we successfully transfected our plasmid into our virus, we tried to identify GFP expression under a fluorescence microscope. As shown in the images, a large proportion of cells exhibit green fluorescence positive, indicating successful transfection of the HEK293T cells and effective packaging of AAV2 (Figure 4a).

Following 48 hours of transfection, HEK293T cells were lysed, and the cell lysates were centrifuged to isolate recombinant AAV2 particles. The harvested virus underwent purification through ultrafiltration and density gradient centrifugation to eliminate cell debris and contaminants. To evaluate the purity of the virus preparation, SDS-PAGE analysis was conducted. The resulting gel electrophoresis revealed distinct protein bands corresponding to the expected viral proteins (Figure 4b). AAV2 viruses are primarily composed of three capsid proteins, with approximate molecular weights of 82 kDa, 67 kDa, and 60 kDa. The presence of well-defined bands indicates successful purification of both the constructed and control AAV2 viruses, with minimal contamination from cellular or extraneous proteins. These results demonstrate the effectiveness of the virus purification process, resulting in a highly purified AAV preparation suitable for subsequent experiments.

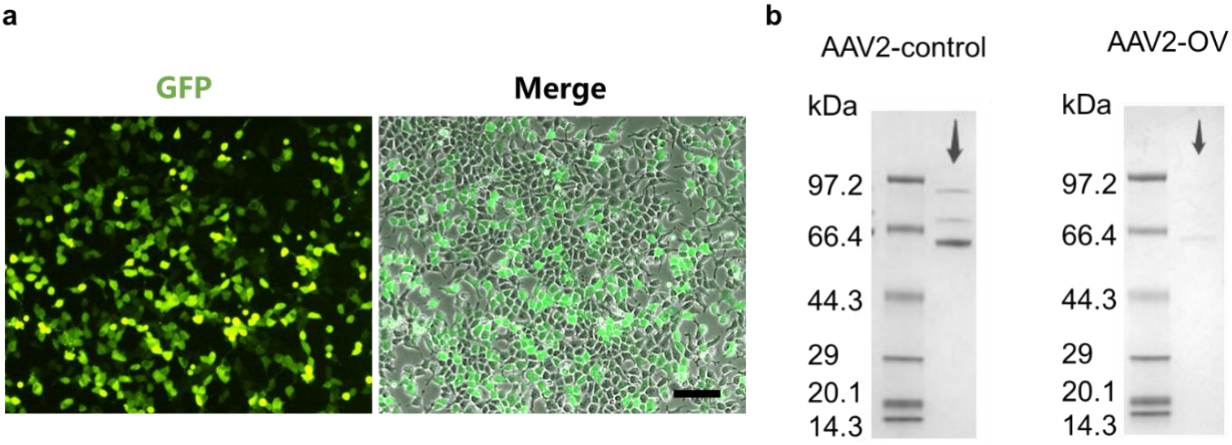

Figure 4. Successfully packaging and validation of AAV2 target virus a, GFP expression of HEK293T cells after 24h transfection of constructed plasmid, indicating successful transfection of the plasmid containing our plasmid. **b**, SDS-PAGE analysis of purified AAV2-control virus and AAV2-AFPpromoter/enhancer-DTA-shGPX4 virus (AAV2-OV). Scale bar represents 100 um.

3.4. AAV2-AFP promoter/enhancer-DTA-shGPX4 virus effectively targets and eliminates HCC Cells

After the successful cultivation of our AAV2-AFPpromoter/enhancer-DTA-shGPX4 virus, we wanted to observe its effectiveness in targeting HepG2 cells, a human HCC cell line with highly expression of AFP. To do so, we ran a CCK8 assay of different HCC cell lines after virus treatment with different MOIs (multiplicities of infection). We also included an AAV2-empty virus as control to see the base effects of viral transfection without our plasmid. The results are shown below (Figure 5a, b). In the AAV2-empty group results. At the same MOI, the control group exhibited significantly higher cell viability (Figure 5a), indicating that the observed cell death in the experimental group is not merely a consequence of viral infection or non-specific effects from the AAV2 vector.

Based on the CCK8 results, our construct virus demonstrates high efficiency in targeting and eliminating HepG2 and Hep3B cell lines, with a negative correlation observed between cell viability and viral MOI. After 72 hours of AAV2- OV treatment, CCK8 analysis revealed minimal decline in normal hepatocyte cell line MIHA cell viability, confirming the construct's safety for non-cancerous cells lacking AFP expression. In contrast, the highly AFP-expression HCC cell lines HepG2 and Hep3B cells, with high AFP expression, showed significant viability reduction, with up to 44.92% ± 5.79% in HepG2 and 36.09% ± 3.62% in Hep3B at an MOI of 2000, highlighting the construct's potent cytotoxic effects. It also demonstrates that while low AFP-expression HCC cell lines Huh7 cells exhibit reduced sensitivity compared to HepG2 and Hep3B, the construct still achieves a noticeable decline in cell viability at higher MOIs (Figure 5b). The results also suggest that the construct retains some activity against Huh7, indicating broader applicability, while maintaining its specificity for high-AFP-expressing HCC cells. Microscopy panels further supported these findings. Compared with control group, MIHA cell line with AAV2-OV treatment shows little to no green fluorescence, suggesting a lack of activation of the construct. This demonstrates how the construct does not target normal cells. And for HepG2 and Hep3B respectively, demonstrates a significant expression of the green marker across the cells, indicating successful activation of the construct. The disorganized clusters and signs of cell fragmentation also indicate the highly effective potent ability of the construct. In the most rightward image, Huh7 cells displayed reduced fluorescence due to low AFP expression, reaffirming the construct's AFP-dependent specificity (Figure 5c).

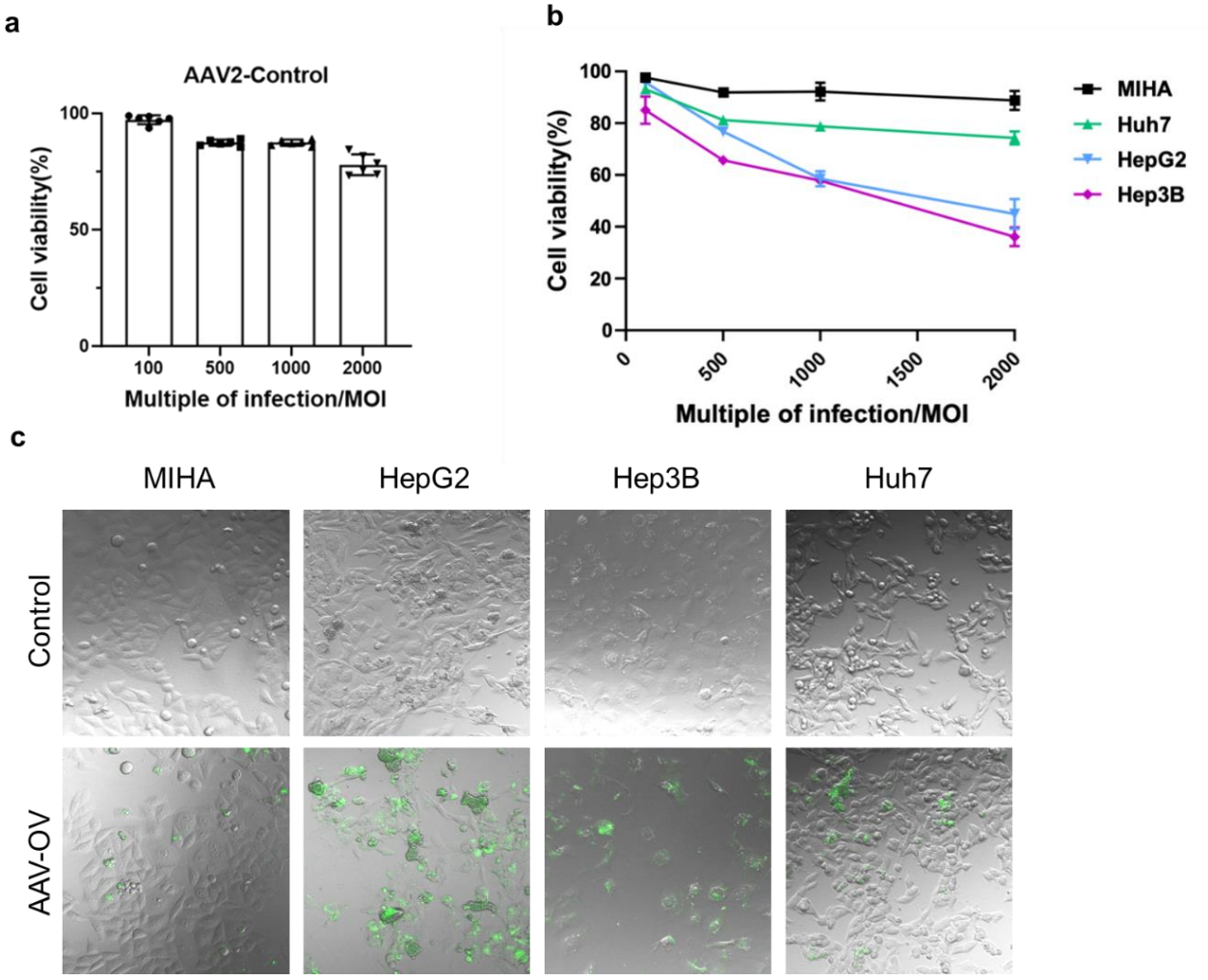

Figure 5. AAV2-AFP promoter/enhancer-DTA-shGPX4 virus effectively targets and eliminates HCC Cells. a, CCK8 cell viability test of HepG2 cells treated with control AAV2 virus. **b**, Cell viability of MIHA cells (Immortal hepatocyte with low expression of AFP), HepG2 cells (Hepatoblastoma with highly expression of AFP), Hep3B (Poorly differentiated HCC with highly AFP expression) and Huh7 (Well differentiated HCC with low AFP expression) at different MOIs (100, 500, 1000, 2000) after being exposed to AAV2-OV for 72 hours. **c**, Represent GFP images of different cell lines treated with AAV-OV (MOI= 2000) after 72 hours, compared with untreated cell lines. Data are presented as mean ± SEM; t test; each experiment n>2. Scale bar represents 100 um.

3.5. AAV2-AFPpromoter/enhancer-DTA-shGPX4 virus promote HCC drug response based on combination therapy with Cisplatin.

Finally, I also used the combination therapy to test the AAV2-OV function in anti-resistance. I treat the HCC cell line with Cisplatin and oncolytic virus for 72 hours, and to see, if AAV2-OV addition can decrease cisplatin resist cells. The bar graph highlights the synergistic effects of my construct with varying concentrations of cisplatin (Figure 6). In the HepG2 and Hep3B cell lines, the combination therapy significantly reduces cell viability at every MOI when compared to the effect of Cisplatin when used alone. This indicates that the inclusion of shGPX4 can overcome natural chemical resistance by disrupting redox homeostasis and inducing ferroptosis. The Huh7 cell line, similarly, to results of the CCK8 analysis, shows a minimal response. This is once again due to its low AFP expression and thus my construct is less likely to target it. This once again shows convincing evidence of the specificity of my construct. This type of therapy overall also highlights the synergy between targeted oncolytic therapy and conventional chemotherapy, suggesting a promising strategy for improving treatment outcomes in patients with HCC.

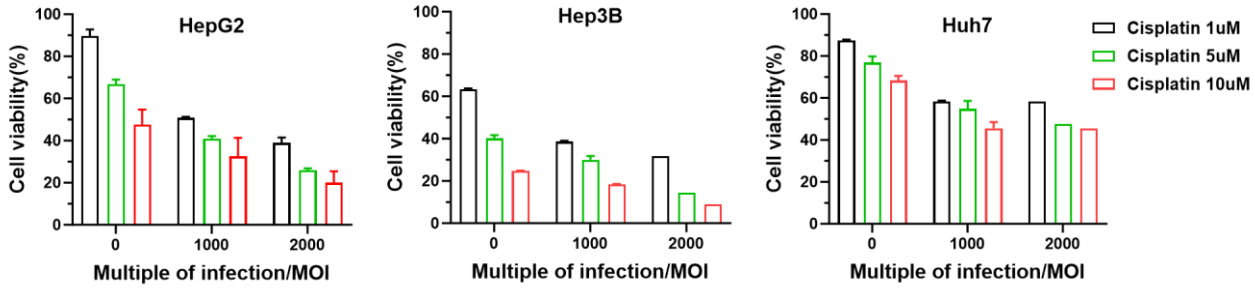

Figure 6. AAV2-AFPpromoter/enhancer-DTA-shGPX4 virus promote HCC drug response based on combination therapy with Cisplatin. Cell viability of 72 hours combination treatment in HepG2 cells, Hep3B and Huh7 at different AAV2-OV MOIs (0, 1000, 2000) with various dosage cisplatin (1uM, 5uM, 10uM). Data are presented as mean ± SEM; t test; each experiment n>2.

4. Discussion

This study presents a novel approach to treating Hepatocellular Carcinoma (HCC) using a genetically engineered oncolytic virus (OV) that combines the specificity of the AFP promoter/enhancer, the cytotoxicity of the DTA gene, and the anti-resistance mechanism of shGPX4. The results demonstrate the potential of this approach in selectively targeting and eliminating HCC cells while minimizing damage to normal liver tissue.

4.1. Specificity and safety of the OV construct based on AFP promoter/enhancer system

The use of the AFP promoter/enhancer in our AAV2 construct addresses one of the primary challenges in cancer therapy: specificity. By exploiting the elevated AFP expression characteristic of HCC cells, our OV demonstrates a high degree of selectivity. Unlike previously published AFP-based strategies that solely utilized the AFP promoter to mediate downstream gene expression[21,22], our approach incorporates both the promoter and enhancer elements. This combination results in more robust expression of downstream genes and enhanced targeting specificity. This is evidenced by the significant reduction in viability of HepG2 and Hep3B cells (which express high levels of AFP) compared to the minimal effect on MIHA cells (normal liver cells) or Huh7(HCC cells with low AFP expression). This specificity is crucial for minimizing off-target effects and potential toxicity to healthy liver tissue, a common concern with traditional chemotherapeutic approaches.

4.2. Efficacy of the DTA-shGPX4 Combination

The combination of DTA-mediated cytotoxicity and GPX4 silencing represents a novel dual-action approach in our study. While previous research has explored DTA-based therapies[23] or GPX4 inhibition[24] independently, our study is among the first to integrate these mechanisms into a single construct for HCC treatment. This innovative combination potentially offers enhanced therapeutic efficacy by simultaneously inducing direct cell death and sensitizing cancer cells to oxidative stress-induced ferroptosis.

The incorporation of the DTA gene provides a potent mechanism for inducing cell death in targeted HCC cells. The dose-dependent reduction in HepG2 and Hep3B cells showed significant viability reduction, with up to 44.92% ± 5.79% in HepG2 and 36.09% ± 3.62% in Hep3B at an MOI of 2000, demonstrates the effectiveness of this approach.

Furthermore, the inclusion of shGPX4 addresses the critical issue of treatment resistance, a significant challenge in HCC therapy. By targeting the GPX4 pathway, our construct potentially enhances the susceptibility of HCC cells to oxidative stress and ferroptosis, which could be particularly beneficial in eliminating resistant cancer stem cells and preventing tumor recurrence. According to my combination experiment, shGPX4 enhances susceptibility to ferroptosis by inhibiting antioxidant defenses in HCC cells. Target GPX4 enhanced HCC response to standard

clinical chemotherapy Cisplatin and decreased the remind cell ratio of resistance cells in HepG2 and Hep3B, not in Huh7 cells.

This dual-targeting strategy not only amplifies the cytotoxic effect but also provides a potential solution to the problem of acquired resistance often encountered in cancer therapies. The synergistic action of DTA-induced cell death and GPX4 inhibition-mediated ferroptosis represents a significant advancement in the field of oncolytic virotherapy for HCC, potentially offering a more robust and durable therapeutic response compared to single-mechanism approaches.

4.3. Advantages of the AAV2 Vector System

The choice of the AAV2 vector for delivering our therapeutic construct offers several advantages. Unlike traditional adenovirus (Ad)[21] or vesicular stomatitis virus (VSV)[25]based OV for HCC, AAV2's low immunogenicity and inability to self-replicate contribute to the overall safety profile of the treatment. This is particularly important given the concerns about severe immune responses associated with some OV therapies. The successful packaging and purification of the AAV2 particles, as evidenced by the GFP expression in transfected cells and the clean protein bands in SDS-PAGE analysis, indicate the feasibility of large-scale production for potential clinical applications.

4.4. Limitations and Future Directions

While the results of our study are promising, several limitations and areas for future research should be addressed to further validate and optimize our approach:

1. Long-term efficacy: Future studies should assess the prolonged effects of the treatment, including its capacity to prevent tumor recurrence and metastasis. This will provide valuable insights into the durability of the therapeutic response.

2. In vivo studies: As our current study primarily focuses on in vitro results, the implementation of animal models will be crucial for evaluating the efficacy and safety profile of our oncolytic virus in a complex biological system. This step is essential for translating our findings towards clinical applications.

3. Combination therapies: Investigating potential synergistic effects between our oncolytic virus and other treatment modalities, such as immunotherapies or targeted drugs, could significantly enhance its therapeutic efficacy. This approach may lead to more comprehensive and effective treatment strategies for HCC.

4. Resistance mechanisms: While the inclusion of shGPX4 addresses one mechanism of resistance, further research into other potential resistance pathways is warranted. This will help in developing strategies to overcome or prevent the emergence of treatment-resistant tumor cells.

5. Optimization of dosing: More detailed dose-response studies and comprehensive pharmacokinetic analyses will be necessary to determine optimal treatment regimens. This will ensure maximum therapeutic benefit while minimizing potential side effects.

Addressing these aspects in future research will be crucial for advancing our oncolytic virus therapy towards clinical trials and potential therapeutic use in hepatocellular carcinoma patients.

4.5. Implications for HCC Treatment

The development of this highly specific and potentially effective OV represents a significant step forward in HCC treatment. The ability to selectively target cancer cells while sparing normal tissue addresses a critical need in liver cancer therapy, where preserving liver function is paramount. Moreover, the potential to overcome treatment resistance through the GPX4 pathway inhibition could provide a valuable tool in managing advanced or recurrent HCC, for which current treatment options are limited.

5. Conclusion

In conclusion, our AAV2-based oncolytic virus incorporating AFP-driven DTA expression and RNAi mediated GPX4 silencing demonstrates promising results in selectively targeting and eliminating HCC cells. The high specificity, potent cytotoxicity, and potential to overcome treatment resistance make this approach a valuable candidate for further development in HCC therapy. While additional research is needed to fully elucidate its efficacy and safety in vivo, this study lays a strong foundation for the development of more effective and targeted treatments for hepatocellular carcinoma.

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