Oncolytic viruses in Cancer Therapy

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EXMD 610

April 2020

Introduction

History of OVs

Cancer is the second cause of death worldwide, with 9.8 million deaths reported in 2018 [1]. Cancer is an ancient illness. The term 'cancer' itself has been around for over 2000 years, and descriptions of cancer-like disease in humans dates back to 1600-1500 BC [2]. Up until the early 20th century, the only treatment option available for Cancer was surgery, which meant alternative and more effective treatments options were a necessity. Observations that patients suffering from Cancer had temporary remission after encountering viral infections drew attention to the possibility of using viruses against Cancer. During the mid 20th century, different viruses were being used in clinical trials against different cancers, however, patient outcomes were not improved, success rate was low, and adverse side effects were common. These factors coupled with the inability to modify viruses at the time lead to the abandonment of research that involved the use viruses to treat Cancer. Later advances in molecular biology and virology opened the door for new possibilities and brought back interest in viruses as Cancer fighting agents [3]. Today, viruses capable of selectively replicating and killing cancer cells are known as oncolytic viruses (OVs) [4]. Majority of these OVs are genetically engineered to increase their selectivity, efficacy and safety.

The global burden of Cancer is significant, and the available treatment options are not enough. Current treatment options include surgery, chemotherapy, radiation therapy, immunotherapy, targeted therapy, and hormone therapy amongst others. These treatments are often used in combination to boost their effectiveness [5]. Nevertheless, the clinical outcomes of these treatment regimens are not optimal, safety and adverse side effects are a concern, and the number of annual Cancer deaths is still increasing [6, 7]. Due to these limitations, the pursue of new treatments that are safer and more effective continues. Oncolytic viruses offer a unique and a promising candidate in the fight against Cancer.

OVs in the field

One of the earliest attempts to test OVs in clinical trials dates back to 1949. The trial was carried on 22 patients with Hodgkin's lymphoma. 63.6% of patients got hepatitis, 31.8% showed clinical improvement that lasted a month or more, and one patient died. Hepatitis B virus was introduced to these patients by injecting them with infectious serum/tissue extracts from patients suffering from Hepatitis. The outcomes of this trial and many that followed presented OVs as unsafe and ineffective treatment against cancer, causing a setback in OVs related research. However, as science progressed, interest was reignited in OVs as cancer therapeutics. In 2005, China was the first county to approve the OV adenovirus H101 for the treatment of head and neck cancer [3]. It took 10 more years to develop talimogene laherparepvec (T-VEC); the first FDA approved OV for the treatment of nonresponsive melanoma. T-VEC is an oncolytic herpes simplex virus that showed promising results in treating inoperable melanoma during phase III clinical trials [8]. Although OVs have proven to be a powerful therapeutic tool for cancer, their clinical effectiveness is limited to a low number of patients. In order to increase their efficacy, it is proposed that OVs are used in combination with other drug to achieve maximum clinical improvement for a larger number of patients [9]. Several combination therapies using OVs and immune check-point blockers (ICB) are currently undergoing clinical trials to assess their effectiveness [10]. Despite the presence of over 150 clinical study on clinicaltrials.gov dedicated to evaluating OVs as cancer therapeutics, only four studies are at phase 3. Two out of four of the aforementioned studies tested the effectiveness or safety of T-VEC, while the other two are combination therapies testing the effectiveness of OVs and other drugs [11]. This could be a direct result of the difference between the promising outcomes observed in pre-clinical studies versus the unsatisfactory outcomes in human trials

Cancer-killing mechanisms of OVs

The cancer-killing function of OVs consists of two arms; the direct lysis of tumor cells, and the induction of antitumor immunity which mediated the indirect killing of tumor cells. OVs directly destroy tumor cells by exploiting the altered pathways in these cells. They utilize the defective antiviral response of cancer cells to ensure viral propagation. Generally, when a virus infects healthy cells, it can cause the activate Toll-like receptors (TLRs) initiating the antiviral response. TLRs activate a series of responses that lead to the release of antiviral elements, including interferons (IFN). IFN works to activate protein kinase R (PKR), an enzyme that plays an integral role in controlling viral infections by inducing the death of infected cells. Cancer cells, however, have altered IFN pathway and PKR activity, these alterations delay cell death and create the window needed for viral replication [12].
Cancers are able to escape the surveillance of the immune system due to the complex tumor composition. Tumors are more than uncontrollably dividing cells. Several other cells types, such endothelial cells and various types of immune cells, are present in the tumor mass. These cell types work together to create an environment known as the tumor microenvironment (TME), which plays a major role in determining the outcome of many anticancer treatments. Tumors with immunologically active immune cells (such as CD 4+ and CD8+) in their TME are known as immunologically "inflamed" tumors, whereas those with immunosuppressive cells (such as regulatory T cells (Treg) and M2 macrophages) are known as immunologically cold tumors. The lack of immune response to cold tumors makes them and resistant to conventual cancer treatments. Cold tumors need to become inflamed to allow the initiation of anticancer immunity. OVs are able to manipulate the TME and activate a potent immune response against tumor cells [13]. Cell death caused by viral infections results in and the release of tumor associated antigens (TAAs),viral antigens, danger signals and cytokines, which work to stimulates an innate and adaptive immune response; effectively

turning a cold tumor to an inflamed tumor. Cell death that induce an immune response is known as immunogenic cell death (ICD). Cytokines and danger signals released during viral infection, including pathogen-associated molecular pattern molecules (PAMPs) and Damage-associated molecular patterns (DAMPs), activate different compartments of the immune system. Innate immunity is initiated when natural killer (NK) cell are activated mainly by type I IFN & DAMPs. Adaptive immunity is carried out by the activation of a T cell response against tumor cells. Cytokines, DMAPs, PAMPs, and TAAs activate antigen presenting cells (APCs), including dendritic cells (DCs), which results in the activation of a CD 4^+ and $CD8^+$ cells that work to eliminate both infected and uninfected tumor cells, causing an overall all reduction of the primary tumor and targeting metastatic tumor cell (Fig. 1) [12].

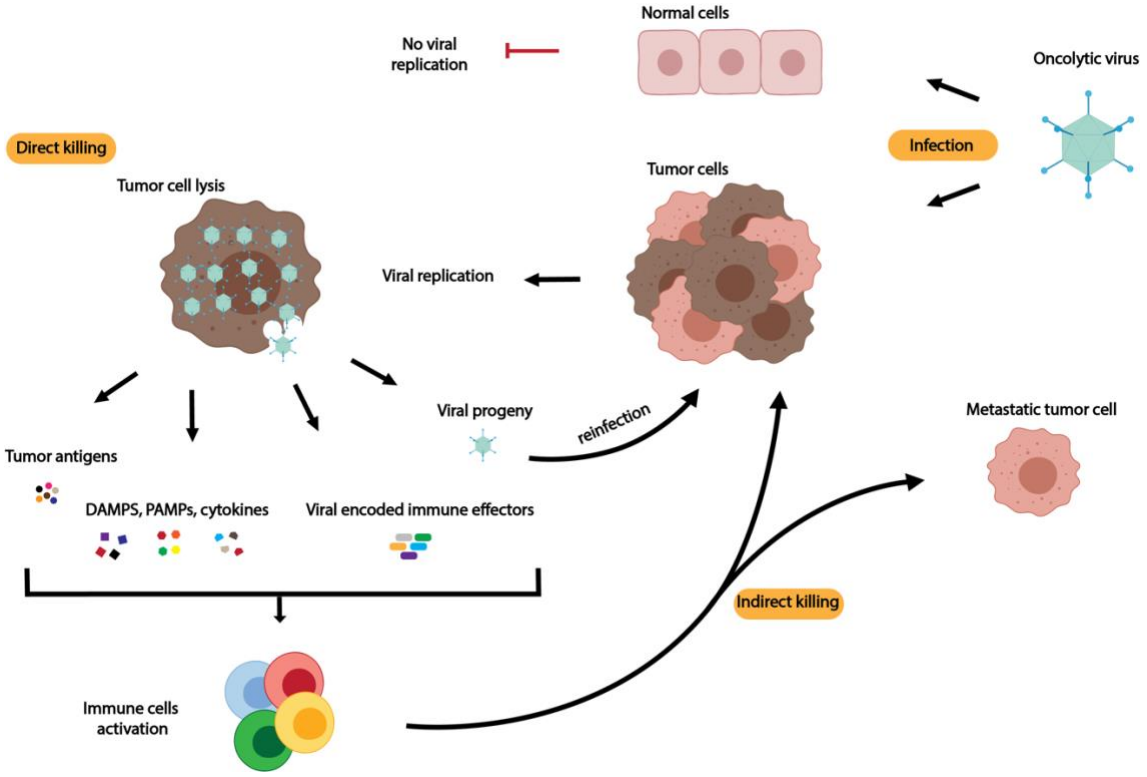

Figure 1: An overview of the mechanisms in which tumor cells are destroyed by the action of OVs. The OV infects target cells based on viral tropism and selectively replicates within cancer cells but not in normal cells. Viral replication causes the lysis of tumor cells, and the consequent release of progeny viruses, tumor antigens, immune effectors (in viruses augmented with immune effector genes), DAMPs, PAMPs and cytokine. Progeny viruses re-infect tumor cells. The other factors work together to stimulate a robust antitumor immune response capable destroying local and metastatic tumor cells.

Methodology

Enhancing the selectivity of OVs to tumor cells:

Generally, viruses infect specific cell types, a characteristic known as viral tropism. For example, Hepatitis B virus infects liver cells, rabies virus infect neuronal cells, and HIV infects T helper cells [14]. Some OVs have a natural tropism for tumor cells, such as herpes simplex virus type 1 (HSV-1) and reovirus, while others are engineered to selectively infect tumor cells, for example, adenovirus and lentivirus [14, 15].

Naturally occurring OVs preferentially infect cancer cells due to the presence of surface receptors that permit viral entry to the cell. For example, HSV-1 has a higher affinity to cells expressing herpes virus entry mediator (HVEM), nectin-1, and nectin-2. These molecules are overexpressed in some types of tumor cells, leading the tumor selectivity of HSV-1. In addition, some naturally occurring OVs infect both healthy and malignant cells. However, the propagation of these viruses is halted in healthy cells and promoted in tumor cells. This is due to the distorted biology of tumor cells. In order to ensure survival, tumor cells manipulate cellular pathways that enhance cell division and resist cell death. This provides some OVs with an environment that promotes their propagation. For example, reovirus infects normal cells as well as tumor cells, however, it is only capable of successfully replicating in Ras-dependent tumor cells, ultimately leading to their destruction [15].

Viruses that lack natural tropism for tumor cells could be adapted to target cancer cells by introducing genes that code for proteins that have high affinity to receptors highly expressed by tumor cells. An example of such engineered virus is a lentivirus vector that was modified to target melanoma cells. This was achieved by using the Sindbis virus envelop, which has a natural affinity for melanoma cells, to create pseudotyped lentivirus vector capable of targeting melanoma cells. Furthermore, an alternative way to control the tropism of OVs is to use cancer-specific promotors to control the transcription of genes responsible for viral

replication. [15, 16]. For example, an engineered adenovirus known as CV706 uses the prostate-specific antigen (PSA) promoter to control the expression of the viral E1A, a protein essential for viral replication. PSA is only expressed in prostate cells and is overexpressed in prostate tumor cells. This made the CV706 effective against prostate tumor cells, with minimal systemic toxicity [17]. In addition, tropism for tumor cells can be controlled by integrating miRNA response elements (MREs) at the 3'UTR to control the transcription of the viral genome. MREs are sequences complementary to microRNA transcripts found in the cell. They serve in post-transcriptional regulation. When microRNAs bind their complementary MREs, the messenger RNA (mRNA) can no longer be translated [18]. For example, the oncolytic Coxsackievirus A21 (CVA21), although very effective against cancer cells, causes sever myositis, that leads to the death of immunocompromised and suckling mice. To overcome this side effect, an MRE was introduced to the 3' UTR of the viral construct, which stopped the propagation of the virus within muscle tissue and the development of myositis [19].

Construction of OVs as cancer therapeutics

This section is dedicated to dissecting two methods used to program effective oncolytic adenoviruses against hepatocellular carcinoma (HCC) and glioblastoma respectively. Adenoviruses are double stranded DNA viruses, with a genome of approximately 36 kilobases (kb). They have low pathogenicity risk and has a DNA loading capacity of up to 8.5 kb, making them a good candidate for OV development [20].

Huang et al (2019) constructed a simple sensory synthetic gene circuit to create a highly selective oncolytic adenovirus with improved efficacy. Gene circuits are the biological analogues of electrical circuits. They are constructed by applying engineering principals, mathematical models, and computational tools to make circuit diagrams that outline cellular

pathways. In addition to providing insight to the complex gene regulatory processes happening in a cell, gene circuits provide a tool to control and manipulate biological processes at the gene level [21]. In this study, the gene circuit consisted of several compartments. The switch consisted of two mutually inhibiting transcription activator-like effector repressors (TALERs) and was controlled by microRNA input. Following thorough research and testing, miR-21 was shown to be a selective marker for HCC cells, while miR-199-3p showed specificity to normal liver cells. In addition, miR-142 was also included to ensure that the sensory circuit remains off in normal cells, specifically tumor infiltrating lymphocytes. The alpha fetoprotein (AFP) promoter, an HCC specific promoter, controlled the OV's replication and the expression of virally encoded immune effectors. AFP was placed upstream of the transcription activator Gal4VP16, which activated the expression of E1A, repressor-a (LacI), and repressor-b (tetR:Krab). Expression of the EA1 gene is a sufficient driver of viral replication, while LacI and tetR encode two repressors that work to inhibit each other. LacI and tetR are controlled by miR-199a-3p/miR-142 and miR-21 respectively. In HCC cells, the AFP promoter activates the transcription of Gal4VP16, leading to the transcription of EA1, LacI, and tetR repressors. The presence of miR-21 prevents the expression of the tetR repressor, allowing the expression of EA1, and LacI repressor, which works to further inhibit the transcription of tetR repressor, ensuring successful viral replication. In contrast, normal liver cells have high levels of miR-199-3p, which prevents the expression of EA1 and LacI repressor, while tetR is expressed normally, preventing further transcription EA1, and halting viral replication (Figure 2).

The adenovirus was constructed through a hierarchical framework. Several gene parts were assembled to construct three functional circuit compartments. These compartments were further assembled together to construct the circuit. The circuit was loaded into the viral backbone. Circuit assembly and viral backbone loading were done using Golden gate and

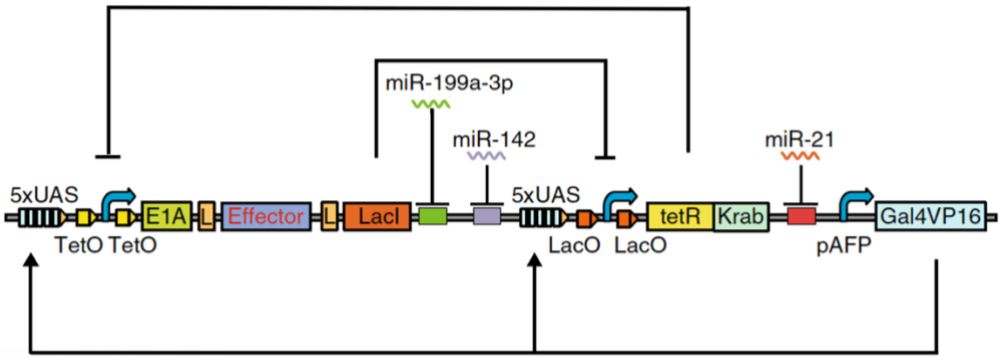

Figure 2: 5X tandem repeats of upstream activations sites (UASs) were placed upstream of the binding of site of repressor-b binding site (tetO), the E1A gene, the immune effector and the LacI repressor. Another 5X tandem repeats of UASs were placed upstream of the binding of site of repressor-a binding site (LacO), and the tetR:Krab gene. The circuit is switch on in cancer cells when the AFP promotor is active, there is a high level of miR-21 and low level of miR-199a-3p and miR-142. pAFP; alpha fetoprotein promoter, L; self-cleavage 2A linker, Effector; immune effector. (This diagram was adapted from Huang et al (2019) with modifications)

Gibson cloning method. The generation of virus particles (VPs) was achieved by transfecting HEK239 with the linearized adenovirus backbone and collecting the virus progeny from the supernatant. To assess the functionality of the sensory circuit, several experiments were carried out using different cell lines and mouse models. Chang liver cells were used to represent normal liver, and the HCC cell lines used were HepG2, Huh7, and Hepa1-6. The oncolytic activity of the OV was tested in the three cells lines and 8 different multiplicities of infection (MOIs). The OV was able to eliminate 50% of HepG2 and Huh7 cells at MOI between 0.1 and 1, while MOI of 100 or more was needed to eliminate 50% of Chang cells. Hepa1-6 cells required an approximate MOI of 300 to eliminate 50% of the cell. This could be a result of the low expression of AFP in these cells, as well as the low viral replication seen in Hape1-6 cells. OVs used in this experiment were not augmented with immune effectors. An enhanced blue fluorescence protein (EBFP) gene was put in place of the effector genes.

Moreover, nude mice were used as an immunocompromised animal model and C57BL/6 mice as an immunocompetent model to test the efficacy of the OV in vivo. Xenograft tumors

of the 3 HCC cell lines were allowed to reach 100 mm^3 after which the mice were intratumorally injected with $1x10^9$ VPs twice a week (at day 0 and day 6). The growth of HepG and Huh7 tumors was delayed up to 39 days, proving the potent oncolytic capacity of the OV. Hapa1-6 cells tumors treated under the same conditions showed a reduced growth rate when compared to the PBS control and the non-replicating adenovirus (Ad-GFP) (Fig. 3a). Furthermore, viral DNA & RNA content in different tissues was assessed using RT-PCR or PCR to monitor the viral distribution in the mice. The highest amount of the viral DNA and RNA were found in tumor tissue (Fig. 3b). Additionally, immunocompetent C57BL/6 mice harboring Hepa1-6 xenografts were injected intratumorally with $1x10^9$ VPs of different synthetic OVs twice a week after the size of the tumor reached 100 mm^3. In consistence with the in vitro results, the different OV constructs effectively stopped or delayed HCC tumor growth in C57BL/6 mice (Fig. 3c left). Moreover, 80% of the mice infected with an OV construct containing single-chain variable fragment (scFvs) against PD-1 showed a durable tumor response or complete elimination of the tumor 60 days after infection (Fig. 3c right). PD-1 is a protein responsible for self-tolerance and the prevention of T-cells reactivity against one's own cell. The protein is highly expressed in tumor cells to escape T-cell killing. Furthermore, OV constructs, especially those with immune effectors, were able to stimulate a cytotoxic T-cell response in the tumor infiltrating lymphocytes (TIL). Measurement of $Ki\text{-}67^+$ & $IFN\text{-}gamma^+$ using flowcytometry confirmed the presence of proliferative and cytotoxic $CD8^+$ T cells (Fig. 3d). In addition, OV constructs encoding immune effectors were able to recruite a higher number of lymphocytes into the tumor. Immunocompetent mice re-challenged with Hepa1-6 tumor cells introduced to the mice in a new site far from the original xenograft site were able to reject and eliminate the tumor cells. This suggests that the immune system was successfully able to create memory cells capable of targeting distant tumor cells even in the absence of the OV.

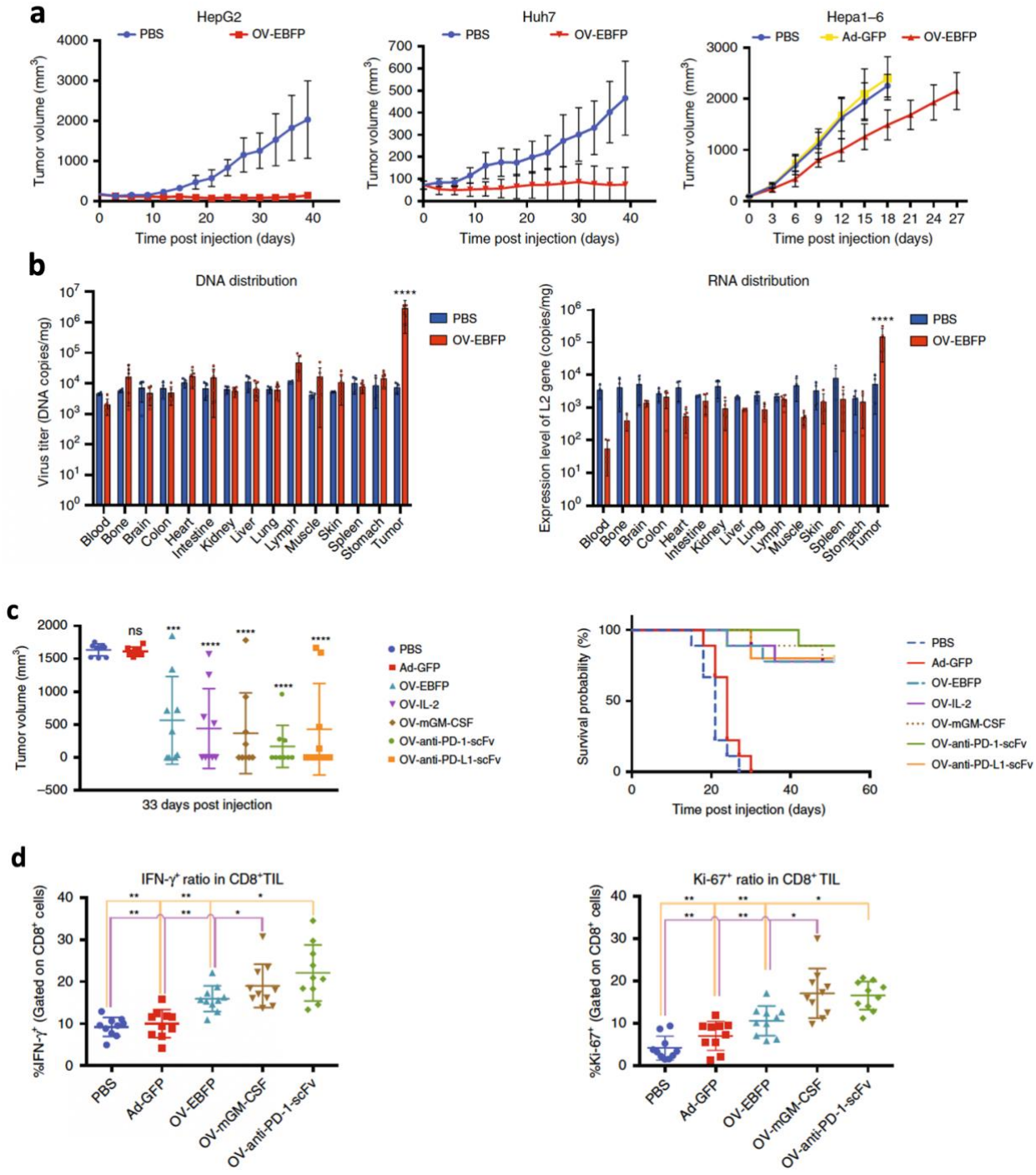

Figure 3: Efficacy of the constructed OV in animal model. **a** Tumor size after the injection of 1x10$_9$ VPs of OV-EBFP (or Ad-GFP in Hape1-6 tumor) at day 0 (D0) and day 6 (D6), after tumor size reached 100mm. Each datapoint represents the mean ± SD (n= 9 or 10) at the specified day. **b** The distribution of DNA (left) and RNA (right) of OV-EBFP in various HepG xenografted nude mice tissues 1 week after the injection of 1x10$_9$ OV-EBFP VPs (n=7 left, n=5 right). PBS (n=4) was used as a negative control **c** Tumor size (left) and survival probability (right) of C57BL/6 mice injected with 1x10$_9$ VPs of different OV constructs at D0 and D6 after the Hepa1-6 tumor size reached 100 mm$_3$. **d** Ratios of INFɣ$_+$ (left) and Ki-67$_+$ (right) T-cells in Hepa-16 tumor infiltrated with CD8$_+$ 14 days post infection with 1x10$_9$ VPs of different OV constructs. PBS was used as a negative control in all experiments, data represents mean ± s.d., *P<0.05, **P<0.01, ***P<0.001 ****P<0.0001 [20].

Overall, this study provided a simple framework to engineer an effective and a highly selective synthetic OV using a sensory switch circuit. The addition of immune effectors to the construct significantly increased the antitumor immune response and improved the tumor regression after OV administration. The circuit design is flexible and could be used to program different types of oncolytic viruses. However, several concerns need to be addressed. The promoter activity and microRNA levels differ from one patient to another, which may cause inconsistent therapeutic outcomes among patients. The effect of the non-human proteins LacI, tetR, and Gal4VP16, which are used to control the circuit switch is not known and their safety must be assessed. The amount of immunomodulators released by the OV must be closely monitored to ensure they are not causing undesirable side effects. [20].

Oh et al (2017) constructed an oncolytic adenovirus that was specific to glioblastoma. The selectivity of the OV was achieved by incorporating a modified telomerase reverse transcriptase (mTERT) promoter. In addition, the efficacy of the virus was increased by encoding trimeric tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a potent apoptotic cell death inducer, into the viral construct. The authors took their OV construct a step further and included a hypoxia responsive region to account for the strongly hypoxic glioblastoma TME, to overcome the reduced viral replication due to the hypoxic environment. The mTERT promoter was synthesized by incorporating 5X c-Myc binding sites upstream of the TERT promoter (5CmTERT), followed by upstream addition of 6 copies of hypoxia responsive element (HREs) to the 5CmTERT (Figure 4)

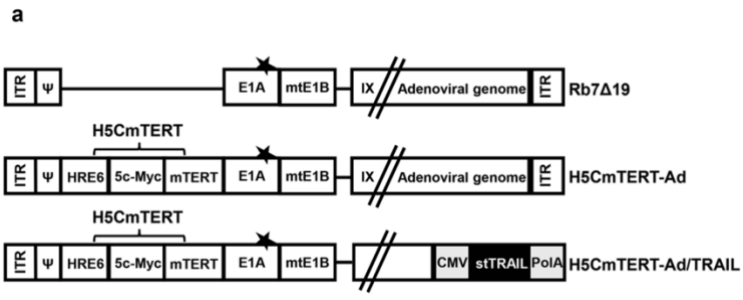

Figure 4: A schematic representation of three viral constructs used to test and validate the effectiveness of oncolytic H5CmTERT-Ad/TRAIL adenovirus. Rb7Δ19 represents the control (Figure adapted from Oh et al (2017)).

TERT promoter was chosen to control viral replication due to its activation in about 90% of cancers and minimal activity in normal human cells. To increase the transcription rate of the promoter, 5X c-Myc binding sites were incorporated, which resulted in a significant increase in tumor killing activity of the OV due to the higher rate of transcription. Furthermore, a truncated but functional TRAIL gene (stTRAIL) was added to selectively induce apoptosis in tumor cells. The specificity of the OV was tested in glioblastoma and normal cell lines under normal and hypoxic conditions at different MOIs. The OV constructs showed stronger killing activity under hypoxic conditions when compared to the control oncolytic adenovirus Rb7Δ19. The oncolytic effect of 5CmTERT-Ad/TRAIL was 4.2 or 2 folds stronger than that of 5CmTERT-Ad only, demonstrating the effectiveness of the stTRAIL. Although treated with higher doses of VPs (MOI of 2 and 5 for BJ cells and 2 and 5 for SVG cells) there was no significant cytotoxicity observed in normal cells, confirming the cancer specificity of the 5CmTERT-Ad constructs and the safety of stTRAIL expression (Fig. 5).

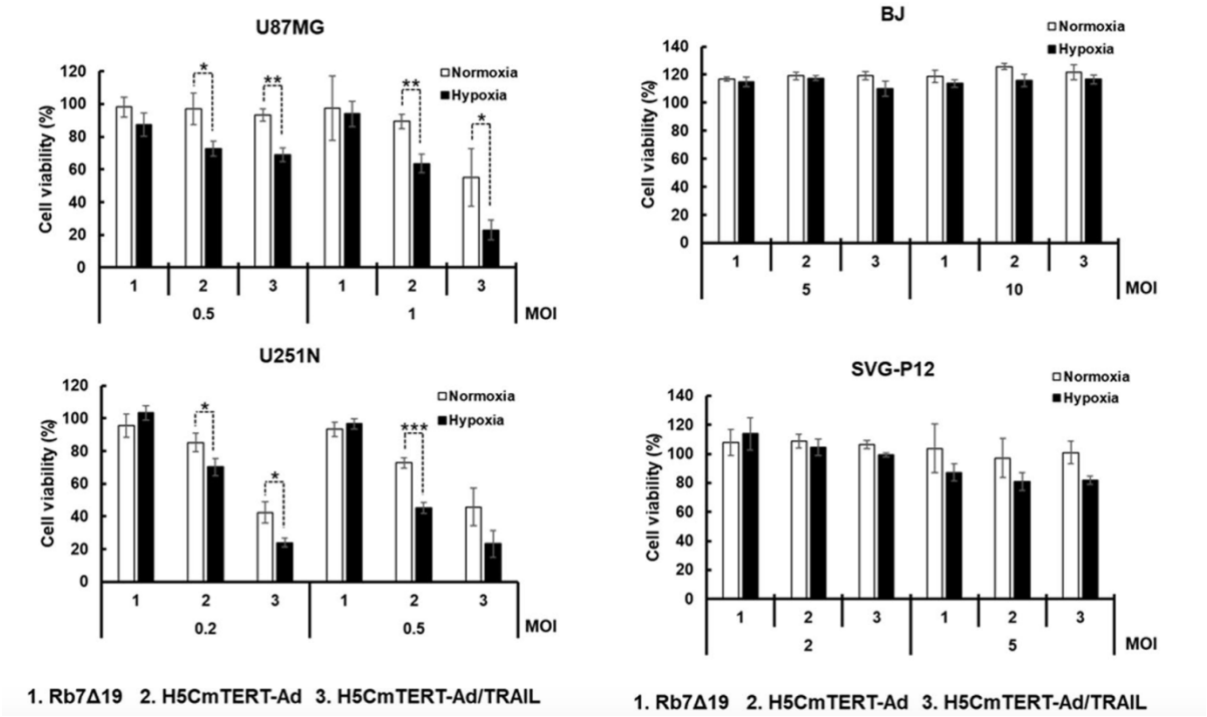

Figure 5: Cell viability after 48hrs infection with different OV constructs in glioblastoma (U87MG and U251N) and normal (BJ and SVG-P12) cell lines at different MOIs under normal or hypoxic conditions. data represents mean ± SD of n=3 for each cell line, *P<0.05, **P<0.01, ***P<0.001 [22].

Moreover, to validate that TRAIL induced apoptotic cell death, a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay and transmission electron microscopy (TME) were performed to detect DNA fragmentation and view the apoptotic changes in infected glioblastoma cell lines. Tumor cells treated with 5CmTERT-Ad/TRAIL showed stronger induction of apoptotic cell death when compared to Rb7Δ19 and control and 5CmTERT-Ad. In addition, TME imaging revealed cell changes indicative of necrosis and the virus was visible within the nucleus and the cytoplasm of infected cells.

To confirm the in vivo antitumor effect of 5CmTERT-Ad/TRAIL nude mice with U87MG xenografts were used. The mice were treated with intratumoral injections of OV constructs or PBS and the tumor volume was measured every two day for 21 days. Mice treated with 5CmTERT-Ad/TRAIL showed 80.9% tumor growth inhibition versus a 54.2% inhibition in 5CmTERT-Ad when compared to PBS (Fig 6).

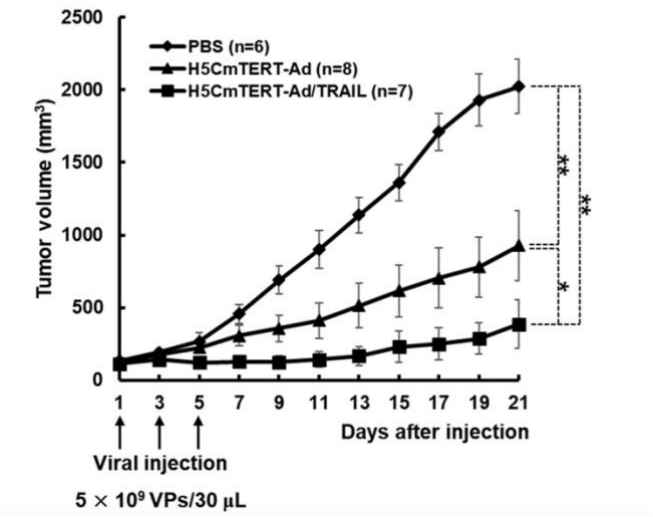

Figure 6: Size of U89MG xenograft tumors in nude mice after the injection with of 5x10$_9$ VPs on day 1, 3 and 5. *P<0.05, **P<0.01 [22]

To attempt to test the OV constructs in a preclinical setting, an orthoptic U89MC glioblastoma xenograft model was chosen due to its close resemblance to the clinical cancer.

Tumors were injected with intracranial injection of OV constructs or PBS 7 days after the xenografts were established. Treatment with 5CmTERT-Ad and 5CmTERT-Ad/TRAIL lead to a 61.7% and 89.8% reduction in tumor growth respectively. In addition, the rate of long-term survival (35 days post treatment) was improved by 33.3% in mice treated with

5CmTERT-Ad/TRAIL versus 16.7% in those treated with 5CmTERT-Ad. Furthermore, to confirm the oncolytic activity of 5CmTERT-Ad/TRAIL in orthotopic tumors models, brain tissues were collected 10 days after treatment. Immunohistological analysis revealed high expression of TRAIL and EA1 (indicative of viral replication). In addition, TUNEL assay revealed a significantly higher induction of apoptotic cell death in tumors treated with 5CmTERT-Ad/TRAIL. These results corroborate the effectiveness of the oncolytic properties of the 5CmTERT-Ad/TRAIL construct [22].

All in all, this study was able to demonstrate the potent oncolytic activity of 5CmTERT-Ad/TRAIL against aggressive glioblastoma that are difficult to treat. However, the effect of the OV construct on the immune response needs to be evaluated and a safety assessment needs to be conducted.

Choosing the right OV

Several factors need to be included when selecting an oncolytic virus. These include the genome structure and size, the pathogenicity, the selectivity, and the immunogenicity. OVs could have a single stranded or a double DNA or RNA genomes. The genome size determines the loading capacity and hence, the ability to modify these OVs. DNA viruses have large stable genomes, while RNA viruses are smaller genomes that more prone to mutation. RNA viruses are able to kill tumor cells faster than DNA viruses. Some OVs have a wide range of tropism while others are more specific to a single type of cancer cells. Moreover, some OVs are highly pathogenic and require attenuation or the removal of the virulence genes before they could be utilized as cancer therapeutics [23]. Different oncolytic viruses harbor different properties and the choice of the OV to be used in a clinical setting greatly depends on the resources available and the desired clinical outcome.

Challenges of oncolytic virotherapy

Despite its effectiveness, some aspects of oncolytic virotherapy such as selectivity, pathogenicity, delivery, require further improvement. Tumor targeting could be improved significantly by employing strategies thoroughly discussed in the methodology section. Viral pathogenicity could be reduced by attenuating or genetically modifying the viruses. Lastly, developing innovative OV delivery methods is necessary to overcome viral clearance by the immune and ensure maximum efficacy [10].

Conclusion:

Oncolytic viruses offer a powerful strategy to treat Cancers, especially those that are resistant to other forms of therapy. Their unique selectivity for cancer cells is key for a safe and a specific tumor elimination. The ability of OVs to induce a potent antitumor immune response by activating the immune system or releasing immune modulators offers a wider range of treatment possibilities.

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