Engineered Oncolytic Herpes Simplex Virus as Treatment for Glioblastoma

Rúbies Bedós, Marta
Biomedical Sciences Bachelor's Thesis
Universitat Autònoma de Barcelona, Cerdanyola del Vallès, 08193, Spain.
Correspondence: marta.rubies@e-campus.uab.cat

Date of submission: June 2018

Keywords: oncolytic immunotherapy, HSV, glioblastoma, combination therapy, oncolytic virus.
ABSTRACT

Since they were discovered over a century ago, oncolytic viruses have been expected to produce a cure for cancer. Although results so far have not been promising, the FDA approval of an oncolytic virus (T-VEC) for malignant melanoma in the United States in 2015 has yet opened again the door for this therapy as a potential treatment for cancer. With improved technologies and more knowledge on the matter, scientists have engineered oncolytic viruses and combined them with current therapies. Results obtained in preclinical trials are promising, and some of them are ready to be translated into clinical trials. Oncolytic immunotherapy is particularly promising for glioblastoma, as there are currently no successful treatment available. Moreover, while glioblastoma tumours constantly develop resistance to more classic treatments, no resistance has been observed towards any oncolytic therapy. In this review, oncolytic Herpes Viruses of three subsequent generations that are currently in clinical trials for glioblastoma are examined.

INTRODUCTION

Glioblastoma, or glioblastoma multiforme (GBM) is one of the most lethal human tumours. There’s no successful treatment available, and average life expectancy is 15-18 months post diagnosis. Glioblastoma is the only grade IV tumor of the central nervous system (CNS), and the most common and malignant in adults’ brains, predominantly in patients aged 50 an over (1). As seen in figure 1, glioblastomas are characterised by hypercellularity, pleomorphism, and nuclear atypia (1). It differentiates from other less malignant gliomas due to the presence of necrosis (according to the World Health Organisation (WHO) grading system (3)).

From both their molecular behaviour and histological appearance, glioblastomas can be subdivided into two different kinds. Primary glioblastoma – the most lethal and common – arises by the key driver mutation that produces Epidermal Growth Factor Receptor (EGFR) ligand-independent activity (4). Secondary glioblastoma is an evolving tumour, which progresses from a lower-grade astrocytoma or oligodendroglioma due to an accumulation of genetic alterations, most of them due to a starting mutation in p53 (3). Secondary glioblastomas contain the characteristic molecular marker 1p/19q co-deletion, which is used as a predictive biomarker for better prognosis (3, 4).

The current therapeutic approach is surgery, followed by radiotherapy and/or chemotherapy with temozolomide (TMZ) (5). Standard treatment doesn’t differentiate by the origin of the tumour, but rather it only depends on the age of onset as well as the methylation state of O-6-methylguanine-DNA methyltransferase (MGMT) (figure 2). This treatment, although being the best approach, only lengthens survival of the patients for a few months. Which is why research on new and alternative treatments is so much needed in glioblastomas.

MGMT is a prognosis biomarker, as its hypermethylation is usually an indicator of longer survival (1, 4). MGMT is a DNA repair protein that counteracts the damage induced by alkylating agents such as TMZ, which is the standard approved chemotherapeutic agent. Temozolomide acts by inducing DNA damage so that the cells can’t proliferate any further. MGMT’s presence means a reduced effect of TMZ because the DNA damage is counteracted by MGMT (4). However, MGMT loss, paradoxically increases genetic instability and facilitates the acquisition of new mutations (6).

A characteristic hallmark of glioblastoma is the high levels of angiogenesis, which result in aberrant vascularisation. The tumour has an initial rapid growth, which creates multiple hypoxic areas, and thus the apparition of aberrant blood vessels (1). Hypoxic cells have an upregulation of HIF1 and HIF2, and therefore the secretion of angiogenic factors will be enhanced, the most important out of them being VEGF (1). Other internal transduction pathways that may become dysregulated during transformation of GBM include Raf, MEK, PI3K, Akt and mTOR pathways (1).

Figure 1 Glioblastoma cells display high chromatin density, and some have multinucleated forms. Pleomorphism, necrosis and high vascularity can also be observed. Image from “Glioblastoma. Molecular Mechanisms of Pathogenesis and Current Therapeutic Strategies” (1).
Unfortunately, these tumours have a highly infiltrative nature, and the tumour cells invade the neural tissue in single cells or small groups – making them elusive to all treatments available (7). Removal of the entire tumour by surgery is almost impossible, and although radiation therapy is effective, it only lengthens the survival of patients. Chemotherapy provides little survival benefit, and clinical trials using single or combination regimens have failed to cure glioblastoma (1). Immunotherapy seems to not be the answer either, as glioblastomas are cold tumours with little immune activity (8). There’s several explanations for the poor results: the nonspecific and non-targeted nature of chemotherapy, the high resistance of tumour cells, and the added blood-brain barrier that the drugs must trespass in order to arrive at their target.

In the last ten years, the existence of glioblastoma stem cells (GSC) has been discovered and its implication thoroughly studied. These GSC are the biggest contributors to both tumor initiation and therapeutic resistance, and evidence reports that targeting these cells is essential for effective treatment (5, 7). GSC don’t necessarily come from neural stem and progenitor cells (NSPC), as there’s evidence that different glial cell types can undergo oncogenic transformation (9). Six mechanisms regulate GSC: genetics, epigenetics, metabolism, tumor microenvironment and the immune response (figure 3). Several molecular mechanisms have been identified as culprits for resistance to cytotoxic therapies, like the upregulation of the DNA damage response pathways, dysregulation of tyrosine kinase pathways, and other survival and proliferating signals (9). Moreover, this resistance is not only intrinsic, but also extrinsic – as said before, the tumor has an immunosuppressive environment, with limited nutrients and oxygen, which increases the resistance mechanisms of these cells (5).

Novel therapeutic strategies that can potentially target both cancer cells and glioblastoma stem cells are the use of molecular targeted therapies like anti-VEGF, anti-EGFR, tumor vaccines, gene therapy and alternative immunotherapies (7). In order to treat glioblastoma, it is imperative that patients have a molecular characterization of the tumour so therapies can be targeted in the future (1).

Figure 2 Standard treatment for glioblastoma. RT = radiotherapy. TMZ = Temozolomide. From the Lancet “Molecular neuro-oncology in clinical practice: a new horizon” (4).

Figure 3 GSC regulation by intrinsic (genetics, epigenetics and metabolism) and extrinsic (microenvironment, niche factors and immune) mechanisms. From Genes & Development “Cancer stem cells in glioblastoma (9)."
ONCOLYTIC IMMUNOTHERAPY

Oncolytic immunotherapy, also called oncolytic immunovirotherapy or viro-immunotherapy, is the use of oncolytic virus as a treatment for cancer (10). Oncolytic viruses (OV) are natural or engineered virus that selectively infect, replicate and kill cancer cells (11). Infection with an OV enhances a positive feedback treatment, whereby the infection of a tumor cell produces more therapeutic virus, which in its turn will infect and kill more tumor cells (12).

At first, it was thought that it was only the OV intrinsic lytic mechanisms that targeted and killed off the cancer cells, however as research in the subject has expanded, it has been accepted that OV act via multiple mechanisms to achieve its effect (11). Its main asset is the ability to activate the immune system, recruiting innate and adaptive immune cells that will then attack the tumor (figure 4). GBM are tumours with little immune activity due to their cold tumor microenvironment. This is useful for OV, as they can replicate, spread and kill tumor cells without being cleared out of the system (8); however, it makes it more difficult for the anti-tumour response to happen. The advantage of OV is that they naturally convert the environment from suppressive into inflamed, due to the natural release of pro-inflammatory cytokines, which recruit and activate the innate and adaptive immune cells (13).

Moreover, oncolysis is a form of immunogenic death, and therefore it results in the release of immune-stimulatory molecules like neoantigens and tumor-associated antigen (14). Cross-presentation of such antigens on Major Histocompatibility Complex I (MHC-I) activates tumor-specific cytotoxic T lymphocytes, and promotes the release of Interleukin 2 (IL-2) and Gamma Interferon (IFNγ) by lymphocytes T_{H}1, potentiating the antitumor immune response and allowing development of antitumoral memory (13).

---

**Figure 4** Oncolytic virus mediate tumor cell death by direct lysis of the infected cells and by inducting the antitumor immune response. Image from Frontiers in Oncology “Immune system, friend or foe of oncolytic virotherapy?” (8).

**Figure 5** Immune barriers. (1) Viral transport to tumor can be hindered by neutralising antibodies or sequestration in organs; (2) early cellular response can clear the virus before successful tumor infection; (3) danger of developing tumor-induced immunosuppression, which would inhibit the antigen-specific antitumor activity. Image from Frontiers in Oncology “Immune system, friend or foe of oncolytic virotherapy?” (8).
The immune response exhibited by the host cells have been shown to be a barrier to viral replication and spread after its initial infection (figure 5). Tumor-associated macrophages (TAM) can either enhance anti-tumor immunity – and reduce viral oncolysis – or reduce the immune response and allow viral replication (8). It has been proven that although M1 polarisation leads to a greater virus clearance, it increases the therapeutic effect of oncolytic immunotherapy (10). This cellular immune response is a complex phenomenon, as enhancing the innate immunity reduces the lytic efficacy, but also increases the antitumoral response by activating CD8⁺ T-cells. The early clearance of the virus by destroying the infected tumor cells can imply the termination of the therapeutic effects of OV; this is the reason why engineering OV to transiently evade this early immune response has the potential to improve the overall therapeutic efficacy of oncolytic immunotherapy (8).

Evidence is now suggesting that OV not only target cancer cells, but also can kill tumor-associated cells (13). This selectivity further proves that OV don’t target a specific pathway, but rather infect cells that are in a cancerous state (13).

ENGEMGING ONCOLOGYHERPES VIRUS TO TARGET GLIOBLASTOMA

HSV is a dsDNA virus that naturally infects humans, and can provoke severe brain pathologies such as viral encephalitis (1). Its natural tropism for the brain, and its high cytotoxicity makes it a perfect candidate for oncolytic immunovirotherapy for glioblastoma (5,15).

The HSV-1 virus is made up of an envelope, a tegument, a viral nucleocapsid and the DNA core (Figure 6). The envelope contains viral glycoproteins, which mediate the attachment and delivery of the virus to the cell’s cytoplasm (12).

The viral capsid core is then transported to the nucleus, where replication will take place (16). The multiple proteins in the tegument will be freed into the cytosol to prepare the cell for the process, which will take place in three sequential steps (11, 16) (figure 7):

1. Transcription and translation of Immediate Early genes (IE) or alpha genes: the encoded proteins have the function of regulating gene expression and disabling the immune responses (12).
2. Transcription and translation of Early genes (E) or beta genes: their function is to allow the replication of the virus’ genome (12).
3. Transcription and translation of Late genes (L) or gamma genes: they are transcribed after the viral DNA has been synthesised. They are involved in the viral pathogenesis (12).

It is possible to engineer non-pathogenic HSV, as they have a large stable genome that can safely be manipulated (15). Many of the 84 viral genes are non-essential, which means they can be manipulated without damaging the virus ability to infect and replicate (12). Since Martuza engineered the first generation of oHSV as an antitumor agent, oHSV have undergone several genetic manipulations that have led to very different approaches of oncolytic viroimmunotherapy (5).

Artificial Chromosome (BAC) technology has been used for over a decade to engineer OV (17). The Flip-Flop HSV-BAC system enables for rapid generation of oHSV. It introduces the whole HSV genome in the BAC plasmid, followed by manipulation of the DNA in E. coli and it finishes with the isolation of the recombined virus (17). Two site-specific recombination systems, Cre/loxP and FLP/FRT, are used to introduce the shuttle vector with the transgene into the HSV-BAC. The shuttle
also contains a stuffer sequence, which allows for
the integration of the DNA into the virion –
otherwise, the genome is too big to fit (17). This
way all the virus isolated are recombinant HSV.
These viruses are co-transfected into mammalian
cells, where the FLP recombinase removes the BAC
and stuffer sequence, obtaining a pure preparation
of oHSV (12,17). In case of wanting to introduce a
specific mutation, the lambda phage Red
recombinase is used instead (12).

The recent development of CRISPR/Cas
mutagenesis system is getting appreciation thanks
to the ability of quickly inserting or removing
sequences directly into the HSV genome, without
using intermediate plasmids. The only problem is
that the accuracy of the cleavage is not perfect, and
mismatches could result in aberrant DNA cleavage
(12).

### THE OHSV GENERATIONS

**Table 1** Three subsequent generations of oHSV in clinical trials as treatment for glioblastoma

<table>
<thead>
<tr>
<th>Generation</th>
<th>oHSV</th>
<th>Engineered mutations</th>
<th>Clinical Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1716</td>
<td>Deletion of γ34.5</td>
<td>UK: phase II and III</td>
</tr>
<tr>
<td>2</td>
<td>G207</td>
<td>Deletion of γ34.5</td>
<td>USA: phase I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inactivating mutation of UL39 (ICP6-LacZ fusion in ICP6)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>G47Δ</td>
<td>Deletion of γ34.5</td>
<td>Japan: phase I and II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inactivating mutation of UL39 (ICP6-LacZ fusion in ICP6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Deletion of α47 and insertion of ICP47pro-Us11.</td>
<td></td>
</tr>
</tbody>
</table>

1716 is one of the first generation of oHSV that
made it into clinical trials (table 1). It contains only
one modification, which consists in the deletion of
both copies of the γ34.5 gene (18). γ34.5 codes for
ICP34.5, the main determinant of the neurovirulence of HSV-1. By erasing it from the
genome, the virus ceased to be dangerous when
injected into the human brain (5). Multiple ICP34.5
are present in the mature virions, as they are the key
factor that inhibits the host translational shutoff
induced by viral infection. They promote the
deprophosphorylation of the host’s eIF2α, as they
activate protein phosphatase 1 (PP1α). Elf2α, when
released of its inactivating phosphate, allows for
protein production, favouring the sustained
synthesis of viral proteins (12).

Deletion of γ34.5 implies that healthy cells will shut
off the viral infection, while uncontrolled cancerous
cells won’t, due to lacking the ability to respond to
stress responses and having most cellular processes
dysregulated. Unfortunately, the deletion of γ34.5
results in a very poor replication of the virus (19).

The deletion of γ34.5 was later on combined with
an inactivating insertion of the E. Coli lacZ in gene
UL39 (table 1), which encodes for the Infected Cell
Protein 6 (ICP6) (12), generating the second-
generation oHSV named G207 (15). ICP6 is a large
subunit of ribonucleotide reductase, a key enzyme
for nucleotide metabolism and viral DNA synthesis
in non-dividing cells (15).

Knocking out γ34.5 and inserting UL39 implied a
slower growth of HSV, but it enhanced its safety
and decreased the already minimal chance of
reverting to wild type (19). G207 provokes a
systemic immune response and infiltration of
tumor-specific CD8⁺ T lymphocytes when
inoculated intratumorally. The problem with both
G207 and 1716, along with the other first and
second generation oHSV, is that the viral infection
causes down-regulation of MHC-I on the cell
membrane of the infected cells (19). This implies
greater antitumor response by NK than T-cells,
which, as explained before, is counterproductive.

The third generation of oncolytic virus introduces
another mutation in the HSV-1 genome (table 1).
G47Δ, derived from G207, includes a deletion in the
α47 gene, a non-essential gene that transcribes for
ICP47 (15,19). ICP47 blocks the TAP protein
channel, and therefore prevents the loading of MHC-I with peptides and therefore MHC-I expression in the cell membrane (15). This deletion enhances the immune response against virus-infected tumor cells, as CD8+ T-cells are activated against the tumor-infected cells. Moreover, the deletion of α47 places Us11 under its promoter, which leads to its increased production. Us11 production synergises with the deletion of γ34.5 by keeping eIF2α dephosphorylated (12,15). Unlike its previous oHSV partners, G47Δ has been shown to be able to also counteract the stem-cell properties of GSC, as G47Δ has been shown to replicate better in hypoxic conditions, where GSC phenotype is enhanced (3,19). This gives G47Δ the potential of being able to overcome the resistance to more traditional therapies.

OBSTACLES AND STRENGTHS OF OHSV: COMBINATION THERAPY

OV have been shown to be extremely safe and non-toxic. The maximum safe dose has never been registered, and the OV have never reverted to a pathogenic state (15). However, the excitement of OV being the future has been hindered, as its efficacy is nowhere near optimal (13). Oncolytic viruses alone are unable to improve patients’ outcome in most cases, and therefore, they are now being studied as an adjunct to other cancer therapies instead of an alternative to the traditional therapies (15). This is called combination therapy, and it has reported to reasonably increase treatment efficacy and prolonged survival in many pre-clinical trials (5,15,20).

oHSV – chemotherapy

Most GBM cells are either resistant to chemotherapeutic agents, or quickly develop resistance to them. GSCs play an important role in this resistance, as well as the chemotherapeutic drugs’ rather narrow therapeutic index and the difficulties for the drugs to arrive at the tumor (9). To the date, there hasn’t been any report on oHSV-resistant GSC. The multiple mechanisms of actions and the fact that they are independent to the many genomic alterations found in resistant cells might be the reason why (13).

Temozolomide, as previously explained, is the only chemotherapeutic agent with proven efficacy in treating glioblastoma (1). It is currently being administered as a first-line treatment, and therefore, combination therapy with oHSV is feasible. G47Δ administered with TMZ proved to be synergistic for MGMT-negative glioblastomas, due to the increased intratumoral DNA damage from the combination (4,21). As seen in Figure 8, the G47Δ viral proteins sequestrate activated ATM, inhibiting this alternative DNA repair mechanism (21). Without either ATM or MGMT, there is a greater DNA damage, the GSC can’t function correctly, can’t replicate, and in the end, are killed by the virus’ oncolytic mechanisms and the immune cells (5,21). Instead, in MGMT-positive GSC the effect of the combination was found to be antagonistic (21). This different response to the same treatment due to epigenetic mechanisms further calls for the need of personalised medicine (13).

Etoposide is a topoisomerase II inhibitor. It is reserved for recurrent glioblastoma resistant to the standard therapy, as etoposide has significant adverse effects, such as nausea, weight loss, alopecia, leukopenia and thrombocytopenia (15). Combination therapy using G47Δ and etoposide was tested in different tumor xenografts, with different sensitivity to etoposide treatment (22). Combining G47Δ with low doses of etoposide resulted in extended survival in all the mice due to the increased rate of intratumoral apoptosis (22).
Molecularly targeted drugs are advancing as cancer treatment because they are highly specific. Immune checkpoint-blockade is now the standard treatment for many cancer types, as they are highly effective in selectively targeting tumor cells and guiding the immune response against them (15). Immune checkpoint molecules are used to maintain homeostasis, however, in cancer tissues, these signals are usually dysregulated, allowing for the growth of the tumor because the suppressive immune response is evaded (20).

Glioblastomas are cold tumours, where immune responses are typically suppressed. This is the reason why immune checkpoint inhibitors (ICI) haven’t been successful in treating them. Combining the ICI with an oncolytic virus could potentially overcome the immune suppression, as OV are able to boost and recruit effector T cells into the tumor and tumor microenvironment (20). After viral infection and replication, the ICI would be delivered in the microenvironment, where it would be able to exert its function and allow for the antitumor T CD8+ response to be sustained (20).

Angiogenesis is a hallmark of glioblastoma, and its targeting has been thoroughly studied over the last decades. Bevacizumab, an anti-VEGF antibody, is approved for recurrent GBM due to being able to control peritumoral oedemas, and therefore, improves performance of patients, although no survival benefits have been observed (15). When combined with G207, but not G47Δ, the tumours have been shown to reduce the growth and reduce the angiogenesis (13). G47Δ and bevacizumab are successfully combined in a further combination treatment, which includes the loading of G47Δ with angiostatin (23).

Antiangiogenic treatments are useful to combine with oncolytic immunotherapy because not only it hinders tumour growth, but it also prevents the infiltration of immune cells that would clear the virus (14).

Armed oHSV are virus with an immune stimulatory gene inserted in the viral genome. Many investigators have armed oHSV, as these viruses are able to further stimulate the antitumor immunity and induce the infiltration of immune cells into the tumor microenvironment (24). These genes are mostly either costimulatory molecules, cytokines or chemokines (20).

One of the most promising advances in combinatorial therapy for glioblastoma immunotherapy is the insertion of murine IL-12 in the viral G47Δ backbone (24). IL-12 is an immune-stimulating and anti-angiogenic cytokine, however, it is toxic if it is administered systemically (25). Todo designed the G47Δ-mIL12 so there would be production of IL-12 within the tumor microenvironment, avoiding the adverse systemic toxic reactions. Saha used the same G47Δ- mIL12, and complemented it with immune checkpoint inhibitors (26). Although the viral therapy alone had little positive impact in the outcome, combination of G47Δ-mIL12 with the ICIs anti-CTLA-4 or anti-PD1 had a positive effect in the survival of the animals in the preclinical study (25). Further combination therapy studies proved that the triple combination of anti-CTLA-1 and anti-PD1 with the armed G47Δ is even more effective (26). While dual combination extended survival of the mice, the triple play therapy was able to cure most of the glioblastomas.
Oncolytic HSV as Treatment for Glioblastoma

**FUTURE DIRECTIONS FOR OHSV**

It has been clear for years that the future of cancer treatment is personalised medicine. Patients with malignant brain tumours will have their molecular profile determined, and the profile resultant from this analysis will be critical to administer the optimal treatment for the individual patient. Standard care has been proven to be futile for the treatment of glioblastoma, only lengthening the survival for few months. The wide window of different mechanisms by which oHSV can act against brain tumours should be used in our favour and help in specifically targeting every individual tumor. The molecular profile would also be helpful in predicting resistance and sensitivity to different therapeutic agents and prognosis. Microarrays and proteomic technologies will be crucial for this targeted approach.

Oncolytic immunotherapy has both created great expectations and produced huge disappointments. It is quite clear now that OV on their own are unable to treat cancer, however, combined with the right treatments, they synergise and produce greater results than when the tumour is treated with the standard treatment alone. Despite the good results, many problems arise when dealing with virus. The host, tumour and virus have very complex interactions, which are yet to be completely understood. The viral delivery has to be improved. Intratumoral injection is not a desirable standard administration route; however, intravenous administration is limited by hepatic and splenic sequestration, plus pre-existing antibodies against the virus. Moreover, antiviral immunity can prematurely clear the OV, reducing the efficacy of the therapy. New delivery methods that can avoid these obstacles have yet to be investigated and further tested, such as carrier cells.

Future research in this area has to be focused in identifying new compounds for combination therapy, as it seems to be the key for glioblastoma treatment and potential cure. The combination of multiple treatments along with oHSV holds great promises. However, great care has to be taken and the interactions between treatments has to be greatly studied and understood in order to optimise the efficacy.

In the field of glioblastoma, oHSV have been proven to be safe and efficient in preclinical and phase I clinical trials. As the knowledge and understanding of the oHSV-host interactions grows, we should be able to engineer new oncolytic virus, identify combinatorial strategies and discover new molecules with increased therapeutic benefit. It is now only by progressing further in this new exciting field of oncolytic immunotherapy that we will be able to obtain a cure for glioblastoma.
BIBLIOGRAPHY


