Bioengineering of G47Δ HSV-1 Combined with Stem Cell Delivery as an Alternative Virotherapy against Colon Cancer

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Abstract: Oncolytic virus (OV) therapy is a recently developed strategy in cancer treatment, especially towards patients who are unresponsive to conventional therapies. Numerous viruses have been identified efficiently lysing tumor cells both in vitro and in vivo and eliciting anti-tumor immunity, including but not limited to Herpes Simplex Virus (HSV), adenovirus, and Newcastle Disease Virus (NDV). However, there are some caveats with present OV therapies. The delivery of the virus to the human body and its replication before immune response are essential to the effectiveness of the therapy. Therefore, to maximize the efficacy of existing OV therapies, we propose a hypothetical herpes simplex virus (HSV)-based OV which combines several engineered traits to tackle colon cancer. The re-designed virus conceivably limits HSV neutralization by pre-existing antibodies. It is also conceivable that the engineered oncolytic virus can secrete chimeric molecules that specifically bind to colon cancer cells. Also, we aim to activate neoantigen-specific T cell responses through the synergy of PD-L1 inhibition, GM-CSF activation, and viral immunogenic oncolysis. We hypothesize that a combination of OV therapies with the usage of mesenchymal stem cells (MSCs) as carriers could enhance the overall efficacy in tumor cell targeting and systemic immune response stimulation. MSC delivery is likely to aid in the migration of the engineered OV to tumor sites. In summary, the modifications of HSV enable more effective injection of oncolytic viruses and more accurate binding with tumors, improving therapeutic outcomes of existing HSV-based immunotherapies.

1 INTRODUCTION

1.1 Colon Cancer

Colorectal cancer develops in the colon or rectum. It was estimated that 5 to 10 percent of colon cancers are hereditary (UT Southwestern Medical Center. 2020). The risk of colorectal cancer increases drastically due to aging and certain lifestyles; almost 90% of colorectal cancer cases result from dietary contributions. More than 70% of colon cancers can be prevented by simple dietary and lifestyle modifications. Sigmoidoscopy and colonoscopy can be used for the diagnosis of colon cancer, preventing colon cancer mortality. There are multiple treatments for colorectal cancer, including surgery of full excision, chemotherapy, radiation therapy, immunotherapy with checkpoint inhibitors, and palliative care, etc (Cancer Net 2021). It is found that there is recurrent deregulation of STING signalling and loss of p-53 function in colorectal carcinoma.

1.2 Chemotherapy for Colon Cancer

Chemotherapy is treatment with anti-cancer drugs like cytotoxins which travel through the bloodstream and reach most parts of the body (American Cancer Society 2020). It is often used to treat colorectal

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cancer. Chemotherapy will be used at different times during treatment for colon cancer. For example, neoadjuvant and adjuvant chemo are given to the patients before and after surgery, respectively. However, these drugs have strong side effects since regular normal cells could also be damaged, resulting in hair loss, sores, and infection (Cancer Net 2021)

1.3 Oncolytic Virus

Oncolytic viruses (OVs) are viruses engineered to replicate in and selectively destroy cancer cells (Chiocca, Rabkin 2014). The common principle of OV therapy is to reduce or eliminate viral virulence factors to prevent the viruses from replicating in normal tissues while retaining the capacity to reproduce within cancer cells and kill them.

There are many viruses have been studied to become promising agents for cancer therapy, such as type 1 herpes simplex virus (HSV), measles virus (MV), oncolytic adenoviruses, Newcastle disease virus (NDV), Zika virus and vesicular stomatitis virus (VSV) (Zheng, Huang, Tong, Yang 2019). Oncolytic HSV (oHSV) is widely used in clinical trials. For example, the U.S. Food and Drug Administration (FDA) approve the use of T-VEC in biological cancer therapy, which is an HSV-based oncolytic virus completed phase Ш clinical trial (Mondal, Guo, He, Zhoub 2020).

Limitations exist in these current oncolytic therapies. One limitation is the pre-existing neutralization of the HSV-based oncolytic virus. Of the more than 100 known herpesviruses, 8 routinely infect only humans (Whitley 1996). Thus, the oncolytic effect of this therapy is compromised. The pre-existing antibodies neutralize the oHSV upon its administration. The oncolysis is compromised and the therapeutic effect is reduced.

In our study, we plan to employ G47Δ, a third-generation oncolytic herpes simplex virus type 1 (Sugawara, Iwai, Yajima, Tanaka, Yanagihara, Seto, Todo 2020). As HSV has a large, double-stranded DNA genome in its core (Whitley, 1996), it is suitable for multiple gene insertions. G47Δ has three mutations in the γ34. 5, ICP6 and α47 genes which make the virus replicate only in dividing cells, such as tumor cells (Cancer Treatments - from Research to Application. 2019).

1.4 Hypothesis

To address the limitations in the current therapies, we propose a new model of oncolytic virus design based on some primary research articles. We plan to employ the genome of G47Δ oncolytic virus. Modifications will be made to the virus genome, including point mutations on the genes that encode for antibody-binding surface proteins of G47Δ, insertion of gene segments of specific chimeric molecules, PD-L1 inhibitor, and granulocyte-macrophage colony-stimulating factor (GM-CSF).

We will use mesenchymal stem cells as a carrier to deliver our oncolytic virus. Additionally, this oncolytic virotherapy will be especially effective in treating advanced cancer with suppressed STING expression.

We hypothesize that using point mutations on surface proteins, triple gene insertion, and mesenchymal stem cell carriers will increase the general accuracy of tumor targeting and oncolysis efficacy for colon cancer.

In our research paper, we draw insights from six primary research articles on the existing oncolytic viral therapies. Following the summary of those primary research articles, we will present our study design of the proposed new OV model, methods and anticipated results.

2 PRIMARY RESEARCH

2.1 Redirecting Innate Immunity to Target Tumor Cells by Arming HSV-based Oncolytic Viruses

A major obstacle of cancer immunotherapy is the patients' innate immunity. This problem has been resolved by redirection of innate immune cells like macrophages or NK (natural killer) cells (Fu, Tao, Wu, Zhang 2020). This is achieved by arming the HSV with secreted chimeric molecules. These chimeric molecules consist of a tumor-associated antigen (TAA) binding moiety at their N terminus and protein L (PL) that binds to immunoglobulins (Igs) at their C terminus. The binding ability of Igs to PL exists among a variety of classes, such as IgM, IgG, IgA, IgE, and IgD (Bjorck 1988). The binding of Igs to the Ig-binding domains exposes Fc to the Fc receptors on the surface of innate immune cells, triggering them to attach to the tumor cells.

Oncolytic viruses, which were based on HSV-1 and HSV-2 were built in this experiment. They inserted EGF-PL chimeric gene cassette into the genome of Synco-2D, an oncolytic virus based on HSV-1. FusOn-H2, an HSV-2-based oncolytic virus was engineered with an insertion of the affibody-PL chimeric gene cassette. Synco-4 and FusOn-PL were

developed from Synco-2D and FusOn-H2, respectively. The results show that CT26-EGFR cells (a kind of colon cancer cells) was tightly bind with the supernatant containing EGF-PL. In addition, it was found that SKOV3 (ovarian cancer) and MCF7 (breast cancer) cell lines expressed high and medium levels of the human epidermal growth factor receptor type 2 affibody (HER2), respectively. A murine colon tumor model was used on mice, Synco-4 and FusOn-PL treatments demonstrated a more efficient therapeutic effect. This suggests that these chimeric molecules successfully redirect the innate immunity in attacking the specific tumor cells.

Moreover, this research also showed that neoantigen-specific antitumor immunity can be enhanced by the combination effect of the innate immune responses and oncolytic virus, rather than the virotherapy alone.

2.2 Third-Generation Oncolytic Herpes Virus G47Δ Used in Human Gastric Cancer

A research paper goes over how certain types of cancer such as Scirrhous gastric cancer are resistant to common treatments and how G47Δ could be a potential solution (Sugawara, Iwai, Yajima, Tanaka, Yanagihara, Seto, Todo 2020). G47Δ was developed through a deletion mutation to the genome of HSV-1, G207. This oncolytic virus has had success with being inoculated into the human brain suggesting it is a safe course of treatment.

Many clinical trials have been conducted indicating that G47Δ has a higher success rate in comparison to common courses of treatment for other types of cancer such as chemotherapy radiation.

The G47Δ treatment can successfully modify immunosuppressive molecules of the tumor including regulatory T cells and macrophages which reduces function. Through clinical trials, it has been discovered that NK cells are significant in the earlier phases as they are able to support healthy cells.

There is not enough data at the moment to support the hypothesis that the G47Δ third generation mutated oncolytic virus is able to become a successful cure to Scirrhous gastric cancer however further testing of high and low doses of treatment on tumors of different sizes are being conducted on mice and scientists are hopeful for more data soon.

2.3 STING Signaling in Cancer Cells

Stimulator of interferon genes (STING) is an adaptor protein that mediates type I INF activation responsive to cytosolic DNA ligands. During infection, STING in infected cells senses the nucleic acids of intracellular pathogens, thereby stimulating the production of multiple interferons. It is observed that STING expression is down-regulated more frequently than up-regulated in advanced diseases (Sokolowska, Nowis 2018), leading to poor prognosis of cancer and less effective immunotherapy (Kol et al 2021).

The oncolytic virus Talimogene laherparepvec (T-VEC), which was approved for melanoma treatment, has shown great efficacy in treating murine tumors with high levels of STING expression. However, in advanced melanoma, STING expression is usually suppressed, rendering PD-1 blockade therapy ineffective. Under these situations, a negative correlation between melanoma cell STING expression and sensitivity to T-VEC was found, suggesting that T-VEC oncolytic treatment would be particularly effective when treating advanced cancer with lower STING expression and is stubborn to PD-1 blockade therapy. It is shown that T-VEC can recruit CD8+ T cells and induce a pro-inflammatory gene expression, generating a systemic anti-tumor immune response. T-VEC infection also promotes the release of damage-associated molecular patterns; since immunogenic cell death is signified by the release of DAMP, which suggests that T-VEC infection would induce ICD. In summary, T-VEC could induce cytokine production, immunogenic cell death, and induction of inflammatory gene expression both *in vitro* and *in vivo.* Besides, a combinatorial treatment of anti-PD1 and T-VEC has a great potential to control systemic diseases with a higher overall response rate and complete response rate (Sun et al 2018).

Future studies can target other signalling pathways such as Toll-like receptor (TLR) signalling and investigate ways of optimizing intratumoral delivery (Sokolowska, Nowis 2018).

2.4 Induction of Antitumor Innate and Adaptive Immune Response by Oncolytic Newcastle Disease Virus

A challenge most Oncolytic Virus (OV) therapies face is to replicate in the human circulation for a sufficient amount of time to achieve efficacy. Usually, there are pre-existing immunities, causing most OVs constricted to intratumoral delivery. A promising solution to overcome this obstacle is the utilization of the Newcastle disease virus (NDV). Naturally, NDV does not infect humans, implying no

pre-existing immunity, ensuring the engineered NDV remains in the human circulation long enough.

By inserting granulocyte-macrophage colony-stimulating factor (GM-CSF), MEDI5395, a recombinant NDV, presents potent oncolytic and immunostimulatory activities. In a previous study (Burke et al 2020), MEDI5395 was shown to exhibit characteristics of preferential virus uptake and non-productive infection in myeloid cells, inducing upregulations of cell surface activation markers and releases of proinflammatory cytokines. Consequently, DCs with NDV infection stimulate higher levels of allogeneic T cell proliferation in comparison with non-infected DCs. Furthermore, infected myeloid cells in a co-culture system are demonstrated to be virus vectors, transferring the viruses to enter and reproduce in tumor cells, causing cell death. Following cell lysis, the antigens are released and cross-presented by the DCs, activating specific autologous T cells.

MEDI5395's capability to promote immune responses suggests the potentials to tackle therapy and anti-viral immunity resistance. However, application of MEDI5395 will cause the upregulation of the protein PD-L1. To add on, these experiments are only tested in vitro, indicating the necessity of further investigations, as well as a lack of standardized protocol for the application of MEDI5395 to patients (Schirrmacher, van Gool, Stuecker 2019).

2.5 Tumor Neoantigen-Specific T Cell Responses Activated by PD-L1 Inhibition

Tumor cells can only express a small proportion of nonsynonymous mutations, which enables neoantigen-specific T cell responses stimulation. Besides, PD-1/PD-L1, as T cell checkpoint molecules should be responsible for the immunosuppressive tumor microenvironment in tumor cells, which inhibits anti-tumor T cell responses. There was a study designing an oncolytic vaccinia virus, co-expressing a PD-L1 inhibitor and a GM-CSF, for investigating the ability of engineered oncolytic viruses in activating tumor neoantigen-specific T cell responses (Wang et al 2020).

In the engineered oncolytic virus, IgG1 Fc was fused with a murine soluble PD-1 extracellular domain as a PD-L1 inhibitor (iPDL1). iPDL1 co-expressed with murine GM-CSF, with the backbone of a tumor-selective double-deleted oncolytic vaccinia virus, with the deletion of thymidine kinase and viral growth factor genes. Two recombinant controls were generated: only with GM-CSF or marker RFP expression. MC38 cell lines carried oncolytic viruses inoculated into the C57BL/6 mice, with PBS (negative control), anti-PD-L1 antibody (positive control).

It was found that the newly engineered oncolytic virus can produce PD-L1 inhibitors and bind to PD-L1+ tumor cells and immune cells (Wang et al 2020). In addition, the intratumoral injection of the (VV)-iPDL1/GM promotes maturation of the dendritic cells and presentation of neoantigen on tumor cells via PD-1/PD-L1 inhibition, leading to active neoantigen-specific T cell responses. Therefore, this study indicates that the synergy of PD-L1 inhibitors, GM-CSF, and viral replication activates neoantigen-specific T cell responses in tumor cells, resulting in effective tumor-specific oncolytic immunotherapy (Wang et al 2020).

2.6 Delivery of Mesenchymal Stem Cell by Oncolytic Virus and Prodrug Activation in Colorectal Cancer Therapy

Although oncolytic virus therapy has been used in various clinical approaches, many factors have been proven to prevent them from reaching the tumor sites. A new strategy of OV therapy improved efficacy by using the mesenchymal stem cells (MSCs) to deliver the oncolytic viruses (Ho, Wu, Chen, Lin, Yen, Hung 2021). This ability of MSCs to target tumor cells makes them suitable carriers for oncolytic viruses (OVs). The MSCs can further undergo numerous modifications to carry the viruses, improving tumor homing, especially for p-53 pathway deficient cancer.

The MSCs are primed with trichostatin A (TSA) under hypoxia conditions to maintain their properties, then loaded with oncolytic viruses to encode an enzyme called E. coli nitroreductase (NTR) for tumor cell targeting (Ho, Wu, Chen, Lin, Yen, Hung 2021). The primed MSCs can increase tumor tropism, which makes tumor cells more susceptible for viral infections and protects NTR from neutralisation of the immune system. The priming also leads to upregulation of CXCR4--a chemokine receptor, further contributing to targeting accuracy since CXCR4 is involved in tumor tropism for cancer homing.

Furthermore, the gene-directed enzymes-prodrug therapy (GDEPT), also known as suicide gene therapy, was used together with the stem cell delivered OV therapy. The mechanism included a combination of the prodrug-activating enzyme gene, here NTR, with a non-toxic prodrug (NTR+CB1954)

that induces apoptosis in tumor cells. The prodrug is converted into cytotoxic metabolites to trigger oncolysis and inhibit tumor growth without damaging other vital tissues and organs.

2.7 Limitations

One limitation in the primary research is the neutralization of HSV-based oncolytic viruses by the pre-existing antibodies. HSV infection is pretty common, as it was estimated that 67% people under age 50 have HSV-1 infection globally (World Health Organization 2020). This would result in high anti-HSV seroprevalence in the general population. The human immune system can respond in a short time. Thus, the killing effect of the virus will be compromised.

One major setback of using MSCs is that some of their previously proved tumor-promoting properties could lead to the opposite results when used with OVs. Another limitation is that MSCs from different tissues can produce varying results, thus it is difficult to predict the effectiveness of this therapy on different patients.

We decided to design a new hypothetical model to minimize setbacks and optimize the immunotherapy effects in oncolytic virus treatment.

3 STUDY DESIGN

3.1 Overview

As shown in Figure 1, the proposed oncolytic virotherapy consists of three sections, engineering of glycoprotein, triple gene insertions, and stem cell carrier. In our design, we plan to use the genome of G47Δ, an HSV-based OV, and target specifically colon cancer.

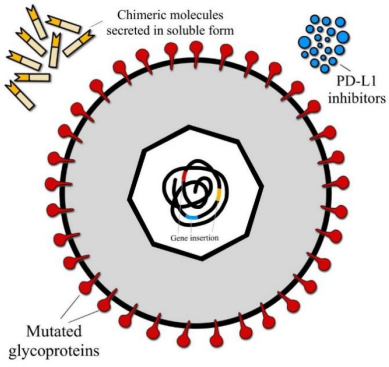

Figure 1: The schematic structure of the new model of G47Δ oncolytic virus.

The first section of approaches is the engineering of the glycoprotein of the G47Δ. We plan to perform point mutations on the gene encoding the binding site of the glycoprotein of HSV. Thus, the pre-existing neutralization by the immune system can be avoided. Our proposal of triple gene insertion includes insertion of genes encoding for special chimeric molecules, PD-L1 inhibitors and GM-CSF, aiming to enhance the killing effect of G47Δ and direct innate immune cells. The last method in our proposal is the stem cell carrier. Stem cell delivery can elevate the accuracy of OV migration to tumor sites and reduce the off-target issue.

The overall purpose is to improve the efficacy of the existing oncolytic virotherapies. We want to achieve this goal by combining the effective methods of each and maximally reduce the limitations.

3.2 Approaches

3.2.1 Engineering of Glycoproteins to Avoid the Pre-Existing Neutralization

One problem we aim to resolve is the pre-existing immunity against HSV in human populations, which poses a difficulty for the HSV to remain long enough in the human body to achieve efficacy. According to the National Health and Nutrition Examination Survey conducted in 2015-2016, HSV-1 infects 47.8% of the U.S. population, and HSV-2 infects 11.9% of the U.S. population (McQuillan, Kruszon-Moran, Flagg, Paulose-Ram 2018). To prevent the virus from the swift elimination by the immune system in these populations, we first proposed the solution of transferring the genes encoding the protein coating of the NDV onto HSV. As humans are not natural hosts of the NDV, if the engineered HSV expresses NDV surface proteins, it will endure longer in the human body to pass down genetic information. However, this plan has considerable flaws. Firstly, the execution of this process will be challenging. Similar efforts have been made without great success (Davidsson et al 2019). As changing the entire genetic makeup of the protein coating is a difficult and inefficient task. Secondly, excessive gene modifications will drop overall efficacy of the virus, impacting other genes that we want to implement on this virus. Thirdly, glycoproteins are encoded by the essential genes of HSV (Nishiyama 2004), and a complete altercation of its protein coat could affect the virus's ability to survive and replicate (Synthego | Full Stack Genome Engineering 2020).

Therefore, we abandoned the concept of combining the two viruses, and decided to do point

mutation on the glycoproteins of the HSV. A recent research proved that Human adenovirus-C5 (HAdv-C5) with mutated hypervariable region 1 (HVR1) of the capsid are more resistant to complement-mediated inactivation (Atasheva, Emerson, Yao, Young, Stewart, Shayakhmetov 2020), indicating the possibility of modifying the glycoproteins on HSV to elude the immune system. To achieve this, identification and artificial mutation of the principal antigenic determinant (epitope) of HSV is required, while ensuring no impacts on the entry of human cells. Using targeted mutagenesis and the screening process, determining modifiable amino acid sequences, the ideal point mutation might be found through trial and error. Other modifications on the HSV will continue after the most suitable glycoprotein mutation is discovered.

3.2.2 Gene Insertions to Enhance Binding and Oncolytic Effect

In our model, we choose to use EGF-PL chimeric molecules. The genes encoding this specific chimeric molecule will be inserted into the genome of G47Δ, an HSV-based OV. The key features of the EGF-PL molecules are its single protein (sp), human epidermal growth factor (EGF), and protein L (PL).

A soluble form of the chimeric molecules can be secreted with the help with single protein - EGF, which is critical to trigger the intermolecular reaction (Fu, Tao, Wu, Zhang 2020). EGF is a TAA-binding moiety that specifically targets colon cancer cells. PL can bind to the Igs, including IgG, IgM, IgA, IgE, and IgD (Bjorck 1988). The Fc region of the binded Igs is available to bind to the Fc receptors on the surface of natural killer cells or macrophages. In this conformation, the EGF-PL molecule can redirect the innate immune cells to attach to colon cancer cells, instead of the viruses.

T cell responses to tumor cells are usually inhibited in immunosuppressive tumor environment, due to the presence of T cell checkpoint molecules (Gray, Gong, Hatch, Nguyen, Hughes, Hutchins, Freimark 2016). The first discovered checkpoint molecule is cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), a T cell receptor with high homology to CD28 binding with B7 molecules to impede CD28 co-stimulation and downregulate T cell responses (Sharma, Allison 2015). Unlike CTLA-4, programmed cell death protein 1 (PD-1), as another checkpoint molecule, interacts with its ligands (PD-L1) blocking inflammatory reactions of T cells by mediating signaling pathways (Grabie, Lichtman, Padera 2019). For dealing with T cell checkpoint inhibition caused by PD-1/PD-L1, relative blockade therapy has been issued, during which there appears some challenges like the failure of tumor cells to provoke spontaneous T cell responses to mutant tumor neoantigens (Wang et al 2020). However, it was proved that synergy of PDL1 inhibition, GM-CSF stimulation, and viral replication can activate neoantigen-specific T cell responses (Wang et al 2020). Therefore, another modification is an insertion of PD-L1 inhibitor, GM-CSF, and a marker RFP to explore the synergistic functions.

PDL1 inhibitor can be formed with the fusion of a murine soluble PD-1 extracellular domain and IgG1 Fc. The extracellular domain functions as a PD-L1 binding site, and the IgG1 Fc is a tracing signal for iPDL1 to be tested by using anti-IgG Fc. iPDL1 impedes PD-L1 to partially reverse the immunosuppressive tumor microenvironment, indirectly activating specific T cell responses for neoantigen (Karabajakian et al 2020). Besides, GM-CSF will act as a stimulator to promote intratumoral T cell infiltration and the anti-PD-1/CTLA immunotherapy (Puzanov 2016).

3.2.3 Stem Cell Carrier to Improve Targeting

The purpose of using Mesenchymal Stem Cells (MSCs) delivery is to solve the problem of possible failures of oncolytic viral migrations to tumor sites (Ho, Wu, Chen, Lin, Yen, Hung 2021), using their inherent tumor tropism, through numerous aspects. First, MSCs can provide shielding protection of the internalized OVs by avoiding immune recognition and further neutralization with their low immunogenicity (Hadryś, Sochanik, McFadden, Jazowiecka-Rakus 2020). In addition, MSCs can migrate directionally to specific tissues that release an “unusual signal”, triggered by a signalling mechanism, to improve the general accuracy of targeting. Moreover, MSCs are suited for gene transduction, thus can be transduced with therapeutic genes for viral vectors (Amara, Touati, Beaune, de Waziers 2014), they are treated with TSA before being administered to the OVs for priming (Ho, Wu, Chen, Lin, Yen, Hung 2021). Not only an up-regulation of CXCR4 level and improved migration ability towards targeted regions are shown after priming but also results in increased susceptibility of OV infections and enhanced capacity of CRAdNTR loading. Afterward, the primed MSCs with OVs expressed will be intravenously injected into tumors.

Although several pieces of research have proven that MSCs have undesired tumor-promoting

characteristics that can be presented via many mechanisms (Lin, Huang, Li, Fang, Li, Chen, Xu 2019, Mahasa, de, Rachid, Amina, Maini, A-Rum, Chae-Ok, 2020), the preclinical studies have shown promising safety and efficacy of MSCs carriers for OV therapies since they have also revealed abilities that contributed to tumor growth inhibitions. In the primary research, the prodrug activation is also used to assist the OV:MSC combination. However, we choose not to use active prodrugs in our design since another insertion of genes encoding for a prodrug-activating enzyme will be risky for the overall presentation of the engineered model due to the increased possibility of flaws occurring. However, without the prodrug being used, a pathway for oncolysis of the tumor cells might not be efficiently presented, and the inhibition of metastasis may not be carried to an ideal extent.

3.3 Experimental Design

3.3.1 Experiments in Vitro

We plan to use CT26-EGFR, a derivative of CT 26 (murine cell line) for our in vitro experiment. Parental CT26 cells with the human EGFR gene will be transduced to established our cell line: CT26-EGFR (Fu, Tao, Wu, Zhang 2020). We will test for oncolytic virus count, the number of antibodies and complement proteins found on the virion surface, the expression and the binding effect of chimeric molecules, iPDL1 and GM-CSF with the help of RFP.

To test the performance of the mutated virus, wild-type HSV, mock HSV, and the mutated HSV will be incubated individually in human serums containing antibodies and complement proteins, and CT26-EGFR, the tumor cells to provide comparisons. Assays, ELISA, will be conducted for the number of antibodies and complement proteins deposition found on the virion surfaces (Atasheva, Emerson, Yao, Young, Stewart, Shayakhmetov 2020). We use western blotting to confirm the transgene expression from the engineered G47Δ virus. We will use flow cytometry analysis to measure the selective binding efficacy of the EGF-PL chimeric molecules to CT26-EGFR. Virus counts will be registered over time for survival and replication rates, and the number of tumor cells will also be recorded.

Furthermore, in order to explore the synergistic function of iPDL1, GM-CSF, and viral replication, it is necessary to test the expression of those molecules, as well as the binding efficacy of iPD-L1. iPDL1 expression can be tested using anti-IgG Fc and anti-PD-1 in western blot (Wang et al 2020). Serum purification can re-confirm the expression of iPDL1 and test the presence of GM-CSF at the same time. Flow cytometric analysis will be conducted to investigate the target binding of iPDL1 to tumor cells with PD-L1 expression, with IgG-Fc at the x-axis and iPDL1 at the y axis. CT26-EGFR with PD-L1-knock down acts as the control.

3.3.2 Experiments in Vivo

To evaluate the therapeutic effect of our new model, we plan to use the chemical approach to induce a colon tumor in mice models. We will inject 1,2-dimethylhydrazine (DMH), a carcinogen to the mice. We will inject each mouse subcutaneously (s.c.) with 15 μg of DMH per gram of body weight (Gurley, Moser, Kempn2015). We will perform each injection once weekly for 12 weeks. When tumors reach the size of about 5mm in diameter, grouped mice will be rejected with the new oncolytic virus inside an MSC or PBS as a mock control (Fu, Tao, Wu, Zhang 2020). We will also vaccinate mice with recombinant HSV glycoproteins to induce anti-HSV antibodies, thereby imitating pre-existing immunity against the HSV virus as seen in patients.

We will test whether an intratumoral injection of engineered OV functions in enhancing T cell responses against neoantigen epitopes. We will use non-engineered OV as a control group. Four groups of mice with tumors are required, which will be treated with PBS, anti-PD-L1, non-engineered OV, and engineered OV. After ten days of the last injection of them, tumor-infiltrating T cells from the tumor-bearing mice should be isolated and cultured with a mixture of 11 neoepitope peptides (Yadav et al 2014), covering the majority of MHC-I restricted neoepitopes in MC38 cells (a colon cancer cell line of mice). Eighty hours incubation later, supernatants will be collected for IFN-gamma ELISA, which reveals the concentration of IFN-gamma, a chemokine released with splenic T cells activation (Wang et al 2020). Also, [3H] thymidine incorporation will be measured to figure out the T cell proliferation. If anti-PD-L1 cannot induce specific T cell responses to neoantigen as efficiently as iPD-L1, the potency of iPD-L1/GM-CSF will be confirmed in stimulating neoantigen-specific T cell responses.

In addition, by carrying out a combination cytotoxicity assay, we can test if MSCs are successfully infected by our engineered virus.

Tumor size will be recorded, and mice treated with mutated variations are expected to have a decrease in tumor size faster than the wild-type HSV

and the mock infected mice. The population count for the virus will be registered.

3.4 Anticipated Results

3.4.1 In Vitro Characterization of Our Engineered Oncolytic Virus

We hypothesize that the virus with the desired mutations will be resistant against neutralizing antibodies and complement proteins while remaining effective in killing cancer cells. We anticipate that the chimeric molecules, iPD-L1 and GM-CSF will function properly and are effective against cancer cells.

A previous study on adenovirus with engineered capsid protein hexon resulted in reduced immune response (Atasheva, Emerson, Yao, Young, Stewart, Shayakhmetov 2020). A beneficial mutation in the glycoproteins HSV will hinder recognition by the immune system, while not compromising the OV's abilities to enter tumor cells and replication to cause cell death. Accordingly, the results should display relatively lower levels of antibodies and complement proteins detected, while the tumor cell counts are similar or lower than the wild-type and mock HSV.

Other studies might support our prediction for chimeric molecules, iPD-L1, and GM-CSF's efficacy. Evidence from the primary research paper showed that the EGF-PL molecules was found in the supernatants from cells infected with OVs, indicating that EGF-PL can be produced and released into the mulieu (Fu, Tao, Wu, Zhang 2020). Therefore, it can be hypothesized that EGF-PL molecules will be successfully produced during virus infection in our experiment.

From similar experiments, iPD-L1 and GM-CSF mount with engineered OV effectively co-express those genes (Wang et al 2020). Thus, it can be estimated that iPD-L1 and GM-CSF might function in colon cancer cells with the engineered OV infection.

3.4.2 In Vivo Characterization of the New Oncolytic Virus

We hypothesize that iPD-L1 and GM-CSF will induce T cell responses with higher levels of chemokine released, while MSC-delivery should show effectiveness in helping the engineered virus to target cancer cells. The gene mutation should also improve the virus's survival in the mice. It can be inferred that iPD-L1/GM-CSF infection is likely to provoke neoantigen-specific T cell responses with elevated proliferation and chemokine (IFN-gamma) secretion (Wang et al 2020).

It has been proven that the MSC-delivered OV therapy shows an impressive improvement in successful migration and targeting to the tumor sites without being attacked by cellular and humoral immune systems. Therefore, we predict that the conduction of our new engineered oncolytic virus will give out similar results. The result should show that the administration of our new virus contained in MSC slows down the growth of tumors and increases the survival rate of mice in the comparison with the control. By the end of our experiment, we should achieve tumor-free in the mice group injected with the new virus.

The data should present a higher amount of mutated HSV in the mice relative to the other two conditions, indicating higher survival and replication rates. ELISA measurement of antibodies and complement proteins binding to the viruses will be conducted. With the right mutation, the antibody and complement protein counts should show a lower figure in the mice injected with mutated HSV.

4 DISCUSSION

In our study design, we addressed the limitations in current oncolytic virotherapies. We addressed the issue of pre-existing neutralizing antibodies by editing the glycoproteins on the surface of G47Δ oncolytic virus. Point mutation in gene encoding for glycoproteins results in the conformational change in glycoprotein. Thus, the hypothetical oncolytic virus tends to evade the immune system and survive for a longer duration. The OVs can successfully replicate their genetic materials and secrete chimeric molecules and iPD-L1/GM-CSF. Moreover, the chimeric molecules direct the immune system to facilitate virotherapy. This helps the hypothetical OVs further evade the attack of the immune system. The chimeric molecules can also recruit the innate immune cells, attack the tumor cells and increasing the efficacy of our proposed therapy.

The modifications of the virus can have some downsides. Point mutations and testing can be highly time-consuming without producing desired results. The mice model may not well resemble human pre-existing immunity, as mice are not natural hosts for HSV, and antibodies do not create a perfect imitation. Reversion of the engineered OVs to wildtype by spontaneous mutation is also possible (Paquet et al 2011). Furthermore, the editing of surface protein might affect the virus entry into stem cells and tumor

cells. Viral replication might be compromised. Thus, the oncolytic effect of the engineered OV is reduced. Additionally, some previous studies have shown the proliferative effects of MSCs on tumor cells after being recruited into tumor sites; however, the precise mechanism of MSCs' cancer-promoting properties remains unknown.

Our study design suggests several possible improvement strategies in the future. One of them is to replace the EGF with other TAA-binding molecules. In this way, we can target a wide range of tumors, broadening the scope of our virotherapy. For example, we can replace the EGF by the human epidermal growth factor receptor (EGFR) type 2 (HER2) affibody. Evidence shows that SKOV3 and MCF7 were found to express HER2 (Fu, Tao, Wu, Zhang 2020), indicating that affibody-PL chimeric molecules can bind to ovarian and breast cancer cells. A thorough investigation of the genetic makeup of HSV is required for further mastery of gene editing, improving the initial gene mutation to a greater extent. Further investigations can also target other cancer types, with high STING signalling or normal p-53 function.

5 CONCLUSION

We raised a new HSV-based therapy model in this paper, combining cell surface protein point mutations, multiple gene insertions including chimeric molecules, PD-L1 inhibitors, and GM-CSF, and delivered through an MSC carrier. We believe our design has a promising future, but there may still be some possible factors limiting the efficacy due to the lack of practical experiments being carried out.

More experiments are required to examine feasibility of the point mutations of the glycoprotein-encoding gene, the successful expression of PD-L1 inhibitor and chimeric molecules after gene insertions, and the comparison of effectiveness between primed MSCs and unprimed MSCs. Further in-vivo studies should be conducted to test the practicality of this new model, even on other cancer types. Additional experimentations can also focus on using prodrug with the MSC delivery. Since the precise mechanism of MSCs' cancer-promoting properties remains unknown, the results of further experiments might reveal their pathway. This might lead to the promotion of developing a new treatment based on the inhibition of tumor promotion on MSC carriers, contributing to more therapeutic improvements based on the manipulation of immune systems.

REFERENCES

Amara, I., Touati, W., Beaune, P., de Waziers, I. (2014). Mesenchymal stem cells as cellular vehicles for prodrug gene therapy against tumors. Biochimie, *105*, 4-11. DOI: https://doi.org/10.1016/j.biochi.2014.06.016.

American Cancer Society. (2020) Colorectal Cancer Chemotherapy | Chemo for Colon & Rectal Cancer. https://www.cancer.org/cancer/colon-rectal-cancer/treating/chemotherapy.html.

Atasheva, S., Emerson, C.C., Yao, J., Young, C., Stewart, P.L., Shayakhmetov, D.M. (2020). Systemic cancer therapy with engineered adenovirus that evades innate immunity. Science Translational Medicine, 12(571), eabc6659. DOI: https://doi.org/10.1126/scitranslmed.abc6659.

Bjorck, L. (1988). Protein L. A novel bacterial cell wall protein with affinity for ig L chains. The Journal of Immunology (1950), 140(4), 1194-1197.

Burke, S., Shergold, A., Elder, M.J., Whitworth, J., Cheng, X., Jin, H., Wilkinson, R.W., Harper, J., Carroll, D. K. (2020). Oncolytic Newcastle disease virus activation of the innate immune response and priming of antitumor adaptive responses in vitro. Cancer immunology, immunotherapy: CII, 69(6), 1015–1027. DOI: https://doi.org/10.1007/s00262-020-02495-x.

Cancer Net. (2021) *Chemotherapy Side Effects*. https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/chemotherapy/chemotherapy-side-effects.html.

Cancer Net. (2021) Colorectal Cancer – Diagnosis. https://www.cancer.net/cancer-types/colorectal-cancer/diagnosis.

Cancer Treatments - from Research to Application. (2019) G47Δ (Delta) Oncolytic Virus: A Breakthrough Therapy for Glioblastoma from Prof. Todo in Japan. https://www.cancertreatmentsresearch.com/g47%CE%B4-delta-virus-a-breakthrough-therapy-for-glioblastoma-from-prof-todo-in-japan/.

Chiocca, E.A., Rabkin, S.D. (2014). Oncolytic viruses and their application to cancer immunotherapy. Cancer Immunology Research, 2(4), 295-300. DOI: https://doi.org/10.1158/2326-6066.CIR-14-0015.

Davidsson, M., Wang, G., Aldrin-Kirk, P., Cardoso, T., Nolbrant, S., Hartnor, M., Mudannayake, J., Parmar, M., Björklund, T. (2019). A systematic capsid evolution approach performed in vivo for the design of AAV vectors with tailored properties and tropism. Proceedings of the National Academy of Sciences - PNAS, 116(52), 27053-27062. DOI: https://doi.org/10.1073/pnas.1910061116.

Fu, X., Tao, L., Wu, W., Zhang, X. (2020). Arming HSV-based oncolytic viruses with the ability to redirect the Host's innate antiviral immunity to attack tumor cells. Molecular Therapy. Oncolytics, 19, 33-46. DOI: https://doi.org/10.1016/j.omto.2020.09.002.

Grabie, N., Lichtman, A.H., Padera, R. (2019). T cell checkpoint regulators in the heart. Cardiovascular

Research, 115(5), 869-877. DOI: https://doi.org/10.1093/cvr/cvz025.

Gray, M.J., Gong, J., Hatch, M.M.S., Nguyen, V., Hughes, C.C.W., Hutchins, J.T., Freimark, B. D. (2016). Phosphatidylserine-targeting antibodies augment the anti-tumorigenic activity of anti-PD-1 therapy by enhancing immune activation and downregulating pro-oncogenic factors induced by T-cell checkpoint inhibition in murine triple-negative breast cancers. Breast Cancer Research: BCR, 18(1), 50-50. DOI: https://doi.org/10.1186/s13058-016-0708-2.

Gurley, K.E., Moser, R.D., Kemp, C.J. (2015). Induction of colon cancer in mice with 1,2-dimethylhydrazine. Cold Spring Harbor Protocols, 2015(9). DOI: https://doi.org/10.1101/pdb.prot077453.

Hadryś, A., Sochanik, A., McFadden, G., Jazowiecka-Rakus, J. (2020). Mesenchymal stem cells as carriers for systemic delivery of oncolytic viruses. European Journal of Pharmacology, 874, 172991-172991. DOI: https://doi.org/10.1016/j.ejphar.2020.172991.

Ho, C.T., Wu, M.H., Chen, M.J., Lin, S.P., Yen, Y.T., Hung, S.C. (2021). Combination of Mesenchymal Stem Cell-Delivered Oncolytic Virus with Prodrug Activation Increases Efficacy and Safety of Colorectal Cancer Therapy. Biomedicines, 9(5), 548. DOI: https://doi.org/10.3390/biomedicines9050548.

Karabajakian, A., Garrivier, T., Crozes, C., Gadot, N., Blay, J., Bérard, F., Céruse, P., Zrounba, P., Saintigny, P., Mastier, C., Fayette, J. (2020). Hyperprogression and impact of tumor growth kinetics after PD1/PDL1 inhibition in head and neck squamous cell carcinoma. Oncotarget, 11(18), 1618-1628. DOI: https://doi.org/10.18632/oncotarget.27563.

Kol, A., Lubbers, J.M., Terwindt, A.L.J., Workel, H.H., Plat, A., Wisman, G.B.A., Bart, J., Nijman, H.W., & De Bruyn, M. (2021). Combined STING levels and CD103+ T cell infiltration have significant prognostic implications for patients with cervical cancer. Oncoimmunology, 10(1). DOI: https://doi.org/10.1080/2162402X.2021.1936391.

Lin, W., Huang, L., Li, Y., Fang, B., Li, G., Chen, L., Xu, L. (2019). Mesenchymal stem cells and cancer: Clinical challenges and opportunities. BioMed Research International, 2019, 2820853-12. DOI: https://doi.org/10.1155/2019/2820853.

Mahasa, K.J., de, P.L., Rachid, O., Amina, E., Maini, P., A-Rum, Y., Chae-Ok, Y. (2020). Mesenchymal stem cells used as carrier cells of oncolytic adenovirus results in enhanced oncolytic virotherapy. Scientific Reports (Nature Publisher Group), 10(1). DOI: https://doi.org/10.1038/s41598-019-57240-x.

McQuillan, G., Kruszon-Moran, D., Flagg, E.W., Paulose-Ram, R. (2018). Prevalence of herpes simplex virus type 1 and type 2 in persons aged 14-49: United states, 2015-2016. NCHS Data Brief, (304), 1-8.

Mondal, M., Guo, J., He, P., Zhou, D. (2020). Recent advances of oncolytic virus in cancer therapy. Human vaccines & immunotherapeutics, 16(10), 2389–2402. DOI: https://doi.org/10.1080/21645515.2020.1723363.

Nishiyama, Y. (2004). Herpes simplex virus gene products: The accessories reflect her lifestyle well. Reviews in Medical Virology, 14(1), 33-46. DOI: https://doi.org/10.1002/rmv.409.

Paquet, A.C., Baxter, J., Weidler, J., Lie, Y., Lawrence, J., Kim, R., Bates, M., Coakley, E., Chappey, C. (2011). Differences in reversion of resistance mutations to wild-type under structured treatment interruption and related increase in replication capacity. PloS one, 6(1), e14638. DOI: https://doi.org/10.1371/journal.pone.0014638.

Puzanov, I., Milhem, M.M., Minor, D., Hamid, O., Li, A., Chen, L., Chastain, M., Gorski, K.S., Anderson, A., Chou, J., Kaufman, H.L., Andtbacka, R.H.I. (2016). Talimogene laherparepvec in combination with ipilimumab in previously untreated, unresectable stage IIIB-IV melanoma. Journal of Clinical Oncology, 34(22), 2619-2626. DOI: https://doi.org/10.1200/JCO.2016.67.1529.

Schirrmacher, V., van Gool, S., Stuecker, W. (2019). Breaking Therapy Resistance: An Update on Oncolytic Newcastle Disease Virus for Improvements of Cancer Therapy. Biomedicines, 7(3), 66. DOI: https://doi.org/10.3390/biomedicines7030066.

Sharma, P., Allison, J.P. (2015). The future of immune checkpoint therapy. Science (American Association for the Advancement of Science), 348(6230), 56-61. DOI: https://doi.org/10.1126/science.aaa8172.

Sokolowska, O., Nowis, D. (2018). STING Signaling in Cancer Cells: Important or Not? Archivum immunologiae et therapiae experimentalis, 66(2), 125–132. DOI: https://doi.org/10.1007/s00005-017-0481-7.

Sugawara, K., Iwai, M., Yajima, S., Tanaka, M., Yanagihara, K., Seto, Y., Todo, T. (2020). Efficacy of a third-generation oncolytic herpes virus G47Δ in advanced stage models of human gastric cancer. Molecular Therapy. Oncolytics, 17, 205-215. DOI: https://doi.org/10.1016/j.omto.2020.03.022.

Sun, L., Funchain, P., Song, J.M., Rayman, P., Tannenbaum, C., Ko, J., Mcnamara, M., Marcela Diaz-Montero, C., Gastman, B. (2018). Talimogene Laherparepvec combined with anti-PD-1 based immunotherapy for unresectable stage III-IV melanoma: a case series. Journal for immunotherapy of cancer, 6(1), 36. DOI: https://doi.org/10.1186/s40425-018-0337-7.

Synthego | Full Stack Genome Engineering. (2020) Why Are Some Genes Difficult to CRISPR Edit? https://www.synthego.com/blog/genes-difficult-crispr-edit.

UT Southwestern Medical Center. (2020) Hereditary Colon Cancer: Guide for Health Pros | Guide to Hereditary Cancer for Health Pros | UT Southwestern Medical Center. https://utswmed.org/conditions-treatments/genetics-and-hereditary-cancers/guide-hereditary-cancer-health-pros/hereditary-colon-cancer-guide-health-pros/.

Wang, G., Kang, X., Chen, K.S., Jehng, T., Jones, L., Chen, J., Huang, X.F., Chen, S. (2020). An engineered oncolytic virus expressing PD-L1 inhibitors activates

tumor neoantigen-specific T cell responses. Nature Communications, 11(1), 1395-1395. DOI: https://doi.org/10.1038/s41467-020-15229-5.

Whitley R.J. (1996) Herpesviruses. In: Baron, S. (Eds.), Medical Microbiology. University of Texas Medical Branch at Galveston, Galveston (TX).

World Health Organization. (2020). Herpes simplex virus. https://www.who.int/news-room/fact-sheets/detail/herpes-simplex-virus.

Yadav, M., Jhunjhunwala, S., Phung, Q.T., Lupardus, P., Tanguay, J., Bumbaca, S., Franci, C., Cheung, T.K., Fritsche, J., Weinschenk, T., Modrusan, Z., Mellman, I., Lill, J.R., Delamarre, L. (2014). Predicting immunogenic tumour mutations by combining mass spectrometry and exome sequencing. Nature (London), 515(7528), 572-576. https://doi.org/10.1038/nature14001.

Zheng, M., Huang, J., Tong, A., Yang, H. (2019). Oncolytic viruses for cancer therapy: Barriers and recent advances. Molecular Therapy. Oncolytics, 15, 234-247. DOI: https://doi.org/10.1016/j.omto.2019.10.007.