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Oncolytic viruses as a viable treatment for breast cancer

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Thesis

ONCOLYTIC VIRUSES AS A VIABLE TREATMENT FOR BREAST CANCER

by

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B.S., Bucknell University, 2019

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Master of Science

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DEDICATION

In loving memory of Pamela Allen.

ACKNOWLEDGMENTS

To my friends and family: thank you for all of the love and support over the past two years. You have gotten me through some very difficult times, and I am very grateful to have you in my life.

To my professors and mentors: thank you for your support, guidance, and lessons. I continue to think about your words of advice, and I will carry them into my future endeavors. Thank you for pushing me to do better.

ONCOLYTIC VIRUSES AS A VIABLE TREATMENT FOR BREAST CANCER

MATTHEW BARTON

ABSTRACT

This article is a systemic review of the breast cancer subtypes, the disparities in breast cancer treatment, and the various oncolytic viruses currently in development for breast cancer treatment. In the event that breast cancer is not diagnosed and treated early, certain types of breast cancer can rapidly spread to other parts of the body causing life-threatening health problems. In light of the decrease in breast cancer screenings following the initial wave of COVID-19, effective systemic treatments for later diagnosed breast cancers are critical now more than ever. Currently, systemic treatments are often associated with severe adverse side effects, resistance, variable specificity, and possible recurrences. Certain subtypes of breast cancer, including triple-negative breast cancer (TNBC), are more aggressive, do not respond to some treatment methods, and disproportionately affect black women. Oncolytic virotherapy aims to produce a systemic treatment that is specific to cancer cells, elicits an immune response, and only causes minor side effects. Further studies are necessary to increase the possibility of developing a virotherapy that can safely and effectively treat systemic breast cancers.

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LIST OF ABBREVIATIONS

ATAP	Advanced Therapy Access Program
AJCC	American Joint Committee on Cancer
CMR	Complete Metabolic Response
CP	Cyclophosphamide
CSC	Cancer Stem Cell
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DRR	Durable Response Rate
dsDNA	Double-stranded Deoxyribunucleic Acid
DCIS	Ductal Carcinoma in Situ
ER	Estrogen Receptor
E1A	Early Region 1A
E1B	Early Region 1B
FDA	Food and Drug Administration
GMCSF	Granulocyte-Macrophage Colony-Stimulating Factor
G0	Quiescent Phase
G1	Gap Phase 1
G2	Gap Phase 2
hCAR	Human Coxsackievirus and Adenovirus Receptors
HER2	Human Epidermal Growth Factor Receptor 2
HR	Hormone Receptor
HSV	Herpes Simplex Virus

HSV-1	Herpes Simplex Virus Type 1
IFN	Interferon
IL-24	Interleukin-24
LCIS	Lobular Carcinoma in Situ
M	Mitosis
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
oHSV	Oncolytic Herpes Simplex Virus Type 1
OS	Overall Survival
OV	Oncolytic Virus
PBS	Phosphate Buffer Saline
PD-1	Programmed Cell Death Protein 1
PET-CT	Positron Emitted Tomography-Computed Tomography
PFS	Progression-Free Survival
PMR	Partial Metabolic Response
PR	Progesterone Receptor
rbp	Retinoblastoma-associated protein
RECIST	Response Evaluation Criteria In Solid Tumors
RFS	Recurrence-Free Survival
S	Synthesis
scFv	Single Chain Fragment Variable
TK	Thymidine Kinase

TNBC	Triple-Negative Breast Cancer
TNM	Tumor Node Metastasis
T-VEC	Talimogene Laherparepvec
VEGF	Vascular Endothelial Growth Factor
VGf	Vaccinia Growth Factor
VV	Vaccinia Virus
WT	Wild-Type

Section 1: Introduction

1.1 Breast Cancer

Breast cancer is one of the leading causes of cancer death among women, second only to lung cancer.^[1] Globally, breast cancer is the most frequently diagnosed cancer. Worldwide more than 2.3 million women were diagnosed with, and 685,000 women died from, breast cancer in 2020. By the end of 2020, approximately 7.8 million women living with breast cancer had been diagnosed within the previous five years.^[2] This high incidence is also seen in the United States, accounting for 3.8 million Americans with a history of breast cancer as of January 1st, 2019.^[3] While men are diagnosed with breast cancer, the disease predominantly affects women as men only account for 0.5-1% of cancer diagnoses.^[2] Additionally, the average lifetime risk for women developing breast cancer is approximately 1 in 8, with a 1 in 39 probability of dying.^[3,4]

Breast cancer arises from the epithelial cells lining the ducts (ductal carcinoma in situ, DCIS) 85% of the time or in the lobules (lobular carcinoma in situ, LCIS), which accounts for the remaining 15% of pre-cancer that arises in glandular breast tissue.^[2] Initially, ductal and lobular cancers are limited to "in situ" pre-cancerous growth, defined as increased and abnormal growth of cancer cells isolated to their location or origin.^[2,5,6] While DCIS and LCIS were historically referred to as the two main types of in situ breast cancer, only DCIS is a potential precursor to invasive breast cancer as LCIS is now believed to be a benign lobular neoplasia that lacks the capacity to metastasize, and only indicates an increased risk for breast cancer.^[3,7]

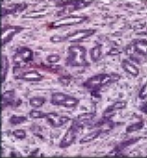

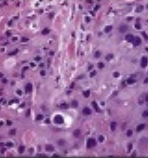
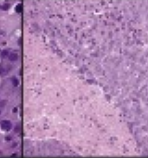
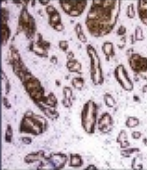
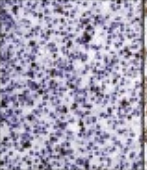

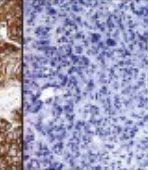
Dysregulation of the cell cycle is a hallmark of tumor development. Normal cells will progress through the cell cycle in a controlled and regulated process, growing during the gap 1 phase (G1), synthesizing DNA in the synthesis (S) phase, preparing to divide in the gap 2 phase (G2), and undergoing cell division in the mitosis (M) phase. Three highly regulated checkpoints normally occur during this cycle toward the end of G1 (G1/S checkpoint), during S phase, and prior to initiating mitosis (G2/M checkpoint). Most cells then exit the cell cycle into a quiescent phase (G0) where the cell is not actively growing and dividing.^[8] Two proteins that play an important role in the regulation of this cycle are the p53 and pRb proteins. p53 is a tumor suppressor protein that plays an important role in both the G1/S checkpoint and the G2/M checkpoints, where the cell arrests prior to DNA synthesis and mitosis, respectively. The activation of p53 following DNA damage causes the expression of subsequent proteins that function in cell cycle arrest and DNA repair, as well as proteins that function in apoptosis if the DNA damage is beyond repair.^[9] Similar to p53, pRb is a tumor suppressor protein involved in regulating the cell cycle at the G1/S checkpoint that also responds to DNA damage. Activation of pRb blocks transcription of the cell's DNA and holds the cell in arrest at this checkpoint until the damage is repaired or the cell is aborted.^[10] TP53, the gene that encodes for p53 protein, is the most commonly mutated gene in all cancers, with approximately 50-60% of cancers involving a mutated TP53 gene.^[11,12] Inactivation of tumor suppressor genes is a critical aspect of uncontrolled cell proliferation and tumor development.

1.2. Breast Cancer Staging

The system most often used for staging breast cancers in recent years is the American Joint Committee on Cancer (AJCC) TNM system, which consists of surgical and clinical components. In this system, T refers to the primary tumor size, and if it has grown into other areas, N refers to the number of nearby lymph nodes cancer cells have spread to, and M refers to metastasis or if cancer has spread to distant parts of the body or other organs. These individual factors are then assigned a value corresponding to their degree of progression (T0-T4, N0-N3, M0-M1), with higher numbers indicating further progression. From the descriptions and criteria mentioned, breast cancer in situ is designated stage 0. Additionally, the staging system takes into account estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor receptor 2 (HER2) status, the grade of the cancer cells, blood levels of tumor markers, tumor location, cell type, and other factors in conjunction with TNM staging prior to assigning an overall stage. These individual factors are then combined into the overall staging of cancer, graded as stages I-IV (with several subtypes), with higher value stages associated with further progression and worse outcomes.^[13,14] Stage IV cancer is indicative of cancer that has traveled to other parts of the body, of which breast cancer will preferentially spread to the brain, bones, lungs, liver, and pleura.^[15,16] Prognosis tends to vary significantly between stages, as depicted by the overall 5-year breast cancer survival rates of patients from 2009-to 2015, where stage I through stage IV had survival rates of 98%, 92%, 75%, and 27%, respectively.^[2]

The staging system alone shows the complexity and vastness of the disease. Breast cancer should then be defined as a grouping of diseases consisting of 4 main molecular subtypes and at least 21 distinct histological subtypes of breast cancer.^[3,17] With most breast cancers being invasive (81%), it is essential to correctly classify each of the individual factors precisely, as this will ultimately shape the presentation, prognosis, and treatment options.^[3]

Table 1. Breast Cancer Molecular Subtypes. Classification and treatment prognosis of the four main molecular subtypes of breast cancer based on distinguishing features in genetics, histology, and biomarkers.^[18]

Molecular subtype	Luminal (A and B)		HER2	Basal
Genetic profile	↑Luminal CKs and ER-related genes (A>B) B↑ in proliferation-related genes		↑HER2-related genes	↑Basal CKs
Histologic correlates				
	A Lower-grade ER+	B Higher-grade ER+	High-grade, ± apocrine features	High-grade, sheet-like, necrosis inflammation
Surrogate markers				
	A Strong ER+, PR±, HER2-, low Ki67	B Weaker ER+, PR±, HER2±, ↑Ki67	HER2+, ± ER/PR	ER/PR-HER2- CK5/6± EGFR±
Prognosis	Good	Intermediate	Worse	Worse
Response to chemotherapy	Lower	Intermediate	Higher	Higher
Targeted therapies	Hormone therapies		HER2-targeted therapies	Currently investigational

1.3. Molecular Subtypes

Molecular subtyping of breast cancer is vital in determining prognosis and effective treatment for patients. While breast cancer subtypes are best determined through gene expression analysis, it has become standard clinical practice to approximate breast cancer subtypes by evaluating biological markers.^[3] The four main breast cancer molecular subtypes include Luminal A (HR+/HER2-), Luminal B (HR+/HER2+), HER2 Enriched (HR-/HER2+), and Basal-Like (HR-, HER2-) (Table 1). Here, HER2+ refers to the presence of an abnormally high presence of human epidermal growth factor receptor 2, and HR+ indicates the presence of estrogen receptor (ER), progesterone receptor (PR), or both. These molecular subtypes are not mutually exclusive.^[18]

Luminal A is the most common subtype of breast cancer, accounting for 73% of all breast cancers. Luminal A tumors tend to be lower grade, slower-growing, and less aggressive when compared to other subtypes. These tumors exhibit ER+, PR±, HER2- and low Ki67 surrogate markers. Estrogen and progesterone can act as mitogens in HR+ cancers, that is that estrogen and progesterone induce mitosis and increase the rate of cell division, which can also lead to an increase in the rate of replication errors and mutations made.^[19] Cancers that are ER+ or PR+ grow in response to estrogen and progesterone, respectively. Ki67 is a nuclear protein marker, whereby a high score of Ki67 indicates a large number of actively dividing cells and has been clinically shown to correlate with metastasis and the clinical stages of tumors. While they have a lower response to chemotherapy, they are still associated with a good prognosis as they are often responsive

to hormone therapies that reduce the amount of estrogen or progesterone in the body.^[3,17,18,20]

Luminal B subtype accounts for 11% of all breast cancers. This subtype is defined by ER+, PR±, HER2±, and highly positive in Ki67. Luminal B cancers tend to be higher grade than luminal A cancers, indicating that they are more likely to grow and spread more rapidly and be more aggressive.^[3,13,18] Estrogen and progesterone also act as mitogens in Luminal B breast cancer. Additionally, the excess HER2 protein, a transmembrane receptor tyrosine kinase, on the surface of these cells does not require a ligand for activation and can undergo spontaneous dimerization and activation.^[21] Downstream effectors of this constitutively active protein provide cancer cells with potent proliferative and anti-apoptosis signals that contribute to the rapid development of the tumor.^[22] The high Ki67, indicating increased levels of actively dividing cells, is likely attributed to these factors. For these reasons, Luminal B cancers are associated with an intermediate prognosis, have an intermediate response to chemotherapies, and are typically targeted using hormone therapies.^[18]

HER2-Enriched cancer accounts for approximately 15-20% of all breast cancers. HER2-Enriched is defined by an over expression of the HER2 protein, with or without the presence of ER or PR. These cancers are known to have high-grade histological features, thus growing and spreading more rapidly, and until recently have been associated with poor outcomes. HER2-Enriched cancers have responded to chemotherapy more favorably than Luminal A and B cancers, but major advancements in treating these

cancers have been through HER2-targeted immunotherapy.^[3,18] Due to the high efficacy of targeted therapies against HER2 protein, the prognosis for HER2-Enriched cancers is good. Since the prognosis of HER2-Enriched cancers relies almost entirely upon targeted therapies, it is recommended that all women with invasive breast cancers be tested for HER2, and the practice of testing all newly diagnosed cancers for HER2 has become a standard practice for pathologist.^[3,23]

Lastly, Basal-Like cancers account for approximately 12-15% of all breast cancers. Basal-Like cancers are commonly referred to as triple-negative breast cancer (TNBC), as are ER-, PR-, and HER2-. These cancers are associated with a poor prognosis, as they are high grade, do not respond to the hormone therapies that are effective against Luminal A and B, and are also unresponsive to immunotherapies developed against HER2-Enriched cancers as they lack ER, PR, and HER2. While TNBC is more responsive to chemotherapy, targeted therapies and treatment advances have lagged behind treatments of other cancer subtypes.^[1,18,24-26] Compared to non-TNBC, TNBC has decreased 3-year progression-free survival rates, decreased 3-year overall survival rates, increased chance of visceral metastasis, and shorter post-recurrence survival rates.^[27] The latency in developing practical, targeted treatments for TNBC becomes apparent when comparing the survival rates of overall survival rates of metastatic Luminal A and B cancers (approximately five years) to metastatic TNBC (approximately one year).^[28] TNBC risk is also elevated in premenopausal women and those with a germline BRCA mutation.^[1,24]

Cancer subtypes provide details on the aggressiveness of the cancer's progression and preferential treatment options for each subtype. Knowing the cancer subtype can also provide information about where the cancer is most likely to metastasize or relapse. For example, Luminal cancers exhibit a bone-seeking phenotype and are less frequently observed in the brain, lung, pleural or multisystem metastasis. In contrast, HER2 and TNBC were found to be more often associated with lung and central nervous system metastasis, and TNBC was also more often associated with pleural metastasis compared to the Luminal subtypes. Breast cancer subtypes can then be used to strategize treatment and follow-ups.^[16]

1.4. Systemic Therapies

Effective treatment of metastatic cancers requires systemic treatments as cancerous cells are no longer limited to their site of origin and have spread to other organs of the body. Current systemic treatments will fall under cytotoxic, humoral, or immunotherapeutic categories. While systemic treatments have substantially decreased the recurrence rate in early-stage breast cancer patients over the last two decades (approximately 30% recurrence to 7-11%), patients with cancer-specific tumor characteristics are still at an increased risk of recurrence.^[29,30] Patients with larger tumors or who have had the cancer travel to other parts of the body are at an increased risk of recurrence. It has been shown that classic systemic agents are much less effective in treating breast cancer metastasis than primary tumors.^[29,31] Similarly, patients with

TNBC or Luminal B breast cancer are nearly 65% more likely to experience a relapse compared to Luminal A breast cancer during five years following initial treatment.^[32]

1.5. Systemic Treatment Side Effects

In addition to setbacks with recurrences in late-stage cancer treatment, systemic treatments are also associated with significant adverse effects. Cancer therapy side effects encompass grade 1 (mild, no intervention needed), grade 2 (moderate, no or minimal intervention needed), grade 3 (severe, medical intervention required, possible hospitalization) and grade 4 (potentially life threatening, significant medical intervention needed) side effects. Thus, the side effects in addition to treatment are important to consider.^[33] With the severity of common systemic treatment side effects, it is becoming increasingly important to limit these adverse effects as women with breast cancer are more frequently offered systemic treatment for early-stage breast cancer and are more frequently receiving one or more systemic therapies throughout their treatment.^[34] This shift is cause for concern as cardiotoxicity is one of cancer adjuvant therapy's most critical side effects.^[35] Chemotherapy, endocrine therapy, and HER2 targeted therapy have been shown to induce cardiotoxicity. Chemotherapy treatments (anthracyclines, alkylating agents, microtubule-targeting drugs, antimetabolites) all come with short-term side effects. Among the list of short-term cardiotoxic side effects are arrhythmias, hypertension, myocardial ischemia, bradycardia, pericarditis, heart failure, and sudden death. A long-term cardiotoxic side effect associated with anthracyclines is the progressive decline in left ventricular function. Additionally, endocrine therapies have

side effects associated with venous thromboembolism and strokes, and HER2-directed therapies have side effects associated with left ventricular dysfunction and heart failure.^[36,37] Many of these side effects can be life-threatening and irreversible, and due to the nature of oncological treatment regimens, often including a combination of therapies, the deleterious effects of these agents can be additive.^[36] Other common side effects of cancer treatment include, but are not limited to, anemia, appetite loss, thrombocytopenia, diarrhea, edema, fatigue, flu-like symptoms, fertility issues, alopecia, memory and concentration problems, mouth and throat problems, nausea, vomiting, skin and nail changes, constipation, pain, sleep problems, and more.^[38]

1.6. Disparities

The rate of breast cancer deaths has decreased by 40% from 1989 to 2017, translating to an estimated 375,900 breast cancer deaths averted.^[4] This trend is attributed to widespread access to mammography screening and improved treatment methods.^[39,40] While the general population has seen a downtrend in breast cancer mortality, not all women have benefited equally from these advances. On average, Black women have a death rate 40% higher than White women (28.4 vs. 20.3 deaths per 100,000), and Black women under 50 years old have a death rate that is 1.9-2.6 times that of White women. Furthermore, Black women are the only group that is more likely to be diagnosed with a high-grade tumor rather than a low- or intermediate-grade tumor, are more likely to be diagnosed at a later stage, are more likely to die from breast cancer at any age, and have higher death rates in all 50 US states when compared to all other racial groups.^[4] Part of

this may be because when Black women are diagnosed with breast cancer, approximately 20% of the time this diagnosis is the TNBC subtype, which is 2-4 times greater than Hispanic (11%), White (9%), Asian and Pacific Islander (6%), and American Indian/Alaskan Native women (5%) diagnosed with breast cancer.^[3,4,41,42] ER- cancers are significantly less likely to be screen-detected than ER-positive cancers (35.1% vs. 51.2%), making screening for cancer disproportionately less effective for Black women.^[40] The aggressiveness of TNBC may account for part of the disparity, as TNBC is a high-grade tumor subtype of breast cancer that grows and spreads quickly, is unresponsive to hormone therapy, and whose targeted treatments have lagged behind those of other cancer subtypes.^[4,26,43,44] However, Black women also have 5-year survival rates that are 5-7% lower than White women for every subtype of breast cancer, not just TNBC.^[4] While more frequent breast cancer screenings for Black women may help diagnose cancer at earlier stages and have a positive impact on 5-year survival rates, healthcare would have to become more readily accessible as Black women currently have less access to timely and high-quality prevention, early detection, and treatment services.^{[4,44][40]}

In light of the disparities seen in healthcare and the COVID-19 global pandemic, a systemic therapy targeting local and distant cancerous cells (including triple-negative) may help alleviate the discrepancies in breast cancer mortality rates among races and decrease breast cancer mortality rates as a whole.

Objectives

This thesis reviews current research on virotherapy in the light of its potential use as a systemic therapy in breast cancer treatment. This research was conducted using the keywords virotherapy, oncolytic virus, systemic therapy, metastatic breast cancer, triple-negative breast cancer, herpes simplex virus, adenovirus, poxvirus, reovirus, measles virus, and vaccinia virus. The majority of research articles referenced are from the years 2000 through 2022 in order to gain a contemporary understanding of virotherapy in late-stage cancer. This review considers the complexity of breast cancer subtypes, disparities in treatment, efficacy and adverse effects of systemic treatments, and limitations of virotherapy to determine the current state and future directions of this research field. A significant amount of background information on breast cancer and current systemic treatments is provided to highlight oncolytic viruses' significance as a novel therapy.

Section 2. Virotherapy and Oncolytic Viruses

2.1. Oncolytic Viruses - Overview

While oncolytic virotherapy is an emerging field, the idea of using viruses as therapeutic agents for cancer treatment is not new, with reports as early as 1912 documenting the apparent regressive effects of rabies vaccine on carcinoma of the cervix.^[45] Subsequent research from 1950-to 1980 attempted using wild-type or naturally attenuated viruses, including hepatitis, West Nile fever, dengue fever, and adenoviruses. However, these were not deemed therapeutic agents as the process of specifically targeting cancer cells while not harming non-cancerous cells had yet to be developed.^[46,47]

In recent decades, significant progress has been made in engineering viruses to exhibit antitumor properties. During that time, it has come to light that viruses can only infect specific cells within certain host organisms. The molecular reasons for this are that viruses can only enter cells with a particular viral receptor on the cell's surface, which allows it to attach, along with a hospitable metabolic activity that will allow the virus to replicate.^[48] Oncolytic viruses (OVs) are derived from naturally occurring viruses, which seek to take advantage of virus specificity to viral receptors on particular types of cells.¹ OVs are a new class of anticancer drugs designed to preferentially propagate in tumor cells and eliminate them without causing harm to surrounding non-cancerous tissues.^[49] Key design features of OVs include their ability to promote tumor regression by selectively targeting and infecting only cancerous cells, self-replicate, induce immunological cell death, and stimulate host antitumor activity (**Figure 1**).^[50,51]

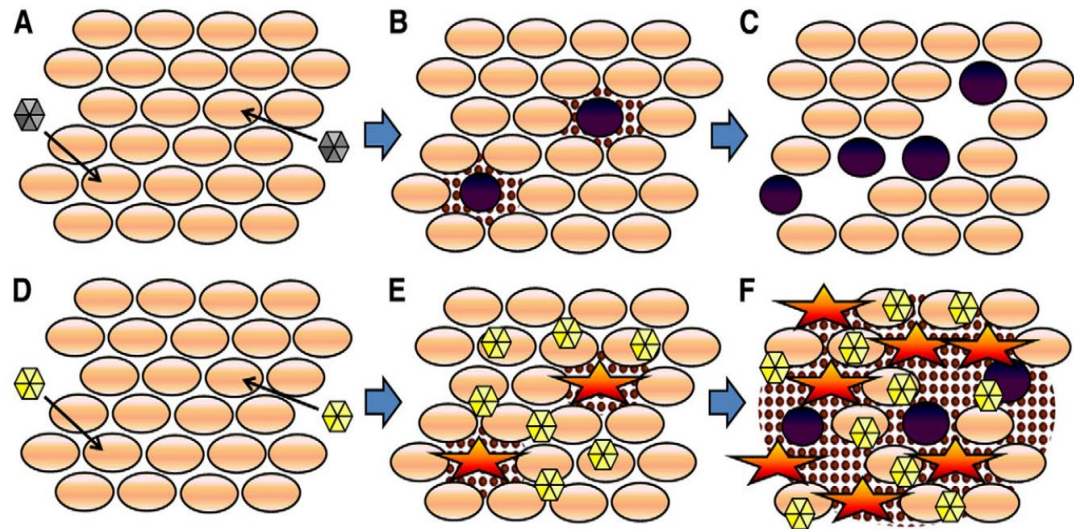
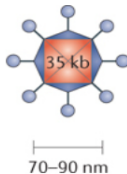
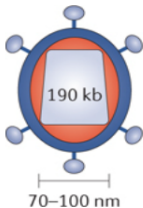
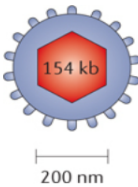


Figure 1. Principles of Classical Gene Therapy vs Oncolytic Virotherapy. Classical gene therapy (A-C) uses replication incompetent viral vectors to target and infect specific cells (A). Viral vectors deliver anti-tumor genes, which typically causes apoptosis (B) and can harm nearby tumor cells (C). Oncolytic viruses (D-F) use replication competent viral vectors to target and infect tumor cells (D). Oncolytic viruses then hijack host cell replication and transcription machinery to produce more oncolytic viruses and anti-tumor transgenes until the cell lyses, releasing more oncolytic viruses into the surrounding tumor environment (E). Oncolytic virus progeny target and infect subsequent tumor cells, repeat the cycle of producing more anti-tumor and oncolytic virus products, and lyse tumor cells, which can also cause harm to surrounding uninfected cells (F).^[52]

According to the Baltimore Classification System, there are seven different groups (6 originally proposed groups plus one added later on) of animal viruses based on how they synthesize messenger RNA (mRNA). How these viruses synthesize mRNA depends mainly on the structure, with each group of viruses having key features to describe their genomes.^[53] Because the majority of oncolytic viruses studied between 2000-2020 are Group I viruses, that is what will be focused on here. Group I viruses contain a double-stranded deoxyribonucleic acid (dsDNA) genome, allowing them to

carry out the asymmetric transcription of their DNA to create mRNA.^{[53][54]} Of all viruses studied within these twenty years, the two most common viruses studied were adenoviruses (30.9%) and herpes simplex virus type 1 (HSV-1, 23.7%).^[54] Of all the Group I viruses studied for their potential use as oncolytic viruses, the three most popular were adenoviruses, herpes simplex virus type 1 (HSV-1), and vaccinia virus (VV) (**Table 2**).^[50]

Table 2. Characteristics of adenovirus, herpesvirus, and vaccinia virus. Reproduced table from citation ^{[51][51]}

	Adenovirus	Vaccinia virus	Herpesvirus
			
Baltimore classification	Group I: dsDNA	Group I: dsDNA	Group I: dsDNA
Family	Adenoviridae	Poxviridae	Herpesviridae
Virion	Naked	Complex coats	Enveloped
Capsid symmetry	Icosahedral	Complex	Icosahedral
Replication site	Nucleus and cytoplasm	Cytoplasm	Nucleus and cytoplasm
Cell receptor	CAR	Unknown	HVEM, nectin 1, nectin 2
Nuclear integration	+	-	+
Transgene capacity	++	+++	+++
Wild-type virus infects non-replicating cells	-	-	-
Virulence of wild-type virus	+/-	+/-	-
Antivirals	+	+	+
Immunogenicity	-	-	-
Haemagglutination	+/-	-	-
Blood-brain barrier penetration	-	-	-

OV research is still a developing field, with numerous proof-of-concept preclinical studies forming the basis of these clinical trials, including developing targeted and armed viruses, best delivery practices, safety, and potential outcomes. In addition to

preclinical studies, during the same 2000-2020 timeframe previously mentioned, the majority (51%) of reporting clinical trials were in the phase I stage of research, while only 2% of studies were in phase III clinical trials, once again alluding to the early stages of this emerging field.^[54] Virotherapy studies on breast cancer made up only about 5% of these clinical trials, while most OV's targeted melanoma or GI cancers (Figure 2). OV's are also tested against many other forms of cancer (including brain, bladder, colorectal, head/neck, liver, lung, kidney, ovarian, pancreatic, prostate, skin, and more) at various stages of the disease progression. As seen with other cancer treatments, clinical trials were performed to assess OV's as a single agent therapeutic and in combination with other therapeutic agents. 61 of the 97 total trials reported assessed OV's alone, while the remaining 36 trials assessed OV's with chemotherapy (36), radiation therapy (5), immunotherapy (5), pro-drugs (7), or targeted therapies (4).^[54]

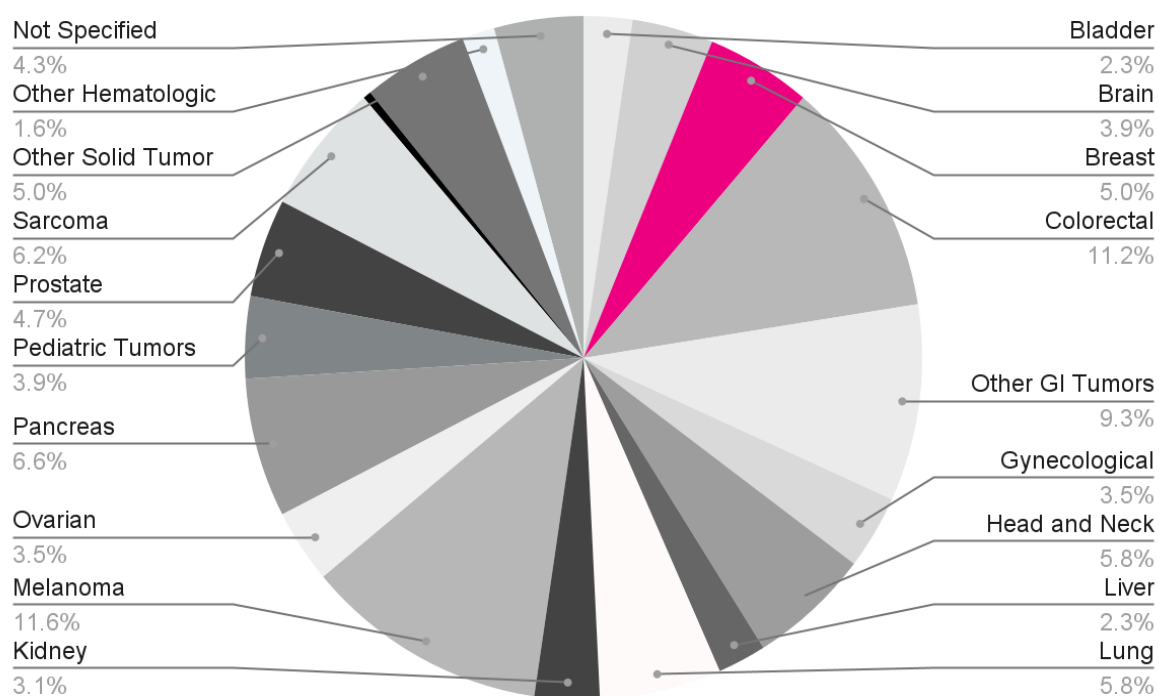


Figure 2. Types of cancers targeted in OV clinical trials. **Original figure produced using data from citation.**^[54]

As this is still a maturing field of study, many clinical trials are in early stages, have small patient sample sizes, and have a relatively short time following treatment interventions to assess the long-term effects of OVs. As OVs are further explored, especially through the few OVs that are currently used in practice, greater amounts of data will be collected and used to assess the viability of OVs in cancer treatment.

2.2. Adenovirus

Adenoviruses are approximately 70-90nm non-enveloped, icosahedral, Group I dsDNA viruses belonging to the Adenoviridae family (Table 2).^[52,55] Of the 51+ adenovirus serotypes that comprise the Adenoviridae family, Ad5 is the serotype most commonly used in oncolytic virotherapy as most oncolytic adenoviruses involve modifications built upon an Ad5 backbone.^[56] Ad5 vectors typically target cells through the human Coxsackievirus and adenovirus receptors (hCAR), which are present on the surface of most cells, followed by interaction with a second receptor responsible for internalization.^[50,51,57] After binding to hCAR and the secondary receptor, the virus enters the cell via endocytosis. The viral DNA is then transported to the nucleus, where it undergoes transcription and expression of early adenoviral genes (E1A and E1B), which are essential for propagation. E1A and E1B target and inactivate the cell's tumor-suppressor gene products, p53 and retinoblastoma-associated protein (Rb), allowing for adenovirus replication within the cell. When adenoviruses that lack functional E1A/E1B genes infect normal cells and target these antitumor gene products, Rb-induced arrest and p53-induced apoptosis are triggered and prevent replication and spreading of the virus.^[58,59] However, deletion or mutation of the p53 gene is seen in most types of human cancer, making the cancer cells intrinsically resistant to apoptosis. While this does allow cancer cells to grow without normal cell cycle controls, it is also a characteristic adenoviruses can exploit. Since cancer cells lacking p53 cannot undergo apoptosis and develop an anti-viral state in response to viral infection, the deletion of E1B in adenoviruses leads to attenuation of the virus vector for normal cells. In contrast,

cancerous cells are left highly susceptible to viral infection.^[60] This is just one approach to designing oncolytic adenoviruses to selectively replicate in cancerous cells while leaving normal cells unharmed by the virus.

For many reasons, adenoviruses were selected for use in oncolytic virotherapy. First, oncolytic adenoviruses can infect various cell types, both dividing and non-dividing, *in vitro* and *in vivo*. Since the adenovirus genome is so well-studied and frequently manipulated, the practice of limiting these viruses to target and infect specific tissues and cell types is well documented. Second, the large dsDNA genome and its amenability to alterations significantly improves the viability of adenoviruses as therapeutic agents. Adenovirus vectors have a high capacity for taking on large or complex transgene expression cassettes, allowing them to express a variety of artificially introduced genes that can be used in various conditions and treatment combinations.^[55,56,61] For example, in 2019, nearly 30 unique transgenes were expressed in adenoviral vectors used in 55 clinical trials for cancer treatment. These transgenes were combined in different assortments in different vectors, used as a monotherapy or combined with one or more traditional antitumor treatments (radiation, surgery, chemotherapy, immunotherapy), administered in various ways, and were tried against numerous types of cancers.^[55] The versatility of these adenovirus vectors creates many potential solutions for treating a disease as diverse as cancer. Third, adenoviruses can be produced in high titers that meet clinical good manufacturing practice standards.^[55,56] Additionally, adenoviruses are immunogenic viruses as they have been shown to elicit a powerful anti-viral and antitumor response. This immune response stimulated by viral

infection is not limited to the site of infection but instead creates a systemic antitumor response against uninfected metastases. Furthermore, this systemic immune response may stimulate an adaptive immune response against these cancer cells.^[55] Lastly, adenoviruses have an extensive safety record in preclinical and clinical trials, as most trials have only mild side effects and show excellent specificity for replicating only in cancer cells, while additional studies continue to enhance their safety profile.^[52,55,56,61]

As a consequence of these characteristics, the human adenovirus is the most extensively studied oncolytic virus.^[52] Recent clinical trials have yielded results for oncolytic adenoviruses in phase I, II, and III clinical trials that tested several oncolytic adenovirus vectors (Onyx-015, H101, DNX-2401, VCN-01, Colo-Ad1, ProstAtak, Ad5/3-E2F- Δ 24-GMCSF, CG0070, and more) against ovarian, bladder, colorectal, pancreatic, lung, breast and other types of cancers.^[51] There are currently 40 ongoing, recruiting, or recently completed clinical trials using oncolytic adenoviruses, 5 of which pertain to breast cancer treatment.^[62] Additionally, the adenovirus Oncorine (H101) vector was approved for treatment use in China as early as 2005 for head and neck cancer and esophagus cancer, though use and clinical data so far have been limited to China.^[46,63]

Ad5/3- Δ 24-GMCSF (CGTG-102)

The first objective of developing an oncolytic virus is getting the virus to target tumor cells selectively. Ad5 is the most common adenovirus serotype used in virotherapy, and it targets the hCAR receptor on most cell surfaces.^[57] However, this is

not true for cancer cells. Cancer cells often express low levels of the hCAR receptor used in Ad5 entry of the cell, which limits the therapeutic potential of adenovirus vectors.^[64] To remedy this problem, many adenovirus vectors use genetic fiber pseudotyping to change the tropism of the virion. For Ad5/3- Δ 24-GMCSF, the knob protein of an Ad3 serotype, which is responsible for the initial cellular attachment to a non-CAR binding site, was inserted into the backbone of the Ad5 serotype to form an Ad5/3 chimera. This modification has been shown to have improved delivery and antitumor efficacy in preclinical studies.^{[65-67][68]} This increase in efficacy results from the enhanced delivery using Ad3, as Ad3 binds to a non-coxsackie and adenovirus receptor that is highly expressed on the surface of human cancer cells.^[69] In addition to the modification made to the knob protein, Ad5/3- Δ 24-GMCSF also has a Δ 24 mutation for tumor selectivity. This 24 base-pair deletion in the viral E1A gene that is responsible for pRb protein binding renders Ad5/3- Δ 24-GMCSF unable to replicate in normal cells with a functional Rb pathway while remaining replication-competent in tumor cells.^[70]

In addition to selective and controlled replication in cancer cells, Ad5/3- Δ 24-GMCSF are also armed oncolytic viruses. Ad5/3- Δ 24-GMCSF includes granulocyte-macrophage colony-stimulating factor (GMCSF), a widely used immunostimulatory molecule in oncolytic virus clinical settings. GMCSF has been shown to promote a systemic antitumor immune response by increasing tumor-antigen presentation of dendritic cells, inducing tumor-specific cytotoxic T-cells, and stimulating an innate immune response by recruiting natural killer cells and neutrophils.^[71,72] Treatment of patients with GMCSF is well tolerated and results in tumor-specific and virus-specific

immunity.^[71] The safety and efficacy of GMCSF have also been demonstrated in a phase III trial using the HSV-1 vector T-VEC (Table 4), where objective measures of durable response rate, overall survival rates and median survival rates were all shown to be significantly higher for this treatment group.^[73]

In an early clinical study using Ad5/3-Δ24-GMCSF in an Advanced Therapy Access Program (ATAP), the viral vector was used as a monotherapy in the treatment of 115 patients with varying types of advanced cancers. 65 of the 115 patients enlisted in the therapy trial were evaluable via imaging and were subsequently evaluated via Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 guidelines. Of the 65 patients, 3% achieved partial response, 11% minor response, 40% stable disease and 46% had progressive disease whereby the cancer continued to grow and spread.^[74,75] These results were obtained with most patients only experiencing grade 1 and 2 adverse effects, including fever, nausea, and fatigue. Notably, approximately 5.2% of patients did incur grade 4 adverse effects, but some of these could be attributed to the underlying disease. It was also found that Ad5/3-Δ24-GMCSF was most effective against breast cancer, melanoma, soft tissue sarcoma, mesothelioma, and ovarian cancer.^[74]

While this first ATAP study using Ad5/3-Δ24-GMCSF as a monotherapy in late-stage cancers showed some encouraging results, other studies have looked at using the adenovirus vector in combination with the chemotherapeutic drug cyclophosphamide (CP). CP is one of breast cancer treatment's most commonly used alkylating agents.^[76] CP is known to have immunostimulatory and anti-angiogenic properties at continuous

low doses, but like many other chemotherapeutic agents, CP in high doses can lead to cytotoxicity and immunosuppression.^[77] Following preclinical testing of Ad5/3- Δ 24-GMCSF, one study used this viral vector in conjunction with CP to treat 21 patients with various advanced, progressing solid tumors, including ovarian, sarcoma, pancreatic, melanoma, colorectal and other types of cancers that were refractory to standard treatment. Of the 21 patients in the trial, 12 were evaluable for radiological benefit using RECIST 1.1 criteria. Of those evaluated, two patients achieved minor response, six stable disease, and four had progressive disease, equating to a radiological benefit of 67%, as the benefit is described as disease control, and all patients entering the study had progressing tumors. Blood samples showed an increase in Ad5-specific CD8+ lymphocytes in 9/14 patients and survivin-specific CD8+ lymphocytes (a classic pancreatic carcinoma marker reported present in nearly all tumor cells) in 8/14 patients, which suggests an adaptive anti-adenoviral response and likely an antitumor response at the site where GMCSF is produced. Once again, no severe adverse effects were observed.^[67]

In a second study using Ad5/3- Δ 24-GMCSF and CP against exclusively breast cancer, including TNBC, results were less supportive. Only 3/14 patients (21.4%) assessed by PET-CT imaging showed radiological benefit by RECIST 1.1 criteria (1 minor response, 2 stable disease). Additionally, of the four patients with TNBC included in this ATAP study, 4/4 showed progressive disease by RECIST 1.1 criteria, indicating no objective clinical benefit by radiological assessment. While preclinical trials did show significantly better efficacy, these lower benefit rates directly show how

aggressive and resistant TNBC can be, even in response to multiple therapeutic strategies.

[78]

Ad5/3-E2F- Δ 24-GMCSF (CGTG-602)

A study using a Ad5/3-E2F- Δ 24-GMCSF (CGTG-602) vector also showed promising results in *in vitro* models, as well as in phase I clinical trials against various metastatic cancers. CGTG-602 is an adenovirus vector similar to CGTG-102 with the same Ad5/3 chimera, Δ 24 mutation, and GMCSF expression. Earlier constructs similar to Ad5/3-E2F- Δ 24-GMCSF, such as CGTG-101, CGTG-102, and CGTG-103, contained only a 24-base pair deletion (Δ 24) for tumor selectivity. Tumor selectivity with Δ 24 takes place after E1A expression, causing E1A to be expressed in normal cells in addition to cancerous cells. The expression of unnecessary E1A in normal cells, which causes p53-induced apoptosis, can potentially result in toxicity and anti-viral immunity. Though these have been shown to be safe in patients, Ad5/3-E2F- Δ 24-GMCSF takes tumor selectivity and control one step further. Since the human E2F1 promoter is active in most tumor cell lines with a mutation in the pRb pathway, facilitating human E2F1 as the promoter of E1A instead the regular E1A promoter would cause tumor-specific expression of E1A in most if not all tumors and spare the expression of E1A in normal cells. Preclinical studies using Syrian hamsters showed that these modifications worked as designed, as viral replication was recorded in tumor cells, while particles in normal liver cells remained low through all time points. These results suggest that Ad5/3-E2F- Δ 24-GMCSF selectively replicated in tumor cells and not normal cells.^[79]

In a small clinical trial (n=13) using Ad5/3-E2F- Δ 24-GMCSF against advanced metastatic tumors in various types of cancer, including breast cancer, signs of antitumor efficacy were seen among 9/12 evaluable patients when evaluated by PET-CT or by tumor markers. Of the patients that were evaluable via PET-CT, a 67-year-old female with metastatic breast cancer showed a partial metabolic response (PMR, 49% decrease in metabolic activity) in the injected liver tumor and a complete metabolic response (CMR) in the non-injected mediastinal tumor (Figure 3a). Additionally, a 50-year-old female patient with metastatic fibrosarcoma had a CMR to treatment (Figure 3b), two other patients showed a minor metabolic response to treatment, and two others had stable metabolic disease. When assessed using biomarkers, 60% of these patients showed some possible treatment benefit, indicated by a reduction in tumor markers at some point during treatment. In addition, tumor biopsies indicated the accumulation of T-cells to tumor sites following treatment, and adverse effects were limited to grade 3 or below.^[79] These results are significant in light that most patients had received multiple rounds of chemotherapy and continued tumor progression prior to Ad5/3-E2F- Δ 24-GMCSF treatment, signifying a highly treatment-refractory patient population.

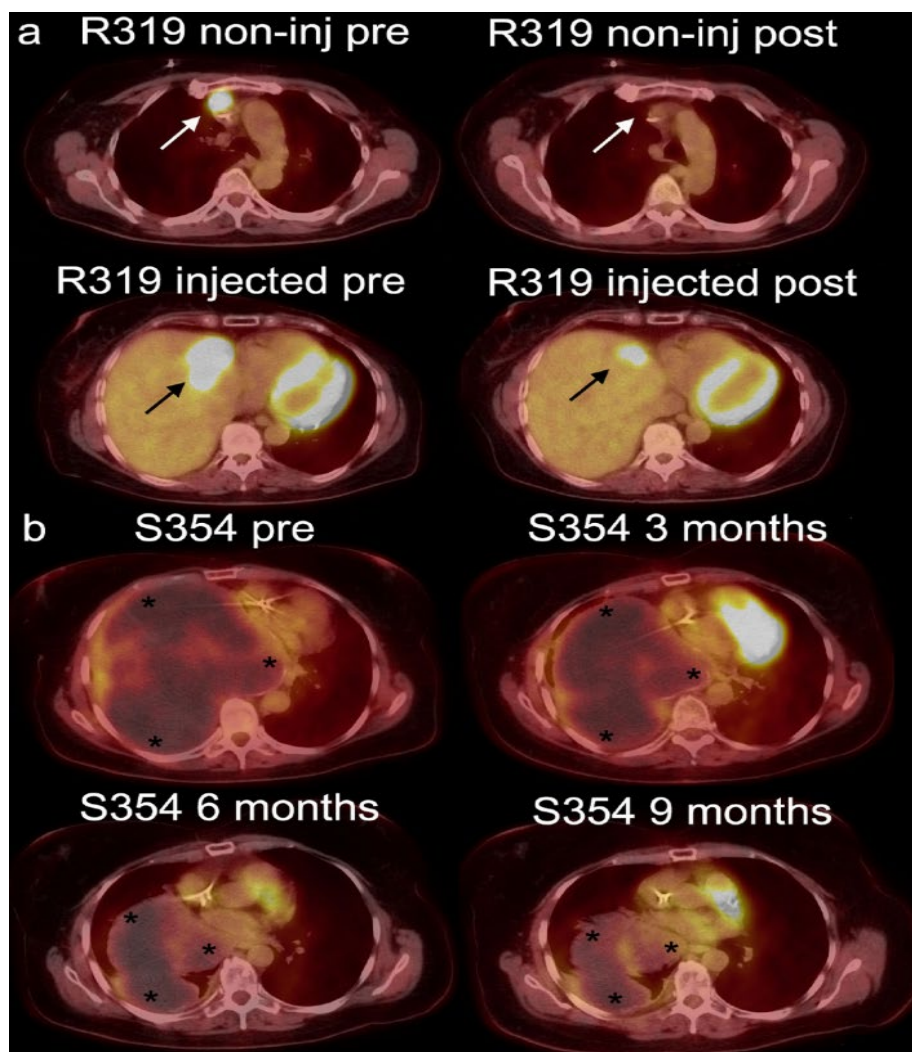


Figure 3. Positron emitted tomography-computed tomography (PET-CT) fusion images before and after Ad5/3-E2F- Δ 24-GMCSF treatment. (a) Images from a metastatic breast cancer patient showing complete metabolic response (CMR) of a non-injected mediastinal tumor (white arrows) and a partial metabolic response (49% reduction in metabolic activity) in the injected liver tumor (black arrows) after treatment with Ad5/3-E2F- Δ 24-GMCSF. (b) Images from a metastatic fibrosarcoma patient showing a 46% decrease in tumor (stars) volume after 3 months, 76% reduction in tumor volume after 6 months, and a stable situation 9 months after initial treatment.^[79]

Research involving oncolytic adenoviruses in TNBC is still an emerging field.

While few adenovirus vectors have been tested against very advanced-stage cancers

through ATAP studies, many novel oncolytic adenoviruses still have yet to reach this

stage of development. Many of these viral vectors have unique characteristics specific to targeting and killing TNBC and have shown efficacy in preclinical trials.^[80] A summary of these vectors and modifications can be found in **Table 3**.

Table 3. Summary of oncolytic adenoviruses for TNBC treatment. Modifications and functions of oncolytic adenoviruses used in preclinical trials against TNBC models. Original table created using information from citation ^[80]

Vector	Added Modification	Function
p55-hTERT-HRE-TRAIL	p55-hTERT-HRE-TRAIL	-Target/Induce TNBC apoptosis - Restrict tumor growth and metastasis
CNHK600-IL24	IL-24	-Elicit adaptive immune response -Induce TNBC apoptosis
OBP-401 adenovirus	hTERT	-Increase targeting to cancer cells
Ad5-10miR145T	Ten miR-145-5p target sequences	- Target miR-145-5P - Replication selectivity in cancer cells
Ad.DCN	Decorin protein expression	- Decorin expression - Regulate pathways - Elicit antitumor inflammatory and immune responses
SG400-E2F/IL-15	Replace endogenous promoter with E2F-1 promoter, arm with IL-15	- Target cancer cells through E2F - Induce adaptive immune response

2.3. Herpes Simplex Virus (HSV)

HSV-1 is a large Group I virus belonging to the Herpesviridae family, consisting of a linear double-stranded DNA genome containing approximately 80 genes (**Table 2**).^[53,81] This virus most often spreads through direct contact at mucosal membranes with or abraded skin with infected bodily fluids. Primary infection typically occurs in the oropharynx, with the course of severe infections causing irritability, sores or ulcerations, fever, tender submandibular lymphadenopathy, flu-like discomfort, and widespread mucocutaneous eruption. However, most infections appear mild or asymptomatic.^[82-86] A unique characteristic of HSV-1 is its capacity to establish a latent infection by infecting dorsal root ganglia, the sensory nerve cells responsible for transmitting signals from thermoreceptors, nociceptors, proprioceptors, and chemoreceptors to the central nervous system.^[87] HSV-1 virions that establish a footing in dorsal root ganglion can evade the host's immune system, allowing the viral genome to remain in infected host neurons for the entirety of the host's life.^[82] A summary of oncolytic HSV-1 (oHSV) vector applications relevant to this current research can be found in **Table 4**.

Table 4. Summary of relevant oHSV vectors in current research.

Vector	R-LM249	G47 Δ	T-VEC
Modifications	γ 34.5 gene deletion; Trastuzumab scFV in gD domain	IPC6 gene mutation; α 47 gene mutation; γ 34.5 gene deletion	Deleted Herpes Virus IPC34.5; Deleted viral IPC47; inserted 2 copies of human GM-CSF,
Modification Aim	Target HER2 Receptors	Restricted replication to dividing cells; immune stimulation;	Eliminate neuropathogenesis, preferential replication in tumor cells; antigen presentation for anti-viral/anti-tumor immunity; Recruitment of dendritic cells/macrophages
Clinical Phase	Preclinical studies	<i>In vivo</i> solid tumor models against several cancers; Few phase I-II clinical trials; Temporary approval for malignant glioma/primary brain cancer	Phase 1-3 with results for various cancers; Approval for Melanoma stage IIIB and IV
Clinical Trial Range of Applications	Ovarian cancer, breast cancer	Glioblastoma, prostate cancer, olfactory neuroblastoma	Skin, pancreatic, ovarian, breast, lung cancer, bladder, head/neck, and liver cancers
Side Effects	No signs of toxicity or side effects were observed in mice	Well-tolerated; No severe adverse effects attributed to G47 Δ to date	Grade 1-2: Fatigue, fever, flu-like symptoms, injection site reactions; Grade 3-4: Cellulitis (2.1% of participants)
Market	No	Yes (Temporary approval); Since 2021 in Japan; malignant glioma/primary brain cancers	Yes Since 2015; stage IIIB and IV Melanoma
References	[50,88,89]	[46,50,90–94]	[73,95,96]

R-LM249

To make a successful oncolytic virus for the treatment of malignant breast cancer, HSV-1 must be reprogrammed to target cancerous cells within the body instead of its natural receptors on host cells. A clear cause for concern is the fact that HSV-1 has the potential to infect host neurons. To circumvent this problem, after it was determined that the neurovirulence gene, γ 34.5, is non-essential for viral growth in culture, subsequent studies deleted this gene from the HSV-1 genome and began reprogramming the virus to target cancer cells specifically.^[97] One such study did so using a naked mouse model where mice exhibited a human tumor expressing HER2, HSV-1 was reprogrammed by de-targeting its natural receptors (nectin1 and HVEM) by replacing its natural Ig-folded core with the Ig-folded core single-chain fragment variable (scFv) antibody, which is directed to target HER2. This switch in protein domains allowed the modified HSV-1 to target human HER2 expressing tumor cells selectively. After just a single administration of HER-2-retargeted HSV, named R-LM249, in vivo growth of HER2-expressing tumor cells was effectively and selectively inhibited. This single dose in mice provided therapeutic effects lasting several weeks as tumor growth was significantly delayed, while multiple administrations of R-LM249 resulted in a high and stable proportion (60%) of tumor-free mice through to the conclusion of the study 5-months later. This study also showed that R-LM249 had a great degree of safety as the mice displayed no signs of toxicity, and the R-LM249 spread of infection was limited to HER2-expressing cells, self-exhausting itself after lysing all HER2-expressing tumor cells and leaving surrounding non-tumor cells uninfected.^[98] This study touched on many important

concepts related to OVs, as it demonstrated safety and efficacy, showed that viruses could be reprogrammed to select specific cell receptors, and showed that the modifications made to the virion would not revert. These findings are of great significance as the 15-20% of HER2-Enriched cancers may be able to benefit from such treatment in the future. HER2-Enriched cancer that is unresponsive to trastuzumab, a monoclonal anti-HER2 antibody immunotherapy and most common targeted drug therapy for HER2-Enriched breast cancer, can be difficult to treat effectively.^[99,100] The specificity of these viruses opens the door to targeting biomarkers specific to various types of cancers, even those that have developed resistance to current targeted treatments.

[50]

G47 Δ

R-LM249 was an excellent proof of concept, but further studies explored these concepts. In a particular case, the G47 Δ vector has had several modifications made to the original HSV-1 virus to increase its efficacy and specificity. G47 Δ is a triple-mutated third-generation oncolytic Herpes Simplex Virus, with mutations in the IPC6 gene and deletions of α 47 and γ 34.5 genes.^[90] As mentioned previously, deleting the γ 34.5 gene from the HSV genome prevents the oHSV vector from entering nerve cells.^[97] To further develop this oHSV, the deletion of α 47 had multiple effects. First, the α 47 gene product is associated with inhibiting the transporter responsible for the presentation of antigens, so the deletion of this gene led to increased MHC class I expression in infected human cells and increased matched antitumor T-cell activity. Additionally, deletion of α 47 in

this oHSV led to the suppression of the reduced-growth properties seen in γ 34.5 deficient mutants, which subsequently produced higher virus yields and enhanced cytopathic activity in tumor cells in both immune-competent and immune-deficient animal models.^[90] Lastly, the mutation in the IPC6 gene, encoding for ribonucleotide reductase, prevents HSV from replicating in non-dividing cells.^[91,101]

Due to its safety and specificity, G47 Δ has gained significant attention in its potential use as an antitumor therapy and has been tested against various human cancers in preclinical trials. In one preclinical study, the safety and efficacy of G47 Δ were tested in human models to determine if the viral vector could be used to treat human esophageal cancer. Results from this study displayed consistent results: efficient cytotoxic effects were observed in all eight cell lines of esophageal cancers tested, injections into mouse models yielded significant inhibitory effects against tumor growth, and mice displayed no remarkable symptoms throughout the study. G47 Δ showed specificity as it was only found in esophageal cells following intraesophageal injections and was not found in any major organs following oral administration.^[102] Similar findings were reported for preclinical trials testing the efficacy of G47 Δ against thyroid carcinoma cells.^[51] Additionally, as of June 2021, DELYTECT® (teserpaturev/G47 Δ) became the first third-generation oHSV to be evaluated in humans as it received temporary approval by the Japanese Ministry of Health, Labour and Welfare for the treatment of patients with malignant glioma or any type of primary brain cancer.^[103] This approval came following recent phase II trial results that included limited side effects, and the 1-year-survival rate reached 92.3% for the small clinical study.^[93,94]

As it relates to breast cancer, one preclinical study describes the efficacy of G47 Δ in treating metastatic breast cancer through the use of breast cancer and healthy cell lines in vitro and a pulmonary metastatic murine model with systemic delivery of G47 Δ to assess the oHSV in vivo. Starting with the in vitro focus, 85-95% of all three breast cancer cell lines were killed by a multiplicity of infection (MOI), or the ratio of infectious virions to cells in a culture, of 0.01 and 0.1 by the fifth day of infection. Conversely, normal cells were viable five days post-infection, exhibiting the same specificity of G47 Δ seen in other preclinical studies.^[104] A similar study reported G47 Δ to be effective against cell line representations of luminal, HER2, and basal-like breast cancer cells, killing >98% of these cells within five days with an MOI of 0.01, demonstrating that G47 Δ is capable of targeting and killing all of the main subtypes of breast cancer, regardless of ER/PR/HER2 expression.^[105] These results are significant as it shows that G47 Δ effectively kills different cancer cell lines in vitro, especially given that it has been previously demonstrated that cancer cell lines effectively model primary tumors.^[106] In vivo, it has previously been determined that G47 Δ was cytotoxic against MDA-MB-435 cancer cells and that intratumoral injections of G47 Δ were effective in treated MDA-MB-435 breast tumor xenografts (to the point where some subcutaneous tumors regressed completely).^[104] From this current study on G47 Δ and advanced cancer, mice treated with phosphate buffer saline (PBS) had lung weights significantly heavier than those treated intravenously with G47 Δ (0.60g \pm 0.01g vs. 0.47g \pm 0.01g) due to tumor burden. Additionally, mice treated with G47 Δ experienced an over 9x reduction in the number of tumor nodules on the surface of their lungs compared to the PBS treatment group (1.27 \pm

0.46 vs. 11.87 \mp 2.23 tumor nodules)^[104]. This study shows the specificity, safety, and effectiveness of G47 Δ delivered systemically against advanced breast cancer tumors.

In terms of metastatic breast cancer and recurrences, G47 Δ has also been shown to target and kill cancer stem cells (CSCs) in a murine model.^{[105][52][107,108][109][47][105]}

T-VEC

Of note, HSV-1 is responsible for two of the four global OV_s that have been approved for treating late-stage cancers. In 2015, the Food and Drug Administration (FDA) approved using Talimogene laherparepvec (T-VEC) in the local treatment of unresected stage IIIB and IV melanoma lesions. T-VEC has been modified to selectively replicate in tumors, enhance antigen loading of MHC class 1 molecules, and encode for two copies of GM-CSF.^[73,110] In a randomized open-label phase III trial comparing the efficacy of T-VEC and GM-CSF, results were assessed according to durable response rate (DRR), an objective measure defined as the progression-free survival lasting continuously for at least six months. T-VEC was found to have a significantly higher DRR (16.3% vs. 2.1%), overall response rate (26.4% vs. 9.5%), and median overall survival (23.3 months vs. 18.9 months) compared to GM-CSF. T-VEC was also well-tolerated as the most common adverse events included fatigue, fever, and chills, with only 2.1% experiencing grade 3 or 4 adverse events.^[73,96] While T-VEC has shown single-agent efficacy, especially in untreated or late-stage melanoma patients, early clinical trials have also shown favorable results when using T-VEC in combination therapy. A 2017 phase 1b clinical study used T-VEC in combination with pembrolizumab

to treat metastatic melanoma.^[111] Pembrolizumab is a humanized antibody that targets programmed cell death protein 1 (PD-1), an inhibitory receptor expressed in some cancers that down-regulates immune responses by suppressing T-cell activity.^[112] This combination of virotherapy and immunotherapy yielded high objective overall response rates and complete response rates. Patients who responded to the therapy showed increased levels of CD8⁺ T-cells, elevated IFN- γ expression, and altered tumor microenvironment. Once again, the most common side effects included fever, chills, and fatigue.^[111] A 4-year interim analysis of this study showed the continued benefits of the combination therapy over pembrolizumab immunotherapy alone, as combination therapy patients showed improved progression-free survival (PFS) and DRR (13.5 months vs. 6.4 months and 33.7% vs. 13.0%, respectively).^[113] Similarly, a phase II trial using T-VEC virotherapy in combination with another immunotherapy, ipilimumab. Ipilimumab is an anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody that can regulate T-cell activation, enhance immune responses, and contribute to anti-tumor immunity.^[114,115] This combination of treatments showed improved overall response rates, longer median progression-free survival rates, and a higher rate of complete reduction in visceral tumor burden (23% vs. 0%) when compared to ipilimumab treatment alone.^[114] Additionally, T-VEC has been shown to improve recurrence-free survival (RFS) and overall survival (OS) in patients with advanced-stage melanoma when used in combination with surgery as compared to surgery alone.^[116] Evidence for T-VEC and surgery in combination providing therapeutic benefits is supported by a 3-year follow-up study.^[117] While T-VEC is marketed for the treatment of melanoma, these findings act as a proof of concept

that HSVs can be used effectively in combination therapy for late-stage cancers.

Currently, there is recruitment of HER2- breast cancer patients for studies that seek to determine if T-VEC in combination with chemotherapy or endocrine therapy provides an added therapeutic benefit over chemotherapy or endocrine therapy on their own.^[118]

Ongoing studies include T-VEC in pancreatic cancer, angiosarcoma of the skin, advanced non-CNS tumors, unresectable stage IIIB-IV malignant melanoma, soft tissue sarcoma, melanoma, and breast cancer.^[95]

2.4. Vaccinia Virus (VV)

Vaccinia virus (VV) is a large, enveloped virus belonging to the poxviridae family. VV is a unique Group I dsDNA virus as it only replicates in the cytoplasm and does not enter the cell nucleus (Table 2).^[51]

While VV is less extensively studied for oncolytic virotherapy than adenovirus and HSV-1, several VV vectors (**Table 5**) have been used in preclinical and clinical trials showing encouraging results in cancer treatment. In one preclinical study, VV vector VG9-IL-24 coding for interleukin-24 (IL-24) was tested against breast cancer cell lines MDA-MB-231 (TNBC), MDA-MB-453 (TNBC), MDA-MB-468 (TNBC), MCF-7 (ER+, PR+), SK-BR-3 (HER2+). MCF-10A and HEK-293 (healthy human breast epithelial cell line and human embryonic kidney cell line, respectively) were used as controls. Results from this study indicated that VG9-IL-24 was able to selectively replicate in cancerous cells, causing inhibition of growth, cellular arrest in the G2/M phase of the cell cycle, and apoptosis in cancerous cell lines while no significant changes

were observed in control groups.^[119] Another preclinical trial tested the VV vector GLV-1h164, armed with the single-chain antibody GLAF-2, against TNBC in vitro and xenograft models. GLAF-2 targeted Vascular endothelial growth factor (VEGF), which is overexpressed in TNBC cells compared to other cancers. In these models, GLV-1h164 was shown to replicate effectively in TNBC cell lines, decrease tumor volume nearly 6-fold and decrease tumor vasculature 2-fold compared to the negative control.^[120] Other preclinical studies focused on the vector Vvdd, with modifications for transcription targeting. Vvdd modifications include deleting thymidine kinase (TK-) and vaccinia growth factor (VGF-) for enhanced tumor selectivity and cytotoxicity. When used as a monotherapy in nude mice, the Vvdd treatment group did not show toxicity as survival time for this group (>100 days) was substantially longer than wild-type (WT), VGF-, and TK- control groups (6, 17, and 29 days, respectively). The Vvdd treatment group also showed significant tumor regression compared to the control group, alluding to potential efficacy in cancer treatment.^[121] Vvdd used in combination with an agonist antibody specific for 4-1BB (CD137), an important cellular receptor that mediates T-cell activation, has also shown significant effects in cancer models.^[122] Prior studies have indicated that engagement of 4-1BB with a receptor agonist antibody will promote T-cell proliferation, cytokine production, and cytolytic effector functions, thus promoting adaptive antitumor immune function.^[123] When Vvdd and H-1BB were tested in combination on subcutaneous tumors in immune-competent mice, tumor growth inhibition was shown to be significantly enhanced compared to either treatment alone. This treatment group also showed greater survival rates and reduced metastatic tumor

growth compared to controls.^[122] These results suggest that oncolytic virotherapy may be beneficial in combination with other antitumor agents rather than alone as a monotherapy.

JX-594

JX-594 is a novel virotherapy vector that has progressed to clinical trials for several cancer types and in combination with other therapeutic agents.^[124] Similar to Vvdd, JX-594 has a mutation in the TK gene that enhances its selectivity to cancer cells. Like many other vectors mentioned, JX-594 also encodes for GM-CSF to elicit an antitumor immune response. In addition to these modifications, this vector also includes a lac-Z transgene as a marker for visualization. Interestingly, JX-594 was found to use multiple selectivity mechanisms for replicating only in cancer cells, including both inherent and engineered methods. In one study, JX-594 replication was activated by epidermal growth factor receptor or Ras pathway signaling, cellular TK levels, and cancer cell resistance to type-I interferons (IFNs).^[125]

In a preclinical trial using intravenous delivery of JX-549 to two live liver cancer models, results showed high efficacy of the vector, including complete responses, while being well-tolerated in both models. Furthermore, in one live model, lung metastases did not develop in any of the subjects administered with JX-549, while all subjects in the control group developed lung metastases from primary liver tumors.^[126] In addition to preclinical data, JX-549 has also been evaluated in clinical trials. In a randomized phase 2 dose-finding trial, 30 patients with advanced hepatocellular cancer were administered

either low-dose or high-dose JX-594 intratumoral injections. Viral replication and GMCSF expression were noted before therapeutic responses. Results of this study showed a 15% objective response rate to both injected and distant/non-injected tumors according to RECIST criteria, as well as patient survival times being strongly correlated to dosing intensity (median survival times of 14.1 months vs. 6.7 months for the high and low-dose groups, respectively).^[127]

While much of the research on JX-549 done so far has been focused on liver cancers, it is currently involved in 5 recruiting or active clinical studies looking to treat renal cancer, colorectal cancer, melanoma, soft-tissue sarcoma, metastatic tumors, and breast cancer. The breast cancer study is a phase I/II trial that has been actively recruiting since 2015. This study looks to treat patients with a combination of JX-549 and CP, a chemotherapeutic alkylating agent used in breast cancer treatment, similar to the Ad5/3- Δ 24-GMCSF ATAP study on breast cancer.^{[76][128]} This combination could provide a viable treatment for breast cancer given the efficacy of both JX-549 and CP in previous trials, as well as the synergistic effects combination therapies often exert.

Table 5. Summary of VV vectors used in oncolytic therapy.

Vector	Modification	Function
VG9-IL-24	IL-24 expression	- Selective apoptosis of cancer cells ^[119]
GLV-1h164	GLAF-2 expression	- Target VEGF and inhibit growth of TNBC ^[120]
Vvdd	-TK deletion & VGF deletions	- Tumor selectivity and cytotoxicity ^[121]
JX-594	-TK mutation - GMCSF & LacZ expression	-Enhanced cancer cell selectivity -Induce adaptive immune response ^[125]

Section 3. Cancer in the Post-Covid Era

One thing to consider is the implications that the COVID-19 pandemic has had on breast cancer screenings and treatment. As the country sought to decrease the spread of COVID-19 infections, hospitals allocated resources to fighting COVID-19 and limited the number of non-emergency patients entering hospitals. Due to these circumstances, radiological practices experienced a significant decline in the screening procedures in the early stages of the pandemic, with some sites experiencing 40-70% drops to that same timeframe in previous years, to other places seeing a 99% drop or even a complete stop in diagnostic procedures.^[129-133] Of these diagnostic procedures, it was found that mammographic breast cancer screenings experienced the largest decrease in volume among all radiological procedures.^[130] Once screening volume started again, there was an apparent shift in the demographic returning to screenings. It was determined that younger, non-white, uninsured patients who had to travel further distances for mammographic screenings were all less likely to return for screening mammograms following the pause.^[129]

Additionally, following the initial decrease in cancer screenings, there was a shift in the stage of cancer diagnoses. Some areas reported a 1.4% increase in stage III and a 3.5% increase in stage IV cancers.^[24] In an area that was reported to have a nearly 44% drop in mammograms from May through July 2020 (compared to that same period of the previous year), it was reported that there were significant decreases in in-situ breast cancer diagnoses (-10.4%), and an increase in node-positive (+11.2%) and stage III

(+10.3%) breast cancers. A subgroup of this population diagnosed with breast cancer that has high proliferative rates had an even more significant increase in node-positive (+18.5%) and stage III (+11.4%).^[131] Similarly, a cohort study assessed patients enrolled during the COVID-19 era compared to those enrolled in treatment during the pre-COVID-19 era. It was found that the COVID-19 era patients had larger tumor diameters, advanced N-staging, and significantly higher incidence of post-surgical radiation-therapy and severely advanced disease.^[132] In line with these findings, it has been hypothesized that the shift to more advanced staged cancer associated with these missed cancer screenings will result in additional deaths.^[134]

The development of targeted, systemic treatments to deal with aggressive and late-stage cancers may be needed now more than ever. With lulls in screenings and aggressive cancers (like TNBC) being diagnosed at later stages, there will be a greater demand on the healthcare system to treat patients with worse prognoses than in the pre-pandemic era. Until screening rates improve, and more effective systemic treatments for late-stage aggressive cancers are developed, the disparities in treatment among women of different races and socioeconomic backgrounds will likely continue to widen in the years following the COVID-19 era.

CONCLUSION

After decades of preclinical and clinical trials, oncolytic viruses have finally started making their way to the market. While OV's have produced favorable results, especially in preclinical tests, it is unlikely that they will be a "silver bullet" monotherapy in the near future. That being said, OV's have consistently shown improved results in combination therapy settings compared to their use as individual monotherapies, alluding to potential future use in combination therapy treatments for advanced cancers. Multiple studies have also advocated for their safety in clinical use, with most OV treatments only eliciting flu-like symptoms as side effects. These side effects are especially favorable in comparison to many other antitumor therapies, which include but are not limited to severe heart problems, anemia, hair loss, fertility problems, diarrhea, fatigue, kidney dysfunction, sores developing in the oral cavity, and peripheral neuropathy.^[135]

Additionally, many OV vectors are undergoing increasing amounts of mutations to achieve greater potency for killing cancer cells. While this may increase the likelihood that OV vectors can be used as monotherapies and may even be able to be applied to multiple types of cancer, additional modifications for specificity will likely need to be made as a safety precaution. As vectors become increasingly cytotoxic, further restrictions to replication will need to be made to ensure replication in cancerous cells alone.

Systemic, targeted treatments of breast cancer have proved difficult due to the heterogeneity of cancer tumors. Cancer heterogeneity, whereby cancer cells of different tumors within the body, or even cancer cells within the same tumor in the body, may

have distinct morphological and phenotypical characteristics from one another. Cancer cells, even within the same tumor, may differ in gene expression, receptor presentation, metabolism, and metastatic potential.^[136] Because of this, it may be difficult to solely target only one protein expressed in or on cancer cells, and treatments may appear to be effective even though some cancerous cells continue to grow as they do not respond to treatment. OV's may be part of a solution. Due to their versatility and large capacity for adding gene cassettes, OV's can be made to target several proteins specifically expressed or overexpressed in cancer cells. Additionally, OV's may work in combination with other therapeutic agents to target different proteins solely expressed by these cancerous cells. It's also possible that multiple OV vectors could be used simultaneously in order to target various cancer profiles, especially since OV's have shown to only replicate within cancer cells and normally only elicit mild to moderate (grade 1-2) side effects. Finally, because OV's lyse cells and cause damage to surrounding tumor cells, it's possible that this may help to eliminate cancerous cells that are not directly targeted by viral vectors. Further studies using OV's in combination with other cancer therapies, or even in combination with other OV therapies, will be necessary to determine if OV's are effective in treating the various cancer profiles that can arise from cancer heterogeneity.

One additional note: most of the clinical studies mentioned included patients with progressing late-stage cancer that had already shown resistance to other antitumor therapies. While response rates may have appeared low, the fact that primary and metastatic tumors did show response to OV treatment is remarkable. With this in mind,

along with how rapidly the field has developed over the past two decades, future OV breast cancer treatment may lead to improved prognoses and outcomes.

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CURRICULUM VITAE

