

# Oncolytic virotherapy using herpes simplex virus: how far have we come?

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**Abstract:** Oncolytic virotherapy exploits the properties of human viruses to naturally cause cytolysis of cancer cells. The human pathogen herpes simplex virus (HSV) has proven particularly amenable for use in oncolytic virotherapy. The relative safety of HSV coupled with extensive knowledge on how HSV interacts with the host has provided a platform for manipulating HSV to enhance the targeting and killing of human cancer cells. This has culminated in the approval of talimogene laherparepvec for the treatment of melanoma. This review focuses on the development of HSV as an oncolytic virus and where the field is likely to head in the future.

**Keywords:** herpes simplex virus, cancer, immunity, combination therapy, oncolysis

## Introduction

Oncolytic virotherapy began with the observation that patients with cancer and a viral infection could, on the rare occasion, briefly enter cancer remission.<sup>1</sup> Although such cases were noted as early as the mid-1800s (before viruses were known to exist), it was not until the 1950s and 1960s that an earnest effort was mounted to put this observation into practice. Clinical trials using different human viruses including hepatitis,<sup>2</sup> Epstein–Barr,<sup>3</sup> adenovirus,<sup>4</sup> and rabies<sup>5</sup> were undertaken with mixed results. None of the studies were able to overcome the risk that infecting humans with wild type virus posed. Attempts were also made to identify and adapt animal viruses for oncolytic virotherapy. Animal viruses that lacked pathogenicity in normal human tissue, yet still retained a propensity for replicating in human cancer cells, were identified in order to circumvent the side effects of human oncolytic viruses. However, the potential for these viruses to alter their tropism to normal human cells was an impediment that halted the majority of virotherapy research with animal viruses.<sup>6</sup>

Interest in oncolytic virotherapy was reborn in the 1990s with the advent of genetic engineering. Combined with the extensive knowledge that had been accumulated on viruses, genetic engineering allowed the generation of viruses with specific attenuations. The deletion of viral genes that, while essential for replication in normal tissue, were not required for replication in cancerous cells, allowed viruses to be retargeted toward cancer cells.

Herpes simplex virus (HSV)<sup>7</sup> was identified as a highly attractive candidate for oncolytic virotherapy due to several characteristics including: 1) a naturally cytolytic life cycle with the ability to infect a broad range of cell types; 2) a highly prevalent human pathogen which in the vast majority of cases causes a self-limiting disease that can be treated with antivirals in life-threatening cases; 3) a very large genome, with

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many nonessential genes that can be replaced with foreign genes; and 4) an envelope with separate attachment and fusion glycoproteins which can be modified for improved cancer cell targeting.

The initial focus of oncolytic HSV (oHSV) virotherapy involved demonstrating the safety of oHSVs for the treatment of cancer. Subsequent research has focused on confirming that oHSV mutants can be effective in various cancers and enhanced by targeting areas such as host immunity, tumor microenvironment, and cancer-specific cell functions, either by insertion of human genes into the oHSV and/or combining with other therapies. This has culminated in the approval by the US Food and Drug Administration (FDA) of the oHSV talimogene laherparepvec (T-VEC) for the treatment of melanoma.<sup>8–10</sup> This review highlights the development of oHSV for the treatment of cancers and potential improvements for the activity and use of oHSV. For a perspective, the reader should consult a number of recent reviews on the field of oncolytic virotherapy as a whole.<sup>11–13</sup>

## Generation of oHSVs

Development of oHSVs involved initial deletion of a single viral gene and subsequently multiple viral gene deletions and modifications. Key examples of this development are

discussed further. A summary of the viral genes modified in oHSVs and the functions of viral proteins encoded by these genes is provided in Table 1. A comprehensive list of deletion mutation oHSVs can be found in Table 2 and altered gene regulation or receptor retargeted oHSVs in Table 3.

## Deletion mutants

HSV (types 1 and 2) infects nondividing cells such as neurons and therefore encodes viral homologues of various nucleotide metabolism and DNA synthesis enzymes.<sup>7</sup> The *dlsptk* HSV-1 mutant contains a deletion within the unique long (*UL*)23 gene which encodes the viral homologue of thymidine kinase (TK).<sup>14–16</sup> The hrR3 HSV mutant contains a *LacZ* (encodes  $\beta$ -galactosidase) insertion mutation of the HSV-1 large subunit of ribonucleotide reductase (RR), also designated infected cell protein (ICP) 6, encoded by gene *UL39*.<sup>17,18</sup> Consequently, *dlsptk* and hrR3 HSV mutants only replicate in dividing cancer cells which overexpress TK and RR. Deletion of viral TK though does result in resistance of HSV *dlsptk* to current nucleoside analog inhibitor antivirals (eg, acyclovir), which are activated by the action of viral TK.<sup>14</sup>

The HSV-1 R3616 mutant contains deletions within both copies of the major neurovirulence determinant gene repeat long (*RL*)1 (encodes neurovirulence determinant

**Table 1** Summary of viral genes modified in oHSVs

Gene	Protein	Function	Reference
<i>RL1</i>	ICP34.5	Major neurovirulence gene. Prevents cellular inhibition of protein synthesis by mediating dephosphorylation of eIF2 $\alpha$ . Binds to Beclin-1, inhibiting autophagy.	19
<i>RL2</i>	ICP0	Multifunctional. Involved in transcription of viral genes. Has ubiquitin ligase activity. Inhibits interferon response. Alters the cellular environment to promote viral replication.	45
<i>RS1</i>	ICP4	Represses expression of viral immediate early genes and activates expression of viral early and late genes.	53
<i>UL2</i>	Uracil DNA glycosylase	Removes uracil from DNA.	36
<i>UL22</i>	gH	Binds to integrins. With gL and gB, enables fusion of the envelope with the cell membrane.	69
<i>UL23</i>	Thymidine kinase	Involved in the synthesis of deoxyribonucleotide thymidine triphosphate.	16
<i>UL27</i>	gB	Part of initial attachment of the virus to the cell by binding to heparan sulfate. With gH/gL, enables fusion of the envelope with the cell membrane.	69
<i>UL39</i>	ICP6	Major subunit of ribonucleotide reductase.	18
<i>UL44</i>	gC	Forms the initial attachment of the virus to the cell by binding to heparan sulfate.	69
<i>UL48</i>	pUL48/VP16	Initiates transcription of immediate early genes.	43
<i>UL53</i>	gK	Essential for cytoplasmic envelopment, egress, and cell fusion.	32
<i>UL54</i>	ICP27	Multifunctional. Inhibits cellular mRNA splicing. Recruits necessary proteins involved in viral transcription and translation. Activates cellular pathways to promote viral replication.	62
<i>UL55</i>	pUL55	Tegument protein. Function unknown.	26
<i>UL56</i>	pUL56	Binds to neuron-specific kinesin KIF1A, an axonal transport motor protein.	28
<i>US6</i>	gD	Binds to HVEM and/or nectin-1, leading to a conformation change that initiates fusion.	69
<i>US11</i>	pUS11	Binds to and is phosphorylated by PKR, preventing cellular inhibition of protein synthesis and autophagy.	41
<i>US12</i>	ICP47	Inhibits TAP/MHC class I presentation.	77

**Abbreviations:** eIF2 $\alpha$ , elongation initiation factor 2 $\alpha$ ; g, glycoprotein; HVEM, herpesvirus entry mediator; ICP, infected cell protein; MHC, major histocompatibility complex; oHSV, oncolytic herpes simplex virus; p, protein; PKR, protein kinase R; RL, repeat long; RS, repeat short; TAP, transporter associated with antigen processing; UL, unique long; US, unique short; VP, viral protein.

**Table 2** Summary of oHSVs incorporating viral gene mutations

oHSV name	Parental HSV-1	Gene(s) mutated	Description	Reference
<i>dl</i> sptk	KOS	<i>UL23</i>	Internal deletion within <i>UL23</i> .	15
hrR3	KOS	<i>UL39</i>	Insertion of <i>LacZ</i> (encodes $\beta$ -galactosidase) in <i>UL39</i> .	17
R3616	F	<i>RL1</i>	Fragment of both copies of <i>RL1</i> deleted.	19
1716	17	<i>RL1</i>	Spontaneously occurring deletion within <i>RL1</i> .	22
NV1020 (R7020)	F	<i>UL23, UL55, UL56, RL1, RL2, RS1</i>	Deletion of <i>UL23</i> , as well as the region encoding <i>UL55, UL56</i> , and one copy of <i>RL1, RL2</i> , and <i>RS1</i> (though not the <i>RS1</i> promoter). HSV-2 region encoding pUS2, pUS3, and glycoproteins D, G, I, J, and part of E inserted in the deletion. <i>UL23</i> reinserted in the deletion such that expression is under the <i>RS1</i> promoter.	27
3616UB	R3616	<i>RL1, UL2</i>	Insertion of <i>LacZ</i> within <i>UL2</i> .	35
HF10	HF	<i>UL53, UL54, UL55, UL56</i>	Spontaneous deletion of <i>UL56</i> as well as duplication of <i>UL53, UL54</i> , and <i>UL55</i> .	34
G207	R3616	<i>RL1, UL39</i>	Essentially R3616 with the hrR3 <i>LacZ</i> insertion in <i>UL39</i> .	37
MGH-1	R3616	<i>RL1, UL39</i>	Same as G207.	38
SUP	Patton	<i>RL1, US11, US12</i>	<i>RL1</i> replaced with $\beta$ -glucuronidase under control of the <i>RL1</i> promoter. Deletion within <i>US12</i> which places expression of the normally late <i>US11</i> gene under the immediate early <i>US12</i> promoter.	39
G47 $\Delta$	G207	<i>RL1, UL39, US11, US12</i>	Deletion of the overlapping <i>US11</i> promoter/ <i>US12</i> region, putting expression of the normally late <i>US11</i> gene under the immediate early <i>US12</i> promoter.	42
KM100	KOS/17	<i>RL2, UL48</i>	Insertions within <i>RL2</i> and <i>UL48</i> . Expressed pUL48 lacks C-terminal transactivation domain.	44
Fu-10	G207	<i>RL1, UL39, glycoprotein(s)?</i>	Random mutagenesis resulted in unidentified mutations, most likely in glycoprotein(s).	48
Baco-1	17	<i>RL1, packaging signal</i>	<i>RL1</i> deleted. DNA packaging signal is restored.	49
Synco-2	Baco-1	<i>RL1, packaging signal</i>	Gibbon ape leukemia virus fusogenic glycoprotein under the late <i>UL38</i> promoter inserted.	50
Synco-2D	Baco-1	<i>RL1, packaging signal, glycoprotein(s)?</i>	Essentially Synco-2 with the Fu-10 glycoprotein(s) mutation.	51

**Abbreviations:** HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; oHSV, oncolytic herpes simplex virus; p, protein; RL, repeat long; RS, repeat short; UL, unique long; US, unique short.

ICP34.5).<sup>19</sup> Host protein kinase R (PKR), in response to various stressors, including the presence of viral dsRNA, phosphorylates elongation initiation factor 2 (eIF2 $\alpha$ ), preventing the synthesis of proteins. One of the functions of ICP34.5 is to mediate dephosphorylation of eIF2 $\alpha$ .<sup>20</sup> Therefore, HSV-1 R3616 targets cancer cells that are characterized by uncontrollable protein synthesis.<sup>21</sup> HSV-1 1716 mutant was also unable to express ICP34.5.<sup>22</sup> It was generated by a recombination between 1714, which has the same inactivating deletion in both copies of *RL1* (among other deletions), and wild type HSV-1 strain 17. While these four first generation oHSV mutants achieved mixed results in vivo,<sup>23–25</sup> they highlighted the potential of oHSVs for the treatment of cancer.

Another HSV-1 mutant generated around the same time as hrR3, NV1020 (R7020), replaced five HSV-1 genes (*UL55*, encodes tegument protein (p)UL55;<sup>26</sup> *UL56*, encodes envelope pUL56; and one copy each of repeat short (*RS*)1, encodes ICP4, *RL1* and *RL2*, encodes ICP0) with the HSV-2 genomic region encoding several viral glycoproteins.<sup>27</sup> The *UL56* deletion, in addition to the deletion of *RL1*, most likely further reduces neurovirulence, as HSV-2 pUL56 associates with the neuron-specific kinesin KIF1A, a protein involved in the

axonal transport of synaptic vesicle precursors.<sup>28</sup> Although unsuccessful for its original purpose as a live attenuated vaccine for HSV-1 and -2,<sup>29</sup> NV1020 showed potential as an oHSV in mice models of head and neck squamous cell carcinoma,<sup>30</sup> epidermoid carcinoma, and prostate adenocarcinoma.<sup>31</sup> The spontaneously generated oHSV HF10, which also includes a deletion of *UL56* as well as duplication of *UL53* (encodes glycoprotein gK),<sup>32</sup> *UL54* (encodes ICP27) and *UL55*, has had some success in animal models and human trials.<sup>33,34</sup>

The next generation oHSV combined gene mutations to reduce the chances of reversion to a virulent strain. The R3616 mutant (deleted *RL1*) was utilized to generate 3616UB, which also has an insertion of the *LacZ* gene into the *UL2* gene which encodes uracil DNA deglycosylase.<sup>35,36</sup> G207 (also known as MGH-1) combined the R3616 *RL1* deletion and the hrR3 *LacZ* inactivating insertion in *UL39*.<sup>37,38</sup> In *RL1* deletion mutants, PKR inhibits the expression of late viral genes including unique short (*US*)11. Passaging of an *RL1* deletion mutant in non-permissive cells leads to the natural generation of a mutant (known as SUP) with enhanced replication. SUP contains an additional deletion within *US12* (encodes ICP47), thus losing expression of

**Table 3** Summary of oHSVs incorporating gene regulated or receptor retargeting mutations

oHSV name	Parental HSV-1	Gene(s) mutated	Description	Reference
G92A	KOS	<i>UL23, RSI</i>	<i>UL23</i> gene replaced by <i>LacZ</i> driven by the unremoved <i>UL23</i> promoter. Mouse albumin enhancer/promoter driving expression of <i>RSI</i> in liver cancer.	54
d12.CALP	KOS	<i>UL23, RSI</i>	Same as G92A except 4F2 heavy chain enhancer and calponin promoter driving expression of <i>RSI</i> in soft tissue and bone cancer.	55
LCSOV	SCI16	<i>UL22</i>	Apolipoprotein E enhancer/ $\alpha$ 1 antitrypsin promoter and four copies each of target sequences for miR-122a, miR-124a, and miR-let-7a driving expression of <i>UL22</i> in liver cancer.	56
Myb34.5	MGH-1	<i>RL1, UL39</i>	<i>LacZ</i> insertion in <i>UL39</i> replaced with B-myb promoter driving expression of <i>RL1</i> .	57
rQNestin34.5	MGH-1	<i>RL1, UL39</i>	<i>LacZ</i> insertion in <i>UL39</i> replaced with Nestin enhancer, hsp68 promoter driving expression of <i>RL1</i> in glioma.	58
AU27	KOS	<i>UL54</i>	Probasin promoter and rat fibroblast growth factor 5'UTR driving expression of <i>UL54</i> in prostate cancer.	63
CMV-ICP4-143T/145T	I7	<i>RSI</i>	CMV promoter and five copies of target sequences for miR-143 or miR-145 driving expression of <i>RSI</i> in prostate cancer.	66
AP27i145	KOS	<i>UL54</i>	CMV promoter and four copies of the target sequence for miR-145 driving expression of <i>UL54</i> in non-small-cell lung cancer.	67
R5141	F	<i>UL27, UL44, US6</i>	Heparan sulfate binding in gB and gC abolished. IL13 inserted into gC and gD to allow binding to IL13R $\alpha$ 2 receptor overexpressed in glioblastoma and astrocytoma. gD no longer binds viral entry receptors HVEM and nectin.	71
R-LM249	F	<i>US6</i>	Sequence for trastuzumab antibody inserted in gD allows binding to HER-2 receptor overexpressed in breast and ovary cancer. gD no longer binds viral entry receptors HVEM and nectin.	73
HSV1716EGFR	I716	<i>US6</i>	Sequence for single chain antibody for EGFR replaced N-terminus of gD.	74
KNE	KOS	<i>UL27, US6</i>	Sequence for single chain antibody inserted in gD allows binding to overexpressed EGFR in glioblastoma. gD no longer binds viral entry receptors HVEM and nectin. Substitutions in gB lowered threshold for initiation of fusion.	75
R-VG809	F	<i>UL22, US6</i>	Sequence for trastuzumab antibody inserted in gH allows binding to HER-2 receptor overexpressed in breast and ovary cancer. gD no longer binds viral entry receptors HVEM and nectin.	76

**Abbreviations:** CMV, cytomegalovirus; EGFR, epidermal growth factor receptor; g, glycoprotein; HER-2, human epidermal growth factor receptor 2; HSV-1, herpes simplex virus type 1; HVEM, herpesvirus entry mediator; ICP, infected cell protein; IL, interleukin; oHSV, oncolytic herpes simplex virus; RL, repeat long; RS, repeat short; UL, unique long; US, unique short.

ICP47, but placing *US11* under the immediate early *US12* promoter.<sup>39,40</sup> pUS11 is phosphorylated by PKR, which thus prevents phosphorylation of eIF2 $\alpha$ .<sup>41</sup> Hence, earlier expression of pUS11 allows it to inhibit PKR before PKR has the chance to inhibit pUS11 expression. This *US12* deletion was also utilized in G207 to produce G47A.<sup>42</sup>

The KM100 mutant has insertions in *UL48* (encodes the transactivator tegument protein pUL48 [VP16])<sup>43</sup> and *RL2* genes.<sup>44</sup> KM100 no longer expresses the multifunctional protein ICP0<sup>45</sup> and while pUL48 is expressed it lacks the C-terminal transactivation domain. The resulting loss of expression of immediate early viral genes means KM100 only replicates well in cancer cells. Furthermore, KM100 activates antitumor immunity through interferon pathways normally suppressed by ICP0.<sup>46,47</sup>

The deletion of genes, whilst governing which cells oHSV can replicate in, also tends to attenuate the mutant virus. To boost virulence while maintaining selectivity for cancer cells, oHSVs with the capability to fuse cells have

been generated. Fu-10 was created by inducing random mutations in G207 and selecting for mutations in the viral glycoproteins that enable syncytia formation.<sup>48</sup> The lack of fusion in normal cells is due to the reduced replication of the HSV-1 genome, which strongly inhibits late gene expression (eg, glycoproteins). Synco-2, derived from Baco-1,<sup>49</sup> utilized an *RL1* deletion genome to insert a modified gibbon ape leukemia virus glycoprotein (constitutively fusogenic), under the control of a late HSV *UL38* promoter.<sup>50</sup> As both fusogenic mechanisms require different cellular receptors to enable syncytia formation, Synco-2D, also derived from Baco-1,<sup>49</sup> employed both methods to ensure resistance to one would not inhibit cellular fusion.<sup>51</sup> All three fusogenic oHSVs (Fu-10, Synco-2, and Synco-2D) demonstrated greater cytotoxicity in cancer cells than the HSV-1 parental strain.<sup>48,50,52</sup>

## Gene regulated mutants

While deletion mutants have been proven to greatly limit virus replication to cancer cells, the attenuation caused by

deleting one or more genes limits viral effectiveness. In an effort to retain the effectiveness of wild type HSV, oHSVs have been engineered to limit transcription and/or translation of an essential viral gene by replacing the viral promoters with tissue- or cancer-specific promoters.

G92A was targeted toward liver cancers by expressing the essential viral gene transcription regulator *RS1* (encodes ICP4)<sup>53</sup> from the exclusively liver expressed albumin promoter.<sup>54</sup> Similar to G92A, *d12.CALP* has *RS1* under the calponin promoter, which is aberrantly expressed in a variety of human soft tissue and bone tumors.<sup>55</sup> However, due to the complicated nature of gene transcription regulation by *RS1* encoded ICP4,<sup>53</sup> both viruses have delayed and slow replication. Another liver targeted oHSV, LCISOV, was generated by placing *UL22* (encodes viral glycoprotein gH) under the apolipoprotein E promoter.<sup>56</sup>

In an effort to boost replication of G207 in cancer cells, two mutants, Myb34.5 and rQNestin34.5, were generated by reinsertion of a copy of *RL1* into the *UL39/LacZ* region, with expression of *RL1* encoded ICP34.5 controlled by either the *B-myb* promoter or a nestin enhancer/heat shock protein 68 promoter cassette, respectively.<sup>57,58</sup> *B-myb* is a transcriptional regulator which appears to be involved in cellular proliferation and differentiation and is downregulated in quiescent cells.<sup>59</sup> Nestin is an intermediate filament protein whose expression is mostly switched off in adults, but expression is upregulated in malignant gliomas.<sup>58</sup>

Additional stringency in viral gene expression has been achieved by exploiting the overexpression of eukaryotic initiation factor 4E (eIF4E) in most cancers. eIF4E is the 5' cap binding and rate limiting protein of the eIF4F complex that is required to initiate capped translation of messenger RNAs (mRNAs). The 5' untranslated region (UTR) of genes associated with malignant progression and metastasis is often rich in GC residues, forming hairpin structures that inhibit binding to eIF4E. While expression of these genes is normally regulated by expression of eIF4E,<sup>60</sup> its constitutive overexpression in cancer allows unabated expression of these genes.<sup>61</sup> The rat fibroblast growth factor gene has a GC rich 5'UTR that was inserted behind a prostate-specific probasin promoter, which replaced the promoter of the essential HSV-1 gene *UL54*<sup>62</sup> to form the mutant AU27. Although *UL54* was transcribed in both cancerous and noncancerous prostate cells, ICP27 protein expression was strongly limited to cancerous cells.<sup>63</sup>

An alternative viral gene translation control strategy takes advantage of the downregulation of certain miRNAs in cancers when compared to noncancerous tissue.<sup>64,65</sup> miRNAs are short

noncoding RNAs that regulate gene expression by binding to the 3'UTR of complementing mRNAs, repressing their transcription. The aforementioned LCISOV also contained four copies of target sequences for three different miRNAs (miR-122a, miR-124a, and miR-let-7a) in the 3'UTR of *UL22* encoding gH to inhibit its translation in nonhepatocellular carcinoma cells.<sup>56</sup>

Multiple copies of target sequences for miR-143 and miR-145 were separately inserted into the 3'UTR of *RS1* to generate CMV-ICP4-143T and CMV-ICP4-145T.<sup>66</sup> AP27i145 was generated by inserting four copies of miR-145 target sequences into the 3'UTR of *UL54*.<sup>67</sup> All three of these viruses were created by the amplicon helper virus system, whereby the HSV genome has been separated into two genomes.<sup>68</sup> The helper genome comprises the complete HSV genome minus the gene that is to be controlled. The amplicon genome contains the missing gene (ie, *RS1* or *UL54*) under control of a promoter from cytomegalovirus with the miRNAs target sequence in the 3'UTR. It also includes a viral origin of replication and DNA packaging signal so that the amplicon genome is synthesized and packaged when the helper genome is present.

## Receptor retargeted mutants

The entry of HSV-1 into cells and the role of viral glycoproteins is well understood.<sup>69</sup> Numerous attempts have been made to maintain wild type HSV effectiveness while limiting cell specificity by altering the receptors that HSV binds to enter cells. In the first instance, the region encoding binding to heparan sulfate in HSV-1 glycoproteins gC and gB was deleted and the sequence encoding interleukin (IL)-13 inserted into gC.<sup>70</sup> The IL-13 sequence also replaced the N-terminal region of HSV-1 glycoprotein gD, which binds to herpesvirus entry mediator (HVEM). IL-13 was used to target the oHSV toward the IL-13R $\alpha$ 2 receptor that is overexpressed on malignant gliomas and high-grade astrocytomas.<sup>70</sup> An additional single amino acid mutation in gD was also required to abolish binding to nectin-1, another entry receptor, with the final mutant known as R5141. However, the retargeting of R5141 caused an attenuation of the virus when compared to the parental virus.<sup>71</sup>

While creating another retargeting mutant, with urokinase plasminogen inserted into gD instead of IL-13, it was discovered that an 159 amino acid region in gD was dispensable for its function.<sup>72</sup> Using this knowledge, R-LM249 was created by replacing this gD dispensable region with the sequence for the single chain antibody trastuzumab, which targets human epidermal growth factor receptor 2 (HER-2).



HER-2 is overexpressed in approximately a quarter of breast and ovary carcinomas. Although R-LM249 displayed no binding to the normal HVEM or nectin-1 receptors, it also had a reduced replicative ability.<sup>73</sup>

In demonstrating that HSV tropism could be altered by replacing the 274 amino acid N-terminus of gD with a single chain antibody, HSV1716EGFR was created by replacing this region with a single chain antibody that targets human epidermal growth factor receptor.<sup>74</sup> Another mutant, KNE, also incorporated the epidermal growth factor receptor antibody to replace the HVEM binding region of gD to target glioblastoma. KNE also required a single amino acid substitution in gD to ablate binding to nectin-1; however, as it did not effectively infect cells, it also needed a pair of entry enhancing mutations in the fusion glycoprotein gB.<sup>75</sup>

The most successful receptor retargeted mutant involves insertion of the trastuzumab antibody (targets HER-2) sequence into the N-terminal region of viral glycoprotein gH. This mutant, known as R-VG809, also contained deletions in gD to prevent binding to HVEM or nectin-1. R-VG809 was the first receptor retargeted mutant to show replication equal to the parental virus, at least in HER-2 positive ovarian cancer cells.<sup>76</sup>

## Enhancing oHSVs

With the abundance of varied oHSVs that have been created, focus has shifted, to an extent, onto improving these viruses. The augmentation of these viruses utilizes two strategies, incorporating host genes into the virus and/or combining the oHSV with other treatments. The broad areas targeted by these strategies include: host immunity; the tumor microenvironment; and cancer cell replication and function.

## Immune enhancement

Oncolytic virotherapies are occasionally viewed as immunotherapies due to the enhanced antitumor immune response that may be seen in patients after oncolytic vector treatment. Such a response could be due to numerous factors including greater release and thus detection of cancer antigens, an increase in infiltration of antitumor immune cells, and disruption of the immune toleragenic environment characteristic of many tumors. For this reason, significant effort has been made to further enhance the immune response during treatment with oncolytic viruses including oHSV.<sup>12,13</sup>

ICP47 (encoded by *US12*) normally inhibits major histocompatibility complex class I antigen presentation by binding to transporter associated with antigen presentation, blocking transport of antigenic peptides in the endoplasmic reticulum.<sup>77</sup>

Thus, deletion of *US12* not only enhances viral replication, but also potentially allows a greater immune response.<sup>42</sup> oHSV has also been used for expression of antitumor antigens. Following the success of dendritic cells loaded with mouse prostatic acid phosphatase (PAP) in eliciting an immune response to prostate cancer in a human clinical trial,<sup>78</sup> the oHSV bPΔ6-hPAP was utilized to induce an antitumor immune response in mice bearing mouse prostate cancer via expression of human PAP.<sup>79</sup> The combination of these two approaches remains an enticing prospect.

Numerous immune stimulating genes have been inserted into various oHSVs including IL-12,<sup>80–83</sup> IL-15,<sup>84</sup> IL-18,<sup>81</sup> tumor necrosis factor alpha,<sup>85</sup> granulocyte macrophage-colony stimulating factor,<sup>80,86</sup> CD80 (B7.1),<sup>81</sup> and fms-like tyrosine kinase 3 ligand.<sup>87</sup> These genes function to attract, activate, proliferate, differentiate, and mature immune cells such as natural killer, cytotoxic CD8+ T, helper CD4+ T, dendritic, macrophage and B cells, as well as their progenitors. The local production of these proteins also proves an advantage where systemic injection proves to be quite toxic.<sup>85</sup>

These cytokine producing oHSVs have proven to be more efficacious and just as safe as their non-cytokine producing parents in mice models through various measures including overall survival rate,<sup>80,82,83,87</sup> tumor reduction (especially in contralateral non-treated tumors),<sup>80,81,83,86</sup> immune cell infiltration,<sup>80,82</sup> and successful rejection of tumors when rechallenged.<sup>80,86</sup> Hopefully, the success shown by T-VEC, a granulocyte macrophage-colony stimulating factor-producing oHSV with a deletion of *RL1* and *US12*,<sup>86</sup> in clinical trials will lead to more trials with other immune stimulating oHSVs. In Phase III clinical trials in late stage melanoma, T-VEC produced an objective response rate of 26%, a durable response rate of 16%, and a complete response rate of 11%.<sup>88</sup>

## Tumor microenvironment

The composition of the microenvironment in tumors remains a barrier to effective treatment of cancer. Most tumors are characterized by a large interstitial area, high levels of collagen, and lack of or a poor lymphatic system, leading to high interstitial pressure.<sup>89</sup> Despite the overexpression of angiogenic factors, the vascular system of these tumors is leaky, dilated, and haphazardly connected with highly varied and abnormal structures. The poor blood flow through these tumors minimizes the amount of drug which reaches the cancer cells, while the higher content of extracellular matrix inhibits the passage of larger molecules.<sup>90</sup>

A natural side effect of HSV infection is the induction of angiogenesis and hyperpermeability in infected cells. It

has been shown that HSV infected cells suppress synthesis of extracellular matrix proteins fibronectin and collagen, as well as the antiangiogenesis factor thrombospondin.<sup>91</sup> HSV infection also leads to increased expression of angiogenic vascular endothelial growth factor and extracellular matrix degrading matrix metalloproteinase-9.<sup>92,93</sup> Combined with the general leakiness of tumor vessels, this increase in angiogenesis leads to rapid infiltration of antiviral immune cells that clear the virus. Since angiogenesis is a requirement for tumor growth, an argument can be made that suppressing angiogenesis benefits oHSVs directly.

A few of the oHSVs generated have shown antiangiogenic effects on their own. The 1716 and hrR3 mutants have demonstrated reduction in microvessel density by infecting and lysing proliferating endothelial cells lining the blood vessels.<sup>94,95</sup> Unlike wild type or the parental G207 mutant, G47Δ does not exhibit downregulation of thrombospondin.<sup>96</sup>

Several oHSVs that express antiangiogenic factors such as thrombospondin-1,<sup>97</sup> endostatin,<sup>95,98</sup> angiostatin,<sup>98</sup> and vaculostatin<sup>99</sup> have also been constructed. These genes have a diverse role in inhibiting angiogenesis including regulating proangiogenesis elements, inhibiting endothelial cell migration, attachment and proliferation as well as inducing apoptosis. These antiangiogenic viruses generated mixed results, with significant antiangiogenesis and delay in disease progression predominantly seen in central nervous system tumors.<sup>98</sup>

Disruption of the extracellular matrix has been shown to be a great benefit to the spread of oHSV. Tumors overexpressing metalloproteinase-1, -8, or -9 proteins, which vary in the extracellular matrix fibers they degrade, demonstrate wider oHSV infection of the tumor.<sup>100,101</sup> Furthermore, coinjection of oHSV with bacterial collagenase enables the virus to diffuse further throughout the tumor.<sup>102</sup>

## Cancer cell replication and function

Chemotherapeutic agents are cytotoxic chemicals used to treat cancer. These agents act by enhancing stress conditions (eg. double stranded breaks in DNA, high levels of unfolded proteins) and promote cell apoptosis. Chemotherapeutic agents are nonspecific however, and rapidly proliferating, normal cells in the bone marrow, digestive tract, and hair follicles can undergo cell death in response to these cytotoxic agents. Another limitation of chemotherapy is that it often requires a functional apoptotic pathway, and cancer cells with mutations in this pathway are often resistant to chemotherapy-induced cell death.

oHSVs can reduce the limitations of other drug treatments by acting synergistically when combined. Such synergy is generally due to two possible factors, enhanced replication of the oHSV and/or enhanced induction of apoptosis. The order and timing of each treatment, the type of oHSV mutant utilized, and the cancer type being treated are all important considerations. Discussed below are some examples of combination treatments with others of note also in the literature.<sup>47,103</sup>

Etoposide, temozolomide, and doxorubicin are DNA damaging agents that lead to double stranded breaks and apoptosis if the DNA damage is not repaired. Combining these drugs with oHSVs leads to greater levels of apoptosis due to oHSV's natural ability to downregulate the DNA damage repair pathway. Furthermore, cell lines that showed resistance to these drugs were still sensitive to oHSV treatment.<sup>104-106</sup>

The taxanes, docetaxel and paclitaxel, are microtubule stabilizing agents, preventing microtubule breakdown during mitosis, a necessary process for cell cycle progression. HSV-1 inhibits cell cycle progression at the G<sub>1</sub> phase. Concomitant treatment of oHSV with taxanes induces greater levels of apoptosis through increased cell cycle arrest.<sup>107</sup>

Certain mutant oHSVs combine better with some treatments. Dipyridamole and dilazep are used for inhibiting clot formation and vasodilation. One of their mechanisms of action is to inhibit equilibrative nucleoside transporter 1, reducing the intracellular concentration of adenosine. Such an action increases the activity of cellular RR, which boosts replication of oHSV mutants lacking *UL39* (encodes viral RR).<sup>108</sup>

Radiation therapy induces DNA damage and apoptosis. Its combinational effect with oHSV also varies with the mutant utilized and the cell line treated. Radiation treatment increases RR activity and upregulates growth arrest and DNA damage-inducible protein (GADD34), a cellular protein with homology to ICP34.5, improving replication of *UL39* and/or *RL1* mutants.<sup>109-111</sup> However, in the absence of increased viral production, enhanced induction of apoptosis is the cause for synergy.<sup>112</sup> oHSVs that produce IL-12 or tumor necrosis factor have an even greater effect when combined with radiation therapy.<sup>85,113</sup>

Bortezomib, a proteasome inhibitor, leads to a buildup of unfolded proteins, an increase in endoplasmic reticulum stress, and apoptosis. One of the cellular responses to proteasome inhibition is upregulation of molecular chaperones, including Hsp90, which is used by HSV-1 DNA polymerase to translocate to the nucleus. Cells pretreated with bortezomib lead to an increase in oHSV replication.<sup>114</sup>

The discovery that cancers often have aberrant histone acetylation patterns has led to a significant interest in the use of histone deacetylase inhibitors for treatment. Pretreatment with histone deacetylase inhibitors increases replication of oHSV<sup>115</sup> with inhibition of interferon stimulated genes of the innate antiviral immunity and activation of NF- $\kappa$ B shown to be involved.<sup>116,117</sup> However, the order of treatment (and thus increased oHSV replication) appears to have no effect on synergistic cell killing, with an increase in cell cycle arrest and decrease in angiogenesis implicated in this synergism.<sup>118</sup>

Alternatively, the specificity of effective replication in cancer cells of oHSVs means they can be used for activating or enhancing the local effect of a combination treatment. Examples of such a mechanism include production of norepinephrine transporter<sup>119</sup> or sodium iodide symporter<sup>120</sup> to enhance uptake of radioactive Iodine-131 labeled molecules for radiation therapy and expression of nitroreductase,<sup>121</sup> rat CYP2B1,<sup>122</sup> and yeast cytosine deaminase<sup>123</sup> for bioactivation of CB1954, cyclophosphamide, and 5-fluorocytosine, respectively.

## oHSV delivery

The route of delivery of oHSV to tumors is another area that is being addressed. Currently, oHSVs are either directly injected into the tumor or given intravenously. While direct tumoral injection ensures all the virus is delivered to the tumor, in the majority of cases, it is limited to the needle track into the tumor and the virus rarely spreads beyond the injected tumor. Intravenous injection gives the virus an opportunity to infect all tumors, which is especially important in the case of metastasis. However, considering that most oHSVs are nonspecific for the cell they infect, less virus generally infects cancer cells compared to normal cells.<sup>124</sup> Intravenous virus must also face the host innate immune system, which can neutralize virions before they reach the target cells.<sup>125</sup>

A few approaches to improve delivery have already been discussed. Retargeting of the receptor oHSVs use for binding to and entering cells ensures the virus has a greater predisposition for entering cancer cells. Coinjection of oHSV with collagenase degrades part of the extracellular matrix in the tumor, allowing the virion to spread further away from the site of injection.

The method for delivery of the oHSV can be enhanced or reduced by pretreatment with antiangiogenesis molecules. When oHSV was administered by direct injection, prior injection of cyclic RGD peptide, an antiangiogenic agent, reduced tumor vascular permeability and infiltration of leukocytes.<sup>126</sup> Alternatively, systemic administration of oHSV after pre-

treatment with vascular endothelial growth factor antibody bevacuzimab reduced the amount of virus taken up by the tumor. However, the opposite treatment regime (oHSV prior to bevacuzimab) led to increased survival of mice with Ewing's sarcoma.<sup>127</sup>

Targeting of tumors in the brain when oHSV is administered intravenously carries the added difficulty of having to cross the blood–brain barrier. In order to overcome this, it has been shown that blood–brain barrier disruption through a hypertonic solution of mannitol enhances the amount of virus that reaches the tumor.<sup>124</sup> Alternatively, mesenchymal stem cells loaded with oHSV have been used to target glioblastoma. These cells are easily isolated from patients, routinely propagated in culture, and have been shown to home to tumors.<sup>128</sup> However, these cells are also known to be immunosuppressive.<sup>129</sup> Whether these cells would help (by enhancing the virus effect) or hinder (by inhibiting the antitumor immune response) treatment of the tumor was not determined as immunocompromised mice were used.

Another cell population that has been tested for delivery of oHSV is tumor antigen-specific lymphocytes. These cells were generated in a mouse host from exposure to inactivated cancer cells. After being harvested, they were infected with the oHSV before being reinjected into new mice bearing the same cancer. Cancer bearing mice injected with oHSV loaded lymphocytes survived longer than those treated with either single agent.<sup>130</sup>

## Conclusion

Significant progress has been made in adapting HSV for the treatment of cancer. From the creation of numerous mutants to enhancing the efficacy of these mutants and the ability to deliver them to tumors, we have come a long way from early oncolytic virotherapy attempts utilizing wild type viruses. This progress is highlighted by several oHSVs that are or have been in Phase I and II clinical trials. These oHSVs have demonstrated excellent safety, with no deaths attributed to oHSVs to date, no encephalitis reported in clinical trials for brain tumors,<sup>131–134</sup> and side effects predominantly limited to flu-like symptoms in nonbrain cancers.<sup>135,136</sup> However, the majority of clinical trials have also shown limited long-term efficacy, as evident by lack of progression to Phase III trials, indicating there is still work to be done. The exception to this is T-VEC, which recently completed Phase III clinical trials in melanoma and just gained FDA approval.<sup>9,10,88</sup>

Given the safety of oHSVs observed in clinical trials to date, a detailed discussion of the testing of oHSVs in animal models for toxicity has not been included in



this review. Though, it should be acknowledged that for oHSVs two predominantly used animal models for toxicity studies include owl monkeys and immunocompromised mice (mainly BALB/c) as both are extra sensitive to HSV encephalitis.<sup>22,29,35,37,42,86</sup>

As with much cancer research, the greatest limitation has been the models available. The immune response to both the virus and tumor seems to be a critical determinant to the effectiveness of oncolytic virotherapy, and hence models must incorporate this interplay. Immunocompromised mice are a poor model while syngeneic mice-cancer systems, with their intact immune system, offer a better view of how these factors interact and can be manipulated to enhance the antitumor effect. However, with such vast differences between preclinical and clinical results being evident, even these mice models still appear inadequate.

Zebrafish are a potential alternative model that is yet to be tested in oHSV virotherapy. Zebrafish have the ability to generate spontaneous tumors that are histopathologically and genetically similar to human tumors, or can be implanted with transgenic cells or xenotransplants.<sup>137</sup> Additionally, due to a delay in developing an adaptive immune system, zebrafish can be used for metastatic and angiogenesis models of human cancers.<sup>138</sup> Studies have revealed that zebrafish can be used as a model for HSV-1 infection, with similar infection and recognition mechanisms as in mice and humans.<sup>139,140</sup>

Better patient outcomes appear to be characterized by breaking the immune tolerance that is preventing clearance of the tumor by the host immune system itself. The virus could potentially achieve this through destruction of the immunosuppressive cells within the tumor and/or by inducing a more effective immune response.<sup>11</sup> Stimulation of an antitumor immune response is epitomized by the aforementioned immune stimulating T-VEC, which demonstrated in melanoma patients not only an increase in melanoma-specific T cells, but also a decrease in regulatory and suppressor T cells, an effect which resulted in 15% of measurable visceral (non-injected tumors) shrinking by more than 50% in the Phase III trials.<sup>88,141</sup>

However, it is perhaps pertinent to also focus on patients in whom oncolytic virotherapy fails. What factors are limiting the response? Rapid clearance of the oHSV by the innate immune system may not allow adequate time for the virus to illicit an effective antitumor immune response, or patients may be incapable of mounting an effective antitumor response. In these cases, suppression of the innate immune system, even temporarily, may allow a more potent adaptive immune response to be mounted, or possibly allow the virus to eradicate the tumor itself.

The enhanced replication and spread of oHSV when combined with immunosuppressive cyclophosphamide or rapamycin would indicate the potential feasibility for such a strategy.<sup>49,142</sup>

Oncolytic virotherapy is a complex treatment, whose success depends on the intricate interactions between the tumor and its microenvironment, as well as the virus and host antiviral and antitumor immune responses. HSV has proven to be a worthy virus for oncolytic virotherapy as it is amenable to genetic alterations, synergizes well with many current cancer treatments, is reasonably safe for patients, and has demonstrated some effectiveness. Future studies need to concentrate on improving outcomes through a combination of oHSV with targeted chemotherapies.<sup>143</sup>

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## Disclosure

The authors report no conflicts of interest in this work.

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