Herpes Simplex Virus Oncolytic Therapy for Pediatric Malignancies

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Despite improving survival rates for children with cancer, a subset of patients exist with disease resistant to traditional therapies such as surgery, chemotherapy, and radiation. These patients require newer, targeted treatments used alone or in combination with more traditional approaches. Oncolytic herpes simplex virus (HSV) is one of these newer therapies that offer promise for several difficult to treat pediatric malignancies. The potential benefit of HSV therapy in pediatric solid tumors including brain tumors, neuroblastomas, and sarcomas is reviewed along with the many challenges that need to be addressed prior to moving oncolytic HSV therapy from the laboratory to the beside in the pediatric population.

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INTRODUCTION

Although pediatric cancer survival rates have improved greatly over the past 30 years, there remains a significant subset of children, ~25%, who succumb to their disease.[1,2] Deaths occur when tumors progress in the face of optimal therapies and acquire resistance to current treatment modalities like chemotherapy or radiotherapy, or the toxicities from such therapies become too great. Augmenting the dose of current therapies is likely to increase toxicity without improving survival significantly. Therefore, novel, targeted treatment strategies that evince effective oncolysis are desperately needed to decrease toxicity and, thereby, improve quality of life and survival rates for children with malignancies. This review will focus on the potential benefit of adjunctive oncolytic virotherapy that utilizes genetically engineered herpes simplex viruses, type-1 (HSVs-1).

HSV-1 is a unique virus that offers promise in treating several pediatric cancers including brain tumors, neuroblastomas, and sarcomas. The virus can be employed as oncolytic viral therapy, a direct, targeted attack or via gene therapy in which foreign genes are expressed therapeutically in cancer cells.[3] Oncolytic viral therapy relies on virus replication in infected tumor cells that die and release infectious virus. In gene therapy, expression of therapeutic foreign gene products either directly or indirectly leads to cell death. Oncolytic viruses that utilize gene therapy are perceived to be more effective at killing tumors.

Genetically engineered HSV-1 has a number of advantages in the treatment of a subset of pediatric malignancies. HSV-1 is a large (152 kb, 89 genes, multiple open-reading frames), double-stranded DNA, enveloped virus that does not integrate into host DNA. HSV is a neurotropic virus thus making cancers of neural origin, including brain tumors and neuroblastomas, ideal targets.[4] However, it is equally able to infect and kill cells from a variety of cancers including sarcomas, melanomas, colon, breast, lung, prostate, and hepatic tumors.[5–10] Normal cells are spared, whereas tumor cells are targeted by deleting genes critical for viral replication in normal cells but not necessary in tumor cells such as the "neurovirulence" gene, $\gamma_1$34.5.[11,12] It has been estimated that up to 30 kb of the HSV genome is nonessential for replication in tumor cells and, therefore, can be replaced with foreign DNA for gene therapy without affecting the virus' ability to infect tumor cells and replicate. From a safety standpoint, antiviral agents in clinical use are readily available in the unlikely event that the mutant HSV produces toxicity to normal tissues.

Currently, four different $\gamma_1$34.5-deleted viruses have been used in adult phase I trials (G207, 1716, NV1020, OncoVex$^{GM\text{-}CSF}$). G207 and HSV1716 have been used to treat patients with recurrent glioblastoma multiforme (GBM) (**Table 1**).[12,13] G207 was derived from R3616 that had been created by deleting both copies of $\gamma_1$34.5 gene from the wild-type isolate, HSV-1(F) strain. G207 was created by inserting the *lacZ* gene encoding β-galactosidase into the U_L39 locus, thus disabling the expression of ICP6, the heavy chain for ribonucleotide reductase (**Figure 1**). HSV1716 was created by deleting both copies of the $\gamma_1$34.5 gene (also known as R_L) from the wild-type isolate, strain 17 (**Figure 2**). HSV-1(F) is a temperature-sensitive isolate, whereas strain 17 is not. These two HSV-1 parents likely have different degrees of virulence, as these $\gamma_1$34.5-deleted variants have different LD_{50} values for mice.[14,15]

In the first trial of G207, a maximum tolerated dose was not reached and no definitive dose-limiting toxicities occurred with stereotactic injection of up to 3×10^9 plaque-forming units (PFUs) of virus directly in up to 5 loci within the enhancing portions of recurrent tumors.[16] A phase Ib trial of stereotactic

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Table 1 Summary of engineered HSV discussed in the text

Virus	Parent virus	Deletions/mutations	Foreign gene insertions/ promoter	Citation number
R3616	HSV-1(F)	1 kb deletion in $\gamma_1$34.5 gene (both copies)	None	11
3616UB	R3616	1 kb deletion in $\gamma_1$34.5 gene (both copies) and an interruption of the uracil DNA glycosylase gene	None	73
*d*12.CALP	D120	ICP4 deletion	Calponin promoter	130
G207	R3616	1 kb deletion in $\gamma_1$34.5 gene (both copies) and disabling *lacZ* insertion in U_L39	None	12
G47Δ	G207	Same as G207 with 312 bp deletion in ICP47 locus and promoter region of U_S11	None	7
HF10	F	3.9 kb deletion in the right end of the U_L and U_L/IR_L junction, and it expression of U_L56 and latency-associated transcripts	None	10
HL	HF10 and L1BR1	Lacks both U_L56 and U_S3	None	129
HSV1716	17	759 bp deletion in $\gamma_1$34.5 gene (both copies)	None	13
MtHSV	F	Disabling *lacZ* insertion into both copies of $\gamma_1$34.5	None	128
NV1020	R7020	700 bp deletion in thymidine kinase (tk) locus and a 15 kb deletion across the joining region of the long and short components of the HSV-1 genome	HSV-1 DNA fragment encoding the tk gene fused to the α gene promoter	6
NV1042	R7020	Same as NV1020	Murine IL-12, hybrid α4-tk promoter	81
NV1066	F	Same at NV1020	Enhanced green fluorescent protein, CMV promoter	100
OncoVex^{GM-CSF}	JS-1	Complete deletions of the genes encoding ICP34.5 and ICP47	GM-CSF, CMV promoter	21
rRp450	hrR3	U_L39 deletion	Rat CYP2B1 gene encodes enzyme to activate oxazophosphorines	30
TIMP3	rHSVQ1	Deletions in ICP6 and $\gamma_1$34.5	Tissue inhibitor of metalloproteinases-3, HSV-1 immediate early 4/5 promoter	103

catheter inoculation of G207 followed by resection and reinoculation into the tumor bed of recurrent GBMs likewise confirmed safety.[17] Although the trials were only designed to determine safety, response was seen in some patients, and two patients were long-term survivors (>5 years). A third trial utilizing stereotactic injection of G207 into recurrent tumors in five loci followed within 24 hours by a single fraction of irradiation (5 Gy) has finished accrual and will be reported shortly.

Similar results were seen in trials using HSV1716, although the highest dose achieved was 1×10^5 PFU, due to its more aggressive behavior in mice. At this dose, the virus was safe with direct injection into tumors or tumor beds after tumor resection, and resulted in several long-term survivors.[18–20]

NV1020 is a rederived virus based on the construction of R7020 that was developed for HSV immunization. NV1020 has only one of the $\gamma_1$34.5 loci deleted and contains an insertion of HSV-2 sequences (**Figure 3**). It is a replication-competent, attenuated-engineered virus, but due to its remaining $\gamma_1$34.5 sequence is believed to have greater potential for neurotoxicity. Nevertheless, NV1020 was infused into the hepatic artery of patients with metastatic colorectal carcinoma to the liver, safely with minimal and self-limited serious adverse events.[6] A multi-institutional phase II trial is ongoing. Similarly, a second generation oncolytic HSV expressing granulocyte macrophage colony–stimulation factor (GM-CSF), OncoVex^{GM-CSF}, was safe and well tolerated when injected intratumorally in patients with recurrent cutaneous or subcutaneous deposits of breast, head and neck, gastrointestinal cancers or malignant melanoma.[21] Several patients had stable disease without progression.

Potential causes for the failure of genetically engineered HSVs to be uniformly curative are legion, but can be broadly grouped into general categories that include, but are not limited to: (i) host antiviral immune responses, (ii) tumor genotype heterogeneity, and (iii) extracellular matrix/tumor microenvironment. Seropositivity to HSV may pose a barrier to repeated administration, however, preclinical and clinical studies suggest that it does not have a deleterious impact on the initial intratumoral administration of HSVs.[16,22–26] More concerning is the observation that infiltrating mononuclear inflammatory cells may destroy virally infected cells prematurely, limiting virus production and eventually the scope of the anti-tumor effect.[27,28] Strategies that utilize agents (*e.g.*, cyclophosphamide) to block bone marrow–derived generation of inflammatory cells seem to benefit the oncolytic effect.[29,30] Likewise, use of agents either exogenously administered (cilengitide, bevacizumab) or re-engineered into the virus (vasculostatin) to inhibit vascular permeability or formation of neovasculature in the tumor could significantly limit the extravasation of inflammatory cells

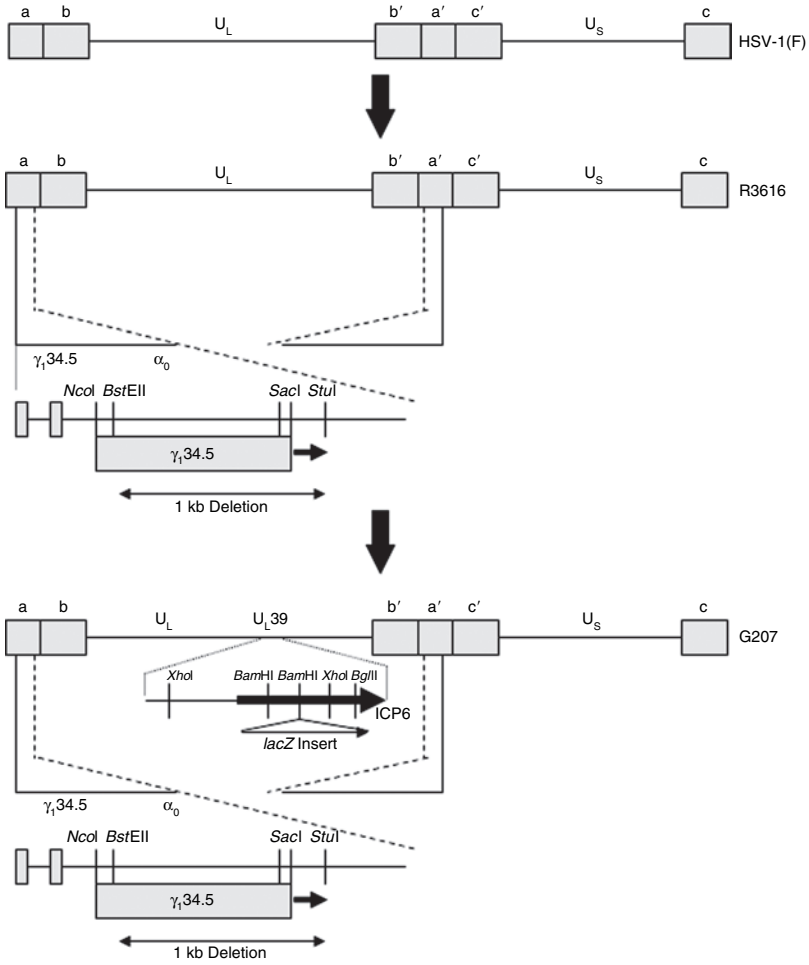

Figure 1 Schematic representation of the derivation of G207 HSV. Chou *et al.* deleted both copies of the $\gamma_1$34.5 gene from HSV strain F, a temperature-sensitive clinical isolate (HSV-1(F)) to create recombinant virus R3616.[11] Only one of the two deletions is shown for simplicity. This deletion ablated the ability of the virus to overcome interferon resistance in normal cells and made the virus aneurovirulent. Mineta *et al.*[12] modified R3616 by inserting the *Escherichia coli* β-galactosidase gene (*lacZ*) to produce a functional deletion of the HSV U_L39 gene, which encodes the heavy chain or ribonucleotide reductase (infected cell protein 6—ICP6), as described by Goldstein and Weller.[137] This second disabling deletion was performed to ensure added safety for intracerebral human trials.[7]

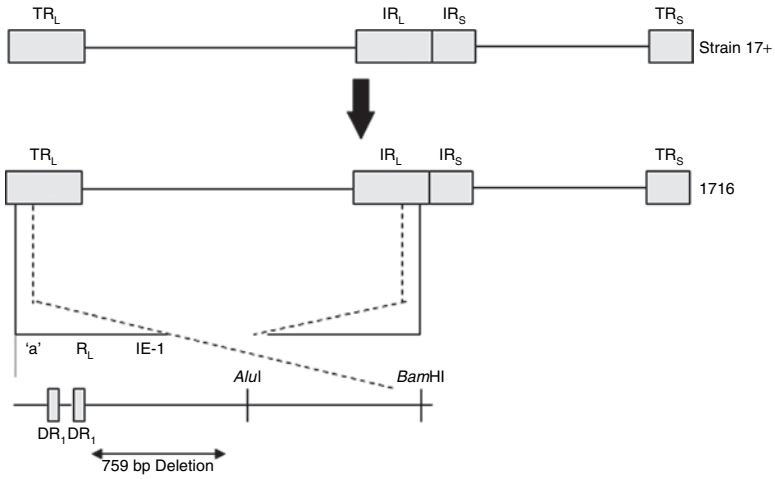

Figure 2 Schematic representation of the derivation of 1716 HSV. In clinical isolate strain 17+ HSV, a 759 bp deletion was produced that extended from the DR_1/Ub boundary in the "a" sequence to remove 105 bp on the 5′ end of the R_L open reading frame.[13] This deletion was produced in both long-repeat regions (terminal repeat-long, inverted repeat-long) of the HSV genome, but only the deletion for the TR_L is shown for simplicity. The strain 17+ HSV had a LD_{50} of <10 PFU, whereas the 1716 mutant was significantly neuroattenuated with a LD_{50} of 7×10^6 PFU.

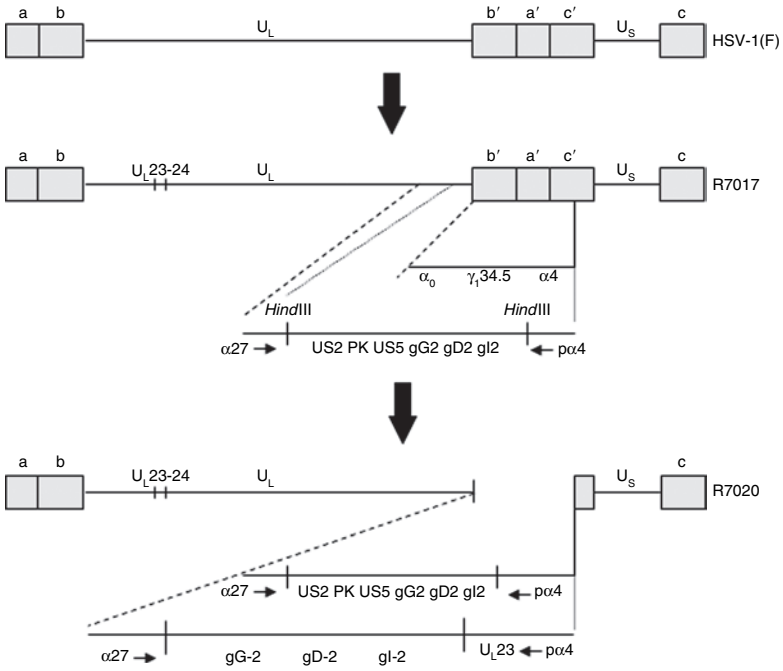

Figure 3 Schematic representation of the derivation of R7020 (aka NV1020). This virus was constructed for the purpose of creating a vaccine for HSV-1 and HSV-2 that would be administered by intramuscular injection of living virus.[138] HSV-1(F) was initially deleted for thymidine kinase (U_L23) and U_L24. The internal set of the inverted repeats, containing α0, α4, $\gamma_1$34.5, ORF O, and ORF P, was replaced with a novel construct that contained U_L23 fused to the α4 promoter-regulatory region and 3′ to a series of HSV-2 genes (U_S2, HSV protein kinase, U_S5, glycoproteins G, D, and I, as well as a portion of E). The terminal inverted repeat remained intact. Thus, this chimeric virus, containing both HSV-1 and HSV-2 sequences, has been extensively tested in both rodent and primate species for safety (LD_{50} = 2.7 × 10^6 PFU versus 3.8 × 10^2 PFU for HSV-1(F)) and genetic stability.[138,139]

into the tumor bed and permit greater virus production in the tumor cells.[31,32]

Tumor genotype heterogeneity poses an inherent problem for an oncolytic virus that has been seriously attenuated to make it safe and aneurovirulent. These differences include (i) reduced or complete lack of expression of the key HSV entry molecule, nectin-1 (CD111), or its family members and (ii) a nonpermissive environment for late gene expression and virus replication events. Solutions include (i) re-engineering the virus to express ligands that bind receptors expressed exclusively on tumor cells or (ii) modulating the cellular replication machinery with irradiation or DNA-damaging agents to engage the DNA repair response, which supports virus replication.[33–38] In some instances, it may be possible to re-engineer the virus to express mutant proteins that activate signaling pathways that would provide a more hospitable virus replication environment.[39] Production of a chimeric HSV that contains a gene from human cytomegalovirus, a related β-herpes virus also enhances late gene expression without compromising safety of the virus.[40]

Finally, to be effective, infectious HSV must disseminate widely throughout the tumor. Extracellular matrix generated by the tumor provides a critical barrier. A potential solution is to re-engineer the virus to express ECM-proteolyzing enzymes to enhance percolation of infectious virus particles through the interstitial spaces of the tumor.[41] Many of these immune, genetic and physical barriers to effective virus oncolysis are being resolved almost as rapidly as they are identified. However, it is the lag between developing the next-generation virus and its application in a phase I clinical trial that remains the most serious impediment in the quest for more effective therapies for children with cancer. To date, no pediatric trials employing engineered HSV have been conducted. However, several preclinical studies have shown promise. The potential benefit of HSV therapy in pediatric solid tumors including brain tumors, neuroblastomas, and sarcomas is reviewed in the following section.

BRAIN TUMORS

Central nervous system tumors account for ~25% of all childhood malignancies, and brain tumors are the leading cause of cancer-related morbidity and mortality in children (**Table 2**). Although survival rates for patients with low-grade, localized tumors have improved to >80%, there is a poor outcome subset of children with disseminated medulloblastoma, high-grade gliomas, intrinsic pontine gliomas, atypical teratoid-rhabdoid tumors (AT/RT), and incompletely resected or metastatic ependymomas. Medulloblastomas are the most common malignant pediatric brain tumor (~20% of cases) and an estimated one-third of these are disseminated at diagnosis.[42] Progression-free survival in patients with high risk, metastatic medulloblastomas only approaches 50%, despite surgery, radiation, and chemotherapy.[43] Similarly, outcomes for children with high-grade gliomas are poor with 5-year event-free survival rates ~20% despite multimodality therapy.[44,45] With current chemotherapy and radiotherapy, intrinsic pontine gliomas are still uniformly fatal with a median survival rate of ~9 months.[46–48] Patients with AT/RT, a recently recognized distinct tumor that tends to occur in children <3 years, have dismal median survival rates of <1 year.[49] Ependymomas most often arise in the posterior fossa from the fourth ventricle with

Table 2 Incidence of histologically defined intrinsic CNS tumors of childhood

Histologic diagnosis	New cases/year[a]	Cell type of origin	Most common site	Median survival[b]	References
Juvenile pilocytic astrocytoma	566	Glial	Cerebellar hemispheres	90–95% survival at 10 years	140
Anaplastic astrocytomas	48	Glial	Cerebral hemispheres	5 years	44,45
Astrocytoma, NOS	148	Glial	Cerebral hemispheres	70–75% survival at 10 years	44,45,140
Pontine gliomas	≈125	Glial	Brainstem	9 months	46–48
Glioblastoma	103	Glial	Cerebral hemispheres	1 year	44,45
Ependymoma	171	Ependymal	Fourth ventricle	10 years	50–53
Medulloblastoma	447	Unknown	Cerebellar vermis	10 years	42,43
Supratentorial PNET	≈100	Unknown	Cerebral hemispheres	2.4 years	141
Atypical teratoid-rhabdoid tumors	≈30	Unknown	Cerebellar hemispheres	<1 year	49
Germ cell tumors	143	Germ	Pineal (males) suprasellar (females)	>90% at 10 years	142

[a]CBTRUS (2008). Supplement Report: Primary Brain Tumors in the United States, 2004. Published by the Central Brain Tumor Registry of the United States, Hinsdale, IL.
[b]CBTRUS (2008). Supplement Report: Primary Brain Tumors in the United States, 2000–2004. Published by the Central Brain Tumor Registry of the United States, Hinsdale, IL.

brainstem involvement occurring in up to 50% of cases making gross total resection difficult.[50,51] Over a quarter of patients are <2 years of age and experience significant morbidity if potentially curative radiation doses are used.[52] Outcomes for children with ependymomas, who have a subtotal resection, who are <3 years of age, or who have disseminated disease to the cord are grim with survival rates of <20%.[53] Because of poor outcomes, children with disseminated medulloblastomas, high-grade gliomas, intrinsic pontine gliomas, AT/RT, and ependymomas may benefit from a novel, targeted therapy such as mutant oncolytic HSV. Although phase I adult trials have confirmed safety of mutant HSV stereotactic injections in the brain, safety in the developing human brain is of paramount concern.

The safety of G207 was confirmed first in CBA/J, BALB/c, and A/J strain mice and then in nonhuman primate studies in New World owl monkeys (*Aotus nancymae*), as young as 1 year old.[54,55] Different strains of mice have widely differing levels of sensitivity to genetically engineered HSV injected into the brain, and this largely follows the patterns of sensitivity defined years ago by injecting wild-type HSVs intraperitoneally.[56,57] Owl monkeys were chosen because of their extreme sensitivity to HSV, similar to that of neonatal children. Magnetic resonance imagings obtained between 10 days and 12 months, after initial injection of HSV into the brain, showed no abnormalities that could be attributed to HSV toxicity, which suggested that G207 injection did not result in short- or long-term changes in the brain, other than those induced by the mechanical trauma of injection. Importantly, Radbill *et al.* found no significant difference in long-term physical development (body weight, brain weight, limb strength), cognitive performance (eight arm radial maze), or exploratory behaviors (open-field maze) between groups of mice that had received a 2 µl intracerebral inoculation of 10^5 PFU of G207 versus control saline at 4 days of life.[58] However, five of seven mice in the G207 group had histological and magnetic resonance imaging evidence of unilateral ventriculomegaly ipsilateral to the site of injection as compared to only one of eight control mice. Free-hand injection was used, which likely resulted in intraventricular delivery of the virus and led the authors to conclude that G207 is unlikely to cause significant neurodevelopmental changes. However, an initial study in children should exclude patients with tumors in or near the ventricles and children under two. Currently, there have been no animal studies examining the safety of engineered HSV on the developing cerebellum or brain stem.

The initial focus in using mutant HSV has been on cerebral tumors because brain stem tumors pose additional patient safety challenges. Damage to the brain stem can affect the body's integrative functions resulting in severe brain injury and death. Additionally, cranial nerves III through XII emerge from the brain stem and are in danger of being injured by local injection trauma, virus infection, or a localized immune response. Gene therapy oncolytic viruses that utilize a local immune response such as those expressing interleukins may not be the best choice on the sensitive brain stem. Studies need to be performed to elucidate whether or not engineered HSV can be safely delivered locally to brain stem tumors.

One way to circumvent potential injury to patients via localized injection is to inject the virus systemically. However, systemic delivery of virus for treatment of brain tumors or metastases to the brain is complicated by the blood-brain barrier (BBB). The BBB consists of brain endothelial cells, which are strongly juxtaposed via high-resistance tight junctions and have minimal pinocytosis, which together results in a physical barrier to drugs and other blood-borne molecules like viruses.[59,60] This barrier can be disrupted by osmotic solutions like mannitol or inflammatory mediators such as bradykinin to allow delivery of intracarotid or intra-arterial injected herpes virus to the brain.[61–64] Using intracarotid delivery of an engineered oncolytic herpes vector, G47Δ, after 25% mannitol, Rabkin *et al.* significantly increased survival of nude mice with metastatic breast cancer in the brain.[7] G47Δ is a variant of G207 with the additional deletion of both the gene that blocks TAP protein presentation of antigens on the cell surface and the promoter region of U_S11. Deletion of that promoter region places the late U_S11 gene under control of the immediate-early ICP47 promoter; thereby, blocking shutoff of protein synthesis and creating a more oncolytic

virus than G207.[65] They found extensive virus replication in intracerebral tumors after BBB disruption, and importantly, staining of peripheral organs was minimal. Disrupting the BBB followed by systemic injection of HSV may prove to be a novel approach to treating tumors difficult to completely resect, such as intrinsic pontine gliomas.

As virus safety and route of delivery in pediatric patients continue to be refined, the efficacy of HSV oncolytic therapy in pediatric brain tumors needs further study. We have shown that a pediatric frontal lobe GBM, D456MG, was more sensitive to infection and killing by engineered HSV than six adult glioblastomas in tissue culture.[66] Additionally, we found that glioma stem cells, cells believed to be responsible for the inherent resistance of GBMs to traditional therapies, were equally sensitive to killing by HSV as nonstem cell tumor cells. Further studies on pediatric high-grade gliomas are planned.[67] Otsuki *et al*. have suggested that glioma stem cells grown in tumorspheres are more resistant due to intact interferon pathways, and thus have devised a virus to target these cells by placing expression of ICP34.5 under control of the nestin enhancer regulatory element.[68] Nestin is a neural stem cell marker and its expression level along with CD133 has recently been shown to correlate with worse clinical outcomes in glioma patients.[69] A potential concern is that any virus that targets CD133 or nestin-expressing tumor cells may also be effective in killing normal neural stem cells. Whether this would be detrimental in the (developing) pediatric brain remains to be determined. A potential benefit is suggested by mouse brain tumor model studies by the Holland and Canoll laboratories in which they found that spontaneously occurring gliomas attract normal neural stem/glial progenitor cells that may eventually comprise as much as half of the tumor mass; however it is unknown if this occurs in human brain tumors.[70,71]

Several human medulloblastoma cell lines have been tested for sensitivity to engineered HSV. Lasner *et al*. found that D283 cells were sensitive to HSV1716 infection and D283 tumor-bearing mice had a statistically significant increased survival advantage compared to mock-treated tumor-bearing mice.[72] DAOY cells were efficiently killed *in vitro* by a double-mutant engineered HSV, 3616UB, and intratumoral injection of DAOY cells in C.b-17 SCID mice resulted in significant growth arrest and tumor regression suggesting that human medulloblastomas may be sensitive to mutant HSV.[73]

To date, no studies examining the sensitivity of intrinsic pontine gliomas, AT/RT, or ependymomas have been published. On a related note, Guzman *et al.* found weak focal expression of nectin-1, an important entry receptor for HSV, in ependymomas, suggesting that ependymomas may be sensitive to infection by HSV.[74] Nectin-1 is a cell surface adhesion molecule that is widely expressed in cell lines of different lineages including neuronal cells, and recently has been shown to predict sensitivity to herpes oncolytic therapy in several different types of tumors including thyroid cancer and invasive squamous cell carcinoma.[8,75,76] Future studies are needed to further define which brain tumor types are sensitive to engineered HSV, however based on current *in vitro* and *in vivo* data, a phase I trial utilizing engineered HSV in children with recurrent supratentorial tumors should begin without delay.

NEUROBLASTOMA

Neuroblastoma is a neural crest-derived neuroendocrine tumor, making it an ideal target for a neurotropic virus like HSV. It is the most common malignancy in infancy and the most common extracranial solid tumor in children accounting for an estimated 8% of all childhood malignancies.[77] Approximately 60% of patients older than 1 year and 45% of all patients with neuroblastoma have disseminated disease at diagnosis.[78] Patients with stage 4, metastatic disease have 3-year event-free survival rates of 55% despite autologous transplantation and 13-*cis*-retinonic acid therapy.[79] However, longer follow-up confirms that many high-risk patients relapse after transplantation suggesting that further dose augmentation of current therapies is unlikely to be beneficial.[80] Therefore, novel therapeutics are needed for high-risk patients, and engineered HSV has shown promise in treating neuroblastoma.

Although local tumor injection may be beneficial for solitary tumors, high-risk neuroblastoma tends to be widely disseminated and would require systemic treatment with virus. Systemic administration of oncolytic HSV has proven safe and efficacious in several mouse models. A murine IL-12-expressing virus, NV1042, was found safe when injected systemically into the tail vein of mice with squamous cell carcinoma pulmonary metastases.[81] No animals developed clinical signs attributed to virus administration, and importantly, histological examination of nontumor-bearing areas of lung, brain, liver, spleen, and pancreatic tissue all appeared completely normal without cytopathic effects. Significantly, improved survival was seen in NV1042-treated mice, and the long-term survivors had no evidence of weight loss, poor grooming, neurotoxicity, mucosal ulcerations, or any other visible morbidity. Using the same virus inoculated via the tail vein of transgenic C57BL/6-TRAMP mice with spontaneous primary and metastatic prostate cancer, Varghese *et al.* found that multiple injections did not seem toxic to the animals.[9,82] However, as Lopez has shown, this particular strain of mice is very resistant to HSV toxicity, presumably because of low nectin-1 expression. Infected tumor cells were detected and persisted in the prostates, periaortic lymph nodes, and lungs of the mice but not in healthy organs. Intraperitoneal injection of HF10, a spontaneously occurring, highly attenuated virus, in immunocompetent mice with peritoneal disseminated melanoma was similarly deemed safe, and all mice survived as compared to 100% fatality in control mice, who did not receive virus.[10] Successful treatment may also be a function of tumor genotype. Veerapong *et al.* have demonstrated localization and spread of systemically administered $\Delta\gamma_1$34.5 HSV in tumors with high levels of expression of activated MAP kinase (MEK).[83]

From these studies, it would appear that disseminated tumor cells can be killed via systemic injections of mutant HSVs. However, in the human population, 70–90% of adults have significant pre-existing immunity with detectable seropositivity due to subclinical infections with HSV-1. This has the potential to impact systemic therapy negatively, although preclinical studies have not demonstrated this uniformly. Although gene transfer to brain tumors using a HSV-1 vector was decreased in HSV-1 immunized rats, several groups demonstrated that prior immunization with HSV-1 and/or HSV seropositivity in mice did not significantly affect viral oncolysis, particularly, when the virus

Table 3 Incidence of histologically defined sarcomas of childhood

Histologic diagnosis	Approximate new cases/year[a]	Cellular features/ differentiation	Most common sites	5-year overall survival (%)	References
Alveolar RMS	50–100	Skeletal muscle	Extremities, head, neck	49	143
Embryonal RMS	210–275	Skeletal muscle	Head, neck, genitourinary	67	143
NRSTS	500	Various mesechymal tissues	Various sites	76	144
Osteosarcoma	400	Bone	Femur, tibia, humerus	62	145
ESFT	200	Neural crest, postganglionic	Extremities, pelvis, chest wall	60	146

[a]Approximate number of cases in persons <20 years of age derived form Surveillance and Epidemiology and End Results (SEER) data.

was given at suitable doses and in proximity to tumor targets.[22–25] Thus, systemic administration of HSV could remain a challenge for adult cancer therapy. The proportion of pediatric cancer patients with HSV premunition, however, is considerably lower and systemic therapy could be more successful.[84] The prevalence of HSV-1 seropositivity rises with age: estimated under 20% for children 1–4; 26% by age 6–7 years; 36% at age 12–13 years; and 39% for adolescents age 14–19 years in the United States.[85,86] Thus, younger children may be better candidates for systemic therapy.

A number of newly constructed viruses have been tested in a murine neuroblastoma model, Neuro-2a. Neuro-2a cells are syngeneic to A/J strain mice (an HSV-sensitive strain), are poorly immunogenic, and support effective HSV infection, providing a convenient model for testing newly constructed mutant viruses.[87–92] However, Neuro-2a only contains single-copy MYC-N, which is typical of lower-risk, favorable neuroblastoma.[93] MYC-N amplification is an important prognostic factor associated with advanced, rapidly progressive disease and a poor prognosis.[94,95] Therefore, Neuro-2a cells are likely not the best model of resistant, difficult to treat neuroblastoma. Nevertheless, several groups have shown sensitivity of Neuro-2a to infection and killing by novel viruses including chimeric viruses, which express the human cytomegalovirus PKR-evasion genes, vectors expressing interleukin 18 and B7-1 immunoglobulin ± interleukin 12, and virus expressing bacterial cytosine deaminase.[40,96–99]

Importantly, eight separate human neuroblastoma cell lines, including several MYC-N amplified tumors and tumors established after patients had received standard chemotherapy and radiation, were uniformly sensitive to infection and killing with the attenuated HSV mutant NV1066 in tissue culture.[100,101] Two human neuroblastoma cell lines were used for *in vivo* studies including one MYC-N nonamplified and one MYC-N amplified cell line.[102] A single dose of NV1066 injected into flank tumors of nude mice resulted in significant antitumor responses and prolonged survivals of mice as compared to PBS-treated controls for both cell lines tested. Recently, Mahller *et al.* showed increased cytotoxicity both *in vitro* and *in vivo* on human neuroblastoma cell lines, including a MYC-N amplified line, by an engineered HSV that expressed human tissue inhibitor of metalloproteinase-3.[103] Increased metalloproteinase expression has been correlated with worse prognosis in pediatric neuroblastomas.[104–106] Furthermore, they demonstrated that several human neuroblastoma cell lines contain tumor-initiating cells that express neurogenic stem cell markers CD133, ABCG2, and nestin, similar to gliomas.[107] When grown in tumorspheres, these tumor-initiating cells were sensitive to a nestin-targeted virus. These promising preclinical studies suggest that HSV therapy may be beneficial for local control of unresectable neuroblastoma tumors, but further studies are needed to establish any potential benefits of oncolytic HSV for metastatic disease.

SARCOMAS

Pediatric sarcomas represent a diverse group of tumors that include rhabdomyosarcoma (RMS), non-RMS soft-tissue sarcomas (NRSTS), osteosarcoma, and Ewing sarcoma family of tumors (ESFT) including Ewing sarcoma and its counterpart primitive neuroectodermal tumor (PNET) (**Table 3**). RMS is the most common soft-tissue sarcoma in children accounting for ~5% of all pediatric malignancies and half of soft-tissue sarcomas of childhood.[108] There are two main histological subtypes of RMS, embryonal (E-RMS) and alveolar (A-RMS). Most A-RMS tumors are found to have the *t(2;13) PAX3-FOX01* translocation.[109] The majority of E-RMS tumors display loss of heterozygosity (LOH) at the chromosome 11p15.5 locus.[110] The A-RMS subtype is more common in older patients and ~15% of A-RMS patients have metastatic disease at diagnosis, which is associated with a 5-year survival rate of <25%.[111–113] Augmentation of conventional chemotherapy regimens has not significantly improved survival for such patients. Further, surviving patients often suffer from lifelong treatment-related morbidity. Therefore, well-tolerated and effective novel targeted therapies are critically needed for A-RMS tumors. NRSTS represent a heterogeneous group of tumors that collectively account for ~4% of all childhood malignancies.[114] Similar to the poor outcomes for patients with high-risk RMS, children with large, high-grade, or unresectable NRSTS have less than a 50% chance of survival, and those with metastatic disease at diagnosis have survival rates under 20%.[115–117] Osteosarcoma and ESFT are the two most common malignant bone tumors in childhood and adolescence. Osteosarcoma tumors often have alterations of *p53* and *RB1* tumor suppressor genes, whereas ESFT are unified by the *t(11;22) EWS-FLI* or similar translocations.[118–120] Despite dose-intensified chemotherapy, outcomes for patients with recurrent or metastatic osteosarcoma or ESFT are poor with survival rates under 30%.[121,122] Recent studies have shown that intensified treatment regimens with more agents or higher dose chemotherapy only increases toxicity and secondary malignancies, without improving survival.[123,124] Very few salvage therapies are available for these malignancies, and newer, less toxic, targeted approaches such as mutant HSV, are desperately needed.

Children with sarcomas that are localized and fully resected, generally, have favorable outcomes with well-tolerated treatment

regimens and would not be candidates for engineered HSV-based therapies. However, children with large, unresectable, metastatic, or relapsed tumors could potentially benefit from such therapy. The proposed approach, like neuroblastoma, would involve local injection of unresectable tumors or systemic delivery of virus for metastatic disease. Several *in vitro* studies and *in vivo* intratumoral and intravenous injection studies have demonstrated that mutant HSV is oncolytic for sarcomas.

Initial studies by Bharatan *et al*. found that mutant HSV one embryonal RMS human cancer line, RD, and two alveolar lines, RhRKM-P4 and RH18, were very sensitive with complete cytopathic effects seen using NV1020 and G207 at low multiplicities of infection (≤0.5 PFU/cell).[5] Currier *et al.* confirmed that immunocompromised mice with a flank human alveolar or embryonal tumor <250 mm^3 showed a complete response to a single-intratumoral injection of NV1020. In contrast, only half of those with bulky disease (>250 mm^3) had complete regression of their tumor with a single injection.[125] Using five fractionated injections in multiple sites, they obtained more widespread intratumoral distribution of virus and improved control of large tumors, suggesting that patients with bulky disease may benefit from oncolytic HSV delivered to multiple sites of a tumor.

Confirming sensitivity of RMS to HSV, Cinatl *et al.* found marked cytotoxic and replicative effects of G207 in three human embryonal and four human alveolar RMS cell lines in tissue culture.[126] Intratumoral injection of G207 in the flanks of mice xenotransplanted with human RMS cell lines resulted in marked tumor regression as well as complete resolution of the tumor in 25% of mice. Intravenous G207 administration in similar mice led to significant tumor-growth inhibition. They found complete tumor regression of alvelolar RMS in five of eight animals who received intravenous G207 combined with vincristine, a drug used as part of frontline therapy for patients with RMS. Similarly, an oncolytic herpes vector rRp450, which expresses a prodrug-converting enzyme for cyclophosphamide, another frontline chemotherapeutic for patients with RMS, prolonged survival in athymic nude mice with flank human alveolar RMS tumors, when the virus was combined with intraperitoneal cyclophosphamide.[30] The virus was deemed safe when given both intravenously and intracranially alone or in combination with cyclophosphamide. Cyclophosphamide has also been shown to increased HSV efficacy by suppressing monocyte influx into infected tumors.[127] Taken together, these data suggest that mutant HSV alone or combined with current chemotherapy may offer significant therapeutic benefits for children with RMS.

Several different NRSTS appear to be sensitive to oncolytic HSV including fibrosarcoma, angiosarcoma, synovial sarcoma, and leiomyosarcoma. Two groups have shown sensitivity in murine adult-type fibrosarcoma models, S-180 and NfSa Y83. S-180 bearing BALB/c mice received a single-intratumoral injection of mtHSV, an oncolytic double $\gamma_1$34.5 deleted virus, or PBS.[128] Significant growth inhibition was seen in mice receiving the virus, and viral replication was limited to tumor cells. Likewise, immunocompetent C3H mice (HSV-resistant) with the highly aggressive sarcoma NfSa Y83, injected into the neck or flank, had marked tumor regression after intratumoral injection with a multimutated HSV, HL.[129] Approximately 75% of flank tumors and 50% of neck tumors completely resolved. There are no reports of human fibrosarcoma cell lines being tested. Intratumoral injection of 3616UB into human angiosarcoma xenografts in SCID mice produced significant growth arrest and some tumor regression.[73] The herpes vector *d*12.CALP, which utilizes calponin to drive expression of a major *trans*- activating factor for HSV viral genes, was tested against human synovial sarcoma and leiomyosarcoma cell lines.[130] Calponin is normally expressed in mature smooth muscle cells but has been shown to be aberrantly expressed in a variety of NRSTS and osteosarcoma.[131–136] Yamamura *et al.* found that *d*12.CALP selectively killed calponin-positive human synovial sarcoma, leiomyosarcoma, and osteosarcoma cells.[130] Additionally, injection of the virus into human leiomyosarcoma in the flanks of athymic nude mice resulted in cure in four of five mice by day 35, and virus replication was noted in a nontreated tumor distant to the site of intratumoral virus inoculation. Importantly, vascular smooth muscle cells as well as normal cells in the brain, lung, liver, kidney, heart, small intestine, and uterus were not infected by the virus. These studies suggest that NRSTS can be targeted and killed by engineered HSV, while normal cells are unharmed.

There is less data regarding the efficacy of mutant HSV in pediatric bone tumors. The sensitivity of two human osteosarcoma cell lines and four Ewing sarcoma/PNET cell lines to G207 and NV1020 was investigated by Bharatan *et al*.,[5] who found intermediate sensitivity of osteosarcoma cells as compared to RMS. Ewing sarcoma/PNET cell lines were the least sensitive with one cell line, 5838, resistant to both viruses. The resistance mechanism is not yet known as HSV entry and gene transduction occurred in resistant cell lines. Further studies examining sensitivities of osteosarcoma and Ewing sarcoma/PNET cell lines are needed, and resistance mechanism(s) need to be elucidated so that circumvention strategies can be developed.

CONCLUSION

Despite improving survival rates for children with cancer, subsets of patients exist with disease resistant to traditional therapies such as surgery, chemotherapy, and radiation. These patients require newer, targeted treatments used alone or in combination with more traditional approaches. Oncolytic HSV affords a candidate therapy with promise for several difficult-to-treat pediatric malignancies including certain brain tumors, neuroblastomas, and sarcomas. Despite many encouraging *in vitro* and *in vivo* studies, unfortunately, no pediatric trials utilizing engineered HSV have been conducted to date.

Many challenges still exist and need to be addressed in order to maximize the benefit of HSV therapy in pediatric patients. Further studies should be conducted to confirm viral safety in the developing human cerebrum, cerebellum, brain stem, and other developing organs. Virus delivery via direct inoculation needs to be tested and perfected especially in sensitive areas like the brain stem, and continued work on improving systemic delivery is necessary. As newer combined modality viruses for adult cancer therapies are created, they should also be tested against pediatric malignancies, and more effort needs to be made at improving tropism of HSV to individual pediatric tumor tissues. Hopefully, with continued advances in virotherapy, oncolytic HSV therapy will become a viable and effective adjuvant treatment for pediatric patients with traditionally dismal outcomes.

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