

# Multimechanistic Tumor Targeted Oncolytic Virus Overcomes Resistance in Brain Tumors

Kaoru Tamura<sup>1,2</sup>, Hiroaki Wakimoto<sup>1-3</sup>, Aayush S Agarwal<sup>1,2</sup>, Samuel D Rabkin<sup>3</sup>, Deepak Bhere<sup>1,2</sup>, Robert L Martuza<sup>3</sup>, Toshihiko Kuroda<sup>4,5</sup>, Randa Kasmieh<sup>1,2</sup> and Khalid Shah<sup>1,2,6</sup>

<sup>1</sup>Molecular Neurotherapy and Imaging Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA; <sup>2</sup>Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA; <sup>3</sup>Department of Neurosurgery, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA; <sup>4</sup>Department of Surgical Oncology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA; <sup>5</sup>Present address: Tokorozawa Proctologic Hospital, Tokorozawa, Japan; <sup>6</sup>Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

Only a subset of cancer patients inoculated with oncolytic herpes simplex virus (oHSV) type-1 has shown objective response in phase 1 and 2 clinical trials. This has raised speculations whether resistance of tumor cells to oHSV therapy may be a limiting factor. In this study, we have identified established and patient derived primary glioblastoma multiforme (GBM) stem cell lines (GSC) resistant to oHSV and also to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) that has recently shown promise in preclinical and initial clinical studies. We created a recombinant oHSV bearing a secretable TRAIL (oHSV-TRAIL) and hypothesized that oHSV-TRAIL could be used as a cancer therapeutic to target a broad spectrum of resistant tumors in a mechanism-based manner. Using the identified resistant GBM lines, we show that oHSV-TRAIL downregulates extracellular signal-regulated protein kinase (ERK)-mitogen-activated protein kinase (MAPK) and upregulates c-Jun N-terminal kinase (JNK) and p38-MAPK signaling, which primes resistant GBM cells to apoptosis via activation of caspase-8, -9, and -3. We further show that oHSV-TRAIL inhibits tumor growth and invasiveness and increases survival of mice bearing resistant intracerebral tumors without affecting the normal tissues. This study sheds new light on the mechanism by which oHSV and TRAIL function in concert to overcome therapeutic-resistance, and provides an oncolytic virus based platform to target a broad spectrum of different cancer types.

Received 10 April 2012; accepted 27 July 2012; advance online publication 28 August 2012. doi:10.1038/mt.2012.175

## INTRODUCTION

Glioblastoma multiforme (GBM) is a high-grade glioma and the most common primary malignant brain tumor.<sup>1</sup> GBMs are diffuse and infiltrating with no clear border between normal brain and tumor. Current treatment regimens that include temozolomide have significantly improved the median, 2- and 5-year survival compared to radiotherapy alone in patients with newly diagnosed

GBM.<sup>2,3</sup> Nevertheless, GBM patients have a poor prognosis with a median survival of 14.6 months.<sup>2</sup> The inherent or acquired resistance of tumor cells to antitumor agents and the highly invasive nature of tumor cells are the major impediments to the currently employed anti-GBM therapies and pose an urgent need for novel therapeutics with substantial efficacy. Oncolytic herpes simplex virus (oHSV) and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) have recently shown promise in both preclinical and clinical trials.<sup>4-13</sup> Oncolytic viruses are genetically modified viruses that, upon infection, selectively replicate in and kill neoplastic cells while sparing normal cells.<sup>4,8,14</sup> Among them, oHSV type 1-derived virus is one of the most extensively studied and considered a promising agent for treating brain tumors as well as other types of cancer.<sup>4,15</sup> Recombinant oHSV vectors such as G207 and G47 $\Delta$  have been previously investigated in both preclinical and clinical studies.<sup>9,16-18</sup> Unlike replication-incompetent vectors, replication-competent or conditional vectors can amplify to produce virus progeny that then infects surrounding tumor cells resulting in multiple waves of infection in situ, virus spread and extensive cell death. In a direct comparison between oncolytic adenovirus and oHSV in GBM cell lines, oHSV was shown to be more efficacious.<sup>19</sup> Mutations of specific HSV genes, namely  $\gamma$ 34.5 and UL39, have been shown to confer selectivity to cancer cells, which has enabled translational studies to humans.<sup>4,15</sup> Although phase 1 and 1b clinical trials for oHSV proved its safety, the efficacy for human GBMs seems marginal as only a subset of patients showed decrease in tumor volume<sup>9</sup> which could in part be due to the insensitivity of a subset of GBM cells to HSV mediated oncolysis. TRAIL has emerged as a promising antitumor agent due to its tumor-specific induction of apoptosis in a death receptor-dependent manner.<sup>20</sup> Both recombinant human TRAIL ligand and TRAIL receptor agonist monoclonal antibodies are currently being evaluated in clinical trials,<sup>21</sup> however, short half-life and off-target toxicity of systemically delivered TRAIL pose challenges in the clinic.<sup>22</sup> We have previously established that a secreted form of TRAIL (S-TRAIL) exerts more potent apoptotic effects compared to TRAIL itself and when delivered by viruses or different stem cell types has significant antitumor effects as compared to systemically administrated TRAIL in different mouse models of

**Correspondence:** Khalid Shah, Department of Radiology, Massachusetts General Hospital, Massachusetts General Hospital, Harvard Medical School, 149, 13th Street, Boston, Massachusetts 02129, USA. E-mail: kshah@mgh.harvard.edu

GBMs.<sup>5,7,10–12,23</sup> However, malignant GBMs show heterogeneity in their response to TRAIL; with ~50% showing sensitivity to TRAIL-mediated apoptosis and others showing varying resistance to TRAIL-mediated apoptosis.<sup>7,24</sup>

In this study, we screened a panel of established and patient derived primary GBM stem cell lines for their sensitivity to a recombinant version of G47Δ (referred to oHSV in this study) and TRAIL. In an effort to develop anti-GBM therapies that target a broad spectrum of GBMs that are either resistant to TRAIL-mediated apoptosis or resistant to both oHSV-mediated oncolysis and TRAIL, we have engineered oHSV-bearing secretable-TRAIL (oHSV-TRAIL) and extensively studied a mechanism-based therapeutic approach to target resistant GBMs *in vitro* and in malignant and invasive GBM models in mice.

## RESULTS

### Screening of different GBM lines reveals differential sensitivities to oHSV-mediated oncolysis and S-TRAIL-mediated apoptosis

We screened a cohort of both established GBM cell lines (Gli36, U87, U251, and LN229) and primary glioma stem cell (GSC) lines obtained from surgical specimens (GBM4, GBM6, BT74, and GBM8F) for their sensitivity to purified S-TRAIL- or oHSV-mediated cell death. While three established lines had varying sensitivity to TRAIL-induced apoptosis-mediated by caspase-3 and -7, one established line (LN229) was fully resistant to TRAIL-mediated apoptosis. Among the primary GSC, GBM8F was fully resistant to TRAIL whereas other GSC lines had varying sensitivity to TRAIL-induced apoptosis. Next, we evaluated the sensitivity of established GBM lines and GSC lines to oHSV (G47Δ empty) mediated oncolysis. Among the established lines, TRAIL resistant LN229 line was also resistant to oHSV-mediated oncolysis whereas all the GSC lines were sensitive to oHSV-mediated oncolysis. The amounts of virus released by the oHSV-infected cells greatly varied among the GBM cell lines tested, and did not necessarily correspond to the sensitivity to oHSV-mediated oncolysis. These results reveal the identification of GBM lines that are either resistant to TRAIL-mediated apoptosis (LN229, GBM8F) or resistant to both oHSV-mediated oncolysis and TRAIL (LN229). Based on these results, we used LN229 and GBM8F GSC for further therapeutic evaluation (Figure 1a).

To evaluate oHSV replication and spread in oHSV and TRAIL resistant GBM cells and corresponding changes in cell viability, LN229 and GBM8F cells engineered to express *Renilla* luciferase (Rluc)-mCherry (LN229-RmC, GBM8F-RmC) were infected with oHSV-bearing firefly luciferase (oHSV-Fluc, Supplementary Figure S1), and monitored by *in vitro* and *in vivo* dual bioluminescence imaging. *In vitro*, oHSV-Fluc replicates in both LN229 and GBM8F cells as indicated by firefly luciferase (Fluc) expression, but oHSV replication was more robust and peaked earlier in GBM8F cells than LN229 cells (Figure 1b and Supplementary Figure S2). Accordingly, while oHSV killed GBM8F cells efficiently (indicated by Rluc expression), LN229 cells exhibited apparent resistance to the killing effect by oHSV (Figure 1c). *In vivo* monitoring of virus infection demonstrated the ability of intratumorally injected oHSV-Fluc to replicate in intracerebral tumors generated with these GBM cells, and revealed patterns of

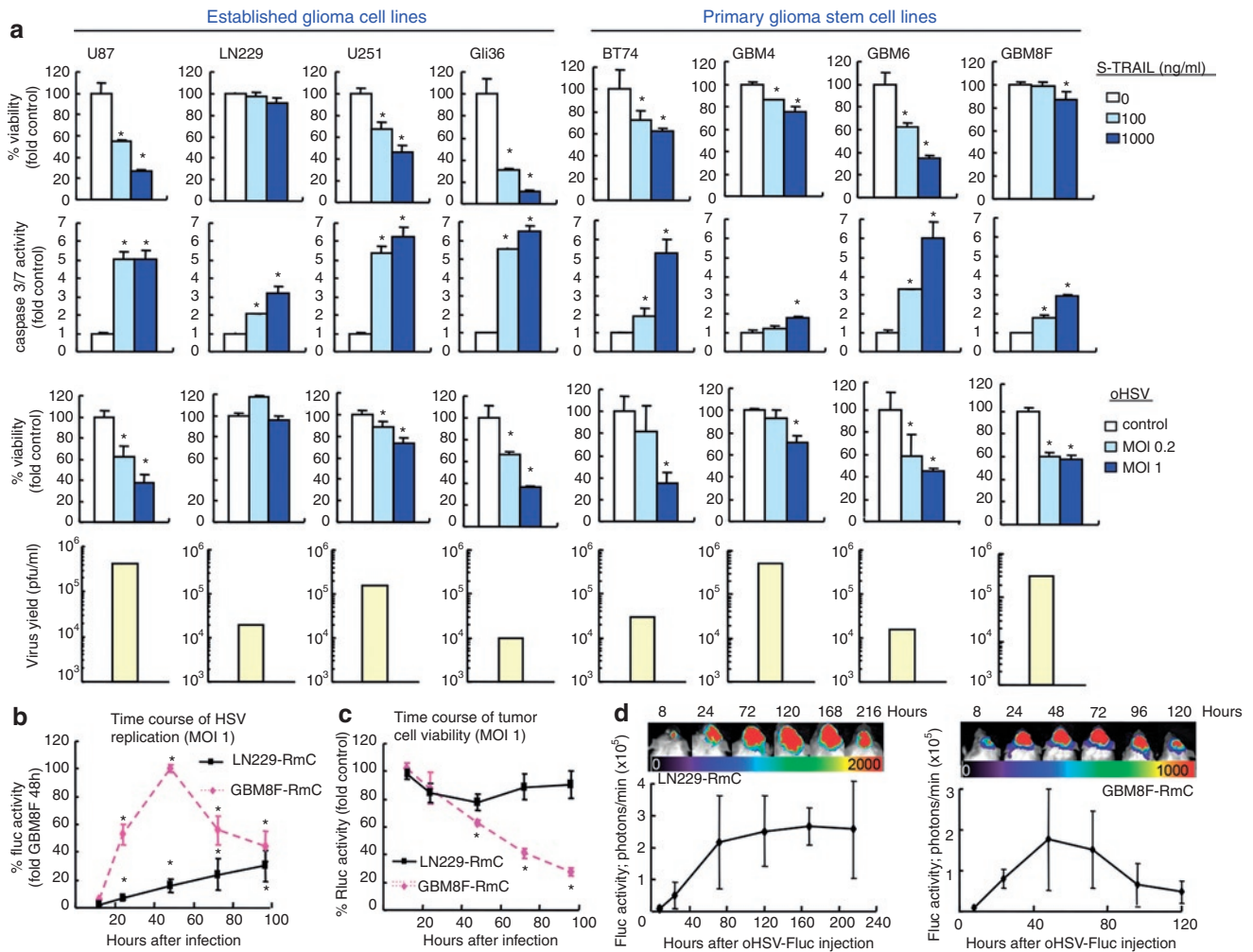
virus replication similar to the ones observed *in vitro* (Figure 1b and d). These data reveal that although oHSV replicates in both oHSV resistant and sensitive lines but results in killing only the sensitive GBM8F line and suggest that GBMs might be heterogeneous in the responses to oHSV.

### Combination of oHSV and S-TRAIL leads to apoptosis in TRAIL resistant GBM cells *in vitro*

In order to evaluate the therapeutic potential of oHSV and S-TRAIL in resistant GBMs, established LN229 GBM cells and primary GBM8F (TRAIL resistant) GBM cells were infected with oHSV and treated with purified S-TRAIL 6 hours post-infection. oHSV infection and subsequent S-TRAIL treatment resulted in significant decrease in viability of LN229 and GBM8F cells in culture, which was associated with increased caspase-3/7 activation (Figure 2a–d). A significantly impaired activation of caspase-3/7 activity was observed when LN229 and GBM8F cells were infected with oHSV and treated with S-TRAIL in the presence of pan-caspase inhibitor, Z-VAD-FMK (Figure 2a, b). This resulted in complete reversal of both LN229 and GBM8F cell death in oHSV-infected and S-TRAIL-treated cells (Figure 2c–d). Western blotting revealed that cleavage of poly-ADP ribose polymerase (PARP), one of the main downstream targets of caspase-3, was undetectable by either S-TRAIL or oHSV treatment in both LN229 and GBM8F cells, indicating that oHSV-mediated cell death is primarily not dependent on caspase/PARP activation. However, cleaved PARP was increased in cells treated with combined oHSV and S-TRAIL (Figure 2e, f). A significant reduction in PARP cleavage was observed when LN229 and GBM8F cells were infected with oHSV and treated with S-TRAIL in the presence of pan-caspase inhibitor, Z-VAD-FMK (Figure 2e, f). These results revealed that oHSV and TRAIL combination leads to caspase-mediated apoptosis in TRAIL resistant and both TRAIL and oHSV resistant GBM cells.

### oHSV-TRAIL targets both cell proliferation and death pathways in resistant GBM cells

In order to target both TRAIL resistant and oHSV resistant cell lines, we engineered an oHSV-bearing S-TRAIL (oHSV-TRAIL) using a BAC containing the backbone structure of G47Δ, G47Δ-BAC<sup>C25–27</sup> (Supplementary Figure S1). The replicating ability of oHSV-TRAIL was similar to that of oHSV as revealed by the virus yield quantified using plaque assay on Vero cells (Supplementary Figure S3a). The secretion of TRAIL in oHSV-TRAIL infected LN229 GBM cells was confirmed by ELISA (Supplementary Figure S3b). To evaluate the effect of oHSV-TRAIL, LN229-RmC cells and GBM8F-RmC cells were infected with oHSV or oHSV-TRAIL at multiplicity of infection (MOI) = 1 for 24, 48, 72, and 96 hours. The changes in cell viability (Rluc activity) revealed that oHSV-TRAIL infection resulted in significantly more potent cell killing in both LN229 and GBM8F cells as compared to oHSV infection (Figure 3a and b and Supplementary Figure S3c, d). Annexin V staining analysis revealed that the number of apoptotic cells (Annexin V positive, propidium iodide negative) was considerably increased in both LN229 and GBM8F cell populations post-oHSV-TRAIL infection (Supplementary Figure S3e). oHSV-TRAIL also resulted in significantly greater killing in oHSV and TRAIL sensitive human Gli36 GBM line as compared to oHSV infection (Supplementary Figure S4). Western blot analysis



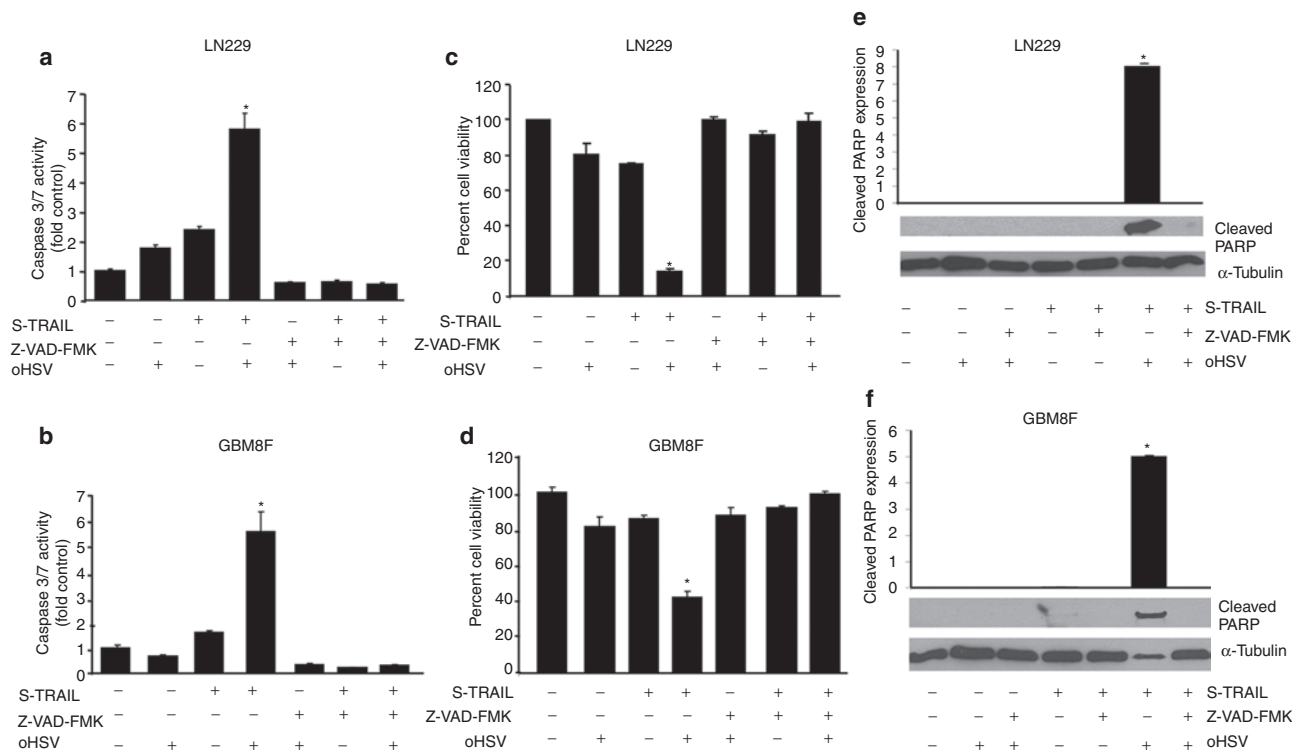
**Figure 1** Differential sensitivities of glioblastoma multiforme (GBM) cells to S-TRAIL-mediated apoptosis and oncolytic herpes simplex virus (oHSV)-mediated oncolysis. **(a)** Screening of different GBM lines reveals differential sensitivities to S-TRAIL-mediated apoptosis and oHSV-mediated oncolysis. Established GBM lines (U87, LN229, U251, and Gli36) and primary glioma stem cell (GSC) lines (BT74, GBM4, GBM6, and GBM8F) were treated with different concentrations of purified S-TRAIL and assayed for viability at 48 hours post-treatment (top row), and for caspase-3/7 activity at 72 hours postinfection (second row). Established GBM lines and primary GSC lines were infected with oHSV at multiplicities of infection (MOIs) 0.2 and 1 and assayed for viability at 72 hours postinfection (third row), and virus titration on Vero cells using the supernatant of oHSV-infected GBM cell lines (MOI1, bottom row). **(b,c)** Pharmacodynamics of oHSV *in vitro*. LN229-RmC and GBM8F-RmC were infected with oHSV-Fluc at MOI = 1. Viral replication indicated by **(b)** firefly luciferase (Fluc) activity and tumor cell viability indicated by **(c)** *Renilla* luciferase (Rluc) activity were monitored by dual Fluc and Rluc bioluminescence imaging, respectively at different time points. **(d)** Pharmacodynamics of oHSV *in vivo*. Mice ( $n = 3$  per line) bearing intracranial LN229-RmC (left) or GBM8F-RmC (right) GBMs were injected with oHSV-Fluc intratumorally and viral distribution was followed by Fluc bioluminescence imaging at different indicated times. One representative image of mice and the average Fluc bioluminescence intensities are shown. \* $P < 0.05$ . Error bars indicate SD.

of oHSV-TRAIL-infected LN229 and GBM8F cell lysates showed cleavage of caspases-8, -9 and PARP and no significant difference was observed in Bcl2 expression (**Figure 3c**) and death receptor (DR)4/5 expression (**Supplementary Figure S5**). These results reveal that oHSV-TRAIL infection induces caspase-mediated apoptosis in both TRAIL resistant LN229 and GBM8F glioma lines.

We next examined the mechanism of oHSV-TRAIL induced apoptosis in resistant GBM cells. As such, we assessed the activation of molecules involved in the cell proliferation pathways, mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated protein kinase 1 and 2 (ERK 1/2), c-Jun N-terminal kinase (JNK) and p38 in both LN229 and GBM8F. A significantly impaired activation of ERK 1/2 was observed in

LN229 cells infected with oHSV, which was further impaired by oHSV-TRAIL (**Figure 3c**). There was a complete shutdown of ERK 1/2 phosphorylation in both oHSV and oHSV-TRAIL infected GBM8F cells. JNK and p38 were activated in LN229 and GBM8F cells after oHSV and oHSV-TRAIL infection.

We next sought to determine the significance of the JNK/ERK MAP kinase signaling alterations in oHSV-TRAIL-mediated cytotoxicity of both TRAIL and oHSV resistant GBM cells. To examine the role of JNK upregulation, we treated resistant GBM cells with JNK inhibitor, SP600125. LN229 were infected with oHSV or oHSV-TRAIL in the absence and presence of SP600125. Western blotting analysis showed SP600125 treatment resulted in the inhibition of JNK phosphorylation in oHSV and oHSV-TRAIL-infected



**Figure 2** Combination of oncolytic herpes simplex virus (oHSV) and S-TRAIL leads to caspase-3/7-mediated apoptosis in resistant glioblastoma multiforme (GBM) cells. (**a–d**) Caspase-3/7 activity and cell viability of (**a,c**) LN229 and (**b,d**) GBM8F treated with purified S-TRAIL (100 ng/ml), oHSV (multiplicity of infection (MOI) = 1) or both oHSV and S-TRAIL in the presence or absence of pan-caspase inhibitor, Z-VAD-FMK (20  $\mu$ mol/l). \* $P < 0.05$  in the comparison of oHSV plus S-TRAIL treatment group with other groups. (**e,f**) Immunoblot analysis using cleaved poly-ADP ribose polymerase (PARP) antibody on whole cell lysates prepared from LN229 and GBM8F cells treated with purified S-TRAIL (100 ng/ml), oHSV (MOI = 1) or both oHSV and S-TRAIL in the presence or absence of pan-caspase inhibitor, Z-VAD-FMK (20  $\mu$ mol/l). Cleaved PARP expression was normalized to  $\alpha$ -tubulin expression. \* $P < 0.05$  in the comparison of oHSV plus tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to TRAIL. Error bars indicate SD.

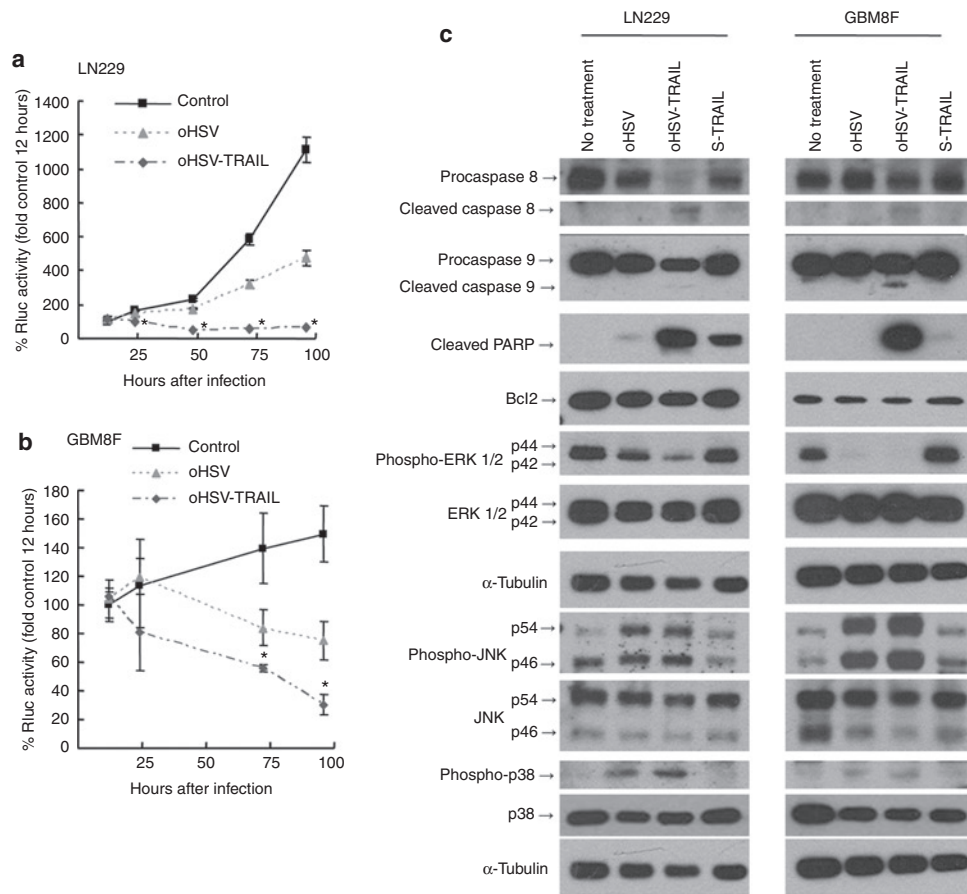
cells and also a significant reduction in the cleavage of PARP in oHSV-TRAIL-infected cells (Figure 4a). Caspase-3/7 activity assays showed a significant decrease in the caspase-3/7 activation in oHSV-TRAIL-infected cells in the presence of SP600125 as compared to the infected cells in the absence of SP600125 (Figure 4b). We also investigated whether inhibition of ERK1/2 with MEK-ERK inhibitor, U0126, could mimic oHSV-mediated inhibition of ERK1/2 and sensitize TRAIL resistant GBM cells to TRAIL-induced apoptosis. Western blotting analysis showed that U0126 treatment of LN229 cells inhibited ERK phosphorylation and subsequent addition of purified S-TRAIL markedly increased PARP cleavage (Figure 4c). Combined treatment with U0126 and S-TRAIL decreased cell viability in resistant LN229 GBM cells compared with single treatment (Figure 4d). These results thus suggest that oHSV-mediated downregulation of the ERK-MAPK and upregulation of JNK signaling may contribute to apoptotic cell death in oHSV-TRAIL-infected resistant GBM cells.

### oHSV-TRAIL inhibits GBM growth and invasion *in vivo* and prolongs survival of mice bearing both TRAIL and oHSV resistant GBM

We assessed the therapeutic efficacy of oHSV-TRAIL in intracranial GBMs, using LN229 and GBM8F glioma lines which were engineered to express Fluc-mCherry (LN229-FmC, GBM8F-FmC). Mice bearing established intracranial LN229-FmC GBMs

were administered intratumorally with oHSV, oHSV-TRAIL, or phosphate-buffered saline (PBS) and followed for changes in tumor volumes by Fluc imaging. As expected, oHSV injection had no impact on both tumor burden (Supplementary Figure S6a) and survival (Figure 5a, PBS: 50.5 days, oHSV: 49 days) in this resistant GBM model. In contrast, oHSV-TRAIL injection resulted in significant decrease in tumor volumes compared to both control (PBS) and oHSV treated mice (Supplementary Figure S6a) and prolonged survival of mice-bearing intracranial GBMs (Figure 5a). The median survival of oHSV-TRAIL-treated mice was 69 days, which was significantly longer than PBS- and oHSV-treated mice (Figure 5a;  $P = 0.038$  oHSV and oHSV-TRAIL comparison, log-rank test). X-gal staining of the brain sections collected 48 hours post oHSV or oHSV-TRAIL injection showed an extensive distribution of reporter  $\beta$ -galactosidase positivity, which overlapped with the tumor area, suggesting that the spread of virus infection was comparable between oHSV and oHSV-TRAIL (Figure 5b). Immunohistochemical analysis and confocal microscopy showed significantly increased cleaved caspase-3 staining in sections from oHSV-TRAIL-treated tumors as compared to oHSV and PBS treated tumors. Furthermore, no cleaved caspase-3 staining was seen in normal brain cells in all treated groups revealing the tumor specificity of oHSV-TRAIL treatment (Figure 5c, d).

To examine the effect on GBM cell invasion, we used matrix-coated assays *in vitro* and intracranial mouse GBM model



**Figure 3** Oncolytic herpes simplex virus (oHSV)-tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediates potent cytotoxicity in resistant glioblastoma multiforme (GBM) cells by altering both cell proliferation and death pathways *in vitro*. **(a,b)** Cell viability of GBM cells assessed by *Renilla* luciferase (Rluc) bioluminescence at different time points. **(a)** LN229-RmC and **(b)** GBM8F-RmC were infected with oHSV or oHSV-TRAIL at multiplicity of infection (MOI) = 1. \* $P < 0.05$  in the comparison of oHSV-TRAIL to control and to oHSV. Error bars indicate SD. **(c)** Immunoblot analysis using antibodies against caspase-8, -9, cleaved poly-ADP ribose polymerase (PARP), Bcl2, phospho-ERK, ERK, phospho-c-Jun N-terminal kinase (JNK), JNK, phospho-p38, and p38 on whole cell lysates prepared from LN229 and GBM8F cells untreated, or treated with oHSV, oHSV-TRAIL (MOI = 1) or S-TRAIL for 18 hours.  $\alpha$ -Tubulin was used as a loading control.

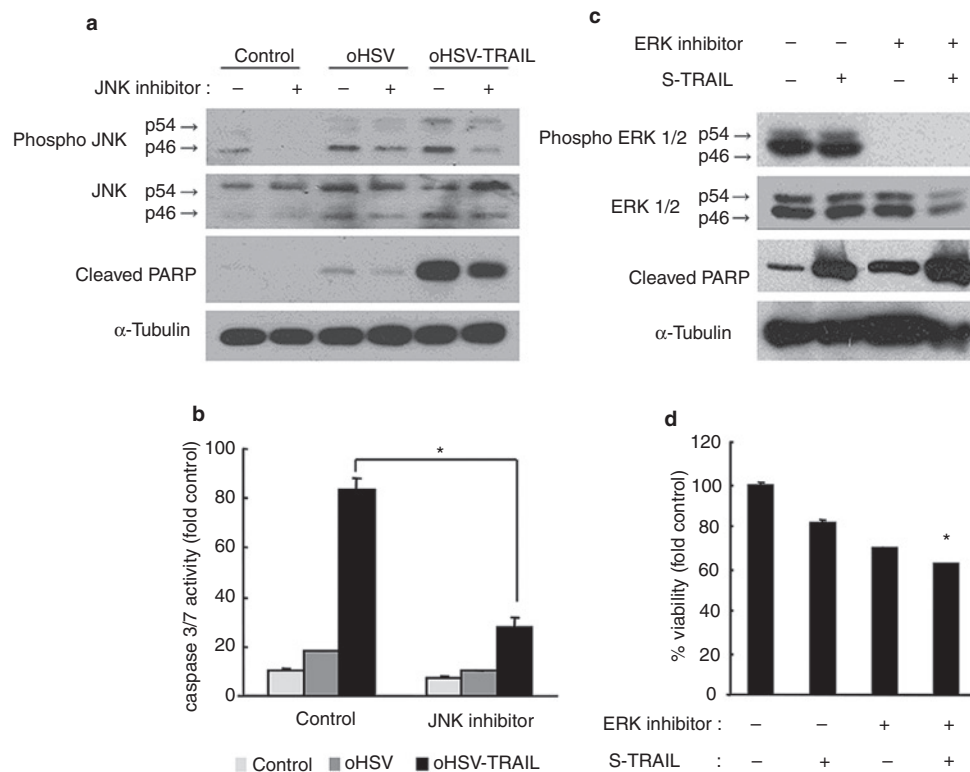
using invasive GBM8F as opposed to LN229 GBM line which is a noninvasive line (**Supplementary Figure S7**). oHSV and oHSV-TRAIL treatment reduced the number of GBM8F cells that had invaded in matrigel-coated invasion assay, with oHSV-TRAIL having a stronger inhibitory effect than oHSV (**Figure 6a**). *In vivo*, both oHSV and oHSV-TRAIL injection into intracranial GBM8F tumors reduced the number of invading GBM8F cells as compared to the control, with oHSV-TRAIL having a stronger inhibitory effect than oHSV (**Figure 6b**). These results demonstrate that oHSV-TRAIL induces apoptosis in TRAIL and oHSV resistant intracranial GBM, inhibits GBM growth and invasiveness and significantly increases survival in mice.

## DISCUSSION

In this study, we identified GBM lines that are either resistant to TRAIL-mediated apoptosis or resistant to both oHSV-mediated oncolysis and TRAIL, and created a novel oHSV-bearing secretable-TRAIL (oHSV-TRAIL) to study the mechanism-based targeted therapy in resistant GBM lines *in vitro* and *in vivo*. We show that oHSV-TRAIL induces apoptosis in TRAIL and oHSV resistant GBMs by targeting both cell proliferation and death

pathways. Furthermore, oHSV-TRAIL inhibits GBM growth and invasion and prolongs survival of mice-bearing intracranial brain tumors.

GBMs are molecularly heterogeneous tumors usually containing a subset of tumor cells that are resistant to a number of currently used anti-GBM therapies.<sup>1</sup> These cells rapidly take over and ultimately result in tumor progression. oHSV as a single agent has been tested in preclinical studies and shown to result in oncolysis in most GBM cells including GBM stem cells.<sup>13,28,29</sup> However, while phase I clinical trials using intracranial oHSV inoculation demonstrated safety in GBM patients, they showed only partial radiologic responses.<sup>9,30,31</sup> These results suggest that GBMs might be heterogeneous in the responses to oHSV and contain a subset of cells resistant to oHSV-mediated oncolysis. In this study, we screened a panel of eight GBM cell lines that covers established GBM cell lines and patient derived primary GSCs. We found that GBM cells have differential sensitivities to oHSV-mediated oncolysis as well as TRAIL-mediated apoptosis. To our knowledge, this is the first study which identifies GBM lines resistant to oHSV. Interestingly, our screening indicated that the yields of oHSV do not always parallel the efficiency of oHSV-mediated cell killing,



**Figure 4** Oncolytic herpes simplex virus (oHSV)-tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in resistant glioblastoma multiformes (GBMs) depends on c-Jun N-terminal kinase (JNK) activation and ERK inhibition. **(a)** Immunoblot analysis using antibodies against JNK, phospho-JNK, and cleaved poly-ADP ribose polymerase (PARP), on whole cell lysate prepared from LN229 cells treated with oHSV, oHSV-TRAIL (multiplicity of infection (MOI) = 1) or control in the absence (-) and presence (+) of JNK inhibitor (SP600125, 20 μmol/l) for 18 hours. **(b)** Caspase 3/7 activity of LN229 cells treated with oHSV, oHSV-TRAIL (MOI = 1), or control for 20 hours in the absence (-) and presence (+) of JNK inhibitor. \* $P < 0.05$  in the comparison of oHSV-TRAIL treated cells in the absence and presence of JNK. **(c)** Immunoblot analysis using antibodies against ERK, phospho ERK, and cleaved PARP on whole cell lysate prepared from LN229 cells treated with S-TRAIL (100 ng/ml) in the absence (-) and presence (+) of ERK inhibitor (U0126, 20 μmol/l) for 18 hours. α-Tubulin was used as a loading control. **(d)** Cell viability assay showing the % viable LN229 cells after treatment with different combinations of U0126 (20 μmol/l) and TRAIL (100 ng/ml) for 48 hours. \* $P < 0.05$  JNK inhibitor group in the comparison with control (in **b**) and ERK inhibitor and S-TRAIL treatment group in the comparison with other treatment groups (in **d**). Error bars indicate SD.

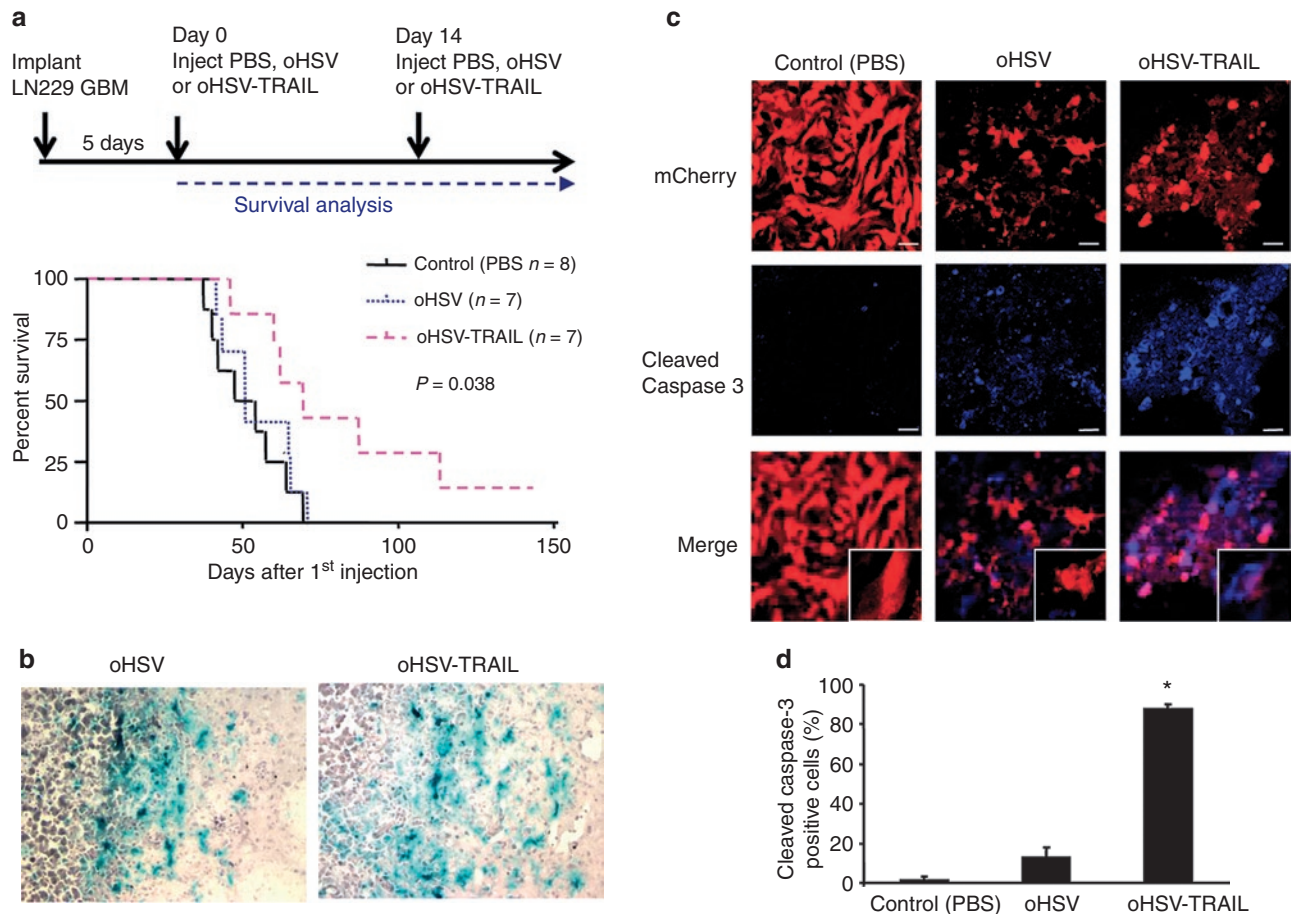
implicating a presence of cell death mode other than oncolysis that affects oHSV efficacy.

A number of previous studies by others and our laboratory have shown that dual bioluminescence imaging of tumor cells and therapeutics enables rapid and noninvasive measurement of both tumor load and fate of therapeutics *in vitro* and in mice-bearing intracranial tumors.<sup>10,12,32-34</sup> Utilizing oHSV-Fluc, GBM cells expressing Rluc and dual bioluminescence imaging, our studies reveal that two distinct biological events, oHSV replication and its effects on cell viability can be monitored. Our results indicate that oHSV can replicate in a GBM line which is resistant to oHSV, even though the viral replication and spread is greatly decreased and slow when compared to the GBM lines which are sensitive to oHSV. The ability of oHSV to replicate in GBM lines resistant to oHSV provides a great rationale of using oHSV to deliver cytotoxic agents like TRAIL and studying both the molecular mechanism and therapeutic effect of oHSV and TRAIL treatments in GBMs that are resistant to oHSV-mediated oncolysis and TRAIL-mediated apoptosis.

Various strategies have been employed to overcome resistance of tumor cells to different drug regimens. The ability of TRAIL to selectively target tumor cells while remaining harmless to most normal cells makes it an attractive candidate for an apoptotic therapy

for highly malignant brain tumors. However, a large percentage of primary GBM lines are resistant to TRAIL-induced apoptosis.<sup>35</sup> Our results reveal that oHSV infection and subsequent S-TRAIL treatment results in a caspase-3/7-mediated cell killing in a line that is resistant to both oHSV and TRAIL. Although TRAIL is a potent tumor-specific agent, its short biological half-life and limited delivery across the blood-brain barrier limit its applicability in treating brain tumors when delivered systemically. oHSV can replicate *in situ*, spread and exhibit oncolytic activity via a direct cytotoxic effect, and at the same time can deliver substantial quantities of therapeutic molecules *in situ*. As compared to the other oncolytic viruses, the capacity to incorporate large transgenes into its genome is one of the advantages of HSV-1.<sup>36</sup> The intratumoral delivery of oHSV-TRAIL can overcome the limitations posed by systemic administration of TRAIL as it remedies the short half-life of drugs, the use of multiple drug regimens to target resistant GBMs, in addition to circumventing the restrictions posed by the blood-brain barrier to deliver drugs to brain.

HSV like other viruses have genes such as Us3 and Rs1 (ICP4) whose products have an anti-apoptotic function.<sup>37,38</sup> These genes appear to facilitate HSV replication by preventing premature cell death. Therefore, it is possible that combining proapoptotic molecule with oHSV dampens viral replication and reduces oHSV

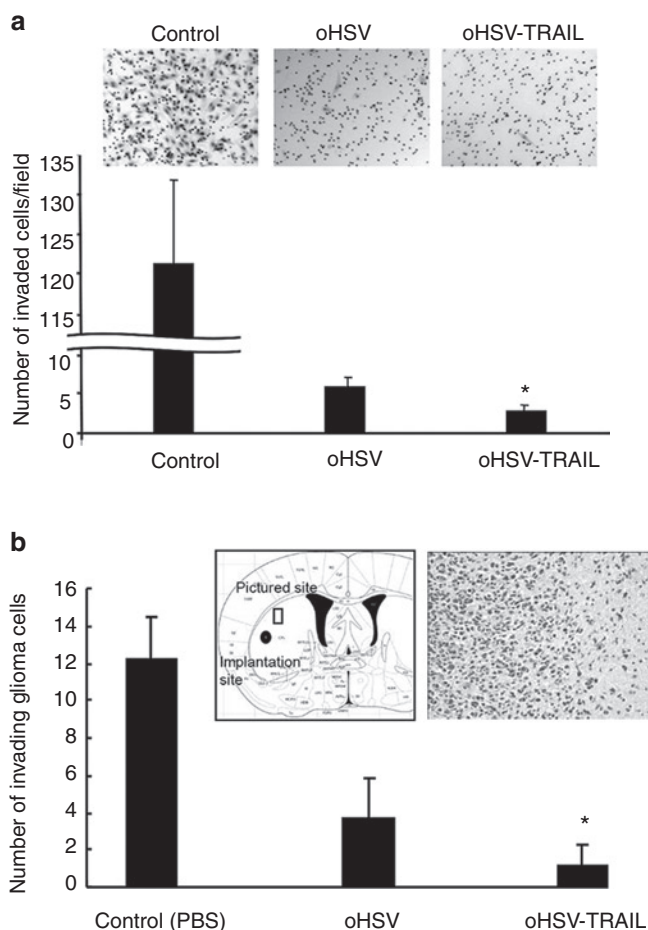


**Figure 5** Oncolytic herpes simplex virus (oHSV)-tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) prolongs survival of mice-bearing both TRAIL and oHSV resistant glioblastoma multiforme (GBM). **(a)** Timeline and survival curves of LN229-FmC GBM-bearing mice treated with oHSV, oHSV-TRAIL, or control (phosphate-buffered saline (PBS)).  $P = 0.038$  in oHSV and oHSV-TRAIL comparison, log-rank test. **(b)** X-gal staining revealing virus-infected areas. oHSV-injected (left) and oHSV-TRAIL-injected (right) tumor sections. Original magnification  $\times 20$ . **(c)** Immunofluorescence of cleaved caspase-3 staining on brain sections from LN229-FmC GBM-bearing mice injected with oHSV, oHSV-TRAIL, or PBS (control). Original magnification  $\times 20$ . **(d)** Plot showing the percentage of cleaved caspase-3 positive LN229-FmC GBM cells on brain sections.  $*P < 0.05$  in the comparison of oHSV-TRAIL to control and to oHSV.  $n = 3$  in each group. Error bars indicate SD.

potency. Our data, however, showed that replication of oHSV and oHSV-TRAIL in glioma cells is comparable (**Supplementary Figure S3a**), indicating that expression of TRAIL does not suppress viral replication. We show that oHSV-TRAIL downregulates ERK phosphorylation, upregulates p38 and JNK phosphorylation and induces cleavage of caspases in two GBM cell lines either TRAIL or TRAIL and oHSV resistant. Downregulation of ERK phosphorylation and upregulation of P38 and JNK phosphorylation have been previously reported following infection with HSV-1.<sup>39,40</sup> ERK1/2, members of the MAPK super family, can mediate cell proliferation and apoptosis<sup>41</sup> while activation of ERK is primarily associated with promoting cellular proliferation. Activation of cell proliferation signals that block apoptosis is associated with tumorigenesis and resistance to chemotherapeutic drugs.<sup>42</sup> Activation of ERK is reported to prevent Fas-induced apoptosis in activated T cells<sup>43</sup> and inhibit activation of caspases despite the release of cytochrome c from mitochondria.<sup>44–46</sup> In contrast to ERK, the JNK and p38-MAPK signaling is activated by proinflammatory cytokines and a variety of cellular stresses, and is typically linked to differentiation and apoptosis. Xia *et al.* reported that activation

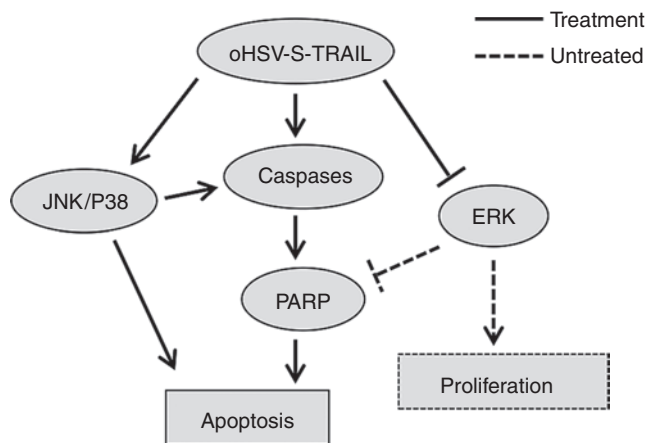
of JNK and p38 and concurrent inhibition of ERK are critical for induction of apoptosis.<sup>47</sup> We show that: (i) the inhibition of JNK activation suppresses oHSV-TRAIL-mediated activation of caspase pathway, and (ii) a small-molecule inhibitor of MEK/ERK sensitizes resistant cells to TRAIL-mediated apoptosis. Therefore, our results suggest that oHSV-mediated downregulation of the ERK signaling and upregulation of the JNK and p38 signaling play a vital role in priming resistant cells so that S-TRAIL expressed in oHSV-TRAIL infected GBM cells promotes activation of caspase-3, -8, and -9, leading to apoptotic cell death (**Figure 7**). Our studies thus underscore targeting both cell proliferation and death pathways as a crucial mechanism underlying oHSV-TRAIL-mediated robust induction of apoptosis in resistant GBMs.

Our *in vivo* studies reveal that oHSV-TRAIL results in marked attenuation of intracranial tumor growth and survival prolongation in mice-bearing TRAIL and oHSV resistant GBM. This makes a clear contrast to the lack of long-lasting antitumor efficacy mediated by an oncolytic adenovirus-expressing TRAIL.<sup>48</sup> Although established GBM lines are the most commonly used models *in vitro* and *in vivo*, they fail to recapitulate the clinical properties of tumors. Given this



**Figure 6** Oncolytic herpes simplex virus (oHSV)-tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) inhibits glioblastoma multiforme (GBM) invasion *in vitro* and *in vivo* in TRAIL resistant GBM. **(a)** *In vitro* invasion assay. Photomicrographs and graph showing the change in cell invasion after treatment with oHSV or oHSV-TRAIL in GBM8F glioma line. Multiplicity of infection (MOI) = 1. \* $P < 0.05$  in the comparison of oHSV-TRAIL to control and to oHSV. **(b)** *In vivo* invasion assay. Mice-bearing intracranial GBM8-FmC gliomas were injected with oHSV, oHSV-TRAIL, or phosphate-buffered saline (PBS) (control) and mice were sacrificed on day 14 and invasion of the GBM cells on brain sections was evaluated. Photomicrograph of hematoxylin and eosin (H&E) staining of GBM8F tumor cell invasion towards adjacent normal brain tissue and illustration of brain revealing GBM8F implantation-site and pictured-site.  $n = 3$  per group. \* $P < 0.05$  in the comparison of oHSV-TRAIL to control and to oHSV. Error bars indicate SD.

limitation of GBM lines as a representative GBM model, recent studies have focused on primary GBM lines and indicated a role for tumor-initiating cells in these lines. In an effort to test the effect of oHSV-TRAIL in such models, we used GBM8F primary GBM line that contains a subpopulation of CD133<sup>+</sup> cells<sup>13</sup> and exhibits highly invasive behavior *in vivo*. Our *in vitro* studies showed that despite resistance to TRAIL, oHSV-TRAIL is significantly more effective in killing and inhibiting invasiveness of GBM8F cells than oHSV. Furthermore, the number of invading cells *in vivo* was strongly reduced by a single inoculation of oHSV-TRAIL into GBM8F-generated tumors. This suggests that the oHSV spread in migrating tumor cells combined with *in situ* release of S-TRAIL can cooperate to block invasiveness of these cells. Future studies will need



**Figure 7** Schematic presentation showing the mechanism underlying the efficacy of oncolytic herpes simplex virus (oHSV)-tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on resistant glioblastoma multiforme (GBM) cells.

to address whether this is solely due to oHSV-TRAIL-mediated increased cell death or its additional but unknown functions that inhibit cellular migratory or invasive machineries.

In conclusion, our findings shed a new light on targeting oHSV and TRAIL resistant GBMs and pave the way for how oHSV and TRAIL can function in concert to target both cell proliferation and death pathways in heterogeneous GBM cells. A recent promising report on oncolytic virus targeting of metastatic cancer cells of multiple cancer types in humans highlighted the feasibility of achieving high concentrations of anticancer molecules *in situ* in the context of oncolytic virus therapy.<sup>49</sup> Therefore, this study may provide the key to ultimately develop novel oHSV-based therapies for patients with different tumors presenting different molecular profiles.

## MATERIALS AND METHODS

**Parental and engineered cell lines.** Established human GBM lines (Gli36, U87, U251, and LN229) and GBM8F were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. GBM stem cells (GBM4, GBM6, and BT74) were cultured in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 3 mmol/L L-glutamine (Mediatech, Manassas, VA), B27 (Invitrogen, Carlsbad, CA), 2  $\mu$ g/ml heparin (Sigma-Aldrich, St Louis, MN), 20 ng/ml human EGF (R&D Systems, Minneapolis, MN), and 20 ng/ml human FGF-2 (Peprotech, Rocky Hills, NJ) as described previously.<sup>13</sup> Two lentiviral vectors were used: (i) Pico2-Fluc-mCherry, a kind gift from Dr Andrew Kung (Dana Farber Cancer Institute; Boston, MA), (ii) Pico2-Rluc-mCherry, which is created by ligating Rluc fragment (the cDNA sequences encoding Rluc were amplified by PCR) into *Bam*H1/*Bst*B1-digested Pico2-Fluc-mCherry. Lentiviral packaging was performed by transfection of 293T cells as previously described.<sup>33</sup> LN229 and GBM8F were transduced with LV-Pico2-Fluc-mCherry and LV-Pico2-Rluc-mCherry at a MOI of 2 in medium-containing protamine sulfate (2  $\mu$ g/ml) and LN229-Fluc-mCherry (LN229-FmC); GBM8-Fluc-mCherry (GBM8-FmC) LN229-Rluc-mCherry (LN229-RmC); GBM8-Rluc-mCherry (GBM8-FmC) lines were obtained after puromycin (1  $\mu$ g/ml) selection in culture.

**Recombinant oHSVs and viral growth assay.** G47 $\Delta$ BAC contains the genome of G47 $\Delta$  ( $\gamma$ 34.5, ICP6, ICP47) and a cytomegalovirus promoter driven enhanced green fluorescent protein (EGFP) in place of lacZ in G47 $\Delta$ .<sup>17</sup> Recombinant oHSV vectors, G47 $\Delta$ -empty (oHSV; referred to oHSV in this study), G47 $\Delta$ -TRAIL (oHSV-TRAIL), and G47 $\Delta$ -Fluc (oHSV-Fluc), were



generated using the methods described previously.<sup>25–27,50</sup> Briefly, the respective shuttle plasmids were integrated into G47ΔBAC using Cre-mediated recombination in DH10B *Escherichia coli*, and proper recombination confirmed by restriction analysis of BAC clones. Next, the resulting BAC and an Flpe-expressing plasmid were cotransfected to Vero cells, to remove the BAC-derived sequences and the *EGFP* gene, and allow virus to be produced. Each recombinant virus was plaque purified and expanded. All the recombinant oHSVs, oHSV, oHSV-TRAIL, and oHSV-Fluc, express *E. coli* lacZ driven by endogenous ICP6 promoter (**Supplementary Figure S1a**). oHSV bears no additional transgene sequences, and oHSV-TRAIL carries S-TRAIL driven by herpes simplex virus immediate early 4/5 promoter. Cytomegalovirus immediate early promoter was used to express Fluc-by oHSV-Fluc. For the viral growth assay, cells plated on 96-well plates were infected with oHSV at MOI = 1. After virus adsorption, media was replaced and culture continued. Forty eight hours after infection, culture supernatant was harvested. Titters of infectious virus were determined by plaque assay on Vero cells (American Type Culture Collection, Manassas, VA). The concentrations of TRAIL in conditioned media of GBM cells infected with oHSV-TRAIL at various MOIs were determined by ELISA using a TRAIL Immunoassay Kit (Biosource International, Camarillo, CA) using recombinant hTRAIL expressed in *E. coli* as a standard.

**In vitro bioluminescence assays.** To determine the effects of S-TRAIL, oHSV, and oHSV-TRAIL on GBM viability and caspase activation, GBM cells were seeded on 96-well plates ( $0.5 \times 10^4$ /well) and treated with different doses S-TRAIL (0–1,000 ng/ml) or different MOIs of oHSV or oHSV-TRAIL 24 hours after plating. Cell viability was measured by determining the aggregate cell metabolic activity using an ATP-dependent luminescent reagent (CellTiter-Glo; Promega, Madison, WI) and caspase activity was determined using a DEVD-aminoluciferin (Caspase-Glo-3/7, Promega) according to manufacturer's instructions. For dual-luciferase imaging of GBM cell viability and oHSV-Fluc distribution in the cells, Fluc and Rluc activity were measured in LN229-RmC and GBM8F-RmC cells by Dual-Glo luciferase assay system (Promega) according to manufacturer's instructions. All experiments were performed in triplicates.

**Detection of apoptosis by flow cytometry using annexin V staining.** After a 24 hours treatment with oHSV, oHSV-TRAIL, or PBS (control), cells were stained with FITC-conjugated annexin V (Invitrogen) and propidium iodide (2 μg/ml) in accordance with the manufacturer's instructions. Cells were subjected to FACS analysis with a FACSCaliber (Becton Dickinson, Franklin Lakes, NJ). Data acquisition and analysis were performed by CellQuest program (Becton Dickinson).

**Western blot analysis.** Following treatment, GBM cells were lysed with NP40 buffer supplemented with protease (Roche, Indianapolis, IN) and phosphatase inhibitors (Sigma-Aldrich). Twenty micrograms of harvested proteins from each lysate were resolved on 10% SDS-PAGE, and immunoblotted with antibodies against caspase-8 (Cell Signaling), cleaved PARP (Cell Signaling, Danvers, MA), p-44/42MAPK (ERK 1/2) (Cell Signaling), phospho-p44/42MAPK (ERK 1/2) (Thr202, Thr204) (Cell Signaling), SAPK/JNK (Cell Signaling), phospho-SAPK/JNK (Thr183/Thr185) (Cell Signaling), p38-MAPK (Cell Signaling), phospho-p38 MAPK (Cell Signaling), caspase-9 (Stressgen, Pharmingdale, NY), Bcl2 (Abcam, Cambridge, MA) or  $\alpha$ -tubulin (Sigma-Aldrich); and blots were developed by chemiluminescence after incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, Santa Cruz, CA). Blots were then exposed to film (3 seconds to 5 minutes) and quantification of western blot signals was performed using Image J. The data was normalized to  $\alpha$ -tubulin expression. For inhibition studies, pan-caspase inhibitor, Z-VAD-FMK (Promega) JNK inhibitor SP600125 (Sigma-Aldrich), and the MEK inhibitor, U0126, (Promega Corporation) were used.

**Intracranial GBM cell implantation and in vivo bioluminescence imaging.** To follow viral distribution in intracranial GBMs, LN229-RmC, and GBM8F-RmC GBM ( $5 \times 10^5$  cells per mouse;  $n = 3$  each GBM line)

were stereotactically implanted into the brains (right striatum, 2.5-mm lateral from bregma and 2.5-mm deep) of SCID mice (6 weeks of age; Charles River Laboratories, Wilmington, MA). Five days later, mice-bearing intracranial GBMs were injected with oHSV-Fluc ( $6 \mu\text{l}$  of  $2.0 \times 10^8$  plaque-forming unit/ml) intratumorally at the same coordinate as the tumor implantation, and viral distribution was followed by Fluc bioluminescence imaging over time as described previously.<sup>33</sup> To follow changes in tumor volume and mice survival after treatment, LN229-FmC GBM cells ( $5 \times 10^5$  per mouse;  $n = 31$ ) were stereotactically implanted into the brains (right striatum, 2.5-mm lateral from bregma and 2.5-mm deep) of SCID mice (6 weeks of age). Mice were imaged for the presence of tumors by Fluc bioluminescence imaging and mice-bearing tumors were injected with  $6 \mu\text{l}$  of  $2.0 \times 10^8$  plaque-forming unit/ml of oHSV ( $n = 10$ ), oHSV-TRAIL ( $n = 10$ ), or PBS ( $n = 11$ ) intratumorally. Two days post-treatment, mice ( $n = 3$  in each group) were sacrificed for immunohistochemical analysis as described below. Fourteen days post-treatment, mice were again injected intratumorally with oHSV, oHSV-TRAIL, or PBS as described above. Mice were imaged for Fluc bioluminescence imaging and followed for survival and sacrificed when neurological symptoms became apparent. All *in vivo* procedures were approved by the Subcommittee on Research Animal Care at MGH.

**Tissue processing and immunohistochemistry.** Mice were perfused by pumping ice-cold 4% paraformaldehyde directly into the heart and the brains were fixed in 4% paraformaldehyde and frozen sections were obtained for hematoxylin and eosin staining and immunohistochemistry. 5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) staining was performed to identify lacZ-expressing infected cells. For cleaved-caspase-3 staining, sections were incubated for 1 hour in a blocking solution (0.3% bovine serum albumin, 8% goat serum, and 0.3% Triton-X100) at room temperature, followed by incubation at 4°C overnight with anti-cleaved-caspase-3 (Cell Signaling) diluted in blocking solution. Sections were incubated in Alexa Fluor 649 goat anti-rabbit secondary antibody (Invitrogen), and visualized using confocal microscope (LSM Pascal; Zeiss, Oberkochen, Germany). The percentage of cleaved caspase-3 positive cells was calculated by counting the positive cells in randomly selected field of views under a microscope.

**In vitro invasion assay.** The invasive capacity of GBM8F cells was tested using two chamber *in vitro* invasion assays (BD BioCoat Matrigel Invasion Chambers). GBM8F cells were seeded in the matrigel-coated upper chamber, infected with oHSV and oHSV-TRAIL at MOI = 1 and 24 hours later the noninvading cells were removed from the upper surface of the invasion membrane and the cells on the lower surface were stained with Diff-Quick staining kit (IMEB Inc, San Marco, CA). The average number of cells/field was determined by counting the cells in 8 random fields/well in  $\times 10$  images of each well captured.

**In vivo invasion study.** GBM8F-FmC GBM cells ( $3 \times 10^5$  per mouse;  $n = 9$ ) were stereotactically implanted into the brain (right striatum, 2.5-mm lateral from bregma and 2.5-mm deep) of SCID mice (6 weeks of age; Charles River Laboratories). Tumor-bearing mice were intratumorally injected with oHSV, oHSV-TRAIL, or PBS ( $n = 3$ , each group) and 14 days post injection, mice were perfused and brains were removed and sectioned for hematoxylin and eosin staining and mCherry visualization. Brain sections on slides were visualized for mCherry expression and the number of GBM8F tumor invading toward adjacent normal brain tissue was counted and compared in different mice groups.

**Statistical analysis.** Data were analyzed by Student *t*-test when comparing two groups. Data were expressed as mean  $\pm$  SD and differences were considered significant at  $P < 0.05$ . Kaplan–Meier analysis was used for mouse survival studies, and the groups were compared using the log-rank test.

## SUPPLEMENTARY MATERIAL

**Figure S1.** Schema of oHSV-bearing Fluc or S-TRAIL transgene.

**Figure S2.** Pharmacodynamics of oHSV *in vitro* at different MOIs.

**Figure S3.** oHSV-TRAIL virus yield is similar to oHSV yield and mediates potent cytotoxicity in resistant GBM cells.

**Figure S4.** oHSV-TRAIL mediates potent cytotoxicity in sensitive GBM cells.

**Figure S5.** oHSV-TRAIL-mediated potent cytotoxicity is independent of DR4/DR5 expression.

**Figure S6.** oHSV-TRAIL inhibits growth of both TRAIL and oHSV resistant LN229-Fluc-mCherry GBMs *in vivo*.

**Figure S7.** H&E stained mouse brain sections harboring intracerebral tumors.

## ACKNOWLEDGMENTS

We thank Dr Howard Chen for his help with Annexin-V staining and FACS analysis; Dr Andrew Kung for providing pico2-Fluc-mCherry lentiviral vector. This work was supported in part by Alliance for Cancer Gene Therapy (K.S.) National Institutes of Health (NS03677; R.L.M. and CA138922-01; NS076873; K.S.) American Cancer Society (K.S.) and James McDonnell Foundation (K.S.). The authors declared no conflict of interest.

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