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Effective Radiovirotherapy for Malignant Gliomas by Using Oncolytic Measles Virus Strains Encoding the Sodium Iodide Symporter (MV-NIS)

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Abstract

Engineered measles virus (MV) strains deriving from the vaccine lineage represent a promising oncolytic platform and are currently being tested in phase I trials. In this study, we have demonstrated that MV strains genetically engineered to express the human sodium iodide symporter (NIS) have significant antitumor activity against glioma lines and orthotopic xenografts; this compares favorably with the MV strain expressing the human carcinoembryonic antigen, which is currently in clinical testing. Expression of NIS protein in infected cells results in effective concentration of radioactive iodine, which allows for *in vivo* monitoring of localization of MV-NIS infection by measuring uptake of ^{123}I or ^{99m}Tc. In addition, radiovirotherapy with MV-NIS followed by ^{131}I administration resulted in significant increase of MV-NIS antitumor activity as compared with virus alone in both subcutaneous ($p = 0.0003$) and orthotopic ($p = 0.004$) glioblastoma models. In conclusion, MV-NIS-based radiovirotherapy has significant antitumor activity against glioblastoma multiforme and represents a promising candidate for clinical translation.

Introduction

Glioblastoma is the most common primary brain tumor, and it is associated with a dismal prognosis. Even with the best available treatment, including surgery, radiation, and chemotherapy, the median survival is between 12 and 18 months (Stupp *et al*., 2005; Grossman *et al*., 2010). Despite recent advances in the field of oncology, glioblastoma remains an incurable disease, and novel therapeutic approaches are needed. Because it rarely metastasizes, glioblastoma is a good candidate for locoregional delivery of novel therapeutics, including gene and virus therapies.

Virotherapy is a promising novel therapeutic approach in the treatment of malignancies. Measles virus (MV) is an RNA virus belonging to the family of Paramyxoviridae. Derivatives of the Edmonston (Edm) vaccine strain of the MV have a long history of safe administration to patients through vaccination programs (Griffin *et al*., 2008). MV-Edm vaccine strains enter cells primarily through the CD46 receptor, and the ability of the virus to induce a cytopathic effect, cell-to-cell fusion, and formation of multinucleated aggregates (syncytia) (Dörig *et al*., 1993; Naniche *et al*., 1993) is primarily determined by receptor density (Anderson *et al*., 2004). The MV receptor CD46 is highly expressed in multiple solid tumors, including gliomas, resulting in the ability of MV to preferentially infect tumor cells (Phuong *et al*., 2003; Anderson *et al*., 2004; Ulasov *et al*., 2006).

Our laboratory has previously shown that MV-Edm derivatives engineered to express the human carcinoembryonic antigen (CEA; MV-CEA virus) have significant antitumor activity against glioma cell lines and mouse glioblastoma models (Phuong *et al*., 2003). These results have led to the initiation of a phase I clinical trial in recurrent glioblastoma patients, which is currently ongoing (Allen *et al*., 2008a; Msaouel *et al*., 2009a). Although MV-CEA could allow monitoring of viral replication based on detection of the measurable marker CEA in the blood, it does not allow for localization of viral spread. To address this concern, the MV-Edm virus has been engineered to express the human sodium iodide symporter (NIS) (Dingli *et al*., 2004). Expression of the NIS protein, a sodium/iodine transporter, can allow noninvasive monitoring of viral infection *in vitro* and *in vivo*

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by using ^{123}I, ^{124}I, ^{125}I, or ^{99m}Tc isotopes (Spitzweg *et al*., 2000; Dingli *et al*., 2004; Msaouel *et al*., 2009b).

In addition, MV-NIS represents a promising platform to allow augmentation of a virus-induced cytopathic effect through radiovirotherapy, because the NIS transgene can allow uptake of the β- and γ-emitter ^{131}I by cancer cells infected by the virus (Dingli *et al*., 2004; Msaouel *et al*., 2009b). We have previously shown that the activity of oncolytic MV-Edm strains against glioblastoma can be enhanced by external beam radiation therapy (EBRT) (Liu *et al*., 2007). We therefore hypothesized that MV-NIS activity against glioblastoma could be enhanced by local radiation treatment, accomplished via accumulation of ^{131}I in infected cancer cells expressing the NIS protein. This approach can be especially attractive in glioma treatment, because radiation therapy represents a key therapeutic modality for patients affected by this disease, and use of ^{131}I at recurrence could represent a less toxic alternative to re-irradiation using EBRT. Therefore, we designed this study to evaluate the efficacy of MV-NIS against glioma lines and xenografts, as well as the impact of the addition of ^{131}I on the observed antitumor effect.

We demonstrated that MV-NIS has significant cytopathic effect against glioma lines *in vitro* and xenografts *in vivo*, with increased viral proliferation and faster tumor-cell killing as compared with MV-CEA. The addition of ^{131}I further improved the efficacy of MV-NIS and prolonged survival in both flank and intracranial mouse models. Furthermore, we demonstrated that NIS enables noninvasive tracking of MV-NIS infection and localization in both subcutaneous and orthotopic xenografts. These promising results underline the significant translational potential of the MV-NIS-mediated radiovirotherapy in the treatment of gliomas.

Materials and Methods

Viral construction

Construction of MV-CEA (Peng *et al*., 2002) and MV-NIS (Dingli *et al*., 2004) have been previously described (Fig. 1). The viruses were propagated in Vero cells and titrated as previously described (Msaouel *et al*., 2009b).

Comparison of MV-CEA and MV-NIS cytopathic effects in vitro

Cells were plated in six-well plates in duplicate at a density of 5×10^5 cells/well (U87, U251, GBM6, GBM10, GBM12, GBM39, GBM43, and GBM44). Twenty-four hours after plating, cells were infected at a multiplicity of infection (MOI) of 1.0 or 0.1 with either MV-CEA or MV-NIS in 1 ml of Opti-MEM for 2 hr at 37°C. At the end of the incubation period, the virus was removed and the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1×penicillin/streptomycin. The number of viable cells in each well was determined using a hemacytometer at 3, 5, 7, and 9 days after infection using the trypan blue exclusion assay. The percentage of surviving cells was calculated by dividing the number of viable cells in the infected well by the number of viable cells in uninfected wells corresponding to the same time point.

Assessment of radioactive iodine uptake in vitro

Cells were plated in six-well plates in duplicate at a density of 5×10^5 cells/well (U251, GBM12, GBM39, and GBM43). Twenty-four hours after plating, cells were infected at an MOI of 1.0 or 0.1 with MV-NIS in 1 ml of Opti-MEM for 2 hr at 37°C; uninfected wells served as controls. At the end of the incubation period, the virus was removed and the cells were maintained in DMEM containing 10% FBS and 1×penicillin/streptomycin. Forty-eight hours post infection, either 0.9 ml Hanks' balanced salt solution (HBSS) with HEPES (pH 7.3) or 0.8 ml HBSS with HEPES and 0.1 ml of 0.01 m*M* potassium perchlorate were added per well. $Na^{125}I$ (0.1 μCi/ml, pH 7.3) was then added to each well, and activity was determined in a gamma counter.

Comparative assessment of virotherapy efficacy in orthotopic tumor models

All animal experiments were approved by the Mayo Institutional Animal Care and Use Committee. Glioblastoma orthotopic xenografts were established by implantation of 3×10^5 primary glioblastoma cells (GBM43 and GBM39) into the right caudate nucleus of 5-week-old BALB/c nude mice, using the small animal stereotactic frame (ASI Instruments, Warren, MI) and a 26-gauge Hamilton syringe. Treatment was initiated 1–2 weeks post implantation by intratumoral injection using the same coordinates as for implantation 1.5×10^5 $TCID_{50}$ per dose was administered in 10 μl three times per week for a total of four doses. The following groups were included (10 animals each): UV-inactivated MV-CEA, MV-CEA, or MV-NIS. Mice were observed daily and were euthanized when neurological impairment or greater than 10% weight loss was observed. The experiment was terminated at 120 days post implantation. Brains of euthanized animals were fixed in paraformaldehyde and embedded in paraffin for subsequent analysis.

Assessment of NIS expression in vivo *in a flank xenograft glioblastoma model*

U251 flank tumors were developed by implantation of 5×10^6 U251 cells mixed 1:2 with Matrigel (BD Biosciences, Bedford, MA) in BALB/c nude mice. Water for these mice was supplemented with levothyroxine (5 mg/ml) to suppress thyroidal NIS expression. On days 12 and 13 post tumor implantation, mice received an intratumoral dose of

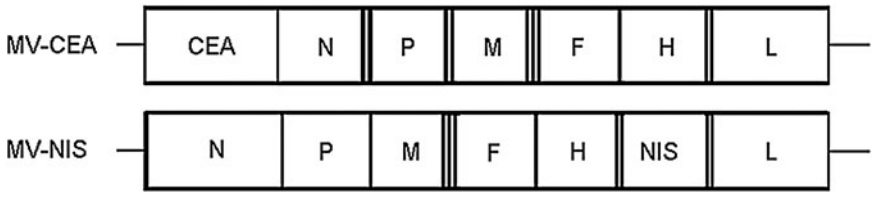

FIG. 1. Schematic representation of MV-Edm strains. F, fusion protein gene; H, hemagglutinin gene; L, large protein gene; M, matrix protein gene; N, nucleoprotein gene; P, phosphoprotein gene; CEA, human carcinoembryonic antigen gene; NIS, sodium iodide symporter gene.

1.5×10^6 $TCID_{50}$ MV-NIS or UV-inactivated MV-NIS. Daily tumor imaging was performed 60 min after the mice were injected with ^{123}I (1 mCi) intraperitoneally using a gamma camera (Helix System; Elscint, Haifa, Israel).

Assessment of NIS expression in vivo *in an orthotopic glioblastoma model*

Orthotopic xenografts were established in female BALB/c nude mice by stereotactic implantation of 3×10^5 GBM43 cells. Mice were treated orthotopically on days 5, 7, 9, 12, 14, and 16 post implantation with MV-NIS (2.5×10^6 $TCID_{50}$ per treatment dose) or UV-inactivated MV-NIS. Mice were imaged twice weekly until day 20 after completion of viral treatment. Images were obtained using a microSPECT/CT imaging system 1 hr after intraperitoneal injection of 1 mCi of ^{123}I (group 1) or 1 mCi of ^{99}Tc (group 2).

Assessment of combination therapy with MV-NIS and ^{131}I in vivo *in flank and orthotopic glioblastoma xenografts*

U251 tumors were developed by implantation of 1×10^7 U251 cells mixed 1:2 with Matrigel (BD Biosciences) in the flank of BALB/c nude mice. Seven or eight mice were randomized into four groups for treatment with either UV-inactivated virus, ^{131}I, MV-NIS, or MV-NIS in combination with ^{131}I. The UV group was treated with UV-inactivated virus intratumorally on days 12 and 13 post tumor implantation. The ^{131}I group received 1 mCi of ^{131}I intraperitoneally on day 16 post tumor implantation. The MV-NIS group was treated with MV-NIS on days 12 and 13 post tumor implantation (2×10^6 $TCID_{50}$ per dose). The MV-NIS/^{131}I group was treated with MV-NIS on days 12 and 13 (2×10^6 $TCID_{50}$ per dose in 0.1 ml volume) and received 1 mCi of ^{131}I intraperitoneally on day 3 after the last viral treatment. Mice were euthanized when any tumor dimension exceeded 2.0 cm or ulceration was noted.

Orthotopic xenografts were established in BALB/c nude mice by stereotactic implantation of 3×10^5 GBM43 cells. Mice were treated orthotopically (^{131}I administered intraperitoneally) with either UV-inactivated virus, MN-NIS, ^{131}I, or MV-NIS/^{131}I. MV-NIS was administered intratumorally using the same stereotactic coordinates used for tumor cell implantation on days 8, 13, and 15 following implantation (0.6×10^6 $TCID_{50}$ per dose in 10 μl). ^{131}I (1 mCi) was administered intraperitoneally on day 18 post implantation. Mice were followed daily and were euthanized when signs of CNS-related symptoms or greater than 10% body weight loss was observed.

Histology and in situ *hybridization for MV-NIS N (nucleoprotein) mRNA*

BALB/c nude mice had GBM43 tumors implanted as described above. After NIS expression was confirmed via ^{131}I imaging, the mice were killed and paraffin-embedded brains were analyzed. Serial sections (12 μm) were used for either hematoxylin and eosin (H&E) staining or *in situ* hybridization (ISH) for MV-NIS N mRNA as previously described (Allen *et al*., 2008b). A custom-designed digoxigenin-labeled oligonucleotide probe (GeneDetect) was used for ISH. The samples were treated with proteinase K (20 mg/ml) for 20 min at 37°C, washed twice with phosphate-buffered saline/glycine, and prehybridized for 2 hr at 39.4°C. The sections were then washed and incubated overnight with the digoxigenin-labeled oligonucleotide probe (250 ng) at 39.4°C in a humidified chamber. The next day the slides were washed in saline-sodium citrate/dithiothreitol followed by the detection step performed using anti-digoxigenin-AP, Fab fragments (Roche Diagnostics, Indianapolis, IN) at 4°C in a humidified chamber, followed by serial washes, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate detection, and counterstaining with nuclear fast red (Sigma, St. Louis, MO).

Results

The MV-CEA (Peng *et al*., 2002), MV-NIS (Dingli *et al*., 2004), and MV-Edm strains were used in these comparative experiments (Fig. 1).

MV-NIS infects glioblastoma cell lines more effectively than MV-CEA

The infectivity and cytopathic effect of MV-NIS were tested in several glioblastoma cell lines, both established and primary [derived from glioblastoma (GBM) patients and maintained as xenografts]. MV-NIS demonstrated significant cytopathic activity in all tested glioma cell lines (U87, U251, GBM6, GBM10, GBM12, GBM39, GBM43, and GBM44) (Fig. 2A and B). When compared with MV-CEA, MV-NIS had enhanced cytopathic activity *in vitro*, even at very low MOI (MOI of 0.1; Fig. 2C and D), and increased replication in one-step viral growth curves performed in Vero and U87 glioma cells (Fig. 2E and F).

MV-NIS infection results in increased uptake of ^{125}I in vitro

We also assessed the ability of MV-NIS-infected glioma cells to concentrate radioactive iodine. As Fig. 2G illustrates, glioblastoma cell lines (U251, GBM12, GBM39, and GBM43) infected with MV-NIS *in vitro* were able to effectively concentrate radioactive iodine (^{125}I). This effect was abrogated with the addition of potassium perchlorate ($KClO_4$), which specifically blocks the NIS transporter, thus demonstrating that ^{125}I uptake in these lines was due to functional NIS protein expression (Fig. 2G).

MV-NIS shows increased antitumor activity as compared with MV-CEA in orthotopic glioblastoma xenograft models

These *in vitro* results suggested that MV-NIS could be a superior oncolytic agent compared with MV-CEA against glioblastoma. This hypothesis was tested in the orthotopic GBM39 and GBM43 GBM models. These models are derived from Mayo Clinic GBM patients, kept as xenografts, and maintain the molecular characteristics and invasiveness of the primary tumor they derive from (Giannini *et al*., 2005; Carlson *et al*., 2011). Treatment was initiated at 1 week (GBM43) or 2 weeks (GBM39) after intracranial implantation of primary glioblastoma cell lines. Mice received a total of four treatments of the virus (1.5×10^5 $TCID_{50}$ per dose). As seen in Fig. 3, the groups treated with MV derivatives had a statistically significant prolongation of survival as compared with

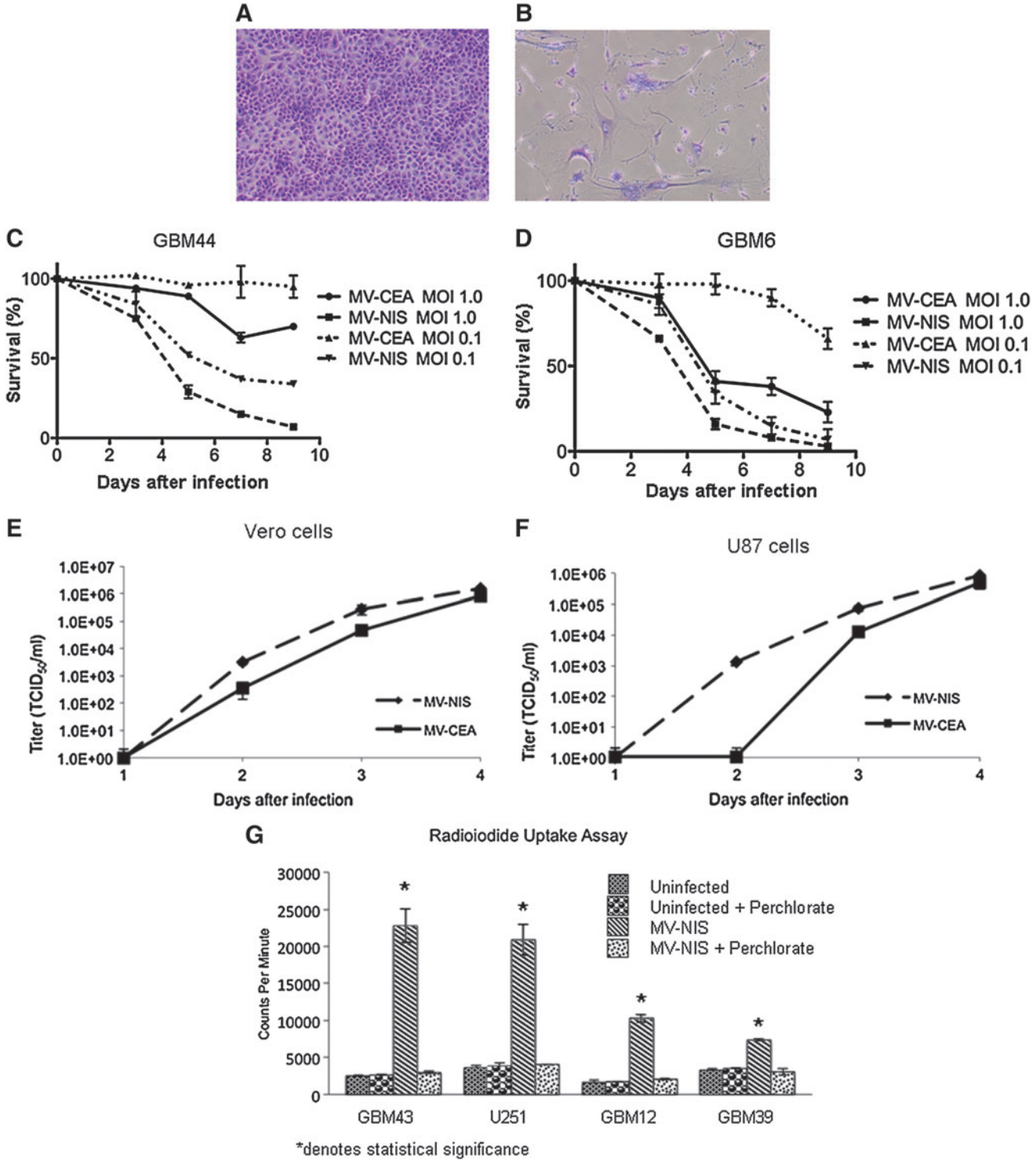

FIG. 2. Increased cytopathic effect and replication of MV-NIS as compared with MV-CEA in primary and established glioma lines *in vitro*. GBM43 cells 5 days after either mock infection **(A)** or MV-NIS infection at MOI of 1.0 **(B)** (crystal violet stain) are shown. Infected cells demonstrate the characteristic cytopathic effect with formation of syncytia. Cytopathic effect of MV-CEA and MV-NIS on GBM44 cells **(C)** and GBM6 cells **(D)** at different MOIs, was determined by trypan blue exclusion assay. MV-NIS infection led to faster elimination of tumor-cell monolayers at comparable MOIs. One-step viral growth curves demonstrate increased replication of MV-NIS as compared with MV-CEA in Vero cells **(E)** and U87 glioma cells **(F)** ± **(G)** *In vitro* ^{125}I uptake by glioblastoma cell lines infected by MV-NIS at an MOI of 1.0. Cells infected with MV-NIS effectively accumulated ^{125}I. Uptake was significant (above 7,000 CPM) even in the glioma line (GBM39) with the lowest ability to concentrate iodine. Uninfected controls did not concentrate ^{125}I. Infected cells exposed to $KClO_4$, a competitive inhibitor of iodine uptake by NIS, were unable to concentrate iodine, similar to the uninfected controls.

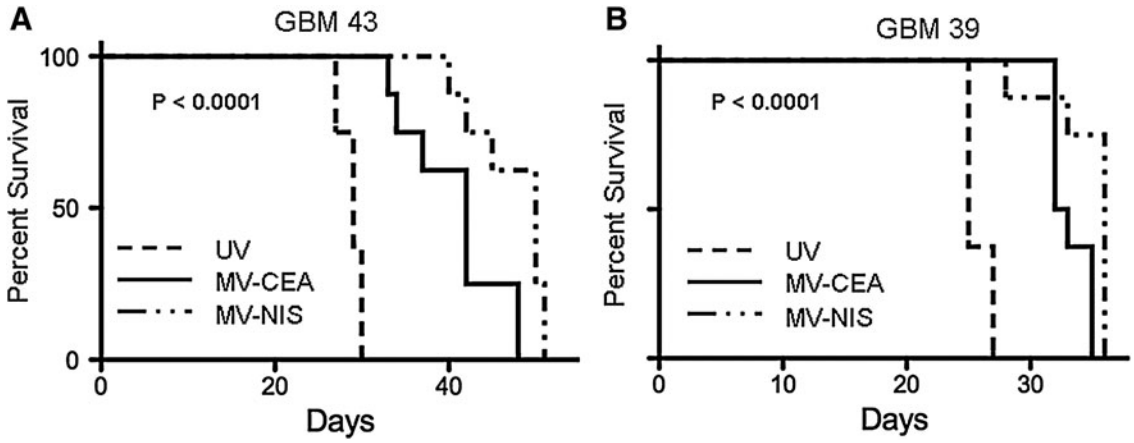

FIG. 3. Oncolytic activity of MV-NIS as compared with MV-CEA in orthotopic xenograft glioblastoma model. **(A)** GBM43 cells. **(B)** GBM39 cells. Mice ($n=8$ per group) received four treatments with MV-NIS or MV-CEA for a total dose of 6×10^5 $TCID_{50}$ control animals received UV inactivated virus. Mice treated with MV-NIS had a statistically significant prolongation of survival as compared with UV-inactivated control ($p<0.001$ in both models), and a modest but significant prolongation of survival as compared with MV-CEA-treated mice (A, $p=0.012$; B, $p=0.009$).

the corresponding group treated with UV-inactivated virus ($p<0.001$) in both glioma models. When the two treatment groups were compared, the MV-NIS-treated group had a modest but statistically significant prolongation of survival when compared with the MV-CEA-treated group ($p=0.009$ in the GBM39 model, $p=0.012$ in the GBM43 model).

MV-NIS efficiently infects glioblastoma xenografts and facilitates noninvasive imaging of tumor infection

To evaluate expression of the NIS protein after viral infection *in vivo,* both mouse flank xenografts and orthotopic GBM models were used. Mice with U251 flank tumors were treated with two intratumoral injections (1.5×10^6 $TCID_{50}$) of MV-NIS with UV-inactivated MV-NIS treated animals serving as control. NIS expression was monitored daily 1 hr after injection of 1 mCi of ^{123}I with a gamma-camera. Significant iodine uptake was demonstrated within the tumor (Fig. 4A). In contrast, there was no ^{123}I uptake in the tumors of UV-inactivated MV-NIS-treated animals (Fig. 4B). The signal, as expected, was also seen in thyroid and stomach, which normally express the NIS transporter protein (Josefsson *et al.*, 2002), as well as bladder due to excretion of the radioisotope in urine. Peak signal was observed at 3 days after the last intratumoral treatment.

To evaluate NIS expression in an intracerebral GBM43 model, 5 days following orthotopic implantation of GBM43 cells, treatment was initiated with MV-NIS or UV-inactivated MV-NIS for a total of six treatments (2.5×10^6 $TCID_{50}$ per dose). Mice were imaged twice weekly 1 hr after intraperitoneal injection of 1 mCi of ^{123}I or ^{99m}Tc. There was increased tumor uptake of radioisotope in MV-NIS-treated mice, which peaked at 3 days and persisted up to 20 days following viral administration (Fig. 4C). In contrast, there was no tracer uptake in the tumors of UV-inactivated MV-NIS-treated animals (Fig. 4D). Therefore, *in vivo* infection with MV-NIS results in expression of NIS protein in tumor tissue and concentration of ^{123}I or ^{99m}Tc, which could be used for assessment and localization of viral infection, and could serve as the basis for therapeutic application of ^{131}I.

Combined radiovirotherapy with MV-NIS and ^{131}I results in improved antitumor efficacy as compared with virotherapy alone in both subcutaneous and orthotopic mouse glioblastoma models

To evaluate the added benefit of ^{131}I to MV-NIS treatment *in vivo*, we tested the efficacy of this combination radiovirotherapy approach in both subcutaneous and orthotopic glioblastoma models. After establishment of U251 flank tumors, mice were treated with MV-NIS or UV-inactivated control on days 12 and 13 post implantation (2×10^6 $TCID_{50}$ per dose). Low doses of MV-NIS were chosen to facilitate the demonstration of additional benefit derived from the radioisotope treatment. The combination therapy group received 1 mCi of ^{131}I intraperitoneally 3 days after the last viral treatment, coinciding with maximal NIS expression as determined by the ^{123}I imaging study in the subcutaneous xenograft model. As Fig. 5A demonstrates, treatment of tumors with only two MV-NIS injections did not improve the survival of the animals. Furthermore, there was no significant difference in survival between ^{131}I-treated animals and UV-inactivated virus-treated controls ($p=0.26$). The addition of ^{131}I, however, resulted in considerable improvement in survival of the animals compared with MV-NIS or UV-inactivated MV-NIS-treated groups ($p=0.011$). Comparison of the ^{131}I/MV-NIS treated group with the MV-NIS treated group showed a significant survival advantage for the combination treated animals ($p=0.0003$). Mice treated with ^{131}I did exhibit late gastrointestinal toxicity and failure to thrive, however, likely related to the radioisotope treatment, which was manifested as weight loss around day 60.

The potential additional benefit of ^{131}I administration following MV-NIS infection was also tested in an orthotopic GBM model (Fig. 5 B). Mice were inoculated intracranially with GBM43 cells and then treated with MV-NIS or with UV-inactivated control on days 8, 11, and 13 after cell inoculation (6×10^5 $TCID_{50}$ per injection). The combination (MV-NIS plus ^{131}I) therapy group received 1 mCi of ^{131}I intraperitoneally, at 3 days following the last viral dose, timing was determined based on the imaging study. Mice were then

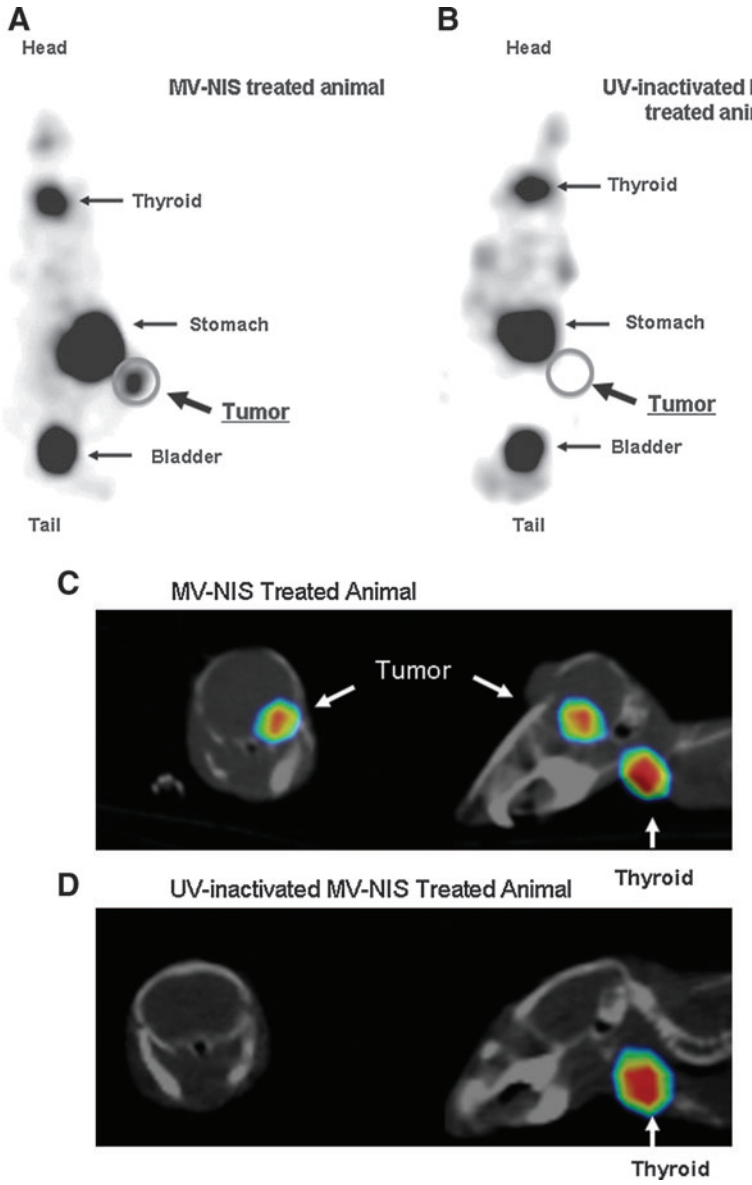

FIG. 4. *In vivo* imaging of glioblastoma xenografts infected with MV-NIS. **(A)** Mice with U251 subcutaneous tumor xenografts received two intratumoral treatments of 1.5×10^6 $TCID_{50}$ of the MV-NIS. Expression of the NIS protein in the tumor was confirmed by ^{123}I uptake. A representative image is shown at 3 days following the viral treatment. **(B)** No tracer uptake was observed in tumors of UV-inactivated MV-NIS-treated animals. **(C)** GBM43 xenografts were established orthotopically by stereotactic implantation of 3×10^5 cells. Five days later, mice were treated with six intratumoral injections of the MV-NIS with UV-inactivated virus serving as control for a total dose of 1.5×10^7 $TCID_{50}$. Gamma camera imaging was performed at multiple time points following administration of ^{123}I or ^{99}mTc. A representative image of MV-NIS-treated tumor at 20 days following viral administration is shown. **(D)** No tracer accumulation was observed in orthotopic tumors of UV-inactivated MV-NIS-treated animals.

followed and observed for any CNS symptoms and overall health status. Although the MV-NIS treatment group had superior survival compared with the UV-inactivated control ($p=0.0004$), the addition of ^{131}I further improved the efficacy of the MV-NIS treatment as compared with MV-NIS alone ($p=0.004$), indicating a synergistic effect of radiovirotherapy. There was no significant difference between ^{131}I-treated animals and UV-inactivated virus-treated controls ($p=0.46$). Similar to the subcutaneous model, mice treated with ^{131}I did exhibit gastrointestinal toxicity, manifested as weight loss after day 55, with no neurological symptoms.

MV-NIS efficiently infects glioblastoma orthotopic brain xenografts

To further document MV-NIS infection *in vivo,* animals implanted with GBM43 cells and treated with MV-NIS were euthanized following demonstration of MV-NIS expression by imaging. H&E staining revealed visible syncytia in tumor samples from the MV-NIS-treated group, confirming viral infection and cytopathic effect (Fig. 5C). *In situ* hybridization for MV N mRNA was also performed, which was positive in the MV-NIS-treated group, further confirming active replication of the virus (Fig. 5D).

Discussion

Despite its initial short-term response to radiation and chemotherapy, glioblastoma tumors invariably progress (Wallner *et al*., 1989; Stupp *et al*., 2005; Grossman *et al*., 2010). They rarely metastasize, however, which makes them good candidates for locoregional delivery of virotherapy agents. Our laboratory has demonstrated *in vitro* and *in vivo* antitumor activity of MV strains against glioma lines and xenografts, which has led to initiation of a phase I clinical trial of MV-CEA in recurrent GBM patients (Phuong *et al*., 2003; Allen *et al*., 2008).

Radiotherapy has been the mainstay of treatment for glioblastoma for many years (Shapiro *et al*., 1989; Galanis

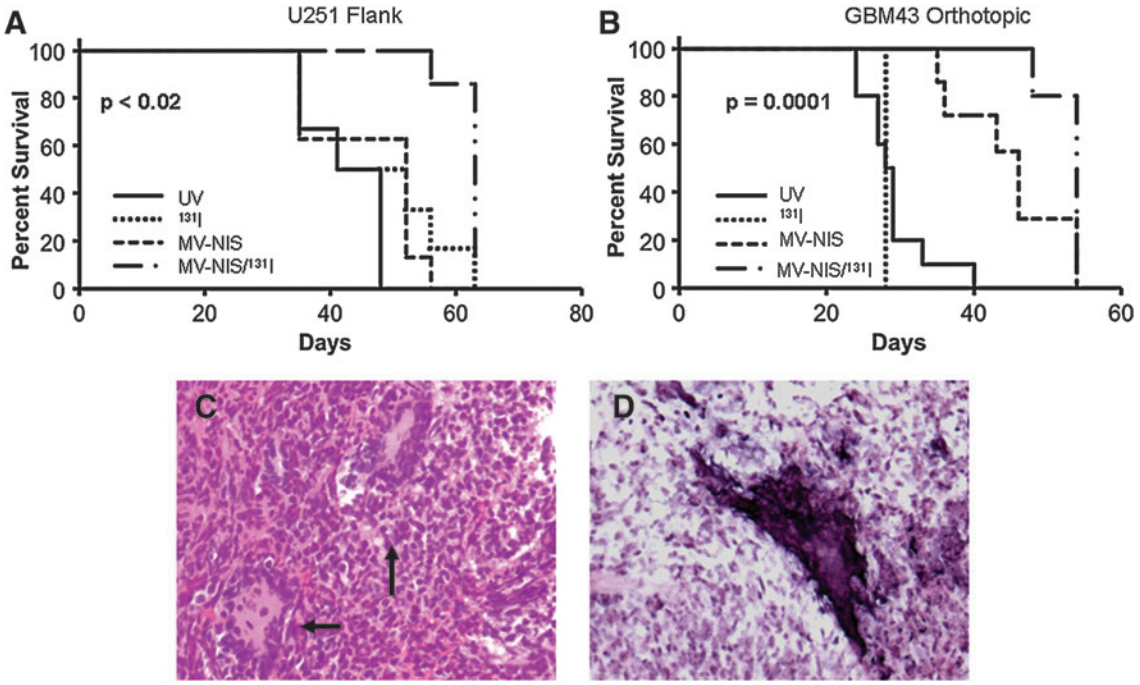

FIG. 5. Therapeutic effect of radiovirotherapy in glioblastoma xenograft mouse models. **(A)** Mice ($n=8$ per group) with established right flank U251 tumors received two injections with MV-NIS or matched UV-inactivated control (4×10^6 $TCID_{50}$ total dose). Three days later, they were treated with 1 mCi of ^{131}I through intraperitoneal injection or PBS only. Mice in the radiovirotherapy group had significantly longer survival ($p=0.0003$) over mice treated with MV-NIS or ^{131}I alone. **(B)** Mice ($n=8$ to 10 per group) with established GBM43 orthotopic brain tumors received four treatments starting 8 days post implantation with MV-NIS or matched UV-inactivated control (1.8×10^6 $TCID_{50}$ total dose). The radiovirotherapy group received ^{131}I intraperitoneally 3 days after the last viral treatment, with the timing determined based on imaging studies. MV-NIS treatment extended the survival of the animals ($p=0.0004$) as compared with the UV-MV-treated control group, and addition of ^{131}I significantly extended survival of the animals as compared with the MV-NIS-only treated group ($p=0.039$). **(C)** Sections from brains from GBM43-implanted mice were stained with H&E: extensive syncytia formation was observed (arrow). Original magnification, 200×. **(D)** *In situ* hybridization for MV N mRNA is positive, confirming active viral replication in tumors.

and Buckner, 2000; Simpson and Galanis, 2006). Our laboratory has previously demonstrated synergistic activity when combining MV therapy with EBRT. Irradiation of glioma cells is associated with increased replication of MV (Liu *et al.*, 2007), and resulted in augmentation of the antitumor effect *in vitro* and *in vivo* through an increase in viral replication, activation of the extrinsic caspase pathway, and an increase in apoptosis (Liu *et al.*, 2007).

The NIS is a plasma membrane glycoprotein, most commonly studied in connection with the thyroid gland, where NIS mediates the active transfer of iodine into the thyroid follicular cells as a crucial first step in thyroid hormone biosynthesis (Carrasco, 1993; Dadachova and Carrasco, 2004). NIS expression from thyroid tissue allows the use of ^{131}I for treatment of thyroid cancer metastasis (Dadachova and Carrasco, 2004), including brain metastases (Hjiyiannakis *et al.*, 1996). In proof-of-principle experiments, Cho and colleagues demonstrated that stable transduction of the F98 glioma lines with the NIS gene with a retroviral vector allowed tumor imaging using ^{99m}Tc or ^{123}I scintigraphy. Use of radioactive iodine prolonged animal survival in this model (Cho *et al.*, 2002; Cho, 2004). Despite these initial proof-of-principle studies, no follow-up work had been performed exploiting the concept of NIS-gene delivery as a therapeutic modality in the treatment of gliomas. The MV-Edm derivative expressing NIS (MV-NIS) virus allows us to capitalize on the potential of this novel therapeutic gene, the NIS gene, and NIS-mediated radiovirotherapy in glioma treatment, thus further augmenting the activity of this promising oncolytic platform by ^{131}I administration.

^{131}I-based therapeutics, usually in the form of ^{131}I-labeled monoclonal antibodies, have shown promise in the treatment of recurrent gliomas. In a phase II trial in recurrent glioma patients, intracavitary administration of 100 mCi of ^{131}I-m81C6, a murine monoclonal antibody targeting tenascin, in combination with chemotherapy was associated with superior median survival as compared with historic controls (Reardon *et al.*, 2006). In another trial, resection cavity administration of ^{131}I-labeled TM-601 (a synthetic version of chlorotoxin) in 18 adult recurrent glioma patients (17 with GBM) was well tolerated (Mamelak *et al.*, 2006) with some preliminary evidence of activity. We therefore hypothesized that radiovirotherapy with MV-NIS and radioactive ^{131}I would be more effective than either agent alone in glioma treatment.

In this study, we have demonstrated that MV-NIS has significant cytopathic activity against glioblastoma cells *in vitro* and antitumor effect *in vivo*, in both settings antitumor activity was improved as compared with MV-CEA. This increase in cytopathic effect is most likely due to more

effective viral replication, as demonstrated in Fig. 2. A potential explanation for the improved antitumor efficacy of MV-NIS pertains to the position of transgene [position 1 for MV-CEA versus position 6 for MV-NIS (Fig. 1)]. MV genome is a single-stranded nonsegmented RNA of negative polarity. During transcription, mono- and bicistronic messenger RNAs are produced with a transcriptional gradient from the N to L gene, *i.e.*, transcription is less efficient as it progresses from the 3′ to the 5′ end of the viral genome (Griffin and Bellini, 1996). An MV strain encoding the transgene downstream from the N, P, M, F, and H genes, such as MV-NIS, would therefore be expected to have a proliferation advantage, resulting in superior antitumor activity, as compared with MV-CEA (encoding the CEA transgene in position 1), because transcription of its genome results in higher copy numbers of MV structural genes. This is supported by the 1 to 2 log higher titers of MV-NIS as compared with MV-CEA obtained in one-step viral growth curves in both glioma and Vero cells, and the *in vivo* efficacy data in GBM39 and GBM43 (Fig. 2).

In vitro expression of NIS protein and significant incorporation of iodine by infected cells were observed following MV-NIS infection. This effect was blocked by the specific NIS inhibitor, potassium perchlorate. Furthermore, NIS expression in tumor cells allowed for monitoring and localization of the viral replication *in vivo.* NIS expression was detected both in subcutaneous and in orthotopically implanted glioma xenografts following intratumoral administration of MV-NIS. These findings support the promise of this method in the monitoring of MV-NIS infection in future glioblastoma trials.

Detectable levels of NIS expression *in vivo* allowed us to proceed with testing of the radiovirotherapy combination. We showed that addition of radioactive iodine therapy to treatment with MV-NIS improved survival in both flank and intracranial orthotopic murine glioblastoma models ($p < 0.05$). This result can be attributed to improved viral replication and cytopathic effect of MV-NIS on glioblastoma cells exposed to radiation (Liu *et al*., 2007) in combination with the ^{131}I radiation treatment effect. Our study is the first to demonstrate the therapeutic potential of MV-NIS-based radiovirotherapy in a glioblastoma model. Delivering targeted radiation treatment directly to glioblastoma tumors has the potential advantage of achieving high radiation doses in the tumor, minimizing the side effects of external radiation treatment to normal tissue, and resulting in therapeutic benefit even in patients who have previously failed EBRT. It is of note that single-agent administration of MV derivatives such as MV-CEA in recurrent GBM patients has not resulted into any dose-limiting toxicity to date (Galanis *et al*., 2008). Although formal toxicology studies would be required prior to clinical translation, given the safety track record of both MV-virotherapy and dosimetry-based radioisotope administration (Mamelak *et al*., 2006; Reardon *et al*., 2006), we expect that the combination treatment will be well tolerated.

In summary, we have demonstrated that MV-NIS is a more effective oncolytic agent against gliomas than MV-CEA both *in vitro* and *in vivo*. In addition, NIS expression can be used to noninvasively monitor viral infection. The expression of NIS can also be used to further increase the efficacy of the virotherapy via ^{131}I administration. MV-NIS therefore represents an attractive candidate as a virotherapy agent against glioblastoma, and clinical translation is warranted.

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Author Disclosure Statement

The authors have nothing to disclose.

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