Retargeted Oncolytic Measles Strains Entering via the EGFRvIII Receptor Maintain Significant Antitumor Activity against Gliomas with Increased Tumor Specificity

Cory Allen, Sompong Vongpunsawad, Takaufumi Nakamura, C. David James, Mark Schroeder, Roberto Cattaneo, Caterina Giannini, James Krempski, Kah-Whye Peng, Jenny M. Goble, Joon H. Uhm, Stephen J. Russell, and Evanthia Galanis

Abstract

Among the best-characterized genetic alterations in gliomas is the amplification of the epidermal growth factor receptor (EGFR) gene, present in ~40% of glioblastoma multiforme, and frequently associated with the EGFRvIII gene rearrangement. We have previously shown that attenuated vaccine strains of measles virus have potent antitumor activity against gliomas, and identified H protein mutations, which ablate recognition of the natural measles virus receptors CD46 and SLAM. Retargeted recombinant viruses were generated from the measles Edmonston-NSe vaccine strain displaying a single-chain antibody against EGFRvIII at the COOH terminus of H and containing the marker green fluorescent protein (GFP) gene in position 1. Two different H mutants were employed: HSS (Y451S, Y481N, and A527S)-CD46 blind, and HAA (Y481A and A532A)-CD46 and SLAM blind. MV-GFP virus was used as a positive control. Both EGFRvIII-retargeted viruses had significant antitumor activity against EGFRvIII-expressing glioblastoma multiforme but no cytopathic effect against normal cells. In an orthotopic model of EGFRvIII-expressing GBM39 xenografts, there was comparable therapeutic efficacy between retargeted strains and unmodified MV-GFP and statistically significant prolongation of survival in treated animals compared with the control group \((P = 0.0001)\). Formation of syncytia was observed in tumors treated with retargeted viruses, with a surrounding infiltrate consisting of macrophages and natural killer cells. In summary, EGFRvIII-retargeted oncolytic measles virus strains have comparable therapeutic efficacy with the unmodified MV-GFP strain against EGFRvIII-expressing glioma lines and xenografts with improved therapeutic index, a finding with potential translational implications in glioma virotherapy. (Cancer Res 2006; 66(24): 11840-50)

Introduction

Glioblastoma multiforme is the most frequent primary brain tumor in adults and accounts for the majority of the 18,500 primary brain tumor cases diagnosed each year in the United States (1). It is one of the most lethal malignancies with a median survival of 12 to 16 months despite multimodality treatment, including surgery, chemotheraphy, and radiation therapy. Gliomas represent a good target for gene transfer or virotherapy approaches given their limited ability to metastasize, but despite promising preclinical data, significant clinical benefit has not materialized to date. We have recently shown that measles virus vaccine strain derivatives represent potent oncolytic agents in the treatment of gliomas (2). Measles virus enters the cells via two known receptors: CD46 (3, 4), a regulator of complement activation that is found on all human nucleated cells but overexpressed in tumors, and SLAM (5–7), the signaling lymphocyte activated molecule, which is expressed on activated T and B cells and macrophages.

Potential challenges in clinical application of measles virus strains in the treatment of gliomas include the ubiquitous, although low level expression, of CD46 in normal brain (8, 9) and the potential interaction between vaccine strains of measles virus and SLAM, the other measles virus receptor, which can be associated with immunosuppression (6, 7). Ablation of viral entry via CD46 and SLAM could, therefore, have significant translational implications.

Attachment of the measles virus H protein to its receptor represents the first step in measles virus entry. We have previously identified mutations that ablate entry through CD46 or SLAM (10, 11). In addition, we have shown that fully retargeted oncolytic measles viruses can be generated by combining CD46- and SLAM-ablating mutations with single-chain antibody display on the COOH terminus of H (12, 13).

Among the best-characterized genetic alterations in gliomas is the amplification of the gene encoding epidermal growth factor receptor (EGFR), which is frequently accompanied by EGFR gene rearrangements resulting in the expression of mutant proteins, such as EGFRvIII. EGFRvIII is the most common of the receptor mutants and lacks amino acids 7 to 273 of the wild-type receptor extracellular domain (14). The EGFRvIII receptor is tumor specific, expressed in 50% to 60% of glioblastoma multiforme with EGFR amplification, and therefore represents an excellent target for viral retargeting in gliomas (15, 16).

A major concern pertaining to the construction of retargeted viruses is the possibility of reduced efficacy resulting from entry via a non-natural receptor (17). The likelihood of decreased efficacy could theoretically be higher for measles strains containing ablating mutations for both natural receptors CD46 and SLAM compared with only the CD46 receptor. We therefore decided to test both natural receptor-ablating strategies (CD46 and SLAM entry ablation versus only CD46 entry ablation) in combination with a single-chain antibody display on the COOH terminus of H, to allow selective entry via the EGFRvIII receptor.
In this work, we have shown that retargeted oncolytic measles virus strains, displaying a single-chain antibody against EGFRvIII in combination with H mutations that ablate entry via the natural receptors CD46 or SLAM, can infect glioma cells in a receptor-specific manner and exhibit significant antitumor efficacy in EGFRvIII-expressing glioma lines and xenografts that it is comparable with unmodified measles virus strains. Furthermore, the retargeted strains led to complete protection of normal cells, such as astrocytes and fibroblasts, even at high viral multiplicities of infection (MOI). Retargeted measles strains against EGFRvIII therefore possess excellent translational potential in the viral therapy of gliomas.

Materials and Methods

Cell Culture

Vero (African green monkey kidney) cells, Chinese Hamster Ovary (CHO), and U118 glioma cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). All cell lines were grown at 37°C in media recommended by the ATCC in a humidified atmosphere of 5% CO2. U118-EGFRvIII cells were generated by stable transfection of the parental U118 cells using a pCDNA3.1 (Invitrogen, Carlsbad, CA) plasmid for expression of the EGFRvIII protein. Single-cell isolates were expanded in media containing 1 mg/mL G418 (Life Technologies, Carlsbad, CA). CHO transfectants stably expressing CD46, SLAM, and EGFRvIII receptors were generated as previously described (11, 12). Primary glialblastoma lines GBM6, GBM14, and GBM39 were generated as previously described (18) and maintained as s.c. xenografts. To establish short-term cultures, xenografts were excised and placed in culture dishes where the tissue was initially minced with a scalpel and then mechanically disrupted to create a cell suspension. Following short-term culture of the tumor cells (3–7 days) in DMEM with 2.5% fetal bovine serum (FBS), cells were either used for orthotopic implantation or further maintained in culture in DMEM/10% FBS. Normal human astrocytes (NHA) and normal human dermal fibroblasts (N HDF; Cambrex, Baltimore, MD) were grown in AGM (Cambrex) and FGM (Cambrex) media, respectively, as recommended by Cambrex.

Construction of MV-GFP-HSSN-scEGFRvIII

The single-chain EGFRvIII antibody DNA was PCR amplified from pMR1ENI (19) using the following primers: 5′-GCCGCGCGAGCTCAGGACCTGAACTGCAAGATG-3′ and 5′-CTATGGCGCGCTTATGAACTGCGTACGAGT-3′. The purified PCR product was ligated into pcR2.1 (Invitrogen), and the sequence of the resultant plasmid (pcR2.1-scEGFRvIII) was verified by sequencing. The PacI-SpeI fragment, containing the measles H sequence and incorporating three CD46-ablating mutations (V451S, Y481N, and Y481A) and the SLAM-ablating mutation R533A was rescued, employing the pseudoreceptor STAR system virus containing the CD46-ablating mutation Y481A and the SLAM-ablating mutation R533A. The single-chain EGFRvIII plasmid was named pcG-HSSN. The pcG-HSSN-scEGFRvIII plasmid was constructed by subcloning the EGFRvIII single-chain antibody sequence, derived from pcR2.1-scEGFRvIII, into the COOH terminus of HSSN in the pcG-HSSN plasmid with SfiI NotI digestion/ligation. After PacI SpeI digestion of pcG-HSSN-scEGFRvIII, the PacI-SpeI fragment was gel purified and subcloned into the full-length plasmid p(+)-MV-GFP-NSE, using PacI SpeI digestion/ligation. The resultant full-length plasmid [p(+)-MV-GFP-HSSN-scEGFRvIII] was used to rescue the virus as described by Radeke et al. (20).

Briefly, 293-3-4 cells with helper cells (20) were transfected with p(+) MV-GFP-HSSN-scEGFRvIII and pEMCA plasmid, using the Profection Mammalian Transfection System Calcium Phosphate kit (Promega, Madison, WI). Seventy-two hours after transfection, the cells were harvested and overlayed on SLAM-transfected Vero cells (Vero-SLAM). The MV-GFP-HSSN-scEGFRvIII virus containing the CD46-ablating mutation Y481A and the SLAM-ablating mutation R533A was rescued, employing the pseudoreceptor STAR system modification, as described by Nakamura et al. (11). A six-histidine tag at the COOH terminus of H allowed rescue and propagation of the virus in Vero-HIS cells transduced with a pDisplace (Invitrogen) plasmid to express a membrane-anchored single-chain antibody that recognizes the six-histidine peptide (21). The MV-GFP virus was rescued as previously described (22) and propagated in Vero cells. Virus stocks were prepared by infecting the appropriate Vero cell line with measles virus at an MOI of 0.02 and incubating at 32°C, 5% CO2. Virus was harvested by three freeze-thaw cycles from cellular substrate and resuspended in Opti-MEM (Life Technologies) after the third serial passage. Titers were determined by 50% tissue culture infective dose (TCID50; ref. 2) titration or Vero-SLAM, Vero-HIS, or Vero, respectively.

Immunoblot Analysis for Detection of Viral H Protein

Viral samples (5×10^7 TCID50) were lysed (23) and separated by SDS-PAGE. The gels were transferred to nitrocellulose (Bio-Rad, Hercules, CA) and blocked overnight at 4°C in 5% bovine serum albumin, 5% skim milk powder in TBST [10 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.05% Tween 20]. The blot was then incubated with rabbit anti-H cytoplasmic tail antisera (1:10,000; ref. 24). After washing, the blot was probed with peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Calbiochem, EMD Biosciences, Darmstadt, Germany) and visualized with Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Immunoblot Analysis for Detection of Viral N Protein

One million NHDF, NHA, GBM14, or GBM39 cells were plated in 10-cm dishes. The next day, the cells were infected at an MOI of 1.0 with MV-GFP, MV-GFP-HSSN-scEGFRvIII, and MV-GFP-HAA-scEGFRvIII, whereas one plate per cell line served as uninfected control. Forty-eight hours after infection, cells were rinsed with PBS and lysed in 1 mL of lysis buffer [50 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 50 mmol/L sodium chloride, 5 mmol/L EDTA, 5 mmol/L EGTA, 100 mmol/L sodium orthovanadate, 10 mmol/L HEPES (pH 7.4), 0.1% Triton X-100 containing complete protease inhibitor cocktail tablets; Roche, Penzberg, Germany]. Samples were subsequently sonicated, and 20 μg total protein was separated by SDS-PAGE. The gels were transferred to nitrocellulose (Bio-Rad) and blocked overnight at 4°C in 1× Casein (Vector, Burlingame, CA) in Tween-TBS [10 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.05% Tween 20]. The blots were then incubated with rabbit anti-N (1:10,000, kindly supplied by R. Cattaneo) in Tween-TBS containing 0.5% nonfat dry milk at room temperature for 60 minutes. Blots were washed for 60 minutes in Tween-TBS and incubated with goat anti-rabbit hors eradish peroxidase (1:2,000; Pierce) in Tween-TBS containing 0.5% nonfat dry milk at room temperature for 60 minutes.

Immunoblot Analysis for EGFRvIII Levels

GBM6, GBM14, and GBM39 xenograft samples were mechanically dispersed in 1 mL of lysis buffer consisting of 50 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 50 mmol/L sodium chloride, 5 mmol/L EDTA, 5 mmol/L EGTA, 100 mmol/L sodium orthovanadate, 10 mmol/L HEPES (pH 7.4), 0.1% Triton X-100 containing complete protease inhibitor cocktail tablets (Roche, Penzberg, Germany). U118 and U118-EGFRvIII cell lines NHA and NHDF were washed in PBS and lysed in 3 mL of lysis buffer. Protein samples were subsequently sonicated, and 20 μg total protein was separated by SDS-PAGE. Twenty micrograms of total protein were used for GBM6, GBM14, and GBM39, whereas 10 μg of total protein were used for U118 and U118-EGFRvIII cell lines. The gels were transferred to nitrocellulose (Bio-Rad) and blocked overnight at 4°C in 1× Casein in Tween-TBS (Vector). The blot was then incubated with rabbit anti-EGFR [Cell Signaling Technology, Danvers, MA; 1:1,000 in Tween-TBS: 10 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.05% Tween 20 (Pierce) containing 0.5% nonfat dry milk]. After a final 2-hour wash, the blot was developed with Supersignal West Femto Chemiluminescent Substrate (Pierce). A β-actin control blot was developed in parallel by loading 5 μg total protein from each specimen. β-Actin was detected with a monoclonal anti-β-actin antibody (Sigma, Saint Louis, MO) followed by filter incubation with Supersignal West Femto Chemiluminescent Substrate (Pierce).
Determination of CD46 Expression Levels

One million U118, GBM6, GBM14, GBM39, CHO cells, NHA, and NHDF were harvested, washed, and then incubated with FITC-labeled mouse anti-human CD46 (PharMingen, San Diego, CA). Washed cells were fixed in PBS containing 0.3% paraformaldehyde and analyzed on a Becton Dickinson FACScan Plus cytometer. Analysis was done using the CellQuest software (BD Biosciences, San Diego, CA).

Assessment of Cytopathic Effect In vitro

Cells were plated in six-well plates at a density of either 10^5 per well (U118, U118-EGFRvIII, CHO, CHO-CD46, CHO-SLAM, and CHO-EGFRvIII) or 5 x 10^5 per well (GBM6, GBM14, GBM39, astrocytes, and fibroblasts). Twenty-four hours after seeding, the cells were infected at different MOIs from 1 to 10 in 1 mL of Opti-MEM for 2 hours at 37°C. At the end of the incubation period, the virus removed, and the cells were maintained in their standard medium. The same number of uninfected cells in six-well plates was used as controls. The number of viable cells in each well was counted using a hemocytometer at 3, 5, 7, and 11 days after infection. Viable cells were identified using trypan blue exclusion. 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 the uninfected well corresponding to the same time point. Infection was confirmed using fluorescent microscopy at the corresponding time points.

Assessment of Viral Replication in Glioma Cell Lines

Glioma lines were plated in six-well plates at a density of 10^5 per well or 5 x 10^5 per well (depending on cell line as described above). The cells were infected as described above at an MOI of 1 and harvested at 2, 3, 4, and 5 days after infection. The viruses were released with two cycles of freeze/thawing. The viral titer was determined by 50% end point dilution assay on Vero, Vero-SLAM, or Vero-HIS cells in a 96-well plate as described above.

Orthotopic In vivo Experiments

GBM39 cells (3 x 10^6) resuspended in PBS were orthotopically implanted into the right caudate nucleus of 5-week-old BALB/c mice using the small animal stereotactic frame (ASI Instruments, Warren, MI) with a 26-gauge Hamilton syringe (2). Treatment was initiated 7 days after implantation by i.t. injection using the same coordinates as implantation; 5 x 10^6 TCD50 per dose were given in 10 μL of normal saline and repeated every 2 to 3 days over a 3-week period for a total dose of 4 x 10^7 TCD50. The following four groups were included (nine animals each): MV-GFP, UV-inactivated MV-GFP-ΔHsNS-scEGFRvIII, MV-GFP-ΔHsNS-scEGFRvIII, and MV-GFP-HAA-scEGFRvIII. Two mice per group were euthanized at 4 days after completion of treatment. The brains of animals were harvested and were either fixed in paraformaldehyde and embedded in paraffin (one animal per group) or harvested and flash frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC; one animal per group) and used for subsequent correlative analysis as described below. Mice were observed daily and were euthanized when they developed neurologic deficits or when >10% weight loss was observed. Experiment was terminated at 120 days. Brains of the animals that had to be euthanized were either fixed in paraformaldehyde and embedded in paraffin or flash frozen in Tissue Freezing Medium.

Molecular Analysis of Orthotopic Brain Tumor Samples

Assessment of enhanced GFP positivity in brain tumor sections. Brains from mice of each treatment arm were harvested and frozen in Tissue Freezing Medium (Triangle Biomedical Sciences). Ten-micrometer

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**Figure 1.** A, schematic representation of recombinant targeted MV genome showing the mutated H protein. The single-chain antibody against EGFRvIII is displayed as a COOH-terminal extension of the H-glycoprotein. The H protein contains either CD46-ablating mutations (Y451S, Y481N, and A527S) or both CD46- and SLAM-ablating mutations (Y481A and R533A_HAA). B, expression levels of viral H protein were determined by Western immunoblotting of equal viral infectious units. The chimeric H proteins of the two EGFRvIII-retargeted strains have higher molecular weights (96 kDa compared with 75 kDa for the unmodified H). C, specificity of infection with EGFRvIII-retargeted strains was shown in measles virus non-permissive CHO cells after stable transfection with the EGFRvIII receptor (CHO-EGFRvIII). In contrast to MV-GFP, the two EGFRvIII-retargeted strains enter and infect efficiently CHO-EGFRvIII cells (MOI of 1, 48 hours after infection).
frozen sections were cut, individually thawed, and immediately examined for enhanced green fluorescent protein (eGFP) expression using a Zeiss Axiosvert 100 M fluorescent microscope.

**In situ hybridization for N protein.** Measles N mRNA was transcribed using the T7 promoter and digoxigenin labeled using the DIG RNA Labeling kit SP6/T7 (Roche, Mannheim, Germany) corresponding to base pairs 825 to 1676 (852 bp). Ten-micrometer paraffin sections were incubated at 50°C for 30 minutes and dewaxed in CitriSolv (Fisher, Pittsburgh, PA), rehydrated in graded ethanol baths, washed with PBS, and incubated in proteinase K for 7 minutes. Slides were washed in 2× SSC buffer, incubated in 0.1 mol/L triethanolamine, 0.0625% acetic anhydride for 10 minutes, and washed in 2× SSC. Slides were dehydrated in graded ethanol baths, hybridized with 500 pg probe, incubated for 3 minutes at 95°C, and then incubated overnight at 60°C in a humid environment. Sections were washed and incubated with anti-digoxigenin-AP (Roche, Mannheim, Germany) as per the manufacturer’s directions. Signal was visualized using the Dig Nucleic Acid Detection kit (Roche, Mannheim, Germany), and counterstained with Accustain (Sigma).

**Brain immunohistochemistry for natural killer cell lectin-like receptor (KLRG1), CD64, and neutrophil elastase.** Ten-micrometer sections were deparaffinized with CitriSolv (Fisher) and then rehydrated in graded ethanol baths. Sections were incubated in 0.6% H2O2 in methanol for 20 minutes followed by 3-minute incubation in 75% ethanol, 3-minute incubation in 50% ethanol, and 3-minute incubation in H2O2. Sections were placed in 50 mL of Antigen Retrieval Citra (Biogenex, San Ramon, CA) at room temperature and then warmed in 95°C water bath for 30 minutes and placed on ice for 20 minutes. Sections were removed and placed into H2O2 and then PBS. Sections were blocked with 10% Normal Donkey Serum (NDS) in PBS for 20 minutes and washed with PBS. They were subsequently incubated with primary antibody, either KLRG1 (SC-22929; concentration 1:50) that recognizes the killer cell lectin-like receptor G1 [present on natural killer (NK) cells and activated CD8+ T cells], the anti-CD64 antibody (SC-15364; dilution 1:50) that recognizes monocytes and macrophages, or the neutrophil elastase antibody SC-25621 (dilution 1:50) that recognizes neutrophils. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody was diluted in 1.5% NDS in PBS for 60 minutes and then washed for 35 minutes in PBS. Slides were incubated with a goat anti-rabbit IgG-TR (SC-2780; dilution 1:100) in 1.5% NDS in PBS for 60 minutes followed by three brief washes in PBS and then PBS. Slides were blocked with 10% Normal Donkey Serum (NDS) in PBS for 20 minutes and washed with PBS. They were subsequently incubated with primary antibody, either KLRG1 (SC-22929; concentration 1:50) that recognizes the killer cell lectin-like receptor G1 [present on natural killer (NK) cells and activated CD8+ T cells], the anti-CD64 antibody (SC-15364; dilution 1:50) that recognizes monocytes and macrophages, or the neutrophil elastase antibody SC-25621 (dilution 1:50) that recognizes neutrophils. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody was diluted in 1.5% NDS in PBS for 60 minutes and then washed for 35 minutes in PBS. Slides were incubated with a goat anti-rabbit IgG-TR (SC-2780; dilution 1:100) in 1.5% NDS in PBS for 60 minutes followed by three brief washes in PBS and then PBS. Slides were blocked with 10% Normal Donkey Serum (NDS) in PBS for 20 minutes and washed with PBS. They were subsequently incubated with primary antibody, either KLRG1 (SC-22929; concentration 1:50) that recognizes the killer cell lectin-like receptor G1 [present on natural killer (NK) cells and activated CD8+ T cells], the anti-CD64 antibody (SC-15364; dilution 1:50) that recognizes monocytes and macrophages, or the neutrophil elastase antibody SC-25621 (dilution 1:50) that recognizes neutrophils. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody was diluted in 1.5% NDS in PBS for 60 minutes and then washed for 35 minutes in PBS. Slides were incubated with a goat anti-rabbit IgG-TR (SC-2780; dilution 1:100) in 1.5% NDS in PBS for 60 minutes followed by three brief washes in PBS and then PBS. Slides were blocked with 10% Normal Donkey Serum (NDS) in PBS for 20 minutes and washed with PBS.

**Vimentin staining.** Ten-micrometer sections were deparaffinized in CitriSolv (Fisher). Slides were then rehydrated in graded ethanol baths, washed briefly with water, and incubated with 0.6% H2O2 in methanol for 20 minutes. Sections were washed in 75% ethanol for 3 minutes, 50% ethanol for 3 minutes, and distilled water for 3 minutes. Sections were placed into 50 mL of Antigen Retrieval Citra (Biogenex, San Ramon, CA) at room temperature and then warmed in 95°C water bath for 30 minutes and placed on ice for 20 minutes. Sections were removed and placed into H2O2 and then PBS. Sections were blocked with 10% Normal Donkey Serum (NDS) in PBS for 20 minutes and washed with PBS. They were subsequently incubated with primary antibody, either KLRG1 (SC-22929; concentration 1:50) that recognizes the killer cell lectin-like receptor G1 [present on natural killer (NK) cells and activated CD8+ T cells], the anti-CD64 antibody (SC-15364; dilution 1:50) that recognizes monocytes and macrophages, or the neutrophil elastase antibody SC-25621 (dilution 1:50) that recognizes neutrophils. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody was diluted in 1.5% NDS in PBS for 60 minutes and then washed for 35 minutes in PBS. Slides were incubated with a goat anti-rabbit IgG-TR (SC-2780; dilution 1:100) in 1.5% NDS in PBS for 45 minutes, washed thrice in PBS, mounted with 4.6-diamidino-2-phenylindole containing vectamount (1:10), and visualized with a Zeiss Axiosvert 100 M fluorescent microscope. Positive controls included the J477 mouse macrophage line and the U937 human monocyte line as well as mouse spleen smears. For negative controls, slides underwent similar processing, except that the primary antibody step was omitted.

**Vimentin staining.** Ten-micrometer sections were deparaffinized in CitriSolv (Fisher). Slides were then rehydrated in graded ethanol baths, washed briefly with water, and incubated with 0.6% H2O2 in methanol for 20 minutes. Sections were washed in 75% ethanol for 3 minutes, 50% ethanol for 3 minutes, and distilled water for 3 minutes. Sections were placed into 50 mL of Antigen Retrieval Citra (Biogenex) at room temperature, placed into a 95°C water bath for 30 minutes, and subsequently placed on ice for 20 minutes and blocked with 10% NDS in PBS. Mouse anti-human vimentin was diluted 1:250 in PBS containing 1.5% normal donkey serum, applied to each section for 60 minutes followed by three brief washes in PBS. Sections were exposed to a universal secondary antibody (Vector) in PBS containing 1.5% NDS for 45 minutes, washed thrice briefly in PBS and were developed using the Nova-Red developing reagent (Vector) as per the manufacturer’s directions. Slides were next rinsed in tap water and counterstained with hematoxylin for 30 seconds and rinsed with tap water. Sections were dehydrated in graded ethanol baths, followed by a 5-minute incubation in CitriSolv, coverslipped, and examined for staining.

**Statistical analysis.** To assess animal survival, Kaplan-Meier curves were generated. The survival of mice in the different treatment groups was compared using the log-rank test. P < 0.05 was considered statistically significant.
infection with both retargeted strains but not with unmodified MV-GFP.

*In vitro* infection of tumor cells with retargeted strains is dependent on the expression of the EGFRvIII receptor and results in comparable cytopathic effect with the unmodified strain MV-GFP in tumor cells expressing EGFRvIII. Levels of expression of EGFRvIII in U118, U118-EGFRvIII, GBM6, GBM14, GBM39 cells, NHA, and NHDF were characterized using Western immunoblotting (Fig. 2). Expression of the EGFRvIII receptor (145 kDa) was readily detectable in U118 cells stably transfected with EGFRvIII (U118-EGFRvIII) and the primary lines GBM6 and GBM39. In contrast, there was no EGFRvIII receptor expression in U118 cells, GBM14 cells, NHA, and NHDF. Tumor cell expression of the measles virus CD46 receptor was characterized by fluorescence-activated cell sorting. Similarly to what we have previously reported (2), high levels of CD46 overexpression in tumor cell lines was observed in contrast to low expression levels in normal human astrocytes and fibroblasts (data not shown).

As Fig. 3A and B indicates, the two retargeted strains infect and replicate efficiently in U118-EGFRvIII glioma cells and the EGFRvIII-expressing primary lines GBM6 and GBM39 (GFP positivity and syncytia formation). Cytotoxicity against EGFRvIII-expressing glioma lines was also compared by trypan blue exclusion assays in multiple time points (Fig. 3D, F, and G) and was comparable with the unmodified strain MV-GFP. In contrast, the two EGFRvIII-retargeted strains did not cause significant infection, syncytia formation, or cytopathic effect in the EGFRvIII-negative lines U118 and GBM14 (Fig. 3A, B, C, and E).

EGFR-retargeted measles virus strain replicates comparably with the unmodified strain MV-GFP in tumor cells expressing the EGFRvIII receptor. To assess the effect of receptor expression on virus replication *in vitro*, one-step growth curves were

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Figure 3. A, U118 cells not expressing the EGFRvIII receptor cannot be infected with the retargeted measles virus strains MV-GFP-HSNS-scEGFRvIII and MV-GFP-HAA-scEGFRvIII, as shown by minimal or no green fluorescence (MOI of 1, 72 hours after infection,  ×100). Stable transfection of U118 cells with the EGFRvIII receptor (U118-EGFRvIII) restored infectivity with the EGFRvIII-retargeted strains; thus pointing to EGFRvIII receptor specific entry and mediation of fusion. B, infection of primary glioma lines, either expressing EGFRvIII, such as GBM6 and GBM39, or not expressing EGFRvIII, such as GBM14, shows that infection with the retargeted strains and resultant cytopathic effect is specific for cells expressing the receptor (MOI of 1, 72 hours after infection,  ×100).
performed. U118 cells that do not express EGFRvIII and U118 cells stably transfected with EGFRvIII and expressing the EGFRvIII receptor EGFRvIII (U118-EGFRvIII) were infected with MV-GFP and the two EGFRvIII-retargeted strains at an MOI of 1. Viral titers were determined at multiple time points as shown in Fig. 4A and B. Viral replication of the two EGFRvIII-retargeted strains was increased by at least two logs in U118-EGFRvIII cells compared with U118 cells. In contrast, replication of the nontargeted MV-GFP was comparable between the U118 and U118-EGFRvIII cells and independent of the EGFRvIII receptor status.

EGFRvIII retargeting of measles virus results in complete protection of normal cells, such as normal human astrocytes and fibroblasts. The EGFRvIII mutation is tumor specific; therefore, normal cells, such as astrocytes, fibroblasts, or neurons, do not express this receptor (25). In contrast to infection with MV-GFP, normal cell lines, such as astrocytes and fibroblasts,

Figure 3 Continued. C to G, CPE of MV-GFP, MV-GFP-H\(^{\text{HsN}}\)-scEGFRvIII, and MV-GFP-H\(^{\text{Haa}}\)-scEGFRvIII in U118 (C), U118-EGFRvIII (D), GBM14 (E), GBM6 (F), and GBM39 (G) at an MOI of 1. Cell viability was determined by trypan blue exclusion and presented as percentage of uninfected cells. There was lack of significant cytopathic effect of the EGFRvIII-retargeted strains against either U118 cells (C) or GBM14 cells (E) that do not express EGFRvIII. In contrast, transfection of U118 cells with EGFRvIII resulted in significant cytopathic effect of the retargeted strains (D), which is comparable with unmodified MV-GFP. Similarly, significant cytopathic effect after infection with retargeted strains was observed against the primary glioma lines GBM6 (F) and GBM39 (G), which express EGFRvIII.
were resistant to infection with the two EGFRvIII-retargeted strains, showing no fusion or cell death. There is lack of GFP positivity and lack of significant cytopathic effect, as assessed by trypan blue exclusion in multiple time points (Fig. 5A–C). In addition, and in contrast to infection with MV-GFP, there was no expression of the measles virus nucleocapsid (N) protein in astrocytes or fibroblasts treated with the retargeted strains, thus indicating lack of infection (Fig. 5D).

Retargeted strains show comparable efficacy with the unmodified virus in EGFRvIII-expressing xenografts. A significant obstacle in studying the efficacy of EGFR/EGFRvIII–targeted therapeutics against glioblastoma has been the difficulty in developing optimal animal models. We have recently, however, described the generation of animal models derived from glioblastoma patients that exhibit a diverse collection of genetic lesions, have reproducible growth characteristics, and maintain in vivo the invasiveness and the molecular characteristics of the patient tumor that they are derived from (18). GBM39 represents such a model for EGFRvIII receptor overexpression at a total dose of $4 \times 10^5$ TCID$_{50}$. The dose employed in these experiments was the maximum viral dose that could be delivered, given administration volume constraints and titers of the viral preparations, as discussed in Materials and Methods. Intratumoral treatment of EGFRvIII-overexpressing GBM39 xenografts with the two retargeted EGFRvIII strains resulted in significant antitumor efficacy, which was comparable with the efficacy of the unmodified strain MV-GFP ($P = 0.51$ for MV-GFP-H$\Delta$AA-scEGFRvIII versus MV-GFP and $P = 0.85$ for MV-GFP-H$_{SSN}$scEGFRvIII versus MV-GFP) and resulted in an ~40% cure rate (Fig. 6A). There was statistically significant prolongation of survival in MV-GFP–, MF-GFP H$_{SSN}$scEGFRvIII–, and MV-GFP-H$\Delta$AA-scEGFRvIII–treated mice compared with UV-inactivated virus-treated controls ($P = 0.0019$, $P = 0.0028$, and $P = 0.0280$, respectively). The characteristic cytopathic effect (i.e., formation of syncytia) was observed in tumors treated with the EGFRvIII-retargeted strains (Fig. 6B1). In situ hybridization for measles virus N-mRNA showed viral replication in the syncyia (Fig. 6BII and BIII). A cellular infiltrate consisting of small round cells surrounded the syncyia (Fig. 6BII). Similar infiltrate was not observed in UV-inactivated virus-treated tumors or in active virus-treated tumors in the absence of syncyia. The cells of the infiltrate were negative for human vimentin (Fig. 6C), thus pointing against a human origin, but positive for the macrophage marker CD64 and the NK marker KLRG1, thus indicating mouse macrophage and NK cell origin (Fig. 6D). In contrast, there was only very limited focal staining for mouse neutrophil elastase (data not shown). Viral propagation in tumors was also shown by GFP expression in tumors treated with MV-GFP, MV-GFP-H$_{SSN}$scEGFRvIII, and MV-GFP-H$\Delta$AA-scEGFRvIII, but not in tumors treated with UV-inactivated virus (Supplementary Figure).

Discussion

Amplification of the gene encoding EGFR is one of the best-characterized genetic alterations in gliomas and is observed in ~40% of glioblastoma multiforme (26, 27). EGFR amplification is often accompanied by EGFR gene rearrangements resulting in the expression of mutant proteins. EGFRvIII is the most common of these receptor mutants, lacks 267 amino acids of the wild-type receptor’s extracellular domain (exons 2–7; ref. 14), and is expressed in the majority of glioblastoma multiforme with EGFR amplification (26). The EGFRvIII receptor does not bind ligand, but it is displayed on the cell surface (15, 26), is constitutively activated, and is tumor cell specific (28). EGFRvIII, therefore, represents an excellent target for glioma-targeted therapeutics in the significant subset of high-grade glioma patients expressing the receptor (29), with most EGFRvIII-targeting efforts to date focusing on monoclonal antibodies (30, 31).

Here, we have shown that retargeting of the measles virus against the EGFRvIII receptor with ablation of its tropism for the natural receptors CD46 and SLAM is feasible and results in retargeted strains with significant in vitro and in vivo therapeutic efficacy against EGFRvIII-expressing glioma tumors, which is comparable with the unmodified MV-GFP strain. This has been accomplished by displaying a single-chain antibody against EGFRvIII at the COOH terminus of H and introducing CD46- and/or SLAM-ablating mutations in the H protein.

Measles virus entry is mediated by two envelope glycoproteins: the attachment protein H and the F protein resulting in fusion. Because the attachment and fusion function are mediated via two different entities, retargeting strategies have mainly focused on H
protein modification (32). The H protein mediates attachment to one of the two known measles virus receptors CD46 (3) or SLAM (5) on the cell surface and signals to the F protein to trigger cell fusion (32). In order to effectively retarget fusion, the new specificity domain displayed on H should preserve its ability to trigger conformational changes in the F protein. Our results show that retargeted measles strains against the EGFRvIII receptor maintain fusogenic properties and antitumor activity, which is

Figure 5. A, in contrast to infection with MV-GFP, treatment of normal human astrocytes and fibroblasts with the two EGFRvIII-retargeted strains did not cause infection or fusion (MOI of 1, 72 hours after infection). B and C, cell viability of normal human astrocytes and fibroblasts in response to infection with MV-GFP-Hsna-scEGFRvIII, MV-GFP-HAa-scEGFRvIII, and MV-GFP was determined by trypan blue exclusion assays and presented as percentage of uninfected cells. In contrast to MV-GFP, the EGFRvIII-retargeted strains had no significant cytopathic effect (MOI of 1). D, Western blot for the measles virus nucleocapsid (N) protein was performed in cell lysates from primary glioblastoma multiforme lines, normal human astrocytes, and fibroblasts, 48 hours after viral infection (MOI of 1): (A) uninfected control, (B) MV-GFP, (C) MV-GFP-Hsna-scEGFRvIII, (D) MV-GFP-HAa-scEGFRvIII. Actin was used as a loading control. There is no expression of measles virus N protein after treatment of normal human astrocytes or fibroblasts with the EGFRvIII-retargeted strains, thus indicating lack of infection. Similar results were observed in the EGFRvIII-negative GBM14 line. In contrast, in the EGFRvIII-positive line GBM39, there is abundant N protein expression after infection with the EGFRvIII-retargeted strains.
comparable with the unmodified strain MV-GFP. This represents the first example of successful retargeting of viral entry via the EGFRvIII receptor without loss in therapeutic efficacy not only in vitro but also in vivo. In contrast, attempts to retarget other viruses against EGFRvIII were hampered by low infectivity, such as in the case of retroviruses (Moloney leukemia virus; ref. 19). Furthermore, titers of the measles EGFRvIII retargeted strains in the range of $10^7$ to $6 \times 10^7$ TCID$_{50}$ were obtained, which represent satisfactory titers for clinical applications.

It is of note that tumor cell lines expressing different, although high, EGFRvIII levels developed comparable cytopathic effect in response to infection with EGFRvIII-retargeted strains without loss in therapeutic efficacy not only in vitro but also in vivo. In contrast, attempts to retarget other viruses against EGFRvIII were hampered by low infectivity, such as in the case of retroviruses (Moloney leukemia virus; ref. 19). Furthermore, titers of the measles EGFRvIII retargeted strains in the range of $10^7$ to $6 \times 10^7$ TCID$_{50}$ were obtained, which represent satisfactory titers for clinical applications.

It is of note that tumor cell lines expressing different, although high, EGFRvIII levels developed comparable cytopathic effect in response to infection with EGFRvIII-retargeted strains. We have previously shown that, both as it pertains to entry of unmodified measles virus strains via the CD46 receptor (33) and entry of retargeted strains via alternative receptors (34), when a certain receptor threshold is reached, infection leads to extensive cell fusion and cytopathic effect, which does not increase linearly with further increase in receptor density (33, 34). Receptor expression is one of the factors affecting the observed cytopathic effect after measles virus infection. Other factors include intrinsic tumor cell membrane fusogenicity and the innate response of infected cells to viral infection that can result to inhibition of viral protein synthesis. The latter is coordinated via different mechanisms, with IFN-α/β being a predominant mechanism in measles virus-infected cells (35–38).

In normal brain, the measles virus receptor CD46 is predominantly expressed in endothelial cells and especially at the blood-brain barrier, emphasizing the important role played by the macrovasculature in the immune surveillance of the brain (8, 9). In contrast, much weaker CD46 staining is observed in subgroups of neurons and oligodendrocytes (8). SLAM is expressed in activated B and T cells and macrophages (5). Entry of the EGFRvIII retargeted strains was specific for tumor cells expressing EGFRvIII. In contrast to the unmodified virus, retargeted strains were unable to infect normal cells that do not express the EGFRvIII tumor-specific receptor, such as normal human astrocytes and fibroblasts. Use of retargeted strains could therefore further increase the therapeutic index of measles virus oncolytic strains, especially if a perfusion-based methodology, such as convection enhanced delivery, is employed, or systemic administration via the cerebral circulation is contemplated to address glioma cell infiltration in the vicinity of the tumor.

A significant challenge in studying the efficacy of EGFRvIII-targeted therapeutic against glioblastoma has been the difficulty
in identifying appropriate animal models. Different in vivo models have been developed in an attempt to study the tumor biology of EGFR mutations and amplification, including stable expression of mutant EGFR in glioma cell lines or in transgenic mice (41). However, these systems frequently do not reflect glioma biology accurately. EGFR alterations in these models are clonal, which is not always the case for EGFR amplification/mutation in human glioblastomas, the latter being often heterogeneous at the cellular level. Based on previous observations showing that amplification of EGFR can be maintained by propagation of xenografts in nude mice (42), we have developed a panel of cell lines deriving from glioblastoma patients, which are maintained in mice as s.c. xenografts. When used for the establishment of orthotopic models, these primary lines generate orthotopic tumors that preserve the histopathologic characteristics and invasiveness of the primary tumor they were derived from (18). One of these lines (GBM39) expresses EGFRvIII and has been used in the in vivo experiments presented here. Our study represents one of the first examples of employing these patient cell line–derived models to develop and validate targeted therapeutics. Although final validation will require testing of the targeted agents in the clinic and correlation of the observed response with the patient’s molecular characteristics, our data support the potential usefulness of these models in preclinical evaluation of EGFRvIII-targeted therapeutics.

Histopathologic analysis of measles virus treated orthotropic GBM39 xenografts showed that the EGFRvIII retargeted strains produce the characteristic cytopathic effect (i.e., formation of syncytia), thus indicating that our retargeting strategy allows maintenance of viral fusogenicity not only in vitro but also in vivo. In situ hybridization showed viral replication in formed and forming syncytia. Syncytia were surrounded by an immune infiltrate consisting of mouse macrophages and NK cells. In contrast to a prior report involving a measles virus–treated lymphoma model (43), significant neutrophilic infiltration was not observed in treated glioma tumors, which likely reflects differences in tumor type, mouse strain, and transgene engineered in the measles genome (GMCSF in the lymphoma study).

Our experiments do not allow us to conclude that this macrophage and NK cell infiltrate has contributed to the observed antitumor effect, especially given the naturally elevated NK activity in nude mice (44). It is of interest, however, that tumor infiltration by macrophages has been also observed in clinical trials after treatment of brain tumors with viral vectors, such as an HSV-tk–encoding adenovirus (45). Our recently activated phase I trial of the measles virus derivative MV-CEA in patients with recurrent glioblastoma multiforme incorporates tumor resection after viral administration, thus allowing us to address the role of macrophage infiltration after measles virus treatment of GBM tumors in humans.

In summary, we have generated recombinant measles virus strains, retargeted against the glioma-specific target EGFRvIII, and we have shown that they maintain the activity of the unmodified strain, but with increased tumor specificity. These viruses could have significant translational potential in glioma virotherapy.

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Cory Allen, Sompong Vongpunsawad, Takafumi Nakamura, et al.


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