Safe and effective treatment of experimental neuroblastoma and glioblastoma using systemically delivered triple microRNA-detargeted oncolytic Semliki Forest virus

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Running Title: SFVmiRT for treatment of glioblastoma and neuroblastoma

Key words: Semliki Forest virus, Glioblastoma, Neuroblastoma, Oncolytic virus immunotherapy, Type-I antiviral response

Funding: ME: The Swedish Children Cancer Foundation, the Swedish Cancer Society, the Swedish Research Council supported this work, AM: Estonian Research Council (Eesti Teadusagentuur)
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Conflict of Interest: The authors declare no financial conflict of interest.

Word Counts: Abstract: 249; Main text: 5141

Number of figures and tables: 6

Number of Supplementary figures and tables: 6
Statement of translational relevance:

With Talimogene laherparepvec, the first oncolytic virus approved for treatment of cancer, oncolytic virus immunotherapy has a very potential future. Glioblastoma multiforme (GBM) is a rapidly growing neoplasm that is very difficult to cure with the current standard therapies. Oncolytic viruses have been previously tested in the clinic, but without much therapeutic benefit. We have engineered a safe oncolytic Semliki Forest virus (SFV) detargeted using three central nervous system (CNS)-related microRNAs (SFV4miRT) that has potential to kill malignant gliomas and neuroblastomas in preclinical murine models. Because SFV4 is partly type-I interferon insensitive the SFV4miRt virus could kill also tumors cells with moderate secretion of type-I interferons. By using the newly established Human Glioblastoma Cell Culture (HGCC) resource (www.hgcc.se) we demonstrate that SFV4miRT could kill human glioblastoma cell cultures of all molecular subtypes (proneural, classical, mesenchymal, neural), which indicates the potential for its clinical translational.
**Abstract**

**Background:** Glioblastoma multiforme (GBM) and high-risk neuroblastoma are cancers with poor outcome. Immunotherapy in the form of neurotropic oncolytic viruses is a promising therapeutic strategy for these malignancies. Here we evaluate the oncolytic potential of the neurovirulent and partly interferon (IFN)-β-resistant Semliki Forest virus (SFV)-4 in GBMs and neuroblastomas. To reduce neurovirulence we constructed SFV4miRT, which is attenuated in normal CNS cells through insertion of microRNA target sequences for miR124, miR125, miR134.

**Methods:** Oncolytic activity of SFV4miRT was examined in mouse neuroblastoma and GBM cell lines and in patient-derived human glioblastoma cell cultures (HGCC). *In vivo* neurovirulence and therapeutic efficacy was evaluated in two syngeneic orthotopic glioma models (CT-2A, GL261) and syngeneic subcutaneous neuroblastoma model (NXS2). The role of IFN-β in inhibiting therapeutic efficacy was investigated.

**Results:** The introduction of microRNA target sequences reduced neurovirulence of SFV4 in terms of attenuated replication in mouse CNS cells and ability to cause encephalitis when administered intravenously. A single intravenous injection of SFV4miRT prolonged survival and cured 4 of 8 mice (50%) with NXS2 and 3 of 11 mice (27%) with CT-2A, but not for GL261 tumor bearing mice. *In vivo* therapeutic efficacy in different tumor models inversely correlated to secretion of IFN-β by respective cells upon SFV4 infection *in vitro*. Similarly, killing efficacy of HGCC lines inversely correlated to IFN-β response and interferon-α/β receptor (IFNAR)-1 expression.

**Conclusion:** SFV4miRT has reduced neurovirulence, while retaining its oncolytic potential. SFV4miRT is an excellent candidate for treatment of GBMs and neuroblastomas with low IFN-β secretion.
Introduction

Malignant tumors arising from the central nervous system (CNS) are among the most feared types of cancer. Glioblastoma multiforme (GBM) is the most common, malignant form of primary brain tumor with poor prognosis and 1-year survival rate of only 35.7% (1). Surgical resection combined with radiotherapy and adjuvant temozolomide is the current standard of care therapy, which extends survival but is not curative (2). GBM is more common in adults but can also arise in children. Neuroblastoma is the most common extracranial solid cancer in children arising from the neural crest cells. Low-risk neuroblastomas are associated with good outcome but children with high-risk neuroblastoma often relapse even after multimodal therapy (3). Conditionally replicating oncolytic viruses are an attractive option for treatment of cancers, as viruses have a natural ability to replicate inside dividing tumor cells and kill them and simultaneously induce an immune response against the tumor. Herpes simplex virus, adenovirus and Newcastle disease virus have been evaluated in phase I and II clinical trials for treatment of GBM. They show excellent safety data but have so far not resulted in successful tumor shrinkage and prolonged survival (4). Adenovirus has been used to treat children with neuroblastoma with encouraging results (5, 6).

Semliki Forest virus (SFV) is an *Alphavirus* belonging to the *Togaviridae* family (7). It is an enveloped, positive strand RNA virus, having high replication efficacy and ability to kill a variety of tumor cells (7-10). It has a natural neurotropism (11), making it an attractive candidate for use as an oncolytic immunotherapy agent to treat neuroblastoma and GBM. Several strains of SFV have been described, including neurovirulent strain SFV4 and avirulent strain SFV-A(7)74 (referred to as A7/74 from now) (7). The neurovirulence of SFV4 is mainly due to initial replication in neurons and oligodendrocytes, and subsequent spread to rest of the brain resulting in encephalitis (12, 13). A7/74 is usually preferred for use.
as an oncolytic agent, due to its natural avirulence in adult rodents. However, type-I interferons α/β (IFN-α/β) responses elicited by the host in response to virus infection impede A7/74 efficacy (14, 15). The non-structural proteins nsP3-nsP4 of SFV4 are involved in reducing STAT1 Tyr701 phosphorylation (P-STAT1) upon stimulation of virus infected cells with exogenous IFN-β, and it confers its ability to partly resist type-I antiviral defense (16). Thus, SFV4 can be a promising alternative to treat IFN-α/β responsive tumors if its neurovirulence can be attenuated.

MicroRNAs (miRNA) are small non-coding RNAs involved in post-transcriptional regulation of gene expression. miRNA expression is cell type restricted, which has been taken advantage of when designing oncolytic virus to prevent their replication in healthy tissues (17-20). This is achieved by introduction of microRNA target sequences (miRT) in the viral genome, leading to that viral messenger RNA (mRNA; in the case of positive stand RNA viruses it is also genomic RNA) degradation in cells where the matching miRNA are expressed. Regarding microRNAs in the CNS, miR124 and miR125 are highly expressed in mouse brain (21), whereas, miR134 is expressed in the dendrites present in the hippocampal neurons (22). Their expression in GBM and neuroblastoma are partly unknown and investigated herein.

In this article we evaluate the efficacy of SFV immunotherapy with respect to type-I IFN response in patient-derived human glioblastoma cell cultures (HGCC) along with preclinical in vivo glioma and neuroblastoma models. We improved the safety of SFV4 immunotherapy by insertion of target sequences for miR124, miR125 and miR134 in its genome (SFV4miRT) to reduce neurovirulence in mice but retain replication potential and oncolytic capacity in glioma and neuroblastoma cells.
Materials and Methods

Quantitative real-time PCR

Total RNA from cell lines for miRNA detection was isolated using miRNeasy mini kit (Qiagen). To isolate RNA from uninfected mouse tissues: brain, spinal cord and dorsal root ganglion (DRG) were excised, dissected, immersed in RNAlater (Ambion, Thermo Fisher Scientific, Waltham, MA) and homogenized before total RNA was prepared by Trizol isolation (Invitrogen, Carlsbad, CA). cDNA was synthesized using QuantiMir RT kit (System Biosciences, Mountain View, CA). miRNAs were quantified from cDNA by SYBR green based qRT-PCR using the forward primers miR124.F: 5’-TAAGGCACCGTGAATGCC-3’, miR125.F: 5’-TCCCTGAGCCTTAACTTGTA-3’, miR134.F: 5’-TGACTGTTGACCAGAGGG-3’ and the universal reverse primer provided in the QuantiMir RT kit. Human (hU6.F: 5’-CGCAAGGATGACACGCAATTC-3’) and mouse (mU6.F: 5’-TGGCCCCTGCGCAAGGATG-3’) U6 snRNA was used to normalize expression levels of miRNAs of interest.

Total RNA from 15mg mouse brain tissue infected with SFV4 or SFV4miRT was extracted using RNeasy-Mini Kit (Qiagen, Hilden, Germany). RNA samples (1µl) at the concentration of 100ng/µl were used directly to determine viral genome copies, IFN-α and IFN-β mRNA by real-time PCR analysis using iScript One-Step RT-PCR Kit (Bio-Rad). Viral genomes were determined by using primers annealing to nsP1 encoding region: nsP1.F 5’-CGCCAAAAGATTTTGTCA-3’, nsP1.R, 5’-CCATCGTGGGTGGTTAATCT-3’. Primers used for murine IFN-α detection were mIFN-α.F: 5’-AGGACAGGAAAGTTTGGA-3’, mIFN-α.R: 5’-GCTGCTGATGGAGGTCATT-3’, for murine IFN-β detection were mIFN-β.F: 5’-CACAGCCCTCTCCATCAACT-3’, mIFN-β.R:
5'- GCATCTTCTCCGTCATCTCC-3’ and for house-keeping murine hypoxanthine-guanine phosphoribosyltransferase (HPRT1) detection were HPRT.F: 5’- CATAACCTGGTTCATCATCGC -3’, HPRT.R: 5’- GGAGCGGTAGCACCTCCT -3’. All primers were from Sigma-Aldrich (St. Louis, MO). Relative expression of murine IFN-α and murine IFN-β was calculated relative to HPRT1 expression levels. Data were evaluated using the \(2^{-\Delta\Delta CT}\) method (23).

**In vitro cell killing assay using GL261, CT-2A, NXS2 and HGCC cells**

Mouse glioma cell lines GL261, CT-2A and mouse neuroblastoma cell line NXS2 were seeded at densities 20,000 cells/well (96 well plate) and infected with SFV4, SFV4miRT, A7/74 or A7/74miRT at MOIs 0.001-10 PFU/cell. Cells infected with SFV4miRT were pretreated with murine IFN-β (mIFN-β) (R&D Systems, Minneapolis, MN) at concentrations 1ng/ml, 0.01ng/ml or only medium. Cell viability was analyzed at 3 days post virus infection using MTS aqueous cell titer reagent (Promega).

Human GBM HGCC cell lines were seeded at density 5000 cells/well (96 well plate) and infected with SFV4miRT at multiplicity of infection (MOIs) 0.01-1 plaque forming units (PFU)/cell 24 hours after seeding. Cells were pretreated with human IFN-β (hIFN-β) (Peprotech, Rocky Hill, NJ) at concentrations 10 ng/ml, 1 ng/ml, 0.01 ng/ml or only medium before virus infection. Cell viability was analyzed at 2 and 3 days post-infection using AlamarBlue Cell viability reagent (Thermo Fisher Scientific).

**IFN-β ELISA and Flow cytometry**

Murine GL261, CT-2A, NXS2 and human HGCC cells were plated at density 50,000 cells/well (24 well plate) in 250μl medium, infected with SFV4 at MOI-0.01 or 1
PFU/cell; control cells were uninfected. Supernatants were collected after 24 hours. mIFN-β was quantified using LEGEND MAX Mouse IFN-β ELISA Kit (BioLegend, San Diego, CA). IFN-β secretion by HGCC cells was quantified using VeriKine Human Interferon Beta ELISA Kit (PBL Assay Science, Piscataway, NJ). Murine cells were stained at 24 hours post-infection using biotin-labeled anti-mouse interferon-α/β receptor (IFNAR)-1 antibody (BioLegend) and streptavidin-conjugated AlexaFluor 488 (Thermo Fisher Scientific) while HGCC cells were stained with phycoerythrin (PE)-conjugated human IFNAR1 antibody (R&D Systems). Stained cells were analyzed in BD FACS Canto II (BD Biosciences, Franklin Lakes, NJ).

Animal experiments

4-5 weeks old outbred NMRI female mice (Janvier Labs, France) were injected intravenously (i.v.) or intraperitoneally (i.p.) with SFV4-nLuc or SFV4-nLucmiRT (1×10^7 PFU in 100μl PBS/mouse). Mice were sedated using isoflurane (Isofluran Baxter, Baxter Medical AB, Sweden); sites of virus replication were revealed by measuring nano-luciferase (nLuc) activity using Nano-Glo® Luciferase Assay substrate (100μl i.v., diluted 1:40 in 1xPBS) (Promega) and NightOWL in vivo imaging system (Berthold Technologies, Bad Wildbad, Germany). Some mice were sacrificed 3, 4 or 5 days post virus injection and brain samples were either snap frozen or fixed with cold 4% paraformaldehyde overnight. Fixed tissues were stored in 70% ethanol until paraffin embedment.

4-5 weeks old female, A/J mice (Taconic, Denmark) were implanted with NXS2 cells (2×10^6 cells in 100μl PBS) subcutaneously (s.c.) in the hind flank and were treated intratumorally (i.t.) (5×10^7 PFU in 50μl) or i.v. (1×10^7 PFU in 100μl) injections of either SVF4miRT or A7/74miRT on day 7 post tumor inoculation. Tumor growth was monitored by
caliper measurements and tumor size was calculated using the ellipsoid volume formula
\[(\text{Length} \times \text{Width} \times \text{Depth}) \times \pi/6].\]

4-5 weeks old female C57BL/6JRj mice (Janvier labs, France) were used for the orthotopic glioma models. GL261-Fluc \((2 \times 10^4 \text{ cells in } 2\mu l \text{ DPBS})\) or CT-2A-Fluc \((5 \times 10^4 \text{ cells in } 2\mu l \text{ DPBS})\) were injected intracranially 1 mm anterior and 1.5 mm right from bregma at 2.7 mm depth using a Hamilton syringe and stereotactic equipment (Agnthos AB, Lidingö, Sweden). Tumor growth was measured as bioluminescence signal using D-luciferin \((150 \text{ mg/kg i.p.; PerkinElmer, Waltham, MA})\) and NightOwl imaging. All mice that had stable luciferase signal 7 or 8 days after tumor inoculation received a single dose of \(4 \times 10^7 \text{ PFU or } 1 \times 10^8 \text{ PFU of SFV4miRT virus or PBS i.v.}\) Mice were sacrificed upon appearance of symptoms like paralysis, hunchback, loss of over 20% of body weight, or notable distress.

**Immunohistochemistry**

Paraffin-embedded brain tissues were sliced into 6\(\mu\)m sections and deparaffinized. Antigen revival was done by heating the slides for 20min at 121°C in antigen revival solution (Vector Laboratories, Burlingame, CA). Sections were blocked with goat serum (Vector Laboratories, Burlingame, CA) and stained with rabbit antibody (diluted 1:3000 in PBS) against SFV structural proteins (kind gift form Dr. Ari Hinkkanen, University of Eastern Finland). Mouse brain cell types were detected using mouse anti-MAP2 (Sigma), mouse anti-GFAP (Sigma) and mouse anti-CNPase (Sigma) antibodies. IFN-\(\beta\) was detected using rabbit anti-IFN-\(\beta\) polyclonal antibody (Thermo Fisher Scientific). Primary antibody staining was detected by probing with goat-anti-rabbit-HRP or goat-anti-rabbit-AF647 or donkey-anti-mouse-AF555 secondary antibodies (Thermo Fisher Scientific). The sections were imaged in Zeiss AxioImager microscope (Zeiss, Oberkochen, Germany).
Statistical analysis

One-way ANOVA post-hoc Tukey test for multiple comparisons was used for statistical comparison of means between more than two experimental groups in an experiment. Statistical comparison of Kaplan-Meier survival curves of mice treated with different viruses was performed by Log-rank (Mantel-Cox) test. Association of cell viability with IFN-β secretion and IFNAR1 expression was evaluated by fitting a linear regression model by backward selection. Associations with p-value <0.05 was considered as statistically significant. Results were analyzed using GraphPad-Prism 6 (Graph-Pad Software, San Diego, CA) and R statistical programming software.

Biosafety level and ethics declaration

Swedish Work Environment Authority has approved the work with genetic modification of SFV (ID number 202100-2932 v66a14 (laboratory) and v67a10 (mice)). All experiments regarding modified SFV were conducted under Biosafety level 2. The local animal ethics committee in Stockholm (N164/15, N170/13) approved the animal studies. All human GBM samples were collected in accordance with protocols approved by the regional ethical review board (2007/353) and after obtaining written consent by all of the patients.

Other related material and methods can be found in the supplementary document.
Results

MicroRNAs miR124, miR125 and miR134 are expressed in healthy CNS cells but not in GBM and neuroblastoma cells

Expression of miR124, miR125 and miR134 were examined in a variety of cells and tissues. All healthy murine tissues from the CNS (brain, spine and DRG) expressed miR124 and miR125 while miR134 was expressed only in healthy mouse brain. In vitro cultured murine neural stem cells (NSC) had low expression of all three miRNAs, differentiated neurons expressed miR124, oligodendrocytes expressed miR134 and astrocytes had relatively low expression of all three miRNAs (Fig 1A, miR expression values normalized to U6 snRNA - Supplementary Table 1). Most human and mouse neuroblastoma and GBM cells had lower expression of all three miRNAs in comparison to healthy murine neural tissues. Exceptions were the U3034 HGCC cells, which expressed moderate levels of all three miRNAs, and U3024 with expression of miR134 (Fig 1A). Non-CNS-related tumor cell lines Hela (cervical cancer) and mel526 (Melanoma), had low expression of all three miRNAs as expected (Fig 1A).

MiRT-detargeted SFV retains replication ability in tumor cells but is attenuated in in vitro differentiated murine CNS cells

To prevent neurovirulence of SFV, we inserted 2 copies of sequences complementary to each of miR124, miR125 and miR134 (hereafter referred to as target sequences, SFV4miRT or 74miRT) in the beginning of 3’UTR of the SFV genome that was modified to contain duplicated subgenomic (SG) promoter (Fig 1B). The oncolytic capacity of SFV4miRT was compared to its wild-type counterpart (SFV4) in vitro.
Neuroblastoma and glioma cells were infected with SFV4miRT or SFV4, using amount of virus corresponding to MOIs from 0.001 to 10 PFU/cell (titrated on BHK-21 cells). No difference was observed between viruses with and without miRTs to kill murine neuroblastoma NXS2 (Fig 1C), murine glioma GL261 (Fig 1D) or CT-2A cells (Fig 1E). CT-2A cells are more susceptible to viral oncolysis, which is probably due to more susceptibility to virus infection while GL261 were more resistant to virus killing, which may reflect that these cells are poorly infected by SFV viruses. The same trend was observed when A7/74 and A7/74miRT strains were tested (Supplementary Figure S3A-C). Also, all four viruses efficiently killed human neuroblastoma cell lines in a dose dependent manner (Supplementary Figure S2A-D).

In vitro cultured NSC, differentiated neurons, astrocytes and oligodendrocytes were infected with SFV4-GFP or SFV4-GFPmiRT to assess toxicity in healthy CNS cells. Virus replication (GFP expression as an indirect measure of viral mRNA translation) for the SFV4-GFPmiRT virus was largely attenuated in neurons, astrocytes and oligodendrocytes but not in NSC (Fig. 1F-G). There was also a significant reduction in the percentage of relative GFP positive cells for the SFV4-GFPmiRT and A7/74-GFPmiRT infected BHK-21 when transfected with miR124, miR125 or miR134 were added, whereas SFV4-GFP and A7/74-GFP were not affected by the addition of microRNAs (Supplementary Figure S1).

SFV4miRT has reduced replication in adult mouse brain, reduced neurovirulence and reduced type-I interferon response compared to SFV4

Infection and replication characteristics of SFV4miRT were assessed in vivo by infecting i.v. with 1x10^7 PFU of SFV4 and SFV4miRT. Mice infected with SFV4 start developing virus-related symptoms like mild paralysis at day 3 post-infection and had to be
sacrificed due to severe neurological symptoms at day 5 (Fig 2A, C). Mice infected with
SFV4miRT did not develop any visible virus-related symptoms (Fig 2A, D). Infected mice
were sacrificed on day 3 or 5 post-infection and virus replication in mouse brain was
quantified. SFV4-infected mice had very high viral RNA copy numbers at day 3 (>10^6 viral
genome copies/gram) and that increased even further by day 5 (>10^7 viral genome
copies/gram) (Fig 2B). The average viral genome copy number in the brain for SFV4miRT-
infected mice was in the range of 10^3 viral genome copies per gram on day 3 and 10^4 viral
genome copies on day 5 (Fig 2B), which is around 1000 times lower compared to the
neurovirulent SFV4. Mice infected with Nanoluciferase-encoding virus SFV4-nLuc either i.p.
(Fig 2E) or i.v. (Fig 2F) displayed wide distribution of virus infection at day-1 post-infection,
which remained on day-4. SFV4-nLucmiRT infected mice had very low luminescence signal
day-1 post-infection and luminescence signal was completely lost by day-4 except in the tail
region of the mice, who were i.v.-injected in the tail vein (Fig 2E, F). SFV4miRT induced
significantly lower IFN-α and IFN-β mRNA levels in the brain as compared to the wild type
SFV4 counterpart both at 3 and 5 days post-injection (Fig 2G, H). IFN-β protein levels were
also verified by IHC in SFV4 and SFV4miRT-infected mouse brain at day 5 (Fig 2I, J). IHC
analysis also confirmed the observation made by qRT-PCR analysis of the IFN-β mRNA
levels in the brain.

Brain sections from SFV4-infected, non-tumor-bearing mice had positive
staining for SFV proteins (Fig 3B). Mice infected with SFV4miRT did not have any strong
staining patterns for viral proteins above the background staining observed in PBS-treated
mice (Fig 2A, C). SFV-infected mouse brains were also co-stained for neuron-specific
(MAP2), oligodendrocyte-specific (CNPase) and astrocyte-specific (GFAP) markers. SFV4
primarily infected neurons (Fig 3E), seen as co-localized SFV (red) and MAP2 (green)
staining, also infected oligodendrocytes to some extent (Fig 3H) but clearly did not infect
astrocytes (Fig 3K). The SFV4miRT replication was attenuated in all the 3 cell types; neurons (Fig 3F), oligodendrocytes (Fig 3I) and astrocytes (Fig 3L), and the staining pattern resembled that of brain from PBS-treated mice (Fig 3D, G, J). This data confirms that miRT-tagged virus replication was strongly attenuated in mouse brain.

SFV4miRT prolonged survival of mice bearing subcutaneous neuroblastoma tumors

Therapeutic efficacy of SFV4-miRT was evaluated in A/J mice implanted s.c. with syngeneic murine neuroblastoma NXS2. Tumors grew vigorously in the PBS-treated group and all mice were sacrificed before day 20. Tumor growth for individual mice (Fig 4A, C) was significantly delayed and a significant survival benefit (Fig 4B, D; 50% remained tumor free) was observed in mice treated with SFV4miRT irrespective of route of virus administration. None of SFV4-miRT treated animals developed encephalitis and the mice were sacrificed due to tumor growth (volume> 1000mm³). All mice that remained tumor free after day 60 were re-challenged with NXS2 cells and none of them developed tumors (data not shown). The microRNA-detargeted IFN-sensitive SFV strain A7/74-miRT also showed similar therapeutic efficacy on curing mice bearing NXS2 tumors, regardless of administration route (Supplementary Figure S3D-G).

SFV4miRT prolongs survival of mice bearing orthotopic CT-2A glioma but not GL261 glioma

Therapeutic efficacy of SFV4miRT was evaluated in two orthotopic murine gliomas, GL261 and CT-2A, in syngeneic C57BL/6 mice. All mice inoculated with GL261 had tumor growth at day 8, the time of treatment. GL261 tumor-bearing mice treated with
SFV4miRT did not have statistically significant survival benefit compared to PBS treated group. Only one mouse in the SFV4miRT treatment group survived (Fig 5A) and one mouse had tumor regression but later relapsed (Fig 5C). Around 85% of mice inoculated with CT-2A cells developed tumors at day 8 after tumor implantation, and only those with tumors were included and treated. CT-2A tumor-bearing mice treated with SFV4miRT had a significant survival benefit and delayed tumor growth (3/11 mice survived, 27.3% survival) compared to the PBS treated group (Fig 5B). Representative pictures of mice with luminescence signal and immunohistochemistry from GL261 and CT-2A tumor-bearing mice treated with SFV4miRT are shown (Fig 5C-H). CT-2A tumor-bearing mice treated with SFV4miRT had strong staining for SFV proteins (Fig 5H) in the tumor, while the GL261 tumor bearing mice did not have any staining for SFV proteins (Fig 5G).

**Therapeutic efficacy of SFV4miRT treatment negatively correlates with IFN-β secretion by tumor cells upon virus infection.**

To analyze mechanisms leading to difference in susceptibility to SFV-killing *in vitro* and correspondingly very different therapeutic efficiencies in the three *in vivo* models, the abilities of NXS2, GL261 and CT-2A cell lines to mount type-I IFN response upon SFV4miRT infection was measured. Cells were infected with SFV4miRT at MOI-0.01 or 1 PFU/cell and 24 hours later their ability to secrete IFN-β and regulate IFNAR1 expression was analyzed. Uninfected cells and cells infected at MOI-0.01 secreted un-detectable amounts of IFN-β. Upon infection at MOI-1 GL261 cells secreted high amounts of IFN-β while CT-2A cells secreted moderate amounts of IFN-β and NXS2 cells secreted undetectable amounts of IFN-β (Fig 6A). There was no significant change in the surface expression of IFNAR1 upon infection of cells with SFV4miRT for both MOIs evaluated (Fig 6B). The reduced expression
of IFNAR1 observed on CT-2A cells at MOI-1 is due to cell death caused by virus infection (Fig 6B).

Previous studies have revealed that SFV4 is partly resistant to antiviral effects of type-I IFN (16). Therefore, cell-killing assays were performed on NXS2, GL261 and CT-2A cells pretreated with high amounts of exogenous recombinant murine IFN-β. Lower concentration of IFN-β (0.01ng/ml) was sufficient to reduce death of CT-2A cells at MOI-0.01 and 0.001 but had virtually no effect on survival of NXS2 cells (Fig 6C). At a high virus dose (MOI-10), no protective effect of exogenous IFN-β was observed for NXS2 and CT-2A (Fig 6C). GL261 cell line was more resistant to virus killing compared to NXS2 and CT-2A even in the absence of exogenous IFN-β. However, in the presence of exogenous IFN-β (0.01ng/ml and 1ng/ml), GL261 cells were protected from SFV4miRT killing even at the highest virus dose (MOI-10) (Fig 6C).

**SFV4miRT efficiently kills patient-derived primary human GBM cell cultures**

Oncolytic effect of SFV4miRT in eleven HGCC lines either in the presence or absence of exogenous human IFN-β was also evaluated. The cohort of cell lines used represented all four subtypes of GBM and detailed information is mentioned in Supplementary Table 2. After 72 hours, most HGCC cell lines were efficiently killed in a dose dependent manner by SFV4miRT (Supplementary Figure S3). The efficacy of virus-mediated tumor cell killing decreased in the presence of exogenous hIFN-β in a concentration dependent manner (Supplementary Figure S3). Fig 6D represents mean-relative viability across different viral doses (MOI-0.01 to 1 PFU/cell) in the absence or presence of 1ng/ml exogenous hIFN-β. The presence of IFN-β inhibited cell killing, with U3024 being an
exception having unchanged cell viability when hIFN-β was added. We believe that this is attributed to the high miR134 expression of U3024, leading to virus genome degradation (Fig 1A).

IFN-β secretion from the HGCC cell line was measured before and after SFV4miRT infection. None of the uninfected HGCC lines secreted IFN-β (Fig 6E). Only U3024 and U3213 secreted detectable levels of IFN-β when infected by SFV4miRT at low virus dose (MOI-0.01). However, at high virus dose (MOI-1), a majority of the HGCC lines (U3013, U3021, U3024, U3028, U3034, U3053, U3054 and U3213) secreted varied amounts of IFN-β (Fig 6F). Most of the HGCC lines that secreted IFN-β were less effectively killed by SFV4miRT in the absence of exogenous hIFN-β (black squares above dotted line, Fig 6D). Furthermore, the mean relative viability of cells after SFV4miRT infection correlated with the ability of cells to secrete IFN-β (p=0.01164) (Fig 6G). The HGCC line U3034 secreted very low IFN-β upon virus infection, but had high cell viability (Fig 6G, grey dot), which may be because U3034 had expression of all three miRNAs (Fig 1A). Change in IFNAR1 expression was measured 24 hours after SFV4miRT infection by flow cytometry. All HGCC cells expressed basal levels of IFNAR1 (Fig 6F, white bars) and had only feeble change in receptor expression after virus infection. Mean relative cell viability after SFV4miRT infection correlated to IFNAR1 expression (MFI values) after SFV4miRT infection (p=0.00205) (Fig 6H). Taken together, efficacy of SFV4miRT to kill HGCC lines was negatively associated with the amount of IFN-β secreted by cells upon virus infection and levels of IFNAR1 expression.
Discussion

Many oncolytic viruses have been tested and proven to be safe in the clinic for GBM treatment (4, 24). Despite encouraging safety results there are many factors like virus delivery, anti-virus immune response, intratumoral spread of infection that still need to be addressed to significantly improve efficacy and make oncolytic virus therapies for GBM a reality (25, 26). An important factor impeding development of successful therapies for GBM is lack of models closely representing the characteristics of tumor in patients. We utilized the HGCC biobank to test efficacy of oncolytic SFV therapy. HGCC lines are cultured in serum-free medium and retain the characteristics of their original tumors as compared to the traditionally used human GBM cell models (27, 28). They also express NESTIN and SOX2, markers that are usually associated with GBM stem cells. It is commonly believed that GBM stem cells are not efficiently killed by conventional therapies (29, 30). Hence, efficacy of SFV and other potential therapies to kill HGCC lines may aid in better evaluating its clinical translational ability.

The SFV A7/74 strain has been successfully tested safe for use as an oncolytic agent in several preclinical models for GBM and other cancer types (8, 31, 32). However, type-I IFN responsive gliomas are resistant to A7/74 virotherapy in murine models (14). The neurovirulent strain SFV4 is interesting because it has the ability to partly resist type-I antiviral defense (33) and this phenotype is important for treatment of IFN-α/β responsive tumors (16). To attenuate neurovirulence of the lethal SFV4 strain, we inserted target sequences complementary to miR124, miR125 and miR134 into the SFV genome (Fig 1B). The initial choice of these miRTs was based on their expression in CNS reported in the literature. miR124 is highly expressed in all brain regions, predominantly in the neurons except for the pituitary gland (21, 34, 35), miR125 is expressed in mid-hindbrain boundary and neurons in mouse (21, 36) and miR134 is a brain-specific miRNA, localized in dendrites.
and synaptic sites (22). We also confirmed that these miRNAs are highly expressed in normal
mouse CNS cells, including in vitro-differentiated neurons, and are downregulated in several
murine gliomas and neuroblastomas (Fig 1A). Most HGCC lines also had low expression of
these miRs, which makes these miRNAs suitable targets to be used to attenuate virus
replication in normal CNS cells however, expression profiles in healthy human brain cells
need to be confirmed before clinical translation. Previously, miRT124-tagged SFV4 was
shown to possess significantly reduced neurovirulence (16, 20). But, a few mice still
developed severe neurological symptoms, with virus replication detected in the
oligodendrocytes of corpus callosum and spine (16, 20). The triple miRT de-targeted SFV4
virus (SFV4miRT) presented herein had attenuated replication in mouse brain cells
specifically neurons, and oligodendrocytes (Fig 3). SFV4 did not infect astrocytes (Fig 3J-L),
which is in accordance with previous reports (37). In immune competent mice SFV4 is also
known not to infect any other organ except CNS (37), therefore we believe that SFV4miRT
has an improved safety profile.

Insertion of miRTs did not inhibit virus replication in tumor cells lines that did
not express any of the miRNAs (Fig 1C-E, Supplementary Figure S2, 3). But in cell lines
where one or more of these miRNAs were expressed (U3024 and U3034), virus replication
was attenuated when administered at low MOIs (Supplementary Figure S3). Non-tumor
bearing brain samples from SFV4miRT infected mice had no or very little IFN-α/β secretion
compared to samples from mice infected with SFV4 (Fig 2G-J). The levels of IFN-α/β
secretion also correlated with virus load in the brain, i.e., more IFN-α/β on day 5 post-
infection, where the titers of the SFV4 virus in the brain were also higher (Fig 2B, G-J). This
finding is in accordance with previous reports, where miRT124 attenuated SFV4 was
used (20) and confirms that IFN-β secretion depends on the efficiency of virus replication in
the brain (38).
Successful biological outcomes of type-I IFN response depends on expression of all components in the IFN signaling pathway like IFNα/β itself, IFN receptor (IFNAR), signaling molecules JAK1, TYK2, STAT1, STAT2 and Interferon Stimulated Genes (ISGs) (39). In three murine tumors models tested, the neuroblastoma cell line NXS2 did not secrete IFN-β upon virus infection, but the two glioma cell lines CT-2A and GL261 secreted IFN-β upon virus infection, with GL261 secreting the highest amount of IFN-β (Fig 6A). In the murine tumor models, IFN-β secretion levels (autocrine effect) negatively correlated with therapeutic efficacy in vivo (Fig 4-6). NXS2-bearing mice benefitted the most (50% survival) from treatment with SFV4miRT (Fig 4) followed by CT-2A tumor bearing mice, then GL261 tumor bearing mice (Fig 5A, B). It should be noted that NXS2 model used was s.c., while orthotopic brain models were used for GL261 and CT-2A, and therapeutic efficiencies can differ because of the location of the tumor. GL261-bearing mice treated with the IFN-β sensitive strain A7/74miRT did not have any survival benefit (data not shown). This is in accordance with previously published data showing that even low amount of IFN-β is enough to inhibit replication of A7/74 strain in both GL261 and CT-2A models (14, 16). SFV4miRT efficiently killed most of the HGCC lines in vitro and efficacy depended on the amount of IFN-β secreted by the cells upon virus infection (autocrine effect) (Fig 6G), which is in accordance with the murine cell line data.

In the presence of exogenous IFN-β, SFV4miRT killed NXS2 and CT-2A cells in a dose dependent manner in vitro (Fig 6C). However, GL261 were resistant to SFV4miRT oncolysis in the presence of exogenous IFN-β, even at the highest virus dose tested (Fig 6C). As previously observed, in clones of mouse colon carcinoma, CT26WT and CT26LacZ differential expression of ISGs can contribute to dramatic difference in the efficacy of SFV therapy (40). ISG expression in CT-2A and GL261 is unknown, which may be a reason for the observed difference in oncolytic response. In HGCC lines, addition of exogenous IFN-β
decreased SFV4miRT oncolysis, indicating that all HGCC lines are responsive to IFN-β (Fig 6D). However, at high SFV4miRT dose, IFN-β did not inhibit viral oncolysis, confirming the ability of SFV4 to partly resist type-I antiviral defense (Supplementary Figure S3), as previously reported (33).

The ability of tumors to initiate antiviral defense is an important factor that determines the therapeutic efficacy of SFV4miRT. This emphasizes the need to test oncolytic potential of SFV in immune competent models as compared to xenografts where excellent efficacy is observed (41). It may still be possible to use the avirulent, type-I IFN sensitive A7/74 strain for treatment of non-IFN-β responsive tumors like NXS2 but our data stresses the need for an IFN-insensitive SFV strain for successful treatment of IFN-responsive tumors. Recent studies suggest that viral-vaccines combined with checkpoint inhibition (αPD-1 or αCTLA4 antibodies) is beneficial for treatment of aggressive tumors like GL261 (42). Oncolytic viruses are naturally immunogenic; combination of SFV4miRT with checkpoint inhibition for treatment of GBM would be worth to investigate. In conclusion, our results show that, insertion of multiple miRTs reduces SFV4 neurovirulence and yields an improved safety profile of SFV4 as an anti-tumor agent.
Funding

The Swedish Children Cancer Foundation (PROJ12/082), the Swedish Cancer Society (CAN2013/373) and the Swedish Research Council (K2013-22191-01-3) supported this work (to ME). Estonian Research Council (Eesti Teadusagentuur) and grant number 20-27 (to AM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgement

The authors wish to thank Patrik Johansson (Uppsala University) for tips and guidance with statistical analysis, Berith Nilsson, Grammatiki Fotaki, Chuan Jin and Tina Sarén (Uppsala University) for assistance in the lab, Dr. Miika Martikainen for fruitful discussions and reviewing the manuscript. We also thank Prof. Ari Hinkkanen (University of Eastern Finland) for providing us α-SFV antibodies and Dr. Markus Vähä-Koskela (Ottawa Hospital Research Institute) for providing the CT-2A cell lines.

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Figure Legends

Figure 1. MicroRNA screening and oncolytic potential of miRT-tagged SFV in murine tumor cell lines. (A) Heat map representing expression pattern of miR124, miR125 and miR134 in normal mouse CNS tissues and in several murine and human neuroblastoma and GBM cell lines. Expression levels of miRNA were normalized to control murine or human U6 snRNA respectively. The heat map was plotted by taking Z-scores for each miRNA across all the samples to equalize the scale. (B) Schematic representation of SFV vector construction. Two copies of sequences complementary to miR124, miR125 and miR134 were inserted at the 3' UTR of the modified SFV genome to construct miRT-tagged viruses. Reporter transgenes, when included, were inserted after a duplicated copy of the subgenomic (SG) promoter. (C-E) In vitro killing ability of SFV4 and SFV4miRT on murine neuroblastoma cells NXS2 (C), murine glioma cells GL261 (D) and murine glioma cells CT-2A (E) at MOIs 0.001-10. Cell viability was measured using MTS at 72 hours post-infection and values represent viability normalized to that of un-infected cells. Data are presented as mean±SD (n=2, with 3 internal replicates). (F) In vitro cultured and differentiated primary murine CNS cells were infected with SFV4-GFP or SFV4-GFPmiRT (2000 PFU). GFP-positive cells were quantified 16 hours post-infection using flow cytometry. Data are presented as % mean±SD GFP+ cells of total (n=3). Statistical comparison of means was assessed using Two-way ANOVA, with Sidak post-test for correcting multiple comparisons (***=p<0.0001, ***=p<0.001, **=p<0.01, n.s =p>0.05) (G) Representative images of murine neural stem cells (Nestin), neurons (MAP2), astrocytes (GFAP) and oligodendrocytes (CNPase) infected with either SFV4-GFP or SFV4-GFPmiRT (63X magnification, Scale Bar 20 μm). NSC-murine neural stem cells.
Figure 2. SFV4miRT has reduced neurovirulence, does not cause virus-associated neurological symptoms to adult mice and elicits lower host IFN-β response compared to SFV4. (A) Survival curves of mice infected i.v. with SFV4-nLuc or SFV4-nLucmiRT at dose 1x10^7 PFU (n=8). (B) Virus genome copy numbers measured by qRT-PCR in brain tissues collected from groups of mice infected i.v. with SFV4 or SFV4miRT (n=4) at day 3 and 5 post-infection. (C-D) Evaluation of virus associated symptoms in mice infected i.v. with 1x10^7 PFU/mice SFV4 (C) or SFV4miRT (D). Score for the symptoms are as follows: 0- no symptom, 0.1- mild limping or minor in coordination, 0.2- paralysis and/or marked in coordination, ≥0.3- severe paralysis or death of the mouse. (E-F) Biodistribution of virus in adult mice injected with SFV4-nLuc or SFV4-nLucmiRT at dose 1x10^8 PFU either i.p. (E) or i.v. (F) at day 1 and 4 post-infection. nLuc expression was monitored following i.v. injection (100μl) of Nano-Glo® Luciferase Assay substrate and imaging mice using NightOWL in vivo imaging system. (G-H) Relative mRNA expression of IFN-α (G) and IFN-β (H) compared to the HPRT1 housekeeping gene in brain tissues collected from groups of mice infected i.v. with SFV4 or SFV4miRT (n=4) at day 3 and 5 post-infection. Baseline (BSL) means relative mRNA expression of IFN-α and IFN-β in brain tissues collected from un-infected mice. (I-J) Immunohistochemical analysis for IFN-β protein expression in brain tissue of mice injected i.v. with SFV4 (I) or SFV4miRT (J). Samples were collected 5 days post-infection and stained with anti-mouse IFN-β monoclonal antibody (Tile scan images: 10X magnification, Scale bar: 2 mm, Zoomed images: 20X magnification, Scale bar: 100 μm). Red arrows indicate positive staining for IFN-β. Statistical comparison of means was assessed using One-way ANOVA, with Tukey post-test for correcting multiple comparisons (****=p<0.0001, ***=p<0.001, **= p<0.01, n.s =p>0.05).
Figure 3. SFV4miRT has reduced replication in mouse brain. (A-C)

Immunohistochemical analysis for SFV proteins in mouse brain tissue in mice injected i.v. with PBS (A), SFV4 (B), or SFV4miRT (C). Samples were collected 5 days after injection and stained with a polyclonal anti-SFV antibody (Tile scan images: 10X magnification, Scale bar: 2 mm, Zoomed images: 20X magnification, Scale bar: 100 μm). (D-L)

Immunofluorescent analysis for SFV proteins (in red) and brain cell types (in green), neurons (D-F), oligodendrocytes (G-I) and astrocytes (J-L) in mice injected i.v. with PBS (D, G, J), SFV4 (E, H, K), or SFV4miRT (F, I, L). Samples were collected 5 days after injection and stained with a polyclonal anti-SFV and anti-MAP2 (neuron), anti-CNPase (oligodendrocyte) or anti-GFAP (astrocyte) antibodies (Tile scan images: 20X magnification, Scale bar: 200 μm, Zoomed images: 40X magnification, Scale bar: 20 μm).

Figure 4. NXS2 tumor growth and survival of mice after a single treatment with SFV4miRT. NXS2 neuroblastoma cells were injected s.c. in the right hind flank of female A/J mice. Mice were treated when they had palpable tumors 7 days after tumor inoculation. (A) Tumor size of individual mice and (B) Kaplan-Meier survival curves for mice treated i.t. either with 50 µl PBS (n=10) or SFV4miRT (n=7, 5×10⁷ PFU). (C) Tumor size of individual mice and (D) Kaplan-Meier survival curves for mice treated i.v. with either with 100 µl PBS (n=10) or SFV4miRT (n=8, 1×10⁷ PFU). The survival curves were compared to PBS-treated mice by performing a Log-rank (Mantel-Cox) test (**** = p< 0.0001).

Figure 5. Orthotopic glioma tumor growth and survival of mice after a single i.v. treatment with SFV4miRT. GL261-Fluc or CT-2A-Fluc murine glioma cells were injected intracranially in female C57BL/6NRj mice. Mice that developed tumors (as detected by
luminescence) were treated i.v. with SFV4miRT (1×10⁸ PFU/in 100 μl) or PBS 7 days after tumor inoculation. Tumor growth was monitored by measuring luciferase expression using NightOWL imaging system. (A) Kaplan-Meier survival curves for GL261 tumor-bearing mice treated with either SFV4miRT (n=15) or PBS (n=13). (B) Kaplan-Meier survival curves for CT-2A tumor-bearing mice treated with either SFV4miRT (n=11) or PBS (n=7). The survival curves were compared by performing a Log-rank (Mantel-Cox) test (** = p<0.001, n.s = p >0.05). (C-D) Representative in vivo luminescence images of mice bearing GL261 (C) and CT-2A (D) tumors treated with SFV4miRT having complete response (CR), relapse or no response. (E-H) Immunohistochemical analysis for SFV proteins in tumor tissue, GL261 (E, G) or CT-2A (F, H) injected i.v. with PBS (E-F), or SFV4miRT (G-H). Samples were collected 3 or 4 days after injection and stained with a polyclonal anti-SFV antibody (Tile scan images: 20X magnification, Scale bar: 200 μm, Zoomed images: 63X magnification, Scale bar: 10 μm).

Figure 6. The tumor cell killing ability of SFV4miRT negatively correlates to IFN-β secretion by tumor cells. NXS2, GL261 and CT-2A cells were left uninfected or infected with SFV4miRT at MOIs 0.01 and 1. (A) IFN-β secretion was measured 24 hours post infection in cell culture supernatant by ELISA. Data are presented as mean±SD (3 internal replicates, supernatants from 2 individual experiments were pooled). (B) IFNAR1 expression was measured 24 hours post-infection by flow cytometry through staining of cells with a biotinylated anti-mouse IFNAR1 antibody followed by a streptavidin-conjugated AlexaFluor 488 secondary antibody. Cells from 2 individual experiments were pooled and analyzed by flow cytometry. (C) In vitro killing ability of SFV4miRT on NXS2, GL261 and CT-2A cells pre-treated with murine IFN-β (1ng/ml, 0.01ng/ml or no IFN-β) at MOIs 0.01-10. Cell viability was measured using MTS at 72 hours post-infection and values represent viability.
normalized to un-infected cells. Data are presented as mean±SD (n=2, with 3 internal replicates). (D) In vitro killing ability of SFV4miRT on HGCC pre-treated with human IFN-β (1ng/ml or no IFN-β). Cell viability was measured using Alamar Blue reagent at 72 hours post-infection and values represent mean cell viability across MOIs 0.01-1 of SFV4miRT. Data are presented as mean±SD (n=2, with 3 internal replicates). (E) IFN-β secretion was measured in cell culture supernatant by ELISA 24 hours post-infection. Data are presented as mean±SD (2 internal replicates, supernatants from 2 individual experiments were pooled). (F) IFNAR1 expression was measured 24 hours post-infection in a flow cytometer by staining cells with PE-conjugated antibody against human IFN-α/βR1. Cells from 2 individual experiments were pooled and analyzed in the flow cytometer. (G) Scatter plot representing mean viability of cells and IFN-β secretion after SFV4miRT infection (MOI-10) for the respective HGCC lines. (H) Scatter plot representing mean viability of cells at MOI-1 infection and IFNAR1 expression for the respective HGCC lines.
Figure 2

A: Percent survival over time post virus injection for SFV4 and SFV4miRT.

B: Viral genome copies per gram brain over time post virus injection for SFV4 and SFV4miRT.

C: Number of mice surviving at different time points post virus injection for SFV4 and SFV4miRT.

D: Number of mice surviving at different time points post virus injection for SFV4miRT.

E: Imaging of mice on Day 1 and Day 4 post i.p. and i.v. virus injection.

F: Imaging of mice on Day 1 and Day 4 post i.p. and i.v. virus injection.

G: Relative mRNA expression of IFN-α.

H: Relative mRNA expression of IFN-β.

I: Histological sections of tissue post virus injection.

J: Histological sections of tissue post virus injection.
Figure 4

A. Tumor size (mm$^3$) over days post tumor inoculation for single dose i.t. injection.

B. Percent survival comparison between PBS and SFV4miRT.

C. Tumor size (mm$^3$) over days post tumor inoculation for single dose i.v. injection.

D. Percent survival comparison between PBS and SFV4miRT.
Figure 6

(A) IFN-β Conc. (pg/mL)

(B) MFI of IFNAR1

(C) Relative cell viability (%)

(D) Mean Relative Viability

(E) IFN-β Conc. (pg/ml)

(F) MFI of IFNAR1

(G) Mean Relative Viability

(H) Mean Relative Viability

Author Manuscript Published OnlineFirst on September 16, 2016; DOI: 10.1158/1078-0432.CCR-16-0925
Clinical Cancer Research

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Clin Cancer Res  Published OnlineFirst September 16, 2016.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-16-0925
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