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Gene Therapy Using Therapeutic and Diagnostic Recombinant Oncolytic Vaccinia Virus GLV-1h153 for Management of Colorectal Peritoneal Carcinomatosis

Clarisse Eveno, M.D.[1], **Kelly Mojica, B.S.**[1], **Justin W. Ady, M.D.**[1], **Daniel L.J. Thorek, PhD**[2], **Valerie Longo, B.S.**[3], **Laurence J. Belin, M.D.**[1], **Sepideh Gholami, M.D.**[1], **Clark Johnsen, B.F.A.**[1], **Pat Zanzonico, PhD**[3], **Nanhai Chen, PhD**[4], **Tony Yu, PhD**[4], **Aladar A. Szalay, PhD**[4,5], and **Yuman Fong, M.D., F.A.C.S.**[1]

[1]Department of Surgery, Memorial Sloan–Kettering Cancer Center, New York, NY 10065

[2]Division of Nuclear Medicine, Department of Radiology and Radiological Science, The Johns Hopkins School of Medicine, Baltimore, MD, 21287

[3]Department of Medical Physics, Memorial Sloan–Kettering Cancer Center, New York, NY 10065

[4]Genelux Corporation, San Diego Science Center, San Diego, CA 92109

[5]Department of Biochemistry, University of Würzburg, Würzburg, Germany, D-97074

Abstract

Background—Peritoneal carcinomatosis (PC) is a terminal progression of colorectal cancer (CRC). Poor response to cytoreductive surgery and chemotherapy, coupled with the inability to reliably track disease progression using established diagnostic methods make this a deadly disease. This paper examines the effectiveness of the oncolytic vaccinia virus GLV-1h153 as a therapeutic and diagnostic vehicle. We believe that viral expression of the human sodium iodide transporter (hNIS) can provide both real-time monitoring of viral therapy and effective treatment of colorectal peritoneal carcinomatosis (CRPC).

Methods—Infectivity and cytotoxic effect of GLV-1h153 on CRC cell lines was assayed *in-vitro*. Viral replication was examined by standard viral plaque assays. Orthotopic CRPC xenografts were generated in athymic nude mice, and subsequently administered GLV-1h153 intraperitoneally. Reduction of tumor burden was assessed by mass. Orthotopic tumors were visualized by SPECT/CT after Iodine (^{131}I) administration and by fluorescence optical imaging.

Results—GLV-1h153 infected and killed CRC cells in a time and concentration dependent manner. Viral replication demonstrated greater than a 2.35 log increase in titer over 4 days.

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Address for correspondence: Yuman Fong, MD, FACS, Murray F. Brennan Chair in Surgery, Department of Surgery, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10065, Phone: (212) 639-2016, Fax: (212) 639-4031, fongy@mskcc.org.

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Intraperitoneal treatment of orthotopic CRPC xenografts resulted in a significant reduction of tumor burden. Infection of orthotopic xenografts was both therapeutic and facilitated monitoring by ^{131}I-SPECT/CT via expression of hNIS in infected tissue.

Conclusions—GLV-1h153 effectively kills CRC *in-vitro* and dramatically reduces tumor burden *in-vivo*. We demonstrate that GLV-1h153 can be used as an agent to provide accurate delineation of tumor burden *in-vivo*. These findings indicate that GLV-1h153 has significant potential for use as theragnostic agent in the treatment of CRPC.

Keywords

Vaccinia; human sodium iodide symporter; virus-based imaging; cell-based imaging; Colorectal Peritoneal Carcinomatosis

INTRODUCTION

In 2012 it is estimated that in the United States alone, there will be over 150,000 new colorectal cancer (CRC) cases diagnosed, and over 50,000 deaths.[1] Peritoneal carcinomatosis (PC) is a fatal evolution of colorectal cancer that is believed to occur due to peritoneal seeding that occurs following an attempt at curative surgery for primary CRC. Estimates place peritoneal seeding events at approximately 3% to 28% of surgical cases.[2] When feasible, cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC), is the commonly used treatment at major medical centers,[3, 4] with five-year absolute survival rates ranging from 20% to 40%.[5] These poor outcomes underline the critical need for novel therapy options to combat this fatal disease.

Among the key challenges towards treatment of PC is the lack of reliable abdominal imaging methodologies to accurately estimate disease burden. Diagnostic imaging is essential to improving outcomes in PC because it allows clinicians to stratify and select patients for whom complete cytoreduction can be achieved. Further, techniques that would enable monitoring of disease could elucidate extent of therapy response.[6–8]

Numerous preclinical studies have utilized recombinant Vaccinia virus (VACV) as an oncolytic vector for the treatment of cancer.[9] Oncolytic viruses selectively infect, replicate within and lyse cancer cells. They can also be used to selectively deliver selective gene therapy to cancer cells. The Vaccinia virus has a well-established safety profile having been used as a live vaccine in tens of millions of people through the World Health Organization's smallpox vaccination program. GLV-1h153 was derived from the parental recombinant Vaccinia virus, GLV-1h68, which is currently under investigation in three Phase I clinical trials world-wide (http://www.clinicaltrials.org).

The recombinant Vaccinia virus GLV-1h153 has been modified from the parental GLV-1h68 to express both green fluorescent protein (GFP) and the human sodium iodine symporter (hNIS). This strategy has been shown in numerous preclinical studies to be an effective treatment modality in triple-negative breast cancers,[10] anaplastic thyroid cancer[11] and pancreatic cancers.[12] GLV-1h153 has been altered from the parental. Via hNIS (an intrinsic plasma membrane glycoprotein) expression, GLV-1h153 facilitates enhanced

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uptake and concentration of radioiodine by infected cancer cells,[13] The hNIS mediated radioiodine uptake enables imaging of infected cancer cells via single-photon emission computed tomography (SPECT/CT).[14, 15] In this study, we look at the ability of GLV-1h153 to effectively treat and facilitate imaging of CRPC in an established metastatic orthotopic murine model.

MATERIAL & METHODS

Cell Lines

Human colorectal LS-174 and C85 cell lines and African Green Monkey Cells (CV-1) were purchased from the American Type Culture Collection (Manassas, VA). Both cell lines were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, and 100 IU/mL penicillin/streptomycin. CV-1 cells for the viral plaque assay were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum and 100 IU/mL penicillin/streptomycin. All cells were grown in a 5% CO_2 humidified incubator at 37 °C.

Virus

GLV-1h153 is a replication-competent, GFP-expressing recombinant vaccinia virus based on the vaccinia virus LIVP strain (Lister strain from the Institute for Research on Virus Preparations, Moscow, Russia). Its construction has been described in detail previously.[12] Insertion of the human sodium iodide symporter to facilitate deep tissue imaging does not alter oncolytic or replication capability of a novel vaccinia virus.

Green Fluorescent Protein Expression

LS174 and C85 cells lines were seeded at a density of 8×10^4 cells/well in a 12-well plate and incubated for 24 h. After 24 h, cells were infected with oncolytic vaccinia virus GLV-1h153 at a Multiplicity of Infection (MOI) of 0.01, 0.1 and 1. To trace viral infection, 24 h after infection, cells were examined using an inverted fluorescence microscope (Nikon Eclipse TE300; Nikon, Tokyo, Japan) for GFP expression and imaged for 5 days.

Viral Plaque Assay

After infection of cells with the virus and prior to daily cytotoxicity assays, the supernatant of each infected well were collected for five days and immediately frozen at −80 °C. After thawing, serial dilutions of each supernatant sample were made to perform standard viral plaque assays on confluent CV-1 culture plates. All samples were measured in triplicate and the results averaged.

Cytotoxicity Assay

LS174 and C85 cells lines were seeded at a density of 3×10^4 cells/well in a 24-well plate and incubated for 24 hours. After 24-h of incubation, cells were infected with oncolytic vaccinia virus GLV-1h153 at a MOI of 0 (control), 0.01, 0.1 and 1. Viral cytotoxicity was measured every 24 h for 5 days using the lactate dehydrogenase assay. Cells were washed with phosphate buffered saline (PBS), followed by lysis with Triton X-100 (1.35%; Sigma, St. Louis, MO). The intracellular lactate dehydrogenase release was measured with a

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Cytotox 96 kit (Promega, Madison, WI) on a spectrophotometer (EL321e; Bio-Tek Instruments) at 490 nm. Results are expressed as the percentage of surviving cells as measured by lactate dehydrogenase release by infected cells as compared with uninfected negative controls. All samples were analyzed in triplicate, and assays were repeated to ensure reproducibility.

Immunofluorescence (IF) for hNIS in cell cultures

To visualize the expression and membrane localization of the hNIS protein following infection with GLV-1h153, LS174 and C85 cells were cultured on coverglass slides and subsequently either infected with_1 plaque forming unit (PFU)/cell or mock-infected with PBS. At 24 h after infection, cells were fixed with 4% paraformaldehyde, permeabilized with methanol, blocked with PBS containing BSA, and incubated with a mouse anti-hNIS monoclonal antibody (AbCam Inc., Cambridge, MA, USA) at a dilution of 1:50 and anti-GFP chicken polyclonal antibody (AbCam) at a dilution of 1:200. This was then followed by incubation with a secondary Alexa Fluor 568-conjugated goat anti-mouse antibody (Invitrogen, Grand Island, NY, USA) at a dilution of 1:1000 and a secondary Alexa Fluor 488-conjugated goat anti-chicken antibody (Invitrogen) at a dilution of 1:000, respectively. IF images were taken with a Zeiss AxioPlan 2 imaging widefield microscope with _20/0.5 NA objective and AxioCam MRM CCD camera (Carl Zeiss, Oberkochen, Germany). Slides plated with mock-infected cells were used as negative controls.

Treatment of Metastatic Disease in an Orthotopic Colorectal Peritoneal Carcinomatosis Xenograft Model

All mice were cared for and maintained in accordance with animal welfare regulations under an approved protocol by the Institutional Animal Care and Use Committee of the Memorial Sloan-Kettering Cancer Center (MSKCC). Forty female athymic nude mice (Harlan) aged 6 to 8 weeks were injected with 2×10^6 LS174 cells suspended in 500 μL of DMEM into the peritoneal cavity. One week after tumor inoculation animals were randomized into 4 groups. Xenografts were treated with 100 μL of intraperitoneal (IP) GLV-1h153 (1×10^7 PFUs) or PBS. Mouse weight was recorded 3 times per week. Two weeks after treatment, mice were sacrificed and tumors were harvested, weighed, embedded in paraffin and stained as detailed above.

Radiopharmaceuticals

^{131}I was obtained from Radiopharmacy of the Molecular Imaging and Therapy Service of MSKCC. The maximum injected activity of the ^{131}I was approximately 500 μCi/mouse.

In Vivo SPECT/CT Imaging

Four groups of animals (n=3) bearing peritoneal LS-174 xenografts were injected with 140 μCi of ^{131}I via the tail vein. The dedicated small animal NanoSPECT/CT (Bioscan Inc., Washington, DC, USA) was used with a multi-pinhole focused collimator and a temperature controlled animal bed unit (Minerve equipment veterinaire). Nine pinhole apertures with a diameter of 2.5 mm were used on each of the 4 gamma-cameras, with a field of view (FOV) of 24 mm. Twenty hours after radiotracer administration, mice were anesthetized with 1% to

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2% isofluorane and heated by an warmed-air circulator to maintain stable body temperature during the entire scanning period. CT images were obtained for anatomical orientation immediately before SPECT imaging. SPECT datasets were acquired wit 64 projections with 1 min per projection at the shortest radius of rotation (30 mm) in a 140 keV ± 10% energy window. SPECT images were reconstructed using ordered subset expectation maximization (5 iterations, 4 subsets) without attenuation correction using the Mediso Suite (Mediso Medical Imaging Systems, Budapest, Hungary). Reconstructed datasets were consistent with 80 × 80 image matrices and had a spatial resolution of 0.8 mm.

In vivo Fluorescent Imaging

Fluorescent images were obtained with the Maestro small animal imaging system (CRI, Woburn, MA). An excitation/emission filter set appropriate (575 to 605 nm/645 nm long-pass filter). After each image was obtained, it was spectrally unmixed to remove the background fluorescence and overlay images were produced.

Statistical Analysis

All results are reported as means with standard errors. The significant differences between different groups were determined using the Student t test. A p value of less than 0.05 was considered significant.

RESULTS

Viral Infection Is Time and Concentration Dependent *In vitro*

GFP expression was assessed in both LS-174 and C85 cell lines every 24 h for 5 days post-viral infection at MOIs of 0.01, 0.1 and 1 by fluorescence microscopy. GFP expression was demonstrated in both cell lines after 24 h, confirming sensitivity to viral infection. Viral infection was time dependent as evidenced by increasing number and signal intensity of green cells with increasing time after infection. In addition, viral infection was concentration dependent as demonstrated by higher levels of GFP expression at the higher viral concentration (MOI 1) than the lower viral concentration (MOI 0.01). Representative images of cell line LS174 are shown in Figure 1A.

GLV-1h153 Replicates in LS174 colon cancer Cell Line Effectively

Viral proliferation was assessed after infection with GLV-1h153 at a MOI of 1. All cell lines efficiently supported the replication of GLV-1h153, with peak viral titers measured in infected supernatants of LS174 cells on day 4 (Fig. 1C). The peak titer of 2.35×10^5 plaque-forming units at day 4 represents a 11-fold increase in viral titer as compared with the initial viral treatment dose.

GLV-1h153 Shows Time- and Concentration-Dependent Cytotoxicity in CRC Cell Lines *In Vitro*

CRC cell lines showed significant sensitivity to GLV-1h153, which killed all cell lines in a dose-dependent fashion. All cell lines showed near complete cytotoxicity (with <5% cell viability) at a multiplicity of infection of 1 by day 5 (Fig. 1B). At the lowest viral

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concentration, significant viral sensitivity was shown in cell line LS174, with more than 50% cytotoxicity at a multiplicity of infection of 0.01 within 5 days of viral infection.

Infected LS174 and C85 cells express hNIS on IF imaging

GLV-1h153 carries the GFP reporter gene and the hNIS gene. At 24 h after viral infection (at an MOI of 1) with GLV-1h153, infected LS174 and C85 cells expressed GFP and stained positive for hNIS (Fig. 1A, bottom panel) in vitro compared to uninfected controls (Fig. 2).

Intra-peritoneal GLV-1h153 Can Effectively Treat LS174 Colorectal Peritoneal Carcinomatosis *In Vivo*

Treated xenografts demonstrated progressive tumor volume regression 3 weeks after a single viral injection. (Fig. 3A) By day 28 (after tumor implantation), the mean tumor volume of the treatment with GLV-1h153 group was only 0.64g as compared with 2.87g for controls animals ($P<0.01$). (Fig. 3B) All mice tolerated the treatment well without significant morbidity demonstrated by the stable mean body weights of all animals at the end of the treatment.

Intra-peritoneal Treatment with GLV-1h153 Facilitates Molecular Imaging of CRPC

Three animals per treatment group were administered ^{131}I by tail vein injection. Four hours following radio-iodine administration, mice were imaged on SPECT/CT. Mice that had received intra-peritoneal GLV-1h153 demonstrated distinct ^{131}I uptakes at sites of peritoneal disease. (Fig. 3A) Uptake was specifically mediated by hNIS transporter accumulation of the radiotracer as no uptake was visualized in tumor bearing mice that had not received the viral treatment. As expected, some background uptake of ^{131}I in the small bowel is seen in both sets of animals. GFP expression was assessed before sacrifice by fluorescence imaging. Imaging of the green fluorescence was not possible without surgical intervention. After the abdominal organs were revealed for imaging following a midline incision the virally induced production of GFP was detectable. In vivo fluorescence imaging demonstrated disseminated and intense GFP signal throughout the abdominal cavity. Signal was restricted to the GLV-1h153 treated group. The QTracker vascular label injected was also detectable in both animal groups (Fig. 3B)

DISCUSSION

Colorectal peritoneal carcinomatosis (CRPC) is generally considered to be extremely difficult to effectively cure, with few treatment options beyond palliative care. As a curative treatment option, systemic chemotherapy has proven inefficient with an increase of survival of patients with isolated PC from 5.2 to 12.6 months.[4, 16–18] While modern chemotherapy regimens were able to improve this to 24 months,[19] cytoreductive surgery with HIPEC has allowed an increase in median survival from 13 months to 63 months[19–23] and has become the standard of care for patients with limited PC. Unfortunately, HIPEC is only offered at specialized centers, limiting its availability to patients. In addition, both the concentration and combination of chemotherapeutic agents used varies widely between medical centers. Most importantly, with no randomized control trials comparing complete cytoreduction

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alone or combined with HIPEC, it is difficult to clearly evaluate the true effect of heated chemotherapy HIPEC on survival in patients with CRPC.

Due to a lack of accurate and reliable imaging modalities to estimate tumor burden and evaluate response to treatment, PC is a disease that remains extremely difficult to stage and treat. X-ray computed tomography (CT) and multimodal positron emission tomography-CT (PET/CT) frequently under-stage peritoneal carcinomatosis.[24] This inadequate detection results in disseminated and incurable disease at diagnosis. A recent study that looked at the ability of radiologists to accurately predict outcome of patients treated with HIPEC via preoperative CT demonstrated large differences in the ability to accurately predict presence, size, and location of peritoneal tumor implants.[25] This discrepancy strongly brings into question the value of preoperative evaluation with CT imaging alone.

The oncolytic viral theragnostic approach demonstrated in this work utilizes a single vector in order to enhance imaging and therapy of disseminated disease. One important advantage of therapy with GLV-1h153 lies in its ability to deliver the hNIS gene to infected tumor cells and insert the corresponding symporter into their membranes. This modification allows for inter-cellular concentration of radioactive isotopes such as ^{99m}Tc, ^{188}Re and iodine (^{124}I, ^{131}I). We have recently studied the use of GLV-1h153 to detect malignant pleural mesothelioma and positive surgical margins in triple negative breast cancer via hNIS-mediated ^{131}I (for imaging and therapy) or ^{124}I uptake for quantitative PET imaging.[26, 27]

In this study, we were able to use GLV-1h153 with ^{131}I to successfully visualize tumor tissue infected with GLV-1h153 and to demonstrate the presence of PC *in-vivo*. GLV-1h153 also expresses GFP, which can be utilized to noninvasively detect disease by fluorescent imaging. This is useful because it provides another means for noninvasive detection of viral distribution and tumor localization. In particular, emerging real-time fluorescence imaging to guide surgical resection is an area of intense research and clinical interest.[28, 29]

CRPC is a heterogeneous disease that has a wide variety of clinical presentations. These range from solitary metastatic lesions to diffuse metastases involving multiple organs. In our study, we use a murine xenograft model that closely mimics key features of the clinical disease. This was utilized in order to enable demonstration of engineered, replication-competent vaccinia virus to effectively infect, replicate within, and kill colon cancer both *in vitro* and *in vivo*. In two colorectal cell lines *in-vitro*, GLV-1h153 infection occurred in a time dependent manner demonstrated by a progressive increase in GFP expression visualized via fluorescent microscopy over 5 days. This infectivity correlated with the ability of GLV-1h153 to both replicate within and effectively kill CRC as shown in the standard assays discussed above. Two weeks following IP administration of GLV-1h153 *in vivo*, we observed significant regression of peritoneal tumors. GLV-1h153 mediated tumor regression strongly suggests that recombinant vaccinia virus can be used to treat CRPC. Further, this theragnostic approach may also be a valuable tool in the radiological armamentarium to more accurately stage patients.

Vaccinia virus as a viral vector to treat CRPC has been previously investigated.[30] This work suggests that recombinant vaccinia virus has several key advantages as an oncolytic vector.

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First, it has a well-documented safety profile in clinical applications and was used by the World Health Organization in its campaign to eradicate smallpox.[31] It has a low toxicity rate (<1%) and vaccinia immunoglobulin can be given in situations of systemic dissemination in immunocompromised cancer patients. Second, vaccinia virus has a large genome that easily accepts recombination of large cDNA fragments and specific genetic deletions without compromising viral replication. We have utilized these features of the virus in the present form of the recombinant vaccinia virus GLV-1h153 by inserting the hNIS transporter into the viral construct.

CONCLUSION

In this study, we demonstrate that GLV-1h153 has significant oncolytic activity against human CRC cells *in vitro*. *In vivo*, we are able to show that GLV-1h153 can reach peritoneal carcinomatosis sites and effectively kill malignant cells in an orthotopic murine colorectal peritoneal carcinomatosis model. We also report on two different imaging modalities that make use of GLV-1h153 to facilitate early detection, localization and estimation of PC burden. These data support further investigation of this novel virus for the treatment of patients with colorectal peritoneal carcinomatosis.

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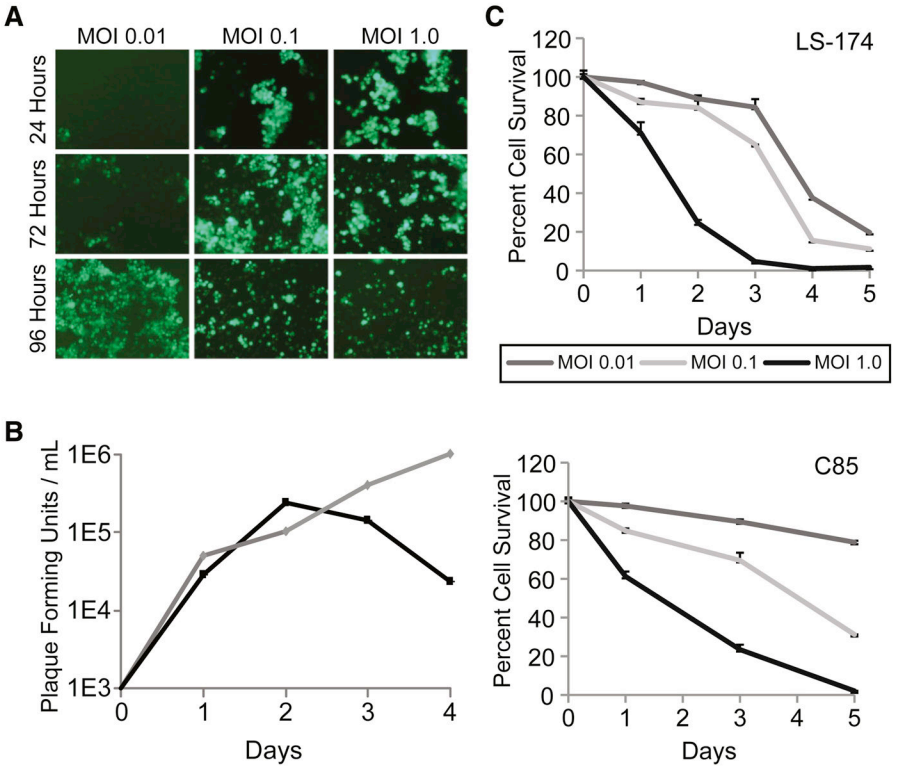

Figure 1. GLV-1h153 infects, replicates within, and shows oncolytic activity against multiple colon cancer cells in a dose and time dependent fashion *in-vitro*

(A) Viral infection in a representative cell line, LS174, is time and dose dependent. (B) Efficient replication of GLV-1h153 was demonstrated in all cell lines with more than a 11-fold increase in viral copy numbers within 4 days of treatment. (C) GLV-1h153 infection resulted in near complete cell death in all cell lines at a multiplicity of infection of 1 within 5 days. Dose of virus measured in multiplicities of infection (MOI).

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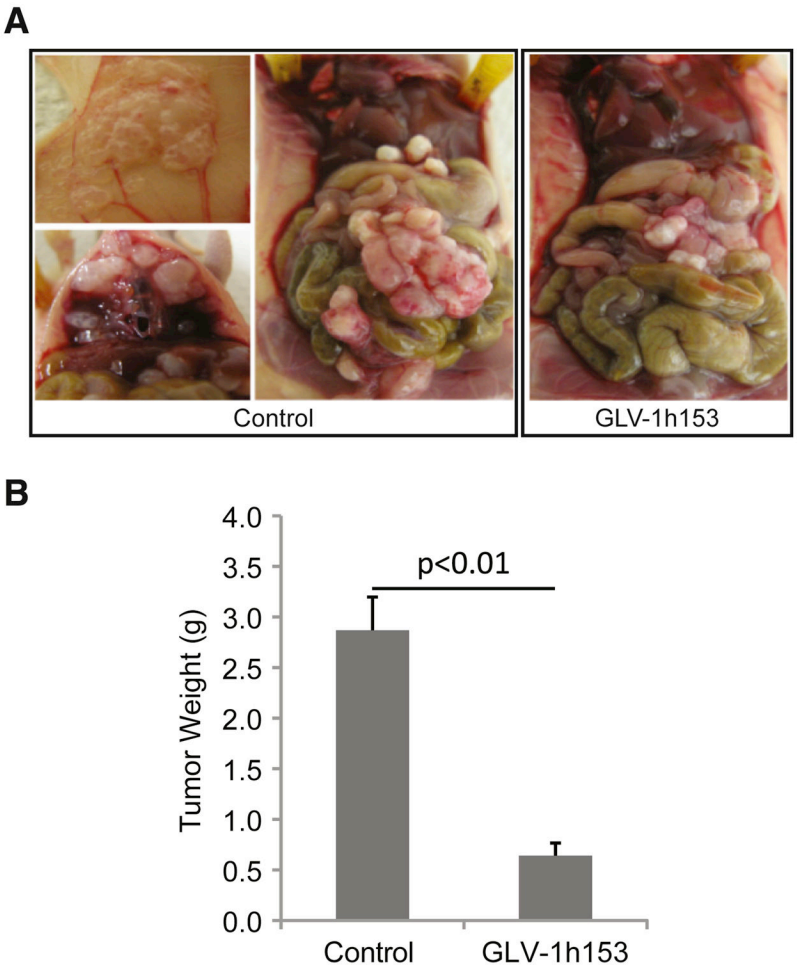

Figure 2. Intra-peritoneal treatment with GLV-1h153 reduces tumor burden in a mouse model of colorectal peritoneal carcinomatosis

(A) On the left, a representative view of control mice euthanized at two weeks after treatment with PBS demonstrated macroscopic tumor deposits on all visceral surfaces and the abdominal wall. Shown on the right, mice treated with intraperitoneal injection of 1×10^7 plaque forming units of GLV-1h153 demonstrated reduced disease. (B) Untreated mice exhibited 4 times the peritoneal CRC tumor burden as compared to mice treated with GLV-1h153

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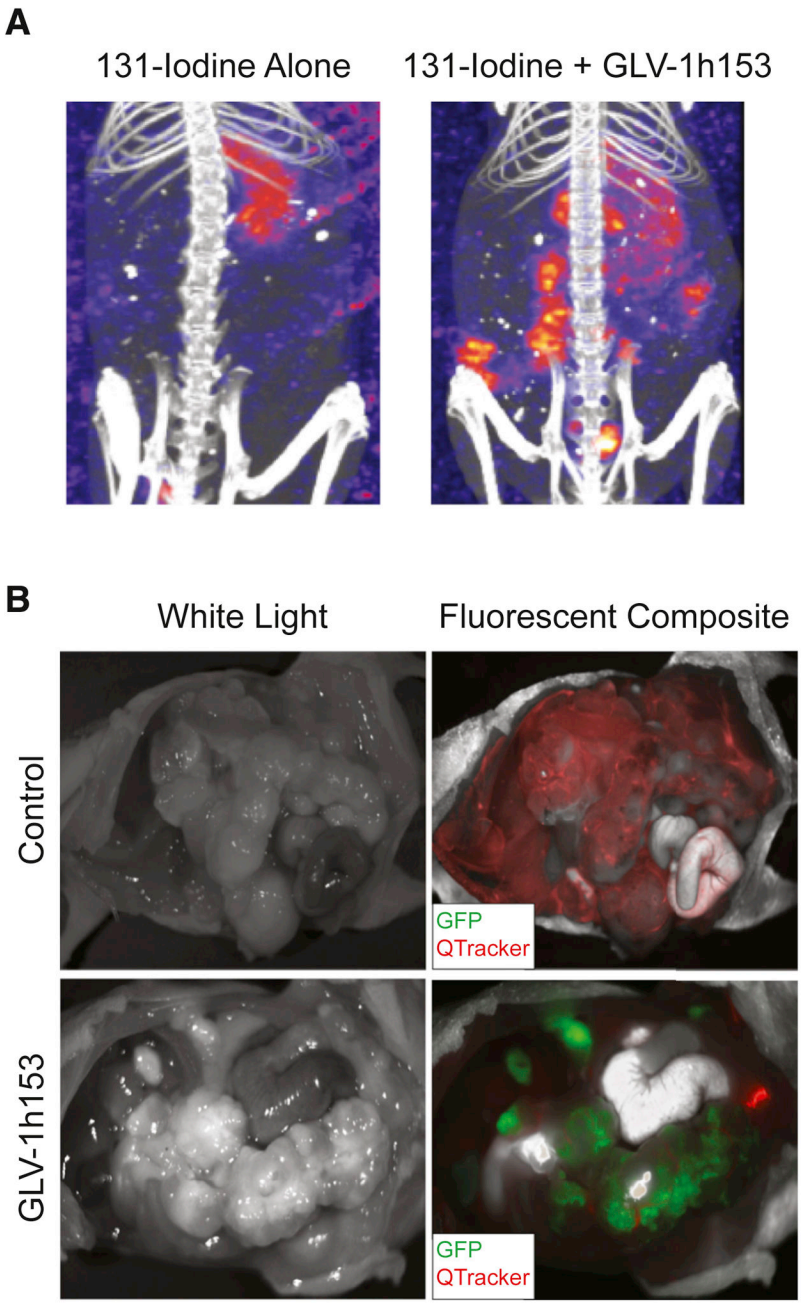

Figure 3. Imaging of enhanced radiouptake in GLV-1h153-infected LS174 cells *in-vivo*

(A) Fourteen days after treatment with GLV-1h153, mice (n=4) were given intravenous ^{131}I and after 4 hours were imaged via SPECT/CT. Viral mediated hNIS expression resulted in the concentration of ^{131}I in infected tumor cells and allowed for imaging of disease burden. No concentration of ^{131}I was seen in the tumors of control animals. (B) Left: white light photographs, right: fluorescent composite images. GFP expression visualized via fluorescent imaging with Maestro demonstrated GLV-1h153 infection of CRPC tumors. As expected,

uninfected control animals demonstrate no expression of GFP. Green, GFP; Red, QTracker 655 vascular label; White, tissue autofluorescence.

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