ORIGINAL ARTICLE

Recombinant vaccinia virus GLV-1h68 is a promising oncolytic vector in the treatment of cholangiocarcinoma

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Although early stage cholangiocarcinoma (CC) can be cured by surgical extirpation, the options for treatment of advanced stage CC are very few and suboptimal. Oncolytic virotherapy using replication-competent vaccinia virus (VACV) is a promising new strategy to treat human cancers. The ability of oncolytic VACV GLV-1h68 to infect, replicate in, and lyse three human CC cell lines was assayed *in vitro* and in subcutaneous flank xenografts in athymic nude mice. In this study, we have demonstrated that GLV-1h68 effectively infects and lyses three CC cell lines (KMC-1, KMBC, and KMCH-1) *in vitro*. Expression of the viral marker gene *rac-gfp* facilitated real-time monitoring of infection and replication. Furthermore in athymic nude mice, a single dose of GLV-1h68 significantly suppressed tumor growth. The treatment was well tolerated in all animals. Recombinant VACV GLV-1h68 has significant oncolytic ability against CC both *in vitro* and *in vivo*. GLV-1h68 has the potential to be used clinically as a therapeutic agent against CC.

Cancer Gene Therapy (2015) 22, 591–596; doi:10.1038/cgt.2015.60; published online 20 November 2015

INTRODUCTION

Cholangiocarcinoma (CC) is an aggressive malignancy that arises from biliary epithelium, it can develop anywhere along the biliary tract. CC is subdivided into three distinct categories; intrahepatic (within the liver), at the confluence of the right and left hepatic duct (perihilar), and distal bile duct tumors. Although it is a rare malignancy, its incidence has increased by 22% between 1979 and 2004.¹ CC accounts for 3% of all tumors of the gastrointestinal tract cancer.² The best chance for cure is surgical extirpation, but unfortunately <30% of patients with CC are resectable due to advanced presentation.³ Surgical treatment involves major resections of the liver, pancreas and bile duct, with considerable morbidity and mortality. In spite of adequate surgery establishing negative margins, absence of nodal disease the 5 year survival ranges from 13 to 44%.^{4,5} The role of adjuvant chemotherapy in CC is unclear with conflicting data on survival. The effectiveness of radiation therapy is also very low.^{6,7} Therefore there is an imperative need to explore novel therapies to improve survival in this highly fatal disease.

The use of genetically engineered, tumor targeting viruses as oncolytic vectors has proven to be promising modalities for cancer therapies. Since 1950s there had been many attempts to develop viruses that specifically target cancer cells. Several viruses like adenovirus, West Nile virus, herpes simplex virus and vaccinia virus (VACV) have shown potential as oncolytic viruses that can effectively infect and kill cancer cells. VACV has shown great promise as an oncolytic virus. Replication-competent recombinant VACV GLV-1h68 has shown anti-tumoral efficacy and tumor selectivity in different canine tumors^{8,9} as well as human hepatocellular carcinoma,¹⁰ pancreatic tumor,¹¹ breast cancer¹²

and anaplastic thyroid cancer.¹³ VACV has a well-established safety profile as a live attenuated vaccine to eradicate smallpox by the World Health Organization in humans.¹⁴ In addition, it replicates within the cytoplasm, thereby reducing the risk of recombinant events with the host cellular DNA. VACV also has a large, 192-kb genome that can easily accept insertions of foreign DNA without compromising viral replication, thus allowing clinicians to deliver targeted gene therapy to tumor cells and the tumor microenvironment.

GLV-1h68 is a replication-competent oncolytic VACV that has been genetically modified by generating interruptions in the thymidine kinase, F14.5L and hemagglutinin genes. These interruptions confer tumor selectivity to the virus and decrease its virulence in normal tissue. Recently the first clinical Phase I trial of GLV-1h68 was completed¹⁵ and three additional Phase I trials are currently ongoing (<http://www.clinicaltrials.gov>, 2012, keyword: Genelux). In this study, we report the efficacy of oncolytic VACV GLV-1h68 to infect, replicate and lyse various CC cells in culture and in tumor xenograft models.

MATERIALS AND METHODS

Cell culture

African green monkey kidney fibroblast cells (CV-1) were obtained from American Type Culture Collection (Rockville, MD, USA) and grown in minimum essential medium. Three human CC lines, KMCH-1, KMC-1 and KMBC were tested. KMCH-1 (originally established at the Department of Pathology, Kurume University School of Medicine, Japan) was derived from combined hepatocellular-CC growth.¹⁶ KMC-1 was established from mucin producing tubular type intrahepatic CC,¹⁷ and KMBC was established from extrahepatic CC.¹⁸ All three cell lines were cultured in Dulbecco's modified

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Received 7 June 2015; revised 19 July 2015; accepted 24 September 2015; published online 20 November 2015

Figure 1. Viability of human cholangiocarcinoma cancer cell lines after GLV-1 h68 infection. Cell lines KMCH-1, KMC-1 and KMBC were infected in culture at MOIs 0.01, 0.1, 1, 5 and assayed for viable cells daily for 7 days. Viability was measured in triplicates and averaged. Averages were normalized against the uninfected controls at each time point that were considered to be 100% viable. MOI, multiplicity of infection.

Eagle medium. All media were supplemented with 10% fetal calf serum, along with 100 units per ml penicillin G, and 100 units per ml streptomycin. Cells were cultured at 37 °C under 5% CO₂ and tested for mycoplasma.

Virus

The recombinant VACV GLV-1h68 is a genetically stable, live attenuated virus with oncolytic activity. It was derived and purified from the parent VACV LVP strain (Lister strain from Institute for Research on Virus Preparations, Moscow, Russia) as described earlier.¹² Three expression cassettes encoding for *Renilla luciferase-Aequoria green fluorescent protein* (GFP) fusion protein, β -galactosidase and β -glucuronidase were inserted into the F14.5L, J2R and A56R loci, respectively, of the viral genome of the LVP strain.

Viral proliferation assay

Standard plaque assays were performed to quantify viral replication following infection of the three CC cell lines with GLV-1h68. Twenty four hours after infection of the cells with virus and before daily cytotoxicity assay, supernatants from each infected well was collected daily for 7 days and immediately frozen at -80 °C for storage. After thawing, 1:10 serial dilutions of supernatants were titrated in triplicates on confluent layers of CV-1 cells in 24-well plates and a standard viral plaque assay was performed. Viral titers were calculated and plotted overtime.

GFP expression

Cells were plated in 96-well flat bottom plates in triplicates and allowed to adhere for 24 h. Cells representing intrahepatic CC (KMC-1) and extrahepatic CC (KMBC) histologic subtypes were plated at a concentration of 1×10^4 cells per well, whereas the combined hepatocellular-CC (KMCH-1) cells were plated at of 3×10^3 cells per well. Cells were then treated with media alone (control) or infected with GLV-1h68 at multiplicities of infection (MOIs; the ratio of plaque-forming units to cell in culture) of 0.01, 0.1, 1 and 5. To monitor and follow the course of viral infection, cells were examined with an inverted fluorescence microscope (Nikon Eclipse TE300, Nikon, Tokyo, Japan) for GFP expression. Bright field and fluorescent images were obtained daily for seven consecutive days.

Cell viability assay

The three cell lines KMCH-1, KMC-1 and KMBC were plated as described above. After 24 h of incubation, cells were infected with GLV-1h68 at MOIs of 0 (control), 0.01, 0.1, 1 and 5. Viral cytotoxicity was measured daily for 7 days using the Dojindo cell counting kit (CCK-8; Rockville, MD, USA). CCK-8 dye was added to phenol red free medium at a ratio of 1:10 and the medium in each well was replaced with 110 μ l of the dye solution and incubated for 4 h. The plate was then read on a spectrophotometer (EL321e; Bio-Tek Instruments, Winooski, VT, USA) for absorbance at a wavelength of 450 nm. Uninfected cells were used as a reference and

considered as 100% viable. Survival curves were generated showing the percent of viable cells as compared with uninfected controls. All samples were analyzed in triplicate.

Treatment of CC xenografts with GLV-1h68

Mice were cared for in accordance with approved protocols by the Institutional Animal Care and Use Committee of the Memorial Sloan Kettering Cancer Center. Homozygous athymic female nude mice aged 6–8 weeks were purchased from Taconic Farms Inc (Hudson, New York, USA). Flank tumors were established by injecting KMC-1 cells suspended in 50 μ l Matrigel (BD Biosciences, San Jose, CA, USA) into the subcutaneous tissue of mice. When tumors reached ~75 mm³–150 mm³ the animals were randomized into two groups with equitable tumor volumes by envelope method. An even amount of small (75–100 mm³), medium (100–125 mm³) and large (125–150 mm³) sized tumors were present in each group. No mice were excluded. Variance was similar between the groups and within each group. Subsequently, one group of mice ($n=12$) was injected intratumorally with 1×10^7 plaque-forming units (PFUs) of GLV-1h68 while the other group (control, $n=12$) was injected with equal volume of phosphate-buffered saline. Tumor dimensions were measured three times a week using a digital caliper. The investigator was not blinded to the groups. Tumor volumes were calculated with the equation $V = (4/3) \times \pi \times \{(a/2)^2 \times (b/2)\}$ where a is the smallest diameter (mm) and b is the largest diameter (mm) of the tumor. Average tumor volume was used to monitor therapeutic efficacy. The experiment was terminated 2 weeks after injection.

Statistics

Significance differences among the tumor volume growth rates of the two groups (control and GLV-1h68) were determined via nonparametric one-way analysis of variance (Kruskal–Wallis test). Sample size in the study was determined to ensure a power of 80% with type I error, α at 0.05. Values of $P < 0.05$ were considered significant. Statistical analyses were performed using IBM Statistical Package for Social Sciences (SPSS) software version 20 (Chicago, IL, USA).

RESULTS

GLV-1h68 demonstrates both time and concentration-dependent cytotoxicity in all three CC Cell lines *in Vitro*

GLV-1h68 successfully infected all three CC cell lines *in vitro*. Cell lines KMCH-1 and KMC-1 showed near complete cytotoxicity (with $< 10\%$ cell viability) in all MOIs by day 7 (Figure 1). Cell line KMBC showed the least sensitivity to GLV-1h68 among the three cell lines. In KMBC cell line at MOI of 5 and 1, GLV-1h68 demonstrated a killing over 80% of cells by day 7, at MOI 0.1, 50% cell death, and at the lowest MOI 0.01 it showed no measurable cytotoxicity by day 7.

Figure 2. KMC-1 cells were infected with GLV-1h68 at MOIs of 0.01, 0.1, 1 and 5. GFP expression in infected cells was visualized by direct fluorescence. Images shown were taken at $\times 10$ magnification on days 1, 3, 5 and 7. GFP, green fluorescent protein; MOI, multiplicity of infection.

The virus-mediated GFP expression in CC cells *in vitro* is both time and concentration dependent

GFP expression was confirmed via fluorescent microscopy to assess viral infection in all cell lines daily for 7 days at MOIs of 0.01, 0.1, 1 and 5. By 24 h, GFP expression was observed in all cell lines, confirming susceptibility of the cells to viral infection. Infectivity occurred in a time- and concentration-dependent manner as demonstrated by earlier GFP expression at higher MOIs and an increasing number of GFP expression visualized overtime. Representative images under fluorescent microscopy and bright field microscopy of CC cell line KMC-1 are shown in Figures 2 and 3, respectively. At higher MOIs, viral cytotoxicity and subsequent cell death at later time points resulted in decline of GFP expression.

GLV-1h68 efficiently replicates in all CC Cell Lines

Viral proliferation for all cell lines was assessed after infection with GLV-1h68 at a MOI of 1. All cell lines efficiently supported the replication of GLV-1h68, with peak viral titers in KMBC reaching 6×10^4 PFUs by day 7. GLV-1h68 showed similar replication capability in KMC-1, reaching close to 6×10^4 PFUs by day 7. Titers for both of the above cell lines showed a threefold increase over 7 days. KMBC, consistent with diminished cytotoxicity results, showed limited replication capacity, reaching approximately a peak of 3.2×10^4 PFUs by day 5 (Figure 4).

In vivo, a single dose of GLV-1h68 effectively kills KMC-1 tumors. We examined the efficacy of GLV-1h68 to target and affect the growth of KMC-1 in flank tumors in nude mice. KMC-1 flank xenografts treated with a single dose of intratumoral injection of GLV-1h68 at a concentration 1×10^7 PFUs demonstrated

significant tumor volume regression over a 2-week period of time. Fourteen days post-infection, controls ($n=12$) nearly increased by $\sim 250\%$ of original tumor volume; while tumors treated with a single dose of GLV-1h68 ($n=12$) shrank to $\sim 46\%$ of the starting volume ($P < 0.05$) (Figure 5). All mice tolerated the treatment well without significant morbidity.

DISCUSSION

CC is the second most common cancer of the liver. Surgery is the only curative option available for early disease. Unfortunately surgery is curative in $< 30\%$ of cases because of advanced presentation.³ Even after curative surgical resection, the median survival is 36 months, with a high recurrence rate of 62% after a median follow-up of 26 months.¹⁹ The options available for recurrent and advanced disease are limited. Currently there is no established standard chemotherapy for locally advanced or metastatic biliary tract cancer. No single randomized study to date have been proven effective; fluropyrimidines,²⁰ cisplatin,²¹ Gemcitabine²² in trials have shown limited activity. There has been a paucity of adequately powered phase III studies in advanced biliary tract cancer to show enough evidence to provide guidelines for treatment. Newer and more efficient treatment strategies are needed to reduce treatment failures especially in recurring cases or advanced stages of CC.

Genetically engineered tumor targeting virus has emerged as a promising area for novel cancer therapies. There are several mechanisms by which viruses attack cancer cells. First, the viruses can destroy tumor cells directly by replicating in them. Second mechanism for example; adenovirus generates death proteins E3 11.6kD and the E4ORF4 that are lethal to cells. The third mechanism is that they can initiate non-specific and specific anti-tumor immune responses. One of the best studied oncolytic

Figure 3. KMC-1 cells were infected with GLV-1h68 at MOIs of 0.01, 0.1, 1 and 5. Infected cells were visualized under bright field microscopy demonstrate lysis of cells with increasing MOI. Images shown were taken at $\times 10$ magnification on days 1, 3, 5 and 7. MOI, multiplicity of infection.

Figure 4. Viral proliferation assay. GLV-1h68 at an MOI of 1.0 effectively replicated in all three CC cell lines and demonstrated at least a threefold increase in viral titers within 7 days of treatment. CC, cholangiocarcinoma; MOI, multiplicity of infection.

viruses is VACV. It is established that VACV depends on proliferating cells for its replication.²³ The antitumor effects are mediated directly by viral infection, replication, and lysis of cancer cells and indirectly by inducing antivascular effects²⁴ as well as stimulation of the host immune response.²⁵ GLV-1h68 is a replication-competent oncolytic VACV that has been genetically modified by creating interruptions in the thymidine kinase, F14.5L and hemagglutinin genes. These interruptions confer tumor selectivity to the virus and decrease its virulence in normal tissue.

In this study, we demonstrate that VACV GLV-1h68 efficiently infected, replicated and lysed three different CC cell lines in culture. The observed oncolytic activity occurred in cells representing intrahepatic CC (KMC-1), extrahepatic CC (KMBC)

and the combined hepatocellular-CC (KMCH-1) histologic subtypes (Figure 1). Expression of the virus encoded *Ruc-GFP*, (Figure 2) and viral replication, measured by plaque formation (Figure 4) correlated with cell killing. Fluorescent microscopy confirmed the GFP expression and was coinciding with or preceded cell death in the treated cell lines. The three cell lines varied in degree of replication and susceptibility, but these differences were MOI dependent. Our results concur with reports that GFP marker gene expression intensity after infection is dependent on cell lines.²⁶

GLV-1h68 successfully killed all three CC cell lines *in vitro*. Cell lines KMCH-1 and KMC-1 showed near complete cytotoxicity (with $< 10\%$ cell viability) in all MOI by day 7 (Figure 1). Cell line KMBC showed the least sensitivity to GLV-1h68 among the three cell lines. In KMBC cell line at MOIs of 5 and 1, GLV-1h68 demonstrated a killing over 80% of cells by day 7, at MOI 0.1, 50% cell death and at the lowest MOI 0.01 it showed no measurable cytotoxicity by day 7. *In vivo* GLV-1h68 suppressed the growth of CC in the xenograft model. KMC-1 flank xenografts treated with a single dose of intratumoral injection of GLV-1h68 at a concentration 1×10^7 PFUs demonstrated significant tumor volume regressions over a 2-week period of time. Fourteen days post-infection, controls ($n = 12$) nearly increased by $\sim 250\%$ of original tumor volume; while tumors treated with single dose of GLV-1h68 ($n = 12$) shrank to $\sim 46\%$ of the starting volume ($P < 0.05$) (Figure 5). All mice tolerated the treatment without significant morbidity.

Our lab has investigated VACV as a potentially viable oncolytic viral agent. We have demonstrated the effectiveness of VACV in the treatment of several cancers such as mesothelioma,²⁷ anaplastic carcinoma of thyroid,²⁸ hepatocellular cancer²⁹ pancreatic tumor¹¹ and breast cancer¹² both *in vitro* and *in vivo*. To date only a very limited number of oncolytic viruses have shown potential against CC. Adenovirus,³⁰ herpes simplex¹⁸ and super

Figure 5. Effect of tumor development after single administration of GLV-1h68 on KMC-1 tumor-bearing mice. KMC-1 cells were implanted subcutaneously in the flank of athymic nude mice and treated by injection of GLV-1h68 or PBS. Virus treatment was tested versus PBS treatment. Mean tumor volume of mice in mm³ ($n = 12$ for PBS treated controls, $n = 12$ for GLV-1h68) is plotted and one-way analysis of variance, was used to compare the data; $P < 0.05$). PBS, phosphate-buffered saline.

cytosine deaminase armed measles vaccine virus³¹ have shown promise as oncolytic vectors to treat CC. Combined radiotherapy along with virotherapy is an additional option that may enhance the therapeutic efficacy to treat CC.¹⁸ Virotherapy has been combined with chemotherapy (5-FU) and has shown greater efficacy.³¹

CC is a fatal disease with a great need to develop novel therapeutic options. Intravenous reovirus is currently the largest oncolytic virus program ongoing worldwide and now is being used to treat refractory head and neck cancers along with paclitaxel and carboplatin.³² HSV-1 strain using T-Vec (talimogene laherparepvec) in melanoma has advanced to a Phase III trial.³³ Wyeth strain of vaccinia, JX-594, has been involved in randomized studies, is currently tested against hepatocellular carcinoma.²⁴ As with many cancer agents, viruses seem to work better in combination with other treatment modalities rather than single agents. In a recent clinical study combination of cisplatin or oxaliplatin along with gemcitabine showed greater activity as evidenced by improved response rate and progression-free survival.³⁴ Critical to future rational treatment of biliary tract cancer is a molecular map with which targeted therapies can be directed, similar to that which is evolving for the common cancer. Little is known about the biology of biliary tract cancers, but it appears to lie in the spectrum of other gastrointestinal cancers with similar mutations.^{35,36} Preclinical and clinical data support combination strategies and co-administration of virus along with chemotherapy or radiotherapy does not increase toxicity or damage the virus.^{37,38} Soon we will be able to define optimal combinations, disease targets, dose and response assessment.

So far, GLV-1h68 has been tested in at least 50 tumor xenograft models, and is being tested in 4 different phase I or phase I/II clinical trials. The attempt to understand the mechanism of action of GLV-1h68 has been made. All the results on GLV-1h68 have been published in over 40 peer-reviewed papers. Our data indicate that GLV-1h68 eradicates tumors in tumor xenograft models by two mechanisms: direct tumor cell lysis and induction of anti-tumor innate immune responses.²⁵ In immune-competent mouse models, GLV-1h68 promotes infiltration of T cells into treated tumors (unpublished data). The extensive study on the mechanism of action of GLV-1h68 has been ongoing in our lab.

This manuscript represents the first report on the treatment of CC using GLV-1h68 in preclinical models. Because of the encouraging results in this study, we are currently planning on starting a phase I trial of GLV-1h68 in patients with CC. The

mechanistic study on GLV-1h68 in this tumor model has been started in our lab and will be published in a follow-up paper.

CONFLICT OF INTEREST

NWC, RJA and AAS are employees of Genelux Corporation, San Diego Science Center, San Diego, CA, USA. The remaining authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Technical services provided by the Research Animal Resource Center (RARC) and the Small-Animal Imaging Core Facilities at Memorial Sloan Kettering Cancer Center are greatly acknowledged. We would like to thank Valerie Longo at the imaging center for her valuable assistance. This study was supported in part by grants IMG0402501 from the Susan G. Komen Foundation and grant 032047 from the Flight Attendant Medical Research Institute (Prof Yuman Fong).

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