Oncolytic Vaccinia Virus Therapy of Salivary Gland Carcinoma

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**Objective:** To examine the therapeutic effects of an attenuated, replication-competent vaccinia virus (GLV-1h68) against a panel of 5 human salivary gland carcinoma cell lines.

**Design:** The susceptibility of 5 salivary gland carcinoma cell lines to infection and oncolysis by GLV-1h68 was assessed in vitro and in vivo.

**Results:** All 5 cell lines were susceptible to viral infection, transgene expression, and cytotoxic reactions. Three cell lines were exquisitely sensitive to infection by very low doses of GLV-1h68. Orthotopic parotid tumors exhibited more aggressive behavior compared with flank tumors. A single intratumoral injection of GLV-1h68 induced significant tumor regression without observed toxic effects in flank and parotid tumor models; controls demonstrated rapid tumor progression.

**Conclusion:** These promising results demonstrate significant oncolytic activity by an attenuated vaccinia virus for infecting and lysing salivary gland carcinomas, supporting future clinical trials.

**ALIVARY GLAND CARCINOMAS** are relatively rare malignant tumors, accounting for less than 5% of all cancers of the head and neck. They encompass a wide spectrum of histologic abnormalities with varied biologic behavior. Initial therapy of localized disease consists of complete surgical excision. The risk of recurrence and metastasis is significantly higher in patients with locally advanced salivary carcinomas. Individuals with high-grade salivary carcinomas have a 5-year survival of roughly 40%; those with low- and intermediate-grade tumors have a 5-year survival of 85% to 90%. For these patients, complete surgical resection followed by postoperative radiation therapy is recommended. Unfortunately, patients with recurrent and/or unresectable disease have few effective treatment options. Clinical trials exploring the role of chemotherapy in the management of salivary gland carcinomas failed to show survival benefit. Chemotherapy, therefore, is generally reserved as a palliative measure for patients with symptoms and/or rapid disease progression. Clearly, novel therapies are needed to improve outcomes for patients with salivary carcinomas.

Oncolytic viruses have emerged as versatile therapeutic agents that can selectively infect, replicate within, and ultimately lyse a host cancer cell. A variety of viruses have been reported to possess oncolytic antitumoral activity, including herpes simplex type 1, adenovirus, reovirus, vesicular stomatitis virus, measles virus, poliovirus, West Nile virus, and Newcastle disease virus. Vaccinia virus, which is a double-stranded DNA member of the genus Orthopoxvirus, has many unique characteristics that make it an excellent tool in cancer treatment. In addition to its natural tropism for tumor tissues and its abilities for efficient entry, replication, and lysis, vaccinia virus has a remarkable safety record in its historical widespread human application as a vaccine for smallpox. The vaccinia replication cycle occurs exclusively in the cytoplasm, which eliminates the possibility of chromosomal integration. Furthermore, there are many Food and Drug Administration-approved antiviral agents available to limit viral spread and control the unlikely possibility of viral toxicity. Recombinant, replication-competent vaccinia virus (GLV-1h68) has been reported as effective in breast cancer, thyroid cancer, mesothelioma, pancreatic cancer, pros-
tate cancer, human hepatocellular carcinoma, and head and neck cancer models.

The aim of this study was to assess the usefulness of GLV-1h68 as a therapeutic agent against salivary gland carcinoma in vitro and in vivo. Murine flank and orthotopic parotid tumor models were used.

### METHODS

#### CELL LINES

Five human salivary gland carcinoma cell lines were studied: H292 (lung mucoepidermoid), H3118 (parotid mucoepidermoid), HTB-41 (submaxillary gland undifferentiated mucoepidermoid carcinoma), HSG (submandibular adenocarcinoma), and HSY (parotid adenocarcinoma). The H292 and H3118 cell lines were grown in Roswell Park Memorial Institute medium containing 10% fetal calf serum and 1% penicillin and streptomycin. The HSY and HSG cell lines were grown in Ham F12 nutrient medium and Dulbecco Modified Eagle Medium containing 10% fetal calf serum and 1% penicillin and streptomycin. All cells were grown in a humidified incubator at 5% carbon dioxide and 37°C.

#### VIRUS

A recombinant, replication-competent vaccinia virus, GLV-1h68 is derived from the LIVP strain (Lister strain from the Institute of Viral Preparations). It contains 4 inserted cassettes encoding Renilla luciferase-green fluorescent protein fusion (RUC-GFP cassette), β-galactosidase, and a reverse inserted human transferrin receptor (rTfr), β-glucuronidase, into F14.5L, J2R (thymidine kinase), and A56R (hemagglutinin) loci of the viral genome, respectively.10

#### IN VITRO GFP VISUALIZATION

Cells were seeded in 12-well plates for 6 hours and exposed to GLV-1h68 at multiplicity of infection (MOI) 1.0. At 12, 24, and 48 hours, cells were imaged under a microscope with a GFP filter at ×10 magnification.

#### IN VITRO QUANTIFICATION OF β-GALACTOSIDASE ACTIVITY

The β-galactosidase assay was performed (enhanced β-galactosidase assay kit; Gene Therapy Systems) with 2 × 10⁴ cells per well seeded in 100 μL of corresponding medium in a 96-well plate for 6 hours. The GLV-1h68 cell line in 100 μL of corresponding medium was added to each well at MOI 1.0. The medium was aspirated at 12 hours and lysis buffer was added to each well. A standard curve was created with serial dilutions of β-galactosidase. Substrate was added to each well and plates were read by spectrophotometry at 570 nm. Samples were assayed in triplicate.

#### IN VITRO QUANTIFICATION OF LUCIFERASE ACTIVITY

Each cell line was plated in 96-well plates at 1 × 10⁴ cells per well for 6 hours, with GLV-1h68 added at MOI 1.0. Coelenterazine 0.25 μg (Biotium) in 50 μL of phosphate-buffered saline (PBS) was added to each well 24 hours after infection. The plates were incubated for 10 minutes, and emitted photons were measured with a cooled charge-coupled device camera (Xenogen IVIS; Xenogen) for 1 minute. Images were analyzed by using commercial software (Living Image; Xenogen). Samples were assayed in triplicate.

### VIRAL PLAQUE ASSAY

Each cell line was plated in 12-well plates at 2 × 10⁴ cells per well for 6 hours, with GLV-1h68 added to each well at MOI 0.1. Supernatant from each well was collected at 72 hours and frozen. The CV-1 cells were grown to confluence on 6-well plates. Supernatant samples were thawed and underwent serial 10-fold dilutions. Samples were incubated on CV-1 cells for 4 hours, washed with medium, and covered with 1% agarose. After 48 hours of incubation, 2 mL of neutral red solution was added and viral plaques were counted in 24 hours. Samples were assayed in triplicate.

### CYTOTOXICITY ASSAYS

Each cell line was plated in 12-well plates at 2 × 10⁴ cells per well for 6 hours, with GLV-1h68 in 100 μL of corresponding medium added to each well at MOI 0, 0.01, 0.1, 1.0, and 5.0. At daily intervals, the cells were washed with PBS and lysed with 1.35% Triton X-100 (Sigma-Aldrich Co). Lactate dehydrogenase (LDH) was measured (Cytotox96 kit; Promega), including spectrophotometry at 450 nm. Results were expressed as the percentage of surviving cells by comparing the LDH of each test sample with that of untreated samples. Samples were assayed in triplicate.

### MURINE FLANK TUMOR THERAPY

In vivo experiments were performed under an approved animal protocol by the Memorial Sloan-Kettering Institutional Animal Care and Use Committee. Six-week-old male athymic nude mice were purchased from the National Cancer Institute. With the mice under general isoflurane anesthesia, HTB-41 cells (5 × 10⁹) in 50 μL of PBS were injected into the subcutaneous flanks. Established HTB-41 flank tumors were injected with a single dose of GLV-1h68 (3 × 10⁵ plaque-forming units [PFU]) in 50 μL of PBS or with 50 μL of PBS alone as a control. Tumor dimensions were measured serially with calipers, and tumor volume was calculated using the following formula: volume = length × width² × 0.4. Body weights were measured serially. Photographs of representative animals were taken on day 26 (flank group) and day 31 (parotid group). At the end of the experiment, the animals were killed using carbon dioxide inhalation.

### MURINE ORTHOTOPIC TUMOR THERAPY

With the mice under general isoflurane anesthesia, HTB-41 cells (5 × 10⁹) in 50 μL of PBS were injected into the parotid gland. Established HTB-41 parotid tumors were injected with a single dose of GLV-1h68 (3 × 10⁵ PFU) in 50 μL of PBS or with 50 μL of PBS alone as a control. Tumor dimensions and body weights were measured serially. Photographs of representative animals were taken on day 31. At the end of the experiment, the animals were killed using carbon dioxide inhalation.
IN VIVO GLV-1H68 GENE EXPRESSION

For luciferase assessment, 2.5 μg of coelenterazine (Biotium) in 95 μL of PBS was injected via the retro-orbital sinus. Luciferase activity was detected with a cooled charge-coupled device camera (Xenogen IVIS). Emitted photons were measured for 1 minute. Images were analyzed using commercial software (Living Image; Xenogen).

Figure 1. Effective early infection by GLV-1h68 of human salivary carcinoma cell lines. Five cell lines were infected by GLV-1h68 at multiplicity of infection 1.0 and visualized under a green fluorescent protein (GFP) filter. All 5 cell lines showed progressively increasing GFP marker gene expression within 24 hours (hematoxylin-eosin, original magnification ×10).
HISTOLOGIC EXAMINATION

After the mice were euthanized, subcutaneous flank and parotid tumors were harvested and embedded in optimal cutting temperature compound (Miles Laboratories, Inc), rapidly frozen in liquid nitrogen, and stored at –80°C. Histologic examination was performed with routine hematoxylin-eosin (HE) staining.

RESULTS

IN VITRO GFP VISUALIZATION

Assessment of GFP by microscopy of 5 human salivary carcinoma cell lines demonstrated evidence of efficient infection by GLV-1h68 as early as 12 hours after infection (Figure 1). Expression of GFP peaked at 24 hours for 4 cell lines (H292, H3118, HTB-41, and HSG) and at 48 hours for HSY. The decrease in GFP expression at 48 hours for H292, H3118, HTB-41, and HSG was due to GLV-1h68-mediated cell death.

IN VITRO QUANTIFICATION OF β-GALACTOSIDASE AND LUCIFERASE ENZYMATIC ACTIVITIES

Quantitative assays for β-galactosidase gene expression demonstrated infection by GLV-1h68 of all the human salivary carcinoma cell lines. The H292 cell line showed the highest gene expression level and HSY showed the lowest by β-galactosidase and luciferase enzymatic activity assays (Figure 2A and B). Quantitative assays correlated with qualitative visual findings from in vitro GFP visualization.

VIRAL REPLICATION

To assess the ability of human salivary carcinoma cell lines to support viral replication, GLV-1h68 at an MOI of 0.1 was added to cell lines for a 3-day course for measurements of viral titer. The 3 cell lines (H292, H3118, and HTB-41) most sensitive to viral infection as determined by β-galactosidase and luciferase enzymatic activity assays also supported robust viral proliferation (Figure 2C). In contrast, the less-sensitive cell lines (HSG and HSY) demonstrated lower viral titers.

CYTOTOXICITY ASSAYS

The GLV-1h68 cell line was incubated for a 7-day period with each human salivary carcinoma cell line to assess cytotoxicity (Figure 3). The H292, HTB-41, and H3118 cell lines were exquisitely sensitive to GLV-1h68, demonstrating significant cell death by day 7 at even a very low MOI of 0.01. Less-sensitive cell lines (HSG and HSY) showed partial cytotoxic reactions at MOI 5.0, with 7% and 32% viability by day 7, respectively. The relative cytotoxic effects in these 5 cell lines reflected their relative susceptibility to GLV-1h68 infection as determined by β-galactosidase and luciferase enzymatic activity assays.

IN VIVO GLV-1H68 GENE EXPRESSION

Luciferase imaging demonstrated focal activity localized specifically to the site of HTB-41 tumor inoculation in the murine flank and orthotopic parotid tumor models (Figure 4 and Figure 5). In the flank xenograft tumor model, luciferase activity levels peaked at days 3 to 6. The activity subsequently declined by day 10. Increased levels of luciferase activity were sustained for a longer time in the orthotopic parotid tumor model. Lu-
ciferase activity peaked at days 3, 7, and 13 and declined by day 15. No luciferase activity was detected in other sites of the animal.

**THERAPY OF HUMAN SALIVARY CARCINOMA FLANK XENOGRAFT IN VIVO**

Established HTB-41 flank tumors with a mean starting volume of 65 mm$^3$ were treated with a single intratumoral injection of GLV-1h68 (5 x 10$^6$ PFU). Treated tumors demonstrated progressive tumor regression for a 26-day period (Figure 4D). Four of 5 animals had complete tumor regression, with the remaining animal demonstrating just a 0.6-mm$^3$ tumor remnant. By day 26, the mean (SE) tumor volume was 0.4 (0.1) mm$^3$. In contrast, all of the control tumors progressively increased in size to a mean volume of 384 (107) mm$^3$ during the same period ($P=.004$, unpaired 2-tailed t test). Un-

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Figure 3. GLV-1h68 exhibited significant oncolytic effects in human salivary carcinomas in vitro. Five cell lines were infected by GLV-1h68 at varying multiplicity of infection (MOI) (0.01, 0.1, 1.0, 5.0), and lactate dehydrogenase assay was performed for 7 days to assess cell viability. All 5 cell lines were susceptible to significant cytotoxic effects at MOI 5.0. Three cell lines demonstrated exquisite sensitivity to GLV-1h68, even at MOI 0.01. Limit lines represent standard error.
treated tumors remained freely mobile on clinical examination and well encapsulated on HE stain (Figure 6). Mean body weights remained stable in both groups (Figure 4E). There were no measurable toxic reactions associated with GLV-1h68 treatment.

Figure 4. Therapy of human salivary carcinoma flank tumor in vivo. A, Established undifferentiated mucoepidermoid carcinoma flank tumors were injected with GLV-1h68 (5×10^6 plaque-forming units [PFU]), and luciferase activity was measured following retro-orbital coelenterazine injections during a 16-day period. GLV-1h68 bioluminescence imaging in vivo showed selective prolonged virus-mediated luciferase activity in flank tumors. B, Quantification of luciferase activity during a 16-day period using software assessment of emitted photons was performed (n=3). The late reduction of luciferase activity correlated with tumor size regression. C, A single injection of GLV-1h68 resulted in complete regression of established undifferentiated mucoepidermoid flank tumor in vivo. D, Established flank tumors (n=5 per group) were treated with a single intratumoral injection of GLV-1h68 (5×10^6 PFU). Measured tumor volumes for the GLV-1h68 group demonstrated nearly complete regression. In contrast, measured tumor volumes for the control phosphate-buffered saline–treated group demonstrated significant tumor progression. E, There was no observed change in animal weight related to viral administration. B and D, Limit lines represent standard error.

THERAPY OF HUMAN SALIVARY CARCINOMA ORTHOTOPIC XENOGRAFT IN VIVO

Established HTB-41 parotid tumors with a mean starting volume of 161 mm³ were treated with a single intratumoral injection of GLV-1h68 (5×10^6 PFU). Treated tumors demonstrated progressive regression during a 31-day period (Figure 5). By day 31, the mean (SE) tumor volume was 90 (32) mm³. In contrast, all of the control tumors progressively increased to a mean volume of 1032 (270) mm³ (P = .008, t test). Untreated tumors were poorly mobile and exhibited significant extracapsular spread, as well as bone and perineural invasion on HE stain (Figure 6B-D). Mean body weights remained stable for the control and treatment groups. There were no measurable toxic reactions attributable to GLV-1h68 therapy. The experiment was concluded at day 31 because of the large size of the control tumors.
Novel therapies are necessary to improve outcomes for patients with salivary gland carcinomas. This is especially true in a subset of patients with advanced disease not amenable to local therapies or for those with recurrent disease. In this setting, oncolytic viral therapy is emerging as an attractive option. Several oncolytic viruses have been reported to selectively infect, replicate within, and ultimately lyse a host cancer cell. One concern when considering clinical implementation of oncolytic viral therapy is its potential for exhibiting pathologic behavior. Vaccinia virus is an orthopoxvirus that, in addition to its natural tropism to tumors, has a remarkable safety record in its widespread historic use in humans as a vaccine for smallpox. Vaccinia possesses many favorable characteristics as an oncolytic virus, including a large genome that can accommodate multiple gene insertions, a broad host range suitable for preclinical evaluation, and rapid replication.

GLV-1h68 is a recombinant, replication-competent vaccinia virus genetically engineered to enhance tumor specificity and introduce diagnostic reporter genes. Introduction of thymidine kinase mutation renders viral
replication exquisitely dependent on host cell thymidine kinase for the synthesis of deoxyribonucleotides. Because cancer cells have a relatively high concentration of deoxyribonucleotides in comparison with healthy cells, thymidine kinase mutation enhances tumor-specific replication while attenuating viral ability to replicate in healthy cells. Inactivation of the hemagglutinin gene by insertional mutation further reduces the virulence of vaccinia. In addition, 3 reporter genes—RUC-GFP, β-galactosidase, and β-glucuronidase genes—were inserted in the F14.5L to allow real-time imaging of tumor-specific viral entry and replication.17

The purpose of this study was to explore the potential usefulness of GLV-1h68 as a therapeutic agent for human salivary gland carcinomas in vitro and in vivo. The effects of GLV-1h68 were examined in a panel of 5 salivary carcinoma cell lines in vitro. These cell lines represent a spectrum of salivary carcinomas, including poorly and well-differentiated mucoepidermoid carcinomas and adenocarcinomas of both minor and major salivary gland origin. All 5 cell lines were susceptible to viral entry, viral gene expression (β-galactosidase, GFP, and luciferase), and cell lysis. Notably, the mucoepidermoid cell lines were exquisitely sensitive to GLV-1h68, demonstrating significant cell death by day 7 at a very low MOI of 0.01. The less-sensitive adenocarcinoma cell lines showed significant cytotoxic reactions at a higher MOI of 5.0. The relative sensitivity of 5 cell lines reflects their susceptibility to viral entry and gene expression. This correlation suggests that viral entry and early gene expression by GLV-1h68 may define the subsequent ability of the virus to lyse the host cancer cell.

To examine the therapeutic efficiency of GLV-1h68 in vivo, we used 2 different models: murine flank and orthotopic parotid tumors. Traditionally, a subcutaneous xenograft tumor model has been used in most preclinical studies on the basis of several studies18-20 reporting its ability to predict the clinical activity of cytotoxic agents. However, other studies21 had called the usefulness of the subcutaneous xenograft model into question. The National...
Cancer Institute examined 39 agents with xenograft data and available phase 2 clinical trial results. This analysis revealed that, with the exception of lung non–small-cell cancer, in vivo activity tested in a subcutaneous xenograft model does not closely correlate with therapeutic response in clinical trials. Therefore, orthotopic xenograft models were introduced as more physiologically relevant with respect to organ-specific abnormalities, microenvironment, and growth, with hopes to recapitulate the biologic behavior of human tumors. In fact, it has been shown that tumors grown orthotopically reflect the clinical effects of drugs more closely than tumors grown subcutaneously. We therefore used an orthotopic tumor model, in addition to a subcutaneous xenograft, to demonstrate the efficacy of oncolytic vaccinia virus in vivo.

An orthotopic parotid tumor model has been established and characterized. To evaluate the therapeutic efficiency of GLV-1h68, we established a subcutaneous model and a parotid gland tumor model using an undifferentiated mucoepidermoid human carcinoma cell line (HTB-41). In contrast to subcutaneous tumors, which were freely mobile, smaller (average maximum size, 384 mm³), and well encapsulated on HE, parotid tumors were poorly mobile, larger (1032 mm³), and exhibited extracapsular spread as well as bone and perineural invasion. More rapid tumor growth and aggressive local invasion correlate closely with the biologic behavior of human poorly differentiated mucoepidermoid carcinoma. Therefore, an orthotopic parotid tumor model might predict more accurately the clinical response of human tumors to oncolytic vaccinia virus therapy.

A single intratumoral injection of GLV-1h68 (MOI 1.0) in vivo into HTB-41 xenografts in mice exhibited localized intratumoral luciferase activity, peaking on day 3, in the flank and parotid tumor models. However, in the parotid tumor model, increased luciferase activity was maintained for a longer time. Because the magnitude of luciferase activity normally correlates with viral entry and proliferation, sustained luciferase activity in parotid tumors could be a function of a larger tumor burden. In addition, a contribution of elements in the parotid microenvironment, such as increased microvessel density and higher expression of growth factors and cytokines, could contribute to enhancement of viral entry and proliferation compared with flank tumors. Treated animals exhibited essentially complete tumor regression during a 26-day period in a flank model and a 31-day period in a parotid tumor model without any observed toxic effects; control animals demonstrated rapid tumor progression. There was a lag period between the resolution of luciferase activity and tumor volume regression in both groups, which could be explained by the additional time needed for the resorption of necrotic tissue. No animal treated with GLV-1h68 exhibited any morbidity attributable to the viral therapy.

Clearly, the limitation of our in vivo flank and orthotopic tumor nude mouse models is the inability to study the immune response to viral replication. The effect of an intact immune system could be 2-fold. An intact immune system might limit viral delivery to the target tumors and suppress viral replication within the tumor, thus limiting the effectiveness of oncolytic vaccinia virus therapy. However, it has been shown that the immune response induced by the virus can provide a beneficial antitumor effect. This is not unexpected, since viral replication will typically lead to direct lysis of tumor cells, releasing tumor antigens and other danger signals into the extracellular environment. This production and release of tumor antigens may be able to induce an adaptive immune response against the tumor. This immunotherapeutic potential of oncolytic vaccinia virus has only recently been appreciated and is currently under investigation. Ideally, the effects of the immune system could be studied in the immunocompetent mouse model with mouse salivary tumor cell line xenograft. Unfortunately, to our knowledge, there is no mouse salivary gland tumor line available from a known mouse strain to study the effects of oncolytic vaccinia virus in this model.

In conclusion, the promising results of this study demonstrate significant oncolytic activity by an attenuated vaccinia virus for infecting, replicating within, and causing regression of human salivary gland carcinomas in vitro, in vivo in a subcutaneous flank xenograft tumor model, and in vivo in an aggressive and physiologically relevant orthotopic parotid tumor model. These data support further evaluation in clinical trials of GLV-1h68 as a therapeutic agent to treat aggressive salivary cancers.

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Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Chernichenko, Szalay, Fong, and Wong. Acquisition of data: Chernichenko, Linkov, Li, Bakst, and He. Analysis and interpretation of data: Chernichenko, C.-H. Chen, Yu, N. G. Chen, and Wong. Drafting of the manuscript: Chernichenko, Linkov, Li, and Bakst. Critical revision of the manuscript for important intellectual content: Chernichenko, Linkov, C.-H. Chen, He, Yu, N. G. Chen, Szalay, Fong, and Wong. Statistical analysis: Chernichenko. Obtained funding: Chernichenko, Szalay, and Wong. Administrative, technical, and material support: Chernichenko, Linkov, Bakst, C.-H. Chen, He, Yu, N. G. Chen, and Wong. Study supervision: Fong and Wong.

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