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Peritoneal-directed chimeric oncolytic virus CF17 prevents malignant ascites and improves survival in gastric cancer peritoneal metastases

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Gastric cancer (GC) peritoneal metastasis (PM) is fatal without effective therapy. We investigated CF17, a new replication-competent chimeric poxvirus, against GC cell lines *in vitro* and PM in an aggressive GCPM mouse model. We performed viral proliferation and cytotoxicity assays on intestinal-type and diffuse-type human GC cell lines following CF17 treatment. At lower MOIs of 0.01, 0.1, there was >80% killing in most cell lines, while in the more aggressive cell lines, killing was seen at higher MOIs of 1.0 and 10.0. We observed reduced peritoneal tumor burden and prolonged survival with intraperitoneal (i.p.) CF17 treatment in nude mice implanted with the resistant GC cell line. At day 91 after treatment, seven of eight mice were alive in the CF17-treated group vs. one of eight mice in the control group. CF17 treatment inhibited ascites formation (0% vs. 62.5% with PBS). Thus, CF17 efficiently infected, replicated in, and killed GC cells in a dose- and time-dependent manner *in vitro*. *In vivo*, i.p. CF17 treatment exhibited robust antitumor activity against an aggressive GCPM model to decrease tumor burden, improve survival, and prevent ascites formation. These preclinical results inform the design of future clinical trials of CF17 for peritoneal-directed therapy in GCPM patients.

INTRODUCTION

Oncolytic viruses (OV) as anti-cancer agents have evolved from their ability to kill tumors directly without damaging normal tissues to the genetically engineered ability to selectively deliver therapeutic transgenes to cancer cells.[1–3] OVs exhibit preclinical promise against many solid tumors and offer hope for improving the survival of cancer patients. However, the excitement of the preclinical success of many OVs has yet to be translated into clinically effective therapeutic strategies.[4] While the safety of OVs has been well-established, the results of the early-phase efficacy trials have been disappointing. Most OVs exhibit poor potency, lack tumor selectivity, and generate low tumor response rates. With improved genetic engineering of OVs with novel molecular targets and efficient delivery methods, investigators seek better oncolytic viral agents and immunotherapeutic strategies against solid tumors.

To overcome these shortcomings, we have developed a new generation of chimeric orthopoxviruses capable of tumor-selective replication. CF17 is a new viral chimera with a unique genome created by the random recombination of nine orthopoxvirus species. We selected it among 100 different chimeric clones for its superior oncolytic potency through high-throughput screening in an NCI-60 panel.[5,6] Based on its high cytotoxic potential in cancer cells, we studied its therapeutic potential against gastric cancer (GC) peritoneal metastasis (PM).

GC accounts for over 1 million cancer diagnoses annually, and an estimated 770,000 lives were lost in 2020 worldwide.[7] The varying responses to more effective chemotherapeutic regimens and an emerging arsenal of biomarker-targeted immunotherapeutic agents such as trastuzumab (anti-HER2), pembrolizumab, and nivolumab (anti-PD-1) highlight the molecular heterogeneity of GC and the disparity of tumor-specific response to therapy.[8] GC has higher cytologic, genomic, and architectural heterogeneity compared with other gastrointestinal cancers, which limits the clinical impact of currently available treatment options and underscores the challenges in improving the survival of GC patients.[9]

Particularly resistant to standard systemic treatment strategies, PM is the leading end-stage manifestation of GC.[10] PM now accounts for over 48% of recurrences after curative surgery despite perioperative chemotherapy and affects approximately 60% of GC patients at the time of death. Therapeutic failure and peritoneal progression of GC are associated with significant life-impairing complications such as

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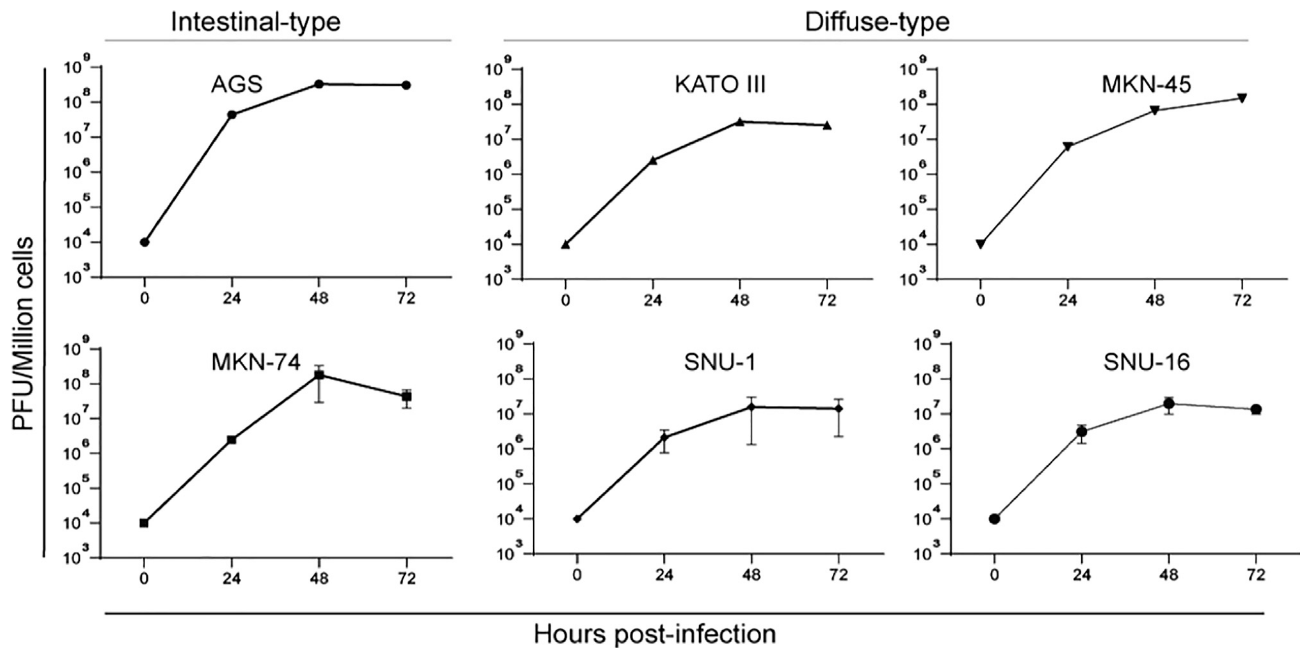


Figure 1. CF17 viruses infect and replicate in human gastric cancer cell lines

After infection over times, CF17 viruses (MOI = 0.01) replicate efficiently in human gastric cancer cell lines AGS, MKN-45, MKN-74, KATO III, SNU-1, and SNU-16, achieving 3–4 log increases in titer within 72 h. Mean ± SEM, n = 3.

malignant small bowel obstruction, large volume malignant ascites, malnutrition, cachexia, and death with a median survival of approximately 3–6 months.[11] Genomic stability, immune inactivity, and diffuse anatomic distribution are the hallmarks of GCPM posing distinct therapeutic challenges, as peritoneal tumors are protected from systemically delivered drugs by the peritoneal-blood barrier and an immunosuppressive peritoneal tumor microenvironment (TME).[9]

To overcome these challenges and develop an effective oncolytic viral therapy, we investigated for the first time the antitumor activity of CF17 against GC cells *in vitro* and in an *in vivo* GCPM mouse model.

RESULTS

CF17 viruses infect and replicate in human GC cell lines

To verify if CF17 viruses can infect and replicate in human GC cell lines not included in the NCI-60 cancer cell panel, we investigated two intestinal-type (AGS and MKN-74) and four diffuse-type (KATO III, MKN-45, SNU-1, and SNU-16) GC cell lines. After treatment with CF17 (MOI = 0.01), cell lysates were collected daily for 3 days to determine virus growth kinetics using a standard plaque assay. Our results show that CF17 successfully infected and replicated in all six GC cell lines. Virus titers plateaued at 48 or 72 h after infection and increased by at least 3-log compared with the input virus (0-h time point) in all six GC cell lines (Figure 1). Virus titers reached a 4-log scale higher than control in intestinal-type AGS and MKN-74 and diffuse-type MKN-45. These results show that CF17 robustly replicates in all the tested human GC cell lines.

CF17 is cytotoxic in human GC cell lines

Next, we analyzed the cytotoxicity of CF17 in these cell lines. All six cell lines were treated with CF17 at four MOIs (0.01, 0.1, 1, and 10) for 8 days. We observed a dose and time-dependent cytotoxicity, with greater effects at higher MOIs (MOI >1) of CF17 (Figure 2). At the lower MOIs (0.01 and 0.1), the virus-induced cytotoxicity differed in cell lines; the intestinal type (AGS and MKN-74) and diffuse type (MKN-45) were killed to a greater extent compared with other cells. This observed cytotoxicity was in accordance with the virus-replication data (Figure 1), i.e., the cell lines supporting higher levels of virus growth were more sensitive to virus-mediated killing. At lower MOIs, SNU-16 cells were resistant to CF17, with 33% cell survival at MOI = 0.1 and 84% at MOI = 0.01 on day 8. At the higher MOIs (1 and 10), CF17 killed 100% of five GC cell lines except for 90% of SNU-16 cells. These results demonstrate that CF17 effectively kills GC cells in a dose- and time-dependent manner.

Xenograft mouse models of GC cell lines KATO III, MKN-74, and SNU-16

Next, we developed a GCPM xenograft model to analyze the potential of CF17 therapy. We tested the KATO III-ffluc, MKN-74-ffluc, and SNU-16-ffluc cell lines for their ability to form tumors and ascites in the peritoneal cavity of nude mice. KATO III-ffluc cells generated tumors in only a fraction of mice. Furthermore, those KATO III tumors underwent spontaneous regression, making it an unsuitable model for our study (Figure S1). Likewise, tumors made by MKN-74-ffluc cells were highly variable, and the mice did not develop ascites. Intraperitoneal (i.p.) injections of 10 million SNU-16-ffluc cells in

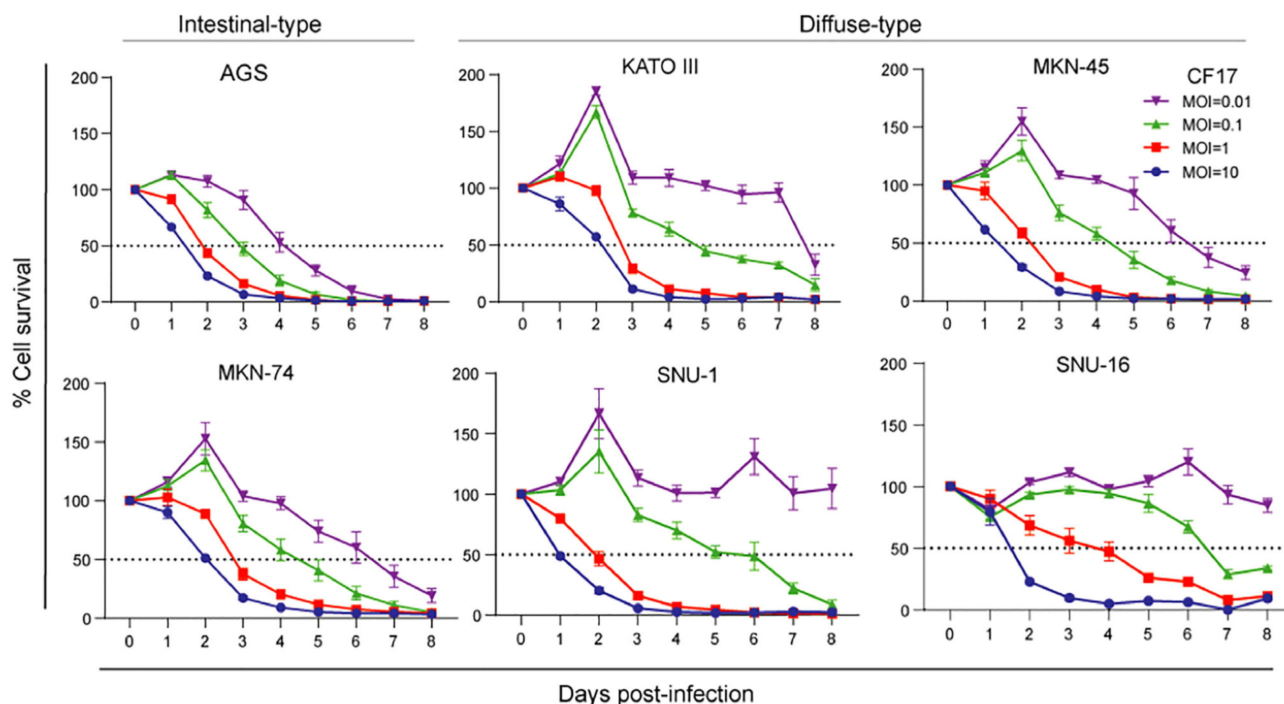


Figure 2. Cytotoxicity of CF17 in human gastric cancer cell lines *in vitro*

Six human gastric cancer cell lines AGS, KATO III, MKN-45, MKN-74, SNU-1. and SNU-16 were treated with CF17 virus with MOI shown in the figure. Cell survival was determined 8 days post-infection by the MTS cell proliferation assay with CellTiter 96 Aqueous One solution. Survival was calculated relative to mock-infected wells. Mean ± SEM, n = 3.

the mice resulted in somewhat homogeneous tumors with diffuse peritoneal progression and ascites formation (37.5%, three of eight mice) with one mouse death within 9 weeks (Figures 3A–3C). While all three cell lines could generate tumors in the peritoneal cavity, the SNU-16-ffluc model appeared the best as the tumors were more homogeneous, mice developed ascites, and demonstrated the later stage of peritoneal dissemination of GC. Hence, we selected this model to evaluate the oncolytic efficacy of CF17.

CF17 treatment reduces GCPM tumor burden

Seven days after inoculation of GC cells, the SNU-16-ffluc-GCPM xenograft mice were divided into two groups: PBS control i.p. treatment and CF17 i.p. treatment (Figure 4A). Peritoneal tumor burden decreased significantly in the CF17-treated group compared with the PBS control group on days 24, 28, and 42 ($p < 0.05$) (Figures 4B and 4C). Figure 4D shows the tumor burden for individual mice. During the 91-day treatment period, mice started to die after day 42, with three surviving on day 84 and only one on day 91 in the PBS control group. The one surviving mouse on day 91 in the control group had a minimum tumor burden on day 7, which grew very slowly (Figure 4B). In contrast, seven out of eight mice treated with CF17 survived until the end of the study (day 91).

Peritoneal tumor nodules were harvested and stained with H&E and anti-vaccinia virus antibody. H&E staining showed cell nuclei as purplish blue and the extracellular matrix and cytoplasm as pink in both treatment groups. Anti-vaccinia virus antibody staining showed only CF17 positive on the edge of the tumor nodule in the CF17-treated group but not in the PBS-treated group (Figure 4E). Further, mouse organs, including stomach, intestine, kidney, liver, spleen, adrenal gland, testis/ovary, heart, lung, and brain, collected simultaneously, and examined using plaque formation assay (pfu g/organ) did not show presence of the virus, indicating no off-target toxicity (data not shown).

CF17 treatment prolongs survival and prevents ascites formation

Kaplan-Meier survival analysis showed that CF17 treatment significantly increased overall survival ($p < 0.01$) (Figure 5A). Further, CF17 treatment prevented the formation of malignant ascites (0%) compared with the PBS control group (62.5%) (Figure 5B). The weight of CF17-treated mice was similar to that of PBS-treated mice, further confirming that the virus dose was well tolerated in these mice (Figure 5C). These data suggest that CF17 treatment can treat GCPM in an aggressive SNU-16-ffluc mouse model.

DISCUSSION

Newly created chimeric OVs, including CF17, possess superior oncolytic characteristics and enhanced antitumor activity compared with other viruses that share segments of their genome.[6] We have

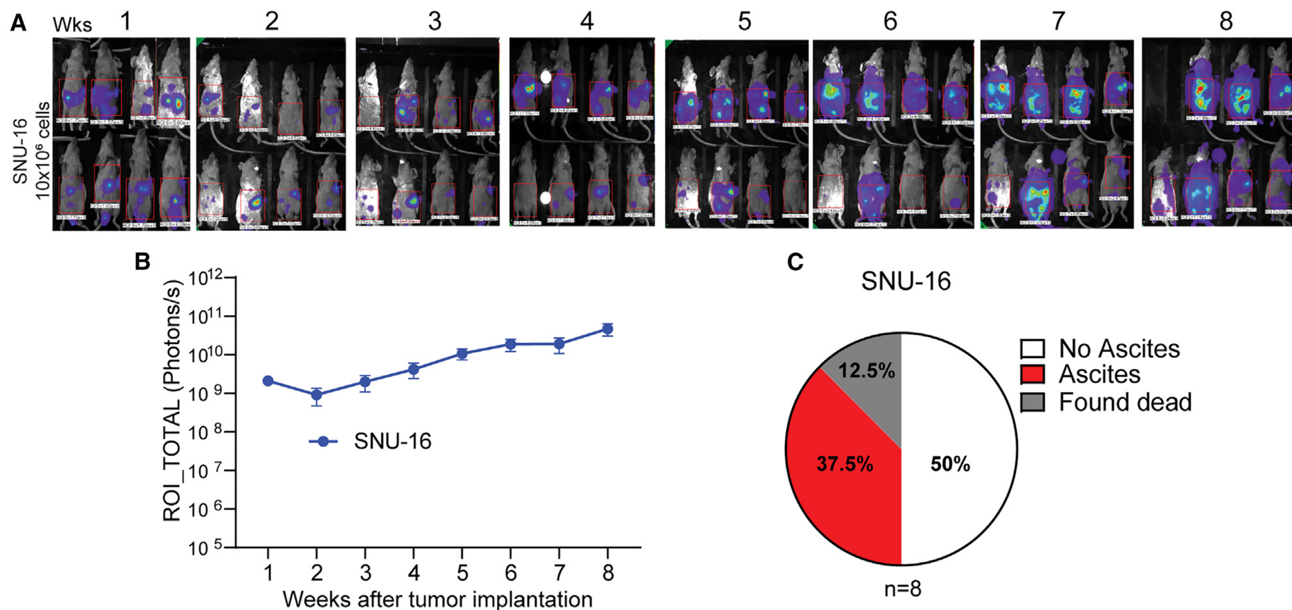


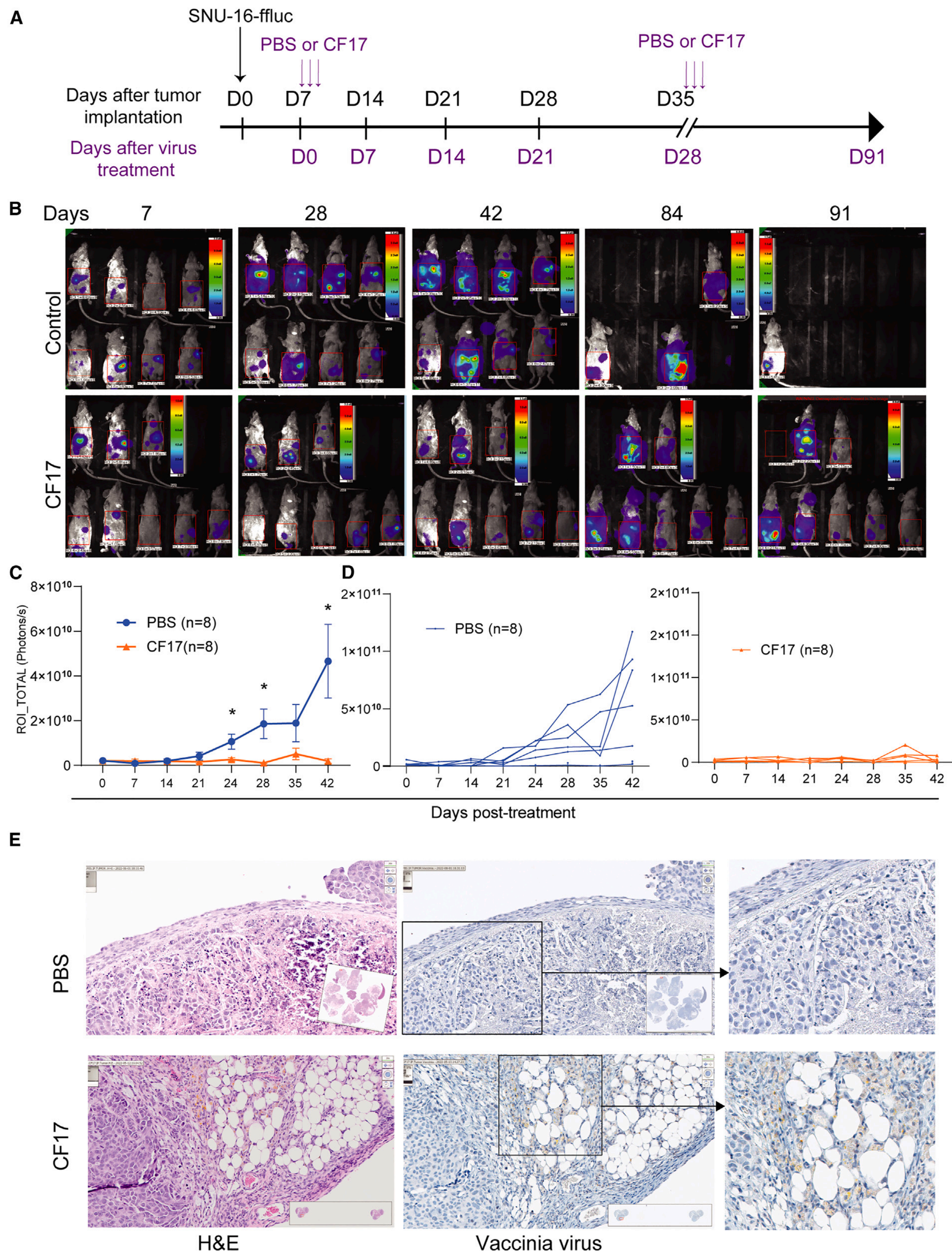
Figure 3. Peritoneal dissemination xenograft mouse model by using human gastric cancer cell line SNU-16-ffluc
Nude mice received i.p. injection of SNU-16-ffluc (10×10^6 cells/mouse, n = 8). (A) Bioluminescence imaging was performed weekly and the tumor burden is shown at time points in the figure. (B) Bioluminescence photons/s of ROI of i.p. tumor burden are shown as mean ± SEM. (C) Ascites formation was evaluated as percentage. Wks = weeks.

previously shown that CF17 has superior oncolytic characteristics and enhanced antitumor activity compared with its parental viruses in ovarian cancer.[6] Here, we demonstrated for the first time the safety and potent oncolytic activity of CF17 against GC *in vitro* and *in vivo*. CF17 replicated in GC cells in a dose- and time-dependent manner to achieve at least a 3- to 4-log increase in titer (PFU/million cells) within 72 h and resulted in the killing of 90%–100% GC cells within 8 days. Varying MOIs of CF17 revealed that GC cancer cells have different sensitivity to CF17 treatment; the AGS intestinal-type cell line showed the highest sensitivity, and SNU-16 diffuse-type cell line derived from the malignant ascites obtained from a GC patient had the lowest sensitivity to both viral replication and oncolysis.

PM is one of the most aggressive and fatal manifestations of GC. To obtain a reliable peritoneal dissemination model of human GC cell lines in nude mice for testing CF17 i.p. treatment, we compared three human GC cell lines, KATO III, MKN-74, and SNU-16, in the peritoneum of nude mouse models. Tumors in the peritoneal cavity of the SNU-16 xenograft model showed linear growth and formed malignant ascites. In contrast, tumors that developed in KATO III cells spontaneously regressed over time, and MKN-74 cells did not form malignant ascites. KATO III cells were established from a pleural effusion of a 55-year-old stomach cancer patient,[12–14] and MKN-74 from a well-differentiated adenocarcinoma that showed no response to hEGF.[15] The SNU-16 cell line was isolated from malignant ascites of a 33-year-old Asian female patient with poorly differentiated stomach carcinoma who had received chemotherapy before.[16] The SNU-16 cell line also expresses carcinoembryonic antigen (CEA), TAG-72, and overexpresses fibroblast growth receptor 2 (FGFR2), which appears to be a putative driver of tumorigenesis.[17] In the SNU-16 mouse model, CF17 i.p. treatment significantly reduced peritoneal tumor burden at days 24, 28, and 42 post-treatment than the control group, prevented malignant ascites formation (0% CF17 vs. 62.5% PBS), and significantly prolonged animal survival.

Safety has been a long-standing concern limiting progress in oncolytic viral therapy with replication-competent viral agents. To address safety concerns, many OVs have been attenuated with deletions in neurovirulent genes (HSV-OVs) and thymidine kinase (vaccinia-based OVs) to decrease unwanted side effects. Unfortunately, such alterations in the OV genome reduce its oncolytic potency. For successful clinical translation of CF17 as effective anti-cancer immunotherapy with a wide therapeutic window, it must overcome the limitations of the previous generation of OVs and possess highly potent oncolytic properties and an exclusive tumor-selective safety profile. After 4 weeks of CF17 i.p. treatment, although weak staining of vaccinia virus was observed in the peritoneal tumor, the presence of the virus was not detected in tested organs. In summary, CF17 is not only effective in preventing malignant ascites and improving survival, but it also demonstrates an exclusive safety profile.

PMs are treatment-resistant and carry a dismal prognosis with a median survival of 3.1 months.[18] Up to half of the patients with advanced GC have tumor recurrence with PM despite radical surgery.[19] GCPM patients may develop complications such as intestinal obstruction, poor nutrition, electrolyte disorders, increasing abdominal distention, pain, and dyspnea, which significantly lower their quality of life and preclude them from systemic treatment. Paracentesis and diuretics are first-line



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treatments for managing malignant ascites but only provide temporary symptom relief. Other options include indwelling peritoneal catheters; however, these are only for symptom management. Although these interventions improve quality of life, none improve overall survival. Several systemic therapies, such as combinations of chemotherapeutic agents, targeted therapies such as trastuzumab or ramucirumab, and immune checkpoint inhibitors have been introduced to treat PM. Systemic chemotherapy improved survival up to 12 months in advanced GC[20,21]; however, a similar survival benefit has not been reported in GCPM. The ineffectiveness of systemic therapy could be attributed to several factors, such as the presence of the peritoneal-plasma barrier, poor cancer tissue vascularity, and the low apoptotic potential of hypoxic tumor cells.[22] This has led to the need to develop i.p. treatment strategies such as catheter-based i.p. chemotherapy, hyperthermic i.p. chemotherapy (HIPEC), and pressurized i.p. aerosol chemotherapy (PIPAC). Laparoscopic HIPEC has been proposed as palliative therapy for GCPM. A systematic review analyzing survival in the treatment of GCPM demonstrated that following cytoreductive surgery (CRS) and HIPEC, the overall median survival was 7.9 months. Completeness of cytoreduction scores of 0/1 improved the median survival to 15 months. The 1- and 5-year survival were 43% and 13%.[23] Despite the widespread use of HIPEC, a standard guideline has not been established.[24] In general, although various approaches to therapy have been attempted, the prognosis of patients with PM is still inferior, and no effective treatment exists so far.[25]

Oncolytic virotherapy is an emerging form of immunotherapy exploited as direct cancer-killing and antitumor immune-activating strategies.[26] Worldwide, four OVs have been approved for clinical use and are available for cancer treatment. RIGVIR (ECHO-7), approved in Latvia (2004), Georgia (2015), and Armenia (2016), a strain from the Picornaviridae family, is a non-genetically engineered virus employed to treat melanoma.[27,28] Oncorine, approved by China's state Food and Drug Administration in 2005, is a genetically modified type 5 human adenovirus (HAdV-C5) to stimulate selective virus replication in p53-impaired cells and enhance the safety of the treatment for head and neck squamous cell carcinoma.[29] T-VEC, a modified form of herpesvirus type 1 (HSV-1) virus, was the first OV approved by the U.S. Food and Drug Administration in 2015 to treat melanoma.[30] Teserpaturev, a third-generation (triple-mutated) recombinant oncolytic herpes simplex virus type 1, was approved for treating malignant glioma in June 2021 by the Japanese Ministry of Health, Labor and Welfare.[31] While many types of viruses, including adenoviruses, HSVs, vaccinia virus, Newcastle disease virus, coxsackievirus, measles virus, Seneca Valley virus, poliovirus, parvovirus, vesicular stomatitis virus, and the Maraba virus have been studied for their oncolytic potential,[26] few such as Pexa-Vec, GL-ONC1, vvDD, VAXINIA, and CHECKVacc, are in clinical trials.[4] While OH2 (HSV2) is in a clinical trial for i.p. therapy of patients with PM from all gastrointestinal cancers, there is no successful report of oncolytic virotherapy in the clinical trial of GCPM. The preclinical results of CF17 in GCPM models are promising and inform the design of future trials and the development of oncolytic viral therapeutic strategies for peritoneal-directed therapy in GCPM patients.

One major limitation of the study is the inability to study the effects of CF17 in the presence of an active adaptive immune system in nude mice xenograft models. Immunocompetent GCPM models are a continuing challenge in preclinical immunotherapeutic investigations in GC mouse models. This is partly due to the difficulty in reproducibility of such models, the time required to develop the number of animals needed, and the high cost of their use. Moreover, CF17 is exclusively tropic for human cancer cells and is not as effective against mouse GC cells, which further compounds the challenges in interpreting the efficacy data. However, syngeneic orthotopic GCPM in immunocompetent mouse models is being considered for immune-associated studies.

In summary, CF17 demonstrates encouraging potential as a peritoneal-directed therapy against both intestinal-type and the more treatment-resistant diffuse-type GC. Intraperitoneal CF17 treatment was safe, prevented malignant ascites formation, decreased peritoneal tumor burden, and improved survival in mice with GCPM. Further translation of CF17 is required to prepare early-phase trials of peritoneal-directed therapy of GCPM patients.

MATERIALS AND METHODS

Generation of CF17

CF17 is a genomically distinct chimeric poxvirus without any gene deletion. We have previously reported the detailed method of generating CF17.[5,6] Briefly, nine strains of orthopoxvirus, including raccoonpox virus (strain designation: Herman), cowpox virus (strain Brighton, catalog# VR-302), rabbitpox virus (strain Utrecht, catalog# VR-1591), and vaccinia virus strains AS (catalog# VR-1508), Connaught Laboratories, Elstree (Lister vaccine, Catalog# VR-1549), vaccinia virus (strain IHD, catalog# VR-156), vaccinia virus strain CL (catakig#VR-1774), vaccinia virus strain Lederle-chorioallantoic (catalog #VR-118), and vaccinia virus strains Western Reserve (catalog# VR-1354) were purchased from American Type Culture Collection (ATCC, Manassas, VA). All nine strains of orthopoxvirus were used to co-infect CV-1 cells (ATCC, catalog# CCL-70) to foster

Figure 4. CF17 intraperitoneal treatment significantly decreases GCPM tumor burden

(A) Timeline shows intraperitoneal (i.p.) inoculation of SNU-16-ffluc cells (10×10^6 cells) in nude mice (black: days after tumor implantation) and treatment with CF17 or PBS (purple: days after virus treatment). Mice were treated with PBS or 3×10^5 pfu CF17 in 100 μL volume on days 7, 9, and 11 after tumor cell implantation. The same multi-dosing was repeated for a second cycle on days 35, 37, and 39. (B) Bioluminescence imaging were performed weekly and the difference of tumor burden is shown at time points in the figure. (C). Bioluminescence photons/s of ROI of peritoneal tumor burden post-treatment with PBS control (blue) or CF17 (orange) are shown as mean ± SEM (n = 8). (D) Individual tumor burden change of post-treatment with PBS control (left) or CF17 (right) are shown in the figure. *$p < 0.05$, in comparison with PBS control group. (E) IHC staining of H&E and anti-vaccinia virus in peritoneal tumors after treatment CF17 for 4 weeks (D35).

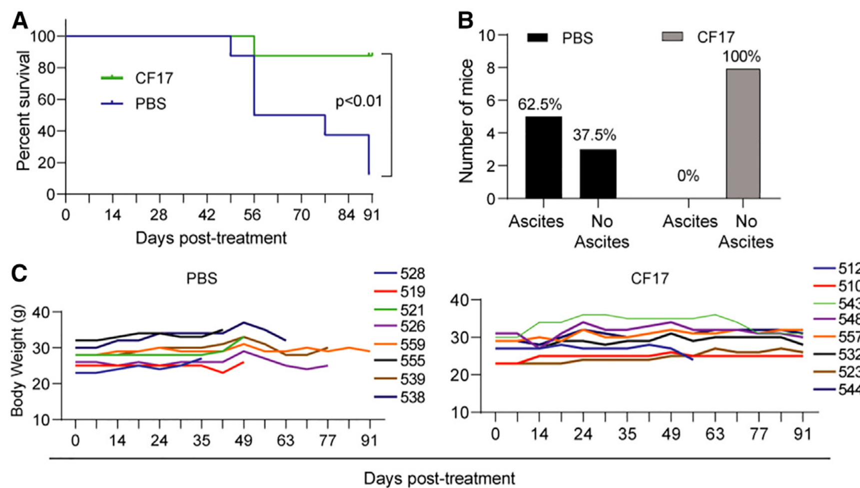

Figure 5. CF17 intraperitoneal treatment significantly prolongs survival and prevents malignant ascites formation

(A) Kaplan-Meier survival analysis of the survival of mice with SNU16-ffluc peritoneal tumor (10×10^6 cells i.p.) treated with PBS control or CF17. $^{**}p < 0.01$. (B) Bar graph showing prevention of malignant ascites formation following CF17 treatment in an SNU-16-ffluc GCPM mouse model. (C) Body weight change of GCPM mouse after treated with PBS control or CF17.

chimerization. Then, viral plaques were chosen and purified through three rounds of plaque purification to obtain 100 unique clonally purified chimeric orthopoxviruses. High-throughput screening was used to compare the cytotoxic efficacy against the NCI-60 panel. CF17 was one of the clones exhibiting the highest cell-killing ability.

Cell culture and cell lines

Human GC cell lines, AGS (ATCC, catalog# CRL-1739), KATO III (ATCC, catalog# HTB-103), MKN-74 (ACCEGEN, catalog# ABC-TC0689), MKN-45 (ACCEGEN, catalog# ABC-TC0687), SNU-1 (ATCC, catalog# CRL-5971), SNU-16 (ATCC, catalog#CRL-5974), and African green monkey kidney fibroblast CV-1 (ATCC, catalog# CCL-70) were purchased from the ATCC or ACCEGEN (Fairfield, NJ). KATO III, MKN-74, MKN-45, SNU-1, and SNU-16 were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (AA) solution. AGS and CV-1 were cultured in DMEM supplemented with 10% FBS and 1% AA solution. All the media and supplements were purchased from Corning (Corning, NY). The cells were maintained in a humidified incubator at 37°C and 5% CO_2. For all adherent cell lines, when adherent cells reached 80% confluency, they were passaged using 0.05% trypsin and EDTA solution (Corning, catalog# 25-051-Cl). Media were changed every 2–3 days.

Virus infection and proliferation assay

Six GC cell lines were plated separately in six-well plates at 5×10^5 cells/well and incubated overnight. The next day, cells were counted and infected with the virus. Media from the wells was removed, and virus diluted in the medium containing 2.5% FBS was added to each well in a total volume of 0.5 mL such that the ratio of cells to the virus was 100:1, i.e., an MOI of 0.01 plaque-forming units (pfu)/cell. Cells were incubated at 37°C for 1 h, followed by aspiration of inoculum and addition of 2 mL media containing 10% FBS to each well. The plates were placed back in the incubator. Cell lysates were collected by scraping at the time points shown in Figure 1, and virus titers in the lysates were determined by the standard plaque assay technique.

Cytotoxicity assay

Cells were seeded at 3,000 cells/well in 96-well plates with 100 μL/well of medium supplemented with 10% FBS plus 1% AA solution and incubated overnight. Virus was thawed on ice and sonicated for 1 min, and appropriate MOIs (0.01, 0.1, 1.0, and 10.0) were calculated and prepared for infection in a medium with 2.5% FBS for 20 μL/well. Cell viability was measured in triplicate every 24 h for 8 days using MTS cell proliferation assay with CellTiter 96 Aqueous One solution (Promega, Madison, WI) according to manufacturer protocol by using a spectrophotometer (Tecan Spark 10M, Mannedorf, Switzerland) at 490 nm.

Establishment of KATO III-ffluc, MKN-74-ffluc, and SNU-16-ffluc cell lines

KATO III, MKN-74, and SNU-16 cells were modified to stably encode firefly luciferase using lentiviral transduction to quantitate tumor volume and dissemination *in vivo* using non-invasive optical imaging (Xenogen). Briefly, KATO III, MKN-74, or SNU-16 cells were incubated with polybrene (4 mg/mL, Sigma) in RPMI-1640 (Lonza catalog# BE12-702F) containing 10% FBS (Hyclone defined FBS, Cytiva catalog# SH30070.03) and 1X AA solution (Gibco catalog#15240062), and infected with lentivirus carrying ffluc cDNA under the control of the EF1α promoter. Expression of ffluc in KATO III, MKN-74, and SNU-16 cells was confirmed, and single-cell subcloning was performed by using the limiting dilution method.[32]

Xenograft mouse models of GC cell lines KATO III, MKN-74, and SNU-16

Animal studies were performed under the City of Hope Institutional Animal Care and Use Committee-approved protocol (IACUC #15003). Six-week-old Hsd:Athymic Nude-Foxn1nu female and male mice (Envigo, Indianapolis, IN) were purchased and acclimatized for 2 weeks. To allow for imaging of peritoneal tumor burden and evaluate the mouse model among three cell lines, the peritoneal xenograft mouse models were generated by peritoneal injection of KATO III-ffluc (10×10^6 cells), MKN-74-ffluc (5×10^6 cells or 10×10^6 cells), or SNU-16-ffluc cells (10×10^6 cells). Injection of cells in PBS in a total volume of 100 μL into the peritoneal cavity was performed for each mouse.

Bioluminescence imaging

All animals were imaged with bioluminescence for luciferase activity in the peritoneum to identify peritoneal tumor implantations and

growth after intraperitoneal KATO III-ffluc, MKN-74-ffluc, or SNU-16-ffluc cells, and the tumor burden was quantified once a week after inoculation. D-luciferin solution was prepared by dissolving 1 g of VISbrite D-Luciferin Potassium Salt Bioluminescent Substrate (PerkinElmer, catalog#122799–5, Waltham, MA) in 35 mL PBS at 28.5 mg/mL concentration. Intraperitoneal delivery of VISbrite D-Luciferin Potassium Salt (200μL/mouse) was performed in all groups, and the mice were imaged using Lago X optical imaging system (Spectral Instruments Imaging, Tucson, AZ). Bioluminescence imaging was analyzed using Aura64 software and presented as photons/second for regions of interest [ROIs]).

Multi-dose i.p. delivery of CF17 in the treatment of GCPM *in vivo*

Seven days after inoculation of 1×10^7 SNU-16-ffluc cells into the peritoneal cavity, mice were divided into two treatment groups according to the average tumor burden: i.p. CF17 treatment group and i.p. PBS control group. Mice in the virus group were treated with 3×10^5 pfu CF17 in 100 μL volume on days 7, 9, and 11 after tumor cell implantation. The same multi-dosing was repeated for a second cycle on days 35, 37, and 39 with the same. The mice in the control group received 100 μL PBS on the same dosing schedule. Starting day 7 after treatments, the mice were evaluated every 7 days to verify i.p. tumor burden using bioluminescence imaging for luciferase activity. Mice were observed and evaluated for tumor burden (luciferase imaging of peritoneal tumor and weight of tumor at death), body weight, jaundice, peritoneal ascites, cachexia, and survival. Animals were euthanized if they demonstrated >20% body weight loss, jaundice, peritoneal ascites, cachexia, or inability to groom and eat, as per institutional guidelines.

Immunohistochemistry

Tumors were harvested at day 35 after tumor implantation (day 28 post virus injection), fixed with 10% formalin, embedded in paraffin, and cut into 5-μm-thick sections. Sections were stained with H&E and anti-vaccinia virus antibody (Abcam, catalog# ab35219, Cambridge, UK). Images were obtained using Ventana Image Viewer.

Statistical analysis

Assay results are expressed as means ± SEM. Statistical analyses comparing the two groups were performed using paired or unpaired Student's t test. All p values were two-sided, and p values ≤ 0.05 were deemed significant. The probability of survival was determined using the Kaplan-Meier survival curve of the log rank Mantel-Cox test. GraphPad Prism 8 (GraphPad Software, La Jolla, CA) was used to calculate statistical values.

DATA AND CODE AVAILABILITY

All data have been presented in the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2023.100734.

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AUTHOR CONTRIBUTIONS

A.Y., Z.Z., and Y.W. conceived and designed the experiments. A.Y. and Z.Z. performed the experiments. S.C., S.K., J.L., A.K.P., and H.V. helped to perform the experiments. A.Y., Z.Z., Y.F., and Y.W. analyzed and interpreted data. Y.W. and Y.F. secured funding. A.Y., Z.Z., and Y.W. drafted the manuscript. All authors edited and approved the final manuscript.

DECLARATION OF INTERESTS

Y.W. is a member of the scientific advisory board of Imugene LTD; Y.F. owns the patent for CF33-Ovs; and Y.F., A.K.P., and S.C. own the patent to CF33-CD19t, both licensed to Imugene LTD. Y.F. owns the patent for CF17.

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