Novel Chimeric Poxvirus CF17 Improves Survival in a Murine Model of Intraperitoneal Ovarian Cancer Metastasis

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Despite improvements in surgical techniques and chemotherapy, ovarian cancer remains the most lethal gynecologic cancer. Thus, there is an urgent need for more effective therapeutics, particularly for chemo-resistant peritoneal ovarian cancer metastases. Oncolytic virotherapy represents an innovative treatment paradigm; however, for oncolytic viruses tested from the last generation of genetically engineered viruses, the therapeutic benefits have been modest. To overcome these limitations, we generated a chimeric poxvirus, CF17, through the chimerization of nine species of orthopoxviruses. Compared with its parental viruses, CF17 has demonstrated superior oncolytic characteristics. Here, we report the oncolytic potential of CF17 in ovarian cancer. Replication of CF17 and its resulting cytotoxicity were observed at multiplicities of infection (MOIs) as low as 0.001 in human and mouse cancer cell lines *in vitro*. Furthermore, CF17 exerted potent antitumor effects in a syngeneic mouse model of ovarian cancer at doses as low as 6×10^6 plaque-forming units. Together, these data merit further investigation of the potential use of this novel chimeric poxvirus as an effective treatment for aggressive intraperitoneal ovarian cancer.

INTRODUCTION

Despite improvements to standard-of-care de-bulking surgery and chemotherapy, ovarian cancer remains the most lethal gynecologic malignancy in the world,[1] with dismal survival rates for older patients and those with advanced-stage disease[2] who have few therapeutic options.[3] Consequently, around 140,000 patients with ovarian cancer (2/3 of those diagnosed) die annually,[4–6] mainly because of intraperitoneal (i.p.) organ failure. Therefore, there is a dire need to generate safer and more effective i.p. therapeutic approaches. Toward this end, many ongoing clinical trials are testing novel approaches, including the use of oncolytic viruses (OVs), to treat ovarian cancer, especially in patients who have developed resistance to standard-of-care treatments.[7–12]

OVs selectively kill tumor cells and have shown promise in clinical trials.[13,14] Importantly, they cause cancer cell lysis, irrespective of chemoresistance,[15] exposing novel tumor antigens to be recognized by the immune system.[16] To date, several OVs have advanced to phase I/II clinical trials for ovarian cancer patients and have demonstrated safety, but their antitumor efficacy has been modest so far.[14]

We have previously generated a promising new chimeric poxvirus, CF17, through the chimerization of nine species of orthopoxviruses, including multiple strains of vaccinia virus (VACV). CF17 has been shown to have superior oncolytic characteristics and enhanced antitumor activity compared with its parental viruses.[17–19] In the current study, we evaluated the cytotoxicity of CF17 *in vitro* using the murine ovarian cancer cell line ID8 and also human high-grade serous ovarian cancer cell lines OVCAR8 and SKOV3, the most prevalent ovarian tumor type (>50% of ovarian malignancies).[20–22] Also, we performed *in vivo* studies using a syngeneic mouse model to evaluate the preclinical utility of CF17 in the context of i.p. ovarian cancer metastases. Our results show that i.p. administered CF17 targets and penetrates sites of tumor metastases, where it replicates within ovarian cancer cells, causing oncolysis. Overall, this study demonstrates the potential of CF17 as a novel therapeutic agent for stage III ovarian cancer patients.

RESULTS

CF17 Infects and Replicates in Human and Mouse Ovarian Cancer Cell Lines *In Vitro*

We infected murine (ID8) and human (OVCAR8, SKOV3) ovarian cancer cells with CF17 at various multiplicities of infection (MOIs). We performed an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay to analyze tumor cell viability 3 days post-CF17 infection. For all cell types tested, non-infected cells were used as negative control. We observed that infection with CF17 eliminated these cancer cells at an MOI of 10
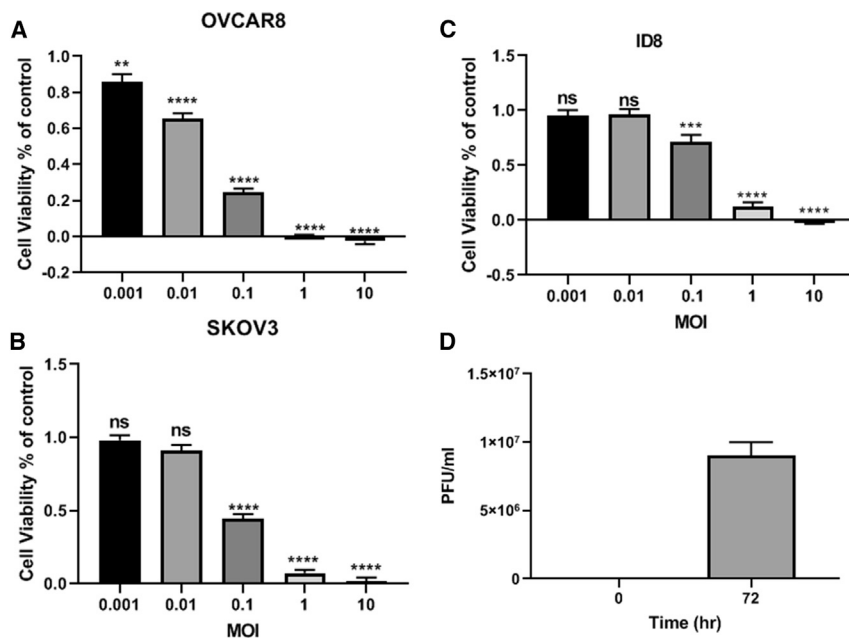
Figure 1. CF17 Infects, Replicates in, and Kills Ovarian Cancer Cells *In Vitro*

(A–C) OVCAR8 (A), SKOV3 (B), and ID8 (C) ovarian cancer cells were infected with CF17 at the indicated MOIs, and cell survival, relative to non-infected control cells, was determined 72 h post-infection. (D) OVCAR8 cells infected with CF17 at an MOI of 0.03 were harvested 72 h post-infection, and virus titers in the harvested cell lysates were determined using a standard plaque assay. Data are shown as mean ± SEM for at least two repeated experiments. **p < 0.01, ***p < 0.001, ****p < 0.0001, as determined by one-way ANOVA comparing infected versus non-infected cells. ns, no significant difference.

(Figures 1A–1C) and significantly reduced the viability of all cell lines even at an MOI of 0.1. These results demonstrate that CF17 can infect, replicate in, and kill ovarian cancer cell lines *in vitro*.

CF17 Demonstrates Significant Antitumor Activity in a Syngeneic Mouse Model of Ovarian Cancer

Based on its promising potency *in vitro*, we further tested the *in vivo* efficacy of CF17 using an institutionally approved and supported murine model of i.p. ovarian carcinomatosis. This model was generated by i.p. injection of immunocompetent C57BL/6J mice with firefly luciferase-expressing ID8 mouse ovarian cancer cells (5×10^6 cells/mouse). On days 1 and 7 post-tumor implantations, mice received i.p. injections with CF17 (6×10^6 plaque-forming units [PFU]/injection) or PBS control and were observed for 23 weeks (Figure 2A). We observed that CF17 treatment reduced the luciferase signal over an 8-week imaging period following tumor implantation (Figures 2B and 2C). By week 8, CF17 significantly suppressed tumor burden, as assessed by the average luciferase signals detected for each group (****p < 0.0001; Figure 2C).

CF17 Treatment Improves Survival and Reduces Ascites in a Syngeneic Mouse Model of Ovarian Cancer

We also tested the toxicity, if any, of CF17, in addition to its potency, using this ID8 model of carcinomatosis. By week 9, some vehicle-treated animals were removed from the study because these had reached a humane endpoint (Figure 3A). By week 11, all vehicle-treated mice were euthanized. In contrast, for the 23-week duration of the study, only one CF17-treated mouse was euthanized (at week 18; Figure 3A).

Furthermore, we did not observe any overt signs of toxicity in the CF17-treated mice, and all continued gaining weight at a rate similar to that of mice without tumors. Meanwhile, vehicle-treated mice exhibited an abnormal increase in body weight because of ascites (Figure 3B). Collec-

tively, these data suggest that CF17 can be used safely and effectively as an antitumor treatment in the current mouse model. Further studies are warranted toward translation to clinical trials.

DISCUSSION

Studies have shown that oncolytic virotherapy is clinically safe and non-toxic in different cancer patients.[1,14,23] Despite their high safety profiles, the last generation of OVs has shown limited efficacy.[23,24] Oncolytic poxviruses have demonstrated encouraging results in multiple preclinical tumor models, as well as some clinical trials for the treatment of various cancers.[25]

For ovarian cancer, this lack of efficacy can be attributed to different factors. First, ovarian tumors are highly heterogeneous; thus, effective treatments must be designed to eliminate the entire spectrum of tumor subpopulations. Second, even within the same subpopulation, several microenvironmental factors, such as various cytokines, chemokines, and angiogenic factors, can promote peritoneal dissemination,[26] including ovarian cancer metastasis, and therefore may influence the efficacy of OVs.[27] Hence to accurately evaluate the clinical potential of novel OVs, it is necessary to administer them i.p. in immunocompetent preclinical models. This allows the evaluation of both the oncolytic effect from viral replication and a potential secondary immune response.

Recently, Ricordel et al.[28] reported the generation of a chimeric poxvirus through the recombination of four different VACV strains: WR, Wyeth, MVA, and Copenhagen. The authors showed that the chimeric virus had greater cancer cell-killing capacity and tumor selectivity compared with the parental VACV strains *in vitro*. Similarly, we previously reported the construction of a chimeric poxvirus, CF17, which was more efficient at killing cancer cells than its parental poxviruses, including the WR strain.[17,18] In this study, the pronounced efficacy of CF17 against human and mouse ovarian cancer cell lines with distinct genotypes, along with the absence of obvious toxicity, lends support to the idea that it can be effective against different ovarian tumor subpopulations. Furthermore, we observed significant antitumor effects and improved survival of mice after
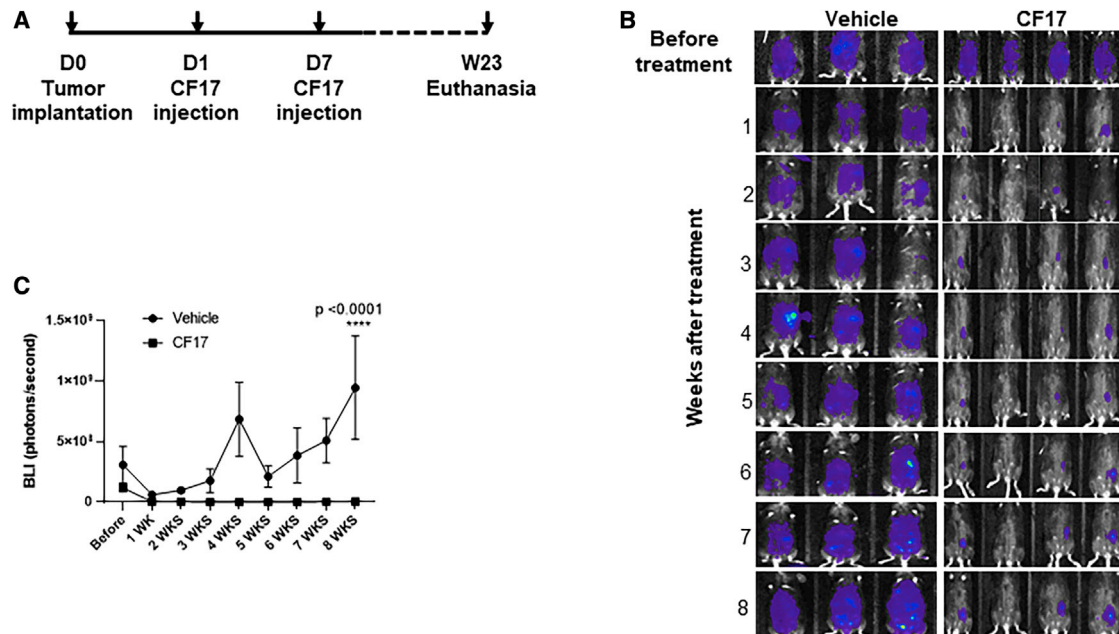
Figure 2. CF17 Shows Antitumor Efficacy in an ID8 Syngeneic Murine Model of Ovarian Cancer

(A) Experimental timeline for *in vivo* studies. Immunocompetent C57BL/6J mice received i.p. injections of 5×10^6 firefly luciferase-labeled ID8 cells. One day later, mice received i.p. injections of PBS (vehicle, n = 3) or 6×10^6 PFUs of CF17 (n = 4). (B) Quantification of luciferase expression. (C) ID8 ovarian tumors were monitored weekly by bioluminescence imaging (BLI) for the first 8 weeks following tumor implantation. Error bars indicate ± SEM.

only two injections of 6×10^6 PFUs, at a dose far lower than that commonly reported for other oncolytic poxviruses.[29–32]

In summary, CF17 is safe and exerts antitumor effects against human ovarian cancer *in vitro* and murine ovarian cancer *in vivo*. Further studies are needed to determine whether CF17 can be used either as a monotherapy or in combination with compatible therapeutics. The data presented here warrant further testing of CF17 for clinical use.

MATERIALS AND METHODS
Generation of CF17 nChimeric Virus
To generate CF17, we used nine strains of orthopoxvirus in co-infecting CV-1 cells and fostering chimerization. These included raccoon-pox virus strain Herman, cowpox virus strain Brighton, rabbitpox virus strain Utrecht, and vaccinia virus strains AS, Connaught Laboratories, Elstree, IHD, Lederle-Chorioallantoic, and Western Reserve, all purchased from ATCC. Following the chimerization, 100 individual plaques were chosen and then purified through three rounds of plaque purification to obtain 100 clonally purified chimeric orthopoxviruses. High-throughput screening was used to compare the cytotoxic efficacy against the NCI-60 panel. CF17 was selected as a chimeric isolate, which demonstrated superior cell killing in the NCI-60 panel when compared with all parental viruses.

Cell Culture
The OVCAR8 human ovarian cancer cell line was generously provided by Dr. Carlotta Glackin (City of Hope). The SKOV3 human ovarian cancer cell line was obtained from ATCC. The ID8 murine ovarian line was obtained from Dr. Katherine Roby (University of Kansas) and then transduced to express firefly luciferase. Ovarian cancer cell lines were cultured in RPMI basal media with 10% fetal bovine serum (Gemini Bio), 1% L-glutamine (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). Cells were maintained at 37°C in a humidified incubator (Thermo Electron Corporation) containing 6% CO_2 and then were harvested. For all cell lines, when cells reached 80% confluency, they were passaged using 0.25% trypsin and EDTA solution (Invitrogen); media were changed every 2–3 days.

Virus Proliferation and Cytotoxicity Assays
To determine the ability of viruses to replicate in cultured cells, we infected cells at an MOI of 0.03, and virus titers in the lysates were determined using a standard plaque assay, as described previously.[18] A CellTiter 96 AQueous colorimetric assay (Promega) was used to measure cell survival after viral infection. In brief, cells were infected in 96-well plates at various MOIs (0.001–10), the substrate was added 72 h post-infection, and absorbance was measured at 490 nm using a plate reader (Tecan Spark). The survival of CF17-treated cells was calculated relative to that of non-infected control cells.

In Vivo Efficacy of CF17 in an Orthotopic Ovarian Cancer Model
All animal experiments were conducted in accordance with NIH *Guidelines for the Care and Use of Laboratory Animals* and City of Hope regulations after review and approval by the City of Hope Institutional Animal Care and Use Committee (protocol #18002).
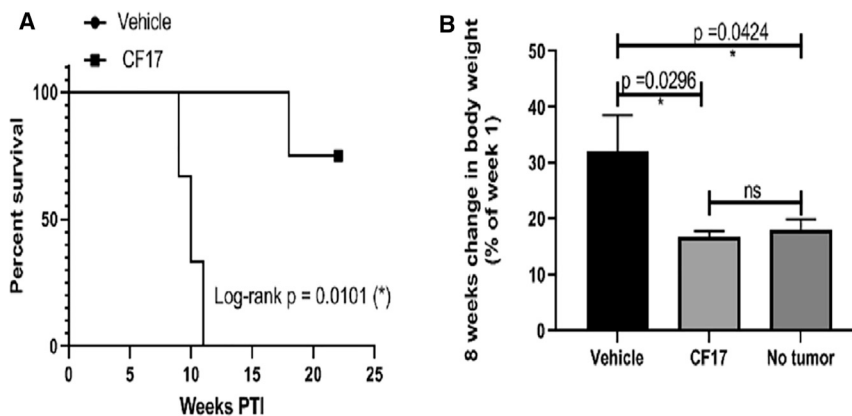
Figure 3. CF17 Improves Survival and Reduces Ascites in an ID8 Syngeneic Murine Model of Ovarian Cancer

(A) Survival curves of ID8 tumor-bearing mice treated with PBS (vehicle) or CF17. (B) All mice were monitored daily for the development of peritoneal ascites and weighed weekly, with results expressed as the change in body weight at 8 weeks post-tumor implantation. Data are shown as mean ± SEM.

Female C57BL/6J mice (6–8 weeks old; The Jackson Laboratory) were inoculated with 5×10^6 firefly luciferase-labeled ID8 cells via i.p. injection. At 1 and 7 days post-inoculation, mice were administered i.p. 6×10^6 PFUs of CF17 (n = 4) or vehicle (n = 3). Bioluminescence imaging (BLI) was performed once a week after tumor implantation using the Lago imaging system (Spectral Instruments Imaging). Prior to imaging, mice were injected i.p. with D-luciferin (Xenogen; 150 μL/mouse). Anesthesia was induced with 2% isoflurane (Abbott Laboratories) in a transparent airtight box for 5–7 min before the mice were moved to the light-tight chamber of the charge-coupled device (CCD) camera in the imaging position. The images were analyzed using Aura software version 2.2.0 (Spectral Instruments Imaging).

Statistical Analysis

Data are presented as mean ± SEM unless otherwise stated. Statistical significance for tumor flux at each time point between the groups was examined using one-way ANOVA (*p < 0.05 was deemed to be significant). Survival analysis was carried out using Kaplan-Meier curves. The survival distributions of vehicle- and CF17-treated mice were compared using log-rank tests.

AUTHOR CONTRIBUTIONS

Conceptualization, K.S.A., M.H., T.H.D., N.G.C.; Methodology, M.H., Y.C.; Formal Analysis, M.H., M.L.; Investigation, M.H., Y.C., J.L.; Writing – Original Draft, M.H.; Writing – Review & Editing, M.H., K.S.A., Y.F., R.M.; Visualization: M.H., Y.C., K.S.A.; Funding Acquisition, M.H., Y.C., K.S.A.; Resources, Y.F., K.S.A.; Supervision, K.S.A., Y.F.

CONFLICTS OF INTEREST

K.S.A. is CSO with an advisory role and stock ownership in TheraBiologics, Inc. All other authors declare no competing interests.

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REFERENCES

1. Cannistra, S.A. (2004). Cancer of the ovary. N. Engl. J. Med. *351*, 2519–2529.

2. Lowe, K.A., Chia, V.M., Taylor, A., O'Malley, C., Kelsh, M., Mohamed, M., Mowat, F.S., and Goff, B. (2013). An international assessment of ovarian cancer incidence and mortality. Gynecol. Oncol. *130*, 107–114.

3. English, D.P., Menderes, G., Black, J., Schwab, C.L., and Santin, A.D. (2016). Molecular diagnosis and molecular profiling to detect treatment-resistant ovarian cancer. Expert Rev. Mol. Diagn. *16*, 769–782.

4. Siegel, R., Naishadham, D., and Jemal, A. (2013). Cancer statistics, 2013. CA Cancer J. Clin. *63*, 11–30.

5. Znaor, A., van den Hurk, C., Primic-Zakelj, M., Agius, D., Coza, D., Demetriou, A., Dimitrova, N., Eser, S., Karakilinc, H., Zivkovic, S., et al. (2013). Cancer incidence and mortality patterns in South Eastern Europe in the last decade: gaps persist compared with the rest of Europe. Eur. J. Cancer *49*, 1683–1691.

6. Bray, F., Ren, J.S., Masuyer, E., and Ferlay, J. (2013). Global estimates of cancer prevalence for 27 sites in the adult population in 2008. Int. J. Cancer *132*, 1133–1145.

7. Buller, R.E., Runnebaum, I.B., Karlan, B.Y., Horowitz, J.A., Shahin, M., Buekers, T., Petrauskas, S., Kreienberg, R., Slamon, D., and Pegram, M. (2002). A phase I/II trial of rAd/p53 (SCH 58500) gene replacement in recurrent ovarian cancer. Cancer Gene Ther. *9*, 553–566.

8. Aghi, M., and Martuza, R.L. (2005). Oncolytic viral therapies—the clinical experience. Oncogene *24*, 7802–7816.

9. Vasey, P.A., Shulman, L.N., Campos, S., Davis, J., Gore, M., Johnston, S., Kirn, D.H., O'Neill, V., Siddiqui, N., Seiden, M.V., and Kaye, S.B. (2002). Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. J. Clin. Oncol. *20*, 1562–1569.

10. Bourke, M.G., Salwa, S., Harrington, K.J., Kucharczyk, M.J., Forde, P.F., de Kruijf, M., Soden, D., Tangney, M., Collins, J.K., and O'Sullivan, G.C. (2011). The emerging role of viruses in the treatment of solid tumours. Cancer Treat. Rev. *37*, 618–632.

11. Hartkopf, A.D., Fehm, T., Wallwiener, D., and Lauer, U. (2011). Oncolytic virotherapy of gynecologic malignancies. Gynecol. Oncol. *120*, 302–310.

12. Liu, T.C., Galanis, E., and Kirn, D. (2007). Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. Nat. Clin. Pract. Oncol. *4*, 101–117.

13. Kim, J., Hall, R.R., Lesniak, M.S., and Ahmed, A.U. (2015). Stem Cell-Based Cell Carrier for Targeted Oncolytic Virotherapy: Translational Opportunity and Open Questions. Viruses *7*, 6200–6217.

14. Li, S., Tong, J., Rahman, M.M., Shepherd, T.G., and McFadden, G. (2012). Oncolytic virotherapy for ovarian cancer. Oncolytic Virother. *1*, 1–21.

15. Ding, J. (2014). Oncolytic virus as a cancer stem cell killer: progress and challenges. Stem Cell Investig. *1*, 22.

16. Russell, S.J., and Barber, G.N. (2018). Oncolytic Viruses as Antigen-Agnostic Cancer Vaccines. Cancer Cell *33*, 599–605.

17. O'Leary, M.P., Choi, A.H., Kim, S.I., Chaurasiya, S., Lu, J., Park, A.K., Woo, Y., Warner, S.G., Fong, Y., and Chen, N.G. (2018). Novel oncolytic chimeric orthopoxvirus causes regression of pancreatic cancer xenografts and exhibits abscopal effect at a single low dose. J. Transl. Med. *16*, 110.

18. O'Leary, M.P., Warner, S.G., Kim, S.I., Chaurasiya, S., Lu, J., Choi, A.H., Park, A.K., Woo, Y., Fong, Y., and Chen, N.G. (2018). A Novel Oncolytic Chimeric Orthopoxvirus Encoding Luciferase Enables Real-Time View of Colorectal Cancer Cell Infection. Mol. Ther. Oncolytics *9*, 13–21.

19. Chaurasiya, S., Chen, N.G., Lu, J., Martin, N., Shen, Y., Kim, S.I., Warner, S.G., Woo, Y., and Fong, Y. (2020). A chimeric poxvirus with J2R (thymidine kinase) deletion shows safety and anti-tumor activity in lung cancer models. Cancer Gene Ther. *27*, 125–135.

20. Koshiyama, M., Matsumura, N., and Konishi, I. (2017). Subtypes of Ovarian Cancer and Ovarian Cancer Screening. Diagnostics (Basel) *7*, 12.

21. Domcke, S., Sinha, R., Levine, D.A., Sander, C., and Schultz, N. (2013). Evaluating cell lines as tumour models by comparison of genomic profiles. Nat. Commun. *4*, 2126.

22. Bacchetti, S., and Graham, F. (1993). Inhibition of cell-proliferation by an adenovirus vector expressing the human wild type-p53 protein. Int. J. Oncol. *3*, 781–788.

23. Galanis, E. (2010). Therapeutic potential of oncolytic measles virus: promises and challenges. Clin. Pharmacol. Ther. *88*, 620–625.

24. Zheng, M., Huang, J., Tong, A., and Yang, H. (2019). Oncolytic Viruses for Cancer Therapy: Barriers and Recent Advances. Mol. Ther. Oncolytics *15*, 234–247.

25. Torres-Domínguez, L.E., and McFadden, G. (2019). Poxvirus oncolytic virotherapy. Expert Opin. Biol. Ther. *19*, 561–573.

26. Sakamoto, S., Kagawa, S., Kuwada, K., Ito, A., Kajioka, H., Kakiuchi, Y., Watanabe, M., Kagawa, T., Yoshida, R., Kikuchi, S., et al. (2019). Intraperitoneal cancer-immune microenvironment promotes peritoneal dissemination of gastric cancer. OncoImmunology *8*, e1671760.

27. Ilkow, C.S., Marguerie, M., Batenchuk, C., Mayer, J., Ben Neriah, D., Cousineau, S., Falls, T., Jennings, V.A., Boileau, M., Bellamy, D., et al. (2015). Reciprocal cellular cross-talk within the tumor microenvironment promotes oncolytic virus activity. Nat. Med. *21*, 530–536.

28. Ricordel, M., Foloppe, J., Antoine, D., Findeli, A., Kempf, J., Cordier, P., Gerbaud, A., Grellier, B., Lusky, M., Quemeneur, E., and Erbs, P. (2018). Vaccinia Virus Shuffling: deVV5, a Novel Chimeric Poxvirus with Improved Oncolytic Potency. Cancers (Basel) *10*, 231.

29. Pugalenthi, A., Mojica, K., Ady, J.W., Johnsen, C., Love, D., Chen, N.G., Aguilar, R.J., Szalay, A.A., and Fong, Y. (2015). Recombinant vaccinia virus GLV-1h68 is a promising oncolytic vector in the treatment of cholangiocarcinoma. Cancer Gene Ther. *22*, 591–596.

30. Brun, J., Mahoney, D.J., Le Boeuf, F., Lefebvre, C., Sanaei, C.A., Falls, T., Mccart, J.A., and Stojdl, D.F. (2013). Oncolytic Vaccinia virus safely and effectively treats skin tumors in mouse models of xeroderma pigmentosum. Int. J. Cancer *132*, 726–731.

31. Guse, K., Sloniecka, M., Diaconu, I., Ottolino-Perry, K., Tang, N., Ng, C., Le Boeuf, F., Bell, J.C., McCart, J.A., Ristimäki, A., et al. (2010). Antiangiogenic arming of an oncolytic vaccinia virus enhances antitumor efficacy in renal cell cancer models. J. Virol. *84*, 856–866.

32. Yu, Z., Li, S., Brader, P., Chen, N., Yu, Y.A., Zhang, Q., Szalay, A.A., Fong, Y., and Wong, R.J. (2009). Oncolytic vaccinia therapy of squamous cell carcinoma. Mol. Cancer *8*, 45.