DOCTORAL THESIS

Imaging and Antitumoral Effect of a Cyclo-oxygenase 2-
specific Replicative Adenovirus for Small Metastatic
Gastric Cancer Lesions.

（制限増殖型アデノウイルスによる
胃癌微小転移巣の可視化と抗腫瘍効果）

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Abstract. Background: Long-term outcomes of patients with peritoneal dissemination of gastric cancer remain unsatisfactory despite advances in treatment modalities. Internal luminescence conditionally replicative adenovirus (CRAd) presents a novel approach for cancer treatment and imaging. Materials and Methods: 3CL is a modified cyclo-oxygenase-2 (COX2) promoter-driven CRAd which contains the luciferase expression gene for bioluminescence imaging. The visualizing and therapeutic effect of 3CL was evaluated in a mouse model of peritoneal dissemination. Results: Intraperitoneal injection of 3CL achieved the shrinkage and reduction of lesions of peritoneal dissemination. Six model mice treated with 3CL had a significantly longer mean survival time than 6 mock-treated mice (85.7 versus 34.3 days, p=0.0005). By whole-body bioluminescent imaging, the sensitivity and specificity of peritoneal dissemination detection through macroscopic inspection were 58.1% and 83.2%, respectively, whereas 3CL viral imaging modality yielded corresponding values of 78.8% and 99.3%. Peritoneal lesions detected by imaging histologically contained cancer cells and necrotic tissue, which originated from viral oncolytic effects. Conclusion: Cox2 CRAds with S/3 chimeric-fiber modification, therefore, appear to be a promising imaging and therapeutic tools for peritoneal dissemination of gastric cancer.

The prognosis of patients with peritoneal dissemination (PD) of gastric cancer (GC) is dismal (1). Several modalities have been tried to treat these condition, such as systemic chemotherapy, intraperitoneal chemotherapy, hyperthermia and aggressive surgery. However, these therapeutic strategies did not show any satisfactory clinical outcome (2, 3). Recently, chemotherapy for gastric cancer has progressed and there have been some trials to show survival benefit in patients whose peritoneal lesions were controlled by intravenous/ intraperitoneal chemotherapy and for whom R0 resection was performed (4-6). These therapies actually improved the treatment results of PD. The 1-year survival rate was 77.3-78% and the median survival time (MST) was 20.3-21.3 months. To further improve prognosis, a more effective therapeutic strategy is required for PD of GC.

Although accurate preoperative diagnosis of PD is crucial, current conventional imaging modalities are inadequate for detecting small lesions (7). Preoperative exploratory laparoscopy has recently been widely applied for the inspection of peritoneal lesions; (8) however, its accuracy is limited because of difficulties in identifying small lesions by visual inspection (9). Indeed, several studies have revealed unidentified peritoneal lesions in 7-13% of patients with serosa-invading GC after staging laparotomy led to an initial diagnosis of no peritoneal dissemination (10-12). A method for specifically labeling and visualizing disseminated lesions could, therefore, improve the accuracy of preoperative staging laparoscopy.

Conditionally replicative adenoviruses (CRAds) with fluorescent reporter genes have been used to develop such an imaging modality (13-15). These viruses are designed to infect both tumor cells and normal cells, but selectively replicate only in the former, which are consequently specifically labeled and visualized with the help of a
fluorescent reporter gene. However, these CRAdS express fluorescent reporter genes in a replication-independent manner, and there is the potential for false-positive results due to viral infection of normal tissue. To overcome these limitations, we developed a CRAd with a fluorescent reporter gene under the control of the natural adenoviral major late promoter (MLP) in the E3 region. Adenoviral MLP-driven expression of enhanced green fluorescent protein (EGFP) accurately reflects viral replication both in vitro and in vivo (16). Moreover, we have previously shown that the cyclo-oxygenase 2 (COX2) promoter is highly selective for GC cells (17-20), and demonstrated that adenoviral vectors with the 5/3 chimeric fiber (an Ad5 tail and shaft combined with the Ad3 knob) exhibit improved infectivity of cancer cells (18-20). The present study evaluated the efficacy of this novel adenovirus in visualizing small or microscopic metastatic GC lesions during laparotomy in a mouse model of PD of GC.

Materials and Methods

Cell lines and cell culture. The MKN-1, MKN-45, and MKN-74 gastric cancer cell lines (Human Science Research Resources Bank (HSRRB) JCRB0252, JCRB0254, and JCRB0255, respectively; Osaka, Japan), the A549 COX2-positive lung cancer cell line (HSRRB JCRB0076), and the BT-474 COX2-negative breast cancer cell line (THB-20; American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C with 5% CO2 under humidified conditions.

Establishment of gastric cancer cell lines expressing Discosoma strirata Red Fluorescent Protein (DsRed2). For the easy evaluation and detection of small peritoneal lesions by using fluorescent imaging, establishment of GC cell lines was firstly considered. The DsRed2 expression plasmid pDsRed2/Zeo was constructed from pcDNA3.1/Zeo(+) (Invitrogen Japan K.K., Shibaura, Tokyo, Japan) and pDsRed2 (Clontech, Mountain View, CA, USA). pcDNA3.1/Zeo(+) and pDsRed2 were cleaved with EcoRI and BamHI, and 4,993 and 716 base-pair fragments were ligated to create pDsRed2/Zeo. Five hundred nanograms of pDsRed2/Zeo was transfected into MKN-1, MKN-45 and MKN-74 cells in 24-well plates (2.0x10^4 cells/well) using Lipofectamine LTX (Invitrogen Japan, Tokyo, Japan) according to the manufacturer’s instructions.

Generation of shuttle plasmids for E3 modification. The construction of pShuttle-AE3-ADP-KanF has been described previously (16). pGL3-Basic (Promega, Madison, WI, USA) was cleaved with NheI and SalI, and a 1,989-base-pair (bp) fragment of the firefly luciferase (luc) gene was cloned into the XbaI–SaiI site of the shuttle plasmid to obtain pShuttle-ΔE3-ADP-Lac.

Adenoviral vector construction. Five adenoviral vectors were used in the present study. SW was an unmodified serotype-5 adenovirus. 5AEI (Ad5 ΔE1 CMV Lac; a non-replicative control virus containing the luc gene under the control of the cytomegalovirus promoter in deleted E1 region) and 5C (Ad5 Cox2CRAds; a control virus with intact E3 the EIA expression of which was under the control of the Cox2 promoter) have been reported previously (17). 5CL (Ad5 Cox2CRAd AΕ3 ADP Lac; a serotype 5 fiber control virus with the E3 region deleted while maintaining the adenovirus death protein (Adp) gene in its native position and the luc gene inserted next to the Adp gene) and 3CL (Ad5/3 Cox2CRAd ΔE3 Adp Lac; an infectivity enhanced virus with a serotype 5 or serotype 3 chimeric fiber consisting of the tail and shaft of Ad5 in combination with the Ad5 knob and ΔE3 Adp Lac construct) were generated as shown in Figure 1A using the adenovirus type 5 backbone plasmid pTG3602 (21) and adenovirus type 5 with Ad5/3 chimeric-fiber modification plasmid pMG552 (containing an Ad5 tail, shaft, and Ad3 knob) (22). pTG3602, pMG552, and Aat II-linearized pShuttle-ΔE3-ADP-Lac were recombined in BJ5183 electroporation-compotent cells (Stratagene, Cedar Creek, TX, USA). After confirming correct homologous recombination, the resultant plasmids were cleaved with SvaI to remove the kanamycin-resistance gene, and then self-ligated to produce adenovirus backbone plasmids with the E3 deletion and Adp-Lac modification. These ΔE3-Adp-Lac plasmids and PacI-linearized pShuttle-Cox2L/H-E1-px-plF-X-F (18) were recombined in BJ5183 cells to generate pAd5 Cox2CRAd ΔE3 Adp Lac and pAd5/3 Cox2CRAd ΔE3 Adp Lac, respectively.

In vitro analysis of cytopathic effects by crystal violet staining. The viability of the MKN-1, MKN-45, MKN-74, A549, and BT-474 cells (5.0x10^4 cells/well) was assessed after infection with adenoviruses at 10 viral particles (VP) per cell. Cytoidal effects were evaluated using crystal violet staining on days 5 (A549), 8 (MKN-1 and MKN-74), 9 (MKN-45), and 10 (BT-474) post-infection, when the 5W (Ad5 wild-type) positive control exhibited notable cell death.

In vitro analysis of cytopathic effects by fluorescence monitoring. To analyze the time course of cytidial effect, cells in 96-well black plates (1.0x10^4 cells/well) were infected with adenoviruses at 10 VP per cell. The fluorescence intensity of DsRed-expressing cell lines was monitored longitudinally after adenovirus infection using a Sureview remote-R plate reader (Tecan Japan, Kanagawa, Japan).

In vitro analysis of viral replication. 5AEI, 5CL, and 3CL replication in MKN-1, MKN-45, and MKN-74 cells was evaluated by a bioluminescent assay, as well as quantitative real-time polymerase chain reaction (PCR) in MKN-45 cells. Half of the collected samples were lysed by Cell Lysis Buffer (Promega), and the resultant lysates were analyzed using the Luciferase assay kit (Biothema AB, Handen, Sweden) and Luminescence-JNR-111-1200 luminometer (ATTO, Tokyo, Japan). According to the manufacturer's instructions. Half of the collected samples were lysed with 20 μl Cell Lysis Buffer (Promega) per well of 96-well plates and were analyzed using the Luciferase assay kit (Biothema AB). 100 μl of Luciferase Assay Reagent was added to the resultant lysates and mixed by pipetting. Luminescence was measured using a Luminescence-JNR-111-1200 luminometer (ATTO). Viral and genomic DNA from MKN-45 cells was prepared using the Wizard SV Genomic DNA Purification System (Promega). Viral E4 DNA was quantified by Taqman real-time PCR (qPCR) using IQ™ Cycler and IQ™ Supermix (Bio-Rad, Tokyo, Japan). The viral E4 primers and probes used were as reported previously (17).

Visualization of PD during laparotomy. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the National Cancer Center Research Institute.
Tokyo, Japan. (No. 000011521) MKN-45 cells (1.0x10^5) expressing DsRed2 were injected into the peritoneal cavity of 12 female SCID mice (Charles River Japan, Kanagawa, Japan). Two days after cell inoculation, the establishment of fluorescent peritoneal tumor dissemination was confirmed noninvasively using the IVIS 100® bioimaging system (Xenogen, Alameda, CA, USA). 3CL virus (1.0x10^10 VP) was injected into the peritoneal cavity of mice with peritoneal disease. Laparotomy was performed on day 6 after viral injection. The mice were firstly illuminated with white light and PD was inspected by a gastrointestinal surgeon (TK) and a technician (F.T.). The number of metastatic tumors was recorded as the gross image count. Next, D-luciferin (150 mg/kg) was sprayed into the peritoneal cavity and luminescent emission was captured by the bioimaging system. Finally, the mice were illuminated by a xenon lamp and fluorescent emission was also captured. The images were processed solely by adjusting globally for contrast and brightness using the IGOR Pro Imaging software (Xenogen). Very low luminescent values (<1.0x10^5 luminescent units) and fluorescent values (<1.0x10^4 fluorescent units) were removed from this analysis and from the figure. The same surgeon and a technician counted the peritoneal lesions in luminescent (virus) and fluorescent (cancer) images and the number of lesions was recorded. The number of tumor lesions by fluorescent imaging was assumed to represent all detectable lesions in the abdominal cavity because tumor cells were engineered to express DsRed2. These data was therefore used as the basis for comparing gross and luminescent images in order to determine sensitivity and specificity. All lesions detected by fluorescent imaging were observed again and the diameter of lesions was measured by the surgeon under direct vision. The sensitivities of macroscopic and bioluminescence imaging relative to lesion diameter were also determined.

**Histological analysis and viral DNA quantification in tumor lesions.** Peritoneal tumors from mice treated with 3CL were excised at day 6 after viral injection. The tumors were embedded in paraffin, and a portion of the fresh lesions was stored for qPCR analysis. Viral and genomic DNA was extracted by DNA Wizard SV Genomic DNA Purification System (Promega). The viral E4 DNA copy number was measured by Taqman quantitative real-time PCR.

**In vivo anti-tumor effect in a mouse model of PD.** 3CL virus (1.0x10^10 VP) was inoculated into the peritoneal cavity of mice with PD 2 days after the inoculation of MKN-45 cells (1.0x10^5) expressing DsRed2. Fluorescent peritoneal tumor dissemination was similarly confirmed without laparotomy using the IVIS 100® bioimaging system. Survival time was compared between the mock group (n=6) and the 3CL group (n=6).

**Statistical analysis.** Differences were evaluated for statistical significance by the Student’s t-test and the Wilcoxon rank-sum test. The anti-tumoral effect of the applied viruses in the mouse model of PD was summarized by plotting survival curves according to the Kaplan–Meier method and the Log-rank test. p-Values less than 0.05 were considered statistically significant.

**Results**

**In vitro analysis of cytotoxic effects.** Out of the viruses examined, 3CL showed the strongest cytotoxic effect, whereas the oncolytic activity of 5CL was comparable to that of 5W. The intact E3 replicative control 5C showed weak cytotoxic effects against all three gastric cancer cell lines. Cox2 CRAds showed equivalent cytotoxic effects against COX2-positive A549 cells, but no cytotoxic effect against COX2-negative BT474 cells (Figure 1B). DsRed2-expressing cell lines infected with a mock or a non-replicative vector grew with increasing fluorescence intensity over time. By contrast, the growth of cell lines treated with 3CL was significantly reduced (Figure 1C; left lane). The fluorescence assay also revealed that the oncolytic effect of 3CL was the strongest among the viruses examined in all three gastric cancer cell lines. The cytotoxic activity of 5CL was comparable to that of 5W. These results were similar to those obtained with crystal violet staining.

**In vitro analysis of viral replication.** Luciferase expression following 3CL infection was highest at day 2 and peaked at day 3 following the infection of MKN-45 cells with 5CL. Similarly, infection with a CRAd with Ad5/3 fiber modification led to earlier luciferase expression than infection with a CRAd with Ad5 wild-type fiber in MKN-1 and MKN-74 cells. In all three gastric cancer cell lines, 5AE1 infection resulted in extremely low levels of luciferase expression (Figure 1C; middle lane). qPCR analysis revealed that the E4 copy number following 3CL infection increased at day 4 in MKN-45 cells, lagging behind viral luciferase expression by 2 days (Figure 1C; right lane).

**Evaluating the use of replicative viruses to detect PD by laparotomy.** Small peritoneal lesions were analyzed by laparotomy based on macroscopic appearance, and bioluminescent and fluorescent images (Figure 2). The surgeon macroscopically observed 110 lesions in 12 mice with white light; 150 and 189 lesions were detected by bioluminescent and fluorescent imaging, respectively. There were 21 false-positive lesions by macroscopic-appearance analysis, shown to comprise normal tissue on further inspection. By contrast, bioluminescent inspection resulted in only one false-positive result. The sensitivity and specificity achieved with macroscopic-appearance inspection were 58.1% and 83.2%, respectively, whereas the viral-imaging modality yielded corresponding values of 78.8% and 99.3%. The majority (38 out of 40) of the lesions that were not identified by bioluminescent inspection were 3 mm or less in diameter (Table I).

**Histological findings of peritoneal dissemination treated with CRAds.** Peritoneal lesions detected by macroscopic appearance and bioluminescence imaging were evaluated histologically. These contained cancer cells and areas of necrosis consistent with viral oncolytic effects (Figure 3A, top). Cancer cells and necrotic changes were also observed in tumor lesions that were not identified by macroscopic.
Figure 1. DNA structure of adenoviruses and assessment of viral cytopathic effects in gastric cancer cell lines, and cyclooxygenase-2 (COX2)-positive and -negative cell lines. A: DNA structure of adenoviruses. Adp, Adenoviral death protein; Ad5 fiber, serotype-5 fiber; Ad5/3 fiber, a serotype-5 or serotype-3 chimeric fiber; CMV, cytomegalovirus; COX2, cyclooxygenase-2; CRAd, conditionally replicative adenovirus; Luc, firefly luciferase. B: Evaluation of viral cytoidal effects in gastric cancer cell lines, A549 and BT-474 cells. Cells were infected with 5W, 5ΔE1, 5C, 5CL, and 3CL at 10 viral particles (VP) per cell. Cytopathic effects were evaluated by crystal violet staining at day 5 (A549), 8 (MKN-1 and MKN-74), 9 (MKN-45), and 10 (BT-474) post-infection. Time points were chosen based on when the control 5W showed notable cytolytic effects. C: Time course of viral cytoidal effects and viral replication in Discosoma striata Red Fluorescent Protein (DsRed2)-expressing gastric cancer cell lines. Cell viability is indirectly represented by total fluorescence intensity resulting from reporter gene expression [red fluorescent protein (RFP)]. Viral replication is represented by total bioluminescence activity resulting from viral replication-mediated luciferase expression (Luciferase). Viral E4 DNA copy number was quantified by quantitative polymerase chain reaction (qPCR) in MKN-45 (virus copy number).
appearance but were detected by bioluminescence imaging (Figure 3A, middle). Lesions that were not detected by macroscopic appearance or bioluminescence imaging showed only necrotic changes with few or no viable cancer cells (Figure 3A, bottom). The copy numbers of viral E4 DNA were elevated in the three fluorescent lesions shown in Figure 3A, and confirmed the presence of replicating virus in these samples (Figure 3B).

In vivo antitumor effect of 3CL in a mouse model of PD. 3CL- and mock-infected mice underwent non-invasive imaging analysis for one hundred days. Mock-infected lesions continued to grow and reached lethal status at day 32 after viral injection. Lesions treated with 3CL were prevented from enlarging by a viral oncolytic effect (Figure 4A). Although two mice treated with 3CL died at 40 days and 68 days, respectively, after viral injection, the mean
survival time of the 3CL-treated mice was significantly higher than that of the mock-treated mice (85.7 versus 34.3 days, \(p=0.0005\); Figure 4B).

**Discussion**

In the present study, a CRAd with a Cox2 promoter, an E3 modification containing a bioluminescent reporter, an intact Adp gene and Ad5/3 fiber alteration (3CL), was successfully used to visualize small metastatic peritoneal GC lesions by laparotomy. These lesions were histologically confirmed, and were found to contain metastatic cancer cells. There were 39 lesions not detected by viral bioluminescence imaging. These lesions were examined histologically and showed almost complete response. Cancer cells were lysed as a result of viral replication, and there were few or no viable cells in these nodules. The virus was designed to express the luciferase gene in a replication-dependent manner by placing
Figure 4. In vivo anti-tumor effects in a mouse model of peritoneal dissemination of gastric cancer. The 3CL virus (1.0x10^10 viral particles) was inoculated into the peritoneal cavity of mice with peritoneal dissemination two days after the inoculation of MKN-45 cells (1.0x10^6) expressing DsRed2. A: Fluorescent peritoneal tumor dissemination was similarly confirmed without laparotomy using the IVIS 100® biolimaging system. B: Mean survival time was significantly higher in the 3CL-treated group (85.7 days, n=6) compared to mock-treated group (34.3 days, n=6; p=0.0005).

it in the deleted E3 region under the control of the adenoviral major late promoter (16). Viral bioluminescence activity was not, therefore, present in the necrotic tissues without viable cancer cells. If a lesion contained viable cells, the sensitivity of this system would probably increase. This method can be also used for the real-time monitoring of treatment of cancerous lesions and has been actually applied in a xenograft mouse model of pancreatic cancer (23).

Several imaging modalities that do not need invasive procedures to identify cancer lesions have been widely reported (24). These technologies have the potential to visualize small lesions that are undetectable by other conventional modalities. Among these molecular imaging devices, adenoviral imaging tools have a high level of resolution for malignancies (25-27). Previous experimental studies using adenoviruses to detect cancer cells showed comprehensive outcomes in terms of detection and treatment. Fluorescence-guided surgical navigation was used to follow telomerase-dependent CRAd infection that inserted GFP, in order to illuminate human colon cancer cells in mouse models of peritoneal or pleural carcinomatosis (25). However, auto-fluorescence from adjacent non-cancerous organs and blood clots around the cancer lesion can occur leading to false-positive results that can cause clinical
Table I. True-positive and false-negative counts and sensitivity of gross, viral luminescent and tumoral fluorescent images relative to the diameters of lesions of peritoneal dissemination in 12 mice.

<table>
<thead>
<tr>
<th>Tumor size (mm)</th>
<th>Gross image</th>
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<th>Luminescent image</th>
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<td>True-</td>
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<td>3</td>
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<td>78.8</td>
<td>189</td>
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</table>

confusion. A particular advantage of our viral imaging tool is its high specificity, due to the fact that bioluminescent inspection does not require for excitation light and that its background is relatively low (26, 27).

Currently, the diagnosis of PD is typically determined by intraoperative frozen-section analysis of small foci detected by macroscopic examination. This requires invasive resection or biopsy and is time-consuming, requiring up to an hour in the operating room (28). In addition, the diagnostic accuracy of intraoperative frozen-section analysis of sentinel lymph-node biopsy specimens for metastases was reported to be only 38.74% (29, 30). By contrast, our present study showed that viral-bioluminescence imaging can detect microscopic disseminated lesions in just a few minutes, with higher sensitivity and without invasive biopsy.

Another study evaluated anti-tumor effects after intratumoral injection of telomerase-dependent adenoviral GFP (15), which resulted in shrinkage of orthotopically implanted human head and neck cancer cells in the mouse. In our previous study, Cox2 CRAdS with 5/3 chimeric-fiber modifications showed the strongest cytotoxic effect against gastric cancer cell lines and subcutaneous xenograft model (20). Moreover, this replicative virus produced satisfactory effects for gallbladder carcinoma and pancreatic carcinoma both in vitro and in vivo (17, 19). In the present study, 3CL was firstly used to treat PD of GC in mice. The survival time of mice treated with 3CL was significantly longer than that of mock-treated mice. Two mice treated with 3CL died at 40 and 68 days after viral injection. Although longitudinal whole-body imaging showed the growth of peritoneal lesions of six mice with 3CL was similarly suppressed, the status of peritoneal lesions did not reach complete response. Thus, the cause of death of these two mice was speculated to be cachexia due to remaining cancer lesions.

The viral replicative activities here depended on gastric cancer cell COX2 promoter status. Enhancement of COX2 expression in several gastric cancer cell lines was confirmed by previous studies (20). Moreover, cytoplasmic COX2 staining and overexpression of COX2 mRNA are observed in more than 50% of gastric cancer specimens (31), and beta-catenin expression level, which closely correlated with COX2 expression, was enhanced in 72% of several cancer types, including GC (32). However, this virus does not work in COX2-negative cell lines. COX2 expression should be confirmed in biopsy specimens from primary gastric lesions by using endoscopy and adenovirus equipped with another promoter should be selected for COX2-negative GC in the clinical setting.

COX2 is an inducible enzyme associated with the regulation of inflammation and was confirmed to be expressed in inflammatory cells in response to inflammatory stimuli (33). Such inflammatory cells infiltrated cancer lesions on pathological specimens in this study. Similarly, there were inflammatory cells in the 39 lesions with complete response. If the viral luminescence was mainly expressed due to COX2 from inflammatory cells, these lesions should also have been detected in luminescent images. However, there was no luminescent expression in these lesions. Therefore, this suggests that the luminescence was expressed from COX2 protein of cancer cell origin.

The 3CL virus could potentially be used for diagnostic purposes as well as cancer therapy. Its therapeutic and diagnostic efficacy was clearly demonstrated in the present study, suggesting that it could be applicable for use in clinical treatment for the PD of human GC. However, some problems remain to be resolved. Although the sensitivity of viruses carrying luciferase results in fewer false-positives in the detection of tumor cells compared to those carrying GFP,
the safety aspects of luciferase administration to humans remain controversial. Our viral system is a promising modality for the determination of therapeutic planning and anticancer agents for advanced GC, whose prognosis is dismal in several countries, including the USA, and Europe. The risk of luciferin administration seems to be within the permissible range, considering the clinical significance of this luminescent viral system. A phase I study should be carried out to clarify the safety of administration of luciferin and use of this luminescent imaging system. In regard to the safety of viral administration, several phase I clinical trials of an intraperitoneal and intratumoral dispensation of CRAd have already been conducted in patients with ovarian (34), pancreatic (35) and prostate (36) cancer. These patients did not experience significant toxicity with viral administration.

In conclusion, Cox2 CRAd with a 5/3 chimeric-fiber modification is a promising virotherapy for PD of GC. Although the bioluminescence imaging using an adenovirus employed in the present study could not immediately be applied to humans, advances in technologies in this field might improve diagnosis and treatment outcomes for PD in patients with advanced GC.

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Conflicts of Interest
The Authors have no financial disclosures to make in regard to this study.

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