

Original Research

**Phase I dose-escalation study of endoscopic intratumoral injection of OBP-301
(Telomelysin) with radiotherapy in esophageal cancer patients
unfit for standard treatments**

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Running Title: Combination of OBP-301 with radiotherapy for esophageal cancer.

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Abstract

Purpose: OBP-301 (Telomelysin) is an attenuated type-5 adenovirus that contains the human telomerase reverse transcriptase (hTERT) promoter to regulate viral replication. OBP-301 sensitizes human cancer cells to ionizing radiation by inhibiting DNA repair, and radiation enhances coxsackievirus and adenovirus receptor (CAR)-mediated OBP-301 infection on the contrary. We assessed OBP-301 with radiotherapy in esophageal cancer patients unfit for standard chemoradiation treatments.

Methods: A phase I dose-escalation study of OBP-301 with radiotherapy was conducted in 13 histologically confirmed esophageal cancer patients deemed unfit to undergo surgery or chemotherapy. Study treatment consisted of OBP-301 administration by intratumoral needle injection using a flexible endoscope on days 1, 18, and 32. Radiotherapy was administered concurrently over 6 weeks, beginning on day 4, to a total of 60 Gy.

Results: Of the 13 patients, 7, 3, and 3 patients were treated with 10^{10} , 10^{11} , and 10^{12} virus particles, respectively. Study group comprised 10 males and 3 females, with a median age of 82 years (range, 53 to 91 years). All patients developed a transient, self-limited lymphopenia. Distribution studies revealed transient virus shedding in the plasma. Eight patients had local complete response (CR); all of them exhibited no pathologically viable malignant cells in biopsy specimens, and 3 patients had a partial response. The objective response rate was 91.7%. The clinical CR rate was 83.3% in stage I and 60.0% in stage II/III. Histopathologic examination revealed massive infiltration of CD8⁺ cells and increased PD-L1 expression.

Conclusion: Multiple courses of endoscopic intratumoral OBP-301 injection with radiotherapy are feasible and provide clinical benefits in patients with esophageal cancer unfit for standard treatments.

1. Introduction

Esophageal cancer is the sixth leading cause of cancer-related death worldwide, affecting more than 50,000 people [1, 2]. Overall 5-year survival rates have improved up to 20%; however, the prognosis for patients diagnosed with esophageal cancer, regardless of histologic type, remains poor [3]. Disease recurrence even after a complete response might be common and if follow-up surveillance detects recurrence, salvage treatments are required [4]. Multimodality therapy has been reported to achieve better treatment results [5]; however, complications after radical surgery are known to be associated with poor prognosis [6]. The incidence of esophageal cancer increases with age, peaking in the seventh and eighth decades of life. In general, elderly patients may have limited ability to tolerate intensive treatments such as radical surgery or definitive chemoradiotherapy [7, 8]. Therefore, it is important to determine the best approaches to treat frail or elderly esophageal cancer patients.

A conditionally replicative oncolytic virus has emerged as a clinically effective translational treatment platform for less-invasive cancer therapy [9]. It is highly effective at controlling locoregional disease; however, systemic effects are limited, typically necessitating combination approaches [10]. Many clinical trials examining combinations of viral agents with chemotherapy [11, 12], radiotherapy [13, 14], or immune checkpoint inhibitors [15] have been reported. OBP-301 (Telomelysin) is a novel, attenuated type-5 adenovirus (Ad5) with oncolytic potency, in which the tumor-specific human telomerase reverse transcriptase (hTERT) gene promoter regulates the *E1A* and *E1B* genes linked with an internal ribosome entry site (IRES) [16, 17]. In an effort to determine the effectiveness of OBP-301 in human cancer treatment, a phase I clinical trial of locoregional administration of OBP-301 as monotherapy was conducted in patients with a variety of advanced malignancies

such as head and neck cancer or metastatic melanoma in the US. The safety profile of OBP-301 itself after intratumoral delivery was also confirmed in this trial [18]. However, no data regarding evaluation of this agent in combination with other modalities have been reported. We elucidated the mechanistic basis for the synergy between OBP-301 treatment and radiotherapy [19]. Tumor cells infected with OBP-301 are rendered more sensitive to ionizing radiation.

On the basis of these promising preliminary findings, in the current study, we enrolled patients diagnosed with various stages of esophageal cancer who were deemed unfit for standard treatments because of advanced age or severe frailty in order to characterize the safety and clinical benefit of OBP-301 plus radiotherapy in this specific population.

2. Methods

2.1. Study design

The protocol was approved by the Institutional Review Board of Okayama University Hospital as well as by the Ministry of Health, Labour and Welfare (MHLW). The trial was an open-label, phase I dose-escalation study of OBP-301 with radiotherapy (UMIN 000010158, NCT03213054). Three patients were treated per dose level, starting with a dose of 1×10^{10} virus particles (vp) and escalating in log increments to a maximal dose of 1×10^{12} vp. In the 1×10^{10} vp cohort, an additional 4 patients were treated because of adverse events (AEs).

2.2. Patients

Patients meeting the following criteria were included in the study: age 20 years or older; histologically confirmed esophageal cancer, including squamous cell carcinoma (SCC) or

adenocarcinoma, for whom effective standard therapies such as radical surgery or definitive chemoradiotherapy were not available due to advanced age or frailty; lesions accessible for injection; measurable disease; tumor area $<25 \text{ cm}^2$ and $>1 \text{ cm}^2$; Eastern Cooperative Oncology Group (ECOG) performance status of 2 or lower; and adequate bone marrow, liver, and renal function. All patients gave written consent for study participation.

2.3. Procedures

The construction and generation of OBP-301 was reported previously [16, 17]. Clinical-grade OBP-301 was manufactured at Lonza Houston, Inc., Houston, TX, USA. Study treatment consisted of intratumoral OBP-301 injections on days 1, 18, and 32 (Fig. 1). Virus was administered via intratumoral needle injection of the primary tumor using a flexible endoscope. A 1-mL volume of OBP-301 was injected into 5 lesions of the tumor (200 μL per lesion). A unique endoscopic injection method was shown (Supplementary Video 1). Radiation therapy was administered concurrently over 6 weeks, beginning on day 4, to a total of 60 Gy. Patients were monitored for toxicity, vector distribution, antibody formation, and tumor response.

Saliva, sputum, urine, and plasma specimens were monitored for shedding of OBP-301 using a vector-specific DNA-PCR assay. We collected stools only in the 10^{12} vp cohort. Vector-specific primers for the E1A region (E1A-Forward 5'-CCT GTG TCT AGA GAA TGC AA-3'; E1A-Reverse 5'-ACA GCT CAA GTC CAA AGG TT-3') or IRES region (IRES-Forward 5'-GAT TTT CCA CCA TAT TGC CG-3'; IRES-Reverse 5'-TTC ACG ACA TTC AAC AGA CC-3') were used. Serum samples were collected at baseline and day 18 post-injection and tested for the presence of neutralizing anti-adenovirus antibodies.

Neutralizing antibodies to Ad5 were detected based on their ability to inhibit the cytopathic effect of adenoviruses in 293 cells.

2.4. Outcomes

The primary end point was incidence of dose-limiting toxicities and identification of the maximum tolerated dose and the recommended phase 2 dose of OBP-301 when combined with conventional radiotherapy. Patients were monitored with regard to safety and tolerability according to the Common Terminology Criteria for Adverse Events, version 4.0 (CTCAE v4.0), for a minimum of 12 months or until death. Indicator lesions were measured serially by endoscope or radiographic scanning and underwent biopsy for histology and immunohistochemical analyses. Secondary endpoints include the objective response rate, overall survival, disease-free survival, and pharmacokinetics. Indicator lesions were measured serially by endoscope or radiographic scanning and underwent biopsy for histology and immunohistochemical analyses. Standard response criteria were used to evaluate target tumor response. Briefly, complete and partial responses were defined, respectively, as 100% or at least 30% decrease in the sum of the longest diameters of target lesions compared with baseline (the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1).

Pre- and post-treatment tumor specimens were obtained by endoscopic core biopsy of the virus-injected tumors. In addition to conventional histopathologic analyses, immunohistochemical staining for CD8 and PD-L1 was performed. Antibodies to human CD8 (clone C8/144B; Dako, Agilent Technologies, Santa Clara, CA, USA) and PD-L1 (E1L3N; Cell Signaling Technology, Danvers, MA, USA) were used.

2.5. Statistical analysis

Safety was evaluated among all patients who received at least one intratumoral injection of OBP-301. Patients were evaluable for efficacy if they completed both the 3 intratumoral injections of OBP-301 and a planned dose of radiation. Survival curves were calculated using the Kaplan-Meier method.

3. Results

3.1. Proposal of most effective synergistic protocol

OBP-301 infection has been confirmed to interrupt the cellular DNA repair mechanism induced by ionizing radiation. Conversely, radiation enhanced adenoviral uptake in human cancer cells due to an increased expression density of coxsackievirus and adenovirus receptor (CAR) [19]. Based on these encouraging preclinical findings, in the current study protocol, radiotherapy was initiated on day 4 following intratumoral injection of OBP-301 on day 1 in order to ensure sufficient viral replication for 3 days. In contrast, OBP-301 was administered on the same day immediately after radiotherapy on days 18 and 32 in expectation of increased viral infectivity (Fig. 1). This treatment schedule is thus considered ideal for stimulating the bidirectional synergistic effects of OBP-301 and radiotherapy.

3.2. Patients

Thirteen patients with histologically confirmed esophageal cancer who were deemed unfit to undergo standard treatments such as surgery or chemotherapy were enrolled in this clinical trial from November 2013 to November 2017 (Table 1). The study group comprised 10 males and 3 females, with a median age of 82 years (range, 53 to 91 years). Of these, 12 patients suffered from SCC and one from adenocarcinoma. All 13 patients were included in the safety

analysis; however, one patient (patient 005) received only two injections of OBP-301 before withdrawing; therefore, the efficacy analysis included only 12 patients. The median follow-up time for survival analysis was 10.5 months (range, 3.0 to 63.0 months).

3.3. Safety

As none of the 13 treated patients withdrew from the study as a result of toxicity, no dose-limiting toxicities were observed (Table 2). Therefore, 1×10^{12} vp/mL, the highest concentration of virus that could be manufactured, was deemed the maximal achieved dose. The frequent treatment-related AEs included self-limited fever (61.5%), esophagitis (30.8%), gastroesophageal reflux disease (30.8%), leukopenia (69.2%), lymphopenia (100%), and anorexia (38.5%). Self-limited fever developed within 12 hours after OBP-301 injection, regardless of dose, with a highest reported grade of 2. The most AEs occurred predominantly at grade 1 or 2 severity; however, grade 3 and 4 lymphopenia occurred in 8 (61.55%) and 2 (15.4%) patients, respectively. These patients exhibited a rapid decline in lymphocytes 24 hours after OBP-301 administration with complete recovery by day 4, followed by a gradual decline in lymphocytes (Supplementary Fig. 1). Patients reporting grade 4 lymphopenia generally recovered by the end of treatment, without any clinical symptoms.

3.4. Clinical outcome

Twelve of the 13 patients (92.3%) could be evaluated for target tumor responses via clinical findings and endoscopy, computed tomography (CT), or positron emission tomography (PET) scan. Objective responses included complete response (CR) in 8 patients (66.7%), partial response (PR) in 3 patients (25.0%), and stable disease (SD) in 1 patient (8.3%) (Table 1, Fig. 2A, B). Therefore, the objective response rate (ORR), as based on the CR and PR, was

91.7%. The clinical CR rate was 83.3% for stage I disease (5 of 6 patients) and 60.0% for stage II or III disease (3 of 5 patients). Biopsy specimens obtained from the sites where the primary tumors existed contained no viable malignant cells in these 8 patients with CR (66.7%). Improvement of symptoms, including decreased nausea and recovery of oral food intake, was observed in one patient with PR (patient 003) following reopening of the esophageal stenosis. Another patient (patient 008) received additional endoscopic submucosal dissection (ESD) following PR of the entire circumferential esophageal cancer and survived for 45.7 months without recurrence. Of the 8 patients exhibiting a CR, 3 patients survived for 63.0, 41.4, and 29.2 months without any recurrence; 3 patients died with other diseases, such as chronic obstructive pulmonary disease (COPD) or Parkinson disease; and 2 patients died due to distant metastasis, although they experienced no recurrence of primary tumors. The long-surviving patients received no additional chemotherapy during the follow-up period. Only one patient (patient 001) who died with malignant pleural effusion received S-1 administration for approximately 3 months after distant metastasis to the chest wall was detected. The median time of response duration was 25.3 months (range, 3.0 to 63.0 months) (Fig. 2B), suggesting that a prolonged response duration could be expected in primary tumors once patients exhibit CR. The median overall survival (OS) was 16.0 months (95% CI 4.3 to not reached) (Fig. 2C). Among patients with CR, median disease-free survival (DFS) was not reached (95% CI 3.0 to not reached) (Fig. 2D).

Patient 007 was a 53-year-old female with locally advanced SCC of the thoracic esophagus. She was ineligible for treatment with conventional chemotherapy because of severe liver dysfunction, with a Child-Pugh score of 8. After endoscopic intratumoral injection of OBP-301, she developed massive ulceration and reduction of the tumor (Fig. 3A). By the time of the third injection on day 32, the tumor could not be identified by

endoscopy. A biopsy specimen obtained 1 month after treatment showed no viable malignant cells in the tissues. A PET-CT scan 8 months after treatment demonstrated that both the primary tumor and metastatic lymph nodes were negative (Fig. 3B). Patient 012 was an 82-year-old male who presented with locally advanced adenocarcinoma of the thoracic esophagus. He had severe cardiovascular disease and therefore was enrolled in this study. Following 3 courses of endoscopic OBP-301 injection with radiotherapy, complete tumor eradication was observed (Fig. 3C), resulting in pathologic confirmation of no malignant cells. Symptomatic improvement was documented in a 91-year-old female (patient 003) with SCC at the esophagogastric junction. At the time of enrollment, she reported nausea and vomiting due to the obstruction in the gastrointestinal tract; however, treatment resulted in marked tumor regression and relief of her symptoms (Supplementary Fig. 2).

3.5. Pharmacokinetics

A total of 178 plasma, 37 saliva, 62 sputum, and 38 urine samples from the 13 patients enrolled in the study were examined for virus shedding by quantitative real-time DNA-PCR. Viral DNA was detected in 5 of 6 patient plasma samples of the 10^{11} and 10^{12} vp cohorts but not in samples of the 10^{10} vp cohort (Supplementary Table 1, 2). Viral DNA was detected in the plasma predominantly 30 minutes after OBP-301 injection at concentrations ranging from 1.221×10^5 to 1.160×10^6 vp/mL, and the titers decreased over the next 60 minutes (Fig. 4A). No viral DNA was detected 24 hours post-treatment in samples from these patients, suggesting that shedding of the virus into the blood circulation was transient. No signs of virus shedding were observed in other body fluids, including saliva, sputum, and urine. Stool samples were confirmed to be virus free. Serum levels of the cytokines interleukin-6 (IL-6), interleukin-10 (IL-10) and interferon- γ (IFN- γ) were determined by enzyme-linked

immunosorbent assay. Although most of the samples were positive, the levels were below the lower limit of quantification (Supplementary Table 3-5).

3.6. Immunological activity

To monitor systemic immunologic activity against OBP-301, patients were tested for neutralizing antibody to Ad5. All patients exhibited a pre-existing neutralizing antibody titer. Titers of specimens collected at day 18 after the first injection of OBP-301 were higher than the baseline titer in all 12 assessable patients; however, the magnitude of the titer increase did not correlate with viral dose or any clinical outcome (Fig. 4B). To also validate the immunologic response following intratumoral injection of OBP-301, tumor biopsy samples obtained before and 1 month after treatment from 2 patients who exhibited a PR were assessed for CD8-positive tumor infiltrating lymphocyte (TIL) density and PD-L1 expression. Immunohistochemical analysis revealed an apparently higher density of infiltrating CD8-positive cells. Of note, OBP-301 injection induced scattered PD-L1 expression within tumors exhibiting negative PD-L1 staining before treatment (Fig. 4C), suggesting that OBP-301-mediated cell death is highly immunogenic. In patients exhibiting a CR, biopsy specimens from the site where tumor existed also contained no residual tumor cells and exhibited slightly enhanced CD8-positive TIL infiltration (Supplementary Fig. 3).

4. Discussion

No dose-limiting toxicities were observed in the study, and OBP-301 could be safely combined with radiotherapy at the highest dose, 1×10^{12} vp/mL, limited by manufacturing considerations. Only the frequency of fever (affecting 61.5% of the patients) was higher in

our trial compared with the OBP-301 monotherapy trial (37.5%).[18] In contrast, all patients developed lymphopenia with a severity of grade of 2 to 4. As adenovirus infection is correlated with lymphopenia [20, 21], OBP-301 likely contributed to the acute onset of lymphopenia, which recovered within 3 days after virus injection, whereas the gradual decline in lymphocyte counts could be attributed to radiotherapy, as with previous studies [22]. We decided not to recognize grade 4 lymphopenia as a severe AE unless some other clinical symptoms were observed.

While shedding of intratumorally administered oncolytic viruses into the body fluids has been investigated, the incidence of viral DNA detection varies with the type of virus and route of administration [23-25]. In the current study, OBP-301 DNA was shed in the plasma within 30 minutes after intratumoral injection in a dose-dependent manner, as expected [18]. Neutralizing antibodies against adenoviruses are also a major determinant of the fate of circulating OBP-301 [26]. Pre-existing and increased antibody titers might contribute to the complete clearance of OBP-301 from the plasma within 24 hours after dosing; however, we previously reported detection of non-replicative adenovirus-mediated transgene expression in most patients despite the presence of neutralizing anti-adenovirus antibodies, suggesting that circulating neutralizing antibodies do not inhibit viral infection when directly injected into the tumors [27].

By exploiting the bidirectional synergistic effects of OBP-301 and radiotherapy, an antitumor effect was observed in the present study, with tumor eradication or regression noted in 11 of 12 evaluable patients, resulting in an ORR of 91.7%. One patient received only two injections of OBP-301 before withdrawing due to progressive disease and, therefore, he was not evaluated for the clinical outcome. Of particular note is that 8 patients (66.7%) achieved clinical CR and 3 experienced durable responses continuing 29.2 to 63.0 months

from the first dose of OBP-301. In addition to these long-surviving patients, one patient sustained a recurrence-free status for 45.7 months with additional ESD to remove residual tumor. It is also notable that the study treatment was effective against regional lymph node metastasis. We previously reported that GFP-expressing OBP-301 (OBP-401) traffics to the adjacent regional lymphatics and selectively replicates in metastatic lesions in orthotopic xenograft models [28, 29]. The organ distribution data for a deceased patient in our previous study also demonstrated the presence of non-replicative adenovirus DNA in tumor tissues as well as proximal lymph nodes after intratumoral injection, suggesting regional spread of the virus via the lymphatic vessels in humans [27]. Thus, intratumorally administered OBP-301 would be expected to spread into the lymphatic vessels and synergistically kill metastatic tumor cells in conjunction with radiation. A variety of oncolytic viruses have been explored as a new class of immunotherapy agents [30]. We confirmed that telomerase-specific replicative adenoviruses could provide danger signals as well as immunogenic cell death molecules such as adenosine triphosphate (ATP) and high-mobility group box protein 1 (HMGB1) that promote an efficient antitumor immune response [31, 32]. Moreover, as patients with PD-L1-positive tumors are typically more responsive to immunotherapy incorporating checkpoint blockade [33], the observed increases in intratumoral CD8-positive T cells and PD-L1 expression in biopsied tissues obtained from patients exhibiting a PR support the immunologic activity of OBP-301. Ultimately, these two pharmacokinetic and immunologic mechanisms provide a strong rationale for combination therapy involving OBP-301 and radiotherapy.

Our efficacy results demonstrating clinical benefits are encouraging, but confirmatory studies in a randomized phase III setting are needed to prove that addition of OBP-301 to conventional radiotherapy provides significant benefit. However, such trials would be

difficult to conduct because a suitable target population that includes vulnerable esophageal cancer patients is very limited. Therefore, we comparatively analyzed the data reported here with the database of the Comprehensive Registry of Esophageal Cancer in Japan as historical controls [34]. A total of 388 patients had received radiotherapy alone among a total of 2,352 registered cases, even though the reason for selecting radiotherapy instead of chemoradiotherapy was unknown. The clinical CR rate was 83.3% in stage I and 60.0% in stage II/III in our current trial, whereas the database indicated clinical CR rates of 55.8% in stage I and 25.5% in stage II/III, respectively. Thus, the addition of OBP-301 was associated with a higher CR rate, with improved outcomes in comparison with radiation alone in historical data.

In conclusion, the present study demonstrates that repeated intratumoral injections of OBP-301 with radiotherapy is feasible and well tolerated in esophageal cancer patients unfit for standard treatments. Of note, the patients experienced increased CR rates and durable responses once they had achieved CR, suggesting that intensification of conventional radiotherapy by administration of less invasive OBP-301 may be appropriate for sufficiently robust vulnerable patients. The results from this trial led to OBP-301 receiving SAKIGAKE Designation (an expedited review program) for the treatment of esophageal cancer patients by the Japan Ministry of Health, Labour and Welfare. Moreover, OBP-301 received an orphan drug designation for esophageal cancer from the US Food and Drug Administration (US FDA). A confirmatory study of endoscopic OBP-301 injection and radiotherapy as a first-line treatment for esophageal cancer is underway as a multi-center, phase II trial.

Author contributions

Yasuhiro Shirakawa: Methodology, Investigation, Resources, Data curation, **Hiroshi Tazawa:** Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, **Shunsuke Tanabe:** Methodology, Investigation, Resources, Data curation, **Nobuhiko Kanaya:** Validation, Investigation, Data curation, **Kazuhiro Noma:** Investigation, Resources, **Takeshi Koujima:** Investigation, Resources, **Hajime Kashima:** Investigation, Resources, **Takuya Kato:** Investigation, Resources, **Shinji Kuroda:** Validation, Formal analysis, Investigation, **Satoru Kikuch:** Validation, Formal analysis, Investigation, **Shunsuke Kagawa:** Validation, Formal analysis, Investigation, **Kuniaki Katsui:** Methodology, Investigation, Resources, Data curation , **Susumu Kanazawa:** Methodology, Investigation, Resources, Data curation, Supervision, **Yasuo Urata:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Funding acquisition, **Toshiyoshi Fujiwara:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data Curation, Writing - Original draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition

Role of the funding sources

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Conflict of interest statement

YU is the president and CEO of Oncolys BioPharma, Inc., the manufacturer of OBP-301 (Telomelysin). HT and TF are consultants for Oncolys BioPharma, Inc. The other authors have no other conflicts of interest to declare.

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Table 1 Patient characteristics and clinical activity outcomes for injected lesion

Patient No.	Sex	Age (years)	ECOG PS	Reason for enrollment	Histology	Location of tumor	TNM	Stage	Prior therapy	Viral dose	Response
001	Female	82	0	High age	Squamous	Lt	cT1bN0M0	cStage I	None	10 ¹⁰ vp	CR
002	Male	85	0	High age	Squamous	Mt, Lt	cT1bN0M0	cStage I	None	10 ¹⁰ vp	CR
003	Female	91	2	High age	Squamous	Lt, Mt, Ae	cT3N0M0	cStage II	None	10 ¹⁰ vp	PR
004	Male	68	0	Chemoresistance	Squamous	Ut	cT4N1Mo	cStage IVa	Chemotherapy (DCF)	10 ¹⁰ vp	SD
005	Male	79	0	Chemoresistance	Squamous	Mt, Lt, Ut	cT3N2M0	cStage III	Chemotherapy (CF)	10 ¹⁰ vp	*Not evaluable
006	Male	88	1	High age	Squamous	Lt	cT1bN0M0	cStage I	None	10 ¹⁰ vp	CR
007	Female	53	0	Liver cirrohsis	Squamous	Ut	cT2N1M0	cStage II	None	10 ¹⁰ vp	CR
008	Male	89	0	High age	Squamous	Mt, Ut, Lt	cT1bN0M0	cStage I	None	10 ¹¹ vp	PR
009	Male	74	2	Neural disease	Squamous	Mt	cT1bN0M0	cStage I	None	10 ¹¹ vp	CR
010	Male	85	0	High age	Squamous	Mt	cT1bN0M0	cStage I	None	10 ¹¹ vp	CR
011	Male	78	1	Renal dysfunction	Squamous	Mt	cT4aN1M0	cStage III	None	10 ¹² vp	CR
012	Male	82	0	High age, Cardio-vascular disease	Adeno	Mt	cT2N0M0	cStage II	None	10 ¹² vp	CR
013	Male	81	0	High age, Cardio-vascular disease	Squamous	Mt	cT3bN0M0	cStage II	None	10 ¹² vp	PR

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group Performance Status; Squamous, squamous cell carcinoma; Adeno, adenocarcinoma; Ut, upper thoracic; Mt, middle thoracic; Lt, lower thoracic; Ae, abdominal esophagus; DCF, docetaxel, cisplatin, and fluorouracil; CF, cisplatin and fluorouracil.

*Not evaluable because the patient was withdrawn due to PD and recieved only 2 injections of OBP-301.

Table 2 Summary of adverse events by toxicity grade

Adverse Events	Grade 1 No. (%)	Grade 2 No. (%)	Grade 3 No. (%)	Grade 4 No. (%)	Total (n=13) No. (%)
Fever	5 (38.5)	3 (23.1)	0	0	8 (61.5)
Chills	2 (15.4)	0	0	0	2 (15.4)
Edema limbs	2 (15.4)	0	0	0	2 (15.4)
Malaise	2 (15.4)	0	0	0	2 (15.4)
Non-cardiac chest pain	2 (15.4)	0	0	0	2 (15.4)
Nausea	2 (15.4)	0	0	0	2 (15.4)
Esophagitis	3 (23.1)	1 (8.3)	0	0	4 (30.8)
Dysphagia	1 (7.7)	0	0	0	1 (7.7)
Gastroesophageal reflux dise	2 (15.4)	2 (15.4)	0	0	4 (30.8)
Gastric hemorrhage	1 (7.7)	0	0	0	1 (7.7)
Dyspepsia	2 (15.4)	0	0	0	2 (15.4)
Constipation	3 (23.1)	0	0	0	3 (23.1)
Leukocytopenia	5 (38.5)	3 (23.1)	1 (7.7)	0	9 (69.2)
Lymphocytopenia	0	3 (23.1)	8 (61.5)	2 (15.4)	13 (100)
Anorexia	4 (30.8)	1 (7.7)	0	0	5 (38.5)
Arthritis	1 (7.7)	1 (7.7)	0	0	2 (15.4)
Back pain	3 (23.1)	0	0	0	3 (23.1)
Buttock pain	2 (15.4)	0	0	0	2 (15.4)
Headache	1 (7.7)	0	0	0	1 (7.7)
Aspiration	0	2 (15.4)	0	0	2 (15.4)
Cough	2 (15.4)	1 (7.7)	0	0	3 (23.1)
Dyspnea	1 (7.7)	0	0	0	1 (7.7)
Hoarseness	1 (7.7)	0	0	0	1 (7.7)
Pharyngeal mucositis	1 (7.7)	0	0	0	1 (7.7)
Pleural effusion	0	1 (7.7)	0	0	1 (7.7)
Pneumonitis	2 (15.4)	1 (7.7)	0	0	3 (23.1)
Pulmonary fistula	0	1 (7.7)	0	0	1 (7.7)
Wheezing	0	1 (7.7)	0	0	1 (7.7)
Postoperative hemorrhage	1 (7.7)	0	0	0	1 (7.7)
Erythema multiforme	0	1 (7.7)	0	0	1 (7.7)
Rash	1 (7.7)	0	0	0	1 (7.7)
Dermatitis	1 (7.7)	0	0	0	1 (7.7)

Figure Legends

Figure 1 Overview of study schedule. (A) OBP-301 injection and radiotherapy schedule. Intratumoral injection of OBP-301 was performed following 2 Gy/day external-beam radiation on days 18 and 32. (B) Schematic showing OBP-301 and radiotherapy to mutually enhance the efficacy. Sufficient viral replication sensitizes cancer cells to radiotherapy, and radiation enhances viral infectivity through increased coxsackievirus and adenovirus receptor (CAR) expression.

Figure 2 Clinical activity of OBP-301 and radiotherapy. (A) Waterfall plots showing best overall response to OBP-301 and radiotherapy in 12 patients with esophageal cancer. One patient (patient 005) received only two injections of OBP-301 and was omitted from the figure. Colors indicate viral doses. (B) Swimmer plot depicting the time from the start of therapy to time of death or last known follow-up of patients enrolled in the study. Arrows indicate surviving patients. Kaplan-Meier curve for (C) overall survival (OS) of all patients that completed the full treatment protocol and (D) disease-free survival (DFS) of complete responders.

Figure 3 Complete responses in patients with advanced esophageal cancer treated with OBP-301 plus radiotherapy. (A) Representative endoscopic images of locally advanced squamous cell carcinoma of the thoracic esophagus treated with 1×10^{10} vp of OBP-301 in patient 007, demonstrating scattered ulcer formation followed by complete regression of the tumor, which is currently continuing at over 63 months. Endoscopic biopsy at the tumor site continuously revealed no viable malignant cells. White-light endoscopy

(*upper panels*). Narrow band imaging or Lugol chromoendoscopy (*lower panels*). (B) PET/CT imaging at baseline and 8 months after treatment for patient 7 demonstrated radiographic as well as metabolic complete responses of the primary tumor injected with OBP-301. Complete response was also observed in the uninjected metastatic lesion at the right recurrent nerve lymph nodes (106recR), suggesting a locoregional or systemic antitumor efficacy. (C) Patient 012 presented with locally advanced adenocarcinoma of the thoracic esophagus at baseline. After combination therapy with intratumoral injection of 1×10^{12} vp of OBP-301 plus radiotherapy, he achieved endoscopic complete tumor destruction, and pathologic examination of biopsy specimens demonstrated no residual malignant tumor cells at 3 months post-treatment.

Figure 4 Pharmacokinetics and immunologic assessments of patient samples

obtained before and after OBP-301 injections. (A) Quantitative detection of OBP-301 viral DNA in patient plasma samples at various time points post-treatment in cohorts 2 and 3. Patients in cohort 1 exhibited levels below the detection limit. Viral shedding was noted more frequently and to a greater extent in patients in the higher-dose cohorts. (B) Neutralizing anti-adenovirus antibody titers. All patients exhibited an increase in neutralizing antibody titer on day 18 after injection compared to baseline (*upper panel*). No correlation was noted between the absolute titer and dose of OBP-301 (*lower panels*). (C) Representative examples of hematoxylin and eosin staining (*left panel*) and immunostaining for CD8 and PD-L1 (*right panel*). Intratumorally delivered OBP-301 promoted CD8-positive T-cell recruitment to biopsy tissues obtained from partially responsive patients (patients 008 and 013). Scale bar, 100 μm .

Fig. 1

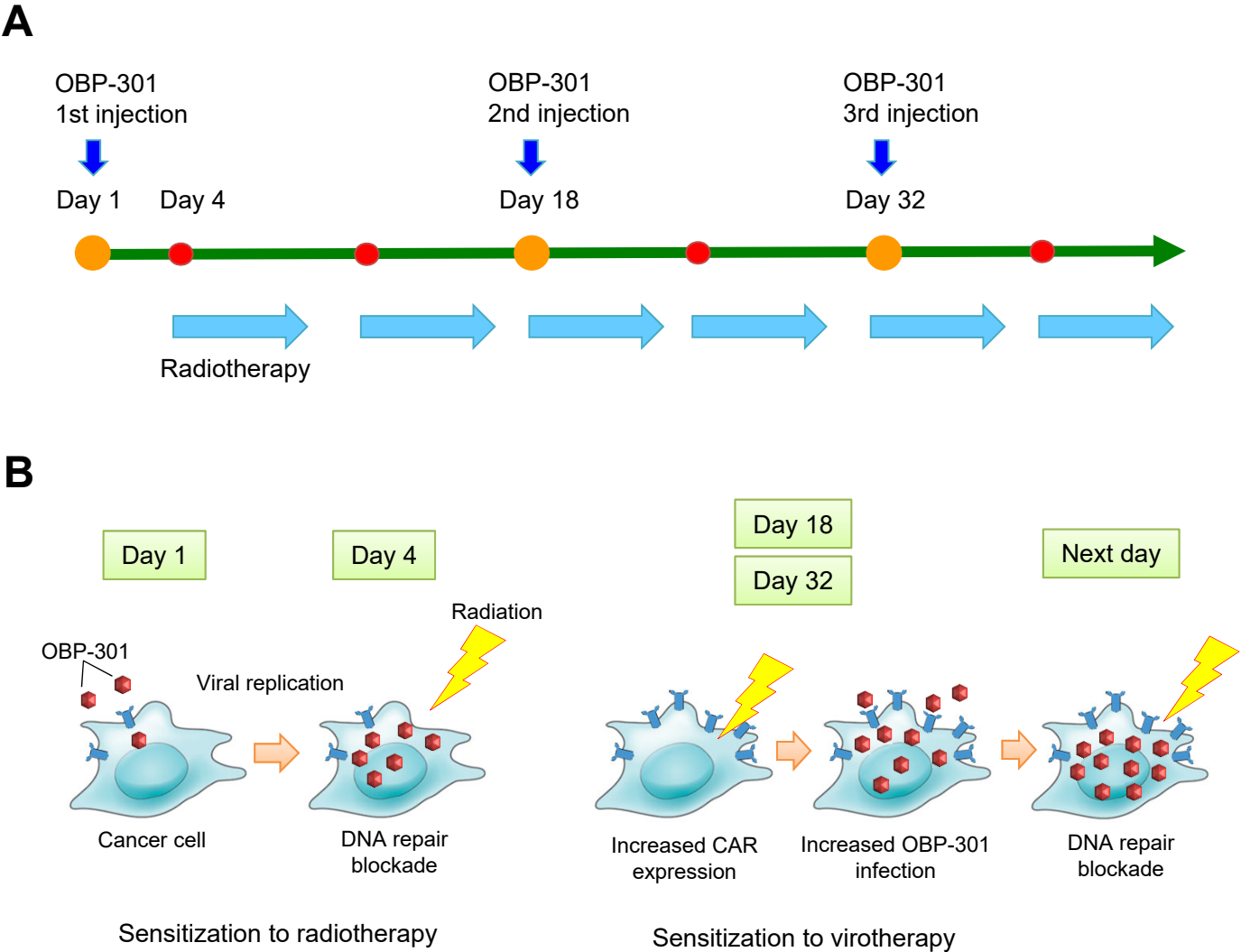


Fig. 2

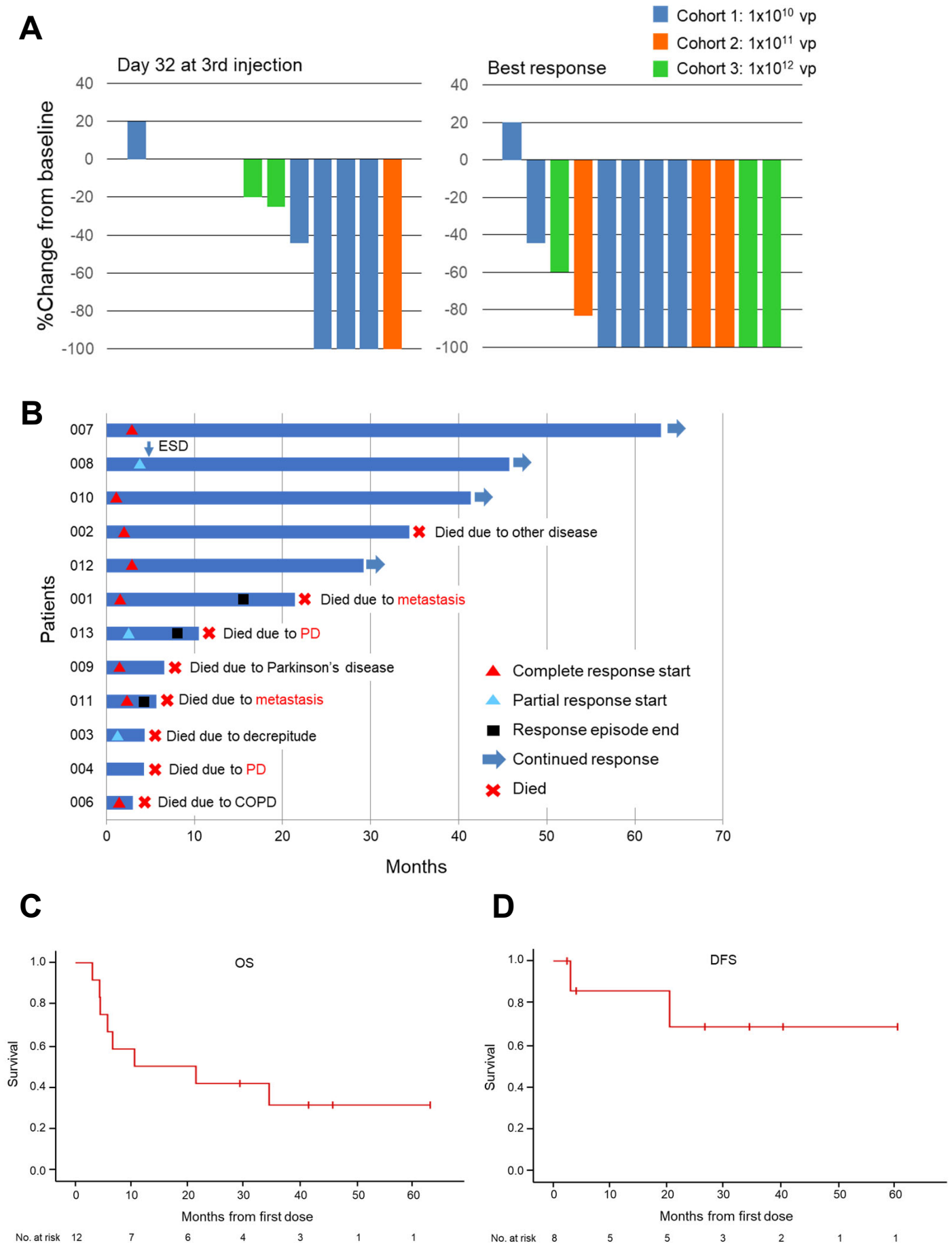


Fig. 3

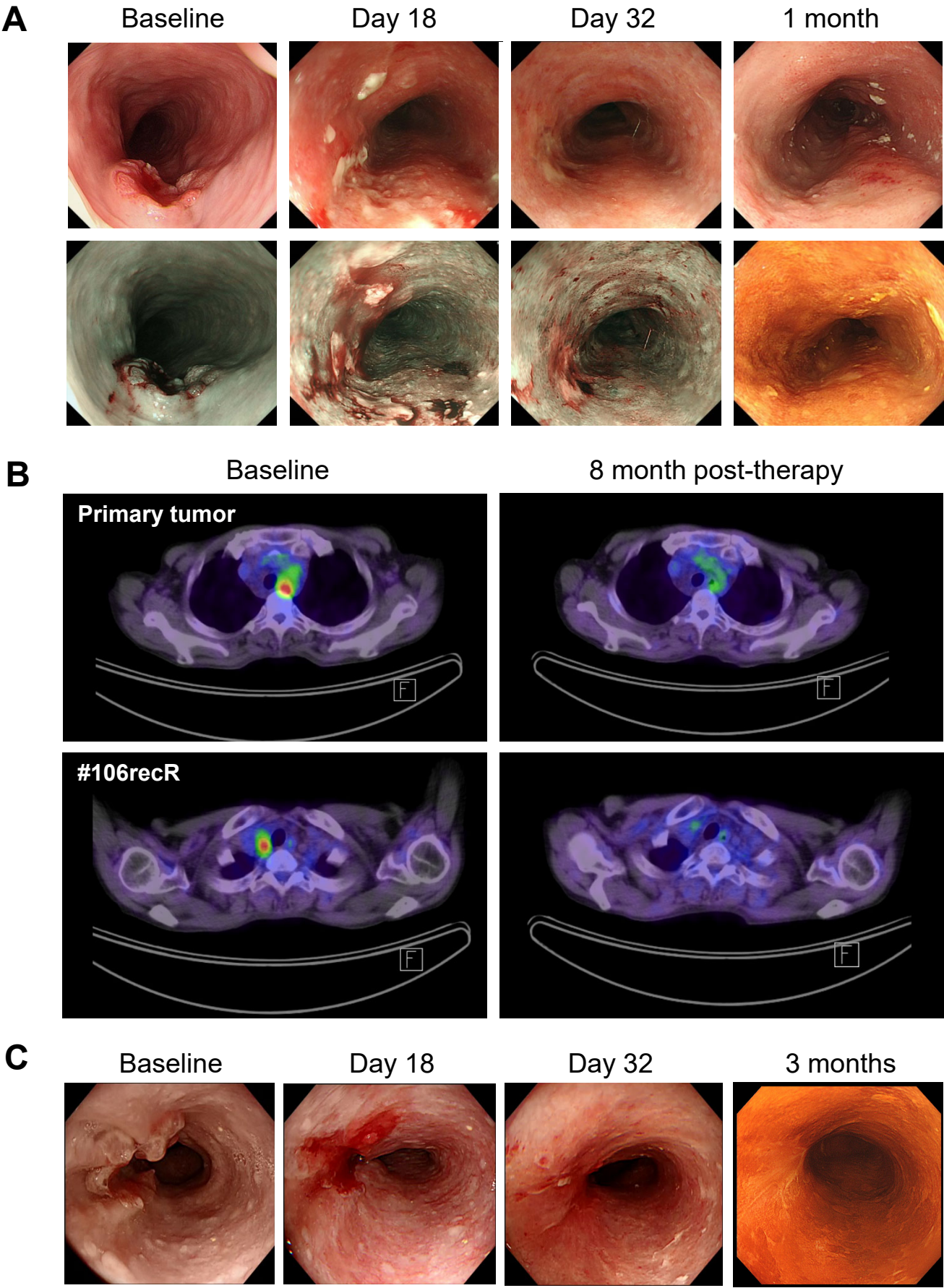
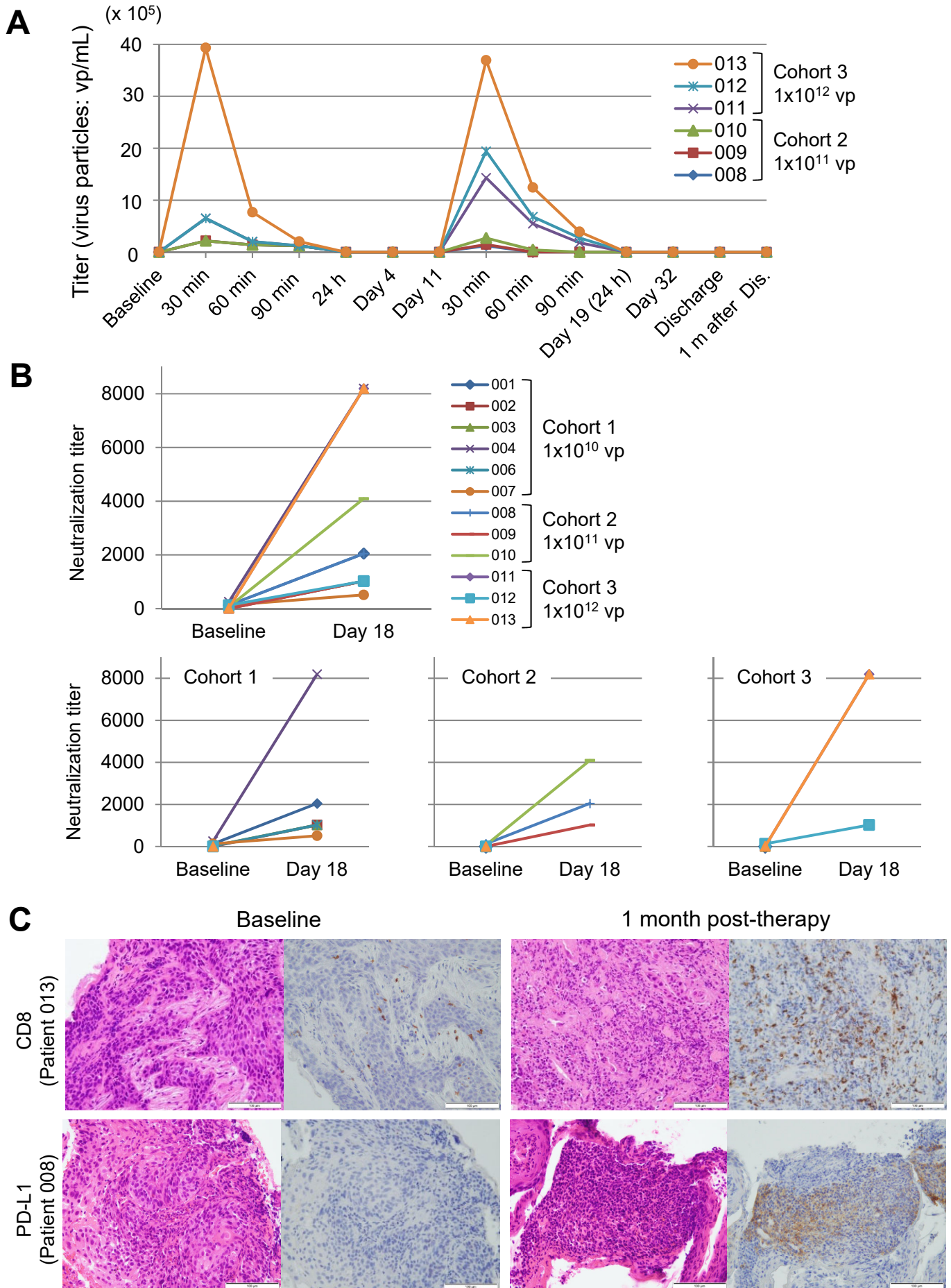


Fig. 4

Phase I dose-escalation study of endoscopic intratumoral injection of OBP-301 (Telomelysin) with radiotherapy in esophageal cancer patients unfit for standard treatments

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Supplementary Figure 1

Lymphocyte counts of patients treated with intratumoral injection of OBP-301 and radiotherapy.

Supplementary Figure 2

Endoscopic images of regressed tumor in the patient who had PR.

Supplementary Figure 3

Immunohistochemical staining of biopsied tissues for CD8 in the patients who had CR .

Supplementary Table 1

Quantitative determination of OBP-301 DNA in human plasma (Cohort 2)

Supplementary Table 2

Quantitative determination of OBP-301 DNA in human plasma (Cohort 3)

Supplementary Table 3

Actual Concentrations of Human Cytokines in Serum Samples: Interleukin 6 (IL-6)

Supplementary Table 4

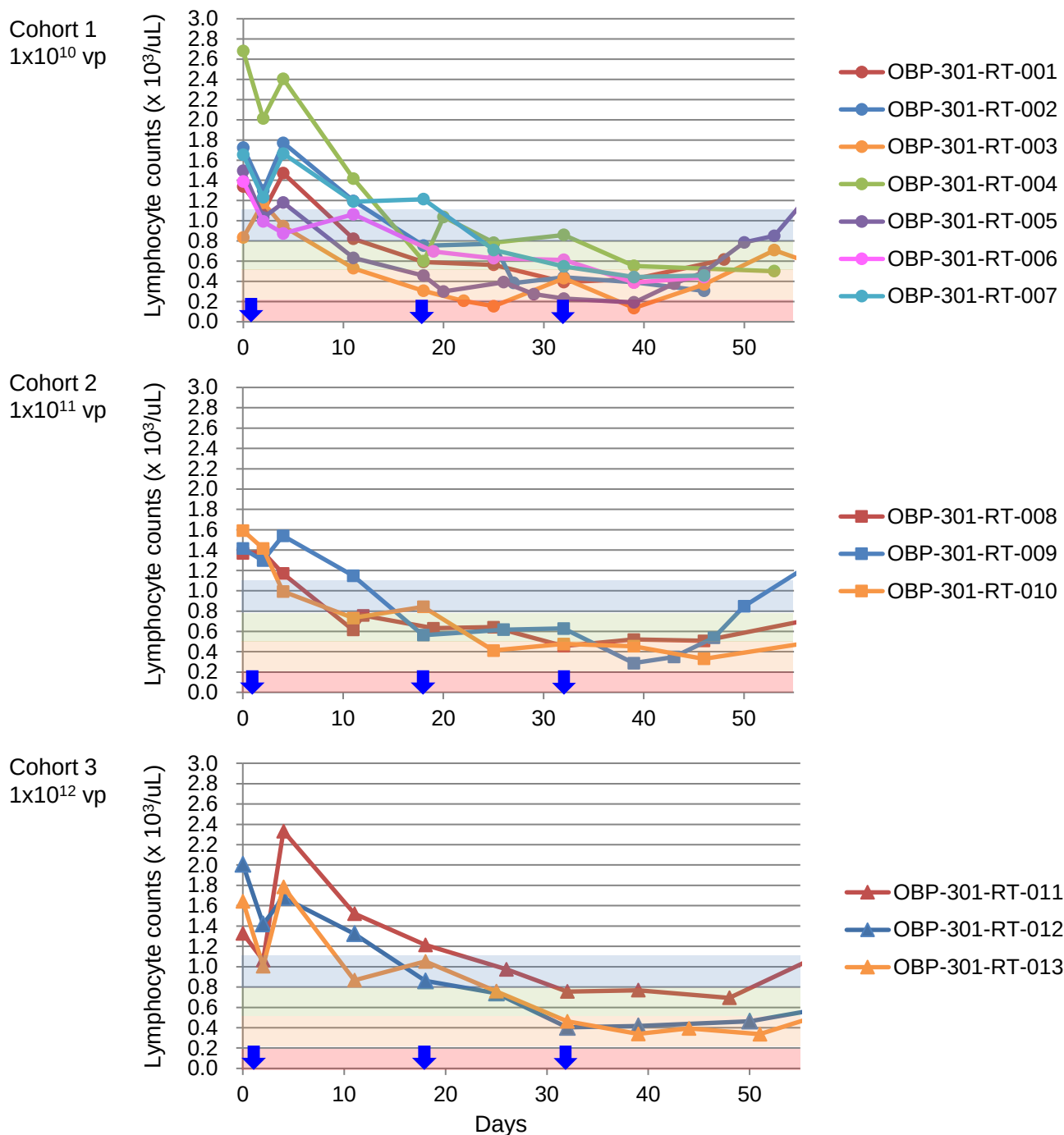
Actual Concentrations of Human Cytokines in Serum Samples: Interleukin 10 (IL-10)

Supplementary Table 5

Actual Concentrations of Human Cytokines in Serum Samples: Interferon γ (IFN- γ)

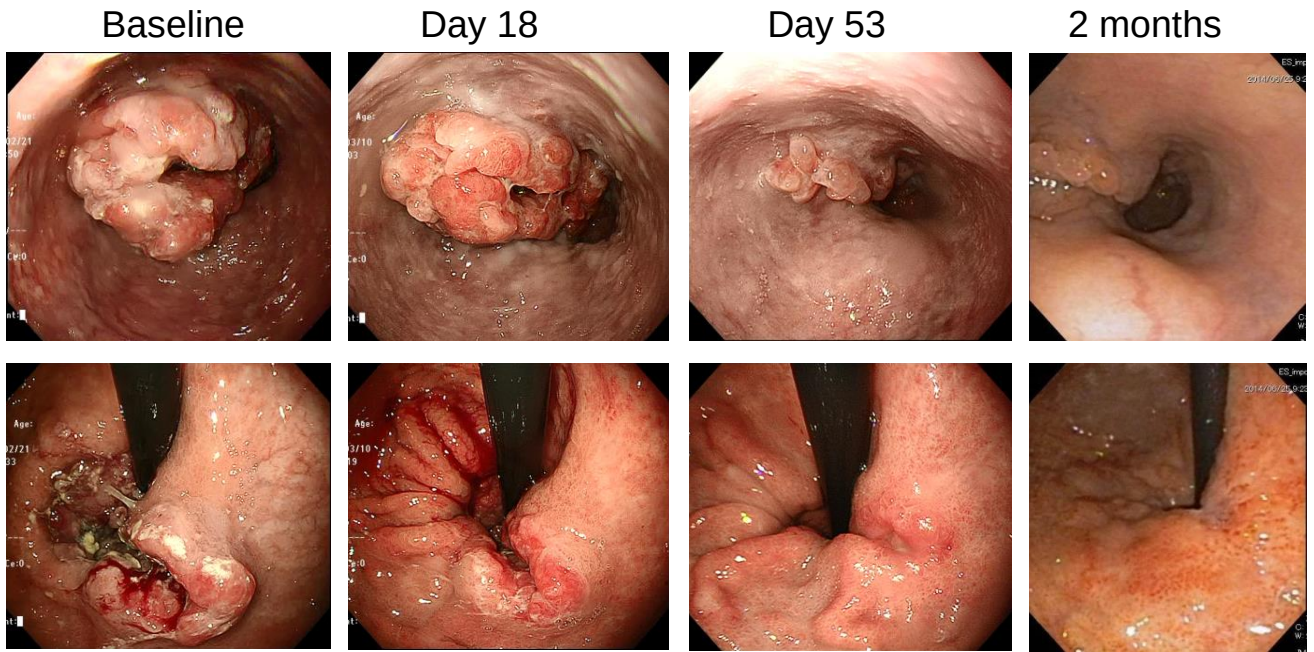
Supplementary Video 1

A unique endoscopic injection method of OBP-301



Supplementary Figure 1

Lymphocyte counts of patients treated with intratumoral injection of OBP-301 and radiotherapy. Arrows indicate OBP-301 administration. Colored areas represent Grades 1 to 4 lymphopenia ranges; Grade 1 in blue, Grade 2 in green, Grade 3 in beige and Grade 4 in red. All patients exhibited transient lymphopenia. We speculate that OBP-301 administration induced a rapid decrease just after virus injection, with the gradual decrease potentially due to radiation. In the current protocol, radiotherapy is not stopped unless the patient shows some clinical symptoms.



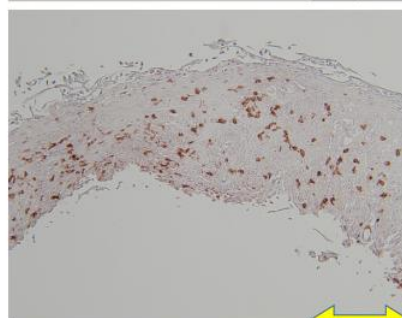
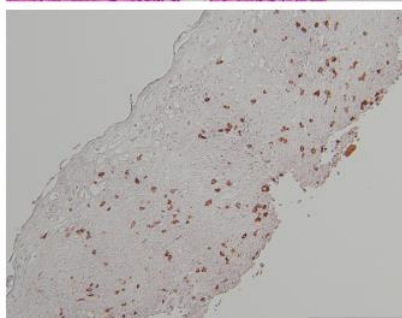
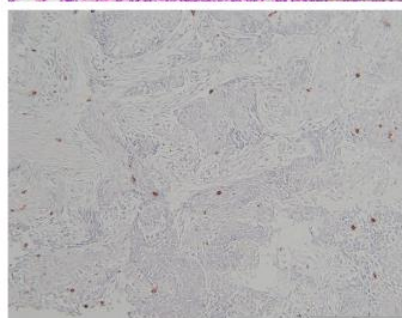
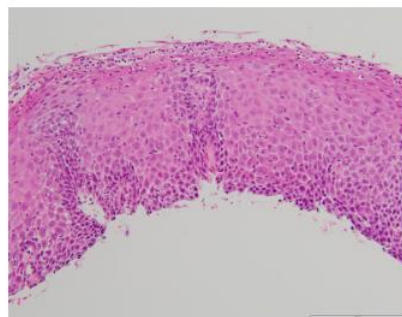
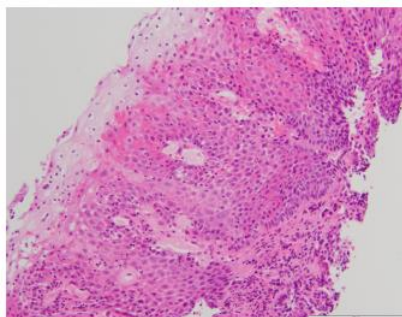
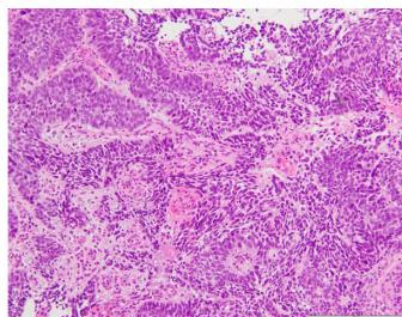
Supplementary Figure 2

Endoscopic images of the patient who exhibited a PR. A 91-year-old female (patient 003) presented with locally advanced squamous cell carcinoma that was almost obstructing the esophagogastric junction. Following 3 courses of endoscopic OBP-301 injection and radiotherapy, tumor destruction was observed at the esophagogastric junction, resulting in reopening of the gastrointestinal tract. All clinical symptoms completely resolved; however, the patient died due to senility but without regrowth of the tumor.

A

Baseline

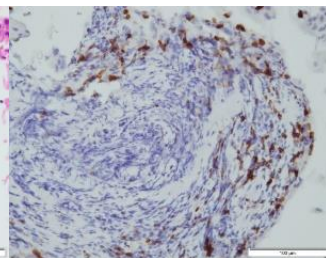
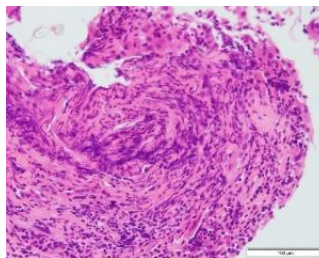
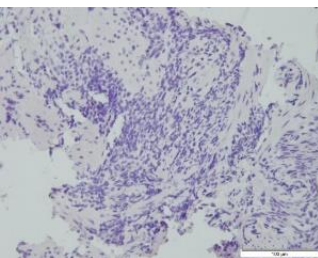
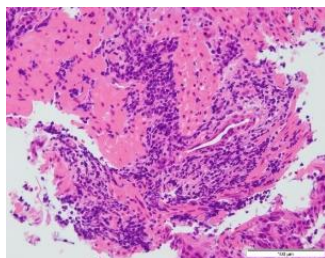
1 month post-therapy (No malignant cells)



100μm

B

Baseline

1 month post-therapy
(No malignant cells)

Supplementary Figure 3

Immunohistochemical staining for CD8 in biopsied tissues obtained from patients exhibiting CR. Patients 007 (A) and 009 (B). There were no residual or recurrent malignant cells, and CD8-positive cell infiltration was low to moderate.

Supplementary Table 1

Quantitative Determination of OBP-301 DNA in Human Plasma (Cohort 2)

Cases	008		009		010	
Primers	E1A (vp/mL)	IRES (vp/mL)	E1A (vp/mL)	IRES (vp/mL)	E1A (vp/mL)	IRES (vp/mL)
Baseline	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
30 min after injection	2.168×10^5	2.545×10^5	Undetect.	Undetect.	8.459×10^4 (below LLOQ)	1.227×10^5
60 min after injection	1.389×10^5	1.727×10^5	Undetect.	Undetect.	2.624×10^4 (below LLOQ)	2.680×10^4 (below LLOQ)
90 min after injection	1.216×10^5	6.852×10^4 (below LLOQ)	Undetect.	Undetect.	Undetect.	Undetect.
24 h after injection	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
Day 4	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
Day 11	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
30 min after 2 nd injection	1.221×10^5	1.130×10^5	2.303×10^4 (below LLOQ)	3.536×10^4 (below LLOQ)	1.252×10^5	1.277×10^5
60 min after 2 nd injection	5.417×10^4 (below LLOQ)	6.105×10^4 (below LLOQ)	Undetect.	Undetect.	4.424×10^4 (below LLOQ)	3.585×10^4 (below LLOQ)
90 min after 2 nd injection	4.004×10^4 (below LLOQ)	4.295×10^4 (below LLOQ)	Undetect.	Undetect.	Undetect.	Undetect.
Day 19 (24 h)	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
Day 32	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
Discharge	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
1 month after Discharge	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.

10^3 vp/mL = 1 vp/reaction

LOD (lower limit of detection): 20 vp/reaction (2×10^4 vp/mL)

LLOQ (lower limit of quantitation): 100 vp/reaction (10^5 vp/mL)

Undetect. = below LOD or undetermined

Supplementary Table 2

Quantitative Determination of OBP-301 DNA in Human Plasma (Cohort 3)

Cases	011		012		030	
Primers	E1A (vp/mL)	IRES (vp/mL)	E1A (vp/mL)	IRES (vp/mL)	E1A (vp/mL)	IRES (vp/mL)
Baseline	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
30 min after injection	4.333×10^5	4.539×10^5	Undetect.	Undetect.	3.282×10^6	3.420×10^6
60 min after injection	6.468×10^4 (below LLOQ)	1.727×10^5	Undetect.	Undetect.	5.639×10^5	6.513×10^5
90 min after injection	Undetect.	5.882×10^4 (below LLOQ)	Undetect.	Undetect.	8.190×10^4 (below LLOQ)	8.566×10^4 (below LLOQ)
24 h after injection	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
Day 4	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
Day 11	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
30 min after 2 nd injection	1.160×10^6	1.144×10^6	5.104×10^5	4.662×10^5	1.751×10^6	2.032×10^6
60 min after 2 nd injection	5.022×10^5	5.539×10^5	1.340×10^5	1.653×10^5	5.640×10^5	5.577×10^5
90 min after 2 nd injection	1.808×10^5	1.609×10^5	9.122×10^4 (below LLOQ)	8.006×10^4 (below LLOQ)	1.187×10^5	1.147×10^5
Day 19 (24 h)	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
Day 32	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
Discharge	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.
1 month after Discharge	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.	Undetect.

10^3 vp/mL = 1 vp/reaction

LOD (lower limit of detection): 20 vp/reaction (2×10^4 vp/mL)

LLOQ (lower limit of quantitation): 100 vp/reaction (10^5 vp/mL)

Undetect. = below LOD or undetermined

Supplementary Table 3

Actual Concentrations of Human Cytokines in Serum Samples: Interleukin 6 (IL-6)

Viral Dose	Patient No.	Actual Concentration (pg/mL)			
		Baseline	Day 2 (24 h after injection)	Day 4	Day 18 (Before 2 nd injection)
Cohort 1	001	0.052	0.442	0.951	0.147
	002	0.136	1.069	0.167	0.030
	003	0.998	2.061	0.884	1.117
	004	2.055	1.894	2.249	5.002
	005	0.000	0.073	0.012	0.010
	006	0.540	0.476	0.197	0.000
	007	0.162	0.475	0.164	0.665
Cohort 2	008	36.144	4.285	45.352	10.852
	009	0.259	5.581	5.771	0.898
	010	0.000	0.000	0.153	0.000
Cohort 3	011	2.686	2.864	6.305	4.025
	012	0.823	0.876	2.143	1.297
	013	1.699	1.752	1.750	1.599

Note: The values of actual measured concentration in bold and italic are indicating that the measured concentrations of IL-6 are below LLOQ (1.56 pg/mL).

Supplementary Table 4

Actual Concentrations of Human Cytokines in Serum Samples: Interleukin 10 (IL-10)

Viral Dose	Patient No.	Actual Concentration (pg/mL)			
		Baseline	Day 2 (24 h after injection)	Day 4	Day 18 (Before 2 nd injection)
Cohort 1	001	0.000	0.000	0.000	0.000
	002	0.695	1.298	1.496	1.219
	003	0.000	0.000	0.000	0.000
	004	0.000	0.000	0.000	0.000
	005	0.000	0.000	0.000	0.000
	006	0.000	0.000	0.000	0.000
	007	0.038	0.000	0.297	0.283
Cohort 2	008	0.000	0.000	0.000	0.000
	009	0.000	0.000	0.000	0.000
	010	0.000	0.000	0.000	0.000
Cohort 3	011	8.345	13.438	7.170	7.385
	012	0.000	0.000	0.000	0.000
	013	0.000	0.000	0.000	0.000

Note: The values of actual measured concentration in bold and italic are indicating that the measured values without dilution factor of IL-10 are below LLOQ (3.1 pg/mL).

Supplementary Table 5
 Actual Concentrations of Human Cytokines in Serum Samples: Interferon γ (IFN- γ)

Viral Dose	Patient No.	Actual Concentration (pg/mL)			
		Baseline	Day 2 (24 h after injection)	Day 4	Day 18 (Before 2 nd injection)
Cohort 1	001	0.176	0.468	0.520	0.516
	002	0.665	0.737	0.889	1.337
	003	1.023	0.928	0.923	1.119
	004	0.268	1.230	0.457	1.100
	005	0.643	0.986	1.026	1.428
	006	0.279	0.348	0.486	0.562
	007	0.440	2.396	0.831	0.769
Cohort 2	008	0.000	1.733	0.000	2.141
	009	0.000	1.475	0.000	0.000
	010	0.000	1.593	1.285	1.982
Cohort 3	011	31.811	34.221	30.603	33.841
	012	0.553	0.363	0.717	0.549
	013	1.539	2.221	1.864	1.334

Note: The values of actual measured concentration in bold and italic are indicating that the measured values without dilution factor of IFN- γ are below LLOQ (1.6 pg/mL).

Supplementary Video 1

A unique endoscopic injection method of OBP-301

After an original solution of OBP-301 was diluted to the indicated concentration, the syringe was moved to the treatment room on ice. Then, OBP-301 was endoscopically injected. The syringe of OBP-301 was connected to the endoscopic injection needle. OBP-301 was injected endoscopically through this injection needle by using a flexible endoscope. A solution of 1 ml was injected into the 5 lesions of the tumor with 200 μ L each.