

The outcome in CY1 patients has been reported to be poor in the East as well as in the West [8, 9], but the recent introduction of novel anticancer agents has changed the picture to some extent. We conducted a phase II trial, named CCOG0301, exploring D2 dissection followed by treatment with 1 M tegafur–0.4 M gimestat–1 M otastat potassium (S-1), in which 48 patients were registered, of whom 47 were eligible for analysis, and we achieved a two-year survival rate of 46 %, which exceeded the initial expectations [4]. The choice of a sample size of 50 patients in this study had been based on the hypothesis that the two-year survival rate would be 36 % and the lower limit of the 90 % confidence interval would exceed 23.5 %, which was the upper limit of the 90 % confidence interval for the historical controls whose two-year survival rate had been 13.3 %. While this phase II trial was ongoing, a pivotal phase III trial comparing postoperative adjuvant S-1 monotherapy with treatment by surgery alone in patients with stage II/III gastric cancer turned out to show positive results for the S-1 monotherapy arm. Moreover, the incidence of relapse, as peritoneal carcinomatosis, was found to be significantly lower in the S-1 monotherapy group [10]. This finding suggests that S-1 is effective against microscopic residual disease in the peritoneal cavity that is undetectable by peritoneal lavage cytology, and also suggests the potential of S-1 to control micrometastases in the peritoneal cavity. Encouraged by these findings, we were motivated to follow the patients for longer, to see what proportion of the patients who were entered in the CCOG0301 trial were actually cured, in order to reconsider the indication for curative surgery in patients with CY1 disease.

Patients and methods

Forty-seven patients who were registered between February 2002 and July 2006 for the CCOG0301 study underwent further follow up (for a median of 2,337 days or until death) to evaluate the long-term outcome.

Characteristics of the patients who were enrolled in the CCOG0301 study

Eligible patients had to meet all of the following criteria: (1) a confirmed diagnosis of gastric adenocarcinoma and age less than 80 years; (2) gastrectomy with systemic D2 lymphadenectomy performed (splenectomy could be omitted at the discretion of the surgeons); (3) no distant metastasis, with the exception of minimal peritoneal deposits that were completely resected; (4) no prior treatment besides surgery; (5) positive cytologic results for cancer cells on examination of peritoneal washings (CY1);

and (6) adequate organ function [4]. Of the 47 eligible patients, 7 patients had peritoneal deposits, which were co-resected at surgery. Seven patients were intraoperatively confirmed to have invasion to adjacent organs (T4), and 38 others had serosal invasion. All but five patients were confirmed to have nodal involvement on pathological examination; six patients had metastasis to the paraaortic lymph nodes [4].

Treatment protocol of the CCOG0301 study

The interval from surgery to the start of therapy was not to exceed 6 weeks. Patients received S-1 at an oral dose of 40 mg per square meter of body-surface area twice daily for 4 weeks, followed by 2 weeks without chemotherapy. Patients with a body-surface area of less than 1.25 m² received 80 mg daily; those with a body-surface area of 1.25 m² to less than 1.5 m² received 100 mg daily; and those with a body surface area of 1.5 m² or greater received 120 mg daily. This 6-week cycle was repeated in an out-patient setting under medical supervision until disease progression, unacceptable adverse events, or the patient's withdrawal of consent. Adequate dose modification and changes in the treatment schedule were conducted, as described previously [4, 10].

Disease status was assessed once every 3 months on the basis of serum tumor markers and at least once every 6 months by computed tomography (CT) scanning for the first 2 years. Follow-up visits including a CT scan were performed once every 6 months until the patients were considered disease-free at 5 years after the surgery.

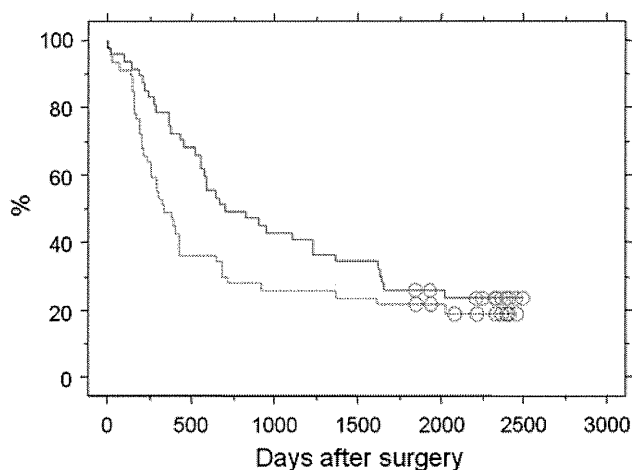


Fig. 1 Overall survival (*solid line*) and relapse-free survival (*dotted line*) of patients with gastric cancer who had free cancer cells in the peritoneal cavity and underwent surgery followed by S-1 monotherapy

Results

Overall survival and relapse-free survival from the day of surgery are shown in Fig. 1. Median overall survival time was 705 days, and relapse-free survival time was 376 days. The 2- and 5-year survival rates were 46 and 26 %, respectively, and the 5-year relapse-free survival rate was 21 %. The most frequent pattern of disease recurrence was peritoneal carcinomatosis, occurring in 29 patients (62 % of all patients enrolled). Other patterns of recurrence were hepatic in 4 patients, lymphatic in 4, locoregional in 2, pulmonary in 1, and osseous in 1. Three patients died of disease other than gastric cancer, at 5, 1371, and 2023 days after surgery. Details on treatment compliance, dose intensity, and toxicity have been reported previously [4].

Conclusions

Patients with CY1 disease with no other non-curative factors could be indicated for surgery with curative intent, provided adequate chemotherapy is given. Although post-operative treatment with S-1, now a standard of care for stage II/III gastric cancer, remains as an option, further trials are warranted to decide on the optimal chemotherapeutic regimen and whether to deliver it before or after surgery.

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STUDY PROTOCOL

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A randomized phase II trial to elucidate the efficacy of capecitabine plus cisplatin (XP) and S-1 plus cisplatin (SP) as a first-line treatment for advanced gastric cancer: XP ascertainment vs. SP randomized PII trial (XParTS II)

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Abstract

Background: On the basis of international clinical trials, capecitabine plus cisplatin (XP) as a first-line treatment of advanced gastric cancer is considered a global standard regimen. However, the usefulness of XP as compared with S-1 plus cisplatin (SP), which is considered standard therapy in Japan, has not yet been assessed.

Methods/design: This is a multicenter randomized phase II trial to elucidate the efficacy of XP as compared with SP for first-line treatment of advanced gastric cancer. Patients with unresectable metastatic or recurrent gastric cancer, 20–74 years of age and human epidermal growth factor 2 (HER2)-negative status, will be assigned in a 1:1 ratio to receive either S-1 40 mg/m² bid for 21 days plus cisplatin 60 mg/m² (day 8) every 5-week cycle or capecitabine 1000 mg/m² bid for 14 days plus cisplatin 80 mg/m² (day 1) every 3-week cycle. Patients will be also asked to the analysis of tumor tissues for translational investigations. The Primary endpoint is progression-free survival and secondary endpoints are overall survival, time to treatment failure, tumor response rate and safety. These comparisons will also be evaluated in terms of biomarkers. Planned sample size is 100 (50 in each arm), which is appropriate for this trial.

Discussion: Fluoropyrimidine plus cisplatin combination is the standard regimen of the first line treatment for advanced gastric cancer. Both S-1 and capecitabine are the prodrug of 5-FU but differ from their process of metabolism. Result of this trial and translational research will provide the important clues to prepare the individualized therapy for advanced gastric cancer in the near future.

Trial registration: ClinicalTrials.gov Identifier NCT01406249

Keywords: Biomarker, Capecitabine, Cisplatin, Clinical trial, Gastric cancer, S-1

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Background

Gastric cancer is the fourth most common malignancy in the world (988 602 cases in 2008, 7.8% of total) and the second leading cause of cancer death (737 419 deaths, 9.7% of total) [1]. For the treatment of advanced or recurrent gastric cancer (AGC), the most commonly used regimens are combination chemotherapy consisting of a fluoropyrimidine (5-fluorouracil or oral fluoropyrimidine) plus a platinum agent with or without docetaxel or anthracyclines [2-6].

S-1 is an oral anticancer drug composed of the 5-fluorouracil (5-FU) prodrug tegafur and two 5-FU modulators; it has achieved high response rates in patients with gastric cancer in phase II studies [7,8]. In a phase III trial (SPIRITS trial) that compared S-1 alone to S-1 plus cisplatin (SP), SP showed a significantly longer overall survival (OS; 13 months vs. 11 months; HR = 0.77, 95% CI 0.61–0.98, $p = 0.04$) and longer progression-free survival (PFS; 6.0 months vs. 4.0 months; HR = 0.57, 95% CI 0.44–0.73, $p < 0.0001$) [4]. Therefore, SP is now considered to be one of the standard first-line regimens for AGC in Japan.

Capecitabine is also an oral fluoropyrimidine, which is metabolized primarily in the liver and converted in tumor tissues to 5-FU by the enzyme thymidine phosphorylase (TP), which is associated in higher concentrations in tumor cells than in normal cells [9]. Kang and colleagues evaluated the non-inferiority of capecitabine plus cisplatin (XP) compared with 5-FU plus cisplatin (FP). The median PFS showed significant non-inferiority (5.6 months vs. 5.0 months; HR = 0.81, 95% CI 0.63–1.04, $P < 0.001$) [5]. On the basis of these results, XP is now considered one of the standard treatments of AGC [10], and XP was adopted as the reference arm in two recent global studies of molecular targeting agents [11,12]. However, data is scarce with respect to XP treatment in Japanese patients, and also the usefulness of XP as compared with SP has not yet been assessed.

As another issue, these 2 types of oral fluoropyrimidine show some different characteristics in the mechanisms of their antitumor effect. A subset analysis of the FLAGS trial showed that S-1 seemed to be better than 5-FU in the subgroup with diffuse-type gastric cancer [6]. This result was consistent with the results of a subset analysis of the JCOG9912 trial, which showed that S-1 was better than 5-FU in patients with diffuse-type gastric cancer or with gastric cancer associated with high dihydropyrimidine dehydrogenase (DPD), with diffuse-type tumors associated more commonly than intestinal type with high DPD [13]. This result was expected, since S-1 consists of tegafur, otastat potassium, and gimestat which is a potent competitive inhibitor of DPD. Capecitabine is transformed to 5-FU in several steps, to be finally converted by TP as above [9]. A phase II trial in Japan showed that response rate (RR) was significantly higher (Fisher's exact test, $p = 0.028$) in patients with TP-positive and DPD-negative tumors (60%, 6/10) than in the remaining

patients (13%, 2/15) [14]. In contrast, high expression of TP is reported to be negatively associated with efficacy of 5-FU or S-1 in gastric cancer [15,16].

On the basis of the above reports, histological type (diffuse or intestinal) and biomarkers (TP, DPD, and others) may be candidates to select whether S-1 or capecitabine be used for each patient, although validation with a randomized study is necessary. We planned the current clinical trial to elucidate the efficacy of XP and SP for the first-line treatment of AGC. This comparison will be also evaluated in terms of several biomarkers.

Method/design

Study objective

This randomized phase II trial is planned to elucidate the efficacy of SP and XP and also to explore predictive or prognostic biomarkers with additional research. This trial protocol has been approved by the Institutional Review Board (IRB) of each participating institution and the Kanagawa Cancer Center.

Study endpoints

Primary endpoint is PFS and secondary endpoints are OS, RR, time to treatment failure (TTF), and incidence of adverse events (safety).

Eligibility criteria

Inclusion criteria

- (i) Histologically confirmed gastric adenocarcinoma with unresectable metastatic or recurrent disease
- (ii) Lesions confirmed by imaging no more than 28 days before registration (not required for measurable lesions as defined in RECIST version 1.1)
- (iii) No previous chemotherapy or radiotherapy.
However, prior adjuvant chemotherapy is allowed if more than 6 months has passed since the end of adjuvant chemotherapy
- (iv) Eastern Cooperative Oncology Group (ECOG) Performance Status of 0, 1, or 2
- (v) Life expectancy of at least 3 months after registration
- (vi) Written informed consent
- (vii) Between the ages of 20 and 74 years at the time informed consent is obtained
- (viii) Adequate major organ function including:
 - (a) Neutrophil count: $\geq 1500/\text{mm}^3$
 - (b) Platelet count: $\geq 10.0 \times 10^4/\text{mm}^3$
 - (c) Hemoglobin: $\geq 9.0 \text{ g/dL}$
 - (d) AST, ALT: $\leq 2.5 \times$ upper limit of normal (ULN) in each institution (≤ 5 times in cases of metastases to liver)
 - (e) ALP: $\leq 2.5 \times$ ULN in each institution (≤ 5 times in cases of metastases to liver; and ≤ 10 times in cases of metastases to bone)

- (f) Total bilirubin: $\leq 1.5 \times \text{ULN}$ in each institution
- (g) Creatinine clearance: $\geq 60 \text{ mL/min}$ (as estimated by Cockcroft-Gault equation)

Exclusion criteria

- (i) HER2- positive status
- (ii) Previous history of fluoropyrimidine therapy within 6 months prior to registration
- (iii) Previous treatment with platinum agents within 12 months prior to registration
- (iv) Previous treatment with cisplatin more than total dose of 120 mg/m^2
- (v) Previous history of serious hypersensitivity to fluoropyrimidines or platinum agents
- (vi) Previous history of adverse reactions suggestive of dihydropyrimidine dehydrogenase (DPD) deficiency
- (vii) More than 1 cancer at the same time or more than 1 cancer at different times separated by a 5-year disease-free interval. However, multiple active cancers do not include carcinoma *in situ* or skin cancer which is determined to have been cured as a result of treatment.
- (viii) Obvious infection or inflammation (pyrexia $\geq 38.0^\circ\text{C}$)
- (ix) Active hepatitis
- (x) Heart disease that is serious or requires hospitalization, or history of such disease within the past year
- (xi) Having a complication that is serious or requires hospitalization (intestinal paralysis, intestinal obstruction, interstitial pneumonia or pulmonary fibrosis, poorly controlled diabetes mellitus, renal failure, liver disorders, or hepatic cirrhosis)
- (xii) Being treated or in need of treatment with flucytosine, phenytoin, or warfarin potassium
- (xiii) Chronic diarrhea (watery stools or ≥ 4 times/day)
- (xiv) Active gastrointestinal bleeding
- (xv) Body cavity fluids requiring drainage or other treatment
- (xvi) Clinical suspicion or previous history of metastasis to brain or meninges
- (xvii) Women who are pregnant, breastfeeding, or potentially (hoping to become) pregnant
- (xviii) Unwillingness to practice contraception
- (xix) Poor oral intake
- (xx) Psychiatric disorders which are being, or may need to be, treated with psychotropics
- (xxi) Otherwise determined by investigators or site principal investigators to be unsuitable for participation in study

Registration

Physicians or coordinators will send a Case Registration Form to the data center (Epidemiological and Clinical

Research Information Network, ECRIN) with all the required items filled out. Enrollment has started from July 2011.

Startification

Eligible patients will be randomized to either Arm-A (SP treatment) or Arm-B (XP treatment) by dynamic allocation via a centralized randomization method using 5 stratification factors as balancing variables:

- (i) baseline ECOG Performance Status (0–1/2)
- (ii) measurable lesion (yes/no)
- (iii) prior adjuvant chemotherapy (yes/no)
- (iv) histopathological classification (intestinal/diffuse)
- (v) institution.

Statistical analysis

PFS has been set as the primary endpoint and is defined as the time from date of registration until the date that progression is determined or the date of death for any reason, whichever is sooner. "Progression" will be evaluated on the basis of Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1 [17]. More information about the definition of PFS and Progression are pre-specified (Table 1).

The primary objective of this trial is to evaluate the PFS of SP and XP as the first-line treatment for advanced gastric cancer. The 24-week progression-free rate (PFR) will be estimated for each group, calculating point estimates and 2-sided 90% confidence intervals. The 2-sided 90% confidence interval of the difference between the 2 groups will be also estimated. Exploratory analysis will be done to test the null hypothesis that PFS is equal in both groups. Cumulative PFS curves will be constructed as time-to-event plots by the Kaplan-Meier method.

With respect to secondary endpoints, efficacy endpoints OS and TTF will be evaluated according to the method of analysis of the primary endpoint. Overall response rate (RR) is defined as the proportion of patients with complete response (CR) or partial response (PR) by RECIST out of the patients with measurable lesions, and the chi-square test will be used to compare the 2 groups. The 2-sided 95% confidence interval of the difference between the 2 groups will also be estimated. For the analysis of safety, Fisher's exact test will be used if necessary, and the exact confidence intervals for the binomial distribution will be estimated.

Sample-size calculation

Assuming a threshold 24-week PFR of 40% and an expected 24-week PFR of 55% (clinically promising), and a 1.5-year registration period and a 1.5-year follow-up period, 49 patients are required in each group to ensure a 1-sided alpha of 5% and statistical power of 90%. Assuming that the 24-week PFR of the biomarker-positive (any FU-related enzyme or expression of intestinal type) population

Table 1 Definition of PFS and progression

Definition of PFS and progression are predefined as below
1.) PFS will be determined as the time from the date of registration until the date that progression is determined or the date of death for any reason, whichever is sooner.
2.) "Progression (PD)" means both PD confirmed by routine diagnostic imaging in each course and PD confirmed by as-needed diagnostic imaging in the case that there is clinical suspicion of PD. In the latter case, it is preferable that there is at least objective evidence.
3.) When progression is determined based on diagnostic imaging, the date of progression will be the date on which imaging is assessed. When clinical progression is first determined independently of diagnostic imaging, and then later objectively determined on the basis of diagnostic imaging, the date of progression will be back-dated to the date of determination of clinical progression. If no objective evidence is obtained, it will be treated as a censoring event in the formal analysis, and sensitivity analysis will be also conducted as if this were PD.
4.) When considering tumor regrowth and determining PD according to RECIST, it is considered a PD as PFS event regardless of tumor diameter. But even if it is decided as PD according to RECIST, investigators can continue the protocol treatment if they consider continued treatment to be beneficial to the patient.
5.) If treatment discontinuation is needed due to symptomatic deterioration without any objective evidence at that time, it is reported as "symptomatic deterioration". Investigators should endeavor to obtain objective evidence of the progression even after discontinuation of treatment. In this case, the event shall be judged to be clinical PD and handled as mentioned in 2) above. When progression is determined on the basis of diagnostic imaging, the date of progression will be back-dated to the date of diagnosis of symptomatic deterioration.
6.) Survivors for whom progression has not been determined will be censored based on the last date on which the absence of progression was clinically confirmed (the last day that PFS was confirmed).
7.) Cases of discontinuation of protocol treatment because of toxicity or patient refusal, even if another therapy is added as a post-treatment, will be censored at the date of discontinuation or the date that post-treatment was started.
8.) In cases where progression is diagnosed on the basis of imaging, the event will be determined based not on evaluation dates where the result is "suspected" on imaging but on a subsequent evaluation date where progression is "confirmed" on imaging.
9.) Secondary cancer (multiple cancers in metachronous) will not be regarded as either an event or censored.

in the SP arm is 45%, and the risk reduction rate in the XP arm is 40%, 46 patients in total are needed to ensure a 2-sided alpha of 10% and statistical power of 70%. Under the hypothesis that the targeted biomarker-positive population is 50%, 92 patients in total are required. Considering the likelihood of some ineligible cases in the whole setting outlined above, the total sample size is set to 100. A following Phase III study will be designed for both randomized comparison and biomarker-oriented comparison of XP and SP (4 groups).

Treatment program

Patients who allocated SP will be treated with S-1 and cisplatin every 5-week cycle. S-1 will be administered orally at

a dose of 40 mg/m² twice-daily (equivalent to a total daily dose of 80 mg/m²) for 3 weeks (day 1 to 21). Cisplatin 60 mg/m² on day 8 of each cycle will be given by intravenous infusion over 2 hours. On the other hand, patients who allocated XP will be treated with capecitabine and cisplatin every 3-week cycle. Capecitabine will be administered orally at a dose of 1000 mg/m² twice-daily (equivalent to a total daily dose of 2000 mg/m²) for 2 weeks (day 1 to 14). Cisplatin 80 mg/m² on day 1 of each cycle will be given by intravenous infusion over 2 hours.

Treatment continuation is intended until disease progression or unacceptable toxicity. If treatment continuation with cisplatin is determined to be unfeasible before any progression is confirmed, continuously monotherapy of S-1 or capecitabine will be continued until PD.

Follow-up

During treatment under this protocol, patients will have a physical check-up and a blood examination before every drug administration. PFS and RR will be monitored by using abdominal CT or MRI every 6 weeks and by measuring levels of tumor markers CEA and CA19-9.

Translational research project

Translational research will be conducted to elucidate the clinical utility of the following biomarkers. These biomarkers will be analyzed Immunohistochemistry (IHC) and mRNA expression by using tissue specimen. Tumor tissue samples from primary lesions and/or biopsy material will be collected and centralized assessment.

- (i) Immunohistochemistry (IHC): Expression of TP, DPD, ERCC1, Ki67, LGALS4, and CDH17
- (ii) mRNA: Expression of TP, DPD, thymidylate synthase (TS), orotate phosphoribosyltransferase (OPRT), and excision repair cross-complementation group1 (ERCC1)

Discussion

Recently, molecular target drugs has resulted in the opportunity to provide individualized treatment in the field of AGC. Especially in patients with HER2-positive AGC (defined as assessed by IHC 3+ on a scale of 0 to 3+, and/or fluorescence in-situ hybridization; FISH, *HER2:CEP17* ratio ≥ 2.0), ToGA study showed that adding trastuzumab was significantly improved overall survival comparing with standard chemotherapy consists of cytotoxic drugs [11]. This study excludes HER2-positive gastric cancer since these patients should be recommended trastuzumab containing regimen. The individualized treatment for cytotoxic agents also needs to be developed to have more effect and less toxicity.

This is the first study to compare two standard regimens for AGC. Additionally, the translational research is

performed to explore the biomarker for chemo-sensitivity and make the individualized treatment possible. When the difference of treatment is found in efficacy or safety from this analysis, we will conduct a phase III trial to examine the possibility of individualized treatment. We believe the result of this study will play the important role to prepare the individualized therapy for advanced gastric cancer in the near future.

Competing interests

All authors declare that they have no competing interest.

Authors' contributions

AT drafted the manuscript and wrote the original protocol for the study. All authors participated in the design of the study. SM performed the statistical analysis. All authors read and approved the final manuscript.

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Combination of the Tumor Angiogenesis Inhibitor Bevacizumab and Intratumoral Oncolytic Herpes Virus Injections as a Treatment Strategy for Human Gastric Cancers

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Key Words:

Oncolytic virus;
Herpes virus; Vastin; Bevacizumab;
Gastric cancer.

Abbreviations:

Vascular Endothelial Growth Factor A (VEGFA); Dulbecco's Modified Eagle's Medium (DMEM); Fetal Calf Serum (FCS); Dendritic cell (DC); Enzyme Linked Immunosorbent Assay (ELISA).

ABSTRACT

Background/Aims: Advanced gastric cancer is difficult to treat due to the frequency of liver metastases and peritoneal dissemination. A combination of two new strategies, including the anti-angiogenesis inhibitor Bevacizumab and an oncolytic herpes virus is a promising treatment for advanced cancer. **Methodology:** The effects of Bevacizumab on oncolytic herpes virus replication and viral cytotoxicity were examined at varying Bevacizumab concentrations and viral titers. In addition, the ability of these two new promising anticancer agents to inhibit tumor growth was studied. Histological examinations of CD31 and LacZ were used to assess angiogenesis and virus distribu-

tion within the tumor, respectively. **Results:** Bevacizumab did not affect viral replication or viral cytotoxicity *in vitro*. The combination of Bevacizumab and the oncolytic herpes virus hrR3 significantly reduced tumor growth *in vivo* in an experimental gastric cancer model. Bevacizumab inhibited angiogenesis caused by local injection of hrR3 and induced virus spread. Bevacizumab increased the distribution of the intratumorally injected oncolytic herpes virus within the tumor. **Conclusions:** Combination therapy consisting of Bevacizumab and an oncolytic herpes virus is a promising new treatment strategy for gastric cancer.

INTRODUCTION

Bevacizumab (Avastin) is a genetically engineered humanized monoclonal antibody that was derived from the murine anti-human vascular endothelial growth factor A (VEGFA) monoclonal antibody A4.6.1. It specifically binds to human VEGFA thereby blocking the ability of VEGFA to bind to the VEGF receptors expressed on vascular endothelial cells. By blocking the biological activity of VEGFA, Bevacizumab and its murine equivalent, A4.6.1, inhibit neovascularization in tumor tissues and thus suppress tumor growth (1-3). Gastric cancer is the most common gastroenterological cancer in the world. Advanced gastric cancer can easily disseminate into the peritoneum and metastasize to the liver. Even when treated surgically with radiation and chemotherapy, advanced gastric cancer is difficult to treat. Currently, anti-angiogenic strategies that block tumor vasculature are being tested in patients as a therapeutic strategy for gastric cancer (4-5). Although these studies have provided encouraging results, it is becoming increasingly obvious that multiple therapeutic approaches in combination with Bevacizumab are essential to treat many types of

cancer. Another promising new strategy is the use of oncolytic viruses, which generally infect cancer cells due to tumor-specific properties. Aghi *et al.* described the possibility that wild type and oncolytic herpes virus lead to tumor angiogenesis, and thrombospondin-derived anti-angiogenesis peptide enhanced tumor growth inhibition. Therefore anti-angiogenesis effects should be considered when designing oncolytic herpes virus therapies (6). Thus, we examined the efficacy of combination therapy with Bevacizumab and a representative oncolytic herpes virus, hrR3 (7-9), in an experimental model of human gastric cancer. Previous studies have examined the effects of Bevacizumab on oncolytic viruses. Silvanina *et al.* reported that Bevacizumab increased the distribution of an intratumorally injected oncolytic adenovirus in human anaplastic thyroid cancer xenografts and enhanced the therapeutic effects of this virus (10). Generally, one major obstacle in successfully applying therapeutic strategies that are based on replicating oncolytic virus is poor virus distribution. Bibrao *et al.* showed that the blood-tumor barrier limits gene transfer to the tumor in an experimental liver cancer (11) and McKee

et al. showed that degradation of fibrillar collagen in a human melanoma xenograft improves the efficacy of an oncolytic herpes simplex virus (12). Similar data have been reported in clinical studies with retroviruses (13). The vascular supply of necrotic areas, the distorted functional properties of tumor vessels and elevated tumor interstitial fluid pressure may contribute to unequal viral distribution within the tumor and thereby reduce the uptake of the oncolytic virus by malignant cells. Administering monoclonal antibodies specific for vascular endothelial growth factor A (VEGFA) or VEGF receptor 2 has been shown to increase the uptake and efficacy of chemotherapy in experimental tumor models and clinical studies (14,15). It has been suggested that vascular remodeling or the normalization of tumor vessels after anti-angiogenic treatment of solid tumors improves the delivery of chemotherapeutic drugs to the tumor tissue (16). It was previously shown that the humanized anti-VEGFA monoclonal antibody lowered the extracellular tumor fluid volume, reduced the interstitial fluid pressure and modulated inflammation in xenograft tumors of human anaplastic thyroid cancer cells (17). In addition, several papers have examined the efficacy of combination therapy with oncolytic viruses and Bevacizumab (18,19), but there are only a few studies on oncolytic herpes viruses and Bevacizumab. Eshun *et al.* reported that Bevacizumab enhanced the therapeutic efficacy of an oncolytic herpes virus but decreased virus distribution in tumors (18). Although they showed that this combination therapy resulted in decreased tumor uptake of the systemic oncolytic herpes virus, our present data indicate that Bevacizumab enhances oncolytic herpes virus distribution within the tumor after direct and local injection of the virus. Therefore, it is important to present our findings illustrating the efficacy of combination therapy with an oncolytic herpes virus and Bevacizumab in a model of experimental human gastric cancer.

METHODOLOGY

Virus and cells

Vero cells, an African Green monkey kidney cell line, as well as cancer cell lines Capan-1 and MIA PaCa-2, derived from a human pancreatic cancer; Hep3B and PLC/PRF/5 derived from a human hepatic cancer; AZ521 and MKN45 derived from a human gastric cancer; WiDr, derived from a human colon cancer; and SKOV-3, derived from a human ovarian cancer; were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Sigma, Tokyo, Japan) at 37°C in a humidified atmosphere with 5% CO₂. hrR3 is a mutated HSV-1 that has the LacZ gene inserted into UL39 (ICP6), which inactivates the ribonucleotide reductase activity associated with UL39, and was kindly provided by Dr. Sandra K. Weller (University of Connecticut, Storrs, CT, USA). The virus was propagated and tittered on Vero cells and stored at -80°C until further use.

VEGFA enzyme linked immunosorbent assay (ELISA)

For the quantitative determination of human VEGF concentrations in cell culture supernatants; 5X10⁶ cells were culture at 48 hours before assay under the condition of DMEM containing 3% FCS. The supernatants were collected and the determination of VEGF concentrations was performed by ELISA using the Quantikine

Human VEGFA Immunoassay kit from R&D Systems (R&D Systems, Tokyo, Japan) according to the protocol provided by the manufacturer.

Determination of VEGFA mRNA levels by quantitative RT-PCR

Tumor cells (2×10⁵) were cultured in 2mL DMEM or RPMI 1640 with 10% FCS for 48 hours. Cellular VEGF expression was determined by reverse transcription-PCR (RT-PCR). Total cellular RNA was isolated using the RNeasy Mini kit and RNase-free DNase kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocols. Total RNA was reverse transcribed using an Omniscript RT kit (Qiagen, Tokyo, Japan). The VEGF and β -actin primers were as follows: VEGF sense (5'-TCCAGGAGTACCCTGATGAG-3') and anti-sense (5'-CTTCCTGGTGAGAGATCTGG-3'); β -actin sense (5'-AAGAGAGGCATCCTCACCT-3') and anti-sense (5'-TACATGGCTGGGGTGTGAA-3').

Proliferation of tumor cells

Cell proliferation was measured using the MTT dye reduction method. Briefly, the tumor cells (5000/well) were plated in 96-well plates and incubated in DMEM containing 5% FCS for 24 hours. Next, the cells were washed and incubated for 24, 48 or 72 hours with several concentrations of Bevacizumab (0, 0.01, 0.1, 1 or 10 μ g/mL) and infected with hrR3 at several MOIs (MOI of 0, 0.01, 0.1, 1 or 10) in fresh MEM containing 5% FCS. Then, 50 μ L of the stock MTT solution (2mg/mL; Sigma) was added to all of the wells and the cells were incubated for 2 hours at 37°C. The media containing the MTT solution was removed and the dark blue crystals were dissolved by adding 100 μ L DMSO. Absorbance was measured with an MTP-120 micro plate reader at the test and reference wavelengths of 550 and 630nm, respectively. Cell survival was calculated as a percentage of the control (mock-infected) cells. Tests were performed in triplicate.

Viral replication assay

Viral proliferation was measured by plating 1×10⁵ cells in 6-well plates overnight and then treating them with hrR3 at an MOI of 1 and several concentrations of Bevacizumab (0, 0.01, 0.1, 1 or 10 μ g/mL). Each day for three days, the cells were scraped and collected and the culture supernatants were harvested. The cells were subjected to three freeze-thaw cycles to release intracellular virus. Diluted samples were added to plates of confluent Vero cells for standard viral plaque assays. All conditions were measured in triplicate.

Orthotopic implantation model

To determine whether the combination of hrR3 and Bevacizumab enhances antitumor activity *in vivo*, cultured MKN45 cells were harvested by pipetting. The cells were washed twice and re-suspended in PBS. The tumor cell suspension (1×10⁶/100 μ L) was injected subcutaneously into the lower right flank of nude mice. Eight- to ten-week-old BALB/c-nu/nu mice were obtained from Charles River, Tokyo, Japan. The mice were divided into groups and treated when the tumor size reached 20-30mm³. The treatment groups were PBS (n=6), hrR3 (n=6), Bevacizumab (n=6) and hrR3 plus Bevacizumab (n=6). hrR3 was administered intratumorally (1×10⁷pfu/mL) and Bevacizumab (100mg/mouse) was administered intracisternally. hrR3 and

Bevacizumab were administered twice weekly on the same day (Monday and Thursday) for two weeks. The animals were weighed weekly and tumor growth was measured twice weekly. The tumor size was calculated as $L \times W \times 3/4$. The animals were sacrificed *via* CO₂ inhalation when the experiment was terminated.

In a separate set of experiments, the animals were treated as described above and sacrificed two days after they received the last treatment. The tumors were harvested from each mouse and were snapped frozen in liquid nitrogen and examined by β -galactosidase (LacZ) histochemistry and CD31 immunohistochemistry. Animal experiments were conducted following the regulations of the Nagoya University Animal experiment approval committee.

Immunohistochemical analysis

Fast frozen tumors from mice that received the various treatments were cut in 4–6 μ m thick sections and subjected to immunohistochemical staining for CD31 and LacZ. CD31 was detected using an anti-CD31 primary antibody (dilution 1/100, BD Pharmingen, San Jose, CA, USA). For LacZ staining, the sections were fixed with 1% glutaraldehyde and LacZ expression was examined by staining with X-gal solution. Angiogenesis was determined by counting the number of CD31-stained foci per high power field. Virus distribution was estimated by LacZ staining based on the relative fold induction area and expressed as a percentage.

Quantification of immunohistochemistry

The five areas containing the greatest staining within a section were selected for histological quantification by light microscopy with 40-fold magnification. The results were evaluated by two authors.

Statistical analysis

Statistical analysis was performed using an unpaired two-tailed *t*-test (SPSS 17.0).

RESULTS

Determination of VEGF protein levels

The VEGFA expression levels of eight representative tumor cells were estimated by examining the VEGFA RNA levels in the cells and the VEGFA protein levels in the cell supernatant. SKOV-3 cells (human ovarian cancer) are known to secrete high levels of VEGFA and were used as positive controls for both cellular VEGFA gene expression and secreted VEGFA in the supernatant. MKN45 cells (human gastric cancer) had the second highest levels of VEGFA gene expression and secreted VEGFA protein by ELISA. Based on the ELISA, WiDr cells (human colon cancer) had the lowest levels of secreted VEGFA in the supernatant and AZ521 (Human gastric cancer) had the lowest cellular VEGFA gene expression (Figure 1).

Viral cytotoxicity assay

We selected MKN45 cells and AZ521 as examples of gastric cancer cell lines that secrete high and low levels of VEGFA respectively, as well as the human ovarian cancer SKOV-3 for the viral cytotoxicity assays with varying concentrations of Bevacizumab. After 24 hours of infection, viral cytotoxicity was examined in these three cell lines. Virus MOIs of 0 and 0.01 did not induce cytotoxicity in these three cell lines. At an MOI of 10, approximately 40% of AZ521 and MKN45 cells survived, and 60% of SKOV cells survived. At an MOI of 1, approximately 60% of AZ521 and MKN45 cells survived, and 80% of SKOV cells survived. Bevacizumab did not have any effects on viral cytotoxicity, even at high doses (10mg/mL) and there were no changes in viral cytotoxicity with the different concentrations of Bevacizumab. After 48 hours of infection, viral cytotoxicity gradually increased with viral replication but Bevacizumab did not cause any significant effects at the examined MOIs. After 72 hours of viral infection, MOIs of 0.1–10 resulted in approximately 20% cell survival in AZ521 and MKN45 cells, and 30% cell survival in SKOV cells, while an MOI of 0.01 resulted in approximately 20% cell survival for AZ521 cells, 40% survival for MKN45 cells and 40% survival for SKOV cells. Bevacizumab did not have any effects on viral cytotoxicity (Figure 2).

Viral replication assay

MKN45 cells, a gastric cancer cell line that secretes high levels of VEGFA, AZ521 cells, a gastric cancer cell line that secretes low levels of VEGFA, and the human ovarian cancer cell line SKOV-3, were selected for the viral replication assay. The viral titers were determined after 72 hours of infection for each concentration of Bevacizumab. The titers revealed approximately 10^{7-8} pfu/mL viral replication in AZ521 and SKOV-3 cells and 10^{5-6} pfu/mL viral replication in MKN45 cells. When virus was infected at 10^5 pfu/mL, the titers were 100 times higher on AZ521 and SKOV-3 cells and 10 times higher on MKN45 cells. The virus replication capacity was not very high on MKN45 cells at 72 hours post infection. Bevacizumab did not have any effects on viral replication at each of the examined concentrations ranging from 0 to 10mg/mL (Figure 3).

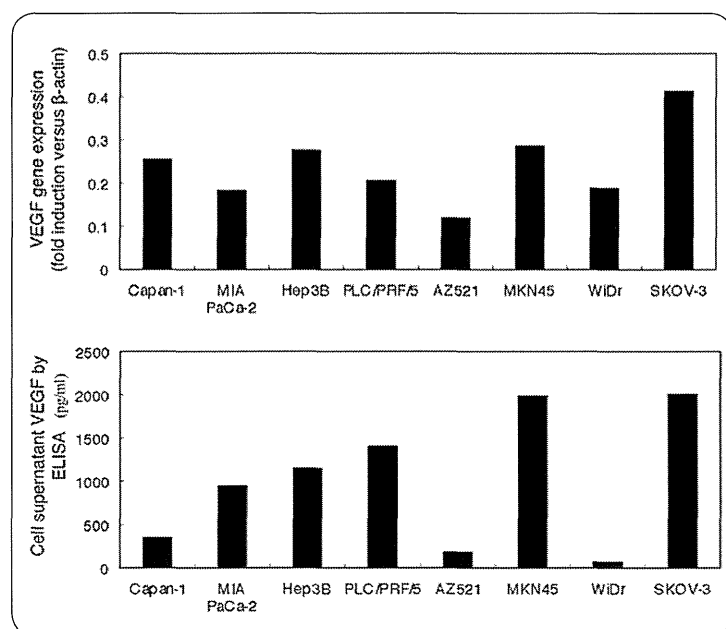


FIGURE 1. Determination of VEGFA protein levels. Eight representative tumor cells were examined for VEGFA RNA levels in cells and VEGFA protein levels in the supernatant. SKOV-3 cells are known to secrete high levels of VEGFA and were therefore used as a positive control for cellular and secreted VEGFA. MKN45 had the second highest levels of VEGFA gene expression and secreted VEGFA protein by ELISA. Based on the ELISA assay, WiDr cells secreted the lowest levels of VEGFA protein into the supernatant and AZ521 cells expressed the lowest levels of the VEGFA gene.

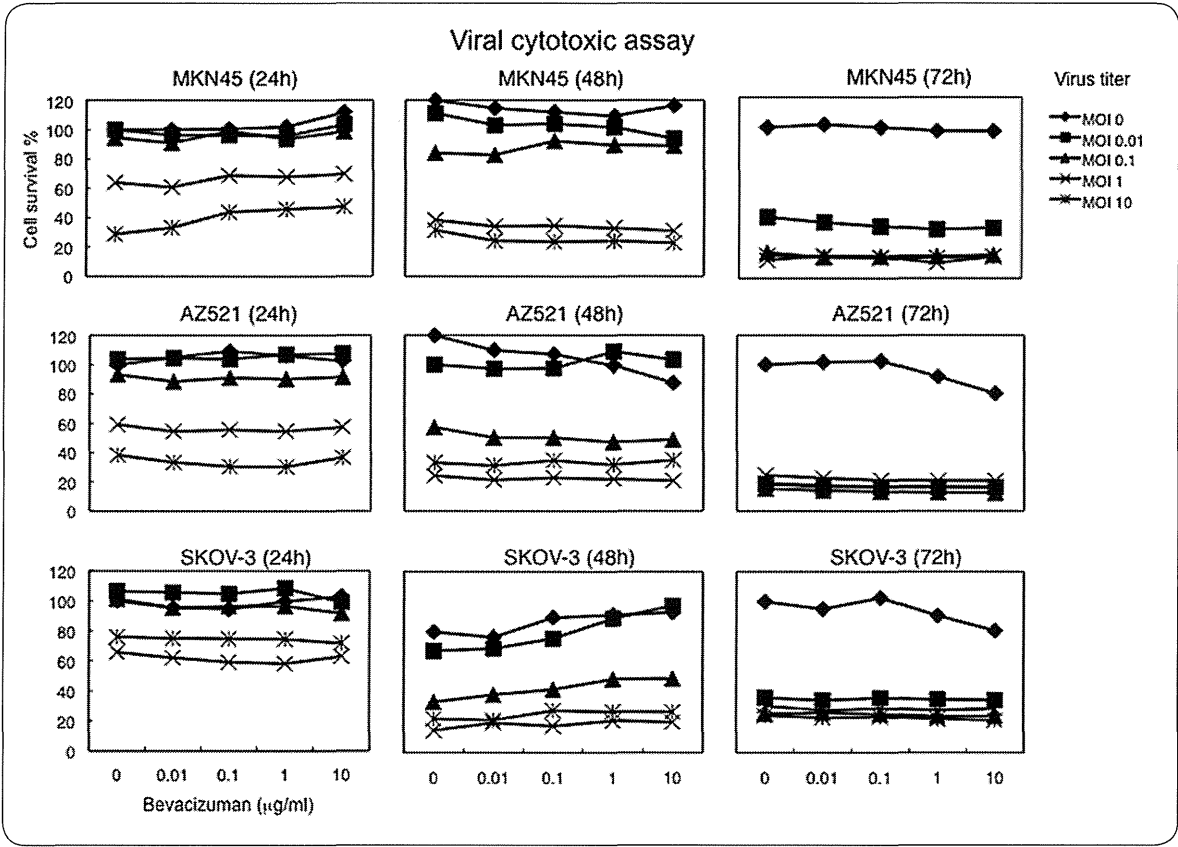


FIGURE 2. Viral cytotoxic assay. MKN45 cells (gastric cancer), which secrete high levels of VEGFA, AZ521 cells (gastric cancer), which secrete the lowest levels of VEGFA, and SKOV-3 cells were used for the assay. After 48h of viral infection, viral cytotoxicity gradually increased with viral replication. After 72h of viral infection, MOIs of 0.1-10 resulted in approximately 20% survival for AZ521 and MKN45 cells, and 30% survival for SKOV cells. At an MOI of 0.01, the cell survival was approximately 20% for AZ521 cells, 40% for MKN45 cells and 40% for SKOV cells. Bevacizumab did not have any effects on viral cytotoxicity, even at high doses (10 $\mu\text{g/ml}$). There were no changes in viral cytotoxicity across the different Bevacizumab concentrations.

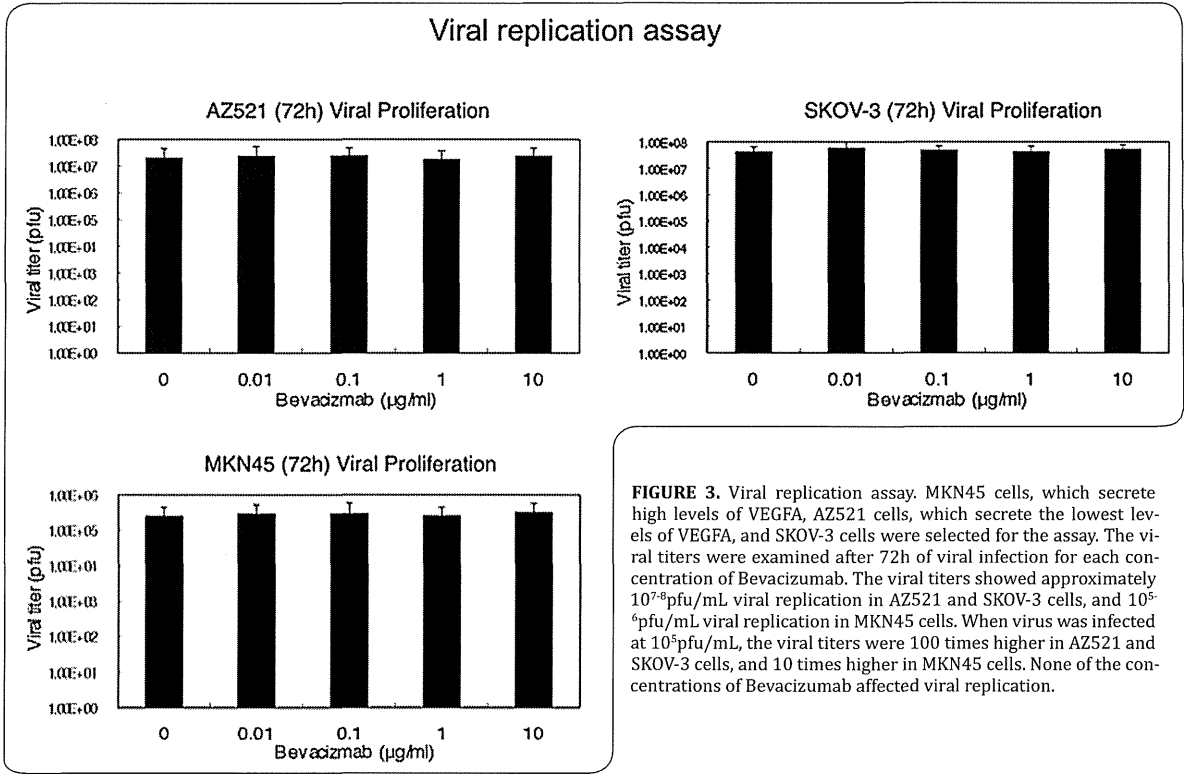


FIGURE 3. Viral replication assay. MKN45 cells, which secrete high levels of VEGFA, AZ521 cells, which secrete the lowest levels of VEGFA, and SKOV-3 cells were selected for the assay. The viral titers were examined after 72h of viral infection for each concentration of Bevacizumab. The viral titers showed approximately 10^{7-8} pfu/mL viral replication in AZ521 and SKOV-3 cells, and 10^5 pfu/mL viral replication in MKN45 cells. When virus was infected at 10^5 pfu/mL, the viral titers were 100 times higher in AZ521 and SKOV-3 cells, and 10 times higher in MKN45 cells. None of the concentrations of Bevacizumab affected viral replication.

Tumor growth inhibition assay

The gastric cancer cell line MKN45, which secretes high levels of VEGFA, was used for the tumor growth inhibition assay. Mice were injected subcutaneously with 1×10^6 MKN45 cells. hrR3 and Bevacizumab were administered twice weekly on the same day (Monday and Thursday) for two weeks. The combination of hrR3 and Bevacizumab significantly inhibited tumor growth and the tumors disappeared in two of six mice. hrR3 alone inhibited tumor growth better than Bevacizumab or PBS alone, even though the virus did not replicate to high levels in MKN45 cells *in vitro*. There were statistically sig-

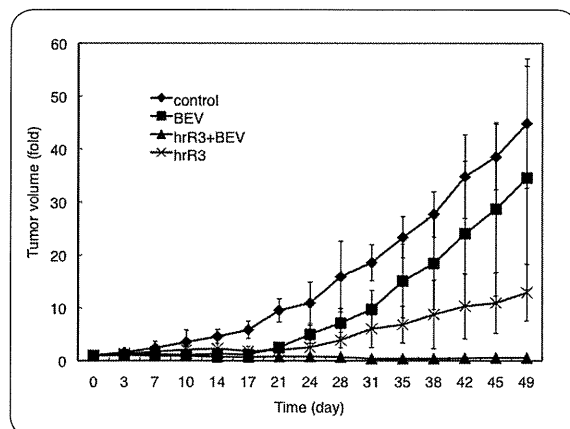


FIGURE 4. Tumor growth inhibition assay. The gastric cancer cell line MKN45, which secretes high levels of VEGFA, was used for tumor growth inhibition assays. Mice were injected with 1×10^6 MKN45 cells subcutaneously.

nificant differences in tumor growth on day 49 between hrR3 and Bevacizumab combination therapy and hrR3 alone ($p < 0.001$) or Bevacizumab alone ($p < 0.003$) (**Figure 4**).

Angiogenesis assay

Bevacizumab inhibited tumor angiogenesis to a greater degree than the control ($p < 0.001$) or hrR3 alone ($p < 0.001$). hrR3 infection alone resulted in a significantly higher rate of angiogenesis compared to the control ($p < 0.001$). However, hrR3 and Bevacizumab combination therapy resulted in a significantly reduced rate of angiogenesis compared to hrR3 alone ($p < 0.001$) and this combination therapy also tended to reduce angiogenesis compared to the control ($p < 0.07$). Bevacizumab inhibited tumor angiogenesis that was induced by hrR3 (**Figure 5**).

Virus distribution assay

Because hrR3 encodes the galactosidase gene, LacZ staining can be used to examine virus distribution. An area of hrR3 infection was seen surrounding the virus inoculation site. The combination of hrR3 and Bevacizumab increased the number of infectious foci and enlarged the virus infection area within the tumor. A combination of hrR3 and Bevacizumab resulted in a significantly higher rate of virus distribution compared to hrR3 alone ($p < 0.001$) (**Figure 5**).

DISCUSSION

We examined how the tumor angiogenesis inhibitor Bevacizumab affects the therapeutic efficacy of the on-

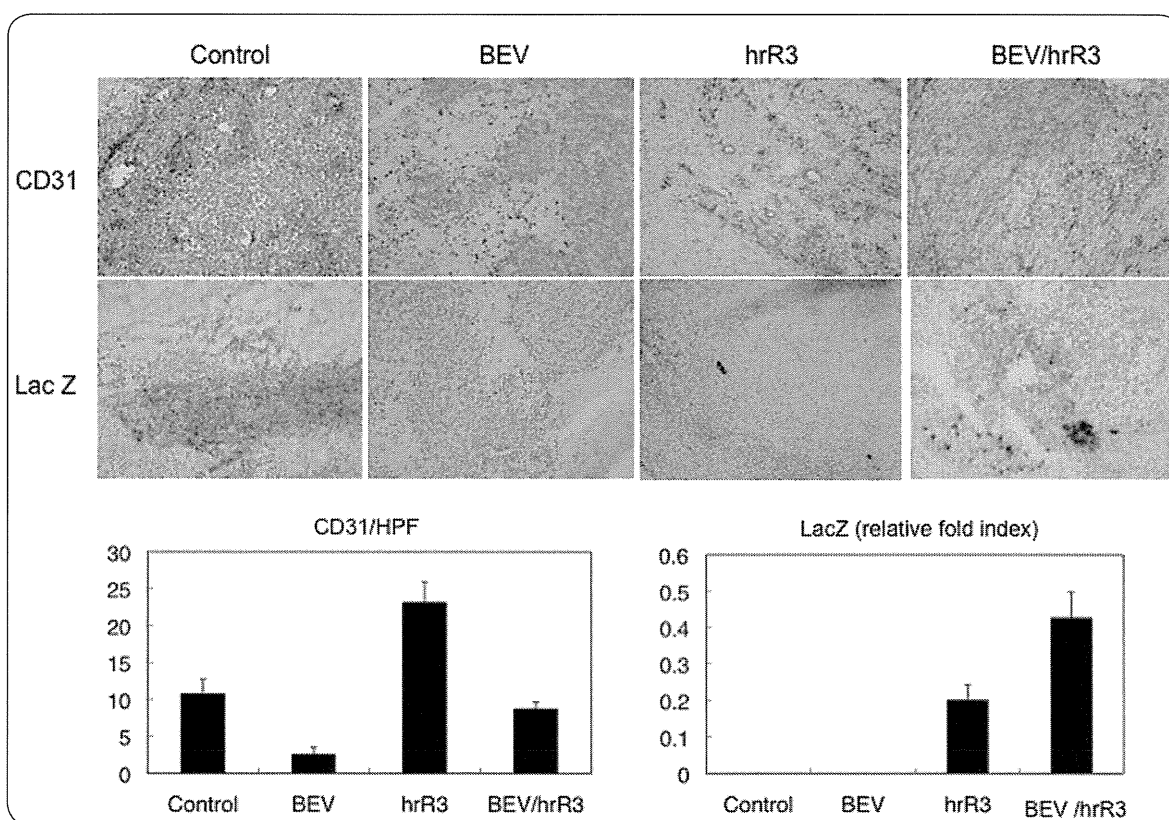


FIGURE 5. Angiogenesis and virus distribution assay. Tumors from mice that received various treatments were immunohistochemically examined for CD31 expression. The combination of hrR3 and Bevacizumab resulted in a significantly reduced rate of angiogenesis compared to hrR3 alone ($p < 0.001$), and this combination therapy tended to reduce the rate of angiogenesis compared to the control ($p < 0.07$).

colytic herpes virus hrR3 and investigated the therapeutic effects of Bevacizumab and hrR3 combination therapy in human gastric cancers. The human gastric cancer cell line MKN45 secretes high levels of VEGFA, while AZ521 cells secrete low levels of VEGFA based on ELISA and RT-PCR analyses. The angiogenesis inhibitor Bevacizumab did not affect viral cytotoxicity or viral replication *in vitro* assays with AZ521 and MKN45 cells, even at the highest concentration. However, the combination of Bevacizumab and hrR3 significantly inhibited tumor growth *in vivo* in a subcutaneous gastric cancer model. Histological examinations were conducted to examine differences *in vitro* and *in vivo*. The combination of Bevacizumab and hrR3 significantly inhibited tumor angiogenesis compared to hrR3 alone and there was a trend for reduced angiogenesis compared to the control after two days of treatment. Bevacizumab and hrR3 combination therapy resulted in significantly greater virus distribution in the tumor tissue compared to hrR3 alone. In the present study, Bevacizumab appeared to enhance both virus distribution and the virus-mediated reduction in tumor growth in a gastric cancer model. However, Eshun *et al.* showed that the distribution of an oncolytic herpes virus within the tumor site was inhibited by Bevacizumab (18). Importantly, both analyses (our data and Eshun *et al.*) showed that combination therapy with Bevacizumab and an oncolytic herpes virus inhibited tumor growth to a greater extent than the virus alone or Bevacizumab alone, although the treatment method and timing as well as the type of oncolytic herpes virus (rRp450 vs. hrR3) differed between these two studies. Eshun *et al.* first injected Bevacizumab intraperitoneally followed by systemic virus intravenously after three days (or systemic virus intravenously followed by Bevacizumab intraperitoneally after three days), while we administered the virus intratumorally and Bevacizumab intraperitoneally at the same time. Several different types of interference could result in differences in virus distribution. Tong *et al.* reported that anti-angiogenic agents typically reduce the interstitial pressure to the lowest point within 2-4 days (20-22). The interstitial fluid pressure in the tumor might be elevated due to the leakiness of the tumor vessels (23). Stohrer *et al.* noted that differences in the oncotic and hydrostatic pressure control the movement of fluid and large molecules across the microvascular walls of normal and tumor tissues. Recently, their studies have shown that tumors have increased interstitial fluid pressure that is approximately equal to the microvascular pressure (24). In addition, Tong *et al.* demonstrated that the normalization process prunes immature vessels and improves the integrity and function of the remaining vasculature by enhancing perivascular cell and basement membrane coverage (20). The size of the virus and expression of the

relevant receptor on the vessel may also affect virus invasion into the tumor vasculature and tissue. If the virus is injected into the vessel systemically, Bevacizumab-induced normalization could change the structure of endothelial surface receptor and the environment of immature perivascular cells. In the present study, we directly injected the oncolytic herpes virus into the tumor instead of administering the virus through the immature perivascular cells of the tumor. The interstitial pressure of the tumor tissue definitely impacts viral distribution and the pressure would increase with the liquid volume of the virus injection. The Bevacizumab-induced decrease in interstitial pressure might significantly impact the spread of the virus into the tumor tissue, particularly when the virus is locally injected into the tumor.

In addition, another aspect is that VEGFA and other angiogenic factors are involved in tumor immune evasion (25-26). VEGFA has been shown to act as an immunosuppressant (27). Treating with an anti-VEGFA antibody such as Bevacizumab might induce an immune response, leading to antitumor immunity (28-29). Because VEGFA regulates dendritic cell (DC) function, inhibiting VEGFA activates DCs and shifts the immune response to cellular (Type 1) immunity, which is thought to favor cancer rejection (30). Bellati *et al.* reported a patient who benefited from complete resolution of ascites after being intraperitoneally administered low doses of Bevacizumab. Immunological analyses showed an initial increase in the proportion and function of CD8 (+) effector T cells and a reduction in circulating T (reg) cells (31).

It is widely established that infecting tumors with an oncolytic herpes virus results in the activation of the innate immune system at the tumor site (32-33). Upon infection with an oncolytic virus, the microenvironment may no longer favor tumor growth and shift to an antitumor growth environment that includes the recruitment of important immune effector cells (34-35). Therefore, a combination of stimuli that induces antitumor immune responses, such as an oncolytic herpes virus and Bevacizumab, has great potential to induce and expand innate antitumor immunity. Improving the interstitial fluid pressure of the tumor and inducing innate antitumor immunity will lead to long-lasting inhibition of tumor growth. The data presented here clearly show that hrR3 and Bevacizumab combination therapy significantly decreases xenograft tumor growth and indicates that this treatment is superior to treatment with the oncolytic virus or Bevacizumab alone.

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The efficacy and safety of bevacizumab beyond first progression in patients treated with first-line mFOLFOX6 followed by second-line FOLFIRI in advanced colorectal cancer: a multicenter, single-arm, phase II trial (CCOG-0801)

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Abstract

Purpose The aim of this study was to evaluate the efficacy and safety of the planned continuation of bevacizumab beyond first progression (BBP) in Japanese patients with metastatic colorectal cancer (mCRC).

Methods Previously untreated patients with assessable disease were treated with mFOLFOX6 plus bevacizumab

until tumor progression, followed by FOLFIRI plus bevacizumab. The primary endpoint of the study was the second progression-free survival (2nd PFS), defined as duration from enrollment until progression after the second-line therapy. Secondary endpoints of the study were overall survival (OS), survival beyond first progression (SBP), progression-free survival (PFS), response rate (RR), disease control rate (DCR), and safety.

Results In the first-line setting, 47 patients treated with mFOLFOX6 plus bevacizumab achieved RR of 61.7 %, DCR of 89.4 %, and median PFS of 13.1 months (95 % CI, 8.7–17.5 months). Thirty-one patients went on to receive a second-line therapy with FOLFIRI plus bevacizumab and achieved RR of 27.6 %, DCR of 62.1 %, and median PFS of 7.3 months (95 % CI, 5.0–9.6 months). Median 2nd PFS was 18.0 months (95 % CI, 13.7–22.3 months). The median OS and SBP were 30.8 months (95 % CI, 27.6–34.0 months) and 19.6 months (95 % CI, 13.5–25.7 months), respectively. No critical events associated with bevacizumab were observed during the second-line therapy.

Conclusion The planned continuation of bevacizumab during a second-line treatment, BBP strategy, is feasible for the Japanese mCRC patients.

Keywords Colorectal cancer · Chemotherapy · Bevacizumab beyond progression (BBP)

Trial registration This trial was registered in University Hospital Medical Information Network (UMIN), UMIN000006818.

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Introduction

Colorectal cancer is one of the most common cancers worldwide and remains the third leading cause of cancer-related mortality in Japan [1, 2]. For several years, first- and second-line chemotherapy with 5-fluorouracil (5-FU) and folinic acid (FA) in combination with either irinotecan

(FOLFIRI) or oxaliplatin (FOLFOX) had been the standard therapy for metastatic colorectal cancer (mCRC) [3, 4]. More recently, these combinations are used together with bevacizumab, a humanized monoclonal antibody that binds to and neutralizes vascular endothelial growth factor (VEGF). Benefits of adding bevacizumab to either the established first-line or second-line chemotherapeutic regimens have been robustly documented in previous clinical trials. Regarding the first-line treatment, Hurwitz et al. reported that addition of bevacizumab to fluorouracil-based combination chemotherapy showed significantly better clinical outcomes as compared with chemotherapy alone (overall survival [OS]: 20.3 vs. 15.6 months [hazard ratio [HR]: 0.66; $P < 0.001$], progression-free survival [PFS]: 10.6 vs. 6.2 months [HR: 0.54; $P < 0.001$], and response rate [RR]: 44.8 vs. 34.8 % [$P = 0.004$]) [5]. Kabbinavar et al. reported that addition of bevacizumab to fluorouracil/leucovorin (FU/LV) improved survival as compared with FU/LV alone (OS: 17.9 vs. 14.6 months [HR: 0.74; $P = 0.008$], PFS: 8.8 vs. 5.6 months [HR: 0.63; $P < \text{or} = 0.0001$], RR: 34.1 vs. 24.5 % [$P = 0.019$]) [6]. Furthermore, Saltz et al. reported that addition of bevacizumab to oxaliplatin-based chemotherapy significantly improved PFS, although OS did not reach statistical significance, and the RR was not improved (PFS: 9.4 vs. 8.0 months [HR: 0.83; $P = 0.0023$], OS: 21.3 vs. 19.9 months [HR: 0.89; $P = 0.077$]) [7]. In the second-line setting, the RR rate of various chemotherapeutic regimens has not been satisfactory, ranging from 4 % for FOLFIRI after the first-line FOLFOX6 to 15 % for FOLFOX6 after the first-line FOLFIRI and 20 % for XELOX after irinotecan-based therapies [8, 9]. Again, benefit of adding bevacizumab was demonstrated in several clinical trials in this setting. Giantonio et al. reported that bevacizumab plus FOLFOX4 showed significantly better survival data compared with FOLFOX4 alone after the first-line irinotecan-based treatment (OS 12.9 vs. 10.8 months [HR: 0.75; $P = 0.011$], PFS 7.3 vs. 4.7 months [HR: 0.61; $P < 0.001$], RR: 22.7 vs. 8.6 % [$P < 0.001$]) [10]. Bennouna et al. showed that bevacizumab plus irinotecan-based regimens showed efficacy with acceptable safety profile after the first-line oxaliplatin-based treatments (PFS: 7.8, OS: 22.4, and RR: 33 %) [11].

More recently, a survival benefit associated with the continuous use of bevacizumab beyond progression (BBP) was generated by two large studies. A large observational cohort study that evaluated the efficacy and safety of bevacizumab in combination with chemotherapy (BRiTE study) indicated that the BBP could contribute to prolong the OS [12]. The Avastin registry: investigation of effectiveness and safety (ARIES) also looked at the role of BBP and indicated trend toward longer OS among patients who

received bevacizumab beyond first progression compared with patients who received bevacizumab only after progression (27.5 vs. 18.7 months) [13]. However, these are observational studies, and true benefits and risks of BBP are yet to be shown in a prospective clinical trial, particularly in Japan. This prompted us to conduct a multicenter phase II study of mFOLFOX6 plus bevacizumab followed by FOLFIRI plus bevacizumab in mCRC to explore the BBP strategy for the first time in the Japanese population.

Patients and methods

Patients

The study inclusion criteria were histologically confirmed colorectal adenocarcinoma; unrespectable metastatic disease; age 20 years or older; Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; no previous chemotherapy for mCRC; bidimensionally measurable disease; a life expectancy of at least 3 months; adequate organ function (white blood cell count 3,000–12,000 cells per μL , neutrophilic cell count $\geq 1,500$ cells per μL , platelet count $\geq 100,000$ per μL , aspartate aminotransferase [AST] ≤ 100 IU/L, alanine aminotransferase [ALT] ≤ 100 IU/L, total bilirubin ≤ 25.7 $\mu\text{mol/L}$ [≤ 15 mg/L], and creatinine ≤ 106.1 $\mu\text{mol/L}$ [≤ 12 mg/L]). Exclusion criteria were pregnancy or lactation; second non-colorectal cancer; complications such as ileus, uncontrolled diabetes mellitus, or hypertension; severe diarrhea; clinically evident gastrointestinal hemorrhage; and ascites or pleural effusion needing treatment. The protocol of this study was approved by the institutional review board or ethics committee of each institution. The study was conducted in compliance with the Declaration of Helsinki. Written informed consent was obtained from all patients participating in the study.

Treatment plan

As the first-line setting for mCRC, the patients received bevacizumab plus mFOLFOX6 therapy (consisting of bevacizumab [5 mg/kg], oxaliplatin [85 mg/m²], and folinic acid [200 mg/m²] followed by bolus infusion of fluorouracil [400 mg/m²] and subsequent continuous infusion of fluorouracil [2,400 mg/m²], repeated every 2 weeks) until disease progression, unacceptable toxicity, or patient's wish to terminate the treatment. In the subsequent second-line setting, the patients received bevacizumab plus FOLFIRI therapy (consisting of bevacizumab [5 mg/kg], irinotecan [150 mg/m²], and folinic acid [200 mg/m²] followed by bolus infusion of fluorouracil [400 mg/m²] and subsequent continuous infusion of fluorouracil [2,400 mg/m²],

repeated every 2 weeks) until disease progression, unacceptable toxicity, or patient's wish to terminate the treatment.

Surgical treatment of the metastatic lesions was allowed in patients with sufficient objective response that rendered the lesions resectable.

Assessments

The primary objective of this study was the second progression-free survival (2nd PFS), defined as the time duration from the date of initiation of the first-line therapy until investigator-assessed disease progression or patient death due to any cause after starting the second-line treatment. If the patient could not receive second-line treatment for medical reasons or refusal, progression-free survival (PFS) on first-line therapy was used. Secondary objectives were OS (the time duration from the date of initiation of each therapy to death due to any cause), survival beyond first progression (SBP) (the time duration from the date of first disease progression to death due to any cause), PFS (the time duration from the date of initiation of each therapy to disease progression or death due to any cause), RR (the proportion of patients who achieved a best response of either a complete response [CR] or partial response [PR] during each therapy), disease control rate (DCR) (the proportion of patients with CR, PR, or stable disease [SD] during each therapy), and safety. Schematic of patients observation periods is presented in Fig. 1b. Adverse events were assessed using National Cancer Institute Common Toxicity Criteria (NCI-CTC), version 3.0. In addition, the frequency of bevacizumab-related adverse events (gastrointestinal perforation, wound healing complications, bleeding, hypertension, proteinuria, and thromboembolic events) was assessed.

Statistical analysis

Assuming a threshold for 2nd PFS of 10.5 months and an expected 2nd PFS of 15.8 months, referring to data from the previous clinical trials, and a 2-year enrollment period and a 2-year follow-up period, 44 patients in total were required to ensure an alpha error of 0.05 (one-sided) and detection power ($1-\beta$) of 80 %. Taking possible dropouts into consideration, the sample size of this study was determined as 50. The 2nd PFS, the primary objective of this study, was estimated using the Kaplan–Meier method, and the median 2nd PFS and its 95 % confidence interval were estimated. Other time-to-endpoint data, PFS and OS, were also estimated in the same manner. RR, DCR, and the toxicities were calculated as proportions with exact confidence intervals.

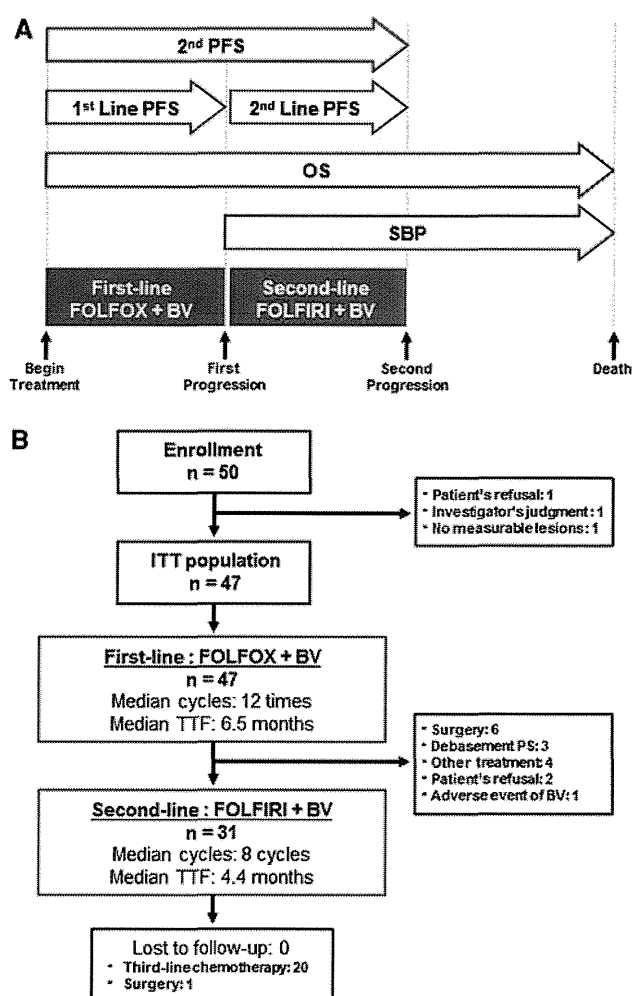


Fig. 1 Schematic of patient observation periods (**a**) and consort chart of the patients (**b**). **a** The second progression-free survival (2nd PFS) is measured from the start of first-line treatment to disease progression after second-line treatment. Progression-free survival (PFS) of each therapy is measured from the start of each therapy to disease progression. Survival beyond first progression (SBP) is measured from the first progression to death. Overall survival (OS) is measured from the start of first-line treatment to death. **b** Fifty patients were enrolled in this study. Three patients were excluded from the study. Forty-seven patients who received the protocol treatment were included in the safety evaluation

Results

Patient characteristics

Fifty patients from 12 institutions in Japan were enrolled in this study from August 2008 to May 2010. Three patients were excluded from the study: one due to the patient's refusal, one due to the investigator's decision, and one due to no measurable lesions as per the inclusion criteria. Forty-seven patients who received the protocol treatment were included in the evaluation of efficacy and safety. Baseline characteristics and consort chart of the patients are presented in Table 1 and Fig. 1b.

Table 1 Patient characteristics

Parameter	No. of patients (<i>N</i> = 47)	%
Age, years		
Median	63	
Range	40–74	
Sex		
Male	31	66.0
Female	16	34.0
Performance status WHO		
0	29	61.7
1	18	38.3
Primary site		
Colon	31	66.0
Rectum	16	34.0
Metastases		
Synchronous	7	14.9
Metachronous	40	85.1
Metastatic sites		
Liver	21	44.6
Lung	21	44.6
Peritoneum	1	2.1
Lymph nodes	10	21.3
Adjuvant chemotherapy		
No	27	57.4
Yes	20	42.6
5FU-based	20	42.6
Oxaliplatin-based	0	0

No/*N* number, WHO World Health Organization, 5FU 5-fluorouracil

Treatment status

As the first-line treatment, 47 patients received a median of 12 cycles (range 2–39) of bevacizumab plus mFOLFOX6 therapy. Median time-to-treatment failure (TTF) was 6.5 months (95 % CI, 4.0–9.0 months). The median relative dose intensity (RDI) for bevacizumab and oxaliplatin was 88 and 76 %. Treatment was discontinued because of disease progression in 21 patients (44.7 %), adverse events in 14 patients (29.8 %), and patient’s refusal in two patients (4.3 %). Secondary surgery to remove metastases was performed in six patients (12.8 %).

As for the second-line treatment, 31 patients received a median of eight cycles (range, 2–28) of bevacizumab plus FOLFIRI therapy. Median TTF was 4.4 months (95 % CI, 2.4–6.4 months). The median RDI for bevacizumab and irinotecan was 80 and 76 %. Treatment was discontinued because of disease progression in 20 patients (64.5 %) and adverse events in two patients (6.5 %). Secondary surgery to remove metastases was performed in one patient (3.2 %). After undergoing the second-line protocol treatment, 20

Table 2 Treatment status

	First-line therapy (mFOLFOX6 + BV) (<i>N</i> = 47)	Second-line therapy (FOLFIRI + BV) (<i>N</i> = 31)
Treatment cycle (times)		
Median	12	7
Range	2–39	2–26
Time-to-treatment failure (month)		
Median	6.5	3.8
95 % CI	4.0–9.0	2.7–4.5
Median relative dose intensity (%)		
Bevacizumab	88	80
Oxaliplatin	76	–
Irinotecan	–	76
Reasons for discontinuation (%)		
Progression of disease	44.7	64.5
Toxicity	29.8	6.5
Secondary surgery for metastasis (%)	12.8	3.2

BV bevacizumab, *N* number, CI confidence interval

patients (64.5 %) received a third-line chemotherapy, of which the regimen delivered to six patients (19.4 %) was cetuximab.

There was no therapy-related death in this study. Treatment status is summarized in Table 2.

Clinical outcomes

After a median follow-up period of 35.9 months (range, 24.2–44.8 months), 39 disease progressions (83.0 %) and 26 deaths (55.3 %) occurred in the 47 patients enrolled.

Median 2nd PFS, the primary endpoint, was 18.0 months (95 % CI, 13.7–22.3 months) (Fig. 2a).

Median OS was 30.8 months (95 % CI, 27.7–34.0 months) (Fig. 2c), and median SBP was 19.6 months (95 % CI, 13.5–25.7 months) (Fig. 2d).

In the first-line bevacizumab plus mFOLFOX6 therapy, RR and DCR of the 47 patients were 61.7 and 89.4 %, respectively (five patients had CR, 24 patients had PR and 13 patients had SD) (Table 3). The median PFS from the initiation of the first-line therapy was 13.1 months (95 % CI, 8.7–17.5 months) (Fig. 2b).

In the second-line bevacizumab plus FOLFIRI therapy, RR and DCR of the 31 patients who went on to the second-line therapy were 29.0 and 64.5 %, respectively (two patients had CR, seven patients had PR, and 11 patients had SD) (Table 3). The median PFS from the initiation of the second-line therapy was 7.3 months (95 % CI, 5.0–9.6 months) (Fig. 2b).

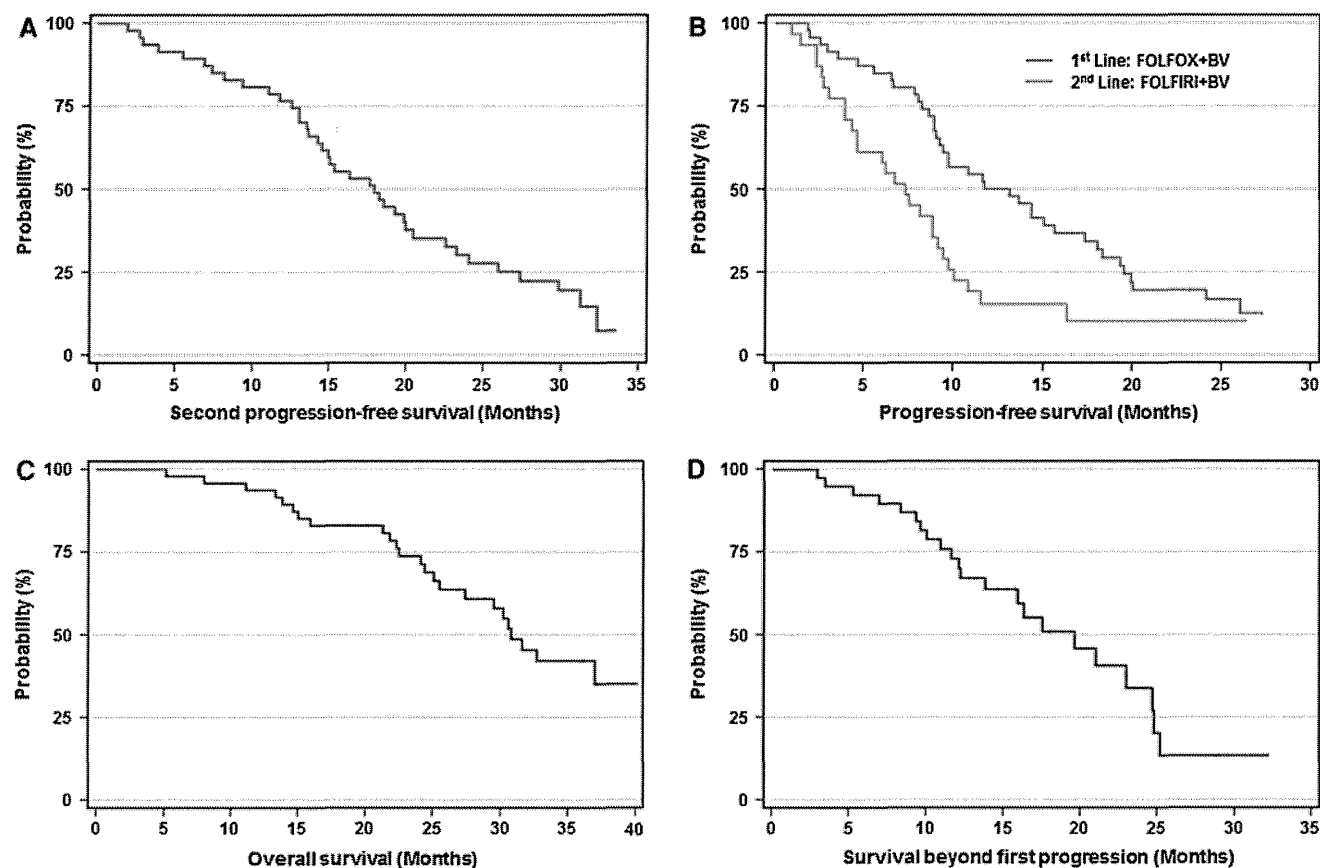


Fig. 2 Survival outcomes. **a** Median second progression-free survival, the primary endpoint, was 17.7 months (95 % CI, 13.4–22.0 months). **b** Median progression-free survivals were 13.1 months (95 % CI, 8.7–17.5 months) in the first-line setting and 7.5 months (95 % CI, 4.9–10.2 months) in the second-line setting. **c** Median overall survival

was 30.6 months (95 % CI, 13.4–22.0 months). **d** Median survival beyond the first progression was 17.7 months (95 % CI, 13.4–22.0 months). Survival curves were estimated using Kaplan–Meier methods

Adverse events

Frequency of common toxicities is presented in Table 4. The incidences of hematologic and non-hematologic > grade 3 toxic events were 44.4 and 16.7 %. The hematologic toxic events (>grade 3) occurred in 10 patients (50 %) in the first-line therapy and six patients (37.5 %) in the second-line therapy. The non-hematologic toxic events (>grade 3) occurred in five patients (25 %) in the first-line therapy and no patient (0 %) in the second-line therapy.

Severe adverse events associated with bevacizumab during the first-line therapy were grade 3 GI perforation in one case (2 %), grade 2 venous thromboembolic event in one case (2 %), and grade 2 bleeding event in one case (2 %). However, no critical events associated with bevacizumab were observed during the second-line therapy. There was a higher incidence of new or worsening hypertension in the second-line therapy as compared with the first-line therapy (26 vs. 45 %).

Discussion

This is the first prospective study to examine the continuous use of bevacizumab in combination with FOLFIRI after failing the first-line treatment with mFOLFOX/bevacizumab combination in the Japanese patients with mCRC. There are several issues regarding the use of BBP that needs to be clarified; the response and survival benefit obtained through adding bevacizumab to each line of chemotherapy, the survival benefit of the BBP strategy *per se*, and the adverse effect of long-term exposure to bevacizumab among patients who received BBP. Of these, benefits in terms of response rate and survival by adding bevacizumab to either the first-line oxaliplatin-based chemotherapy or second-line irinotecan-based chemotherapy have been well documented in previous clinical trials [7, 11]. In the current study, the response and survival data observed both in the first-line and second-line settings seem to compare favorably with these studies, with a RR of

Table 3 Objective tumor response

Response	First-line therapy (<i>N</i> = 47)		Second-line therapy (<i>N</i> = 31)	
	No. of patients	%	No. of patients	%
CR	5	10.6	2	6.5
PR	24	51.1	7	22.6
SD	13	27.7	11	35.5
PD	5	10.6	11	35.5
RR (%)	61.7		29.0	
DCR (%)	89.4		64.5	

No/N number, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *RR* response rate (*CR* + *PR*), *DCR* disease control rate (*CR* + *PR* + *SD*)

61.7 % and a PFS of 13.1 months in the first-line setting, and a RR of 29.0 % and a PFS of 7.5 months in the second-line setting.

In general, failure to respond to chemotherapy with cytotoxic agents implies inherent or acquired resistance to the therapy and leads to a change in the therapeutic regimen. The mechanisms of the resistance to cytotoxic agents are typically consequences of genetic instability inherent in cancer that renders mutant cells insensitive to chemotherapeutic agents. In contrast, the mechanisms of resistance to biologic targeted agents, including bevacizumab, are not well understood. One hypothesis that forms the basis of BBP is that persistent VEGF suppression continues to have clinical benefit when given in combination with the secondary and tertiary cytotoxic regimens. This hypothesis was supported by the results of several clinical trials exploring benefit of BBP. The first evidence of a survival benefit associated with BBP was generated by a large, observational study, BRiTE study. In this study, the patients who had been treated with BBP had a superior median SBP and OS (19.2 and 31.8 months, respectively) as compared with those who were treated without BBP (9.5 and 19.9 months, respectively) [12]. The ARIES study examined the role of bevacizumab after disease progression in patients who had received first-line bevacizumab and in those who were bevacizumab-naïve at the time of second-line treatment. The authors observed a trend toward longer SBP and OS in patients who had received first-line and second-line bevacizumab (median SBP: 14.1 and OS: 27.5 months) when compared with patients who received bevacizumab only after the disease progression (median SBP: 7.5 and OS: 18.7 months), while PFS of the second-line treatment was similar in both groups [13].

The primary objective of the current study was to assess the efficacy of BBP determined in terms of the 2nd PFS, defined as the time duration from the initiation of the first-line therapy until disease progression during the second-

Table 4 Frequency of common toxicities

Toxicity	First-line therapy (<i>N</i> = 47)		Second-line therapy (<i>N</i> = 31)	
	All grades (%)	>Grade 3 (%)	All grades (%)	>Grade 3 (%)
Hematologic toxicity	72.3	27.7	51.6	32.3
Neutropenia	57.4	23.4	41.9	22.6
Thrombocytopenia	12.8	0	9.7	0
Anemia	23.4	0	9.7	0
Febrile neutropenia	–	4.3	–	3.2
Non-hematologic toxicity	85.1	25.5	51.6	12.9
Diarrhea	0	0	12.9	3.2
Nausea/vomiting	27.7	4.3	19.4	0
Mucositis	10.6	2.1	12.9	3.2
Hand-foot syndrome	2.1	0	0	0
Alopecia	2.1	0	3.2	0
Fatigue	6.4	0	3.2	3.2
Neuropathy	72.3	17.0	19.4	3.2
Allergy	12.8	2.1	3.2	0
Bevacizumab-associated toxicity	51.1	2.1	45.2	3.2
Hypertension	25.5	0	45.2	3.2
Proteinuria	21.3	0	16.1	0
Bleeding	2.1	0	3.2	0
Infection	2.1	0	0	0
Thrombosis	2.1	0	0	0
GI perforation	2.1	2.1	0	0

N number, *GI* gastrointestinal

line of chemotherapy. Tournigand et al. reported that the median 2nd PFS was 10.9 months when the first-line FOLFOX and second-line FOLFIRI were administered, both without bevacizumab, and this was a historical benchmark to design our study. The median 2nd PFS of 17.7 months as shown in this study met our expectations and clearly pointed to an improvement in the outcome compared with the historical precedent setting without bevacizumab. There could be an argument that the end-point of a chemotherapeutic strategy such as BBP that constitutes from several lines of treatment should be OS. In this aspect, the median OS and SBP in this study were 30.8 and 19.6 months, respectively. These survival data are potentially comparable with the results observed in the BBP population from the previous studies.

Safety of a long-term exposure to bevacizumab among patients who received BBP is another issue explored in this study. The safety outcomes in the BRiTE study showed no apparent increase in serious adverse events reported in the BBP group compared with the no-BBP group [12, 14], with the exception of thromboembolic event in the elderly