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/m^2 twice-daily (equivalent to a total daily dose of 80 mg/m^2) for 3 weeks (day 1 to 21). Cisplatin 60 mg/m^2 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/m^2 twice-daily (equivalent to a total daily dose of 2000 mg/m^2) for 2 weeks (day 1 to 14). Cisplatin 80 mg/m^2 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.

Acknowledgements and funding
We like to thank all other participating investigators and referral centres for their efforts. Research funding will be supported in part by a non-profit organization: Epidemiological and Clinical Research Information Network (ECRIN).

Author details
[1]Department of Gastrointestinal Surgery, Kanagawa Cancer Center, 1-1-2 Nakao, 241-0815, Yokohama, Asahi-ku, Japan. [2]Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, Yokohama, Japan. [3]Department of Surgery II, Nagoya University Graduate School of Medicine, Nagoya, Japan. [4]Department of Surgery, Kochi Medical School, Kochi, Japan. [5]Department of Clinical Oncology, Aichi Cancer Center Hospital, Nagoya, Japan. [6]Department of Gastroenterology, Saitama Cancer Center, Saitama, Japan. [7]Department of Surgical Oncology, Gifu Graduate School of Medicine, Gifu, Japan. [8]Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Graduate School of Medicine, Ube, Japan. [9]Epidemiological and Clinical Research Information Network, Nagoya University Graduate School of Medicine, Aichi, Nagoya, Japan.

Received: 19 April 2012 Accepted: 6 July 2012
Published: 23 July 2012

References
1. International Agency for Research on Cancer: *GLOBOCAN*.: 2008. http://www-dep.iarc.fr/CancerMondial.htm.
2. Van Cutsem E, Moiseyenko VM, Tjulandin S, Majlis A, Constenla M, Boni C, Rodrigues A, Fodor M, Chao Y, Voznyi E, Risse ML, Ajani JA, V325 Study Group: **Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group.** *J Clin Oncol* 2006, **24**(31):4991–4997.
3. Cunningham D, Starling N, Rao S, Iveson T, Nicolson M, Coxon F, Middleton G, Daniel F, Oates J, Norman AR, Upper Gastrointestinal Clinical Studies Group of the National Cancer Research Institute of the United Kingdom: **Capecitabine and oxaliplatin for advanced esophagogastric cancer.** *N Engl J Med* 2008, **358**(1):36–46.
4. Koizumi W, Narahara H, Hara T, Takagane A, Akiya T, Takagi M, Miyashita K, Nishizaki T, Kobayashi O, Takiyama W, Toh Y, Nagaie T, Takagi S, Yamamura Y, Yanaoka K, Orita H, Takeuchi M: **S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial.** *Lancet Oncol* 2008, **9**(3):215–221.
5. Kang YK, Kang WK, Shin DB, Chen J, Xiong J, Wang J, Lichinitser M, Guan Z, Khasanov R, Zheng L, Philco-Salas M, Suarez T, Santamaria J, Forster G, McCloud PI: **Capecitabine/cisplatin versus 5-fluorouracil/cisplatin as first-line therapy in patients with advanced gastric cancer: a randomised phase III noninferiority trial.** *Ann Oncol* 2009, **20**(4):666–673.
6. Ajani JA, Rodriguez W, Bodoky G, Moiseyenko V, Lichinitser M, Gorbunova V, Vynnychenko I, Garin A, Lang I, Falcon S: **Multicenter phase III comparison of cisplatin/S-1 with cisplatin/infusional fluorouracil in advanced gastric or gastroesophageal adenocarcinoma study: the FLAGS trial.** *J Clin Oncol* 2010, **28**(9):1547–1553.
7. Sakata Y, Ohtsu A, Horikoshi N, Sugimachi K, Mitachi Y, Taguchi T: **Late phase II study of novel oral fluoropyrimidine anticancer drug S-1 (1 M tegafur-0.4 M gimestat-1 M otastat potassium) in advanced gastric cancer patients.** *Eur J Cancer* 1998, **34**(11):1715–1720.
8. Koizumi W, Kurihara M, Nakano S, Hasegawa K: **Phase II study of S-1, a novel oral derivative of 5-fluorouracil, in advanced gastric cancer. For the S-1 Cooperative Gastric Cancer Study Group.** *Oncology* 2000, **58**(3):191–197.
9. Miwa M, Ura M, Nishida M, Sawada N, Ishikawa T, Mori K, Shimma N, Umeda I, Ishitsuka: **Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue.** *Eur J Cancer* 1998, **34**(8):1274–1281.
10. *NCCN Clinical Practice Guidelines for Treatment of Cancer by site.*: http://www.nccn.org/professionals/physician_gls/f_guidelines.asp.
11. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK, ToGA Trial Investigators: **Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial.** *Lancet* 2010, **376**(9742):687–697.
12. Ohtsu A, Shah MA, Van Cutsem E, Rha SY, Sawaki A, Park SR, Lim HY, Yamada Y, Wu J, Langer B, Starnawski M, Kang YK: **Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a randomized, double-blind, placebo-controlled phase III study.** *J Clin Oncol* 2011, **29**(30):3968–3976.
13. Yamada Y, Yamamoto S, Ohtsu A, Suzuki Y, Nasu J, Yamaguchi K, Denda T, Tsuji A, Hara Y, Boku N, Gastrointestinal Oncology Study Group/Japan Clinical Oncology Group: **Impact of dihydropyrimidine dehydrogenase status of biopsy specimens on efficacy of irinotecan plus cisplatin, S-1, or 5-FU as first-line treatment of advanced gastric cancer patients in JCOG9912.** *ASCO Meeting Abstracts*, **27**(15s):4535.
14. Koizumi W, Okayasu I, Hyodo I, Sakamoto J, Kojima H, Clinical Study Group of Capecitabine: **Prediction of the effect of capecitabine in gastric cancer by immunohistochemical staining of thymidine phosphorylase and dihydropyrimidine dehydrogenase.** *Anticancer Drugs* 2008, **19**(8):819–824.
15. Ichikawa W, Takahashi T, Suto K, Hirayama R: **Gene expressions for thymidylate synthase (TS), orotate phosphoribosyltransferase (OPRT), and thymidine phosphorylase (TP), not dihydropyrimidine dehydrogenase (DPD), influence outcome of patients (pts) treated with S-1 for gastric cancer (GC).** *J Clin Oncol (Meeting Abstracts) ASCO Meeting Abstracts* 2004, **22**(14_suppl):4050.
16. Napieralski R, Ott K, Kremer M, Specht K, Vogelsang H, Becker K, Müller M, Lordick F, Fink U, Rüdiger Siewert J, Höfler H, Keller G: **Combined GADD45A and thymidine phosphorylase expression levels predict response and survival of neoadjuvant-treated gastric cancer patients.** *Clin Cancer Res* 2005, **11**(8):3025–3031.
17. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J: **New response evaluation criteria in solid tumors: revised RECIST guideline (version 1.1).** *Eur J Cancer* 2009, **45**(2):228–247.

doi:10.1186/1471-2407-12-307
Cite this article as: Tsuburaya *et al.*: 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). *BMC Cancer* 2012 12:307.

Original Paper

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

Tomohiro Deguchi[1], Toshio Shikano[1], Hideki Kasuya[1], Akihiro Nawa[2], Sawako Fujiwara[2], Shin Takeda[1], Tan Gewen[1], Tevfik T Sahin[1], Suguru Yamada[1], Akiyuki Kanzaki[1], Kazuo Yamamura[1], Tsutomu Fujii[1], Hiroyuki Sugimoto[1], Shuji Nomoto[1], Saori Fukuda[1], Yoko Nishikawa[1], Yasuhiro Kodera[1] and Akimasa Nakao[3]

[1]Department of Surgery II and [2]Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[3]Department of Surgery, Nagoya Central Hospital, Nagoya, Japan

Corresponding author: Hideki Kasuya, Department of Surgery II, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan; Tel.: +81-52-842-2322; E-mail: kasuya@med.nagoya-u.ac.jp

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 distribution 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. Silvania *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

Hepato-Gastroenterology 2012; 59:1844-1850 doi 10.5754/hge11566

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% CO_2. 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; $5X10^6$ 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 (2×10^5) 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'-CTTTCCTGGTGAGAGATCTGG-3'); β-actin sense (5'-AAGAGAGGCATCCTCACCCT-3') and anti-sense (5'-TACATGGCTGGGGTGTTGAA-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 10μg/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, 50μL 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 100μL 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 1×10^5 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 10μg/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 (1×10^6/100μL) 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 20-30mm^3. The treatment groups were PBS (n=6), hrR3 (n=6), Bevacizumab (n=6) and hrR3 plus Bevacizumab (n=6). hrR3 was administered intratumorally (1×10^7pfu/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×W×3/4. The animals were sacrificed *via* CO_2 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 4-6μm 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.

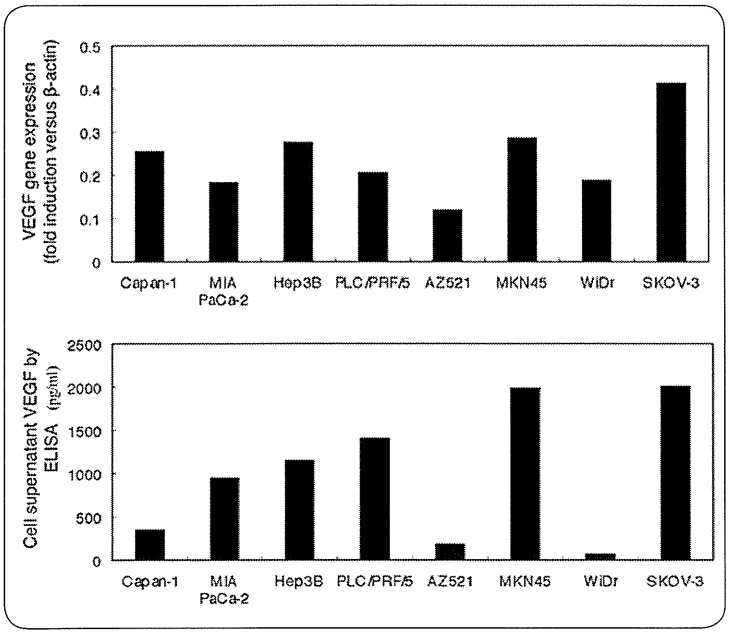

FIGURE 1. Determination of VEGFA protein levels. Eight representative tumor cells were examined for VEGFA RNA levels in cells and VEGFA protein levels in the supernatant. SKOV-3 cells are known to secrete high levels of VEGFA and were therefore used as a positive control for cellular and secreted VEGFA. 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.

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 10^5pfu/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**).

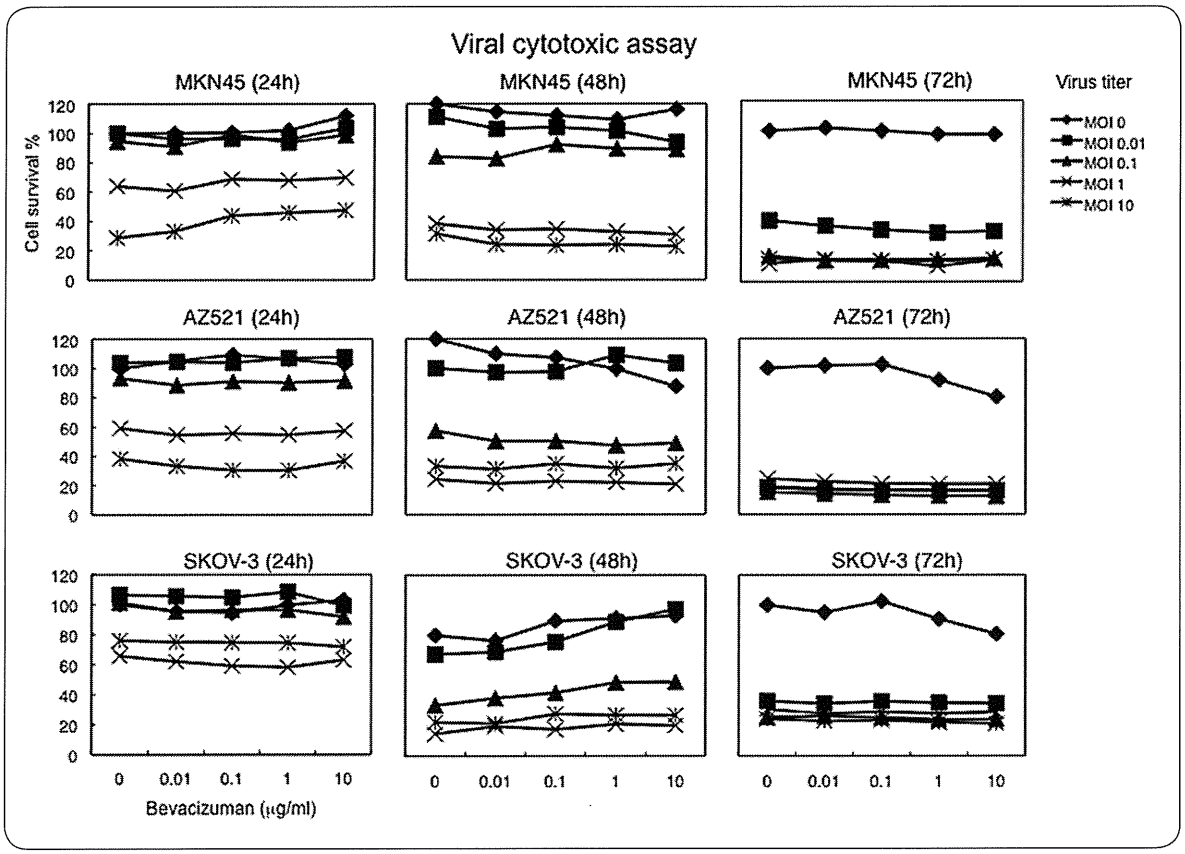

FIGURE 2. Viral cytotoxic assay. MKN45 cells (gastric cancer), which secrete high levels of VEGFA, AZ521 cells (gastric cancer), which secrete the lowest levels of VEGFA, and SKOV-3 cells were used for the assay. After 48h of viral infection, viral cytotoxicity gradually increased with viral replication. 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 (10μg/mL). There were no changes in viral cytotoxicity across the different Bevacizumab concentrations.

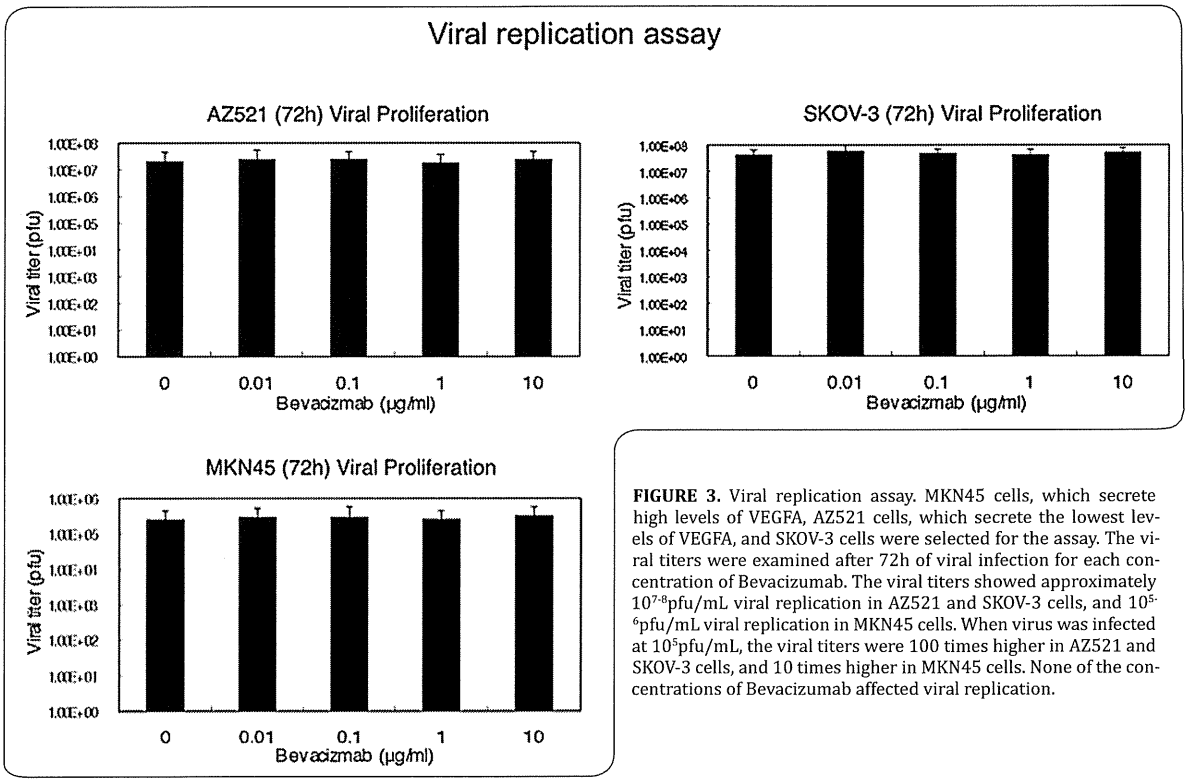

FIGURE 3. Viral replication assay. MKN45 cells, which secrete high levels of VEGFA, AZ521 cells, which secrete the lowest levels of VEGFA, and SKOV-3 cells were selected for the assay. The viral titers were examined after 72h of viral infection for each concentration of Bevacizumab. The viral titers showed approximately 10^{7-8}pfu/mL viral replication in AZ521 and SKOV-3 cells, and 10^{5-6}pfu/mL viral replication in MKN45 cells. When virus was infected at 10^5pfu/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 significant 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**).

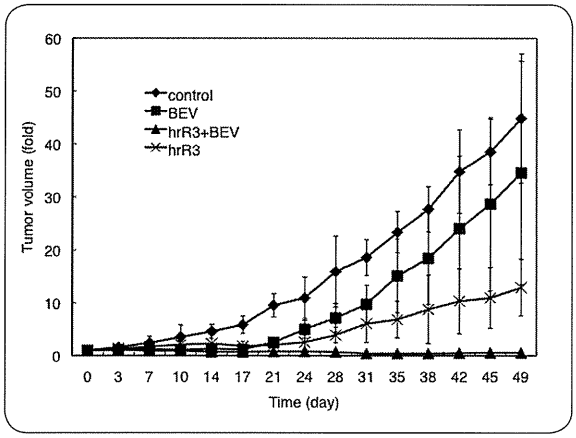

FIGURE 4. Tumor growth inhibition assay. The gastric cancer cell line MKN45, which secretes high levels of VEGFA, was used for tumor growth inhibition assays. Mice were injected with 1×10^6 MKN45 cells subcutaneously.

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-

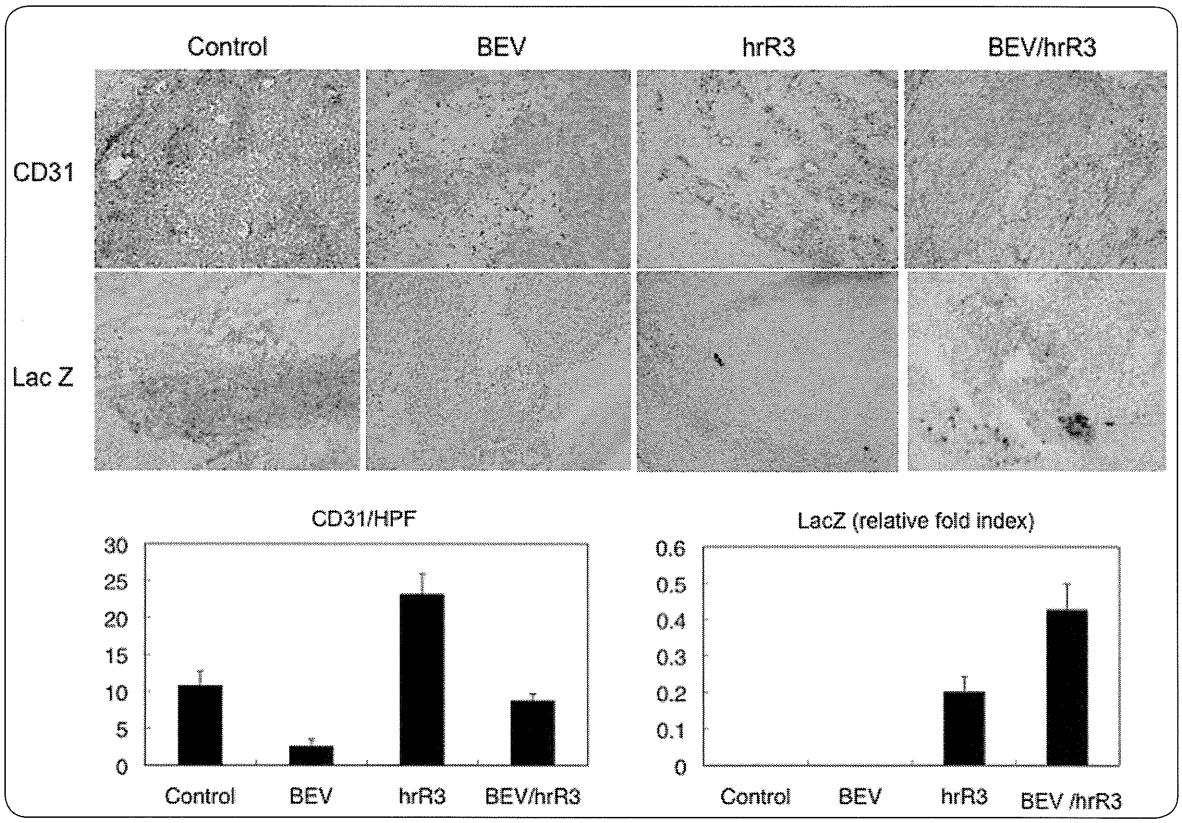

FIGURE 5. Angiogenesis and virus distribution assay. Tumors from mice that received various treatments were immunohistochemically examined for CD31 expression. The combination of hrR3 and Bevacizumab resulted in a significantly reduced rate of angiogenesis compared to hrR3 alone ($p<0.001$), and this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

ACKNOWLEDGMENTS

This work was supported by the Takeda Science Foundation, 2009 Grants-in-Aid for Scientific Research in Japan and Nitto Foundation.

REFERENCES

1. **Liu H, Liu S, Wu Y, et al.:** Cloning and expression of a single human immunoglobulin heavy-chain variable domain with vascular endothelial growth factor binding activity. Sheng Wu Gong Cheng Xue Bao 2010; 26(11):1555-1562.
2. **Teoh DG, Secord AA:** Antiangiogenic therapies in epithelial ovarian cancer. Cancer Control 2011; 18(1): 31-43.
3. **Yamamoto H, Yamada T, Takabayashi T, et al.:** Platelet Derived Endothelial Cell Growth Factor/Thymidine Phosphorylase Enhanced Human IgE Production. Allergol Int 2011; 60(1): 79-85.
4. **El-Rayes B. F, Zalupski M, Bekai-Saab T, et al.:** A phase II study of bevacizumab, oxaliplatin, and docetaxel in locally advanced and metastatic gastric and gastroesophageal junction cancers. Ann Oncol 2010; 21(10):1999-2004.
5. **Yagi Y, Fushida S, Harada S, et al.:** Biodistribution of humanized anti-VEGF monoclonal antibody/bevacizumab on peritoneal metastatic models with subcutaneous xenograft of gastric cancer in mice. Cancer Chemother Pharmacol 2010; 66(4):745-753.
6. **Aghi M, Rabkin SD, Martuza RL:** Angiogenic response caused by oncolytic herpes simplex virus-induced reduced thrombospondin expression can be prevented by specific viral muta-

tions or by administering a thrombospondin-derived peptide. Cancer Res 2007; 67(2):440-444.
7. **Shikano T, Kasuya H, Sahin TT, et al.:** High Therapeutic Potential for Systemic Delivery of a Liposome-conjugated Herpes Simplex Virus. Curr Cancer Drug Targets 2010; 11(1):111-122.
8. **Kulu Y, Dorfman JD, Kuruppu D, et al.:** Comparison of intravenous versus intraperitoneal administration of oncolytic herpes simplex virus 1 for peritoneal carcinomatosis in mice. Cancer Gene Ther 2009; 16(4):291-297.
9. **Nomura N, Kasuya H, Watanabe I, et al.:** Considerations for intravascular administration of oncolytic herpes virus for the treatment of multiple liver metastases. Cancer Chemother Pharmacol 2009; 63(2):321-330.
10. **Libertini S, Iacuzzo I, Perruolo G, et al.:** Bevacizumab increases viral distribution in human anaplastic thyroid carcinoma xenografts and enhances the effects of E1A-defective adenovirus dl922-947. Clin Cancer Res 2008; 14(20):6505-6514.
11. **Bilbao R, Bustos M, Alzuguren P, et al.:** A blood-tumor barrier limits gene transfer to experimental liver cancer: the effect of vasoactive compounds. Gene Ther 2000; 7(21):1824-1832.
12. **McKee T. D, Grandi P, Mok W, et al.:** Degradation of fibrillar collagen in a human melanoma xenograft improves the efficacy of an oncolytic herpes simplex virus vector. Cancer Res 2006; 66(5):2509-2513.
13. **Ram Z, Culver K. W, Oshiro E. M, et al.:** Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. Nat Med 1997; 3(12):1354-1361.
14. **Jain RK:** Delivery of molecular and cellular medicine to solid tumors. Adv Drug Deliv Rev 2001; 46(1-3):149-168.
15. **Minchinton AI, Tannock IF:** Drug penetration in solid tumours. Nat Rev Cancer 2006; 6(8): 583-592.
16. **Jain RK:** Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. Nat Med 2001; 7(9):987-989.
17. **Salnikov AV, Heldin NE, Stuhr LB, et al.:** Inhibition of carcinoma cell-derived VEGF reduces inflammatory characteristics in xenograft carcinoma. Int J Cancer 2006; 119(12):2795-2802.
18. **Eshun FK, Currier MA, Gillespie RA, et al.:** VEGF blockade decreases the tumor uptake of systemic oncolytic herpes virus but enhances therapeutic efficacy when given after virotherapy. Gene Ther 2010; 17(7):922-929.
19. **Wojton J, Kaur B:** Impact of tumor microenvironment on oncolytic viral therapy. Cytokine Growth Factor Rev 2010; 21(2-3):127-134.
20. **Tong RT, Boucher Y, Kozin SV, et al.:** Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 2004; 64(11):3731-3736.
21. **Boucher Y, Leunig M, Jain RK:** Tumor angiogenesis and interstitial hypertension. Cancer Res 1996; 56(18):4264-4266.
22. **Boucher Y, Jain RK:** Microvascular pressure is the principal driving force for interstitial hypertension in solid tumors: implications for vascular collapse. Cancer Res 1992; 52(18):5110-5114.
23. **Hashizume H, Baluk P, Morikawa S, et al.:** Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol 2000; 156(4):1363-1380.
24. **Stohrer M, Boucher Y, Stangassinger M, et al.:** Oncotic pressure in solid tumors is elevated. Cancer Res 2000; 60(15):4251-4255.
25. **Smirne C, Camandona M, Rosso E, et al.:** Emanuelli, G. Vascular endothelial growth factor. From basic research to clinical application. Minerva Med 1999; 90(1-2):15-23.
26. **Tromp SC, oude Egbrink MG, Dings RP, et al.:** Tumor angiogenesis factors reduce leukocyte adhesion in vivo. Int Immunol 2000; 12(5):671-676.
27. **Gabrilovich DI, Ishida T, Nadaf S, et al.:** Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function. Clin Cancer Res 1999; 5(10):2963-2970.
28. **Bourla DH, Dotan A, Weinberger D, et al.:** Consecutive appearance of corneal subepithelial infiltrates after intravitreous bevacizumab injections. Cornea 2010; 29(6):686-687.
29. **Roland CL, Dineen SP, Lynn KD, et al.:** Inhibition of vascular endothelial growth factor reduces angiogenesis and modulates immune cell infiltration of orthotopic breast cancer xenografts. Mol Cancer Ther 2009; 8(7):1761-1771.
30. **Laschos KA, Papazisis KT, Kontovinis LF, et al.:** Targeted treatment for metastatic renal cell carcinoma and immune regulation. J BUON 2010; 15(2):235-240.
31. **Bellati F, Napoletano C, Ruscito I, et al.:** Complete remission of ovarian cancer induced intractable malignant ascites with intraperitoneal bevacizumab. Immunological observations and a literature review. Invest New Drugs 2010; 28(6):887-894.
32. **Todo T:** Oncolytic virus therapy using genetically engineered herpes simplex viruses. Front Biosci 2008; 13:2060-2064.
33. **Shimoyama S, Goshima F, Teshigahara O, et al.:** Enhanced efficacy of herpes simplex virus mutant HF10 combined with paclitaxel in peritoneal cancer dissemination models. Hepatogastroenterology 2007; 54(76):1038-1042.
34. **Mantovani A, Romero P, Palucka AK, et al.:** Tumour immunity: effector response to tumour and role of the microenvironment. Lancet 2008; 371(9614):771-783.
35. **Yao F, Murakami N, Bleiziffer O, et al.:** Development of a regulatable oncolytic herpes simplex virus type 1 recombinant virus for tumor therapy. J Virol 2010; 84(16):8163-8171.

Cancer Chemother Pharmacol (2012) 70:575–581
DOI 10.1007/s00280-012-1948-1

ORIGINAL ARTICLE

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)

Goro Nakayama · Keisuke Uehara · Kiyoshi Ishigure · Hiroyuki Yokoyama · Akiharu Ishiyama · Takehiko Eguchi · Kenji Tsuboi · Norifumi Ohashi · Tsutomu Fujii · Hiroyuki Sugimoto · Masahiko Koike · Michitaka Fujiwara · Yuich Ando · Yasuhiro Kodera

Received: 24 May 2012 / Accepted: 29 July 2012 / Published online: 12 August 2012

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.

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

G. Nakayama (✉) · N. Ohashi · T. Fujii · H. Sugimoto · M. Koike · M. Fujiwara · Y. Kodera
Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Japan
e-mail: goro@med.nagoya-u.ac.jp

K. Uehara
Department of Surgical Oncology (Surgery I), Nagoya University Graduate School of Medicine, Nagoya, Japan

K. Ishigure
Department of Surgery, Konan Kosei Hospital, Konan, Japan

H. Yokoyama
Department of Surgery, Komaki City Hospital, Komaki, Japan

A. Ishiyama
Department of Surgery, Okazaki City Hospital, Okazaki, Japan

T. Eguchi
Department of Surgery, Nakatsugawa City Hospital, Nakatsugawa, Japan

K. Tsuboi
Department of Surgery, Tosei Hospital, Seto, Japan

Y. Ando
Department of Clinical Oncology and Chemotherapy, Nagoya University Hospital, Nagoya, Japan

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

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; $P <$ 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 ≥1,500 cells per μL, platelet count ≥100,000 per μL, aspartate aminotransferase [AST] - ≤100 IU/L, alanine aminotransferase [ALT] ≤100 IU/L, total bilirubin ≤25.7 μmol/L [≤15 mg/L], and creatinine ≤106.1 μmol/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/m^2], and folinic acid [200 mg/m^2] followed by bolus infusion of fluorouracil [400 mg/m^2] and subsequent continuous infusion of fluorouracil [2,400 mg/m^2], 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/m^2], and folinic acid [200 mg/m^2] followed by bolus infusion of fluorouracil [400 mg/m^2] and subsequent continuous infusion of fluorouracil [2,400 mg/m^2],

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-β) 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.

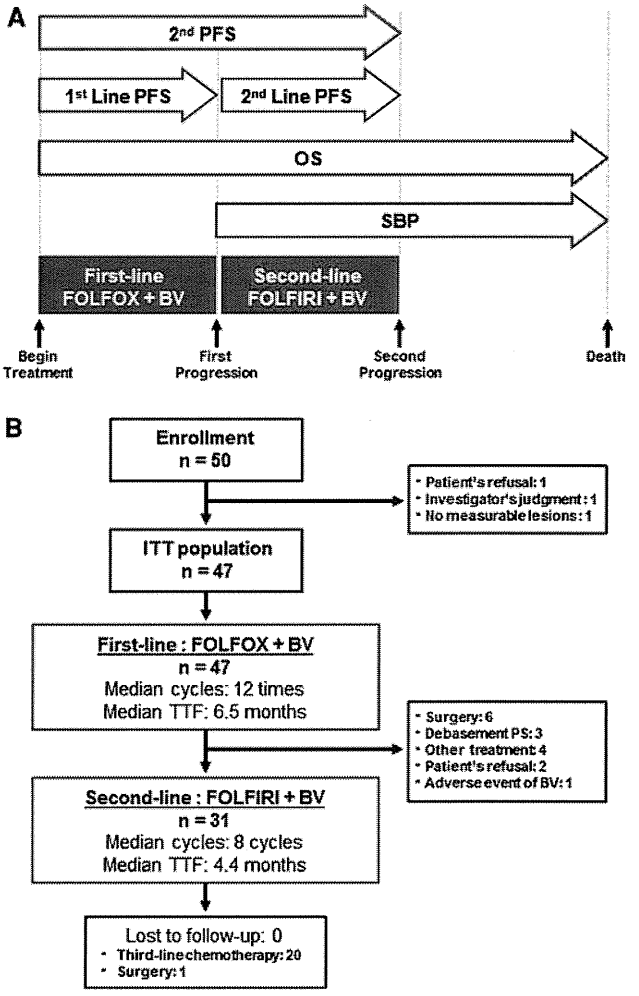

Fig. 1 Schematic of patient observation periods (**a**) and consort chart of the patients (**b**). **a** The second progression-free survival (2nd PFS) is measured from the start of first-line treatment to disease progression after second-line treatment. Progression-free survival (PFS) of each therapy 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 ($N = 47$)	%
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

Table 2 Treatment status

	First-line therapy (mFOLFOX6 + BV) ($N = 47$)	Second-line therapy (FOLFIRI + BV) ($N = 31$)
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

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 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).

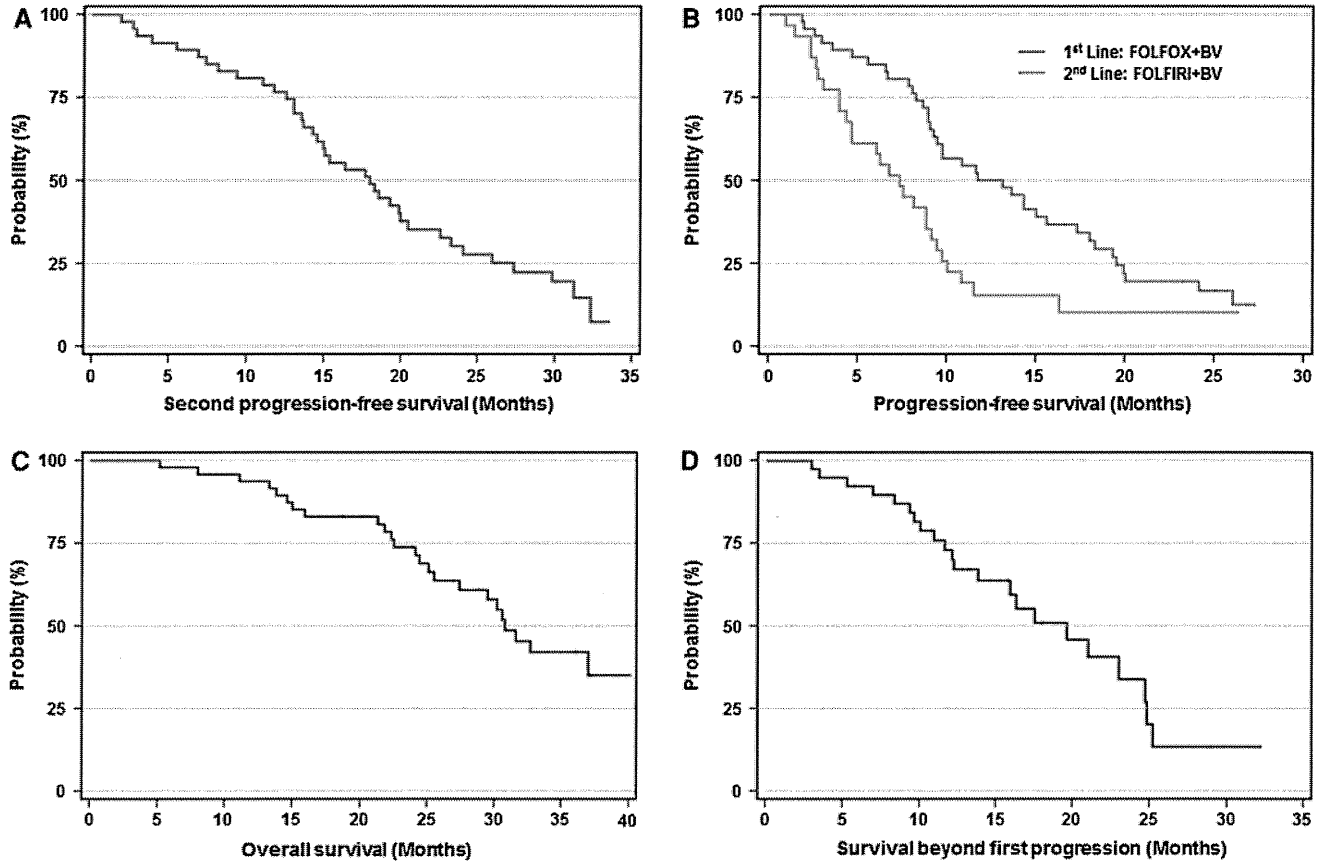

Fig. 2 Survival outcomes. **a** Median second progression-free survival, the primary endpoint, was 17.7 months (95 % CI, 13.4–22.0 months). **b** Median progression-free survivals were 13.1 months (95 % CI, 8.7–17.5 months) in the first-line setting and 7.5 months (95 % CI, 4.9–10.2 months) in the second-line 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 ($N = 47$)		Second-line therapy ($N = 31$)	
	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)

Table 4 Frequency of common toxicities

Toxicity	First-line therapy ($N = 47$)		Second-line therapy ($N = 31$)	
	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

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-naive 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-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 endpoint 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

population [15]. Such thromboembolic event was rare at 2 % in the current Japanese population. Other severe adverse events associated with bevacizumab were grade 2 bleeding event (2 %) and grade 3 GI perforation (2 %), all of which occurred during the first-line chemotherapy. Thus, no critical events associated with bevacizumab were observed during the second-line therapy. It is of note that a higher incidence of new or worsening hypertension was observed during the second-line therapy compared with the first-line therapy. The higher cumulative incidence of hypertension in the BBP group was not unexpected, given that the risk of developing bevacizumab-associated hypertension appears to accumulate over time and that the BBP results in substantially longer bevacizumab exposure. The type and frequency of other grade 3/4 events (including neutropenia, diarrhea, vomiting, and asthenia) were consistent with the known safety profile of the chemotherapy regimens.

Our study is merely hypothesis-generating regarding the efficacy of BBP because of the one-arm design and relatively small sample size. However, it does imply that the BBP strategy is beneficial to the Japanese population with the 2nd PFS nearly 10 months longer than that observed in the Tournigand study and SBP and OS that is similar to the survival data observed in the BRiTE study and the ARIES study. Data regarding safety of the BBP strategy was more robust, in which only hypertension was to be carefully taken care of. From these encouraging data, it can now be recommended that a randomized study involving a larger numbers of patients be performed in Japan to obtain hard evidence regarding the efficacy of BBP.

In summary, the planned continuation of bevacizumab during the second-line treatment is feasible for the Japanese mCRC patients. A prospective randomized control study to confirm the efficacy is warranted.

Conflict of interest The authors have declared no conflicts of interest.

References

1. Boyle P, Levin B (2008) World cancer report. IARC, Lyon
2. Kayama T, Sobue T (2010) Cancer statistics in japan. Foundation for Promotion of Cancer Research, Tokyo
3. Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L, Moore MJ, Maroun JA, Ackland SP, Locker PK, Pirotta N, Elfring GL, Miller LL (2000) Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. N Engl J Med 343:905–914
4. Giacchetti S, Perpoint B, Zidani R, Le Bail N, Faggiuolo R, Focan C, Chollet P, Llory JF, Letourneau Y, Coudert B, Bertheaut-Cvitkovic F, Larregain-Fournier D, Le Rol A, Walter S, Adam R, Misset JL, Lévi F (2000) Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. J Clin Oncol 18:136–147
5. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335–2342
6. Kabbinavar FF, Hambleton J, Mass RD, Hurwitz HI, Bergsland E, Sarkar S (2005) Combined analysis of efficacy: the addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer. J Clin Oncol 23:3706–3712
7. Saltz LB, Clarke S, Díaz-Rubio E, Scheithauer W, Figer A, Wong R, Koski S, Lichinitser M, Yang TS, Rivera F, Couture F, Sirzén F, Cassidy J (2008) Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. J Clin Oncol 26:2013–2019
8. Tournigand C, André T, Achille E, Lledo G, Flesh M, Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, Louvet C, de Gramont A (2004) FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. J Clin Oncol 22:229–237
9. Rothenberg ML, Cox JV, Butts C, Navarro M, Bang YJ, Goel R, Gollins S, Siu LL, Laguerre S, Cunningham D (2008) Capecitabine plus oxaliplatin (XELOX) versus 5-fluorouracil/folinic acid plus oxaliplatin (FOLFOX-4) as second-line therapy in metastatic colorectal cancer: a randomized phase III non inferiority study. Ann Oncol 19:1720–1726
10. Giantonio BJ, Catalano PJ, Meropol NJ, O'Dwyer PJ, Mitchell EP, Alberts SR, Schwartz MA, Benson AB 3rd, Eastern Cooperative Oncology Group Study E3200 (2007) Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern cooperative oncology group study E3200. J Clin Oncol 25:1539–1544
11. Bennouna J, Borg C, Delord JP, Husseini F, Trillet-Lenoir V, Faroux R, François E, Ychou M, Goldwasser F, Bouché O, Senellart H, Kraemer S, Douillard JY (2012) Bevacizumab combined with chemotherapy in the second-line treatment of metastatic colorectal cancer: results from the phase II BEVACOLOR study. Clin Colorectal Cancer 11:38–44
12. Grothey A, Sugrue MM, Purdie DM, Dong W, Sargent D, Hedrick E, Kozloff M (2008) Bevacizumab beyond first progression is associated with prolonged overall survival in metastatic colorectal cancer: results from a large observational cohort study (BRiTE). J Clin Oncol 26:5326–5334
13. Bekaii-Saab TS, Bendell JC, Cohn AL et al. (2010) Bevacizumab (BV) plus chemotherapy (CT) in second-line metastatic colorectal cancer (mCRC): initial results from ARIES, a second BV observational cohort study (OCS). J Clin Oncol 28(15s): abstract 3595
14. Kozloff M, Yood MU, Berlin J, Flynn PJ, Purdie DM, Ashby MA, Dong W, Sugrue MM, Grothey A, Investigators of the BRiTE study (2009) Clinical outcomes associated with bevacizumab-containing treatment of metastatic colorectal cancer: the BRiTE observational cohort study. Oncologist 14: 862–870
15. Kozloff MF, Berlin J, Flynn PJ, Kabbinavar F, Ashby M, Dong W, Sing AP, Grothey A (2010) Clinical outcomes in elderly patients with metastatic colorectal cancer receiving bevacizumab and chemotherapy: results from the BRiTE observational cohort study. Oncology 78:329–339

Cancer Letters 331 (2013) 220–229

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Phosphorylation of 4E-BP1 predicts sensitivity to everolimus in gastric cancer cells

Tetsuo Nishi [a], Kenta Iwasaki [b], Norifumi Ohashi [c], Chie Tanaka [a], Daisuke Kobayashi [a], Goro Nakayama [a], Masahiko Koike [a], Michitaka Fujiwara [a], Takaaki Kobayashi [b,*], Yasuhiro Kodera [a]

[a] Nagoya University Graduate School of Medicine, Department of Gastroenterological Surgery (Surgery II), Nagoya 466-8550, Japan
[b] Nagoya University Graduate School of Medicine, Department of Transplant Immunology, Nagoya 466-8550, Japan
[c] Aichi Medical University, Department of Gastroenterological Surgery, Nagakute 480-1195, Japan

ARTICLE INFO

Article history:
Received 19 September 2012
Received in revised form 25 December 2012
Accepted 3 January 2013

Keywords:
mTOR inhibitor
Gastric cancer
Sensitivity
4E-BP1
ERK

ABSTRACT

We studied the effect of everolimus, an inhibitor of the mammalian target of rapamycin (mTOR) on human gastric cancer cell lines. Cell proliferation in 3 of 8 cell lines was effectively inhibited by everolimus. Basal phosphorylation level of 4E-BP1 (T37/46, T70) was significantly higher in everolimus-sensitive cells than in everolimus-resistant cells. In subcutaneous xenograft model, immunohistochemistry analysis revealed that everolimus-sensitive cells expressed high levels of phospho-4E-BP1 (T37/46). In conclusion, phosphorylation of 4E-BP1 may be a predictive biomarker of everolimus sensitivity in gastric cancer.

1. Introduction

Gastric cancer is the fourth most common cancer worldwide and it is the second most common cause of cancer deaths [1]. Most patients with gastric cancer commonly present with advanced unresectable disease and over 60% of them eventually experience relapse even after curative surgical resection [2]. Systemic chemotherapy has been attempted in patients with unresectable and recurrent gastric cancer [2,3]. At present, although fluoropyrimidine-based therapy is used worldwide, no standard chemotherapeutic regimen has been accepted globally for advanced gastric cancer. Combination regimens including 5-fluorouracil, taxanes, irinotecan, and platinum derivatives achieved median overall survival of only up to 12 months in phase III trials [4–9]. Further efforts to explore new and more effective drugs or treatment regimens, or biomarkers to find a cohort that would benefit from specific drugs are warranted, as exemplarily shown by the recent application of trastuzumab to the HER2-positive gastric cancer.

The mammalian target of rapamycin (mTOR) is a key downstream protein kinase of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, which has been recognized to play a central role in regulating cell growth, cell cycle progression, cell proliferation, cell metabolism and angiogenesis [10–12]. mTOR forms with the adaptor protein raptor named mTOR complex 1 (mTORC1), and a rictor named mTOR complex 2 (mTORC2). mTORC1 regulates mRNA translation by activating ribosomal protein S6 kinase 1 (S6K1) and inhibiting a translational repressor, 4E-binding protein 1 (4E-BP1) [13]. In particular, mutations, deletion or ectopic expression of mTOR signal-related genes affects protein synthesis by eIF4E/4E-BP1 and S6K1, a phenomenon commonly observed in several types of cancer [14]. The translational repressor 4E-BP1 binds tightly to eIF4E. When 4E-BP1 is phosphorylated at multiple sites, it prevents the formation of eIF4E translation initiation complex at the 5′ end of cap-bearing mRNA [15]. eIF4E overexpression in mammalian cell culture is enough to increase cell size, and this is counteracted by co-overexpression of 4E-BP1 [16]. These observations are consistent with the reports that eIF4E expression is high in several types of cancer [14]. Subsequent activation of eIF4E results in translation of multiple

Abbreviations: 4E-BP1, 4E-binding protein 1; DCR, disease control rate; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; PFS, progression-free survival; PI3K, phosphoinositide 3-kinase; S6K1, S6 kinase 1.

* Corresponding author. Tel.: +81 52 744 2303; fax: +81 52 744 2305.
E-mail addresses: ntetsu@med.nagoya-u.ac.jp (T. Nishi), kiwasaki@med.nagoya-u.ac.jp (K. Iwasaki), ohashi.norifumi@gmail.com (N. Ohashi), chtanaka@med.nagoya-u.ac.jp (C. Tanaka), kobadai@med.nagoya-u.ac.jp (D. Kobayashi), goro@med.nagoya-u.ac.jp (G. Nakayama), dockoike@med.nagoya-u.ac.jp (M. Koike), mfuji@med.nagoya-u.ac.jp (M. Fujiwara), takakoba@med.nagoya-u.ac.jp (T. Kobayashi), ykodera@med.nagoya-u.ac.jp (Y. Kodera).

0304-3835/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canlet.2013.01.004

malignancy-associated proteins such as c-myc [17]. Abnormal expression of growth receptors frequently activates PI3K/AKT/mTOR pathway in human gastric cancer [18–22]. The mTOR pathway is, therefore, a promising new therapeutic target in the treatment of gastric cancer.

Rapamycin and its derivatives including everolimus inhibit mTOR, thereby preventing phosphorylation of its downstream molecules including S6K and 4E-BP1. mTOR inhibitors have been shown to exhibit potent preclinical activities against a wide variety of cancers, including neuroblastoma, glioblastoma, small cell lung cancer, renal cell carcinoma, pancreatic cancer and leukemias [17]. Everolimus has recently been approved for treatment of renal cell carcinoma and neuroendocrine tumor of pancreas in Japan. Everolimus has shown to be active also against gastric cancer in the preclinical studies [23–25], and has been investigated in a phase I/II clinical trial for patients with advanced gastric cancer [26,27]. In this phase II trial, although the disease control rate was 56% and a decrease in tumor size from baseline was obtained in 45% of patients, in the phase III trial, the overall survival rate was not superior to that of gastric cancer patients who were treated only with best supportive care [28]. It is imperative that biomarkers for everolimus be found to optimize patients who should be treated with this drug. But little is known about the details of the signaling pathways that predict the effect of everolimus for gastric cancer. The present study was performed to assess the antiproliferative effect of everolimus on gastric cancer cell lines and to determine the predictable molecule which is correlated with sensitivity to everolimus *in vitro* and *in vivo*.

2. Materials and methods

2.1. Reagents

Everolimus, purchased from Sigma–Aldrich (St. Louis, MO), was initially dissolved in ethanol at a concentration of 0.05 mg/ml and stored at 4 °C.

2.2. Cell lines

Eight cell lines derived from human gastric cancer were examined. NUGC-2, NUGC-4 and SC-2-NU were established and maintained at the Department of Surgery II, Nagoya University Graduate School of Medicine. AZ521, MKN28 and MKN1 were provided by the Japanese Cancer Research Resource Bank (Tokyo, Japan). H111 and SC-6-JCK were established and kindly donated by the Department of Surgery, Research Institute for Microbial Disease, Osaka University and Central Institute for Experimental Animals, respectively. All cells were maintained at 37 °C in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and with 1% penicillin–streptomycin (GIBCO, Paisley, UK) in a humidified atmosphere of 5% CO_2 in air.

2.3. MTT assay

Cells (5×10^3 in 200 μl/well) were seeded on 96-well plates and incubated with everolimus at concentrations as indicated. After treatment, medium was replaced by 50 μl medium containing 0.05% 3-[4.5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT] (Sigma–Aldrich) and incubated for 1 h at 37 °C. The 570 nm absorbance was recorded by a kinetic microplate reader Vient XS (DS Pharma Biomedical, Osaka, Japan). All experiments were performed in triplicate.

2.4. Flow cytometric analysis for cell apoptosis

2×10^5 cells were treated with 10 and 100 ng/ml everolimus for 48 h on 6-well plates. Cells were collected and stained with FITC labeled Annexin-V and PI (BD, Franklin Lakes, NJ). Apoptotic cell death was measured by counting the number of cells that stained positive for Annexin V by flow cytometry. All experiments were performed in triplicate.

2.5. Western blot analysis

2×10^5 cells were treated with various amounts of everolimus and lysed in 150 μl lysis buffer (Cell Signaling Technology, Danvers, MA). In xenograft tumor tissue study, frozen tumor tissue was sonicated on ice in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% SDS) with protease and phosphatase inhibitor (Sigma–Aldrich). Samples separated on a 10–12.5% SDS–PAGE were transferred to nitrocellulose membranes (Millipore, Billerica, MA). Membranes were incubated with antibody against AKT, phospho-AKT (Ser473), mTOR, phospho-mTOR (Ser2448), S6 K, phospho-S6 K (Thr389), phospho-S6 (Ser235/236), 4E-BP1, phospho-4E-BP1 (Thr37/46), phospho-4E-BP1 (Ser65), phospho-4E-BP1 (Thr70), 4E-BP1, ERK, phospho-ERK (Thr202/Tyr204), GAPDH (all are from Cell Signaling Technology) and S6 (Santa Cruz Biotechnology, Santa Cruz, CA) with a working dilution in Can Get Signals Solution 1 (TOYOBO, Tokyo, Japan) at 4 °C overnight. Primary antibodies were detected by HRP-anti-rabbit IgG (Cell Signaling Technology). Signals were observed via ECL® Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK). Antibody concentrations were 1:2000 for the anti-Akt antibody and 1:1000 for the others.

2.6. Tumor xenograft studies

1×10^7 cells (SC-2-NU and NUGC-4) in 0.2 ml PBS were injected subcutaneously into the left shoulder of 6–7 week-old male nude mice of KSN/Slc strain (Chubu Kagaku Shizai, Nagoya, Japan) maintained under specific-pathogen-free conditions. Mice ($n = 8$) were orally administered everolimus via a gastric tube at a dose of 0 or 5 mg per kg per day from day 10 after inoculation, five times per week for 4 weeks. Tumor maximum diameter (L) and the right angle diameter to the axis (W) were measured 3 times a week. Tumor volume was estimated by the following formula, $L \times W \times W \times 1/2$. Animal welfare was strictly monitored by the Committee for Ethics of Animal Experimentation, and the experiments were carried out in accordance with the Guidelines for Animal Experiments at Nagoya University.

2.7. Immunohistochemistry

Paraffin-embedded tissue sections were heated in a microwave at 500 W for 10 min in citric acid buffer, pH 6.0, for antigen retrieval and then incubated in 0.3% H_2O_2 in methanol for 15 min at room temperature to suppress the endogenous peroxidase activity. After blocking nonspecific reactions, these slides were further incubated with primary antibodies overnight in a moist chamber at 4 °C. Anti-4E-BP1 and anti-p-4E-BP1 (T37/46) antibodies (Cell Signaling Technology; 1:200 dilution) were used for primary antibodies. The sections were incubated for 30 min at room temperature with the secondary antibodies (biotinylated goat anti-rabbit IgG; Dako, Denmark) followed by incubation with streptavidin-peroxidase complex (HISTOFINE SAB-PO Kit, Nichirei Biosciences, Tokyo, Japan). The chromogen was developed with 0.03 mol/L diaminobenzidine, and the sections were counterstained with hematoxylin.

2.8. Statistical analysis

For comparison between groups, data were analyzed by t test. Differences in western blot analysis between the groups were analyzed with Mann–Whitney-U test. Differences between groups were considered statistically significant at $p < 0.05$ except for the tumor xenograft studies, in which differences between groups were considered statistically significant at $p < 0.01$.

3. Results

3.1. Antiproliferative and apoptotic effects of everolimus in gastric cancer cells

Eight gastric cancer cell lines were incubated with serial concentrations of everolimus (0.1–100 ng/ml) for up to 4 days. Everolimus inhibited proliferation of these cells in a concentration dependent manner, but with varying effectiveness (Fig. 1A). For almost all cell lines, the growth inhibitory effect diminished at 4 days after initiation of the treatment. The 50% growth inhibition of each cell line by everolimus was therefore determined after 3 days of exposure. The concentration of everolimus required to inhibit growth of AZ521, H111 and SC-2-NU cells was less than 100 ng/ml (10 ng/ml, 25 ng/ml, 100 ng/ml, respectively) whereas other cells (NUGC-2, SC-6-JCK, NUGC-4, MKN1, MKN28) did not reach the point of 50% inhibition even with 100 ng/ml (Fig. 1B). We therefore classified the cell lines AZ521, H111 and SC-2-NU as sensitive to everolimus and the other 5 cell lines as resistant. Apoptosis analysis by flow cytometry was performed in both the everolimus-sensitive cells (AZ521, SC-2-NU) and the everolimus-resistant cells (NUGC-4, MKN28) (Fig. 1C and D). Regardless of the sensitivity to everolimus, no increase in apoptotic cells was observed. Taken together, we confirmed that everolimus exhibits

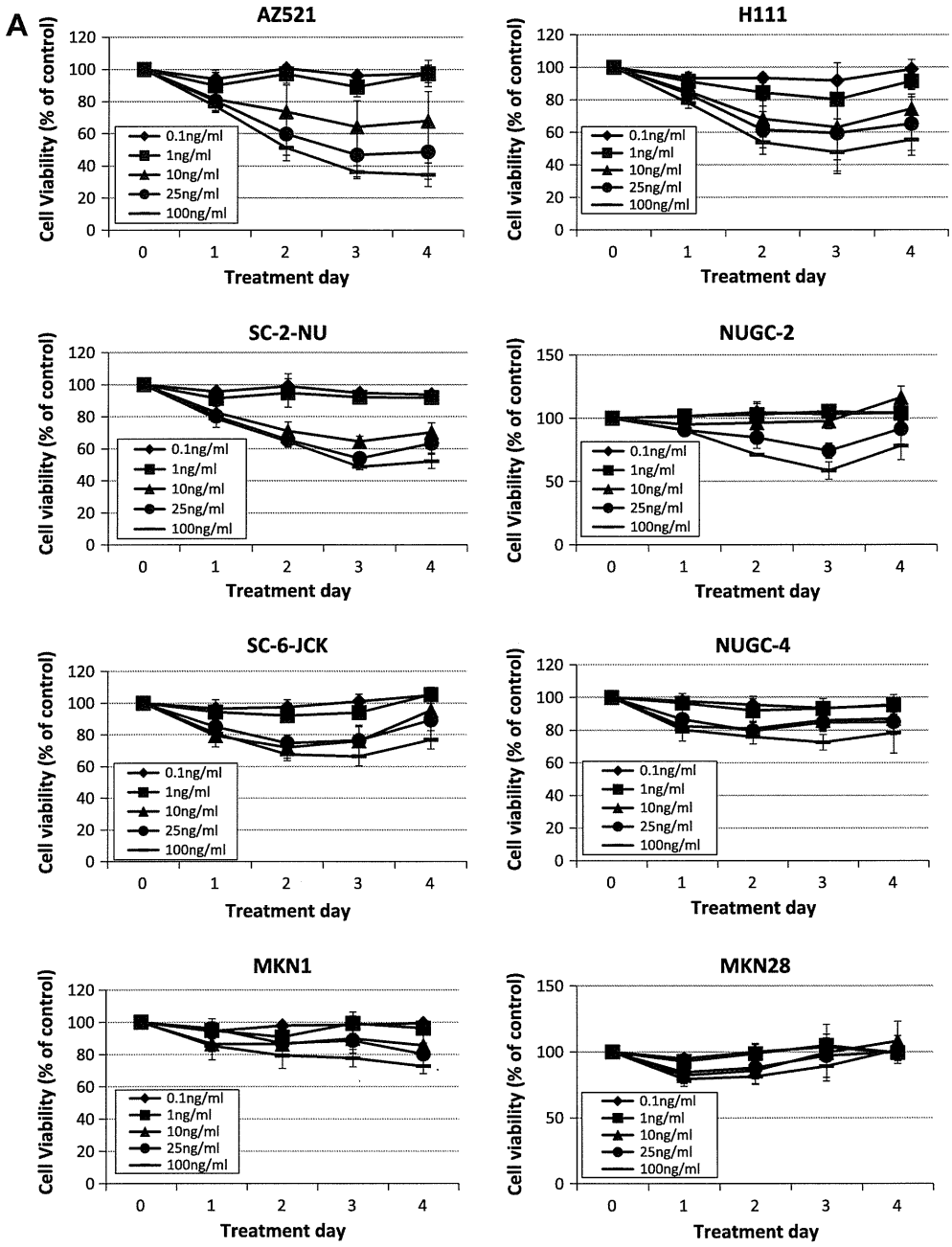

Fig. 1. Inhibitory effects of everolimus on gastric cancer cell growth and proliferation. (A) Eight different gastric cancer cells were seeded into a 96-well plate. Cells were treated with different amounts of everolimus from 0.1 to 100 ng/ml for 4 days. The data represents mean ± SEM ($n = 3$). (B) Cell viability was determined daily, and compared at 3 days by MTT assay. The data represents mean ± SEM ($n = 3$). (C) Gastric cancer cells were treated with 10 and 100 ng/ml everolimus for 48 h. Apoptotic cells were analyzed by Annexin-V/PI staining assay. Representative flow cytometry results using AZ521, H111, SC-2-NU, NUGC-4 were shown. Each population was separated by Quadrant, and each percentage was calculated. (D) Bars compare differences in the percentage of apoptosis cells between controls and everolimus-treated cells. Values are expressed as mean ± SD ($n = 3$). Everolimus did not induce apoptosis nor showed protective effect, although the differences in apoptosis levels were observed among cancer cells.

various levels of growth inhibitory effect on gastric cancer cell lines, and this is not through induction of apoptosis.

3.2. Effect of everolimus on mTOR-related genes and ERK in gastric cancer cell lines

To investigate the effect of everolimus on mTOR pathway, genes related to the pathway were analyzed. AZ521, SC-2-NU, NUGC-4 and MKN28 cells were treated for 24 h by various concentrations of everolimus. Cells were then harvested and subjected to western blotting to estimate the respective levels of phosphorylated and non-phosphorylated forms of S6K, S6 and 4E-BP1. Fig. 2 shows that the treatment with everolimus reduces phosphorylation levels of S6 and 4E-BP1 (T70) in a dose-dependent manner in all cell lines. But there were no significant effects of everolimus on the phosphorylated S6K and 4E-BP1 (T37/46). In addition, everolimus at 100 ng/ml concentration resulted in a decrease of the phosphorylated 4E-BP1 (S65) only for MKN28. We then examined the levels of phosphorylated and non-phosphorylated ERK, the downstream component of the Ras/MEK signaling which has been considered to be one of major pathways involved in cell proliferation and tumorigenesis. In AZ521, the phosphorylation of ERK was increased in a dose-dependent manner after the treatment. But in other cell lines, the phosphorylation of ERK was not affected by

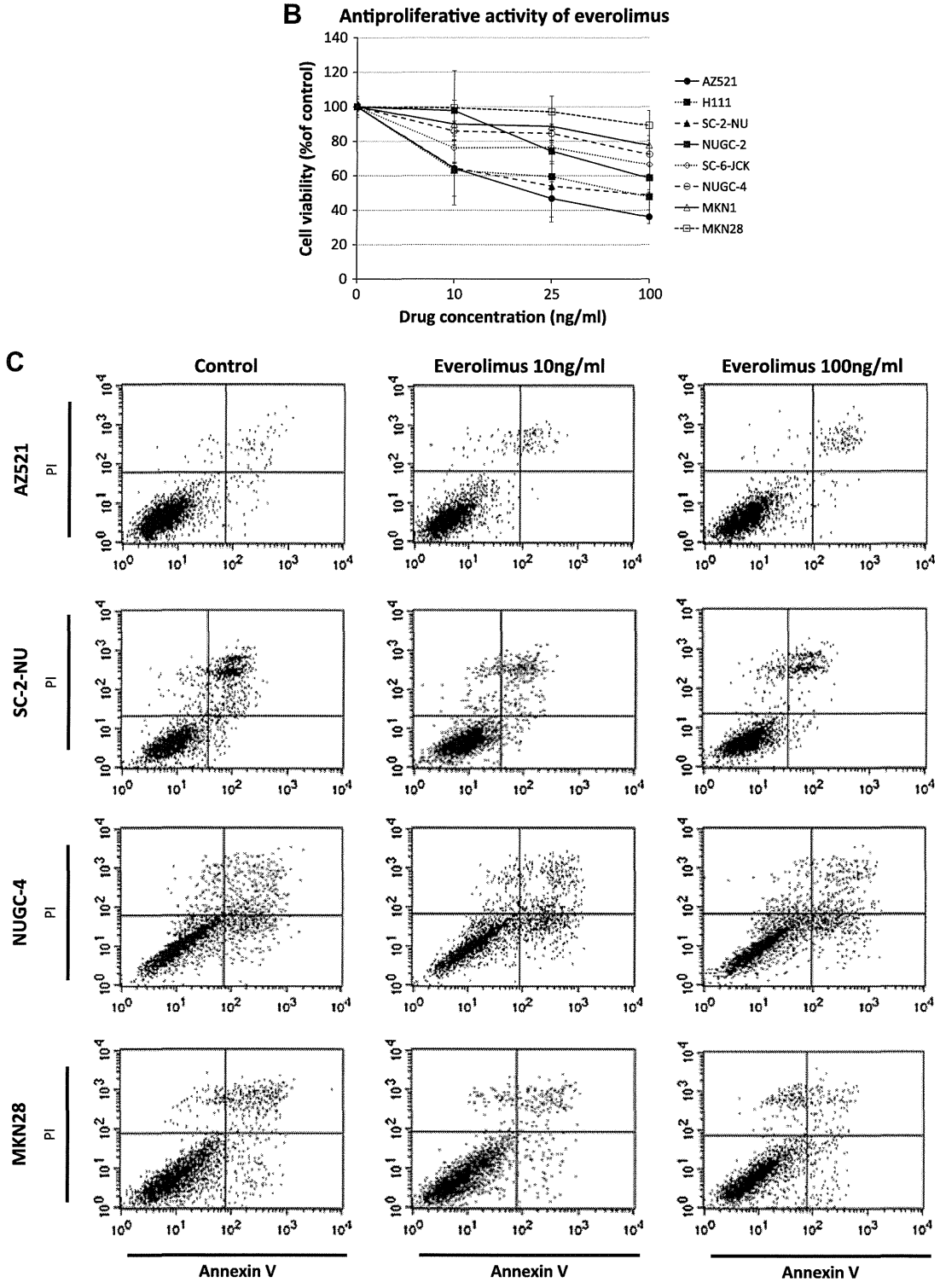

Fig. 1. (*continued*)

everolimus. Taken together, the effects of everolimus on the phosphorylation levels of proteins related to mTOR signaling pathway did not correlate with the sensitivity of tested cell lines to the growth inhibitory effect of everolimus.

3.3. Different expression of 4E-BP1 and ERK phosphorylation between everolimus-sensitive and everolimus-resistant gastric cancer cells

To further characterize sensitivity to mTOR inhibition, we conducted a conventional quantitation-approach for component proteins of mTOR signaling pathway. All 8 cell lines were lysed and subjected to western blotting to estimate basal phosphorylation levels of AKT, mTOR, S6K, S6, 4E-BP1 and ERK (Fig. 3A). The levels of AKT, mTOR, S6K and S6 did not exhibit any relation with everolimus sensitivity. Statistical analysis showed significant positive correlations between everolimus sensitivity and phosphorylation levels of 4E-BP1 (T37/46) and 4E-BP1 (T70) (Fig. 3B). In contrast, the level of phosphorylation of ERK tended to be lower in everolimus-sensitive cell lines than in resistant ones, although not significantly so.