Our study limitations should be noted. There likely were unmatched variables between the two groups, and these variables should be identified and addressed in future, randomized studies to reduce the potential selection bias. Furthermore, whether advanced colon cancer, transverse colon cancer, and rectal cancer are indicated for SLC should be evaluated as well as the long-term oncologic outcomes, the costs, training for SLC, and the stress levels of surgeons performing the procedure.

In conclusion, our study revealed that SLC with CME is feasible and safe when performed by experienced surgeons for selected patients. This procedure provides improved cosmesis and possible reduced postoperative pain with acceptable short-term outcomes and certain oncologic clearance. We hope that the short-term outcomes reported here will encourage future, prospective, randomized analysis to validate SLC with CME as a preferable alternative to conventional laparoscopy.

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ORIGINAL ARTICLE

Safety of fondaparinux to prevent venous thromboembolism in Japanese patients undergoing colorectal cancer surgery: a multicenter study

Taishi Hata · Masayoshi Yasui · Kohei Murata · Masaki Okuyama · Masayuki Ohue · Masataka Ikeda · Shigeyuki Ueshima · Kotaro Kitani · Junichi Hasegawa · Hiroshi Tamagawa · Makoto Fujii · Atsushi Ohkawa · Takeshi Kato · Shunji Morita · Takayuki Fukuzaki · Tsunekazu Mizushima · Mitsugu Sekimoto · Riichiro Nezu · Yuichiro Doki · Masaki Mori · Multi-Center Clinical Study Group of Osaka, Colorectal Cancer Treatment Group (MCSGO)

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Abstract

Purpose To investigate the safety and efficacy of fondaparinux (FPX) for venous thromboembolism (VTE) prophylaxis in Japanese patients undergoing colorectal cancer surgery.

Methods The subjects of this multicenter, open-label, prospective observational study were patients undergoing resection of the colon/rectum for colorectal cancer. All patients were given FPX 2.5 or 1.5 mg by subcutaneous injection, once daily for 4–8 days, starting 24 h after surgery. The primary endpoint was any major bleeding event and the secondary endpoint was any symptomatic VTE event.

Results Between February 2009 and December 2010, 619 patients from 23 institutions were enrolled in this study. The median duration of FPX prophylaxis was 4 days. The incidence of major bleeding was 0.81 % [5/619, 95 % confidence interval (CI) 0.3–1.9] and the incidence of minor bleeding was 9.5 % (59/619, 95 % CI 7.3–12.1). There was no fatal bleeding or symptomatic VTE. Multivariable analysis revealed the following to be risk factors for bleeding events: preoperative platelet count $<15 \times 10^4/\mu l$ [odds ratio (OR) 4.521], male sex (OR 2.078), and blood loss during surgery <50 ml (OR 2.019).

Conclusion The administration of 2.5/1.5 mg FPX 24 h after colorectal cancer surgery is safe and effective.

Keywords Venous thromboembolism · Prophylaxis · Colorectal cancer patients · Fondaparinux · Japan

This trial is registered with UMIN, UMIN000007073.

T. Hata · T. Mizushima · Y. Doki · M. Mori
Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan

M. Yasui
Department of Surgery, Kaizuka City Hospital, Kaizuka, Japan

K. Murata
Department of Surgery, Suita Municipal Hospital, Suita, Japan

M. Okuyama
Department of Surgery, Higashiosaka City General Hospital, Higashiōsaka, Japan

M. Ohue
Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

M. Ikeda (✉) · M. Sekimoto
Department of Surgery, National Hospital Organaization, Osaka National Hospital, 2-1-14 Hoenzaka, Chuo-ku, Osaka 540-0006, Japan
e-mail: mikeda@onh.go.jp

S. Ueshima
Department of Surgery, Osaka Police Hospital, Osaka, Japan

K. Kitani
Department of Surgery, Nara Hospital, Kinki University Faculty of Medicine, Osaka, Japan

J. Hasegawa
Department of Surgery, Osaka Rosai Hospital, Osaka, Japan

H. Tamagawa
Department of Surgery, Osaka General Medical Center, Osaka, Japan

M. Fujii
Department of Surgery, Osaka Kouseinenkin Hospital, Osaka, Japan

A. Ohkawa
Department of Surgery, Higashi Takarazuka Satoh Hospital, Takarazuka, Japan

Introduction

Venous thromboembolism (VTE) is a common complication of abdominal surgery [1]. According to a recent study in Japan, VTE occurred in 24.3 % of abdominal surgery patients, including one with symptomatic pulmonary embolism (PE) [2]. A comparable incidence has been reported in Western countries [3]. These findings support active VTE prophylaxis after abdominal surgery.

Two randomized studies have been conducted in Japan, using two different pharmacological agents to prevent VTE: enoxaparin and fondaparinux (FPX). In both studies, pharmacological VTE prophylaxis proved more effective than mechanical prophylaxis, such as intermittent pneumatic compression, alone and did not increase bleeding events [4, 5]. However, these two Japanese prospective studies assessed only the safety of these pharmacological agents in 187 patients. Moreover, both studies comprised patients undergoing gastroenterological, gynecological, or urological procedures, so that patient heterogeneity did not allow stratification of bleeding risk for any specific condition, such as major cancer surgery requiring lymph node dissection and bowel anastomosis.

Postoperative bleeding is a concern for patients receiving pharmacological prophylaxis for VTE. Bleeding is easily detectable but can cause serious complications. On the other hand, VTE is clinically silent unless actively searched for and seldom causes serious conditions; however, once discovered, VTE requires many medical resources for treatment, which is why it is important to establish safe VTE prophylaxis. Bleeding complications after surgery sometimes depend on the type of surgical procedure, such as whether it is open or laparoscopic and if there is bowel anastomosis. Precise analysis of bleeding events can enable surgeons to use pharmacological agents and to better prepare for bleeding events.

This prospective study evaluates the safety of FPX for the prevention of VTE in Japanese patients undergoing colorectal cancer surgery, using the dosage regimen already approved for abdominal surgery in this country.

T. Kato
Department of Surgery, Kansai Rosai Hospital, Amagasaki, Japan

S. Morita
Department of Surgery, Toyonaka Municipal Hospital, Toyonaka, Japan

T. Fukuzaki
Department of Surgery, Ikeda Municipal Hospital, Ikeda, Japan

R. Nezu
Department of Surgery, Nishinomiya Municipal Central Hospital, Nishinomiya, Japan

Methods

We conducted a multicenter, open-label, observational study at 23 affiliated medical institutions between February 2009 and December 2010. This study was approved by the appropriate institutional review boards and undertaken according to the ethical principles stated in the Declaration of Helsinki (1964).

Study protocol and patient recruitment

The inclusion criteria for this study were that patients underwent elective surgery for colorectal malignancy and that they gave written informed consent. Exclusion criteria were as follows: active bleeding; thrombocytopenia, defined as a platelet count of $<10 \times 10^4/\mu L$; disorders associated with an increased risk of bleeding, such as gastrointestinal tract ulcers, diverticulitis, colitis, acute bacterial endocarditis, severe uncontrolled hypertension, or severe uncontrolled diabetes mellitus; severe hepatic dysfunction (Child C); a known history of hypersensitivity to unfractionated heparins, low-molecular-weight heparins, or heparinoids; a history of intracranial bleeding; a history of surgical intervention of the central nervous system or ocular surgery within the past 3 months; unexpected bleeding or difficulty of hemostasis during surgery; severe renal dysfunction, defined as a creatinine clearance of <20 ml/min; a history of major orthopedic, abdominal, or cardiovascular surgery within the past 3 months; any treatment with anticoagulants, dextran, thrombolytics, or antiplatelet agents within the past week; clinical signs of VTE; a preoperative D-dimer >1 μg/ml or twice the institutional limit; a history of arterial thromboembolism; drug abuse or alcohol dependence; another elective surgical intervention during the study period; pregnancy or lactation; several attempts at, or bleeding during, epidural catheter insertion; and being deemed by the attending physician as unfit for the study. Because patients with high D-dimer levels might be at risk of thrombosis preoperatively, they were excluded from the study, and VTE prophylaxis was left to the discretion of the physician.

The study protocol included approved use of epidural anesthesia when necessary. The catheter had to be removed after 20 h of FPX administration, and FPX was required to be administered for 2 h after catheter withdrawal.

VTE prophylaxis

FPX administration was started 24 h after surgery, once hemostasis was established, following the Japanese regimen for VTE prevention. FPX (2.5 or 1.5 mg) was given once daily for 4–8 days. Mechanical VTE prophylaxis, such as intermittent pneumatic compression (IPC), elastic stockings

(ES), and elastic bandage (EB), was not prohibited by the protocol, with their use and duration left to the discretion of the investigators or institutions. An FPX dose of 1.5 mg was administered when creatinine clearance was <50 ml/min, body weight was <40 kg, or age was ≥80 years.

Assessment and outcome definitions

The primary endpoint was major bleeding, and the secondary endpoint was the incidence of symptomatic VTE. Patients who met the inclusion criteria and received at least one dose of FPX were analyzed for these primary and secondary endpoints. Bleeding was classified as major if the event met at least one of the following definitions: fatal bleeding, retroperitoneal or intracranial bleeding, bleeding of critical organs (intraocular, adrenal, endocardial, or spinal bleeding), surgical site bleeding that required surgical intervention, or clinically overt bleeding with a decrease in hemoglobin (Hb) by at least 2 g/dl, or the need for transfusion of ≥800 ml red blood cells or whole blood. Minor bleeding was defined as bleeding that did not meet any of the major bleeding criteria.

If clinically suspicious symptoms of VTE were noted, such as dyspnea, chest pain, or decreased percutaneous arterial oxygen saturation (SpO_2), enhanced multi-detector helical computed tomography (MDCT) with contrast media, pulmonary scintigraphy, or pulmonary arteriography was performed to look for PE. If there was lower extremity swelling, ultrasonography, MDCT, or ascending phlebography was done for the diagnosis of deep vein thrombosis. Primary and secondary endpoints were assessed during the period between when FPX was started and 1 day after its completion. Clinical symptoms as well as SpO_2, plasma D-dimer, platelet count, and liver function were prospectively recorded preoperatively and on postoperative days (PODs) 1, 3, and 7.

Statistical analysis

This trial was designed to demonstrate the safety of FPX in Japanese patients with colorectal cancer. Because we had no background data for patient recruitment, we referred to the APOLLO trial for the sample size calculation in terms of safety assessment [6]. Therefore, the recruitment target was set at 600 patients. All continuous data are expressed as the median (range). Frequency distributions between categorical data were compared using χ^2 tests. The association between a major or minor bleeding event and the bleeding risk factors was assessed using multivariate logistic regression models. Results are expressed with odds ratios (ORs) and 95 % confidence intervals (CI). All statistical tests were two-sided, and all analyses were performed with SPSS 11.0J (IBM SPSS, Chicago, IL).

Results

Clinical characteristics of the study population

Between February 2009 and December 2010, 665 patients from 23 institutions were registered for this study, 619 (93.1 %) of whom met the inclusion criteria. These 619 patients received at least one dose of FPX and were included in the safety and efficacy analyses. The reasons for exclusion from the study included increased D-dimer ($n = 23$), no D-dimer values ($n = 17$), no histological evidence of malignancy ($n = 5$), and bleeding before FPX administration ($n = 1$). Table 1 shows the baseline clinical characteristics of the 619 patients and Table 2 summarizes the surgical procedures and related operational information. Two-hundred patients underwent open surgery and 419 patients underwent laparoscopic surgery, which was converted to open surgery in 27 (6.4 %).

The mechanical prophylaxes used with FPX were as follows: EB for a median duration of 1 day (range 1–3 days) in 10 patients (1.6 %), 2 of whom received only EB; ES for a median duration of 1 day (range 0–7 days) in 518 patients (83.7 %); and IPC for a median duration of 0 days (mean 0.46 days, range 0–4 days) in 572 patients (92.4 %). In many institutions, IPC was discontinued after the patient began to ambulate on postoperative day (POD) 1 and ES were removed after the first injection of FPX. One patient did not receive any type of mechanical prophylaxis.

For pharmacological VTE prophylaxis, FPX was given at a dosage of 1.5 mg to 83 patients and at a dosage of 2.5 mg to 536 patients. The total median duration of FPX treatment at both 1.5 and 2.5 mg was 4 days (range 1–10 days).

Safety outcomes

The incidence of major bleeding during the treatment period was 0.81 % (5/619) with a 95 % CI of 0.3–1.9 %.

Table 1 Background clinical characteristics of the patients ($n = 619$)

Age (years), mean (SD)	66.6 (9.5)
Sex (M/F)	371/248
Weight (kg), mean (SD)	57.8 (11.0)
BMI (kg/m^2), mean (SD)	22.4 (3.3)
Diagnosis, *n* (%)	
Cancer	615 (99.4)
Carcinoid	4 (0.6)
Site of disease, *n* (%)	
Right-side colon	184 (29.7)
Left-side colon	192 (31.0)
Rectum	243 (39.3)

BMI body mass index (kg/m^2)

Table 2 Operational procedure and surgical characteristics

	Open surgery ($n = 200$)	Laparoscopic surgery ($n = 419$)
Partial resection	5	21 (3)[a]
Ileocecal resection	11	40 (3)[a]
Right colectomy	38	70 (3)[a]
Left colectomy	10	28 (2)[a]
Sigmoidectomy	32	110 (6)[a]
Anterior resection	38	65 (3)[a]
Low anterior resection	48	82 (7)[a]
Abdominoperineal resection	12	2 (0)[a]
Total pelvic exenteration	1	1 (0)[a]
Subtotal colectomy	1	0
Colostomy	2	0
Trans-anal resection	1	0
Other	1	0
Operation time in minutes, median (range)	169.5 (30–651)	225 (72–586)
Blood loss in ml, median (range)	107.5 (0–7440)	25 (0–3635)
Use of epidural catheter, *n* (%)	170 (85)	107 (25.6)

[a] Values in parentheses indicate the number of laparoscopic procedures converted to open surgery

There was no death related to bleeding or from other causes during the treatment period. Table 3 summarizes the five cases of major bleeding. One patient with ascending colon cancer who underwent open surgery suffered a fall, sustaining a subdural hematoma after hitting his head against the floor. The other patient underwent low anterior resection as open surgery for rectal cancer. He exhibited an Hb decrease of greater than 2 g/dl and received an 800 ml transfusion for anemia 2 days after FPX administration. An upper gastrointestinal fiberscope revealed a bleeding ulcer in the gastric tube used for reconstruction after esophagectomy. Three patients from the laparoscopic surgery group suffered major bleeding at the anastomosis, after resection of ascending colon cancer in two patients and of descending colon cancer in one patient. All three patients had an Hb decrease >2 g/dl and one had concomitant anastomotic leakage necessitating re-operation.

The incidence of minor bleeding during the treatment period was 9.5 % (59/619). Most minor bleedings occurred at the surgical site, including the wound, the drain insertion site, and the anastomosis site (Table 4). Subcutaneous hemorrhage or hematoma was the most frequent event; followed by melena, caused mainly by bleeding of the anastomosis site. One patient had bleeding of the epidural catheter insertion site, but no subsequent symptoms of epidural hematoma were noted.

Table 3 Occurrences of major bleeding

	Open surgery ($n = 200$) *n* (%)	Laparoscopic surgery ($n = 419$) *n* (%)	Total ($n = 619$) *n* (%)
Major bleeding	2 (1)	3 (0.72)	5 (0.81)
Fatal bleeding	0	0	0
Bleeding in a critical organ	1 (0.5)	0	1 (0.16)
Bleeding at the surgical site leading to re-operation	0	1 (0.24)	1 (0.16)
Bleeding at the surgical site with Hb decrease >2 g/dl	0	2 (0.48)	2 (0.32)
Bleeding at a non-surgical site with a Hb decrease >2 g/dl	1 (0.5)	0	1 (0.16)

Hb hemoglobin

Table 4 Occurrences of minor bleeding

	Open surgery ($n = 200$) *n* (%)	Laparoscopic surgery ($n = 419$) *n* (%)	Total ($n = 619$) *n* (%)
Minor bleeding	12 (6.0)	47 (11.2)	59 (9.5)
Subcutaneous hemorrhage/hematoma	6 (3.0)	18 (4.3)	24 (3.9)
Melena anastomotic hemorrhage	2 (1.0)	21 (5.0)	23 (3.7)
Bloody drain discharge hemorrhage at drain site	4 (2.0)	6 (1.4)	10 (1.5)
Bleeding of gastric ulcer	0	1 (0.24)	1 (0.16)
Bleeding of epidural catheter insertion site	0	1 (0.24)	1 (0.16)

Risk factors for bleeding events

To assess the risk factors for bleeding, univariable analysis was performed for major and minor bleeding events, and patient-related factors (age, sex, body weight, and BMI), surgery-related factors (mode of surgery, duration, and blood loss), FPX dose, and patient laboratory data (pre- and postoperative platelet count, D-dimer level, and liver function). Table 5 shows that sex (male), blood loss during surgery (<50 ml), preoperative platelet count ($<15 \times 10^4/\mu l$), and platelet count on POD 1 ($<15 \times 10^4/\mu l$) were

Table 5 Univariable and multivariable analysis of factors associated with bleeding events

Factor	*n*	Incidence of bleeding events (%)	*p* value	Odds ratio	*p*	95 % CI
Age						
<80	572	10.3	1.000			
≥80	47	10.6				
Sex						
Male	371	12.7	0.022	2.078	0.016	1.143–3.778
Female	248	6.9		Reference		
Body weight (kg)						
≤40	19	15.8	0.434			
>40	600	10.2				
BMI (kg/m^2)						
≤18	41	17.0	0.177	2.170	0.092	0.881–5.349
>18	578	9.9		Reference		
Surgery						
Open	200	7.0	0.067	Reference		
Laparoscopic	419	11.9		1.674	0.126	0.865–3.238
Operation time (min)						
<180	203	8.4	0.325			
≥180	416	11.3				
Blood loss (ml)						
<50	328	13.1	0.017	2.019	0.020	1.120–3.640
≥50	291	7.2		Reference		
FPX dose (mg)						
1.5	83	12.0	0.563			
2.5	536	10.1				
Pre-op D-dimer (μg/ml)						
<0.5	253	12.3	0.227			
≥0.5	366	9.0				
Pre-op platelet count (×10^4/μl)						
<15	41	29.3	<0.0001	4.521	0000	2.081–9.822
≥15	578	9.0		Reference		
Platelet count on POD 1 (× 10^4/μl)						
<15	109	16.5	0.023			
≥15	507	8.9				
Pre-op AST (U/L)						
≤40	582	10.5	0.781			
>40	36	11.1				
Pre-op ALT (U/L)						
≤40	583	9.9	0.162	Reference		
>40	35	17.1		1.628	0.336	0.603–4.398

CI confidence interval, *BMI* body mass index, *FPX* fondaparinux, *POD* postoperative day, *AST* aspartate amino transferase, *ALT* alanine transaminase

associated with a significantly greater incidence of bleeding events. The threshold of the platelet count was defined as 15 × 10^4/μl, being the lower limit of most institutional normal ranges of 13–14 × 10^4/μl. We then performed multivariate analysis using factors with *p* values of <0.2, excluding the platelet count on POD 1. This revealed that a preoperative platelet count of <15 × 10^4/μl, male sex, and intraoperative blood loss of less than 50 ml were independent risk factors.

Efficacy outcomes

There was no incidence of symptomatic VTE or fatal VTE in this study.

Discussion

In this series of patients undergoing surgery for colorectal cancer, no fatal bleeding occurred, although the incidences of major and minor bleeding were 0.81 and 9.5 %, respectively. In the APOLLO trial comparing FPX + IPC with IPC alone, incidences of major and minor bleeding were 1.6 % (10/635) and 0.8 % (5/635), respectively [6]. In another study comparing FPX with dalteparin, there were two cases (0.1 %) of fatal bleeding and a 2.0 % incidence of bleeding necessitating reoperation or intervention, with an incidence of major bleeding of 3.4 % [7].

On evaluating other agents, a previous study on general surgery found incidences of major hemorrhage and wound hematoma of 3.2 and 6.1 %, respectively, in patients treated with unfractionated heparin prophylaxis [8]. In a report comparing enoxaparin and unfractionated heparin for the prevention of VTE in cancer surgery, incidences of major bleeding were 4.1 and 2.9 %, respectively, and those of minor bleeding were 14.6 and 14.3 % [9]. Taken together, in the current group of patients treated with FPX, the safety profile was comparable with those of these studies.

In evaluating the efficacy endpoint, we found no incidence of symptomatic VTE in these 619 patients. This incidence is comparable with those in the FPX prophylaxis arms of two previous studies, reporting 0.2 % (1/650) and 0.4 % (6/1465), respectively [6, 7].

We identified three randomized studies on the prevention of VTE in patients with colorectal surgery [10–12]. A randomized phase III trial reported incidences of 1.5 % (10/643) and 2.7 % (18/653) for major bleeding and 0.6 % (3/468) and 0.4 % (2/468) for symptomatic VTE, respectively, in patients receiving low-dose unfractionated heparin and enoxaparin [10]. Another phase III study compared nadroparin and enoxaparin in colorectal cancer surgery, and reported incidences of 7.3 % (47/643) and 11.5 % (72/628) for major bleeding and 0.2 % (1/643) and 1.4 % (9/628) for symptomatic VTE, respectively [11]. The high incidence of major bleeding in that study was attributed to the definition of blood loss during the operation, which was not included in the study. In Singapore, Ho et al. [12] investigated the efficacy of enoxaparin in colorectal surgery and found that the patients given enoxaparin prophylaxis vs. those not given prophylaxis had VTE incidences of 0 and 5 %, respectively. Bleeding events were more common in the enoxaparin prophylaxis group (6.7 %) than in the no-prophylaxis group (1.8 %), with three cases (2.2 %) of major bleeding events in the enoxaparin prophylaxis group. Considering these data on colorectal surgery, our present data demonstrate that VTE prophylaxis with FPX in patients with colorectal cancer is safe and effective.

Several randomized phase III trials of VTE prophylaxis have used pharmacological agents; however, the bleeding risk during pharmacological prophylaxis has rarely been analyzed [13]. This may be due to the fact that most studies include a wide variety of patient conditions. Because only patients with colorectal cancer were included in the present study, we sought to find risk factors for bleeding mainly in terms of patient-related and operational factors. We found that a preoperative platelet count $<15 \times 10^4/\mu l$, male sex, and bleeding <50 ml during the operation were independent risk factors for postoperative bleeding. Male sex was previously identified as a risk factor for bleeding in abdominal surgery as men have a small pelvic cavity rich in visceral fat, which makes hemostasis difficult [13, 14]. Moreover, in the Japanese population, being female is a risk factor for VTE in abdominal surgery [2], which may explain why women bleed less. It is not clear why less bleeding during the operation is a risk factor for postoperative bleeding. It is possible that a very small amount of bleeding will not induce sufficient natural coagulability to prevent postoperative hemorrhage. This rationale would also explain why laparoscopic surgery is associated with a lower rate of VTE [15, 16].

Of the five major bleeding events in this series, three were anastomotic bleeding, one of which was accompanied by anastomotic leakage and required re-operation. Those anastomoses were performed in a so-called "functional end-to-end" fashion using a mechanical stapler, in the right colon [17]. In this situation, precautions should be taken, especially on the first stapling of intraluminal mucosal edges. Any bleeding from the mucosal edges should be stopped by suturing or electro-coagulation. Bleeding events from this site tend to be major because they manifest more slowly than from left-side colon anastomoses.

In Japan, FPX and enoxaparin are used as VTE prophylaxis after abdominal high-risk surgery. It is very important to know which agent is safer, but there is no evidence to distinguish these two agents in terms of their safety profile. In comparison with enoxaparin, FPX has a longer half-life and no antidote [18], so it is given once a day, and if bleeding occurs, all we can do is to stop its administration. The fact that all bleeding events in this series were controlled by stopping FPX demonstrates that its prompt discontinuation is necessary in the case of bleeding.

The weaknesses of this study are that there was no control group, the duration of VTE prophylaxis was only 4–8 days, and the observation period was only up until POD 5. Without a control arm, the incidence of bleeding and symptomatic VTE will be descriptive; however, more than 600 patients is sufficient to evaluate the safety of FPX. Because the ENOXACAN II study showed that 4 weeks of enoxaparin prophylaxis significantly reduced the incidence of venographically detected thrombosis, longer prophylaxis seems warranted [19]. However, only 4–8 days of FPX is approved by the government in Japan, and the observational period of this study was thus necessarily short. We plan to evaluate the usefulness of longer prophylaxis in the next trial.

In conclusion, thromboprophylaxis in cancer patients is complicated by the fact that they are at increased risk of both VTE and bleeding [20]. Thus, it is important to identify the best way to prevent thrombosis while minimizing bleeding complications. Ample information about the bleeding risks of specific surgical procedures may help surgeons use pharmacological prophylaxis more effectively. The findings of the present study suggest that FPX given once daily at a dose of 2.5 mg, initiated 24 ± 2 h after an operation, is safe and effective for Japanese patients undergoing colorectal cancer surgery.

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Conflict of interest Masataka Ikeda received lecture fees from GlaxoSmithKline.

Appendix

The following institutions and investigators participated in this study, in no particular order.

Kimimasa Ikeda and Masakazu Miyake, Toyonaka Municipal Hospital; Hideyuki Mishima and Masakazu Ikenaga, Osaka National Hospital; Yoshihito Ide, Suita Municipal Hospital; Kazuya Sakata, Higashiosaka City General Hospital; Shingo Noura and Tatsushi Shingai, Osaka Medical Center for Cancer and Cardiovascular Diseases; Hirofumi Yamamoto, Ichiro Takemasa, Junichi Nishimura, and Mamoru Uemura, Osaka University Hospital; Hiroki Akamatsu, Osaka Police Hospital; Chu Matsuda, Osaka General Medical Center; Keigo Yasumasa, Osaka Kouseinenkin Hospital; Atsushi Ohkawa, Higashi Takarazuka Satoh Hospital; Shunji Morita, Yao Municipal Hospital; Hitoshi Mizuno, Rinku General Medical Center; Yasunori Watanabe, Osaka Sen-in Hospital; Seiji Kawasaki, Kobe Ekisaikai Hospital; Osamu Takayama, Itami City Hospital; Masato Sakon, Takayuki Ichihara, and Masakazu Murakami, Nishinomiya Municipal Central Hospital; Yasuhiro Miyake, Minoh City Hospital; Takamichi Komori and Yoshio Uemura, Kinki Central Hospital of Mutual Aid Association of Public Teachers; Mutsumi Fukunaga and Hiroyoshi Takemoto, Sakai City Hospital.

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FULL PAPER

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Phase II study of trastuzumab in combination with S-1 plus cisplatin in HER2-positive gastric cancer (HERBIS-1)

Y Kurokawa*[,1], N Sugimoto[2], H Miwa[3], M Tsuda[4], S Nishina[5], H Okuda[6], H Imamura[7], M Gamoh[8], D Sakai[9], T Shimokawa[10], Y Komatsu[11], Y Doki[1], T Tsujinaka[12] and H Furukawa[13]

[1]*Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2-E2, Yamadaoka, Suita 565-0871, Japan;* [2]*Department of Clinical Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases, 3-3-1, Nakamichi, Osaka 537-8511, Japan;* [3]*Department of Upper Gastroenterology, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya 663-8501, Japan;* [4]*Department of Gastroenterological Oncology, Hyogo Cancer Center, 13-70, Kitaoji-cho, Akashi 673-8558, Japan;* [5]*Department of Medical Oncology, Kinki University Faculty of Medicine, 377-2, Ohnohigashi, Osakasayama 589-8511, Japan;* [6]*Deparment of Medical Oncology, Keiyukai Sapporo Hospital, 1-1-14, Hondori, Sapporo 003-0027, Japan;* [7]*Deparment of Surgery, Sakai Municipal Hospital, 1-1-1, Yasui-cho, Sakai 590-0064, Japan;* [8]*Deparment of Medical Oncology, Osaki Citizen Hospital, 2-3-10, Furukawasenjujicho, Osaki 989-6183, Japan;* [9]*Deparment of Frontier Science for Cancer and Chemotherapy, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Osaka 565-0871, Japan;* [10]*Graduate School of Medicine and Engineering, University of Yamanashi, 4-4-37, Takeda, Kofu 400-8510, Japan;* [11]*Deparment of Cancer Center, Hokkaido University Hospital, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan;* [12]*Deparment of Surgery, Kaizuka City Hospital, 3-10-20, Hori, Kaizuka 597-0015, Japan and* [13]*Deparment of Surgery, Kinki University Faculty of Medicine, 377-2, Ohnohigashi, Osakasayama 589-8511, Japan*

Background: S-1, an oral fluoropyrimidine, plus cisplatin (SP) is a standard regimen for advanced gastric cancer (AGC) in East Asia. To date, no studies have evaluated the efficacy and safety of trastuzumab combined with SP in patients with human epidermal growth factor receptor type 2 (HER2)-positive AGC.

Methods: Patients with HER2-positive AGC received S-1 (80–120 mg per day) orally on days 1–14, cisplatin (60 mg m^{-2}) intravenously on day 1, and trastuzumab (course 1, 8 mg kg^{-1}; course 2 onward, 6 mg kg^{-1}) intravenously on day 1 of a 21-day cycle. The primary end point was response rate (RR); secondary end points included overall survival (OS), progression-free survival (PFS), time to treatment failure (TTF), and adverse events.

Results: A total of 56 patients were enrolled. In the full analysis set of 53 patients, the confirmed RR was 68% (95% confidence interval (CI) = 54–80%), and the disease control rate was 94% (95% CI = 84–99%). Median OS, PFS, and TTF were estimated as 16.0, 7.8, and 5.7 months, respectively. Major grade 3 or 4 adverse events included neutropaenia (36%), anorexia (23%), and anaemia (15%).

Conclusions: Trastuzumab in combination with SP showed promising antitumour activity and manageable toxic effects in patients with HER2-positive AGC.

*Correspondence: Dr Y Kurokawa; E-mail: ykurokawa@gesurg.med.osaka-u.ac.jp

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Gastric cancer is the second leading cause of cancer deaths worldwide (Ferlay *et al*, 2010). A global standard regimen for to treat advanced gastric cancer (AGC) has not been established (Macdonald *et al*, 2001; Cunningham *et al*, 2008). In Western countries, regimens containing a fluoropyrimidine (fluorouracil or an oral preparation) plus a platinum compound, and usually including docetaxel or epirubicin, have been most widely used. In East Asia, including Japan and Korea, a fluoropyrimidine plus a platinum compound has been used as standard therapy (Koizumi *et al*, 2008; Kang *et al*, 2009).

Recent studies have shed new light on the molecular mechanisms underlying the development and progression of gastric cancer. Trastuzumab is a monoclonal antibody targeting human epidermal growth factor receptor type 2 (HER2) with two antigen-specific sites that bind to the juxtamembrane portion of the extracellular domain of the HER2 receptor, thereby preventing activation of its intracellular tyrosine kinase (Hudis, 2007). The Trastuzumab for Gastric Cancer (ToGA) study, an international phase III trial comparing chemotherapy consisting of cisplatin plus capecitabine or fluorouracil *vs* trastuzumab plus chemotherapy in patients with HER2-positive AGC, demonstrated a survival benefit with the addition of trastuzumab (Bang *et al*, 2010). Currently, both the US Food and Drug Administration and the European Medicines Agency approved trastuzumab for the treatment of patients with HER2-positive AGC, and trastuzumab in combination with cisplatin plus capecitabine or fluorouracil is a standard treatment for HER2-positive AGC in the West.

S-1 is a fluoropyrimidine preparation combining tegafur, a prodrug of 5-fluorouracil (5-FU), gimeracil, and oteracil potassium in a molar ratio of 1:0.4:1. Gimeracil is a dihydropyrimidine dehydrogenase inhibitor, allowing high concentrations of 5-FU to be maintained (Shirasaka *et al*, 1996; Diasio, 1999). Two phase II studies (Sakata *et al*, 1998; Koizumi *et al*, 2000) in patients with AGC showed response rates (RRs) exceeding 40%. The S-1 Plus cisplatin versus S-1 In RCT In the treatment for Stomach cancer (SPIRITS) phase III trial established S-1 plus cisplatin (SP) as a standard first-line regimen for AGC in the East (Koizumi *et al*, 2008; Japanese Gastric Cancer Association, 2011). However, SP plus trastuzumab has not been evaluated in patients with HER2-positive AGC to date. We therefore conducted this phase II study to evaluate the efficacy and safety of SP plus trastuzumab in HER2-positive AGC.

PATIENTS AND METHODS

Patients. We enrolled patients with histologically proven unresectable or recurrent HER2-positive tumours in the stomach or gastroesophageal junction. Human epidermal growth factor receptor type 2 status of tumours was evaluated using immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH). In the IHC testing, HER2 tumour cell-membrane immunostaining was scored using a four-grade scale (0/1+/2+/3+) according to scoring scheme (ToGA score): 0, no staining or membranous reactivity in <10% of tumour cells; 1+, weak, barely perceptible membranous reactivity in >10% of tumour cells; 2+, complete or basolateral membranous reactivity either nonuniform or weak in ⩾10% of cells; and 3+, complete or basolateral membranous reactivity of strong intensity in ⩾10% of tumour cells (Hofmann *et al*, 2008; Bang *et al*, 2010). FISH analyses for HER2 status were carried out according to the manufacturer's procedure. The total numbers of HER2 and chromosome 17 signals were counted in at least 20 tumour cell nuclei in two different areas. The case with HER2/chromosome 17 ratio of ⩾2.0 was defined as FISH positive. In this study, only patients with IHC 3+, or IHC 2+ and FISH positive were eligible. Patients were required to have measurable lesions according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 (Eisenhauer *et al*, 2009). Eligibility criteria also included: age between 20 and 75 years; Eastern Cooperative Oncology Group performance status score of 0 or 1; leukocyte count between 3500 and 12 000 mm^{-3}, neutrophil count ⩾2000 mm^{-3}, hemoglobin ⩾9.0 $g dl^{-1}$, platelet count ⩾100 000 mm^{-3}, serum bilirubin <1.5 $mg dl^{-1}$, creatinine clearance ⩾60 $ml min^{-1}$ calculated using the Cockcroft–Gault formula, serum creatinine ⩽1.2 $mg dl^{-1}$, serum aspartate aminotransferase and alanine aminotransferase <100 $IU l^{-1}$; and baseline left ventricular ejection fraction ⩾50%. Patients were excluded from the study if they could not maintain sufficient oral intake, have massive ascites or pleural effusions, or had received prior chemotherapy or radiotherapy within 6 months before enrollment. The study protocol was approved by the Osaka Gastrointestinal Cancer Chemotherapy Study Group (OGSG) Steering Committee and the institutional review boards of all participating hospitals. All patients provided written informed consent before enrollment. This study was registered with UMIN-CTR, UMIN000005739.

Treatment. Trastuzumab was commercially obtained in this study. Patients received cisplatin (60 $mg m^{-2}$) plus trastuzumab (course 1, 8 $mg kg^{-1}$; course 2 onward, 6 $mg kg^{-1}$) intravenously on day 1 and oral S-1 twice daily at a dose based on body surface area (<1.25 m^2, 40 mg; ⩾1.25 to <1.5 m^2, 50 mg; ⩾1.5 m^2, 60 mg) on days 1–14 of a 21-day cycle.

This schedule was repeated until disease progression, development of unacceptable toxicity, or patient withdrawal of consent. If patients had a neutrophil count less than 1000 mm^{-3}, platelet count less than 75×10^3 mm^{-3}, serum creatinine more than 1.2 $mg dl^{-1}$, infection with fever, or anorexia, diarrhoea, oral mucositis, or rash of grade 2 or higher, treatment with S-1 was suspended. In patients with febrile neutropaenia, grade 4 neutropaenia, grade 3–4 thrombocytopaenia, serum creatinine >1.2 $mg dl^{-1}$, or grade 3–4 diarrhoea, oral mucositis, or rash, doses of S-1 and cisplatin were reduced starting from the next cycle. In patients who had grade 3–4 vomiting or anorexia because of cisplatin, the dose of cisplatin was reduced. If heart failure or severe infusion reactions occurred, treatment with trastuzumab was discontinued.

Evaluations. The primary end point was RR. The secondary end points were overall survival (OS), progression-free survival (PFS), time to treatment failure (TTF), and adverse events. Tumours were assessed every 6 weeks until disease progression, and objective responses were evaluated according to the RECIST guidelines (version 1.1). For complete response (CR) or partial response (PR), confirmation 4 weeks after initial evaluation was necessary. An independent review committee assessed responses in all patients. OS was defined as the time from the date of enrollment to the date of death from any cause. PFS was defined as the time from the date of enrollment to the date of disease progression or death from any cause. TTF was defined as the time from the date of enrollment to the date when the treating physician decided to discontinue treatment for any reason. Physical examination and blood test were mandatory before each course, and left ventricular ejection fraction was assessed every 3 month during treatment. Adverse events were evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0.

Statistical analysis. The required sample size was estimated based on a threshold RR of 35% and an expected RR of 50%, 80% power, and an alpha value of 0.1 (one-sided) using the binomial test. Given 2% of ineligible patients, the target sample size was determined to be at least 50 patients. Efficacy was evaluated in all patients who received at least one dose of the study treatment.

We used the Kaplan–Meier method to estimate survival curves and Greenwood's formula to calculate 95% confidence intervals (CIs) for survival rates. Statistical analyses were conducted with R, version 3.0.1.

RESULTS

Patient background. Between July 2011 and May 2012, a total of 56 patients were enrolled from 29 hospitals in Japan. Two patients were ineligible because of inadequate renal function or the absence of measurable lesions. The characteristics of the 54 eligible patients are listed in Table 1. The median age was 66 years (range = 34–75 years). Two-thirds of patients had differentiated adenocarcinoma. Only three patients (6%) had recurrent disease; the others had unresectable lesions. The most frequent sites of metastasis were the lymph nodes (81%), followed by the liver (59%). The proportions of IHC 3+ and IHC 2+/FISH-positive tumours were 83% and 17%, respectively.

Table 1. Eligible patient characteristics

Characteristic	*n* = 54
Age, years	
Median	66
Range	34–75
Sex	
Male	42 (78%)
Female	12 (22%)
Performance status	
0	42 (78%)
1	12 (22%)
Histological type	
Differentiated	36 (67%)
Undifferentiated	18 (33%)
Previous gastrectomy	
No	45 (83%)
Yes	9 (17%)
Unresectable/recurrent	
Unresectable	51 (94%)
Recurrent with adjuvant chemotherapy	2 (4%)
Recurrent without adjuvant chemotherapy	1 (2%)
Metastatic sites[a]	
Lymph nodes	44 (81%)
Liver	32 (59%)
Lung	5 (9%)
Peritoneum	5 (9%)
Bone	2 (4%)
Other	1 (2%)
HER2 status	
IHC 3+	45 (83%)
IHC 2+/FISH positive	9 (17%)

Abbreviations: FISH = fluorescence *in situ* hybridization; HER2 = human epidermal growth factor receptor type 2; IHC = immunohistochemistry.
[a]Some patients had multiple metastatic sites.

Efficacy. Of the 54 eligible patients, 1 patient did not receive any treatment per protocol because of a decrease in serum hemoglobin levels after study enrollment. Efficacy and safety analyses were therefore conducted in the full analysis set of the remaining 53 patients.

The median number of cycles was 6 (range = 1–27), and the median relative dose intensity for S-1, cisplatin, and trastuzumab was 76%, 83%, and 96%, respectively. At the time of analysis (August 2013), 51 patients had discontinued treatment. The main reason for discontinuation was progressive disease (31 patients), followed by adverse events (16 patients). Four patients underwent surgery because of a prominent response.

The confirmed RR based on RECIST (version 1.1) was 68% (95% CI = 54–80%; 80% CI = 58–76%; Table 2), so the null hypothesis for the primary end point (RR ⩽35%) was rejected ($P < 0.001$). The confirmed RRs in the differentiated type cases ($n = 35$) and the undifferentiated type cases ($n = 18$) were 69% (95% CI = 51–83%) and 67% (95% CI = 41–87%), respectively. Among 36 patients with CR or PR, the median time to response and duration of response were 41 days (range = 33–91 days) and 208 days (range = 42–630 days), respectively. The disease control rate, that is, the proportion of patients who had a CR, PR, or stable disease, was 94% (95% CI = 84–99%). Two patients (4%) had a CR. A waterfall plot of the confirmed best overall response for each patient is shown in Figure 1.

The median duration of follow-up at the time of analysis (August 2013) for the 53 patients was 13.5 months. The median OS was 16.0 months (95% CI = 13.3–not applicable), and the 1-year OS rate was 67.9% (95% CI = 56.5–81.7%; Figure 2). The median PFS was 7.8 months (95% CI = 6.0–8.8 months), and the 1-year PFS rate was 17.0% (95% CI = 9.4–30.8%; Figure 2). The median TTF was 5.7 months (95% CI = 4.2–7.1 months), and the 1-year TTF rate was 5.1% (95% CI = 1.4–18.6%).

Safety. All adverse events that occurred in three or more patients are shown in Table 3. Among the haematological adverse events, the proportions of grade 3–4 neutropaenia and anaemia were 36% and 15%, respectively. The most frequent common non-haematological toxicity was anorexia (any grade, 79%; grade 3–4, 23%). Except for anorexia, there were no grade 3 or 4 toxicities that occurred in more than 10% of patients. Creatinine was elevated in 24 of 53 patients (45%). Grade 2 infusion-related reactions occurred in three patients (6%). Heart failure did not occur in any patients.

There was one treatment-related death attributable to myelosuppression. This patient was judged as an ineligible case afterwards, because creatinine clearance before enrollment was 47.4 ml min^{-1}. Furthermore, S-1 administration continued despite a serum creatinine level of 2.31 mg dl^{-1} on day 7. Renal dysfunction led to myelosuppression that progressed to death. Upon review of the patient's records, the data and safety

Table 2. Objective response with confirmation based on RECIST

Total	*n* = 53
Complete response	2 (4%)
Partial response	34 (64%)
Stable disease	14 (26%)
Progressive disease	3 (6%)
Response rate (95% confidence interval)	68% (54–80%)
Disease control rate (95% confidence interval)	94% (84–99%)

Abbreviation: RECIST = Response Evaluation Criteria in Solid Tumor.

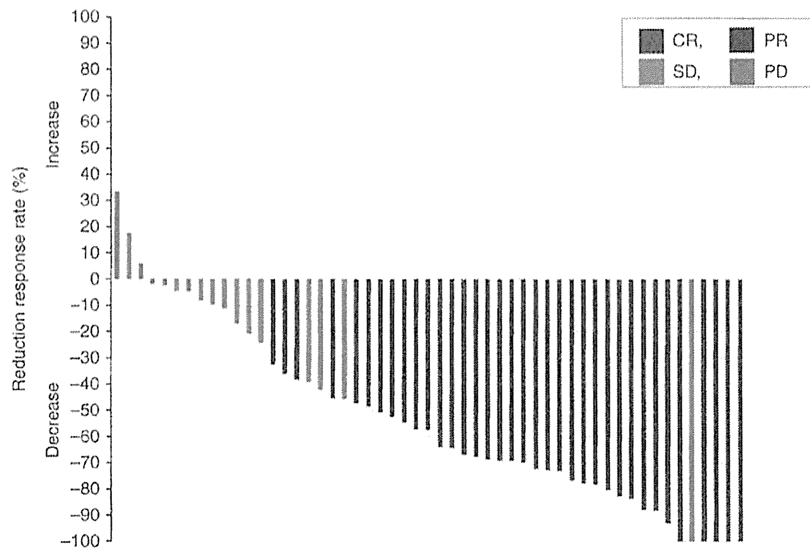

Figure 1. Waterfall plot of confirmed best overall response.

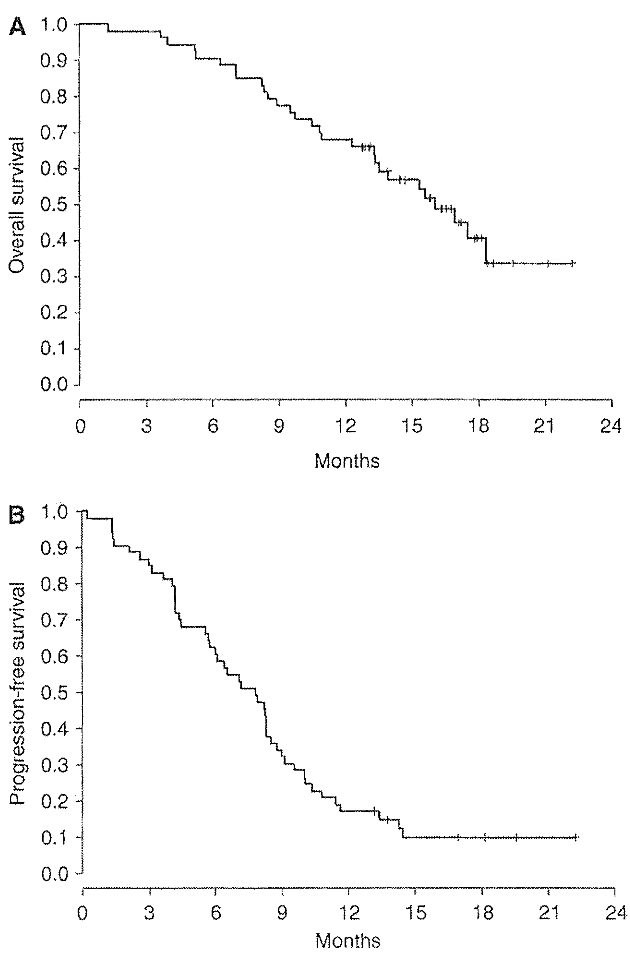

Figure 2. (A) The Kaplan–Meier overall survival and (B) progression-free survival.

monitoring committee determined that the patient died from critical deviations from the eligibility criteria and treatment protocol.

DISCUSSION

This multicenter phase II study is the first clinical trial reporting the efficacy and safety of SP plus trastuzumab in patients with HER2-positive AGC. We obtained a much higher RR (68%) than expected. The toxicity profile of our regimen was tolerable, and the incidence of grade 3–4 adverse events were similar to those of the SP regimen in the SPIRITS study (Koizumi *et al*, 2008). These results suggest that SP plus trastuzumab is a potential new treatment option for patients with HER2-positive AGC.

The ToGA study demonstrated that trastuzumab in combination with cisplatin plus capecitabine or fluorouracil was superior to cisplatin plus capecitabine or fluorouracil alone (Bang *et al*, 2010). The RR was 35% in the chemotherapy group and 47% in the trastuzumab plus chemotherapy group. In the aforementioned phase II study of a 3-week cycle of SP, the RR was 48%, compared with 68% in the present study, suggesting that trastuzumab considerably enhanced the effectiveness of chemotherapy, which is consistent with the results of the ToGA study. In addition, the median OS and PFS in our study were 16.0 and 7.8 months, respectively, whereas the subgroup of Japanese patients in the trastuzumab arm of the ToGA study had a median OS and PFS of 15.9 and 6.2 months, respectively (Sawaki *et al*, 2012). Although these results must be interpreted with caution because of the differences between the ToGA study and our study in terms of patient characteristics, especially histologic type, the proportion of patients with HER2 IHC 3+ tumours, and exclusion of patients with performance status ⩾2, trastuzumab may be a good addition to a S-1-based regimen. Experimental studies have reported that trastuzumab induces downregulation of thymidylate synthase expression. This mechanism has been implicated in the synergistic antitumour effect of S-1 plus trastuzumab against gastric cancer cell lines that overexpress HER2 (Tanizaki *et al*, 2010). Capecitabine and S-1 are both 5-FU derivatives, but were developed based on different concepts. Further studies of biomarkers and other predictors of outcomes are necessary to optimise the use of these drugs.

During the planning phase of this trial, a 5-week cycle of SP therapy was the mainstay of chemotherapy for AGC in Japan, based on the results of the SPIRITS study (Koizumi *et al*, 2008). As a molecular-targeted agent was combined with SP, the development of a 3-week cycle was planned. Results of phase II studies of a 3-week regimen of SP have been reported in gastric

Table 3. Adverse events ($n=53$)

Event	Grade 1	Grade 2	Grade 3	Grade 4	Any (%)	Grade 3–4 (%)
Leukopaenia	17	18	3	1	74	8
Neutropaenia	8	5	14	5	60	36
Febrile neutropaenia	0	0	1	1	4	4
Anaemia	5	22	6	2	66	15
Thrombocytopaenia	20	6	0	0	49	0
Anorexia	15	15	12	0	79	23
Fatigue	18	14	2	0	64	4
Nausea	20	12	1	0	62	2
Hypoalbuminaemia	14	6	5	0	47	9
Hypertension	9	12	1	0	42	2
Creatinine increased	21	0	3	0	45	6
Diarrhoea	10	7	4	0	40	8
Oral mucositis	10	6	1	0	32	2
Skin rash	12	1	0	0	25	0
Vomiting	7	3	3	0	25	6
ALT increased	11	2	0	0	25	0
Constipation	7	4	0	0	21	0
Dysgeusia	7	3	0	0	19	0
AST increased	9	0	0	0	17	0
Blood bilirubin increased	6	2	0	0	15	0
Edema	6	2	0	0	15	0
Peripheral sensory neuropathy	1	5	0	0	11	0
Epistaxis	3	1	0	0	8	0
Hiccups	4	0	0	0	8	0
Fever	2	2	0	0	8	0
Infusion-related reaction	0	3	0	0	6	0
Alopecia	2	1	0	0	6	0
Abdominal pain	1	2	0	0	6	0
Skin hyperpigmentation	2	1	0	0	6	0

Abbreviations: ALT = alanine aminotransferase; AST = aspartate aminotransferase.

cancer and lung cancer (Lee *et al*, 2008; Choi *et al*, 2010; Kubota *et al*, 2010). Recently, a phase III trial comparing the standard 5-week cycle of SP with a 3-week cycle of SP was conducted in patients with AGC. This trial showed that the median PFSs in the 3-week and 5-week cycle groups were 5.5 and 4.9 months, respectively, and it concluded that a 3-week cycle of SP was superior to a 5-week cycle of SP in terms of PFS ($P=0.042$) (Ryu *et al*, 2013). We therefore expected that a 3-week regimen of SP plus trastuzumab would be more effective than a 5-week regimen of SP plus trastuzumab. Although the dose intensity of cisplatin (20 mg m^{-2} per week) in a 3-week SP regimen was 25% lower than that (26.7 mg m^{-2} per week) in the ToGA study regimen, the RR (48%) of 3-week SP regimen was higher than that (35%) of the ToGA regimen. Thus, we considered that the dose (60 mg m^{-2}) of cisplatin was adequate in this 3-week SP regimen.

In this study, we limited subjects to patients with measurable lesions assessable according to RECIST guidelines (version 1.1). In clinical practice, however, many patients with gastric cancer have no measurable lesions, such as those with peritoneal metastasis. We are therefore conducting another phase II study in patients who have HER2-positive AGC without measurable lesions (HERBIS-1B; UMIN000007941) to confirm the usefulness of this regimen in this subgroup.

In conclusion, although this was not a randomised controlled study, our results suggest that SP plus trastuzumab has a good toxicity profile and promising efficacy, justifying the further study of regimens that contain SP and trastuzumab.

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CONFLICT OF INTEREST

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Association between ephrin-A1 mRNA expression and poor prognosis after hepatectomy to treat hepatocellular carcinoma

HIROSHI WADA[1], HIROFUMI YAMAMOTO[1], CHIWAN KIM[1], MAMORU UEMURA[1], HIROFUMI AKITA[1], YOSHITO TOMIMARU[1], NAOKI HAMA[1], KOICHI KAWAMOTO[1], SHOGO KOBAYASHI[1], HIDETOSHI EGUCHI[1], KOJI UMESHITA[2], YUICHIRO DOKI[1], MASAKI MORI[1] and HIROAKI NAGANO[1]

[1]Department of Surgery and [2]Division of Health Sciences, Graduate School of Medicine, Osaka University, Osaka, Japan

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Abstract. Hypoxia regulates the expression of genes that promote tumor growth, angiogenesis and invasion. We previously studied hypoxic tumor cells *in vitro* and from hepatic metastases of colorectal cancer and determined several potential prognostic factors for hepatocellular carcinoma (HCC). In this study, we evaluated the prognostic impact of the expression of ephrin-A1 (EFNA1) and its receptor, EPHA2, in patients with HCC after curative resection. Samples from a total of 139 HCC patients were analyzed by either microarray alone (n=86) or by microarray and quantitative PCR (n=53). There was no correlation between *EFNA1* expression and clinicopathological factors. *EPHA2* expression was not significantly correlated with any clinicopathological factors, except for microscopic portal invasion. *EFNA1* was an independent prognostic factor for HCC (p=0.0277). These findings suggest that *EFNA1* expression may be a useful marker for predicting high risk of recurrence in patients who have undergone curative resection for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the fifth leading cause of cancer-related death worldwide. Despite recent advances in diagnostic technology and new therapeutic modalities for HCC, the prognosis for patients with advanced-stage HCC is still poor (1). Thus, it is crucial to find novel cancer-related genes that may serve as diagnostic markers and molecular targets in HCC therapy, especially after curative treatment.

Correspondence to: Professor Hirofumi Yamamoto, Department of Surgery, Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Yamadaoka 2-2, Suita, Osaka 565-0871, Japan
E-mail: hyamamoto@gesurg.med.osaka-u.ac.jp

Abbreviations: RT-PCR, reverse transcription PCR

Key words: hepatocellular carcinoma, ephrin-A1, hypoxia, prognosis

Hypoxia is a central feature of solid tumors, and it regulates the expression of a diverse group of genes that promote tumor growth, invasion, angiogenesis and cell survival (2-5). In tumor cells under hypoxic conditions, the hypoxia-inducible factor-1 (HIF-1) pathway is activated and leads to upregulation of many hypoxia-response genes, which are associated with an aggressive tumor phenotype (5-7). We previously reported that these hypoxia-related genes include several angiogenic factors that play important roles in cancer biology (3,8-10). The anti-VEGF antibody bevacizumab is used clinically for treatment of several human cancers (11), and the multi-tyrosine kinase inhibitor sorafenib was shown to have survival benefits for patients with advanced HCC in two phase III clinical trials (12,13). These findings support the use of hypoxia-induced genes as clinically relevant therapeutic targets.

Ephrin-A1 (EFNA1) is known as an angiogenesis factor and is induced through an HIF-1-dependent pathway (14,15). EFNA1 was originally isolated as a secreted protein in conditioned media from cultures of human umbilical vein endothelial cells treated with tumor necrosis factor-α (16,17). Binding of EFNA1 ligand to its receptor EPHA2 promotes autophosphorylation, which triggers downstream signals that regulate cell growth and migration. EFNA1 expression has been observed in tumor cells and in endothelial cells and has been shown to induce endothelial cell migration (18), capillary assembly *in vitro* and corneal angiogenesis *in vivo* (19). EFNA1 and EPHA2 expression is associated with carcinogenesis, angiogenesis (18,20-22), and tumorigenesis in various types of cancer (23-28).

We previously reported that HIF1A expression is correlated with tumor angiogenesis in HCC and that high nuclear expression of HIF-1 is a significant predictive factor for recurrence after curative resection in HCC patients (9). Previously, we detected several potential prognostic factors and therapeutic targets in hypoxic tumor cells from hepatic metastases of CRC *in vivo* (8). Of the 3,000 genes ranked in the microarray data, the top 30 were identified as hypoxia-inducible genes. Among these hypoxia-inducible genes, Jumonji domain containing 1A (*JMJD1A*, also known as *KDM3A*) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) were novel prognostic factors of HCC (3,10). In these experiments, *EFNA1* expression was highly induced in hypoxic regions of liver metastases. Thus, we hypothesized that *EFNA1* expression may be a novel prognostic factor in patients with HCC. In the present study, we examined the correlation between *EFNA1* expression and prognosis in HCC patients and analyzed the biological significance of *EFNA1* expression in human HCC.

Materials and methods

Cell culture. The human hepatoma cell lines PLC/PRF/5, HuH7, and HpeG2 were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), and the Hep3B cell line was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. For hypoxic conditions, cells were maintained in a continuously monitored atmosphere of 1% O_2, 5% CO_2, and 94% N_2 in a multigas incubator (model 9200; Wakenyaku Company, Kyoto, Japan).

Patients and clinical sample collection. A total of 139 HCC patients who underwent hepatectomy at Osaka University Hospital and its associated hospitals were enrolled in this study. All aspects of our study protocol were approved by the ethics committee of the Graduate School of Medicine, Osaka University. All patients provided written informed consent to use their surgical specimens and clinicopathological data for research purposes. Clinical staging was based on the TNM classification of the Union for International Cancer Control (UICC), and histological grading was based on World Health Organization classification.

Immediately after surgical resection, a tissue sample was collected from the fresh specimens and stored in RNA Stabilization Reagent (RNA Later; Ambion, Inc., Austin, TX, USA) at -80°C until RNA extraction.

RNA extraction and real-time quantitative RT-PCR analysis. Total RNA was extracted by a single-step method with TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA) at Osaka University. Complementary DNA (cDNA) was generated by using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA), as described previously (3). Real-time monitoring of PCR reactions was performed with the LightCycler system (Roche Applied Science, Indianapolis, IN, USA) for quantification of mRNA expression, as described previously (29). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The sequences of the GAPDH primers were as follows: sense primer, 5'-CAACTACATGGTTTACATGTTC-3' and antisense primer, 5'-GCCAGTGGACTCCACGAC-3'. *EFNA1* primer sets were designed to flank one intron and were tested to ensure amplification of only cDNA to avoid amplification of possible contaminating genomic DNA. The sequences of these PCR primers were as follows: *EFNA1* sense primer, 5'-TGCC GTCCGGACGAGACAGGC-3' and antisense primer, 5'-CTG GAGCCAGGACCGGGACTG-3'.

Microarray experiment. Microarray results were evaluated in accordance with previously described methods (30). Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions supplied by the manufacturer. The integrity of RNA was assessed with Agilent 2100 Bioanalyzer and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNA was used for analysis. Seven RNA extractions from different normal liver tissue samples were mixed and used as the control reference. Next, 2 μg of total RNA was used to synthesize double-stranded cDNA that contained a promoter for T7 RNA polymerase. Amplified antisense RNA was synthesized by *in vitro* transcription of the cDNA templates using the Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX, USA). The reference and test samples were labeled with Cy3 and Cy5, mixed, and hybridized on a microarray covering 30,336 human probes (AceGene Human 30K; DNA Chip Research Inc. and Hitachi Software Engineering Company, Yokohama, Japan). The microarrays were scanned using ScanArray Lite, and signal values were calculated using DNASIS array software (Hitachi Software Engineering Company). The local background was subtracted from each spot, and the ratio of the intensity of fluorescence from the Cy5 channel to the intensity of fluorescence from the Cy3 channel was calculated for each spot. The ratio of expression levels of each gene was converted to a logarithmic scale (base 2), and the data matrix was normalized.

Statistical analysis. For clinicopathological analyses, study samples were divided into high- and low-expression groups based on the median *EFNA1* mRNA expression levels in tumor tissue. All statistical analyses were carried out using the StatView J-5.0 program (Abacus Concepts, Inc., Berkeley, CA), USA. The post-operative period was measured from the date of surgery to the date of the last follow-up or death. Differences were estimated using Fisher's exact probability test. Survival curves were calculated by the Kaplan-Meier method and compared statistically using the log-rank test. To estimate relative risk (RR) and 95% confidence intervals (95% CI), univariate and multivariate analyses were performed using the Cox proportional hazards regression model. Data are reported as mean ± standard deviation. Mean values were compared using the Mann-Whitney test. A probability value of <0.05 was deemed to be statistically significant.

Results

Expression of EFNA1 under hypoxic conditions. First, we evaluated expression of *EFNA1* under hypoxic conditions. *EFNA1* was expressed in all four hepatoma cell lines and gradually increased under hypoxia in HuH7, HepG2 and Hep3B cell lines, but not in PLC/PRF/5 cells (Fig. 1). This result suggests that hypoxic conditions are associated with increased *EFNA1* expression in HCC.

Patient profiles. Next, we evaluated the expression of *EFNA1* in clinical samples by using microarray analysis. The patients selected for microarray analysis included 113 (81.3%) men and 26 (18.7%) women. Twenty-six patients had hepatitis B virus infection, and 85 patients were positive for hepatitis C virus antibody. A total of 102 patients had a single tumor in the liver, and 65 patients had a tumor <3 cm in diameter. Macroscopic vascular invasion was seen in 15 patients. With regard to TNM

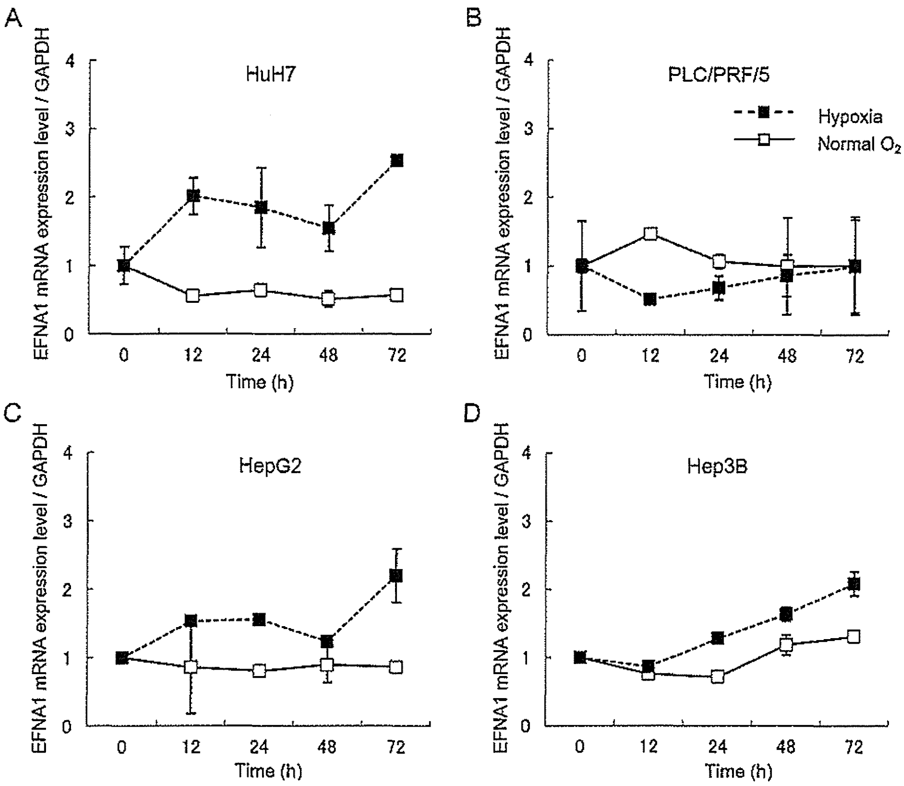

Figure 1. *In vitro* assay to measure *EFNA1* expression under hypoxic conditions in hepatoma cell lines. Comparison of *EFNA1* mRNA expression under hypoxic and normal conditions in (A) HuH7, (B) PLC/PRF/5, (C) HepG2 and (D) Hep3B cell lines.

staging, 96 patients (69.1%) were stage I, 31 patients (22.3%) were stage II, and 12 patients (8.6%) were stage III. The characteristics of the 139 patients are summarized in Table I.

Microarray analysis of EFNA1 mRNA expression. We examined the correlation between expression levels of *EFNA1* and *EPHA2* and the clinicopathological factors of the 139 HCC patients who had undergone hepatic resection. The 139 patients were divided into two groups, a high-expression group (n=70) and a low-expression group (n=69), based on median expression levels from the microarray data for each gene in Table II. There was no correlation between *EFNA1* expression and clinicopathological factors including tumor size, vascular invasion and number of tumors. *EPHA2* expression was not significantly correlated with any clinicopathological factors, except for microscopic portal invasion. Tumors with high expression of *EPHA2* had a tendency to have microscopic vascular invasion, although this result was not statistically significant (p=0.0786) (Tables I and II).

Correlation between EFNA1 and EPHA2 expression levels. We next evaluated the correlation between *EFNA1* and *EphA2* expression levels using microarray data. We found that *EFNA1* expression levels were significantly correlated with those of *EPHA2* (Fig. 2).

EFNA1 expression measured by quantitative RT-PCR correlated with microarray data. We next examined the correlation between expression data from the microarray and quantitative RT-PCR (qRT-PCR) analysis of *EFNA1* to validate the microarray data. qRT-PCR analysis was performed on 53 HCC tissue samples that were randomly selected from among the 139 HCC tissue specimens. Individual mRNA levels were normalized to GAPDH. In the 53 samples, qRT-PCR data for *EFNA1* were significantly correlated with the results obtained from the microarray data (Fig. 3).

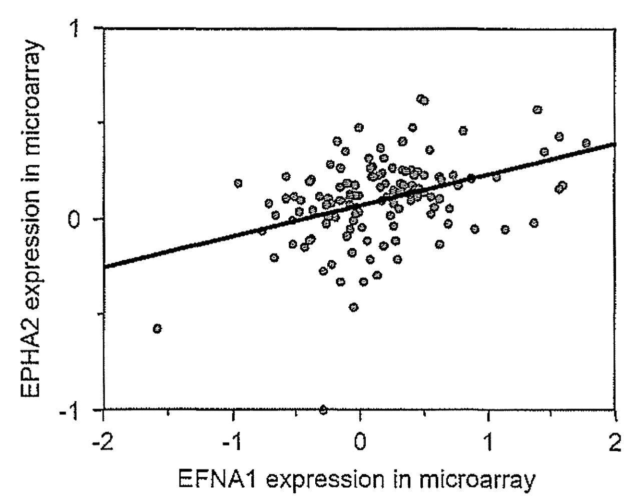

Figure 2. Correlation between *EFNA1* and *EPHA2* expression based on microarray data. The Pearson correlation coefficient was 0.455.

Survival analysis stratified by EFNA1 and EPHA2 mRNA expression. Kaplan-Meier survival curves demonstrated that

Table I. Association between clinicopathological factors and EFNA1 expression.

Characteristics	Low expression (n=69)	High expression (n=70)	p-value
Age (years)			0.9999
<65	31	32	
≥65	38	38	
Gender			0.1271
Male	60	53	
Female	9	17	
HBV infection			0.5150
Present	11	15	
Absent	58	55	
HCV infection			0.9999
Present	42	43	
Absent	27	27	
Child-Pugh grade			0.0761
A	53	62	
B	16	8	
Cirrhosis			0.4955
Absent	41	37	
Present	28	33	
α-fetoprotein (ng/ml)			0.1519
<100	42	51	
≥100	27	19	
PIVKA-II (mAU/ml)			0.2829
<40	26	20	
≥40	43	50	
Tumor size (cm)			0.9999
<3	32	33	
≥3	37	37	
Tumor multiplicity			0.2500
Single	54	48	
Multiple	15	22	
Macroscopic portal invasion			0.1829
Absent	59	65	
Present	10	5	
Stage (TNM)			0.7055
I/II	64	63	
IIIA/IIIB	5	7	
Histological grade			0.2796
Well/moderately	42	40	
Poorly	27	30	
Microscopic portal vein invasion			0.292
Absent	47	41	
Present	22	28	
Microscopic intrahepatic metastasis			0.8469
Absent	51	53	
Present	18	17	

HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence or antagonist II; well/moderately, well or moderately differentiated hepatocellular carcinoma; poorly, poorly differentiated hepatocellular carcinoma.

Table II. Association between clinicopathological factors and EphA2 expression.

Characteristics	Low expression (n=69)	High expression (n=70)	p-value
Age (years)			0.2349
<65	35	34	
≥65	34	42	
Gender			0.8283
Male	57	56	
Female	12	14	
HBV infection			0.6689
Present	14	12	
Absent	55	58	
HCV infection			0.999
Present	42	43	
Absent	27	27	
Child-Pugh grade			0.6596
A	56	59	
B	13	11	
Cirrhosis			0.8650
Absent	38	40	
Present	31	30	
α-fetoprotein (ng/ml)			0.2829
<100	43	50	
≥100	26	20	
PIVKA-II (mAU/ml)			0.1519
<40	27	19	
≥40	42	51	
Tumor size (cm)			0.8656
<3	33	32	
≥3	36	38	
Tumor multiplicity			0.2500
Single	54	48	
Multiple	15	22	
Macroscopic portal invasion			0.9999
Absent	62	62	
Present	7	8	
Stage (TNM)			0.3472
I/II	65	62	
IIIA/IIIB	4	8	
Histological grade			0.3309
Well/moderately	45	37	
Poorly	24	33	
Microscopic portal vein invasion			0.0786
Absent	49	39	
Present	20	31	
Microscopic intrahepatic metastasis			0.4353
Absent	54	50	
Present	15	20	

HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence or antagonist II; well/moderately, well or moderately differentiated hepatocellular carcinoma; poorly, poorly differentiated hepatocellular carcinoma.