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Multicenter Phase I/II Study of Docetaxel, Cisplatin and Fluorouracil Combination Chemotherapy in Patients with Advanced or Recurrent Squamous Cell Carcinoma of the Esophagus

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Key Words
Chemotherapy · Cisplatin · Docetaxel · Fluorouracil · Squamous cell carcinoma of esophagus

Abstract
Objective: Esophageal squamous cell carcinoma (ESCC) is refractory to current therapeutic regimens and more effective therapies are imperative. To this end, we conducted a multicenter phase I/II trial of docetaxel, cisplatin, and fluorouracil (DCF) combination chemotherapy for ESCC. ***Methods:*** The study subjects were 46 patients with advanced or recurrent ESCC. Treatment included docetaxel at 60, 70, and 75 mg/m^2, cisplatin at 70 mg/m^2 on day 1, and daily fluorouracil at 700 mg/m^2 on days 1 through 5. The recommended dose of docetaxel was determined in phase I, while the response rate (RR) and progression-free survival rates were analyzed in phase II. ***Results:*** The recommended dose was determined to be 70 mg/m^2 in phase I. In phase II, the RR was 72.5%. Interim analysis showed median and 1-year progression-free survival of 14 months and 55.6%, respectively. Grade 3/4 toxicities of leukopenia and neutropenia occurred in 72.5 and 90% of patients, respectively. No treatment-related death was recorded. Surgical resection was subsequently performed in 20 patients after chemotherapy, and curative resection was achieved in 19. ***Conclusion:*** DCF was tolerable and effective for advanced and recurrent ESCC. Such findings might encourage a change in the treatment strategy for ESCC.

Introduction

Advanced esophageal squamous cell carcinoma (ESCC) is one of the most refractory cancers and is associated with poor outcome. Systemic chemotherapy is regarded as one of the most effective treatments for ESCC, and currently forms an important part of the multidisciplinary treatment approach for advanced and metastatic disease. In particular, fluorouracil and cisplatin (FP) combination therapy has become a standard choice. FP regimens result in partial response rates of 25–35% [1, 2], with a 1-year survival rate ranging from 27.8 to 37.6% [3–5], and median survival time of 9.2 months for responders and 5.3 months for nonresponders. To improve the prognosis of patients with advanced ESCC, more effective regimens are urgently needed.

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Docetaxel (DTX) is a relatively new cytotoxic antineoplastic agent currently used for the treatment of various cancers. Many studies have reported that taxanes have significant activity in patients with advanced and metastatic esophageal carcinoma [6–10]. As a single agent, DTX is reported to have achieved a partial response rate (RR) of 20.4% in patients with metastatic esophageal cancer, raising hope that this agent could be useful in esophageal cancer [6]. Several studies of head and neck cancer and gastric cancer further indicated that the regimen of DTX plus cisplatin and fluorouracil (DCF) produced a higher RR and improved overall survival than the conventional FP regimen [11–14]. However, there are only a few reports on the efficacy and feasibility of DCF for esophageal cancer, especially ESCC. To evaluate the safety and efficacy of DCF, we conducted a multicenter phase I/II trial for ESCC.

Patients and Methods

Eligibility Criteria

Patients were regarded as eligible if they had histopathologically confirmed ESCC. The selection criteria were as follows: (1) clinically confirmed T4 and/or M1 tumors including nonregional lymph node metastases according to the 6th edition of the Tumor-Node-Metastasis Classification of the International Union Against Cancer (UICC) or recurrent tumor after esophagectomy; (2) assessable lesions according to the criteria of the Japanese Society for Esophageal Diseases; (3) no prior treatment for ESCC in primary cases and no prior chemotherapy and/or radiotherapy for ESCC in recurrent cases; (4) age 20–75 years; (5) Eastern Cooperative Oncology Group (ECOG) performance status within 0–1; (6) normal function of the major organs (indicated by leukocyte count >4.0 × 10^9/l, platelet count >100 × 10^9/l, hemoglobin >9.0 g/dl, serum AST/ALT less than 2× upper limit of normal, serum bilirubin <1.5 mg/dl, and calculated creatinine clearance >60 ml/min); (7) a life expectancy of at least 3 months, (8) lack of serious complications such as heart disease, pulmonary fibrosis, interstitial pneumonia, and bleeding tendency; (9) lack of overt infection (fever above 38°C); (10) lack of active double primary cancer; (11) lack of symptoms related to peripheral nerve lesions or edema of more than grade 2 according to the Common Terminology Criteria for Adverse Events of the National Cancer Institute (NCI-CTCAE, version 3); (12) negative history of drug hypersensitivity; (13) absence of current or previous brain metastases, and (14) absence of pregnancy, breast-feeding, or consideration of pregnancy.

All patients provided written informed consent before enrollment. The study protocol was approved by the instruction review board of each participating hospital.

Pretreatment Evaluation

Before treatment, the patients underwent enhanced computed tomography (CT) of the chest and abdomen, esophagogastroscopy, and positron emission tomography to assess the pretreatment tumor stage. Positive lymph nodes were defined as those with a diameter of at least 1 cm on CT scans [15].

Treatment Plan

We conducted a brief phase I trial to determine the maximum tolerated dose (MTD) and recommended dose (RD), followed by a larger phase II study using the regimen determined by the phase I trial to evaluate the response rate, survival, and toxicities of the DCF treatment. The following supportive therapy was routinely used in this study: patients received sufficient hydration and premedication consisting of dexamethasone infusion plus administration of cimetidine 1 day before infusion of DTX. Furthermore, antiemetics and 5-hydroxytryptamine-3 antagonist were routinely administered twice a day on days 1–3 with adjustment of dose and period as needed. No prophylactic granulocyte colony-stimulating factor (GCSF) or antibiotics were provided. However, GCSF was administered subcutaneously when febrile neutropenia or grade 4 neutropenia was encountered, and the use of GCSF in such cases was continued until the leukocyte count increased to more than 5,000 cells. GCSF was also administered upon the development of grade 3 neutropenia in patients who required GCSF in previous cycles.

Phase I Study. The dose of DTX was escalated using the following protocol: level 1: 60 mg/m^2, level 2: 70 mg/m^2, and level 3: 75 mg/m^2, intravenously infused over 60–120 min on day 1. Cisplatin and fluorouracil were administered at a dose of 70 mg/m^2 infused over 120 min on day 1 and 700 mg/m^2 continuously infused from day 1 to day 5, respectively. The above course was repeated every 21 days for up to two cycles. The dose-limiting toxicities (DLTs) during chemotherapy were defined as follows: (1) grade 4 leukopenia or neutropenia persisting for ≥5 days; (2) grade 3 or higher neutropenia with fever (febrile neutropenia); (3) grade 4 thrombocytopenia; (4) grade 3 or higher nonhematological toxicity except for anorexia, vomiting, nausea, constipation, fatigue, stomatitis, electrolyte abnormality, and diarrhea that resolved after 3 days, and (5) the need to delay therapy for more than 14 days because of toxicity. The MTD was defined as follows: if none of the 3 patients at level 1 developed DLTs, the dose was escalated to the next level. If 1 of the 3 patients had DLTs, 3 additional patients received the dose at the same level. If 2 or fewer patients showed DLTs, the dose was escalated to the next level. If 3 or more patients showed DLTs, that dose was defined as the MTD.

We defined the dose below the MTD as the RD, if the DCF chemotherapy in this dose was tolerable, and this regimen would be advanced into the phase II study. If the MTD was the level 1 dose, this trial was stopped and the phase II study would not proceed.

Phase II Study. After completion of the phase I study, the phase II study started at the RD defined by the phase I study. The treatment regimen was repeated every 3 weeks for up to two cycles according to the protocol, unless progression, patient's refusal, or unacceptable toxicity occurred. The next course commenced when the patient maintained particular biological parameters to meet the following criteria: (1) <grade 1 leukopenia, neutropenia, or thrombocytopenia; (2) urea nitrogen, creatinine, alanine aminotransferase, and aspartate aminotransferase remain within twice the normal limits, and (3) performance status within 0–1. If these criteria were not satisfied for more than 6 weeks after day 1 of the first cycle of chemotherapy, the patient was withdrawn from the study. The subsequent treatment was not defined.

Table 1. Patient characteristics

	Phase I	Phase II
Number	9	40
Median age (range), years	65 (53–71)	64 (43–75)
Sex (male:female)	9:0	34: 6
ECOG performance status (0:1)	6:3	29:11
Disease status (initial:recurrent)	9:0	39:1
Location of primary tumor (Ut:Mt:Lt)	1:4:4	10:18:11
Depth of invasion of primary tumor (T2:T3:T4)	0:4:5	4:13:22
Metastatic organ		
Nonregional LN	5	15
Liver	0	2
Lung	1	3
Adrenal	1	0
Cancer stage (III:IV)	2:7	18:21
Location of recurrent tumor		regional LN

Ut = Upper thoracic; Mt = middle thoracic; Lt = lower thoracic; LN = lymph nodes.

Evaluation

Toxicity was evaluated according to NCI-CTCAE, version 3. Clinical tumor responses were evaluated by esophagoscopy and CT after every cycle of chemotherapy. The responses of primary tumors and metastatic tumors in lymph nodes and distant organs to chemotherapy were evaluated according to the criteria of the Japanese Society for Esophageal Disease [16]. The primary tumor was defined as a measurable lesion and the response was evaluated by CT scan when the longest diameter of primary tumor was at least 20 mm. The response of primary tumor was evaluated by esophagoscopy when there was no measurable lesion. Partial response was defined as ≥50% decrease in the products of the two perpendicular diameters (TPD) of measurable lesions or marked morphological improvements (i.e., tumor regression, flattening of a raised ulcer margin, and shallowing and clearing of ulcer base) confirmed by esophagoscopy. Stable disease was defined as <50% reduction and <25% increase in the TPD of a measurable lesion, or a slight decrease or no change in tumor size confirmed by esophagoscopy. Progressive disease was defined as ≥25% increase in the TPD of measurable lesions, enlargement of the tumor, and/or the appearance of new lesions confirmed by esophagoscopy. Complete response was defined as the complete disappearance of all evidence of tumor, including negative biopsy results. The final response was assigned based on the evaluation after two cycles of the combination chemotherapy. If the treatment was not repeated past two cycles because of tumor progression, due to the patient's refusal or an unacceptable outcome, the assignment after two cycles was defined as the clinical tumor response.

In patients who underwent surgery, the primary tumors were examined for histopathological changes, which were classified as grade 3 (markedly effective; no viable cancer cells), grade 2 (moderately effective; 'viable' cancer cells account for less than 1/3 of tumor tissue, while other cancer cells showed severe degeneration or necrosis), and grade 1 (slightly effective, where apparently 'viable' cancer cells accounted for 1/3 or more of the tumor tissue, but there was some evidence of degenerating cancer tissue or cells). Grade 1 lesions were also subclassified into grade 1a (viable cancer cells accounted for 2/3 or more of tumor tissue), grade 1b (viable cancer cells accounted for ≥1/3, but<2/3, of tumor tissue), and grade 0 (ineffective, denoting no discernible therapeutic effect on cancer tissue or cells).

Statistical Analysis

The primary objective of the phase I study was to determine the MTD and RD. In phase II study, the primary objective was to assess the objective response rate (ORR) using the defined RD, and the secondary objective was to evaluate overall survival and treatment-related toxicity. The sample size was assumed based on findings that the ORR with previous FP therapies ranged from 33 to 36%, and we hypothesized that this regimen (DCF) would achieve an ORR of 55%. The phase II study enrolled 38 patients, a number that was required to confirm the null hypothesis that 95% confidence interval of the expected ORR (55%) would be less than 35% under conditions of an α error of 0.05 and a β error of 0.2.

Results

Patient Characteristics

Between October 2008 and June 2010, the phase I study enrolled 9 patients followed by the enrollment of 40 patients in the phase II study. Of the total number, 86, 15 and 9% received treatment at Osaka University Hospital, Osaka Medical Center for Cancer and Cardiovascular Disease, and Kinki University Hospital, respectively. The baseline characteristics of all patients are listed in table 1. In the phase I study, the median age was 65 years (53–71), all enrolled patients were males, and they were all new cases. The location of the primary tumor was the upper esophagus in 1, middle in 4, and lower thoracic esophagus in 4. Two and 7 patients had stage III and IV disease, respectively, at enrollment. In the phase II study, the median age was 64 years (43–75) and 34 of 40 patients were males. Of the 40 patients, 39 were new cases and 1 patient had recurrent tumor in the cervical paraesophageal lymph nodes. The location of the primary tumor in patients of the phase II study was the upper esophagus in 10, the middle esophagus in 18, and the lower thoracic esophagus in 11. At enrollment, 18, 21, and 1 patients had stage III disease, IV disease, and recurrence, respectively.

Phase I Study

Hematological and nonhematological toxicities encountered in the phase I study are summarized in table 2. At level 1, 2 patients had grade 3 leukopenia and neutro-

Table 2. Toxicities recorded in phase I trial

	NCI-CTC grade				Grades 3/4 %
	1	2	3	4	
Hematological toxicities					
Level 1 (n = 3)					
Leukopenia	0	1	2	0	66.7
Neutropenia	0	1	2	0	66.7
Anemia	0	0	1	0	33.3
Thrombocytopenia	0	0	0	0	0
Level 2 (n = 3)					
Leukopenia	1	1	0	1	33.3
Neutropenia	0	0	2	1	100
Anemia	1	0	0	0	0
Thrombocytopenia	0	0	0	0	0
Level 3 (n = 3)					
Leukopenia	0	0	0	3	100
Neutropenia	0	0	0	3	100
Anemia	1	0	1	0	33.3
Thrombocytopenia	1	0	0	0	0
Nonhematological toxicities					
Level 1 (n = 3)					
Anorexia	0	2	0	0	0
Nausea	0	3	0	0	0
Diarrhea	0	0	1	0	33.3
Stomatitis	0	1	1	0	33.3
Febrile neutropenia	0	0	0	0	0
Level 2 (n = 3)					
Anorexia	0	2	0	0	0
Nausea	1	0	0	0	0
Diarrhea	0	0	0	0	0
Stomatitis	0	2	0	0	0
Febrile neutropenia	0	0	0	0	0
Level 3 (n = 3)					
Anorexia	0	2	0	0	0
Nausea	0	1	0	0	0
Diarrhea	0	0	1	0	33.3
Stomatitis	1	1	0	0	0
Febrile neutropenia	0	0	1	0	1

Data represent number of patients. NCI-CTC = National Cancer Institute Common Toxicity Criteria.

Table 3. Toxicities recorded in phase II trial

	NCI-CTC grade				Grades 3/4 %
	1	2	3	4	
Hematological toxicities					
Leukopenia	1	7	18	11	72.5
Neutropenia	1	1	11	25	90.0
Anemia	4	7	0	0	0
Thrombocytopenia	1	3	1	1	5.0
Nonhematological toxicities					
Anorexia	2	13	9	0	22.5
Nausea	4	3	5	0	12.5
Diarrhea	1	6	4	1	12.5
Stomatitis	3	9	2	0	5.0
Fatigue	2	2	3	0	4.5
Dementia	0	0	1	0	2.5
Venous thrombus	0	0	1	0	2.5
Febrile neutropenia	0	0	4	0	10.0

Data represent number of patients. NCI-CTC = National Cancer Institute Common Toxicity Criteria.

penia, and 1 patient had grade 3 anemia, while 1 patient developed grade 3 diarrhea and stomatitis as nonhematological toxicities, which did not meet the criteria of DLT. At level 2, 2 patients developed grade 3 neutropenia, and 1 patient had grade 4 leukopenia and neutropenia, which did not meet the criteria of DLT because recovery occurred within 3 days. No patient showed grade ≥3 nonhematological toxicity. At level 3, 3 patients developed grade 4 leukopenia and neutropenia, and 1 of these developed grade 3 febrile neutropenia. One patient developed grade 3 diarrhea persisting for more than 3 days. Consequently, we defined level 3 as the MTD and level 2 as RD because 2 of the latter patients met the criteria of DLT.

Phase II Study

Based on the phase I study, level 2 was determined as the RD that was used to treat the subsequent 37 patients in the phase II study, with the 3 patients designated as level 2 in the phase I study making up the total of 40 patients evaluated in the phase II study.

Hematological and nonhematological toxicities in the phase II study are summarized in table 3. Grade 3/4 leukopenia, neutropenia, and thrombocytopenia were observed in 29 (72.5%), 36 (90%), and 2 (5%) patients, respectively. The nadir of myelosuppression such as leukopenia and neutropenia was registered at days 8–10 after treatment in 30 (83%) of 36 patients, and continued for a median (minimum to maximum) of 3 (1–5) days. Febrile neutropenia of grade 3 occurred in 4 patients (10%), with anorexia and diarrhea in 9 and 5 patients, respectively, and grade 3 venous thrombus associated with percutaneous central venous catheter was reported in 1 patient. No death associated with the toxic effects occurred in the phase I or II study.

Of these, 33 (82.5%) patients completed the chemotherapy as defined by the protocol. The other 7 patients did not receive the second cycle of chemotherapy be-

Table 4. ORR for the recommended dose

	Primary tumor (n = 39)	Lymph nodes (n = 35)	Organ metastases (n = 5)	Overall (n = 40)
Complete response	7 (17.9%)	8 (22.9%)	0 (0%)	4 (10%)
Partial response	24 (61.5%)	17 (48.6%)	5 (100%)	25 (62.5%)
Stable disease	8 (20.5%)	9 (25.7%)	0 (0%)	10 (25%)
Progressive disease	0 (0%)	1 (2.9%)	0 (0%)	1 (2.5%)

Data represent number of patients with the percentage in parentheses.

Table 5. Subsequent treatments

Treatment modality	Number
Surgery	20
Chemotherapy	6
Chemoradiotherapy	6
Chemoradiotherapy and surgery	7
Best supportive care	1

cause of the chemotherapy as defined by the protocol: patient refusal by 2, grade 3 dementia in 1, grade 2 renal dysfunction in 1, and stable or progressive disease in 3 patients. The median number of treatment cycles was 2 cycles (range 1–6). The ORR of primary tumors was 79.4% (31/39 patients), the ORR of lymph nodes was 71.5% (25/35 patients), and the overall RR was 72.5% (29/40 patients) (table 4). Four patients showed a complete response (10%), 25 partial responses (62.5%), 10 patients stable disease (25%), and 1 progressive disease (2.5%).

Of the 40 patients in the phase II study, 39 (97.5%) received one or more subsequent treatments (table 5), while 1 patient remained untreated at his request. Six patients with organ metastases and/or extensive lymph node metastases, such as axillary or abdominal para-aortic nodes, received continuous DCF chemotherapy. Chemoradiotherapy was performed in 13 patients who were diagnosed as T4 in the postchemotherapy evaluation. Surgical resection was performed in 20 patients and all were considered to have achieved curative resection by postchemotherapy evaluation. Subtotal esophagectomy via right thoracotomy with two- or three-field lymphadenectomy was performed in 20 patients, of whom 19 received a curative resection (R0) and only 1 patient underwent a noncurative resection (R1). Postoperative complications (grade 2 or more according to NCI-CTCAE, version 3) occurred in only 1 of 20 patients in the form of grade 2 vocal cord palsy. There was no postoperative death. The histological effects in the primary tumors or recurrent lesions were grade 3 in 5 patients, grade 2 in 3, grade 1b in 5, and grade 1a in 7. These results also showed that 6 of 16 patients who were clinically diagnosed as positive for lymph node metastasis were pathologically node-negative.

We performed interim analysis with a median follow-up period of 12.4 months. The minimum follow-up period in surviving patients was 6.5 months. The median survival time was not reached, but the 1-year survival rate was 74.6%, the median progression-free survival (PFS) was 14 months, and the 1-year PFS was 55.6%.

Discussion

The present study was designed to evaluate the safety and efficacy of DTX, cisplatin, and fluorouracil used in combination (DCF) for T4 and/or metastatic ESCC. The overall ORR in this study was 72.5%, which was nearly twice that reported previously using the FP regimen, and about 1.5 times that of RRs reported using the FAP regimen [1, 17, 18]. These results highlight DCF as a promising successor of first-line chemotherapy for T4 and/or metastatic ESCC.

Interestingly, our results showed that there was no progression of disease in primary tumors when the disease control rate (DCR) was 100%. In this study, 22 of 40 patients (55%) were clinically diagnosed with invasion of adjacent organs (cT4): trachea or left main bronchus in 17 patients, aorta in 6 patients, and liver in 1 patient. These patients often underwent chemoradiotherapy as the first-line therapy because it was considered superior to chemotherapy in achieving local control. Previous studies indi-

cated that chemoradiotherapy resulted in RRs of 68–76% and DCRs of 88–100% [19–21]. The DCF regimen used herein for the patients with T4 disease resulted in 82% RR and a DCR of 100%. This indicates that the DCF chemotherapy was functionally active and that this approach might lead to more curative cases of unresectable advanced esophageal cancer. Recently, Takahashi et al. [22] used the DCF combination chemotherapy for metastatic ESCC and reported satisfactory tolerance and outcome. The dosage of DTX used in their study was 50 mg/m^2, which was relatively small compared with the 70 mg/m^2 dose used in our study. The difference in the dose between the two studies is probably related to differences in the rank of this regimen in the treatment strategy for advanced ESCC between their and our trial. Their study focused on the effectiveness and continuity of DCF, similar to standard phase I/II trials. On the other hand, our study was designed not only to determine the effectiveness but also usefulness of DCF as a first-line treatment regimen that would be applied in neoadjuvant or induction setting. Therefore, we set the dosage similar to that used by Vermorken et al. [11], which was fixed at 75 mg/m^2 as induction chemotherapy, rather than at that used by Takahashi et al. [22]. Our results showed that our regimen was tolerated well and was effective enough to be applied in neoadjuvant or induction therapy, although our regimen was highly toxic compared to that used by Takahashi et al. [22].

One limitation of this study was the assignment of the final response on evaluation conducted after two cycles of DCF, rather than evaluation at a subsequent time point. In nonrandomized trials where the response is the primary endpoint, the overall response is generally confirmed by evaluation at a subsequent time point [23]. Bogaerts et al. [24] indicated that removing the requirement for response confirmation led to a significant increase in the number of patients classified as responders, resulting in a relative increase in the response rate.

However, at the risk of overestimating the response rate, we developed a protocol in which the therapeutic regimen was repeated for up to two cycles and the subsequent treatment was not defined. This approach was selected because we focused on the application of DCF therapy as a first-line treatment in a multidisciplinary approach for advanced ESCC as well as the evaluation of the toxicity and response rates. Indeed, 12 patients with cT4 underwent standard surgery used for clinically resectable tumors, when they achieved downstaging after two cycles of DCF (i.e., from cT4 to <ycT3). On the other hand, 10 patients with ycT4 who did not achieve downstaging after two cycles of DCF received chemoradiotherapy as a secondary treatment.

Curative resection was subsequently achieved in 95% of these patients. There were no differences in perioperative outcome (e.g., operation time, blood loss, and complications) between the present and our previous study [25]. Therefore, it seems that surgical resection after DCF is safe and feasible. DCF might also be suitable as neoadjuvant treatment for patients scheduled for resection surgery. Analyses of data of cT4 patients showed promising results with 1-year overall survival and PFS rates of 72.7 and 54.5%, respectively. These results are encouraging regarding the potential clinical impact on future treatment strategies for ESCC.

The DCF regimen described herein produced side effects with high frequency, especially leukopenia and neutropenia, as reported previously [11–14]. However, it should be noted that the myelosuppression improved rapidly after GCSF administration and did not necessarily cause treatment delays. Posner et al. [12] also reported that although grade 3 or 4 neutropenia occurred in 83% of patients in the DCF group and in 56% of patients in the FP group, prolonged neutropenia was responsible for treatment-associated delays in only 1% of patients of the DCF group and 39% of patients of the FP group [12]. The frequency of nonhematological toxicities such as anorexia, nausea, and stomatitis noted in our protocol was similar to that reported in other studies [11–14] and caused no treatment delays. Although febrile neutropenia occurred in 10.8% of patients, these patients improved within 5 days and there were no treatment-related deaths. Thus, careful monitoring for adverse events, especially myelosuppression, should ensure safe completion of the DCF regimen.

We did not report the long-term outcome in the present study because the minimum (median) follow-up time for surviving patients was 6.5 (12.4) months. Interim analysis provided promising results with a median PFS of 14 months, which is more than twice longer than that of patients treated by the FP regimen [3–5]. Follow-up studies are needed to assess the long-term prognosis.

In conclusion, DCF was tolerable and useful for advanced and recurrent ESCC. This regimen could enhance the treatment strategy for not only unresectable and inoperable ESCC, but also resectable ESCC, although larger prospective studies are required.

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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Tyrosine Kinase Inhibitor PTK/ZK Enhances the Antitumor Effects of Interferon-α/5-Fluorouracil Therapy for Hepatocellular Carcinoma Cells

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ABSTRACT

Purpose. There is no standardized treatment for hepatocellular carcinoma (HCC) with portal vein tumor thrombus. We previously reported the efficacy of interferon-alpha and 5-fluorouracil combination (IFN/5-FU) therapy for these patients and the potential mechanism via the regulation of vascular endothelial growth factor (VEGF). In this study, we showed the VEGF-related effects of IFN/5-FU therapy using VEGF-receptor (VEGFR) selective inhibitor, PTK787/ZK222584 (PTK/ZK), in HCC cells.

Methods. Using two VEGF secreting and VEGFR expressing human HCC cell lines, PLC/PRF/5 and HuH7, we performed growth inhibitory assays in vitro and in vivo, apoptosis assay, cell cycle analysis, and Western blot analysis for the mechanism, with or without PTK/ZK in IFN/5-FU therapy.

Results. The combination of PTK/ZK and IFN/5-FU significantly inhibited cell growth in vitro and tended to reduce tumor growth in vivo in a HuH7 xenograft model in nude mice—in both cases without affecting VEGF secretion. PTK/ZK enhanced the IFN/5-FU induced apoptosis, based on increased proteins levels of Bax and reduced Bcl-xL and Bcl-2. Cell cycle analysis showed different results between the HCC cell lines following the combination therapy, possibly due to differences in p21 protein.

Conclusions. VEGF signaling inhibition would support an antitumor effect of IFN/5-FU therapy against HCC cell lines via induction of apoptosis and cell cycle delay.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Recent advances in surgical resection and liver transplantation have improved the local control of HCC; however, there is no standardized treatment for locally advanced HCC, defined by the presence of portal vein tumor thrombus. The prognosis for such patients remains extremely poor with surgery alone, and the reported median survival times are 6–14 months.[1–3] We recently started administering interferon-alpha (IFN-α) and 5-fluorouracil (5-FU) combination (IFN/5-FU) therapy as an adjuvant in cases of advanced HCC, with good clinical efficacy (1-survival rate: 100% vs. 41% in patients without IFN/5-FU historical controls).[4–7] One of our previous biochemical analyses revealed that this therapy controls tumor-associated angiogenesis by regulating endothelial growth factor (VEGF).[8,9] However, VEGF inhibition in this therapy is limited to only 30–40% and control of VEGF may contribute more tumor suppression under the therapy.[8,9]

On the other hand, recent advances in drug development targets angiogenic factors, in the field of HCC, because of its hypervascularity.[10,11] Among these factors, VEGF plays a central role, and VEGF-targeted agents have some clinical benefits for HCC, via tyrosine kinase blocking of VEGF-receptors (VEGFRs; Flt-1, Flk-1/KDR, and Flt-4).[12–16] We thus hypothesized that VEGF inhibition would enhance the VEGF-related antitumor effects of IFN/5-FU therapy. Actually, IFN/5-FU therapy partially inhibits VEGF secretion; therefore, the purpose of this study was

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evaluation for an additional benefit under VEGF inhibition in the IFN/5-FU therapy. In this study, we used this VEGFR selective inhibitor, PTK787/ZK22584 (PTK/ZK, Vatalanib®), rather than sorafenib, which is commonly used but targets several tyrosine kinases.[15,16] PTK/ZK is a selective potent inhibitor of all known VEGFR tyrosine kinases, particularly potent against Flt-1 and Flk-1/KDR, and the efficacy against human HCC cell lines in vitro and in vivo was reported.[17] Our results showed that PTK/ZK enhanced the direct effect of IFN/5-FU therapy on human HCC cells both in vitro and in vivo. Furthermore, we investigated the possible additional effects of such therapy on apoptosis and cell cycle, previously reported as the main mechanisms of IFN/5-FU.[18]

MATERIALS AND METHODS

Reagents and Cell Lines

Purified human IFN-α was obtained from Otsuka Pharmaceutical Co. (Tokyo, Japan), 5-FU was obtained from Kyowa Hakko Kirin Co. (Tokyo), and PTK/ZK was obtained from Bayer Schering Pharma (Berlin, Germany). The two human HCC cells lines, PLC/PRF/5 and HuH7, expressing IFN receptor type 2 (IFNAR2), were purchased from the Japanese Cancer Research Resources Bank.[18] Both lines were maintained as adherent monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture at 37°C in a humidified incubator with 5% CO_2 in air.

Growth Inhibitory Assays using PTK/ZK Combined with IFN/5-FU Therapy

The growth inhibitory effects were tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously.[18] The cells were incubated in medium with or without PTK/ZK (5 or 10 μM) and/or IFN (0.5 μg/ml)/5-FU (500 U/ml) for 96 hours, based on previous reports.[17,18] These assays were repeated at least three times, with similar results obtained. The proportion of MTT-positive cells incubated without drugs was denoted as 100% viability.

Flow Cytometric Analysis of Annexin V-FITC Binding

Apoptosis was measured based on the binding of FITC-conjugated annexin, as described previously.[19] Briefly, after treatment with IFN-α and 5-FU with or without various concentrations of PTK/ZK, the cultured cells (1×10^6) were incubated with binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) containing saturating concentrations of annexin V-FITC (BioVision, Mountain View, CA) and propidium iodide (PI) for 15 minutes at room temperature. After incubation, the cells were pelleted and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson Immunocytometry Systems, BD, San Jose, CA), and data were processed using Cell Quest_software (BD).

Cell Cycle Analysis

Flow cytometric analysis was performed to assess the cell cycle, as described previously.[18] Briefly, after treatment with IFN-α and 5-FU with or without PTK/ZK (10 μM), cells were washed twice with phosphate-buffered saline (PBS) and then fixed in 70% cold ethanol for 4 hours before being washed and resuspended in 1 ml of PBS. PI (50 ml of 1 mg/ml solution in PBS) and RNase were added for 30 minutes at 37°C, and data were acquired on the FACSCalibur. Analysis of the cell cycle was performed using ModFIT software (BD).

Concentration of VEGF in Cell Culture Supernatants

After treatment with IFN-α and 5-FU with or without PTK/ZK (15 μM) for 48 hours, this conditioned medium was collected, and VEGF levels were analyzed using the human VEGF enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Camarillo, CA) as recommended by the manufacturer, as described previously.[8]

Western Blot Analysis

Cells were washed twice with ice-cold PBS and harvested from the culture dish. After centrifugation, the cell pellets were resuspended and lysed in RIPA buffer [25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 1 mM phenylmethylsulphonyl fluoride, and 500 KIE/ml aprotinin] containing phosphatase inhibitor. The extracts were centrifuged and the supernatant fractions were collected for Western blot analysis, performed as described previously.[20] The antibodies were used at 1:500 for anti-human Flt-1 antibody, 1:500 for anti-human KDR/Flk-1 antibody, 1:1000 for anti-human Bcl-xL antibody, 1:500 for anti-human Bcl-2 antibody, 1:400 for anti-mouse Bax antibody, 1:500 for anti-human cyclin D1 antibody, 1:300 for anti-human p27 antibody, and 1:500 for anti-human p21 antibody from Santa Cruz Biotechnology (Santa Cruz, CA), and 1:1000 for anti-human β-actin from Sigma (St Louis, MO), and 1:2000 for all secondary antibodies. The protein band intensities were analyzed densitometrically with the values for each protein band expressed relative to the density of the actin band.

Inhibitory Effect on Subcutaneous Xenograft Model in Nude Mice

Four- to 6-week-old female mice (BALB/c nu/nu, CLEA Japan, Tokyo) were used for subcutaneous xenograft models as described previously, under specific pathogen-free conditions in accordance with the institutional guidelines for animal care.[9] The doses and schedules of PTK/ZK and IFN/5-FU therapy were based on the results of previous studies.[9,17] Mice were assigned at random to one of the following four groups (5 mice per group): a) mice of the PTK/ZK group were administered PTK/ZK as an oral instillation (PO) at 20 mg/kg daily; b) each mouse of the IFN/5-FU group was injected subcutaneously (SC) 20,000U of IFN-α, three times per week, and an injected intraperitoneally (IP) 30 mg/kg 5-FU three times per week; c) mice of the IFN/5-FU+PTK/ZK group were administered PTK/ZK PO combined with IFN/5-FU SC/IP; and d) mice of the control group were administered SC/IP and PO injections of PBS. Tumor volume (TV) and body weight were measured twice per week, and TV was calculated using the following formula: (longest diameter) x (shortest diameter)^2 x 0.5. Four weeks after the initial treatment, all mice from each group were killed and tumors were harvested for examination.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). The unpaired Student's t test was used to examine differences in growth inhibitory effects in vitro. $P < 0.05$ was considered statistically significant.

RESULTS

VEGFR Expression and VEGF Secretion in Human HCC Cells

Flt-1 (VEGFR1) and KDR/Flk-1 (VEGFR2) were both expressed and in similar amounts in the two human HCC cells, PLC/PRF/5 and HuH7 (Fig. 1a). Incubation of both cells with IFN/5-FU alone and IFN/5-FU plus PTK/ZK, resulted in significant reductions of supernatant VEGF to 68.5% and 65.8%, respectively (Fig. 1b). These results indicated that PTK/ZK did not enhance the effect of IFN/5-FU on VEGF secretion through VEGFRs.

Inhibitory Effects of PTK/ZK and IFN/5-FU on Human HCC Cells In Vitro

To evaluate whether the combination of PTK/ZK and IFN/5-FU has an antiproliferative effect on human HCC

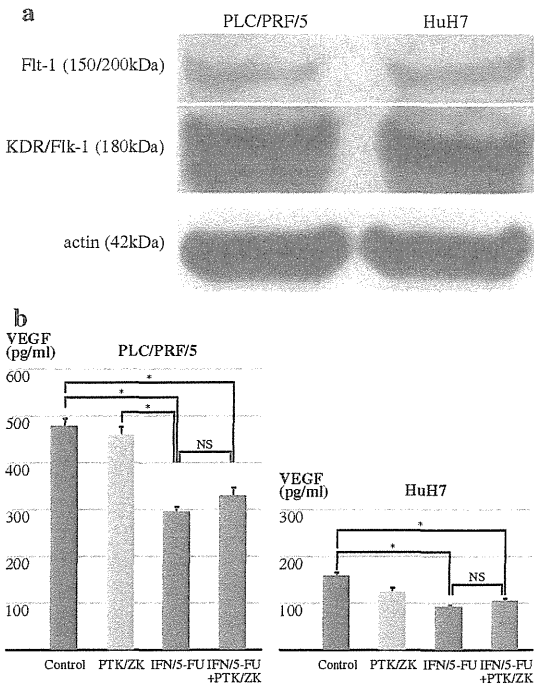

FIG. 1 VEGFRs expression and VEGF secretion in human HCC cells. a Expression of Flt-1 (VEGFR1) and KDR/Flk-1 (VEGFR2) in human HCC cells. b Secretion of VEGF from human HCC cells. PTK/ZK combined with IFN/5-FU significantly reduced the concentration of secreted VEGF in culture supernatants compared with the control group, whereas IFN/5-FU alone had no such effect. Data are mean ± SEM of triplicate assays; * $P < 0.05$

cells, we measured first the growth inhibition by the MTT assay. The data showed PTK/ZK concentration-dependent inhibition of cell growth, and PTK/ZK augments the inhibitory effect of IFN/5-FU; the addition of PTK/ZK (10 μM) to IFN/5-FU reduced the percentage of viable cells by 22.2% and 45.9% in PLC/PRF/5 and HuH7 cells, respectively (Fig. 2). The cooperative effect was statistically significant ($P < 0.05$).

PTK/ZK and IFN/5-FU Therapy Enhances Apoptosis of Human HCC Cells

To determine the mechanism of the antiproliferative effects of PTK/ZK combined with IFN/5-FU therapy on human HCC cells and whether it is related to the induction of apoptosis, we evaluated the extent of apoptosis using the annexin V assay to detect pre-apoptotic cells. The combination of PTK/ZK and IFN/5-FU increased the rate of apoptotic PLC/PRF/5 cells in a concentration-dependent manner and to a greater extent than IFN/5-FU alone (Fig. 3a, b; $P < 0.05$). The HuH7 cells showed similar results (data not shown).

Next, we assessed the expression levels of Bcl-xL and Bcl-2 proteins for anti-apoptosis and Bax for pro-apoptosis

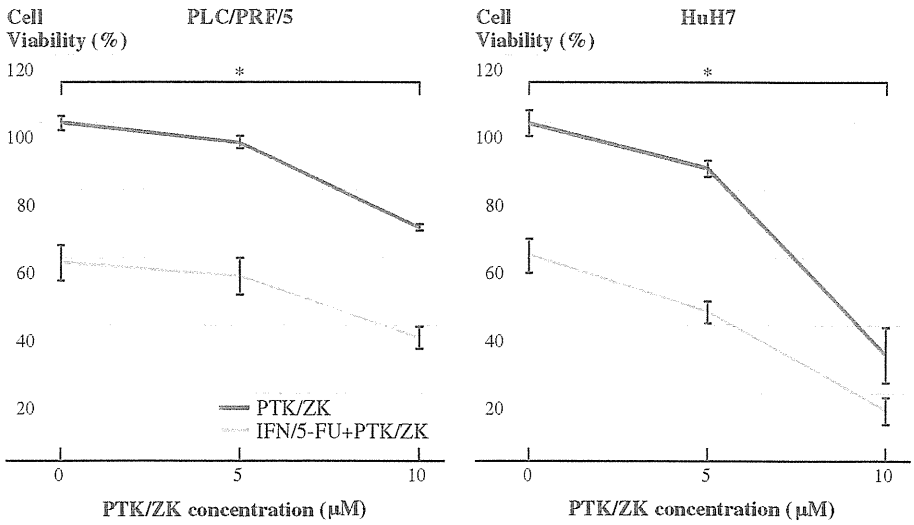

FIG. 2 Effect of PTK/ZK combined with IFN/5-FU on cell growth inhibition in human HCC cells lines. PLC/PRF/5 and HuH7 cells were incubated with PTK/ZK and/or IFN/5-FU, and then assayed using the MTT method. The proportion of viable cells incubated without drugs was defined as 100% viability. Data are mean ± SEM of four assays per condition. * $P < 0.05$. PTK/ZK combined with IFN/5-FU reduced cell growth more than IFN/5-FU alone in both PLC/PRF/5 and HuH7 cells

(Fig. 3c), which were reported previously to be associated with the apoptotic effect of IFN/5-FU therapy.[20] Figure 3d shows the relative expression levels of each protein compared with actin. PTK/ZK combined with IFN/5-FU decreased the expression levels of Bcl-xL and Bcl-2 to half of those in control, although there was no statistical difference in Bcl-2 expression of PLC/PRF/5 cells. In Bcl-2 expression, the addition of PTK/ZK to IFN/5-FU alone attenuated 10–30%, although there was no statistical difference. On the other hand, PTK/ZK combined with IFN/5-FU upregulated the expression of Bax compared with control by 1.5–3.4 times. The effect of the additional PTK/ZK to IFN/5-FU was 10–30% increments, although there was no statistical difference.

Effects of PTK/ZK and IFN/5-FU on the Cell Cycle in Human HCC Cells

Flow cytometric analysis was used to examine cell cycle progression of treated and untreated human HCC cells. Before any treatment, all cells were synchronized in G0-G1 phase by serum starvation for 72 hours. The cells were then put back in the growth medium with 10% FBS and the treatments were started. Cells were collected 12, 24, 48, and 72 hours later. Flow cytometric data confirmed that after serum starvation, the majority of cells (PLC/PRF/5, 77.9%; HuH7, 50.3%) were in G0-G1 phase. At 24 hours after the addition of either PTK/ZK combined with IFN/5-FU or IFN/5-FU alone, the PLC/PRF/5 cells showed increased S-phase-DNA content. This S phase accumulation and G0/G1 phase degradation was maintained at 48 and 72 hours (Fig. 4a). In contrast, HuH7 cells treated with PTK/ZK combined with IFN/5-FU showed more cells with G0/G1-phase-DNA content at 24, 48, and 72 hours; a similar pattern was seen with PTK/ZK alone (Fig. 4b). We evaluated the cell cycle-related protein expression levels: cyclin D1 for promotion of cell cycle, p27 and p21 for delay (Fig. 4c). PTK/ZK combined with IFN/5-FU decreased the expression level of cyclin D1 in PLC/PRF/5 cells by 0.84 times that in control cells and increased the expression of p27 by 0.65 times than in control HuH7 cells; however, the differences were not significant. On the other hand, the expression of p21 protein was significantly different between PLC/PRF/5 and HuH7 cells. PTK/ZK combined with IFN/5-FU significantly decreased the expression of p21 by 0.71 times than in control PLC/PRF/5 cells, whereas the relative decrease was only small in HuH7 cells.

Inhibitory Effects of PTK/ZK and IFN/5-FU on Human HCC Xenografts In Vivo

The serial changes in implanted tumor volume in each treatment group are shown in Fig. 5. HuH7 cells were injected SC into nude mice, which were then treated for 4 weeks according to their group (n = 5 each). On day 30, the mean TV in the control group was 4.8 ± 1.1 cm^3, whereas TVs in the single-treatment groups were 3.2 ± 0.1 cm^3 (PTK/ZK group) and 2.0 ± 0.4 cm^3 (IFN/5-FU group). PTK/ZK combined with IFN/5-FU therapy significantly reduced the mean TV to 1.3 ± 0.3 cm^3 at 30 days. There were no significant differences in body weights among the different mice groups after removing the xenografts on the 27th day in each group compared with the respective pretreatment weight. Considered together, the above findings indicate that the combination of PTK/ZK and IFN/5-FU therapy inhibited the growth of human HCC cells both in vitro and in vivo, and that PTK/ZK enhanced the inadequate effect of IFN/5-FU therapy.

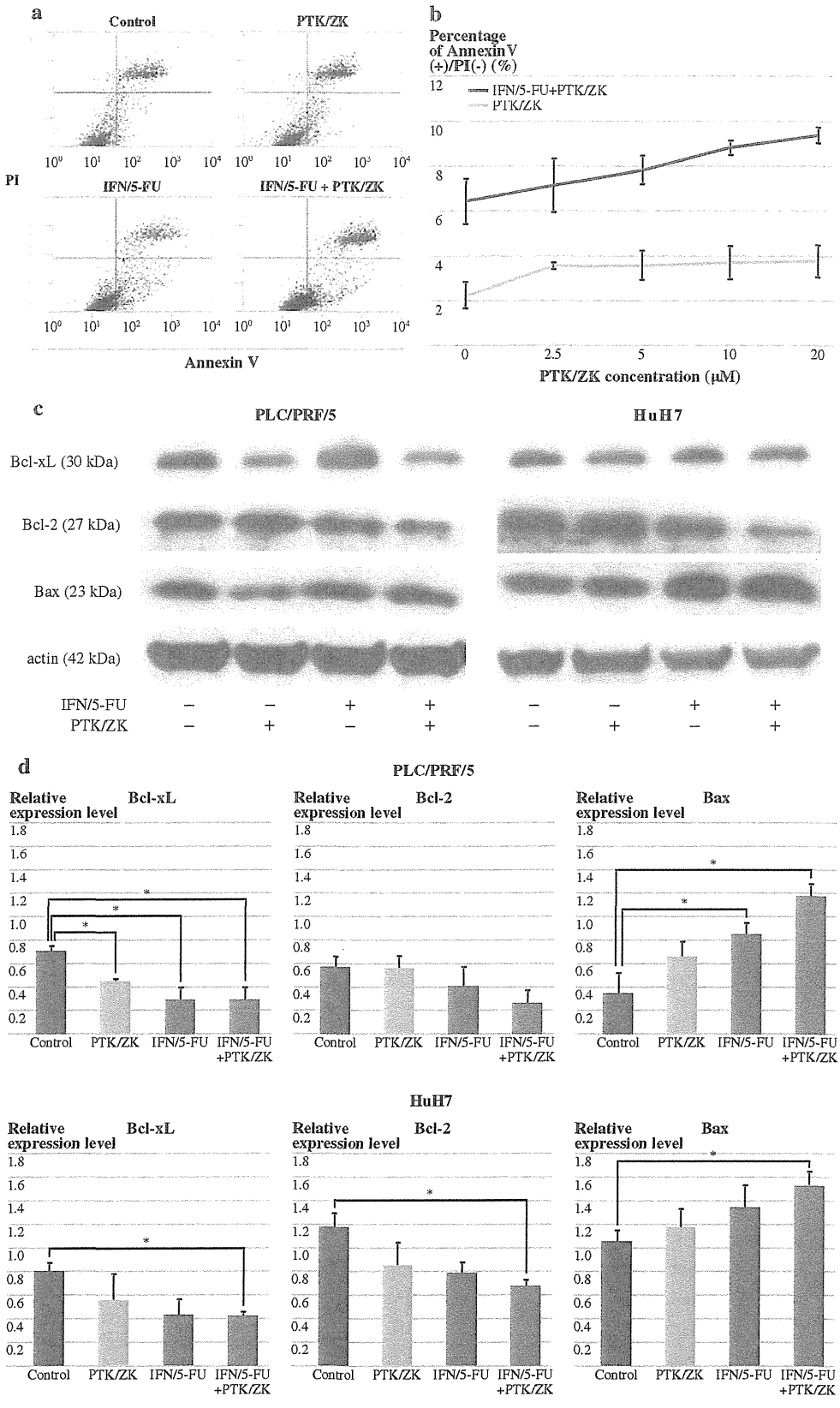

FIG. 3 Effects of PTK/ZK combined with IFN/5-FU on cell apoptosis, using the annexin V assay. Cells were harvested and double stained for annexin V-FITC and PI, with apoptosis defined by annexin V-positive/PI-negative cells. **a** Representative figure of flow cytometry. **b** Percentage of apoptosis at the indicated PTK/ZK concentration with or without IFN/5-FU. Data are mean ± SEM of triplicate assays. PTK/ZK combined with IFN/5-FU increased apoptosis in a concentration-dependent manner in PLC/PRF/5 cells to a greater extent than IFN/5-FU alone (P <0.05). **c** Expression of apoptosis-related proteins (Bcl-xL, Bcl-2, Bax) in PLC/PRF/5 and HuH7 cells treated for 6 hours, assessed by Western blot analysis. **d** Relative expression levels of apoptosis-related proteins. The band intensities were analyzed by densitometry and expressed relative to actin. Data are mean ± SEM of triplicate assays. * $P < 0.05$

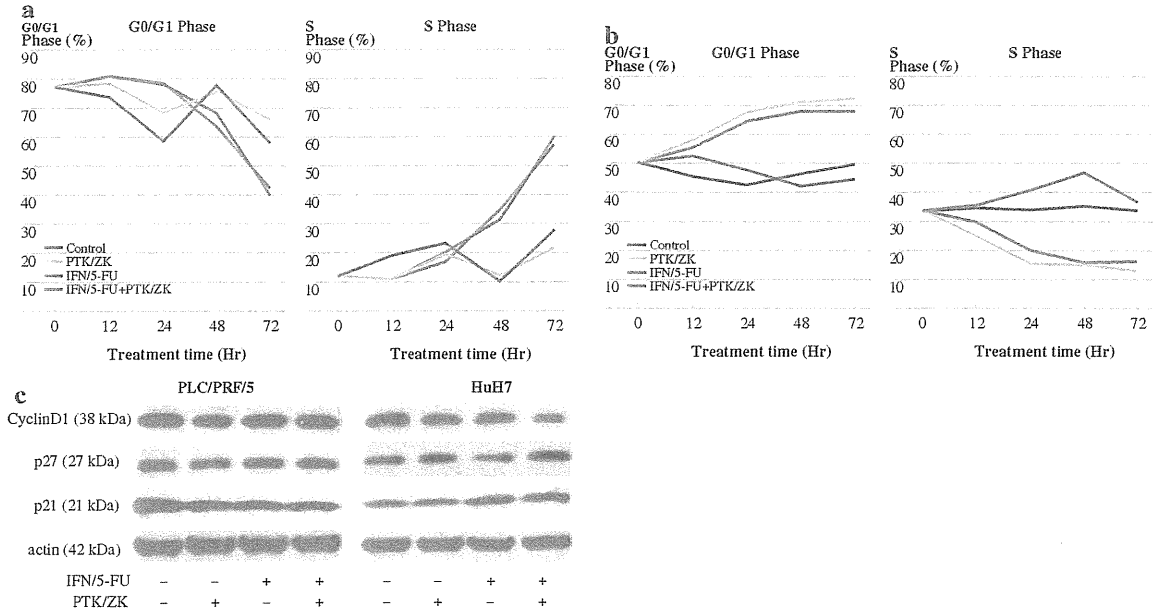

FIG. 4 Effects of PTK/ZK combined with IFN/5-FU on cell cycle in PLC/PRF/5 (**a**) and HuH7 (**b**) cells. The percentages of cells in G0-G1 phase (*left panel*) and S phase (*right panel*) are indicated for each treatment time course. **c** Expression of cell cycle-related proteins (cyclin D1, p27, p21) in PLC/PRF/5 and HuH7 cells treated for 12 h, assessed by Western blot analysis

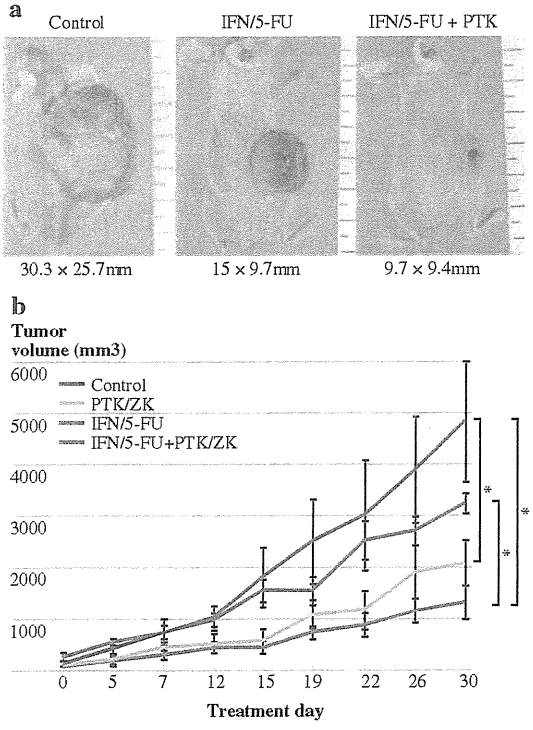

FIG. 5 Effect of PTK/ZK combined with IFN/5-FU therapy on tumor volume in xenografted nude mice. **a** Representative figure. **b** Change of tumor volume in each treatment. Data are mean volume of tumors ± SEM. * $P < 0.05$. Tumor volume of the IFN/5-FU+PTK/ZK therapy group was significantly decreased compared with the other two groups (control and PTK/ZK group); however, there was no significant difference between the IFN/5-FU and IFN/5-FU+PTK/ZK groups

DISCUSSION

We reported previously the potential mechanisms of the antitumor effects of IFN/5-FU therapy, both in vitro and in vivo, namely, the synergistic inhibition of cell proliferation and induction of apoptosis. The cell growth inhibition, including regulation of cell cycle progression, was orchestrated by increasing the S-phase fraction and thereby cell cycle arrest, whereas the apoptotic effect was mediated via IFNAR2 signaling to regulate the expression of apoptosis-related molecules.[18,20] Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and/or the Fas/Fas-L pathway also seemed to partially mediate the antitumor effects of IFN/5-FU therapy.[19,21] In addition, this therapy showed significant antitumor activity through the inhibition of angiogenesis in vitro and in vivo.[8,9] On the other hand, IFNAR2 protein is expressed on the cell surface in human HCC cell lines, although it is relatively weak in HuH7 cells and no cooperative effects were seen in these cells.[18] There are some nonresponders to IFN/5-FU therapy clinically, possibly due to activation of Wnt/β-catenin signaling pathway inducing chemoresistance to the IFN/5-FU therapy.[22] Thus, it is desirable to further examine the antitumor effects of this therapy to identify different potential targets.

Our previous study also showed that IFN/5-FU therapy inhibits VEGF secretion by tumor cells.[8] In the current study, we anticipated a stronger inhibitory effect on VEGF signaling with supplemented IFN/5-FU therapy, therefore we chose PTK/ZK because it potently inhibits all known VEGFR tyrosine kinases (including Flt-4, which is

associated with the lymph system), selectively.[23] PTK/ZK works by binding to the ATP-binding sites of VEGFRs inhibiting tyrosine kinase phosphorylation.[23]

The major findings of the present study were as follows: a) PTK/ZK did not enhance the effect of IFN/5-FU therapy on secreted VEGF; b) PTK/ZK combined with IFN/5-FU inhibited cell growth in vitro and in vivo; c) the combination of PTK/ZK and IFN/5-FU enhanced the induction of apoptosis, but had different effects on cell cycle between two cell lines; and d) Bcl-2 family protein-related apoptosis plays a key role in the effect of these therapies, and the expression of p21 protein related to the cell cycle changed adversely in cells incubated with PTK/ZK and IFN/5-FU.

The findings suggested that pathways other than the VEGF secretion could be involved in the antitumor effects of PTK/ZK combined with IFN/5-FU. In considering the mechanisms of this effect related to apoptosis and cell cycle, we found that the addition of PTK/ZK increased the apoptotic effect of IFN/5-FU therapy dose-dependently. To elucidate the molecular mechanisms underlying this additional effect in human HCC cells, we examined the expression of key apoptotic regulators, Bcl-xL, Bcl-2, and Bax, which were regulated by IFN/5-FU therapy in the previous report.[20] Our findings suggested that VEGF binding to VEGFR activates a phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway leading to the upregulation of Bcl-2 protein.[24,25] On the other hand, evidence suggests that IFN induces apoptosis with activation of the Bcl-2-family members Bak and Bax, and that it activates several signaling pathways, including mainly the canonical Janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, but also the p38 mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways.[26] In addition, since PTK/ZK potently inhibits the activities of all known VEGFR tyrosine kinases, it also is active against other receptors, such as platelet-derived growth factor receptor beta and c-kit, although at higher concentrations.[23] It is therefore probable that PTK/ZK acts in different apoptotic pathways following IFN/5-FU treatment and that the combined therapy tested herein enhanced such effect.

Second, we evaluated the effect of PTK/ZK combined with IFN/5-FU on the cell cycle. Interestingly, we obtained different results on cell cycle analysis between the two cell lines tested, with the findings suggesting that IFN/5-FU has a stronger effect on PLC/PRF/5 cells than on HuH7 cells, whereas PTK/ZK works the other way. This phenomenon could be related to p21 protein in the cells, which reacted adversely in the present study. This protein is a cyclin-dependent kinase inhibitor acting mainly to induce G1 arrest,[27–29] thus our results could be understood within this functional context. A number of studies showed that p21 plays a role in cell cycle progression and growth via p53-dependent and -independent pathways.[27,28,30] The two cell lines used in the present study harbor p53 mutations, and therefore such effects must be p53-independent via stimuli, such as IFNs and transforming growth factor-β (TGF-β).[31–33] However, HuH7 cells are associated with a p53 point mutation, which results in mutated proteins expressed with a prolonged half-life and thus protracted effects on apoptosis and cell cycle arrest.[31,34,35] Such properties could explain the observed changes in cell response and action site among the different drug treatment of the two cell lines. Thus, combining several drugs that act by different mechanisms should enhance these effects, and expand the possibility of potent therapy against HCC.

In these results, PTK/ZK could synergistically enhance the antitumor effects of IFN/5-FU, particularly in nonresponders, but the potential side effects must be managed. In our in vivo experiments, we used PTK/ZK alone at 20 mg/kg. Although this dose was lower than the effective doses used previously [50 or 100 mg/kg; effective concentration for human HCC cells xenografts as a single agent[17]], IFN/5-FU therapy combined with low-dose PTK/ZK therapy would sufficiently inhibit tumor growth and had no obvious adverse effects on the mice. Such combination therapy for advanced HCC might provide sufficient antitumor effects with fewer side effects. Sorafenib, multikinase inhibitor including VEGF, is widely used against HCC, and this also may be useful in this combination therapy.

In conclusion, we showed that PTK/ZK combined with IFN/5-FU therapy had antitumor effects on human HCC cells in vitro and in vivo, and that these effects were related to upregulated apoptosis and the complementary effects on cell cycle delay, without any change on VEGF secretion.

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The Roles of Surgical Oncologists in the New Era – Minimally Invasive Surgery for Early Gastric Cancer and Adjuvant Surgery for Metastatic Gastric Cancer

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Key Words
Gastric cancer · Laparoscopic surgery · Chemotherapy · Adjuvant surgery

Abstract
In the new era of technical development in surgery, operative devices, molecular targeting and chemotherapeutic agents, surgical oncologists have two main roles in the treatment of gastric cancer. One is to provide patients with minimally invasive surgery, including laparoscopy- or robot-assisted surgery in early gastric cancer patients, and the new concept of surgical intervention toward advanced and metastatic disease. Since recently, laparoscopy-assisted distal gastrectomy has become prevalent in Japan as a surgery which is minimally invasive for the patients and provides them with a good quality of life afterwards. However, the provision of advanced surgical techniques, including lymph node dissection and reconstruction, is more important for patient survival. The second role of surgical oncologists is to evaluate the significant values of the aggressive treatment which we term 'adjuvant surgery' for stage IV gastric cancer patients who have successfully responded to initial chemotherapy for curative intent. Stage IV gastric cancer patients are now being informed about the possibility of longer survival with the new chemotherapeutic and surgical strategic approach.

Introduction

Gastric cancer is the fourth most commonly diagnosed cancer and the second highest in terms of mortality rate. It is a global disease and a type of cancer frequently found in Asian countries. Recent demographic surveys have demonstrated that the mortality rate is notably decreasing, in spite of an only gradual decrease of the occurrence rate [1, 2]. The major causes of this phenomenon in Japan might be the broad reach of the general screening system of gastric cancer, and secondly, the innovation of newly developed diagnostic systems for the early detection of cancer and the high standard of operative techniques and chemotherapy [3].

According to the Japanese General Rules and Guidelines for gastric cancer [4, 5], intramucosal cancers are treated by endoscopic submucosal dissection or endoscopic mucosal resection, and minimally invasive surgery, including laparoscopic gastrectomy, is often performed for the rest of the early gastric cancers which are limited to within the submucosal layer [6–8].

The surgical treatments for stage II and III gastric cancer are well established, as demonstrated by Songun et al. [9] after a 15-year follow-up of the randomized nationwide Dutch D1D2 trial. That is to say, D2 lymphadenectomy is the recommended surgical approach for

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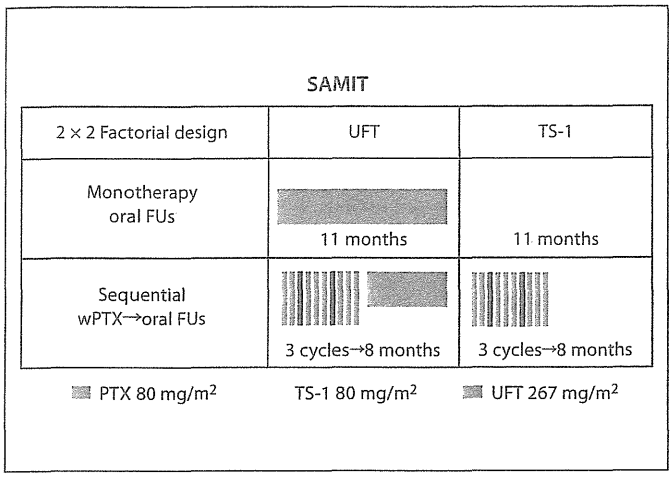

Fig. 1. The rationale of the SAMIT trial. PTX = Paclitaxel.

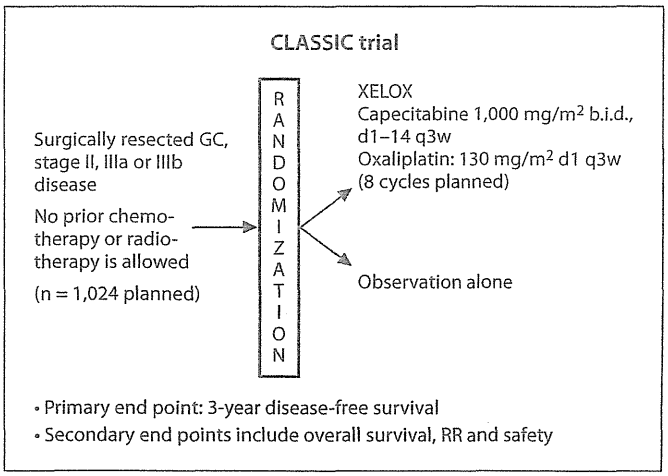

Fig. 2. The rationale of the CLASSIC trial. GC = Gastric cancer; XELOX = capecitabine in combination with oxaliplatin.

Table 1. Consensus of perioperative chemotherapy of gastric cancer

USA	SWOG 9008/Intergroup 0116 5-FU/leucovorin + radiation
Europe	MAGIC trial Perioperative ECF
Japan	ACTS-GC Postoperative S-1

patients with resectable (curable) gastric cancer, and is popular in Japan, Korea and other Asian countries. However, postoperative or perioperative treatments remain a controversial issue between the East and the West. Perioperative ECF (epirubicin/cisplatin/5-FU) therapy is regarded as the standard treatment in the UK and in some European countries [10] and intraoperative radiation with postoperative chemotherapy is the widely accepted treatment in the USA [11]. D2 lymph node dissection was not performed in most of the cases in these trials. What was interesting is that the postoperative survival of the patients who underwent D2 lymph node dissection without postoperative chemotherapy in Japan was far better than for those in the Medical Research Council Adjuvant Gastric Cancer Infusional Chemotherapy (MAGIC) trial and Intergroup study [12]. According to the results of the standard procedure of curative surgery, the Adjuvant Chemotherapy Trial of Thymidine Synthase (TS-1) for Gastric Cancer (ACTS-GC) was performed for 1 year on stage II and III patients to establish the postoperative S-1 treatment; it was accepted with significant survival benefit of the treatment group in Japan [13, 14]. The consensus of the perioperative strategies is summarized in table 1. The Stomach Cancer Adjuvant Multi-Institutional Trial (SAMIT) is currently ongoing; it compares the benefits of S-1 and UFT and also the benefits of adding paclitaxel as adjuvant chemotherapy for curatively resected patients with serosal invasion of a tumor [15]. In Korea, the CLASSIC trial is underway to establish a standard postoperative adjuvant chemotherapy with capecitabine in combination with oxaliplatin after curatively resected stage II and III gastric cancer patients have undergone D2 lymph node dissection [16] (fig. 1, 2).

There is no established global standard chemotherapy for metastatic or recurrent gastric cancer. A combination therapy of fluoropyrimidine and platinum is commonly used [17]. Data is also available about a triplet regimen. ECX (epirubicin/cisplatin/capecitabine) [18], EOX (epirubicin/oxaliplatin/capecitabine) [19] and DCF (docetaxel/cisplatin/5-FU) [20] (or modified DCF [21]) are used as standard care in certain areas of the US and the UK. S-1 combination chemotherapy (S-1 + CDDP) is currently regarded as the standard first-line treatment in metastatic gastric carcinomas in Japan [22]. The median survival time (MST) was prolonged to 13.0 months in the SPIRITS trial conducted in Japan.

As reported at ASCO 2009, a targeted therapy for HER2, Herceptin, was approved for HER2-positive gastric cancer in Europe, Korea and other areas [23]. Other targeted therapies are now under investigation in clinical

trials. Under these circumstances, we need, via clinical trials, to provide a new treatment which is more effective and has fewer adverse events throughout the world and especially in Asian countries. The data relating to gastric cancer should be obtained by collaboration between Asian countries and transmitted globally to establish a standard treatment because gastric cancer is the most prevalent and common disease in this part of the world.

What is interesting in the recent trend of chemotherapeutic treatment in stage IV gastric cancer is that downstaging of the tumors is often observed with high response rate (RR) regimens with newly developed chemotherapeutic agents, and as a result, R0 resection (complete resection with no residual microscopic tumor) has been performed on quite a few patients after chemotherapy [24, 25]. These cases can broadly be called 'adjuvant surgery' or oncosurgery (conversion therapy as it is often described in the treatment of liver metastasis in colorectal surgery) after neoadjuvant chemotherapy [26–28].

Considering the present observations described above, minimally invasive surgery, including laparoscopic surgery or robotic surgery for early gastric cancer [29], and aggressive surgery with curative intent in stage IV, or recurrent gastric cancer with perioperative chemotherapy are the main themes of surgical oncology in the new era. These points are highlighted in this article.

Minimally Invasive Surgery

Laparoscopic Surgery and Its Indication for Gastric Cancer

Laparoscopy-assisted distal gastrectomy (LADG), a minimally invasive surgery, has recently become prevalent in Japan, and provides patients with a good quality of life [30–32]. However, it is more important to provide them with advanced surgical techniques including lymph node dissection and reconstruction. According to the Japanese guidelines for gastric cancer treatment, LADG is not regarded as the standard procedure. In order to establish the safety and noninferiority of the method compared to open surgery, randomized control studies in Japan and Korea are ongoing [33, 34]. For the technical assurance of the laparoscopic surgery, a certification system has been adopted by the Japanese Society of Endoscopic Surgery.

In our institution, the indication for the operation is restricted to patients with early gastric cancer which includes: carcinomas of the mucosal layer (T1), no evidence of lymph node metastasis (N0), not suitable for endoscopic mucosal resection (with a size of more than 2 cm and with ulcer scar formation), or invaded to the submucosal layer with no clinical lymph node metastasis. Up to October 2010, we performed 204 laparoscopic gastrectomies including 152 LADG, 8 laparoscopy-assisted pylorus-preserving gastrectomies, 21 laparoscopy-assisted proximal gastrectomies, 6 laparoscopy-assisted total gastrectomies and 17 simple resections of the stomach.

Surgical Techniques

In order to perform complete laparoscopic gastrectomy, resection and anastomosis should be performed in the abdominal cavity. In this section, we describe our standard procedure of LADG and gastroduodenostomy.

We performed 103 cases of gastroduodenostomy using the delta anastomosis technique [30, 35]. Under general anesthesia, the patient was placed in the supine position with legs apart. Initially, a trocar was inserted under the umbilical portion via a 2-cm incision by the open method. Flexible laparoscopy (Olympus) was used in the operation and the camera operator stood between the legs of the patient. Four other trocars were inserted in the flank and subcostal regions.

The operation consisted of 9 parts: (1) ligation of the left gastroepiploic artery and vein [dissection of lymph node (LN) 4d and 4sb], (2) ligation of the right gastroepiploic artery and vein (LN 6), (3) transection of the duodenum, (4) ligation of the right gastric artery (LN 5), (5) dissection of LN 8a, (6) ligation of the left gastric artery and vein (LN 7, 9 and 11p), (7) dissection of LN 1 and 3, (8) transection of the stomach and (9) reconstruction by the Billroth I method with the delta anastomosis technique. The surgeon stood on the right side except during step (2) and (3).

(1) Ligation of the Left Gastroepiploic Artery and Vein (Dissection of LN 4d and 4sb)

The greater omentum was dissected with harmonic scalpel (Ethicon) about 5 cm away from the epiploic vessels; LN 4d and 4sb were removed. The left gastroepiploic artery and vein were dissected with clips.

(2) Ligation of the Right Gastroepiploic Artery and Vein (LN 6)

The surgeon stood on the left side of the patient to continue the procedure. The greater omentum of the right side was divided in the same manner towards the hepatic flexure of the colon and the gastroduodenal artery was visualized. Dissection of LN 6 was performed, with liga-

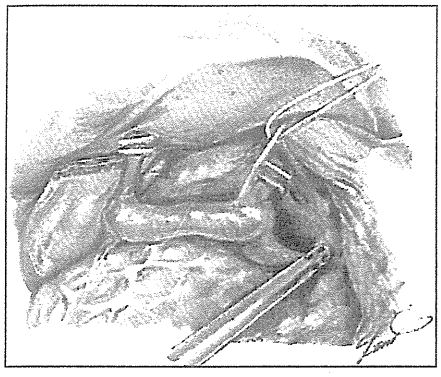

Fig. 3. Lymph node dissection by laparoscopic approach (illustrated by Leon Sakuma [30]).

tion and division of the right gastroepiploic artery and vein by harmonic scalpel.

(3) Transection of the Duodenum and (4) Ligation of the Right Gastric Artery (LN 5)

The surgeon stood on the right side of the patient again. The antrum was lifted and the duodenum was transected close to the pylorus ring using an Echelon (Ethicon) from the left lower port and then the right gastric artery was divided and dissected with the harmonic scalpel with clips cleaning the LN 5.

(5) Dissection of LN 8a

The stomach was lifted towards the left flank and the lesser omentum was divided visualizing the hepatic branch of the vagus nerve near the liver bed. Preserving the branch, the dissection was performed towards the cardia. The serosa of the right crus was dissected with the harmonic scalpel.

LN 8a was dissected using the harmonic scalpel visualizing the common hepatic artery preserving the hepatic plexus of the autonomic nerve. The dissection was performed from the right side towards the celiac axis (fig. 3).

(6) Ligation of the Left Gastric Artery and Vein (LN 7, 9 and 11p)

The fat tissue and connective tissues of LN 8a, 7, 9 and 11p were dissected with the harmonic scalpel. The left gastric artery and vein were visualized, then ligated with 2 clips and divided with the harmonic scalpel. The dissection was performed along the curus towards the esophagogastric junction.

(7) Dissection of LN 1 and 3

The dissection of the LN 1 and 3 was performed along the lesser curvature of the stomach, dissecting the anterior and posterior branch of the vagus nerves toward the stomach. The dissection was performed towards the transection line of the stomach.

(8) Transection of the Stomach and
(9) Reconstruction by the Billroth I Method with the Delta Anastomosis Technique in the Abdominal Cavity

The proximal resection margin was estimated by the serosal side carbon ink color which was injected in the submucosal layer the day before the operation by endoscopy and transected from the left lower port using Echelon (60 mm). The resected stomach was captured by the end catch and taken out through the camera port with an additional abdominal muscle fascia incision but without an additional skin incision.

For the gastroduodenostomy, the edge of greater curvature of the remnant stomach and the duodenum were opened with the harmonic scalpel and the linear stapler (endcutter 45 mm) was inserted via each hole and connected and fired. The V-shape anastomosis of gastroduodenostomy was performed with the entry hole opened. The final step was the closure of the hole with 3 firings of the linear stapler by lifting up the 3 stitches (3-0 monocryl) of incomplete closure of the entry hole (fig. 4).

The mean operative time was 253 min and blood loss was 50 ml. Thirty-seven lymph nodes were harvested. Patients started to walk the next day, started the oral intake treatment on day 3 after the operation and were discharged on day 9. Among 103 cases of delta anastomosis, there was no anastomotic leakage and no reoperation; there were, however, 2 cases of anastomotic stenosis.

Although this procedure requires time and the precise knowledge of the anatomy of the upper abdominal regions, it provides patients with several advantages including improved cosmetics, shorter hospitalization, minimal operative pains and a low incidence of bowel movability and pancreas functions (demonstrated elsewhere). Moreover, the postoperative complications can be reduced. The LADG with lymphadenectomy can be one of the most effective therapeutic methods for early gastric cancer patients.

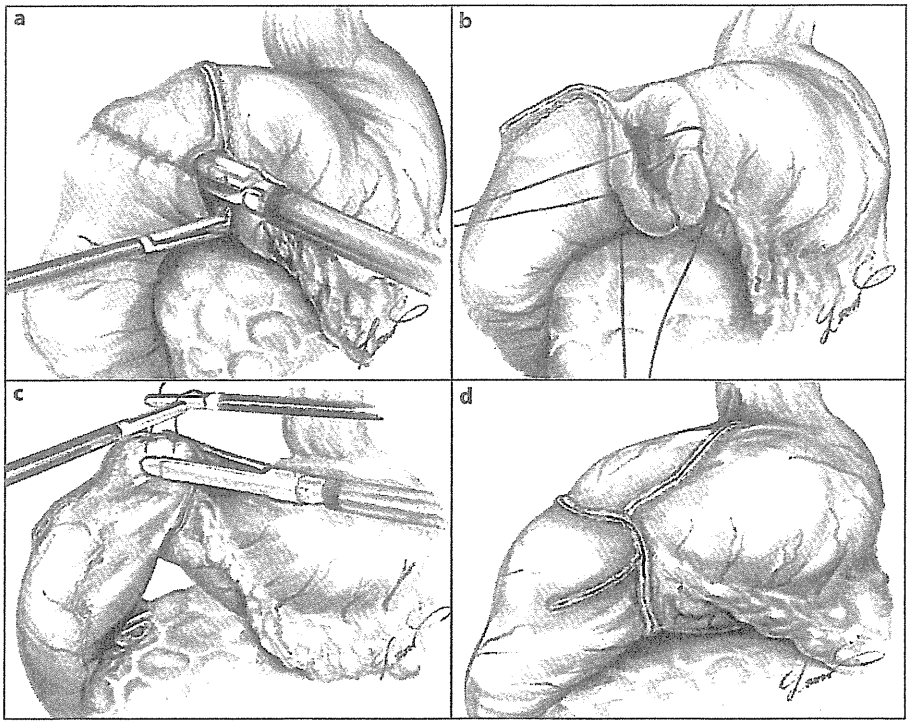

Fig. 4. Delta anastomosis (illustrated by Leon Sakuma [30]). **a** Anastomosis by linear stapler. **b** Ligation of entry hole. **c** Closure of entry hole by linear stapler. **d** Final anastomosis image.

New Therapeutic Approach for Stage IV Gastric Cancer

Establishment of New Chemotherapeutic Regimens for Gastric Cancer

Several combination regimens with S-1 have been established in Japan in this decade and randomized phase III studies have been conducted. They are S-1 + CDDP, S-1 + CPT-11 and S-1 + docetaxel as reported by Fujii et al. [36].

Cisplatin at a dose of 60 mg/m^2 on day 8 was combined with S-1 for 3-weeks-on and 2-weeks-off treatment [37]. This was repeated every 5 weeks, unless disease progression was observed. The RR was 74% (14/19; 95% confidence interval (CI) 54.9–90.6) and the MST was 383 days. Komatsu et al. [38] reported the results of a phase I/II study with CPT-11 + S-1 (IRIS study) in AGC patients. S-1 was given orally twice a day for 14 days and CPT-11 was administered as a 90-min intravenous infusion on days 1 and 15. This regimen was repeated every 4 weeks. The overall RR was 54.2% in the phase II study. The MST achieved with this regimen was 581 days. Yoshida et al. [39, 40] performed a phase I study and a phase II study of docetaxel in combination with S-1 in patients with AGC. In the phase II study, the RR was 52.1% and the MST was 434 days. Moreover, the biochemical modulations of docetaxel enhanced the sensitivity of 5-FU in vitro and in vivo [41]. More interestingly, the mTOR inhibitor downregulated the expression of TS and enhanced the reactivity of 5-FU on TMK-1 gastric cancer cells [42].

Based on the results obtained in the above phase II studies, 3 large randomized phase III studies, the SPIRITS trial [22], the TOP-002 trial [43] and the JACCRO GC03 trial [36, 44] were conducted independently to compare the data with that of S-1 monotherapy, the results of which are summarized in table 2.

In the SPIRITS trial, chemotherapy-naïve patients with AGC were randomly assigned to receive either S-1 plus cisplatin or S-1 alone. The primary end point was overall survival and the secondary end points were progression-free survival, proportion of responders and safety. Median overall survival was significantly longer in the patients assigned to receive S-1 plus cisplatin than in those assigned to receive S-1 alone (13.0 vs. 11.0 months, respectively; hazard ratio (HR) 0.77; 95% CI 0.61–0.98; $p = 0.04$). Progression-free survival was significantly longer in the patients assigned to receive S-1 plus cisplatin than in those assigned to receive S-1 alone (median progression-free survival 6.0 vs. 4.0 months, respectively; $p < 0.0001$). Moreover, of the 87 patients with target tumors assigned to receive S-1 plus cisplatin, 1 showed a complete response (CR) and 46 showed a partial response