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Ⅲ　.　研究成果の刊行物・別刷

Clinical Application of Immunoreactivity of Dihydropyrimidine Dehydrogenase (DPD) in Gastric Scirrhous Carcinoma Treated with S-1, a New DPD Inhibitory Fluoropyrimidine

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Abstract. *Background: A highly specific antibody against recombinant human dihydropyrimidine dehydrogenase (DPD) has been developed to immunohistochemically assess DPD expression in tumors. A new oral DPD inhibitory fluoropyrimidine (DIF), S-1, is reportedly effective against gastric scirrhous carcinoma. Patients and Methods: In this study, the relationship between immunoreactivity to DPD in biopsy specimens and the effects of chemotherapy were investigated in 61 patients treated with first-line fluoropyrimidine-based chemotherapy (S-1:DIF, 5-FU:non-DIF) for gastric scirrhous carcinoma. Results: The response rate was significantly higher in patients with DPD-positive tumors than in those with DPD-negative tumors in the S-1 group (45.5%, 10.0% : $p<0.05$), as compared to the 5-FU group (0%, 5.6%: $p=0.398$). According to the median survival time, there was no significant difference between patients with DPD-positive tumors (364 days) and those with DPD-negative tumors (406 days; $p=0.626$) in either the S-1 group or the 5-FU group (181 days and 256 days, respectively; $p=0.543$). Conclusion: This study indicates that S-1 may be effective even in gastric scirrhous carcinoma with a high level of DPD activity.*

Borrmann-type-4 gastric cancer, clinically synonymous with gastric scirrhous carcinoma, is generally resistant to systemic chemotherapy. This type of gastric cancer is characterized by diffuse malignant lesions with indistinct borders, and is usually diagnosed at a very advanced stage. High rates of lymph node metastasis, invasion of neighboring structures and peritoneal dissemination pose a great challenge for medical care. The outcome is usually poor, with 5-year survival rates ranging from 0% to 20% (1). Although most gastric scirrhous carcinomas are resistant to conventional 5-fluorouracil (5-FU)-based regimens, several recent case studies have reported a good response to S-1 (2, 3) Many studies have demonstrated that dihydropyrimidine dehydrogenase (DPD) is a biomarker for response in patients treated with 5-FU-based chemotherapy (4-7). DPD is a rate-limiting enzyme in the metabolism of 5-FU, and its expression by tumors is thought to attenuate the response to 5-FU (8-10). Since more than 80% of the administered dose of 5-FU is degraded in the liver by DPD to fluorinated β-alanine, the level of DPD activity is also a major determinant of 5-FU toxicity (11).

Recently, encouraging clinical results have led to the development of a new generation of oral fluoropyrimidines, commonly referred to as DPD inhibitory fluoropyrimidines (DIF) (12, 13). S-1 is a combined preparation consisting of 1 M tegafur, 0.4 M 5-chloro-2,4-dihydroxypyridine (CDHP), and 1 M potassium oxonate (Oxo). CDHP is a potent inhibitor of DPD, approximately 180 times more active than uracil in inhibiting DPD *in vitro*, and maintains prolonged 5-FU concentrations in plasma and tumors (14-16) Oxo protects against 5-FU-induced gastrointestinal toxicity. Two phase II studies of S-1 monotherapy in patients with metastatic gastric cancer yielded response rates of about 50%, with minimal toxicity (17-19). S-1 is now used to treat advanced gastric cancer as a single agent or in combination with other anticancer agents, including cisplatin, CPT-11, paclitaxel and docetaxel (20).

A technique using highly specific antibodies against recombinant human DPD (rhDPD) has been developed to immunohistochemically assess DPD expression in tumors (21-23) and thereby predict the clinical response to 5-FU-based chemotherapy. Several studies have examined the relationship between the DPD immunoreactivity of tumors

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Key Words: Gastric scirrhous carcinoma, S-1, dihydropyrimidine dehydrogenase (DPD), DPD inhibitory fluoropyrimidines (DIF).

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and the response to oral fluoropyrimidines, but the clinical impact of DPD activity on response remains unclear for new drugs such as S-1, and there are no reports on the treatment of gastric scirrhous carcinoma. In this study, intra-tumoral levels of DPD were assessed immunohistochemically using anti-DPD polyclonal antibodies, and the relationship between the immunoreactivity of DPD and the antitumor effects of S-1 were investigated. We propose that S-1 might circumvent the resistance to 5-FU in gastric scirrhous carcinoma with a high level of DPD activity. Our aim was to clarify the differences between the antitumor activities and mechanisms of action of S-1 as a DIF and 5-FU as a non-DIF.

Patients and Methods

Patients. Sixty-one patients with Borrmann-type-4 gastric scirrhous carcinoma, who received S-1 or 5-FU as first-line chemotherapy at the National Cancer Center Hospital (Tokyo, Japan) between February 2000 and January 2003, were studied retrospectively. Thirty-one patients were given S-1 and 30 were given 5-FU. Tumor biopsy specimens were obtained from all patients before chemotherapy.

Treatment schedule and evaluation of response. S-1 was administered at a dose of 40 mg/m^2 of body surface area (BSA) twice daily in one of the following doses: 40 mg (BSA<1.25 m^2), 50 mg (1.25 m^2≤BSA <1.50 m^2), or 60 mg (BSA ≥1.50 m^2). S-1 was given for 28 consecutive days, followed by a 14-day rest period. This period was defined as one course of treatment. S-1 was purchased from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) in the form of 20 and 25 mg capsules. 5-FU (800 mg/m^2/day) was administered as a 5-day (120 h) intravenous continuous infusion, repeated every 28 days, comprising one course of treatment.

Both treatments were continued until tumor progression, unacceptable toxicity, or refusal by the patient to continue further therapy. The response of measurable target lesions to chemotherapy was objectively evaluated according to the WHO criteria after each course of treatment. The response of primary lesions was also evaluated according to the roentgenographic and endoscopic criteria proposed by the Japanese Research Society of Gastric Cancer for "c-lesions" (24). Complete response (CR) was defined as the disappearance of all invasive findings. Partial response (PR) was defined as a decrease of 50% or greater in the affected area on X-ray films after barium administration, obtained in the same position as that before treatment. Progressive disease (PD) was defined as a 25% or greater increase in lesions or the appearance of new lesions. Responses not falling into any of these categories were classified as stable disease (SD). The survival time was calculated from the start date of the first course of treatment to the date of death or to the final date of confirmed survival.

Immunohistochemical examination. DPD immunoreactivity in the tumor biopsy specimens was examined with the use of an anti-recombinant human DPD polyclonal antibody (diluted at 1:1000, The Second Cancer Laboratory, Taiho Pharmaceutical Co., Ltd., Saitama, Japan). The tissues were routinely fixed in 10% formalin and embedded in paraffin wax. Sections 3 μm thick were cut and mounted on aminopropyltriethoxysilane-coated slides, and were deparaffinized with xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxidase in methanol for 30 min at room temperature. Representative specimens were evaluated by the following antigen retrieval procedure. Three types of soaking solutions were employed: 10 mM citrate buffer, pH 6.0, 10 mM citrate buffer, pH 7.0 and 1mM EDTA solution, pH 8.0. After pressure cooking, the sections were left at room temperature for 30 min. The sections were incubated with polyclonal antibody against DPD overnight at room temperature. The specificity of this antibody has been reported previously (21). After rinsing in phosphate-buffered saline (PBS), pH 7.2, the sections were incubated with universal immunoperoxidase polymer, anti-mouse and rabbit (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan), at room temperature for 30 min. The reaction products were visualized in 50 mM Tris-HCl buffer, pH 7.6, containing 50 mg/dl diaminobenzidine tetrahydrochloride and 0.006% hydrogen peroxidase. The nuclei were lightly counterstained with Mayer's hematoxylin, and the specificity of immunostaining with the polyclonal antibody was checked by preabsorption experiments using representative samples. Before immunostaining, the diluted antibody was combined with recombinant human DPD (Taiho Pharmaceutical Co., Ltd.) at final concentrations of 0.01, 0.1, 1.0 and 10 μg/ml, at 37°C for 1 h. As a positive control, we employed tumor tissue obtained from a xenograft of the human pancreatic cancer cell line MIAPaCa-2 in nude mice, established to have high DPD expression. Negative

Table I. *Patient characteristics in both regimen (S-1 : DIF, 5-FU : non-DIF) groups.*

Characteristics	S-1	5-FU	*p*-value
Total noumber of patients	31	30	
Age, years, median (range)	53.7 (30-73)	58.2 (39-70)	0.387
Gender (men/women)	16/15	18/12	0.592
ECOG performance status			
0	10	4	0.214
1	20	22	
2	1	4	
Histological type			
Intestinal type	2	1	0.978
Diffuse type	29	29	
Number of organs involved			
1	8	12	0.151
2	17	11	
3	6	7	
Site of metastatic disease			
Peritoneum	29	16	0.117
Distant lymph nodes	17	20	0.672
Liver	9	12	0.867
Lung	2	2	0.978
Others	3	5	0.330
Surgery (total gastrectomy)			
yes	17	11	0.126
no	14	19	
Treatment duration, days, median (range)	217 (27-767)	76 (25-258)	0.006
Number of chemotherapy cycles, mean (range)	5.0 (1-16)	2.4 (1-5)	0.045

controls were prepared by substituting PBS for the primary antibody (Rabbit Immunoglobulin Fraction: DAKO ENVISION). The slides were counterstained with hematoxylin.

Evaluation of immunostaining. Immunohistochemical staining intensity was semiquantitatively graded (– to 3+) on the basis of the proportion of positively-stained cancer cells in the lesions: –, negative; 1+, less than 1/3 of cancer cells positive; 2+, from 1/3 to less than 2/3 of cancer cells positive; 3+, 2/3 or more of cancer cells positive. A staining intensity of – to 1+ was considered negative, and that of 2+ to 3+ was considered positive. Immunohistochemical staining was evaluated independently by four investigators blinded to clinical outcomes. Any disagreement was resolved by consensus.

Statistical analysis. The statistical significance of the relationships of DPD immunoreactivity and TS immunoreactivity to the patients' responses to chemotherapy was evaluated with χ^2-tests. Survival curves were calculated with the Kaplan-Meier method and analyzed with the use of log-rank tests.

Results

Patients' characteristics. The patients' characteristics are provided in Table I. Thirty-four men and 27 women, with a median age of 55 years (range, 30-73 years) were included. Fifty-six patients (91.8 %) had a performance status of 0 or 1 on the Eastern Cooperative Oncology Group scale, and all patients received S-1 or 5-FU chemotherapy as first-line treatment, including preoperative neoadjuvant chemotherapy.

DPD immunoreactivity. DPD immunoreactivity was diffusely distributed in the cytoplasm of tumor cells, with some differences in staining intensity within a given tumor. All grading patterns of DPD immunoreactivity using anti-recombinant human DPD polyclonal antibody are shown in Figure 1 .

Immunoreactivity and response to chemotherapy. The overall response rate was 22.6% (7/31) in the S-1 group and 3.3% (1/30) in the 5-FU group. Positive rates for DPD were, respectively, 35.5% (11/31) in the S-1 group and 40.0% (12/30) in the 5-FU group. Response rates were 45.5% (5/11) in patients with DPD-positive tumors and 10% (2/20) in those with DPD-negative tumors (p=0.044) in the S-1 group, as compared with 0% (0/12) and 5.6 % (1/18) (p=0.398), respectively, in the 5-FU group.

Relationship between survival and DPD activity. The median survival time of all patients was 340 days (S-1: 393 days, 5-FU: 226 days). The median survival times were 364 days in patients with DPD-positive tumors and 406 days in those with DPD-negative tumors in the S-1 group (p=0.626), as compared with 181 days and 256 days, respectively, in the 5-FU group (p=0.543). The median survival time did not differ significantly between patients with DPD-positive tumors and those with DPD-negative tumors in either treatment group.

Discussion

Our study indicates that S-1 may be effective in the treatment of gastric scirrhous carcinoma with higher DPD activity. Several studies focusing on human cancer cell lines have suggested that intratumoral DPD levels, assessed on the basis of either enzymatic activity or mRNA expression, are good predictors of the response to 5-FU-based chemotherapy (25-27). Previous studies have also shown that inhibition of intratumoral DPD increases sensitivity to 5-FU, and that thymidylate synthase (TS) overexpression plays a major role in the resistance. Here, we focused on the antitumor effect of S-1 as a newly-developed DIF, and examined the correlation with a DIF antitumor effect and a biomarker (DPD). Immunohistochemical analysis has several important advantages over measuring protein and mRNA levels, since it is labor-saving, low-cost and can be used for tissue specimens fixed in formalin. We believe that it would be valuable to establish a simple and reliable method to assess DPD expression in biopsy specimens, since this is the only available material capable of providing information on the biological properties of tumors before chemotherapy. Antibodies against DPD have recently become available for immunohistochemical analysis, and studies have shown that DPD immunoreactivity correlates with DPD activity and the level of mRNA expression in cancer tissue. Cancer cells that express higher levels of DPD are considered more resistant to 5-FU and may be unresponsive to chemotherapy. However, our findings suggest that S-1 may be effective against gastric scirrhous carcinoma with higher DPD activity. Although there was no significant difference in median survival time between DPD-positive patients and -negative patients in the S-1 group (p=0.626) as compared with those of the 5-FU group (p=0.528), S-1 showed a higher response rate in tumors with a high DPD activity (p<0.05). These results indicates that S-1 could be more effective in gastric scirrhous carcinoma patients resistant to 5-FU only and with high DPD activity. One remarkable point was that all patients who responded to S-1 in the DPD-positive group showed shrinkage of primary lesions. Although DPD has been documented as an important determinant of chemosensitivity to 5-FU, most previous studies have found that the levels of DPD mRNA, protein and activity in tumors are unrelated to outcome. Our results, which showed no correlation of the DPD score in biopsy specimens with survival or time to progression, are in agreement with these findings.

In tumors with low DPD activity, inhibition of DPD by CDHP did not enhance cytotoxicity, even if tumor DPD activity was further reduced. In contrast, maximum

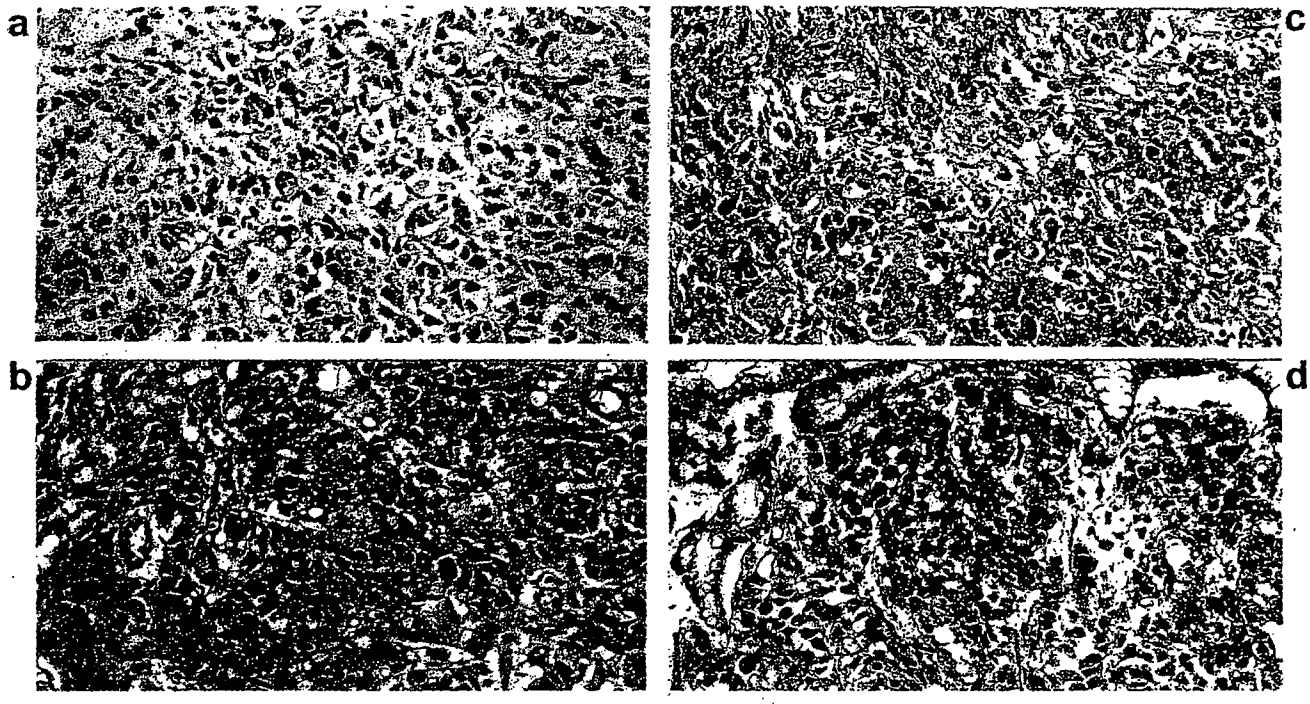

Figure 1. *All grading pattern of immunohistochemical staining for DPD with polyclonal antibody (x400 magnification). Positive staining for DPD is observed in the cytoplasm of cancer cells, with some differences in staining intensity within a given tumor. (a. DPD -, b. DPD 1+, c. DPD 2+, d. DPD 3+). A staining intensity of – to 1+ was considered negative, and that of 2+ to 3+ was considered positive.*

enhancement of the antitumor effect of S-1 would be expected in patients whose tumors have high DPD activity (28). Although the proportion of intratumoral DPD activity inhibited by CDHP is not clinically known, S-1 is expected to show antitumor effects, regardless of the status of intratumoral DPD. Similar to our results, several recent case studies have reported that S-1 is associated with shrinkage of primary lesions of Borrmann-type-4 gastric scirrhous carcinoma (2, 3). Although the mechanism of the response of primary lesions to S-1 remains unclear, strong inhibition of DPD, resulting in prolonged active concentrations of 5-FU in plasma and tumors, may be responsible for the shrinkage of these lesions.

In conclusion, our results suggest that S-1 may be effective against gastric scirrhous carcinoma, even in tumors with high levels of DPD activity. The relationship between DPD and the clinical response to other chemotherapeutic regimens should be investigated to determine whether intra-tumoral DPD activity is useful for selecting the best suited chemotherapeutic regimen. Further immunohistochemical studies on DPD with larger numbers of patients will hopefully contribute to the development of tailor-made DIF-based regimens designed to optimize response.

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ORIGINAL ARTICLE

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A phase I/II study of nedaplatin and 5-fluorouracil with concurrent radiotherapy in patients with T4 esophageal cancer: Japan Clinical Oncology Group trial (JCOG 9908)

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Abstract
Background. Nedaplatin is an analogue of cisplatin with less nonhematologic toxicity. The combination of nedaplatin and 5-fluorouracil showed a promising response rate in a previous phase II study for metastatic esophageal cancer. The purpose of this study was to determine a recommended dose and to evaluate the efficacy of nedaplatin and 5-fluorouracil combined with concurrent radiotherapy.
Methods. Eligibility criteria included squamous cell carcinoma of the thoracic esophagus; T4 disease without distant organ metastasis; age 20–70 years; performance status 0–2; and adequate organ functions. Patients received two cycles of nedaplatin (80 mg/m^2 or 90 mg/m^2) on day 1 and continuous infusion of 5-fluorouracil 800 mg/m^2/day on days 1–5, every 5 weeks with concurrent radiotherapy 60 Gy in 30 fractions.
Results. Between December 1999 and April 2002, 26 patients were accrued. The recommended dose of nedaplatin was 90 mg/m^2. Common grade ≥3 toxicities included leukopenia 9, neutropenia 5, thrombocytopenia 4, esophagitis 4, and esophageal fistula 3. Three of 26 patients achieved complete response (12%; 95% confidence interval, 2%–30%). With a minimum follow-up of 26 months for surviving patients, the median survival time was 12 months (95% confidence interval, 9–22 months), and the 2-year overall survival was 31% (95% confidence interval, 13%–49%).
Conclusions. This combined therapy is active with acceptable toxicity, however, the survival figure remains poor. Further investigation into more effective treatment is needed.

Key words Esophageal neoplasms · Drug therapy · Radiotherapy · Clinical trial

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Introduction

Esophageal cancer has become an important disease in the fight against cancer. In recent years, the number of patients with stage I disease has been increasing, but most patients are diagnosed with advanced disease and their prognoses are still daunting.

Over the last decade, chemoradiotherapy (CRT) for esophageal cancer has revealed promising results [1,2]. After the report of an intergroup randomized controlled trial (Radiation Therapy Oncology Group 85-01) that compared CRT with radiotherapy alone, the combined-modality treatment became a standard for patients with esophageal cancer who received nonsurgical treatment [3,4]. Most reports of CRT used cisplatin (CDDP) and fluorouracil (FU) with concurrent radiotherapy, and this combination is thought to be standard [1–6].

Nedaplatin (NDP; *cis*-diammine-glycolatoplatinum), a novel second-generation platinum compound, has shown promising antitumor activity with less nephrotoxicity, gastrointestinal toxicity, and neurotoxicity than CDDP in some preclinical and clinical studies [7–11]. The combination of NDP and FU also showed promising results in a phase II study for metastatic esophageal cancer [12]. Following the results of this phase II study, we decided to investigate this combination with concurrent radiotherapy in locally advanced disease. To determine a recommended dose and to evaluate the efficacy of NDP and FU combined with concurrent radiotherapy, we conducted a phase I/II study in patients with T4 (according to the International

Union Against Cancer tumor-node-metastasis system, 1997) esophageal cancer.

Patients and methods

Eligibility criteria

Eligibility criteria included previously untreated patients with pathologically proven squamous cell carcinoma of the thoracic esophagus; clinical tumor-node-metastasis system T4 disease without distant organ metastasis but supraclavicular and celiac nodes metastases were allowed; age, 20–70 years; performance status (PS; based on the Eastern Cooperative Oncology Group scale), 0–2; adequate hematologic [white blood cell count (WBC) count ≥ 4000/mm^3, platelet count ≥ 100000/mm^3, and hemoglobin ≥ 9.5 g/dl], hepatic [aspartate amino transferase (AST) and alanine amino transferase (ALT) level ≤ 2.5 times the upper limit of normal, and total bilirubin ≤ 1.5 mg/dl], and renal (creatinine ≤ 1.2 mg/dl and creatinine clearance ≥ 50 ml/min) functions; PaO_2 ≥ 70 torr; no esophageal fistula; no pleural and pericardial effusion; and no serious comorbidity. All patients gave written informed consent in accordance with institutional review boards.

Pretreatment evaluation

Pretreatment evaluation included history and physical examination; complete blood cell count; serum chemistries; chest radiograph; barium swallow; endoscopy of the esophagus; computed tomography (CT) scan of the neck, the chest, and the abdomen; and electrocardiogram. Endoscopic ultrasonography of the esophagus was optional. Bronchoscopy was performed if tracheobronchial involvement was suspected and surgical resection was under consideration. The tracheobronchial tree was judged to be involved if the tumors extended into the lumen or caused deformity of the lumen. The descending aorta was judged to be involved if the contact angle of the tumor was 90° or greater on the CT scan. Metastatic lymph nodes were defined if they were ≥1 cm in longest diameter on any imaging.

Treatment details

The treatment consisted of two cycles of NDP (level 1, 80 mg/m^2; level 2, 90 mg/m^2) on day 1 and continuous infusion of FU 800 mg/m^2/day on days 1–5, every 5 weeks, with concurrent radiotherapy at 60 Gy in 30 fractions over 6 weeks. The dose level of NDP was set referring to the results of a preceding phase I/II study in patients with metastatic esophageal cancer (data not shown). The second cycle of chemotherapy was set in the 6th week referring to a CDDP/5-FU chemoradiation regimen used in our institutions [5]. Radiotherapy was delivered with megavoltage equipment using anterior/posterior opposed fields up to 40 Gy including the primary tumor and the metastatic lymph nodes. An additional dose of 20 Gy was given to the primary tumor and the metastatic lymph nodes for a total dose of 60 Gy using bilateral oblique or multiple fields. The clinical target volume for the primary tumor was defined as the gross tumor volume plus 3 cm craniocaudally, and the clinical target volume for the metastatic nodes was the same as the gross tumor volume. The planning target volumes for the primary tumor and the metastatic lymph nodes were determined with 0.5- to 2-cm margins, taking account of setup variations and internal organ motion. Elective nodal irradiation was not intended in this study. Lung heterogeneity corrections were not used.

Toxicity assessment

Patients were observed weekly during treatment to monitor toxicity. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0) [13]. Late toxicity was graded according to the Radiation Therapy Oncology Group (RTOG)/European Organisation for Research and Treatment of Cancer (EORTC) late radiation morbidity scoring scheme. Late toxicity was defined as that occurring more than 90 days after treatment initiation.

Follow-up evaluation

The following evaluations were performed until disease progression every 3 months for the first years and every 6 months thereafter: physical examination, toxicity assessment, complete blood cell count, serum chemistry profile, endoscopy of the esophagus, and CT scan of the neck, the chest, and the abdomen. Biopsy of the primary tumor site was routinely performed at each follow-up examination. Pulmonary function test, electrocardiogram, and cardiac ultrasound were performed when indicated.

Response assessment

Complete response (CR) for the primary tumor was defined by endoscopy when all visible tumors, including ulcerations, disappeared with negative biopsy and lasted for ≥4 weeks.

Responses of the metastatic lymph nodes were assessed using the World Health Organization response criteria for measurable diseases. Briefly, CR was defined as the complete disappearance of all measurable and assessable disease for ≥4 weeks. Uncertain CR (uCR) was defined when small nodes (≤1 cm) persisted with no evidence of progression for ≥3 months after completion of treatment, and it was also included in CR.

Patterns of failure

Patterns of failure were defined as the first site of failure. Local/regional failure included the primary tumor and

regional lymph nodes. Distant failure included any site beyond the primary tumor and regional lymph nodes.

Study design and statistics

Two dose levels were set following the results from a preceding phase I study for metastatic esophageal cancer. A recommended dose for phase II was determined using the conventional 3 × 3 method. Dose-limiting toxicities (DLTs) were defined as follows: treatment-related death; grade 4 thrombocytopenia; grade 4 vomiting; PS 3; grade 3 febrile neutropenia persisting ≥4 days; and grade 3 nonhematologic toxicities excluding anorexia, nausea, vomiting, esophageal fistula, esophagitis, and infection due to esophageal fistula. It was also regarded as DLT if radiotherapy could not be completed within 60 days or if protocol treatment could not be completed because of any adverse event. For exploratory evaluation of the efficacy of this treatment, the sample size for phase II part was determined following the assumption that a CR rate of less than 20% would not be promising and a CR rate of 40% or greater with α error of 0.10 and β error of 0.20 would warrant further investigation of this regimen. Taking into account that 10% of the patients may be ineligible, the total sample size including phase I part was determined to be 25 to 40. Survival was measured from the first day of treatment. Death from any cause was included as an event in the overall survival, and any failure and any cause of death were included as events in the progression-free survival. The overall and the progression-free survival curves were calculated by the Kaplan–Meier method [14].

Results

Patient population

Between December 1999 and April 2002, 26 patients were enrolled in the study: 3 patients at level 1 and 23 patients at level 2. Their median age was 60 years (range, 45–69 years), 25 were male, and 1 was female. Patient and tumor characteristics are summarized in Table 1.

Table 1. Patient and tumor characteristics

Number of patients	26
Age	
Median	60
Range	45–69
Sex	
Male	25
Female	1
Performance status	
0	14
1	12
Location	
Ut	13
Mt	12
Lt	1
TNM	
T4	26
N0	5
N1	21
M0	17
M1a	5
M1b	4
Stage	
III	17
IV	9
Involved sites in T4	
Aorta	4
Bronchial tree	19
Both	3

Ut, upper thoracic esophagus; Mt, middle thoracic esophagus; Lt, lower thoracic esophagus; TNM, tumor-node-metastasis classification

Treatment compliance and toxicity

One of 3 patients in the level 1 group and none of the first 3 patients in the level 2 group experienced DLT, and level 2 was determined to be the recommended dose. In total, including patients in the phase II part, 3 of 23 patients in the level 2 group experienced DLT. Twenty-four patients completed the protocol treatment, and 2 patients in the level 2 group could not complete the treatment due to DLT. Eight patients had treatment delay before delivering the second cycle of chemotherapy as a result of hematologic toxicity in 7 patients and pneumonia caused by esophageal fistula in 1 patient. The median overall treatment time of radiotherapy was 44 days (range, 42–56 days), and 21 patients completed radiotherapy within 49 days.

Common grade 3 or greater acute toxicities were leukopenia, 9 (35%); neutropenia, 5 (19%); thrombocytopenia, 4 (15%); esophagitis, 4 (15%); and esophageal fistula, 3 (12%). There was no treatment-related death. The toxicity profile is shown in Table 2. As of the date of this analysis, 1 case with grade 3 pericardial effusion, 1 with grade 3 pleural effusion, and 2 with esophageal stenosis were observed as late toxicities.

Response and survival

Of all 26 registered patients, 3 achieved CR with a CR rate of 12% [95% confidence interval (CI), 2%–30%]. With a minimum follow-up period of 26 months for surviving patients, the median survival and the 1- and 2-year survivals were 12 months (95% CI, 9–22 months), 50% (95% CI, 31%–69%), and 31% (95% CI, 13%–49%), respectively (Fig. 1). The median progression-free survival and the 1-year progression-free survival were 6 months (95% CI, 5–8 months) and 27% (95% CI, 10%–44%), respectively. Two of 3 CR patients and 6 of 23 non-CR patients survived more than 2 years.

Patterns of failure

At the time of this analysis, 22 of 26 patients (85%) showed tumor progression, and 4 patients (15%) were alive without disease progression. The patterns of first failure were local/

Table 2. Acute toxicities[a]

	Level 1 ($n = 3$)					Level 2 ($n = 23$)					Total
	Grade					Grade					≥Grade 3 (%)
	0	1	2	3	4	0	1	2	3	4	
Hemoglobin	0	3	0	0	0	1	13	9	0	0	0
Leukocytes	0	0	2	1	0	1	3	11	7	1	35
Neutrophils	0	0	2	1	0	4	7	8	3	1	19
Platelets	2	0	0	1	0	8	7	5	2	1	15
Creatinine	3	0	0	0	0	22	1	0	0	0	0
Performance status	1	2	0	0	0	4	16	3	0	0	0
Infection	2	0	0	1	0	14	2	5	2[b]	0	12
Diarrhea	1	2	0	0	0	17	5	1	0	0	0
Esophagitis	1	2	0	0	0	5	11	3	4	0	15
Esophageal fistula	3	–	–	0	0	20	–	–	3	0	12
Mucositis/stomatitis	3	0	0	0	0	14	4	3	2	0	8
Nausea	2	1	0	0	0	10	10	3	0	0	0
Vomiting	2	1	0	0	0	14	7	2	0	0	0
Dyspnea	3	0	0	0	0	21	0	2	0	0	0
Pneumonitis	3	0	0	0	0	22	0	1	0	0	0

[a] National Cancer Institute–Common Toxicity Criteria version 2
[b] Both cases were caused by esophageal fistula

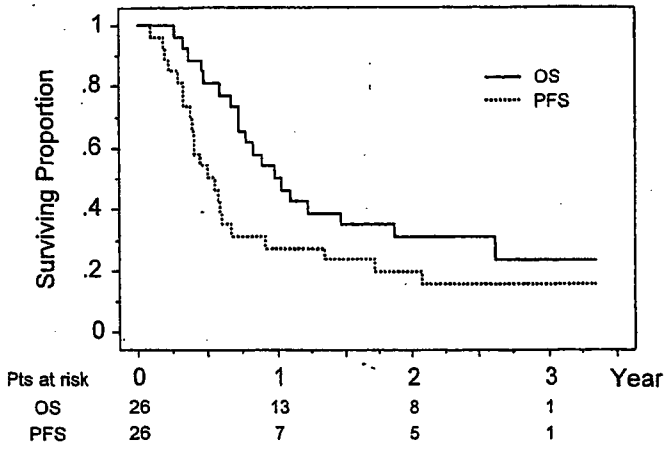

Fig. 1. Overall survival (*OS*) and progression-free survival (*PFS*) for all patients (*Pts*) enrolled in this study

regional only, 12 (46%); local/regional and distant, 3 (12%); and distant only, 7 (27%). Four patients developed local/regional progression after the occurrence of distant metastasis, and two patients developed distant metastasis after local/regional failure. In total, 19 (73%) patients developed local/regional failure and 12 (46%) patients developed distant failure.

Discussion

In the past decade, chemoradiotherapy consisting of CDDP and FU with concurrent radiotherapy has become a standard of care in selected patients with unresectable locally advanced esophageal cancer. Ohtsu et al. [5] reported median progression-free survival, median survival, and 1-year overall survival in patients with T4 and/or M1 lymph node disease as 6 months, 9 months, and 41%, respectively. Grade ≥3 toxicities were also reported as follows: leukopenia, 24%; anemia, 24%; thrombocytopenia, 17%; esophagitis, 15%; and esophageal fistula, 10%. In our study, median progression-free survival, median survival, and 1-year survival were 6 months (95% CI, 5–8 months), 12 months (95% CI, 9–22 months), and 50% (95% CI, 31%–69%), respectively. Grade ≥3 toxicities were observed as follows: leukopenia, 31%; thrombocytopenia, 15%; esophagitis, 15%; and esophageal fistula, 12%. These results seemed comparable with CDDP and FU with concurrent radiotherapy, showing that the treatment regimen of NDP and FU with concurrent radiotherapy is effective in selected patients with T4 disease. However, these survival figures are far from satisfactory, and patterns of failure showed that about three-fourths of patients developed local/regional failure and about one-half of patients developed distant failure. We should make further efforts to improve local control and to prevent distant metastasis.

The dose-escalation strategy of radiotherapy was one way but was not proven to be effective in the INT 0123 study [15], and current approaches of escalating dose of radiotherapy with CDDP and FU could achieve incremental benefit but seem to have reached a plateau. Different combinations with a novel cytotoxic drug are another way to improve survival. Paclitaxel is active for both adenocarcinoma and squamous cell carcinoma of the esophagus. A phase II trial of paclitaxel in patients with advanced esophageal cancer showed a 34% response rate in adenocarcinoma and a 28% response rate in squamous cell carcinoma [16]. There is evidence of synergism between paclitaxel and CDDP or FU [17], and paclitaxel combined with CDDP and FU in patients with advanced esophageal cancer was tested in a phase II study [18]. This trial showed a 46% response rate in adenocarcinoma and a 50% response rate in squamous cell carcinoma. These encouraging results led to trials

employing induction chemotherapy followed by concurrent chemoradiotherapy with paclitaxel, CDDP, and FU, but the advantage of this approach has yet to be proven.

Recently, molecular targeted drugs have been enthusiastically investigated in various malignant diseases [19–22]. Epidermal growth factor receptor is one of the targets, and this has been shown to be effective in patients with head and neck cancer when combined with radiotherapy in a phase III study [23]. It seems reasonable to investigate whether the combination of these agents has a survival impact for esophageal cancer.

There is another concern about the response criteria in the treatment of esophageal cancer. We employed response criteria using endoscopy for the primary tumor, which seemed to be reliable in patients who received nonsurgical treatment [6]. In this trial, the CR rate obtained was far less than expected, and this treatment regimen should be deemed ineffective according to the predefined hypothesis. However, 2 of 3 CR patients and 6 of 23 non-CR patients survived more than 2 years, 3 of these 6 non-CR patients did not show any disease progression, and the median survival obtained was not worse than historical data. This discrepancy suggests that the CR criteria used in this trial was not applicable to T4 disease and thus the CR rate failed to be a surrogate endpoint for survival. We think that overall survival will be appropriate as a primary endpoint in future phase II trials for this patient population.

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Novel Genetic Polymorphisms in the NR3C1 (Glucocorticoid receptor) Gene in a Japanese Population

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SNP Communication

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Summary: Glucocorticoid receptor, encoded by *NR3C1*, is a transcriptional regulator of many drug metabolizing enzymes and anti-inflammatory molecules. In order to identify genetic variations of the *NR3C1* gene, genomic DNA from 265 Japanese individuals was sequenced. Fifty genetic polymorphisms were identified, including 32 novel ones [3 were in coding exons, 17 in the introns, 4 in the 5′-untranslated region (UTR), and 8 in the 5′-flanking region]. The novel nonsynonymous variation was 420G>T (Lys140Asn), and the allele frequency was 0.004. We did not detect any nonsynonymous polymorphism reported previously in other races, including a relatively frequent SNP Asn363Ser found in Caucasians and African-Americans. Thus, ethnic differences between Japanese and other races are suggested to exist in *NR3C1*.

Key words: ***NR3C1*; glucocorticoid receptor; genetic polymorphisms; non-synonymous alteration**

Introduction

Glucocorticoid receptor (GR), encoded by *NR3C1*, is a member of the nuclear hormone receptor family of transcription factors. In the cytosol, GRs are associated with heat-shock and other proteins, and the binding of glucocorticoid leads to their nuclear translocation and positive or negative regulation of various genes.[1,2] It is well known that GR causes anti-inflammatory effects through transcriptional activation of glucocorticoid-induced leucine zipper genes or transcriptional suppression of genes of inflammatory cytokines induced by NF-κB or AP-1.[3-5] In addition, GR regulates expression of many drug metabolizing enzymes. For instance, it is reported that GR activates the transcription of drug metabolizing enzymes CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP3A4, and CYP3A5 through GREs in the promoter/enhancer regions of these genes, or induction of both pregnane X receptor and constitutive androstane receptor.[6-11]

The human *GR* gene is located on chromosome 5p31

On October, 18, 2004, these variations were not found in the Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), PharmGKB (http://www.pharmgkb.org/do/) or GeneSNPs (http://www.genome.utah.edu/genesnps/) databases.

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and contains 9 exons, including exon 9α.[12] GRβ is an alternatively spliced form with exon 9β replacing exon 9α, which was initially identified in glucocorticoid-resistant human multiple myeloma cells and functions as a dominant negative type.[13] Recently, three promoters and three 5′-noncoding exon 1′s (exons 1A, 1B, and 1C) were identified in *NR3C1*.[14] Interindividual differences of cytochrome P450 enzymes are thought to result from differences in their expression levels and/or activities.[11] Therefore, it is possible that the altered transcriptional activity of GR associated with polymorphisms of *NR3C1* might affect the expression levels of target genes including P450 enzymes.

Several genetic polymorphisms have been reported in *NR3C1*.[15] A common single nucleotide polymorphism (SNP), IVS2-646G>C (*Bcl*I polymorphism; allele frequencies, 0.15–0.45.[15]) and a relatively frequent SNP, 1088A>G (Asn363Ser; allele frequencies, 0.03–0.07.[15]), were found in Caucasians and African-Americans and reported to increase cortisol sensitivity and insulin response, and to cause other metabolic disturbances.[16,17] Moreover, it has been reported that the Ile559Asn (1676T>A) GRα variant has a trans-dominant effect on the wild-type GR by inhibiting its nuclear translocation.[18] Furthermore, the Ile747Met (2241T>G) GRα variant causes autosomal dominant glucocorticoid resistance through abnormal interactions with the p160 steroid receptor coactivator.[19] We previously reported that the Ser651Phe (1952C>T) and 2314insA variants showed reduced and almost abrogated transcriptional activities, respectively, correlating with their protein expression levels.[20] Thus, it is suggested that the genetic polymorphisms in *NR3C1* at least partly contribute to the interindividual differences in GR transcriptional activity. However, little information on promoter and coding SNPs in the entire *NR3C1* gene has been available in any race.

In this study, the promoters, exons, and surrounding introns of *NR3C1* were sequenced from 265 Japanese subjects. Sequence analysis identified 50 genetic polymorphisms, including 32 novel polymorphisms.

Materials and Methods

Human DNA samples: DNA was extracted from the blood leukocytes of 114 Japanese cancer patients (with lung, stomach, and colon cancers) administered irinotecan. Additional 151 Japanese subjects were patients with allergy. Their peripheral lymphocytes were immortalized using the Epstein-Barr virus, and genomic DNA was extracted from the immortalized cells. Written informed consent was obtained from all participating patients. The ethical review boards of the National Cancer Center, the National Center for Child Health and Development, and the National Institute of Health Sciences approved this study.

Polymerase chain reaction (PCR) conditions and DNA sequencing: First, the entire *NR3C1* gene was amplified by mixed primer sets (Mix 1 or Mix 2 in the "1st PCR" section) shown in **Table 1**. Amplification was performed from 200 ng of genomic DNA using 1.25 units of Z-Taq (Takara Bio Inc., Shiga, Japan) with 0.2 μM of the primer sets. Since exon 1B and 1C are highly GC-rich, this exon was amplified by utilizing the GC-buffer Kit (LA-Taq, Takara Bio, Inc.). The first round PCR conditions were 94°C for 10 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 3 min, and then a final extension at 72°C for 7 min. Then, each exon was amplified by Ex-Taq (0.625 units) with a set of primers (0.2 μM) listed in the "2nd PCR" section of **Table 1** (primers were designed in the intronic regions). The second-round PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. Thereafter, the PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the primers listed in the "Sequencing" section of **Table 1**. Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). For exons 3 through 7 and 9β, the primer sets for the 2nd PCR were also used for sequencing. As for exons 1A, 1B, 1C (with the 5′-flanking region), 2, and 8–9α, different primer sets were used for sequencing. All the detected variations were confirmed by repeating the PCR from the genomic DNA and sequencing the newly generated PCR products. All nucleotide changes were clearly discernable in the electropherograms. Hardy-Weinberg equilibrium for all the detected genetic polymorphisms was statistically analyzed by SNPAlyze Ver. 3.2 (DYNACOM Co., Kanagawa, Japan).

Results and Discussion

NT_029289.10 (Genbank Accession number) was utilized as a reference sequence of *NR3C1*. Fifty genetic variations were identified, including 32 novel ones, from 265 Japanese individuals (see **Table 2**). Of the 32 single nucleotide variations, 3 were in the coding exons, 17 in the introns, 4 in the 5′-untranslated region (UTR), and 8 in the 5′-flanking region. All 50 genetic polymorphisms were in Hardy-Weinberg equilibrium.[21,22]

In the coding region, a novel nonsynonymous variation, 420G>T (Lys140Asn), was detected in exon 2. 420G>T (Lys140Asn) was detected from 2 cancer patients (stomach and small cell lung cancers) as heterozygotes. The electropherograms of the variation is shown in **Fig. 1**. The allele frequency was 0.004.