Figure 2. Macroscopic findings of the resected stomach. Multiple erosions and oedematous mucosa are evident in the whole stomach. Irregularly shaped depressions are indicated by green arrows.

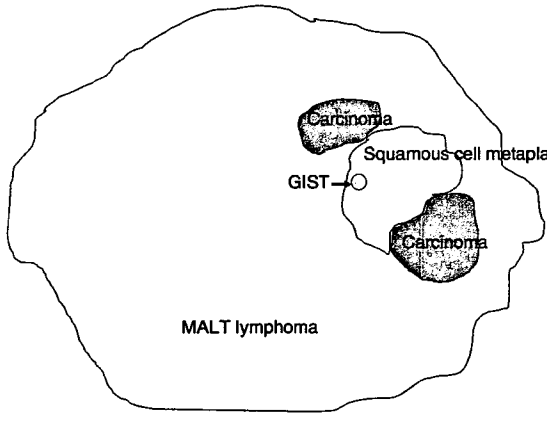

Figure 3. Schematic illustration of Figure 2 based on the histological findings. The brown area shows involvement of the mucosa-associated lymphoid tissue (MALT) lymphoma. The two depressed, red lesions represent gastric carcinoma surrounded by the MALT lymphoma. The whitish mucosa between the two gastric carcinomas represents squamous cell metaplasia, under which MALT lymphoma tissue has invaded. The gastrointestinal stromal tumour (GIST), located at the subserosal portion, is shown as a blue circle.

findings. Multiple erosions and oedematous mucosa were evident throughout the stomach. Irregularly shaped depressions measuring 25 × 20 mm and 25 × 20 mm were seen in the fornix and gastric body, as indicated by arrows in the figures. The mucosa between the two lesions was whitish. Figure 3 is a histological schematic illustration of the resected stomach. The brown area indicates MALT lymphoma involvement. Two depressed, red gastric carcinoma lesions were surrounded by the MALT lymphoma. The whitish mucosa between the two

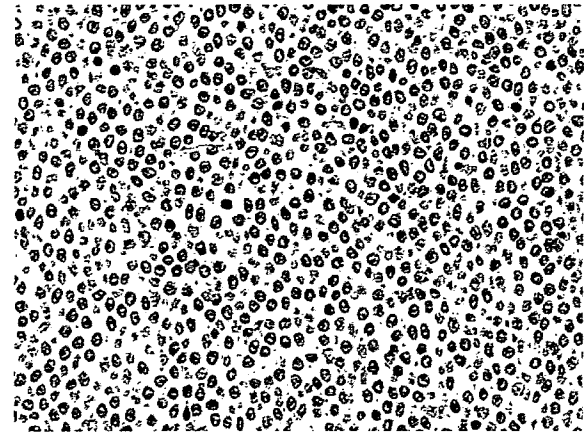

Figure 4. Histology of mucosa-associated lymphoid tissue (MALT) lymphoma. The MALT lymphoma is largely composed of low-grade B-cell lymphoma cells. (H&E.)

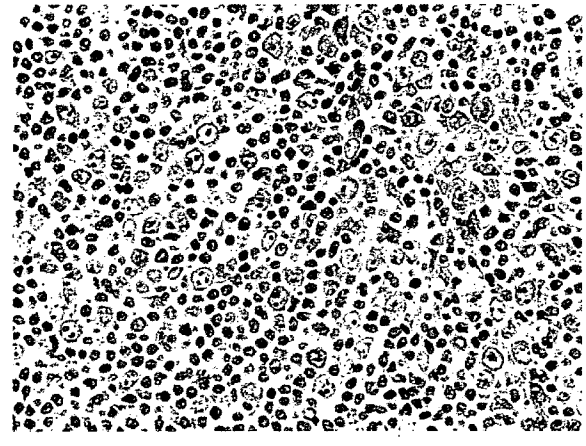

Figure 5. Histology of mucosa-associated lymphoid tissue lymphoma. Diffuse large-cell lymphoma tissue can be seen in the duodenum. (H&E.)

gastric carcinomas represents squamous cell metaplasia, beneath which invasion by the MALT lymphoma was evident. A gastrointestinal stromal tumour (GIST), which was located at the subserosal portion of the stomach, is shown as a blue circle (see below for details).

Histology revealed that invasion by the MALT lymphoma was primarily limited to the submucosal tissue with partial invasion into the subserosal tissue. This lymphomatous proliferation was predominantly occupied by low-grade MALT lymphoma tissue, consisting of centrocyte-like cells (Figure 4) accompanied by small, scattered clusters of blasts in the duodenal portion (Figure 5). Therefore, the postoperative

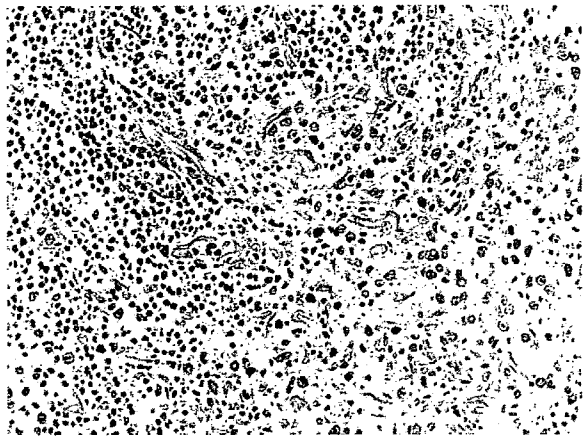

Figure 6. Histology of the collision portion of gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. The gastric carcinoma is composed of poorly differentiated adenocarcinoma or signet-ring cell carcinoma, in the right lower part. The MALT lymphoma is evident in the left upper part. (H&E.)

histological diagnosis was low-grade MALT lymphoma with a high-grade diffuse large B-cell lymphoma component. The tumour cells were immunophenotypically positive for CD20, CD79a and Bcl-2, but not for CD3, CD5, CD10, CD23, CD45RO or cyclin D1, and *API2-MALT1* chimeric transcript was detected in fresh tissue. Intestinal metaplasia and atrophic changes in the mucosa adjacent to the MALT lymphoma were also evident. Microlymphomas were not seen. Lymph node dissection was limited to the lymph nodes surrounding the gastric wall, due to peritoneal dissemination. The regional lymph nodes surrounding the gastric wall were all involved by the MALT lymphoma. On the other hand, involvement of signet-ring cell carcinoma was limited to the lymph nodes in the gastric wall.

Both gastric carcinomas were poorly differentiated adenocarcinoma of diffuse type and/or signet-ring cell carcinoma extending to the serosa and were located within the area involved by the MALT lymphoma with no evidence of normal tissue between the two, indicating synchronous contiguous/collision tumours (Figure 6). Epstein–Barr virus (EBV) infection was not detected by *in situ* hybridization for EBV-encoded non-polyadenylated RNA in either MALT lymphoma or adenocarcinoma tissue.

An intramural nodule, measuring 15 × 10 mm, was eventually found as a synchronous independent tumour at the subserosal portion of the stomach (Figure 3), which showed a uniform proliferation of spindle cells with few mitoses or necrosis. Based on positivity of CD117/KIT, but not of S100 protein, desmin or smooth muscle action, the diagnosis of this nodule was GIST.

Most cases of gastric *API2-MALT1*+ MALT lymphoma are characterized by unresponsiveness to antibacterial treatment against *H. pylori*, and the pathogenesis of this tumour is independent of infection with this microorganism.[5,8,9] The *API2-MALT1* chimeric transcript is also exclusively largely detected in low-grade MALT lymphomas.[10,11] Furthermore, thus far, this transcript has shown few additional gene alterations, indicating that fusion-positive tumours are genetically more stable than negative ones,[11,12] although there is an exceptional case report of *API2-MALT1*+ MALT lymphoma with secondary cytogenetic abnormalities.[13] However, the natural history and actual prognosis of *API2-MALT1*+ MALT lymphoma have yet to be clarified due to the lack of any long-term follow-up observations. Notably in the present patient, gastric MALT lymphoma did not affect the prognosis during a 7-year clinical course, in spite of advanced clinical stage and the presence of a diffuse large B-cell lymphoma component. This is in keeping with previous reports that the prognosis of patients with double primary gastric lymphoma and adenocarcinoma is more closely associated with the adenocarcinoma than the lymphoma.[14,15] As far as the present case is concerned, the *API2-MALT1*+ MALT lymphoma provided no evidence of a relationship with *H. pylori* infection and preceded the development of gastric carcinoma.

In one summary of the literature on synchronous lymphoma and adenocarcinoma of the stomach,[16] the author found that *H. pylori* infection was detected in 78% of 32 patients. The majority of lymphomas were low grade (75%) and were larger than the carcinoma (81%). The majority of carcinomas (65.6%) were at an early stage. The author[16] also suggested that lymphoma might develop before carcinoma or the presence of MALT lymphoma might increase the risk of developing carcinoma. Three cases of metachronous gastric MALT lymphoma with a rearrangement of *API2-MALT1* and early gastric carcinoma have recently been reported by Copie-Bergman *et al.*[17] These cases had a good prognosis after detection of subsequent carcinoma and resection of the stomach. The present case has provided additional evidence for their assertion that prolonged residual gastric MALT lymphoma could constitute an additional risk factor for the development of gastric carcinoma, but it is unique in revealing that the latter rapidly progressed into advanced-stage disease and was the greatest influence on prognosis. Further investigation is required to clarify the possibility that gastric

glandular epithelium presenting within an *API2-MALT1+* MALT lymphoma might be prone to neoplastic transformation.

The findings in this case suggest that the prognosis of *API2-MALT1+* gastric MALT lymphoma is relatively good, even if the clinical stage is advanced. However, if follow-up is selected as the treatment strategy for this particular lymphoma, the physician should be aware of the possibility of the development of concomitant gastric carcinoma.

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Two cases of hepatoid adenocarcinoma of the intestine in association with inflammatory bowel disease

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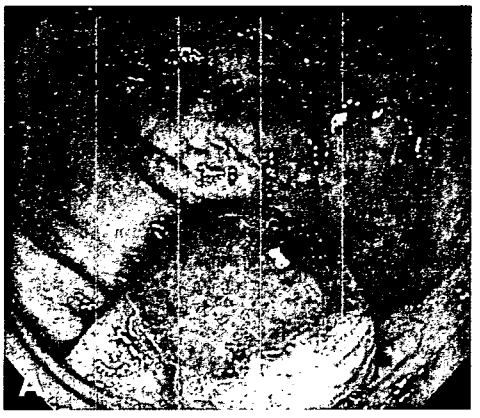
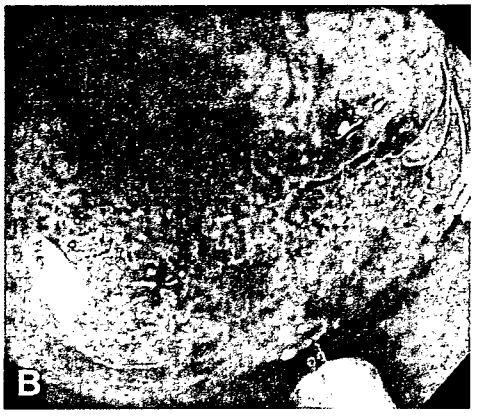
Sir: Hepatoid adenocarcinoma (HAC) is a distinctive type of extrahepatic adenocarcinoma that shows

A case of colonic morule with colitis cystica profunda





C

A 48-year-old man was referred to our institute for evaluation of a positive fecal occult blood test. Colonoscopy with a magnifying videoscope revealed a submucosal tumor with a sharply demarcated central depression in the ascending colon (**A**). After the area was sprayed with 0.2% indigo carmine, the magnified view of the depressed lesion showed a pitted pattern that was difficult to fit into the classification proposed by Kudo et al (**B**, orig. mag. ×50). The pit pattern basically was composed of tubular or roundish pits that were irregular in shape, size, and arrangement. Histologic examination of the biopsy specimen led us to suspect a well-differentiated adenocarcinoma. EUS demonstrated a nonhomogenous, well-circumscribed, hypoechoic lesion including a calcified component in the third hyperechoic layer of the 5-layer structure of the colonic wall. We speculated that early colon cancer developed on a submucosal tumor; however, we could not exclude invasive neoplasia, because an abdominal CT scan showed regional lymph node enlargement. Therefore, a right hemicolectomy was performed. The histologic diagnosis of the resected

specimen was colonic morule in tubular adenoma with severe atypia on a colitis cystica profunda (**C**, H&E, orig. mag. ×4; **D**, H&E, orig. mag. ×40). There was no cancerous involvement in the regional lymph nodes.

DISCLOSURE

None of the authors have any disclosures to make.

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doi:10.1016/j.gie.2006.06.021

Commentary

A morule is a term familiar to us if we think back to our embryology classes and recall one of the early cleavage stages of the zygote (*L morum*, mulberry). Morules are associated with aberrant β-catenin expression and have been reported with both benign and malignant neoplasia, including carcinoma of the lung, uterus, thyroid, colon, and others as well as with nonneoplastic tissue such as pregnancy-related endometrium. Although somewhat controversial, it is believed that, at least in the colon, morules are cell clusters with a basal or stem cell phenotype and that they may have less proliferative and invasive potential than other cancer cells do. Histologically, a morule contains biotin-rich intranuclear inclusions referred to as "optically clear nuclei" (different from the cell nucleus) and that they are composed of cytologically bland cells in a syncytial-like arrangement with a solid or a fenestrated pattern. Colitis cystica profunda is another rare disease, characterized by submucosal mucin-filled cysts. Colitis cystica profunda has been associated with a variety of diseases, including adenocarcinoma of the colon, rectal prolapse, and solitary rectal ulcer. Taken together, the findings in this case teach us that there are a limited number of symptoms and signs the body can exhibit and, therefore, many disease processes from the congenital to the acquired and the inflammatory to the neoplastic may share presentations and appearances. Thankfully, today we have a wide range of techniques that enable us to unravel some of complexities of what we see and to echo Aristotle that "In all things of nature there is something of the marvelous."

Lawrence J. Brandt, MD
Associate Editor for Focal Points

Thymidylate Synthase Gene Expression in Primary Tumors Predicts Activity of S-1-based Chemotherapy for Advanced Gastric Cancer

Hiroya Takiuchi, Shin-ichiro Kawabe, Masahiro Gotoh, Ken-ichi Katsu

ABSTRACT

Purpose: To evaluate the association between dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) levels in primary gastric tumors and clinical response to S-1 or S-1 plus irinotecan in patients with unresectable advanced gastric cancer, and to investigate the molecular mechanism of augmented antitumor activity of the combination using human gastric cancer xenografts with high TS activity.

Materials and Methods: TS mRNA expression and DPD mRNA expression were measured by reverse transcription polymerase chain reaction in initial primary cancer biopsy specimens in 29 patients with advanced gastric cancer who had received S-1 alone (n=18) or in combination with irinotecan (n=11). In an experimental study, antitumor effects of S-1, irinotecan, and the combination were assessed in mice bearing human gastric tumors with high TS expression (4-1-ST and AZ-521 tumors) and low TS expression (SC-2 tumors), and activities of 5-fluorouracil–metabolizing enzymes were measured.

Results: In the clinical study, a strong statistical association between high TS expression and clinical resistance to S-1 alone was found (P = .009). In the experimental studies, S-1 plus irinotecan showed augmented antitumor activity against tumors with high TS activity (P < .01) compared with either agent alone. A potential mechanism for this effect was suggested by the significant reduction in TS activity observed following irinotecan administration in tumors with high TS activity.

Conclusion: This study suggests that, via down-regulation of TS by irinotecan treatment, combination chemotherapy with S-1 and irinotecan could be effective in gastric cancer patients with high TS levels.

Gastrointest Cancer Res 1:172–177.

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Tumors expressing high levels of thymidylate synthase (TS), the rate-limiting enzyme of de novo DNA synthesis, have poor sensitivity to fluoropyrimidine-based chemotherapy,[1–4] and tumor levels of dihydropyrimidine dehydrogenase (DPD), a rate-limiting enzyme of 5-fluorouracil (5-FU) catabolism, have been reported to inversely correlate with sensitivity to 5-FU–based chemotherapy.[5–9] These observations have led to attempts to predict efficacy of 5-FU treatment by assessing TS and DPD levels in gastrointestinal tumors.[5, 8–11]

S-1 is a new oral fluoropyrimidine with high activity in gastric and colorectal cancer.[12–14] The drug contains the potent DPD inhibitor 5-chloro-2, 4-dihydroxypyrimidine (gimeracil, CDHP) and a potassium oxonate (oteracil potassium, Oxo) component that inhibits the phosphorylation of 5-FU, together with the 5-FU prodrug tegafur. The inclusion of CDHP as a component of S-1 is expected to reduce the effect of level of DPD expression on tumor response to fluoropyrimidine treatment. However, as with other fluoropyrimidines, tumors that express high levels of TS mRNA are expected to be relatively resistant to S-1. Ichikawa et al[15] reported a positive correlation between TS mRNA expression and expression of topoisomerase I, and Danenberg et al[16] have reported cases of metastatic colorectal cancer in which the topoisomerase I inhibitor irinotecan was effective in tumors with high levels of TS mRNA. Such findings suggest that the use of irinotecan in combination with S-1 might overcome tumor fluoropyrimidine resistance on the basis of high TS levels, and that selection of S-1 monotherapy or combined therapy could be made on the basis of tumor TS mRNA levels.

In the current study, we analyzed TS mRNA and DPD mRNA levels in initial biopsy samples from patients with un-

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resectable advanced gastric cancer who had been treated with S-1 monotherapy or S-1 plus irinotecan to determine the association of expression levels with response to treatment. We further evaluated the antitumor activity of S-1 plus irinotecan using human gastric cancer xenografts with high or low TS activity in nude mice and investigated the molecular mechanism of the augmented activity seen with the combination.

MATERIALS AND METHODS

Clinical Study

Patient Characteristics and Treatment Outcome

The study population consisted of all 29 patients with unresectable advanced gastric cancer who were treated with first-line S-1 alone (n=18) or S-1 plus irinotecan (n=11) from January 2000 to December 2001 at the Second Department of Internal Medicine, Osaka Medical College (Osaka, Japan). This study was approved by the Institutional Review Board of Osaka Medical College, and all patients gave written informed consent. To be eligible, patients had to have a histologically confirmed diagnosis of gastric cancer; age of 20 to 75 years; performance status of 0, 1, or 2 on the Zubrod scale (Eastern Cooperative Oncology Group); no prior chemotherapy regimens before entry; adequate hematologic, hepatic, and renal function; and estimated survival of at least 3 months. Clinical characteristics of the patients are shown in Table 1.

Oral S-1 was given at 80 to 120 mg/day in two doses for 28 days, followed by a 14-day rest period for 1 course. S-1/irinotecan treatment consisted of S-1 at the same dose for 21 days followed by a 14-day rest period and irinotecan 80 mg/m^2 IV on days 1 and 15 for 1 course.[17] Objective response was determined by World Health Organization (WHO) criteria, and primary lesions were assessed by Japanese Research Society for Gastric Cancer criteria (originally established by WHO). Response was defined as complete or partial response; nonresponse was defined as no change or progressive disease.

Among 18 patients who received S-1 alone, 6 (33.3%) had partial response and 12 were nonresponders (8 with no change, 4 with progressive disease). Among 11 patients receiving S-1/irinotecan, 8 (72.7%) had partial response and 3 were nonresponders (all with no change).

Table 1. Main clinical characteristics of patients.

	S-1 alone (n=18)		S-1 + irinotecan (n=11)	
Sex				
Male	12	(67%)	6	(55%)
Female	6	(32%)	5	(45%)
PS				
0–1	14	(78%)	8	(73%)
2	4	(22%)	3	(27%)
Type				
Type 1	1	(5%)	0	(0%)
Type 2	4	(22%)	0	(0%)
Type 3	4	(22%)	2	(18%)
Type 4	9	(50%)	9	(82%)
Histology				
Differentiated	10	(56%)	2	(18%)
Undifferentiated	8	(44%)	9	(82%)
Response				
Responder	6	(33%)	8	(73%)
Nonresponder	12	(67%)	3	(27%)

[AUTHOR: Please explaing "types" in a footnote.]

Determination of TS mRNA and DPD mRNA in Tumor Specimens

Tumor samples were obtained from primary gastric tumors at the time of initial endoscopy. Immediately after biopsy, the tumor biopsy specimens were fresh frozen in liquid nitrogen until the time of RNA extraction. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using a previously description method, the reliability and validity of which have been reported in detail by Ishikawa et al.[18] Total RNA for each sample was isolated using the RNeasy mini kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instructions. Reverse transcription using 10 g of total RNA was performed in a total volume of 100 L containing 250 pmol oligo $(dT)_{18}$, 80 units of Rnasin (Promega, Madison, WI, USA), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, and 0.5 mM deoxynucleotide triphosphates solution. Initially, RNA and oligo $(dT)_{18}$ were heated to 70°C for 10 minutes and immediately chilled on ice; the remaining reagents were added and then incubated for 15 minutes at 30°C and 60 minutes at 42°C.

cDNA for genes of interest and an internal reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were quantified using a fluorescence-based real-time detection method (ABI PRISM 7700 Sequence Detection System [TaqMan]; Perkin-Elmer Applied Biosystems, Foster City, CA, USA) as described previously.[19]

The PCR reaction mixture consisted of 600 nM of each primer, 200 nM probe, 2.5 units AmpliTaq Gold Polymerase, 200 μM each of dATP, dCTP, and dGTP, 400 μM dUTP, 5.5 mM MgCl2, and 1 X TaqMan Buffer A containing a reference dye, to a final volume of 25 μL (all reagents Perkin-Elmer Applied Biosystems). Primer and probe sequences were those previously described.[20,21] Cycling conditions were 50°C for 10 seconds, 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. TaqMan analyses yield values expressed as ratios between two absolute measurements (gene of interest/internal reference gene).

Statistical Analysis

The Mann-Whitney U test was used to compare the responders and nonresponders in terms of the related gene expressions. To evaluate the association of DPD or TS mRNA with response, DPD or TS mRNA was categorized into low or high values with the cutoff value determined by receiver operating characteristic (ROC) analysis. Association with response was analyzed by two-sided Fisher's exact test.

Experimental Study

The antitumor effects of S-1 and irinotecan

were evaluated in human gastric cancer xenografts with high TS activity (4-1-ST and AZ-521) and low TS activity (SC-2) in nude mice. Human gastric tumors AZ-521, 4-1-ST, and SC-2 were obtained from Dai-Nippon Pharmaceutical Co., Ltd. (Osaka, Japan) and maintained by implantation into the right axilla of nude mice at 3-week intervals. For enzyme assays, [6-^{3}H]-5-FU (525 GBq/mmol), [6-^{3}H]-thymidine (dThd; 2.41 TBq/mmol), [6-^{3}H]-FdUMP (625 GBq/mmol), and [^{14}C(U)]-cytidine-5'-diphosphate (CDP; 2.04 GBq/ mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA, USA). For immunoblot analysis of proteins, anti-TS antibody was provided by Okabe,[20] and all other antibodies used were purchased from Santa Cruz Biochemicals Inc. (San Diego, CA, USA).

Xenografts and Treatments

For the antitumor experiments, 4-1-ST, AZ-521, and SC-2 tumors were prepared by subcutaneous implantation of approximately 3 mm^3 fragments into the right axilla in groups of 5 mice each. After 7 days, animals received oral S-1 at 8.3 mg/kg for 14 consecutive days, irinotecan 40 mg/kg IV on days 1 and 8, or the combination. For studies of the effects of irinotecan on 5-FU–metabolizing enzymes, groups of 5 nude mice were prepared by the same methods and received saline or irinotecan alone 75 mg/kg once weekly for 2 weeks. In studies assessing dose response of TS activity, groups of 3 nude mice with AZ-521 tumors received no irinotecan or irinotecan 20, 40, or 60 mg/kg weekly for 2 weeks.

In the antitumor studies, tumor volume [½ × (the major axis) × (the minor axis)^2] was measured in all groups twice a week throughout the experiments, and relative tumor volume (RTV) was calculated as follows: RTV = (mean tumor volume during therapy)/(mean tumor volume at the start of therapy). The antitumor effects of S-1, irinotecan, and both drugs combined were estimated by the following equation: mean tumor growth inhibition (TGI, %) = [1-(mean RTV of drug-treated group/mean RTV of control group) × 100].

Enzyme Assay

The effects of irinotecan treatment on 5-FU–metabolizing enzymes in AZ-521, 4-1-ST, and SC-2 tumors and the effect of different doses of irinotecan on TS activity in AZ-521 tumors were analyzed. AZ-521, 4-1-ST, and SC-2 tumors were homogenized with 3 volumes of 50 mM Tris-HCl (pH 7.6) containing 10 mM 2-mercaptoethanol, 25 mM KCl, and 5 mM $MgCl_2$, centrifuged at 105,000g for 60 minutes, and the resulting supernatant was used to measure enzyme activity. The enzymes measured were TS, DPD, ribonucleotide reductase (RNR), orotate phosphoribosyltransferase (OPRT), thymidine kinase (TK), and thymidine phosphorylase (TP). TS was measured by [6-^{3}H]-FdUMP binding assay based on the method of Spears et al.[21] DPD and OPRT activity was determined according to the method of Shirasaka et al[22] using [6-^{3}H]-5-FU as the substrate. TK activity was measured by the method of Ikenaka et al[23] except that the reaction product, [6-^{3}H]-thymidine-5'-monophosphate, was separated from [6-^{3}H]-thymidine by Silica gel 60F$_{254}$ (2 × 10 cm) thin layer chromatography with a mixture of chloroform, methanol, and acetic acid (17:3:1, v/v/v) as the mobile phase. TP was measured according to the modified method described by Maehara et al.[24] Ribonucleotide reductase activity was determined using [^{14}C(U)]-CDP as the substrate.[25]

Western Blot Analysis

For analysis of expression of TS proteins, Western blot analysis was performed using the ECL Western blotting detection system and protocol (Amersham Corp., Arlington Heights, IL). Briefly, cells were scraped from the plates, washed with PBS, and lysed in cell lysis buffer. Forty micrograms of protein were electrophoretically separated on SDS-polyacrylamide gels and transferred to polyvinylide difluoride membrane (Millipore, Bedford, MA, USA). Anti-

Table 2. Effect of irinotecan administration on the activities of 5-FU–metabolizing enzymes in 4-1-ST, AZ-521, and SC-2 human gastric cancer xenografts in mice.

Tumor	4-1-ST		AZ-521		SC-2	
Enzyme	Control	Irinotecan (75 mg/kg/wk x 2)	Control	Irinotecan (75 mg/kg/wk x 2)	Control	Irinotecan (75 mg/kg/wk x 2)
TS	2.163±0.281	1.375±0.184*	0.622±0.095	0.082±0.019*	0.089±0.043	0.086±0.033
DPD	2.22 ± 0.27	1.84 ± 0.34	78.10±12.59	53.87±15.40†	8.580±3.45	8.120±1.46
OPRT	8.119±0.663	7.599±0.806	6.649±0.772	4.726±0.600†	21.01±2.68	22.10±4.00
TP	1.011±0.018	1.168±0.157	0.115±0.030	0.103±0.025	0.647±0.154	0.676±0.105
RNR	2.030±0.975	3.076±1.183	0.483±0.282	0.660±0.099	4.330±1.840	4.150±0.730
TK	15.28±1.90	15.29± 3.41	31.10± 3.75	35.97± 6.86	32.01± 4.20	68.70±13.61

For each tumor, mice received saline (n=5) or irinotecan 75 mg/kg (n=5) administered IV weekly for 2 weeks. At 24 hours after last treatment, tumors were removed and activities of 5-FU-metabolizing enzymes were measured. TS = thymidylate synthase; DPD = dihydropyrimidine dehydrogenase; OPRT = orotate phosphoribosyltransferase; TP = thymidine phosphorylase; RNR = ribonucleotide reductase; TK = thymidine kinase. *P< .01, †P< .05 compared with control group by Dunnett's test.

Table 3. Effect of irinotecan 20 to 60 mg/kg on TS activity in AZ-521 human gastric cancer xenografts in mice.

Dose (mg / kg)	N	TS activity* (pmol/mg ± SD)	% of Control
Control	3	0.994 ± 0.188	100.0
20	3	0.526 ± 0.118	52.7
40	3	0.292 ± 0.088	29.3
60	3	0.214 ± 0.054	21.4
Control	5	0.622 ± 0.095	100.0
75	5	0.082 ± 0.019	13.2*

Mice received no irinotecan or irinotecan 20, 40, or 60 mg/kg on days 1 and 8; 24 hours after final treatment, tumors were dissected and TS activity was measured. *Data on 75 mg/kg treatment and control are from Table 2.

Rh TS polyclonal antibody was applied and the membranes were enhanced by chemiluminescence using ECLTM Western blot detection reagents (Amersham Corp.) according to the manufacturer's instructions.

Statistical Analysis

The significance of differences between groups was assessed using Dunnett's test and the IUT test.

RESULTS

Clinical Study

The relationship between tumor response and DPD mRNA and TS mRNA expression was assessed in 29 primary gastric tumor samples from patients with advanced gastric cancer treated with S-1 alone (n=18) or S-1/irinotecan (n=11). The cutoff values for high vs. low mRNA expression, determined by ROC analysis, were 6.76 (range, 0.19–19.77) for DPD mRNA and 4.53 (range, 0.75–17.27) for TS mRNA. Tumor response occurred in 6 of 18 patients receiving S-1 alone and in 8 of 11 receiving S-1/irinotecan.

There was no relationship between high or low DPD mRNA expression and tumor response with either S-1 alone or with S-1/irinotecan (Figure 1). Response in patients receiving S-1 alone was significantly associated with TS mRNA expression, with none of the 6 responses occurring in tumors with high TS mRNA levels ($P = .009$) (Figure 2). There was no relationship between TS mRNA expression and tumor response in the S-1/irinotecan group, with responses being observed in some patients with high TS levels (Figure 2).

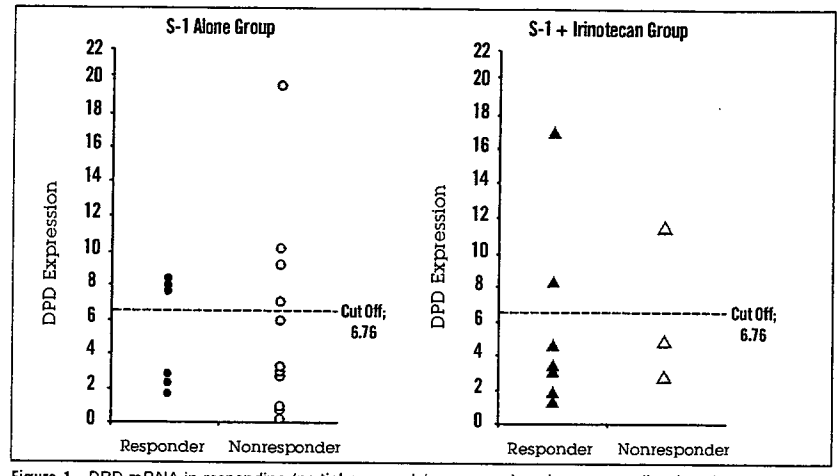

Figure 1. DPD mRNA in responding (partial or complete response) and nonresponding (no change or disease progression) primary gastric tumors in patients receiving S-1 (n=18) or S-1 plus irinotecan. Cutoff value demarcates high vs. low values. **[Author: Difficult to tell, but looks like 17 data points for S-1 and 10 for S-1/CPT-11 on graph? Also, units of measure for DPD expression?]**

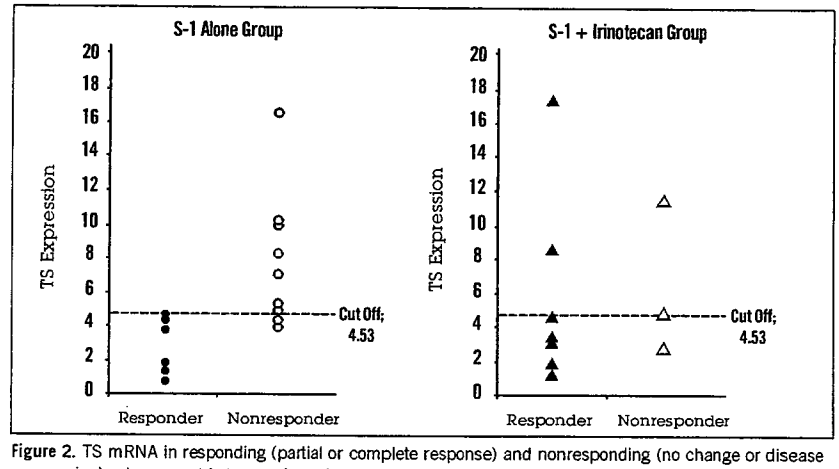

Figure 2. TS mRNA in responding (partial or complete response) and nonresponding (no change or disease progression) primary gastric tumors in patients receiving S-1 (n=18) or S-1 plus irinotecan. Cutoff value demarcates high vs. low values. $P = .009$ for **[Author: Please specify comparison that P value applies to.]**

Experimental Study

Antitumor Effects of S-1 and Irinotecan

The antitumor activities of S-1 and irinotecan were evaluated in nude mice with human gastric cancer xenografts with high TS activity (4-1-ST and AZ-521) or low TS activity (SC-2). As shown in Figure 3, for 4-1-ST and AZ-521 tumors, the tumor growth inhibition rate (IR) with the combination of S-1/irinotecan was significantly augmented ($P < .01$) compared with either agent alone, reaching approximately 60% for both tumor types. For SC-2 tumors, S-1 alone had IR of approximately 70%, with no further augmentation observed with the addition of irinotecan.

Effects of Irinotecan on the Activity of 5-FU–Metabolizing Enzymes

To investigate the mechanism for augmented antitumor activity of the combination of S-1 and irinotecan on 4-1-ST and AZ-521 tumors, activities of the 5-FU–metabolizing enzymes TS, DPD, OPRT, TP, RNR, and TK were assessed in 4-1-ST, AZ-521, and SC-2 tumor xenografts in mice treated with irinotecan or saline. As shown in Table 2, irinotecan 75 mg/kg weekly for 2 weeks significantly reduced TS activity in 4-1-ST and AZ-521 xenografts compared with controls, and produced no change in TS activity in SC-2 tumors. Other 5-FU–metabolizing enzymes were not affected by treatment with irinotecan. Moreover, TS activity in AZ-521 tumor xenografts decreased dose-dependently following administration of 20, 40, and 60 mg/kg of irinotecan (Table 3). Overall, the doses of irinotecan examined in these studies were associated with a dose-dependent antitumor effect, with IR ranging from approximately 30% to 60% (data not shown). Doses of 20 to 40 mg/kg correspond to clinically applicable doses of irinotecan.

Immunochemical Detection of TS Proteins and Rate-Limiting Proteins in G1/S Phase of Cell Cycle

To confirm the decrease in TS activity in 4-1-ST and AZ-521 tumors treated with irinotecan, we assessed expression of TS proteins.

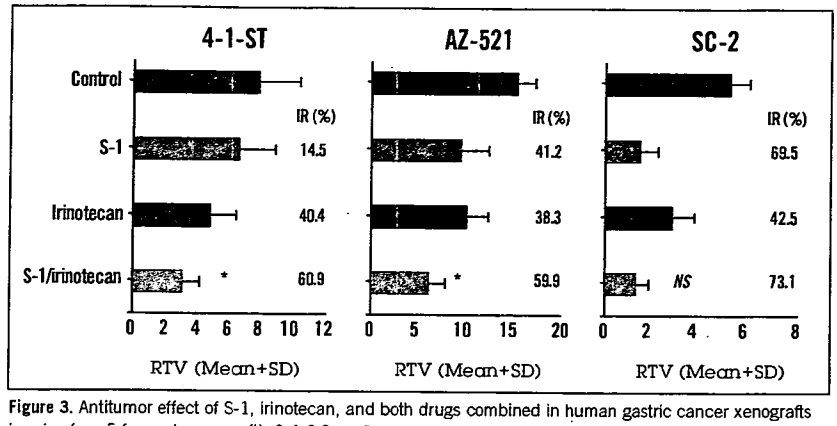

Figure 3. Antitumor effect of S-1, irinotecan, and both drugs combined in human gastric cancer xenografts in mice (n = 5 for each xenograft). S-1 8.3 mg/kg was orally administered once daily for 14 days, and irinotecan 40 mg/kg was injected weekly for 2 weeks to tumor-bearing mice starting 2 weeks after tumor implantation. On treatment day 15, the antitumor activity of S-1, irinotecan, and their combination was evaluated as the inhibition rate of tumor growth (IR, %). * **[Author: P values?] Significantly different from S-1 alone and irinotecan alone by the IUT test.**

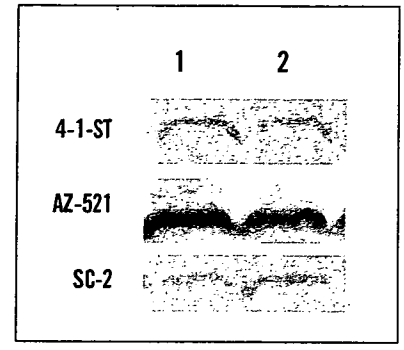

Figure 4. Expression of TS proteins in 3 human gastric cancer xenografts in mice treated with or without irinotecan. The TS aliquot (50 µg protein) was immunochemically analyzed using anti-TS polyclonal antibody by the Western blot method. The left lane shows nontreated tumors and the right lane shows irinotecan-treated tumors.

The expression of TS proteins in 4-1-ST and AZ-521 tumors treated with irinotecan also decreased, whereas TS protein expression in SC-2 tumors did not change with irinotecan treatment (Figure 4).

The expression of TS mRNA and its subsequent protein expression are known to be regulated by active E2F1 proteins released from the RB-R2F complex on which cell cycle-regulating proteins (eg, CDK4 and cyclin D1) would operate. We thus assessed expression of activated phospho-RB, free E2F1, CDK4, and cyclin D1 proteins in AZ-521 tumors treated with irinotecan. As shown in Figure 5, the expression of those proteins seemed to decrease in the tumors treated with irinotecan compared with untreated tumors. This result suggests that a decline in free E2F1 proteins in irinotecan-treated tumors may be connected with down-regulation of TS expression.

DISCUSSION

High expression of TS mRNA and DPD mRNA has been associated with reduced gastrointestinal tumor sensitivity to 5-FU–based chemotherapy.[1-8] Since the fluoropyrimidine S-1 contains the potent DPD inhibitor CDHP, its antitumor activity theoretically should be independent of level of DPD expression. It also has been reported that TS mRNA expression is positively correlated with topoisomerase I mRNA expression and that response can be achieved in tumors with high levels of TS activity by using the topoisomerase I inhibitor irinotecan.[15,16] It is thus plausible to combine S-1 with irinotecan in patients with tumors with high expression of TS mRNA.

The findings in our study using samples from advanced gastric cancer patients receiving first-line therapy with either S-1 or S-1/irinotecan support the absence of effect of DPD level on tumor response to S-1, since there appeared to be no association between response and high or low level of DPD expression in primary tumors in patients receiving S-1 alone; level of DPD expression also did not affect response when S-1 and irinotecan were used together. However, tumor response to S-1 alone was observed only in patients with low TS mRNA expression, whereas response was observed in some patients with high TS expression with the addition of irinotecan.

The augmentation of antitumor activity with the addition of irinotecan to S-1 was confirmed by our findings in gastric cancer xenografts, with tumor growth inhibition being markedly greater with the combination of S-1/irinotecan than with S-1 alone in the high-TS-expressing 4-1-ST and AZ-521 tumors and no difference between S-1 alone and the combination being found in the low-TS-expressing SC-2 tumors.

These findings suggest that patients with advanced gastric cancer may derive greater benefit from the combination of S-1 and irinotecan when high levels of TS expression are present. Our experimental studies indicated that TS activity was down-regulated by irinotecan in a dose-dependent manner in xenografts with high levels of TS expression, suggesting that irinotecan resulted in an environment in which S-1 was more likely to exert its antitumor effect. This finding also suggests that order of treatment with S-1 and irinotecan might make some difference in achieving tumor response. Ongoing preliminary animal studies indeed suggest that treatment with irinotecan followed by

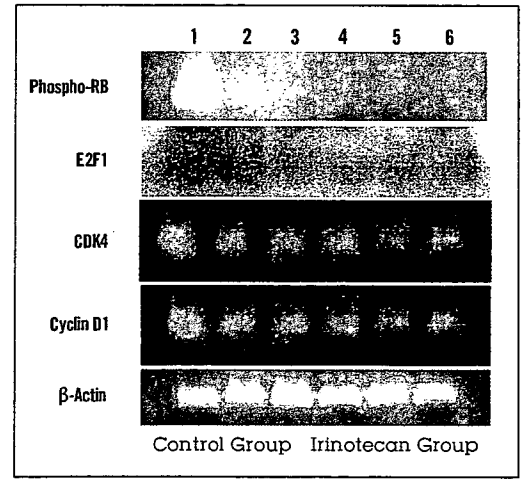

Figure 5. Expression of phospho-RB, E2F1, CDK4 and cyclin D1 proteins in AZ-521 tumors treated with or without irinotecan. Fifty microgram proteins of nuclear extracts from the tumors were immunochemically analyzed using anti-pRB, anti-E2F1, anti-CDK4, anti-cyclin D1, and anti-β-actin monoclonal antibodies, respectively. Lanes 1 to 3 show nontreated tumors and lanes 4 to 6 irinotecan-treated tumors.

S-1 may result in more prolonged tumor inhibition in GI tumors with high TS expression than that observed with S-1 alone, irinotecan alone, or both drugs in combination (data not shown).

Fluoropyrimidines are a mainstay of palliative treatment for unresectable advanced gastric cancer. S-1 has a theoretic advantage over 5-FU in terms of having antitumor activity that appears to be independent of level of DPD expression, suggesting that it should be active in tumors expected to be resistant to 5-FU on the basis of high DPD expression. The potential strategy of using S-1 alone or in combination with irinotecan based on absence or presence of high TS mRNA expression on initial biopsy of tumor tissue should be explored in a prospective clinical setting. Such a strategy might help avoid unnecessary combination therapy and unnecessary toxicity—a primary concern in palliative treatment—in patients with low TS expression, and the combination may improve response rates in patients with high TS expression.

Measuring TS mRNA expression would seem to be a first step toward the individualized treatment of gastric cancer using fluoropyrimidine-based chemotherapy, particularly S-1–based chemotherapy. However, it also needs to be acknowledged that fluoropyrimidine metabolism involves a large number of genes in addition to those examined in the current study, and the effects of different levels of expression of these genes and their products may also be of importance in modulating response to fluoropyrimidine-based treatment.

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Disclosures of Potential Conflicts of Interest

To come.

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Chemotherapy for Advanced Gastric Cancer: A New Milestone Lies Ahead

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Our first goal is not far away. The time-worn debate between the West and Japan over chemotherapy for advanced gastric cancer may be resolved by a couple of well-designed randomized phase III trials worthy of the name, and chemotherapy for advanced gastric cancer may soon enter a new stage. Others may feel the same after reading the review by Koizumi in this issue of *Gastrointestinal Cancer Research.*[1]

As Dr. Koizumi describes in his review, the overall survival time in patients with metastatic gastric cancer has been apparently prolonged after the emergence of a new generation of agents, including irinotecan, taxanes (docetaxel, paclitaxel), oxaliplatin, and novel oral fluoropyrimidines (capecitabine, S-1) in the past decade. The median survival time was only 6–7 months in randomized phase III studies performed before the appearance of these drugs,[2,3] and 8–10 months (a 2–3 month improvement) in randomized phase III studies recently conducted in the West.[4]

To define a new standard, one still needs an appropriate reference regimen. Prior to the appearance of these novel drugs, the most popular regimen was CF (cisplatin plus 5-fluorouracil [5-FU]). The median progression-free survival (PFS) or time to progression (TTP) with CF was about 4 months, which was similar in Japanese and global phase III studies.[5] Based on these results, CF is considered the most appropriate reference regimen for global randomized phase III studies currently under way. In a few European countries, and perhaps Canada, epirubicin, cisplatin, and 5-FU (ECF) was considered the standard regimen.[6] However, ECF is not widely accepted in the East or in the United States, because the addition of epirubicin to cisplatin/5-FU has not been demonstrated to be superior to CF alone.[7]

Combination therapy with novel anticancer drugs has also been investigated worldwide. In Europe and the United States, irinotecan/cisplatin, docetaxel/cisplatin/5-FU (DCF), capecitabine/cisplatin, capecitabine/oxaliplatin, and epirubicin/capecitabine/oxaliplatin have been investigated, and response rates were 50% or higher, with TTP ranging from 5.5 to 7 months in phase II and III studies. In Japan, irinotecan/cisplatin and S-1–based regimens (S-1/cisplatin, S-1/irinotecan, S-1/docetaxel, and S-1/paclitaxel) have been investigated, and response rates were also 50% or higher, and TTP was also 5.5–7 months in phase II settings.[8–11] Doublets and triplets including these novel agents consistently prolonged time to progression by about 2–3 months, compared with CF. This increase in time to disease progression is consistent with improvements in median survival times recently reported from randomized phase III studies conducted in the West.

TAX325 was a large-scale international study, in which a triplet including docetaxel, cisplatin, and 5-FU (DCF) was compared to CF. The primary end point was time to progression, with overall survival being a secondary outcome measure. The final results of this study clearly demonstrated the superiority of DCF to CF. Time to progression was significantly longer for DCF than for CF (median, 5.6 months vs. 3.7 months, respectively; $P = .0004$). Overall survival time was also significantly prolonged by DCF (9.2 months 8.6 months; $P = .0201$).[4] However, many oncologists hesitate to use DCF as the standard regimen because the survival improvement was only 0.6 months, and toxicity is more severe than that of CF. There is no doubt that docetaxel is effective for gastric cancer based on these findings, but the use of docetaxel in second-line and later treatments may have been restricted because the TAX325 study was a sponsored trial aiming at the approval of docetaxel. **[AUTHOR: The meaning of this statement concerning docetaxel in second-line and later treatment is unclear. TAX325 looked at DCF in a first-line setting. What is meant by "restricted" in a second-line setting? Are you suggesting possible official or regulatory restrictions because DCF was approved for first-line therapy only? And what is the significance of sponsorship in this context? As currently worded, a reader could infer that a perception of bias exists regarding the results of TAX352 due to sponsorship and marketing objectives. Please clarify.]**

In gastric cancer chemotherapy, many patients receive second-line treatment or further anticancer drug therapy after they become resistant to first-line treatment. For example, further debate is necessary concerning whether docetaxel should be used as the first-line treatment in simultaneous combination therapy or as second-line treatment in sequential combination therapy. This debate may be resolved by ongoing Japanese studies, and the effects of second-line or later therapies with new agents such as docetaxel can be estimated, since the reference regimen used in Japan is different from that used in other countries. CF is used worldwide, and ECF is also used in some European countries including the United Kingdom, but monotherapy, such as 5-FU or S-1, is used in Japan.

This disparity may yield valuable data in the near future because phase III studies of monotherapy vs. combination therapy — ie, sequential combination therapy vs. simultaneous combination therapy — are currently under way in Japan. The study results will be reported within 2007, which may lead to a conclusion of the debate. We are awaiting the results of the JCOG9912 trial (5-FU alone vs. irinotecan/cisplatin vs. S-1 alone) and the TAIHO trial (S-1 alone vs. S-1/cisplatin). The primary end point of these studies is overall survival. If irinotecan/cisplatin is superior to 5-FU in JCOG9912,

many oncologists will welcome the result not only in Japan, but also in the United States.

A superiority study of S-1/cisplatin compared with CF is now under way in a large-scale international trial (the First Line Advanced Gastric Cancer Study [FLAGS]), in which overall survival is the primary end point. Should S-1/cisplatin prove superior in both the TAIHO trial and FLAGS, the disparity between chemotherapies performed in Japan and other countries may be resolved, and S-1/cisplatin may become a new standard therapy for advanced gastric cancer.

I look forward to reviewing the final results of these studies, and wish to thank all enthusiastic investigators worldwide for choosing overall survival as their primary end point. I do believe that a new milestone in chemotherapy for gastric cancer is around the corner, and it is clearly time for both global and Japanese investigators to pursue further benefits by incorporating molecular targeting agents into a new standard.

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Epidermal Growth Factor Receptor is a Possible Predictor of Sensitivity to Chemoradiotherapy in the Primary Lesion of Esophageal Squamous Cell Carcinoma

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Background: Chemoradiotherapy (CRT) is currently performed for patients with esophageal squamous cell carcinoma (SCC). Some reports have revealed that patients who responded well to CRT had favorable outcomes, whereas poor responders conversely showed a worse prognosis. The aim of this study was to identify molecular markers predicting sensitivity to CRT.
Methods: We reviewed 62 patients with T_{3-4}, N-any, and M-any esophageal SCC treated with definitive CRT. The regimen comprised protracted 5-fluorouracil infusion and a 2-h infusion of cisplatinum combined with radiation therapy (2 Gy/day) at a total radiation dose of 60 Gy. The expressions of epidermal growth factor receptor (EGFR), vascular endothelial growth factor, cyclin D1, and proliferating cell nuclear antigen were investigated immunohistochemically in biopsy specimens obtained before treatment from all 62 patients. The immunoreactivities were compared with responsiveness to CRT, as evaluated by endoscopy.
Results: The complete response rate of the primary tumor estimated by endoscopy was 62% (13/21) in patients in the EGFR-positive group. The difference in the CR rate between EGFR-positive and -negative groups was significant ($p = 0.037$). The immunoreactivities of the other molecular markers did not show a significant correlation with the responsiveness of the primary lesion to CRT. Multiple logistic regression analysis revealed that positive immunostaining for EGFR was significantly correlated with primary CR for CRT in esophageal SCC.
Conclusion: Among 62 patients with esophageal SCC, differences in the responsiveness of primary lesions to CRT were correlated with EGFR immunoreactivity assessed in the biopsy specimens. These results suggest that EGFR may help to predict the response of primary sites to definitive CRT in esophageal SCC, although the results should be confirmed in a larger, more homogeneous series.

Key words: esophageal cancer – chemoradiotherapy – epidermal growth factor receptor

INTRODUCTION

Esophageal cancer is a typical refractory cancer with a poor prognosis among malignant tumors of the gastrointestinal tract. Even in operable cases, the outcomes of surgical treatment alone are poor in Western countries, and the 5-year survival rate has been reported to be 6–24% (1). However, as a local complete response (cCR) is obtained by radical chemoradiotherapy (CRT) in 40–60% of patients (2), CRT is also beginning to be performed widely for resectable esophageal cancers. While there has been no randomized comparative study of esophagectomy and radical CRT for operable esophageal cancers, a retrospective study reported that a survival rate comparable to that after esophagectomy was obtained by radical CRT (3). The prognosis of advanced localized esophageal cancer invading multiple organs has been considered very poor. Otsu et al., however, reported an overall CR (complete disappearance of the tumor) rate of 33% (18/54) and a 3-year survival rate of 23% after CRT without surgery in 54 patients with esophageal cancer clinically staged before treatment as T4/M1LYM (4), suggesting

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that even locally advanced esophageal cancer can be cured by radical CRT. Recently, important comparative studies of preoperative CRT vs radical CRT for locally advanced esophageal cancer (T3–4) were also performed almost simultaneously in Germany and France (5–7) The French study considered that definitive radiochemotherapy may be regarded as a standard treatment in responders. The German study was designed to perform surgery after CRT (40 Gy) in one group and to perform radical CRT (at least 65 Gy) in another group with chemotherapy before CRT in both groups. Since the 3-year survival rate exceeded 50% in the responders to chemotherapy in both groups (58% in the surgery group and 55% in the radical CRT group), it was concluded that surgery may not be necessary in these patients. On the other hand, the 3-year survival rate was low in the non-responders to chemotherapy in both groups (17.9% in the surgery group and 9.4% in the radical CRT group), but some patients in the surgery group survived for a long period after surgery, because the tumor could be resected completely while they did not respond to chemotherapy. Therefore, the study concluded that whether surgery should be performed after CRT or radical CRT for advanced localized esophageal cancer depends on the response to induction chemotherapy or induction chemo-radiotherapy. Therefore, if factors that allow prediction of the effect of CRT are found, a more effective therapeutic strategy can be expected. However, whether primary CR can be achieved by CRT is an important point if CRT is intended to be a radical treatment. Owing to the recent development of molecular biology, various target molecules related to the proliferation, infiltration and metastasis of cancer cells have been identified, and their relations with chemo- or radiosensitivity and the prognosis have been evaluated (8–11).

In this study, we investigated the relationships between the clinical effect (primary CR) of definitive CRT on the primary lesions of advanced esophageal cancer and molecular markers considered to be involved in cell proliferation and angiogenesis by examining biopsy specimens, and evaluated whether these molecular markers are useful as predictors of the effectiveness of treatment.

PATIENTS AND METHODS

Subjects

The source of the study data was a database of esophageal cancer patients who received definitive CRT between July 1994 and July 2003 at Osaka Medical College. Sixty-two patients fulfilled the following criteria: (a) histologically proven esophageal squamous cell carcinoma; (b) no previous treatment; (c) PS on Eastern Cooperative Oncology Group scale 0–2; (d) those with an endoscopically evaluable primary lesion; (e) patients with adequate organ, bone marrow, liver, and renal functions; (f) patients with no severe complications; (g) clinically diagnosed T_{3-4}, N-any, and M-any on the International Union Against Cancer tumor-node-metastasis (TNM) classification; and (h) informed consent was obtained before treatment. All patients were given the same regimen of definitive CRT.

Treatment Schedule

The treatment comprised protracted 5-fluorouracil (5-FU) infusion (400 mg/m^2/day on days 1–5 and 8–12), and a 2 h infusion of cisplatinum (CDDP 40 mg/m^2 on days 1 and 8) combined with radiation therapy (2 Gy/day) delivered for 3 weeks (5 days/week). These schedules were repeated twice every 4–5 weeks and the total radiation dose was 60 Gy. For patients who showed an objective response to treatment, additional chemotherapy was administered and consisted of a protracted infusion of 5-FU (800 mg/m^2/day) on days 1–5 and a 2 h infusion of CDDP (80 mg/m^2/day) on day 1. This treatment was repeated every 4 weeks for two courses. No further treatment was administered if no disease progression was observed.

Evaluation of Response Concerning the Primary Site and Survival

We assessed the primary site by way of the endoscopic response criteria proposed by Tahara et al. (12). Response at the primary site was evaluated as CR (primary-CR) by endoscopic examination when all of the following criteria were satisfied under observation of the entire esophagus: (a) disappearance of the tumor lesion; (b) disappearance of ulceration; and (c) absence of cancer cells in biopsy specimens. When these criteria were not satisfied, a non-CR was designated. Existence of an erosion, ulcer scar, and lugol-voiding lesion did not preclude a CR evaluation. The first evaluation was performed 1 month after the completion of CRT to determine whether or not disease progression was present. Although repeat assessments were not essential to confirm primary-CR after the criteria for a response were first met, endoscopic examinations were performed every 2 or 3 months. All 62 patients were reviewed according to the above criteria. Survival time was measured from the initiation of the first course of treatment to the date of death or the final date of survival confirmation.

Immunohistochemical Staining Methods

Pretreatment endoscopic biopsy specimens from 62 patients were assessed for epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), cyclin D1, and proliferating cell nuclear antigen (PCNA) expression. Immunohistochemical staining was carried out with the labeled streptavidin biotin (LSAB) method using a Dako LSAB kit (Dako, Carpinteria, CA, USA). Primary antibodies used for the immunohistochemical staining were as follows: anti-EGFR rabbit polyclonal antibody (1005; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, dilution 1:100),

anti-Cyclin D1 mouse monoclonal antibody (DOS-6; Novocasta, dilution 1:50), anti- PCNA mouse monoclonal antibody (PC-10; DAKO, Glostrup, Denmark, dilution 1:200), and anti-VEGF rabbit polyclonal antibody (A-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, dilution 1:100).

Formalin-fixed, paraffin-embedded biopsy materials were cut into 4 μm sections. After deparaffinization with three changes of xylene, and then dehydration in a graded alcohol series, the sections were heated in a microwave at 500 W for 5 min three times in 10 mM citrate buffer solution for the retrieval of antigenicity. Endogenous peroxide activity was blocked with 3% hydrogen peroxide at room temperature for 10 min. After being rinsed with phosphate-buffered saline (PBS), the sections were incubated with 10% normal bovine serum albumin (blocking buffer) in PBS for 30 min in order to reduce nonspecific background staining. Sections were then drained off and incubated overnight at 4°C with primary antibodies. After six rinses in PBS, sections were incubated with the secondary biotinylated anti-mouse antibodies for cyclin D1 and PCNA, and anti-rabbit antibodies for EGFR and VEGF for 20 min at room temperature. The primary antibodies were localized by the sequential application of biotinylated anti-mouse–rabbit IgG gout immunoglobins, streptavidin–peroxide conjugate (Dako, Carpinteria, CA, USA). Immunostaining was visualized by developing the slides in diaminobenzidine (DAB) and counterstaining with Meyer-hematoxylin. Finally, the sections were subjected to alcohol and xylene baths, and then mounted for examination. For negative controls, the primary antibody solutions were replaced by the blocking buffer.

Staining Evaluation

Immunohistochemical staining was evaluated by two authors without prior knowledge of the endoscopic response. The immunoreactivity of EGFR was graded into four groups according to the intensity of cell membrane EGFR staining in the whole tumor: high (markedly stronger staining than normal esophageal epithelium), medium (moderately stronger staining), low (the same staining level as normal epithelium), and negative (fainter staining). Strong and moderate staining groups were defined as positive for EGFR expression, in agreement with previous interpretations of EGFR in esophageal squamous cell carcinoma (10,13,14). VEGF staining was graded as follows: (a) +, staining intensity in cancer cells was stronger than that in stromal cells; (b) ±, staining intensity in cancer cells was equal to that in stromal cells; and (c) −, staining intensity in cancer cells was weaker than that in stromal cells. The cases graded as + were defined as positive, as described in previous reports (15). The percentages of cyclin D1-positive tumor cells were calculated by counting the number of brown-stained tumor nuclei/total number of cancer cells in the most highly stained area on a high-power view (×400). Cut-off values were determined by the following estimation: cyclin D1-positive judgment was a more than 30% labeling index (16). The PCNA index was the percentage of nuclei staining positive (17). A PCNA score greater than 40 was taken as PCNA-positive.

Statistical Analysis

The χ^2 test and Student's t-test were used to evaluate the association between the response of primary lesions and clinical variables. A logistic regression analysis was used to control for possible confounding factors. Survival curves of the patients excluding M_1 disease were calculated by the Kaplan–Meier method and analyzed by the log rank test. Significance was defined as $P < 0.05$. Statistical analyses were conducted with the Stat View software (5.0 version)

RESULTS

Patient Characteristics and Response

From July 1994 to July 2003, 62 patients fulfilled the inclusion criteria of our study, and their characteristics are presented in Table 1. There were 54 males and eight females with a median age of 68 years (range, 43–85 years). Twenty-one patients had tumors in the lower third of the esophagus, 28 in the middle third, and 13 in the upper third. All had histologically proven squamous cell carcinoma. In terms of the T stage, 49 patients had T3 invasion and 13 had T4 invasion. In terms of the N stage, 10 had N_0 disease, and 52 patients had N_1 disease. Twenty two cases of M1 disease were as follows: M1 LYM ($n = 6$), liver ($n = 9$), lung ($n = 4$), liver and lung ($n = 2$), and liver and bone ($n = 1$). Sixty-five percent (40/62) belonged to TNM stage II/III and the other 22 to stage IV. Primary-CR to CRT was seen in 44% (27/62) of patients. Primary-CR rates in patients with T3 and T4 were 47% (23/49) and 31% (4/13), respectively.

Immunoreactivity

All 62 specimens were immunohistochemically evaluated for EGFR, cyclin D1, VEGF, and PCNA. Positive cyclin D1,PCNA immunoreactivities were detected in nuclei, whereas VEGF immunoreactivities were observed in the cytoplasm. EGFR expression was seen both on the cell membrane and in the cytoplasm (Figure 1). Positivity for EGFR, cyclin D1, VEGF, and PCNA was observed in 21, 21, 24, and 27 of 62 cases (34, 34, 39, and 44%), respectively. Table 2 shows the correlation between the immunohistochemical study and endoscopic response. Thirteen (62%) of the 21 patients with EGFR positivity achieved primary tumor CR. In contrast, 14 (34%) of the 41 EGFR-negative patients achieved primary tumor CR. The CR of the primary tumors tended to be higher in the EGFR-positive than -negative group, and the difference between the CR of the EGFR-positive and -negative groups was significant ($P = 0.037$). These results suggest that the

Table 1. Patient characteristics

Characteristic	Patients	
	No.	%
Median age, years (range)	68	(43–85)
Sex		
Male	54	87
Female	8	13
Performance status		
0–1	47	76
2	15	24
Location		
Upper	13	21
Middle	28	45
Lower	21	34
T stage		
T3	49	79
T4	13	21
Node classification		
N0	10	16
N1	52	84
Distant metastasis		
M0	40	64
M1a	6	10
M1b	16	26
Stage		
II	7	11
III	33	53
IVA	6	10
IVB*	16	26

Distant metastasis: liver alone 9, lung alone 4, liver + lung 2, liver + bone 1.

immunoreactivity of EGFR in biopsy specimens has a significant correlation with the sensitivity to CRT. Other immunohistochemical markers do not show significant correlations with the sensitivity to CRT. Table 3 shows the correlation

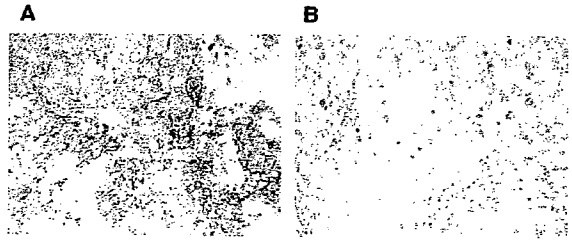

Figure 1. Representative immunohistochemical epidermal growth factor receptor (EGFR) stainings in biopsy specimens before chemoradiotherapy (CRT) A, EGFR-positive; EGFR immunostaining is seen in the cell membranes of tumor cells. B, EGFR-negative; Immunostaining is not seen in any cell membranes.

Table 2. Association of response with markers

	No. of patients (%)	Primary response		*P*-value
		CR ($n = 27$)	Non-CR ($n = 35$)	
EGFR				
High expression	21 (34%)	13	8	
Low expression	41 (66%)	14	27	0.0370
Cyclin D1				
High expression	21 (34%)	10	11	
Low expression	41 (66%)	17	24	0.6436
VEGF				
High expression	24 (39%)	11	13	
Low expression	38 (61%)	16	22	0.7731
PCNA				
High expression	27 (44%)	11	16	
Low expression	35 (56%)	16	19	0.4107

All patients ($n = 62$). CR, complete response; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; PCNA, proliferating cell nuclear antigen.

between each T stage and immunohistochemical markers. In T3 stages, similar results were obtained concerning EGFR ($P = 0.07$). However, there were no significant correlations between immunohistochemical markers and each T stage. On multiple logistic regression analysis, only EGFR was shown to be a significant predictor of primary CR in T_{3-4} esophageal SCC (Table 4).

Survival

Patients with local disease (M_0) comprised 40 cases. With a median follow-up of 11 months, the median survival time (MST) of all patients with $T_{3-4}M_0$ esophageal cancer given CRT was 16 months. Figure 2 shows the survival curves according to EGFR expression using Kaplan–Meier analysis. The MST of the EGFR-positive group was 18 months, and the MST of the EGFR-negative group was 19 months. There was no difference between the groups concerning EGFR expression ($P = 0.645$). Seven patients are still alive. The causes of death in the 33 patients who died consisted of 21 cases of locoregional disease (EGFR+, 6; EGFR−, 15), 11 cases of distant disease (EGFR+, 6; EGFR−, 5), and one case of treatment-related death due to radiation pneumonitis. There were no significant differences in the EGFR status and relapse site ($P = 0.518$).

Table 3. Association between markers and T stage, with CR rate in each stage

Depth	Response (response rate)	EGFR			Cyclin D1			VEGF			PCNA		
		+	−	*P*	+	−	*P*	+	−	*P*	+	−	*P*
T3	CR (47%:23/49)	10	13		8	15		9	14		8	15	
				0.07			0.99			0.96			0.28
	Non-CR (53%:26/49)	5	21		9	17		10	16		13	13	
T4	CR (30%:4/13)	3	1		2	2		2	2		3	1	
				0.16			0.32			0.57			0.16
	Non-CR (70%:9/13)	3	6		2	7		3	6		3	6	

+: positive; −: negative.

DISCUSSION

In this study, we evaluated the usefulness of molecular biological markers for the prediction of the effectiveness of definitive CRT in patients with advanced esophageal cancer (T3–4). In patients with esophageal cancer, definitive CRT is often performed as a radical treatment, and whether primary CR can be achieved is very important. This study focused on the relationships between molecular biological markers and primary CR. Therefore, even M1 patients who underwent CRT for the primary lesion and in whom the responses were evaluable were included in the subjects. In this study, EGFR positive patients correlated with primary CR on multivariate analysis. However, the expression of cyclin D1, VEGF, or PCNA was not correlated with the response to CRT. By nature, EGFR is not only involved in the proliferation of tumor cells when stimulated by EGF, but also involved in their infiltration, metastasis, and angiogenesis. If tumor vessels are generated more vigorously in deep areas of the tumor in EGFR-positive patients among those with T3-4 highly invasive esophageal cancer, tumor cell proliferation is considered to contribute to high radiosensitivity, because cells are reported to be three times more radiosensitive in the presence of oxygen than in a severely hypoxic condition (22). Since the distribution of the drug to the lesion increases if more vessels are supplying the tumor, the increase in oxygen and drug transport to the lesion in the EGFR-positive patients may contribute to high radiochemosensitivity. However, when the relationship between the EGFR expression and outcome was examined in 40 patients, excluding those with distant metastasis (M1), the median survival period was 18 months in the EGFR-positive group and 19 months in the EGFR-negative group, with no significant difference ($P = 0.65$). Also, no difference was observed in the first relapse site between the two groups.

There have been reports on the relationship between the outcome of surgically treated esophageal cancer and EGFR overexpression in surgical specimens (8,9,11), and the outcomes of tumors over-expressing EGFR were poor in all these reports. In particular, Kitagawa et al. reported that the median survival period was 9 months in patients with EGFR gene amplification but 42 months in those without EGFR gene amplification in 107 patients ($P < 0.01$) after radical surgery for esophageal cancer, and concluded that EGFR gene amplification is an independent prognostic factor. In our study, the survival rate of EGFR-positive patients appeared better by radical CRT, while positive EGFR has been regarded as a poorer prognostic factor after surgery.

Table 4. Multiple logistic regression analysis for primary CR of 62 T_{3-4} patients treated with CRT

Factory	Category	Odds ratio	95% CI	*P*
EGFR	− VS +	0.274	0.083–0.906	0.0338
Cyclin D1	− VS +	0.753	0.233–2.431	0.6352
VEGF	− VS +	1.104	0.336–3.626	0.8704
PCNA	− VS +	1.179	0.365–3.810	0.7830
T stage	T_3 VS T_4	2.671	0.608–11.746	0.1934
N stage	0 VS 1	0.503	0.099–2.546	0.4059
M stage	0 VS 1	1.230	0.364–4.153	0.7386

95% CI, 95% confidence interval.

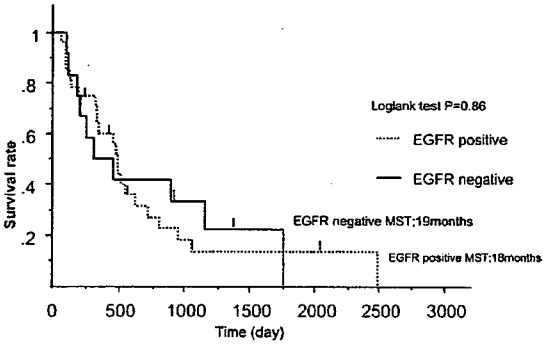

Figure 2. The Survival curves for 40 patients with T_{3-4} M_0 (M1 cases were excluded from the analysis of survival analysis) esophageal squamous cell carcinoma received CRT, according to EGFR. Overall survival is almost same both positive EGFR and negative EGFR. MST, median survival time.

Local recurrence and distant metastasis were observed regardless of the EGFR expression, so that EGFR expression may not be a prognostic factor in radical CRT for locally advanced esophageal cancer.

Recently, various therapeutic strategies incorporating CRT such as neoadjuvant chemotherapy and radical CRT have been evaluated to improve the therapeutic results in esophageal cancer. There have been various opinions put forward regarding preoperative adjuvant CRT. The survival rate improved significantly with preoperative CRT compared with surgery alone in some reports (18), but no significant difference was noted in other reports (19,20), and its significance remains controversial. There is also a report that the outcomes were better in those who obtained a pathologic complete response (pCR) by preoperative CRT than in those who did not (21). Concerning the curability of treatment for advanced localized esophageal cancer, there was no clear difference between surgery and radical CRT, and even local advanced esophageal cancer impossible to curatively resection has been reported to be cured by CRT alone in some patients (3,4). Therefore, if predictive factors of the effectiveness of CRT can be found, more appropriate design of treatment for each patient may become possible, leading to an improvement in the outcome. In our present study, a close correlation was observed between the EGFR expression of biopsy specimens from primary lesions of esophageal cancer before treatment and primary-CR with CRT, but the high probability of recurrence even after CR cannot be ignored. Even in patients who are expected to respond markedly to CRT, salvage surgery must always be considered. On the other hand, surgery without CRT may be a better option in some patients who are not expected to respond sufficiently to CRT to avoid treatment-related death such as radiation pneumonitis. We hope that EGFR helps with the selection of treatment in the future and contributes to improvement in the outcome through optimization of individualized treatment. However, further studies including prospective ones are considered to be necessary to establish the usefulness of this biologic marker.

Conflict of interest statement

None declared.

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Evaluation of Prognostic Factors of Esophageal Squamous Cell Carcinoma (Stage II–III) After Concurrent Chemoradiotherapy using Biopsy Specimens

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Background: Recently, attention has been directed to concurrent chemoradiotherapy (CRT) for the treatment of squamous cell carcinoma of the esophagus with regard to efficacy, quality of life and functional preservation, and survival periods comparable to those after standard surgical therapy have been reported in responders to CRT. However, there are some non-responders to CRT, and the prediction of the outcome after CRT is an important subject for future studies. In this study, using biopsy specimens obtained before CRT, we evaluated the relationships between biological markers and the outcome after CRT in order to determine the prognostic factors of CRT.

Methods: The subjects were 51 patients (42 males and nine females: median age 68 years). who were histologically confirmed to have squamous cell carcinoma of the esophagus at stage II or III (UICC). Concurrent CRT consisting of chemotherapy using 5FU and CDDP and radiation therapy (60 Gy) was performed as the initial treatment, and the relationships of overexpression of EGFR, p53, VEGF, PCNA and CyclinD1 were examined immunohistochemically in biopsy specimens collected before treatment. Overall survival was estimated by multivariate analysis.

Results: The percentages of patients overexpressing p53, VEGF, PCNA, CyclinD1, and EGFR were 33, 31, 37, 31 and 29%, respectively. On multivariate analysis, T stage ($P = 0.0393$) and PCNA ($P = 0.0302$) were found to be significant prognostic factors.

Conclusions: PCNA overexpression appears to be a prognostic factor for squamous cell carcinoma of the esophagus after CRT.

Key words: esophageal squamous cell carcinoma – chemoradiotherapy – PCNA – overall survival

INTRODUCTION

The mortality rate due to esophageal cancer in Japan has remained unchanged in males and has gradually increased in females during the past 20 years, and deaths due to the disease account for 3.6% of all deaths from malignant neoplasms (1). In Japan, more than 90% of esophageal cancers are squamous cell carcinomas, which are relatively sensitive to chemotherapy and/or radiation therapy. However, esophageal cancer is likely to develop lymph node metastases and distant metastases at an early stage, and many patients are treated after the disease has advanced with a poor prognosis. Therefore, an improvement in the therapeutic results is a major clinical target. Presently, the standard treatment for esophageal cancer is surgical resection in Japan. Although the results of surgical treatments such as three-field lymphadenectomy are improving, the 5-year survival rate in all surgically treated patients between 1988 and 1997 (11 642 patients) was only 36.1%, and esophageal cancer remains a disease with poor prognosis (2–4). In the 1980s, chemoradiotherapy (CRT) was introduced for stage I–IV esophageal cancer primarily in Western countries (5), and a subsequent phase III trial (RTOG85-01 study) comparing the outcomes of T1-3/N0-1/M0 esophageal cancers between a chemoradiotherapy group and radiotherapy alone group concluded chemoradiotherapy to be the standard non-surgical treatment on the basis of a significantly longer survival period (6,7). Then, in the 1990s, the therapeutic results of CRT as a non-surgical treatment for esophageal cancer began to be reported from various institutions to attract attention. Ohtsu et al. (8) reported the clinical results in T4 and/or M1

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