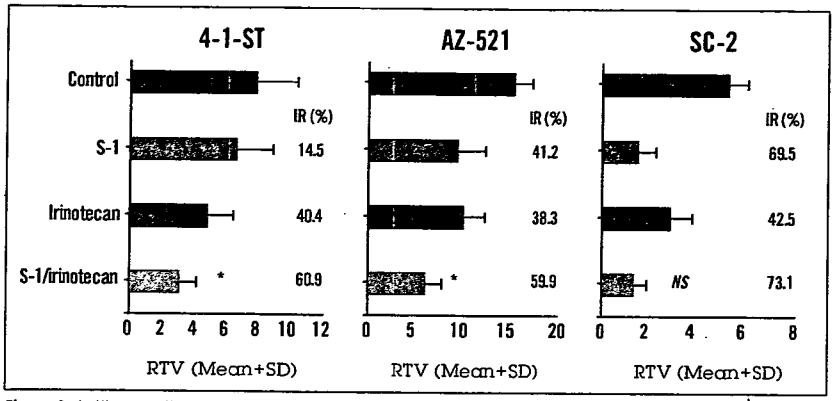

Figure 3. Antitumor effect of S-1, irinotecan, and both drugs combined in human gastric cancer xenografts in mice (n = 5 for each xenograft). S-1 8.3 mg/kg was orally administered once daily for 14 days, and irinotecan 40 mg/kg was injected weekly for 2 weeks to tumor-bearing mice starting 2 weeks after 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.**

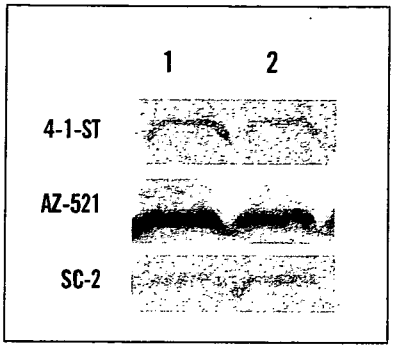

Figure 4. Expression of TS proteins in 3 human gastric cancer xenografts in mice treated with or without irinotecan. The TS aliquot (50 µg protein) was immunochemically analyzed using anti-TS polyclonal antibody by the Western blot method. The left lane shows nontreated tumors and the right lane shows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

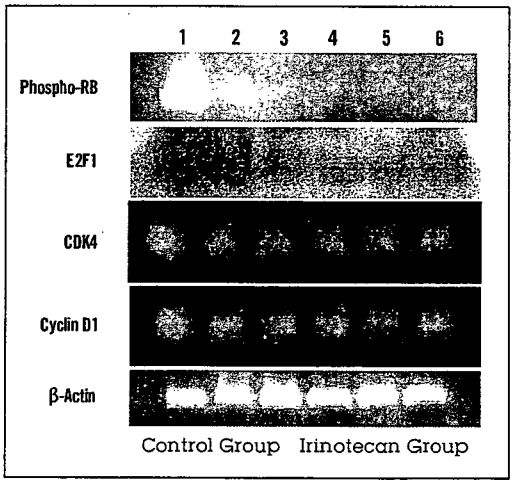

Figure 5. Expression of phospho-RB, E2F1, CDK4 and cyclin D1 proteins in AZ-521 tumors treated with or without irinotecan. Fifty microgram proteins of nuclear extracts from the tumors were immunochemically analyzed using anti-pRB, anti-E2F1, anti-CDK4, anti-cyclin D1, and anti-β-actin monoclonal antibodies,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

REFERENCES

1. Lenz HJ, Leichman CG, Danenberg KD, et al: Thymidylate synthase mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival. *J Clin Oncol* 14:176–182, 1996
2. Johnston PG, Lenz HJ, Leichman CG, et al: Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res* 55:1407–1412, 1995
3. Leichman L, Lenz HJ, Leichman CG, et al: Quantitation of intratumoral thymidylate synthase expression predicts for resistance to protracted infusion of 5-fluorouracil and weekly leucovorin in disseminated colorectal cancers: preliminary report from an ongoing trial. *Eur J Cancer* 31A:1306–1310, 1995
4. Leichman CG, Lenz HJ, Leichman L, et al: Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. *J Clin Oncol* 15:3223–3229, 1997
5. Beck A, Etienne MC, Cheradame S, et al: A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumour sensitivity to fluorouracil. *Eur J Cancer* 30A:1517–1522, 1994
6. Etienne MC, Cheadame S, Fischel JL, et al: Response to fluorouracil therapy in cancer patients: the role of tumoral dihydropyrimidine dehydrogenase activity. *J Clin Oncol* 13:1663–1670, 1995
7. Ishikawa Y, Kubota T, Otani Y, et al: Dihydropyrimidine dehydrogenase activity and messenger RNA level may be related to the antitumor effect of 5-fluorouracil on human tumor xenografts in nude mice. *Clin Cancer Res* 5:883–889, 1999
8. Kirihara Y, Yamamoto W, Toge T, et al: Dihydropyrimidine dehydrogenase, multidrug resistance-associated protein, and thymidylate synthase gene expression levels can predict 5-fluorouracil resistance in human gastrointestinal cancer cells. *Int J Oncol* 14:551–556, 1999
9. Salonga D, Danenberg KD, Johnson M, et al: Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. *Clin Cancer Res* 6:1322–1327, 2000
10. Metzger R, Leichman CG, Danenberg KD, et al: ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. *J Clin Oncol* 16:309–316, 1998
11. Metzger R, Danenberg K, Leichman CG, et al: High basal level gene expression of thymidine phosphorylase (platelet-derived endothelial cell growth factor) in colorectal tumors is associated with nonresponse to fluorouracil. *Clin Cancer Res* 4:2371–2376, 1998
12. Sakata Y, Ohtsu A, Horikoshi N, et al: Late phase II study of novel oral fluoropyrimidine anticancer drug S-1 (1M tegafur-0.4M gimestat-1M otastat potassium) in advanced gastric cancer patients. *Eur J Cancer* 34:1715–1720, 1998
13. Koizumi W, Kurihara M, Nakano S, et al: Phase II study of S-1, a novel oral derivative of 5-fluorouracil, in advanced gastric cancer. *Oncology* 58:191–197, 2000
14. Ohtsu A, Baba H, Sakata Y, et al: Phase II study of S-1, a novel oral fluoropyrimidine derivative, in patients with metastatic colorectal carcinoma. *Br J Cancer* 83:141–145, 2000
15. Ichikawa W, Uetake H, Nihei Z, et al: Topoisomerase I (Topo-I) expression correlates to thymidylate synthase (TS) expression in colorectal cancer (CRC). *Proc Am Soc Clin Oncol* 18:246a, 1999 (abstract 947)
16. Johnston PG, Danenberg KD, Gilmore PM, et al: Thymidylate synthase (TS) expression from formalin fixed paraffin embedded (FFPE) tissues using quantitative RT-PCR correlates with frozen tissue data and predicts for response to 5-fluorouracil (5-FU) in metastatic colorectal cancers. *Proc Am Soc Clin Oncol* 18:617a, 1999 (abstr 2383)
17. Takiuchi H, Narahara H, Tsujinaka T, et al: Phase I study of S-1 combined with irinotecan (CPT-11) in patients with advanced gastric cancer (OGSG 0002). *Jpn J Clin Oncol* 35:520–525, 2005
18. Ishikawa Y, Kubota T, Ohtani Y, et al: Dihydropyrimidine dehydrogenase activity and messenger RNA level may be related to the antitumor effect of 5-fluorouracil on human tumor xenografts in nude mice. Clin Cancer Res 5: 883-889, 1999
19. Yoshinare K, Kubota T, Watanabe M et al: Gene expression in colorectal cancer and in vitro chemosensitivity to 5-fluorouracil: a study of 88 surgical specimens. *Cancer Sci* 9:633–638, 2003
20. Okabe H, Tsujimoto H, Fukushima M: Preparation of the antibodies against recombinant human thymidylate synthase for the detection of its intratumoral levels and the application to sensitivity-study of 5-fluorouracil. *Oncol Rep* 4: 685–690, 1997
21. Spears CP, Shahinian AH, Moran RG: In vivo kinetics of thymidylate synthase inhibition in 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas. *Cancer Res* 42:450–456, 1982
22. Shirasaka T, Shimamoto Y, Fukushima M: Inhibition by oxonic acid of gastrointestinal toxicity of 5-fluorouracil without loss of its antitumor activity in rats. *Cancer Res* 53:4004–4009, 1993
23. Ikenaka K, Fukushima M, Nakamura H, et al: Metabolism of pyrimidine nucleotides in various tissues and tumor cells from rodents. *Gann* 72: 590–597, 1981
24. Maehara Y, Nakamura H, Nakane Y, et al: Activities of various enzymes of pyrimidine nucleotide and DNA synthesis in normal and neoplastic human tissues. *Gann* 73:289–298, 1982
25. Fukushima M, Fijioka A, Uchida J, et al: Thymidylate synthase (TS) and ribonucleotide reductase (RNR) may be involved in acquired resistance to 5-fluorouracil (5-FU) in human cancer xenografts in vivo. *Eur J Cancer* 37: 1681–1687, 2001

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.

REFERENCES

1. Koizumi W: Chemotherapy for advanced gastric cancer: review of global and Japanese status. *Gastrointestinal Cancer Res* 1:193–199, 2007
2. Vanhoefer U, Rougier P, Wilke H et al: Final results of a randomized phase III trial of sequential high-dose methotrexate, fluorouracil, and doxorubicin versus infusional fluorouracil and cisplatin in advanced gastric cancer: a trial of the European Organization for Research and Treatment of Cancer Gastrointestinal Tract Cooperative Group. *J Clin Oncol* 18: 2648–2657, 2000
3. Ohtsu A, Shimada Y, Shirao K, et al: Randomized phase III trial of fluorouracil alone versus fluorouracil plus cisplatin versus uracil and tegafur plus mitomycin in patients with advanced gastric cancer: JCOG study 9205. *J Clin Oncol* 21: 54–59, 2003
4. Van Cutsem E, Moiseyenko VM, Tjulandin S, et al: Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group. *J Clin Oncol* 24: 4991–4997, 2006
5. Ohtsu A, Yoshida S, Saijyo N: Disparities in gastric cancer chemotherapy between the East and West. *J Clin Oncol* 24: 2188–2196, 2006
6. Webb A, Cunningham D, Scarffe JR, et al: Randomized trial comparing epirubicin, cisplatin, and fluorouracil versus fluorouracil, doxorubicin, and methotrexate in advanced esophagogastric cancer. *J Clin Oncol* 15: 261–267, 1997
7. Ajani JA: Standard of care for gastric cancer based on meta-analysis? Treading on thin ice or it is very nice! *J Clin Oncol* 24: 5473–5474, 2006
8. Boku N, Ohtsu A, Shirao K, et al: Phase II study of a combination of irinotecan and cisplatin against metastatic gastric cancer. *J Clin Oncol* 17: 319–323, 1999
9. Koizumi W, Tanabe S, Saigenji K, et al: Phase I/II study of S-1 combined with cisplatin in patients with advanced gastric cancer: *Br J Cancer* 89: 2207–2212, 2003
10. Takiuchi H, Narahara H, Tsujinaka T, et al: Phase I study of S-1 combined with irinotecan (CPT-11) in patients with advanced gastric cancer. *Jap J Clin Oncol* 35: 520–525, 2005
11. Yamaguchi K, Shimamura T, Hyodo I, et al: Phase I/II study of docetaxel and S-1 in patients with advanced gastric cancer. *Br J Cancer* 94: 1803–1808, 2006
12. Fujitani K, Narahara H, Takiuchi H, et al: Phase I and pharmacokinetic study of S-1 combined with weekly paclitaxel in patients with advanced gastric cancer. *Oncology* 69:414–420, 2005

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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 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 $T_{3\text{-}4}$ esophageal SCC (Table 4).

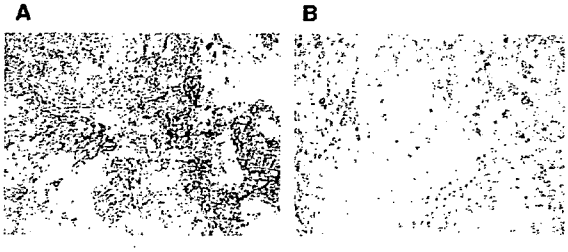

Figure 1. Representative immunohistochemical epidermal growth factor receptor (EGFR) stainings in biopsy specimens before chemoradiotherapy (CRT) A, EGFR-positive; EGFR immunostaining is seen in the cell membranes of tumor cells. B, EGFR-negative; Immunostaining is not seen in any cell membranes.

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.

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 $T_{3\text{-}4}M_0$ 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.

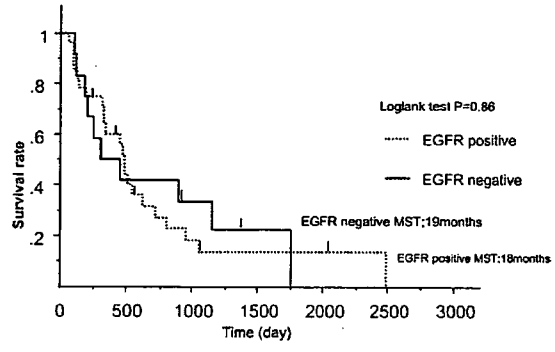

Figure 2. The Survival curves for 40 patients with T_{3-4} M_0 (M1 cases were excluded from the analysis of survival analysis) esophageal squamous cell carcinoma received CRT, according to EGFR. Overall survival is almost same both positive EGFR and negative EGFR. MST, median survival time.

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.

References

1. Roth JA, Putnam JB, Jr. Surgery for cancer of the esophagus. *Semin Oncol* 1994;21(4):453–61.
2. Brenner B, Ilson DH, Minsky BD. Treatment of localized esophageal cancer. *Semin Oncol* 2004;31(4):554–65.
3. Hironaka S, Ohtsu A, Boku N, Muto M, Nagashima F, Saito H, et al. Nonrandomized comparison between definitive chemoradiotherapy and radical surgery in patients with T(2–3)N(any) M(0) squamous cell carcinoma of the esophagus. *Int J Radiat Oncol Biol Phys* 2003;57(2):425–33.
4. Ohtsu A, Boku N, Muro K, Chin K, Muto M, Yoshida S, et al. Definitive chemoradiotherapy for T4 and/or M1 lymph node squamous cell carcinoma of the esophagus. *J Clin Oncol* 1999;17(9):2915–21.
5. Stahl M, Stuschke M, Lehmann N, Meyer HJ, Walz MK, Seeber S, et al. Chemoradiation with and without surgery in patients with locally advanced squamous cell carcinoma of the esophagus. *J Clin Oncol* 2005;23(10):2310–7.
6. Bedenne LPM, Bouche O, Triboulet J-P, Conroy T, Pezet D, Roullet B, Seitz J-F, Lacourt J, Milan C, Service d'Hepato, Gastroenterologie, Chu, Le Bocage, Dijon Cedex, France; CHU Charles-Nicolle, Rouen France; CHU Robert-Debre, Reims, France; CHU Claude-Huriez, Lille, France; Centre Alexis Vautrin, Nancy, France; CHU Hotel-Dieu, Clermont-Ferrand, France; CHU Dupuytren, Limoges, France; CHU la Timone, Marseille, France; Clinique Sainte-Marie, Chalon sur Saone, France; FFCD Statistical Center, Dijon, France. Randomized phase III trial in locally advanced esophageal cancer: radiochemotherapy followed by surgery versus radiochemotherapy alone (FFCD 9102). *Proc Am Soc Clin Oncol* 2002;21: 130a.
7. Bonnetain F, Bouche O, Michel P, Mariette C, Conroy T, Pezet D, et al. A comparative longitudinal quality of life study using the Spitzer quality of life index in a randomized multicenter phase III trial (FFCD 9102): chemoradiation followed by surgery compared with chemoradiation alone in locally advanced squamous resectable thoracic esophageal cancer. *Ann Oncol* 2006;17(5):827–34.
8. Ozawa S, Ueda M, Ando N, Shimizu N, Abe O. Prognostic significance of epidermal growth factor receptor in esophageal squamous cell carcinomas. *Cancer* 1989;63(11):2169–73.
9. Mukaida H, Toi M, Hirai T, Yamashita Y, Toge T. Clinical significance of the expression of epidermal growth factor and its receptor in esophageal cancer. *Cancer* 1991;68(1):142–8.
10. Itakura Y, Sasano H, Shiga C, Furukawa Y, Shiga K, Mori S, et al. Epidermal growth factor receptor overexpression in esophageal carcinoma. An immunohistochemical study correlated with clinicopathologic findings and DNA amplification. *Cancer* 1994;74(3):795–804.
11. Kitagawa Y, Ueda M, Ando N, Ozawa S, Shimizu N, Kitajima M. Further evidence for prognostic significance of epidermal growth factor receptor gene amplification in patients with esophageal squamous cell carcinoma. *Clin Cancer Res* 1996;2(5):909–14.
12. Tahara M, Ohtsu A, Hironaka S, Boku N, Ishikura S, Miyata Y, et al. Clinical impact of criteria for complete response (CR) of primary site to treatment of esophageal cancer. *Jpn J Clin Oncol* 2005;35(6):316–23.
13. Yano H, Shiozaki H, Kobayashi K, Yano T, Tahara H, Tamura S, et al. Immunohistologic detection of the epidermal growth factor receptor in human esophageal squamous cell carcinoma. *Cancer* 1991;67(1):91–8.
14. Gibault L, Metges JP, Conan-Charlet V, Lozac'h P, Robaszkiewicz M, Bessaguet C, et al. Diffuse EGFR staining is associated with reduced overall survival in locally advanced oesophageal squamous cell cancer. *Br J Cancer* 2005;93(1):107–15.
15. Boku N, Chin K, Hosokawa K, Ohtsu A, Tajiri H, Yoshida S, et al. Biological markers as a predictor for response and prognosis of unresectable gastric cancer patients treated with 5-fluorouracil and *cis*-platinum. *Clin Cancer Res* 1998;4(6):1469–74.
16. Samejima R, Kitajima Y, Yunotani S, Miyazaki K. Cyclin D1 is a possible predictor of sensitivity to chemoradiotherapy for esophageal squamous cell carcinoma. *Anticancer Res* 1999;19(6C):5515–21.
17. Jain S, Filipe MI, Hall PA, Waseem N, Lane DP, Levison DA. Prognostic value of proliferating cell nuclear antigen in gastric carcinoma. *J Clin Pathol* 1991;44(8):655–9.
18. Walsh TN, Noonan N, Hollywood D, Kelly A, Keeling N, Hennessy TP. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. *New Engl J Med* 1996;335(7):462–7.
19. Bosset JF, Gignoux M, Triboulet JP, Tiret E, Mantion G, Elias D, et al. Chemoradiotherapy followed by surgery compared with surgery alone in squamous-cell cancer of the esophagus. *New Engl J Med* 1997;337(3):161–7.
20. Urba SG, Orringer MB, Turrisi A, Iannettoni M, Forastiere A, Strawderman M. Randomized trial of preoperative chemoradiation versus surgery alone in patients with locoregional esophageal carcinoma. *J Clin Oncol* 2001;19(2):305–13.
21. Rohatgi P, Swisher SG, Correa AM, Wu TT, Liao Z, Komaki R, et al. Characterization of pathologic complete response after preoperative chemoradiotherapy in carcinoma of the esophagus and outcome after pathologic complete response. *Cancer* 2005;104(11):2365–72.
22. Chapman JD, Dugle DL, Reuvers AP, Meeker BE, Borsa J. Letter: Studies on the radiosensitizing effect of oxygen in Chinese hamster cells. *Int J Radiat Biol Relat Stud Phys Chem Med* 1974;26(4):383–9.

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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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lymph node locally advanced esophageal cancers (JCOG9516) as follows: complete response (CR) rate 15%, response rate 68.3%, median survival period 8.4 months and 2-year survival period 31.5%. These were comparable to those of conventional surgical treatments in similar cases (9–12). Hironaka et al. retrospectively compared the results of CRT and surgery alone in UICC-stage II or III (T4 excluded) patients and reported no difference in the 5-year survival rate between the two groups (46 versus 51%) (13). In the present decade, the results of comparative studies between definitive CRT and surgery have been reported from Europe. In France, a randomized phase III trial was performed by treating the responders with introductory CRT by continued definitive CRT or surgery. In Germany, a randomized comparative trial of introductory chemotherapy followed by CRT + surgery or definitive CRT was performed. In both trials, the survival rate did not differ significantly in the responders to the introductory chemotherapy or CRT regardless of whether they were subsequently treated by CRT or surgery (14,15).

Thus, as the reported results of CRT in patients with esophageal cancer were comparable to those of conventional surgical therapy, evaluation of prognostic factors in patients undergoing CRT as well as those undergoing surgery has become important in order to set out the therapeutic strategy. Various factors have been evaluated as possible prognostic factors of esophageal cancer, primarily in surgically treated patients: p53, bax and bcl-2 related to apoptosis, cyclinD1, P16, P21 and PCNA related to the cell cycle, and EGFR, TGF-α, HER-2neu and KI-67 related to growth regulation, VEGF related to angiogenesis, and ERCC1 related to DNA repair (16). Among them, the expressions of p53 (17), EGFR (18), PCNA (19,20) VEGF have been reported to be associated with poor outcomes after surgery alone (21) and the expression of CyclinD1 has been reported to be associated with poor outcomes after preoperative CRT (22,23).

In this study, we immnohistochemically examined biopsy specimens of stage II or III squamous cell carcinomas of the esophagus obtained before definitive CRT in order to find out if any prognostic factors that have been evaluated in surgical cases are overexpressed. We also retrospectively evaluated the relationships of their overexpression with the total survival time.

PATIENTS AND METHODS

Subjects

The subjects were 51 patients who underwent chemoradiotherapy at Osaka Medical College Hospital between July 1994 and July 2003 who fulfilled the following criteria: (1) those histologically confirmed to have squamous cell carcinoma of the esophagus; (2) those previously untreated for the disease; (3) those aged 80 years or less; (4) those in whom the disease was stage II or III according to the International Union against Cancer Tumor-node-metastasis (TNM) Classification, 6th edn, 2002; (5) those in a 0–2 Eastern Cooperative Oncology Group Performance status; (6) those who retained functions of major organs (bone marrow, heart, liver, and kidney); and (7) those who submitted informed consent.

Treatment Schedule

Chemotherapy consisted of the protracted infusion of 5-FU 400 mg/m^2/day on days 1–5 and 8–12 combined with CDDP 40 mg/m^2 with adequate hydration and antiemetic coverage on days 1 and 8. This schedule was repeated twice every 5 weeks. Radiation therapy using megavoltage X-rays was started on day 1 concomitantly with chemotherapy. The planned target volume for carcinoma of the upper or middle third esophagus included the primary tumor with a 3 cm margin craniocaudally, metastatic nodes with a 1–1.5 cm margin, supraclavicular fossa and mediastinum. For carcinoma of the lower third esophagus, the field was extended to include the perigastric nodes, and the supraclavicular fossa was excluded if the cervical nodes tested negative. When the planned volume included both the supraclavicular fossa and upper abdominal nodes, a daily dose of 2.0 Gy was allowed. A 2-week interval took place after a dose of 30 Gy. Radiation therapy was restarted on day 36 along with the same schedule of chemotherapy as before. The irradiation techniques used were anterior- and posterior-opposed equally weighted beams up to a dose of 40 Gy. Then, the radiation portals were changed to shield the spinal cord and to craniocaudally encompass the primary tumor with a 2–3 cm margin. Metastatic nodes were encompassed with a 1–1.5 cm margin. The radiation dose to the spinal cord was kept at a maximum of 50 Gy. The homogeneity of the dose within the planned target volume was within $\pm$10% of the prescribed dose. For patients treated with prophylactic filgrastim, a daily dose of 75 μg/total body was administered subcutaneously during the period between days 18 and 31. The treatment was discontinued when disease progression, patient refusal or delay of recovery from the toxicity in excess of 6 weeks from the initiation of the treatment occurred (8).

Clinical Response

Clinical responses were assessed by endoscopy, barium esophagogram and CT in accordance with the response criteria given by the Japan Society of Clinical Oncology: complete response (CR), the complete disappearance of clinical evidence of existing lesions for over 4 weeks; partial response (PR), a >50% reduction in the sum of the products of two perpendicular measurements taken of all measurable lesions lasting for over 4 weeks; no change (NC), change in tumor <50% over 4 weeks; progressive disease (PD), a >25% increase in the sum of the products of two perpendicular

measurements taken of an evaluable lesion or the appearance of new lesions (24).

Immunohistochemical Staining Methods

Pretreatment endoscopic biopsy specimens from 51 patients were assessed for p53, EGFR, cyclin D1, PCNA and VEGF 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-human p53 protein mouse monoclonal antibody (DO-7; DAKO, Glostrup, Denmark, dilution 1 : 50); 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, the sections were incubated in a microwave oven for 10 min three times, and incubated on 0.3% H_2O_2. Then these sections were incubated with the primary antibodies. After six rinses in phosphate-buffered saline (PBS), sections were incubated with the secondary biotinylated anti-human p53 protein mouse monoclonal antibody, 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 biotylinated anti-mouse–rabbit IgG gout immunoglobins and 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.

Method for evaluation of immunohistochemical results

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 (18,25,26). 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 (27). 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 (28). PCNA was calculated as the percentage of PCNA-positive cancer cells by counting more than 1000 cancer cells in more than three fields of a specimen with ×400 magnification microscopy without knowing any clinical information. For the endoscopic biopsy specimens, PCNA were counted at the site of the maximum number of positive nuclei in the whole tumor. Only strong nuclear staining was regarded as positive, and weak nuclear or cytoplasmic staining was regarded as negative (19,20,29). The PCNA index was the percentage of nuclei staining positive (30). A PCNA score greater than 40 was taken as PCNA-positive. Also, tumors in which positive nuclei were observed in 20% or more cells were considered to be over-expressing p53. The results of immunohistochemical staining were evaluated by two pathologists without being informed of endoscopic findings.

Statistical Analysis

The survival time was calculated from the date of treatment initiation to that of death from any cause or to the last date of confirmation of survival. We estimated survival curves using the Kaplan–Meier method and compared them with the log–rank test. Relative risks and their 95% confidence intervals (CIs) of chemoradiotherapy were estimated using the univariate Cox regression model adjusting for gender, age, performance status, tumor location, T stage, N stage, p53, EGFR, cyclin D1, PCNA and VEGF, and the multivariate Cox regression model adjusting for T stage, PCNA and VEGF. Statistical analyses were performed using Stat View software 5.0.

Statistical analysis concerning risk factors was performed by Student's *t*-test and the χ^2-test.

RESULTS

Characteristics of Patients and Results of Immunohistochemical Staining

The median age of the patients, comprising 42 males (82%) and nine females (18%), was 68 years (range 43–80 years). The performance status (PS) was 0/1 in 44 patients (84%), and the general condition was good in many patients. The location was the middle in 26 (50%). The T stage was T3/T4 in 40 (78%) and T1/T2 in 11 (22%). Lymph node metastasis was detected by CT or EUS in 39 (76%). The UICC stage was II in 18 and III in 33. The percentages of patients over-expressing various biological markers were 37% (19/51) for

PCNA, 33% (17/51) for p53, 31% (16/51) for cyclinD1, 29% (15/51) for EGFR and 31% (16/51) for VEGF. Clinical response was CR in 55% (28/51), PR in 31% (16/51), SD in 8% (4/51) and PD in 6% (3/51; Table 1). It was PR, SD or PD in 23 patients, of whom five underwent surgery, three gastrostomy, eight chemotherapy and seven best supportive care. Of these patients, PCNA was positive in 48% (11/23) and the T stage was T3/4 in 91% (21/23).

Table 1. Patient characteristics

Factor		Number of patients	%
Age	Range 43–80 (median)(68)		
Sex			
	Male	42	82
	Female	9	18
PS			
	0,1	44	84
	2	7	16
Location			
	Upper	12	24
	Middle	26	51
	Lower	13	25
Primary tumor			
	T1/T2	11	22
	T3/T4	40	78
Regional lymph nodes			
	N0	12	24
	N1	39	76
Stage	II	18	35
	III	33	65
PCNA	High expression	19	37
	Low expression	32	63
p53	High expression	17	33
	Low expression	34	67
CyclinD1	High expression	16	32
	Low expression	35	68
EGFR	High expression	15	29
	Low expression	36	71
VEGF	High expression	16	31
	Low expression	35	69
Clinical response			
	CR	28	55
	PR	16	31
	SD	4	8
	PD	3	6

All patients ($n = 51$). PS, performance status; PCNA, proliferating cell nuclear antigen; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Overall Survival

The median survival time (MST) in all 51 patients with clinical stage II or III squamous cell carcinoma of the esophagus who underwent CRT was 553 days. The MST in clinical stage II patients was 807 days, and that in clinical stage III patients was 495 days ($P = 0.1313$), with no significant difference, but it was 'not reached' in T1/T2 patients and 485 days ($P = 0.0125$) in T3/T4 patients, with a significant difference. Concerning biological markers, the MSTs of patients with low and high VEGF expression were 669 and 352 days ($P = 0.0474$), and those of patients with low and high PCNA expression were 766 and 491 days ($P = 0.0045$), respectively, with significant differences (Figs 1 and 2). The MSTs of patients with low and high EGFR expression were 776 and 553 days ($P = 0.9326$), those of patients with low and high cyclinD1 expression were 553 and 669 days ($P = 0.7275$), and those of patients with low and high p53 expression were 491 and 669 days ($P = 0.9368$), respectively; no significant difference was observed.

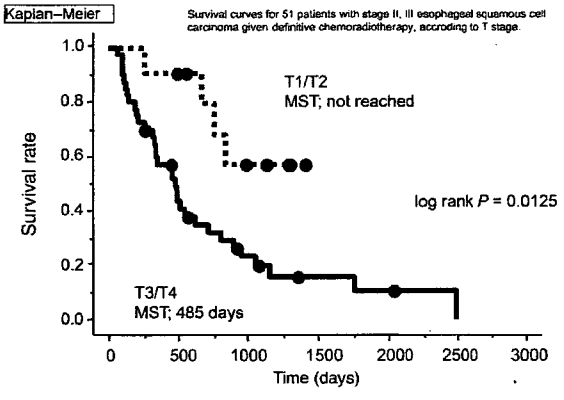

Figure 1. Overall survival according to tumor.

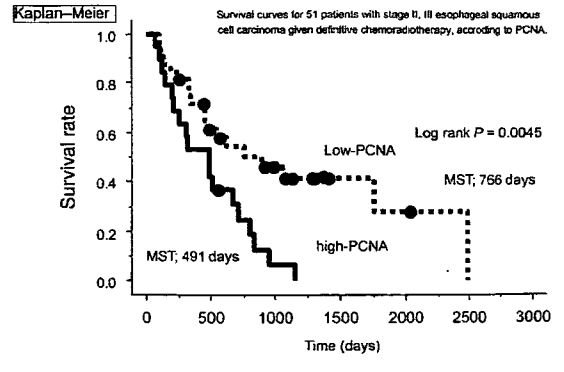

Figure 2. Overall survival according to proliferating cell nuclear antigen.

Univariate Analysis: the Cox Proportional Hazards Model

On univariate analyses, no difference was observed in the outcome according to sex, PS, location, N stage or clinical stage, but, according to the T stage, the outcome was better in T1/T2 patients than in T3/T4 patients ($P = 0.0190$, relative risk = 0.286, 95% CI = 0.101–0.814; Table 2). Concerning the biological markers, the outcome was better in the low VEGF expression group than the high expression group ($P = 0.0515$) and in the low PCNA expression group than in the high expression group ($P = 0.0060$; Table 3).

Multivariate Analysis: yhe Cox Proportional Hazards Model

Multivariate analysis was performed using the T stage and PCNA, which showed significant differences on univariate analysis, and VEGF, which showed a slight change on univariate analysis. Among the T stage, PCNA and VEGF, T stage and PCNA ($P = 0.0302$, relative risk = 0.438, 95% CI = 0.208–0.924) were independent prognostic factors (Table 4).

Relationships of Biological Markers and Clinical Response with Various Factors

Concerning the relationships of PCNA with various clinical factors, eight patients (8/19, 42%) showed high PCNA expression and were positive for lymph node metastasis and 28 (28/32, 87%) showed low PCNA expression and were positive for lymph node metastasis; a significant correlation ($P = 0.0160$) was observed between PCNA and lymph node metastasis. Immunohistochemically, the expression of both PCNA and p53 was high in 10 (10/19, 53%) and low in 25 (25/32, 78%), indicating a correlation between PCNA and p53 ($P = 0.0243$). No correlation was noted between PCNA and the T stage. p53, EGFR, VEGF, PCNA or CyclineD1 showed no correlation with clinical response. Similarly, no correlation was noted between the T stage and clinical response.

Table 2. Relative risk and 95% CIs from univariate analysis

		Univariate		
		Relative risk	95% CI	*P*
Sex	male : female	0.657	0.286–1.508	0.3213
PS	0/1 : 2	0.670	0.234–1.917	0.4555
Location				
	Upper (reference)	1.00		
	Middle	0.661	0.296–1.474	0.7418
	Lower	1.152	0.486–2.729	0.4653
Tumor				
	T1/T2 : T3/T4	0.286	0.101–0.814	0.0190
Lymph nodes	N0 : N1	1.060	0.479–2.343	0.8864
Stage	II : III	0.571	0.274–1.193	0.1363

Univariate analysis for 51 patients with stage II, III esophageal squamous cell carcinoma given definitive chemoradiotherapy, according to clinical factors. CI, confidence interval.

Table 3. Relative risk and 95% CIs from univariate analysis

		Univariate		
		Relative risk	95% CI	*P*
EGFR				
	Low expression:high expression	1.031	0.506–2.101	0.9326
Cycline D1				
	Low expression:high expression	0.883	0.438–1.778	0.7277
P53				
	Low expression:high expression	0.972	0.484–1.952	0.9368
VEGF				
	Low expression:high expression	0.506	0.255–1.005	0.0515
PCNA				
	Low expression:high expression	0.387	0.197–0.762	0.0060

Univariate analysis for 51 patients with stage II, III esophageal squamous cell carcinoma given definitive chemoradiotherapy, according to molecular factors.

DISCUSSION

As therapeutic results similar to those by surgical treatment were reported to have been obtained by definitive CRT in esophageal cancer (8,13), it has become of importance to examine prognostic factors in patients undergoing CRT as well as those undergoing surgery to evaluate the therapeutic strategies against the disease. In this study, we evaluated the relationships between clinical and immunohistochemical biological markers and the outcome in patients with stage II or III squamous cell carcinoma of the esophagus who underwent definitive CRT alone as the initial treatment. The

Table 4. Multivariate analysis of the tumor, VEGF, PCNA for overall survival

	Multivariate		
	Relative risk	95% CI	*P*
T	0.322	0.110–0.946	0.0393
VEGF	0.903	0.417–1.956	0.7957
PCNA	0.438	0.208–0.924	0.0302

Multivariate analysis of 51 patients with stage II, III esophageal squamous cell carcinoma given definitive chemoradiotherapy, according to T stage, VEGF, PCNA.

relationship between the results of surgery and biological markers have already been evaluated, and the outcome of surgery alone has been reported to be poor in those expressing p53 (17). EGFR (18) and VEGF (21). The outcome of preoperative CRT was reported to be poor in those expressing cyclinD1 (22,23). Concerning patients showing high PCNA expression, Kinugasa et al. (20) reported that the outcome after surgery alone was poor, and Yasunaga et al. (19) reported that the outcomes after surgery alone and preoperative chemotherapy + surgery were poor. Also, Okuno et al. (31) reported that the outcome after radiation therapy alone was poor, and Hickey et al. (29) reported that the outcome of preoperative CRT + surgery was poor, in patients showing high PCNA expression. However, there has not been a report on the relationship between PCNA and the prognosis in patients with squamous cell carcinoma of the esophagus who underwent definitive CRT alone. In this study the outcome after definitive CRT was favorable in patients showing low PCNA expression, indicating that the T stage and PCNA were independent prognostic factors.

In definitive CRT for advanced esophageal cancer, the DNA of cancer cells is considered to be damaged by radiation and chemotherapy (5-FU/CDDP), p53 to be expressed, apoptosis to be induced by p53, and p21, which binds to PCNA, to be induced to protract the G1 period. The tumor suppression gene p53 induces apoptosis and regulation of the cell cycle by positively or negatively adjusting the expression of many genes as a transcription factor and causing arrests in the cell cycle in response to DNA damage. Also, PCNA is involved in DNA repair and replication as well as exhibiting other gene control functions and acts as a binding mechanism of other proteins requiring interactions with DNA. PCNA and p53 are considered to be interrelated in the cell cycle and to be associated with each other in the proliferation of cancer cells. In this study, a correlation ($P = 0.0243$) was observed between the expression of PCNA and that of p53. It has been reported that squamous cell carcinomas positive for p53 often show high PCNA expression (32). Since the prognosis is poor in patients showing high PCNA expression, our results are considered to be biologically plausible. While the PCNA expression has been related to the outcome of squamous cell carcinoma of the esophagus, Kinugawa et al. (20), who studied the outcomes of patients after surgery alone, reported a correlation between the T stage and PCNA, but Okuno et al. (31), who performed radiotherapy alone, reported no correlation between PCNA and the T stage. In this study, PCNA was an independent prognostic factor after definitive CRT, but no correlation was observed between PCNA and the T stage. Also, an inverse correlation was observed between PCNA and lymph node metastasis. This result may be explained by the fact that the N stage was determined not pathologically but clinically. However, the number of samples analyzed was small in this study, and our results need to be confirmed by increasing the number of patients.

In conclusion, the outcome was better in patients with low PCNA expression than those with high PCNA expression, indicating that the expression of PCNA affects the total survival time. Also from previous reports, the outcome is considered to be favorable in low PCNA expression patients by either surgery or definitive CRT. In contrast, the outcome of high PCNA expression patients is presently poor by surgery alone, preoperative CRT + surgery, or CRT alone, so that the development of new therapies, particularly the advent of new agents, is awaited.

Conflict of interest statement

None declared.

References

1. Fujita H. Present status of esophageal cancer and its treatment in Japan. *Ann Thorac Cardiovasc Surg* 2004;10(3):135–9.
2. Isono K, Sato H, Nakayama K. Results of a nationwide study on the three-field lymph node dissection of esophageal cancer. *Oncology* 1991;48(5):411–20.
3. Kato H, Watanabe H, Tachimori Y, Iizuka T. Evaluation of neck lymph node dissection for thoracic esophageal carcinoma. *Ann Thorac Surg* 1991;51(6):931–5.
4. Kato H. Lymph node dissection for thoracic esophageal carcinoma. Two- and 3-field lymph node dissection. *Ann Chir Gynaecol* 1995;84(2):193–9.
5. Coia LR, Engstrom PF, Paul A. Nonsurgical management of esophageal cancer: report of a study of combined radiotherapy and chemotherapy. *J Clin Oncol* 1987;5(11):1783–90.
6. Cooper JS, Guo MD, Herskovic A, Macdonald JS, Martenson JA, Jr, Al-Sarraf M, et al. Chemoradiotherapy of locally advanced esophageal cancer: long-term follow-up of a prospective randomized trial (RTOG 85-01). Radiation Therapy Oncology Group. *JAMA* 1999;281(17):1623–7.
7. Al-Sarraf M, Pajak TF, Marcial VA, Mowry P, Cooper JS, Stetz J, et al. Concurrent radiotherapy and chemotherapy with cisplatin in inoperable squamous cell carcinoma of the head and neck. An RTOG Study. *Cancer* 1987;59(2):259–65.
8. Ohtsu A, Boku N, Muro K, Chin K, Muto M, Yoshida S, et al. Definitive chemoradiotherapy for T4 and/or M1 lymph node squamous cell carcinoma of the esophagus. *J Clin Oncol* 1999;17(9):2915–21.
9. Roth JA, Putnam JB, Jr. Surgery for cancer of the esophagus. *Semin Oncol* 1994;21(4):453–61.
10. Baba M, Aikou T, Yoshinaka H, Natsugoe S, Fukumoto T, Shimazu H, et al. Long-term results of subtotal esophagectomy with three-field lymphadenectomy for carcinoma of the thoracic esophagus. *Ann Surg* 1994;219(3):310–6.
11. Fujita H, Kakegawa T, Yamana H, Shima I, Toh Y, Tomita Y, et al. Mortality and morbidity rates, postoperative course, quality of life, and prognosis after extended radical lymphadenectomy for esophageal cancer. Comparison of three-field lymphadenectomy with two-field lymphadenectomy. *Ann Surg* 1995;222(5):654–62.
12. Kato H, Tachimori Y, Watanabe H, Iizuka T. Evaluation of the new (1987) TNM classification for thoracic esophageal tumors. *Int J Cancer* 1993;53(2):220–3.
13. Hironaka S, Ohtsu A, Boku N, Muto M, Nagashima F, Saito H, et al. Nonrandomized comparison between definitive chemoradiotherapy and radical surgery in patients with T(2–3)N(any) M(0) squamous cell carcinoma of the esophagus. *Int J Radiat Oncol Biol Phys* 2003;57(2):425–33.
14. Bonnetain F, Bouche O, Michel P, Mariette C, Conroy T, Pezet D, et al. A comparative longitudinal quality of life study using the Spitzer quality of life index in a randomized multicenter phase III trial (FFCD 9102): chemoradiation followed by surgery compared with chemoradiation alone in locally advanced squamous resectable thoracic esophageal cancer. *Ann Oncol* 2006;17(5):827–34.

15. Stahl M, Stuschke M, Lehmann N, Meyer HJ, Walz MK, Seeber S, et al. Chemoradiation with and without surgery in patients with locally advanced squamous cell carcinoma of the esophagus. *J Clin Oncol* 2005;23(10):2310–7.
16. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100(1):57–70.
17. Aloia TA, Harpole DH, Jr., Reed CE, Allegra C, Moore MB, Herndon JE, 2nd, et al. Tumor marker expression is predictive of survival in patients with esophageal cancer. *Ann Thorac Surg* 2001;72(3):859–66.
18. Gibault L, Metges JP, Conan-Charlet V, Lozac'h P, Robaszkiewicz M, Bessaguet C, et al. Diffuse EGFR staining is associated with reduced overall survival in locally advanced oesophageal squamous cell cancer. *Br J Cancer* 2005;93(1):107–15.
19. Yasunaga M, Tabira Y, Kondo K, Okuma T, Kitamura N. The prognostic significance of cell cycle markers in esophageal cancer after neoadjuvant chemotherapy. *Dis Esophagus* 1999;12(2):120–7.
20. Kinugasa S, Tachibana M, Hishikawa Y, Abe S, Yoshimura H, Monden N, et al. Prognostic significance of proliferating cell nuclear antigen (PCNA) in squamous cell carcinoma of the esophagus. *Jpn J Clin Oncol* 1996;26(6):405–10.
21. Shih CH, Ozawa S, Ando N, Ueda M, Kitajima M. Vascular endothelial growth factor expression predicts outcome and lymph node metastasis in squamous cell carcinoma of the esophagus. *Clin Cancer Res* 2000;6(3):1161–8.
22. Samejima R, Kitajima Y, Yunotani S, Miyazaki K. Cyclin D1 is a possible predictor of sensitivity to chemoradiotherapy for esophageal squamous cell carcinoma. *Anticancer Res* 1999;19(6C):5515–21.
23. Itami A, Shimada Y, Watanabe G, Imamura M. Prognostic value of p27(Kip1) and CyclinD1 expression in esophageal cancer. *Oncology* 1999;57(4):311–7.
24. Hayashi K, Ando N, Watanabe H, Ide H, Nagai K, Aoyama N, et al. Phase II evaluation of protracted infusion of cisplatin and 5-fluorouracil in advanced squamous cell carcinoma of the esophagus: a Japan Esophageal Oncology Group (JEOG) Trial (JCOG9407). *Jpn J Clin Oncol* 2001;31(9):419–23.
25. Yano H, Shiozaki H, Kobayashi K, Yano T, Tahara H, Tamura S, et al. Immunohistologic detection of the epidermal growth factor receptor in human esophageal squamous cell carcinoma. *Cancer* 1991;67(1):91–8.
26. Itakura Y, Sasano H, Shiga C, Furukawa Y, Shiga K, Mori S, et al. Epidermal growth factor receptor overexpression in esophageal carcinoma. An immunohistochemical study correlated with clinicopathologic findings and DNA amplification. *Cancer* 1994;74(3):795–804.
27. Boku N, Chin K, Hosokawa K, Ohtsu A, Tajiri H, Yoshida S, et al. Biological markers as a predictor for response and prognosis of unresectable gastric cancer patients treated with 5-fluorouracil and cis-platinum. *Clin Cancer Res* 1998;4(6):1469–74.
28. Samejima R, Kitajima Y, Yunotani S, Miyazaki K. Cyclin D1 is a possible predictor of sensitivity to chemoradiotherapy for esophageal squamous cell carcinoma. *Anticancer Res* 1999;19(6C):5515–21.
29. Hickey K, Grehan D, Reid IM, O'Briain S, Walsh TN, Hennessy TP. Expression of epidermal growth factor receptor and proliferating cell nuclear antigen predicts response of esophageal squamous cell carcinoma to chemoradiotherapy. *Cancer* 1994;74(6):1693–8.
30. Jain S, Filipe MI, Hall PA, Waseem N, Lane DP, Levison DA. Prognostic value of proliferating cell nuclear antigen in gastric carcinoma. *J Clin Pathol* 1991;44(8):655–9.
31. Okuno Y, Nishimura Y, Kashu I, Ono K, Hiraoka M. Prognostic values of proliferating cell nuclear antigen (PCNA) and Ki-67 for radiotherapy of oesophageal squamous cell carcinomas. *Br J Cancer* 1999;80(3–4):387–95.
32. Goukon Y, Sasano H, Nishihira T, Nagura H, Mori S. p53 overexpression in human esophageal carcinoma: a correlation with tumor DNA ploidy and two parameter flow cytometric study. *Anticancer Res* 1994;14(3B):1305–12.

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Phase I/II Study of CPT-11 plus UFT in Patients with Advanced/Recurrent Colorectal Cancer: Osaka Gastrointestinal Cancer Chemotherapy Study Group (OGSG): Protocol 0102

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Objective: The primary objective of this study was to explore the efficacy and safety of combined chemotherapy with CPT-11 and UFT in patients with advanced/metastatic colorectal cancer.
Methods: Twenty-two patients with metastatic colorectal cancer were enrolled in the phase I trial and 35 patients (including eight patients treated at level 4 during phase I) were evaluated in the phase II trial. Treatment consisted of two 35-day cycles of combination chemotherapy with CPT-11 and UFT. During phase I, CPT-11 was administered on days 1 and 15 as an intravenous infusion over 90 min at four different dose levels, starting from a dose of 80 mg/m^2 (level 1). During phase II, the dose of CPT-11 was fixed at 150 mg/m^2 based on the results of the phase I study. UFT was administered orally at a fixed dose of 300 mg/m^2 on days 1–28, followed by a 1-week drug holiday, during each course (35 days).
Results: The maximum tolerated dose (MTD) of CPT-11 was determined to be 150 mg/m^2 during the phase I trial. The major toxicities detected during phase II in 35 patients receiving CPT-11 at this recommended dose were grade 3/4 neutropenia in nine patients (25.7%) and grade 3/4 anorexia in six patients (11.4%). No severe adverse events occurred. The overall response rate and the median overall survival time was 22.9% (8/35) and 23.9 months for all patients, respectively. For pre-treated patients they were 26.3% (5/19) and 25.1 months, respectively.
Conclusion: This combination of CPT-11 and UFT is considered to be both feasible and relatively safe. The response rate of the patients receiving CPT-11 at a dose of 150 mg/m^2 was comparable to that reported previously for 5-FU-based regimens coupled with CPT-11, and this regimen can probably be beneficial for patients with pre-treated advanced colorectal cancer on an outpatient basis.

Key words: colorectal cancer – chemotherapy – CPT-11 – UFT – oral fluoropyrimidine

INTRODUCTION

The 5-fluoropyrimidines have been key drugs in the treatment of metastatic colorectal cancer for over 50 years (1). With respect to the inhibition of thymidylate synthase (TS), which accounts for the major antitumor effect of 5-fluorouracil (5-FU), numerous studies on the combined administration of 5-FU and leucovorin (5-FU/LV) had been performed and a 5-FU/LV regimen was established as international standard chemotherapy for patients with advanced colorectal cancer in the 1990s (2–5). However, it has not necessarily contributed to prolongation of survival although combination with LV increased response rate (6).

More recently, newer drugs like irinotecan (CPT-11) and oxaliplatin have become available and are expected to contribute to an increase of therapeutic efficacy by combined use with 5-FU. CPT-11, a potent topoisomerase I inhibitor, is a derivative of camptothecin that was developed in Japan (7). It has been shown to be effective for various malignancies, including lung cancer, cervical cancer, ovarian cancer,

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breast cancer and malignant lymphoma, as well as for gastrointestinal tumors such as stomach cancer or colorectal cancer. The response rate to CPT-11 monotherapy as first-line or second-line treatment for colorectal cancer has been reported to be 15–32% (8–13). CPT-11 has also shown activity against 5-FU-resistant colorectal cancer (14,15). The efficacy of CPT-11 in combination with 5-FU (bolus administration or continuous infusion) and leucovorin was examined in several large-scale studies and finally the combination of CPT-11/5-FU/LV was established as first-line chemotherapy for advanced colorectal cancer (16,17). However, intravenous administration of 5-FU and leucovorin, especially by continuous infusion that has been shown to be most effective, is somewhat complex and inconvenient as outpatient therapy. If an alternative to continuous infusion of 5-FU could be developed with the same efficacy, it would be more convenient and beneficial for patients with colorectal cancer.

It is interesting to note in this context that evidence has been accumulating that various oral fluoropyrimidines, including tegafur/uracil (UFT), capecitabine and TS-1, may be as effective as intravenous 5-FU (18–20). Besides intravenous administration of 5-FU, oral 5-FU and its derivatives have long been used to treat cancer in Asian countries, including Japan. Despite previous criticism of the employment of oral fluoropyrimidines as a substitute for intravenous administration of 5-FU, especially in Western countries, the clinical usefulness of these oral drugs have been re-evaluated since the mid 1990s. Among several oral 5-FU derivatives, tegafur/uracil (UFT; Taiho Pharmaceutical Co. Ltd., Tokyo, Japan) is a combined drug that contains tegafur and uracil at a molar ratio of 1:4. It has been widely used in Japan, where it has been demonstrated that UFT at doses of 300–600 mg/day is well tolerated and shows activity against various solid tumors (18). UFT was reported to have the same AUC as equimolar intravenous 5-FU and shows similar pharmacokinetics to those obtained with continuous infusion of 5-FU (21). This is considered to be due to the gradual conversion of UFT into 5-FU and inhibition of the 5-FU degrading enzyme, dihydropyrimidine dehydrogenase (DPD), by the uracil component of UFT (22). Because of these unique characteristics as a DPD-inhibitory fluoropyrimidine, UFT has been expected to become a substitute for intravenous 5-FU in various regimens. Ohtsu et al. performed a phase II study of combination of CPT-11 and infusional 5-FU without LV, and reported promising results with a response rate of 45% and lower toxicity (23). The Spanish TTD group reported that infusional 5-FU plus oxaliplatin without LV (FUFOX) was effective and well tolerated (24). Moreover, oral LV was not commercially available for colorectal cancer treatment in Japan at that time. Therefore, we designed this study to determine the maximum tolerated dose (MTD) of CPT-11 and to explore the preliminary therapeutic efficacy of a combination of CPT-11 and UFT in patients with advanced colorectal cancer. If CPT-11/UFT was as effective as CPT-11/5-FU/LV, while causing less toxicity, it could be better tolerated as first-line or second-line chemotherapy for colorectal cancer, especially when performed on an outpatient basis.

PATIENTS AND METHODS

Eligibility

Patients enrolled in this study were required to have histologically proven adenocarcinoma of the colon or rectum that was considered to be inoperable and to have at least one measurable metastasis (RECIST criteria). Patients also had to be older than 18 years and aged under 75 years, be expected to survive for more than 3 months after starting chemotherapy, have a performance status of 0–1 on the Eastern Cooperative Oncology Study Group (ECOG) scale, and have no problems with oral intake.

Other eligibility criteria included a white blood cell count of 4000–12 000/mm^3, a neutrophil count >2000/mm^3, a platelet count >100 000/mm^3, a hemoglobin >8.9 g/dl, AST and ALT <2.5 times the institutional upper limit of normal (ULN) total bilirubin <1.5 mg/dl, and creatinine < the ULN.

Exclusion criteria included the following: previous CPT-11 treatment; concomitant treatment with other chemotherapy agents or radiation within the previous 2 weeks or failure to recover from adverse effects; interstitial pneumonia or pulmonary fibrosis causing chest X-ray changes or symptoms (or a history of these diseases); a fluid collection in a body cavity that needed treatment; concurrent active cancer originating from a site other than the colorectum or metachronous cancer that was untreated or had a disease free period <5 years (except carcinoma *in situ* or surgically treated skin cancer); infectious disease or intestinal paresis or obstruction; watery diarrhea; poorly controlled diabetes mellitus; uncontrolled medical conditions such as cardiac failure, hepatic failure, or renal failure; symptomatic brain metastasis; actual or potential pregnancy, breast-feeding status, or the intention to become pregnant in the near future; a past history of serious drug allergy; or any other condition that was judged to make the patient ineligible for this study by the responsible physician.

Pretreatment Evaluation and Dose Modification

Pretreatment evaluation included obtaining detailed medical history, performing physical examination and performing standard laboratory tests, including hematology (leucocyte and absolute neutrophil counts, platelet count and hemoglobin) and biochemistry (sodium, potassium, chloride, blood urea nitrogen, creatinine, alkaline phosphatase, total bilirubin, AST and ALT).

The criteria for starting day 1 of the first course were the eligibility criteria above. The criteria for administration of CPT-11 on day 15 of each course included a white blood cell count >3000/mm^3, a platelet count >100 000/mm^3, absence of fever (>38°C) caused by infection, no diarrhea