[Frontiers in Bioscience 16, 1036-1043, January 1, 2011]

TS gene tandem repeats in esophageal cancer patients receiving chemoradiotherapy

Kazuhiro Kaneko[1,2], Maho Nagai[2], Yoshitaka Murakami[3], Mari Kogo[4], Tsunehiro Oyama[5], Takashi Kojima[1], Atsushi Ohtsu[1], Michio Imawari[2]

[1]Department of Gastroenterology and Gastrointestinal Oncology, National Cancer Center Hospital East, Chiba, 277-8577, Japan, [2]Department of Gastroenterology, Showa University School of Medicine, Tokyo, 142-8666, Japan, [3]Department of Health Science, Shiga University of Medical Science, Shiga, 520-2192, Japan, [4]Promotion Center of Pharmaceutical Education, Showa University School of Pharmaceutical Sciences, Tokyo, 142-8666, Japan, [5]Department of Environmental Health, University of Occupational and Environmental Health, Kitakyushu, 807-8555, Japan

TABLE OF CONTENTS

1. ABSTRACT

5-Fluorouracil (5-FU) interferes with tumor-cell proliferation by inhibiting thymidylate synthase (TS). We examined the relationship between tandem repeat (TR) variations in the TS gene and survival following concurrent chemoradiotherapy in patients with esophageal squamous cell carcinoma (ESCC). TS-TR variations were analyzed in 57 stage II-IV ESCC patients undergoing chemoradiotherapy combined with 5-FU and cisplatinum (CDDP), and in 106 controls. Pretreatment non-neoplastic biopsy specimens from ESCC patients and lymphocytes from controls were used for analysis. Variations were identified by the size of DNA fragments amplified by polymerase chain reaction. Two to five TRs were found in Japanese individuals. TR3 homozygotes were predominant in 74% of ESCC patients and 61% of controls. Three-year survival rates were significantly longer in patients with TR2/2 or TR2/3 genotypes (38%) than in patients with TR3/3, 3/4, or 3/5 genotypes (9%; p=0.011). In the Cox proportional hazard model, the TR2/2 or TR2/3 genotypes were the only independent predictor for survival (Hazard ratio, 2.647; 95% confidence interval, 1.271-5.513). The TS-TR variations exert an important influence on survival following chemoradiotherapy in ESCC patients.

2. INTRODUCTION

Standard chemoradiotherapy (CRT) combined with 5-FU and CDDP has the potential to cure locally advanced ESCC, including T4 stage disease (1,2). Previous reports on CRT as a definitive and preoperative treatment have indicated various advantages in managing carcinoma of the esophagus (1-4). One strategy to improve the outcome of patients treated with CRT is to select treatment responders or long-term survivors for further directed therapy. Therefore, greater understanding of the molecular basis of ESCC may ultimately lead to improvements in therapy and patient outcomes. In particular, molecular biomarkers of tumor behavior are potentially powerful new tools for predicting outcome.

5-FU the most important antineoplastic drug in standard chemotherapeutic ESCC regimens, principally works by interfering with the proliferation of cancer cells via inhibition of TS activity. TS is an enzyme that catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), a component of DNA. After 5-FU is metabolized to FdUMP in cells, FdUMP binds to TS in the presence of folate cofactor (5,10-CH2FH4). This inhibits TS activity and dTMP production.

Relationships between the expression of TS and the response to 5-FU treatment or prognosis have been demonstrated (5,6). These studies played crucial roles in outlining the acquisition of resistance to 5-FU that can develop in some patients and that can be avoided with combination of other chemotherapeutic agents including radiation. TS expression has been demonstrated to be related to better prognostic outcome in various organ cancer-patients treated with chemotherapy containing 5-FU (7-11). In contrast, the TS gene also contains a genetic polymorphism that may also be involved in the efficacy of 5-FU-based chemotherapy. It is a 28-bp tandem repeat sequence within the 5'-untranslated region, and the vast majority of individuals show one of the following three genotypes; two tandem repeats (2R/2R), three tandem repeats (3R/3R) or a heterozygous (2R/3R) genotype (12). This tandem repeat seems to function as an enhancer element since *in vitro* studies have shown that stepwise increase of TS mRNA expression and TS enzyme activity are associated with increasing number of tandem repeat sequences. In most of these studies, however, surgical resections were added after chemotherapy, or multimodal therapies utilizing different treatment regimens were administered (5-11). Thus, there have been no investigations into the association between TR variations in the TS gene and the prognosis in ESCC patients using concurrently definitive CRT with 5-FU continuous infusion. To evaluate whether TS TR variations are prognostic biomarkers, one important step is that patients who received treatment of a single regimen should be evaluated.

In the present study, we examined the long-term relationship between TS-TR variations and overall survival in order to determine whether TR variations predict the prognosis in ESCC patients receiving concurrent CRT.

3. PATIENTS AND METHODS

3.1. Patient selection and sample collection

A total of 110 ESCC patients received CRT between May 1996 and March 2002. Fifty seven patients met the following criteria and were included in the study: (a) sufficient biopsy specimens obtainable before treatment; (b) no previous treatment had been received; (c) age ≤ 75 years; (d) PS on the Eastern Cooperative *Oncology* Group (ECOG) scale ≤ 2; (e) adequate bone marrow, hepatic, and renal functions; and (f) stage II to IVA on the International Union against Cancer (UICC) tumor-node-metastasis (TNM) classification. Patients with distant organ metastasis (stage IVB) were excluded.

Control subjects visiting our hospital for a health checkup who did not have active malignant disease, and who had not received treatment for malignancy were enrolled in the study based on the following criteria. Male and female individuals, age ≤ 75 years, with an ECOG performance status of 'zero', and no symptoms of dysphagia, abdominal pain, chest and/or back pain, or vomiting regarding ESCC.

Non-neoplastic esophageal tissue from 57 patients with ESCC and lymphocytes from 106 control subjects were used for TR analysis. The Human Ethical Review Committee in our hospital approved this study. Informed consent was obtained from all subjects.

3.2. Treatment schedule

Chemotherapy consisted of protracted infusion of 5-FU at a dose of 400 mg/m2/day on days 1-5 and 8-12, combined with 2-hour infusion of CDDP at 40 mg/m2 on days 1 and 8. A 10 MV radiation treatment was administered for 3 weeks (5 days/week) at 2 Gy/day, concomitantly with chemotherapy. The targeted area for carcinoma of the upper and middle thirds of the esophagus included the primary tumors with a 3-cm margin craniocaudally and any metastatic nodes with 1- to 1.5-cm margin, in the supra-clavicular fossa and mediastinum. For carcinoma of the lower third of the esophagus, the field was extended to include the perigastric nodes, while the supra-clavicular fossa was excluded if the cervical nodes were found to be negative. The daily fractional dose of radiotherapy was 2 Gy administered 5 days a week. When the planned volume included both the supra-clavicular fossa and upper abdominal nodes, a daily dose of 1.8 Gy was allowed. After a dose of 30 Gy, we allowed a 2-week treatment-free period. Radiotherapy was restarted on day 36, along with the same schedule of chemotherapy as described above. The treatment course included 3 weeks of radiotherapy followed by a 2-week break, and the course was repeated twice, with a total radiation dose of 60 Gy. The irradiation techniques were initially applied in anterior and posterior opposed fields. At 40 Gy, the radiation portals were reduced to shield the spinal cord and to encompass the primary tumor craniocaudally with a 2- to 3-cm margin, usually by using an oblique opposed field. Metastatic nodes were encompassed with a 1- to 1.5-cm margin. The total radiation dose to the spinal cord was kept at a maximum of 40 Gy. The homogeneity of the dose within the planning volume was within 10% of the prescribed dose.

Patients who were evaluated for an objective response to the above treatment received additional chemotherapy consisting of a continuous infusion of 5-FU at a dose of 800 mg/m2 on days 1-5 and CDDP at a dose of 80 mg/m2 on day 1. This treatment schedule was administered for 1-week followed by a 3-week break and was only repeated once in some patients with no further treatment applied if no disease progression was observed. When a single course consisted of treatment followed by > 5-week break, we defined the latter as interruption. All patients receiving CRT were monitored by neck, chest and abdominal computed tomography (CT), and endoscopy every 8 weeks.

3.3. Evaluation of Response

Overall responses to treatment were assessed according to World Health Organization (WHO) criteria. The response was evaluated by endoscopy, and neck, chest and abdominal CT scans during each course.

3.4. DNA extraction and determination of TR number in TS

Non-neoplastic esophageal tissues removed endoscopically from ESCC patients before treatment and

Table 1. Characteristics of patients and controls

	ESCC patients	Controls
Mean age (year)	63	61
Range (year)	46-75	40-75
Gender (male/female)	53/4	58/48
PS (0/1)	43/10	106/0
Location*		
Upper	5	
Middle	30	
Lower	22	
Histological type		
Well differentiated	15	
Moderately differentiated	39	
Poorly differentiated	3	
Stage (UICC)		
II	13	
III	29	
IV	15	
T23/T4	29/28	
M0/M1	42/15	

ESCC, esophageal squamous cell carcinoma; PS, performance status; *, Location of the tumor according to the TNM classification; UICC, International Union Against Cancer

lymphocytes from control subjects were used for analysis. All samples were stored at -80°C until use. DNA from tissues and lymphocytes was extracted using proteinase K and a phenol-chloroform method. The TR number in the 5' noncoding region of TS was determined using a polymerase chain reaction (PCR) according to a previously described protocol (12). Briefly, the amplification reaction contained 100 ng DNA, 1μM each of the forward and reverse primers, and PfuTurbo DNA polymerase (Stratagene, USA) containing 10% dimethyl sulfoxide. The forward and reverse primers used for the analysis were 5'-GTGGCTCCTGCGTTTCCCCC-3' and 5'-CCAAGCTTGGTCGAGCCGGCCACACAGGCATG-3', respectively. After 30 cycles of reactions (95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min), the amplified products were separated electrophoretically on a 2% agarose gel, and the bands visualized with ethidium bromide. The TR number was calculated from the length of each PCR product. To confirm the TR number, we also determined the DNA sequences of the amplified fragments.

3.5. Statistical analysis

Survival time was measured from the initiation of the first course of treatment to the date of death or to the final date of confirmation of survival. Follow-up evaluations after CRT were performed every 3 months for the first 2 years and every 6 months thereafter by endoscopy and CT scan.

The chi-square test and Fisher's exact test were used to determine any association between any of the clinical covariates. Analysis for survival was assessed according to log-rank tests. The influence of each biological variable on patient survival was assessed by the Cox proportional hazards model. P-values of less than 0.05 were considered significant.

4. RESULTS

4.1. Patient Characteristics

Clinicopathological features of the patients and control subjects in this study are shown in Table 1. Of the 57 ESCC patients, 13 were stage II, 29 were stage III, and 15 were stage IVA. In terms of M stage, 42 patients had M0 disease, and 15 patients had M1 LYM disease. There were no patients with distant organ metastasis. Furthermore, 28 patients had T4 disease. Clinically involved sites in the 28 cases with T4 disease were thoracic aorta (18 patients), tracheobronchial tree (10 patients).

Fifty-five patients (96%) completed at least the CRT segment with a total radiation dose of 60 Gy. The remaining 2 patients did not complete CRT; 1 experienced disease progression, and 1 died due to treatment-related esophagoaortic fistula. Eleven patients (19%) received one additional course of only chemotherapy, and 34 patients (60%), 5 patients (9%), and 3 patients (5%) received an additional two, three, and four courses, respectively.

At the end of the follow-up period, 7 of the remaining 57 patients (12.3%) were still alive. The follow-up time for all 57 patients ranged from 2 to 108 months (median: 22 months).

The control subjects had no symptoms of dysphagia, abdominal pain, chest and/or back pain, or vomiting. They had no active malignant disease, and had not received treatment for malignancy.

4.2. TR number in TS in control subjects and ESCC patients

TR number, determined by the lengths of PCR products, ranged from two to five in controls and ESCC patients. Band patterns for a TR2 homozygote, a TR3 homozygote, and TR2/TR3, TR3/TR5, and TR2/TR5 heterozygotes as examples of gel electrophoresis (Figure 1), and the observed allele frequencies of the TR variants in ESCC patients are summarized in Table 2. TR3 was the most frequent allele in both ESCC patients and controls, and TR3 homozygosity occurred in 74% of ESCC patients and 61% of controls. The frequency of each TR variant did not differ significantly between these two groups

Table 2. Frequencies of TS TR polymorphisms

Genotype	ESCC n=57	Controls n=106	p-value
2/2	2 (4%)	2 (2%)	p=0.125*
2/3	11 (18%)	33 (31%)	
3/3	42 (74%)	65 (61%)	
3/4	1 (2%)	1 (1%)	
2/5	0 (0%)	1 (1%)	
3/5	1 (2%)	4 (4%)	

TS, thymidylate synthase; TR, tandem repeat; ESCC, esophageal squamous cell carcinoma; *, p-value between TR variation with TR2/2 or TR2/3 and TR3/3 or more repeats.

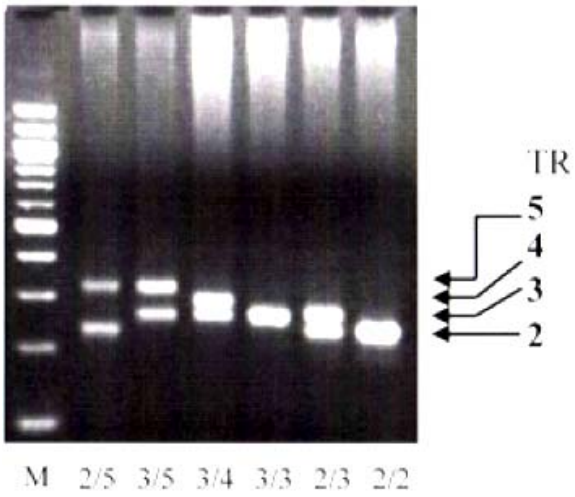

Figure 1. Variations of thymidylate synthase (TS) tandem repeat (TR) number. Typical patterns of amplified DNA fragments showing two to five repeats, a 100-bp ladder is used as a size marker (M).

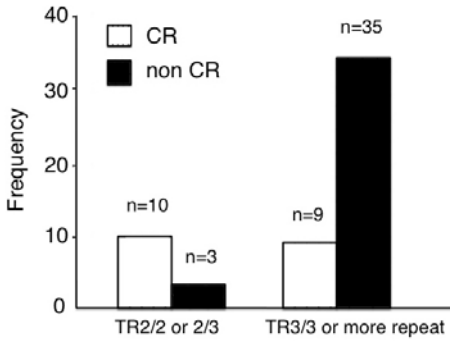

Figure 2. Correlation between TS TR polymorphisms and response. Patients with TR2/2 or TR2/3 genotypes, 10 of 13 (77%) attained CR, whereas only 9 of 44 (20%) patients with TR3 or more repeats attained CR ($p<0.01$, Fisher's exact test).

(p=0.856). In this study, abnormal variations, such as TR3/TR5 or TR2/TR5 heterozygotes, were found in Japanese ESCC patients and controls. In contrast, the frequency of TR2 homozygotes was very low in Japanese individuals with 4% in ESCC patients and 2% in controls.

4.3. TS TR variation and survival

The relationship between TR variations and the clinical stages of ESCCs was analyzed. We examined the occurrence of genotypes with at least one TR2 variant, including TR2/2 and TR2/3, and of TR3/3 or more highly repeated genotypes, because the expression of TS from TS genes containing the TR2 variant is lower than the expression from alleles containing TR3 or more repeats. TR2/2 and TR2/3 genotypes occurred in 23% of ESCC patients and in 33% of controls. TR genotypes did not differ significantly between ESCC patients and controls (p=0.173, chi-square test; Table 2). Furthermore, TR2/2 and TR2/3 genotypes occurred in 13–29% of ESCC patients, in 15% of stage II, in 28% of stage III, and in 13% of stage IVA. TR genotypes did not differ significantly between ESCC patients displaying different tumor stages.

The relationship between TR variations and response to treatment in ESCC patients was also analyzed. The TS TR genotype was the important predictor of response. Among ESCC patients with TR2/2 or TR2/3 genotypes, 10 of 13 (77%) attained CR, whereas only 9 of 44 (20%) patients with TR3 or more repeats attained CR ($p<0.01$, Fisher's exact test; Figure 2). Furthermore, the patients with TR3/4 or TR3/5 genotypes did not attain CR, with resistance for 5-FU.

Three-year survival rates in patients with TR2/2 or TR2/3 genotypes and in patients with TR3/3 or more highly repeated genotypes were 38% and 9%, respectively. Significant difference in 3-year survival was seen between the two groups (p=0.011, chi-square analysis), while the overall survival rate of patients with TR2 variation was not significantly different from that of patients with TR3 variation (p=0.08, log-rank test; Figure 3). In contrast, survival was analyzed using the Cox proportional hazards model to determine whether any of the clinical covariates of age, gender, T factor, M factor, and TS-TR variations predicted survival (Table 3). Although 49% of patients were T4 stage, and 26% were M1 stage, Cox proportional hazard model indicated that only the TR2 variation were independent predictors for survival (Hazard ratio (HR), 2.647; 95% confidence interval (CI), 1.271-5.513; Table 3).

5. DISCUSSION

We examined the relationship between variations in the number of 28-base-pair TRs in the 5' non-coding exon of the TS gene and survival in ESCC patients receiving concurrent CRT combined with 5-FU plus CDDP. The Cox proportional hazard model identified TS gene polymorphisms as independent predictive markers for survival in ESCC patients. Furthermore, ESCC patients with TR2 variant proved to be more sensitive to 5-fluorouracil-cisplatinum CRT than those with only TR3 or more highly repeated alleles. Other reports have also demonstrated that patients with carcinomas in various organs who carried the TR2 variant were more sensitive to 5-FU-based chemotherapy than patients with only TR3 alleles (13-16). Although TR3 has been reported to be the major TS variant worldwide, its frequency differs among

Table 3. Multivariate Cox proportional hazard model

Variable	Hazard ratio	95% CI
Age	0.974	0.939-1.009
Gender	0.780	0.380-1.603
T factors	1.366	0.791-2.359
M factors	1.360	0.736-2.515
TS TR	2.647	1.271-5.513

95% CI, 95% confidence interval; TS TR, thymidylate synthase (TS) tandem repeat (TR) polymorphisms. Age, gender, T factor, M factor, and TS were included in a multivariate Cox proportional hazards model. For each variable, < 65 years, male gender, T4 stage, M1 stage (only M1 lymph node except for distant organ metastasis), and TR3 or more repeats in TS gene were set as reference levels.

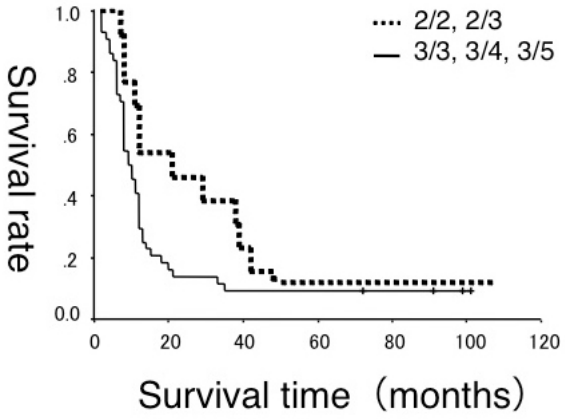

Figure 3. Three-year survival rates in patients with TR2/2 or TR2/3 genotypes (38%) were significantly different from those in patients with TR3/3 or more highly repeated genotypes (9%; p=0.011, chi-square analysis). Survival curves of 13 patients with the variant genotypes of 2/2 or 2/3 and 44 patients with the variant genotypes of 3/3 or more repeats (p=0.08, log-rank test).

ethnic populations. For example, the TR3 variant occurs in 40–50% of native Africans and Caucasians, but in 60–80% of Chinese. In contrast, the TR2 variant is more frequently observed in Caucasians than in other populations, and the frequency of the TR2/2 genotype is approximately 50% in Caucasians (17). Conversely, the TR4, TR5, and TR9 alleles are occasionally found among Chinese and native Africans, while expression of TS containing TR5 or TR9 is essentially the same as the expression of the TR3-containing allele (18,19). Our findings confirmed that the TR3 variant was the most abundant allele, occurring in 67% of Japanese control subjects, a proportion similar to that found in the Chinese population. Furthermore, we detected two to five repeats among ESCC patients and controls. Thus, since large differences in TS-TR variations are seen among different races, these may reflect differences in response or survival for 5-FU based treatments among Caucasians, Asians, and native Africans. We demonstrated that no significant difference was apparent in the allele frequencies of the TR variants in the ESCC patients and controls. We suggested that TR variants were not related to occurrence of ESCC.

Relationships between TS expression of mRNA and TS-TR variations have been reported in basic research. Kaneda *et al.* concluded that the first and the second TR elements upstream of the third element have an inhibitory effect on the translation efficiency of TS mRNA and that the third element has an activator effect (20). The inhibitory effect of the first and the second elements on the translation efficiency of TS mRNA may result from formation of a stem-loop structure by association of the inverted repeated sequence CGCCGCG within the triple TR sequence. In 1995, Horie *et al.* performed a similar study, examining the role of the triple TR in the expression activity of the TS gene. They constructed deletion mutants of this region and examined expression activity using another transient expression assay. They concluded that the three sequence mutant clones had a higher efficiency of expression than did the clones with mutations in the first and second sequences. Therefore, polymorphisms of the triple TR contributed to the efficiency of expression of the TS gene (12). Thus, TS expression is lower from genes containing the TR2 variation than those containing the TR3 variation. Based on these results, a clinical study demonstrated the importance of TS gene polymorphisms (21). Results from this study emphasized that the TS polymorphism status can be a predictive biomarker in colorectal cancer patients receiving monotherapy with the oral fluoropyrimidine prodrug S-1. In both the 5-FU-based treatment and S-1 monotherapy, it appears that TS-TR variations strongly identify prognosis in colorectal cancer patients. Since this study used S-1 monotherapy, we believe that differences in TS polymorphisms are directly reflected in prognosis.

In contrast, it is uncertain whether TS polymorphisms are related to survival in ESCC patients receiving 5FU-based CRT. The concurrent 5FU-based CRT has become a standard regimen in ESCC, not only because of the clinical outcome but also because of the synergism between 5FU and its radiosensitising effects (22-24). There have been some studies regarding relationships between response or survival and TS gene polymorphisms or expression in patients receiving CRT. Two reports identified that TS repetitive-sequence polymorphisms were predictive for tumor downstaging in rectal cancer (14), and that TS gene expression was a predictive marker for survival in esophageal cancer (11). Concurrent CRT was performed using a 5-FU continuous infusion in these two reports. In contrast, another report demonstrated that no prognostic significance of TS gene polymorphisms in ESCC patients (25). In this report, 5-FU was not concurrently administered with radiation, and 5FU was administered by bolus infusion before radiation periods. However, our study regimen used both concurrent CRT and continuous infusion of 5-FU. There is a possibility that radiosensitising effects are enhanced for concurrent CRT with 5-FU continuous infusion. This is the first report that TS-TR variations are identified as predictive biomarkers in cases receiving concurrent 5FU-based CRT.

In the past few years, studies investigating prognosis and overexpression of TS protein using immunohistochemical staining have been increasing. Some studies reported that expression of TS protein was a

significant prognostic factor (7,8), and others reported that expression of TS protein was not related to prognosis (26-28). It seems that relationships between expression of TS protein using immunohistochemical staining and prognosis remain controversial. Limitations associated with immunohistochemical staining are attributable to its semiquantitative nature, tissue aging effects, the staining technique, and the enzyme antibody used, as well as interobserver variation. These points may explain the lack of association between enzyme expression and chemotherapeutic response or survival.

In our previous report, to identify a prognostic marker, p53 mutations were analyzed in pretreatment biopsy specimens removed from ESCC patients who received definitive CRT (29). Multivariate analysis demonstrated that survival was strongly related to the T stage, rather than the presence of the p53 mutation, and that T4 was associated with a poor prognosis. In contrast, T4 patients were found in approximately 50% of stage II-IV patients in our present study, the Cox proportional hazard model showed that the TR2/2 or TR2/3 genotypes were the only independent predictors for survival among clinical covariates including T stage. One strategy to improve the outcome of patients treated with CRT is to select long-term survivors for further directed therapy. We believe that the detection of molecular biomarkers for ESCC will lead to improvements in patient outcomes and suggest that TS-TR variations provide good molecular biomarkers in ESCC.

Some studies of a continuous irradiation course combined with 5-FU plus CDDP indicated that grade 3 and higher leukopenia and esophagitis occurred in 33-54% and 48-50% of patients, respectively (30,31). It is likely that severe leukopenia and esophagitis frequently occur when a course of continuous irradiation is administered. The presence of severe toxicity because of both the extended field of irradiation and a combination of CRT had been expected. Since the periods of recovery from toxicity were not sufficient for a 1-week break in many patients, we used a split course radiation technique with a 2-week treatment-free period rather than using a continuous irradiation course as described in previous studies (3,4). Therefore, we believed that at least a 2-week break would be required to administer CRT without an interruption. Our results suggested that definitive CRT with a split course radiation technique was feasible for locally advanced ESCC.

In our present study, patients with TR2 variation were more likely to be long-term survivors following CRT combined with 5-FU plus CDDP. We emphasize that this treatment was concurrently definitive rather than multimodal CRT, with long-term follow-up of at least 5-years. Furthermore, 55 patients (96%) at least completed the CRT segment, with a total radiation dose of 60 Gy. We were therefore able to investigate valid relationships between TS-TR variations and survival in ESCC patients. In contrast, the incidence of TR2/2 occurred in approximately 50% of Caucasians, while the TR2/2 was found in only 2% to 4% of Japanese individuals in our study. Thus we believe that our investigation between TR2/2 and survival was limited.

In conclusion, TS-TR variation was analyzed in pretreatment biopsy specimens removed from ESCC patients. The present results show that TS-TR variation influenced the survival of ESCC patients following definitive CRT containing 5-FU plus CDDP. Furthermore, our findings confirmed that the TR3 variant was the most abundant allele, with a very low frequency of the TR2/2 genotype in Japanese patients. Although TS-TR variation is just one of many clinical covariates, this variation proves to be an independent biomarker for predicting survival.

6. ACKNOWLEDGMENTS

This work was supported by a Research Grant on the Association between risk of upper aero-digestive tract cancer and alcohol-metabolizing enzymes, and its clinical significance (to K.K.), grant number 20-10.

7. REFERENCES

1. L.R. Coia. Chemoradioation as primary management of esophageal cancer. *Semin Oncol*, 21,483-92 (1994)

2. A.A. Forastiere, M.B. Orringer, C. Perez-Tamayo, S.G. Urba & M. Zahurak. Preoperative chemoradiation followed by trans-hiatal esophagectomy for carcinoma of the esophagus: final report. *J Clin Oncol*, 11,1118-23 (1993)

3. A. Ohtsu, N. Boku, K. Muro, K. Chin, M. Muto, S. Yoshida, M. Satake, S. Ishikura, T. Ogino, Y. Miyata, S. Seki, K. Kaneko & A. Nakamura. Definitive chemoradiotherapy for T4 and/or M1 lymph node squamous cell carcinoma of the esophagus. *J Clin Oncol*, 17,2915-21 (1999)

4. K. Kaneko, H. Ito, K. Konishi, T. Kurahashi, T. Ito, A. Katagiri, T. Yamamoto, T. Kitahara, Y. Mizutani, A. Ohtsu & K. Mitamura. Definitive chemoradiotherapy for patients with malignant stricture due to T3 or T4 squamous cell carcinoma of the oesophagus. *Br J Cancer*, 88,18-24 (2003)

5. P.G. Johnston, H.J. Lenz, C.G. Leichman, K.D. Danenberg, C.J. Allegra, P.V. Danenberg & L. Leichman. Thymidylate syntheses gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res*, 55,1407-12 (1995)

6. N.A. Wong, L. Brett, M. Stewart, A. Leitch, D.B. Longley, M.G. Dunlop, P.G. Johnston, A.M. Lessells & D.I. Jodrell. Nuclear thymidylate syntheses expression, p53 expression and 5FU response in colorectal carcinoma. *Br J Cancer*, 85,1937-43 (2001)

7. M. Ciaparrone, M. Quirino, G. Schinzari, G. Zannoni, D.C. Corsi, F.M. Vecchio, A. Cassano, G. La Torre & C. Barone. Predictive role of thymidylate synthase, dihydropyrimidine dehydrogenase and thymidine phosphorylase expression in colorectal cancer patients receiving adjuvant 5-fluorouracil. *Oncology*, 70,366-77 (2006)

8. R. Soong, N. Shah, M. Salto-Tellez, B.C. Tai, R.A. Soo, H.C. Han, S.S. Ng, W.L. Tan, N. Zeps, D. Joseph, R.B. Diasio & B. Iacopetta. Prognostic significance of thymidylate synthase, dihydropyrimidine dehydrogenase and thymidine phosphorylase protein expression in colorectal cancer patients treated with or without 5-fluorouracil-based chemotherapy. *Ann Oncol*, 19,915-9 (2008)

9. F.V. Negri, N. Campanini, R. Camisa, F. Pucci, S. Bui, G. Ceccon, R. Martinelli, M. Fumagalli, P.L. Losardo, P. Crafa, C. Bordi, S. Cascinu & A. Ardizzoni. Biological predictive factors in rectal cancer treated with preoperative radiotherapy or radiochemotherapy. *Br J Cancer*, 98,143–7 (2008)

10. D. Hua, Z.H. Huang, Y. Mao & J.Z. Deng. Thymidylate synthase and thymidine phosphorylase gene expression as predictive parameters for the efficacy of 5-fluorouracil-based adjuvant chemotherapy for gastric cancer. *World J Gastroenterol,* 13,5030-4 (2007)

11. M.M. Joshi, Y. Shirota, K.D. Danenberg, D.H. Conlon, D.S. Salonga, J.E. Herndon II, P.V. Danenberg & D.H. Harpole Jr. High Gene Expression of TS1, GSTP1, and ERCC1 Are Risk Factors for Survival in Patients Treated with Trimodality Therapy for Esophageal Cancer. Clin *Cancer Res*,11,2215–21 (2005)

12. N. Horie, H. Aiba, K. Oguro, H. Hojo & K. Takeishi. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate syntheses. *Cell Struct Funct*, 3,191-7 (1995)

13. B. Iacopetta, F. Grieu, D. Joseph & H. Elsaleh. A polymorphism in the enhancer region of the thymidylate syntheses promoter influences the survival of colorectal cancer patients treated with 5-fluorouracil. *Br J Cancer*, 85,827-30 (2001)

14. B.E. Villafranca, Y. Okruzhnov, M.A. Dominguez, J. García-Foncillas, I. Azinovic, E. Martı´nez, J.J. Illarramendi, F. Arias, R.M. Monge, E. Salgado, S. Angeletti & A. Brugarolas. Polymorphisms of the Repeated Sequences in the Enhancer Region of the Thymidylate Synthase Gene Promoter May Predict Down staging After Preoperative Chemoradiation in Rectal Cancer. *J Clin Oncol*, 19,1779-86 (2001)

15. S.T. Pullarkat, J. Stoehlmacher, V. Ghaderi, Y.P. Xiong, S.A. Ingles, A. Sherrod, R. Warren, D. Tsao-Wei, S. Groshen & H.J. Lenz. Thymidylate syntheses gene polymorphism determines response and toxicity of 5-FU chemotherapy. *Pharmacogenomics J*, 1,65-70 (2001)

16. D. Edler, B. Glimelius, M. Hallstrom, A. Jakobsen, P.G. Johnston, I. Magnusson, P. Ragnhammar & H. Blomgren. Thymidylate syntheses expression in colorectal cancer: a prognostic and predictive marker of benefit from adjuvant fluorouracil-based chemotherapy. *J Clin Oncol*, 20,1721-8 (2002)

17. S. Marsh, E.S. Collie-Duguid, T. Li, X. Liu & H.L. McLeod. Protein, Nucleotide Ethnic variation in the thymidylate syntheses enhancer region polymorphism among Caucasian and Asian populations. *Genomics*, 58,310-2 (1999)

18. H.R. Luo, X.M. Lu, Y.G. Yao, N. Horie, K. Takeishi, L.B. Jorde & Y.P. Zhang. Length polymorphism of thymidylate syntheses regulatory region in Chinese populations and evolution of the novel alleles. Biochem Genet, 1-2,41-51 (2002)

19. S. Marsh, M.M. Ameyaw, J. Githang'a, A. Indalo, D. Ofori-Adjei & H.L. McLeod. Novel thymidylate syntheses enhancer region alleles in African populations. *Hum Mutat*,16,528 (2000)

20. S. Kaneda, K. Takeishi, D. Ayusawa, K. Shimizu, T. Seno & S. Altman. Role in translation of a triple tandemly repeated sequence in the 5'-untranslated region of human thymidylate synthase mRNA. *Nucleic Acids Res*, 15,1259-70 (1987)

21. K. Uchida, K. Hayashi, K. Kawakami, S. Schneider, J.M. Yochim, H. Kuramochi, K. Takasaki, K.D. Danenberg & P.V. Danenberg. Loss of Heterozygosity at the Thymidylate Synthase (TS) Locus on Chromosome 18 Affects Tumor Response and Survival in Individuals Heterozygous for a 28-bp Polymorphism in the TS Gene. Clin *Cancer Res*, 10,433-9 (2004)

22. E.B. Douple & R.C. Richmond. A review interaction between platinum coordination complexes and ionizing radiation: implications for cancer therapy. In Cisplatin: Current Status and New Developments, Prestayko AW, Crooke ST, Karter SK (eds) 125-147. Orland, FL: Academic Press (1980)

23. K.J. Scanlon, Y.L. Newman & D.G. Priest. Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. *Proc Natl Acad Sci USA*, 83,8923-5 (1986)

24. J.E. Byfield. Combined modality infusional chemotherapy with radiation. In: Cancer Chemotherapy by Infusion, Lokich JJ (ed) 2nd edn, pp 521-551. Chicago, IL (1990)

25. M Sarbia, M Stahl, C von Weyhern, G Weirich and F Pu¨hringer-Oppermann. The prognostic significance of genetic polymorphisms (Methylenetetrahydrofolate Reductase C677T, Methionine Synthase A2756G, Thymidilate Synthase tandem repeat polymorphism) in multimodally treated oesophageal squamous cell carcinoma. *Br J Cancer*, 94,203-7 (2006)

26. S.A. Jensen, B. Vainer & J.B. Sørensen. The prognostic significance of thymidylate synthase and dihydropyrimidine dehydrogenase in colorectal cancer of 303 patients adjuvantly treated with 5-fluorouracil. *Int J Cancer*, 120,694-701 (2006)

27. R. Bendardaf, A. Elzagheid, H. Lamlum, A. Algars, E. Korkeila, R. Ristamäki, Y. Collan, K. Syrjänen & S. Pyrhönen. Thymidylate synthase expression in primary colorectal tumours is correlated with its expression in metastases. *Scand J Gastroenterol,* 42,471-6 (2007)

28. H.C. Kwon, M.S. Roh, S.Y. Oh, S.H. Kim, M.C. Kim, J.S. Kim & H.J. Kim. Prognostic value of expression of ERCC1, thymidylate synthase, and glutathione S-transferase P1 for 5-fluorouracil/oxaliplatin chemotherapy in advanced gastric cancer. *Ann Oncol*, 18,504-9 (2007)

29. T. Ito, K. Kaneko, R. Makino, H. Ito, K. Konishi, T. Kurahashi, T. Kitahara & K. Mitamura. Prognostic value of p53 mutations in patients with locally advanced esophageal carcinoma treated with definitive chemoradiotherapy. *J Gastroenterol*, 36,303-11 (2001)

30. A. Herskovic, K. Martz, M. as-Sarraf, L. Leichman, J. Brindle, V. Vaitkevicius, J. Cooper, R. Byhardt, L. Davis & B. Emami. Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. *N Engl J Med,* 11,1593-8 (1992)

31. E.A. Poplin, P.S. Khanuja, M.J. Kraut, A.M. Hersskovic, P.B. Lattin, G. Cummings, L.E. Gasper, J.L. Kinzie, Z. Steiger & V.K. Vaitkevicous. Chemoradiotherapy of esophageal carcinoma. *Cancer*, 74,1217-24 (1994)

Abbreviations: chemoradiotherapy (CRT); cisplatinum (CDDP); confidence interval (CI); computed tomography (CT); deoxyuridine monophosphate (dUMP); deoxythymidine monophosphate (dTMP); Eastern Cooperative *Oncology* Group (ECOG); esophageal squamous cell carcinoma (ESCC); 5-Fluorouracil (5-FU); folate cofactor (5,10-CH2FH4); Hazard ratio (HR); International Union against Cancer (UICC); polymerase chain reaction (PCR); tandem repeats (TRs); thymidylate synthase (TS); tumor-node-metastasis (TNM); World Health Organization (WHO)

Key Words: thymidylate synthase, 5-Fluorouracil, esophageal squamous cell carcinoma, DNA polymorphism

Send correspondence to: Kazuhiro Kaneko, Division of Digestive Endoscopy and Gastrointestinal *Oncology*, National Cancer Center Hospital East, Kashiwanoha 6-5-1, Kashiwa, Chiba 277-8577, Japan, Tel: 81-4-7133-1111, Fax: 81-4-7134-6928, E-mail: kkaneko@east.ncc.go.jp