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• *BRIEF REPORTS* •

*p*53 negativity, CDC25B positivity, and metallothionein negativity are predictors of a response of esophageal squamous cell carcinoma to chemoradiotherapy

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AIM: Esophageal squamous cell carcinoma is generally sensitive to chemoradiotherapy (CRT), but some cases are not. Using a retrospective analysis, we aimed to identify the predictors of the response by esophageal squamous cell carcinoma to definitive CRT.

METHODS: The intensities of expression of *p*53, Ki67, Bcl-2, Bax, cyclin D1, VEGF, CDC25B, and metallothionein (MT) were evaluated immunohistochemically in the biopsy specimens obtained before CRT, and the intensities of their expression were tested for correlations with the clinical effects of CRT.

RESULTS: The esophageal squamous cell carcinomas with negative *p*53, positive CDC25B, and negative MT expression were found to be significantly more sensitive to CRT. In addition, p53 positivity and CDC25B positivity respond well to CRT.

CONCLUSION: Esophageal squamous cell carcinomas with negative *p*53,positive CDC25B, and negative MT expressions respond well to CRT. Even with p53 positivity, if with CDC25B positivity, CRT can be expected.

Key words: *p*53; CDC25B; Metallothionein; Chemoradiotherapy; Esophageal squamous cell carcinomas

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Chemoradiotherapy (CRT) is one of the most commonly used modalities of treatment for squamous cell carcinoma of the esophagus. Although the response rate to CRT with 5-fluorouracil and cisplatin is high (64%), there is no survival benefit for non-responders[1]. Hence, it would be useful to be able to predict the response to CRT so that the non-responders could avoid the side-effects of CRT. This has not been possible, however, on the basis of the clinicopathological factors that are routinely examined. Recent advances in tumor biology, especially in relation to apoptosis, offer a possible solution to this problem. Apoptosis induced by CRT involves various biological phenomena, such as DNA repair, altered drug metabolism, inflammation, molecular chaperoning and changes to the cell cycle, and a variety of biological markers, including *p*53, Ki67, Bcl-2, Bax, VEGF, cyclin D1, metallothionein (MT), and CDC25B have been investigated for an association with response to CRT[2-5]. *p*53 is a tumor suppressor gene that contributes to the preservation of genetic stability by facilitating both G_1 arrest and apoptosis in response to DNA damage[6,7]. The Bcl-2 family of proteins includes molecules with both the anti-apoptotic effects (e.g., Bcl-2 and Bcl-XL) and pro-apoptotic effects (e.g., Bax and Bak), and cell susceptibility to apoptosis has been found to be determined by competing dimerization of different members of the Bcl-2 family[8,9]. Previous studies have revealed that CDC25B, which activates CDC2 and the G_2-M progression, is significantly associated with radiation sensitivity among various molecules regulating the G_2-M checkpoint[10]. MT is an intracellular metal-binding protein involved in zinc homeostasis and the detoxification of heavy metals[11]. A study has shown that MT affects cisplatin-induced apoptosis[12]. Moreover, another recent study has reported that essential cytotoxic targets of both oxidants and heavy metals exist in the cytoplasm and establish the importance of nucleocytoplasmic partitioning for the function of MT[13], and a positive association has also been found between MT expression and resistance to CRT[14]. Thus, the purpose of our study was to identify the predictors of the response to definitive CRT with a retrospective analysis. Therefore, we have investigated a variety of biological markers, including *p*53, Ki67, Bcl-2, Bax, VEGF, cyclin D1, MT, and CDC25B.

Thirty-six patients with advanced squamous cell carcinoma of the esophagus, who refused surgery and gave informed consent to CRT at Ibaraki Prefectural Central between 1996 and 2001, were included in this study (Table 1). The diagnosis of squamous cell carcinoma was confirmed by histological examination of biopsy specimens obtained before starting CRT (the clinicopathological data are summarized in Table 1). Response to CRT was evaluated clinically after two courses and the evaluation included a barium esophagogram, esophagoscopy, and computed tomography (CT) of the chest and abdomen. A complete response was defined as no visible tumor by esophagoscopy, biopsy specimens free of tumor tissue, and normal CT findings; a partial response as >50% tumor regression as evaluated by CT, and >50% reduction of intraesophageal tumor extension assessed by barium swallow and esophagoscopy; no change as <50% regression of tumor extension, and no evidence of tumor progression; and progressive disease as increasing tumor growth indicated by barium swallow or esophagoscopy and increasing tumor diameter assessed by CT.

Table 1 Characteristics of patients (mean±SD, *n*)

Parameters	Values
Sex (male/female)	33/3
Age (range)	63.3±9.2 (42-78 yr)
Histopathology	
Well-differentiated	6
Moderately differentiated	23
Poorly differentiated	7
Stage (UICC)	
I	4
II	7
III	18
IVa	7

Chemoradiotherapy

Chemotherapy consisted of protracted infusion of 5-FU at a dose of 400 mg/m^2 per d on d 1-5 and 8-12, combined with a 2-h infusion of CDDP at 40 mg/m^2 per d on d 1 and 8, repeated twice every 5 wk. Concurrent radiotherapy was started on d 1 at 2 Gy/d for 5 d/wk. The total radiation dose was 60 Gy, with a 2-wk break after a dose of 30 Gy. The patients were followed up every 3 mo for the first 3 years after the end of treatment, and afterward every 6 mo thereafter. New chemotherapy agent (e.g., docetaxel) was applied for the patient with non-effective CRT.

Immunohistological staining and its evaluation

Immunostainings for *p*53, Ki67, Bcl-2, Bax, cyclin D1, VEGF, MT, and CDC25B were performed using streptavidin-peroxidase complex methods with an EnVision+™ peroxidase kit (Dako, Glostrup, Denmark) on a TeckMate Horizon automated staining system (Dako, Glostrup, Denmark). Primary antibodies were incubated overnight at 4 ℃ with E9 (dilution 1:50; Dako, Kyoto, Japan) for MT, and for 1 h at room temperature with DO-7 (dilution 1:50; Novocastra Laboratories, Newcastle upon Tyne, UK) for *p*53, with Ki-S5 (dilution 1:50; Dako, Kyoto, Japan) for Ki-67, with 124 (dilution 1:50; Dako, Kyoto, Japan) for Bcl-2, with A3533 (dilution 1:50; Dako, Kyoto, Japan) for Bax, with DSC-6 (dilution 1:200; Novocastra) for cyclin D1, with JH 121 (dilution 1:50; Upstate, Lake Placid, NY, USA) for VEGF, and with C45820 (5 μg/mL; Transduction Laboratories, Lexington, KY, USA) for CDC25B. The expressions of *p*53, Bcl-2, Bax, VEGF, cyclin D1, MT, and CDC25B were investigated in consecutive histological sections prepared from the biopsy specimens. After being pretreated thrice, each for 5 min, in a citrate buffer (pH 6.0) at 750 W, the slides were separately incubated overnight at 4 ℃ with the mAbs, and the expressions of *p*53, Ki-67, Bcl-2, Bax, VEGF, cyclin D1, MT, and CDC25B were assessed under light microscope by one observer (Itabashi), who was unknown about the clinical outcome. The percentages of positive tumor cells were determined semiquantitatively, and each sample was assigned to one of the following categories: negative (0-10%) and positive (11-100%). In addition, the intensities of immunostaining of antigens localized in the cytoplasm (Bcl-2, Bax, cyclin D1, VEGF, MT, and CDC25B) were classified as negative or positive. Staining intensity was not determined for *p*53 (nuclear immunostaining), because no significant differences in staining intensity were observed in the *p*53-positive cases.

Statistical analysis

Associations between two parameters were analyzed by the Spearman's rank correlation test. For continuous parameters, the difference between the two groups was analyzed by Student's *t*-test. Determination of the distribution of immunohistochemical staining between groups was analyzed using χ^2 analysis. Cumulative survival of the patients was calculated on July 31, 2002, by the Kaplan-Meier method and the statistical significance was analyzed by the log-rank test. $P<0.05$ was considered statistically significant in all analyses. All statistical analyses were performed using the StatView version 5.0 software package (Abacus Concepts, Berkeley, CA, USA).

The overall proportions of the cells positive for expression of *p*53, Bcl-2, Bax, Ki67, cyclin D1, VEGF, MT, and CDC25B were 58.3%, 27.8%, 30.6%, 86.1%, 41.7%, 58.3%, 27.8%, and 13.9%, respectively (Tables 2 and 3). Not MT negativity, but CDC25B positivity with p53 positivity is a predictor of a response to CRT (respectively p=0.39, 0.027).The median survival time of the *p*53-negative and *p*53-positive patients were 588 and 415 d, respectively ($P<0.001$, Student's *t*-test), but no significant difference was found between the survival curves of the *p*53-negative and *p*53-positive patients and esophageal cancer staging (Figures 1 and 2). However, there was a significant association between *p*53-negative and MT-negative patients and effect of CRT (Table 4). At the end of the follow-up period on July 31, 2002, 25% patients (9/36) were still alive. The follow-up time for all 36 patients ranged from 26.7 to 81 mo (median, 59.5±18.1 mo). In the group treated by CRT, there was a marked difference in mean survival time between the patients with *p*53-negative tumors (19.6±10.3 mo) and with *p*53-postive tumors (13.8±8. 6 mo). Esophageal squamous cell carcinomas relapsed in

Table 2 Relation between immunohistochemical expressions of biological markers and response of esophageal squamous cell carcinoma to CRT (*n*)

Markers	Responder (CR+PR)	Non-responder (NC+PD)	*P*
p53(-)	14	1	0.0095
p53(+)	11	10	
MT (-)	21	5	0.019
MT (+)	4	6	
CDC25B (-)	7	8	0.0129
CDC25B (+)	18	3	

Spearman's rank correlation test.
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Table 3 Relation between expressions of biological markers and response of esophageal squamous cell carcinoma to CRT (*n*)

Markers	Responder (CR+PR)	Non-responder (NC+PD)	*P*
Bax (-)	17	8	0.799
Bax (+)	8	3	0.799
Bcl-2 (-)	18	8	0.836
Bcl-2 (+)	7	3	0.836
Cyclin D1 (-)	15	6	0.305
Cyclin D1 (+)	10	5	0.305
VEGF (-)	10	5	0.936
VEGF (+)	15	6	0.936
Ki67 (-)	4	1	0.586
Ki67 (+)	21	10	0.586

Spearman's rank correlation test.

Table 4 Relation between effects of CRT and immunohistochemical staining

	Immunoreactivity / response				*P*
*p*53	-	-	+	+	0.027
CDC25B	+	-	+	-	
Responder	9	5	9	2	
Non-responder	0	1	3	7	
MT	-	-	+	+	
0.0305					
CDC25B	+	-	+	-	
Responder	16	5	2	2	
Non-responder	2	3	1	5	

χ^2 test.

11 out of 25 (44%) responder cases after CRT.

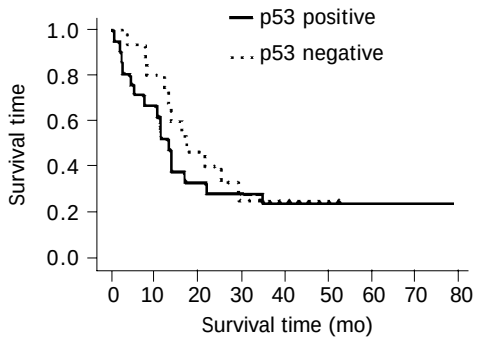

Figure 1 Cumulative survival curve of 36 patients. A significant difference that was not found was observed between the survival rates of the patients with *p*53-negative and *p*53-positive expression.

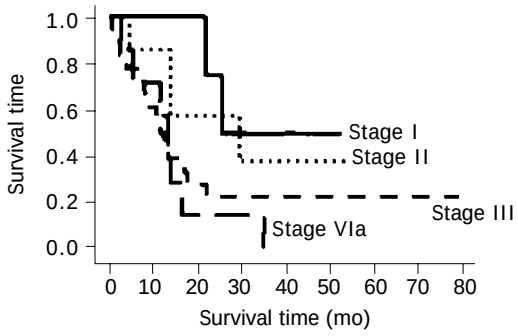

Figure 2 Relation between survival rates of 36 patients and staging of esophageal cancer. No significant difference was seen between survival curves of the *p*53-negative and *p*53-positive patients and esophageal cancer staging.

Esophageal cancer is a relatively uncommon but aggressive disease. Surgical resection has been widely accepted as the standard treatment for esophageal cancer, and techniques have improved during the past decades. However, long-term survival after resection of carcinoma of the thoracic esophagus is generally poor, with rates of only 20-42.4%[15-18]. Some reports on CRT have indicated that it offers various advantages in managing esophageal cancer[19]. Oncologists have advocated a non-surgical approach with definitive CRT as the standard treatment for this disease[20-23]. The value of CRT for the treatment of unresectable esophageal squamous cell carcinoma remains a matter of controversy. Only a few clinical studies have been published since the 1980s, and most patients in those studies had local-regional disease (UICC stage I or II). Several investigators have reported successful results with these modalities, either with or without surgery, against local-regional carcinoma[24-26]. The combination of 5-FU and CDDP has become the standard regimen, not only because of the clinical outcome but also because of the synergism between the two agents and their radiosensitizing effects[27,28]. Recently published results on CRT have indicated that it offers various advantages for the treatment of carcinoma of the esophagus[29,30]. A multicenter study on the indications for CRT as curative therapy for patients with locally advanced disease has suggested that concurrent CRT is potentially curative, even in cases of locally advanced carcinoma of the esophagus (i.e., T4 and/or M1 lymph node metastasis disease)[31]. Some studies have implicated various molecules, including *p*53, CDC25B, and MT, as candidates for biological markers for the response of human esophageal cancer to CRT[10,14]. We evaluated the role of cell-cycle-regulating molecules in the sensitivity of human squamous cell carcinoma of the esophagus to CRT by immunohistochemical methods and found *p*53, MT, and CDC25B to be significant independent markers for predicting sensitivity to CRT. Our results showed that negative

immunostaining for *p*53 in pre-CRT biopsy specimens predicted a good response. In addition to being the only established biological marker for response to CRT in clinical studies, *p*53 is the best characterized and most powerful marker[32,33], and it is commonly acknowledged to be a definite indicator of radiation sensitivity in various cancers[34,35]. The *p*53 immunoreactivity is generally thought to be attributable to the accumulation of abnormal *p*53 protein[36]. In our results, even the tumors with *p*53 positivity were sensitive to the CRT. In most of the esophageal squamous cell carcinoma, p53 are positive immunohistologically. Even with p53 positivity, CDC25B positivity respond well to CRT. So, we think that another pathway independent of *p*53 that regulate CRT sensitivity is sure to exist. G_2-M checkpoint, in which CDC25B is involved, is a candidate for this *p*53-independent pathway. Among the various molecules that regulate G_2-M arrest, overexpression of CDC25B has frequently been observed in human cancer[37,38], and also found to be related with response to CRT[10]. In our cases, no significant difference was observed between the survival curves of the *p*53-negative and *p*53-positive patients. It might be because a new chemotherapeutic agent (e.g., docetaxel) was applied for the patients with non-effective CRT. Docetaxel is an active drug for treating squamous cell carcinoma of head and neck. For the patients with recurrent or metastatic disease of squamous cell carcinoma, docetaxel could be used as a second line chemotherapy[39]. From a clinical standpoint, it is very important that a prospective study based on this result should be done. There are only retrospective studies that have been reported up to now. Standard therapy may be developed from the results of a prospective study based on the immunohistochemical characteristics of the tumor biopsy.

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