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Predictors of Response to Chemo-radiotherapy and Radiotherapy for Esophageal Squamous Cell Carcinoma

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Abstract. *Background: The sensitivity of tumors to chemotherapy and radiotherapy differs from one case to another and may be influenced by the expression of biological molecules. The presence of six potential predictive markers in esophageal squamous cell carcinoma (ESCC) was investigated and the data obtained were related to the response of the tumors to chemo-radiotherapy and radiotherapy. Materials and Methods: Biopsy specimens were obtained from 61 patients with ESCC before treatment with chemo-radiotherapy (31 patients) or radiotherapy (30 patients). External radiotherapy was delivered by a two-field technique to a total of 60-70 Gray. Concurrent chemotherapy consisted of cisplatin or nedaplatin and 5-fluorouracil administered intravenously. The patients were examined before and after treatment by endoscopy, esophagography and computed tomography. The clinical response was classified as effective (>50% decrease in primary lesion), or ineffective. Immunohistochemical staining for p53, p21, bax, bcl2, heat-shock protein (Hsp) 27 and Hsp70 was performed on the biopsy specimens before therapy. Results: The primary tumor response was effective in 73.8% (45/61) and ineffective in 26.2% (16/61) of patients. Tumors with p53-positive expression were less sensitive than p53-negative tumors (p=0.033). p21-positive patients (p=0.027), and Hsp27-negative (p=0.0057) and Hsp70-negative patients (p=0.010) were all good responders. Neither bcl2 nor bax expression was related to the efficacy of therapy. Multivariate analysis revealed that Hsp27 was the most reliable predictor of the effect of chemo-radiotherapy and radiotherapy among the four potential markers. p53-negative and Hsp70-negative patients had a more favorable prognosis than p53- and Hsp70-positive patients (p=0.039, p=0.038, respectively). Conclusion: Expression of Hsp27 was a good predictor of the response of ESCC to chemo-radiotherapy and radiotherapy.*

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Key Words: Esophageal squamous cell carcinoma, chemo-radiotherapy, Hsp27, Hsp70, p21, p53, bax, bcl2.

Esophageal cancer is one of the most aggressive and lethal malignancies. Mortality rates are very similar to the incidence rates (1) due to the relatively late stage of diagnosis and lack of effective treatment; the survival rate at 5 years was reported to be <10% (2), although recent advances in surgical techniques and adjuvant therapy have improved the 5-year survival rate to about 40% (3). Some cases respond very well to radiotherapy or chemo-radiotherapy, whereas others do not. The effects of adjuvant therapy differ between patients, and the survival of responders has been reported to be better than that of non-responders (4). It is important to recognize the probable response of the tumor to treatment. It has been reported that p53, a tumor suppressor gene, is an indicator of chemotherapy and/or radiotherapy resistance in patients with esophageal squamous cell carcinoma (ESCC) (5, 6). Apoptosis-related factors, bax (7, 8), bcl2 (8, 9) and Ki-67 labelling index (10) have also been investigated as prognostic indicators. Heat-shock protein (Hsp) 27 and Hsp70 have a variety of vital intracellular chaperoning functions and have been reported to be good indicators of the tumor response to chemotherapy and/or radiotherapy (11, 12). However, a comprehensive study of these markers has never been carried out. In this study, the predictive value of these markers in the response of ESCC to chemo-radiotherapy and radiotherapy was investigated using biopsy specimens obtained from patients before treatment.

Materials and Methods

Clinical characteristics of patients and ESCC tissue samples. Pretreatment ESCC biopsy specimens were obtained endoscopically from 61 patients before chemo-radiotherapy (31 patients) or radiotherapy (30 patients) at the Gunma University Hospital, Japan, between 1985 and 2000. The patients included 53 men and 8

women, aged 36-86 years (mean age: 65.7 years). Written informed consent to participate in the study was obtained from each patient before treatment, according to the ethical guidelines of our university. All patients underwent chemo-radiotherapy or radiotherapy for advanced tumor invasion, distant metastasis, low performance status, or rejection from surgery. Tumor stage was classified according to the fifth edition of the TNM classification of the International Union against Cancer (UICC). The mean postoperative follow-up period for the 61 patients was 22.6 months (range: 31.9-118.7 months).

Treatment protocol. After diagnosis, the patients underwent radiotherapy, or chemo-radiotherapy, consisting of concurrent radiotherapy and chemotherapy, for 7 weeks. The tumor response was assessed by endoscopy with biopsy and esophagography 2 weeks after the end of treatment. External radiotherapy was delivered by a two-field technique using a 10-15 MV photon beam at 2 Gray/fraction/day, 5 fractions/week, to a total of 60-70 Gray. Concurrent chemotherapy consisted of 80 mg/m^2 cisplatin or nedaplatin administered intravenously over 1 h on days 1 and 29, and 350 mg/m^2 5-fluorouracil administered as a continuous intravenous infusion on days 1-5 and 29-33. Twelve of the 31 patients who underwent chemo-radiotherapy were administered nedaplatin. Nedaplatin is a second-generation platinum complex that has been found, in preclinical (13, 14) and clinical studies (15-17), to have pronounced activity against solid tumors, but is less nephrotoxic than cisplatin.

Clinical features and evaluation of treatment. Before treatment, all patients were evaluated by radiographic examination (chest X-rays, barium swallow), endoscopy (esophagoscopy and, in some cases, bronchoscopy), endoscopic ultrasonography, ultrasonography and computed tomography (CT). Biopsy samples were obtained from 3 or more tumor parts. Tumor stage was classified according to the fifth edition of the TNM classification of the International Union against Cancer (UICC). The clinical response was evaluated 2 weeks after the end of treatment according to the guidelines of the Japanese Society for Esophageal Diseases (JSED) (18). Assessment included repeated endoscopy, esophagography and CT scans. Endoscopy and esophagography were carried out by two investigators who measured the maximal major and minor axes of the tumor before and after treatment. All patients underwent a CT scan of the neck, chest and abdomen. Ten-millimeter continuous scans were obtained from the neck to the bottom of the liver using an intravenous contrast medium. The CT response was assessed by two experienced radiologists who measured the maximum wall thickness before and after treatment. The results of endoscopy, esophagography and CT scans were discussed among the investigators, and the response was classified as complete, partial (>50% decrease), or no change (<50% decrease) in the primary lesion. The patients were divided into two groups: an effective group (EG), that included all patients with a complete or partial response, and an ineffective group (IG), that included patients who showed no change or progressive disease.

Antibodies and immunohistochemistry. Antibodies were purchased from the following manufacturers: monoclonal antibody (Mab) specific for p53 (DO-7; Novocastra Laboratories Ltd., Newcastle, UK), used at a dilution of 1:100; Mab specific for p21 (WAF1; Novocastra Laboratories Ltd.), used at a dilution of 1:100; Mab specific for Hsp27 (G3.1; StressGen Biotechnologies Corporation, Victoria, BC, Canada), used at a dilution of 1:80; Mab specific for Hsp70 (C92F3A-5; StressGen Biotechnologies Corporation), used at a dilution of 1:70; Mab specific for bcl-2 (124; DAKO A/S, Glostrup, Denmark), used at a dilution of 1:40; and Mab specific for bax (B-9; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), used at a dilution of 1:100. Immunohistochemical staining was performed by the standard avidin-biotin-peroxidase complex (ABC) method, as described previously (19-21). Briefly, each 4-μm tissue section was deparaffinized, rehydrated and incubated with fresh 0.3% H_2O_2 in methanol for 30 min at room temperature. After rehydration through a graded ethanol series, the sections were microwaved in zinc sulfate heptahydrate buffer at 90˚C for 10 min for anti-p53 Mab, microwaved in 1 mM EDTA buffer (pH 8.0) at 90˚C for 10 min for p21, or microwaved in 10 mM citrate buffer (pH 6.0) at 90˚C for 10 min for anti-bcl2 Mab and anti-bax

Table I. *Correlation between clinicopathological characteristics and effect.*

Parameters		effective (n=45)	ineffective (n=16)	*p*-value
Gender				
Male	(n=53)	39	14	0.93
Female	(n=8)	6	2	
Age	mean±SD	66.4±10.4	64.0±11.5	0.44
Location				
Ce	(n=3)	3	0	0.20
Ut	(n=10)	7	3	
Mt	(n=35)	23	12	
Lt	(n=13)	12	1	
Histology				
Well	(n=16)	12	4	0.89
Moderately	(n=25)	19	6	
Poorly	(n=20)	14	6	
TNM clinical classification				
Depth of invasion				
T1	(n=4)	4	0	0.12
T2	(n=6)	5	1	
T3	(n=24)	20	4	
T4	(n=27)	16	11	
Regional lymph nodes				0.096
N0	(n=28)	24	4	
N1	(n=33)	21	12	
Distant metastasis				0.64
M0	(n=41)	31	14	
M1	(n=20)	10	6	
Stage				
I	(n=4)	4	0	0.058
II	(n=11)	11	0	
III	(n=26)	16	10	
IV	(n=20)	14	6	

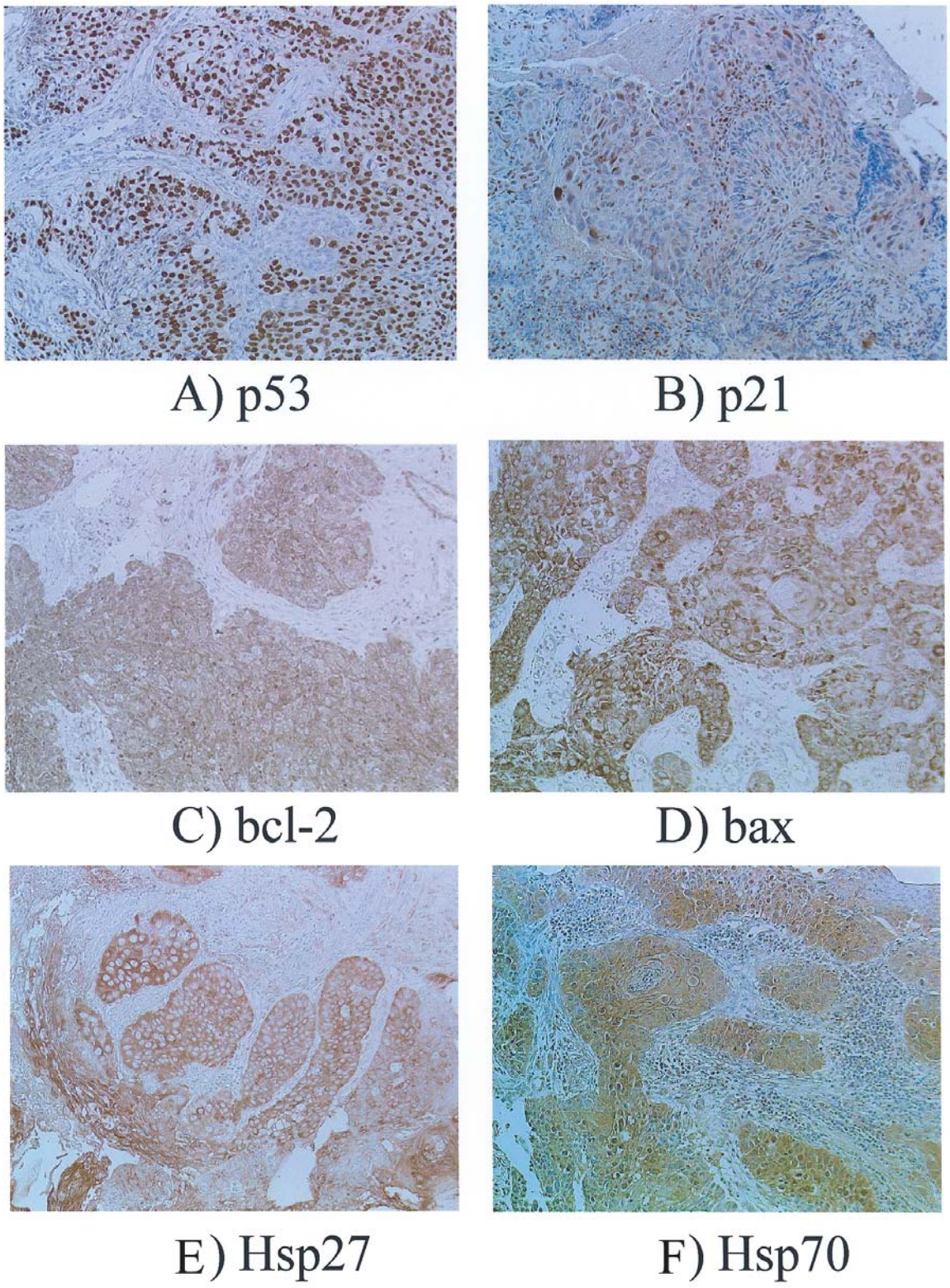

Figure 1. *Representative photomicrographs of tissue sections immunostained for candidate markers (x 100). (A) p53 and (B) p21 were detected in the nuclei of cancer cells. (C) bcl2, (D) bax, (E) Hsp27 and (F) Hsp70 were detected in the cytoplasm of cancer cells.*

Table II. *Correlation between expression of predictors and clinicopathological characteristics or effect of treatment.*

Parameters		p53 positive (n=33)	p53 *p*-value	p21 positive (n=37)	p21 *p*-value	bcl-2 positive (n=24)	bcl-2 *p*-value	bax positive (n=25)	bax *p*-value	Hsp27 positive (n=40)	Hsp27 *p*-value	Hsp70 positive (n=29)	Hsp70 *p*-value
Gender			0.8		0.51		0.37		0.32		0.84		0.88
Male	(n=53)	24		33		22		23		35		25	
Female	(n=8)	4		4		2		2		5		4	
Location			0.93		0.33		0.82		0.76		0.80		0.87
Ce	(n=3)	1		3		1		1		2		1	
Ut	(n=10)	4		7		5		4		7		4	
Mt	(n=35)	17		21		14		13		24		18	
Lt	(n=13)	6		6		4		7		7		6	
Histology			0.19		0.20		**0.0006**		0.64		0.95		0.58
Well	(n=16)	9		7		0		6		11		6	
Moderately	(n=25)	8		18		12		12		16		12	
Poorly	(n=20)	11		12		12		7		13		11	
Depth of invasion			0.21		0.92		0.34		0.34		0.25		0.065
T1	(n=4)	0		3		0		0		1		0	
T2	(n=6)	2		4		3		3		5		1	
T3	(n=24)	13		14		11		11		15		14	
T4	(n=27)	13		16		10		10		19		14	
Regional lymph nodes			0.66		0.60		0.59		0.20		0.73		**0.0012**
N0	(n=28)	12		16		10		9		19		7	
N1	(n=33)	16		21		14		16		21		22	
Distant metastasis			0.92				0.80		0.66		0.52		0.17
M0	(n=41)	19		26	0.53	13		16		28		17	
M1	(n=20)	9		11		11		9		12		12	
effective	(n=45)	17	**0.033**	31	**0.027**	20	0.17	20	0.36	25	**0.0057**	17	**0.010**
ineffective	(n=16)	11		6		4		5		15		12	

Table III. *Multivariative analysis of candidates.*

	F-value	*p*-value
Hsp27	4.75	0.0345
p21	3.41	0.0714
Hsp70	2.36	0.132
p53	0.78	0.781

Mab, and cooled to 30˚C. After incubation with normal horse serum for 30 min, the sections were then incubated with Mabs at their optimum dilution at 4˚C overnight, washed in phosphate-buffered saline and incubated with secondary antibody for 30 min at room temperature. Immunohistochemistry was performed using the ABC method (Vectastain Lab., Inc., Burlingame, CA, USA). The chromogen was 3,3'-diaminobenzidine tetrahydrochloride, applied as a 0.02% solution containing 0.0055% H_2O_2 in 50 mM ammonium acetate-citric acid buffer (pH 6.0). The sections were lightly counterstained with hematoxylin. Negative controls were prepared by substituting normal mouse serum for each primary antibody, and no detectable staining was evident.

Evaluation of staining of markers. When >20% of the carcinoma cells in a given specimen were positively stained, the sample was classified as Hsp27- and Hsp70-positive (+); when <20% of the tumor cells were stained it was considered negative (20). For p53, p21, bcl2 and bax, when >10% of the tumor cells were stained, the sample was graded as positive and when <10% of the cells were stained it was considered negative (–) (19, 21).

Statistical analysis. Statistical analysis was performed using the χ^2 test, Fisher's exact test and the Mann-Whitney *U*-test. Survival curves of the patients were prepared using the Kaplan-Meier method and analysis was carried out by the log-rank test. Multiple analysis of variance (MANOVA) was performed for multivariate statistics.

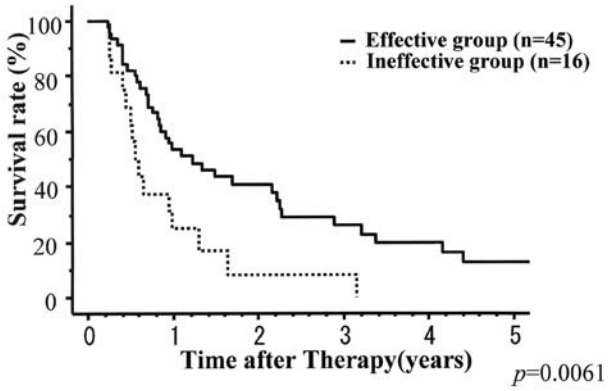

Figure 2. *Overall survival post-treatment related to tumor response. Patients in the effective group had a significantly more favorable prognosis than those in the ineffective group (2-year survival rate: positive, 40%; negative, 6.3%; p=0.0061).*

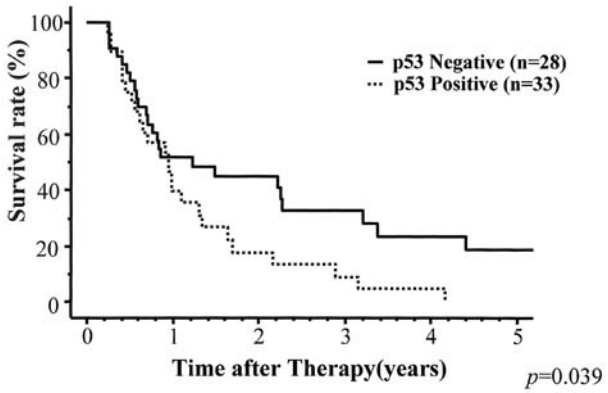

Figure 3. *Overall survival post-treatment related to p53 expression. p53-negative patients had a significantly more favorable prognosis than those with p53-positive expression (p=0.039).*

Results

The characteristics of the study population are shown in Table I. According to the histological differentiation, the incidence of well-differentiated ESCC was 26.2%, moderately-differentiated ESCC was 41.0% and poorly-differentiated ESCC was 32.8%. Adventitial invasion was seen by imaging in 27 patients (44.3%) and lymph node metastasis occurred in 33 (54.1%). The primary tumor response rate was complete response in 26.2% (16/61), partial response in 47.5% (29/61) and no change in 26.2% (16/61). The cases were divided into two groups for analysis: EG and IG. The response rate (complete and partial response) of the primary lesion in the 31 patients treated with chemo-radiotherapy was 74.2% (23/31). The response rate of the primary lesion in the 30 patients treated with radiotherapy was 73.3% (22/30).

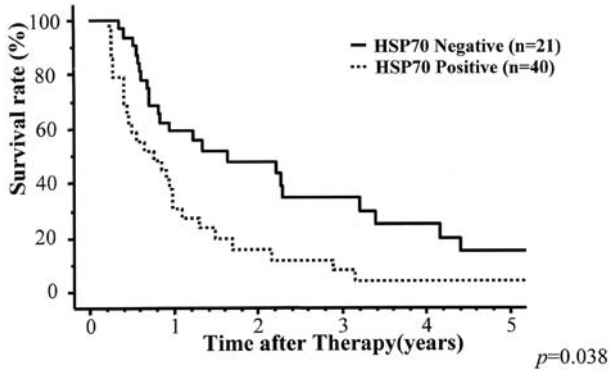

Figure 4. *Overall survival post-treatment related to Hsp70 expression. Hsp70-negative patients had a significantly more favorable prognosis than those with Hsp70-positive expression (p=0.038).*

Relationship between clinicopathological factors and immunohistochemical findings. The expression of each potential predictive marker was investigated by immunohistochemical analysis of formalin-fixed, paraffin-embedded specimens using Mabs. Immunostaining for p53 and p21 proteins was detected in the nucleus of esophageal cancer cells (Figure 1A, B). bcl2, bax, Hsp27 and 70 proteins were detected in the cytoplasm of cancer cells (Figure 1C-F). A summary of the correlation between clinicopathological factors and the expression of each marker is shown in Table II. As the expression tendency for each marker was the same in all patients irrespective of treatment, the expression tendency for each marker was analyzed in all patients together. p53-positive patients had a significantly poorer response to treatment than p53-negative patients ($p=0.033$). However, there was no relationship between clinicopathological factors and expression of p53. p21-positive patients were good responders ($p=0.027$), although there was no correlation between clinicopathological factors and expression of p21. Expression of bcl2 was correlated with tumor differentiation ($p=0.0006$). There was no relationship between clinicopathological factors and expression of bax. Neither bcl2 nor bax expression was related to the efficacy of therapy. Hsp27-negative patients were also good responders to treatment ($p=0.0057$), although there was no correlation between clinicopathological factors and expression of Hsp27. Hsp70-negative patients were good responders ($p=0.010$). Expression of Hsp70 was correlated with lymph node metastasis ($p=0.0012$).

Multivariate analysis of the 4 factors, p53, p21, Hsp27 and Hsp70, which had a significant correlation with efficacy of treatment, was then carried out (Table III). Of these 4 candidates, Hsp27 was found to be the most reliable marker of the efficacy of chemo-radiotherapy and radiotherapy.

The survival rates of the patients given ineffective treatment were significantly lower than those of the group given effective treatment ($p=0.0061$; Figure 2). The 2-year survival rate for the EG was 40%, whereas that for the IG was 6.3%. p53-negative patients had a more favorable prognosis than p53-positive patients ($p=0.039$; Figure 3). Hsp70-negative patients also had a more favorable prognosis than Hsp70-positive patients ($p=0.038$; Figure 4). There was no relationship between expression of the other markers and prognosis.

Discussion

The aim of this study was to evaluate whether it was possible to predict the likely effects of treatment using biopsy specimens from ESCC before treatment was initiated. The results show that cancer tissue from good responders had positive p21 expression and negative p53, Hsp27 and Hsp70 expressions. The p53 and p21 genes are associated with G1 arrest during the cell cycle and with apoptosis. p53 is one of the most common tumor suppressor genes, and shows a mutation in many human cancers (22). p53 mutation was reported with high frequency in esophageal cancer (23). On the other hand, there have been few reports on the genomic alteration of p21. A correlation between accumulation of p53 protein (probably due to a mutated p53 gene) and other types of chemosensitivity or radiosensitivity has been reported (5, 6, 10). Nakashima *et al.* (24) reported that chemotherapy was effective against metastatic lymph nodes which were p53-negative but p21-positive. The results of the current study support these results.

bax and bcl2 have been reported to regulate apoptosis positively and negatively, respectively (25-28). However, the current results showed no relationship between the efficacy of treatment and expression of bax or bcl2. Shimoji *et al.* (8) reported that the effects of radiotherapy were correlated with p53 expression, but that bcl2 and bax expressions showed no relationship with the effect of radiotherapy in ESCC.

Heat-shock proteins are a set of highly evolutionarily conserved proteins found in nearly all organisms. They are known to be induced by various kind of stress, including exposure to non-physiological temperatures, anoxia or chemical agents. One of their most important functions is to act as molecular chaperones, implicated in cellular protection mechanisms (29). Both Hsp27 and Hsp70 could be used to predict the effect of treatment in this study. Multivariate analysis revealed that Hsp27 was the most reliable indicator of the efficacy of chemo-radiotherapy and radiotherapy. Furthermore, 20 out of 21 Hsp27-negative patients underwent effective treatment, indicating that chemo-radiotherapy and radiotherapy may be indicated in Hsp27-negative tumors.

p53-negative and Hsp70-negative patients had a more favorable prognosis than p53- and Hsp70-positive patients. However, Hsp27-negative patients did not have more favorable prognosis than Hsp27-positive patients. The reason for this discrepancy, namely that the most reliable factor did not relate to prognosis, is unclear. However it was thought that this discrepancy was due not only to the response of the primary lesion, but also to many other factors which influenced the prognosis. For example, Hsp70 may have been influenced by lymph node metastasis.

Patients will benefit greatly when there is accurate information available about the likely effects of treatment before treatment is started. Further work is required to elucidate the factor or combination of factors which best reflect the effects of treatment. These factors might be available not only as predictors, but also as targets for treatment. For example, sensitivity to treatment might be increased if these alterations of function and expression are normalized.

In conclusion, expression of Hsp27 was a good predictor of the response of ESCC to chemo-radiotherapy and radiotherapy. This might be useful in helping clinicians to decide on the best clinical policy and treatment in patients with ESCC.

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