Expression of p53 and p21 and the Clinical Response for Hyperthermochemoradiotherapy in Patients with Squamous Cell Carcinoma of the Esophagus

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Abstract. *Background: The p53 and p21 genes are closely related to sensitivity to chemoradiotherapy. Patients and Methods: The expressions of the p53 and p21 genes were immunohistochemically examined in 32 patients with esophageal cancer, who underwent an esophagectomy after hyperthermochemoradiotherapy (HCRT). The significance of the expression of these genes for the effect of HCRT was evaluated. Results: HCRT was markedly effective (grade 3 response: no residual viable cancer cells) in 12 cases (38%). The incidences of the grade 3 were 67% and 20% in the cases with a positive and negative p21 expression, respectively (p=0.0213). A multivariate analysis revealed the p21 expression to be a significant independent factor associated with the histological effects. None of the 10 patients with p53-positive and p21-negative tumors showed a grade 3 response, while 55% showed grade 3 response in other combination groups (p<0.05). Conclusion: The combination of p53 and p21 expressions in biopsy findings can thus predict the histological effectiveness of HCRT.*

The prognosis of patients with esophageal cancer is extremely poor, in comparison to other malignancies of the digestive tract. The disease is frequently too advanced at diagnosis to be curatively resected. Esophagectomy is the most effective treatment modality for a comparatively early stage of carcinoma. For patients with a locally advanced disease who are not indicated for surgery, definitive radiation therapy with concurrent chemotherapy, such as cisplatin/5-FU, has been considered as the standard treatment (1). Combined chemotherapy, radiotherapy, and surgery could improve survival in patients with resectable esophageal cancer, and neoadjuvant chemoradiation offers early treatment of micrometastatic disease and can facilitate a surgical resection by downstaging cancers (2-4). We previously applied and reported hyperthermia in comparison to chemoradiotherapy (CRT) for patients with advanced esophageal carcinoma based on both retrospective and prospective trials (5, 6). CRT has been revealed to be a promising treatment not only as a neoadjuvant therapy but also as a definitive therapy. A meta-analysis has revealed that preoperative CRT improved the three-year survival rate in comparison to surgery alone (7, 8).

The *p53* gene is the most popular tumor suppression gene and either mutation or deletion is recognized in over 50% of all human tumors. An abnormality of *p53* has also been reported to be frequent in esophageal squamous cell carcinoma (ESCC). As the 'guardian of the genome', wild-type p53 mediates cell-cycle arrest or apoptosis in response to DNA damage, and the loss of *p53* function is associated with the resistance to apoptosis induced by chemotherapy and radiotherapy (9, 10). Therefore, *p53* and its downstream *p21* are considered to be candidates for predictors of CRT effects for ESCC (11). Several authors have examined the relationship between *p53* mutation and sensitivity to CRT in patients with ESCC (12, 13), but the results have been inconsistent. Moreover, data on the relationship between the wild-type *p53* function and sensitivity to HCRT, have never been reported. The objective of this study was to investigate the relationship between the *p53* and *p21* status in biopsy specimens of ESCC and the effectiveness of preoperative HCRT, and then to reveal the predictive value of the immunohistochemical evaluation of *p53* or *p21* expression in the pre-treatment biopsy samples.

Patients and Methods

Patients. This retrospective study included 32 patients with ESCC who underwent an esophagectomy at the Department of Surgery and Sciences (Surgery II), Kyushu University Hospital, Fukuoka, Japan, from 1991 to 1996. All of the patients were preoperatively

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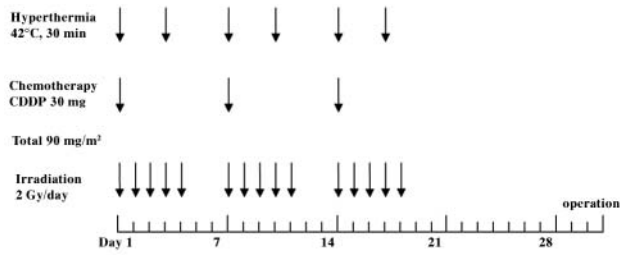

Figure 1. *Regimen of hyperthermochemoradiotherapy for patients with esophageal carcinoma.*

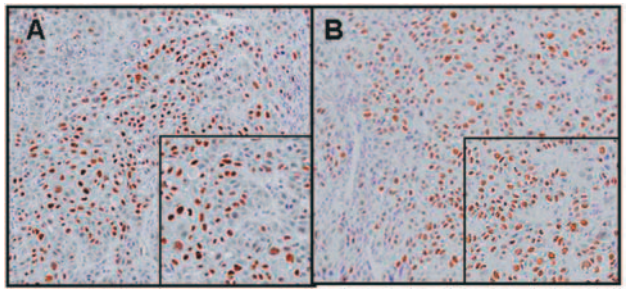

Figure 2. *Immunohistochemistry for the detection of the p53 (A), and p21 antigens (B) (biopsy specimen, x400).*

treated with HCRT and biopsy specimens were also obtained from all of the patients before HCRT therapy.

Preoperative treatment. The schedule of the preoperative treatments is shown in Figure 1. The regimen of preoperative HCRT consists of irradiation given five days per week, and two fractions of hyperthermia per week, combined with chemotherapy. Hyperthermia was clinically applied using this radiofrequency system with an endotract electrode (Endoradiotherm 100A, Olympus Co, Tokyo, Japan), and 30 mg/m^2 of cisplatin was administered three times during the treatment periods (for a total of 90 mg/m^2), along with 30 Gy of irradiation (2 Gy/day, 5 days per week.)

Samples from ESCC. Biopsy samples taken under endoscopy were used for the immunohistochemical (IHC) examination. After the diagnosis of ESCC was confirmed with a section from the paraffin block with hematoxylin and eosin staining, the remaining sections were used for IHC.

Immunohistochemical (IHC) examination. The following monoclonal antibodies were used: p53 (DO-7, Dako, Glostrup, Denmark; dilution 1:100), p21 (4D10, Novocastra, Laboratories Ltd., New Castle, UK; dilution 1:100).

The immunohistochemical status of the p53 and p21 protein was assessed on 5-μm sections prepared from the paraffin blocks. The streptavidin-biotin-peroxidase complex (SAB) method was used for staining. DO-7 is a mouse monoclonal antibody which reacts to human p53 of both wild and mutant forms. The streptavidin-biotin-peroxidase complex (SAB) method was used for staining. All of the sections were initially washed in phosphate-buffered saline (PBS, pH 7.2) and were incubated at room temperature with 10% normal rabbit serum for 10 min. The sections were then incubated with DO-7 and 4D10 both overnight, then with biotinylated mouse anti-mouse IgG+IgA+IgM antibody (10 μm/ml for 15 min), and finally with streptavidin-biotin-peroxidase complex (100 μm/ml for 5 min). A known positive specimen was used as the positive control (6). The omission of the primary antibody served as a negative control. All evaluations for immunostainings were made by two independent observers (M.I. and T.O.) without any knowledge of the patient's clinical status. Both p53 and p21 were seen in the nucleus of esophageal carcinoma cells in the biopsy, the samples with clearly stained nuclei in 20% or more of the cancer cells were regarded as positive (Figure 2).

Histopathological evaluation of the effect of preoperative treatment. The effects of preoperative treatment were evaluated according to the histopathological criteria for the effects of radiation and anticancer chemotherapy as given in the 9th edition of Guidelines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus by the Japanese Society for Esophageal Diseases (14, 15): Grade 0: Ineffective, there is no discernible therapeutic effect on the cancer tissue or cells; Grade 1: Slightly effective, apparently viable cancer cells account for 1/3 or more of the tumor tissue, but there is some evidence of degeneration of the cancer tissue or cells (Figure 3A); Grade 2: Moderately effective, viable cancer cells account for less than 1/3 of the tumor tissue, while the other cancer cells are severely degenerate or necrotic (Figure 3B); Grade 3: Markedly effective, no viable cancer cells are evident (Figure 3C).

Statistical analysis. Either unpaired test or Fisher's exact test were used to evaluate the significance of the difference of clinical backgrounds as well as the expression of *p53* and *p21*. A logistic regression analysis was used to evaluate the independent factors associated with the histological effectiveness after HCRT. The data was analyzed using the Stat View Software Package (Abacus concepts, Inc., Berkeley, CA, USA.). A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Immunoreactivity of p53 and p21. Positive staining of p53 and p21 was observed in 17 (53%) and 12 cases (38%) respectively. There were no significant differences between the expression in relation to the clinical background, *e.g.* such as age, gender, location of the tumor, adventitial invasion, lymph node metastasis or TNM stage (Table I). Among seven patients who showed positive staining for p53, 12 were also p21-positive. Twenty patients showed a negative staining for p21. Ten patients were p53-positive among the 20 with a negative p21 expression. There was no significant difference between the expression of p53 and p21 (Table II).

Histopathological effects of preoperative treatment. Table III shows the correlation between age, gender, location of the tumor, adventitial invasion, nodal involvement, and expression of p53 and p21. There were no significant differences in the histological effects with regard to the clinical factors such as age, gender, location of the tumor,

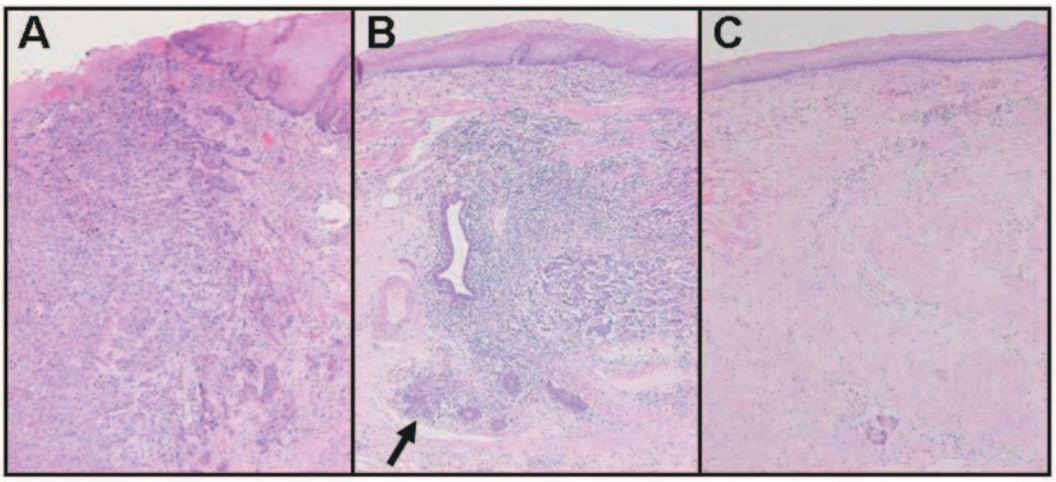

Figure 3. *Histopathologocal effect of hyperthermo-chemo-radiothrapy. A) Grade 1 (slightly effective). There are remaining viable cells. B) Grade 2 (moderately effective). Most remaining cancer cells have degenerated, however some residual cancer cell nests are seen (black arrow). C) Grade 3 (markedly effective). There are no viable cancer cells (biopsy specimen, x100).*

Table I. *p53 and p21 expression and clinicopathological background of the patients.*

Clinicopathological background	p53+/p21+ (n=7)	p53+/p21– (n=10)	p53–/p21+ (n=5)	p53–/p21– (n=10)
Age (years)				
Mean±S.D.	64.7±9.14	62.2±6.73	63.2±10.18	62.0±8.01
Gender				
Male	7	10	3	8
Female	0	0	2	2
Location of the tumor				
Upper esophagus	0	0	1	0
Middle esophagus	5	6	4	5
Lower esophagus	2	4	0	5
Adventitial invasion				
Positive	5	7	2	6
Negative	2	3	3	4
Lymph node metastasis				
Positive	4	6	1	7
Negative	3	4	4	3
TNM stage				
0 or I	0	1	0	1
II	4	3	5	6
III	3	5	0	0
IV	0	1	0	3

adventitial invasion, nodal involvement, or expression of p53 and p21. Regarding p53 expression, HCRT was markedly effective (grade 3 response) in 5 cases among the 12 with a positive expression. However there were no statistical differences. Regarding p21 expression, HCRT was markedly effective (grade 3) in 8 out of the 12 patients with a positive expression, while it was only effective in 4 among the 20 patients with a negative expression ($p=0.0213$). The analyzed the histopathological effectiveness was analyzed paying particular attention to the combined effects of p53 and p21 expression. Regarding correlation between p53 and p21 phenotype and histopathological effects of HCRT, none of the 10 patients with a positive p53 and negative p21 expression showed a grade 3 response to HCRT. Among the 22 cases with another combination subtype (p53+/p21–, p53–/p21+, and p53–/p21–), 12 cases showed a grade 3 response. ($p=0.0044$ by Fisher's test).

Table II. *Relationship between p53 and p21 expression in ESCC patients.*

Expression of p53	Expression of p21		
	Positive	Negative	Total
Positive	7	10	17
Negative	5	10	15
Total	12	20	32

No significant differences were found in analysis using Fisher's exact test.

A logistic regression analysis was also performed in order to identify significantly independent factors regarding treatment efficacy (Table IV). In this multivariate analysis, clinicopathological factors such as age, gender, location of the tumor, adventitial invasion, lymph node metastasis, and p53 and p21 expression were evaluated. As a result, the p21 expression was revealed to be the most significant independent factor associated with the histological effects ($p=0.0244$, odds ratio: 9.73, 95% confidence interval: 1.34-70.6).

Discussion

The optimal treatment for advanced ESCC remains controversial. A surgical resection was once the only curative treatment. However, an esophagectomy with extended lymph node dissection is a procedure accompanied by considerable morbidity and mortality, although perioperative managements have been improved. Recently, on the other hand, definitive CRT has become a promising, less-invasive treatment, but the benefit of this treatment is limited for patients who achieved a complete response. If some markers that predict the effect of CRT are identified, patients and oncologists will be able to choose the optimal treatment modality on an individual basis.

A biopsy under endoscopy is the only (and most convenient) method to obtain pretreatment cancerous tissue from ESCC patients. Several authors have examined some predictive markers for CRT, including p53, p21, Bax, c-erbB2 and cyclin D1, using biopsy specimens. Most of these studies have used immunohistochemical (IHC) analysis (16-22), while others have used a polymerase chain reaction/single-strand conformation polymorphism (PCR-SSCP) method to detect *p53* mutations (14, 23). As biopsy samples are frequently contaminated by necrotic tissue or non-cancerous tissue, a PCR-based analysis may sometimes lead to a misdiagnosis, unless using laser capture microdissection (24). In this study, therefore, we have thus used IHC to detect p53 and p21 expression in cancer cells.

The wild-type p53 protein was recently disclosed to regulate at least two important events as tumor suppressor.

Table III. *Correlation between the clinicopathological factors and histological effectiveness of HCRT treatment of ESCC.*

Clinicopathological background	Effectiveness of preoperative treatment		
	Grade 1 or 2 (n=20)	Grade 3 (n=12)	*P*-value
Age (years)			
≥60	7	3	
<60	13	9	0.2445
Gender			
Male	18	10	
Female	2	2	0.6196
Location of the tumor			
Upper & middle	11	10	
Lower	9	2	0.600
Adventitial invasion			
Positive	13	7	
Negative	7	5	0.9999
Nodal involvement			
Positive	10	3	
Negative	10	9	0.6199
p53			
Positive	12	5	
Negative	8	7	0.4670
p21			
Positive	4	8	
Negative	16	4	0.0213
Combination of p53 and p21			
p53+/p21+	2	5	
p53–/p21+	2 } 10*	3 } 12*	
p53–/p21–	6	4	
p53+/p21–	10	0	0.0044*

All analyses using Fisher's exact test.

Table IV. *Analysis of independent factors with logistic regression analysis.*

Factor	Object	Control	Odds ratio	95% confidence interval	*P*-value
Age (years)	60	60	2.50	0.33-18.8	0.3792
Gender	Female	Male	0.49	0.03-9.72	0.6424
Location of the tumor	Upper & middle	Lower	2.54	0.32-19.9	0.3747
Adventitial invasion	Present	Absent	0.81	0.12-5.59	0.8316
Nodal involvement	Present	Absent	0.45	0.06-3.24	0.4265
p53	Positive	Negative	0.28	0.03-2.40	0.2467
p21	Positive	Negative	9.73	1.34-70.6	0.0244

95%CI: 95% confidence interval.

First, p53 has been shown to induce the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1/CIP1}$, which negatively regulates the cell cycle. Second, *p53* can induce apoptosis through the regulation of genes such as *BAX* (12). We focused on the p53 pathway for predictive markers of HCRT because *p53* is known to regulate both the cell cycle and apoptosis and thus is associated with the sensitivity to chemotherapeutic agents or irradiation *in vitro* (25). The correlations between *p53* status and response to CRT have been investigated in several articles. Michel *et al.* (19) reported that wild-type *p53* in ESCC was associated with a good response to definitive CRT. Gibson *et al.* (13) reported that a *p53* mutation along with an EGF-R mutation may be used as an outcome predictor. These two studies suggest the usefulness of a *p53* analysis for the prediction of CRT effects, although they did not investigate the correlation between *p53* status and histopathological effectiveness. On the contrary, two other studies reported that *p53* status did not influence the clinical outcome (18, 23). In this study, immunohistochemical p53 expression alone had no impact on the histological effects or prognosis in the HCRT groups.

The *p21* gene is one of the major transcriptional targets of *p53* and is a major negative regulator of the G1 checkpoint by binding to and inhibiting the activities of most cyclin/CDK complexes (25). The results for p53 expression combined with p21 expression can predict the response to CRT or radiation in rectal and pancreatic cancer (27, 28). Nakamura *et al.* (29) have reported that p21 expression is potentially useful for predicting the response to chemoradiotherapy and survival of patients with advanced ESCC. In this study, positive staining for p21 was closely associated with a favorable response for HCRT and a multivariate analysis revealed it to be an independent predictive factor of marked effectiveness.

The immunohistochemical expression of the p53 protein was once considered to be nearly equal to the extent of mutation of the *p53* gene. Recently, however, it has been revealed that some other mechanisms to prolong the lifetime of the p53 protein, such as post-translational modifications, exist. Therefore, p53 expression combined with p21 expression has been used for immunohistochemical determination of the wild-type *p53* function. Two different groups have reported that a combination of p53 and p21 expression was useful in predicting the effect of chemotherapy in patients with ESCC (30, 31). According to these reports, the p53–/p21+ phenotype, which preserved the wild-type *p53* function, was associated with favorable effects of chemotherapy. In this study, we demonstrated that the combination of p53 and p21 expression significantly correlated with the histological response to HCRT. None of the 10 patients with the p53+/p21– phenotype showed a grade 3 response. These results suggest that an immunohistochemical analysis of p53 and p21 expression in biopsy samples is useful in estimating the effects of HCRT.

The prognostic significance of the p53 and p21 expression in patients with ESCC preoperatively treated with CRT or chemotherapy has been reported (13, 15). Since the number of patients involved in this study was small, additional studies with squamous cell carcinoma of the esophagus will be needed to verify these results. These findings suggest that not only p21 expression, but also the combinated evaluation of p53 and p21 expression, together with the biopsy can predict the histological effectiveness of HCRT.

Conclusion

An immunohistochemical analysis of p53 and p21 expression in biopsy samples is thus considered to be useful for predicting the effect of HCRT in patients with ESCC. Utilizing such pretreatment information will enable us to select the optimal treatment modality for individual cases.

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