Expression of p21\textsuperscript{WAF1/Cip1} in the p53-dependent Pathway Is Related to Prognosis in Patients with Advanced Esophageal Carcinoma\textsuperscript{1}

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ABSTRACT

The proteins p53 and p21 are important components that regulate G\textsubscript{1}-S transition through the cell cycle. We immunohistochemically investigated p53 and p21 expression in 111 patients with esophageal squamous cell carcinoma. We also evaluated whether the expression of either of these proteins is a prognostic factor according to the p53-dependent and -independent pathways. The positive rates of p53 and p21 expression were 42.8 and 43.2\%, respectively. Clinicopathological findings according to p53 and p21 expression did not differ significantly. The 5-year-survival rates between p21 positive and negative expression did not differ significantly in the p53-positive group. In the p53-negative group, the 5-year-survival rate of patients with p21-positive expression was 22.9\%, which was significantly better than that of patients with p21-negative expression (12.7\%; \textit{P} < 0.05). Multivariate analysis revealed that p21 expression in the p53-dependent pathway was an independent prognostic factor. Accordingly, the prognostic values of p21 expression between the p53-dependent and -independent pathways differed. Examination of p21-positive expression in the p53-dependent pathway will help to estimate the favorable prognosis of patients with advanced esophageal carcinoma.

INTRODUCTION

The tumor suppressor gene p53 is located on the short arm of chromosome 17. The product of wild-type p53 prevents uncontrolled cellular proliferation after DNA damage via a G\textsubscript{1} arrest checkpoint (1, 2). Mutation of the p53 gene is one of the most frequent genetic lesions associated with cancer, including esophageal cancer (3, 4). The p21 protein, which is encoded by the WAF1/Cip1 gene, is a downstream target effector of wild-type p53, which transcriptionally activates p21 (5, 6). The relationship between p21 expression and tumors in the gastrointestinal tract has been reported. Furthermore, p21 expression is an important prognostic factor in various organs (7–10). The p53-dependent expression of p21 plays a central role in cell growth regulation and apoptosis. p21 can be induced in a p53-independent manner (11, 12). Understanding p53 and p21 protein expression is important for gene function in malignant tumors. Both p53-dependent and -independent pathways must be examined when considering p21 expression.

Although p53 gene mutation and p53 protein accumulation are common in esophageal cancer, their clinical significance is controversial (13, 14). In this study, we examined p53 and p21\textsuperscript{WAF1/Cip1} (p21) expression in advanced esophageal squamous cell carcinoma according to these protein accumulation detected by immunohistochemical methods. The aim of this study was to investigate the relationship between p53 and/or p21 expression and to evaluate whether their expression is a prognostic factor according to p53-dependent and -independent pathways.

PATIENTS AND METHODS

Patients. One hundred and eleven consecutive patients with advanced carcinoma of the esophagus underwent esophagectomy with lymph node dissection at Kagoshima University Hospital between 1987 and 1991. The ages of 103 male and 8 female patients ranged from 41 to 81 years (mean, 64.0 years), and none of them had received radiation therapy or chemotherapy before surgery. All patients were followed up after discharge by an X-ray examination and studies of tumor markers (squamous cell carcinoma antigen, carcinoembryonic antigen) every 1–3 months, computed tomography every 3–6 months, and ultrasonography every 6 months. Bronchoscopic and endoscopic examinations were performed when necessary. Follow-up data after surgery were obtained from all patients, with a median follow-up period of 28 months (range, 2–135 months).

On the basis of the Tumor-Node-Metastasis classification of the International Union Against Cancer (15), the 111 patients were divided into 18 with T\textsubscript{2} tumors, 66 with T\textsubscript{3} tumors, and 27 with T\textsubscript{4} tumors. Seventeen tumors were located in the upper third of the esophagus, 59 in the middle third, and 37 in the lower third. Pathologically, all of the tumors were squamous cell carcinoma (51 well-differentiated, 42 moderately differentiated, and 18 poorly differentiated). Lymph node metastases were present in 77 of 111 of the patients (69.4\%). All of the M1 tumors were due to distant lymph node metastases.

Immunohistochemical Staining. Sections were immunohistochemically stained using avidin-biotin-peroxidase as described. In brief, after deparaffinizing in xylene and dehydrating

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in ethanol, the sections were heated in citrate buffer (0.01 M, pH 6.5) at 120°C for 10 min to retrieve antigen, then incubated with either the primary monoclonal antibody anti-p21WAF1/Cip1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-p53 (Transduction Laboratories, Lexington, KY) overnight at 4°C. The sections were then incubated with biotinylated anti-mouse IgG and avidin-biotin-peroxidase (Vector Laboratories, Burlingame, CA) and visualized using diaminobenzidine tetrahydrochloride. The negative control group contained 1% BSA instead of primary antibody.

The immunohistochemical expression of p53 and p21 was evaluated by independent two observers (Sh. N. and Sa. N.). The five representative fields were examined, and a total of 1000 tumor cells (200 for each field) were counted under the microscope with a high power (×200) objective. A distinct nuclear immunoreaction for p53 and p21 was judged positive. When 10% of the cancer cells were positive for nuclear staining, the specimen was scored as positive (Figs. 1 and 2).

The tumors were divided according to p53 expression into positive (58.7% of all patients) and negative (41.3% of all patients). Table 1 shows that the relationship between p53 expression and the clinicopathological findings of age, sex, tumor location, depth of tumor, histology, and pN did not differ significantly. However, the rate of advanced tumors in pM1 and the stage were significantly higher in p53-positive tumors than in p53-negative tumors. The positive rate of p21 was 43.2% (48 of 111) for all patients. Clinicopathological findings and p21 expression did not significantly differ, and neither did p53 and p21 expression (Table 1).

### RESULTS

#### Expression of p53 and p21

The rate of p53 expression was 42.8% (47 of 111) for all patients. The relationship between p53-positive and -negative expression and the clinicopathological findings of age, sex, tumor location, depth of tumor, histology, and pN did not differ significantly. However, the rate of advanced tumors in pM1 and the stage were significantly higher in p53-positive tumors than in p53-negative tumors. The positive rate of p21 was 43.2% (48 of 111) for all patients. Clinicopathological findings and p21 expression did not significantly differ, and neither did p53 and p21 expression (Table 1).

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#### Statistical Evaluation

Statistical analysis was performed by the χ² test and the Kruskal-Wallis test for group differences. The Kaplan-Meier method was used, and analysis was also evaluated by the log-rank test. Prognostic factors were tested by univariate and multivariate analyses (proportional hazard regression model). P < 0.05 was considered statistically significant.
the p53-positive \( [p53(+) \] and p53-negative \( [p53(-) \] groups. With regard to clinicopathological findings in the p53(+) group, there was no significant relationship between the p21(+) and p21(-) group. Similarly, the relationship among clinicopathological findings in the p53(-) group was not significant, irrespective of p21 expression. The categories of M1 and stage were more advanced in the p53(+)p21(+) group than in the p53(-)p21(+) or p53(-)p21(-) groups (Table 2).

**Clinical Outcome**

**The Prognosis of p53 Expression or p21 Expression.** All patients were followed up, and 8 died of postoperative complications within 30 days, leaving 103 patients for survival analysis. The total number of 5-year survivors was 19, and 15 of these patients are still alive. According to the p53 expression, the 5-year survival rates of the p53(+) and p53(-) patients were 14.9 and 18.8%, respectively \( (P = 0.19) \). Concerning the p21 expression, the 5-year survival rates were 22.9% for patients with p21-positive tumors and 12.7% for those with p21-negative tumors \( (P = 0.08) \).

**The Prognosis of p21 Expression in the p53-dependent or -independent Pathway.** In the p53(+) group, the 5-year survival rates of the p21(+) and p21(-) patients were 19.1 and 11.5%, respectively. There was no significant difference between the two groups. On the other hand, in the p53(-) group, the 5-year survival rate of the p21(+) patients (25.9%) was significantly higher than that of the p21(-) patients (13.5% \( ; P < 0.05 \); Fig. 3).

**Prognostic Significance of p21 Expression in the p53-dependent Pathway.** Factors related to prognosis within the p53(-) group were evaluated by univariate and multivariate analysis (Table 3). According to the univariate analysis, age, histology, depth of tumor invasion, lymph node metastasis, lymphatic invasion, venous invasion, and p21 expression were related to prognosis. However, age, stage, and p21 expression

| Table 2 Relationship between p21 expression and clinicopathological findings according to p53 expression |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| p53(+) p21(+) (n = 21, %) | p53(+) p21(−) (n = 26, %) | p53(−) p21(+) (n = 27, %) | p53(−) p21(−) (n = 37, %) | P |
| Tumor location | Tumor depth | Histology | pN | pM | Stage |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Upper | 4 (19.0) | 3 (11.1) | NS |
| Middle | 9 (42.9) | 14 (51.9) | NS |
| Lower | 8 (38.1) | 10 (37.0) | NS |
| T2 | 4 (19.0) | 5 (18.5) | NS |
| T3 | 11 (52.4) | 18 (66.7) | NS |
| T4 | 6 (28.6) | 4 (14.8) | NS |
| Well | 9 (42.9) | 12 (44.4) | NS |
| Moderate | 9 (42.9) | 12 (44.4) | NS |
| Poor | 3 (14.2) | 3 (11.2) | NS |
| pN0 | 3 (14.3) | 12 (44.4) | NS |
| pN1 | 18 (85.7) | 15 (55.6) | NS |
| pM0 | 8 (38.1) | 20 (74.1) | P < 0.05 |
| pM1 | 13 (61.9) | 11 (42.3) | P < 0.05 |
| II A | 3 (14.3) | 10 (37.0) | 6 (16.2) |
| II B | 2 (9.5) | 1 (3.7) | 1 (2.7) |
| III | 3 (14.3) | 9 (33.3) | 19 (51.4) |
| IV | 13 (61.9) | 7 (26.0) | 11 (29.7) |

\( ^{a} \) NS, not significant.  
\( ^{b} \) \( P < 0.05 \).

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*Fig. 3* A comparison of 5-year survival curves in the p53-dependent pathway between patients with p21-positive tumors (thick lines) and p21-negative tumors (broken lines) is shown. The 5-year survival rate of the p21-positive group is significantly higher than that of the p21-negative group \( (P < 0.05) \).
were independent prognostic factors, according to the multivariate regression analysis.

DISCUSSION

The tumor suppressors p53 and p21 are among the most important known gene products involved in cell growth arrest, differentiation, and senescence. The p21 protein is a cyclin-dependent kinase inhibitor that is a downstream effector of p53-dependent cell cycle regulation (19, 20). Although to date, some reports have addressed the relationship between poor prognosis and p53 overexpression in esophageal carcinoma (21, 22), others did not find such correlation (23, 24). Sarbia et al. (21) reported recently that p21-positive expression correlates with the poor prognosis of patients with esophageal carcinoma. In the present study, we compared p53 and p21 protein expression by p53-dependent and -independent pathways.

In this series, p21 expression and clinicopathological findings were not correlated. Histologically, p21 plays a role in regulating the cellular differentiation of various tissues. Heterogeneous components were identified in advanced esophageal carcinoma; some areas were well differentiated, and others were moderately or poorly differentiated. However, we did not find a significant difference between p21 expression and histology. Furthermore, p21 and p53 expression did not correlate in the present study. Factors such as transforming growth factor β (26), cyclin D1 (27), and bcl-2 (28) might influence p21 expression. Barboule et al. (29) also reported that the absence of p21 expression does not correlate with wild-type p53 in ovarian cancer. However, the p53(+)p21(+) tumors were more advanced than those tumors of the p53(−) group. Tumors that expressed p21 progressed faster via the p53-independent than the p53-dependent pathway, suggesting that p53 mutant tumors are highly malignant, even in the presence of p21 protein.

Age, histology, depth of tumor invasion, lymph node metastasis, lymphatic invasion, and venous invasion were important prognostic factors among patients with advanced esophageal carcinoma in the present study. Lymph node metastasis or the number of involved nodes are useful prognostic factors (30, 31). In this study, although the 5-year survival rate of p21-positive patients (22.9%) tended to be better than that of p21-negative patients (12.7%), the difference did not reach significance (P = 0.08). However, when patients were divided according to p53 expression, i.e., the p53-dependent or p53-independent pathway, the prognosis was better in p21-positive than in p21-negative patients in the p53-dependent group. Furthermore, p21 expression was an independent prognostic factor in the wild-type p53 pathway by multivariate analysis. Sarbia et al. (25) reported that the patients with p21 positivity had a poorer prognosis, compared with those with p21 negativity, when p21 expression was categorized into <50% positive cells and ≥50% positive cells. This result was different from ours, which was caused by different criteria of p21 expression. Although the prognosis of patients with advanced esophageal carcinoma remained poor, the examination of the pathway of p53-dependent p21 protein expression is useful for predicting prognosis in addition to the conventional staging system.

We conclude that the prognostic value of p21 expression differed between p53-dependent and -independent pathways. An examination of p53 and p21 expression is useful when estimating the prognosis of patients with advanced esophageal carcinoma.

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