

# p21 Expression as a Predictor for Favorable Prognosis in Squamous Cell Carcinoma of the Lung

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## ABSTRACT

Although p21 WAF1/Cip1 expression has been detected immunohistochemically in non-small cell lung cancer (NSCLC), the associations between p21 expression and clinical characteristics are unknown. To determine the association between p21 expression and clinical features, p21 expression was immunohistochemically analyzed in paraffin-embedded tumor samples from 137 patients with curatively resected NSCLC. p21 expression, indicating normal p21 function, was detected in 48 (35.0%) of the 137 patients with curatively resected NSCLC and was detected more frequently in patients with stage I or II disease (40.2%) than in those with stage IIIA disease (22.5%;  $P = 0.0483$ ). There was no difference in the positive rate between squamous cell carcinoma [SCC; 15 of 48 (31.3%)] and adenocarcinoma [30 of 77 (39.0%)]. For SCC, patients with tumors expressing p21 survived longer than did those with tumors negative for p21 expression; however, the corresponding survival time was not significant for adenocarcinoma. On the other hand, p53 expression, detected in 58 (42.3%) of these patients, did not act as any predictor for prognosis in either SCC or adenocarcinoma. Our findings suggest that the presence of p21 expression is associated with favorable prognosis in SCC and may be useful in obtaining candidates for adjuvant therapies from among patients with SCC.

## INTRODUCTION

As in Western countries, lung cancer will in the future be the leading cause of cancer death in Japan. NSCLC<sup>2</sup> comprises approximately 85% of all lung cancers, and with few exceptions only those patients with NSCLC who undergo curative surgery obtain long-term survival. In general, pathological TNM stage is the most reliable prognostic factor in predicting the outcome of treatment. However, to improve the outcome of treatment for NSCLC, other prognostic factors such as molecular markers should be established.

Mutation of the p53 gene is the most common genetic alteration in a wide spectrum of tumors (1). p53 mutations are present at frequencies of approximately 50% in NSCLCs and 75% in small cell lung cancers (2-5). Although p53 abnormality may play an important role in the development of lung cancer, there is still much conflict regarding its relationship to malignant potential of lung cancer tumors (6-17). The prognostic significance of overexpression of p53 protein in particular is controversial. Several studies have shown that p53 overexpression is associated with a favorable prognosis for patients with NSCLC (14, 16, 17), whereas others have indicated that it is correlated with a poor prognosis (6). For establishment of a molecular staging system, not only p53 abnormality but other molecular factors as well are required.

p21, a CDK inhibitor and product of the WAF1 (18), CIP1 (19) or SDI1 (20) gene, has been considered a critical downstream effector in the p53-specific pathway of growth control in mammalian cells. p53 expression in response to DNA damage promotes the transcription of p21, resulting in growth arrest through inhibition of CDKs, which are required for the G<sub>1</sub>-to-S transition (21, 22). Recently, several studies have shown that p21 can also be induced by p53-independent pathways (23-25) and that p21 expression is associated with tumoral differentiation (26, 27). Although p21 overexpression has been reported to be associated with short disease-free survival for patients with breast cancer (28), there have been no studies demonstrating any prognostic value of p21 expression in patients with NSCLC. We investigated both p21 and p53 expressions in a larger series of curatively resected NSCLCs to evaluate the correlation between them and survival and clinical characteristics and to establish a molecular staging system.

## PATIENTS AND METHODS

**Patients.** A total of 137 patients with histologically confirmed NSCLC were studied. All patients had undergone curative surgery at Osaka Prefectural Habikino Hospital between September 1988 and December 1992 and had stage I-III A

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<sup>2</sup> The abbreviations used are: NSCLC, non-small cell lung cancer; CDK, cyclin-dependent kinase.

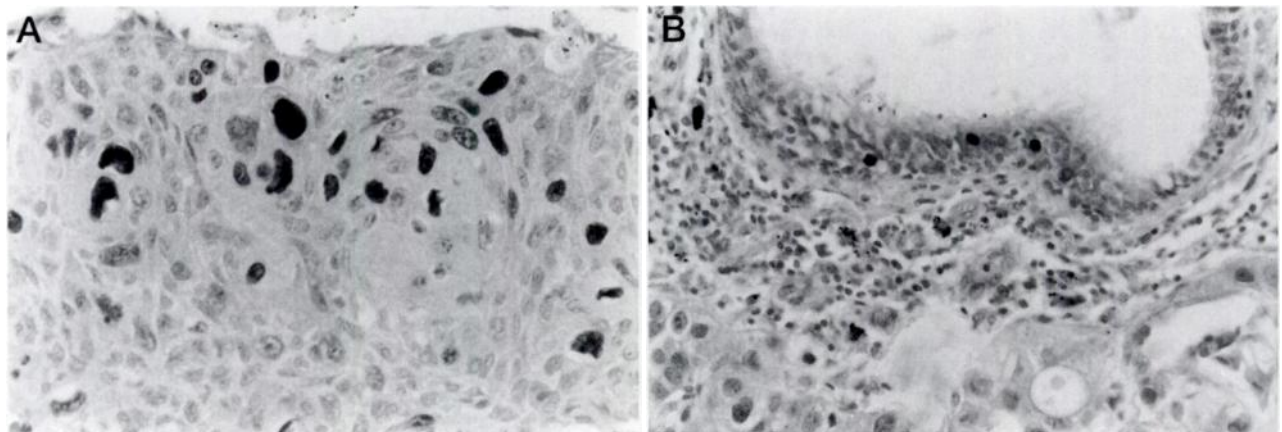


Fig. 1 Immunostaining of squamous cell carcinoma of the lung with an anti-p21 antibody. Nuclei of squamous cell carcinoma cells were clearly stained (A). p21 was also expressed in a few cells in normal tissues surrounding carcinoma (B). Magnification,  $\times 100$ .

Table 1 Patient characteristics

	Number of patients
Male/female	99/38
Age	
$\leq 67$	66
$> 67$	71
Performance status	
0 or 1	132
2	5
Histology	
Squamous cell carcinoma	48
Adenocarcinoma	77
Large cell carcinoma	9
Adenosquamous	3
Pathological stage	
I	68
II	29
IIIA	40

disease (Ref. 29; Table 1). None of the patients received treatment before surgery, whereas 64 received adjuvant chemotherapy or radiotherapy after surgery. The median duration of follow-up for living patients was 1591 days.

**Immunohistochemical Analysis.** p21 immunoreactivity was evaluated in paraffin-embedded tissues from 137 patients using the EA10 monoclonal antibody (Oncogene Science, Cambridge, MA), as described by Marchetti *et al.* (27). Briefly, paraffin sections treated with microwave retrieval were incubated for 1 h at room temperature with the primary antibody (1:50 dilution) and processed with the labeled streptavidin biotin assay method (30). p53 immunoreactivity was also evaluated for these 137 patients using the DO7 monoclonal antibody (DAKO A/S, Glostrup, Denmark; Ref. 17). Sections of resected lung cancer known to express p21 or p53 were used as positive controls. Cells exhibiting distinct nuclear staining were considered positive for p21 and p53. Two observers (T. K. and Y. H.) without knowledge of patient outcome independently evaluated the percentage of immunoreactive nuclei, and the mean of the percentage numbers was used. Cutoff values for both p21 and p53 were defined as 5% in accordance with the previous studies

Table 2 Results of p21 assay

	No. of patients	p21 positive (%) <sup>a</sup>	P <sup>b</sup>
Sex			
Male	99	32 (32.3)	0.2826
Female	38	16 (42.1)	
Age			
$\leq 67$	66	23 (34.8)	0.9645
$> 67$	71	25 (35.2)	
Performance status			
0 or 1	132	47 (35.6)	0.4728
2	5	1 (20.0)	
Histology			
Squamous cell carcinoma	48	15 (31.3)	0.3824
Adenocarcinoma	77	30 (39.0)	
Large cell carcinoma	9	2 (22.2)	
Adenosquamous	3	1 (33.3)	
Pathological stage			
I or II	97	39 (40.2)	0.0483
IIIA	40	9 (22.5)	
Adjuvant chemotherapy			
+	58	22 (37.9)	0.5429
-	79	26 (32.9)	

<sup>a</sup>p21 expression was considered positive if at least 5% of reactive nuclei were observed.

<sup>b</sup> $\chi^2$  and Fisher's exact test.

of Marchetti *et al.* (27) and with our previous studies (17), respectively.

**Statistical Analysis.** The significance of association between variables was tested by the  $\chi^2$  and Fisher's exact test. Differences between survival curves, which were estimated by the Kaplan-Meier method (31), were evaluated using the log-rank test. Cox proportional hazards models were used to identify which independent factors jointly have significant effects on survival (32).

## RESULTS

**p21 Expression.** Fig. 1 shows immunostaining of specimens of resected tumors with an anti-p21 antibody. Nuclei of squamous cell carcinoma were clearly stained (Fig. 1A). p21

Table 3 Results of p53 assay

	No. of patients	p53 positive(%) <sup>a</sup>	P <sup>b</sup>
Sex			
Male	99	41 (41.4)	0.7245
Female	38	17 (44.7)	
Age			
≤67	66	24 (36.3)	0.1726
>67	71	34 (47.9)	
Performance status			
0 or 1	132	57 (43.2)	0.3956
2	5	1 (20)	
Histology			
Squamous cell carcinoma	48	25 (52.1)	0.1134
Adenocarcinoma	77	29 (37.7)	
Large cell carcinoma	9	3 (33.3)	
Adenosquamous	3	1 (33.3)	
Pathological stage			
I or II	97	41 (42.3)	0.9801
IIIA	40	17 (42.5)	
Adjuvant chemotherapy			
+	58	23 (40.0)	0.5864
-	79	35 (44.3)	

<sup>a</sup> p53 expression was considered positive if at least 5% of reactive nuclei were observed.

<sup>b</sup>  $\chi^2$  and Fisher's exact test.

was also expressed in a few cells in normal tissues surrounding carcinoma (Fig. 1B). This is consistent with a report of Marchetti *et al.* (27). As shown in Table 2, frequencies of p21 expression in squamous cell carcinomas and adenocarcinomas were 31.3% and 39.0%, respectively. No correlation was found between the presence of p21 expression and sex, age, or histological subtype. However, p21 expression occurred more frequently in stage I or II disease (40.2%) than in stage IIIA disease (22.5%;  $P = 0.0483$ ).

p53 expression was found in 58 of the 137 patients (42.3%; Table 3). No clinical characteristics were correlated with the presence of p53 expression.

In a univariate analysis for the group including all histological types, the difference in survival between the p21-positive and -negative groups was not significant ( $P = 0.1449$ ; Fig. 2A). We also performed analyses for subgroups of patients with squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma patients with tumors expressing p21 survived significantly longer than did those with tumors not expressing p21 ( $P = 0.0019$ ; Fig. 2B). On the other hand, there was no significant difference in survival between p21-positive and -negative groups for patients with adenocarcinoma ( $P = 0.2217$ , Fig. 2C). In a multivariate analysis, however, p21 status had no independent prognostic value for the group of all patients and for the group with squamous cell carcinoma.

In contrast, no significant difference in survival was found between p53-positive and -negative groups for either patients with squamous cell carcinoma ( $P = 0.1055$ ) or those with adenocarcinoma ( $P = 0.7421$ ; data not shown).

## DISCUSSION

In the present series of NSCLCs, p21 expression was detected less frequently than in the study of Marchetti *et al.* (27),

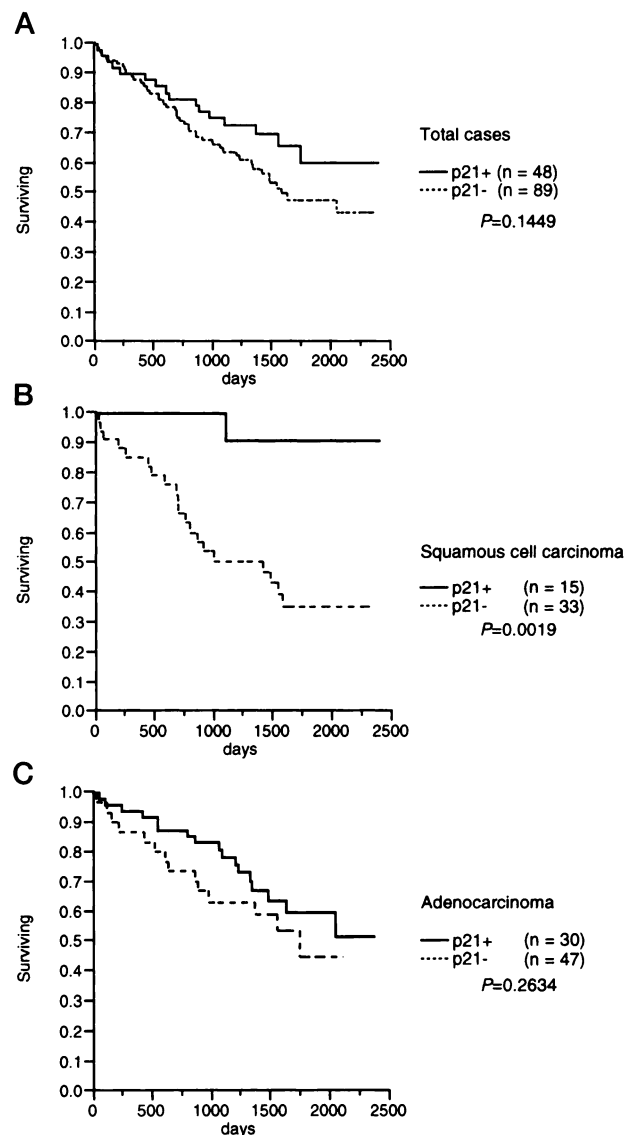


Fig. 2 Survival curves according to p21 for total cases (A), squamous cell carcinoma (B), and adenocarcinoma (C).

although immunohistochemistry was performed in the two studies using similar materials and methods. However, the sample size in the study by Marchetti *et al.* (27) was small, and they found no correlation between p21 expression and clinical features.

p21 expression was associated significantly with earlier (I or II) stage disease not only for the group of all patients but also for those with squamous cell carcinoma. The finding of decreased p21 expression in advanced disease suggests that functional loss of p21 may occur during tumor growth, resulting in accelerated tumor growth via skipping of G<sub>1</sub> arrest. This hypothesis should be tested by immunostaining of biopsy specimens obtained chronologically from the same patients.

The present study demonstrated clearly that patients with squamous cell carcinoma expressing p21 survived significantly

longer than did those with tumors not expressing p21. In adenocarcinoma, however, the corresponding survival time was not significant, resulting in no significant difference in survival time between groups divided according to p21 in all NSCLCs. On the other hand, p53 expression did not act as any important predictor for prognosis in either squamous cell carcinoma or adenocarcinoma in this study. Our previous study (17) obtained a similar finding, that the difference in survival between p53-positive and -negative groups was not statistically significant.

p21 expression is reported to be associated with tumor differentiation in NSCLC (27) and with histological grade in breast cancer (33). However, both the present study and a study of colorectal cancer (34) revealed no correlation between p21 expression and such pathological parameters. These findings suggest that regulation of p21 expression may differ among types of cancer or races.

p21 has been considered a critical downstream effector in the p53-dependent pathway of growth control. However, several studies have shown that p21 can also be induced by p53-independent pathways in p53-null cell lines with anticancer drugs (23), induction of differentiation (35, 36), and growth arrest inducers (24). Thus, several mechanisms may be involved in p21 induction. Although p21 gene mutation might be one of these mechanisms, to the best of our knowledge, there have been no reports of p21 gene mutation in any types of cancer, including NSCLC (37–40).

In the present series, p21 expression was detected more frequently in tumors with p53 expression. To determine the p21 induction pathways, additional studies should be performed to examine the correlation between p21 and other candidate molecules involved in cell-cycle regulation. For example, epidermal growth factor receptor (41) and transforming growth factor  $\beta$  (42, 43), levels of which are correlated with tumor grade and disease progression in breast cancer, are reported to up-regulate p21 expression. It may also be worthwhile to examine the relationships among members of the p21 CDK inhibitor family, such as p27 (44) and p57 (45). These molecules have NH<sub>2</sub>-terminal domains with sequence similarity to p21 and CDK inhibitory activity (46).

In conclusion, our findings suggest that the presence of p21 expression is associated with a favorable prognosis for squamous cell carcinoma of the lung. Patients with squamous cell carcinoma without p21 expression may be good candidates for adjuvant therapies, although additional studies examining the relationship between p21 status and other cell cycle regulators or growth factors should be performed to verify that they are.

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