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Prognostic Significance of p53 and *ras* Gene Abnormalities in Lung Adenocarcinoma Patients with Stage I Disease after Curative Resection

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We investigated the prognostic significance of p53 gene abnormalities and *ras* gene mutations in patients with curatively resected stage I lung adenocarcinoma. Formalin-fixed and paraffin-embedded tissues were obtained from 30 patients who had undergone curative resection for stage I lung adenocarcinoma. Abnormalities of the p53 gene were detected using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis and immunohistochemistry and *ras* mutations were detected using PCR-restriction fragment length polymorphism (RFLP) analysis. Both univariate and multivariate analyses were performed to assess the relationship between the presence of abnormalities of these genes and the patients' disease-free survival. Eleven tumors (37%) had mutated p53 sequences and 11 (37%) showed p53 overexpression. A total of 15 tumors (50%) had p53 gene abnormalities and the concordance rate was 73%. Seven tumors (23%) showed mutated *ras* sequences. The univariate analysis revealed that the disease-free survival of patients with any p53 abnormality was shorter than that of those without abnormalities ($P=0.02$, generalized Wilcoxon test), and survival of those with p53 protein overexpression was more significantly shorter ($P=0.003$, generalized Wilcoxon test). Multivariate analysis using the Cox proportional hazards model indicated that the presence of p53 abnormalities was a significantly ($P=0.01$) unfavorable prognostic factor. There was no significant correlation between the presence of *ras* mutation and survival. These results suggest that analysis of the p53 gene may be helpful for the selection of high-risk patients for clinical trials of adjuvant therapy for stage I lung adenocarcinoma.

Key words: p53 gene — *ras* gene — Stage I lung adenocarcinoma — Prognostic factor

Surgical resection is considered to be the best therapeutic choice for patients with stage I non-small-cell lung cancer (NSCLC). However, about 30 to 40% of such patients who have undergone potentially curative resection will be dead due to cancer recurrence within 5 years of resection[1,2] and the disease has been found to recur more often in the distant organs of patients with non-squamous than squamous disease.[3] Therefore, adjuvant systemic treatments, including chemotherapy, are probably necessary for patients with stage I NSCLC. The results of the study conducted at Helsinki University Central Hospital support this suggestion.[4] Another study conducted by the Lung Cancer Study Group demonstrated no advantage of adjuvant treatment, but the authors considered that the most likely explanations for the lack of treatment efficacy were poor compliance with the protocol and inactivity of the regimen.[5] In order to avoid poor compliance in future studies, we think it would be helpful to identify patients who are at high risk of recurrence, even though they have stage I disease. Identifying unfavorable prognostic factors, other than the TNM system, may stimulate patients to receive adjuvant treatment, and encourage physicians to participate in clinical trials.

Although several prognostic factors other than the TNM system have been proposed,[6,7] none was considered to be related directly to tumor carcinogenesis or progression. Recent molecular biological developments have led us to suggest that carcinogenesis/progression is a multiple process involving a cascade of genetic changes and that alterations in oncogenes and tumor suppressor genes may be the most important among such changes.[8]

To date, the p53 and *ras* genes have been found to be the most frequently mutated genes in adenocarcinoma of the lung. The relationship between abnormalities of these genes and patients' prognoses have been discussed in several reports.[9–22] However, none of these deals extensively with the relationship between these gene abnormalities and stage I disease, for which identification of unfavorable prognostic factors can have a considerable impact on physicians' decision-making about follow-up for patients; everyone knows patients with stage III/IV disease have poor prognoses.

Therefore, the objective of this study was to investigate the prognostic significance of p53 abnormalities (mutation and overexpression) and *ras* gene mutations in 30

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stage I lung adenocarcinoma patients who had undergone curative resection.

MATERIALS AND METHODS

Selection of patients Tumor tissues for this study were obtained from 30 out of 31 consecutive patients with operable stage I lung adenocarcinoma, classified according to the International Staging System for Lung Cancer,[23] who underwent curative resection at Hiroshima University Hospital from 1978 to 1990. The sample from one patient was not available for this study. The patients' ages ranged from 45 to 73 years (yr) (median, 62 yr) and there were 13 men and 17 women. All the tumor tissues were formalin-fixed and paraffin-embedded. Clinical follow-up data was available for all the patients (median follow-up period, 60 months). They were followed up every month for 2 years after surgery and every 3 to 6 months thereafter. Cancer recurrence was diagnosed clinically and confirmed by radiographic evidence of disease. The days of recurrence and death were known for all the patients.

Histological analysis and DNA extraction All the paraffin blocks were cut into sections 5 μm and 10 μm thick. Some were stained with hematoxylin and eosin (H-E), some were used for DNA analysis and others were used for immunohistochemical analysis. The tumor lesions from 2 or 3 unstained 10-μm sections were cut out by comparing them with an adjacent H-E-stained section and placed in a 1.5-ml microfuge tube. The excised lesions were deparaffinized with xylene/ethanol and the genomic DNA was extracted using proteinase K and sodium dodecyl sulfate (SDS).

PCR-DGGE analysis for p53 mutations in exons 5–9 The oligonucleotide primers used to amplify the p53 genes were synthesized on the basis of published sequences,[24, 25] as follows:

exon 5: 5′-TGCCCTGACTTTCAACTCTC-3′
5′-TGGGCAACCAGCCCTGTCGT-3′ (266 bp)
exon 6: 5′-TGGTTGCCCAGGGTCCCCAG-3′
5′-GGAGGGCCACTGACAACCA-3′ (223 bp)
exon 7: 5′-CTTGCCACAGGTCTCCCCAA-3′
5′-AGGGGTCAGCGGCAAGCAGA-3′ (237 bp)
exons 8–9: 5′-TTGGGAGTAGATGGAGCCT-3′
5′-AGTGTTAGACTGGAAACTTT-3′ (445 bp).

The second step PCR using the following internal primers was employed for exons 8–9:

exons 8–9: 5′-TGGGACAGGTAGGACCTGAT-3′
5′-ACTTGATAAGAGGTCCCAAG-3′(393 bp).

The genomic DNA (100 ng) extracted from the paraffin blocks was amplified in a 50-μl reaction mixture containing 200 μM each deoxynucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.25 μM each primer and 1 unit of *Taq* DNA polymerase (Wako, Osaka). A program temperature control system (Astec, Fukuoka) was used for 40 cycles of: 60 s at 95°C, 60 s at 60°C (exon 5), 63°C (exons 6 and 7) or 58°C (exons 8–9) and 90 s at 74°C.

Denaturing gradient gel electrophoresis (DGGE) analysis, following the method of Takahashi *et al.*,[26] of the polymerase chain reaction (PCR)-amplified genomic DNA fragments was carried out to screen them for mutations in exons 5 through 9 of the p53 gene. The PCR products were electrophoresed at 60°C on a 6.5% polyacrylamide gel with a linearly increasing denaturant concentration gradient (100% denaturant = 7 M urea and 40% formamide) as follows: exons 6 and 7, 10 to 40% denaturant at 240 V for 3 h; exons 5 and 8–9, 20 to 60% denaturant at 150 V for 12 h. The gels were stained with ethidium bromide and illuminated with an ultraviolet light transilluminator. Any abnormal bands revealed by DGGE analysis were cut out from the gel, reamplified by the PCR and sequenced using a double-stranded DNA cycle sequencing system, according to the manufacturer's instructions (GIBCO BRL). The primers used to amplify each fragment were used as sequencing primers.

Immunohistochemistry The histological sections from the formalin-fixed and paraffin-embedded tissues were pretreated by microwave-oven processing. The antibody against the p53 gene product we used was DO-7 (Novocastra Laboratories Ltd., Newcastle, England), which reacts with both mutant and wild-type forms of p53.[27] Immunoperoxidase staining was performed using the streptoavidin-biotin method as follows: the endogenous peroxidase activity was blocked with 0.3% H_2O_2 in absolute methanol, the nonspecific binding sites were blocked with normal serum and the sections were incubated with the DO-7 antibody for 18 h at 4°C. The immunoreactive compounds were detected using a Stravigen BSA-PO kit (BioGenex Laboratories, San Ramon, CA) with diaminobenzidine (Sigma, St. Louis, MO) as a chromogen, and the nuclei were counterstained with methyl-green. The immunoreactivity with DO-7 was evaluated as follows: positive, the population of positive cells comprised more than 5% of the carcinoma cells; negative, the population of positive cells comprised less than 5% (including none).

Amplification and detection of *ras* gene mutations The PCR was carried out using H-*ras* codon 12 and K-*ras* codons 12 and 13 with the following mismatched primers, as described elsewhere[28]:

H-*ras* 12: 5′-GACGGAATATAAGCTGGTGG-3′
5′-TGGATGGTCAGCGCACTCTT-3′ (63 bp)
K-*ras* 12: 5′-CATGTTCTAATATAGTCACA-3′
5′-CAAGGCACTCTTGCCTACGGC-3′ (110 bp)
K-*ras* 13: 5′-CATGTTCTAATATAGTCACA-3′
5′-TATCGTCAAGGCACTCTTGCCTAGG-3′ (116 bp).

The genomic DNA (100 ng) extracted from the paraffin blocks was amplified by the PCR in a 50-μl reaction mixture containing 200 μM each dNTP, 1.5 mM $MgCl_2$, 0.5 μM each primer and 1 unit of *Taq* DNA polymerase for 40 cycles of: 60 s at 95°C, 60 s at 50°C for K-*ras* 12 and 13 and 63°C for H-*ras* 12 and 90 s at 74°C.

Mutated *ras* genes were identified using designed restriction fragment length polymorphism (RFLP) analysis (H-*ras* codon 12, *Msp* I; K-*ras* codon 12, *Ban* I; K-*ras* 13, *Hae* III), following the method of Mitsudomi *et al.*[28] Twenty microliters of the PCR product was digested with each restriction enzyme described above and incubated at 37°C for at least 2 h, then the DNA was electrophoresed through 5% polyacrylamide gel, stained with ethidium bromide and illuminated with an ultraviolet light transilluminator. In order to determine the type of mutation, samples which showed an uncleaved band were subjected to direct sequencing, as described above.

Statistical analysis The Kaplan-Meier method was used to estimate the disease-free survival (starting from the date of surgical resection to that of cancer recurrence). The recurrence rates were estimated by dividing the number of events by the person-years of exposure. The significance of differences between disease-free survival rates was evaluated using the generalized Wilcoxon test. The Cox proportional hazards model was used to identify which independent factors had a significant influence on disease-free survival. Differences at P values of less than 0.05 were considered to be statistically significant. All the statistical analyses were performed using CARE software,[29] which was provided by Dr. Seo, Department of Public Health, Hiroshima University School of Medicine.

RESULTS

We were able to amplify the genomic DNA and evaluate the p53 and *ras* gene abnormalities in all 30 tumors. The PCR-DGGE analysis of the p53 gene revealed abnormal DGGE patterns in 11 (37%) lung adenocarcinoma tumors (Fig. 1A); these were detected in exon 5 in 3, in exons 6 and 7 in one each and in exons 8 or 9, including the splicing junctions, in 5.

Eleven of the 30 tumors (37%) were p53 protein overexpression-positive (Fig. 1B). The concordance rate between the PCR-DGGE and immunohistochemical analyses of p53 abnormalities was 73%. The PCR-RFLP analysis showed that 7 tumors (23%) had mutated *ras* gene sequences (H-*ras* codon 12 in 1 and K-*ras* codon 12 in 6). Direct sequencing showed one base substitution in all 7 of the samples with *ras* gene mutation: G-to-T transversion in one and G-to-A transitions in the other 6. Five of the 7 (71%) tumors with *ras* mutations also had p53 abnormalities and 10/23 (43%) with normal *ras* genes had p53 abnormalities (no significant difference).

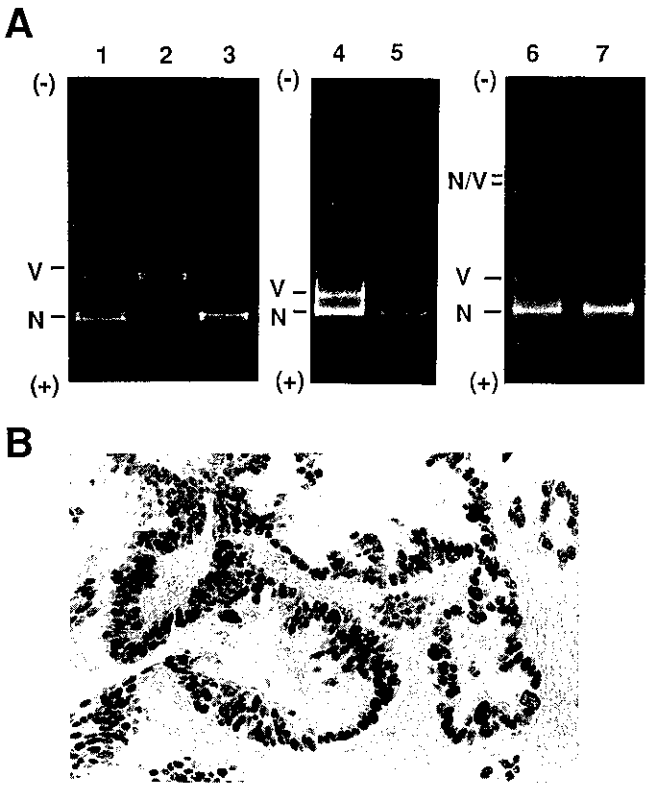

Fig. 1. A: DGGE analysis of the *p53* gene in stage I adenocarcinoma of the lung. Lanes 1–3, exon 5; lanes 4, 5, exon 7; lanes 6, 7, exons 8, 9. The PCR products were electrophoresed on a denaturing gradient gel and stained with ethidium bromide. The abnormal patterns are shown in lanes 2 (T-22), 4 (T-24), and 6 (T-13). Lanes 1 and 5 are DNA from normal lung. Lanes 3 (T-23) and 7 (T-17) are DNA from tumor tissues without mutation. N, normal homoduplex; V, variant homoduplex; N/V, normal/variant heteroduplex. B: Immunohistochemical staining for the p53 protein in case T-13, a well differentiated adenocarcinoma. Most of the tumor cells exhibit strong staining with antibody DO-7.

All the p53 and the *ras* gene abnormalities are summarized in Table I. There were no significant correlations between abnormalities of these two genes and smoking history, sex, tumor size, grade of differentiation or histological subtypes (data not shown).

Twelve patients (40%) were confirmed to have cancer recurrence after complete resection. The first recurrence sites observed were the lung in 8 patients, brain in 3 and bone in one. The recurrence rate was 0.139 in the patients with p53 abnormalities (10/15 with p53 abnormalities had recurrences) and 0.023 in the 15 without them. The Kaplan-Meier survival curves demonstrated that, compared with those with no p53 abnormalities, disease-free survival of the patients with any type of p53 abnormality was shorter ($P = 0.02$, generalized Wilcoxon test), and

Table I. Background of p53 and/or *ras*-positive Stage I Lung Adenocarcinoma Cases

Tumor No.	p53 Abnormalities				*ras* mutation	Sex	Age	Smoking index	Tumor size (mm)	Grade of differentiation	Histological subtype	Site of recurrence (months)
	DGGE	Codon	Immunostaining	Total								
1	N		P	A	N	M	68	880	25	Moderately	Papillary	Lung (10)
4	N		P	A	K12	F	72	0	30	Well	B-A	
6	Exon 5	n.d.	P	A	N	F	62	0	30	Well	Papillary	Lung (28)
7	Exon 8, 9	n.d.	P	A	N	F	63	0	28	Well	Papillary	
8	Exon 5	130 (CTC-GTC)	P	A	N	F	50	0	34	Moderately	Papillary	Brain (24)
9	Exon 6	n.d.	N	A	N	F	73	0	14	Well	Papillary	
10	N		P	A	N	F	65	0	22	Well	Papillary	Lung (48)
13	Exon 8, 9	n.d.	P	A	N	F	52	0	19	Well	Papillary	Lung (20)
14	Exon 5	160 (ATG-ATA)	P	A	K12	M	51	300	40	Well	Papillary	Lung (19)
15	Exon 8, 9	n.d.	N	A	N	F	63	300	40	Moderately	Tubular	Brain (61)
16	N		P	A	N	M	65	450	32	Moderately	Tubular	Brain (51)
19	N		N	N	H12	F	58	0	30	Moderately	Papillary	
22	Exon 5	135 (TGC-TGT)	N	A	N	M	66	0	18	Moderately	Papillary	
24	Exon 7	n.d.	N	A	K12	M	68	750	25	Moderately	Tubular	Lung (70)
26	Exon 9	331 (CAG-CAT)	P	A	K12	M	60	800	35	Moderately	Tubular	Lung (36)
27	Intron 9	splicing site	P	A	K12	F	68	0	30	Well	B-A	
28	N		N	N	K12	F	63	0	17	Moderately	Tubular	
Total	11/30		11/30	15/30	7/30							

N, negative; P, positive; n.d., not detected; A, p53 alterations; M, male; F, female; K12, K-*ras* codon12; H12, H-*ras* codon 12; B-A, bronchiolo-alveolar type.

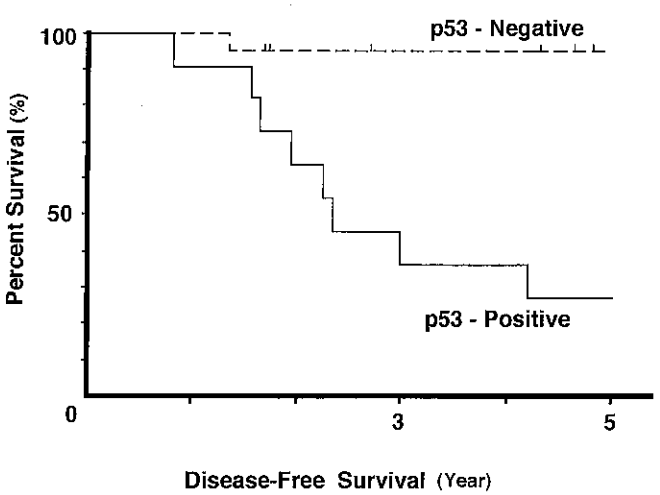

Fig. 2. Kaplan-Meier disease-free survival curves after curative resection according to the presence or absence of p53 protein overexpression.

survival of those with p53 protein overexpression was shorter with higher significance ($P = 0.003$, generalized Wilcoxon test, Fig. 2), whereas survival of those with p53 gene mutations appeared shorter, but the difference failed to reach statistical significance. Univariate analysis showed that neither age, sex, smoking history, tumor size nor tumor differentiation was a statistically significant prognostic factor (data not shown).

Table II. Cox Proportional Hazards Model for Factors Associated with Survival of Patients with Stage I Adenocarcinoma of the Lung

Variable	Relative risk	*P* value
Smoking	5.87 (smoker/non-smoker)	0.04
Tumor size	0.59 (T2/T1)	0.52
ras mutation	0.38 (positive/negative)	0.23
p53 abnormality	10.03 (positive/negative)	0.01

Tumor size (T1, T2) was classified according to the International Staging System for Lung Cancer.

The Cox proportional hazards model using 4 variables (smoking history, tumor size and *ras* and p53 abnormalities) revealed that the presence of p53 abnormalities and a history of smoking were significant unfavorable prognostic factors (Table II). The relative risk ratio for death for the patients with to those without p53 abnormalities was 10.03 ($P = 0.01$). Further multivariate analysis showed that p53 protein overexpression (detected by immunostaining) was a significant unfavorable prognostic factor ($P = 0.002$), whereas p53 mutation (detected by PCR-DGGE analysis) was not a statistically significant factor ($P = 0.08$).

DISCUSSION

In this study, we found that p53 gene abnormality, but not *ras* gene mutation, was a TNM-independent poor

prognostic factor in patients with stage I lung adenocarcinoma who underwent potentially curative resections. To our knowledge, this is the first study to examine the prognostic significance of both p53 and *ras* gene abnormalities in stage I NSCLC patients, in whom clinical oncologists need selection factors to decide whether adjuvant therapy is necessary. In addition to the characteristics of our patients, it should be noted that we used non-radio isotope (RI) methods to detect these gene abnormalities. When applying molecular biological approaches to the clinical situation, it is preferable to use non-RI methods, such as the PCR-DGGE and PCR-RFLP analysis used in this study. Both the PCR-DGGE and PCR-RFLP techniques are now considered to be easy and reliable methods for detecting p53 and *ras* gene mutations, respectively.[28, 30]

It has been recognized that p53 and *ras* genes are frequently mutated and may play important roles in the carcinogenesis and/or progression of NSCLC. With respect to p53 abnormalities, the frequency of gene mutation and/or protein overexpression in resected lung adenocarcinoma has been reported to be 28 to 82%.[9–19] RI methods were used in 5 of those studies: PCR-sequencing in one,[16] PCR-single strand conformation polymorphism (SSCP) analysis in 3[13, 17, 19] and RNase protection assay and cDNA sequencing in one.[9] Immunohistochemical analysis was used in 5 studies[10–12, 14, 15] and both PCR-SSCP and immunohistochemical analysis in one other.[18]

We observed a 50% incidence of p53 abnormality in patients with curatively resected stage I lung adenocarcinoma. As most other investigators (except Hiyoshi *et al.*,[12] who reported that the incidence of p53 abnormality in resected stage I lung adenocarcinoma was 20%) only reported the incidence without providing histological subtype information, we are unable to compare our results accurately with those of others. We performed both molecular (PCR-DGGE) and immunohistochemical analyses to estimate the p53 abnormality precisely and found the concordance rate was 73%. Marchetti and co-workers, who also carried out both molecular and immunohistochemical analyses, reported a similar concordance rate of 54%.[18] Their samples were snap-frozen, resected surgical specimens stored at $-80^{\circ}C$, and they used the PCR-SSCP method and PAb-1801 and PAb-240 monoclonal antibodies to detect p53 gene mutation protein overexpression. In this study, four cases were positive by DGGE but stained negative for p53; such false-negative cases can be explained by considering that the p53 protein overexpression is dependent upon the type of mutation. In fact, one of our case showed a silent mutation at codon 135 (TGC to TGT).

Four cases were positively stained by p53, but no abnormality was observed in DGGE analysis. It is possible that overexpression of a p53 binding protein, such as mdm-2,[31] might have inhibited p53 protein degradation, thus inducing p53 protein overexpression without gene mutation, in these four cases. However, the mechanism of non-mutational overexpression of p53 protein remains to be elucidated. In view of our result that p53 protein overexpression was related more strongly to disease-free survival than gene mutation, the functional status of the p53 gene may be correlated with its protein overexpression. However, it should be noted that this is only a hypothesis and a well-designed correlation study is needed to confirm or refute it.[32]

Our clinical follow-up data (median follow-up period, 5 yr) indicated clearly that a p53 abnormality, especially its protein overexpression, in surgical specimens was a significant poor prognostic factor. To our knowledge, 4 reports describing the relationship between p53 abnormality (gene mutation or protein overexpression) and overall survival in resected NSCLC patients have been published.[14, 15, 17, 19] However, there appear to be several problems with these reports. MacLaren *et al.*[14] investigated the prognostic significance of positive p53 protein staining, using a panel of mono- and polyclonal antibodies, in each histological type but did not consider the disease stage, and found no differences between p53 protein overexpression-positive and negative groups. Quinlan *et al.*, however, showed that survival of a p53 protein accumulation-positive group was shorter than that of a negative group, but their use of the PAb-1801 monoclonal antibody for paraffin-embedded samples appears inappropriate considering the immunoreactivity of this antibody.[27] There are two definitive studies with detailed statistical analysis,[17, 19] but without immunohistochemical analysis, in which the presence of p53 mutation was reported to be associated with a shortened overall survival in all patients and multivariate analysis revealed that such mutation was an independent unfavorable prognostic factor. Although Horio *et al.*[17] concluded that the presence of p53 mutation was associated with a shortened overall survival in patients with stage I and II disease, Mitsudomi *et al.*[19] found no statistically significant difference in such patients. They included all NSCLC histological types in this early-stage disease, whereas we selected only stage I adenocarcinoma cases, in whom distinct factors are required in order to select which patients should receive adjuvant therapy, and we confirmed that p53 protein overexpression was a TNM-independent unfavorable prognostic factor.

Mutations of the *ras* gene have been detected in about 20% of NSCLC samples and their presence, especially the K-*ras* mutation, was reported to correlate negatively with overall and/or disease-free survival.[20–22] Although the incidence of *ras* gene mutation (H-*ras* and K-*ras*) in our study was 23%, which is comparable to those in previous studies, we detected no statistically significant

difference between the disease-free survival of the *ras* gene mutation-positive and negative groups. As the PCR-RFLP method is as sensitive as PCR-SSCP and mutation-specific oligonucleotide probe hybridization,[33] we may have been unable to identify *ras* gene mutation as a poor prognostic factor due to the small sample size. However, as the hazards ratio for *ras* mutation was less than 1.0 and no tendency towards poor prognosis was apparent, *ras* mutation may not be a poor prognostic factor for potentially curatively resected stage I adenocarcinoma. We did find that patients with *ras* gene mutation also tended to have a p53 abnormality (71%, 5/7, with *ras* mutation, and 43%, 10/23, with normal *ras* genes), but this failed to reach significance.

Although the frequency of p53 or K-*ras* gene mutations has been reported to be significantly higher in smokers than non-smokers,[16, 34] we observed no relationship between a history of smoking and the presence of p53 abnormality or *ras* gene mutation. However, in this study, multivariate analysis using the Cox proportional hazards model revealed that a history of smoking was an independent unfavorable prognostic factor in lung adenocarcinoma patients who underwent potentially curative resection and is in good agreement with the study of Sobue *et al.*[35]

In conclusion, in our retrospective study the presence of p53 abnormalities correlated significantly with cancer recurrence and disease-free survival time. As the impact of adjuvant chemotherapy on the survival of NSCLC patients with curatively resected tumors remains to be elucidated,[4, 5] more clinical trials should be carried out in such patients. We believe that p53 gene analysis may be useful for selecting high-risk patients for these clinical trials and will help to increase the motivation of patients to participate in them and that of physicians to conduct them.

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