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Significance of p53 expression as a prognostic factor in oesophageal squamous cell carcinoma

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Abstract. The tumour suppressor gene product p53 is believed to play an important role in the progression of human malignant tumours through mutation and over-expression. Using a microwave oven heating method, we have detected over-expression of p53 in buffered-formalin fixed, paraffin-embedded sections of oesophageal carcinomas immunohistochemically and examined the relationship between the p53 over-expression and postoperative survival. Employing a monoclonal antibody (pAb1801), nuclear p53 was detected in 56 of 105 (53%) tumour specimens. Homogeneous, heterogeneous, and focal immunostaining patterns were noted. No immunostaining was found in adjacent benign tissues. The results in buffered-formalin fixed sections were similar to those in the frozen sections. The cumulative survival rate of patients with p53 expression was significantly lower than that of the patients without expression ($P < 0.05$), even though there were no significant differences between the clinicopathological features of the two groups. The results indicate that the nuclear accumulation of p53 might be an independent prognostic factor in patients with oesophageal squamous cell carcinomas.

Key words: p53 – Tumour suppressor gene product – Oesophageal cancer – Immunohistochemistry – Prognostic factor

Introduction

The nuclear phosphoprotein p53, located on the short arm of chromosome 17p13.1 (Isobe et al. 1986), is a suppressor of cell growth, but this function is abrogated by mutation or deletion of the *p53* gene (Finlay et al. 1989; Hinds et al. 1989; Nigro et al. 1989). Alterations of the *p53* gene are the most common genetic changes detected in various types of cancer (Baker et al. 1989; Hollstein et al. 1991; Takahashi et al. 1989). These changes play a critical role in the multiple stages of carcinogenesis.

Little is known, however, about the role of p53 in the progression of human tumours, including oesophageal cancer. Other investigators have demonstrated that immunohistochemical detection of p53 (Banks et al. 1986) is associated with the presence of *p53* mutation in situ (Bartek et al. 1990a; Iggo et al. 1990; Rodrigues et al. 1990), and several papers have described the nuclear accumulation of p53 in various tumours (Bennett et al. 1991; Cattoretti et al. 1988; Iggo et al. 1990; Martin et al. 1992; Rodrigues et al. 1990) using immunohistochemical analysis. But most of the previous studies were performed on cryostat sections since long-term formalin fixation is not suitable for preserving the antigenicity of p53. To obtain access to well characterized archival material and to patient populations with long-term survival data, however, p53 expression must be examined in routinely processed, formalin-fixed, paraffin-embedded material.

In the present study we applied the microwave oven heating technique, shown to be effective in the retrieval of masked epitopes of many antigens (Kerns et al. 1992; Shi et al. 1991) and detected the accumulations of p53 in buffered-formalin fixed, paraffin-embedded sections of 105 oesophageal carcinomas, using a monoclonal antibody recognizing a p53 domain. We also analysed the relationship between p53 expression and clinical and prognostic factors.

Materials and methods

Surgical specimens were obtained from 105 patients (92 male and 13 female), who had squamous cell carcinoma of the oesophagus and underwent subtotal oesophagectomy with lymph node dissection in the Department of Surgery II, Osaka University Medical School between 1987 and 1991. The age of the patients ranged from 41 to 80 years with a mean age of 61.7 years. None of them received irradiation or chemotherapy prior to surgery. Additional-

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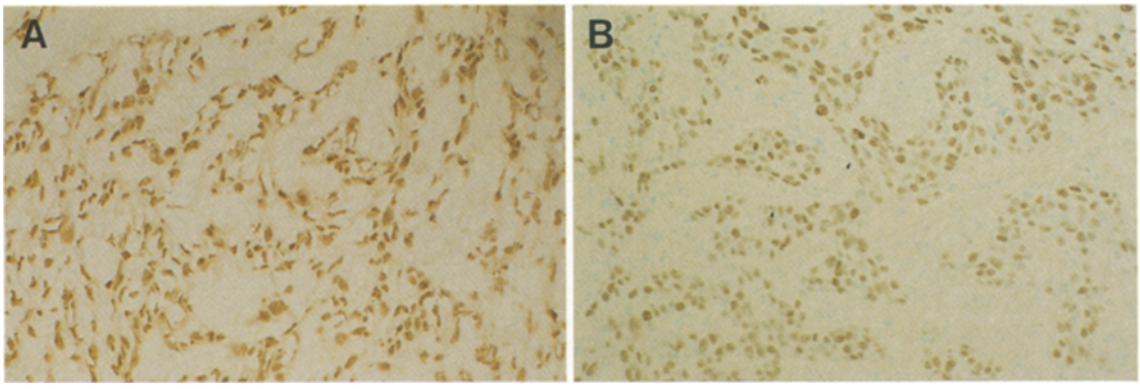

Fig. 1A, B. Immunohistochemical staining of p53 in **A** frozen section and **B** buffered-formalin-fixed section from the same tumour sample. ABC; methyl green counterstain. × 50

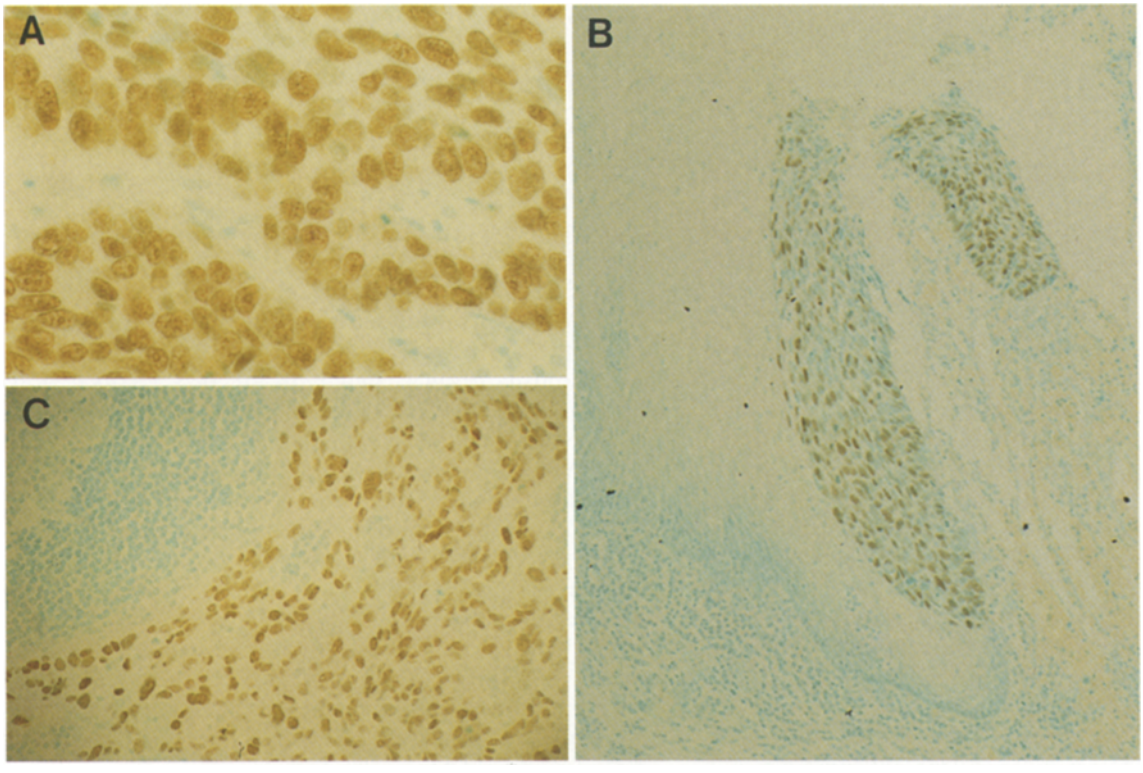

Fig. 2A–C. Immunohistochemical localization of p53 in oesophageal squamous cell carcinoma. **A** p53 staining was confined to the nuclei of cancer cells. No cytoplasmic staining was observed. × 100. **B** Nuclear staining was observed in cancer cells but not in the adjacent normal epithelia. × 33. **C** p53 expression was also observed in metastatic cells in lymph node. × 100. Buffered-formalin fixed, paraffin-embedded sections; ABC; methyl green counterstain

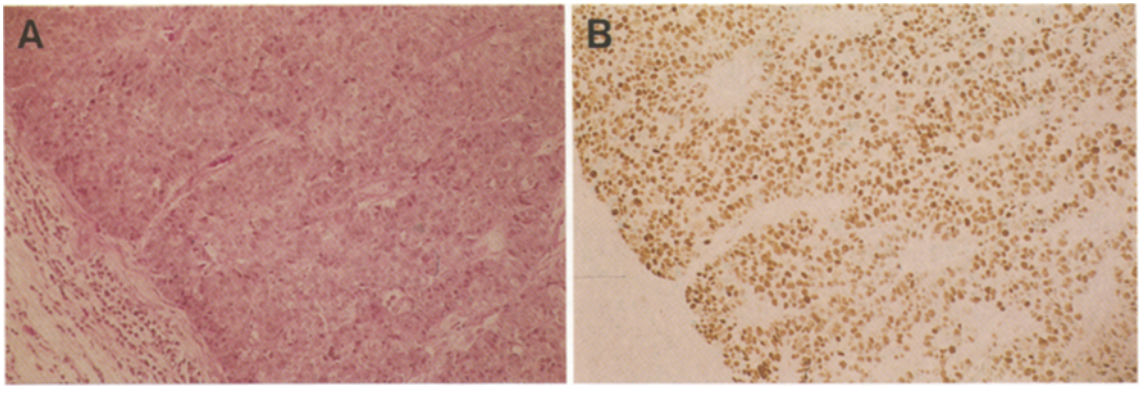

Fig. 3A, B. Homogeneous staining pattern of p53. Virtually all the tumour cells show nuclear p53 expression. Buffered-formalin-fixed, paraffin-embedded sections; **A** H&E staining and **B** immunohistochemical staining, methyl green counterstain. × 50

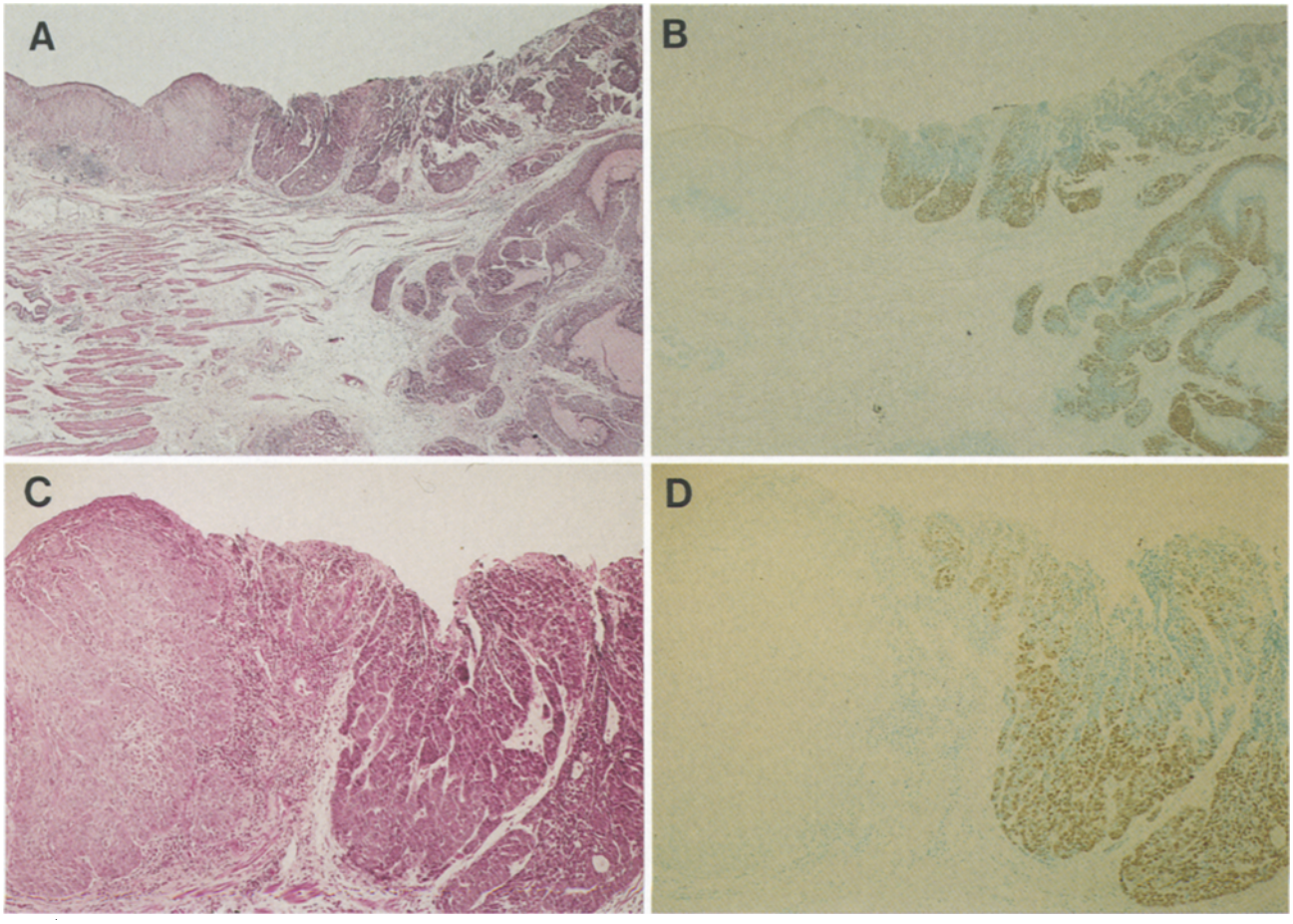

Fig. 4A–D. Heterogeneous staining pattern of p53. **A** There are two morphologically different tumour components. H&E, × 5. **C** Histological evaluations of both components are moderately differentiated squamous cell carcinoma. H&E, × 25. **B**, **D** p53 accumulation was observed in the right half tumour component but not in the left half. Immunohistochemical staining, methyl green counterstain, **B** × 5, **D** × 25

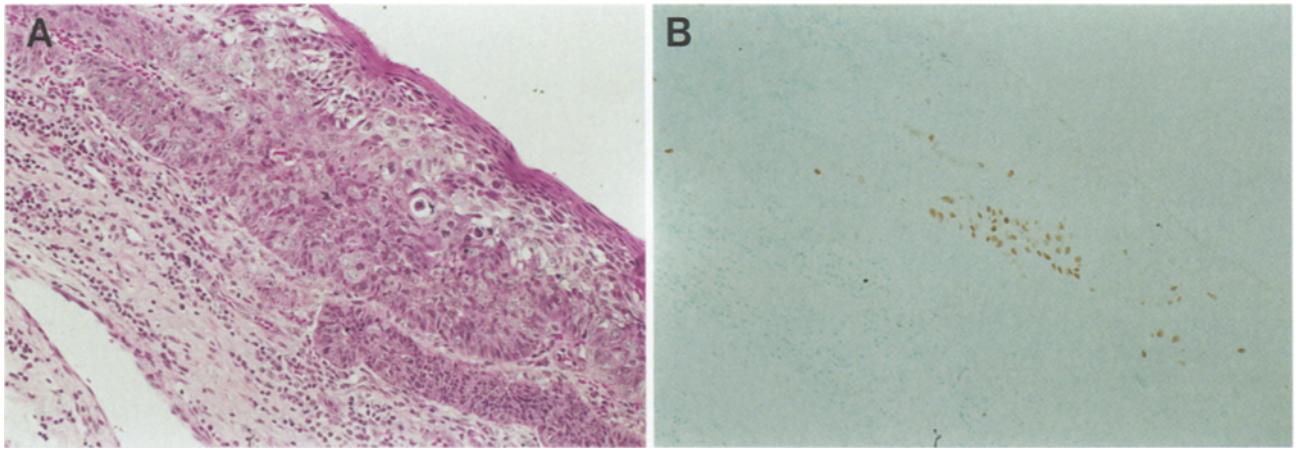

Fig. 5A, B. Focal staining pattern of p53. Few tumour cells show nuclear p53 expression. Buffered-formalin-fixed, paraffin-embedded sections; **A** H&E, **B** immunohistochemical staining. methyl green counterstain, × 50

ly, metastatic lymph nodes were obtained from 72 patients at surgery.

Representative samples of tumour, adjacent benign tissue, and metastatic lymph nodes were fixed with 10% neutral buffered formalin (BF) and embedded in paraffin blocks. To test the usefulness of this method, fresh frozen samples were also obtained from 30 of the patients. Serial sections, 4 μm thick, were prepared from these BF-fixed and frozen samples, and mounted on poly-L-lysine-coated slides (Sigma, St. Louis, Mo.) for immunohistochemistry.

A modified immunoglobulin enzyme bridge technique (avidin-biotin-peroxidase complex method) was used for immunostaining. After blocking the endogenous peroxidase activity for deparaffin-

ized BF-fixed sections, the microwave oven heating technique (Shi et al. 1991) was employed. In brief, the sections were placed in glass jars containing 500 ml distilled water and heated in the microwave oven (Panasonic model NE-M335) for 10 min. After heating they were rinsed in 0.05 mol/l phosphate-buffered saline (PBS) and incubated with 3.0% normal rabbit serum for 15 min to block nonspecific conjugation in the tissues. To detect p53 protein, they were incubated with primary anti-p53 monoclonal antibody (pAb1801, Novocastra Laboratories, Newcastle, UK) at a dilution of 1:50 for 24 h at 4° C. After washing with PBS, they were incubated with biotinylated rabbit anti-mouse immunoglobulin [Histofine SAB-PO (M) Kit, Nichirei Corporation, Tokyo, Japan] for 30 min at room temperature followed by peroxidase-conjugated streptavidin [Histofine SAB-PO (M) Kit] for an additional 30 min. Immune conjugate was visualized with 0.05 mol/l TRIS-hydrochloric acid (pH 7.6) containing both 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride and 0.03% (v/v) hydrogen peroxide. Negative control sections in each run of staining were made using normal mouse IgG instead of anti-p53 antibody in these procedures. Immunostaining for frozen sections was performed using the same method as for the paraffin-embedded sections without the microwave oven heating technique.

The tumours were classified arbitarily as positive for p53 expression if any of the tumour cells manifested nuclear positivity. Histological evaluation was assessed by haematoxylin and eosin staining. The pathological classification was based on the TNM classification of malignant tumours. The data examined in this study were tumour depth (T), lymph node status (N), distant metastasis (M), TNM stage, and histological grade of the tumour.

Postoperative status of the patients was examined on 15 July 1992, at which time the mean term of follow up was 619 days. The cumulative survival rates were calculated by the Kaplan-Meier method and the statistical significance was analysed by the generalized Wilcoxon test. The other statistical differences of the data were analysed by chi-square test.

Results

Reactivity with the anti-p53 antibody could be clearly demonstrated in deparaffinized sections made from BF-fixed, paraffin-embedded tissues of oesophageal carcinomas. A direct comparative study of p53 expression in BF-fixed sections and frozen sections produced almost identical results. As shown in Table 1, 15 tumours (50%) were positive and 13 tumours (43%) were negative in both assays. Overall, identical staining, either positive or negative, was observed in 28 of 30 tumours (93%). Figure 1 shows representative immunostaining in frozen (Fig. 1A) and BF-fixed (Fig. 1B) sections of the same tumour sample. Of the 105 tumour samples from the oesophagus, 56 (53%) contained cells expressing p53 in their nuclei without cytoplasmic staining (Fig. 2A). Normal oesophageal epithelia or non-cancerous epithelia adjacent to the tumours were negatively stained (Fig. 2B) and no immunostaining was noted in the negative control sections (data not shown).

Cases were considered positive if any of the tumour cells manifested nuclear positivity. Three major staining patterns of p53 were evident; homogeneous, heterogeneous, and focal (Figs. 3–5, Table 2). In the homogeneous type tumours, all the tumour cells were stained positive (Fig. 3). In the heterogeneous type tumours, there were clearly p53-positive and negative components (Fig. 4). In the focal staining type tumours, only a few tumour cells were stained positive (Fig. 5).

Table 1. Relationship between p53 expression in buffered-formalin (BF)-fixed and frozen sections

p53 positive[a]	p53 negative[b]	Discordant	Total
15 (50%)	13 (43%)	2 (7%)	30 (100%)

[a] Both BF-fixed and frozen sections stained positive for the p53 protein
[b] Both BF-fixed and frozen sections stained negative for the p53 protein

Table 2. Staining patterns of p53-positive cases

Homogeneous pattern	39	69.6%
Heterogeneous pattern	6	10.7%
Focal pattern	11	19.6%
Total	56	100%

Table 3. Relationship between p53 expression in metastatic lymph node (LN) and that in primary lesion

			p53 expression in metastatic LN	
		No.	(+)	(−)
p53 expression in primary lesion	(+)	39	32	7
	(−)	33	4	29
	No.	72	36	36

Table 4. Relationship between p53 expression and clinicopathological factors in patients with oesophageal squamous cell carcinoma

	No. examined	No. positive (%)
TNM classification[a]		
pT1	25	14 (56.0)
pT2	17	10 (58.8)
pT3	51	26 (50.9)
pT4	12	6 (50.0)
pN0	33	17 (51.5)
pN1	72	39 (54.2)
M0	102	54 (52.9)
M1	3	2 (66.6)
Stage		
I	17	8 (47.1)
IIa	13	8 (61.5)
IIb	22	13 (59.1)
III	50	25 (50.0)
IV	3	2 (66.6)
Tumour grade[b]		
1	31	13 (41.9)
2	41	25 (60.9)
3	33	18 (54.5)

[a] T, Tumour status T_1, T_2, T_3, and T_4 spread to submucosa, muscularis propria, adventitia, and adjacent structures, respectively; N, nodal status; M, metastatic status
[b] Grade 1, well differentiated squamous cell carcinoma; grade 2, moderately differentiated squamous cell carcinoma; grade 3, poorly differentiated squamous cell carcinoma

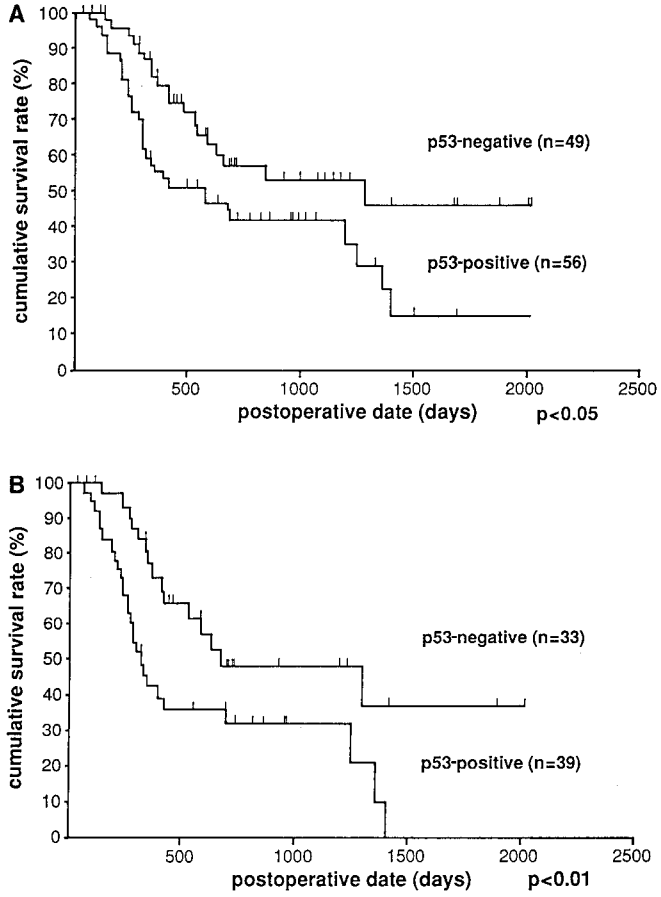

Fig. 6A, B. The cumulative survival curves for patient groups divided by p53 expression (Kaplan-Meier method). **A** Curves for all the patients, **B** curves for patient groups with lymph node metastasis

We also analysed p53 expression in the metastatic lymph nodes of 72 patients and compared the staining results with those of corresponding primary lesions (Fig. 2C, Table 3). Expression of p53 in the metastatic lymph nodes was almost identical to that in the corresponding primary lesions (61/72, 85%), although discordant staining was observed in some cases (11/72, 15%).

The relationship between p53 positivity in primary lesions and clinicopathological factors is shown in Table 4. No statistically significant association was observed between p53 expression and clinicopathological features.

Figure 6A shows cumulative survival curves for p53-positive and negative patients. A significant association between positive nuclear staining and survival time was observed. The survival rate of patients with p53 expression (median survival = 550 days; 5-year survival rate = 15%) was significantly lower than that of patients without p53 expression (median survival = 1219 days; 5-year survival rate = 45%) ($P < 0.05$). Furthermore, in 72 patients with positive node involvement, p53 expression was also significantly correlated with a worse disease outcome (Fig. 6B), although there was no difference between the patient groups without lymph node metastasis (data not shown).

Discussion

In this study, nuclear p53 expression was observed in 53% of the oesophageal carcinomas employing anti-p53 monoclonal antibody in BF-fixed, paraffin-embedded sections using the microwave oven heating technique. This figure is similar to the results of previous studies of human malignancies (Bartek et al. 1990b; Bennett et al. 1991; Van den Berg et al. 1989). The results of p53 staining in BF-fixed sections were identical to those in frozen sections, confirming the usefulness of this method as an alternative.

We were interested to note that some primary tumours showed marked heterogeneous expression of p53 (Thompson et al. 1992) in the present study (heterogeneous pattern, 10.7%; focal pattern, 19.6%). Compared with the staining result with haematoxylin and eosin in a parallel section (Fig. 4), heterogeneous expression is apparently more likely to reflect tumour heterogeneity. The difference between the staining results in the metastatic lymph nodes and the corresponding primary lesions might be an outcome of this heterogeneity in p53 accumulation. It also underlines the importance of care at the time of sampling a tumour tissue for DNA and mRNA studies to avoid faulty evaluation of the tumour.

No significant correlation was found between clinicopathological factors and p53 expression in oesophageal cancer patients. This result suggests that p53 is not associated with determinants of malignant potential such as invasion or metastasis, and that in oesophageal squamous cell carcinoma at least, alteration of *p53* may be an early event in carcinogenesis, since p53 accumulation was observed at almost the same rate in both the later and the earlier stages.

Although there is a lack of correlation with established prognostic indicators such as tumour grade and TNM system, simple grouping of the p53-positive tumours regardless of staining pattern denotes a group of patients with very poor survival. This is consistent with reports on other human tumours (Iwaya et al. 1991; Martin et al. 1992). Furthermore, we showed that p53 expression is correlated with a worse disease outcome in patients with positive node involvement. Additionally, Tamura et al. (1992) reported that short-term relapse of oesophageal cancer is more frequent in patients with a *p53* gene mutation. All of these results in survival or relapse suggest that *p53* mutation may affect growth rate of the tumour in vivo, as expected from results in vitro (Baker et al. 1990; Eliyahu et al. 1989; Finlay et al. 1989).

The results of the present study indicate that nuclear accumulation of p53 may be an independent prognostic factor in oesophageal tumour. We believe that immunohistochemical analysis of nuclear p53 localization is useful as a routine procedure for evaluating the malignant potential of oesophageal squamous cell carcinoma, and that a careful follow-up is necessary for cases showing staining for nuclear p53.

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