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Clinicopathological Significance of Nuclear Accumulation of Tumor Suppressor Gene p53 Product in Primary Lung Cancer

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Since the nuclear accumulation of p53 protein is known to correspond well with mutation of the p53 tumor suppressor gene, the authors examined 88 primary lung cancer specimens immunohistochemically using anti-p53 mouse monoclonal antibody, pAb1801, and analyzed the relationship between the immunohistochemical results and clinicopathological features. Nuclear localization of p53 protein was found in 43/88 (49%) tumor specimens, but not in the corresponding normal lung tissues. The percentage of cases showing nuclear p53 localization varied according to the histological type. In squamous cell carcinoma, nuclear p53 localization was found in 15/26 (57%), appearing more frequently than in other histologic types. However, no obvious correlation was observed between nuclear p53 localization and patients' age, sex, history of smoking, TNM factor, degree of differentiation, or any other clinicopathological features analyzed. In adenocarcinoma, nuclear p53 localization was found in 20/46 (43%). Incidence of positive cases was significantly correlated with regional lymph node metastasis, distant metastasis, and pathological stage ($P<0.05$). These results indicate that mutation of the p53 tumor suppressor gene plays an important role in the development of primary lung cancer, and that nuclear accumulation of p53 protein is a potential prognostic factor in adenocarcinoma of the lung.

Key words: Tumor suppressor gene p53 — Immunohistochemistry — Lung cancer — Tumor progression — Prognostic factor

Recently it has been reported that the tumor suppressor gene p53, located on the short arm of chromosome 17, is frequently mutated in primary lung cancer[1–4] and various other types of cancer.[5–11] It is widely accepted that mutation of the p53 gene plays a critical role somewhere in the multiple stages of carcinogenesis. In colorectal tumors, p53 gene mutation has been detected more frequently in carcinoma rather than in adenoma, and Baker *et al.* have suggested that p53 mutation is a late event in colorectal carcinogenesis.[9] In gastric cancer, we have detected p53 mutation only in carcinomas with an aneuploid DNA content, also suggesting that p53 mutation is a relatively late event.[12] To determine the timing of p53 gene mutation and to understand its role, it is very important to analyze its relationship with clinicopathological features.[13]

On the other hand, it has been found that the wild-type p53 protein is present in the nucleus at a very low level, whereas the mutant p53 protein is metabolically more stable than the wild type and tends to accumulate in the nucleus.[14,15] In colorectal tumors and cell lines, Rodrigues *et al.* detected p53 protein in the tumor cell nucleus immunohistochemically, and showed a good correlation between nuclear p53 localization and p53 gene mutation.[10]

Therefore we examined the nuclear localization of p53 in 88 primary lung cancers immunohistochemically and analyzed its relationship with clinicopathological features.

MATERIALS AND METHODS

Patients Tumor specimens were obtained from primary lung cancers of 88 patients, who were treated surgically between November 1989 and July 1990 at the National Cancer Center Hospital, Tokyo. The patients comprised 69 men and 19 women, with an age range of 32–74 years (mean, 58.7 years). Of these, 8 patients were older than 70 yr and 13 were aged 50 yr or less. Clinical data were available for all patients, including age, sex, history of smoking, TNM stage, and other pathological factors (degree of differentiation, pleural involvement, pleural dissemination, vascular invasion, nuclear atypia, and mitotic index). TNM staging was done according to the General Rules for Clinical and Pathological Recording of Lung Cancer published by the Japan Lung Cancer Society.[16]

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Immunohistochemistry Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin, and histological diagnosis was made according to the WHO classification. The tumor specimen and the lung tissue from each patient were also fixed in cold acetone and embedded in paraffin by the AMeX method, as described previously.[17] Immunohistochemical staining was performed on AMeX sections using an avidin-biotinyl peroxidase complex method. The antibody against p53 protein used was pAb1801 (Oncogene Science, Inc., Manhasset, NY), which is a mouse monoclonal antibody against human p53 protein and reacts with both the wild-type and mutant forms.[18]

Briefly, 5-μm-thick AMeX sections were placed on lysine-coated slides and dried at room temperature. The sections were post-fixed with 4% paraformaldehyde in 0.067 *M* phosphate-buffered saline, pH 7.4 (PBS), for 5 min, washed in water, and preincubated in 2% normal swine serum in PBS. The sections were reacted with the first antibody (pAb1801, diluted 1:100) overnight at 4°C. After washing in PBS, the sections were incubated for 30 min with biotinylated horse anti-mouse immunoglobulin diluted 1:200 as a secondary antibody (Vector Laboratories Inc., Burlingame, CA). Then the sections were incubated for 30 min with avidin-biotinyl peroxidase complex diluted 1:100 using a Vectastain ABC kit (Vector). After washing in PBS, the sections were reacted with 0.025% (w/v) 3,3′-diaminobenzidine tetrahydrochloride, 0.01% H_2O_2, and Tris-HCl buffer, pH 7.4, with addition of 0.01% sodium azide to visualize the site bound with the antibody. Sodium azide was added in order to inhibit endogenous peroxidase activity. Finally, the sections were counterstained with 2% methyl green or Meyer's hematoxylin.

The positive control included in all assays was an AMeX section of the colon cancer cell line SW837, which is known to carry point-mutation of p53.[10] The negative control staining, performed by omitting the exposure to the first antibody, always gave negative results. Cases in which more than 10% of the cancer cell nuclei were stained were considered positive; otherwise a negative result was recorded.

Western immunoblot analysis Crude protein extracts from AMeX sections, obtained by brief sonication after deparaffinization, were lysed in 62.5 m*M* Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.017% phenylmethylsulfonyl fluoride and 0.001% bromophenol blue. The tissue lysate was denatured at 100°C for 2 min, loaded onto 10% polyacrylamide gels and transferred to nitrocellulose membranes (Nihon Millipore Kogyo, Yonezawa) by semi-dry electroblotting at 15 V for 30 min. Western blots were incubated with the anti-p53 protein monoclonal antibody, pAb1801, and p53 protein was detected on the membrane by the avidin-biotinyl peroxidase complex method following the procedures for immunohistochemistry.

Statistical analysis Statistical significance was evaluated by using the chi-squared test with the criterion of $P < 0.05$.

RESULTS

In normal or non-tumorous lung tissues, all the cells including those of bronchoalveolar epithelia and bronchial glands were negative for p53 (Fig. 1). Of all the 88 primary lung cancers, 43 cases (49%) showed a clearly positive reaction for p53. The nucleus of tumor cells was stained predominantly (Fig. 2), but in 4 cases only the cytoplasm was stained (Fig. 3). By Western blot analysis, p53 was not detected in these 4 cases, whereas it was clearly detected in 3 of 3 cases positive for nuclear p53 (Fig. 4).

The percentage of p53-positive cases varied according to the histological type, as shown in Table I. In adenocarcinoma and large cell carcinoma, the p53 staining pattern and its intensity were relatively heterogeneous within each tumor; small areas composed of positive tumor cells were observed within the larger negative area in about half of the cases (Fig. 5). In squamous cell carcinoma, the p53 staining pattern was relatively ho-

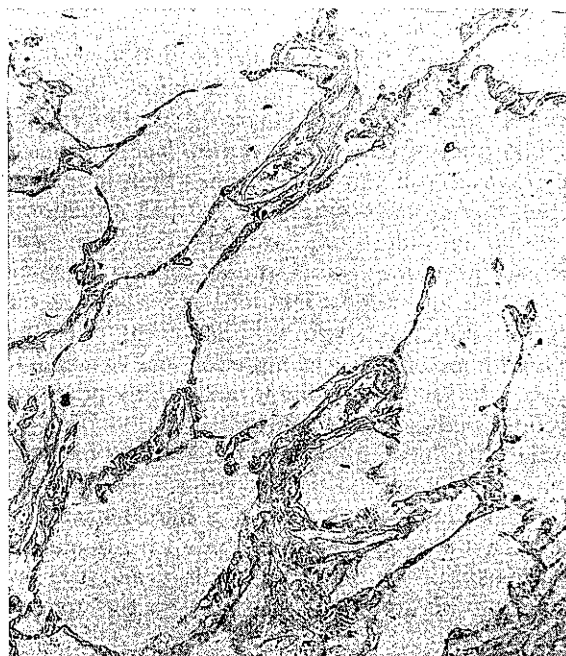

Fig. 1. Immunohistochemical staining of p53 in normal lung tissue.

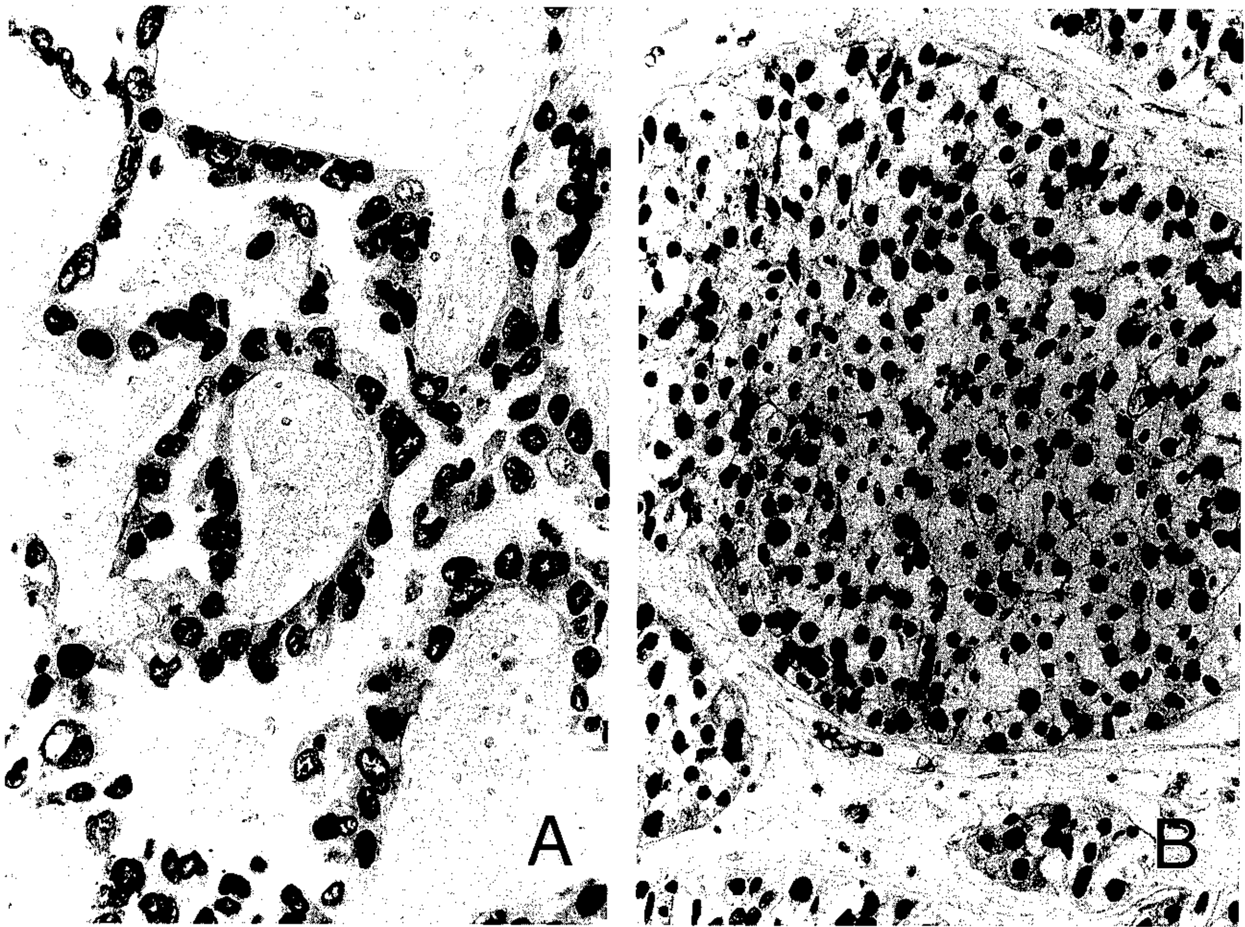

Fig. 2. Immunohistochemical localization of p53. A, moderately differentiated adenocarcinoma. B, poorly differentiated squamous cell carcinoma. Most of the tumor cells show a strong staining reaction indicating accumulation of p53 in nuclei. Stromal cells are completely negative.

mogeneous. However, when zonal differentiation was observed in nests of squamous cell carcinoma, nuclei of less differentiated cancer cells were more strongly stained for p53. In adenosquamous carcinoma, p53-positive cells were localized in the area of squamous cell carcinoma, but not in the area of adenocarcinoma. In small cell carcinoma, most of the tumor cell nuclei showed positive staining.

The relationship between nuclear p53 staining and TNM stage in adenocarcinoma and squamous cell carcinoma is shown in Tables II and III. In adenocarcinoma, nuclear p53 staining was significantly correlated with regional lymph node metastasis, distant metastasis, and pathological stage ($P<0.05$). The incidence of cases positive for nuclear p53 staining showed a tendency to increase with advancing pathological stage. A higher incidence was detected at stages III_A, III_B and IV compared with stages I and II; the difference was statistically significant ($P<0.05$). As to the N factor, the incidence of nuclear p53 staining was significantly higher in cases showing regional lymph node metastasis (58%) than in those without metastasis (27%) ($P<0.05$). With regard to the M factor, the incidence of nuclear p53 staining was significantly higher in cases with distant metastasis (88%) than in those without (37%) ($P<0.05$). No relationship was demonstrated between nuclear p53 staining and patients' age, sex, smoking history, degree of histological differentiation, pleural involvement, pleural dissemination, vascular invasion, nuclear atypia, or mitotic index. In squamous cell carcinoma, no correlation was found between nuclear p53 staining and clinicopathological features.

Fig. 3. Immunohistochemical reaction of anti-p53 antibody in a well differentiated adenocarcinoma. Cytoplasmic staining is evident.

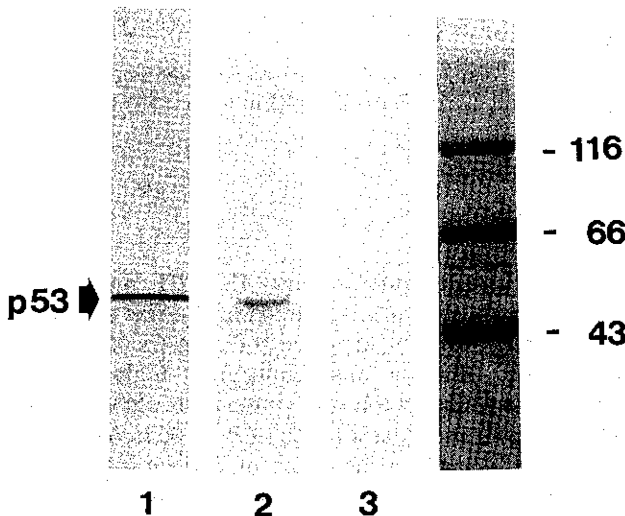

Fig. 4. Western immunoblot analysis of extracts from primary lung cancer and a control colon cancer cell line (SW837) using a mouse monoclonal antibody against human p53, pA1801. Lane 1: SW837. Lane 2: case showing nuclear p53 staining (same case as that shown in Fig. 2-B). Lane 3: case showing cytoplasmic staining. Lane 2 shows a single band of p53 protein with the same molecular size as that of SW837 (Lane 1). No immunoreactive band is seen in Lane 3.

Table I. Incidence of Nuclear p53 Localization in Primary Lung Cancer

	No. examined	No. positive (%)
Adenocarcinoma	46	20 (43)
Squamous cell carcinoma	26	15 (57)
Small cell carcinoma	4	2 (50)
Large cell carcinoma	5	3 (60)
Adenosquamous carcinoma	4	2 (50)
Carcinoid	2	0 (0)
Adenoid cystic carcinoma	1	1 (100)
Total	88	43 (49)

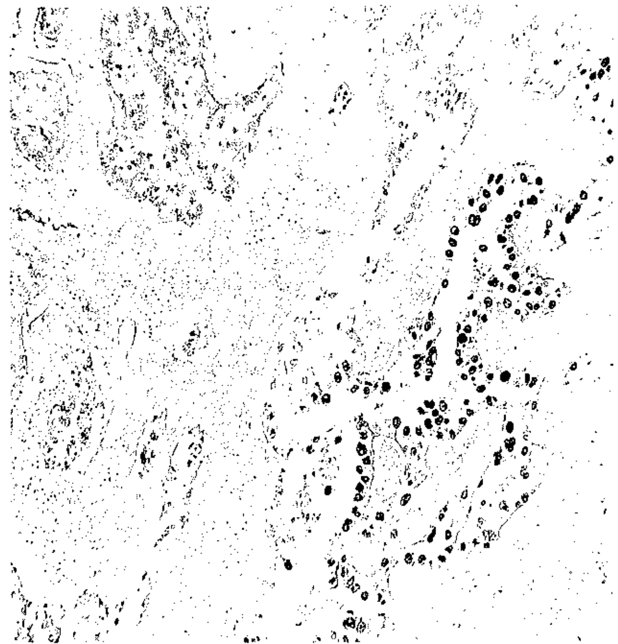

Fig. 5. Immunohistochemical localization of p53 in a moderately differentiated papillary adenocarcinoma. Tumor cells in a focal area are stained positively for nuclear p53.

DISCUSSION

In this study, nuclear p53 staining was observed selectively in tumor cells and not at all in non-tumorous lung tissues. This result is consistent with recent studies on colorectal[10] and primary lung[1] tumors. We showed that nuclear p53 staining was frequently present in primary lung cancer, and its incidence in each histological type agreed with previous reports.[1] The incidence of nuclear p53 staining was also similar to that reported for p53 gene mutation,[4] and this coincidence further suggests that nuclear p53 staining reflects the mutation in its gene.

Table II. Nuclear p53 Localization and TNM Stage of Adenocarcinoma

		No. examined	No. positive (%)	
	T1	16	6 (38)	
	T2	21	9 (43)	
	T3	2	1 (50)	
	T4	7	4 (57)	
	N0	22	6 (27)	*
	N1	9	5 (56)	
	N2	14	8 (57)	
	N3	1	1 (100)	
	M0	38	14 (37)	*
	M1	8	7 (88)	
Stage	I	15	3 (20)	*
	II	6	2 (33)	
	III_A	12	6 (50)	
	III_B	5	2 (40)	
	IV	8	7 (88)	

*, $P<0.05$.

Table III. Nuclear p53 Localization and TNM Stage of Squamous Cell Carcinoma

		No. examined	No. positive (%)
	T1	3	2 (67)
	T2	11	5 (45)
	T3	6	5 (83)
	T4	6	3 (50)
	N0	9	5 (56)
	N1	11	7 (64)
	N2	5	2 (40)
	N3	1	1 (100)
	M0	26	15 (58)
	M1	0	0
Stage	I	7	4 (57)
	II	6	2 (33)
	III_A	6	5 (83)
	III_B	7	4 (57)
	IV	0	0

Since the mutant p53 protein is known to accumulate in the cell nucleus, we considered only those cases showing nuclear staining to be positive in the present study. In fact, the majority of cases showed nuclear staining in tumor cells. Western blot analysis clearly confirmed the accumulation of p53 protein in three representative cases of them, though some discrepancy between immunostain and Western blot analysis cannot be ruled out because we did not analyze all the cases by Western blotting. On the other hand, p53 protein was not detected by Western blot analysis in cases with staining only in the cytoplasm. Although the significance of cytoplasmic p53 staining remains unknown, it may be due to the cross-reactivity of this antibody with cytokeratin intermediate filaments.[1]

In the present investigation, we studied the clinicopathological significance of inactivation of the p53 tumor suppressor gene using the nuclear accumulation of its product as a marker. The results suggested that p53 inactivation occurs at a different stage, and possibly plays a different role in the multi-stage carcinogenesis pathway, in the two major types of lung cancer, adenocarcinoma and squamous cell carcinoma. In squamous cell carcinoma, alteration of p53 seems to be a relatively early event, since cases positive for nuclear p53 protein were frequent not only in the later stages but also in stages I and II. This result is consistent with a similar study of the oral cavity, where precancerous dysplastic epithelium was also positive for nuclear p53 localization (unpublished observation). Thus, p53 inactivation may play an important role at an early stage in the development of squamous cell carcinoma.

In contrast, the incidence of nuclear p53 staining was significantly correlated with advanced N factor, M factor, and pathological stage in adenocarcinoma. These results suggest that p53 alteration is a late event causing progression of adenocarcinoma, conferring aggressive growth and metastatic potential. Moreover, in about half of positively stained cases, the staining pattern of p53 was focal within each tumor, and there were small positively stained areas surrounded by negative areas. This implies that the more malignant cell population, positive for p53 alteration, may originate from the less malignant cell population as a result of stepwise progression of malignancy. This process is also consistent with the stepwise progression of differentiated adenocarcinoma of the lung demonstrated by means of histologic cytofluorometric DNA analysis.[19] Generally, both chromosome 17p allele loss and p53 gene mutation in the remaining allele are observed in p53 inactivation. Nishikawa *et al.* showed that chromosome 17p allele loss occurred more frequently in metastatic lesions in the brain than in the primary lesion of lung adenocarcinoma using restriction fragment length polymorphism (RFLP) analysis (unpublished data). This observation is also consistent with our conclusion.

In comparison with RFLP analysis of chromosome 17p and polymerase chain reaction amplification and sequencing analysis of the p53 gene, immunohistochemical analysis can be performed easily. Therefore immunohistochemical analysis of nuclear p53 localization is considered useful as a routine procedure for evaluation of the malignant potential of lung adenocarcinoma, and we consider that a careful follow-up is necessary for cases showing positive staining for nuclear p53.

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REFERENCES

1) Iggo, R., Gatter, K., Bartek, J., Lane, D. and Harris, A. L. Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet,* **335,** 675–679 (1990).
2) Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F. and Minna, J. D. p53: a frequent target for genetic abnormalities in lung cancer. *Science,* **246,** 491–494 (1989).
3) Takahashi, T., D'Amico, D., Chiba, I., Buchhagen, D. L. and Minna, J. D. Identification of intronic point mutation as an alternative mechanism for p53 inactivation in lung cancer. *J. Clin. Invest.,* **86,** 363–369 (1990).
4) Chiba, I., Takahashi, T., Nau, M. M., D'Amico, D., Curiel, T. D., Mitsudomi, T., Buchhagen, L. D., Carbone, D., Piantadosi, S., Hironobu, K., Reissmann, T. P., Slamon, J. D., Holms, C. E. and Minna, D. J. (For the Lung Cancer Study Group). Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. *Oncogene,* **5,** 1603–1610 (1990).
5) Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., Tuinen, P., Ledbetter, D. H., Baker, D. F., Nakamura, Y., White, R. and Vogelstein, B. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science,* **244,** 217–221 (1989).
6) Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Creary, K., Binger, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Mondail, R., Harris, C. C. and Vogelstein, B. Mutation in the p53 occur in diverse human tumor types. *Nature,* **342,** 705–708 (1989).
7) Menon, A. G., Anderson, K. M., Riccardi, V. M., Chung, R. Y., Whaley, J. M., Yandell, D. W., Farmer, G. E., Freiman, R. N., Lee, J. K., Li, F. P., Baker, D. F., Ledbetter, D. H., Kleider, A., Martuza, R. L., Gusella, J. F. and Seizinger, B. R. Chromosome 17 deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklighausen neurofibromatosis. *Proc. Natl. Acad. Sci. USA,* **87,** 5435–5439 (1990).
8) Mulligan, L. M., Matlashewski, G. J., Scrable, H. J. and Cavenee, W. K. Mechanisms of p53 loss in human sarcomas. *Proc. Natl. Acad. Sci. USA,* **87,** 5863–5867 (1990).
9) Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Wilson, J. K. V., Hamilton, S. and Vogelstein, B. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res.,* **50,** 7717–7722 (1990).
10) Rodrigues, N. R., Rowan, A., Smith, M. E. F., Kerr, I. B., Bodmer, W. F., Gannon, V. J. and Lane, D. P. p53 mutations in colorectal cancer. *Proc. Natl. Acad. Sci. USA,* **87,** 7555–7559 (1990).
11) Bartek, J., Iggo, R., Gannon, J. and Lane, D. P. Genetic and immunochemical analysis of mutant p53 in breast cancer cell line. *Oncogene,* **5,** 893–899 (1990).
12) Tamura, G., Kihana, T., Nomura, K., Terada, M., Sugimura, T. and Hirohashi, S. Detection of frequent p53 gene mutations in primary gastric cancer by cell sorting and polymerase chain reaction single-strand conformation polymorphism analysis. *Cancer Res.,* **51,** 3056–3058 (1991).
13) Iwaya, K., Tsuda, H., Hiraide, H., Tamaki, K., Tamakuma, S., Fukutomi, T., Mukai, K. and Hirohashi, S. Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. *Jpn. J. Cancer Res.,* **82,** 835–840 (1991).
14) Matlashewski, G., Banks, L., Pim, D. and Crawford, L. Analysis of human p53 proteins and mRNA levels in normal and transformed cells. *Eur. J. Biochem.,* **154,** 665–672 (1986).
15) Rogel, A., Popliker, M., Webb, G. C. and Oren, M. p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors. *Mol. Cell. Biol.,* **5,** 2851–2855 (1985).
16) The Japan Lung Cancer Society. "General Rules for Clinical and Pathological Record of Lung Cancer (in Japanese)," pp. 17–21 (1987). Kanehara Shuppan, Tokyo.
17) Sato, Y., Mukai, K., Watanabe, S., Goto, M. and Shimosato, Y. The AMeX method: a simplified technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. *Am. J. Pathol.,* **125,** 431–435 (1986).
18) Banks, L., Matlashewski, G. and Crawford, L. Isolation of human-p53-specific monoclonal antibodies and their use in the study of human p53 expression. *EMBO J.,* **159,** 529–534 (1986).
19) Nomori, H., Hirohashi, S., Noguchi, M., Matsuno, Y. and Shimosato, Y. Tumor cell heterogeneity and subpopulations with metastatic ability in differentiated adenocarcinoma of the lung. *Chest,* **99,** 934–940 (1991).